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Studies On The Human And Bovine Spinal Cord Protein

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STUDIES ON THE HUMAN AND BOVINE SPINAL CORD PROTEIN

by

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of the requirements for the degree of
Doctor of Philosophy

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A B S T R A C T

This report contributes to the knowledge of the anti-encephalitogenic spinal cord protein (SCP) with respect to three major areas of study: (1) the purification and characterization of human SCP; (2) the nature of the association of SCP with nervous tissue components and (3) purification of cyanogen bromide-derived peptides of bovine SCP.

Human SCP and a protein immunochemically identical to SCP (SCP-PN) were purified from spinal cords and peripheral nerves, respectively. Purification involved tissue extraction with 0.15 M sodium chloride, carboxy-methyl cellulose chromatography and gel filtration on Sephadex G-50 superfine. SCP and SCP-PN had estimated molecular weights of I3,700 and 14,700 daltons, respectively and had similar amino acid compositions. The isoelectric point of SCP-PN was estimated to be 9.9. Immunodiffusion analyses with anti-human SCP sera or anti-bovine SCP sera revealed that human SCP and SCP-PN are each composed of two different antigenic forms. Each antigenic form contains a distinct immunogenic domain that is identical to one of the immunogenic sites on bovine SCP.

Bovine SCP-PN is identical to the P_2 protein found in purified peripheral nerve myelin. The bovine SCP-PN content of 0.3 M NaCl extracts of whole tissue was 1.3 mg per g of tissue. Approximately 0.33 mg of SCP-PN was found in the soluble fraction of 0.8 M sucrose homogenates of bovine

peripheral nerves. Densitometry data indicated that SCP-PN decreased from 19% of the total myelin protein to less than 1% when purified myelin was extracted with 0.3 M sodium chloride or 0.05 M hydrochloric acid. The basic proteins SCP-PN and lysozyme bound to myelin and sodium chloride-extracted myelin when they were added to a suspension of myelin in 0.8 M sucrose. Pepsin, an acidic protein, did not bind to myelin. The results suggest that in 0.8 M sucrose, positively charged SCP-PN can bind to negatively charged myelin. Myelin-associated SCP-PN behaves like a peripheral membrane protein.

This interpretation is consistent with earlier research in which bovine SCP-PN was localized by immunohistological techniques in axons of peripheral nerves but not in myelin sheaths surrounding the axons. The histological fixatives acetone and 95% ethanol/ether did not render SCP-PN in whole tissue insoluble in saline. This result indicates that if a tissue section is washed with saline after fixation with acetone or 95% ethanol/ether, then most of the SCP-PN in the tissue section could be solubilized.

Rat SCP-PN was not detected in purified rat peripheral nerve myelin by immunodiffusion analyses and was shown to be distinct from the rat P_2 protein with respect to molecular size.

Cleavage of bovine SCP with cyanogen bromide resulted in three peptides: CN1, CN2 and CN3. Peptides CN2 and CN3

contain approximately 18 and 17 amino acid residues, respectively. The estimated molecular weight of CN1 was 9,000 ± 900 daltons. CN2 was composed of two small peptides joined by a disulfide bond. None of the peptides formed specific precipitates with anti-bovine SCP antibodies.

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In memory of my father,

Gordon Alexander Weir.

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ABBREVIATIONS

SCP	Spinal cord protein
SCP-PN	Spinal cord protein - peripheral nerve
CFA	Complete Freund's adjuvant
CNBr	Cyanogen bromide
EAE	Experimental allergic encephalomyelitis
EAN	Experimental allergic neuritis
IFA	Incomplete Freund's adjuvant
MyBP	Myelin basic protein
PAS-II	Periodic acid Schiff adjuvant
SDS	Sodium dodecyl sulfate
TEMED	N, N, N', N'-tetramethylethylenediamine

CHAPTER 1: INTRODUCTION

The molecular components of the nervous system have been widely studied during the past half century. One of the aims, with respect to the biochemistry of the nervous system, has been to characterize and localize proteins in the nervous system to gain a better understanding of the functioning of the nervous system at the cellular and molecular levels. Special emphasis has been placed on the proteins thought to be involved in autoimmune diseases of the nervous system.

In 1972, Yo and MacPherson described the purification and immunochemical properties of a spinal cord protein (SCP) extracted from non-delipidated bovine spinal cord with 0.1 M NaCl. It has been demonstrated that pre-treatment with bovine SCP can prevent experimental allergic encephalomyelitis (EAE) in guinea pigs and rats (MacPherson and Yo, 1971; MacPherson and Armstrong, 1977; Montgomery and Rauch, 1980). Extracts of human, rabbit, pig, monkey, rat and guinea pig spinal cords contain a protein that cross-reacts with anti-bovine SCP serum. Immunoreactive SCP isolated from the peripheral nervous system (SCP-PN) lacks anti-encephalitogenic activity.

It has been established that bovine SCP-PN is identical to the bovine P_2 protein (Kies *et al*, 1975; Deibler, Driscoll

and Kies, 1978). P_2 is a protein isolated from purified peripheral nerve myelin (Brostoff and Eylar, 1972). Immunodiffusion studies using an anti-bovine SCP-PN serum have revealed that SCP-PN and P_2 are immunochemically identical.

While bovine SCP has been demonstrated to be anti-cephalitogenic, SCP-PN has no anti-encephalitogenic activity (MacPherson, 1978) but has been found to be neuritogenic in one species, the Lewis rat (Kadlubowski and Hughes, 1979; Ishaque, Hoffman and Eylar, 1979; Hoffman *et al.*, 1980a; MacPherson, personal communication). Thus, SCP and SCP-PN have different biological activities both in the guinea pig and in the Lewis rat. The amino acid sequence that differentiates SCP and SCP-PN has not been determined.

There is disagreement in the literature regarding the location of SCP and SCP-PN in the nervous system. SCP and SCP-PN have been localized by the fluorescent antibody technique in axoplasm and glial cells in the central nervous system and in axoplasm and Schwann cells in the peripheral nervous system, respectively, by MacPherson and Wallace (1980). In contrast, Eylar *et al.* (1980) have localized SCP-PN (P_2) in peripheral nerve myelin and have not found SCP in the central nervous system.

This study is an attempt to gain more knowledge of the biochemical properties and the biological activity of SCP and SCP-PN. The specific aims were (1) to purify and characterize human SCP and SCP-PN, (2) to localize the anti-encephalitogenic amino acid sequence in bovine SCP by preparing cyanogen bromide

peptides of SCP, (3) to determine the nature of the association of bovine SCP-PN with purified peripheral nerve myelin and (4) to determine whether rat SCP-PN is associated with purified peripheral myelin.

The value of the present study is that the results could form the basis for future studies on (1) the possible anti-encephalitogenic activity of human SCP, (2) the levels of human SCP in the nervous system of normal subjects and of patients with neurological disorders and (3) the anti-encephalitogenic amino acid sequence of bovine SCP. The results could also resolve the discrepancy in the literature regarding the localization of bovine SCP-PN.

1.1 Experimental Allergic Encephalomyelitis

1.1.1 Induction

During the past twenty years, the study of the immunology of the nervous system has been dominated by intensive research on EAE. EAE is an autoimmune disease of the central nervous system that is mediated by T lymphocytes sensitized to myelin basic protein (MyBP) (Gonatas and Howard, 1974; Ortiz-Ortiz *et al.*, 1976).

Rivers, Sprunt and Berry (1933) and Rivers and Schwentker (1935) demonstrated that encephalomyelitis could be experimentally induced in monkeys with repeated injections of nervous tissue. The animals developed paralysis, ataxia and urological disturbances. Histological lesions characterized by perivascular infiltration of lymphocytes and demyelination

were present in the central nervous system. It was subsequently shown that one injection of nervous tissue emulsified with complete Freund's adjuvant (CFA) produced an acute response within weeks rather than months (Morgan, 1946; Kabat, Wolf and Bezer, 1946; Kabat, Wolf and Bezer, 1948).

The pathological course of EAE in test animals is commonly evaluated by observing the clinical signs and enumerating the histological lesions in the brain and spinal cord. Two of the early histological events of EAE are the perivascular accumulation and infiltration of mononuclear cells in the central nervous system approximately seven days before the onset of clinical signs (Waksman and Adams, 1962; Leibowitz, 1966). The demyelination of axons is the result of the phagocytic action of macrophages on myelin (Lampert, 1967; Prineas, Raine and Wisniewski, 1969).

The central nervous system component responsible for EAE has been shown by Laatsh *et al.*, (1962) to be a basic protein present in myelin. The isolation and purification of myelin basic protein (MyBP) is routinely accomplished by a three-step procedure that involves acid extraction of delipidated nervous tissue, ion exchange chromatography and gel filtration (Kies, Murphy and Alvord, 1960; Nakao, Davis and Roboz-Einstein, 1966; Carnegie, Bencina and Lamoureux, 1967; Eylar *et al.*, 1969). The biochemical characterization of MyBP culminated with the publication of the complete amino acid sequence of the human (Carnegie, 1971) and bovine (Eylar *et al.*, 1971) basic proteins.

Human and bovine MyBP exhibit considerable sequence homology. For example, there are only fifteen substitutions, deletions or additions in a total of 169 amino acid residues when human MyBP is compared to bovine MyBP. It is interesting to note that central nervous system myelin of rodents of the suborders *Myomorpha* and *Sciuromorpha* contains two basic proteins that differ in molecular size (Cotman and Mahler, 1967; Eng *et al.*, 1968). The smaller protein is related to the larger protein by a deletion mutation involving a sequence of 40 amino acid residues (Dunkley and Carnegie, 1974). The larger rat basic protein is comparable to the central nervous system MyBP of other mammalian species with respect to molecular size, and the ability to induce EAE in the guinea pig. The smaller basic protein, is much less encephalitogenic than the larger basic protein (Martenson, Deibler and Kies, 1970). For example, 5 µg of the large basic protein produced considerable encephalitogenic activity (the results being expressed in terms of a combined clinico-pathologic index of severity of EAE), whereas 5 µg of the small basic protein produced very low levels of activity.

In an effort to determine the encephalitogenic regions of MyBP of different species, various peptide fragments have been tested for their ability to induce EAE in guinea pigs, rat and rabbits. The encephalitogenic determinant for the guinea pig was found to be a nine amino acid peptide containing residues 113 to 121 of bovine MyBP (Eylar and Hashim, 1968; Eylar *et al.*, 1970). Martenson *et al.* (1975) demonstrated

that fragments 1 to 42, 37 to 88 and 89 to 152 from bovine MyBP induced EAE in the rat whereas fragment 43 to 88 was the only peptide derived from guinea pig MyBP that was capable of inducing EAE in the rat. Shapira *et al.* (1971) showed that a fragment of bovine, rabbit or human MyBP composed of residues 43 to 87 was encephalitogenic in rabbits. Thus, while the intact protein will induce EAE in most species of mammals tested, the various species respond to different amino acid sequences of the molecule.

Moreover, it is evident that the immunological response to the encephalitogen is different when the sensitizing inoculum contains other antigens. For example, Hoffman, Gaston and Spitler (1973) induced EAE in guinea pigs with spinal cord, MyBP or synthetic encephalitogenic peptides and compared the severity of disease that developed. The animals that received the spinal cord or pure MyBP had an earlier onset of clinical disease, a lower tendency to recover and more inflammatory lesions than animals that were injected with synthetic peptides.

The immune response is expressed by cell-mediated immunity and humoral immunity. EAE is a T cell-mediated response to MyBP. Paterson (1960) and Stone (1961) showed that EAE could be transferred by injecting lymph node cells from rats that had been sensitized to MyBP into unsensitized rats. The correlation between EAE induction and the cell-mediated immune response to MyBP has since been repeatedly demonstrated both *in vivo* (Shaw *et al.*, 1965; Wenk, Levine and

Warren, 1967; Falk, Kies and Alvord, 1968) and *in vitro* (David and Paterson, 1965; Hughes and Field, 1968; Dan and Peterson, 1969; Ellison, Waksman and Ruddle, 1971).

Direct evidence of the involvement of the humoral immune response in the pathogenesis of EAE has not been demonstrated. There are several studies that indicate that there is no correlation between the severity of EAE and the presence of anti-MyBP antibodies (Kibler and Barnes, 1962; Lisak *et al.*, 1969; Falk, Kies and Alvord, 1969; Lennon *et al.*, 1971). However, antibodies directed towards MyBP should not be completely excluded from the pathogenesis of EAE. It is possible that the release of antibody at the target tissue or the rapid removal of antibody from circulation by antigens in the target tissue can not be detected by current techniques (Brostoff, 1977). Lerner and Dixon (1966) demonstrated that such a situation exists in glomerulonephritis. When sheep are made nephritic by immunization with glomerular basement membrane, the serum is found to contain nephritogenic antibodies that are detectable in the serum only when the kidneys are removed. Unfortunately, this type of experiment is not feasible to study the involvement of antibodies in EAE because the test animals would not survive in the absence of a central nervous system.

1.1.2 Treatment of EAE

1.1.2.1 Myelin Basic Protein

Many studies have been concerned with the inhibition of EAE at different stages in the development of the disease.

