IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF THE BOVINE BRAIN MYELIN MARKER ENZYME GLYCEROPHOSPHORYLCHOLINE PHOSPHOCHOLINE PHOSPHODIESTERASE

A Thesis Presented to The

Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfillment of the

Requirements for the Degree of

Master of Science

by

Jun Yuan

Department of Biochemistry

and Molecular Biology

Faculty of Medicine

The University of Manitoba

July 1992

ISBN 0-315-77812-1

IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF THE BOVINE BRAIN MYELIN MARKER ENZYME GLYCEROPHOSPHORYLCHOLINE PHOSPHOCHOLINE PHOSPHODIESTERASE

BY

JUN YUAN

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

To my parents and my wife

Love of knowledge is akin to wisdom.

Strenuous attention to conduct is akin to compassion.

Sensitiveness to shame is akin to courage.

Wisdom, compassion and courage — these are the three

universally recognized moral qualities of people.

Some people are born with the knowledge of these moral qualities;

some acquire it as the result of education;

some aquire it as the result of hard experiences.

But when the knowledge is acquired,

it comes to one and the same thing.

- Confucius, 551-479 B.C.

ACKNOWLEDGEMENTS

First and foremost, I wish to deeply acknowledge my supervisor, Dr. J. N. Kanfer, for providing me with the opportunity and laboratory facilities to deepen and broaden my basic medical education. I appreciate his rigorous scientific attitude, intelligent supervision, and continuous support throughout this project. I will always cherish and remember the wonderful time I spent in his lab.

I would like to express my appreciation to my committee members, Dr. H. K. Jacobs and Dr. R. Bhullar for their suggestions and criticisms during the course of investigation and writing of this thesis.

I would also like to express my thanks to my laboratory colleagues, Dr. I. Singh, Dr. C. J. Andriamampandry, D. G. McCartney, Z. M. Cao, and C. Kuo for their technical assistance, effective discussions, and faithful friendship.

Finally, I am most deeply grateful for the love, support, and patience of my wife, Mai, without her understanding and assistance, things would not have turned out so well. My gratitudes are also extended to my parents for their financial and spiritual support during my earlier stage of education.

This work was supported by a grant from the Multiple Sclerosis Society of Canada.

ABSTRACT

GPC phosphocholine phosphodiesterase is a newly discovered myelin associated enzyme and it is believed to be myelin specific. In order to confirm the localization of this enzyme activity in the myelin sheath, levels of GPC phosphocholine phosphodiesterase, p-NP phosphocholine phosphodiesterase, CNPase, and C gal T activities were estimated with pure cultures of oligodendrocytes and astrocytes; mixed primary glial cell cultures; C-6 cells; and CNS tissue of dysmyelinating md rat, the jimpy mouse and the quaking mouse. The highest activities of GPC and p-NP phosphocholine phosphodiesterase as well as CNPase and C gal T were found in the pure cultured oligodendrocytes. C-6 cells had very low or undetectable activities for these two phosphodiesterases but possessed very high CNPase activity. The activity of GPC phosphocholine phosphodiesterase was significantly decreased in CNS tissue of the md rat, the jimpy and quaking mouse. Similar reductions were observed for the p-NP phosphocholine phosphodiesterase, CNPase, and C gal T activities. By classical procedures, such as enzyme extraction and chromatography, the GPC and p-NP phosphocholine phosphodiesterase was purified 87- and 98-folds from bovine brain myelin with 14% and 15% recovery, respectively. The purified GPC phosphocholine phosphodiesterase had a specific activity of 5750 nmole/mg protein/hour, for p-NP phosphocholine phosphodiesterase, the specific activity was about 35,882 nmole/mg protein/hour. The column fractions with the highest of both phosphodiesterases activities showed a major single band with molecular weight of about 14 kDa on 12% SDS-PAGE by silver staining. The purified enzyme had a pH optimum of 10 for GPC phosphocholine phosphodiesterase, and 11 for p-NP phosphocholine phosphodiesterase, both phosphodiesterases had similar Km values and

required Zn^{2+} or Co^{2+} for their activities. The optimum concentrations of metal required for these two enzymes are similar, 0.25 mM for Zn^{2+} and 1 mM for Co^{2+}. p-NP phosphocholine and GPC were competitive inhibitors of GPC and p-NP phosphocholine hydrolysis with Ki of 28 μM and 0.5 mM, respectively. Phosphocholine also inhibits these two phosphodiesterases. The selective cellular enrichment in oligodendrocytes of the GPC phosphocholine phosphodiesterase activity and decreases of its activities in three dysmyelinating mutants in the same ratio as for CNPase and C gal T suggest that GPC phosphocholine phosphodiestease is myelin specific, and it may reflect the quantity of myelin and oligodendrocytes present. Similar distribution in the cultured cells and in the CNS tissues of the three dysmyelinating rodent mutants, similar degree of purification, and similar enzyme characteristics of these two phosphodiesterases also strongly suggest that GPC and p-NP phosphocholine phosphodiesterase reside on the same enzyme protein that can hydrolyze two different substrates.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

BSA:	Bovine serum albumin
C gal T:	UDP galactose: ceramide galactosyltransferase
CNPase:	2'3' -cyclic nucleotide 3' phosphodiesterase
CNS:	Central nervous system
GPC:	Glycerophosphorylcholine
kDa:	Kilodalton
MAG:	Myelin associated glycoprotein
MBP:	Myelin basic protein
Md rat:	Myelin-deficient rat
MS:	Multiple Sclerosis
NADP:	Nicotinamide adenine dinucleotide phosphate
PAGE:	Polyacrylamide gel electrophoresis
PLP:	Proteolipid protein
PMSF:	Phenylmethylsulfonyl fluoride
pNP:	para-nitrophenyl
PNS:	Peripheral nervous system
OβG:	Octyl β D- glucopyranoside
S.D.:	Standard deviation
SDS:	Sodium dodecyl sulfate
TCA:	Trichloroacetic acid
UDP:	Uridine diphosphate
W-10:	Waymouth's medium supplemented with 10 % calf serum

I. INTRODUCTION

1. CNS Myelin Formation and Composition

1.1. General

The nervous system can be divided into two parts, the central nervous system (CNS), consisting of the brain and spinal cord, and the peripheral nervous system (PNS), consisting of the cranial, spinal and peripheral nerves, together with their motor and sensory nerve endings. The CNS tissue contains i) white matter which is composed of nerve fibers enveloped by the processes (called myelin) of glial cells, glial cell bodies, and capillaries, ii) gray matter which has, in addition, the nerve cell bodies with their extensive dendritic arborizations (Blackwood, 1976). The major difference between white and gray matter is that white matter has a high percentage of myelin sheath. Myelin constitutes approximately 50 percent of the total dry weight of white matter, and is mainly responsible for the gross appearance and chemical differences between white and gray matter.

1.2. CNS myelination

Myelination is a complex, neurobiological process which is developmentally regulated. CNS myelin is a membrane derived from the oligodendroglial cells. Initially, the oligodendrocytes extend processes of their plasma membranes to distant nerve fibers (axon) (Davison and Peters, 1970). Once contact is made between the oligodendroglial and axonal membranes, the glial cell membrane begins to wrap around the axon until a complete circuit is accomplished and the extracellular faces adhere (Figure 1). After that, a myelin

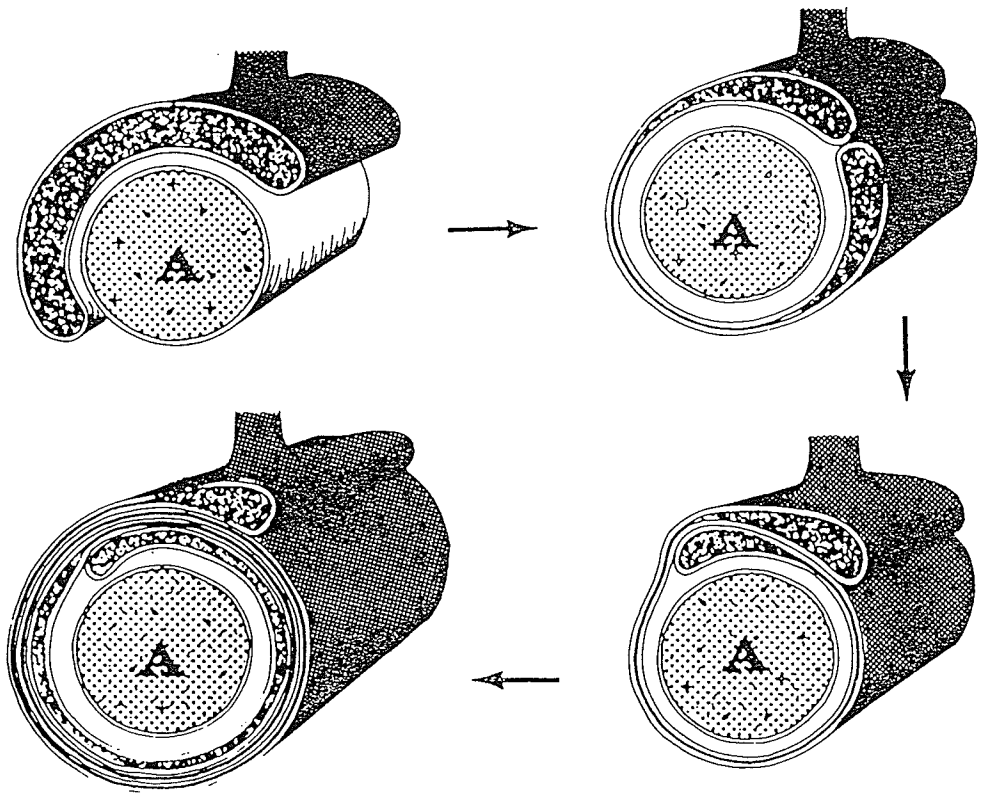

Figure 1. Schematic representation of the CNS myelin formation. A process of an oligodendrocyte becomes flattened and spirally wrapped around the axon (A) of a neuron. This picture is adopted from Raine (1984a).

compaction process occurs within the cytoplasmic space which results in the association of the two apposing bilayers and formation of a major dense line. Cytoplasm is squeezed out toward the periphery of the sheet, forming a thin tube around the edge. Concurrent compaction of the outer leaflets of the oligodendroglial plasma membrane forms the intraperiod line (Norton, 1981a; Raine, 1984a; Deber and Reynolds, 1991). In the mature myelin sheath, a thickness of 5-20 multilayers of this oligodendroglial plasma membrane unit (five-layered structure) can be observed (Figure 2).

The oligodendrocyte in the CNS is at a distance from the myelin sheath. A single oligodendroglial cell produces several myelin sheaths through independent processes at varying distances from the cell body. They are connected and communicate with each other only by a slender cytoplasmic process (Bunge and Glass, 1965). The period at which rapid myelination takes place varies considerably among different species and different regions of the same animals. In most laboratory animals, PNS and CNS myelin formation is fairly complete by 3-4 weeks of age. At the present time, the precise mechanism of initiation and termination of myelination is unknown.

1.3. CNS myelin composition

CNS of many species including human has been studied for myelin composition. Most of the detailed information was estabilshed by 1965. The lipid-to-protein ratio in myelin sheath is higher than that of other cell surface or intracellular membranes. The solids of myelin, depending on its source, contain 70-80% lipid and 15-30% protein. Myelin is a relatively dehydrated

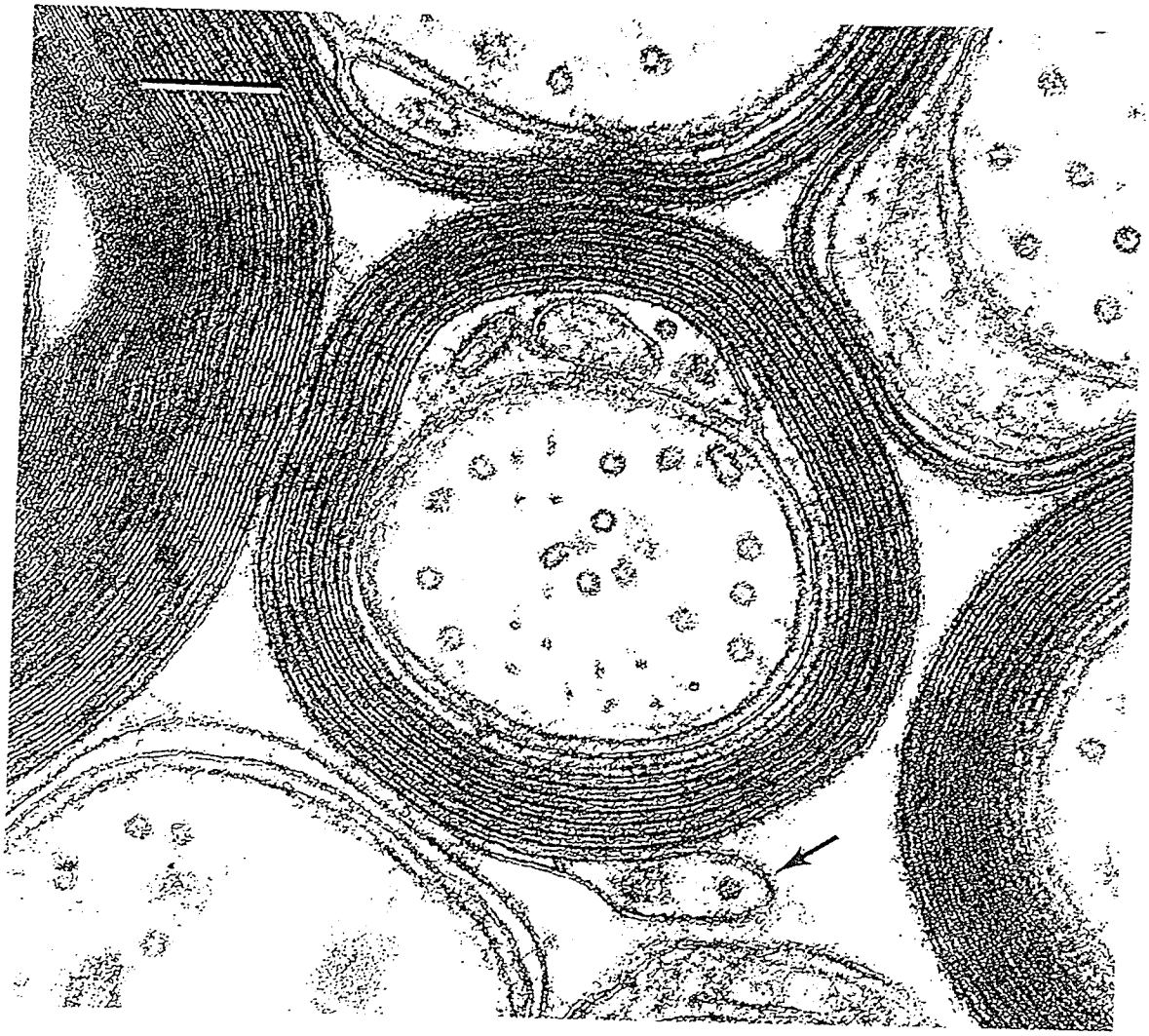

Figure 2. Typical transverse section of a mature CNS myelin sheath. The repetitive multilayers of myelin plasma membrane surrounding an axon can be seen. This picture is adopted from Raine (1984).

structure, containing 40% water, but non-myelin containing parts of white matter have 80% water. This also explains the low water percentage of whole white matter (72%) compared to that of gray matter (82%) (Norton and Cammer, 1984a).

1.3.1. Lipids

Mammalian CNS myelin is composed of 25% to 28% cholesterol, 27 to 30% galactosphingolipid, and 40 to 45% phospholipid (Norton and Cammer, 1984a). These values are very similar to those in the corresponding white matter, even though myelin tends to have less phosphatidylcholine, and sulfatides than white matter. Although there are no myelin specific lipids, galactosylceramide (cerebroside) is regarded as a typical myelin marker. During myelin formation and maturation, the amount of cerebroside in brain is directly proportional to the amount of myelin present (Norton and Poduslo, 1973). In addition to cerebroside, ethanolamine phosphatides in the plasmalogen form and cholesterol are also enriched in myelin compared to other membranes. One third of total phospholipids in mature myelin is plasmalogens which are mainly of the ethanolamine type (Norton and Autilio, 1966; Rapport and Norton, 1962). Phosphatidylcholine is a major myelin lipid, but in mature myelin, there is more phosphatidylethanolamine than phosphatidylcholine. The ratio of these two lipids is an indicator of maturation of myelin. Myelin also has small amounts of phosphatidylinositol and sphingomyelin.

1.3.2. Proteins

The protein composition of CNS myelin is relatively simple compared to

that of other plasma membranes. There are only about six prominent bands when CNS myelin protein of most species is separated on SDS-PAGE, but a synaptic plasma membrane fraction, under the same conditions, would produce 50 or more. The major myelin proteins are the myelin basic proteins (MBP) and the proteolipid proteins (PLP) which together represent 70-80% of the protein content of the membrane, Two less abundant proteins are the myelin-associated glycoprotein (MAG) and 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase).

MBP is a water soluble membrane protein, and comprises 30-40% of the total myelin protein. When this protein is injected into an animal, it causes a CNS autoimmune disease called experimental allergic encephalomyelitis (EAE) which is very similar to the human CNS demyelinating disease called Multiple Sclerosis (Spitler et al., 1972; Maugh, 1977). This protein is mainly located at the major dense line of myelin . The major MBP with molecular weight of about 18.5 kDa was first isolated and reported in the early 1960s. Currently, at least six isoforms have been identified in mice (deFerra et al., 1985) and four in human (Kamholz et al., 1986) with a molecular weight range from 14 to 21.5 kDa. The proteolipid proteins are major myelin components, constituting about 50% of the total protein content of myelin. There are at least two isoforms, PLP and DM-20 with molecular weight of about 25 kDa and 20.5 kDa, respectively. The major proteolipid protein (PLP) is very hydrophobic and forms large aggregates in aqueous solution and interferes with solubilization and purification of some other myelin proteins. Unlike MBP, PLP is mainly located on the extracellular side of the myelin membrane and within the hydrophobic core of the bilayer (Laursen et al., 1984). It is generally believed that MBP and PLP play very important roles in maintaining the structure of the myelin membrane (Lees and Brostoff, 1984; Boggs et al., 1982). MAG is quantitatively a minor protein in CNS myelin. It

constitutes 1% of total myelin protein and has a molecular weight of 100 kDa if the protein is fully glycosylated. The carbohydrate content of MAG is high, accounting for about one third of the moleculear weight. Most part of the MAG is located in the extracellular surface of the unit bilayer (Poduslo et al., 1976). The physiological role of MAG in myelin is not clear, it may be involved in the association of the myelin membrane and the axon of the neuron (Quarles, 1983). For the myelin enzyme purification purpose, the above three proteins and CNPase (see below) are major proteins that need to be removed.

2. CNS Myelin Associated Enzymes

Myelin had been believed for many years to be an inert membrane because of its function as an insulator of the axon. However, presence of several enzymatic activities and a significant turnover rate of major myelin components in the purified myelin have suggested that myelin is a biochemically dynamic membrane, and plays an active role in biosynthesis, transport, and other biochemical reactions. Myelin associated enzymes can be classified into two groups, myelin-specific and myelin non-specific (see Table I).

2.1. Myelin-specific enzymes

Myelin-specific enzymes are those with a several-fold enrichment of their specific activities in purified myelin compared with the starting brain homogenate. The recovered activities of the enzymes should be a sizeable fraction of those in whole brain, and developmental and regional patterns of these enzymes should be similar to the myelin in normal animals. Decreased activities should be observed in the brain tissue of neurological mutant animals, and in plaques of the

brain tissue in patients with Multiple Sclerosis (Suzuki, 1980; Ledeen, 1984). Presently, two enzymes 2'3' -cyclic nucleotide 3' phosphodiesterase (CNPase, EC 3.1.4.37.) and cholesterol ester hydrolase with a pH optimum of 7.2 (EC 3.1.1.13.) are generally recognized as myelin specific enzymes (Suzuki, 1980; Norton, 1981b). (Table I).

2.1.1. 2'3' -cyclic nucleotide 3' phosphodiesterase:

CNPase activity was first demonstrated in bovine spleen (Whitfeld et al., 1955) and pancreas (Davis and Allen, 1956). CNPase was the first enzyme to be found in purified myelin. This enzyme catalyzes the hydrolysis of 2'3' -cyclic nucleotides into the corresponding 2'-nucleotides *in vitro* (Drummond et al., 1962; Sogin, 1976). Although the true substrate and functional significance of this enzyme *in vivo* are still uncertain, it is highly enriched both in purified myelin and in oligodendrocytes (Kurihara and Tsukada, 1967; Takahashi, 1981) and makes up about 5% of the total myelin protein. The level of this enzyme in the CNS seems to be proportional to the amount of myelin formation and it is significantly decreased in brains of three dysmyelinating mutants, the myelin deficient (md) rat, the jimpy mouse, and the quaking mouse (Hof et al., 1984; Kurihara et al., 1970; Neskovic et al., 1970). It is generally believed that CNPase reflects the quantity of myelin present (Sims and Carnegie, 1978; Sprinkle et al., 1978). CNPase and another enzyme C gal T (EC 2.4.1.45., see below) are also regarded as oligodendrocyte markers (for review, see Pfeiffer, 1984).

Localization of CNPase has been studied within CNS tissue, at the cellular level, and in cultured oligodendrocytes in many laboratories by different methods.

Table I. Enzyme Activities of Central Nervous System Myelin.

Enzyme	RSA	References
Myelin specific		
2'3' -Cyclic nucleotide 3' phosphohydrolase (CNPase)	3-6	Kurihara and Tsukada (1967)
Cholesterol ester hydrolase (pH 7.2)	10	Eto and Suzuki (1973)
Myelin-localized or non-specific		
Transport		
Carbonic anhydrase (CA)	0.5-1.0	Cammer et al. (1976)
5'- Nucleotidase	2.5	Kreutzberg et al. (1978)
Lipid metabolism		
UDP-gal: ceramide galactosyltransferase (C gal T)	1	Neskovic et al. (1973)
Phosphoinositide and diphosphoinositide Kinases	0.16	Deshmukh et al. (1978)
Polyphosphoinositide mono- and diphosphoesterases	0.7	Deshmukh et al. (1982)
CDP-choline: 1,2 -diacyl-sn-glycerol-cholinephosphotransferase	NA	Ledeen and Wu (1979)
CDP -ethanolamine: 1,2 -diacyl-sn-glycerol-ethanolaminephosphotransferase	0.9	Wu and Ledeen (1980)
CTP:ethanolaminephosphate cytidylyl-transferase	0.19	Kunishita and Ledeen (1984)
Choline kinase	NA	Kunishita et al. (1983)
Protein kinases and phosphatases		
cAMP- or Mg^{2+}stimulated basic protein kinases	NA	Steck and Appel (1974)
Ca^{2+} -dependent, calmodulin-stimulated protein kinase	NA	Endo and Hidaka (1980)
Nonspecific esterase	NA	Rumsby et al. (1973)
Proteinase	NA	Sato et al. (1982)

Relative specific activity (RSA) was calculated by dividing the specific activity in myelin by the specific activity in the unfractionated homogenate. (NA) Not available. This table is adopted from Norton and Cammer (1984).

In electron micrographs of immunostained ultrathin cryosections, CNPase is associated with oligodendrocytic membranes during the earliest phase of axonal ensheathment (Trapp et al., 1988). Ultrastructural localization by immunocytochemistry shows that CNPase is concentrated within specific regions of the oligodendrocyte and myelin internode. Biochemical studies showed that more than 90% of CNPase activity in unilamellar-myelin vesicles (known to be in inside-out orientation) is trypsin-sensitive, indicating that the CNPase active site is located on the cytoplasmic side (Lin et al., 1987). A cytoplasmic localization of CNPase is further supported by immunohistochemical studies. Reynolds et al. (1989) found that no immunstaining was obtained with anti-CNPase antibodies without prior fixation of oligodendrocytes in cultures. This phenomenon is best explained by intracellular orientation of CNPase. However, CNPase is not exclusively located in either CNS myelin or oligodendrocytes because it is present in peripheral organs, such as spleen and thymus (Weissbarth et al., 1981); and some non-myelin-associated cells, such as erythrocytes (Dreiling, 1981), rat liver (Dreiling et al., 1981), and neuroblastoma cells (B104 cells) (Muller et al., 1981). CNPase activity can also be detected in endoplasmic reticulum of oligodendrocytes (Nishizawa et al., 1981), Schwann cells in the PNS (Yoshino et al., 1983) and serum (Clapshaw et al., 1981).

Research in the field of CNPase has been carried out for over 20 years, however, the biological role of CNPase still remains uncertain. Some evidence recently suggested that CNPase may be related functionally to the biosythesis of myelin proteins (Starich and Dreiling, 1980); it may function as an RNA ligase (Sprinkle et al., 1987); and may play an important role in the early stages of glial cell differentiation (Reynolds et al., 1987), in cell-cell interactions (Weissbarth et

al., 1981), and in membrane stabilization, independent of their specialized structure or functions (Dreling et al., 1981).

