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DEMYELINATING PHASE OF CANINE DISTEMPER.

The Ohio State University, Ph.D., 1972
Veterinary Science

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ENZYME ACTIVITIES ASSOCIATED WITH THE
DEMYELINATING PHASE OF CANINE DISTEMPER

DISSERTATION

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

By

Donald Neil McMartin, D.V.M., M.Sc.

* * * * *

The Ohio State University
1972

Approved by

A handwritten signature in cursive script, appearing to read "F. Koentner", is written over a horizontal line.

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CHAPTER I

BETA-GLUCURONIDASE, ACID AND NEUTRAL PROTEINASES

The biochemical breakdown of myelin in etiologically unrelated demyelinating diseases appears to proceed by common catabolic processes (Eto et al., McMartin et al.). Cholesterol, galactolipids and ethanolamine plasmalogen, which are relatively concentrated in myelin (Norton and Autilio, 1966a; Horrocks), are lost from demyelinated white matter (Gerstl, et al.; Norton, et al., 1966b; Svennerholm; Howell et al., 1964, 1969). Concentrations of esterified cholesterol often increase drastically in demyelinating white matter from the minute levels found in normal adult brain (Norton, et al., 1966b; Svennerholm; Howell et al., 1964). Plasmalogenase activity was increased in partially demyelinated white matter resulting from multiple sclerosis (MS) in a human patient, avitaminosis B₁₂ in monkeys (Ansell and Spanner, 1968) and canine distemper infection in dogs (McMartin et al.).

Myelin protein is also destroyed during demyelination (Einstein et al., 1970). Brain tissue contains at least two enzymes (Marks and Lajtha, 1963, 1965) which may hydrolyze myelin protein. Acid proteinase, the better characterized of the two brain proteinases (Marks and Lajtha, 1965), is capable of cleaving the basic (encephalitogenic) protein of myelin (Einstein et al., 1968). Its activity was shown to be increased on the actively demyelinating edge of multiple sclerosis plaques (Einstein et al., 1970). The other proteolytic enzyme, neutral

proteinase, is more concentrated in white than in grey matter (Ansell and Richter). Seven to 14% of the neutral proteinase in the brain is firmly attached to isolated myelin, suggesting that this enzyme might be trapped between myelin lamellae (Riekkinen et al.).

In addition to acid and neutral proteinases, beta-glucuronidase has been associated with demyelinating conditions. This enzyme was increased in the cerebrospinal fluid (CSF) of rabbits with experimental allergic encephalomyelitis (EAE) (Paterson and Steele), from dogs with distemper-associated demyelination (Long et al.), and from human patients with acute demyelinating diseases (Allen and Reagan, 1964). Beta-glucuronidase, a nonspecific lysosomal enzyme, is useful as an indicator of inflammation (Allen, 1968) or the presence of cells containing phagocytosed material (Meijer and Willighagen).

Parainfectious demyelination resulting from distemper virus infection in dogs shares many common features with demyelination in other diseases of man and animals. Canine distemper virus (CDV) is antigenically related to measles virus (Imagawa), an agent which has been isolated from brains of children with subacute sclerosing pan-encephalitis (SSPE) (Chen, et al.; Horta-Barbosa, et al.; Payne, et al.). CDV antigens were demonstrated in glial cells of dogs within areas of demyelination (Appel) and measles virus antigens occurred in brain biopsies of children with SSPE (Muelen et al.). Demyelination has been produced in vitro by the action of distemper virus on cerebellar explants (Storts et al., 1968b). Histologically, demyelination in SSPE, multiple sclerosis (MS) and canine distemper often is centered around vessels which have perivascular cuffs of lymphocytes and

plasma cells (Peters; Greenfield and Norman; Jubb and Kennedy). This lesion, which is also typical of the lesion produced in experimental allergic encephalomyelitis (EAE) (Peterson et al.; Stone and Lerner), suggests that autoimmunity plays a role in demyelination in multiple sclerosis (Peters) and canine distemper (Trevan). In vitro experiments further support the concept of an autoimmune demyelination. Sera from animals with EAE (Bornstein and Appel, 1961, 1965), from patients with MS (Bornstein and Appel, 1965; Lumsden; Kim et al.) and from dogs with distemper-associated demyelination (Koestner et al., 1970b) cause demyelination of myelinated explants. Lymphocytes from animals sensitized against brain were shown to produce a myelinotoxic serum agent when cultured in vitro (Bornstein and Iwanami, 1971).

Distemper-associated demyelination was used as a model in an attempt to clarify the role of proteinases and lysosomal hydrolases in the process of demyelination. It is apparent that several factors may account for enzymatic changes in the nervous tissue of dogs during distemper-associated demyelination. One must consider invading lymphocytes as a source of enzymes, or that the enzyme activity be directly related to either the effect of distemper virus or of myelinotoxic serum upon brain cells. The enzyme content of isolated lymphocytes can be readily measured but the effects of virus and myelinotoxic serum on the enzyme content of brain cells are more difficult to determine because of secondary changes in the brain not directly related to either the virus or serum during the course of this disease. Enzyme changes resulting from the effects of CDV and myelinotoxic serum can be best evaluated through the use of primary glial monolayer cultures of canine brain.

The cells, which can be grown in quantities sufficient for biochemical determinations, retain many of their in vivo morphological characteristics and are susceptible to injury caused by CDV infection.

Finally, any enzyme changes found in demyelinating brain may be nonspecific events that occur whenever nervous tissue is damaged. Ischemic necrosis was selected as another form of tissue damage to determine whether altered activities of these enzyme are restricted specifically to demyelination. Cerebral ischemia is easily produced by ligation of one carotid artery in a gerbil (Lewis and Payan, 1966). The resultant infarction presents at least two types of tissue reaction in which enzymes can be measured: uncomplicated ischemic necrosis occurring soon after ligation, and invasion of macrophages to digest necrotic tissue several days later.

The first objective of this study was to determine whether the activities of acid and neutral proteinase and beta-glucuronidase are increased in the cerebellums of dogs with distemper-associated demyelination in comparison to normal dogs. Demonstration of elevated activities of these enzymes in distemper-associated demyelination would further support the hypothesis that demyelination in different diseases and various species proceeds by common biochemical processes. The second objective was to evaluate the source and significance of the enzyme changes found in demyelinated tissue. This was attempted in 3 ways: 1) by comparing the content of these enzymes in isolated lymph node cells (lymphocytes) with the enzyme changes found in severely demyelinated tissue; 2) by measuring these enzyme activities in canine glial monolayer cultures infected with CDV or exposed to myelinotoxic

serum and comparing them with the levels of activity in uninfected and unexposed control cultures; and 3) by assessing the activities of these enzymes during early and late cerebral infarction and comparing any enzyme changes with enzyme changes previously demonstrated in areas of demyelination.

Materials and Methods

Dogs.