EAE has successfully been inhibited by injecting MyBP or whole central nervous system tissue emulsified in incomplete Freund's adjuvant (IFA) into test animals prior to the injection of MyBP in CFA. The inhibition of EAE may be accomplished by preventing or suppressing the disease. The term prevention indicates that the inhibiting IFA emulsion is injected prior to the disease-inducing emulsion in CFA. On the other hand, suppression implies that the EAE-inducing injection precedes the inhibiting injections. If the inhibiting injections are administered after the development of cutaneous delayed-type hypersensitivity, but before the appearance of clinical signs, then a form of suppression known as desensitization is said to occur (Shaw *et al.*, 1965). Treatment is also a form of suppression in which the inhibiting injections are given after the onset of clinical signs (Alvord *et al.*, 1965).

The prevention of EAE was first accomplished by Ferraro and Cazzulla (1949a, 1949b). Guinea pigs that were pretreated with injections of whole central nervous system tissue emulsified in IFA failed to develop EAE. Alvord *et al.* (1965) demonstrated that either MyBP emulsified in IFA or MyBP in saline was also effective in preventing EAE. Injections of MyBP in IFA as late as 11 days after the EAE-inducing dose of MyBP in CFA, were shown to be effective in desensitizing the test animal.

The inhibition of EAE after the appearance of clinical signs has been the major goal in devising a therapy for EAE. Eylar *et al.* (1972) showed that under certain conditions MyBP

could be used to effectively treat EAE after the appearance of clinical signs in the monkey. The effectiveness of the treatment was dependent on animal care, which included forced feeding and treatment with antibiotics. However, in these experiments MyBP and penicillin were not tested individually. Shaw Alvord and Hruby (1976) demonstrated that MyBP by itself was almost completely ineffective in treating EAE in monkeys. The conditions under which MyBP could be used to treat EAE became more clearly understood when Alvord, Shaw and Hruby (1979) found that the treatment failed unless an adjunct was used. The adjunct, either an antibiotic or a steroid, varied with the strain of monkey and, interestingly, the adjunct by itself was more effective than MyBP.

The efficacy of treatment with MyBP for guinea pigs was illustrated by Driscoll, Kies and Alvord (1974). However, only in highly inbred strain 13 guinea pigs was MyBP found to be an effective treatment. In non-inbred guinea pigs the response to treatment was inconsistent.

The T lymphocyte participates in a variety of cell-mediated immune reactions. For example, it can carry out a cytotoxic function, aid B cells or suppress cellular immune reactions by other effector cells. Such suppressor T lymphocytes can be used to transfer unresponsiveness to EAE. Swierkosz and Swanborg (1977) demonstrated that the Lewis rat could be rendered unresponsive to the induction of EAE by the transfer of suppressor T lymphocytes from donor animals sensitized to MyBP to normal syngeneic recipients. However, this

effect was transitory, as the unresponsiveness persisted in the recipients for only three weeks. The authors suggested that the suppressor cells act at the inductive phase of the immune response because no inhibitory effect occurred when the disease was transferred by effector lymph node cells. In a subsequent study, Welch, Swierkosz and Swanborg (1978) provided evidence to suggest that MyBP-specific suppressor T-lymphocytes and non-specific splenic adherent suppressor cells are involved in the regulation of self tolerance in EAE.

Despite the extensive research carried out on EAE, the mechanisms of prevention, suppression and treatment of EAE are not yet completely understood. For example, non-encephalitogenic peptides that are derived from MyBP can prevent the disease in guinea pigs (Hashim and Schilling, 1973). Thus, the mechanism of the inhibition of EAE does not appear to depend on an intact encephalitogenic determinant.

1.1.2.2 Synthetic Amino Acid Copolymers

The report of Teitelbaum *et al.* (1971) revealed that several linear random copolymers of amino acids had a suppressive effect on EAE in guinea pigs. Copolymer I, composed of alanine, glutamic acid, lysine and tyrosine, exerted the most marked suppressive effect. Copolymer I was also able to suppress EAE in rabbits (Teitelbaum *et al.*, 1973). Copolymer I was estimated by Teitelbaum *et al.* (1973) to be equally efficient in the suppression of EAE induced in guinea pigs by either human or bovine MyBP. In addition, Copolymer I was shown to be devoid of non-specific immunosuppressive activity.

In 1973, Webb *et al.* demonstrated that T lymphocytes from animals sensitized to MyBP could react with copolymer I. This cross-reactivity was demonstrated both *in vivo* and *in vitro*. Injections of copolymer I provoked delayed-type hypersensitivity reactions in guinea pigs that had been sensitized to MyBP. *In vitro*, copolymer I stimulated the transformation of MyBP-sensitized lymphocytes, as measured by the cellular incorporation of radioactive thymidine. Cross-reactivity was also observed between MyBP and all of the synthetic copolymers that were effective in suppressing EAE. Ineffective copolymers and unrelated proteins did not cross-react with MyBP. Therefore, the authors of this report suggested that the cell-mediated cross-reactivity between the copolymers and MyBP may serve as a basis of suppression.

Copolymer I has also been used to treat monkeys after the appearance of the clinical signs of EAE (Webb *et al.*, 1975). The validity of this study is questionable because only two animals were tested. In a similar report, Arnon and Teitelbaum (1978) found that copolymer I successfully treated EAE in three out of four baboons. In contrast to these results, Alvord, Shaw and Hruby (1979) were unable to confirm the suppressive effect of copolymer I. However, the important use of certain adjuncts was not completely investigated in the latter study. Thus, despite the uncertainty regarding the ability of copolymer I to suppress EAE in monkeys and baboons, the suppressive effect of copolymer I in guinea pigs and rabbits has been established.

1.1.2.3 Bovine and Rat SCP

In addition to copolymer I, there is another protein that is not antigenically related to MyBP by the criteria of immunodiffusion analyses and the passive hemagglutination test (MacPherson, Armstrong and Tan, 1976) and that can be used to prevent and suppress EAE. In 1973, MacPherson and Yo reported that pre-treatment of guinea pigs with bovine SCP prevented the development of the neurological signs of EAE in animals that were subsequently immunized with disease-inducing doses of bovine spinal cord in CFA. It was demonstrated that a dose of 300 µg of bovine SCP, the total amount used for the pre-treatment of each animal, was not encephalitogenic. The significance of this discovery is that a non-encephalitogenic protein which is a normal component of the nervous system and is antigenically unrelated to MyBP has the capacity to prevent EAE. The anti-encephalitogenic activity of bovine SCP has been confirmed by Montgomery and Rauch (1980) and by Teitelbaum (personal communication).

Rat SCP has been shown by MacPherson and Armstrong (1977) to have anti-encephalitogenic activity in the Lewis rat. Two weekly injections of 100 µg of rat SCP commencing 21 days before challenge with rat MyBP resulted in complete protection of all animals tested.

Lewis rats could also be protected from EAE induced with rat MyBP by using bovine SCP (MacPherson and Armstrong, 1977). Approximately seven times as much bovine SCP was needed to give the same degree of protection as 300 µg of rat SCP. The

ability of heterologous bovine SCP to protect against EAE in the rat suggests that rat SCP and bovine SCP share at least one antigenic amino acid sequence recognized by T lymphocytes. At present, there is no evidence that rat SCP and bovine SCP have common immunogenic sites that are recognized by antibody forming cells. For example, immunodiffusion studies using anti-rat SCP serum or anti-bovine SCP serum revealed that rat SCP and bovine SCP react only with their homologous anti-serum.

The successful suppression of MyBP-induced EAE in guinea pigs using bovine SCP was accomplished by MacPherson (1980). Quantitatively, bovine SCP was as effective as MyBP in the suppression of the disease. Suppression was carried out by administering daily intradermal injections of 500 µg of bovine SCP in saline. Doses of 750 µg of bovine SCP in saline were found to reverse the clinical disease when the treatment was initiated within a day of the first sign of disease. Suppression was also accomplished by injections of bovine SCP in IFA. Interestingly, in the same study, bovine gamma globulin, or even saline, appeared to have suppressive activity when three or more injections of these proteins in IFA were administered between sensitization and disease onset. In view of these results, MacPherson (1980) has suggested that the suppressive ability of an antigen may be misinterpreted when the antigen is frequently injected in IFA during the interval between sensitization and the appearance of clinical symptoms.

These results question the validity of the study of Webb *et al.* (1975) and Arnon and Teitelbaum (1978) because these authors used frequent injections of copolymer I in IFA to suppress EAE after sensization. In the study of MacPherson (1980), however, the injection of antigens in saline clearly demonstrated the suppressive activity of bovine SCP and MyBP and the lack of this property in bovine gamma globulin or saline.

It has been suggested by Deibler, Driscoll and Kies (1978) that the preparations of bovine SCP may contain small amounts of degraded bovine MyBP that are effective in preventing the induction of EAE. However, MacPherson and Armstrong (1977) have reasoned that prevention due to contamination of SCP with MyBP is improbable. A competitive inhibition radioimmunoassay (MacPherson, unpublished results) indicated that 1 mg of purified rat SCP contained a maximum of 30 ng of rat MyBP. Similarly, 1 mg of bovine contained no more that 15 ng of bovine MyBP. These results were confirmed in an assay performed by V. Lenon of the Salk Institute, La Jolla, California. Therefore, the amount of SCP required for protection of Lewis rats against EAE induced with rat MyBP did not contain more than 30 ng of bovine MyBP. It is interesting to note that Coates, MacKay and Crawford (1974) reported that at least 10,000 ng of bovine MyBP was required to prevent EAE in the guinea pig.

Therefore, it appears that there is an insufficient amount of undegraded MyBP, by several orders of magnitude, in

the bovine and rat SCP preparations to be responsible for the prevention of EAE.

An anomalous situation occurs in the rat. In this species, the homologous MyBP is at least 100 times less effective in inducing EAE than the guinea pig MyBP. Thus, considerably smaller amounts of encephalitogenic peptides derived from guinea pig MyBP are able to prevent EAE induced with guinea pig MyBP in the Lewis rat. For example, Chou *et al.* (1980) has shown that approximately 200 ng of peptide 66-88 of guinea pig MyBP can prevent EAE in the Lewis rat. Information concerning the minimum amount of peptide derived from bovine or rat MyBP that can prevent EAE is not in the literature. To determine if contaminating peptides of MyBP were responsible for preventing EAE, one would have to determine not only the amount of the peptides in SCP preparations, but also the minimum protective dose of peptides.

Thus, the possibility arises that the protective and suppressive activities of SCP may be due to an amino acid sequence common to SCP and MyBP recognized only by T lymphocytes. This proposal is supported by the results of recent studies (Ramshaw and MacPherson, unpublished results) that indicate that bovine SCP and bovine MyBP cross-react at the T cell level *in vitro*. Thus, SCP and copolymer I are similar in this respect. That SCP and copolymer cross-react with MyBP at the T cell level suggests that this cross-reactivity may be the basis for the mechanism of protection rather than contamination with peptides of MyBP.

1.1.3 EAE and Multiple Sclerosis

Interest in EAE research has been greatly sustained because of similarities in the pathology of EAE and the human disease, multiple sclerosis. Multiple sclerosis is a demyelinating disease of the central nervous system that usually occurs between the ages of 15 and 55. The peak incidence occurs at age 30. It is one of the most prevalent diseases of the central nervous system and, because young adults are most frequently affected, multiple sclerosis is a serious health problem. Multiple sclerosis is more prevalent in the temperate regions than in the tropics. The prevalence rates in low and high risk zones are less than 20 per 100,000 and greater than 40 per 100,000, respectively. The cause of multiple sclerosis is not known and an effective treatment and cure have not been found.

Multiple sclerosis is a polyphasic disease that is characterized by a lengthy time course with exacerbations and remissions (McAlpine, 1972). The lesions of multiple sclerosis are confined to the central nervous system and frequently involve the myelin sheath with the sparing of axons. Over the course of the illness, the formation of new lesions usually correlates with exacerbations.

Tissue typing studies suggest that multiple sclerosis has a genetic component (Jersild *et al.*, 1975; Ebers and Paty, 1979). For example, there is a significant increase in the frequency of HLA-A3 and HLA-B7 histocompatibility antigens in multiple sclerosis patients. Other studies on the etiology

of multiple sclerosis suggest that an environmental factor is involved (Acheson, 1977) and also that multiple sclerosis may occur in epidemic form (Kurtzke and Hyllested, 1979). In keeping with this line of thought, the serum and cerebrospinal fluid of most multiple sclerosis patients contain elevated antibody titers to measles virus and, less commonly, to other viruses (Norrby *et al.*, 1974; Johnson, 1975; Norrby, 1978). These findings have suggested that a latent viral infection may be involved in the etiology of multiple sclerosis. Although there have been frequent attempts to isolate a virus from tissues of multiple sclerosis patients, the isolation of a viral agent has never been convincingly confirmed (Wallen *et al.*, 1979; Huddlestone *et al.*, 1979; Gould *et al.*, 1979; Burks *et al.*, 1980).