2.1.2. pH 7.2 cholesterol ester hydrolase

The myelin-specific cholesterol ester hydrolase has been less well characterized than CNPase. There are three such hydrolases in the brain, which can be distinguished from each other by their pH optima and response to detergents (Eto and Suzuki, 1973; Suzuki, 1980). The relative specific activity of the pH 7.2 cholesterol ester hydrolase of purified rat brain myelin is 10 fold greater compared to that of rat brain homogenate, 70-80 percent of the total activity can be recovered in purified myelin. The activity of this enzyme is much less in young rat brain than that of adults. White matter contains more enzyme activity than the gray matter. The activity of this enzyme is significantly reduced in the brains of jimpy and quaking mice (Eto and Suzuki, 1971). Since there are no cholesterol esters in normal myelin, the function of this enzyme is still unknown.

2.2. Myelin non-specific enzymes

Myelin non-specific enzymes are those enzymes which are not specifically located in myelin. They may have similar specific activity in myelin and in other subcellular fractions. For example, C gal T (EC 2.4.1.45.) was found both in myelin and microsomes (Costantino-Ceccarini and Suzuki, 1975). Some phospholipid-synthesizing enzymes are also believed as myelin non-specific (see Table I).

2.2.1. UDP-galactose: ceramide galactosyltransferase

C gal T (EC 2.4.1.45.) is the enzyme which catalyzes the last step in the biosynthesis of the myelin-specific lipid galactosylceramide. In brain the activity of this enzyme is primarily localized in the microsomal fraction (Morell and Radin, 1969). However, a significant amount of the activity is also associated with the myelin fraction (Neskovic et al., 1973). The developmental pattern of this enzyme in rat brain myelin differed considerably from that in rat brain microsomes (Costantino-Ceccarini et al., 1979). The specific activity of microsomal C gal T reached a maximum when the animals were about 20 days old and then declined. By contrast, the specific activity of the enzyme in the myelin membrane was maximal at 16 days, and it was three to four times higher than that of the microsomal membranes at that age. Sato et al., (1988) reported that the endoplasmic reticulum and Golgi apparatus fractions of isolated oligodendrocytes had the highest activity of C gal T. By using an immunocytochemical technique, Roussel et al. (1987) found that C gal T was mainly located in the cytoplasm and processes of oligodendrocytes and in the myelin sheaths of developing and adult rat brain. No immunostaining was detected in neurons and astrocytes. It is generally believed that C gal T and CNPase are two oligodendrocyte markers.

2.2.2. Phospholipid-synthesizing enzymes

Phospholipid-synthesizing enzymes were preferentially in the microsomal fraction. CDP-ethanolamine: 1,2-diacyl-*sn*-glycerol ethanolamine-phosphotransferase is the first phospholipid-synthesizing enzyme detected in purified myelin (Wu and Ledeen, 1980). The relative specific activity of this enzyme is 0.7-0.9 similar to that of C gal T. Subsequent work showed the

presence of two additional enzymes was demonstrated in purified myelin needed for the synthesis of phosphatidylethanolamine: CTP: phosphoethanolamine cytidylyltransferase (Kunishita and Ledeen, 1984), and ethanolamine kinase (Kunishita et al., 1983) (Figure 3). It was also demonstrated that there were three parallel enzymes that catalyzed the biosynthesis of phosphatidylcholine from DAG in the purified myelin, choline kinase (Kunishita et al., 1983), CTP:phosphocholine cytidyltransferase (Kunishita and Ledeen, 1982), and CDP-choline: 1,2-diacyl-*sn*-glycerol choline phosphotransferase (Ledeen and Wu, 1979) (Figure 4). The enzymes catalyzing the synthesis of the diphosphoinositide and triphosphoinositide: phosphoinositide and diphosphoinositide kinases can also be detected in purified myelin (Deshmukh et al., 1978, 1981).

2.2.3. Phospholipid-catabolizing enzymes

There is much less information available on the phospholipid-catabolizing enzymes compared to that on the phospholipid-synthesizing enzymes present in myelin. The only catabolic activities reported to date are the mono- and diphosphoesterases which catalyze the hydrolysis of phosphate groups from polyphosphoinositides (Deshmukh et al., 1982). Recently a novel form of the enzyme, Glycerophosphorylcholine (GPC) phosphocholine phosphodiesterase (EC 3.1.4.38) was discovered in rat brain myelin in our lab (Kanfer and McCartney, 1989a). This is the first enzyme of the phosphatidylcholine catabolic pathway found in purified myelin and is believed to be a myelin-specific. It will be further discussed in a later chapter.

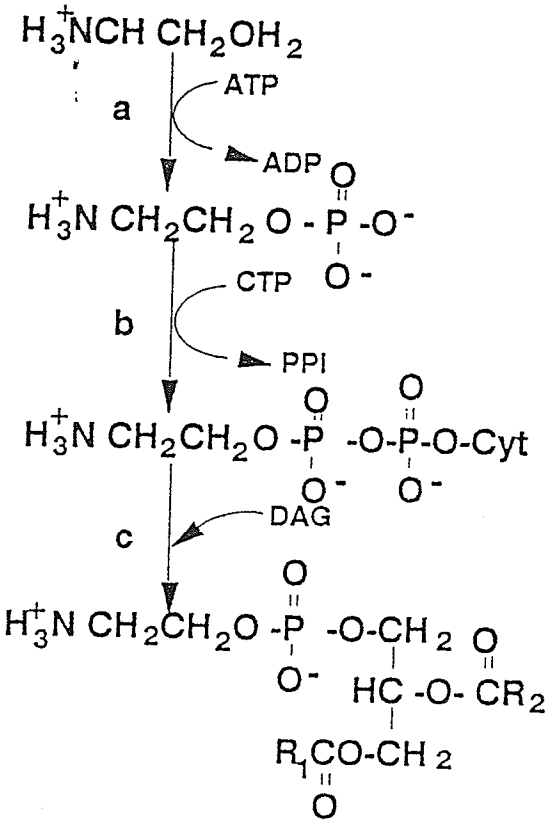

Figure 3. Pathway for conversion of diacylglycerol (DAG) to phosphatidylethanolamine in myelin. a, ethanolamine kinase. b, CTP: phosphoethanolamine cytidylyltransferase. c, CDP-ethanolamine: 1,2-diacylglycerol phosphoethanolamine transferase. This pathway is adopted from Ledeen (1984).

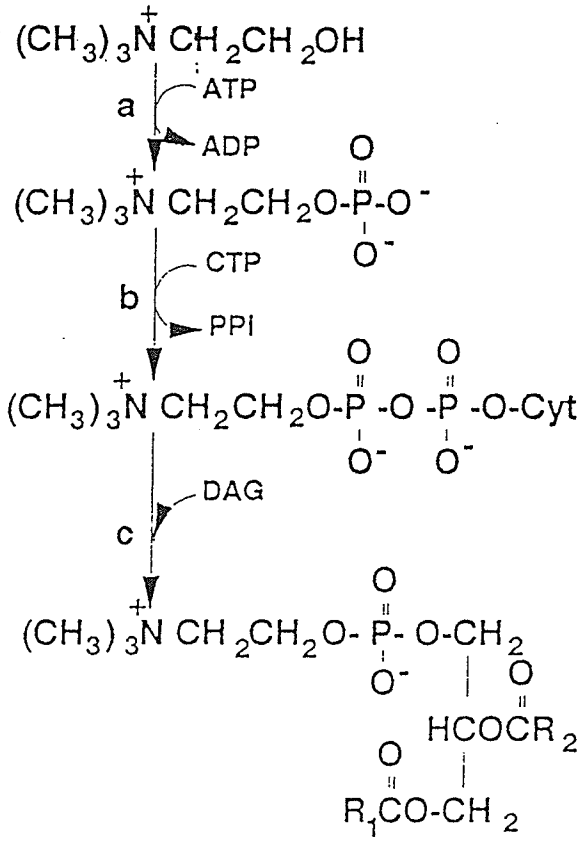

Figure 4. Pathway for conversion of diacylglycerol (DAG) to phosphatidylcholine in myelin. a, choline kinase. b, CTP: phosphocholine cytidyltransferase. c, CDP-choline: 1,2-diacylglycerol phosphocholine transferase. This pathway is adopted from Ledeen (1984).

3. Phospholipid Metabolism in CNS Myelin

The major components of phospholipids in myelin are phosphatidylcholine and phosphatidylethanolamine. These two lipids are mainly synthesized by reaction of CDP-choline or CDP-ethanolamine with 1,2 diacylglycerol (Kennedy, 1961). The enzymes catalyzing these reactions are largely found in the microsomal fraction (Jungalwala, 1974; Miller and Dawson, 1972; Porcellati et al., 1970). The phospholipids needed for CNS myelin formation are synthesized in a similar manner to that in other eucaryotic cells. The majority of myelin phospholipids is synthesized within the oligodendrocytic body and then transferred to the growing plasma membrane by the precursor vesicles or proteins to form myelin (Branmmer, 1978).

The myelin membrane itself may also have the ability to synthesize phospholipid by using substrate from the axon. There is a signifigant turnover rate of phosphatidylcholine in the purified myelin, which is different from that in the microsomal fraction. For example, 10-20 days after injection of [Me-^{14}C]choline into adult rat brain, Jungalwala (1974) found that the specific radioactivity of phosphatidylcholine in myelin remained stable, while that in the microsomes fell, suggesting that the turnover rate of phosphatidlycholine in these two membranes is different. Miller et al. (1977,1978) reported that in the 2-3 week old rat, with respect to the ^{3}H at the C-2 position of the glycerol moiety of phosphatidylcholine and phosphatidylethanolamine, the turnover rates in the fast phase (3 to 4 days) and in the slow phase (13 to 14 days), were similar in microsomes. In myelin, the corresponding values were 6 to 10 days in the fast phase and 25 days in the slow phase. When [^{14}C] choline or [^{14}C] ethanolamine were used as precursor, the half-lives of the base moieties of phosphatidylcholine and

phosphatidylethanolamine were 26 days in microsomes and 39 and 33 days in myelin, respectively (Miller et al., 1977). For the 60 day old rat, the half-lives for the glycerol backbone were 6 days for the microsomes and 11 days for myelin (Miller and Morell, 1978). Finding all six enzymes (see 2.2.2.) which can convert diacylglycerol to phosphatidylcholine and phosphatidylethanolamine in the purified myelin give direct evidence for the biosynthesis of membrane phospholipids within myelin itself. These enzymes may use substrates derived from two possible sources: i) The oligodendrocyte itself, this would require the long distance transport of substrates from glial cell bodies to the adaxonal regions of the membrane. ii) The axon, which is relatively close to the myelin membrane. In both the CNS (Alberghina et al., 1982; Haley and Ledeen, 1979) and PNS (Droz et al., 198; Gould et al., 1982), incorporation of axonally derived isotope labelled substrate into a variety of myelin lipids was observed. These suggest that the axon may be a source of substrate for the myelin-localized phospholipid synthesizing enzymes.

4. GPC Phosphocholine Phosphodiesterase

4.1. General

Glycerophosphocholine (GPC) is a major water-soluble phosphodiester in several tissues. It was first demonstrated as a breakdown product of phosphatidylcholine in liver by Dawson (1955). It is also present in high level in the male reproductive tract (Dawson et al., 1959). By using ^{31}P nuclear magnetic resonance (^{31}P NMR) spectroscopy, Burt et al. (1976) reported that skeletal and cardiac muscle from several species contained high levels of GPC. Significant increases in the level of GPC have been reported in human forearm muscle in

patients with Duchenne's muscular dystrophy (Newman et al., 1982) indicating GPC may play an important metabolic role in skeletal and cardiac muscle. GPC is present in relatively high concentrations in brain tissue (Jope and Jenden, 1979). It is estimated that the concentration of GPC is 0.4 mM to 0.6 mM in whole brain (Ansell and Spanner, 1982).

Mammalian tissues contain various types of phosphodiesterases which are known to cleave the phosphodiester bond (Kelly et al., 1975; Landt and Buther, 1978). The different types can be distinguished by their substrate specificity, pH optimum, metal requirement, and activitor and inhibitor selectivity. Glycerylphosphorylcholine phosphodiesterase was first reported by Dawson (1955) from rat liver. Free choline was rapidly released when rat liver homogenates were incubated with GPC at 37°C, but when incubated with phosphorylcholine, lysophosphatidylcholine or phosphatidylcholine, liberation of free choline could not be observed. This enzyme has a pH optimum of approximately 7.5, is stimulated by the addition of Mg^{2+}, and inhibited by Zn^{2+}. Activity of this enzyme can also be found in other rat tissues (Dawson, 1955). There was significant activity of GPC phosphodiesterase which might require Zn^{2+} as active cation in rat kidney microsomal fraction (Baldwin et al., 1969). Rat brain has two distinct membrane bound phosphodiesterase activities which can catalyze the hydrolysis of GPC. One is called GPC choline phosphodiesterase (EC 3.1.4.2) which can release free choline from GPC and is mainly located in the brain microsomes, this enzyme has a pH optimum of about 8.6 and is Ca^{2+}-dependent (Spanner and Ansell, 1982). The other phosphodiesterase can liberate phosphocholine from GPC and is referred to as GPC phosphocholine phosphodiesterase (EC 3.1.4.38), and was found in homogenates of rat brain by Abra and Quinn (1975), with no activity detected in rat liver homogenates and

P815Y mouse mastocytoma cells. This enzyme was purified 120-fold by DEAE-cellulose, ion-exchange chromatography and sucrose density gradient centrifugation. Purified enzyme was shown to have a pH optimum of 10.5, a high Km of 2 mM and relatively unaffected by EDTA, which causes only a 20% reduction of activity. Ca^{2+} was the only metal ion capable of enzyme activity restoration, the optimum concentration is about 10 mM (Abra and Quinn, 1976). These properties are very similar to that of the enzyme present in the P3 fraction (microsome) of rat brain (Kanfer and McCartney, 1989a).

4.2. GPC phosphocholine phosphodiesterase in myelin

Recently a novel form of the enzyme, GPC phosphocholine phosphodiesterase (EC 3.1.4.38) was discovered in rat brain myelin in our laboratory (Kanfer and McCartney, 1989a) This enzyme can catalyze the hydrolysis of GPC to produce phosphocholine and glycerol (Figure 5) and is different from the enzyme described above. The activity of this enzyme in purified myelin is 3.2-fold higher than that of rat brain homogenate and was shown to have a pH optimum of 9.5. It had a relatively low Km of 0.2 mM, and a Vmax of 150 nmoles/mg protein/hour. The enzyme activity was completely lost if the sample was dialyzed against a 1,000-fold excess of a solution composed of 0.32 M sucrose and 10 mM EDTA overnight in the cold, and it was restored if the EDTA was removed and Zn^{2+} was added, with an optimum concentration of Zn^{2+} of about 0.25 mM. Maximum enzyme activity was observed of 50°C (Kanfer and McCartney, 1989a). They also studied the regional distribution pattern of this enzyme by using rat brain at postnatal day 5 to postnatal day 70 (P5 to P70). At P5, there were detectable levels of GPC phosphocholine phosphodiesterase activity in the mesencephalon, diencephalon, cerebral hemispheres, cerebellum,

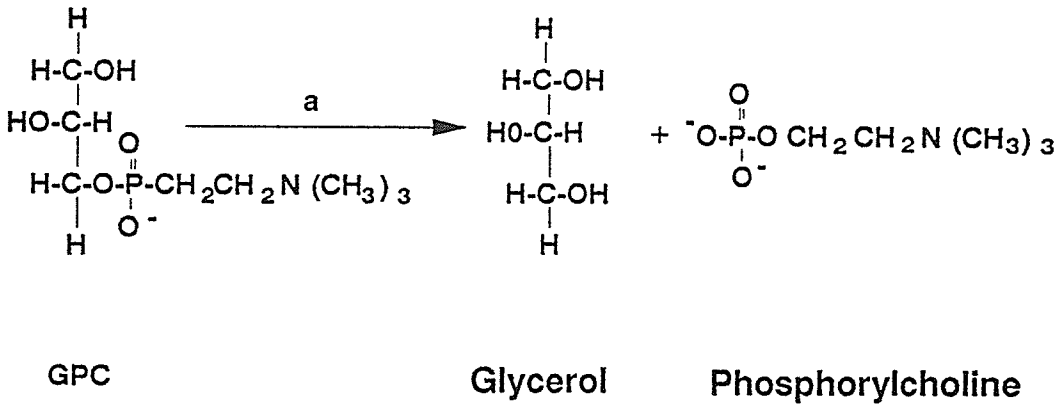

FIGURE 5. Hydrolysis of GPC by (a) GPC phosphocholine phosphodiesterase in myelin

but not in the olfactory bulb. The highest specific activity was found in the homogenates of mesencephalon. The specific activities in all regions increased rapidly until P18 to P25 and then remained constant in the olfactory bulb, diencephalon and mesencephalon, while increased in the cerebral hemispheres and cerebellum until P70. Only the mesencephalon specific activity decreased, 16% by P70. The highest specific activity was associated with mesencephalon followed by diencephalon, cerebellum and cerebral hemispheres with the lowest in the olfactory bulb. The regional developmental pattern of this enzyme is very similar to that reported for myelin specific enzyme CNPase (Kanfer and McCartney, 1989b; Sprinkle et al., 1978). Janzen et al. (1990) reported that the acitvity of GPC phosphocholine phosphodiesterase was significantly decreased in the plaques of brain tissue from patients with multiple sclerosis, but the normal-appearing white matter (NAWM) and control brain tissue had similar activity of this enzyme. The GPC phosphocholine phosphodiesterase in the cerebral spinal fluid (CSF) was not detected by any of the current enzyme assay methods.

Several hydrolytic enzymes can be quantitated by measuring the p-nitrophenol release from a variety of artificial derivatives. For example, Hawley et al. (1984) measured the bovine intestinal phosphodiesterase I activity by using bis p-nitrophenylphosphate. Hynie and Zbarsky (1970) used p-nitrophenyl-thymidine 5-phosphate to measure rat intestinal mucosa phosphodiesterase or nucleotidase activities. Activities of phospholipase C from *Clostridium perfringes* (Kurioka, 1968), or phospholipase D from peanut when coupled with acid phosphatase (Gupta and Wold, 1980) can also be measured by using p-nitrophenylphosphorylcholine. Since these artificial derivatives are chromogenic substrates for these enzymes, the activities of these enzymes are

readily measured with a spectrophotometer. Kanfer and McCartney (1989a) reported that p-nitrophenylphosphocholine can be hydrolyzed by purified myelin and is a competitive inhibitor of GPC phosphocholine phosphodiesterase with a Ki of 0.075 mM. GPC is a non-competitive inhibitor of p-nitrophenylphosphocholine hydrolysis with a Ki of 0.5 mM. At that stage, they could not distinguish between the possibility that of a single enzyme utilizes both of these substrates or two separate enzymes exist. Sok and Kim (1990) also reported that there is a p-nitrophenyl phosphorylcholine phosphodiesterase activity in mouse brain. They solubilzed this enzyme by 1% sodium deoxycholate treatment and partially purified it with HPLC gel chromatography. Their data indicates that this enzyme is tightly bound to membranes and has a Km of 1 mM, a Vmax of 150 nmoles/mg/hour and a pH optimum of about 11. This enzyme is EDTA-insensitive, and divalent ion-independent. Various compounds containing the phosphorylcholine group such as lecithin, lysolecithin, phosphorylcholine, and GPC have no significant effect on the p-NP phosphocholine phosphodiesterase activity indicating that there may be a narrow binding specificity for this enzyme.

5. Cultured Oligodendrocytes and Dysmyelinating Rodent Mutants

5.1. Cultured oligodendrocytes

Tissue culture is a useful system for investigation of the complex biology of oligodendrocytes and their relationship with myelination. It can be used to study the effects of the exogenous factors that may affect the glial cell protein and lipid metabolism or enzyme expression during primary myelination as well as during post-trauma regeneration. Several procedures have been reported for the isolation and culture of oligodendrocytes from young adult animals (Lisak et al.,

1981; Norton et al., 1983; Szuchet et al., 1980). All these procedures depend on enzymatic and mechanical dissociation of the tissue, followed by a sucrose, and a Ficoll or Percoll gradient to separate different types of cells. A variety of serum-containing media are available for long-term cultures of oligodendrocytes, even though there are still some other cell types in these culture systems (for review, see Pfeiffer, 1984). McCarthy and de Vellis (1980) and Espinosa de los Monteros et al. (1986) reported a method for isolating the oligodendrocytes from primary cultures of newborn rat brain, which is different from the procedures described above. They used a serum-free medium which is also called chemically defined media for long-term cultures of oligodendrocytes. This medium eliminates the influence of serum factors, and consists of equal parts of Dulbecco modified Eagle's and Ham's F-12 media supplemented with insulin, sodium selenite, putrescine, and D^+ galactose (Espinosa de los Monteros et al., 1986). Contaminating astrocytes can not survive in this medium. Biochemical data indicate that these purified cultured oligodendrocytes express CNPase and C gal T activities. Electron microscopical examination showed that these cells have morphology similar to cells grown in serum-containing medium. The homogeneous cultured oligodendrocytes prepared by this technique make possible the further investigation of exogenous and endogenous factors involved in oligodendrocyte metabolism. These pure oligodendrocytes can also be used for either myelin or oligodendrocyte associated enzyme studies. The cultured cells prepared by this technique were provided by Dr. Espinosa and were used in my research project.

5.2. Cultured C-6 cells

The C-6 cell is a chemically (N-nitrosomethylurea) induced tumor cell from

a rat glioma (Parker et al., 1980). These cells differentiate to resemble the astrocyte after treatment with amethopterin (Silbert and Goldstein, 1972). C-6 glial cells also express CNPase (Zanetta et al., 1972), a marker enzyme for myelin and oligodendrocytes. These data suggested that C-6 cells represent an undifferentiated glial cell with the potential to differentiate into astrocytes.

5.3. Dysmyelinating rodent mutants

Dysmyelinating rodent mutants are very good research tools to study myelin structural proteins, such as proteolipid protein and myelin basic protein, myelin assembly, myelin lipid metabolism, and myelination in relation to other developmental processes. These mutants can also be used to determine whether a particular enzyme is strictly associated with myelin or not, and further investigation of these mutations provides more information concerning the function of glial cells and myelin in the CNS (Baumann, 1980). These mutants include the myelin deficient rat, the jimpy mouse, the quaking mouse, and the shiverer mouse (for review, see Hogan and Greenfield, 1984).

5.3.1. Myelin-deficient rat

The myelin-deficient rat (md) is a sex-linked recessive mutation of Wistar rats in which normal myelin is not detected in the CNS, while in the peripheral nervous system (PNS) myelin seems normal (Csiza and deLahunta, 1979). One-half of the male offspring develop tremors and ataxia at 10-12 days of age and seizures at 16-21 days. Usually, the animals die 24-28 days postnatally. Morphological studies of CNS tissues from md rats showed lack of myelin or poorly organized, non-compacted, myelin-like loops of membranes in the areas of

frontal cortex, corpus callosum, optic nerves, cerebellum and spinal cord. There are significant amounts of abnormal oligodendrocytes in md. The cytoplasm of these abnormal cells contains dilated rough-surfaced endoplasmic reticulum and the nuclear envelope is widened. Immature oligodendrocytes are present and fail to complete differentiation (Kumar et al., 1988) and also show poor maturation (Espinosa et al, 1990). Therefore, the population of mature oligodendrocytes is decreased during development, although their progenitors are present. (Barron et al., 1980; Dentinger et al., 1982, 1985). Biochemical studies of these mutants show that during the period from two weeks to four weeks of age, there is severe reduction in the amounts of the galactosphingolipids, and the specific activities of C gal T and CNPase (Hof and Csiza, 1982; Hof et al., 1984). Yanagisawa et al., (1986) reported that major myelin proteins such as myelin basic protein (MBP), myelin-associated glycoprotein (MAG), CNPase, and proteolipid protein (PLP) were significantly decreased in the brain and spinal cord of md rats. Their data showed that the levels of MBP, MAG, and CNPase in 25 day-old md brain were 1.1, 1.8, and 11% of those in controls, respectively. PLP content was reduced more than that of the other proteins in the md rats, and was only 0.2% of that in controls. This finding indicated that the expression of PLP may be preferentially affected in the md mutation.

5.3.2. Jimpy mouse

The jimpy mouse was first reported by Phillips (1954). The animals exhibit tremors starting at 11-12 days after they are born, which worsens until the animals die at 30-35 days. Like the md rat, the jimpy disorder is also inherited as sex-linked recessive and seems to affect only the CNS (Billings-Gagliardi and Adcock, 1981). Histological studies of this animal demonstrated that there is

almost complete lack of myelin in the CNS and a significant reduction of the number of oligodendrocytes (Sidman et al., 1964; Knapp et al., 1986). Many studies of this mutant have demonstrated quantitative deficiencies in myelin-associated lipids. The greatest deficits are of cerebrosides and sulfatides (Sidman et al, 1964; Nussbaum et al., 1969). The activities of myelin specific enzyme, CNPase (Kurihara et al., 1970) and cholesterol ester hydrolase (Eto and Suzuki, 1973) in brain of jimpy mouse are significatly lower than those in littermate controls. The CNPase levels in the mutant range from 7 to 25% of normal, depending on the region of the CNS examined (Mikoshiba et al., 1985). Neskovic et al., (1970), Morell and Costantino-Ceccarini (1972) also demonstrated the reduction of the oligodendrocytic marker enzyme C gal T activity in the jimpy mutant. There is a marked reduction in proteolipid protein and a lesser but significant decrease in the myelin basic proteins (Nussbaum and Mandel, 1973; Druse and Hogan, 1972). These findings were further confirmed by immunohistochemical techniques (Mikoshiba et al., 1985), indirect immunofluorescence microscopy (Dupouey et al., 1980), and radioimmunoassay (RIA) (Zimmerman and Cohen, 1979).