Dogs with clinical signs of distemper-associated demyelination were anesthetized with intravenous sodium pentobarbitol and exsanguinated. A 0.5 cm transverse slice was taken from the middle of each cerebellum, divided into blocks of about 0.5 g each and stored at -50°C . Adjacent tissue was fixed in neutral buffered formalin, embedded in paraffin, sectioned at $6\ \mu$, stained with hematoxylin and eosin (H&E) and luxol fast blue-periodic acid Schiff (PAS) stains and examined histologically. Cerebellar tissues from control dogs, which were free from clinical signs of distemper, were obtained and treated in an identical manner. Intranuclear inclusion bodies pathognomonic for canine distemper and areas of demyelination were demonstrated in all diseased dogs. Control dogs were free of clinical and pathological evidence of canine distemper infection.

Tissue blocks from diseased and control dogs were paired so that the age of the dogs and the visually estimated ratio of white to grey matter in the tissue were nearly the same. Cryostat sections of each frozen block of tissue were cut just before enzymatic analysis and the sections were stained with Paragon Multiple Stain (Paragon C. & C. Co., Inc., Bronx, N.Y.). The frozen sections and paraffin sections taken

from the adjacent formalin fixed tissue were used to determine the extent of demyelination and gliosis in each block of tissue. The blocks of tissue were thawed and homogenized in 0.2% octylphenoxy polyethoxyethanol (Triton X-100) (Bingham et al.) for subsequent enzymatic analysis. Both members of each pair of tissue blocks were assayed simultaneously. The entire assay was repeated on another set of paired tissue blocks from the same dogs and the two values obtained from each dog were averaged. Tissue from 10 pairs of dogs was assayed. Enzyme activities from distemper tissues and the paired control tissue were statistically evaluated by the Student t-test for paired samples (Freund et al.).

Canine Lymphocytes.

A normal dog weighing 25 kg was killed by electric shock and both popliteal lymph nodes were removed immediately. The lymph nodes were cooled at 4°C and subsequent manipulations were performed at 4°C. The lymph nodes were minced with scissors, suspended in 5 ml normal saline and centrifuged 4 minutes at 800 RPM (160 xg). The supernate was decanted and the pellet resuspended in 5 ml saline. Debris was allowed to settle out for 4 minutes and the supernate was then decanted and saved (Billingham). The processes of centrifugation, resuspension and sedimentation were repeated once more. The entire procedure yielded 185 million cells which were relatively free of foreign material as judged by light microscopy. Triton X-100 was added to a concentration of 0.2% and the cell suspension was frozen, thawed, homogenized and assayed for enzyme activities.

Monolayers.

Monolayers were prepared according to the method described by Storts et al. (1968b) from cerebral hemispheres of newborn puppies. Leptomeninges and the choroid plexus were removed before the hemispheres were homogenized. The following alterations in media were made: the initial medium contained 2% of the stock 8.8% NaHCO_3 ; the second (maintenance) medium (changed at 6 days) contained $2\frac{1}{2}\%$ of the stock NaHCO_3 and $7\frac{1}{2}\%$ fetal calf serum (FCS); the third medium (changed 1 week later) contained 3% of the stock NaHCO_3 and 5% FCS. The monolayers were grown in 100 ml prescription bottles and in Leighton tubes. Beta glucuronidase and acid and neutral proteinases had the same pH optimums in both the monolayer cells and the canine cerebellums (unpublished data).

Virus Infected Monolayers.

Six day-old cultures were infected with the Lederle strain of canine distemper virus. The virus had been previously adapted to glial monolayers by 3 serial passages in these cells (Storts et al., 1968b) and stored at -70°C (Koestner and Long, 1970a).

The viral titer was 10^2 tissue culture infective doses (50% infectivity) per ml. as indicated by the cytopathic effect (eosinophilic intranuclear and intracytoplasmic inclusion body formation and giant cell production) in cerebral monolayer cultures 21 days after inoculation. Each bottle was infected with two ml. of virus suspension and each Leighton tube with 0.2 ml. of virus as described by Storts et al. (1968b). Control cultures were inoculated with the same volume of an uninfected cell suspension. The medium was changed every 7 days post-infection (p.i.).

Cells from 3 infected and 3 control culture bottles were collected 1, 2 and 3 weeks p.i. These time sequences were chosen because they represent significant stages in the viral replicative cycle (Koestner and Long, 1970a). The medium was removed at these times and centrifuged for 10 minutes at 160 g to remove cellular debris. The supernate was then assayed for beta-glucuronidase activity. The monolayer was rinsed two times with 2 ml. saline, allowed to drain in a vertical position for 2 minutes, and thawed again. Two ml. of 0.2% Triton X-100 was added, the cells were scraped loose with a stainless steel spatula and the suspension poured into an all glass homogenizer. The bottle was rinsed with an additional 2 ml. of 0.2% Triton X-100. This procedure removed essentially all of the cells in a monolayer. The cells were then homogenized and assayed for enzyme activity. Each bottle was treated separately. Coverslips from the infected and control Leighton tube cultures were collected at the same time as the bottles. The coverslips were fixed in Bouin's solution, stained with hematoxylin and eosin (H&E) and examined microscopically.

Myelinotoxic Canine Serum on Monolayers.

Serum was aseptically collected from normal dogs and from dogs with clinical signs and lesions of distemper-associated demyelination. The serum was stored at -50°C . The criterion for myelinotoxicity was the ability of the serum, at a concentration of 25% to cause in vitro demyelination (Koestner et al., 1970b). The test cultures were myelinated canine cerebellar explants grown on collagen coated coverslips in Leighton tubes (Storts and Koestner, 1968a; Storts et al., 1968b, 1969). Control serum lacked any in vitro toxic effect on myelin. The myelino-

toxic serum was pooled from 3 dogs and the control serum from 4 dogs. These pooled batches of sera will hereafter be called distemper and control sera.

Canine serum was added to the maintenance media described above at a concentration of 19%, replacing an equal volume of Hank's balanced salt solution containing 0.5% lactalbumin hydrolysate (Storts et al., 1968b).

A second batch of canine cerebral monolayers was started as described above. After 6 days in vitro growth the initial medium was replaced with maintenance media containing canine serum. The monolayers were incubated for 2 hours at 37°C and then 0.2 ml. of guinea pig complement was added to each bottle and 0.04 ml. to each Leighton tube. Extra complement was added to maximize the opportunity for any complement-dependent reaction to occur in the cultures. Cells from 8 control cultures and from 8 cultures with distemper serum were collected and homogenized separately as described above at 1, 3 and 5 days after the media with dog serum had been added.

The results from the monolayer culture experiments were statistically evaluated by the Student t-test (Freund et al.).

Cerebral Infarction.

Mongolian gerbils (Meriones unguiculatus), 2½ months of age, were obtained from Chick Line, Vineland, New Jersey. The gerbils were anesthetized with ether and their right carotid artery was ligated twice. The skin incision was closed by sutures and the gerbil placed in a 34°C environment for 1 hour. Animals were examined frequently for neurological signs during the first few hours after ligation and

sporadically thereafter. Neurologic signs of infarction developed and persisted in 20 of the 39 animals following ligation. Most of the remaining animals exhibited signs of cerebral ischemia during or immediately after recovery from anesthesia, but all appeared normal 20 minutes later. Of the animals which "recovered", 14 were subjected to nitrogen gas inhalation for 1/2 to 1 minute. This additional treatment, which did not cause the animals to become comatose, induced cerebral infarction (verified later by histology) in 5 more animals. The gas was administered by holding a hose from which compressed nitrogen gently flowed directly in front of the gerbil's nose.