In 1974, Sheremata, Cosgrove and Eylar reported a transient cellular hypersensitivity to MyBP in some multiple sclerosis patients during exacerbations. However, this has not been confirmed and there is no evidence that a cellular immune response to MyBP has a pathogenic role in multiple sclerosis. However, during the active stages of multiple sclerosis certain subsets of T suppressor cells are decreased (Huddlestone and Oldstone, 1979; Reinherz *et al.*, 1980). Thus, the loss of suppressor cells in patients with active multiple sclerosis suggests that immunoregulatory abnormalities may be responsible for the acute exacerbations of multiple sclerosis.

Although there are significant differences between multiple sclerosis and EAE, it is generally believed that there are enough similarities to justify the use of EAE as an experimental model for multiple sclerosis (MacKay, Carnegie and Coates, 1973; Paterson, 1973; Kies, 1978). The clinical symptoms and histological lesions are the two characteristics upon which the comparison of the two diseases is mainly based. Other common features include the demyelinating and synapse-blocking activity of sera in tissue culture and an increased level of spinal fluid immunoglobulin.

Despite the similarities, there are enough differences to suggest that the two diseases are not comparable. For example, multiple sclerosis is characterized by a lengthy time course that includes periods of exacerbations and remissions. EAE usually follows an acute clinical course that leads to paralysis and death rather quickly. Although inflammatory demyelination is characteristic of the histological lesions in both diseases there are significant differences between the lesions. The lesions, or plaques, of multiple sclerosis appear to be of different ages and contain increased amounts of immunoglobulin, suggesting local production of immunoglobulin. Inflammatory demyelination occurs in early lesions along with mononuclear exudates. Older lesions contain plasma cells, mature lymphocytes and astrocytes. The lesions of EAE are all of the same age and contain perivascular mononuclear cell infiltrates with varying degrees of demyelination.

An encephalitogen related to multiple sclerosis has not been identified in humans. Despite the lack of success in identifying an encephalitogen, numerous clinical experiments to treat multiple sclerosis with MyBP have been attempted. Unfortunately, all such attempts have failed (Campbell *et al.*, 1973; Gonsette, Delmotte and Demonty, 1977; Salk *et al.*, 1979). In retrospect, one may criticize these studies in view of the lack of evidence that MyBP is an encephalitogen in multiple sclerosis.

Copolymer I has been shown to be as effective as MyBP in suppressing EAE. Thus, the therapeutic value of copolymer I in multiple sclerosis has been investigated. Unfortunately, copolymer I failed to be effective in the treatment of multiple sclerosis (Abramsky, Teitelbaum and Arnon, 1977).

1.2 Experimental Allergic Neuritis

Experimental allergic neuritis (EAN) is an inflammatory demyelinating disease of the peripheral nervous system. Waksman and Adams (1955) were the first to induce EAN in rabbits by the injection of whole peripheral nervous tissue in CFA. Many animal species have since been used to study EAN (Arnason, 1971). The histological lesions of EAN are confined to the peripheral nervous system in rabbits, chickens, monkeys and mice, while in guinea pigs, rats and sheep, the lesions extend into the central nervous system.

Brostoff and Eylar (1972) reported that rabbit peripheral nerve myelin contains MyBP, which is referred to as the P_1

protein of the peripheral nervous system. The rabbit P_1 protein can produce EAE in rabbits, monkeys or guinea pigs (Kiyota and Egami, 1972; Brostoff *et al.*, 1975b). However, when rabbits or monkeys were challenged with bovine or rabbit peripheral nerve myelin, EAN developed rather than EAE (Brostoff *et al.*, 1972; Wisniewsky *et al.*, 1974; Brostoff, Sacks and DiPaula, 1975a). The authors suggested that the encephalitogenic determinants of P_1 for rabbit and monkey are blocked in the intact myelin membrane.

The proteins P_o, P_1 and P_2 account for approximately 70% of protein of purified peripheral myelin (Greenfield *et al.*, 1973). About half the total protein is the P_o glycoprotein. P_o is an integral membrane protein that has a molecular weight of 28,000 daltons. The rabbit P_1 protein has the same sequence as the MyBP (Brostoff and Eylar, 1972) and has been found in purified peripheral myelin in all species studied thus far.

The P_2 protein is claimed to be unique to peripheral nerve myelin (Kitamura *et al.*, 1980; Eylar *et al.*, 1980). Earlier studies indicated that the bovine, rabbit or human P_2 protein was a major protein of purified peripheral nerve myelin and that it was not capable of inducing EAN in guinea pigs (Uyemura *et al.*, 1972; Brostoff *et al.*, 1975b; Uyemura, Suzuki and Kitamura, 1978). However, in a few instances, purified bovine P_2 induced mild cases of EAN in the rabbit (Kiyota and Egami, 1972; Brostoff *et al.*, 1975). Only the Lewis rat appears to be susceptible to the induction of EAN

by bovine and rabbit P_2 (Ishaque, Hoffman and Eylar, 1979; Kadlubowski and Hughes, 1979; Hoffman *et al.*, 1980a; MacPherson, personal communication). Hoffman *et al.* (1980b) demonstrated that when the half-cystine residues in bovine P_2 were reduced and carboxymethylated, the chemically modified P_2 was a more potent neuritogen than unmodified P_2. Thus, the status of the half-cystines in P_2 may have an effect on the ability of P_2 to induce EAN.

There are several lines of evidence to suggest that factors other than the P_2 protein (SCP-PN) may influence the induction of EAN. The study of Nagai *et al.* (1978) indicates that P_2 exhibits full neuritogenic activity equivalent to PNS myelin when combined with gangliosides isolated from peripheral nerves. The results of this report raise two possibilities concerning the role of gangliosides in the induction of EAN. First, gangliosides may impart a special conformation to P_2 necessary for neuritogenic activity. This proposal is supported by the report of Weise *et al.* (1980a) in which a neuritogenic peptide was isolated from the cyanogen bromide (CNBr) cleavage of P_2. Thus, while P_2 may be neuritogenic by itself, the presence of gangliosides might enhance the activity of the encephalitogenic determinant. Secondly, the gangliosides themselves may contain neuritogenic determinants. Nagai *et al.* (1976) demonstrated that gangliosides GD_{1a} and GM, can induce an immunological disease in rabbits called ganglioside syndrome. This disease is characterized by paralysis and extensive degeneration of the peripheral

nervous system. Evidence supporting the involvement of lipids in EAN was also obtained by Saida *et al.* (1977) who reported that EAN sera contains a high level of anti-galactocerebroside antibodies. EAN sera and antisera directed towards galactocerebrosides both result in demyelination of peripheral nerve cultures. However, the extent to which gangliosides and anti-galactocerebroside antibodies are involved in EAN has not been established.

It is interesting to note that there is a striking difference between P_2 and MyBP with respect to the amount of each required to induce disease. Coates, MacKay and Crawford (1974) reported that as little as 10 µg of MyBP could induce EAE in the guinea pig. However, in the Lewis rat, the only animal in which EAN can be induced by bovine P_2, 250 ug required to induce EAN (Kadlubowski and Hughes, 1979). Ishaque, Hoffman and Eylar (1979) showed that only 100 to 200 µg of bovine P_2 were needed to induce EAN when 1000 µg of acidic peripheral nerve lipids was combined with the protein. Thus, while the capacity of the P_2 protein to induce EAN in the Lewis rat has been demonstrated, the involvement of additional factors, such as lipids, remains unclear at the present time.

EAN is used as a laboratory model for the human disease idiopathic polyneuritis or the Guillain-Barre syndrome (Arnason, 1971). Idiopathic polyneuritis is a demyelinating disease that is characterized by mononuclear cell infiltration of peripheral nerves in areas of inflammation (Asbury, Arnason

and Adams, 1969). The clinical features are characterized by weakness and paralysis of the extremities. In contrast to multiple sclerosis, the prognosis is usually promising, with a gradual return to normal function in approximately nine months.

Kadlubowski, Hughes and Gregson (1980) have suggested that P_2 may be the neuritogen that induces idiopathic polyneuritis. However, if this is the case, then one must explain why the events that induce human idiopathic polyneuritis (infection, fever therapy, surgery or vaccination) give rise to the autoimmune response to P_2. Alternatively, it is entirely possible that an autoimmune response to P_2 is but one cause of idiopathic polyneuritis.

1.3 The Spinal Cord Protein

1.3.1 Bovine SCP

The bovine spinal cord protein (SCP) was first isolated by Yo and MacPherson (1972) by extracting bovine spinal cord with 0.1 M NaCl. Bovine SCP was detected by immunodiffusion analyses in the soluble portion of subcellular fractions of bovine spinal cord homogenates separated by differential centrifugation. High concentrations of immunoreactive SCP were found in peripheral nerves and, in minute amounts, in bovine brain. Extracts of the spinal cords of the human, monkey and rabbit contained two proteins that cross-reacted extensively with an anti-bovine SCP serum. The precipitin lines formed by the two proteins fused without spurring with the precipitin line of bovine SCP.

Initially, DEAE Sephadex A-50 and CM-cellulose chromatography were used to purify bovine SCP. Subsequently, MacPherson, Armstrong and Tan (1976) published a simplified purification procedure and achieved a higher yield of bovine SCP. The current purification procedure involves: (1) extraction of bovine spinal cord or spinal roots with 0.15 M NaCl at pH 4.5; (2) batch absorption on CM-52 cellulose; (3) stepwise elution with sodium acetate buffers, pH 5.8, containing increasing concentrations of NaCl and (4) separation from impurities by gel filtration on Sephadex G-50 superfine at pH 5.8.

Immunoelectrophoretic analyses in agar, at pH 8.6, revealed that SCP had electrophoretic mobilities corresponding to those of a γ-serum globulin and a β-serum globulin. The molecular weight of bovine SCP was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to be 13,200 daltons. The amino acid composition of SCP and SCP-PN were similar to each other, but distinct from bovine MyBP. SCP and SCP-PN each contained at least two half-cystine residues. SCP that was purified by gel filtration at pH 5.8, formed a series of polymers when subjected to SDS-polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol. This observation indicated that at pH 5.8, SCP is in a reduced state.

Using the immunodiffusion technique, bovine SCP and SCP-PN were found to be immunochemically identical. However, important differences exist between SCP and SCP-PN that are

not revealed by comparisons of the amino acid compositions or by studies with anti-bovine SCP antibodies. The most important difference involves the biological activities of bovine SCP and SCP-PN. SCP-PN (P_2) is neuritogenic in the Lewis rat (Ishaque, Hoffman and Eylar, 1979; Kadlubowski and Hughes, 1979; Hoffman *et al.*, 1980a; MacPherson, personal communication), but is not anti-encephalitogenic (MacPherson, 1978). SCP is anti-encephalitogenic in the guinea pig (MacPherson and Yo, 1973; MacPherson, 1980; Montgomery and Rauch, 1980) and in the Lewis rat (MacPherson and Armstrong, 1977). This contrast in the biological activities of SCP and SCP-PN may be caused by minor differences in the amino acid sequence of the two proteins. Although the sequence of bovine SCP-PN (P_2) is known (Kitamura *et al.*, 1980; Weise *et al.*, 1980b; MacPherson and Bradshaw, personal communication), bovine SCP has not been sequenced. Thus, the probable differences in the amino acid sequence have yet to be verified.

1.3.2 Rat SCP

Yo and MacPherson (1972) reported that rat spinal cord contained a protein antigenically similar to bovine SCP. Later, MacPherson and Armstrong (1976) demonstrated that saline extracts of rat nervous tissue reacted with an anti-bovine SCP serum, but that anti-rat SCP serum reacted only with rat SCP. It was not known whether the anti-bovine SCP serum was reacting with rat SCP or with a protein that was different from rat SCP but antigenically similar to bovine

SCP. This question was resolved by MacPherson, Armstrong and Yu (1981) who showed that rat peripheral nerve contains a small amount of a component that reacts with anti-bovine SCP serum. This component is not detected by the immunodiffusion technique in extracts of rat brain or spinal cord. Purified rat SCP from brain, spinal cord or peripheral nerves does not react with anti-bovine SCP serum. Thus, the anti-encephalitogenic rat SCP and the protein that is antigenically similar to bovine SCP are distinctly different proteins.

The differences between rat SCP and bovine SCP are further exemplified by the purification procedure for rat SCP. In 1976, MacPherson and Armstrong purified SCP from rat brain and spinal cord by: (1) extraction of the tissues with 0.05 M ammonium acetate buffer, pH 4.0; (2) batch absorption of impurities on CM-52 cellulose; (3) batch absorption of SCP on SP-Sephadex, pH 5.5 and (4) gel filtration on Sephadex G-50 superfine.

When rat SCP was purified using this method, two closely-migrating bands appeared in the SDS-polyacrylamide gel electrophoretogram of rat SCP isolated from brain or spinal cord, while electrophoretograms of rat SCP-PN contained only one band.

The rat SCP had been purified from tissue that had been frozen at -20^{0}C for at least one year and most of the SCP could be extracted with 0.1 M NaCl. Later, it was discovered that rat SCP could not be extracted from fresh or freshly frozen tissue with 0.1 M NaCl unless the tissue was first

delipidated (MacPherson, Armstrong and Yu, 1981). This result suggests that rat SCP is probably an integral membrane protein because the extraction from fresh tissue is dependent on the disruption of the lipid bilayer (Singer, 1974).