5.3.3. Quaking mouse

The quaking mouse is a mutant of the C57BL/6J strain with a neurologic disease characterized pathologically by a general deficiency of myelin in the central nervous system (only 5-10% of normal), (Sidman et al., 1964, 1965; Greenfield et al., 1971) and inherited as an autosomal recessive trait on chromosome 17. Clinically, the animals have tremors and seizures that develop early in life (12 days postpartum), but the life span is close to normal. The affected females will breed, but affected males are sterile. Similar to the jimpy

mouse, the quaking mouse has deficits in the myelin-associated lipids, such as cerebrosides and sulfatides (Baumann et al., 1968; Kanfer and Stein, 1972). In the adult mutant, the activities of CNPase and C gal T are about 50% (Kurihara et al., 1970) and 35% (Neskovic et al., 1970) of the control values, respectively.

6. Clinical Aspects of Myelin Diseases

6.1. General

Myelin diseases are a heterogeneous group. The clincial findings of these diseases are quite different and include a number of neurological symptoms. Loss of myelin is a common problem resulting from several different conditions. Infarct necrosis, trauma, neoplasia, hemorrhage, abscess, edema, and anoxia are all factors which may result in a demyelinating disease. In some diseases, myelin is the primary target; in others, the loss of myelin is a secondary effect of the disease. Myelin diseases can be classified into five classes: i. Acquired allergic (inflammatory) and infectious diseases of myelin (demyelinating diseases). ii. Hereditary metabolic diseases of myelin. iii. Acquired toxic-metabolic diseases of myelin. iv. Nutritional diseases of myelin, and v. Traumatic diseases of myelin (Raine et al., 1984b). Among the above myelin diseases, Multiple Sclerosis is the most important and prevalent myelin disease of the CNS in humans.

6.2. Multiple Sclerosis

Multiple sclerosis (MS) is a common neurologic disorder of unknown

causes. Epidemiologic studies indicate that this disease is much more prevalent in persons of western European lineage who live in temperate zones. It has its greatest incidence in young adults (under 55 years of age at onset), especially in young women (Aminoff, 1990). It has been estimated that in 1981, there were about 250,000 MS patients in the United States (Waksman, 1981), and more than 50,000 Canadians suffered from this disease.

The common initial clinical presentation is weakness, some other symptoms of this disease are tingling, or unsteadiness in a limb; spastic paraparesis; diplopia; blurred vision and sphincter vesicae disturbance such as urinary urgency or hesitancy. Symptoms may disappear after a few days or weeks and recur after months or years. The total clinical picture indicates involvement of different parts of the CNS at different times. Gross pathology studies have shown that the brain from patients who died with chronic MS contains multiple, disseminated plaques, grossly visible throughout the white matter, ranging in size from about 1.0 mm to several centimeters (Raine, 1984b). Histological studies show that these MS plaques are areas of myelin and oligodendrocyte losses with subsequent infiltration of cells, such as macrophages, neutrophils and lymphocytes (Hauser et al., 1986; Antel and Arnason, 1987). Computer tomography (CT) scanning is sometimes helpful in demonstrating the presence of a multiplicity of lesions. Magnetic resonance imaging (MRI) is the choice for imaging diagnosis (Aminoff, 1990).

The primary cause of MS remains uncertain. There are several hypotheses in terms of etiology of MS. The strong association between MS and specific HLA antigens (HLA-DR_2) provides evidence to support a genetic predisposition theory (Antel and Arnason, 1987). Others believe that MS has an

immunologic basis, which is based on the observation that elevation of IgG and presence of discrete bands of IgG called oligoclonal bands in the cerebrospinal fluid (CSF) of MS patients, even though these findings are not MS specific (Waksman, 1983; Aminoff, 1990). Antibodies against MBP (Carson et al., 1978), and oligodendrocytes (Traugott and Raine, 1981) can also be demonstrated in the CSF of MS patients. Recently, several viruses, such as visna virus (Stowring et al., 1985), cornavirus (Burks et al., 1980), and immunodeficiency virus (HIV) (Johnson and McArthur, 1986) etc. have been found to be associated with MS, These viruses may induce the autoimmune response or affect the immune regulation system of the body (Waksman, 1981,1983). Biochemically (for review, see Norton and Cammer, 1984b), myelin isolated from MS tissue has a normal protein (Wolfgram and Tourtellotte, 1972) and lipid (Suzuki, et al., 1973) composition, but only one half the normal activity of CNPase (Gopfert et al., 1980). Myelin proteins and CNPase are both reduced in the MS plaque (Itoyama et al., 1980; Newcombe et al., 1982) and the normal-appearing white matter (NAWM) by which the MS plaque is surrounded (Newcombe et al, 1982; Riekkinen et al., 1972). Recently, Janzen et al. (1990) found that glycerophosphorylcholine (GPC) phosphocholine phosphodiesterase and p-nitrophenyl (p-NP) phosphocholine phosphodiesterase activities are significantly reduced in Multiple Sclerosis plaques, suggesting that these enzymes may also be related to the etiology of MS.

7. Solubilization and Purification of Myelin Associated Proteins

Several myelin-associated proteins such as proteolipid protein, myelin basic protein, and myelin specific enzymes: CNPase and pH 7.2 cholesterol ester hydrolase have been solubilized and purified from bovine brain myelin. Since the

major proteins of myelin are membrane-bound and extremely hydrophobic, classically, these proteins have been first extracted in organic solvents. Although the use of organic solvents for the extraction of the major myelin protein from the plasma membrane is useful, there are still some disadvantages. They may change the structures of proteins and the interactions between polypeptides. In investigating of some of the characteristics of brain myelin by solubilization in aqueous detergent solutions, Eng et al. (1968) reported a simplified procedure for extracting the major myelin protein (MBP, PLP, and Wolfgram protein) by using Triton X-100 and neutral salt (ammonium acetate). Recently Aveldano et al. (1991) found that octyl glucoside and Lubrol PX proved relatively better in myelin solubilization than other detergents. The detergent concentrations required for myelin solubilization were reduced substantially if the temperature and the salt (sodium sulfate and sodium phosphate) concentration of the media were increased (Aveldano et al., 1990).

7.1. Proteolipid protein

The standard procedures for purification of PLP include extensive dialysis in organic solvents, chromatography on silicic acid or isopropyl Sephadex (LH series), and gel electrophoresis (for review, see Lees and Sakura, 1978). The appropriate method depends on the starting material (myelin, white matter or whole brain) and on the subsequent studies to be carried out. In all cases, however, either acidic solvents or detergent are used to obtain the complete separation of lipid from protein. The standard procedures were further modified by Helynck et al. (1983). They obtained homogeneous PLP (determined by SDS-PAGE) by using chloroform/methanol extraction and fractionation by chromatography on modified (lipophilic) Sephadex, followed by ion-exchange

chromatography on CM-Trisacryl. Eng et al. (1968) and Igarashi and Suzuki (1976) also reported that PLP and MBP (see below), the major components of myelin can be extracted with ammonium acetate and Triton X-100 and this may suggest that these two proteins are bound in the membrane primarily by ionic bonds. Smith et al. (1983) extracted PLP by using deoxycholate or Triton X-100, and further purified it by blue dye-ligand column or gel permeation chromatography.

7.2. Myelin basic protein

Like PLP, MBPs can be obtained from the homogenized CNS tissue by acid extraction after removing lipid by chloroform-methanol (2:1, vol./vol.), even though the MBPs are water-soluble at low and neutal pH. Alternatively, the basic proteins are easily extracted directly from partially delipidated myelin by dilute acid or Triton X-100 and ammonium acetate (Eng et al., 1968). Further purification procedures include gel filtration (Sephadex G75) or ion-exchange chromatography (CM-cellulose) or both (Eylar et al., 1969; Martenson et al., 1971). Currently, the most frequently used methods for purification of MBP are derived from Deibler et al. (1972) and Eylar et al. (1974) (for review, see Dunkley and Carnegie, 1978). Bellini et al. (1986) also reported a rapid method for purification of MBP by using 2-butanol (to remove lipids) and acetate buffer at pH 4.5 (to solubilize the MBP). The entire procedure requires less than 4 hours and gives homogeneous MBP determined by SDS-PAGE.

7.3. CNPase

CNPase has been isolated from the human, rat, and bovine nervous

systems.The first step for purification is extraction of this enzyme from myelin membrane, and several methods have been used for this enzyme extraction. Guha and Moore (1975) and Drummond et al. (1978) reported that the use of guanidinium chloride was very effective for the extraction or solubilization of CNPase without using detergent. Suda and Tsukada (1980) found that CNPase from bovine cerebral white matter could be solubilized using a mixture of ammonium acetate and Triton X-100 at alkaline pH without loss of the enzyme activity which may occur by using guanidinium chloride. A similar result was also obtained by Wells and Sprinkle method (1981) using ammonium acetate in the presence of 2-mercaptoethanol and EDTA. The enzyme was further purified by chromatography on Phenyl-Sepharose CL-4B, CM-Sepharose CL-6B, and 8-(6-aminohexyl) amino-2' AMP-Sepharose 4B (Suda and Tsukada, 1980; Wells and Sprinkle, 1981). After these procedures, a homogeneous enzyme protein was obtained (determined by SDS PAGE). The enzyme purified from bovine brain had a molecular weight of 51kDa, and a Km of 3.13 mM (Suda and Tsukada, 1980). The enzyme purified from rat brain showed two protein bands of approximately 48kDa and 50kDa M.W. on SDS-PAGE. The relative intensities of the bands for CNPase and the molecular weights are similar to those of the Wolfgram proteins W1 and W2. The amino acid analysis of the purified rat enzyme is similar to that of enzyme purified from bovine brain and also similar to the rat Wolfgram proteins W1 and W2 (Wells and Sprinkle, 1984). The CNPase purified from wheat germ (Tyc et al, 1987) immunoprecipated with a polyclonal anti-CNPase antiserum prepared against mammalian brain CNPases. The migration pattern of the enzyme prepared from wheat germ is similar to that of CNP1 and CNP2 of various mammalian brain CNPases on SDS-PAGE (Sprinkle et al., 1988).

7.4. pH 7.2 cholesterol ester hydrolase

pH 7.2 cholesterol ester hydrolase, one of three distinct cholesterol ester hydrolases in rat brain is almost exclusively localized in the myelin sheath (Eto and Suzuki, 1973). This enzyme was solubilized and partially purified by Igarashi and Suzuki (1977). The solubilization procedure required both high ionic strength and an amphoteric detergent, Miranol H2M. The enzyme was reversibly precipitated when the concentration of either Miranol H2M or KCl was below certain critical levels. The solubilized enzyme was further purified by Sephadex G-200 gel filtration chromatography (Igarashi and Suzuki, 1977). It is interesting to note that after solubilization with detergent, KCl and partial purification (Igarashi and Suzuki, 1977), the pH optimum changes from 7.2 to 6.7-6.8, and the enzyme needs the addition of phosphatidylserine for optimal activity.

8. My Research Objectives

The first part of my project is to estimate the level of GPC phosphocholine phosphodiesterase, and p-NP phosphocholine phosphodiesterase in pure cultures of oligodendrocytes and astrocytes; mixed primary glial cell cultures; C-6 cells; and CNS tissue of the dysmyelinating md rat, the jimpy mouse, and the quaking mouse. The aim of these experiments is to obtain additional documentation for the localization of GPC phosphocholine phosphodiesterase activities in the myelin sheath. As discussed above, the CNPase and C gal T are good markers for myelin and/or oligodendrocytes, thus making the cultured oligodendrocytes and dysmyelinating rodent mutants good research tools for studying of myelin protein, myelin metabolism, and myelin associated enzymes. So in my experiments, the activities of CNPase, and C gal T were also measured

measured as a basis for determining the enrichment in myelin or oligodendrocytes. If the results are consistent with previous findings that GPC phosphocholine phosphodiesterase is myelin specific, the subsequent part of my project is to try to solubilize and purify this enzyme from bovine brain myelin, and further characterize it. Homogeneous enzyme obtained from myelin will provide direct evidence that this enzyme is myelin and oligodendrocyte specific. Purification and characterization procedures can also provide some helpful information to distinguish whether there is one enzyme using two substrates (GPC and pNP phosphocholine) or there are two distinct phosphodiesterases in myelin sheath. It is also basic work for further study of this enzyme, such as making antibody for immunohistochemical studies, partially sequencing enzyme protein for preparation of an oligonucleotide probe and gene cloning.

Phosphatidylcholine is one of the major components of myelin. GPC phosphocholine phosphodiesterase is involved in this phospholipid metabolism in myelin. Therefore, the more study is done on this enzyme, the more information will be obtained concerning the homeostasis of myelin.

II. EXPERIMENTAL PROCEDURES

1. Determination of Enzyme Activities

1.1. Materials

[^{3}H] Choline-labelled phosphatidylcholine (37.00 Ci/mmole) was purchased from NEN (Boston, MA). Non-radiolabelled GPC, p-Nitrophenylphosphocholine, nicotinamide adenine dinucleotide 2',3' -cyclic monophosphate (2'3' cyclic NADP), and glycine were obtained from Sigma Chemical Co. (St. Louis, MO). ^{14}C-UDP galactose (290.0 Ci/mmole) was purchased from NEN (Boston, MA), and diluted with nonradiactive UDP galactose to a final specific activity of about 2 x 10^5 cpm/nmole. Hydroxy-fatty-acid-containing ceramide was prepared according to a published method (Carter et al., 1961). All other reagents were of analytical grade.

1.2. Substrate (GPC) preparation

The preparation of [^{3}H] choline-labelled GPC is based on the published method of Spanner and Ansell (1987). A sample containing approximately 2 x 10^8 cpm in 120 µl [^{3}H] choline-labelled phosphatidylcholine and 2.5 ml of a 10 mg/ml solution of phosphatidylcholine (egg) was dried under nitrogen and dissolved in 1.0 ml of 0.125 M LiOH in methanol (2.99 g/l) solution. The solution was kept at room temperature for 45-60 minutes and pH kept alkaline. The total radioactivity present was determined on an aliquot. 2 ml chloroform and 1 ml water were added and the mixture was vortexed and centrifuged at 10,000 g for 10 minutes. The upper and lower phases were separated, their volumes were noted and an aliquot from each was counted. If approximately 75% of the total

starting count were not found in the upper phase, a 1 ml aliquot of TUP-H_2O was added to the lower phase, mixed, centrifuged at 10,000 g for 10 minutes and this upper phase pooled with the previous. The pooled upper phase was passed through a DOWEX H^+column (50 ml bed volume capacity - 15 cm x 5 mm) that had been previously washed with H_2O, and 50% methanol. The DOWEX 50 column was washed with approximately 40 ml of a 50% methanol solution and 1.0 ml fractions were collected and their pH checked to ensure they were neutral. If these fractions were alkaline, a second DOWEX 50 H^+column chromatography was carried out. A 1.0 μl aliquot from each 1.0 ml fraction was counted to locate the fractions with the highest counts containing ^{3}H-GPC. These fractions were pooled, dried under nitrogen and dissolved in 2 ml water which was further diluted with 10 mM non-radiolabelled GPC to obtain final specific activity of about 1000 cpm/nmole.

1.3. Enzyme assay

1.3.1. GPC phosphocholine phosphodiesterase

A sample of tissue homogenates containing 100 μg of protein was incubated with 5 μl (for the characterization of purified enzyme, 8.75 μl was used) of 10mM of [^{3}H] GPC having a specific activity of about 1000 cpm/nmole, 12.5 μl of 0.5 M glycine buffer pH 10, 5 μl of 10 mM $ZnCl_2$, in a total volume of 125 μl in an Eppendorf centrifuge tube. After incubation for 1 hour at 37^{o}C, the reaction was stopped by heating in a boiling water bath for 10 minutes. Each incubation tube was cooled in an ice water bath for 10 minutes and then centrifuged at 10,000 g for 10 minutes. Half of the supernatant (about 60 μl) was extracted directly according to the procedure of Fonnum (Fonnum, 1975) by the addition 2

ml of 10 mM $K_3(PO_4)_2$ buffer pH 7.5, 2 ml of TPB-acetonitrile (5 mg TPB/ml), 10 ml of PPO-Toluene (0.5 g PPO and 0.2 g POPOP/1 liter toluene). Each scintillation vial was then inverted gently 52 times and counted using scintillation counter (Model LS 6000 TA, Beckman, U.S.A) to estimate [^{3}H] choline release(*a*). The remainder of the supernatant was transferred to an Eppendorf centrifuge tube containing 0.16 units of bovine intestinal alkaline phosphatase (Sigma, No. P-8149). Each incubation tube was vortexed, then incubated at 37°C for 60 minutes. The reaction was stopped by cooling each tube in an ice water bath for 10 minutes. This mixture was then transfered to a scintillation vial and the radioactivity was determined following extraction as per the Fonnum method described above to determine the total [^{3}H] choline release(*b*). Phosphocholine released by GPC phosphocholine phosphodiesterase is calculated by subtraction of [^{3}H] choline(*a*) from the total [^{3}H] choline(*b*). Boiled enzyme was used as control.

1.3.2. p-NP phosphocholine phosphodiesterase

A sample of tissue homogenates containing 100 μg protein was incubated with 17.5 μl of 5 mM p-NP phosphocholine, 5 μl of 10 mM $ZnCl_2$, 12.5 μl of 0.5 M glycine buffer pH 11.0 in a total volume 125 μl in an Eppendorf centrifuge tube. After incubation at 37°C for a 1 hour, the reaction was stopped with 125 μl of trichloroacetic acid (TCA) and centrifuged at 10,000 g for 10 minutes. The supernatant was removed and 100 μl of NaOH and 1 ml of 0.25 M glycine buffer pH 10.2 was added. The absorbance of the p-NP released was measured at 420 nm using a Bausch & Lomb Spectronic 2000 spectrophotometer.

1.3.3. 2'3' -cyclic nucleotide 3' phosphodiesterase

The following method is based on the procedure described by Sogin (1976). Aliquots of the homogenates were diluted to a protein concentration of 0.34 mg/ml by using 0.2 M Tris-HCl buffer pH 7.5 containing 1% Triton X-100. The incubation mixture contained about 3.4 μg protein, 1ml of 0.2 M MES buffer pH 6.0, 10 μl of 0.5 M glucose-6-PO4-monosodium salt, 20 μl of 0.1 mg/ml glucose-6-PO_4 dehydrogenase and 10 μl of 0.1 M 2'3' -cyclic NADP. The change in absorbance at 340 nm due to NADPH formed in each sample was measured at frequent intervals (0.5 min., 1 min., 3 min., 5 min., 10 min., and 15 min.) using a Bausch & Lomb Spectronic 2000 spectrophotometer.

1.3.4. UDP galactose: ceramide galactosyltransferase

UDP galactose: ceramide galatosyltransferase activity was measured according to the procedure of Costantino-Ceccarini and Cestelli (1981).

1.3.4.1. Liposome mixture preparation

12.5 mg PE (beef brain), 12.5 mg PC (beef brain) and 1.9 mg hydroxy-fatty acid containing ceramide were combined in a 1.5 ml Eppendorf centrifuge tube and brought to dryness under N2 and 1.0 ml of 10 mM Tris-HCl buffer pH 9.0 containing 1 mM EDTA was added. This liposome mixture remained at room temperature for 30 minutes and was then sonicated in ice at level 3.5 for 30 minutes (Sonicator Model W-385).

1.3.4.2. Procedures for the C gal T assay

A sample of homogenates containing about 100 μg protein in a 13 x 100 cm borosilicate disposable culture tube was incubated with 10 μl of Tris-HCl buffer mixture composed of 500 μl of 1 M Tris-HCl buffer pH 8.8, 50 μl of 0.2 M DTT, and 50 μl of 0.2 M EDTA; 5 μl of 0.3 M $MgCl_2$; 40 μl of liposome mixture; 5 μl of 5 mM ^{14}C-UDP Galactose (Specific activity about 15,000 cpm/nmole); and H_2O to final volume 125 μl. After incubation at 37°C for 60 minutes, the reaction was stopped by adding 3.0 ml chloroform: methanol (2:1) to each tube. The tubes were vortexed and then 0.6 ml of 0.1 M KCl (0.75 g/100 ml) was added. The contents of each tube were mixed, and then centrifuged at 1000 g for 10 minutes. The upper phase was aspirated, discarded and 1.2 ml of chloroform: methanol: 0.1 M KCl (3:48:47) mixture were added to the organic phase in each tube. The content of each culture tube were well mixed and centrifuged at 1000 g for 10 minutes again. The upper phase was aspirated, discarded and 1.2 ml of chloroform: methanol: H_2O (3:48:47) was added to the organic phase in each culture tube. The contents in each culture tube were well mixed. then centrifuged at 1000 g for 10 minutes. The upper phase was aspirated and discarded. A 1.0 ml aliquot of the final lower phase from each culture tube was transferred to a scintillation vial and brought to dryness by hot air, 10 ml of Scintiverse II was added and then the radioactivity of each sample was determined.

2. Cultured Cells and Dysmyelinating Rodent Mutants

2.1. Cultured cell preparation

These cells were grown and provided by Dr. Espinosa for the enzymatic

analysis. Briefly, primary glial cultures (containing astrocytes and oligodendrocytes) were prepared from newborn rat brains as described by Espinosa et al. (1986). Pure cultures of astrocytes and oligodendrocytes were prepared from the same primary glial cultures by using a new technique resulting in the combination of two original techniques (McCarthy and de Vellis, 1980; Espinosa et al., 1986). Briefly, 2 week-old primary glial cultures were vigorously shaken for 10 minutes; supernatants were recovered and centrifuged; the resulting pellet contained about 96% of oligodendrocytes and their progenitors, 2% of type I astrocytes, and 1-2% of macrophages (Espinosa et al., 1986). Cells were plated in new poly-D-lysine-pretreated dishes in Waymouth's medium supplemented with 19% calf serum.

Four hours after plating, the medium was changed for a chemically defined medium called selective defined medium (OSM) as described (Espinosa et al., 1986). This step may allow for the elimination of the remaining type I astrocytes and the macrophages. Twenty-four hours later this medium was replaced by the working medium—either Waymouth's supplemented with 10% calf serum (W-10) or a richer, chemically defined medium for oligodendrocytes. For all the experiments described in the present thesis W-10 was used. After these treatments the astrocytes are free of other cell types. Cultured cells were then homogenized directly in ice-cold distilled water (10% w/v) and ready for enzyme activity measurement.

2.2. Dysmyelinating rodent mutant tissues

2.2.1. Sources

The myelin-deficient rat (+md/Y) and littermate controls (+ +/Y) were bred at U.C.L.A. The mutant mice were principally supplied by Dr. Zalc from INSERM U 134 Paris, France. The jimpy mutants (+jp/Y) were bred on B6CBA/A^{wj} animals. Mutation of it was genetically marked with the Tabby locus (Ta) and recombination between Ta and Jimpy was supressed by the introduction, in repulsion, of an inverted segment of the X chromosome (In (X) 1 H). The quaking mutants (qk/qk) were bred on C57BL6 animals in which the quaking mutation was marked, in repulsion, with the T locus.

2.2.2. Brain tissue dissection and homogenization

Myelin-deficient rat brains were frozen at the source (U.C.L.A.) and sent to Winnipeg packed in dry ice. Cerebral cortex and corpus callosum from the brain tissue of 16 day-old md rats and littermate controls were dissected and homogenized in ice-cold distilled water (10% w/v) as the source for the enzymes. The brains of the jimpy and the quaking mouse and their littermates were dissected at the source into combined diencephalin plus mesencephalon, cerebellum, and cerebral cortex and were homogenized in the same manner as the md brain tissues.

3. Protein Assay

Protein concentration was determined by the method of Lowry et al. (1957), with BSA as standard.

4. Statistics

Duplicate incubations were performed for the enzyme analysis and purified myelin samples available in the laboratory were employed as controls. The Student t-test was used for the statistical analysis.

5. Extraction and Purification of GPC Phosphocholine Phosphodiesterase

5.1. Materials

Bovine brain tissue was purchased from Pel Freez Biologicals (Rogers, Arkansas). Triton X-100 was obtained from Calbiochem (LaJolla, CA). Q-Sepharose fast flow gel was purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden). Octyl β D- glucopyranoside (OβG), glycine, HEPES, PMSF, and pepstatin A were purchased from Sigma Chemical Co. (St. Louis, MO). All other regents were of analytical grade. Coomassie blue reagent was obtained from PIERCE (Rockford, Illinois) and used for determination of protein concentration with BSA as the standard (Sedmak and Grossberg, 1977).