Animals died or were killed following ether anesthesia 1, 2 and 24 hours and 10 days after ligation and their brains were removed immediately (See Table 6). Gerbils with "nitrogen-aggravated" infarction were only included in the 24 hour group (one) and in the 10 day group (three) (Table 6). Gerbils lacking signs of infarction or which died or were killed at times other than those in Table 6 were not included in the study. A 2 to 3 mm. transverse slice was taken from the center of the cerebrum, fixed in neutral buffered formalin, embedded in paraffin, sectioned at 6 μ , stained with hematoxylin and eosin (H&E) and with luxol fast blue-periodic acid Schiff (PAS) stains and examined histologically. The remaining cerebrum was separated into right and left hemispheres and stored at -50°C until assayed for enzymatic activity. The cerebral hemispheres were homogenized in 0.2% Triton X-100 and both hemispheres from each animal were assayed simultaneously for enzyme activities. Enzyme activities of the infarcted (right) cerebral hemispheres and their control (left) cerebral hemispheres were

statistically evaluated by the Student t-test for paired samples (Freund et al.).

Enzyme Assays.

Acid and neutral proteinase were measured by the procedure of Marks and Lajtha (1963, 1965) with the following modifications: the substrate for acid proteinase was 2% hemoglobin (Hemoglobin Substrate Powder, Worthington Biochemical Co.) dissolved in 0.05 M citrate, pH 3.2, containing 0.2% Triton X-100 (Serre and Marks). The substrate for neutral proteinase was prepared the same way except that 0.05 M TES (N-Tris (Hydroxymethyl) Methyl-2-aminoethane sulfonic Acid), pH 7.6 replaced citrate buffer. Release of free amino groups was measured by reaction with ninhydrin as described by Bingham et al.

Beta glucuronidase was determined at pH 5.0 by the method of Talaley et al. as modified for brain homogenates by Dekirmenjian et al., using phenolphthalein β -glucuronide as substrate. Protein was measured colorimetrically by the method of Lowry et al.

Results

Canine Cerebellums.

Acid and neutral proteinase activities were significantly higher in the cerebellums of the diseased dogs than in cerebellums of control dogs (Table 1), while total protein content was essentially the same in both groups. Beta-glucuronidase activities were higher in the distemper group (Table 1). The increase (119%) was found to be statistically significant ($P < 0.05$) if enzyme activities of the distemper tissues were first calculated as percent of their paired controls or if pairs from

the least demyelinated tissue ("+" group, Fig. 1; see below) were not included.

Enzyme activities were highest in tissues with most severe demyelination (Fig. 1). The severity of demyelination was determined microscopically and graded as "+, ++, and +++", according to the extent of demyelination. Tissues of the "+" group had only one or two small foci of demyelination and the lowest enzyme activities. Up to 30% of the myelin was lost from the "+++" group of tissues (Fig. 2). The cellular composition of the "+++" lesions indicated that demyelination was more recent in some animals than in others. The earliest demyelinating lesion contained primarily gitter cells and plump astrocytes (gemistocytes). Numerous, thick perivascular cuffs consisting of lymphocytes were present in older lesions (Fig. 2 and 3). Fibrous astrocytic scarring predominated in the most chronic-appearing lesion of the "+++" group of tissues. The latter lesion contained occasional glial foci and only minimal perivascular infiltration of inflammatory cells (Fig. 4).

Lymphocytes.

The enzyme content of normal canine lymph node cells (lymphocytes) is compared in Table 2 to the mean increase of enzyme activities in severely ("+++") demyelinated cerebellums. The tissue of group "+++" that was most densely infiltrated with lymphocytes was estimated to contain 10^8 lymphocytes per cc (or gram) of tissue. This value was obtained by counting these cells in Fig. 3, multiplying by the magnification and dividing by the section thickness ($5\ \mu$) and the area in the photograph. The enzyme content of 10^8 cells isolated from lymph nodes of a

normal dog (right side of Table 2) is 4 to 20 times less than the mean increase of enzyme activity in the most severely demyelinated cerebellums (left side of Table 2).

CDV Infected Monolayers.

Biochemical changes resulting from virus infection were not detected in cultured brain cells before 14 days p.i. Enzyme activities and protein contents of infected and control cells were similar at 7 days (Table 3). In the control cultures, protein content and acid proteinase and beta-glucuronidase activities increased to near maximum levels at 14 days and then remained constant for the next week. Neutral proteinase activities were more variable in the controls. At 14 days, and more so at 21 days, protein decreased in the infected cultures. Likewise, enzyme activities decreased during the same time. Enzyme activities in Table 1 are expressed as m- or μ moles/g protein/hr, which means that the enzyme content of the entire culture decreased even more than protein.

Virus infected cells released a greater proportion of their total beta-glucuronidase content into the culture medium than did control cells. (See last two rows, Table 4). The beta-glucuronidase activity of the medium increased from 20 nmoles/hr/culture at the time when the medium was added to the culture to a level two to three times higher during the 7 days that the medium was on the culture. Acid and neutral proteinase activities in the medium were too low to be readily detectable above the high background due to amino acids present in the medium.

Morphological changes in the cultured cells paralleled the biochemical changes caused by virus infection. Canine cerebral monolayers and the degenerative changes produced in them have been previously described in detail (Gibson; Storts and Koestner 1968a; Storts et al., 1968b). The morphological findings at 1, 2 and 3 weeks post-inoculation (p.i.) with canine distemper virus will be summarized here.

Control and infected cultures could not be differentiated by morphological criteria at 7 days p.i. The predominant cell type in control cultures 14 days p.i. was a protoplasmic astrocyte (Fig. 5). A few oligodendrocytes were recognized by their small dark nucleus suspended between two or more astrocytes by thin cytoplasmic processes (Storts and Koestner, 1968a).

Viral-induced changes such as the appearance of intranuclear and intracytoplasmic inclusion bodies, syncytial giant cell formation and cellular degeneration were frequent in the cultures infected for 14 days (Fig. 6). Fibrous constriction of astrocytic processes and cell loss gave these cultures a reticulated appearance (Storts et al., 1968b; Gibson).

These degenerative changes appeared more pronounced in cultures infected for 21 days (Fig. 8). Inclusion bodies appeared larger, fewer cells were present and the remaining astrocytes were almost entirely fibrous. The 21 day control cultures (Fig. 7) contained about the same number of astrocytes as 14 day control cultures, but the 21 day cells appeared much more fibrous. Most of the oligodendroglia had disappeared by this time.

Myelinotoxic Canine Serum on Monolayers.

Morphologically the cultures incubated with a distemper serum appeared similar to cultures incubated with control serum.

Incubation of cerebral monolayers with distemper serum decreased the activity of all three enzymes by the third day (Table 5). By 5 days neutral proteinase and beta-glucuronidase activities of cultures with distemper serum had returned to control values. Acid proteinase activity had not yet reached control levels by this time. At no time was the protein content of cultures incubated with distemper serum significantly different from the protein content in control cultures. Beta-glucuronidase activities were 0.016 mmoles/ml in control medium and 0.009 mmoles/ml in the distemper medium before incubation.