MacPherson, Armstrong and Yu (1981) have shown that rat SCP isolated from delipidated tissues has an electrophoretic mobility in agarose, at pH 8.6, corresponding to that of an acidic protein. In fact, the isoelectric point of rat SCP and SCP-PN was estimated by isoelectric focusing to be 4.6.

In order to ascertain which of the two components present in rat SCP preparations purified by gel-filtration chromatography was immunoreactive, SCP was mixed with an anti-rat SCP serum that formed one line with rat SCP in immunodiffusion analyses (MacPherson, Armstrong and Yu, 1981). The SDS-polyacrylamide gel electrophoretograms of the specific precipitate revealed that the band with the slower electrophoretic mobility was immunoreactive rat SCP. The molecular weight of rat SCP and SCP-PN was estimated to be 11,000 daltons by SDS-polyacrylamide gel electrophoresis. Thus, rat SCP-PN is significantly smaller than bovine SCP-PN. Moreover, the amino acid compositions of rat and bovine SCP-PN are different.

It has been established that bovine SCP-PN and bovine P_2 are identical. However, the assumption that the 13,500 dalton component of rat peripheral myelin is rat P_2 is not justified because rat P_2 has never been isolated and compared with bovine P_2 (Singh, Silberlicht and Singh, 1978).

One of the aims of this study is to ascertain if rat SCP-PN is present in purified rat peripheral myelin. This will be approached by comparing the electrophoretic mobilities in SDS-polyacrylamide gels of rat SCP-PN and the proteins of rat peripheral myelin. In addition, an anti-rat SCP-PN serum will be used to determine if purified rat peripheral myelin contains immunoreactive SCP-PN.

1.3.3 Human SCP

Human SCP was detected by Yo and MacPherson (1972) by immunodiffusion analyses in saline extracts of human spinal cord using an anti-bovine SCP serum. Unlike bovine SCP, human spinal cord extracts formed two distinct precipitin lines, each of which fused with the single line that formed opposite bovine SCP. In 1976, knowledge concerning the immunochemical characteristics of human SCP was limited to this observation. The physical and chemical properties were not known as the protein had not been isolated in a purified state. The availability of purified human SCP would enable one to characterize the protein and to assess the anti-encephalitogenic capacity of human SCP.

Thus, the purification and characterization of human SCP was undertaken in this study to ascertain whether human SCP is more closely related to the acidic rat SCP or the basic bovine SCP. This objective could be achieved by determining the molecular size, the isoelectric point and the amino acid composition of human SCP.

1.4 SCP-PN and P_2

1.4.1 Identity of SCP-PN with P_2

In a preliminary report presented in 1975, samples of bovine SCP and SCP-PN were compared by Kies *et al.* (1975) to the BF protein that had been isolated by acid extraction from bovine peripheral nerve myelin by Uyemura *et al.* (1972). That year Brostoff and Eylar isolated the same protein from peripheral nerve myelin and called it P_2. The amino acid compositions of the proteins were very similar and, in immunodiffusion analyses, SCP, SCP-PN, BF and P_2 were shown to be immunochemically identical using an anti-bovine SCP serum. Uyemura, Kato-Yamanaka and Kitamura (1977) have also isolated the BF protein from 0.1 M NaCl extracts of non-delipidated bovine spinal cord, thereby confirming the reports of Yo and MacPherson (1972) and MacPherson, Armstrong and Tan (1976).

Deibler, Driscoll and Kies (1978) subsequently published the details of the 1975 preliminary report. It was shown that bovine SCP-PN, P_2 and BF had the same chemical and physical properties and were identical when compared by immunodiffusion analyses. Recently, the identity has been confirmed by comparing the amino acid sequences of bovine SCP-PN and bovine P_2 (Kitamura *et al.*, 1980; Weise *et al.* 1980b; MacPherson and Bradshaw, personal communication). Nevertheless, while the identity is established, there is disagreement with respect to the cellular location of SCP-PN in the peripheral and central nervous systems.

1.4.2 Localization

Yo and MacPherson (1972), MacPherson, Armstrong and Tan (1976) and MacPherson (1978) showed that immunoreactive SCP exists in bovine brain, spinal cord and peripheral nerves in the proportions of 1:10:100, respectively. However, the original discoverers of P_2 claim that P_2 is unique to the peripheral nervous system and is a component of the myelin membrane (Uyemura *et al.*, 1972; Brostoff and Eylar, 1972). Although Uyemura, Kato-Yamanaka and Kitamura (1977) have published confirmation of the results of Yo and MacPherson (1972) and showed that P_2 could be extracted from non-delipidated bovine spinal cord with 0.1 M NaCl, P_2 is still described as a component that is restricted to the peripheral nervous system (Ishaque, Hoffman and Eylar, 1979; Kitamura *et al.*, 1980; Weise *et al.*, 1980a).

MacPherson (1978) has always considered bovine SCP to be a cytoplasmic protein because it can be rapidly released from fresh bovine tissue by extraction with 0.1 M NaCl. All of the immunoreactive SCP-PN can easily be removed from peripheral nerves. For example, when bovine spinal roots are extracted with 0.1 M NaCl and then delipidated, additional SCP-PN can not be extracted from the delipidated tissue. This property of SCP-PN is characteristic of a cytoplasmic and a peripheral membrane protein (Singer, 1974).

There is no doubt that bovine SCP-PN is a major component of purified peripheral myelin (Uyemura *et al.*, 1972;

Brostoff and Eylar, 1972). That P_2 can be released from purified peripheral nerve myelin by extraction with 0.03 N HCl indicates that P_2 may be classified as a peripheral membrane protein (Singer, 1974).

In an effort to determine whether SCP-PN was localized in myelin or in cytoplasm *in vivo*, MacPherson and Wallace (1980) studied the localization of SCP-PN in bovine peripheral nerve using an indirect immunofluorescent technique. A rabbit anti-bovine SCP serum was first allowed to react with tissue sections which had been fixed in acetone. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was then allowed to bind to the anti-SCP antibodies on the tissue section. It was clearly demonstrated that SCP-PN was localized in axons and in the endoneurial space surrounding the myelinated nerve fibres. SCP-PN was not detected in the myelin sheaths. Appropriate control tissue sections indicated that the staining was specific for SCP-PN. The ability to localize SCP-PN precisely in the area surrounding the myelin sheath was limited because the stained sites surrounding the myelin could not be adequately visualized by this technique. However, the authors concluded that because the anti-bovine SCP antibodies did not react with peripheral nerve collagen or blood vessels, which are the major components of the endoneurial space, it was possible that the staining of the endoneurial space was caused by the presence of SCP-PN in Schwann cells. SCP was also localized in the axons and glial cell processes in the white matter of bovine spinal cord (MacPherson and Wallace, personal communication). Thus, the

results of MacPherson and Wallace (1980) indicate that bovine SCP-PN is present in axoplasm and, possibly, in Schwann cell cytoplasm. This proposal is consistent with the solubilization of SCP-PN from whole tissue with 0.1 M NaCl.

In contrast, Eylar *et al.* (1980) also used the immunohistological technique to localize rabbit P_2. Goat anti-rabbit P_2 antibodies were allowed to react with cryostat sections that had been fixed in 95% ethanol/ether. The sections were then washed with saline for an unspecified period of time before a fluorescein isothiocyanate-conjugated anti-goat antibody antiserum was allowed to react with the tissue section. It was found that rabbit P_2 was localized only in the myelin sheaths of the peripheral nervous system.

It is not known if ethanol/ether can effectively fix P_2 *in situ* in tissue sections so that the P_2 is insoluble in saline. Therefore, it is possible that a saline wash of the ethanol/ether-fixed tissue sections could result in the solubilization of P_2.

Based on the results of this report it was concluded that P_2 is exclusive to peripheral nerve myelin. It is uncertain whether this conclusion refers only to the rabbit P_2 protein or whether it is meant to include the P_2 protein of all species. Whatever the case, there is a discrepancy between the results of the two reports on the localization of bovine SCP-PN and rabbit P_2. At present, there is no confirmation of the localization of SCP-PN or P_2 by an independent

investigation that takes into consideration the solubility of the protein in saline after fixation.

An attempt has been made in this study to explain the conflicting results of the localization studies. The rationale for this attempt is that differences in the experimental technique may have been responsible for the conflicting results. Thus, the effects of the fixatives used in the two studies on the solubility of SCP-PN in whole tissue with saline is examined.

The resolution of the distribution and localization of SCP-PN may be approached in two ways. If one assumes that SCP-PN is present in peripheral myelin *in vivo*, then it must be demonstrated that the localization of SCP-PN in peripheral nerve axons by the immunofluorescent technique is an artifact. Also, the localization of SCP in the axons and glial cells of the spinal cord must be explained. On the other hand, if one assumes that SCP-PN is an axonal protein, then one will have to demonstrate that the presence of SCP-PN in purified myelin is an artifact. This study was undertaken to investigate the validity of the latter assumption. Thus, two of the objectives of this research are to understand why SCP-PN is found in purified myelin and to account for the conflicting results of the immunohistological studies.

It is important to determine whether SCP-PN is a peripheral or integral myelin membrane protein. An integral association would provide evidence that SCP-PN is present in myelin *in vivo*. On the other hand, if SCP-PN is only loosely bound

to myelin and is present in other cellular fractions, then it's alleged location in myelin would be questionable.

If SCP originates from a non-myelin location in the nervous tissue, then it will be necessary to determine the property of SCP-PN or myelin that is responsible for the redistribution of SCP-PN into the myelin fraction and if the mechanism is specific for SCP-PN.

1.4.3 Biological Activity

The central nervous system forms of bovine and rat SCP have been demonstrated to have anti-encephalitogenic activity in guinea pigs and rats (MacPherson and Yo, 1973; MacPherson and Armstrong, 1977). However, bovine SCP-PN has no anti-encephalitogenic activity in guinea pigs (MacPherson, 1978) and is neuritogenic in the Lewis rat. Thus, it is probable that slight, but critical, differences exist in the amino acid sequences of the central and peripheral nervous system forms of bovine SCP. Indeed, the amino acid compositions of bovine SCP and SCP-RN are slightly different, but it is not known whether the differences are quantitatively significant (MacPherson, Armstrong and Tan, 1976). This question can not be answered until bovine SCP is sequenced.

1.5 Protective Sequence of SCP

The sequencing of bovine SCP-PN (P_2) has facilitated the search for the biologically active determinants of SCP and SCP-PN. Recently, a cyanogen bromide (CNBr)-derived peptide from bovine P_2 has been isolated by Weise *et al.*

(1980a). Preparations of this peptide have been shown to cause EAN in Lewis rats.

CNBr cleaves proteins at methionine residues. The amino acid composition of bovine SCP indicates that there are three methionine residues in the protein. Hence, the CNBr cleavage of SCP should result in four peptides. An important consideration is that the fewer the number of points at which SCP is cleaved, the higher the probability of obtaining an intact biologically active site. In this respect, the use of CNBr is preferred to that of an enzyme such as pepsin that hydrolizes proteins at several sites. Thus, cleavage of SCP with pepsin would result in considerably more peptides than cleavage with CNBr.

The search for the amino acid sequence of the anti-encephalitogenic determinant in bovine SCP is initiated in this study. The primary objective will be to establish a purification procedure by which sufficient amounts of CNBr-derived peptides can be obtained for future studies involving the assessment of the anti-encephalitogenic capacity of the peptides.

The study of the anti-encephalitogenic determinant of SCP is important because SCP might be used in the treatment of multiple sclerosis.

1.6 Nomenclature of SCP-PN

SCP-PN is called P_2 protein by Brostoff and Eylar (1972) and has been referred to in the past as the BF protein by

Uyemura *et al.* (1972) and as the P_1 protein (London, 1971). It is evident that this nomenclature can be confusing for present and future investigators. Recently, Eylar *et al.* (1979) have agreed that a general terminology for peripheral myelin proteins be adopted to avoid confusion. The authors have proposed that the 14,000 dalton component present in SDS-polyacrylamide gels of peripheral nerve myelin proteins be referred to as the P_2 protein. In accordance with this proposal, the BF protein is now referred to as P_2 (Kitamura *et al.*, 1980).

Several authors have reported that the P_2 protein is found only in peripheral nerve myelin (Brostoff and Eylar, 1972; Greenfield *et al.*, 1973; Eylar *et al.*, 1980). This claim is made regularly in spite of conclusive evidence that immunoreactive SCP is in the central nervous system (Yo and MacPherson, 1972; Uyemura, Kato-Yamanaka and Kitamura, 1977). Thus, the term P_2 has become synonomous with a peripheral myelin protein, although there is no conclusive evidence that P_2 is localized in peripheral myelin or is exclusive to the peripheral nervous system.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

Human nervous tissues from patients who died of non-neurological causes were received in the laboratory within 12 hours of death. Bovine spinal cords were obtained within 2 hours of death from a local abattoir. The dural membrane was removed from the human and bovine spinal cords and, all the nerve roots were cut at the cord surface. Rat sciatic nerves and spinal nerve roots were dissected from rats within minutes after decapitation. Tissues were either used immediately or wrapped in plastic film and stored at -20 °C.

