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ASSAY AND PROPERTIES OF PLASMALOGENASE
UNDER NORMAL AND PATHOLOGICAL CONDITIONS

DISSertation

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Robert Anthony D'Amato, B.S.

The Ohio State University
1974

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Chapter I  HISTORICAL REVIEW

Plasmalogen: discovery and structure

Plasmalogens (Fig 1) were discovered accidentally by Feulgen and Voit in 1924, when they were staining cell nuclei with fuchsin-sulfurous acid. Kidney slices were dehydrated with alcohol, treated with acid to hydrolyze the deoxyribonucleic acid and finally exposed to the fuchsin-sulfurous acid. This gave a red-violet complex with the liberated deoxyribose. When fresh tissue which had been placed into a solution of HgCl₂ for preservation was erroneously put into fuchsin-sulfurous acid without fixation or acid treatment, Feulgen found that the plasma of the cells was stained instead of the nuclei. Feulgen concluded that an aldehyde must be present in the plasma of the cells. Feulgen named this aldehyde - because of its presence in the plasma of the cell - "plasmal" and the unknown mother substance releasing this aldehyde - "plasmalogen". After this discovery, Feulgen and co-workers examined many other tissues. Plasmalogen was found in nearly every kind of tissue (Stepp et al., 1927), but was particularly concentrated in muscle, cerebellum, and cerebral white matter. Feulgen and Berson (1939) isolated a plasmalogen-like substance, acetalphosphatide (Fig 2), after treatment of the extracted phospholipids with concentrated alkali. Thus, the ester linkages of the choline, ethanolamine or serine phosphoglycerides were hydrolyzed, while the acetal linkages formed from the plasmalogens were stable. Under these conditions, the fatty acids were saponified to
Fig. 1. Ethanolamine Plasmalogen

Fig. 2. Acetalphosphatide
soaps and neither they nor the choline or ethanolamine esters of glycerophosphoric acid were soluble in organic solvents, but the aldehyde-containing phosphatides were. Feulgen and Bersion (1939) realized that their product was not identical to the original plasmalogen. For the next 15 years, researchers attempted various reaction schemes to separate plasmalogens from diacyl phosphoglycerides. Even today, 35 years later, no method is available for separation of plasmalogens from other phospholipids without chemical change of one or the other.

Klenk and Debuch (1954) demonstrated that the ethanolamine plasmalogens of human brain contained a fatty acid moiety as well as an aldehyde moiety in the same molecule. Conclusive evidence for the presence of a fatty acid in ethanolamine plasmalogens of brain was published by Debuch (1956). She treated phospholipids with mild acid which hydrolyzes the aldehyde from the plasmalogens and separated the lysophosphatidylethanolamines (that were formed from the plasmalogens) from the acid-stable diacyl phospholipids. In this way, it was now possible to investigate the fatty acids of the plasmalogens. Debuch (1959a,b) then proved that the aldehydogenic group was at the α-position of the glycerol moiety.

**Plasmalogen: concentration in myelin, cell types and subcellular fractions**

During the last decade, central nervous system (CNS) myelin has been isolated in highly purified form using differential ultracentrifugation (Cuzner, Davison and Gregson, 1965; Norton and Autilio, 1966; Horrocks, 1967, 1968).
The most abundant organic molecules in myelin are lipids. The lipid content as reported by Smith (1967) and Horrocks (1968b) is 75-80% of the dry weight of CNS myelin. Proteins make up the remainder. More recently, values between 62-70% have been obtained for the lipid content of myelin (Eto, Suzuki and Suzuki, 1970; Horrocks, 1972; Sun and Samorajski, 1972). The latter values are closer to the theoretical model of a lipid bilayer which requires at least 33% protein. The major lipid molecules of myelin are cholesterol, cerebroside, cerebroside sulfate, ceramide, sphingomyelin, ethanolamine phosphoglycerides, choline phosphoglycerides and serine phosphoglycerides (Smith, 1967; Eichberg, Hauser and Karnovsky, 1969; Mokrasch, 1969).

The content of phospholipids is 43 percent of the total lipid weight for adult bovine CNS, including 17.4% ethanolamine phosphoglycerides, 10.9% choline phosphoglycerides, 6.5% serine phosphoglycerides and 7.1% sphingomyelin (Norton and Autilio, 1966). From 31-37% of the phospholipids are ethanolamine plasmalogens in mammalian central nervous system myelin (Horrocks, 1968, 1972).

Central nervous system myelin is synthesized and maintained by the oligodendroglia (Davison and Dobbing, 1968). The myelin membranes are continuous with the plasma membrane of the oligodendroglia. Until recently there has not been a reliable procedure for the isolation of oligodendroglia without considerable myelin contamination. Poduslo and Norton (1972) have reported a lipid composition of isolated oligodendroglia which is relatively free of myelin contamination. Methods are now available for isolation of some specific cell types whose lipid compositions are reported in Table 1.
Table 1. Phospholipid composition of isolated cells and myelin

<table>
<thead>
<tr>
<th></th>
<th>Rat Neuronal Perikarya&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rat Astrocyte&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ox oligodendroglia&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ox Myelin&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of Total Phospholipid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline phosphoglycerides</td>
<td>57</td>
<td>53</td>
<td>50</td>
<td>26</td>
</tr>
<tr>
<td>Ethanolamine phosphoglycerides</td>
<td>26</td>
<td>29</td>
<td>24</td>
<td>41</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>5</td>
<td>5</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>Serine phosphoglycerides</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Inositol phosphoglycerides</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Plasmalogen</td>
<td>12</td>
<td>8</td>
<td>-</td>
<td>35</td>
</tr>
</tbody>
</table>

<sup>a</sup>Norton and Poduslo, 1971

<sup>b</sup>Poduslo and Norton, 1972

<sup>c</sup>Norton and Autilio, 1966
Plasmalogen accounts for about 18% of the total phospholipids in microsomes from whole brain of a variety of animals (mouse, Horrocks, 1968a; rat, Toews, 1974; Mandel and Nussbaum, 1966; rabbit, Cuzner et al., 1965b). The corresponding value for mitochondria is 8-10% (mouse, Sun and Horrocks, 1970; rat, Mandel and Nussbaum, 1966).

Plasmalogen: degradation

A role of intrinsic phospholipases in the pathogenesis of demyelinating diseases and other degenerative neurological conditions has been postulated because of the abundant phospholipid composition of brain white matter and myelin and the detergent properties of lyso-phosphatidylcholine (Thompson, 1961). The presence of phospholipase A-like activity in brain was first reported by Gallai-Hatchard, Magee, Thompson and Webster (1962). Evidence was obtained for two positionally specific types of phospholipase A (phosphatide acyl-hydrolase EC 3.1.1.4) (Webster, 1967; Webster and Cooper, 1968). These are phospholipases $A_1$ and $A_2$, which catalyze the hydrolysis of the ester bonds at the 1- and 2- positions of $\text{sn-1,2-diacyl phosphoglycerides}$ and thus form the isomeric 2-acyl and 1-acyl lysoderivatives, respectively.