Cerebral Infarction.

Beta-glucuronidase, acid and neutral proteinase activities were not significantly altered by uncomplicated ischemic neurosis at 1, 2 and 24 hours following arterial ligation (Table 6). Decreased protein concentration during this time reflects the presence of cerebral edema (Plum et al.). Microscopic evidence of infarction first appeared as nuclear pyknosis and cytoplasmic vacuolation at two hours and was prominent at 24 hours (Fig. 9). Leucocytic invasion did not occur within 24 hours following ligation. Development and persistence of severe neurological signs was interpreted as evidence that cerebral infarction was present in those gerbils that died or were killed before detectable lesions developed. These signs, which included rapid circling, twisting of the head and trunk and rolling (all to the right), often terminated in myoclonic seizures. They were followed by a few minutes of calmness

and motor inactivity. Affected gerbils that lived longer than two hours developed massive ischemic necrosis of their right cerebral hemisphere (Fig. 10).

Acid proteinase and beta-glucuronidase activities were greatly increased in infarcted cerebral hemispheres 10 days after arterial ligation. Protein concentration and neutral proteinase activities in these hemispheres were not significantly altered. Necrotic tissue of the 10 day-old infarcts, which involved 1/4 to 1/3 of the right cerebral hemisphere, was invaded and essentially replaced by many particle-laden macrophages (Figs. 11 and 12). Neurological abnormalities in this group of animals were limited to lack of vision in the left eye and a tendency to turn left more often than right.

Discussion

Beta-glucuronidase and acid and neutral proteinase activities were elevated in demyelinated tissue above the levels found in normal cerebellums from dogs of the same age. Pairing the diseased dogs with a control dog of the same age simplified interpretation of the results because such pairing minimized the effect that maturation has on brain enzyme activities (Robins et al., 1961b). Beta-glucuronidase activity decreased with age ($r = 0.72$, $P < 0.02$) in the cerebellum of control dogs (present study) and in maturing rat cerebellums (Robins et al., 1961b). Acid and neutral proteinase activities also decrease in the maturing rat brain (Marks and Lajtha, 1970), but not enough control animals were used in the present study to confirm this decrease in dogs.

Activities of these three enzymes tended to be higher in the most severely demyelinated lesions. Acid proteinase activity increased most

and was almost as accurate an indicator of the extent of demyelination within a given tissue block as microscopic examination for lesions in the frozen section from that block.

The finding of elevated acid and neutral proteinase activities in the more severely demyelinated tissue strongly suggests that both proteinases contribute to the demyelination process by degrading myelin proteins. Acid proteinase has previously been shown to be capable of degrading the basic (encephalitogenic) protein of myelin (Einstein et al., 1968) into subunits small enough to pass through a semipermeable membrane (Nakao and Einstein). Preliminary results using a crude preparation of basic protein indicate that neutral proteinase can also degrade basic protein (McMartin, unpublished results).

Einstein et al. (1970) reported that acid proteinase activity was greatly increased, and basic protein decreased, in the actively demyelinating border of multiple sclerosis plaques. These authors suggested that acid proteinase may cleave the encephalitogenic protein of myelin and allow the products, which are still encephalitogenic (Nakao and Einstein), to reach immunocompetent cells and initiate a process of autoimmunity. Acid proteinase activity was also increased in rat sciatic nerve undergoing Wallerian degeneration (Hallpike et al.).

Neutral proteinase activity was increased in peripheral nerve and spinal cord undergoing Wallerian degeneration (Hallpike, et al.; Porcellati, 1966, 1970). These increases occurred at a time when degenerated myelin was phagocytosed by macrophages and proliferated Schwann cells (Greenfield and Meyer; Robins, 1961a). However, neutral proteinase could degrade myelin directly without phagocytosis being

necessary since this enzyme is partially located within myelin (Reikkinen et al.).

The elevation of beta-glucuronidase in the most severely demyelinated tissues (groups "++" and "+++") was probably related to the presence of inflammation and/or phagocytosis. Highest beta-glucuronidase activities were found in the most severely inflamed (most caudal) portions of the spinal cords of rabbits with EAE (Arnaki and Weissbarth). An increase of beta-glucuronidase and other lysosomal enzymes was also correlated with the appearance of phagocytic cells during Wallerian degeneration of both peripheral nerve and central myelinated tracts (McCaman and Robins, 1959; Robins, 1961a).

The lymphocytes present within perivascular cuffs can probably be excluded as the source of the increased enzyme activities in demyelinated tissue. The mean increase of enzyme activities in the most severely demyelinated tissues (Table 2) was 4 to 20 times greater than enzyme activities measured in isolated lymphocytes comparable in number to the maximal number of lymphocytes present within a given sample of affected cerebellum. This comparison may be misleading because these lymphocytes from normal lymph nodes were not sensitized and had not undergone blastogenic transformation. We observed, however, that tissues with few lymphocytes (such as Fig. 4) had enzyme activities similar to those tissues with many lymphocytes (Fig. 3).

The in vitro studies using canine cerebral monolayer cultures suggest that the actions of canine distemper virus and myelinotoxic serum on glial cells were not the immediate cause of the increased enzyme activities previously found in severely demyelinated cerebellums.

The protein content of infected monolayers and enzyme activities in these cells were less than the respective values in non-infected cultures. Microscopically visible cell damage undoubtedly caused much of the protein and enzyme loss in infected cells. Distemper virus may also act similarly to poliovirus and inhibit protein synthesis in infected cells. The inhibition by poliovirus was not dependent on and could be separated from production of virus and induction of morphological changes within the cell (Bablanian et al., 1965a,b). An inhibition of protein synthesis in oligodendroglia by distemper virus would upset the dynamic balance of protein formation and degradation by which these cells maintain normal myelin (Smith). Demyelination would then proceed without increased activity of catabolic enzymes. Virus infection may also activate the neutral proteinase which is located within myelin (Reikkinen et al.). This possibility could not be excluded because myelin was not present in the monolayers.

Distemper virus infection may also damage cells by release of pre-formed lysosomal hydrolases. Media from infected cultures contained a higher percentage of the total beta-glucuronidase content of the culture than media from control cultures (Table 2) which indicates release of intracellular enzymes. Release of hydrolases from lysosomes ("activation") has been reported in other virus culture systems (Allison and Sandelin; Allison and Mallucci; Sato et al., Sabesin et al.; Mallucci and Allison). Enzyme release preceded (Allison and Sandelin) or occurred at the time when cytopathic effects were first seen microscopically (Allison and Mallucci). Inhibition of protein synthesis did not affect enzyme release which suggests that viral infection causes a re-

distribution rather than de novo synthesis of lysosomal enzymes (Mallucci and Allison). Beta-glucuronidase released from brain cells in vivo by distemper virus may contribute to the high activity of this enzyme in cerebrospinal fluid of dogs with distemper-associated demyelination (Long et al.).

Serum from dogs with distemper-associated demyelination also depressed enzyme activities of cerebral monolayer cells. The serum used was a blend of three sera, each of which was selected for its ability to demyelinate cerebellar explants in the presence of complement (Koestner et al., 1970b). Enzyme activities were lowest at three days after addition of myelinotoxic serum and back to near control values by 5 days. This apparent recovery at 5 days may have resulted from spontaneous deterioration of complement (Humphrey and White) and/or other serum factors.