Bovine SCP was prepared by the method of MacPherson, Armstrong and Tan (1976), except that 0.15 M NaCl, pH 4.5, was used to extract the tissue. The rabbit anti-bovine SCP serum used by MacPherson *et al.* (1976) was used for this study. The rat SCP-PN and a rabbit anti-rat SCP-PN serum were prepared in this laboratory.

Rats were purchased from Woodlyn Laboratories, Guelph, Ontario. Female New Zealand White rabbits, 2.2 - 2.7 kg, were supplied by Riemens Fur Ranches Ltd., St. Agatha, Ontario.

Carboxymethyl cellulose and 3$^{m}_{m}$ chromatography paper were supplied by Whatman Biochemicals Ltd., Kent. Sephadex G-50 superfine was purchased from Pharmacia, Montreal,

Canada. Acrylamide, sodium dodecyl sulfate (SDS) and Bio-Gel P6 were obtained from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Canada. Cyanogen bromide, N,N'-methylene-bisacrylamide and N,N,N',N'-tetramethylethylenediamine (TEMED) were products of Eastman Kodak Co., Rochester, New York.

Most of the proteins used as standards for molecular weight determinations were bought from Sigma Chemical Co., St. Louis, Mo., as was Coomassie Brilliant Blue R and bovine aprotinin. Bovine serum albumin and horse radish peroxidase were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Chen Chin polyamide sheets, dansyl chloride and amino acid calibration standards were supplied by Pierce Chemical Co., Rockford, Il. Carrier ampholytes (pH range 3-10 and 9-11) were purchased from Brinkman Instruments Inc., Westbury, New York. All other chemicals were reagent grade.

Spectropor 1 was obtained from Sepctrum Medical Industries Inc., Los Angeles, Calif. Collodian membranes were purchased from Schleicher & Schuell Inc., Keene, N.H.

2.2 METHODS

2.2.1 Isolation of Human SCP and SCP-PN

2.2.1.1 Extracts of Spinal Cords and Spinal Nerves

Approximately 125 g of frozen spinal cords or spinal nerves were cut into shavings and homogenized with 1,125 ml of 0.15 M NaCl to make a 10% homogenate (w/v). Spinal cords were homogenized in a Tri-R blender at speed 7 for 2 min.

Spinal nerves were homogenized in a Sorval Omni-Mixer at speed 10 for 3 min.

The homogenate was centrifuged at 14,000 x g for 30 min at 0°C and the supernatant was removed by suction. The precipitate was rehomogenized in one-third the original volume of 0.15 M NaCl and centrifuged as above. The supernatants were combined and adjusted to pH 4.5 with 1 M acetic acid. Insoluble material was removed from the supernatant by centrifuging as above. After being concentrated three-fold by flash evaporation, the supernatant was dialyzed in Spectropor 1 against several changes of 0.05 M sodium acetate buffer, pH 4.5, until the conductivity was equal to that of the equilibrated CM-52 cellulose.

2.2.1.2 Absorption of Extracts on CM-52 Cellulose

Preswollen CM-52 cellulose was equilibrated with 0.05 M sodium acetate, pH 4.5. A sufficient amount of CM-52 cellulose, equal to 50 times the weight of the total protein of the extract, was added to the tissue extract and stirred for 2 hours at 4°C. Following collection by vacuum filtration on a Buchner funnel, the CM-52 cellulose was transferred to a 4.5 x 60 cm chromatography column. The column was washed sequentially with (1) 0.05 M sodium acetate buffer, pH 4.5, (2) 0.05 M sodium acetate buffer, pH 5.5, and (3) 0.05 M sodium acetate buffer, pH 5.8, containing 0.05 M NaCl. Each wash buffer was passed through the column until the absorbance at 280 nm (E_{280}) was 0.09 or less for at least 100 ml of eluant.

2.2.1.3 Ion Exchange Chromatography

Human SCP and SCP-PN were each eluted from CM-52 cellulose with 0.05 M sodium acetate buffers, pH 5.8, containing increasing concentrations of NaCl. The two elution buffers, E_1 and E_2, contained 0.1 M and 0.2 M NaCl, respectively. Fractions containing 5 ml of eluant were collected at a flow rate of 90 ml/h at 20°C. Areas of the chromatogram containing SCP-PN, serum proteins or other nervous system antigens were identified by immunodiffusion analyses using a rabbit anti-bovine SCP serum, an anti-human serum serum and an anti-human brain extract serum, respectively.

2.2.1.4 Gel Filtration Chromatography

Fractions which contained human SCP or SCP-PN were pooled and concentrated by flash evaporation. The concentrated fractions were dialyzed in Spectropor 1 against distilled water. The purity of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis as described below. Fractions which contained similar kinds and amounts of impurities were pooled and applied to a 1.8 x 90 cm chromatography column containing Sephadex G-50 superfine. Approximately 25 mg of protein was applied in a volume of 2 ml and eluted with 0.1 M NaCl. Fractions containing 2 ml of eluant were collected at a flow rate of 20 ml/h at 20°C. Fractions containing SCP or SCP-PN were detected by immunodiffusion analyses.

2.2.2 Preparation of Antisera

Anti-human SCP sera were raised in rabbits by injecting them initially in the hind foot pads with 0.25 mg of purified SCP or 0.5 mg of SCP-PN emulsified in complete Freund's adjuvant (CFA). Injections were repeated intramuscularly every 3 to 4 weeks for eight months. Rabbits that were producing suitable amounts of antibodies were bled at 3-week intervals and were re-injected when necessary to maintain the serum antibody concentration at a satisfactory level. All sera were stored at -20°C. Anti-human SCP sera were tested for the presence of antibodies directed towards contaminants by immunodiffusion analyses using various dilutions of human brain extracts and human serum. The sera were absorbed with human serum and a human liver extract.

2.2.3 Immunochemical Analyses

2.2.3.1 Double Diffusion Analyses

Immunodiffusion analyses were performed according to the method of Ouchterlony (1953), except that 7.5 x 2.5 cm glass slides were used. The slides were covered with 1.2% agarose or 1.3% agar containing 0.05 M barbiturate buffer, pH 8.6. Circular wells were cut in the solidified gel in a suitable pattern. An antigen solution and antiserum were added to appropriate wells, and the plates were left at room temperature for 24 h.

In some analyses the agar contained either 10% of an aprotinin solution (10-20 trypsin inhibitor units per ml) or 0.1 M NaCl in addition to the barbiturate buffer to facilitate the diffusion of the antigens.

2.2.3.2 Immunoelectrophoretic Analyses

Immunoelectrophoretic analyses were run according to the method of Scheidegger (1955) on glass microscope slides coated with either 1.3% agar or 1.2% agarose containing 0.05 M barbiturate buffer, pH 8.6, using a constant current of 15 mA per slide for 45 min.

2.2.4 Quantitative Single Radial Immunodiffusion

The human SCP or SCP-PN content of tissue extracts was estimated by the procedure of Mancini, Carbonara and Heremans (1965). Anti-bovine SCP (0.8 ml) was added to 9.2 ml of 1.2% agarose containing 0.05 M barbiturate buffer, pH 8.6, at 56°C. The mixture was pipetted onto a warm glass plate (7.5 x 5 cm). After the gel had solidified, wells were punched in the gel, 1.4 cm apart, using a Vita No. 12 hypodermic needle from which the bevel had been cut. Samples of the tissue extracts were added to duplicate wells and known concentrations of human SCP or SCP-PN were added to selected wells. The plates were stored in a moist chamber at room temperature. After 24 to 48 h the diameters of the precipitin circles formed aound each well were measured. The diameters in mm were plotted against the protein concentration in mg to obtain a standard calibration line.

2.2.5 Physical Analyses

2.2.5.1 Protein Determination

Protein concentration was determined by the method of Lowry *et al.* (1951) using either bovine serum albumin or bovine SCP-PN as a standard.

2.2.5.2 Molecular Weight Determination

Estimation of the molecular size of proteins was determined by SDS-polyacrylamide gel electrophoresis using the method of Weber and Osborne (1969). Gels containing 10% polyacrylamide, 0.1 M sodium phosphate buffer, 0.1% SDS, 0.13% TEMED and 0.75% ammonium persulfate were used. Samples containing 5 to 50 µg of protein were incubated at 37°C for 1 h with 50 µl of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS and 5 µl of 2-mercaptoethanol. One drop of glycerol and 4 µl of 0.05% bromphenol blue were added to the sample. The mixture was applied to the top of a gel and electrode buffer was layered on top of each sample. A constant current of 8 mA/gel was applied until the bromphenol blue had moved 6 cm through the gel. The gels were removed from the glass tubes, fixed overnight in 50% methanol-5% acetic acid then stained for 6 h with 0.25% Coomassie blue containing 5% methanol and 7.5% acetic acid. Destaining was performed with several changes of 5% of methanol-7.5% acetic acid. The relative mobility of proteins was calculated using the following formula:

$$\text{Relative Mobility} = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

Proteins used as standards were bovine serum albumin, α-chymotrypsinogen, myoglobin, ribonuclease and cytochrome c. Relative mobilities were plotted as ordinates against the molecular weights on semi-log paper.

Molecular sizes were also estimated by gel filtration chromatography. A 1.8 x 90 cm chromatography column containing Sephadex G-50 superfine was calibrated according to the method of Andrews (1964) with proteins of known molecular weight. Blue Dextran 2000 was used to determine the void volume. Gel filtration chromatography was performed under the same conditions as described above. The E_{280} was used to measure the elution volumes of HSCP-PN and the standard proteins.

2.2.5.3 Isoelectric Focusing

The isoelectric points of human and bovine SCP-PN were determined in an LKB 8101 electrofocusing column in the manner described by Haglund (1967). A continuous sucrose density gradient containing 1% carrier ampholytes (pH 9-11) and 8 mg of protein was prepared in the column. A constant voltage of 500 volts was applied to the column for 24 h. The column was drained and the E_{280} and pH of 1 ml fractions were measured.

2.2.6 Chemical Analyses

2.2.6.1 Amino Acid Analyses

The amino acid analyses were performed in the laboratory of Dr. B. Smith of the Department of Biochemistry,

University of Western Ontario, in a Beckman 120C automatic amino acid analyzer. The samples were hydrolyzed under vacuum with constant boiling HCl at 110°C for 24 h.

2.2.6.2 NH_2-Terminal Amino Acid Analyses

The dansyl technique, which was introduced by Gray (1970), was used for the NH_2-terminal amino acid identification. Five nanomoles of protein were added to 200 µl of 0.5 M sodium bicarbonate, pH 9.8, containing 1% SDS. The protein solution was added to 100 µl of dansyl chloride (5 mg/ml of acetone). After incubating the mixture at 37°C for 20 min the protein was precipitated by the dropwise addition of trichloroacetic acid while being vigorously mixed. The precipitate was pelleted by centrifugation, washed with 200 µl of 1 N HCl and recentrifuged. The pellet was mixed with 50 µl of 6 N HCl and sealed under vacuum. After 6 h at 105°C, the tubes were opened and dried in a vacuum desiccator at 56°C.

A 100 µl aliquot of an amino acid standard mixture was dansylated according to the method of Weiner, Platt and Weber (1972). The dansyl-amino acid standard mixture and the unknown sample were spotted on opposite sides of a 6 x 6 cm polyamide plate. Two dimensional chromatography of the dansyl derivatives were performed according to the method of Hartley (1970). Chromatograms were viewed under ultraviolet light to reveal the position of the dansylated amino acids.

2.2.7 Preparation of Myelin

The method of Uyemura *et al.* (1972) was used to prepare bovine or rat peripheral nerve myelin by density gradient centrifugation of a 15% (w/v) homogenate of fresh spinal nerves. Myelin was further purified by an additional upward flotation step in 0.8 M sucrose. The myelin interface was washed three times in distilled water to remove the sucrose and lyophilized. In one experiment, 0.8 M sucrose containing 0.15 M NaCl was used to homogenize the tissue.

2.2.8 Electron Microscopy

The purity of myelin was assessed by electron microscopic examination. Myelin was fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide in a 0.1 M sodium cacodylate buffer, pH 7.3, with 5.4% sucrose added. After dehydration, the samples were stained in lead acetate and uranyl nitrate for 1 h prior to embedding in Spurr resin. Uranyl acetate and Reynolds' (1963) lead citrate were used to stain the sections. The micrographs were evaluated by Dr. J. C. Kaufmann of the Department of Pathology, University of Western Ontario.

2.2.9 Analysis of Myelin Preparation Supernatants

The supernatants obtained from the first six centrifugations of the myelin preparation were analyzed for the presence of bovine SCP-PN. Each supernatant was adjusted to pH 4.5, with 1.0 M acetic acid and absorbed batchwise onto CM-52 cellulose as described above. After transfer to

a 1 x 20 cm chromatography column the CM-52 cellulose was washed with 0.05 M sodium acetate buffer, pH 5.8 to remove sucrose. SCP-PN was then eluted from the CM-52 cellulose buffer containing 0.3 M NaCl. The eluates were concentrated by flash evaporation and dialyzed against 0.15 M NaCl. The amount of SCP-PN in each eluate was then determined by single radial immunodiffusion.