With regard to the distribution of phospholipases, Cooper and Webster (1970) have found phospholipase $A_1$ in both gray and white matter of human brain and in peripheral nerve. Phospholipase $A_2$ activity is lower than $A_1$ activity in all regions of the CNS examined and is absent from peripheral nerve. The exact cytological location of these phospholipases is not yet known but Cooper and Webster's data suggest that in the CNS both enzymes are present in glia and probably in neuronal cells.
The distribution of phospholipases A₁ and A₂ in rat brain subcellular particles has been examined by Woelk and Porcellati (1973). Phospholipase A₁ activity is almost exclusively located in the microsomal fraction, whereas phospholipase A₂ activity predominates in mitochondria.

The product of phospholipase A activity is a lysophosphoglyceride. Detergent and lytic properties have been demonstrated for 1-acyl phosphoglycerides but not yet for 2-acyl phosphoglycerides. Hall and Gregson (1971) demonstrated the myelinolytic properties of lysophosphatidylcholines by injecting 1-acyl-sn-glycerophosphorylcholines into peripheral nerve fibers of adult mice. An injection of phospholipase A₂ which produces 1-acyl lysophosphoglycerides gave similar results (Hall, 1972). Neither 1-acyl-sn-glycerophosphoryl ethanolamines, phospholipase A₁ nor 2-acyl lysophosphoglycerides have been tried.

In vivo investigations of the fate of administered plasmalogens (Feulgen et al., 1928; Leupold and Buttner, 1953; Robertson and Lands, 1962) have demonstrated that plasmalogens are removed fairly rapidly from the blood. These results establish the presence of catabolic enzymes for plasmalogens in tissues.

Bergman and co-workers (1957) found that extracts from Escherichia coli and Bacillus subtilis cleaved the vinyl ether moiety of plasmalogens isolated from horse brain, whereas identically prepared extracts from other bacteria and yeast did not affect the ether linkage. Thiele (1959a) reported that plasmalogens were degraded when incubated at 37°C
with extracts of acetone-powders from fresh organs. Aldehydes were produced. Since chelating agents inhibited cleavage of the vinyl ether moiety, Thiele concluded that a stable heavy metal-complex probably catalyzed the reaction. Anderson (1960) reported that rat liver homogenates degraded plasmalogens, but he could not detect aldehydes after incubation.

The first clear description of a specific enzyme capable of cleaving the vinyl ether moiety of ethanolamine plasmalogens was reported by Warner and Lands (1961). They isolated a microsomal preparation from rat liver that hydrolyzed the alk-1-enyl group from 1-alk-1'-enyl-sn-glycero-3-phosphorylcholine. Added cofactors were not necessary. If ethanolamine was substituted for choline or any acyl group for the hydroxyl group, the hydrolysis was prevented. The hydrolytic enzyme was labile to heat, acid, alkali and chymotrypsin, whereas freeze-drying, Mg$^{2+}$, and Ca$^{2+}$, and chelating agents had no effect on its activity. Inactivation of the enzyme caused by exposure of the microsomes to phospholipases A or C, freezing and thawing or imidazole (and derivatives) could be reversed by the addition of exogenous diacyl phosphoglycerides (Ellington and Lands, 1968). Acyl moieties at both the 1- and 2- positions of the glycerol moiety of the phospholipid were required for reactivation. Thus, this liver enzyme is most active within a membrane and is relatively specific for lysoplasmalogens.

Ansell and Spanner (1965) described the enzyme, plasmalogenase, in rat brain that is capable of cleaving the vinyl ether moiety of ethanolamine plasmalogens. The brain enzyme requires Mg$^{2+}$ for
optimal activity and is most active with 1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphorylethanolamine. This hydrolysis is shown in Fig 3. Plasmalogenase is the name commonly used for this hydrolytic enzyme, 1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphorylethanolamine alk-1-enylhydrolase. Although both brain and liver contain an enzyme that hydrolyzes alkenyl groups, the systems exhibit marked differences in their substrate and cofactor specificities.

Ansell and Spanner (1965 and 1968) showed the optimum pH for the assay of plasmalogenase to be 7.4. They reported a subcellular localization in the crude mitochondrial fraction. Yavin and Gatt (1972) studied the optimum deoxycholate concentrations for the incubation of ethanolamine plasmalogens and choline plasmalogens with plasmalogenase and found that 2 mg/ml gave the highest plasmalogenase activity. They also found that the enzyme was indefinitely stable in the dried acetone powder form but was only stable for 48 hours after extraction with glycerol-bicarbonate. They also confirmed Ansell and Spanner's pH optimum. Both groups have reported a substrate saturation of enzyme using 2mM ethanolamine or choline plasmalogens.
Figure 3. The hydrolysis of the vinyl ether moiety of ethanolamine plasmalogen by plasmalogenase.
Chapter II  ENZYME ACTIVITY - NORMAL CONDITIONS

Introduction

The discovery of relatively high concentration of ethanolamine plasmalogens in mammalian white matter and myelin (reviewed by Horrocks, 1972) has led to the hypothesis that the catabolic enzyme plasmalogenase might be involved in demyelination and other responses of the central nervous system to injury (Ansell and Spanner, 1968a; McMartin et al., 1972; Horrocks et al., 1973).

Several methods have been described for the assay of 1-alk-1'-enyl ethers in lipids. These methods have lacked reproducibility or have been too complex to be useful in an enzyme analysis. Prominent among these methods has been the iodine uptake reaction (Gottfried and Rapport, 1962). This reaction is fast and provides a facile approach to the colorimetric measurement of remaining plasmalogens after enzymatic incubation. Another method of direct analysis applicable to the plasmalogenase catalyzed reaction is the p-nitrophenyl-hydrazine reaction with the free long chain fatty aldehydes liberated by the hydrolytic cleavage of the alk-1-enyl ether linkage (Wittenberg et al., 1956). However, this assay has not been unequivocally applicable to all tissues or substances and has given values as much as 10% lower than the actual value (Hanahan, 1972). The only other method for direct analysis is Feulgen's method for quantitative estimation of "plasmal" (Gray and MacFarlane, 1958). Total aldehydes are measured
after liberation from plasmalogen by acid treatment, or free aldehydes may be measured by omitting the acid treatment. After reaction with fuchsin-sulfurous acid, the colored complex is extracted from the water-soluble mixture by an alcohol immiscible with water, such as amyl alcohol. Horrocks (1968a) has reported an indirect method for the determination of plasmalogens using two-dimensional thin-layer chromatography.

One purpose of this study was the selection of a reproducible assay system for the plasmalogens remaining after incubation with plasmalogenase. Up to this time, other investigators have used an incubation system similar to that developed by Ansell and Spanner (1965) together with the iodine addition method for the assay of plasmalogens. In this laboratory, this method has not been reproducible. After development of a reproducible assay using two-dimensional thin-layer chromatography together with the Ansell and Spanner incubation system as modified by Yavin and Gatt (1972), various properties of the enzyme were studied. The constants $K_m$ and $V_{max}$ were determined and lipid inhibitors of the enzyme were investigated. In addition, the activity of plasmalogenase in brain tissue from several species was determined.
EXPERIMENTAL PROCEDURES

1) Methods

a) Analytical

Total lipid phosphorus was determined by the method of Bartlett (1959). Individual lipids on TLC plates were detected with iodine vapor and assayed for phosphorus according to the micro method of Gottfried (1967).

Protein contents were determined by a modification (Lowden, 1966) of the method of Lowry et al. (1951).

b) Thin layer and column chromatography

Thin-layer plates (20 cm square) were prepared from a slurry of 35 g Silica Gel G (E. Merck, Darmstadt, Germany) in a 67 ml of 0.01 M Na$_2$CO$_3$. The slurry was spread to form a 0.5 mm layer.