5.2. Myelin isolation

Myelin was isolated according to the published procedures (Norton and Poduslo, 1973). 12 gram of bovine brain white matter was homogenized in about 40 ml of 0.32 M sucrose and then brought to a total volume of about 100 ml with 0.32 M sucrose. 18 ml of homogenates were layered over 10 ml of 0.85 M sucrose and then centrifuged for 30 minutes at 75,000 g (Beckman SW28 rotor, 25,000 rpm). The layer of crude myelin which formed at the interface of the two sucrose solutions was collected and homogenized in 180 ml of H_2O. The

homogenates were centrifuged again at 75,000 g for 15 minutes (Beckman 60 Ti rotor, 33,000 rpm). The pellets were homogenized in 180 ml H_2O and centrifuged at 10,000 rpm for 10 minutes (Sorvall rotor SS34), this step was repeated again. The pellets were then homogenized in 100 ml of 0.32 M sucrose, and homogenate layered over 0.85 M sucrose. The tubes were centrifuged at 75,000 g for 30 minutes (Beckman SW28 rotor, 25,000 rpm). The pure myelin was collected at the interface and homogenized in 180 ml of H_2O. The final pellets were obtained from myelin homogenates were centrifuged at 10,000 rpm for 10 minutes (Sorvall rotor SS34). These pure myelin pellets were then suspended in a small amount of H_2O and lyophilized. For each step, homogenate contained 0.5 mM PMSF and 10 μg/ml Pepstatin A.

5.3. Extraction of GPC phosphocholine phosphodiesterase

500 mg of lyophilized myelin was homogenized with 500 ml of 1% Triton X-100 in H_2O at 4°C and centrifuged at 37,000 g for 20 minutes (Beckman 60 Ti rotor, 23,000 rpm). The pellet was then extracted with 50 ml of a buffer pH 7.5 containing 1% Triton X-100, 1 M ammonium acetate, 0.2 M HEPES, 1.0 mM EDTA, and 1.0 mM 2-mercaptoethanol at 4°C and then centrifuged at 37,000 g for 20 minutes (Beckman 60 Ti rotor, 23,000 rpm). The pellet was then successively extracted with 10 ml of 25 mM, 50 mM, 100 mM, and 200 mM OβG in 0.05 M HEPES pH 7.5 by homogenization. Each homogenate was rotated for 30 minutes in cold room and then centrifuged at 37,000 g for 20 minutes (Beckman 60 Ti rotor, 23,000 rpm). For each step, aliquots of all supernatants and pellets were saved for enzyme assay and each solution contained 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml Pepstatin A (Figure 6).

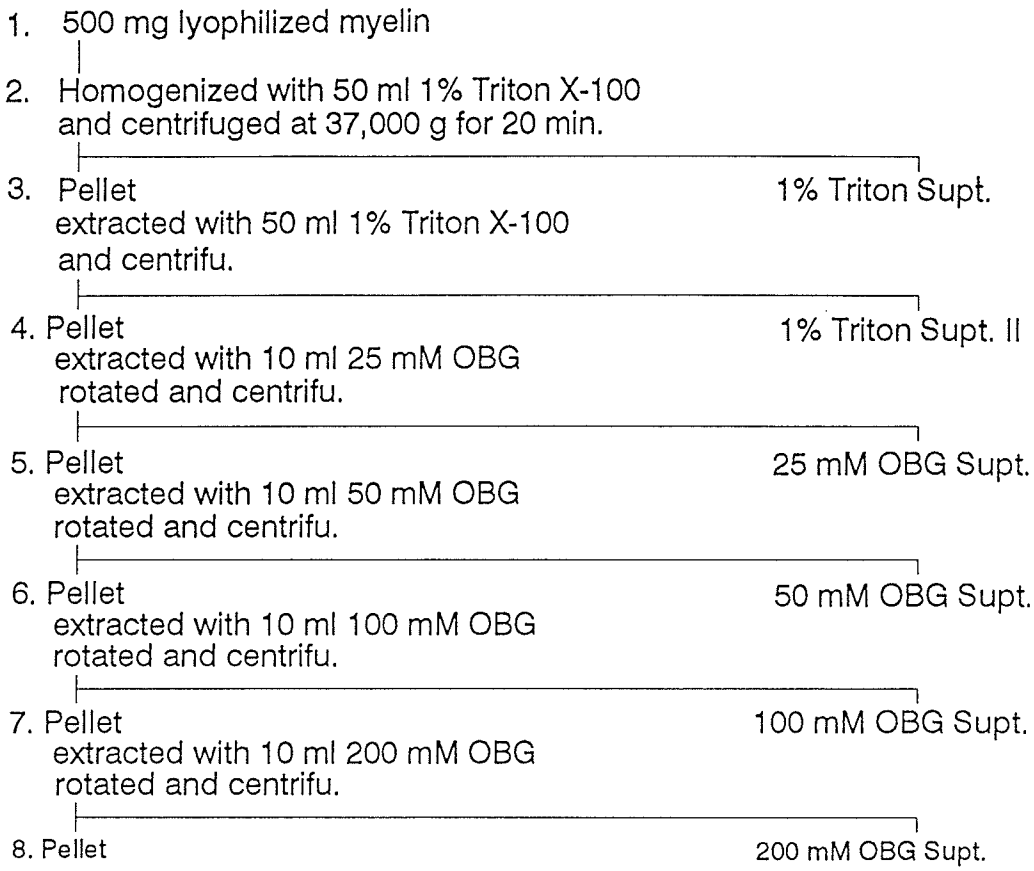

Figure 6. Schematic representation of the extraction of GPC phosphocholine phosphodiesterase from bovine brain myelin (details are as in the text).

5.4. Q-Sepharose column chromatography

The 200 mM OβG supernatant which contained the partially purified enzyme was concentrated to about 2 ml by using polyethylene glycol. The concentrated sample was dialyzed against a buffer containing 0.05 M glycine, 0.5 mM PMSF pH 9.5 for 2 to 3 hours, and then applied immediately to a Q-Sepharose Fast Flow column (2 ml bed volumn) (NB: The sample protein will be aggregated if the dialyzed sample is stored at - 80°C again, but not used immediately. Large aggregation will make the following column study more difficult) which was previously equilibrated with 10 ml of 0.05 M glycine buffer pH 9.5 containing 0.5 mM PMSF. The Q-Sepharose column was washed with 10 ml of same buffer, and then eluted with an increasing linear gradient from 0 to 1 M of NaCl generated with 15 ml of 0.05 M glycine buffer gradually mixed with 15 ml 0.05 M glycine, 1 M NaCl buffer at pH 9.5. The column was further washed with 10 ml of 0.05 HEPES, pH 7.5 buffer. Finally, the column was eluted with 15 ml of 100 mM OβG, 0.05 M HEPES buffer at pH 7.5. The flow rate for this column is about 10 ml/hour, and fractions of 1 ml were collected for each tube. The activities of GPC and pNP phosphocholine phosphodiesterase and the protein were determined for every other fraction.

6. Polyacrylamide Gel Electrophoresis

The SDS-PAGE system of Laemmli (1970) was prepared. The 200 mM OβG supernatant and the Q-Sepharose column fractions with highest activity were dialyzed against a buffer containing 0.05 M HEPES, 0.5% SDS pH 8.5 overnight. The samples containing about 2 μg protein (about 5 μg protein was used for myelin) were dried under nitrogen and 30 μl of sample buffer (contains

glycerol, SDS, and 2-mercaptoethanol) was added. Samples were not boiled except molecular weight marker which was boiled for 3 to 4 minutes. After centrifugation at 10,000 g for 10 minutes, the aliquots of each sample were submitted to SDS-PAGE analysis on 0.75 mm gel slab (8 cm x 10 cm in size, Bio-Rad Mini-Protean II Slab cell apparatus, Richmond, California) using a 12% separating gel. Gels were run at a constant current of 40 mA until the dye front (bromophenol blue) reached the bottom of the gel (about 2 hours). The gel was fixed and stained by Silver Stain Plus (Method derived from Gottlieb and Chavko, 1987) according to supplier's instruction (Bio-Rad Laboratories, Richmond, CA). SDS-PAGE low range molecular weight markers (Bio-Rad) were used as standards and consisted of Rabbit muscle phosphorylase b (97 kDa), BSA (66 kDa), Hen egg white ovalbumin (43 kDa), Bovine carbonic anhydrase (31 kDa), Soybean trypsin inhibitor (21 kDa), and Hen egg white lysozyme (14 kDa).

III. RESULTS

1. Cultured cells and Dysmyelinatng Rodent Mutants

1.1. Cultured cells

Cultures of pure oligodendrocytes, of astrocytes, or of mixed primary glial cell cultures containing both oligodendrocytes and astrocytes derived from newborn brain and C-6 cells established from a rat glioma were used. The levels of CNPase, C gal T, GPC phosphocholine phosphodiesterase, and pNP phosphocholine phosphodiesterase were estimated from homogenates of these cells. The pure cultured oligodendrocytes had the highest activities of CNPase and C gal T which are similar to those previously reported (Espinosa et al., 1988). CNPase activity of mixed cultured cells was slightly lower than that of the pure cultured oligodendrocytes, but higher than that of the cultured astrocytes. CNPase activity in C-6 cells was quite high as compared to cultured astrocytes, which is consistent with the previous findings (Volpe et al., 1975), but the activity of C gal T was very low (Table II). Like CNPase and C gal T, the highest activities of GPC phosphocholine phosphodiesterase and pNP phosphocholine phosphodiesterase was found in cultured oligodendrocytes. The activities of these two phosphodiesterases were low in mixed cultured cells, and substantially lower or undetectable in cultured astrocytes and C-6 cells (Table II). Myelin contained very high activities of CNPase, GPC phosphocholine phosphodiesterase, and pNP phosphocholine phosphodiesterase, but similar C gal T activity to that of cultured astrocytes and C-6 cells (Table II).

Table II. Specific activities of CNPase, GPC and p-NP phosphocholine phosphodiesterase, and UDP galactose:ceramide galactosyl-transferase in cultured oligodendrocytes, astrocytes, mixed glial cell and C-6 cells.

Cells	CNPase	GPC'diesterase	pNPPC'diesterase	C gal T
Oligodendrocytes I	21.3	27.0	170.9	4.8
Oligodendrocytes II	44.7	64.8	332.4	10.4
Mixed cells I	22.1	6.9	50.8	1.2
Mixed cells II	30.7	9.0	75.1	1.2
Astrocytes I	7.1	1.8	0	0.3
Astrocytes II	3.6	2.8	0	0.4
C-6 cells	68.7	0.3	0	0.4
Myelin	189.4+/- 109	66.2+/- 8.5	423.9+/- 109	0.4+/- 0.1

I and II designate two separate cell cultures. Values are expressed as nmole/mg protein/hour, except for CNPase which is expressed as μmole/mg protein/hour. Details are as in the text.

1.2. Myelin-deficient Rats

The homogenates of the cerebral cortex and the corpus callosum of the 16 day old myelin deficient rats and of their littermate controls were assayed for CNPase, GPC and p-NP phosphocholine phosphodiesterase activities. As shown in Figure 7, CNPase activity was diminished in the cerebral cortex of the md rats by 36%, even though this reduction was not statistically significant. The activities of GPC and p-NP phosphocholine phosphodiesterase were significantly decreased in cerebral cortex of md rats by 47% and 92% respectively. There were significant reductions in activities of CNPase, GPC phosphocholine phosphodiesterase and p-NP phosphocholine phosphodiesterase in corpus callosum of md rats. The activities of these three enzymes were only 11%, 24%, 5%, respectively, of those of normal littermate controls (Figure 8).

The level of UDP galactose: ceramide galactosyltransferase was also estimated in the cerebral cortex and corpus callosum of the 16 day old md rats. The activity of this enzyme was significantly reduced in the cerebral cortex and the corpus callosum (data not shown) of md rats by 93% and 96%, respectively. Activities of these four enzymes in the corpus callosum of littermate control rats were higher than that in the cerebral cortex (Figure 7 & 8).

1.3. Jimpy and Quaking mice

The homogenates of the cerebral cortex, the cerebellum, and the combined diencenphalon plus mesencephalon were used to measure the activities of CNPase, C gal T, GPC phosphocholine phosphodiesterase and pNP

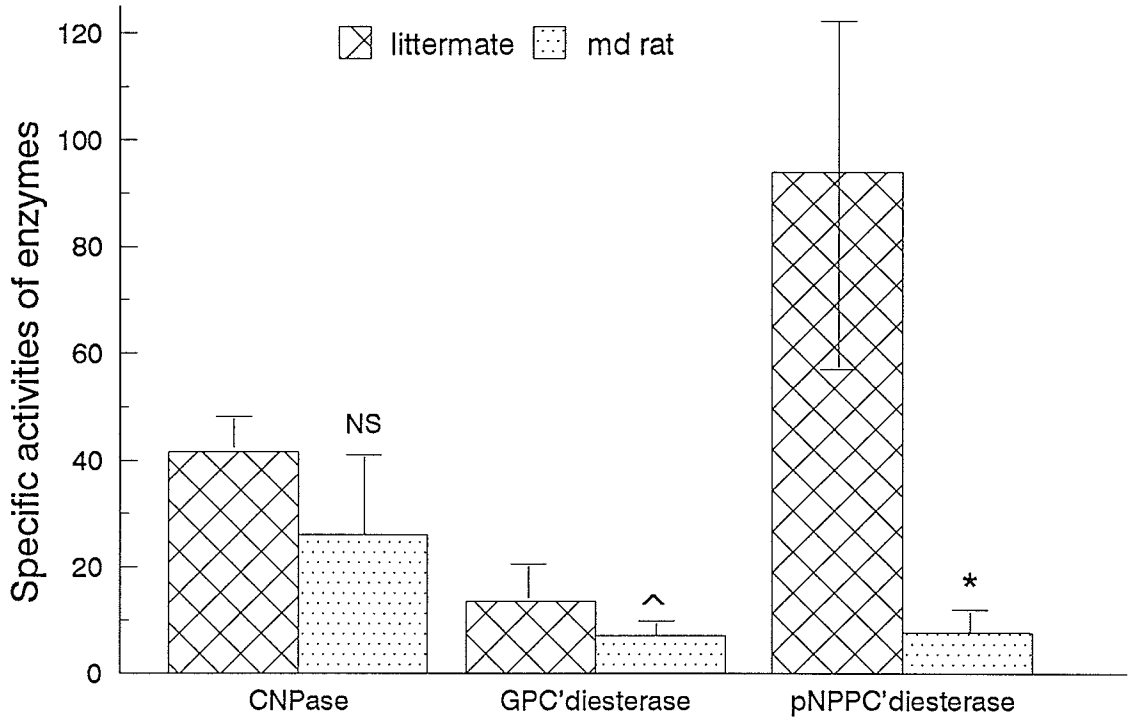

Figure 7. Specific activities of CNPase, GPC & pNP phosphocholine phosphodiesterase in **cerebral cortex** of 16 day old Md rats. Assays were performed in duplicate as described under the experimental procedure. Results are the means +/- S.D. of two to four separate brain samples (one brain for each determination). Values are expressed as nmole/mg protein/hour except CNPase which is expressed as μmole/mg protein/hour. ^P< 0.05, * P< 0.01, NS means not statistically significant.

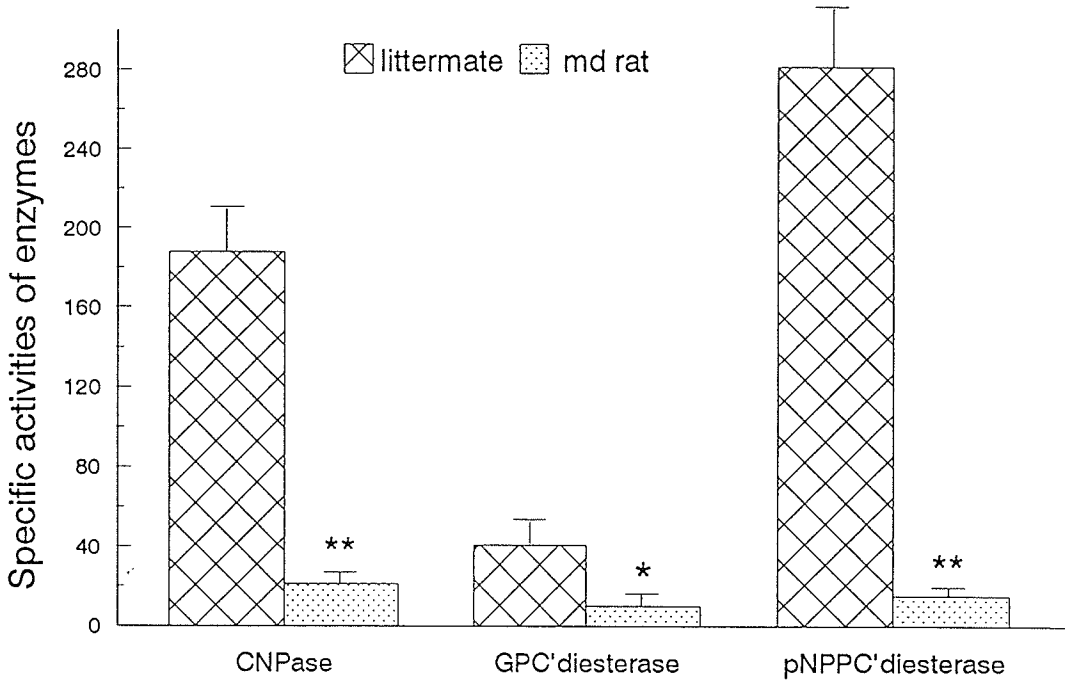

Figure 8. Specific activities of CNPase, GPC & pNP phosphocholine phosphodiesterase in **corpus callosum** of 16 day old Md rats. Assays were performed in duplicate as described under the experimental procedure. Results are the means +/- S.D. of two to four separate brain samples (one brain for each determination). Values are expressed as nmole/mg protein/hour except CNPase which is expressed as μmole/mg protein/hour. * P< 0.01, ** P< 0.001.

phosphocholine phosphodiesterase. The activities of CNPase (Figure 9) and C gal T (Figure 10) are significantly lower in the affected jimpy and quaking mice as compared to those of the littermate controls. The reduction of enzyme activity in the jimpy mouse was greater than that in the quaking mouse consistent with previous reports (Kurihara et al., 1970; Morell and Costantino-Ceccarini et al., 1972; Neskovic et al., 1970). The highest specific activities of these two enzymes were found in the combined diencephalon plus mesencephalon or cerebellum of jimpy or quaking littermate controls, respectively, and the lowest activities in cerebral cortex of controls (Figure 9 &10).

Like CNPase and C gal T, the activities of GPC phosphocholine phosphodiesterase (Figure 11) and p-NP phosphocholine phosphodiesterase (Figure 12) in both affected animals were significantly lower than those of their littermate controls. The regional distributions of these two phosphodiesterases in controls were identical to those of CNPase and C gal T. The magnitude of the reduction of GPC phosphocholine phosphodiesterase, pNP phosphocholine phosphodiesterase, CNPase, and C gal T activities were greater in the jimpy than in the quaking mice and the degree of diminution of the two phosphodiesterases was very similar in each brain region of each mutant (Figure 11 & 12).

2. Enzyme Extraction and Purification

2.1. Enzyme extraction

500 mg of lyophilized myelin containing approximately 120 mg myelin protein was employed for extraction and all steps were carried out at 4°C. The lyophilized myelin was first extracted with 1% Triton X-100, the 1% Triton X-100

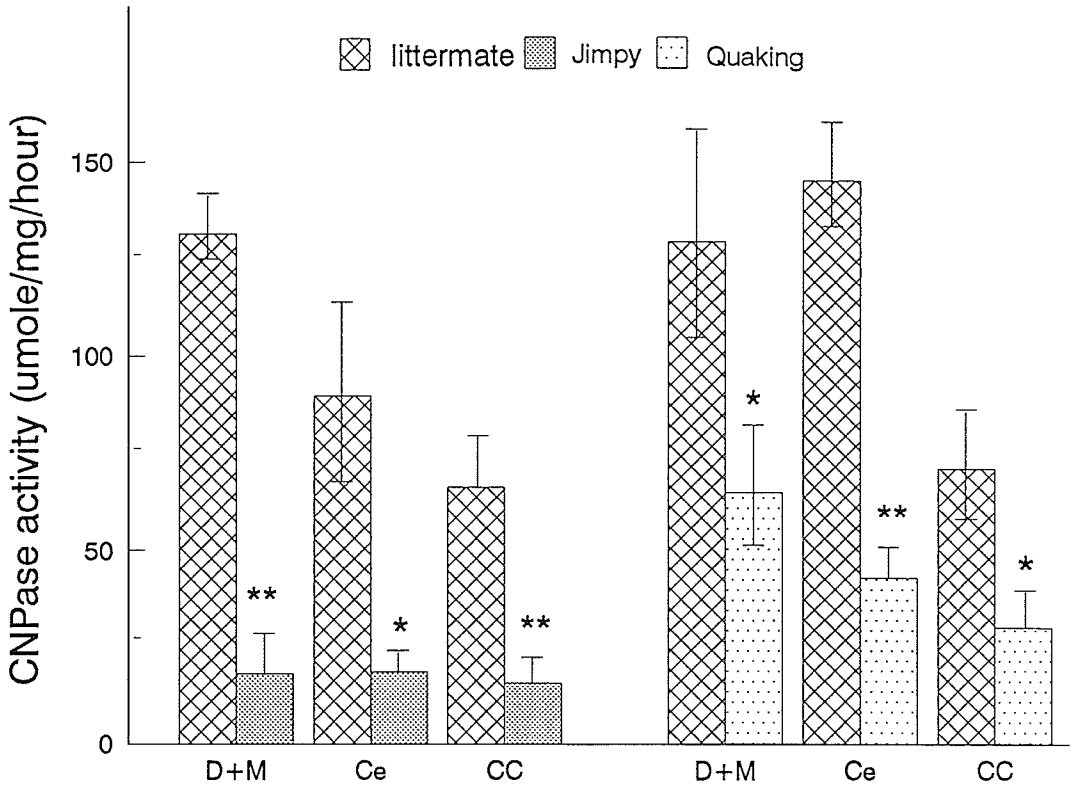

Figure 9. Specific activity of **CNPase** in CNS tissues of jimpy, quaking and littermate control mice. Assays were performed in duplicate as described under the experimental procedure. Results are the means +/- S.D. of five to seven separate brain samples (one brain for each determination). Tissue: D+M, diencephalon plus mesencephalon; Ce, cerebellum; CC, cerebral cortex. * $P<0.01$, ** $P<0.001$.

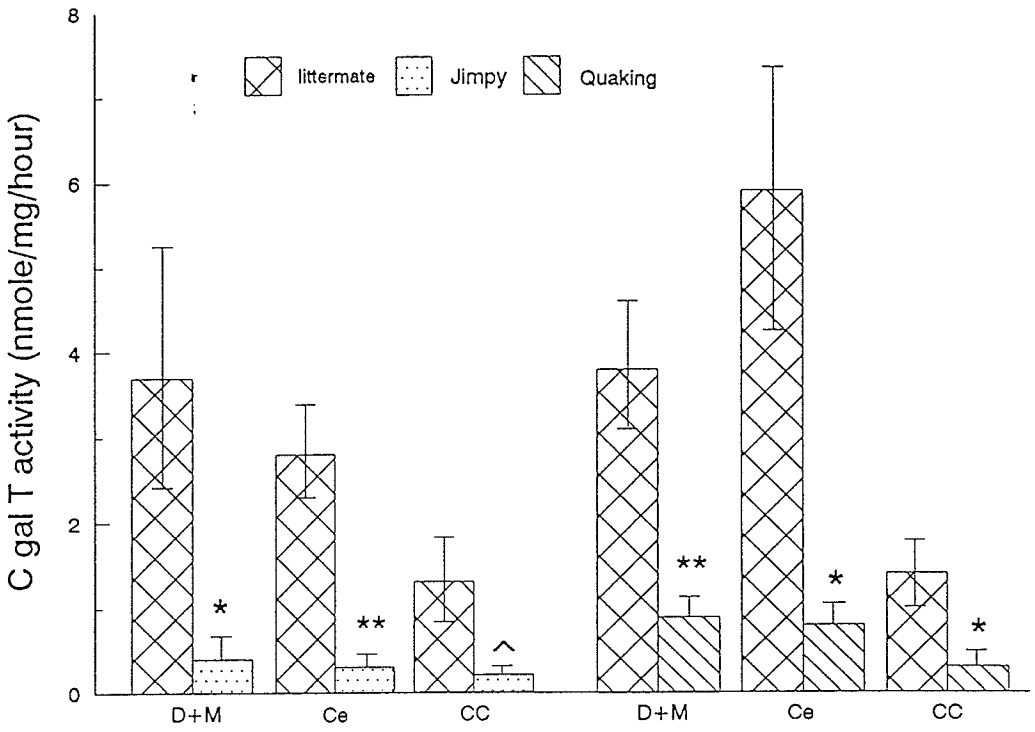

Figure 10. Specific activity of **C gal T** in CNS tissues of jimpy, quaking and littermate control mice. Assays were performed in duplicate as described under the experimental procedure. Results are the means +/- S.D. of five to seven separate brain samples (one brain for each determination). Tissue: D+M, diencephalon plus mesencephalon; Ce, cerebellum; CC, cerebral cortex. ^P< 0.05, * P<0.01, ** P<0.001.