2.2.10 Extraction of Peripheral Nerves

Approximately 5 g of bovine peripheral nerve was added to sufficient 0.3 M NaCl to make a 15% mixture (w/v) and homogenized in an Omni-Mixer at speed 10 for 3 min at 4°C. The homogenate was centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was removed and the bovine SCP content was estimated by quantitative single radial immunodiffusion.

2.2.11 Extraction of Myelin

Ten mg of myelin was added to 5 ml of either 0.3 M NaCl or 0.05 N HCl, pH 1.86. The mixture was homogenized at 4°C using a Tri-R blender at speed 5 for 90 s. The homogenate was centrifuged at 4°C at 170,000 x g for 20 min and the supernatant was removed by suction. The pellet was solubilized in 5 ml of 1% SDS by homogenization in a Tri-R blender as above and heating for 3 min in a boiling water bath. Two additional samples of myelin were extracted two and three times, respectively, as above.

Myelin extracted with NaCl or HCl in the above manner will hereinafter be referred to as NaCl-extracted myelin or HCl-extracted myelin, respectively.

2.2.12 Electrophoretic Analyses

SDS-polyacrylamide gel electrophoretic analyses were performed as above. The quantitative distribution of myelin proteins in SDS-polyacrylamide gel electrophoretograms was determined by densitometric scanning. Stained gels were scanned at 540 nm in a Zeiss PMQ II spectrophotometer using a slit width of 0.10 mm. The distribution of each protein was calculated from the weights of paper cutouts of the protein peaks obtained from the scans.

Analytical and preparative SDS-polyacrylamide slab gel electrophoretic analyses were run in an EC vertical slab gel apparatus as described by Singh, Silberlicht and Singh (1978). Myelin or myelin proteins were solubilized in 1.5% SDS containing 0.75% dithiothreitol, 0.002% bromphenol blue and 20% glycerol in 0.02 M Tris-HCl buffer, pH 6.8. For analytical slab gels, samples containing 40 to 120 µg of protein were applied on a spacer gel which contained 5% polyacrylamide, 1% SDS, 0.15% TEMED and 0.15% ammonium persulfate in 0.5 M Tris-HCl buffer, pH 8.8. For preparative slab gels, approximately 3 mg of myelin proteins were used. Electrophoresis was continued at a constant voltage of 80 volts for 16 h. Slab gels were fixed, stained and destained as above except that the staining time was increased to 8 h.

Glycoproteins were stained using periodic acid-Schiff's reagent as described by Segrest and Jackson (1972).

2.2.13 Recovery of Protein

Protein in the SCP-PN + periodic acid-Schiff II (PAS-II) band was recovered from preparative slab gels or conventional gels. The selected band was macerated with ten volumes of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.5% SDS and stirred for 24 h at 40°C. After centrifugation at 170,000 x g the supernatant was concentrated to 2 ml in Spectropor 1 by ultrafiltration under positive pressure of 100 mm Hg. After dialysis for 24 h against 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS, the supernatant was added dropwise to 100 ml of acetone containing 1.5% HCl and slowly stirred for 24 h at -20°C. Following centrifugation at 2,500 x g, the precipitate was dried under nitrogen and solubilized, at a concentration of 3 mg/ml, in 1% SDS.

2.2.14 Absorption of Proteins on Myelin and NaCl-Extracted Myelin

Myelin, which had been extracted twice with NaCl, was washed once with water and lyophilized. Suspensions of 5 mg of myelin or NaCl-extracted myelin in 12 ml of 0.8 M sucrose were prepared using a Tri-R blender. Either 0.05 ml or 0.25 ml of 0.1 M NaCl containing 1 mg of bovine SCP-PN, lysozyme or pepsin/ml was added to the suspension of myelin or NaCl-extracted myelin. After further homogenization in a Tri-R blender at speed 4 for 1 min the homogenates were incubated at 4°C for 30 min. The homogenates were centrifuged at 170,000 x g for 1 h. The floating myelin was removed, washed

twice with 8 ml of water and centrifuged at 170,000 x g for 30 min. The pellets were solubilized in 1% SDS as above and subjected to SDS-polyacrylamide gel electrophoresis. The stained gels were scanned as above.

2.2.15 Effects of Fixatives on the Solubility of Bovine SCP

Two grams of bovine spinal cord or peripheral nerve was homogenized in an Omni-Mixer at speed 8 for 2 min in 38 ml of either acetone or 95% ethanol-ether (1:1). The homogenates were left at 4°C for 1 h and then centrifuged at 10,000 x g for 20 min at 4°C. The pellets were homogenized as above with 15 ml of 0.15 M NaCl and centrifuged. The supernatant was concentrated by flash evaporation to 1.5 ml and dialyzed against 0.15 M NaCl. The presence of immunoreactive SCP or SCP-PN was established by immunodiffusion analyses using anti-bovine SCP serum.

2.2.16 Cyanogen Bromide-Derived Peptides

2.2.16.1 Cleavage

Cyanogen bromide cleavage of bovine SCP was performed according to the method of Gross (1967) by dissolving 30 mg of SCP and 100 mg of CNBr in 2 ml of 70% formic acid. After 48 h the solution was diluted to 50 ml with distilled water and lyophilized. The lyophilized material was washed with 25 ml of distilled water and relyophilized.

2.2.16.2 Isolation of Peptides

Approximately 30 mg of lyophilized material was solubilized in 1 ml of 0.05 M acetic acid and applied to a 1.8 x 90 cm chromatography column containing Bio-Gel P6. The column was eluted with 0.05 M acetic acid and fractions containing 2 ml of eluant were collected at a flow rate of 15 ml/h.

2.2.16.3 Purity

The purity of the peptides was assessed by three different electrophoretic methods, SDS-polyacrylamide gel electrophoresis, polyacrylamide gel electrophoresis at pH 2.7 and high voltage paper electrophoresis.

Polyacrylamide gel electrophoresis at pH 2.7 was performed according to the procedure of Jackson *et al.* (1975) except that the concentrations of polyacrylamide in the spacer and separation gels were increased to 7.5% and 15%, respectively. The gels were stained with Coomassie Blue as described above.

A method similar to that of Katz, Dreyer and Anfinsen (1959) was used for high voltage paper electrophoresis. Approximately 2 to 4 mg of peptide in 1 or 2 drops of 0.1 N HCl was spotted on the chromatography paper. Electrophoresis was carried out at pH 3.7 in a solution containing acetic acid, pyridine and water, 10:1:89 (v/v), at a constant voltage of 2,500 volts for 1 h. Ascending chromatography in pyridine/butanol/acetic acid/water (21:30:6:24) was carried

out for 18 h. A solution of 0.25% ninhydrin and 0.6% trimethylpyridine in acetone was used to stain the peptides.

CHAPTER 3: RESULTS

3.1 Isolation and Characterization of Human SCP

3.1.1 Purification

Immunodiffusion analyses were used throughout the purification procedure to detect the presence of human SCP in soluble fractions. Saline extracts of human spinal cords or peripheral nerves were found to contain distinct antigenic components both of which cross-reacted extensively with bovine SCP, thereby confirming the results of MacPherson and Armstrong (1976).

Following batch absorption of the saline extracts on CM-52 cellulose, human SCP was not detected in the CM-52 cellulose filtrate. Therefore, human SCP bound to the ion exchanger under the condition specified for absorption. The saline extracts of 100 g of spinal cord or spinal nerves contained 1,150 and 660 mg of protein respectively. Approximately 1,050 and 600 mg of protein from the extracts of spinal cord and spinal nerves, respectively were absorbed on the CM-52 cellulose. The elution profile of SCP from CM-52 cellulose is shown in Figure 1. Human SCP was eluted with 0.05 M sodium acetate buffer, pH 5.8 containing increasing concentrations (discontinuous) of NaCl. The elution pattern of human SCP-PN, illustrated in Figure 2, was similar to that of SCP. However, SDS-polyacrylamide gel electrophoretograms and immunodiffusion analyses revealed that the two

Figure 1. Elution pattern of human SCP from a CM-52 cellulose chromatography column (4.5 x 60 cm). Approximately 1050 mg of protein was absorbed on 53 g of CM-52 cellulose. The column was washed sequentially with (1) 0.05 M sodium acetate buffer, pH 4.5, (2) 0.05 M sodium acetate buffer, pH 5.5 and (3) 0.05 M sodium acetate buffer, pH 5.8, containing 0.05 M NaCl. Stepwise elution of SCP was carried out with 0.05 M sodium acetate buffers, pH 5.8, containing increasing concentrations of NaCl: E_1 was eluted with 0.1 M NaCl and E_2 was eluted with 0.2 M NaCl. Fractions containing 5 ml of eluant were collected at a flow rate of 90 ml/h at 20°C. The shaded areas indicate the fractions containing SCP.

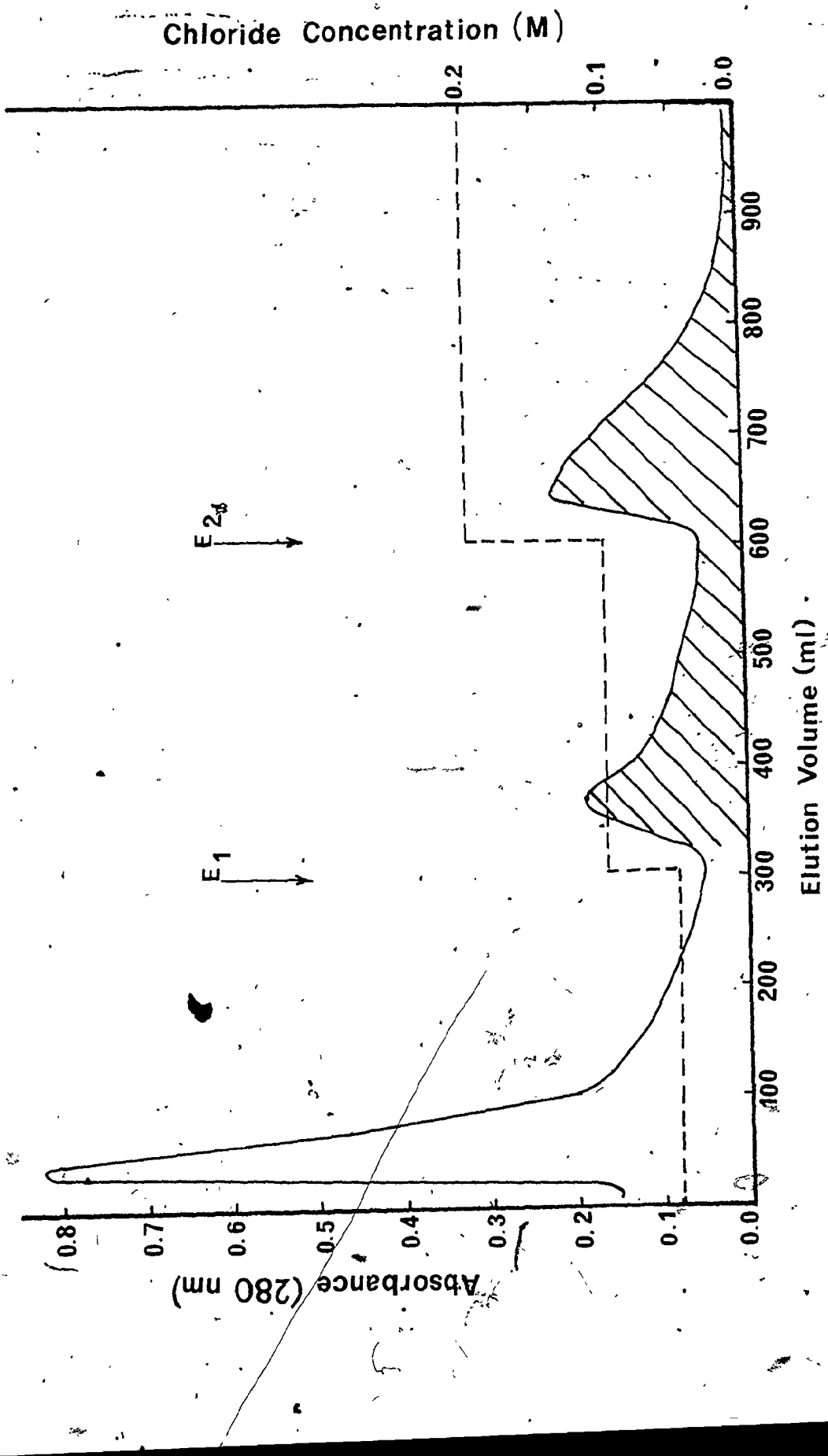
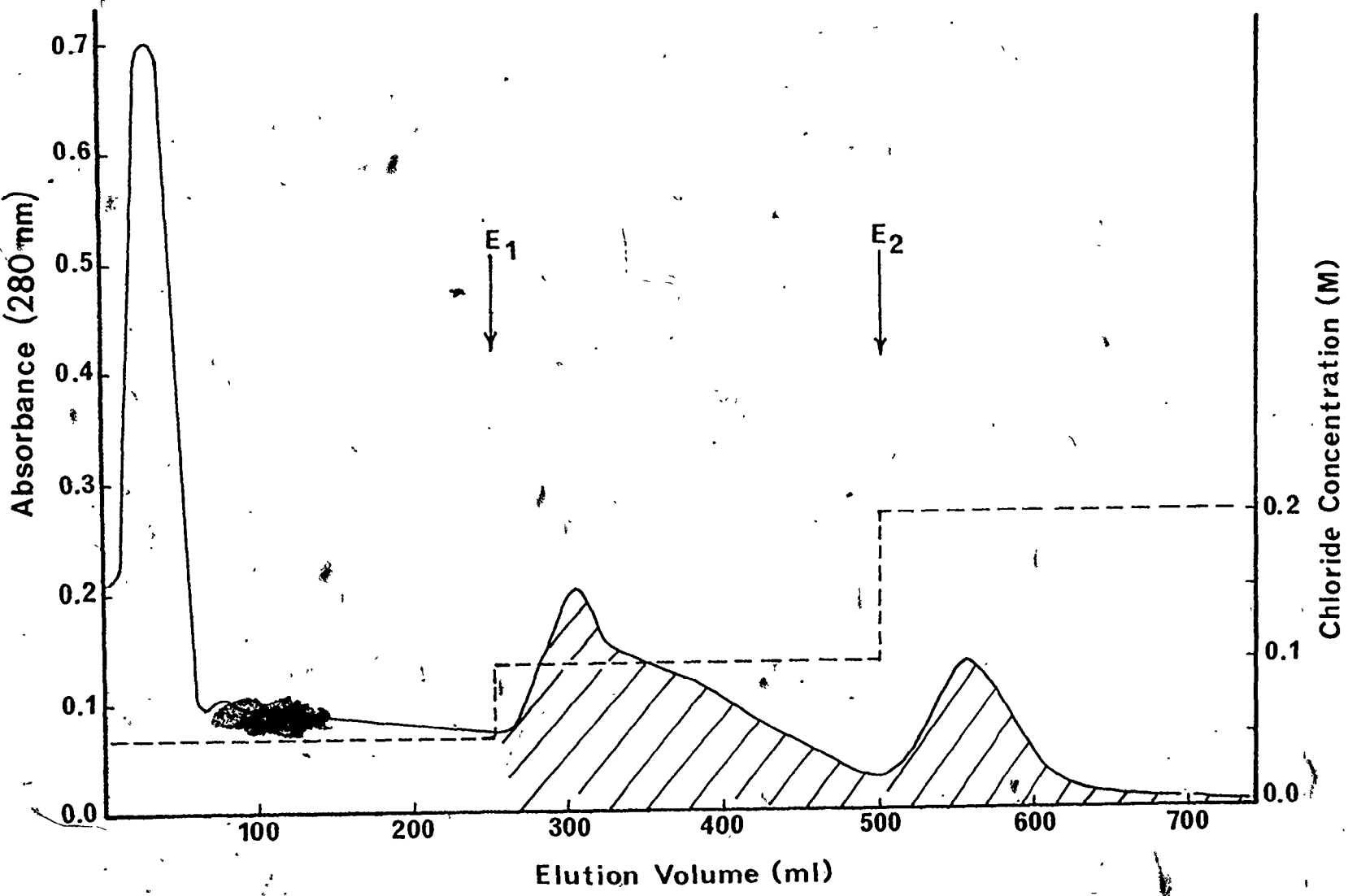