The identity of the toxic agent in distemper serum that caused these enzyme changes is unknown. The measured inhibition did not result from serum factors directly inactivating the enzymes because such inactivation should have been detectable within 1 day. Although the sera used in this study were not assayed for viable virus, it is safe to assume that they did not contain any virus. Samples of the sera used previously on myelinated cultures did not produce any evidence of viral infection although the cultures were maintained for more than 2 weeks after the serum was removed to test for remyelination. Appel (1969) was not able to detect distemper virus in the serum of dogs at the same stage of disease as the dogs from which the myelinotoxic serum was obtained. Finally, infection of similar cultures with a high

titer of CDV did not produce morphological or enzymatic changes at 7 days p.i.

Several factors could have been present in the myelinotoxic distemper serum, some of which may not have been antibody against myelin proteins. Myelinotoxic serum factors capable of destroying myelin in vitro have been found in sera from patients with MS (Bornstein and Appel, 1965; Kim et al.; Lumsden) and from animals with EAE (Bornstein and Appel, 1961, 1965). Demyelination with these sera, as with some distemper sera (Koestner et al., 1970b) is complement dependent. Kim et al. isolated a myelinotoxic factor from MS plaques. This factor was considered to be complement dependent because toxic activity was abolished by heating the extract at 56°C for 30 minutes. This treatment does not rule out the presence of lymphotoxin which is inactivated under similar conditions (Lawrence and Valentine). Lymphotoxin is produced by lymphocytes following attachment of these cells to a target cell. Lymphotoxin, which does not require complement, kills both the target cell and the lymphocytes (Granger and Kalb). Another toxic agent has been found in sera of MS patients. This factor, which withstood heating at 56°C for 45 minutes, inhibited RNA synthesis in lymphocytes (Stjernholm et al.; van den Noort and Stjernholm).

Unilateral cerebral infarction was employed to determine whether degenerative lesions other than demyelination would be associated with increased activities of beta-glucuronidase and acid and neutral proteinases. The gerbil was used because its cerebral circulation allows one to easily produce unilateral cerebral infarction by occluding one carotid artery (Levine and Payan; Levine and Sohn). The non-ischemic hemisphere

served as an excellent control for the ischemic side and permitted discrimination between effects caused by ischemia and other factors such as anesthesia and/or seizures.

Uncomplicated ischemic necrosis occurring within 24 hours after arterial ligation did not increase activities of beta-glucuronidase, acid and neutral proteinase above levels in the control hemisphere (Table 6). This confirms the results of Clendenon et al., who found that lysosomal enzymes were neither increased nor released from lysosomes ("activated") early enough to cause the cell damage found in anoxic-ischemic rats. These findings are useful because they indicate that the increase of enzymes, especially neutral proteinase, in demyelinating tissues (Hallpike et al.) did not result from non-specific factors injurious to glial cells.

The cause of the statistically significant increase of beta-glucuronidase activity between one and two hours and of acid proteinase activity between 2 and 24 hours in the control hemisphere is unknown. This increase may be related to the convulsive seizures experienced by these animals.

Neutral proteinase activity was also not elevated in the 10 day-old lesions. The distribution of lesions in this group of animals was fortuitous because white matter was relatively spared. Hence, enzyme changes associated with demyelination were not present to obscure the finding that macrophage invasion did not affect neutral proteinase activity.

The increase of beta-glucuronidase and acid proteinase activities many days after ischemia correlated with and was probably due to the

presence of macrophages within the area of infarction (Fig.12). This indicates that increased activities of these lysosomal enzymes during demyelination are nonspecific findings which are encountered whenever macrophages invade and digest previously damaged tissue (Meijer and Willighagen; Cohn and Benson). These studies do not eliminate the possibility that the lysosomal enzymes of macrophages help initiate autoimmune demyelination by degrading damaged myelin and releasing its antigens to the immune systems.

The results of the present study contribute to our knowledge of the biochemical breakdown of myelin in canine distemper and other demyelinating diseases. CDV infection of brain cells may start a sequence of biochemical events which, if not stopped or altered by the host, may culminate in demyelination. An early event may be the release of acid proteinase from infected cells. Acid proteinase cannot act on isolated myelin (Marks, 1971) but can degrade protein isolated from myelin (Einstein et al., 1968). Hence, myelin must be altered by plasmalogenase and/or another agent active in the initiation of demyelination (McMartin et al.). Acid proteinase is then capable of acting on its freed substrate. Both lysophosphatidyl ethanolamine (the catabolic product of plasmalogenase) and lysolecithin (a structurally similar compound) have detergent properties and can rupture cell membranes (Gottfried and Rapport). Lysolecithin can also release acid proteinase from intact lysosomes (Porcellati, 1970; Marks and Lajtha, 1965) and dissolve myelin (Gregson et al.). Plasmalogenase may, therefore, act indirectly to release acid proteinase from lysosomes and to expose myelin protein to this catabolic enzyme. The encephalitogenic subunits

that acid proteinase cleaves from myelin basic protein could then escape to immunocompetent cells and the resulting autoimmune encephalomyelitis (Bornstein and Appel; Kim et al.; Dau and Peterson; Koestner et al., 1970) could be responsible for the progressive character of parainfectious demyelination.

During experimentally induced allergic encephalomyelitis, enzymes forming myelin lipids are less active which indicates a failure of the oligodendroglia to maintain normal myelin (Smith). Normal metabolism of myelin components by oligodendroglia may also be impaired in distemper-associated demyelination by one or more toxic agents in the serum and/or by viral infection of these cells. Finally, macrophages, which contain acid proteinase and beta-glucuronidase and other lysosomal enzymes, enter, phagocytose and digest myelin debris.

The role of neutral proteinase in demyelination is not clear. The enzyme could be activated by an agent toxic to oligodendroglia and/or myelin. Since some neutral proteinase is already in myelin (Reikkinen et al., 1970), the enzyme could then initiate demyelination by cleaving adjacent myelin proteins. Conversely, neutral proteinase could be involved only in the Wallerian degeneration which occurs to some extent in both multiple sclerosis (Greenfield and Norman) and canine distemper (Jubb and Kennedy). Further studies of neutral proteinase should be profitable because of the close association of this enzyme with myelin (Reikkinen et al.).

Summary

Beta-glucuronidase, acid and neutral proteinase activities were higher in the cerebellums of dogs with distemper-associated demyelina-

tion than in the cerebellums of age-matched control dogs. Interpretation of results was simplified by pairing dogs according to their age because maturation effects were avoided. The highest elevation corresponded with the most severely demyelinated cerebellums. Evidence was presented that the source of the increased enzyme activities was not from enzymes within the lymphocytes that were present in areas of demyelination.

Canine cerebral monolayer cultures were used to determine the effects of canine distemper virus and canine myelinotoxic serum on activities of these enzymes. Cultures infected with distemper virus had lower protein content and enzyme activities than noninfected control cultures. Media from the infected monolayer contained a greater proportion of the culture's total beta-glucuronidase content than media from control cultures. This suggests that distemper virus releases pre-formed lysosomal enzymes.