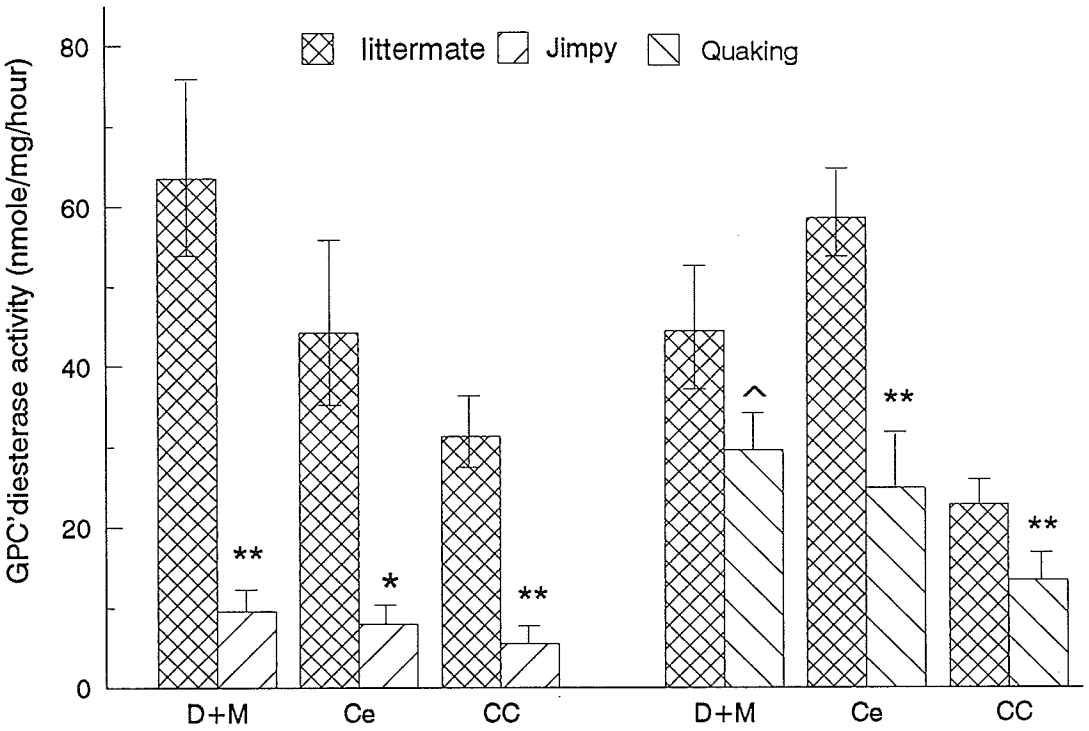

Figure 11. Specific activity of **GPC phosphocholine phosphodiesterase** in CNS tissues of jimpy, quaking and littermate control mice. Assays were performed in duplicate as described under the experimental procedure. Results are the means +/- S.D. of five to seven separate brain samples (one brain for each determination). Tissue: D+M, diencephalon plus mesencephalon; Ce, cerebellum; CC, cerebral cortex. ^ $P< 0.05$, * $P< 0.05$, ** $P< 0.01$.

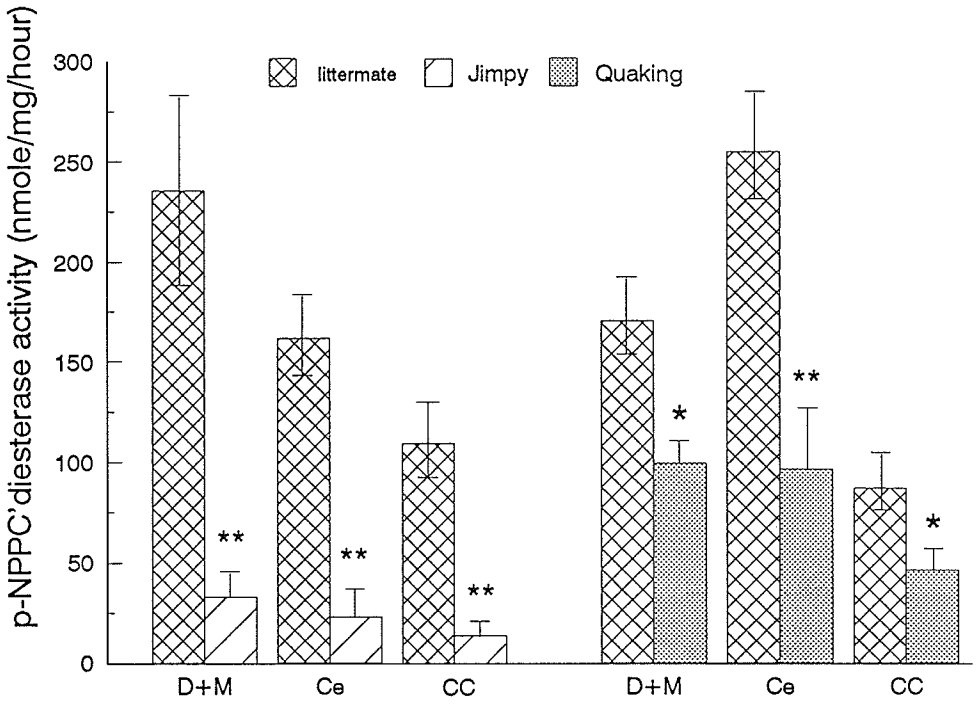

Figure 12. Specific activity of **p-NP phosphocholine phosphodiesterase** in CNS tissues of jimpy, quaking and littermate control mice. Assays were performed in duplicate as described under the experimental procedure. Results are the means +/- S.D. of five to seven separate brain samples (one brain for each determination). Tissue: D+M, diencephalon plus mesencephalon; Ce, cerebellum; CC, cerebral cortex. * $P< 0.01$, ** $P< 0.001$.

pellet was then reextracted with 1% Triton X-100 in a buffer containing 1 M ammonium acetate, 0.2 M HEPES, 1.0 mM EDTA, and 1.0 mM mercaptoethanol pH 7.5 and centrifuged (method of Wells and Sprinkle, 1981). These two extraction steps removed approximately 60% myelin proteins which contain mainly myelin proteolipid protein, CNPase and some of the myelin basic protein. The final 1% Triton X-100 supernatant contains most of CNPase activity (data not shown), little or no GPC or pNP phosphocholine phosphodiesterase activities (Table III). The 1% Triton X-100 pellets were further successively extracted with 25 mM, 50 mM, 100 mM, and 200 mM Octyl β D-glucopyranoside (OβG). The highest activities of these two phosphodiesterases were found in 200 mM OβG supernatant which has about 4% of total protein and 66.3% or 76.9% of the total activity for GPC phosphcholine phosphodiesterase or pNP phosphocholine phosphodiesterase, respectively. The extraction behaviors of these two phosphodiesterases are very similar. The 200 mM OβG pellets contain about 3% of total protein but low phosphodiesterase activity and total activity (Table III).

2.2. Q-Sepharose column chromatography fractionation

The concentrated 200 mM OβG supernatant was dialyzed for 2 to 3 hours to partially remove the detergent (a relatively high concentration of the detergent has to be maintained in order to keep the enzyme in the soluble form), and then applied directly to the Q-Sepharose column. As shown in Figure 13, at least 70% of total protein, but no GPC and pNP phosphocholine phosphodiesterase activities were found after the column was washed with glycine buffer pH 9.5, increasing NaCl gradient, or the HEPES buffer pH 7.5. The major peak of both

Table III. Extraction of GPC and p-NP phosphocholine phosphodiesterase by Triton X-100 and Octyl-β-D Glucopyranoside(OβG).

	Protein[a]	Specific activity[b]		Recovery(%)	
		GPC[c]	p-NPPC[c]	GPC	p-NPPC
Myelin Homogenate	115	70.6	365.9	100	100
1% TritonSupt I	25.0	5.8	0	1.8	0
1% Triton Supt II	44.2	0	0	0	0
25mM OβG Supt	2.9	4	15.3	0.1	0.1
50mM OβG Supt	1.3	186	1333.3	2.9	0.4
100mM OβG Supt	0.9	242.4	2190	2.7	4.7
200mM OβG Supt	**4.2**	**1281**	**7714**	**66.3**	**76.9**
200mM OβG Pellet	3.6	136.9	1296.3	6.1	11.1

500 mg lyophilized myelin was used for this study. [a] mg protein, [b] Specific activity is expressed as nmole/mg protein/hour. [c] GPC and pNP represent GPC and pNP phosphocholine phosphodiesterase. Details are provided in the text.

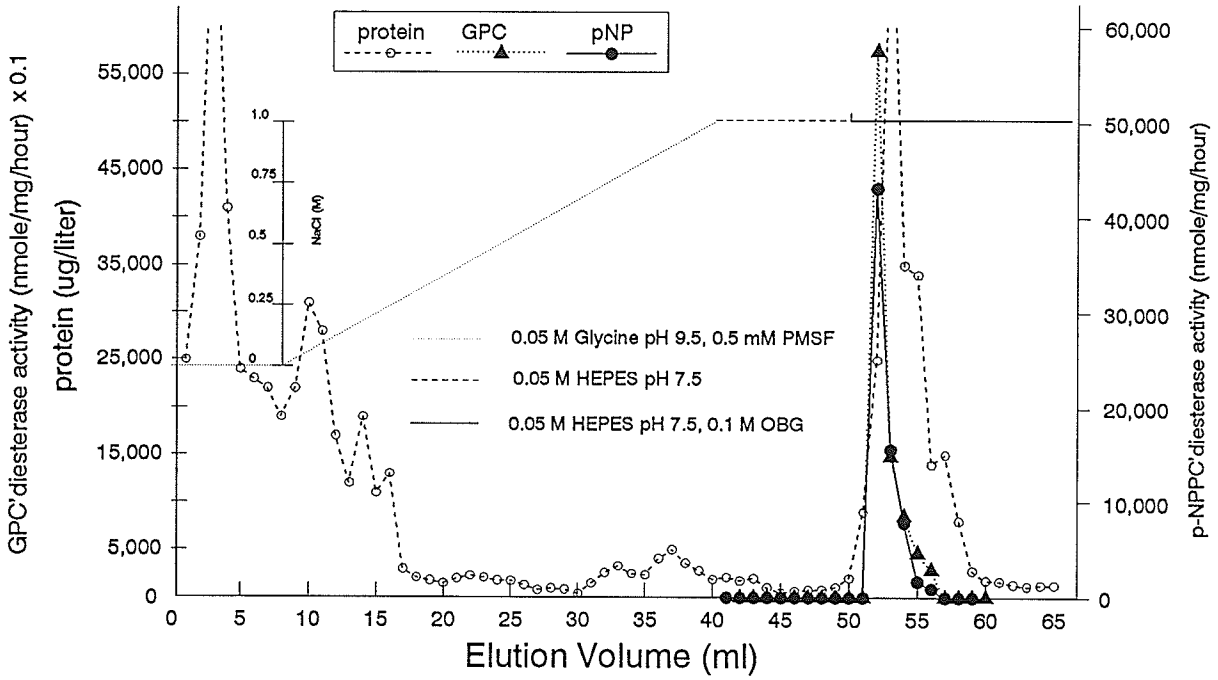

Figure 13. **Q-Sepharose column chromatography** of bovine brain myelin GPC and p-NP phosphocholine phosphodiesterase. Dialyzed 200 OβG supernatant (2 ml) was applied to a Q-Sepharose column and then washed with glycine buffer. The column was further eluted with NaCl gradient, HEPES buffer, and 100 mM OβG as described under the experimental procedure. Protein and enzyme activities were determined in column fractions by methods described under the experimental procedure.

phosphodiesterases can be observed only after the column was eluted with 100 mM OβG in 0.05 M HEPES buffer pH 7.5 (Figure 13). The yields of protein, total activities and specific activities of GPC and pNP phosphocholine phosphodiesterase during each purification step are shown in Table IV. The recovery of protein in pooled Q-Sepharose column fractions which contain the highest enzyme activity for these two phosphodiesterases (No. 53-55) was about 0.16%, the specific activities of GPC and pNP phosphocholine phosphodiesterase were 5750 nmole/mg/hour and 35882 nmole/mg/hour, respectively. Both phosphodiesterase had a similar degree of purification and total recovery of activity during each purification step (Table IV).

3. Purified Enzyme Characterization

3.1. Purity and molecular weight determination by 12% SDS-PAGE

Purity and molecular weight of GPC and pNP phosphocholine phosphodiesterase was determined by 12% SDS-polyacrylamide gel electrophoresis (Figure 14). Prominant myelin proteins, such as CNPase (44 kDa), PLP (25 kDa), DM-20 (20.5 kDa) and MBP (14 kDa) can be seen in Lane 2. Lane 3 is the 200 mM OβG supernatant, the Triton X-100 extractable myelin protein such as CNPase, PLP, DM-20 and part of MBP have been removed. The pooled Q-Sepharose column fractions with highest specific activities of both phosphodiesterases appears as a major single band on SDS-PAGE with molecular weight of about 14 kDa (Lane 4). The apparent band at 66 kDa present in both the 200 OβG and the Q-Sepharose is an artificial band, that is partially removed by addition of iodoacetamide (Beis and Lazou, 1990).

Table IV. Yields of protein, GPC and p-NP phosphocholine phosphodiesterase total activites and specific activities during purification procedures.

	Protein[a]	GPC' diesterase			p-NPPC' diesterase		
		SA[b]	TA[c]	Fold Enrichment	SA[b]	TA[c]	Fold Enrichment
Myelin	115	66.5	7650(100%)	1	366	42090(100%)	1
200mM OβG	3.2	1251	4003(52%)[d]	19	6764	21645(51%)	19
Q-Sepharose	**0.18**	**5750**	**1058(14%)**	**87**	**35882**	**6459(15%)**	**98**

500 mg lyophilized myelin was used for extraction. [a] mg protein, [b] specific activity is expressed as nmole/mg protein/hour, [c] total activity is expressed as nmole, d is percent recovery of enzyme activity compared to the starting myelin. Details are provided in text.

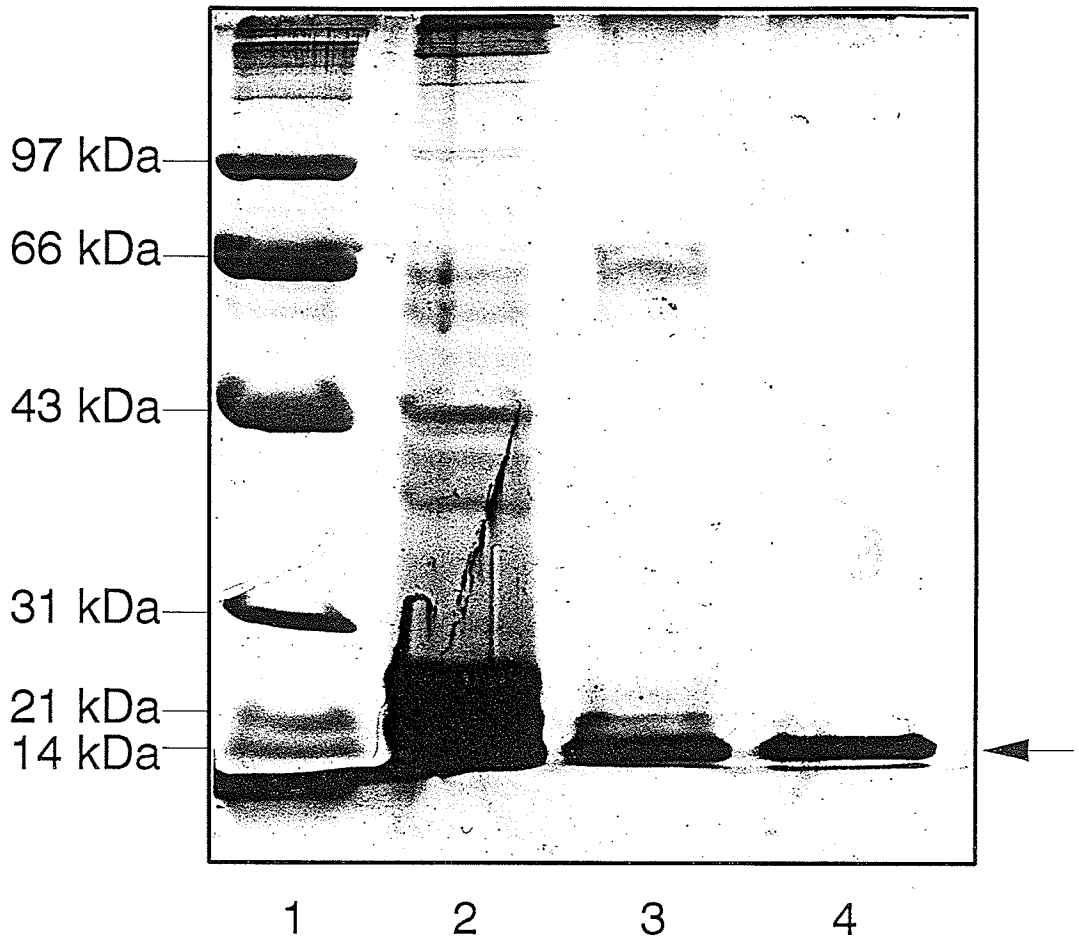

Figure 14. **SDS polyacrylamide gel electrophoresis** on a 12% slab gel. Lane 1, molecular weight standards. Lane 2, myelin homogenate. Lane 3, 200 mM OβG supernatant. Lane 4, pooled Q-Sepharose column fraction (arrow). Aliquot of samples containing 2 μg protein (for myelin 5 μg protein) was used for this study and the gel was visualized by silver staining. Detailed information was given under the experimental procedure.

3.2. Km and pH optimum

The specific activities of GPC and pNP phosphocholine phosphodiesterase were estimated in the presence of different concentrations of GPC and p-NP phosphocholine (0 to 2 mM) in order to determine the Km and Vmax values for these two phosphodiesterases. Lineweaver-Burk plots with the purified enzyme indicated that the Km, and the Vmax of GPC phosphocholine phosphodiesterase and pNP phosphocholine phosphodiesterase were 0.23 mM, 8571 nmole/mg protein/hour (Figure 15) and 0.22 mM, 52632 nmole/mg protein/hour (Figure 16), respectively.

0.5 M HEPES-NaOH buffer for pH 7.5, 8.0, 8.5; 0.5 M glycine-NaOH buffer for pH 9.0, 9.5, 10.0, 10.5, 11.0; and 0.5 M sodium bicarbonate buffer for pH 11.5, 12 were used for study of the effect of pH on the purified enzymes. Figure 17 & 18 showed that the optimum pH for GPC and pNP phosphocholine phosphodiesterase are around 10 and 11, respectively, which are higher than those of the native rat brain myelin-bound enzymes (pH 9.5, Kanfer and McCartney, 1989).

3.3. Metal requirement

The pooled Q-Sepharose column fractions were dialyzed against 1 liter of a solution containing 10 mM EDTA and 0.32 M sucrose overnight in the cold room, and the sample then was dialyzed against 0.32 M sucrose for another night in the cold to remove the EDTA. These twice dialyzed samples were assayed for GPC and pNP phosphocholine phosphodiesterase activities in presence of 1 mM of various cations. As shown in Table V, the non-dialyzed column fraction had

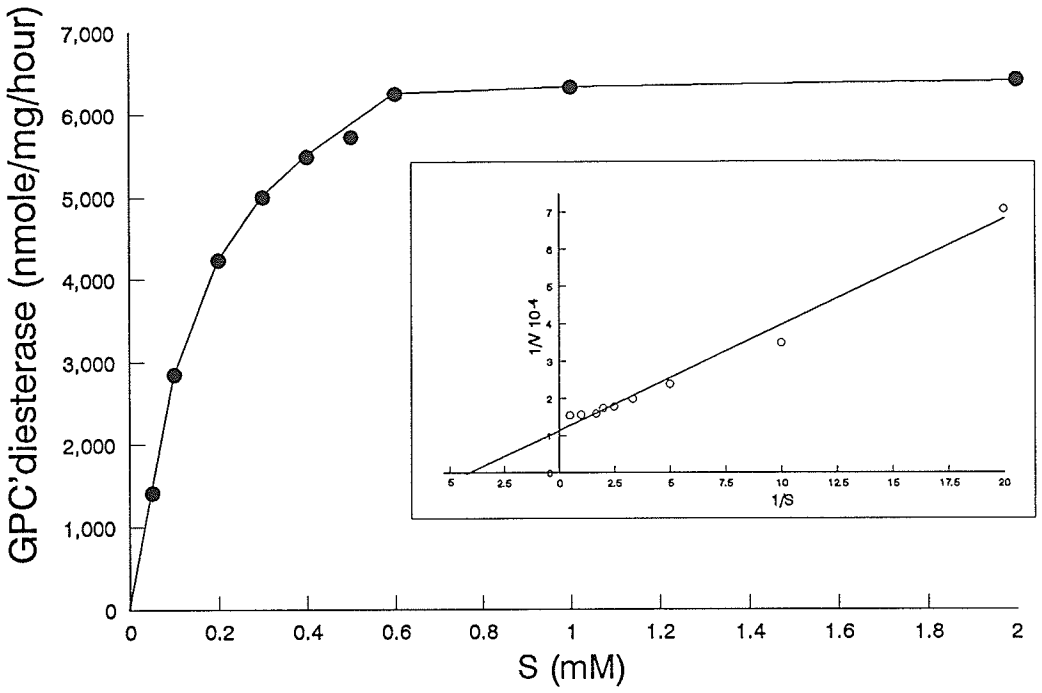

Figure 15. Effect of varying GPC concentration on purified bovine brain myelin GPC phosphocholine phosphodiesterase. Insert is the Lineweaver-Burk plot of these data. Details are as in the text.

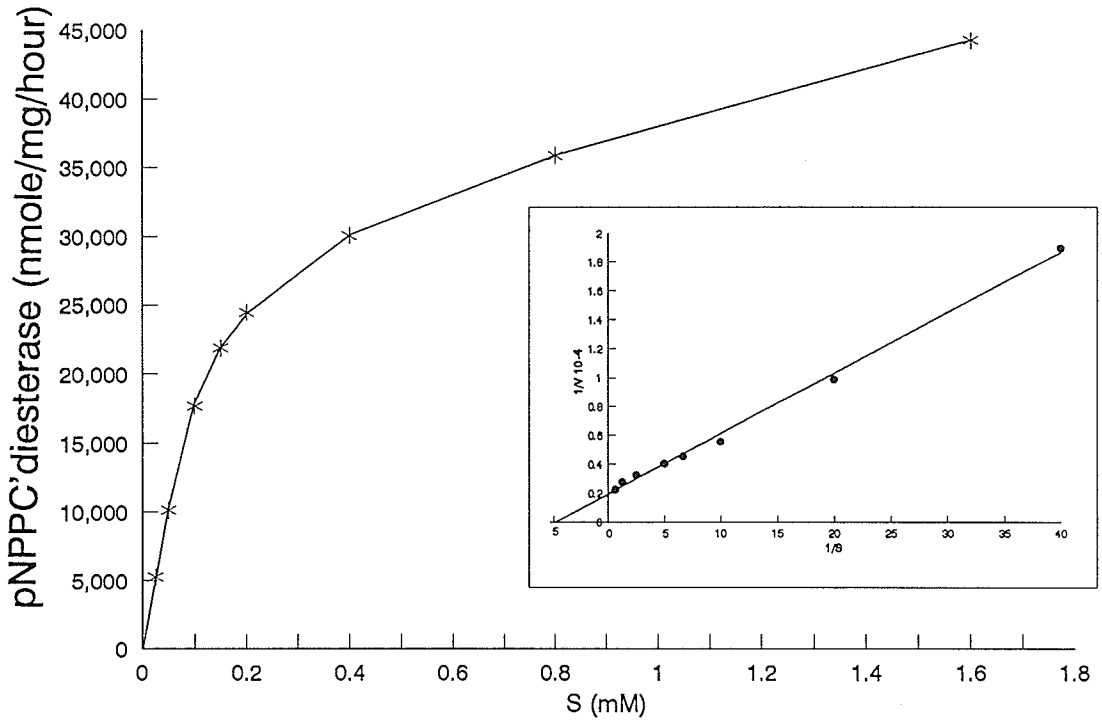

Figure 16. Effect of varing p-NP phosphocholine concentration on purified bovine brain myelin p-NP phosphocholine phosphodiesterase. Insert is the Lineweaver-Burk plot of these data. Details are as in the text.