Alumina columns (4 cm diameter) were prepared with 60 g of aluminum oxide (Chemical Samples Co., Columbus, Ohio), transferred to the columns as a slurry in chloroform.

Silicic acid columns (2 cm diameter) were prepared with 8 g of silicic acid (Clarkson Chemical Co., Williamsport, Pa.), transferred to the columns as a slurry in chloroform.

The columns contained a plug of glass wool at the bottom.

c) Statistical

The hypothesis that the plasmalogenase activity of the large animal (dog) was greater than the activity of the rodents
(gerbil and rat) was tested by a two sample one tailed t test (Armitage, 1971).

2) Preparation of Substrates

a) Plasmalogens (Ethanolamine and Choline Plasmalogens)

A fresh bovine heart (Coil Packing Co., Columbus, Ohio) weighing 1500 grams was packed in ice for transport to the laboratory. After trimming excess fat, portions of 300 grams each were homogenized in a Waring blender in 600 ml of chloroform-methanol (1:1, v/v) and stored overnight at 4°C in a 1 liter flask. After filtration through Whatman no. 1 paper, 1500 ml of chloroform and 1100 ml of water were added (Folch and Lees, 1957). The mixture was shaken, transferred to large cylinders and the phases were allowed to separate overnight at 4°C. The lower phase was taken to dryness with a rotary flash evaporator, and the residue was dissolved in a small volume of chloroform-methanol (2:1, v/v) and stored at -20°C (Yavin and Gatt, 1972). Aliquots were evaporated, dissolved in chloroform, chromatographed on neutral alumina (7 μmol/gr) and eluted according to Ansell and Spanner (1963). Aliquots of each fraction were chromatographed on thin layer Silica Gel G plates (0.5 mm) using chloroform-methanol - NH₄OH (65:25:4, v/v/v). Those fractions which contained ethanolamine phosphoglycerides (1-alk-1'eny1-2-acyl-sn-glycero-3-phosphorylethanolamine and 1,2-diacyl-sn-glycero-3-phosphorylethanolamine) were pooled. Choline phosphoglycerides (1-alk-1'eny1-2-acyl-sn-glycero-3-phosphorylcholine and 1,2-diacyl-sn-glycero-3-phosphorylcholine) were prepared in a similar manner with initial elution of the alumina column with 300 ml chloroform to remove the neutral lipids. The choline and ethanolamine phosphoglyceride
fractions were taken to dryness with a rotary evaporator and redissolved in chloroform. They were individually chromatographed on a Unisil silicic acid column (15 μmol phosphate per g silicic acid) using a continuous gradient of increasing concentrations of methanol in chloroform. Aliquots of the fractions were chromatographed on thin-layer Silica Gel G plates (0.5 mm) as described above. Those fractions which contained ethanolamine phosphoglycerides were combined. The choline phosphoglyceride fractions were similarly combined. The plasmalogen content of the ethanolamine phosphoglyceride and choline phosphoglyceride fractions was assayed by phosphorus determination after 2-dimensional thin layer chromatography (Horrocks, 1968). The ratio of ethanolamine plasmalogens to diacyl glycerophosphorylethanolamines was 3:2.

The diacyl glycerophosphorylcholines and diacyl glycerophosphorylethanolamines were removed from the phosphoglyceride preparations by mild alkaline hydrolysis (Dawson, 1960). An aliquot containing not more than 0.5 mg of lipid phosphorus was dried in a 50 ml Erlenmeyer flask with a rotary evaporator. After addition of 0.5 ml of 0.5 N NaOH in methanol and 2.0 ml of methanol, the mixture was incubated for 20 minutes. The hydrolysis was stopped with 1.0 ml ethyl formate. The incubation mixture was dried with a rotary evaporator. The reaction products were separated by the addition of 1.0 ml upper phase and 2.0 ml lower phase (Dawson, 1960). The phases were mixed and allowed to separate while warming slightly for 1 minute. The mixture was transferred to centrifuge tubes and centrifuged at 3000 rpm for 20 minutes.
The lower phase containing purified ethanolamine or choline plasmalogens was removed and stored at 4°C. Purities were determined by two-dimensional thin-layer chromatography. In both cases the alkylacyl phosphoglyceride accounted for 10% of the total phospholipid, the remainder being either choline or ethanolamine plasmalogens.

b) Diacyl phosphoglycerides

Lipids were isolated from the yolk of a fresh egg according to the method of Rhodes and Lea (1957). The yolk was blended in twenty volumes of chloroform-methanol (2:1, v/v). The homogenate was filtered (Whatman no. 1 paper) and taken to dryness on a rotary evaporator. The extract was dissolved in a small amount of chloroform and placed on an alumina column. Neutral lipids were eluted with 200 ml of chloroform. The diacyl glycerophosphorylcholines were eluted with 200 ml chloroform-methanol (2:1, v/v). This was followed by 250 ml chloroform-methanol-water (2:5:1, v/v/v). The diacyl glycerophosphorylethanolamines were eluted with 270 ml chloroform-ethanol-water (2:5:2, v/v/v). The diacyl glycerophosphorylcholine and diacyl glycerophosphorylethanolamine fractions were taken to dryness and redissolved in chloroform. The purity of each fraction was determined by two-dimensional thin-layer chromatography. Only one spot corresponding to diacyl glycerophosphorylcholines or diacyl glycerophosphorylethanolamines was detected.

c) Lysophosphatidylethanolamine

In order to prepare 2-monoacyl-sn-glycero-3-phosphorylethanolamines, the purified ethanolamine plasmalogens were applied on a line
2 cm from the bottom across a Silica Gel G plate (0.5 mm). The plate was placed over HCl fumes, in order to cleave exclusively the alk-1-enyl groups from the plasmalogens. The plate was developed in chloroform-methanol-acetone-acetic acid-water (75-15-30-15-8 by vol).

d) Purified myelin

Myelin from rat brains was purified by a modification (Toews and Horrocks, personal communication) of the methods of Kurihara (1971) and Horrocks (1968b) as shown in Fig 4.

3) Preparation of Plasmalogencase

a) Acetone powders from beef brain were purchased from Nutritional Biochemical Corporation, Cleveland, Ohio. Three different lots (7773, 6841, and 1649) were used in this study.

b) The crude enzyme preparation was extracted from the acetone powder with 20 volumes of a 1:1 (v/v) mixture of 50% glycerol and 1% (w/v) sodium bicarbonate as described by Magee, Gallai-Hatchard, Sanders and Thompson (1962). The mixture was centrifuged at 40,000 g for 60 minutes using a Beckman preparative ultracentrifuge model L3-40. The supernatant which contained the enzymic activity was decanted and kept at 4°C.

4) Incubation

The solution of the purified lipid substrate (2-3 μmoles per incubation tube) was placed in a 50 ml beaker and taken to dryness with a stream of nitrogen. Glycylglycine (0.1M) buffer, pH 7.4, containing 2 mg per ml of sodium deoxycholate was added to the beaker and the mixture was sonicated (Blackstone model SS-2, Fisher Scientific Co.,
Figure 4. Isolation of purified myelin by ultracentrifugal techniques.
Pittsburgh, Pa.) for one minute. A 0.5 ml portion was transferred to each incubation tube. Samples of the enzyme extract equivalent to 25 mg of acetone-dried powder (0.5 ml) were then added to each incubation tube and the mixture was incubated at 37°C from 0 to 180 minutes in a Dubnoff shaking incubator. The sodium deoxycholate and N-glycylglycine were purchased from Matheson Coleman and Bell Chemical Co., Norwood, Ohio.