Serums collected from dogs with distemper-associated demyelination have been shown previously to demyelinate cerebellar explants. The myelinotoxic serum caused a transient decrease of enzyme activities which occurred three days after serum was added to the culture. These findings suggest that canine distemper virus and myelinotoxic serum from dogs with distemper-associated demyelination contribute to brain damage by releasing lysosomal enzymes and by inhibiting the synthesis of enzymes and probably of other proteins.

Cerebral infarction was produced in gerbils by unilateral carotid artery ligation. The brains were examined for morphologic and enzymatic changes at 1, 2, 24 hours and 10 days following surgery. Uncomplicated

ischemic necrosis occurring within 24 hours of arterial ligation did not alter activities of beta-glucuronidase and acid and neutral proteinases. Decreased protein concentration and early excitatory signs indicated the presence of cerebral edema. Increased beta-glucuronidase and acid proteinase activities ten days after infarction were associated with macrophage invasion and digestion of necrotic tissue. Neutral proteinase activity remained unchanged. These findings suggest that the increased activities of beta-glucuronidase and acid proteinase found in distemper-associated demyelination probably are derived from macrophages present in the tissues and that neutral proteinase activity, which was not increased in ischemic necrosis, may be more specific for demyelination.

TABLE 1. PROTEIN CONCENTRATION AND ENZYME ACTIVITIES IN CEREBELLUMS FROM NORMAL DOGS AND DOGS WITH DISTEMPER-ASSOCIATED DEMYELINATION

	<u>DISTEMPER</u> <u>(n=10)</u>	<u>CONTROL</u> <u>(n=10)</u>	<u>COMPARISON</u>
PROTEIN (mg/g wet weight)	114 $\pm$ 1 ^a	112 $\pm$ 2	t=0.75; N.S. ^b
ACID PROTEINASE (nmoles/hr/g protein)	0.589 $\pm$ 0.053	0.263 $\pm$ 0.016	t=5.25; P<0.001
NEUTRAL PROTEINASE (nmoles/hr/g protein)	0.211 $\pm$ 0.021	0.177 $\pm$ 0.013	t=2.42; P<0.05
BETA-GLUCURONIDASE (μ moles/hr/g protein)	11.8 $\pm$ 1.0	9.95 $\pm$ 0.6	t=2.06; 0.1>P>0.05

^a Mean $\pm$ SE

^b Probability that distemper equals control

TABLE 2. COMPARISON OF ENZYME CHANGES ASSOCIATED WITH DEMYELINATION WITH ENZYME ACTIVITIES OF LYMPHOCYTES

	INCREASE OF ENZYME ACTIVITIES IN "+++" DEMYELINATED TISSUE (umoles/hr/g wet tissue, mean $\pm$ SE)	ENZYME ACTIVITIES OF CANINE LYMPH NODE CELLS (umoles/hr/ 10^8 cells)
ACID PROTEINASE	48.6 $\pm$ 9.0	2.3
NEUTRAL PROTEINASE	9.1 $\pm$ 3.1	0.9
BETA-GLUCURONIDASE	0.40 $\pm$ 0.12	0.1
PROTEIN	3.2 $\pm$ 3.2 mg/g tissue ^a	3.38 mg/ 10^8 cells

^a Protein loss in demyelinated tissue was not statistically significant.

TABLE 3. ENZYME ACTIVITIES OF GLIAL MONOLAYER CELLS INFECTED WITH CANINE DISTEMPER VIRUS

Days post-inoculation		7	14	21
PROTEIN (mg/culture)	Infected	1.50 ± 0.05^a	2.05 ± 0.05^c	1.69 ± 0.08^d
	Control	1.50 ± 0.05	2.74 ± 0.13	2.92 ± 0.07
ACID PROTEINASE (mmoles/hr/g protein)	Infected	1.66 ± 0.20	1.16 ± 0.15^b	1.07 ± 0.09^b
	Control	2.13 ± 0.11	2.06 ± 0.16	2.09 ± 0.25
NEUTRAL PROTEINASE (mmoles/hr/g protein)	Infected	0.31 ± 0.04	0.14 ± 0.06	0.15 ± 0.10^b
	Control	0.41 ± 0.06	0.26 ± 0.05	0.65 ± 0.05
BETA-GLUCURONIDASE (μ moles/hr/g protein)	Infected	45.6 ± 3.8	34.9 ± 1.0^d	26.6 ± 0.6^d
	Control	47.6 ± 2.8	73.1 ± 3.8	76.4 ± 2.8

^a Mean $\pm$ SE; ^b P<0.02 that infected equals control; ^c P<0.01; ^d P<0.001

TABLE 4. BETA-GLUCURONIDASE CONTENT OF VIRUS-INFECTED CULTURES

		7 Days	14 Days	21 Days
CELLS ^a	Infected	69 $\pm$ 6	72 $\pm$ 1	45 $\pm$ 2
	Control	72 $\pm$ 5	201 $\pm$ 19 ^b	223 $\pm$ 5 ^c
MEDIA ^a	Infected	55 $\pm$ 2	74 $\pm$ 4	51 $\pm$ 0
	Control	52 $\pm$ 1	76 $\pm$ 6	66 $\pm$ 1 ^c
MEDIA (% of total)	Infected	44.7 $\pm$ 2.5	50.7 $\pm$ 1.6	53.3 $\pm$ 1.0
	Control	42.3 $\pm$ 1.4	27.5 $\pm$ 0.4 ^c	22.8 $\pm$ 0.1 ^c

^a nmoles/hr/culture, mean $\pm$ SE

^b P<0.01 that infected equals control

^c P<0.001

TABLE 5. ENZYME ACTIVITIES OF GLIAL MONOLAYER CELLS INCUBATED WITH CANINE DISTEMPER OR CONTROL SERA

Days Incubation		1	3	5
PROTEIN (mg/culture)	Distemper	1.51 $\pm$ 0.14 ^a	2.24 $\pm$ 0.24	3.85 $\pm$ 0.25
	Control	1.54 $\pm$ 0.10	2.77 $\pm$ 0.14	3.26 $\pm$ 0.20
ACID PROTEINASE (nmoles/hr/g protein)	Distemper	0.26 $\pm$ 0.05	0.34 $\pm$ 0.12 ^d	1.05 $\pm$ 0.07 ^c
	Control	0.15 $\pm$ 0.08	0.84 $\pm$ 0.09	1.32 $\pm$ 0.05
NEUTRAL PROTEINASE (nmoles/hr/g protein)	Distemper	0.40 $\pm$ 0.08	0.20 $\pm$ 0.05 ^b	0.55 $\pm$ 0.04
	Control	0.25 $\pm$ 0.04	0.37 $\pm$ 0.05	0.57 $\pm$ 0.04
BETA-GLUCURONIDASE (μ moles/hr/g protein)	Distemper	3.8 $\pm$ 0.5	5.2 $\pm$ 0.7 ^d	14.6 $\pm$ 2.1
	Control	3.2 $\pm$ 0.3	8.9 $\pm$ 0.9	17.8 $\pm$ 1.7