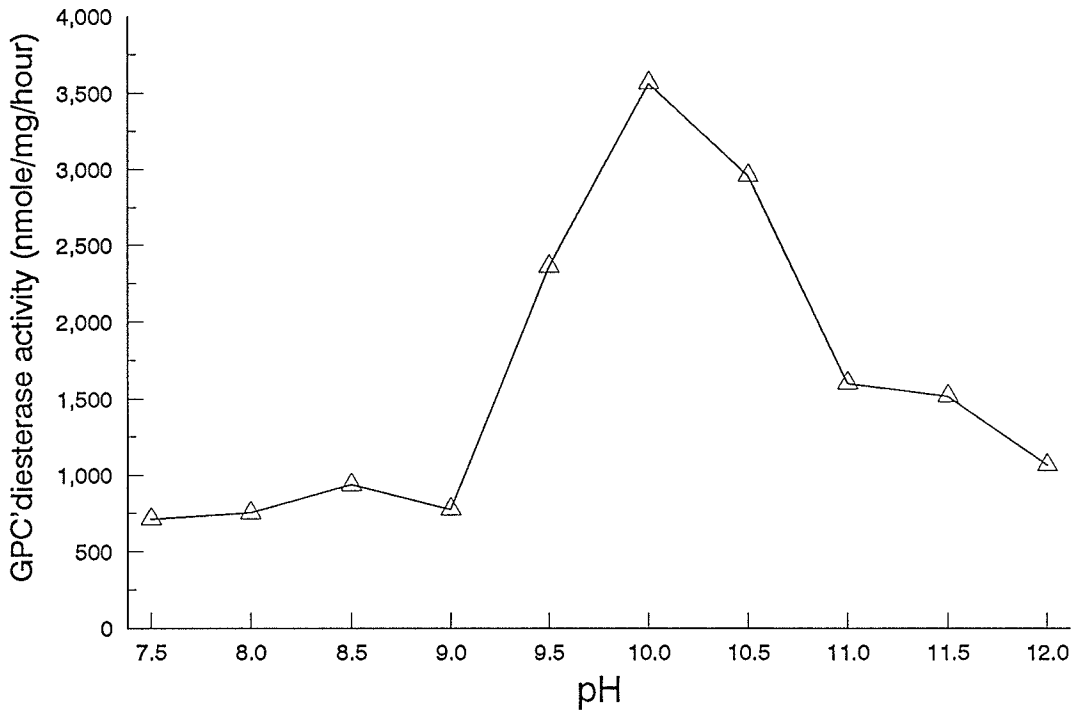

Figure 17. Effect of **pH** change on the activity of purified bovine brain myelin GPC phosphocholine phosphodiesterase. 0.5 M HEPES-NaOH buffer was used for pH 7.5, 8.0, 8.5; 0.5 M Glycine-NaOH buffer was used for pH 9.0, 9.5, 10.0, 10.5, 11.0; and 0.5 M Sodium bicarbonate buffer was used for pH 11.5, 12. Assays were performed in duplicate as described under the experimental procedure.

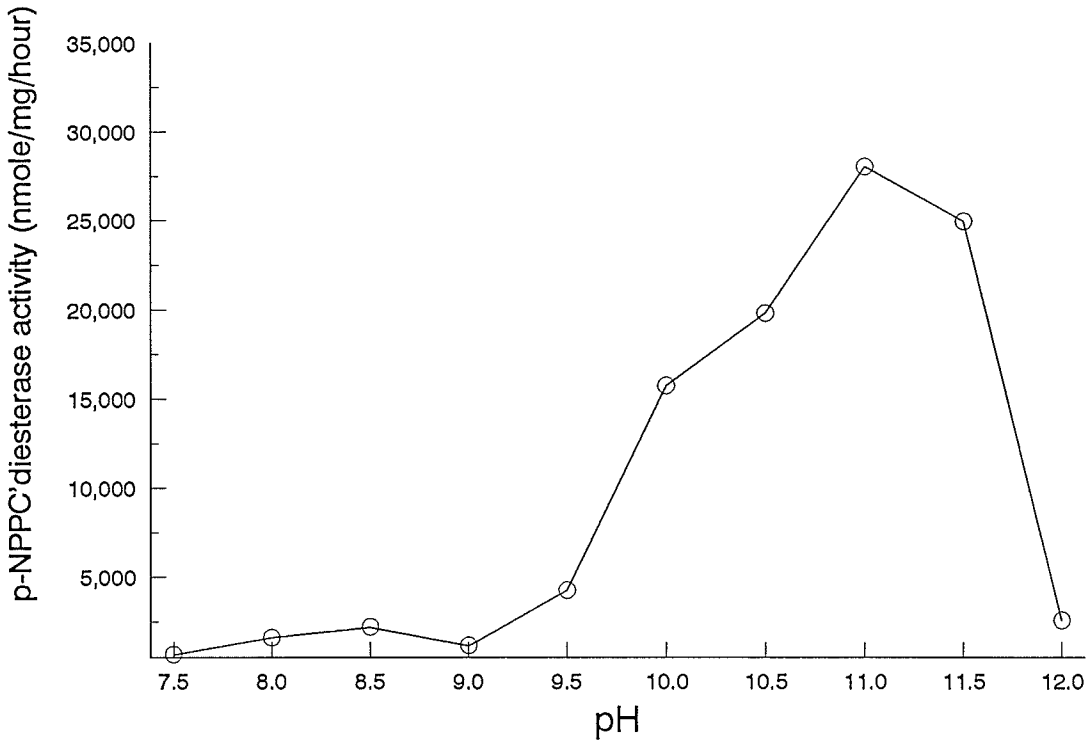

Figure 18. Effect of **pH** change on the activity of purified bovine brain myelin p-NP phosphocholine phosphodiesterase. 0.5 M HEPES-NaOH buffer was used for pH 7.5, 8.0, 8.5; 0.5 M Glycine-NaOH buffer was used for pH 9.0, 9.5, 10.0, 10.5, 11.0; and 0.5 M Sodium bicarbonate buffer was used for pH 11.5, 12. Assays were performed in duplicate as described under the experimental procedure.

very high specific activities for both phosphodiesterases, but the activities were completely lost after samples were twice dialyzed. The activities of these two enzymes could be restored only in the presence with $ZnCl_2$ or $CoCl_2$, other salts had little or no affect on these two phospodiesterase (Table V). The optimum concentrations to restore both GPC and pNP phosphocholine phosphodiesterase were 0.25 mM for $ZnCl_2$, and 1 mM for $CoCl_2$ (Figure 19 & 20). The metal requirements of these two enzymes were nearly identical.

3.4. Substrate specificity, inhibitor selectivity, and Ki

In order to determine the substrate specificity and inhibitor selectivity, GPC phosphocholine phosphodiesterase activity was measured in the presence of 1 mM of various substrate analogues, such as choline, phosphocholine, lysophosphatidylcholine, phosphatidylcholine, p-nitrophenol phosphocholine, bis-p-nitrophenol phosphate, p-nitrophenol thymidine, lysophosphatidyl-ethanolamine, glycerol phosphoethanolamine, and hexadecyl phosphocholine. Inhibition of GPC phosphocholine phosphodiesterase activity was observed only with p-nitrophenol phosphocholine and phosphocholine. Dixon plots were used for determination of the Ki for these two substances. For the GPC phosphocholine phosphodiesterase, p-NP phosphocholine and phosphocholine showed a competitive type of inhibition with a Ki of 28 μM and 30 μM, respectively (Figure 21 and 23). For p-NP phosphocholine phosphodiesterase, GPC and phosphocholine also showed a competitive type of inhibition and the Ki was about 0.5 mM and 1.75 mM, respectively (Figure 22 and 24).

Table V. Effect of various cations on twice dialyzed purified bovine brain myelin GPC and p-NP phosphocholine phosphodiesterase activity.

Cations	Conc. mM	Specific activity[a]	
		GPC ' diesterase	p-NPPC ' diesterase
No additions		2679.31	15142.86
No additions[b]		0	0
Al^{+++}	1	0	0
Ca^{++}	1	150.86	937.5
Co^{++}	**1**	**785.90**	**7187.5**
Cu^{++}	1	29.38	1718.75
Fe^{+++}	1	0	781.25
Hg^{++}	1	161.57	937.5
Mg^{++}	1	188.68	468.75
Mn^{++}	1	173.44	0
Zn^{++}	**1**	**1145.81**	**11093.75**

[a] Activity is expressed as nmole/mg protein/hour. [b] Twice dialyzed sample was used in this and the following assay. The activity for the GPC phosphocholine phosphodiesterase was 2679 and that for the pNP phosphocholine phosphodiesterase was 15142, both of which were the non dialyzed Q-Sepharose fraction. Details are as in the text.

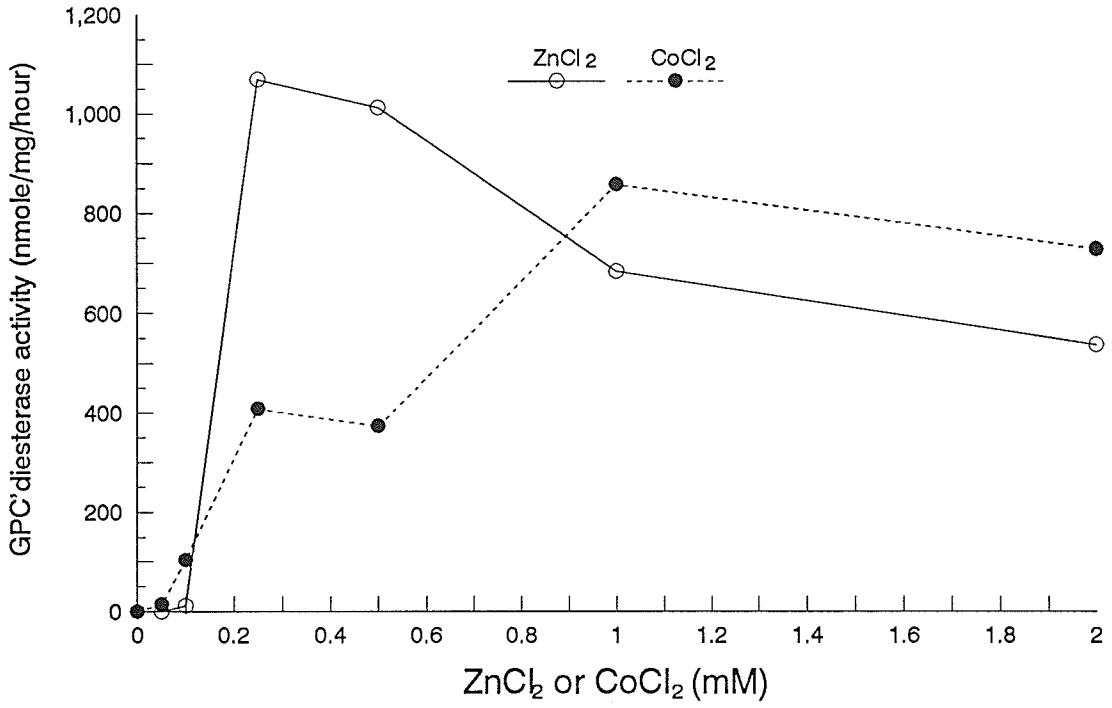

Figure 19. Effect of varying **$ZnCl_2$ or $CoCl_2$** concentrations on dialyzed purified GPC phosphocholine phosphodiesterase. Details are as in the text.

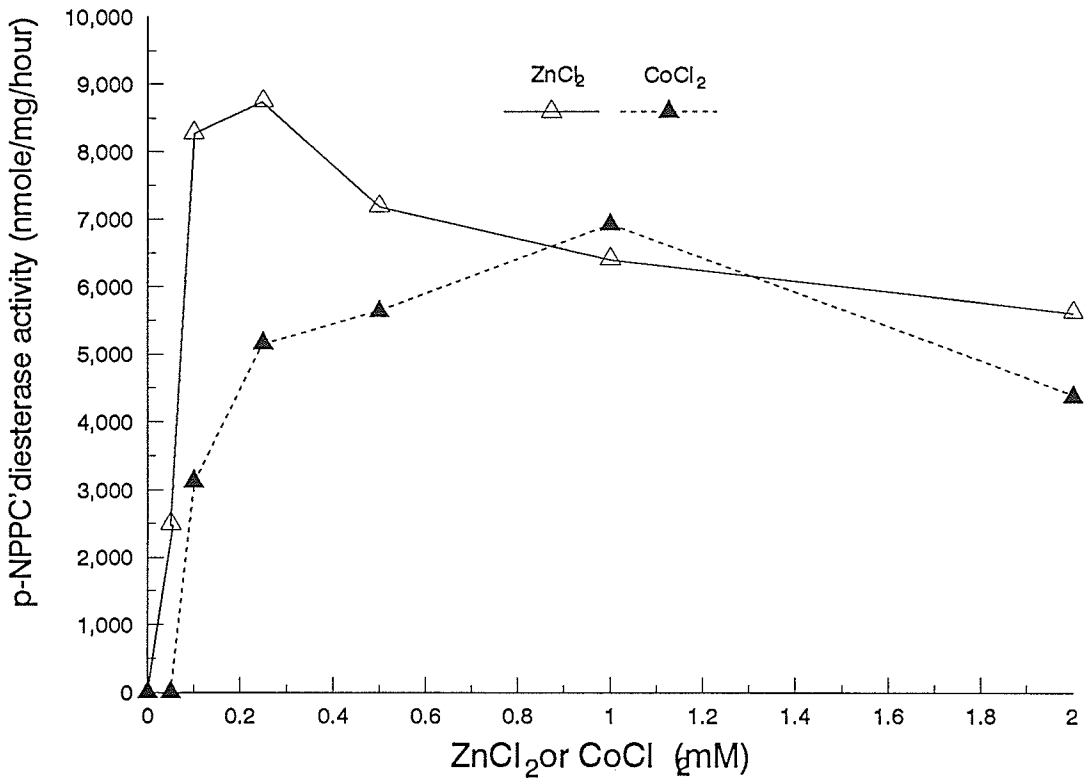

Figure 20. Effect of varying **ZnCl2 or CoCl2** concentrations on dialyzed purified p-NP phosphocholine phosphodiesterase. Details are as in the text.

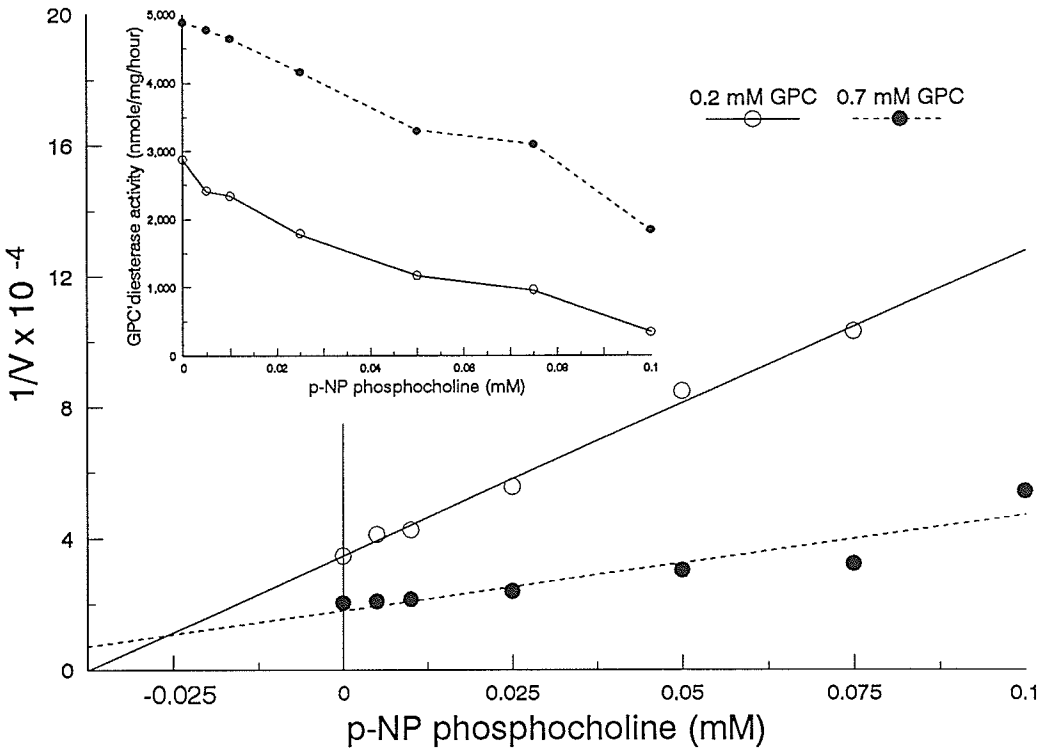

Figure 21. Inhibition of purified **GPC phosphocholine phosphodiesterase** by **p-NP phosphocholine.** A Dixon plot was used for determination of **Ki.** Details are provided in the text.

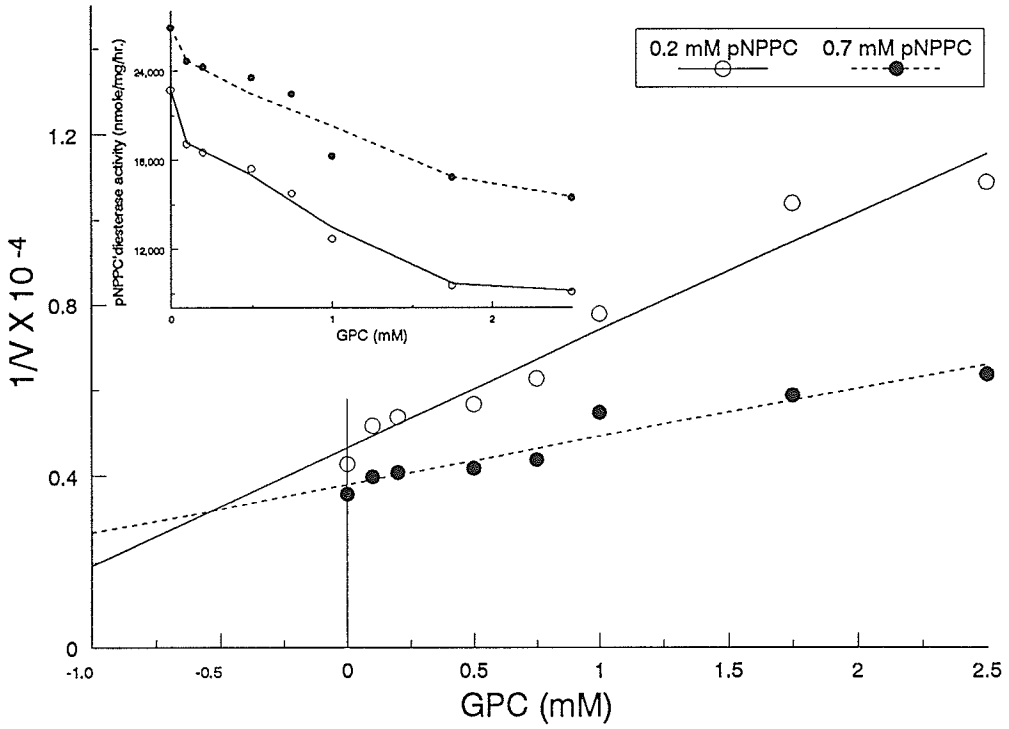

Figure 22. Inhibition of purified **p-NP phosphocholine phosphodiesterase** by **GPC.** A Dixon plot was used for determination of **Ki.** Details are provided in the text.

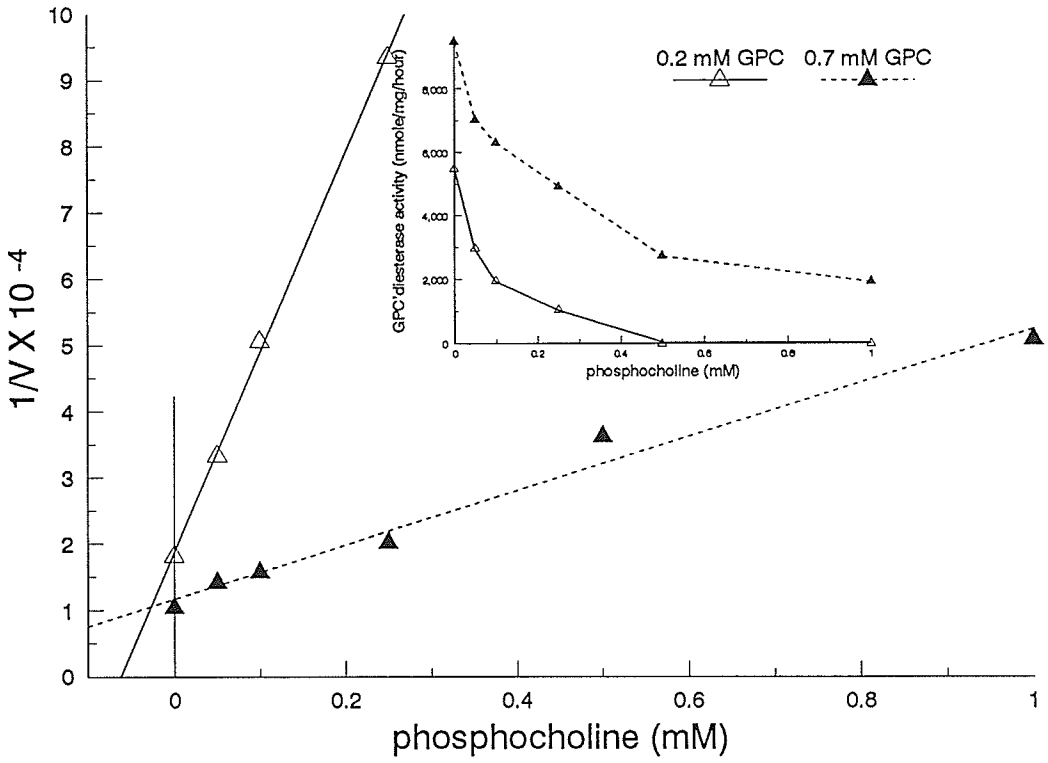

Figure 23. Inhibition of purified **GPC phosphocholine phosphodiesterase** by **phosphocholine**. A Dixon plot was used for determination of **Ki.** Details are provided in the text.

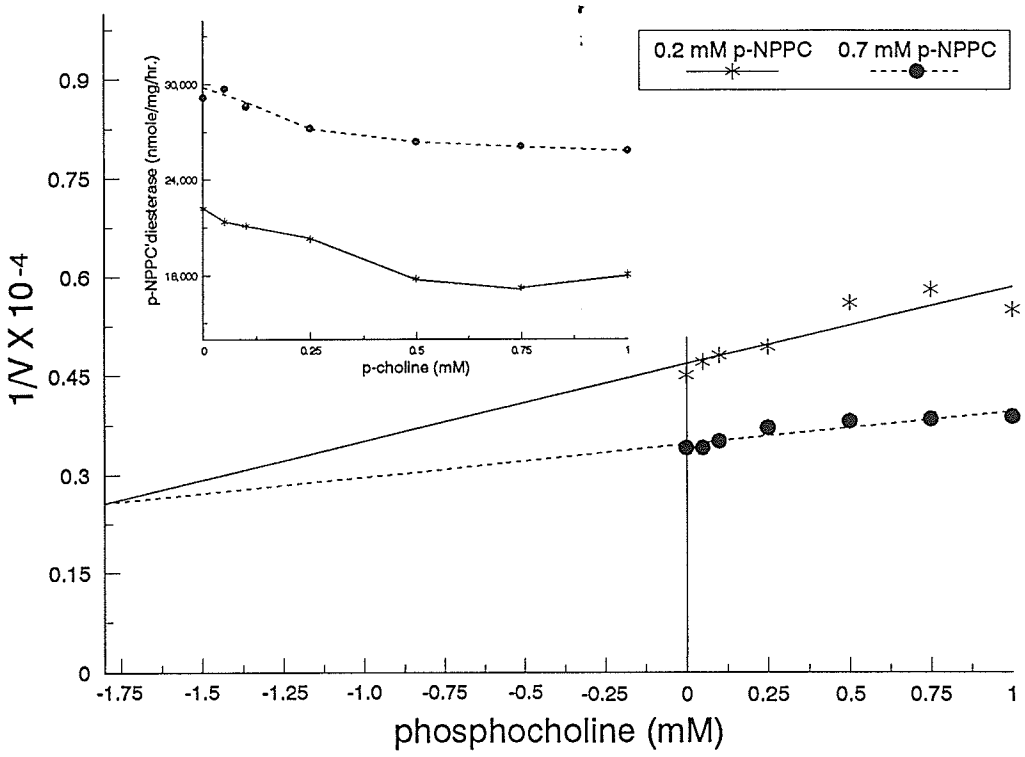

Figure 24. Inhibition of purified **p-NP phosphocholine phosphodiesterase** by **phosphocholine.** A Dixon plot was used for determination of **Ki.** Details are provided in the text.

IV. DISCUSSION

1. Cultured Cells and Dysmyelinating Rodent Mutants

CNPase and UDP galactose: ceramide galactosyltransferase (C gal T) are generally accepted as oligodendrocyte markers (Pfeiffer, 1984), even though the function and true substrate for CNPase *in vivo* remains uncertain (Vogel and Thompson, 1988). It is highly enriched in both oligodendrocyte and purified myelin (Kurihara and Tsukada, 1967; Takahashi, 1981). It is associated with oligodendrocytic membranes during the earliest phase of axonal myelination and is concentrated within specific regions of the myelin formation cells and myelin itself. Trapp et al. (1988) demonstrated that CNPase mRNA was mainly located around the perinuclear regions of oligodendrocytes during all stages of myelin formation, the CNPase gene products were expressed exclusively by CNS oligodendrocytes. C gal T is primarily localized in the cytoplasm and in processes of oligodendrocytes and in myelin sheaths of developing and adult rat brain (Roussel et al., 1987), and the highest activity of C gal T was observed in the endoplastic reticulum fractions of isolated oligodendrocyte (Sato et al., 1988). The activities of both enzymes are significantly decreased in the CNS tissues of the myelin-deficient rat, and of the jimpy and the quaking mice (Hogan and Greenfield, 1984). This evidence supported the view that the levels of CNPase or C gal T reflect the quantity of myelin and/or oligodendrocytes present. In the present study, the activities of CNPase and C gal T were employed as a basis for determining the enrichment in oligodendrocytes or myelin, and it was observed that the activities of these two enzymes were much higher in cultured pure oligodendrocytes as compared to those of mixed glial cell cultures or pure astrocytes. Similar results were obtained for GPC phosphocholine

phosphodiesterase and pNP phosphocholine phosphodiesterase activities showing that these two phosphodiesterases are also enriched in the pure oligodendrocytes (Table II). Based upon this information, it is difficult to distinguish between the possibility that a single enzyme utilizes both of these substrates or separate enzymes exist in myelin. The C-6 cell is a chemically induced tumor cell from a rat glioma possessing both astrocyte and oligodendroctye properties but not myelin properties (Parker et al., 1980) and it had been previously shown to have quite high CNPase activity as compared to cultured astrocytes (Volpe et al., 1975). As shown in the Table II, these C-6 cells had very low levels of C gal T, GPC phosphocholine and pNP phosphocholine phosphodiesterase activities but high CNPase activity (Table II). These enzyme determinations demonstrate that GPC phosphocholine and/or p-NP phosphocholine phosphodiesterase activities may be more reliable than CNPase as oligodendrocyte and myelin markers.