Chloride Concentration (M)
0.2
0.1
0.0
900
800
700
600
500
400
300
200
100
0.0
E2
E1
Elution Volume (ml)
Absorbance (280 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

Figure 2. Elution pattern of human SCP-PN from a CM-52 cellulose column. Approximately 600 mg of protein was absorbed on 33 g of CM-52 cellulose. The column (4.5 x 60 cm) was washed sequentially with (1) 0.05 M sodium acetate buffer, pH 4.5, (2) 0.05 M sodium acetate buffer, pH 5.5 and (3) 0.05 M sodium acetate buffer, pH 5.8, containing 0.05 M sodium chloride. Stepwise elution of SCP-PN was carried out with 0.05 M sodium acetate buffers, pH 5.8 containing increasing concentrations of NaCl: E_1 was eluted with 0.10 M NaCl and E_2 was eluted with 0.20 M NaCl. Fractions containing 5 ml of eluant were collected at a flow rate of 90 ml/h at 20°C. The shaded areas indicate the fractions containing SCP-PN.


Absorbance (280 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
E1
E2
0.2
0.1
0.0
Chloride Concentration (M)
100
200
300
400
500
600
700
Elution Volume (ml)

patterns differed notably. Peak E_1 of the SCP-PN pattern contained more contaminants and significantly less immunoreactive SCP-PN than peak E_2. In contrast, SCP was more evenly distributed throughout peaks E_1 and E_2 than SCP-PN.

Gel filtration chromatography in Sephadex G-50 superfine was used to separate SCP from impurities of differing molecular size. Material in peaks E_1 and E_2 from the SCP chromatogram were combined for gel filtration chromatography because the protein compositions of these eluates were similar. Fractions containing SCP had to be rechromatographed to remove all the impurities. The elution pattern of SCP is shown in Figure 3. SCP-PN-containing fractions derived from peaks E_1 and E_2 were chromatographed separately and combined following gel filtration. Figure 4 contains the elution profile of SCP-PN. It was observed that fractions from the gel filtration chromatograms containing SCP or SCP-PN formed one band when analyzed in SDS-polyacrylamide gels. Photographs of gel electrophoretograms of SCP, after rechromatography, and of SCP-PN, following one gel filtration, are presented in Figure 5. It appears that all impurities differing in molecular size were separated from SCP and SCP-PN during gel filtration.

When human or bovine SCP-PN were subjected to isoelectric focusing in a pH gradient, one narrow peak was obtained in each case. The isoelectrophoretogram of human SCP-PN is illustrated in Figure 6. The SDS-electrophoretogram and the

Figure 3. Elution profile of human SCP on Sephadex G-50 superfine. Fractions E_1 and E_2 from the CM-52 cellulose chromatography were combined, dialyzed and concentrated to contain 28 mg of protein per ml. Approximately 2.5 ml of this solution containing 70 mg of protein was applied to a 18 x 90 cm chromatography column. Elution with 0.1 M NaCl was performed at 20°C at a flow rate of 20 ml/h. Two ml fractions were collected. Fractions 58 to 70 were rechromatographed to remove minor contaminants.

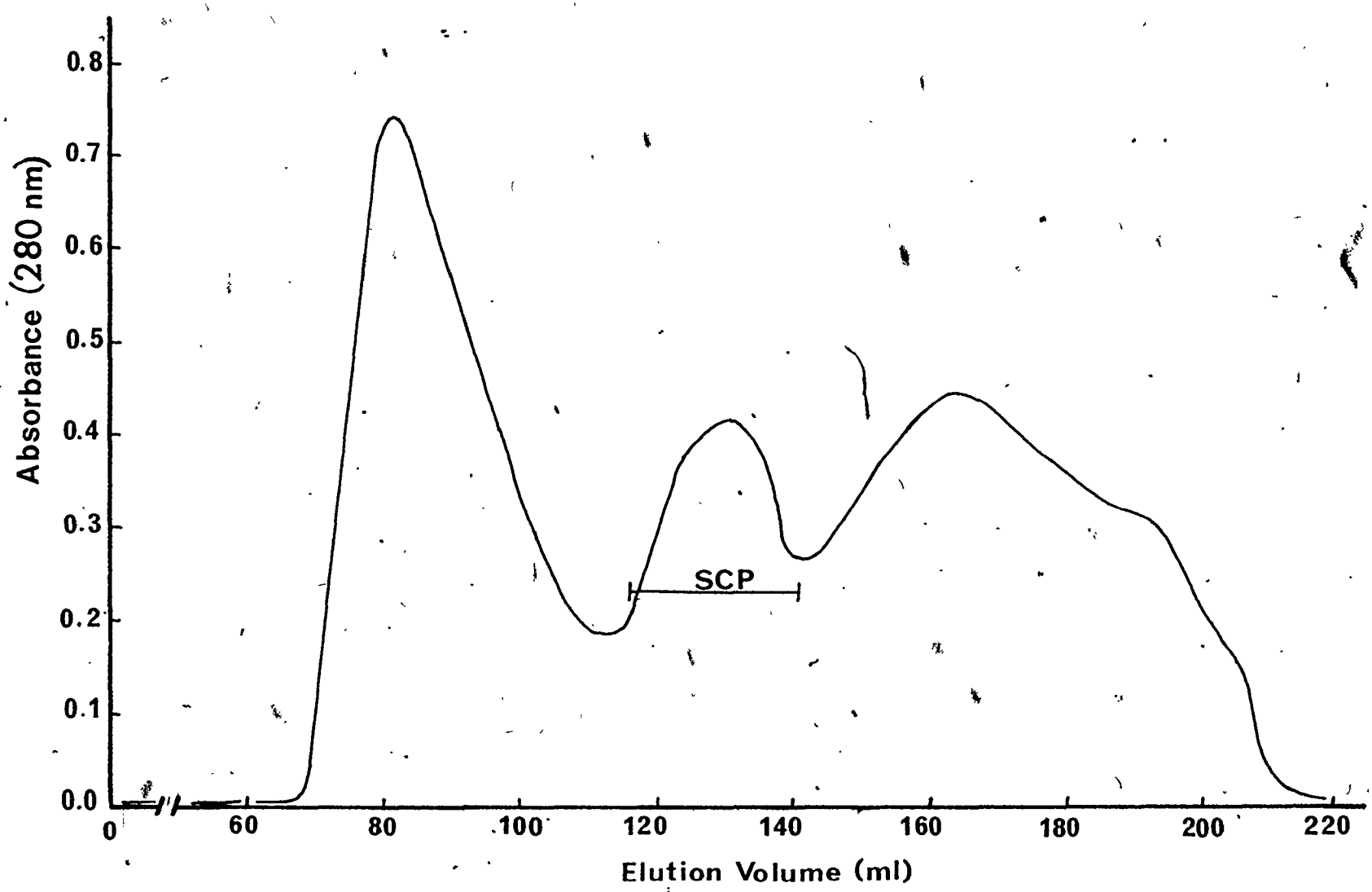

Absorbance (280 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SCP
0
60
80
100
120
140
160
180
200
220
Elution Volume (ml)

Figure 4: Elution pattern of human SCP-PN on Sephadex G-50 superfine. Approximately 25 mg of the protein in fraction E_1 that was eluted from CM-52 cellulose, was applied in a volume of 2 ml to a 1.8 x 90 cm chromatography column. The column was eluted with 0.1 M NaCl at a flow rate of 20 ml/h at 20°C. Two ml fractions were collected and SCP-PN was obtained between fractions 62 and 75.

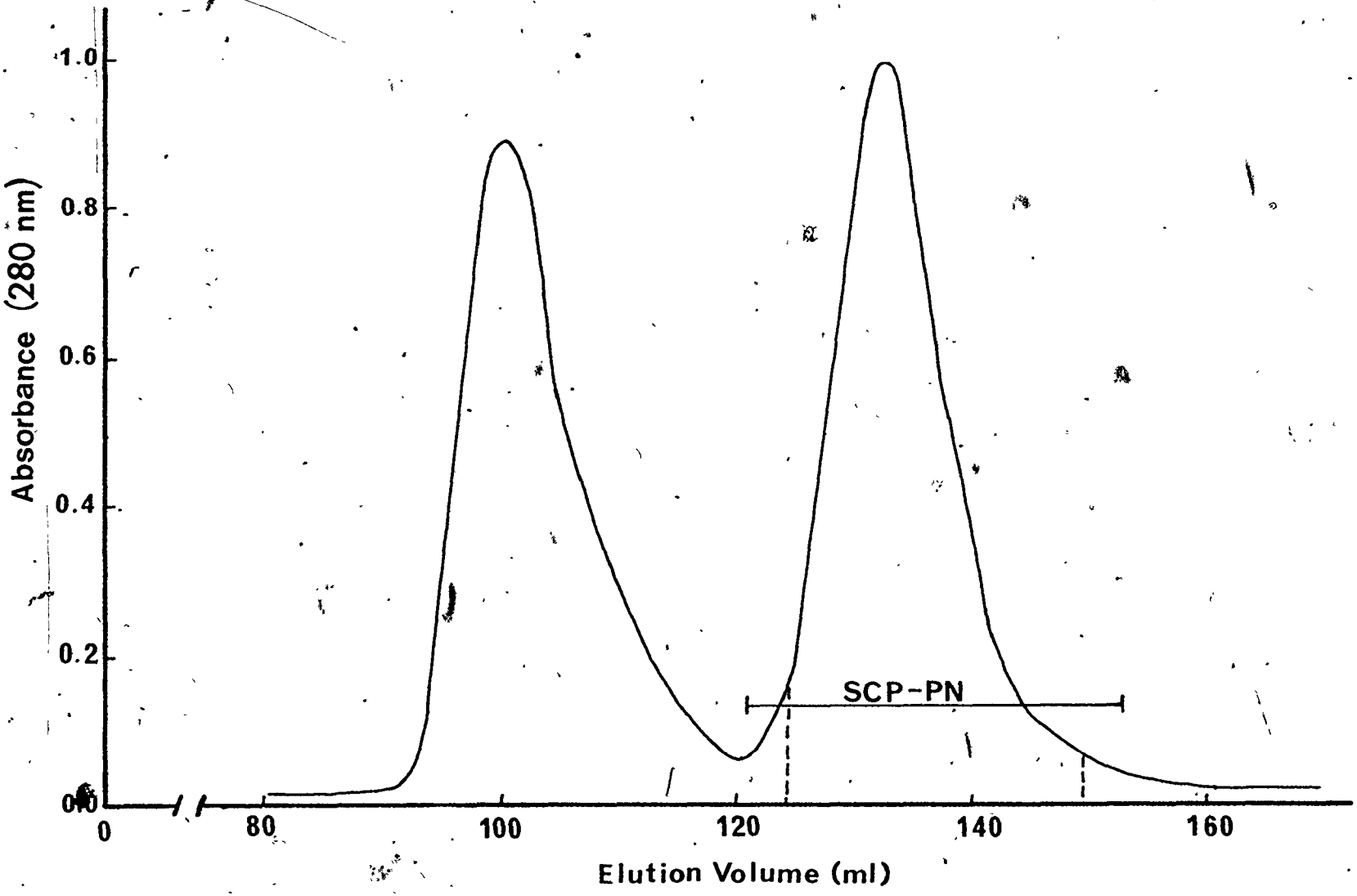

Absorbance (280 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
80
100
120
140
160
Elution Volume (ml)
SCP-PN