^a Mean $\pm$ SE; ^b P<0.05 that distemper equals control; ^c P<0.02; ^d P<0.01

TABLE 6. EFFECT OF UNILATERAL CEREBRAL INFARCTION ON PROTEIN CONCENTRATION AND ENZYME ACTIVITIES

TIME AFTER ONSET		1 HOUR (n=3)	2 HOURS (n=4)	24 HOURS (n=4)	10 DAYS (n=4)
PROTEIN (mg/g wet weight)	INFARCTED	137 $\pm$ 2 ^a	131 $\pm$ 2 ^b	111 $\pm$ 6 ^b	119 $\pm$ 5
	CONTROL	140 $\pm$ 3	139 $\pm$ 3	133 $\pm$ 2	136 $\pm$ 5
ACID PROTEINASE (μ moles/hr/g protein)	INFARCTED	193 $\pm$ 24	193 $\pm$ 20	177 $\pm$ 34	1055 $\pm$ 49 ^c
	CONTROL	183 $\pm$ 23	172 $\pm$ 16	237 $\pm$ 19	222 $\pm$ 23
NEUTRAL PROTEINASE (μ moles/hr/g protein)	INFARCTED	205 $\pm$ 9	228 $\pm$ 11	149 $\pm$ 19	250 $\pm$ 44
	CONTROL	214 $\pm$ 8	248 $\pm$ 15	234 $\pm$ 26	242 $\pm$ 21
BETA-GLUCURONIDASE (μ moles/hr/g protein)	INFARCTED	2.48 $\pm$ 0.27	3.84 $\pm$ 0.18	3.54 $\pm$ 0.27	45.60 $\pm$ 3.42 ^c
	CONTROL	2.42 $\pm$ 0.28	3.70 $\pm$ 0.17	3.79 $\pm$ 0.37	3.75 $\pm$ 0.27

^a Mean $\pm$ SE; ^b P<0.02 that infarcted equals control; ^c P<0.001

Fig. 1. Enzyme activities grouped according to severity of demyelination. Least severely demyelinated tissue is indicated by "+" and most severely by "+++". Activities of each demyelinated tissue were first expressed as percentage of its paired control activity and then the mean (solid bar) and standard error of mean (vertical lines) of these percentages were obtained. The number of paired tissues in each group is indicated below the bars.

Fig. 1

Fig. 2. Cerebellum of a dog with distemper-associated demyelination.
The lighter staining areas (arrows) are foci of demyelination.
Luxol blue-PAS. (X 12)

Fig.2

Fig. 3. Higher magnification of Fig. 2. Note demyelination and perivascular cuffs of lymphocytes. H & E (X 920)

Fig. 3

Fig. 4. Areas of demyelination with astroglial scarring and a microglial nodule. This lesion appears more chronic than the one depicted in Fig. 3. H & E (X 570)

Fig. 4

Fig. 5. 14 day p.i. control culture. Culture consists primarily of protoplasmic astrocytes (A). Oligodendrocytes (O) are recognized by their small dark nuclei. H & E (X 133)

Fig. 5

Fig. 6. Distemper infected culture 14 days p.i. Many multinucleated giant cells (G) are present. Inclusion bodies (arrow) in these cells appear as irregularly-shaped bodies surrounded by a halo. Processes of astrocytes (A) are constricted. Amorphous masses represent degenerated cells. H & E (X 133)

Fig. 6

Fig. 7. Twenty-one day p.i. control culture. Population density of astrocytic nuclei (A) remained nearly the same as in 14 days p.i. control cultures (Fig. 1). The thin lines are astrocytic processes (P). A few oligodendrocytes are present (O). H & E (X 133)

Fig. 7

Fig. 8. Distemper infected culture 21 days p.i. Fewer cells are left and the ones remaining are either fibrous astrocytes (F) or giant cells (G). Inclusion bodies appear larger (arrow) than 14 days p.i. Oligodendroglia are rare and difficult to detect. H & E (X 133)

Fig. 8

Fig. 9. Transverse section of gerbil brain 24 hours after ligation of right carotid artery. Nearly all of the right side of the brain is infarcted. H & E (X 11)

Fig . 9

Fig. 10. Higher magnification of the midline of the section in Fig. 9. Cells to the left of the midline appear normal but those in the infarcted tissue (right side) are necrotic and contain large vacuoles suggestive of intracellular edema. H & E (X 470)

Fig. 10

Fig. 11. Transverse section of gerbil brain 10 days after ligation of right carotid artery. Necrosis and malacia are present in the cortical grey matter of the right hemisphere. H & E (X 11)

Fig. 11

Fig. 12. Higher magnification of the cortex in Fig. 11. Cytoplasm of macrophages is filled with particulate material. Section was fixed in glutaraldehyde, post-fixed in OsO_4 , dehydrated, embedded in Epon, sectioned at $1\ \mu$ and stained with toluidine blue (X 930)

Fig. 12

CHAPTER II

PLASMALOGENASE

The enzymic processes responsible for the initiation of demyelination are poorly understood despite numerous chemical analyses and morphological studies. Normal myelin is rich in lipid, with the remainder of its dry weight consisting of protein. The major components of the lipid fraction are cholesterol (28%), galactolipid (29%) and ethanolamine plasmalogen (12%) (Norton and Autilio; Horrocks, 1967). White matter from which myelin has been lost as a consequence of multiple sclerosis (Gerstl, et al.) and subacute sclerosing panencephalitis (Norton, et al., 1966b; Svennerholm) in man and swayback in sheep (Howell et al., 1964, 1969) contains abnormally low amounts of the myelin lipids cholesterol, galactolipid and ethanolamine plasmalogen. Each of these myelin components must be catabolized or removed from the brain. Plasmalogenase (1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphoryethanolamine alk-1'-enyl hydrolase) initiates the catabolism of ethanolamine plasmalogen by hydrolysis of its vinyl ether linkage to release a fatty aldehyde (Ansell and Spanner, 1965). The enzyme is located primarily in white matter but not within myelin. An elevation of plasmalogenase activity has been found in partially demyelinated white matter resulting from multiple sclerosis in a human patient and avitaminosis B₁₂ in monkeys (Ansell and Spanner, 1968), suggesting a common catabolic mechanism in demyelination caused by apparently un-

related factors (Eto et al.).

Canine distemper is a common viral disease of dogs and is often associated with parainfectious demyelination. This naturally occurring disease may be a useful model for clarification of the role of plasmatogenase in the initiation of demyelination.

The purpose of the present investigation was to determine if plasmatogenase activity in the brains of dogs with demyelination resulting from canine distemper infection is higher than the plasmatogenase activity in the brains of normal dogs. This investigation would further test the hypothesis that plasmatogenase is involved in the enzymic degradation of myelin in all demyelinating diseases.

Materials and Methods

Dogs with a clinical diagnosis of canine distemper encephalomyelitis were received alive from The Ohio State University Veterinary Hospital. Following a thorough neurological examination, the dogs were anesthetized with intravenous sodium pentobarbital and exsanguinated. A 0.5 cm thick transverse section was obtained at the level of the corpora restiforme to include cerebellum, the posterior cerebellar brachia and medulla which are established predilection sites for distemper-associated demyelination (Jubb and Kennedy). The slices of cerebellum and medulla were stored separately at -50°C until assayed for plasmatogenase activity. Adjacent tissue was fixed in neutral buffered formalin for histological examination. The formalin-fixed tissue was embedded in paraffin, sectioned at $6\text{ }\mu$ and stained with hematoxylin and eosin (H&E) and with Luxol fast blue-periodic acid Schiff (PAS). Brain tissue from control dogs, which were of comparable

age but free from clinical signs of distemper, was obtained from the same areas and treated in an identical manner.