5) Isolation of Reaction Products

The incubation mixture (1 ml) was mixed with 4 ml of chloroform-methanol (2:1, v/v) to stop the reaction. A portion of the lower phase containing about 2 μg of phosphorus was applied to a Silica Gel G plate. The phospholipids were separated by two-dimensional chromatography (Horrocks, 1968a). The area containing the 2-acyl-sn-glycerophosphorylethanolamines which were derived from intact ethanolamine plasmalogen was scraped into a digestion tube for phosphorus determination.

6) Determination of Kinetic Constants

For the determination of the kinetic constants $K_m$ and $V_{max}$ and the inhibition of the reaction by membrane components ($K_i$), the reciprocal of reaction velocity was plotted against the reciprocal of the substrate concentration according to Lineweaver and Burk (1934). The slopes and y-intercept of the lines were determined by linear regression and then used for the calculation of $K_m$, $V_m$ and $K_i$ (Lineweaver and Burk, 1934).
RESULTS

1) Reproducibility and Kinetics

The activity of plasmalogenase extracted from a commercial acetone powder from bovine brain was studied with the purified ethanolamine plasmalogen preparation. The activity of the enzyme was determined from the initial 20 minutes (Fig 5) and was proportional to the amount of protein in enzyme preparation (Fig 6). These assays were performed with measurement of the remaining substrate by two-dimensional thin-layer chromatography. A number of determinations (15) of plasmalogenase activity using the Ansell and Spanner incubation system as modified by Yavin and Gatt with the two-dimensional thin-layer chromatography technique gave an activity of 278±11 nmol/hr/mg protein. This method was then used for studies of competitive inhibition and the effects of added Mg^{2+}.

With 1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphorylethanolamines as the substrate, competitive inhibition was obtained with 1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphorylcholines (Fig 7), 1,2-diacyl-sn-glycero-3-phosphorylethanolamines (Fig 8), and 1,2-diacyl-sn-glycero-3-phosphorylcholines (Fig 9). When choline plasmalogens (1-alk-1'-enyl-2-acyl-glycero-3-phosphorylcholines were used as a substrate, the hydrolysis was also competitively inhibited by ethanolamine plasmalogens (Fig 10) and by diacyl glycerophosphorylcholines (Fig 11).

The kinetic constants from the Lineweaver-Burk graphs are given in Table 2. The maximum velocity of the enzyme catalyzed reaction is
Figure 5. The loss of ethanolamine plasmalogens as a function of the time of incubation with plasmalogenase. Incubation mixture: 2.0 μmoles of ethanolamine plasmalogens, 2.0 mmoles glycylglycine (pH 7.4), 0.1 mg deoxycholate in 0.5 ml of water were mixed with 0.5 ml of bovine acetone powder extract in 1:1 (v/v) 50% glycerol - 1% (w/v) sodium bicarbonate. The incubation was for different periods of time at 37°C.
Figure 6. The effect of protein concentration on the hydrolysis of ethanolamine plasmalogens. Incubation mixture: see Figure 5. The different protein concentrations were obtained by using different amounts of beef brain acetone powder in the extraction procedure. Incubation was for 20 minutes at 37°C.
Figure 7. The competitive inhibition of the hydrolysis of ethanolamine plasmalogens (0-0) by choline plasmalogens (X-X, 0-0). Incubation mixture: see Figure 5 except the inhibitor was included. Incubation was for 20 minutes at 37°C.
Figure 8. The competitive inhibition of the hydrolysis of ethanolamine plasmalogens (0-0) by 1,2-diacyl-sn-glycero-3-phosphorylethanolamines (X-X, 0-0). Incubation mixture: same as Figure 7.
Figure 9. The competitive inhibition of the hydrolysis of ethanolamine plasmalogens (0-0) by 1,2-diacyl-sn-glycero-3-phosphorylcholine (X-X, 0-0). Incubation mixture: same as Figure 7.
Figure 10. The competitive inhibition of the hydrolysis of choline plasmalogens (O-O) by ethanolamine plasmalogens (X-X, O-O). Incubation mixture: same as Figure 7.
Figure 11. The competitive inhibition of the hydrolysis of choline plasmalogens (θ-θ) by 1,2-diacyl-sn-glycero-3-phosphorylcholine (X-X, 0-0). Incubation mixture: same as Figure 7.
Table 2. Kinetic constants for the enzymic hydrolysis of ethanolamine plasmalogens and choline plasmalogens

<table>
<thead>
<tr>
<th></th>
<th>Ethanolamine plasmalogens</th>
<th>Choline plasmalogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>285</td>
<td>221</td>
</tr>
<tr>
<td>$K_m$</td>
<td>181 (.999)</td>
<td>208 (.999)</td>
</tr>
<tr>
<td>$K_i$ (CP)</td>
<td>984 (1.00)</td>
<td>----</td>
</tr>
<tr>
<td>$K_i$ (DE)</td>
<td>584 (1.00)</td>
<td>----</td>
</tr>
<tr>
<td>$K_i$ (DC)</td>
<td>778 (.999)</td>
<td>822 (1.00)</td>
</tr>
<tr>
<td>$K_i$ (EP)</td>
<td>----</td>
<td>703 (.999)</td>
</tr>
</tbody>
</table>

The abbreviations are:
(CP) choline plasmalogens; (DE) diacyl glycerophosphorylethanolamines; (DC) diacyl glycerophosphorylcholines; (EP) ethanolamine plasmalogens.

The $K_m$ and $K_i$ values were determined by linear regression. The correlation coefficient is in parenthesis.
about 30% faster with ethanolamine plasmalogens than with choline plasmalogens as substrate. The affinities of the competitive inhibitors of ethanolamine plasmalogen hydrolysis are first diacyl glycerophosphorylethanolamines, followed respectively by diacyl glycerophosphorylcholines and choline plasmalogens.

The $K_i$ values are nearly identical for the competitive inhibition by diacyl glycerophosphorylcholines of the hydrolysis of ethanolamine plasmalogens and choline plasmalogens.

2) Activity of Lysophospholipase

No 2-acyl-sn-glycero-3-phosphorylethanolamines were detected by thin layer chromatography of the phospholipid classes in the lower phase of the separated reaction mixture. This finding suggested that a lysophospholipase was present in the enzyme extract. A product of the lysophospholipase would be glycerophosphorylethanolamine which is water soluble and would be found in the upper methanol phase. The loss of ethanolamine plasmalogen from the lower chloroform phase was equivalent to the increase in phosphorus in the upper methanol phase (Fig 12). A preparation of 2-monoacyl-sn-glycero-3-phosphorylethanolamines was incubated with the enzyme extract with a similar equivalence between the loss of 2-monoacyl-sn-glycero-3-phosphorylethanolamines and the increase of phosphorus in the upper phase (Fig 13). While the maximum velocity for the hydrolysis of ethanolamine plasmalogens was 285 nmol/hr/mg protein, the velocity of the hydrolysis of 2-monoacyl-sn-glycero-3-phosphorylethanolamines was 375 nmol/hr/mg protein.
Figure 12. Equivalence between the cleavage of the vinyl ether linkage of ethanolamine plasmalogen and formation of water soluble phosphorus.
Figure 13. Equivalence between the hydrolysis of the acyl moiety from 2-acyl-sn-glycero-3-phosphorylethanolamine and the formation of water soluble phosphorus.
3) **Inhibition with Diacyl Phosphoglycerides**

When plasmalogenase was incubated with 1,2-diacyl-sn-glycero-3-phosphorylethanolamines or 1,2-diacyl-sn-glycero-3-phosphorylcholines and the corresponding plasmalogen, another type of inhibition was also observed (Fig 14). The reaction proceeded almost linearly with time until the concentrations of the two compounds were nearly equal. The addition of further plasmalogen after the reaction had stopped yielded complete hydrolysis of the added substrate, indicating the availability of enzyme. Also, the addition of further enzyme resulted in additional hydrolysis.