The myelin-deficient rat is a sex-linked recessive mutant. Morphological studies have indicated the presence of immature oligodendrocytes that fail to complete differentiation (Kumar et al., 1988) and also show poor maturation (Espinosa et al., 1990). Although oligodendrocyte progenitors are present, the total amount of mature cells is significantly decreased during development. Molecular biological studies showed that this dysmyelinating condition is similar to the dysmyelinating jimpy mouse (Barron et al., 1980; Dentinger et al., 1982; Boison and Stoffel, 1989), and both these animals have a base substitution in the myelin proteolipid protein gene. Biochemically, the md rat has significantly reduced activity of CNPase (Hof et al., 1984) and C gal T (Hof and Csiza, 1982). The reduction of GPC phosphocholine phophodiesterase and pNP phosphocholine phosphodiesterase activities in this mutant, especially in the

corpus callosum, the region with a high percentage of white matter, suggesting that these phosphodiesterases behave similarly to CNPase in the CNS (Figure 7 & 8). It was reported that the activities of CNPase and C gal T in the jimpy and the quaking mice were markedly diminished and the reduction of enzyme activity in the jimpy mouse was greater than that in the quaking mouse (Kurihara et al., 1970; Morell and Costantino-Ceccarini, 1972; Neskovic et al., 1970). These results are in agreement with those findings (Figure 9 & 10). A reduction was also observed for GPC and p-NP phosphocholine phosphodiesterases in these two mutant mice. There was a greater reduction of GPC phosphocholine phosphodiesterase activity in the jimpy mouse than in the quaking mouse (Figure 11 & 12). The GPC phosphocholine phosphodiesterase activity reductions in the jimpy and the quaking animals for the combined diencephalon plus mesencephalon tissues were 85% and 34%, respectively (Figure 11), and the reductions for p-NP phosphocholine phosphodiesterase were 86% and 42%, respectively (Figure 12). In the two PLP mutants the magnitudes of reduction in these two phosphodiesterases are similar in the areas enriched in white matter, the corpus callosum of the md rat and combined diencephalon plus mesencephalon of the jimpy mouse (Figure 8, 11 & 12).

There is a selective enrichment in oligodendrocyte cells of the GPC phosphocholine phosphodiesterase activity (Table II), and there is decrease in the activity in the three dysmyelinating mutants in the same ratio as for the other myelin marker enzymes.. These observations provide additional evidence that GPC phosphocholine phosphodiesterase is also a myelin marker enzyme. The results presented in Table II confirm that C gal T activity is an acceptable oligodendrocyte marker and that the low activity in isolated myelin is similar to that of astrocytes (Table II). Thus C gal T activity may not be a reflection of

myelin deposition, but rather an index of oligodendrocyte presence. If this is valid the three brain regions studied show that in the jimpy mutant only 11 +/- 2.3% and in the quaking mutant 19.6 +/- 5% of the oligodendrocyte marker C gal T activity remains (Figure 10). However, both the oligodendrocyte and isolated myelin (Table II) are enriched in GPC phosphcholine phosphodiesterase but the astrocyte and C-6 cells are not enriched in this activity. Therefore, if the average activity for the remaining C gal T activity is subtracted from that of the two phosphodiesterases activities, it should provide an estimate of remaining myelin. This value is between 2.1 to 5.1% for the jimpy mutant and 30 to 35% for the quaking mutant (Figure 10, 11 & 12). This comparison of detectable enzyme activities provides a relative quantitative estimation for the recognized morphological differences between the white matter defects in these two mutans.

It was reported that phospholipase C-type enzymes can be quantitated using an artificial substrate p-NP phosphocholine. This substance can be hydrolyzed by purified myelin and was shown to be a competitive inhibitor of GPC phosphocholine phosphodiesterase activity, suggesting that p-NP phosphocholine and GPC are common substrates for the same phosphocholine phosphodiesterase (Kanfer and McCartney, 1989a). This may explain the similar extent of diminution of the two phosphodiesterases activities in the jimpy and the quaking mice (Figure 11 & 12).

2. Enzyme Purification and Characterization

CNS myelin contains several major proteins, proteolipid protein (PLP) and myelin basic protein (MBP) which constitute 70-80% of total myelin protein. PLP is an integral membrane protein which is very hydrophobic and readily

aggregates under the different experimental condition (Lee, 1982), MBP is an extrinsic, hydrophilic membrane protein (Boggs and Moscarello, 1978). CNPase is a major myelin bound enzyme. The major difficulty for purification of an insoluble, myelin enzyme is its solubilization without loss of activity. If the enzyme could be kept in an aqueous environment, it is possible to apply classical chromatographic methods to purify the enzyme. Several methods have been reported for solubilizing the major myelin proteins. In the current study, Triton X-100 and OβG were employed for extraction of GPC phosphocholine phosphodiesterase from myelin. Triton X-100 is a nonionic detergent which in the presence of ammonium acetate, has been used to solubilize major myelin proteins, such as, PLP (Eng et al., 1968), CNPase (Sada and Tsukada, 1980; Wells and Sprinkle, 1981), pH 7.2 cholesterol ester hydrolase (Igarashi and Suzuki, 1977), and MBP (Eng et al., 1968). As shown in Table III, after lyophilized myelin was extracted with 1% Triton X-100 and ammonium acetate at alkaline pH, approximately 70% of the total myelin protein containing mainly PLP, CNPase, and some of MBP was separated from myelin-membrane as determined by SDS-PAGE. GPC phosphocholine and/or pNP phosphocholine phosphodiesterases were not extracted by 1% Triton X-100 even in the presence of ammonium acetate, indicating that this (these) enzyme (s) is (are) very hydrophobic proteins and tightly bound to the myelin-membrane. OβG is a nonionic detergent that can be easily removed by dialysis, and is nondenaturing with high solubilizing power (Baron and Thompson, 1974), has been used for solubilization of many membrane proteins, such as the lactose carrier of E. coli (Newman et al., 1981), H^+ translocating ATPase and the glutamate transport carrier of E. coli (Tsuchiya et al., 1982). For solubilization of the CNS myelin membrane protein, Aveldano et al. (1991) reported that OβG was a better agent

than any other nonionic detergents, and that the high salt concentration reduced the amount of detergent required through decreasing the free water available for interaction with OβG molecules. The GPC and p-NP phosphocholine phosphodiesterases could be extracted only by 200 mM OβG (Table III) which is far in excess of the OβG critical micelle concentration of about 25 mM. High concentration of salt could not decrease the concentration of OβG required for extraction, further supporting the idea that there is a close relationship between GPC phosphocholine phosphodiesterase and the myelin membrane. GPC and p-NP phosphocholine phosphodiesterase are extracted by OβG, but not extracted by Triton X-100 and ammonium acetate, This provides a simple method to separate these phosphodiesterases from the major myelin protein and make the purification easier. Nonionic detergent used in the extraction also makes ion-exchange chromatography, a frequently used technique for purification of membrane protein, possible.

Several chromatographic techniques have been tried to purify GPC phosphocholine phosphodiesterases. There was binding to a hydrophobic column of Octyl-Sepharose CL-4B by GPC and p-NP phosphocholine phosphodiesterase, but the results ware not consistent. Relatively high concentrations of detergent in extracts may contribute to this relatively non-reproducible results (data not shown). p-Aminophenyl phosphoryl choline affinity chromatography was used previously to purify the C-reactive protein which had the ability to bind phosphocholine (Pontet et al., 1977; Oliveira et al., 1980). Because p-NP phosphocholine and p-Aminophenyl phosphocholine have similar structures, p-Aminophenyl phosphoryl choline chromatography was tested as affinity column for the purification of GPC and p-NP phosphocholine phosphodiesterase. Unfortunately, under various conditons, the activities of both

phosphodiesterases were not retarded but found in the effluent. This may be due to the hydrolysis of the phosphodiester ligand of this column by these two phosphodiesterases. Q-Sepharose Fast Flow is an anion exchanger, and as shown in Figure 13, both phosphodiesterases were retarded on this column. Increasing the NaCl gradient from 0 to 1M or changing pH from 9.5 to 7.5 failed to elute the GPC and p-NP phosphocholine phosphodiesterase from the column indicating that Q-Sepharose may not be acting as an ion exchange gel. Non-dialyzed 200 mM OβG extracts did not bin to this column, but dialyzed sample were retarded on it and enzymes were eluted only when the column was washed with 100 mM OβG, strongly suggesting that there is hydrophobic interaction between the enzyme protein and this column gel. The ion exchange group[$-\backslash/\backslash-CH_2-N^+(CH_3)_3$] of Q-Sepharose has a similar structure to that of choline, and the enzyme protein may also bind to this column by affinity. OβG is easily removed by simple dialysis, the dialyzed pooled Q-Sepharose fractions had similar specifc activities to those of non-dialyzed samples for both phosphodiesterases, this suggests that the OβG had no effects on the enzyme activities per se.

The pooled Q-Sepharose fractions which contain both phosphodiesterases showed only one single silver staining band on 12% SDS-PAGE with a molecular weight of 14 kDa under denaturing conditions. This suggested that an homogenous enzyme protein possessing both GPC and p-NP phosphocholine phosphodiesterase activities was obtained. The enzyme was different from microsome GPC phosphocholine phosphodiesterase which has a dimeric structure of 120 kDa molecular weight (monomer 70 kDa) (Abra and Quinn, 1976). Several artificial bands with molecular weights ranging from 50 kDa to 68 kDa were observed when protein samples were analyzed on SDS-PAGE and

these bands were due to the presence of 2-mercaptoethanol or contaminating skin keratins in the sample solution (Tasheva and Dessev, 1983; Ochs, 1983). An artificial band with a molecular weight of 66 kDa was observed in the 200 mM OβG supernatant, the Q-Sepharose column fraction (Figure 14) and commercially purified MBP on 12% SDS-PAGE. This was partially avoided by addition of iodoacetamide to the sample prior to application to the SDS-PAGE gel (Beis and Lazou, 1990). Purified bovine brain myelin GPC phosphocholine and p-NP phosphocholine phosphodiesterases had a similar Km value of about 0.23 mM. These values were also similar to those of native myelin bound enzyme in rat brain (Kanfer and McCartney, 1989a). The concentration of water soluble GPC is about 0.4 mM to 0.6 mM in the brain (Jope and Jenden, 1979) which is 2 to 3 times the Km for these two phosphodiesterases. The pH optimum for solubilized GPC phosphocholine phosphodiesterase is 10 which is different from that of rat brain myelin enzyme, this may be due to removal of lipid surrounding the enzyme. A similar observation was also reported by Igarashi and Suzuki (1977) for the solubilizaton of rat brain cholesterol ester hydrolase pH 7.2. The pH optimum of p-NP phosphocholine phosphodiesterase is about 11 which is similar to that reported previously (Sok and Kim, 1990). As shown in Table V, zinc (micromineral) was definitely required for the activity of the purified bovine myelin GPC and p-NP phosphocholine phosphodiesterase, and the optimum concentration for restoration of activities of purified enzymes is similar to that of rat myelin enzyme. Cobalt had no restorative ability for rat myelin native enzyme (Kanfer and McCartney, 1989a), but could restore the activity of purified bovine phosphodiesterases (Table V). Sok and Kim (1990) reported that there was no endogenous substrate for p-NP phosphocholine phosphodiesterase in the CNS tissue, this conclusion seems to be incorrect, since the incubation system for their

enzyme assays contained 50 mM substrate p-NP phosphocholine, but only 1 mM glycerophosphorylcholine (GPC) as inhibitor. Our data has shown that GPC phosphocholine phosphodiesterase activity was inhibited in the presence of a similar substrate concentration of p-NP phosphocholine. In addition, GPC was a competitive inhibitor of p-NP phosphocholine phosphodiesterase (Figure 22), indicating that GPC is the endogenous substrate of p-NP phosphocholine phosphodiesterase. Phosphocholine inhibits the activities of both phosphodiesterases (Figure 23 and 24), presumably by a product inhibition. There was no inhibition of GPC or p-NP phosphocholine phosphodiesterase activities by glycerophosphorylethanolamine (GPE), phosphatidylethanolamine, phosphoethanolamine, and ethanolamine, indicating that these substances are not substrates for the two phosphodiesterases.

Several enzymes, such as CNPase, and pH 7.2 cholesterol ester hydrolase have been reported to be specifically associated with myelin (Suzuki, 1980; Norton, 1981b), even though the real function of these enzymes in myelin is still unknown. GPC phosphocholine phosphodiesterase is believed to be myelin specific (Kanfer and McCartney, 1989a). This observation was supported by studies in cultured cells and dysmyelinating rodent mutant (Yuan et al., 1992). The homogenous enzyme protein obtained by the current study provides direct evidence for the localization of this enzyme with myelin. In summary: The GPC phosphocholine and/or p-NP phosphocholine phosphodiesterase had similar activity distribution in the cultured cells, similar extent of reduction of activities in the dysmyelinating rodent mutants, similar degree of purification by Triton X-100, OβG, and ion-exchange chromatography, and similar enzymatic characteristics, these suggest that it is very likely that there is only one enzyme protein which can hydrolyze two substrates, GPC and p-NPPC. Although, the physiological

significance of GPC phosphocholine phosphodiesterase, like other myelin specific enzymes, is still unknown at this moment, the purification and characterization of this enzyme have already provided some useful information, and it is also the basis for further work on this enzyme, such as making antibodies for immunohistochemical studies, preparation of oligonucleotide probes for gene cloning. This will give more information concerning the real function of this unique myelin enzyme.

REFERENCES:

Abra RM, Quinn PJ (1975): A novel pathway for phosphatidylcholine catabolism in rat brain homogenates. *Biochim Biophys Acta* 380:436-441.

Abra RM, Quinn PJ (1976): Some characteristics of a glycero-3-phosphocholine diesterase from rat brain. *Biochim Biophys Acta* 431:631-639.

Alberghina M, Viola M, and Giuffrida AM (1982): Transfer of axonally transported phospholipids into myelin isolated from the rabbit optic pathway. *Neurochem Res* 7:139-149.

Aminoff MJ (1990): Multiple sclerosis. In Steven A, Schroeder MA, Krupp LM, Tiertney Jr, and Stephen JM (eds): "*Current medical diagnosis and treatment.*" Norwalk, Connecticut/San Mateo, Califorina. Appleton and Lange medical book. chapter 18, pp671-673.

Ansell GB, Spanner S (1982): Choline transport and metabolism in the brain. In Horrocks L, Ansell GB, Porcellati G (eds): "*Phospholipids in the Nervous System.*" I. Metabolism. New York: Raven Press, pp137-144.

Antel JP and Arnason BGW (1987): Demyelinating disease. In Braunwald E, Isselbacher KJ, Petersdorf RG, Wilson JD, Martin JB, and Fauci AS (eds): "*Harrison's principles of internal medine*" **11th Edition**, pp1995-2000.

Aveldano MI, Diaz RS, Requeiro P, and Monreal J (1991): Solubilization of myelin membranes by detergents. *J Neurochem* 57:250-257.

Baldwin JJ, Lanes P and Cornatzer WE (1968): Glycerylphosphorylcholine diesterase: effects of metal-binding agents. *Biochim Biophys Acta* 164:195-204.

Baron C, and Thompson TE (1974): Solubilization of bacterial membrane proteins using alkyl glucosides and dioctanoyl phosphatidylcholine. *Bichim et Biophys Acta* 382:276-285.

Barron KD, Dentinger MP, Csiza CK (1980): Ultrastructural observations on myelin deficiency (md), a dysmyelinating neurological mutant in the Wistar rat. In Baumann N (ed): "*Neurological Mutations Affecting Myelination.*" INSERM Symposium No. 14. Amsterdam: Elsevier/North-Holland Biomedical Press, pp 99-106.

Baumann N (1980): Mutations affecting myelination in the central nervous system: research tools in neurobiology. *Trends in NeuroSciences* April, pp 82-85.

Baumann NA, Jacque CM, Pollet SA, and Harpin ML (1968): Fatty acid and lipid composition of the brain of a myelin deficient mutant, the "quaking" mouse. *Eur J Biochem* 4:340.

Beis A and Lazou A (1990): Removal of artifactual band associated with the presence of 2-mercaptoethanol in two-dimensional polyacrylamide gel electrophoresis. *Analytical Biochem* 190:57-59.

Bellini T, Rippa M, Matteuzzi M, and Dallocchio F (1986): A rapid method for purification of myelin basic protein. *J Neurochem* 46:1644-1646.

Billings-Gagliardi S, and Adcock L (1981): Hypomyelinated mutant mice. IV. Peripheral myelin in *jp^{msd}*. *Brain Res* 225:309-317.

Blackwood W (1976): Normal structure and general pathology of the nerve cell and neuroglia. In Blackwood W, Corsellis JAN (eds): "*Greenfield's neuropathology*." London: Edward Arnold Ltd., pp 1-42.

Boggs JM, Moscarello MA (1978): Structure organization of the human myelin membrane. *Biochim Biophys Acta* 515:1-21.

Boggs JM, Moscarello MA, Papahadjopoulos D (1982): Structural organization of myelin—role of lipid-protein interactions determined in model systems. In Jost P, Griffith OH, (eds): "*Lipid-protein interactions*". New York: J. Wiley and Sons, Vol 2. pp 1-51.

Boison D, Stoffel W (1989): Myelin-deficicient rat: a point mutation in exon III (A—C, Thr75—Pro) of the myelin proteolipid protein causes dysmyelination and oligodendrocyte death. *EMBO J* 8:3295-3302.

Branmmer MJ (1978): The protein-mediated transfer of lecithin to subfractions of mature and developing rat myelin. *J Neurochem* 31:1435.

Bunge RP, Glass P (1965): Some observation on myelin-glial relationships and on the etiology of the cerebrospinal fluid exchange lesion. *Ann NY Acad Sci* 122:15-22.

Burks JS, De Vald BL, Jankovsky LD, Gerdes JC (1980): Two coronaviruses isolated from central nervous system tissue of two multiple sclerosis patients. *Science* 209:993-994.

Burt CT, Glonek T, and Barany M (1976): Phosphorus-31 nuclear magnetic resonance detection of unexpected phosphodiesterase in muscle. *Biochemistry* 15:4850-4853.

Cammer W, Fredman T, Rose AL, and Norton WT (1976): Brain carbonic anhydrase: Activity in isolated myelin and the effect of hexachlorophene. *J Neurochem* 27:165.

Carson JH, Barbarese E, Braun PE, and McPherson TA (1978): Components in multiple sclerosis cerebrospinal fluid that are detected by radioimmunoassay for myelin basic protein. *Proc Natl Acad Sci U.S.A.* 75:1976.

Carter HE, Rothfus JA, Gigg R (1961): Biochemistry of sphingolipid XII. *J Lipid Res* 2:228-234.

Clapshaw PA, Muller HW, and Seifert W. (1981): Characterization of 2'3' -cyclic nucleotide 3' phosphodiesterase: rapid isolation, native enzyme analysis, identification of serum-soluble activity, and kinetics. *J Neurochem* 36:1996-2003.

Costantino-Ceccarini E, Cestelli A (1981): A novel assay method for the biosynthesis of galactosyl and glucosylceramides. In Lowenstein JM (ed): "*Methods in Enzymology*." Vol. 72. New York: Academic Press, pp 384-391.

Costantino-Ceccarini E, Cestelli A, and DeVries GH (1979): Characterization and developmental changes of UDP-galactose ceramide galactosyl transferase in a rat CNS axolemma-enriched fraction. Differences and similarities of the enzyme associated with the microsomal and myelin fractions. *J Neurochem* 32:1175-1182.

Costantino-Ceccarini E and Suzuki K (1975): Evidence for presence of UDP-galactose:ceramide galactosyltransferase in rat myelin. *Brain Res* 93:358.

Csiza CK, deLahunta A (1979): Myelin deficiency (md): a neurologic mutant in the Wistar rat. *Am J Pathol* 95:215-224.

Davis FF and Allen FW (1956): A specific phosphodiesterase from beef pancreas. *Biochim Biophys Acta* 21:14-17.

Davison AW, Peters A (1970): Biochemistry of the myelin sheath. In Davison AN and Peters A (eds): " *Myelination*." Springfield, IL: Thomas, pp 80-161.

Dawson RMC (1955): The role of glycerylphosphorylcholine and glycerylphosphorylethanolamine in liver phospholipid metabolism. *Biochem J* 59:5-8.

Dawson RMC and Rowlands IW (1959): Glycerophosphorylcholine in the male reproductive organs of rats and guinea pigs. *Quart J Exp Physiol* 44:26-36.

Deber CM and Reynolds ST (1991): Central nervous system myelin: structure, function, and pathology. *Clinical Biochemistry* 24:113-134.

Deibler GE, Martenson RE, and Kies MW (1972): Large scale preparation of myelin basic protein from central nervous tissue of several mammalian species. *Prep Biochem* 2:139-165.

deFerra F, Engh H, Hudson L, Kamholz J, Puckett C, Molineaux S, and Lazzarini RA (1985): Alternative splicing accounts for the four forms of myelin basic protein. *Cell* 43:721-727.

Dentinger MP, Barron KD, Csiza CK (1982): Ultrastructure of the central nervous system in a myelin deficient rat. *J Neurocytol* 11:671-691.

Dentinger MP, Barron KD, Csiza CK (1985): Glial and axonal development in optic nerve of myelin deficient rat mutant. *Brain Res* 344:255-266.

Deshmukh DS, Bear WD, and Brockerhoff H (1978): Polyphosphoinositide biosynthesis in three subfractions of rat brain myelin. *J Neurochem* 30:1191.

Deshmukh DS, Kuizon S, Bear WD, and Brockerhoff H (1981): Rapid incorporation *in vivo* of intercerebrally injected ^{32}Pi into polyphosphoinositides of three subfractions of rat brain myelin *J Neurochem* 36:594.

Deshmukh DS, Kuizon S, Bear WD, and Brockerhoff H (1982): Polyphosphoinositide mono- and diphosphoesterases of three subfractions of rat brain myelin. *Neurochem Res* 7:617-626.

Dreiling CE (1981): Localization of 2'3' -cyclic nucleotide 3' phosphodiesterase in human erythrocyte membranes. *Biochim Biophys Acta* 649:587-594.

Dreiling CE, Schilling RJ, and Keitz RC (1981): 2'3' -cyclic nucleotide 3' phosphohydrolase in rat liver mitochondrial membranes. *Biochim Biophys Acta* 640:114-120.

Droz B, Di Giamberardino L, Koenig HL (1981): Contribution of axonal transport to the renewal of myelin phospholipids in peripheral nerves. I. Quantitative radioautographic study. *Brain Res* 219:57-71.

Drummond GI, Hamil EB, and Guha A (1978): Purification and comparison of 2'3' -cyclic nucleotide 3' -phosphohydrolases from bovine brain and spinal cord. *J Neurochem* 31:871-878.

Drummond GI, Iyer NT, and Keith J (1962): Hydrolysis of ribonucleoside 2'3' -cyclic phosphates by a diesterase from brain. *J Biol Chem* 237:3535-3539.

Druse MJ and Hogan EI (1972): Metabolism *in vivo* of brain galactolipids: The jimpy mutant. *J Neurochem* 19:2435.

Dunkley PR and Carnegie PR (1978): Isolation of myelin basic proteins. In Marks N and Rodnight R (eds): "*Research Methods in Neurochemistry*." Vol. 2 New York: Plenum Press, pp219-245.

Dupouey P, Lucas CV, Gomes D, and Jacque C (1980): Immunohistochemical localization of the myelin basic protein and of the glial fibrillary acidic protein: Comparative study in normal, quaking and jimpy mice. *J Neurosci Res* 5:387.

Endo T, and Hidaka H (1980): Ca^{2+}-calmodulin dependent phosphorylation of myelin isolated from rabbit brain. *Biochem Biophys Res Commun* 97:553.

Eng LH, Chao FC, Gerstl B, Pratt D, and Tavaststjerna MG (1968): The maturation of human white matter myelin. Fractionation of the myelin membrane proteins. *Biochemistry* 7:4455-4465.

Espinosa de los Monteros A, Roussel G, Nussbaum JL (1986): A procedure for long-term culture of oligodendrocytes. *Dev Brain Res* 24:117-125.

Espinosa de los Monteros A, Roussel G, Neskovic NM, Nussbaum JL (1988): A chemically defined medium for the culture of mature oligodendrocytes. *J Neurosci Res* 19:202-211.

Espinosa de los Monteros A, Zhang M, Gordon MN, Kumar S, Scully S, de Vellis J (1990): The myelin-deficient rat mutant: partial recovery of oligodendrocyte maturation in vitro. *Dev Neurosci* 12:326-339.