Fourteen tissue samples from 8 diseased and 8 control dogs were paired according to the age of the dog and anatomical origin of the tissue. Plasmalogenase activities were measured simultaneously in each member of the pair by the procedure of Ansell and Spanner (1965, 1968). Acetone powders of the tissues were extracted with bicarbonate-buffered 25% glycerol. The undialyzed extracts were incubated in triplicate with ethanolamine plasmalogen at 37°C for 3 hours. The loss of plasmalogen was measured by iodine addition (Gottfried and Rapport, 1962).

The ethanolamine plasmalogen substrate was prepared from a human brain lipid extract by alkaline hydrolysis and column chromatography (Ansell and Spanner, 1968). The column fractions were combined to give the best yield of plasmalogen with the least amount of contamination as judged by thin-layer chromatography (Horrocks, 1968; Horrocks and Sun, 1972). The substrate contained 81% 1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphorylethanolamines (plasmalogen), 15% 1-alkyl-2-acyl-sn-glycero-3-phosphorylethanolamines, and 4% of an unknown compound. The unknown phospholipid was chromatographically similar to the N-acetyl ethanolamine plasmalogen reported by Debuch and Wendt (1967). A column fraction enriched in the unknown compound was a substrate for bovine brain plasmalogenase (McMartin, unpublished results). Alkyl-containing compounds cannot be removed from preparations of the corresponding plasmalogens (Horrocks, 1972).

Enzyme activities from distemper tissues were calculated as a percentage of the activity from the paired control tissue and statistically evaluated by the Student t-test for paired samples (Freund et al.).

Results

The neurological signs of the diseased dogs varied considerably. Two dogs were moribund and three other dogs had posterior paralysis. All dogs exhibited rhythmic twitching of various muscle groups at some time during their clinical course. This rhythmic muscle twitching (canine chorea) is nearly diagnostic of the nervous form of canine distemper.

All diseased dogs had microscopically demonstrable brain lesions pathognomonic for canine distemper. Intranuclear inclusion bodies in glial cells and demyelinated areas of various extent were present in all affected dogs. None of the control dogs showed clinical or histological evidence of canine distemper infection.

The plasmatogenase activity of 0.619 ± 0.154 $\mu\text{moles/hr/g}$ of wet tissue (mean $\pm$ S.E.) in brain samples from distemper dogs was 67% greater than the activity of 0.416 ± 0.077 $\mu\text{moles/hr/g}$ in samples from control dogs. The difference was statistically significant ($t = 2.36$, $df = 13$, $P < 0.05$). The relationship of plasmatogenase activity to demyelination can be better understood by separating the brain samples into three groups (Table 1). These three groups, "+", "++" and "+++", were defined by the extent of demyelination present in a histological section immediately adjacent to the sample being assayed for plasmatogenase activity (McMartin et al.). Plasmatogenase activity was highest in the "+" group in which little demyelination was present. These

tissues contained proliferating astrocytes and numerous microglial cells diffusely infiltrating the white matter. Many of the astrocytes contained large intranuclear inclusion bodies. As the extent of demyelination increased (Table I), plasmalogenase activity decreased. It was estimated that up to 25% of the myelin had been removed from the "+++" group of samples and plasmalogenase activity in these tissues averaged only 77% of control levels. Gitter cells and reactive astrocytes were present in or near the areas of demyelination.

Discussion

The present study adds canine distemper to the list of demyelinating diseases in which plasmalogenase activity is elevated and further accentuates the importance of plasmalogenase in the process of demyelination. Ansell and Spanner (1968, 1970) found that plasmalogenase activity was elevated in demyelinating spinal cords of vitamin B₁₂-deficient monkeys and in the cerebral white matter from one human patient with multiple sclerosis. The mechanism of demyelination is unknown in both of these diseases. Vitamin B₁₂-deficiency in monkeys and its prototype, subacute combined degeneration in human vegans, are associated with a lack of dietary vitamin B₁₂. However, the role of this nutrient in the maintenance of myelin is not understood (Girdwood). Likewise, the cause of demyelination in multiple sclerosis is also unknown. Epidemiological evidence suggests that a slow virus may be the etiological agent of multiple sclerosis (Leibowitz).

The mechanism by which the canine distemper virus produces demyelination is also unknown. Acute viral encephalitis was produced in the majority of dogs by experimental infection with a virulent strain of

distemper virus (Appel; Gillespie, et al.). Viral antigen was demonstrated in glial cells and other cell types in most infected animals but demyelination was rarely observed (Appel; Gillespie et al.).

Distemper infection of canine cerebellar explants resulted in myelin destruction before detectable neuronal changes occurred. It was not determined if demyelination was due to a direct action of the virus on the cells or due to secondary influences (Storts, et al.).

The present results suggest that an increase in plasmalogenase is an early event in the process of demyelination. Plasmalogenase activity was highest in infected tissue with the least extent of demyelination ("+" group, Table I). This finding suggests that an early event associated with viral action on nervous tissue and not phagocytic activity following tissue destruction caused this enzyme elevation. Since plasmalogen in nervous tissue is in a dynamic state of being continuously formed and broken down (Horrocks, 1969), any increase in the rate of catabolism by plasmalogenase should then result in a net loss of plasmalogen from myelin. The product of plasmalogenase catabolism is lysophosphatidyl ethanolamine (Ansell and Spanner, 1965). This compound, which has detergent properties and can rupture cell membranes (Gottfried and Rapport, 1963), could dissolve myelin and release the antigenic myelin brain protein (Gregson et al., Seitelberger). Concurrently with the loss of myelin, plasmalogenase activity decreased, perhaps because the source of plasmalogenase, the normal glial elements of white matter (Ansell and Spanner, 1968), had also disappeared.

Summary

Plasmalogenase activity was 67% higher in the brains of dogs with demyelination resulting from canine distemper than in brains of age-matched control dogs. The finding that plasmalogenase activity was highest in the tissues with least severe demyelination suggests that plasmalogenase acts early in the process of demyelination. The elevation of plasmalogenase activity and loss of plasmalogen which occurs in other demyelinating diseases indicates that plasmalogenase is part of a common catabolic mechanism by which demyelination proceeds.

TABLE 1. PLASMALOGENASE ACTIVITIES GROUPED ACCORDING TO SEVERITY OF DEMYELINATION

Severity of Lesion	^a	^b	^c
Plasmalogenase Activity (% of paired control)	100% 315 168 143 166 252 207 60	134% 177 88	118% 60 51
Mean $\pm$ S.E.	177 $\pm$ 29	133 $\pm$ 26	77 $\pm$ 21

^a Little demyelination present in histological section immediately adjacent to sample assayed for enzymatic activity.

^b Demyelination covered about 3 to 10% of white matter area of adjacent section.

^c Demyelination covered 15 to 25% of white matter area.

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