4) **Activity with Purified Myelin**

Purified myelin from rat brains was incubated with an enzyme extract from bovine brain acetone powder (Table 3). The measured rate of hydrolysis is compared with that expected for the content of ethanolamine plasmalogens in the myelin with the presence and absence of the amount of 1,2-diacyl-sn-glycero-3-phosphorylcholines.

5) **Magnesium-ion Effect on Ethanolamine Plasmalogen Solubility**

Ansell and Spanner (1965) reported that the cleavage of the vinyl ether linkage of brain ethanolamine plasmalogens by plasmalogenase was dependent on the presence of magnesium ions. Dialyzed enzyme extracts had no hydrolytic activity. The addition of magnesium ion (between 5 and 10mM) completely restored the ability of the dialyzed extract to cleave the vinyl ether linkage of ethanolamine plasmalogens.

Ethanolamine plasmalogens were sonicated and preincubated with 5mM and 10mM Mg$^{2+}$. The preincubation was for 12 hr (Fig 15), 24 hr
Figure 14. The inhibition of plasmalogen hydrolysis by diacyl phosphoglycerides (X-X). Hydrolysis of plasmalogens without diacyl phosphoglycerides (Θ-Θ). Incubation mixture: same as Figure 5 plus 0.75 μmoles 1,2-diacyl-sn-glycero-3-phosphorylethanolamine.
Table 3. Plasmalogenase activity with purified myelin as the substrate

<table>
<thead>
<tr>
<th>Ethanolamine plasmalogen content (μmoles)</th>
<th>Observed activity with purified myelin as substrate (n = 2)</th>
<th>Calculated activity with purified ethanolamine plasmalogen as substrate (Fig. 8)</th>
<th>Calculated activity with purified ethanolamine plasmalogen competitively inhibited by diacyl glycerophosphorylcholine (Fig. 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/hr/mg protein (range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.113</td>
<td>80 (75-85)</td>
<td>125</td>
<td>87</td>
</tr>
<tr>
<td>0.193</td>
<td>120 (112-128)</td>
<td>156</td>
<td>129</td>
</tr>
<tr>
<td>0.323</td>
<td>158 (149-167)</td>
<td>191</td>
<td>166</td>
</tr>
</tbody>
</table>

The purified myelin was freeze-dried and reconstituted in the glycylglycine (pH 7.4)-deoxycholate solution. The mixture was sonicated for 1 min prior to incubation.
Figure 15. Preincubation for 12 hours with 5 and 10 mM Mg$^{2+}$. Preincubation mixture: 2.0 μmole ethanolamine plasmalogen, 2 mmoles glycylglycine (pH 7.4), 0.1 mg deoxycholate, final volume 0.5 ml (sonicated). Incubation mixture: preincubation mixture plus 0.5 ml enzyme extract. The mixture was incubated at 37°C.
(Fig 16) and 48 hr (Fig 17) in glycylglycine (pH 7.4)-deoxycholate incubation medium followed by incubation with plasmalogenase. For all preincubation time periods, the total recoverable ethanolamine plasmalogen was increased by 10% with pre-incubation with 5mM Mg\(^{2+}\) and 25% with preincubation with 10mM Mg\(^{2+}\). With 5mM Mg\(^{2+}\) and 12 hr preincubation, the plasmalogenase activity decreased 75%. In this same time period, 10mM Mg\(^{2+}\) decreased the activity about 80%. Both Mg\(^{++}\) concentrations with 24 and 48 hours preincubation decreased the activity from 90 to 100 percent. The distribution of phospholipid in upper and lower phase solvents was investigated after 24 hr preincubation with 10mM Mg\(^{2+}\) (Table 4). The effect of incubation with Mg\(^{++}\) was to decrease the amount of phosphorus found in the upper phase and increase the amount of plasmalogens found in the lower phase.

6) Plasmalogenase Activity in Different Animals

The significant differences (P<.05) in enzymic activity (Table 5) between gerbil, rat and dog reflect the relative proportions of white matter in each animal. The dog has a greater proportion of white matter than do the gerbil and rat.
Figure 16. Preincubation for 24 hours with 5 and 10 mM Mg$^{2+}$. Preincubation and incubation mixtures: same as Figure 15.
Figure 17. Preincubation for 48 hours with 5 and 10 mM Mg$^{2+}$. Preincubation and incubation mixtures: same as Figure 15.
Table 4. Phosphorus distribution after preincubation of ethanolamine plasmalogens for 24 hours with 10 mM Mg$^{2+}$

<table>
<thead>
<tr>
<th></th>
<th>Lower phase ethanolamine plasmalogen</th>
<th>Upper phase phosphorus</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Mg$^{2+}$</td>
<td>36.80</td>
<td>4.27</td>
<td>41.07</td>
</tr>
<tr>
<td></td>
<td>38.32</td>
<td>5.03</td>
<td>43.35</td>
</tr>
<tr>
<td>Control: with no</td>
<td>32.67</td>
<td>8.37</td>
<td>41.04</td>
</tr>
<tr>
<td>added cations</td>
<td>34.54</td>
<td>9.02</td>
<td>43.56</td>
</tr>
</tbody>
</table>
Table 5. Plasmalogenase activity in various normal animal brains

<table>
<thead>
<tr>
<th>Animal (number)</th>
<th>nmol/hr/mg protein</th>
<th>nmol/hr/mg wet wt</th>
<th>nmol/hr/mg acetone powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerbil (5)</td>
<td>216 ± 13</td>
<td>3.36 ± .44</td>
<td>25.93 ± .95</td>
</tr>
<tr>
<td>Rat (3)</td>
<td>230 ± 10</td>
<td>3.92 ± .16</td>
<td>21.97 ± 1.02</td>
</tr>
<tr>
<td>Dog (10)</td>
<td>290 ± 10</td>
<td>5.13 ± .31</td>
<td>26.04 ± 3.78</td>
</tr>
</tbody>
</table>

The activity of the enzyme from the dog brain was significantly higher (P<.05) than the activity of the enzyme from the rodent (gerbil and rat) brains.
DISCUSSION

The assay procedure as developed by Ansell and Spanner and modified by Yavin and Gatt was not reproducible in this laboratory when used with the iodine addition procedure for the determination of plasmalogens. The reason for this lack of reproducibility is not known. Our assay procedure, using the Ansell and Spanner incubation system as modified by Yavin and Gatt and measuring the decrease in plasmalogens by two-dimensional thin-layer chromatography was very reproducible. The coefficient of variation for fifteen determinations was 3.7%. Also, the very high linear correlation coefficients (Table 2) for the Lineweaver-Burk relationships suggests a good reliability. The activity found for rat brain in this study, $3.92 \pm 0.16$ μmoles/hr/g wet wt., was somewhat higher than the value of $2.42 \pm 0.4$ μmoles/hr/g wet wt. reported by Ansell and Spanner (1968).