Eto Y and Suzuki K (1971): Cholesterol ester metabolism in rat brain: Properties and subcellular distribution of cholesterol-esterifying enzymes and cholesterol ester hydrolases in adult rat brain. *Biochim Biophys Acta* 239:293.

Eto Y and Suzuki K (1973): Cholesterol ester metabolism in rat brain: A cholesterol ester hydrolase specifically localized in the myelin sheath. *J Biol Chem* 248:1986.

Eylar EH, Kniskern PJ, and Jackson JJ (1974): Myelin basic protein. *Methods Enzymology* 32:323-341

Eylar EH, Salk J, Beveridge GC, and Brown LV (1969): Experimental allergic encephalomyelitis: an encephalitogenic basic protein from bovine myelin. *Arch Biochim Biophys* 132:34.

Fonnum F (1975): Radiochemical assays for choline acetyltransferase and acetylcholinesterase. In Marks N and Rodnight R (eds): "*Research Methods in Neurochemistry*." Vol. 3 pp. 253-275.

Gould RM, Spivack WD, Sinatra RS, Lindquist TD, and Ingoglia NA (1982): Axonal transport of choline lipids in normal and regenerating rat sciatic nerve. *J Neurochem* 39:1569-1578.

Gopfert E, Pytlik S, and Debuch H (1980): 2'3' -cyclic nucleotide 3' phosphohydrolase and lipids of myelin from multiple sclerosis and normal brain. *J Neurochem* 34:732.

Gottlieb M and Chavko M (1987): Silver staining of native and denatured eucaryotic DNA in agarose gels. *Analytical Biochemistry* 165:33-37.

Greenfield S, Norton WT, and Morell P (1971): Quaking mouse: isolation and characterization of myelin protein. *J Neurochem* 18:2119-2128.

Guha A and Moore S (1975): Solubilization of 2'3' -cyclic nucleotide 3' -phosphohydrolase from bovine brain without detergents. *Brain Res* 89:279-286.

Gupta MN, and Wold F (1980): A convenient spectrophotometric assay for phospholipase D using p-Nitrophenylphosphocholine as substrate. *Lipids* 15:594-596.

Haley JE, and Ledeen RW (1979): Incorporation of axonally transported substances into myelin lipids. *J Neurochem* 32:735-742.

Hauser SL, Bhan AK, Gilles F, Kemp M, Kerr C, Heiner HL (1986): Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. *Ann Neurol* 19:578-587.

Hawley DM, Schulz AR, and Hodes ME (1984): A comparative kinetic analysis of 6 substrates widely used for the detection and quantitation of phosphodiesterase I. *Enzyme.* 32:105-109.

Helynck G, Luu B, Nussbaum JL, Picken D, Skalidis G, Trifilieff E, Dorsselaer AV, Seta P, Sandeaux R, Gavach C, Heitz F, Simon D, and Spach G (1983): Brain proteolipids-Isolation, purification and effect on ionic permeability of membranes. *Eur J Biochem* 133:689-695.

Hof L, Csiza CK (1982): Developmental changes in glycolipid synthesizing enzymes in the brain of a myelin-deficient mutant Wistar rat. *J Neurochem* 39:1434-1439.

Hof L, Csiza CK, Dentinger MP, Barron KD (1984): 2'3' -cyclic nucleotide 3' phosphodiesterase and 5' -nucleotidase in the central nervous system of a myelin-deficient rat mutant. *J Neurochem* 42:1767-1770.

Hogan EL, Greenfield S (1984): Animal model of genetic disorders of myelin. In Morell P (ed): "*Myelin*" New York: Plenum Press, pp 489-534.

Hynie I and Zbarsky SH (1970): Some properties of phosphodiesterase I from microsomes of rat intestine. *Can J Biochem* 48:1151-1159.

Igarashi M, and Suzuki K (1976): Effect of exogenous lipids on activities of the rat brain cholesterol ester hydrolase located in the myelin sheath. *J Neurochem* 27:859-866.

Igarashi M, and Suzuki K (1977): Solubilization and characterization of the rat brain cholesterol ester hydrolase localized in the myelin sheath. *J Neurochem* 28:729-738.

Itoyama Y, Sternberger NH, Webster HdeF, Quarles RH, Cohen SR and Richardon EP (1980): Immunocytochemical observation on the distribution of myelin-assciated glycoprotein and myelin basic protein in multiple sclerosis lesions. *Ann Neurol* 7:167.

Janzen L, Tourtellotte WW and Kanfer JN (1990): Glycerylphosphocholine phosphocholine phosphodiesterase activity is reduced in multiple sclerosis plaques. *Exp Neurol* 109:243-246.

Johnson RT, McArthur JC (1986): AIDS and the brain. *Trends Neurol Sci* 9:91-94.

Jope RS and Jenden DJ (1979): Choline and phospholipid metabolism and the synthesis of acetylcholine in rat brain. *J Neurosci Res* 4:69-82.

Jungalwala FB (1974): The turnover of myelin phosphatidylcholine and sphingomyelin in the adult rat brain *Brain Res* 78:99.

Kamholz J, deFerra F, Puckett C, and Lazzarini R (1986): Identification of three forms of human myelin basic protein by cDNA cloning. *Pro. Natl. Acad. Sci USA* 83:4962-4966.

Kanfer JN, McCartney DG (1989a): Glycerophosphorylcholine phosphocholine phosphodiesterase activity of rat brain myelin. *J Neurosci Res* 24:231-240.

Kanfer JN, McCartney DG (1989b): Regional and developmental estimations of glycerophosphorylcholine phosphodiesterase activities in rat brain. *Dev Neurosci* 11:26-29.

Kanfer JN, McCartney DG (1990): An unusual phosphodiesterase activity towards p-nitrophenylphosphorylcholine present in rat brain membranes. *Neurochem Res Vol 15 No 10* pp987-992.

Kanfer JN, and Stein M (1972): Sulfatide biosynthesis by intact microsomes and triton extracts of normal and "quaking" mouse brain. *Lipids* 7:259.

Kelly SJ, Dardinger DE, and Butler LG (1975): Hydrolysis of phosphonate esters catalyzed by 5'-nucleotide phosphodiesterase. *Biochemistry* 14:4983.

Kennedy EP (1961): Biosynthesis of complex lipids. *Fed Proc Fed Am Soc Exp Biol* 20:934-940.

Knapp PE, Skoff RP, and Redstone DW (1986): Oligodendroglial cell death in jimpy mice: an explanation for the myelin deficit. *J Neurosci* 6:2813-2822.

Kreutzberg G, Barron KD, and Schubert P (1978): Cytochemical localization of 5' -nucleotidase in glial plasma membranes. *Brain Res* 158:247.

Kumar S, Gordon MN, Espinosa de Los Monteros A, de Vellis J (1988): Developmental expression of neural cell type-specific mRNA markers in the myelin-deficient mutant brain: inhibition of oligodendrocyte differentiation. *J Neurosci Res* 21:268-274.

Kunishita T, Morrow CW, and Ledeen RW (1983): Detection of ethanolamine kinase and choline kinase in purified rat brain myelin. *J Neurochem* 41:S48A.

Kunishita T, and Ledeen RW (1982): Evidence for the presence of CTP: phosphoethanolamine cytidyltransferase in rat brain myelin. *Trans Am Soc Neurochem* 192 Abstract.

Kunishita T, and Ledeen RW (1984): Phospholipid biosynthesis in mylein: presence of CTP:phosphoethanolamine cytidylyltransferase in purified myelin of rat brain. *J Neurochem* 42:326-333.

Kurihara T, Nussbaum JL, Mandel P (1970): 2'3' -cyclic nucleotide 3' phosphohydrolase in brain of mutant mice with deficient myelination. *J Neurochem* 17:993-997.

Kurihara T, Tsukada Y (1967): The regional and subcellular distribution of 2'3' -cyclic nucleotide 3' -phosphodiestease. *J Neurochem* 14:1167-1174.

Kuriota S (1968): Synthesis of p-nitrophenylphosphorylcholne and the hydrolysis with phospholipase. *Can. J Biochem* 63:678-680.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of head bacteriophage T4. *Nature* 227:680-685.

Landt M and Buther L (1978): 5'-nucleotide phosphodiesterase: isolation of covalently bound 5'-adenosine monophosphate, an intermediate in the catalytic mechanism. *Biochemistry* 17:4130.

Laursen RA, Samiullah M, Lees MB (1984): The structure of bovine brain myelin proteolipid and its organization in myelin. *Proc Natl Acad Sci USA* 81:2912-6.

Ledeen RW (1984): Lipid-metabolizing enzymes of myelin and their relation to the axon. *J Lipid Res* 25:1548-1554.

Ledeen RW, and Wu PS (1979): Evidence for the presence of CDP-choline: 1,2-diradyl-*sn*-glycerol choline phosphotransferase and CDP-ethanolamine: 1,2-diradyl-*sn*-glycerol ethanolamine phosphotransferase in rat CNS myelin. *7th Int Meet Int Soc Neurochem*, Jerusalem 117 Abstract.

Lees MB (1982): Proteolipids. Scand. *J Immunol* 15(9):147-166.

Lees MB, Brostoff SW (1984): Protein of myelin In Morell P (ed): "*Myelin*" New York: Plenum Press, pp 197-211.

Lees MB, and Sakura JD (1978): Preparation of proteolipids. In Marks N and Rodnight R (eds): "*Research Methods in Neurochemistry*." Vol. 4 New York: Plenum Press, pp354-370.

Lin LF, Bartlett C, and Lees MB (1987): Topography of proteolipid protein and CNPase in unilamellar myelin vesicles. (Abstr.) *J Neurochem* 48 (Suppl.), S33A.

Lisak RP, Pleasure DE, Silberberg DH, Manning MC, Saida T (1981): Long term culture of bovine oligodendroglia isolated with a percoll gradient. *Brain Res* 223:107-122.

Lowry OH, Rosebrough NJ, Farr AL, Randall RS (1951): Protein measurement with the Folin phenol reagent. *J Bio Chem* 193:265-275.

Martenson RE, Deibler GE, and Kies MW (1971): Microheterogeneity and species-related differences among myelin basic proteins. In Rowland LP (ed): "*Immunological Disorders of the Nervous System*" Williams and Wilkins, Baltimore.

Maugh TH (1977): The EAE model: a tentative connection to multiple sclerosis. *Science* 195:969-971.

McCarthy KD, de Vellis J (1980): Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 85:890-902.

Mikoshiba K, Kohsaka S, Hayakawa T, Takamatsu K, and Tsukada Y (1985): Immunohistochemical localization of 2'3'-cyclic nucleotide 3'- phosphodiesterase and myelin basic protein in the central nervous system of the jimpy and the normal mouse. *J Neurochem* 44:686-691.

Miller EK and Dawson RMC (1972): Exchange of phospholipids between brain membranes and *in vitro*. *Biochem J* 126:823.

Miller SL, Benjamin JA, and Morell P (1977): Metabolism of glycerophospholipids of myelin and microsomes in rat brain. *J Biol Chem* 252:4025-4037.

Miller SL, and Morell P (1978): Turnover of phosphatidylcholine in microsomes and myelin of young rats. *J Neurochem* 31:771-777.

Morell P, and Radin NS (1969): Synthesis of cerebroside by brain from uridine diphosphate galactose and ceramide containing hydroxy fatty acid. *Biochemistry* 8:506-512.

Morell P, and Costantino-Ceccarini E (1972): Jimpy mouse: *in Vitro* studies of brain sphingolipid biosynthesis. *Lipids* 7:266.

Muller HW, Clapshaw PA, and Seifert W (1981): Intracellular localization of 2'3' -cyclic nucleotide 3' phosphodiesterase in a neuronal cell line as examined by immunofluorescence and cell fractionation. *J Neurochem* 37:947-955.

Neskovic N, Nussbaum JL, Mandel P (1970): A study of glycolipid metabolism in myelination disorder of jimpy and quaking mice. *Brain Res* 21:39-53.

Neskovic NM, Sarlieve LL, and Mandel P (1973): Subcellular and submicrosomal distribution of glycolipid synthesizing transferases in young rat brain. *J Neurochem* 20:1419-1430.

Newcombe J, Glynn P, and Cuzner ML (1982): The immunological identification of brain proteins on cellulose nitrate in human demyelinating disease. *J Neurochem* 38:267.

Newman MJ, Foster DL, Wilson TH, and Kaback HR (1981): Purification and reconstitution of functional lactose carrier from *Escherichia Coli*. *J Biol Chem* 256:11804-11808.

Newman RJ, Bore PJ, Chan L, Gadian DG, Styles P, Taylor D, and Radda GK (1982): Nuclear magnetic resonance studies of forearm muscle in Duchenne dystrophy. *Brit Med J* 284:1072-1074.

Nishizawa Y, Kurihara T, and Takahashi Y (1981): Immunohistochemical localization of 2'3' -cyclic nucleotide 3' phosphodiesterase in the central nervous system. *Brain Res* 212:219-222.

Norton WT (1981a): Formation, structure, and biochemistry of myelin. In Siegel GJ et al., (eds): "*Basic neurochemistry*." (third edition). Boston: Little, Brown and Company, pp 63-92.

Norton WT (1981b): Biochemistry of myelin. In Waxman SG, Ritchie JN (eds): " Demyelinating Disease: Basic and Clinical Electrophysiology." New York: Raven Press, pp 93-121.

Norton WT, Autilio LA (1966): The lipid composition of purified bovine brain myelin. *J Neurochem* 13:213.

Norton WT, Cammer W (1984a): Isolation and characterization of myelin. In Morell P (ed): "*Myelin*" New York: Plenum Press, pp 147-195.

Norton WT, Cammer W (1984b): Chemical pathology of diseases involving myelin. In Morell P (ed): "*Myelin*" New York: Plenum Press, pp 369-403.

Norton WT, Farooq M, Fields KL, Raine CS (1983): The long term culture of bulk-isolated bovine oligodendroglia from adult brain. *Brain Res* 270:295-310.

Norton WT and Poduslo SE (1973): Myelination in rat brain: method of myelin isolation. *J Neurochem* 21:749-757.

Nussbaum JL and Mandel P (1973): Brain proteolipids in neurological mutant mice. *Brain Res* 61:295-310.

Nussbaum JL, Neskovic N, and Mandel P (1969): A study of lipid components in brain of the "jimpy" mouse, a mutant with myelin deficiency. *J Neurochem* 16:927.

Ochs D (1983): Protein contaminants of sodium dodecyl sulfate- polyacrylamide gels. *Analytical Biochem* 135:470-474.

Oliveira EB, Gotschlich EC, and Liu TY (1980): Comparative studies on the binding properties of human and rabbit C-reactive proteins. *J Immunol* 124:1396-1402.

Parker KK, Norenberg MD, and Vernadakis A (1980): Trans-differentiation of C-6 glia cell in culture. *Science* 208:179-181.

Pfeiffer SE (1984): Oligodendrocyte development in culture systems. In Norton WT (ed): "*Oligodendroglia*." New York: Plenum Press, pp 233-298.

Phillips JSR (1954): Jimpy: A new totally sex-linked gene in the house mouse. *Arch Indukt Abstammungs-Vererbungsl* 86:322.

Porcellati G, Biasion MG, and Pirotta M (1970): The labeling of brain ethanolamine phosphoglycerides from cytidine diphosphateethanolaamine *in vitro. Lipids* 5:734.

Poduslo JF,Quarles RH, and Brady RO (1976): External labeling of galactose in surface membrane glycoproteins of the intact myelin sheath. *J Biol Chem* 251:153-158.

Pontet M, Engler R, and Jayle MF (1977): One step preparation of both human C-reactive protein and Clt. *FEBS Lett* 88:172-175.

Quarles RH (1983): Myelin-associated glycoprotein in development and disease. *Dev Neurosci* 6:285-303.

Raine CS (1984a): Morphology of myelin and myelination. In Morell P (ed): "*Myelin*" New York: Plenum Press, pp 1-50.

Raine CS (1984b): Neuropathology of myelin diseases. In Morell P (ed): "*Myelin*" New York: Plenum Press, pp 259-310.

Rapport MM, Norton WT (1962): Chemistry of lipids. *Annu Rev Biochem* 31:103.

Reynolds R, Carey EM, and Herschkowitz N (1987): Differential localization of MBP and CNP in a myelin-like membrane produced by oligodendrocytes in culture. (Abstr.) *J Neurochem* 48 (Suppl), S179B.

Reynolds R, Carey EM, and Herschkowitz N (1989): Differential immunohistochemical localization of myelin basic protein and 2'3' -cyclic nucleotide 3' phosphodiesterase in a myelin-like membrane produced by isolated oligodendrocytes in culture. *Neuroscience* 28 (1):181-188.

Riekkinen PJ, Rinne UK, Arstila AU, Kurihara T, and Pelliniemi TT (1972): Studies on the pathogenesis of multiple sclerosis: 2'3' -cyclic nucleotide 3' phosphohydrolase as a marker of demyelination and correlation of findings with lysosomal changes. *J Neurol Sci* 15:113.

Roussel G, Nussbaum JL, Espinosa de los Monteros, and Neskovic NM (1987): Immunocytochemical location of UDP-galactose:ceramide galactosyltransferase in myelin and oligodendroglial cells of rat brain. *J Neurocytol* 16:85-92.

Rumsby MG, Getliffe HM, and Riekkinen PJ (1973): On the association of non-specific esterase activity with central nerve myelin preparations. *J Neurochem* 21:959.

Sato S, Black JA, Yu RK (1988): Subcellular distribution of UDP-galatose: ceramide galactosyltransferase in rat brain oligodendroglia. *J Neurochem* 50:1887-1893.

Sato S, Quarles RH, and Brady RO (1982): Susceptibility of the myelin-associated glycoprotein and basic protein to the neutral proteinase in highly purified myelin from human and rat brain. *J Neurochem* 39:97.

Sedmak JJ and Grossberg SE (1977): A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal Biochem* 79:544-552.

Silbert SW, and Goldstein MN (1972): Drug-induced differentiation of a rat glioma *in Vitro*. *Cancer Res* 32:1422.

Sidman RL, Dickie MM, and Appel SH (1964): Mutant mice (quaking and jimpy) with deficient myelination in the central nervous system. *Science* 144:309-310.

Sidman RL, Green MC, and Appel SH (1965): Catalogue of the neurological mutants of the mouse. *Harvard University Press*, Cambridge, Mass 40-42.

Sims NR, Carnegie PR (1978): 2'3' -cyclic nucleotide 3' phosphodiestease. *Adv Neurochem* 3:1-41.

Smith R, Cook J, and Dickens PA (1984): Structure of the proteolipid protein extracted from bovine central nervous system myelin with nondenaturing detergents. *J Neurochem* 42:306-313.

Sogin DC (1976): 2'3' -cyclic NADP as a substrate for 2'3' -cyclic nucleotide 3' phosphodiesterase. *J Neurochem* 27:1333-1337.

Sok DE and Kim MR (1990): A p-nitrophenyl phosphorylcholine phosphodiesterase from mouse brain. *Biochem and Biophys Res Commun* 172:(3)1317-1323.

Spanner S, Ansell GB (1982): Activation of glycerophosphocholine diesterase in rat by Ca^{2+}. *Biochem J* 208:845-850.

Spanner S, Ansell GB (1987): The hydrolysis of glycerophosphorylcholine by rat brain microsomes: Activation and inhibition. *Neurochem Res* 12:203-206.

Spitler LE, von Muller CM, Fudenberg HH, Eylar EH (1972): Experimental allergic encephalitis. Dissociation of cellular immunity to brain protein and disease production. *J Exp Med* 136:156-174.

Sprinkle TJ, Tippins RB, Hancock JP (1988): Observation on an interesting apparent relationship between brain and brain CNPase. *Trans Am Soc Neurochem* 19:115.

Sprinkle TJ, Tippins RB, and Kestler DP (1987): Inhibition of bovine and human brain 2'3' -cyclic nucleotide 3' phosphodiesterase by heparin and polyribonucleotides and evidence for an associated 5'-polynucleotides kinase activity. *Biochem Biophys Res Commun* 145:686-691.

Sprinkle TJ, Zaruba ME, Mckhann GM (1978): Activity of 2'3' -cyclic nucleotide 3' phosphodiesterase in regions of rat brain during development: Quantitative relationship to myelin basic protein. *J Neurochem* 30:309-314.

Starich GH and Dreiling CE (1980): Simultaneous inhibition of guinea pig brain 2'3' -cyclic nucleotide 3' phosphohydrolase and myelin protein synthesis by 2'-adenosine monophophate. *Life Sci* 27:567-572.

Steck AJ, and Appel SH (1974): Phosphorylation of myelin basic protein. *J Biol Chem* 249:5416.

Stowring L, Haase AT, Petursson G et al., (1985): Detection of visna virus antigens and RNA in glial cells in foci of demyelination. *Virology* 141:311-318.

Suda H, and Tsukada Y (1980): Improved method for purification of 2'3' -cyclic nucleotide 3' phosphohydrolase from bovine cerebral white matter. *J Neurochem* 34:941-949.

Suzuki K (1980): Myelin-associated enzymes. In Baumann N (ed): " *Neurological Mutations Affecting Myelination.* " INSERM Symposium No. 14 Amsterdam: Elsevier/North-Holland Biomedical Press, pp 333-347.

Suzuki K, Kamoshita S, Eto Y, Tourtellotte WW and Gonatas JO (1973): Myelin in multiple sclerosis. *Arch Neurol* 28:293.

Szuchet S, Stefansson K, Wollmann RL, Dawson G, Arnason BGW (1980): Maintenance of isolated oligodendrocytes in long term culture. *Brain Res* 200:151-164.

Takahashi Y (1981): 2'3' -cyclic nuceotide 3' phosphodiesterase: an oligodendrocyte-myelin marker enzyme. *TINS* 4:8-12.

Tasheva B and Dessev G (1983): Artifacts in sodium dodecyl sulfate— polyacrylamide gel electrophoresis due to 2-mercaptoethanol. *Analytical Biochem* 129:98-102.

Trapp BD, Bernier L, Andrews SB, Colman DR (1988): Cellular and subcelluar distribution of 2'3' -cyclic nucleotide 3' phosphodiesterase and its mRNA in the rat central nervous system. *J Neurochem* 51:859-868.

Traugott U, and Raine CS (1981): Auti-oligodendrocyte-antibodies in cerebrospinal fluid of multiple sclerosis and other neurologic diseases. *Neurology* 31:695.

Tsuchiya T, Misawa A, Miyake Y, Yamasaki K, and Niiya S (1982): Solubilization and reconstitution of membrane energy-transducing systems of Escherichia Coli. *FEBS Lett* 142:231-234.

Tyc L, Kellenberger C, Filipowicz W (1987): Purification and characterization of wheat germ 2'3' -cyclic nucleotide 3' phosphodiesterase. *J Biol Chem* 262:12994-13000.

Vogel US, Thompson RJ (1988): Molecular structure, localization, and possible functions of the myelin-associated enzyme 2'3' -cyclic nucleotide 3' phosphodiesterase. *J Neurochem* 50:1667-1677.

Volpe JJ, Fujimoto K, Marasa JC, Agrawal HC (1975): Relation of C-6 glial cells in culture to myelin. *Biochem J* 152:701-703.

Waksman BH (1981): Current trends in multiple sclerosis research. *Immunol Today* 2:87.

Waksman BH (1983): Immunity and the nervous system basic tenets. *Ann Neurol* 13:587-591.

Weissbarth S, Marker HS, Raes I, Brannan TS, Lapin EP, Lehrer GM (1981): The activity of 2'3' -cyclic nucleotide 3' phosphodiesterase in rat tissues. *J Neurochem* 37:677-680.

Wells MR and Sprinkle TJ (1981): Purification of rat 2'3' -cyclic nucleotide 3' phosphodiesterase. *J Neurochem* 36:633-639.

Whitfeld PR, Heppel LA, and Markham R (1955): The enzymic hydrolysis of ribonucleoside 2'3' phosphates. *Biochem J* 60:15-19.

Wolfgram F and Tourtellotte WW (1972): Amino acid composition of myelin in multiple sclerosis. *Neurology* 22:1044.

Wu PS, and Ledeen RW (1980): Evidence for the presence of CDP-ethanolamine:1,2-diacyl-*sn*-glycerol ethanolaminephosphotransferase in rat central nervous system myelin. *J Neurochem* 35:659-666.

Yanagisawa K, Duncan ID, Hammang JP, and Quarles RH (1986): Myelin-deficient rat: analysis of myelin proteins. *J Neurochem* 47:1901-1907.

Yoshino JE, Griffin JW, and De Vries GH (1983): Identification of an axolemma-enriched fraction from peripheral nerve. *J Neurochem* 41:1126-1130.

Yuan J, McCartney DG, Monge M, Espinosa de Los Monteros A, Zalc B, de Vellis J, and Kanfer JN (1992): Glycerophosphorylcholine phosphocholine phosphodiesterase activity in cultured oligodendrocytes, astrocytes, and central nervous tissue of dysmyelinating rodent mutants. *J Neurosci Res* 31:68-74.

Zanetta JP, Benda P, Gombos G, Morgan IG (1972): The presence of 2'3' -cyclic AMP 3'-phosphohydrolase in glial cells in tissue culture. *J Neurochem* 19:881-883.

Zimmerman TR and Cohen SR (1979): The distribution of myelin basic protein in subcellular fractions of developing jimpy mouse brain. *J Neurochem* 32:817.