The Lineweaver-Burk graphs of the reciprocal of substrate concentration against the reciprocal of plasmalogenase activity indicate that diacyl phosphoglycerides are competitive inhibitors of the plasmalogenase catalyzed hydrolysis of the 1-alk-1'-enyl moiety of plasmalogens. Woelk and Porcellati (1973) reported the exact opposite for the competitive inhibition of phospholipase $A_1$ from rat brain. They found competitive inhibition of the hydrolysis of 1,2-diacyl-sn-glycero-3-phosphorylcholines and competitive inhibition of the hydrolysis of 1,2-diacyl-sn-glycero-3-phosphorylethanolamines by 1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphorylethanolamines. They concluded that this
evidence suggested a possible role of ethanolamine plasmalogens in brain, i.e., that ethanolamine plasmalogens may be important for the maintenance of membrane structure. This is particularly important for the high content of ethanolamine plasmalogens in myelin and white matter and for its protective role in maintaining a certain degree of metabolic stability of myelin itself by competitively inhibiting the hydrolysis of the 1,2-diacyl-sn-glycero-3-phosphorylcholines. The competitive inhibition by diacyl phosphoglycerides of the hydrolysis of the ethanolamine plasmalogens catalyzed by plasmalogenase also suggests the same protective role for diacyl glycerophosphorylcholines in brain. This role is further indicated by the plasmalogenase activity with purified myelin. When the measured rate of hydrolysis is compared with that expected for the content of ethanolamine plasmalogens in the myelin, the measured rate is lower. When the observed activity is compared to that calculated for hydrolysis of ethanolamine plasmalogens in the presence of 1,2-diacyl-sn-glycero-3-phosphorylcholines, the observed and calculated mean activities are very similar.

Ethanolamine plasmalogens, even in the myelin sheath, are actively turned over (Horrocks, 1966). As part of the normal catabolism of ethanolamine plasmalogens by plasmalogenase, compounds such as 2-monoacyl-sn-glycero-3-phosphoryl ethanolamine are most likely produced. The function of the lysophospholipase may be the removal of such lytic compounds before they have damaged any membranes. However, when the plasmalogenase has been activated (Ansell and Spanner, 1967; McMartin et al., 1972), then this protective activity of the lysophospholipase may be overwhelmed resulting in membrane damage including demyelination.
The nearly identical (+5%) $K_i$ values for diacyl glycerophosphoryl-cholines as a competitive inhibitor of the hydrolysis of ethanolamine plasmalogens and choline plasmalogens indicates that the same enzyme may be responsible for the hydrolysis of both substrates. The cross-inhibition by ethanolamine and choline plasmalogens also suggests that the same enzyme is responsible for the hydrolysis of the 1-alk-1'-enyl moiety of both plasmalogens.

Another type of inhibition of plasmalogenase by diacyl phosphoglycerides was also observed. The reaction stopped when the concentrations of the plasmalogens and the diacyl phosphoglycerides were nearly equal. The enzyme was still active because the addition of plasmalogens led to further reaction. The substrate was still available because the addition of plasmalogenase led to further reaction. An end-product inhibition due to phospholipase A reaction products might explain this inhibition. Exhaustion of a cofactor is a possible but less-likely explanation.

Incubations with additional $\text{Mg}^{2+}$ decreased the recovery of phosphorus in the aqueous phase in the presence and absence of plasmalogenase. The inhibition of plasmalogenase by excess $\text{Mg}^{2+}$ and the decreased aqueous solubility with excess $\text{Mg}^{2+}$ were probably due to an interaction of $\text{Mg}^{2+}$ with a polar portion of the plasmalogen molecule. For example, a $\text{Mg}^{2+}$ salt with two molecules of plasmalogen would decrease the polarity and thus decrease the aqueous solubility and might also block a binding site for plasmalogenase.
Chapter III  ENZYME ACTIVITY - PATHOLOGICAL CONDITIONS

Introduction

Although traditionally regarded as metabolically inert, recent experiments (Davison, 1971) indicate that most myelin constituents turn over appreciably in the adult brain (Ansell and Spanner, 1967; Horrocks, 1968, 1973; Norton and Poduslo, 1973). In the steady state, the catabolism is exactly balanced by synthesis. This suggests that any uncompensated decrease in synthesis or uncompensated increase in catabolism would lead, then, to demyelination.

As early as 1906 Marburg proposed that a lipolytic agent which destroyed myelin could be the basis for the demyelinating process. Thompson (1961) suggested that lysolecithin could be the lytic agent. Both phospholipase A and lysophosphatidyl choline can bring about demyelinating changes in vitro in the CNS (Morrison and Zamecnik, 1950). Moreover, lysophosphatidyl cholines produce a rapid "clearing" of brain homogenates in saline, an action accompanied by the release into solution of a number of intracellular enzymes in an active state (Webster, 1957), and a complete solubilization of rat brain myelin (Gent, Gregson, Gammack and Raper, 1964). Hall and Gregson (1971) have studied the demyelinating effect of subperineurial injections of phospholipase A and lysophosphatidyl choline into myelinated peripheral nerve fibers of adult mice. Based on electron microscopy, lysophosphatidyl choline initiated demyelination very rapidly, within 30
minutes after injection. This demyelination is probably produced by a primary attack on the myelin sheath or the oligodendroglia. By 96 hours, all traces of the myelin sheath had disappeared from the area of the lesion which then contained debris-laden cells lying in chains parallel to one another and the long axis of the fiber. Phospholipase A\textsubscript{2} produced a demyelination identical to that produced by lysophosphatidyl choline. Hall (1972) has also injected lysophosphatidyl choline into white matter of the adult mouse spinal cord, a part of the CNS. The subsequent demyelination was identical to that observed in the peripheral nerve.

Lysophosphatidyl ethanolamine is produced by the action of plasmalogenase on ethanolamine plasmalognes (Yavin and Gatt, 1972). Demyelination in the CNS may be the result of the lytic action of lysophosphatidyl ethanolamine on oligodendroglial plasma membranes (Ansell and Spanner, 1968). Agents which increase the activity of the plasmalogenase enzyme must be investigated as possible causes of demyelination. McMartin et al. (1972b) reported plasmalogenase activity that was 67% higher in the brains of dogs coming into the clinic with demyelination resulting from canine distemper than in age-matched control dogs. They found that plasmalogenase activity was highest in the tissues with the least severe demyelination and suggested that plasmalogenase acts early in the process of demyelination.

After the demonstration in tissue culture of myelinolysis by serum from patients with multiple sclerosis, Bornstein (1963) suggested that antibodies to myelin may play a role in the disease. Thus, experimental allergic encephalomyelitis has been used as a model for
multiple sclerosis on morphological and biochemical grounds (Paterson, 1968). This model is of limited value because only lymphocytes sensitized to myelin basic proteins are able to induce the disease. Recent reports indicate that an infectious agent in conjunction with a suspected sensitization to brain components may be involved in the etiology of multiple sclerosis (Field et al., 1972; Johnson and Weiner, 1972). For this reason, canine distemper, a naturally occurring viral disease of dogs, has been chosen as a model to investigate multiple sclerosis.

The role of the immune response in the etiology of demyelinating diseases such as canine distemper and multiple sclerosis remains to be elucidated. Complement-dependent serum demyelinating factors in sera have been described by using the cerebellar explant culture technique and sera from cases of experimental allergic encephalomyelitis, multiple sclerosis and Guillain-Barre syndrome (Dowling et al., 1968; Hughes and Field, 1967). Koestner et al. (1973) reported that some distemper sera were capable of demyelinating explant cultures of canine cerebellum in vitro. The myelinolytic effect was complement dependent.

Complement-fixation and indirect immunofluorescent methods were used to examine sera from dogs with spontaneously occurring and experimentally produced canine distemper (Krakowka et al., 1973). Complement-fixing immunoglobulin M antibodies and non-complement-fixing immunoglobulin G antibodies were found in 97% of the spontaneous cases. In contrast, only 28% of the control sera contained these antibodies. They also found complement-fixing antemyelin antibodies in the sera
of gnotobiotic dogs with experimentally induced distemper virus-associated demyelination. These results indicate that demyelination in canine distemper may have an immune mechanism; perhaps according to the hypothesis described in Fig. 18.

The purpose of this investigation is to test the hypothesis (Fig. 18) that plasmalogenase is involved in the enzymic degradation of myelin in demyelinating diseases. The plasmalogenase activity in control and distemper infected gnotobiotic dogs was determined. Since CNS-myelin specific antibodies have been shown to be involved in distemper-induced demyelination, the role of these antibodies in the activation of plasmalogenase was investigated. Also, the plasmalogenase activity in cerebrospinal fluid of normal and distemper dogs in various stages of the disease was assayed.
Figure 18. Hypothesis of the activation of plasmalogenase and mechanism of demyelination. Complement-fixing antibodies or other agents binding to the oligodendroglia plasma membrane activate the plasmalogenase enzyme. The resulting lysophosphatidylethanolamine, a detergent, lyases the plasma membranes of oligodendroglia and myelin. This is followed by necrosis of cells with lysed plasma membranes and degeneration of myelin that had been supported by the necrotic oligodendroglia.
EXPERIMENTAL PROCEDURES

1) Animal Preparation
   a) Gnotobiotic dogs

   Eight dogs were obtained by hysterectomy from unconditioned conventional bitches supplied by a local dealer. The methods described by Griesmer and Gibson (1963) for raising gnotobiotic dogs in flexible plastic isolators were employed.

   The origin of R252 canine distemper virus was reported by McCullough et al. (in press). To summarize, cerebellar tissue from a spontaneous case of distemper with demyelination (R252) was used to induce demyelination in three gnotobiotic dogs. Cerebellar tissue from these dogs were then prepared as a 10% suspension, clarified by low-speed centrifugation and subsequently stored as 1.0 ml aliquots in liquid nitrogen.

   Inoculation of the dogs with the 10% cerebellar suspension was also described by McCullough. Two control dogs were inoculated intracerebrally with 0.2 ml of a 10% suspension of normal cerebellum obtained from a gnotobiotic dog. All inoculations were made while the dogs were sedated with Innovar (Pitman-Moore, Inc, Washington Crossing, N.J.)

   Dogs were sacrificed when they became moribund. Dogs under Innovar sedation were exsanguinated by cardiac puncture preceding necropsy. Control dogs were sacrificed in the same manner at various
times. The above animal preparation was done by the Department of Veterinary Pathobiology.

b) Rats for CNS-myelin specific antibody injection

Sprague-Dawley rats were injected intracerebrally with CNS-myelin specific antibody (titer 1:276). The calverium was exposed and a hole was made with a 18 gauge needle. A 100 μl portion of the antibody solution was injected with a tuberculin syringe. The antibody was provided by Dr. S. Krakowka, Veterinary Pathobiology, The Ohio State University. Brains were removed at various times after injection.

2) Preparation and Incubation of Plasmalogenase from Animal Tissue

Acetone powders were prepared from rat brains and portions of canine brains by the method of Ansell and Spanner (1965). All operations were carried out at 0°C. Using a Potter-Elvehjem tissue grinder equipped with a Teflon pestle, the tissue was dispersed in 4 volumes of ice-cold acetone and the mixture filtered through a sintered-glass funnel. This residue was resuspended in 4 volumes of acetone and the mixture was filtered. The process was repeated with a further 6 volumes of acetone and then repeated with 6 volumes of diethyl ether. The dried acetone powder was kept in a desiccator at 4°C for at least 1 hour before use. A 1 gram sample of powder was equivalent to approximately 5.6 g fresh weight of tissue. Glycerol-bicarbonate extracts of the acetone powders were incubated with ethanolamine plasmalogen as described in Chapter II.
3) **Cerebrospinal Fluid**

Frozen cerebrospinal fluid that had been obtained from gnotobiotic dogs by lumbar puncture was supplied by Dr. S. Krakowka, Department of Veterinary Pathobiology. The spinal fluid was freeze-dried and re-dissolved in 1.5 ml of the glycerol-bicarbonate mixture as described above for the preparation of the enzyme. A 1.0 ml portion was used for enzyme assay and the remainder was used for protein determination.

4) **Statistical Methods**

Cerebrospinal fluid plasmalogenase values were compared using a Wilcoxon rank sum non-parametric test (Hollander and Wolfe, 1973). The significance of the elevated plasmalogenase activity in canine distemper tissues as compared to normal dog tissues was tested using a two sample one-tailed t test (Armitage, 1971).
RESULTS

1) Canine Distemper

The neurological signs of the inoculated dogs varied according to the time of onset of the disease. The infected dogs were classified into three groups as described by Krakowka et al. (1973).

Group I: became moribund at various intervals after injection and had microscopic lesions of non-inflammatory distemper virus-induced demyelination with accompanying lymphoid depletion.

Group II: survived the observation period (15 wks) with little or no clinical evidence of neurological involvement. In these dogs lesions of demyelinating encephalitis were detected.

Group III: remained asymptomatic throughout the observation period and had no detectable lesions in the nervous system.

The plasmalogenase activity in the histologically normal areas (Fig 19) was 290 ± 10 nmol/hr/mg protein. The plasmalogenase activity was highest in an area judged histologically to be an early demyelinating lesion (Fig 20). This activity of 520 ± 63 nmol/hr/mg protein was 180% of the activity in the corresponding control areas (Table 6). The plasmalogenase activity was significantly (P<.01) higher than the control areas and also significantly higher (P<.001) than control samples from various areas.
Figure 19. Normal white matter surrounding fourth ventricle. Stain: Hematoxylin and Eosinophil. Magnification: 450 X.

Figure 20. Distemper lesion in white matter surrounding fourth ventricle. Stain: Hematoxylin and Eosinophil. Magnification: 450 X.
Table 6. Plasmalogenase activity in normal and distemper gnotobiotic dogs

<table>
<thead>
<tr>
<th></th>
<th>Normal Control Dogs</th>
<th>Inoculated Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>White matter surrounding 4th ventricle</td>
<td>270 (2)</td>
<td>275 (2)</td>
</tr>
<tr>
<td>Optic tract</td>
<td>280 (2)</td>
<td>283 (2)</td>
</tr>
<tr>
<td>Corpus Callosum</td>
<td>260 (2)</td>
<td>272 (2)</td>
</tr>
<tr>
<td>Brain Stem</td>
<td>225 (2)</td>
<td>232 (2)</td>
</tr>
<tr>
<td>Restb # 1</td>
<td>275 (2)</td>
<td>280 (2)</td>
</tr>
<tr>
<td>Rest # 2</td>
<td>278 (2)</td>
<td>273 (2)</td>
</tr>
</tbody>
</table>

*Histologically shown to be an early demyelinating lesion (Fig. 20). Significantly (P<.01) higher than comparable normal areas.

bRest remaining white matter
2) **Cerebrospinal Fluid**

In a blind study of cerebrospinal fluid from normal and distemper dogs of groups I and II, the plasmalogenase activity in the CSF from distemper dogs was significantly higher (P<.05) than that in the CSF from normal dogs (Table 7). There were no significant differences in the albumin content of the CSF, indicating a normal blood-brain barrier in the infected dogs.

3) **Enzyme Activation by CNS-myelin Antibody**

Two experiments with comparable results were done with intracerebral injections of CNS-myelin specific antibody. The plasmalogenase activity was 25% higher in brains injected with normal rabbit serum than in non-injected control brains. In the brains exposed to the CNS-myelin antibody, the plasmalogenase activity had increased by 45% at 70 hours after injection and reached a peak increase of 102% at 140 hours after injection (Fig 21).
Table 7. Plasmalogenase activity in CSF from normal and distemper dogs

<table>
<thead>
<tr>
<th>Normal</th>
<th>μmoles/hr/ml</th>
<th>Distemper</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.05</td>
<td></td>
<td>7.82</td>
</tr>
<tr>
<td>2.54</td>
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<td>5.36</td>
</tr>
<tr>
<td>3.32</td>
<td></td>
<td>3.87</td>
</tr>
<tr>
<td></td>
<td>mean ± S.D.</td>
<td>3.13</td>
</tr>
<tr>
<td>2.97 ± 0.32</td>
<td></td>
<td>5.02 ± 1.80</td>
</tr>
</tbody>
</table>

Each value is the mean of two determinations. The distemper CSF activity was significantly (P<.05) higher than that for the normal CSF.
Figure 21. Activation of plasmalogenase activity by CNS-myelin specific antibodies. Exp. 1: Brain was exposed by drilling through calvarium prior to injection. Exp. 2: Direct injection. Shaded area is mean ± S.D. of non-injected controls.
DISCUSSION

A number of laboratories have reported a decreased content of ethanolamine phosphoglycerides in the brains from patient with multiple sclerosis. Wolfgram et al. (1969) reported the same result for normal-appearing white-matter from three cases of multiple sclerosis. Yanagihara and Cumings (1969) found in two of three cases of multiple sclerosis that the ethanolamine plasmalogen levels were reduced in the normal-appearing myelin. Gerstl et al. (1961, 1970) found in both gray and white matter of patients with multiple sclerosis an appreciably lower plasmalogen to lipid phosphorus ratio, and in the plaque material an especially striking depletion of plasmalogens. Ansell and Spanner (1968) reported an increase in the plasmalogenase activity of normal-appearing white matter in one case of multiple sclerosis. Horrocks et al. (1973) reported a decrease in the proportion of ethanolamine plasmalogens in the myelin during the first hour after spinal cord trauma. Whether the increased plasmalogenase activity is related to the decreased content of ethanolamine phosphoglycerides found in the CNS of patients with demyelinating disorders remains to be proved.

In this study, a significantly increased plasmalogenase activity was found with early distemper lesions with little demyelination, confirming McMartin et al. (1972b). They had also shown that this increase was not associated with an increase of macrophages or
lysosomal enzymes. The latter were not increased until extensive demyelination was evident. McMartin et al. (1972b) used dogs brought to the clinic with distemper of unknown duration. In the present study, inoculated gnotobiotic dogs were used. The dog with the early demyelinating lesion had been exposed to the distemper virus 5 weeks before sacrifice and at the time of sacrifice, exhibited marked neurological signs characteristic of canine distemper. The 80% increase in plasmalogenase activity in the lesion area supports the hypothesis that plasmalogenase plays an integral role in the demyelinating process.

Krakowka et al. (1973) reported an increase in myelin specific antibodies in serum from dogs with canine distemper. By immunofluorescence techniques, they demonstrated binding of these antibodies to myelin lamellae and possibly to oligodendroglia. McMartin et al. (1972a) showed that canine distemper virus induced cellular degeneration and cell loss when applied to glial monolayers.

Although we cannot be completely certain, the work of Krakowka et al. and McMartin et al. implies that the distemper-induced antibodies to CNS myelin also bind to the oligodendroglia plasma membrane. In spinal cord tissue cultures, a rapid degeneration of oligodendroglia is caused by serum from multiple sclerosis patients (Raine et al., 1973). The increase in plasmalogenase activity after CNS myelin specific antibodies have been injected into the brains of young adult rats supports the hypothesis that the enzyme can be activated by CNS myelin specific antibodies. The possibility of a second messenger cannot be ruled out, but the possibility that the enzyme is membrane bound is most inviting. The 25% increase in plasmalogenase activity for the
injected controls as compared to the non-injected controls also supports the hypothesis that the enzyme can be activated by trauma.

Plasmalogenase activity was significantly (P<.05) elevated in the CSF of gnotobiotic dogs injected with canine distemper virus, and the increased enzyme activity directly reflected clinically and histologically diagnosed distemper. The implications for a clinical test for demyelinating diseases cannot be overlooked. The low value of 3.13 μmole/hr/ml of CSF from one of the diseased dogs does not detract from the significance of the assay because this dog may have been in remission.

The higher enzyme activity in CSF than in tissues from comparable animals could be the result of a complete activation of the plasmalogenase secreted into the CSF or a selective release of plasmalogenase from damaged white matter cells. The activity in pathological tissues is about 25% of that found in the CSF. The increase for pathological tissues as compared to activity from control tissue could be the result of an uncovering of a sequestered enzyme or an activation of the available enzyme.

A number of agents and processes have been associated with an increased activity of plasmalogenase which produces lysophosphatidyl ethanolamines. Lyso compounds are detergents that can act on oligodendroglia and myelin membranes to bring about necrosis and demyelination. Hall (1972) and Hall and Gregson (1971) have shown the lytic effect of lysophosphatidylcholine on myelin. We believe that
such agents and processes as trauma, viruses and myelin specific antibodies can increase the activity of plasmalogenase and thus bring about the observed neurological and demyelinating effects observed with spinal cord injury, canine distemper, and perhaps multiple sclerosis.
Chapter IV  SUMMARY

The following facts have been established:

1) Diacyl glycerophosphorylethanolamines and diacyl glycerophosphorylcholines are competitive inhibitors of the enzymic hydrolysis of ethanolamine plasmalogens.
2) Choline plasmalogens are a competitive inhibitor of the enzymic hydrolysis of ethanolamine plasmalogens and vice versa.
3) A lysophospholipase is present in ox brain.
4) Plasmalogenase activity is increased in both CNS tissue and CSF in demyelination due to canine distemper.
5) Plasmalogenase activity is increased by CNS myelin specific antibodies.

The following conclusions have been drawn:

1) The same enzyme hydrolyzes the vinyl ether moiety of ethanolamine plasmalogens and choline plasmalogens.
2) Mg$^{2+}$ increases the lipid solubility of plasmalogens.
3) Plasmalogenase activity in normal CNS tissues reflects the proportion of white matter.
REFERENCES