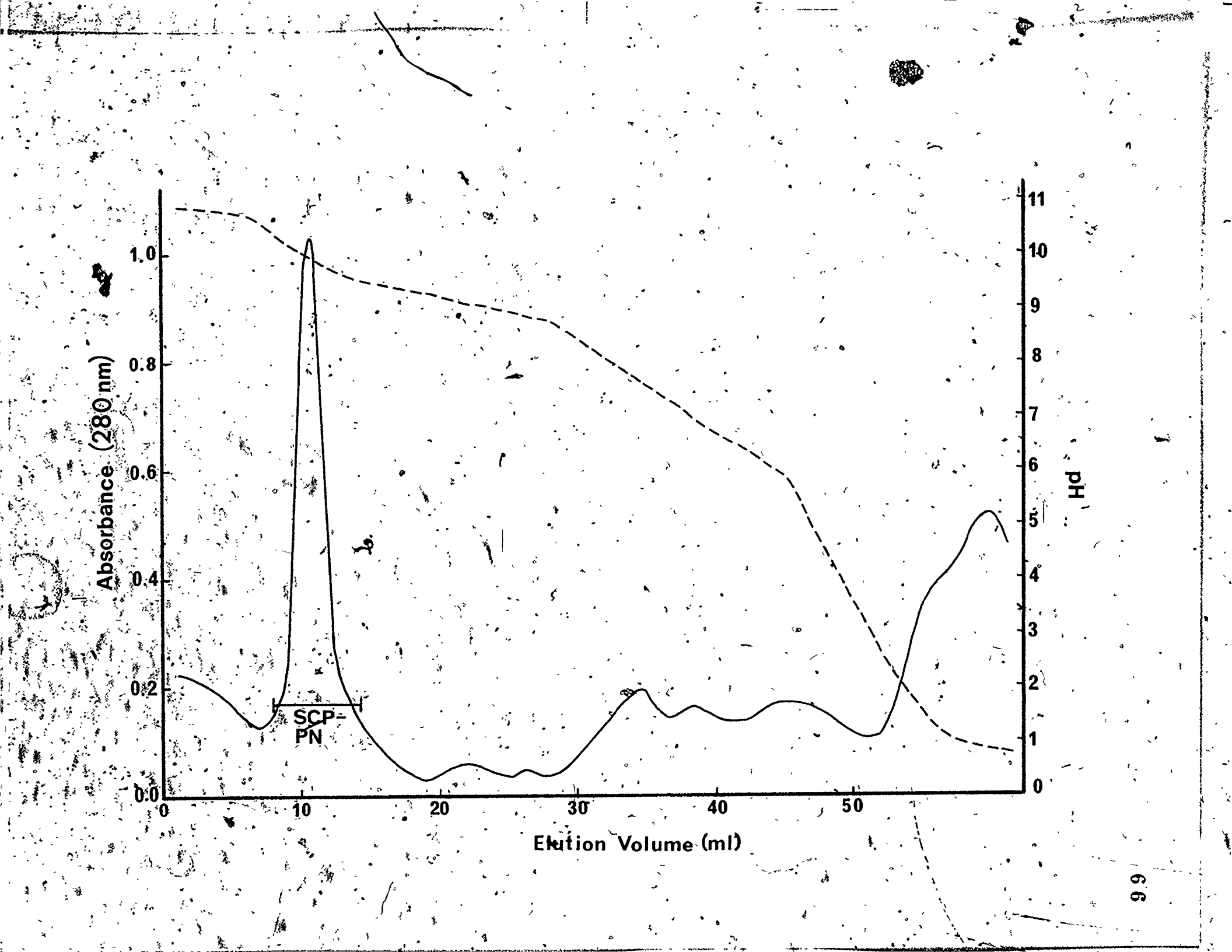
Figure 5 SDS-polyacrylamide gel electrophoretograms of human SCP following rechromatography on Sephadex G-50 superfine and of human SCP-PN following one gel filtration. Each electrophoretogram contained approximately 10 µg of protein.


SCP-PN
SCP

Figure 6. Isoelectric focusing of human SCP-PN. Approximately 8 mg of SCP-PN was analyzed in a sucrose density gradient containing a pH gradient from 9 to 11. Fractions 9 to 14 were found to contain SCP-PN.

———— indicates absorbance at 280 nm.

- - - - - - - indicates the pH of the fractions.


Absorbance (280 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Elution Volume (ml)
SCP-PN
pH
11
10
9
8
7
6
5
4
3
2
1
0

isoelectrophoretogram of human SCP-PN indicate that all major impurities were removed from the SCP-PN preparations.

3.1.2 Physical and Chemical Analyses

The molecular weights of human SCP and SCP-PN were estimated by SDS-polyacrylamide gel electrophoresis to be 13,700 daltons and 14,700 daltons, respectively. A purified sample of human SCP-PN was eluted from a calibrated gel filtration column of Sephadex G-50 superfine at a volume corresponding to a molecular size of 15,000 ± 20% daltons.

A pH gradient from 9 to 11 was established in a sucrose density gradient. The isoelectric point of human SCP-PN and bovine SCP-PN was estimated to be 9.9.

The amino acid compositions of purified human SCP and SCP-PN are presented in Table 1. The value for ammonia was not included and no corrections were made for hydrolytic losses. Although the amino acid compositions are similar, SCP appears to lack half-cystine, while SCP-PN contains 1% of half-cystine.

HCl-hydrolyzates of dansylated human and bovine SCP and SCP-PN were analyzed by thin layer chromatography. In each case, a dansylated amino acid derivative corresponding to the NH_2-terminal amino acid could not be detected. Plates containing up to 50 n moles of human SCP or SCP-PN did not contain dansylated amino acid derivatives other than those of ε-lysine and O-tyrosine. This result is consistant with a high degree of homogeneity of the SCP and SCP-PN preparations

TABLE 1: Amino acid compositions of human SCP and SCP-PN.

Amino Acid	composition (mol/100 mol amino acid)	
	SCP	SCP-PN
Lysine	13.9	13.7
Histidine	0.5	0.4
Arginine	4.1	5.3
Aspartic Acid	8.6	
Threonine	8.0	10.5
Serine	7.5	5.8
Glutamic Acid	16.0	11.9
Proline	1.4	1.4
Glycine	10.7	7.9
Alanine	5.2	4.1
Half cystine	0.0	1.0
Valine	6.4	7.5
Methionine	1.5	1.7
Isoleucine	4.5	5.4
Leucine	5.7	8.8
Tyrosine	2.1	1.2
Phenylalanine	3.9	3.6

and indicate that the two proteins had blocked NH_2-terminal amino acids.

3.1.3 Immunochemical Analyses

The immunodiffusion analyses were performed in 1.3% agar containing 0.05 M barbiturate buffer, pH 8.6. Under these conditions, the precipitin bands that formed opposite human SCP and SCP-PN were often fuzzy and required 48 h to develop, particularly when an anti-human SCP-PN serum was used. However, when either agarose slides or agar slides containing 0.1 M NaCl or aprotinin were used, the precipitin lines were distinct and developed within 24 h. The fuzzy precipitin lines encountered in the agar slides may possibly be caused by interactions between the agar and SCP. Agar contains highly acid sulfate groups which impart a negative charge to the agar in the barbiturate buffer at pH 8.6. Human SCP and SCP-PN are basic proteins. Thus, electrostatic interactions between SCP and the agar might interfere with the diffusion of SCP through the agar support.

Since agarose contains a minimal amount of sulfate, adsorption of basic substances onto the polysaccharide support is lessened considerably. This interpretation agrees with the study of Brishammar, Hjerten and Hofsten (1961) in which agarose gave rise to more distinct lines of precipitation in gel diffusion analyses of basic antigens than did agar. Aprotinin or NaCl in agar appears to improve the diffusion of human SCP and SCP-PN in agar. This may possibly

be accomplished through a mechanism by which aprotinin interacts with the acidic groups of agar thereby decreasing the number of acidic groups that would otherwise be available to interact with SCP or SCP-PN. There is, indeed, evidence that indicates that aprotinin has the property of binding to acidic sugars (Stoddart and Kiernan, 1973). By increasing the ionic strength in the gel, NaCl could decrease the electrostatic attraction between SCP and agar, thereby facilitating diffusion. Factors other than the basicity of the antigen may also determine the sharpness of a precipitin line because bovine SCP, which has the same net charge as human SCP, did not form fuzzy precipitin lines in agar.

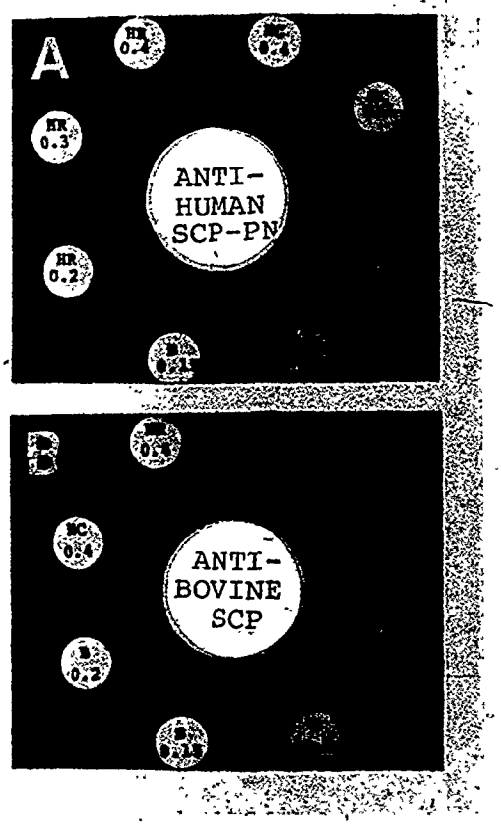
When human SCP and SCP-PN and bovine SCP were allowed to diffuse towards an anti-human SCP-PN serum, two distinct precipitin lines formed opposite each antigen, the outer line being heavier than the inner line. This is shown in Figure 7a. If the concentration of antigens was too high or if the reaction was allowed to proceed for more than 24 h, then the two precipitin bands widened and coalesced to form one broad band.

The major (outer) and minor (inner) precipitin lines formed by human SCP and SCP-PN fused with those of bovine SCP without spurring. Therefore, the three proteins may be considered to be immunochemically identical. However, the proteins cannot be considered to be equally reactive because the homologous human SCP-PN formed heavier precipitin bands than did human SCP and bovine SCP when the proteins were present at similar concentrations.

Figure 7. Immunodiffusion analyses of human SCP and SCP-PN and bovine SCP in 1.3% agarose containing 0.05 M barbiturate buffer, pH 8.6. The concentrations of the pure antigens in the peripheral wells are in mg/ml and are indicated on the photograph. The slides were photographed after 18 h of development. HC, human SCP; HR, human SCP-PN; B, bovine SCP.

A. The center well contained an anti-human SCP-PN serum.

B. The center well contained an anti-bovine SCP serum.


A
HR 0.2
HR 0.3
ANTI-HUMAN SCP-PN
HR 0.2
B
ANTI-BOVINE SCP

When human SCP and SCP-PN and bovine SCP were allowed to react with an anti-bovine SCP serum in immunodiffusion analyses, the major and minor precipitin bands that formed opposite human SCP and SCP-PN fused with the single band that formed opposite the homologous bovine SCP. This is shown in Figure 7b. Thus, it appears that the two immunochemically distinct forms of human SCP and SCP-PN share different antigenic determinants with bovine SCP.

In immunoelectrophoretic analyses in agarose, human SCP and SCP-PN also formed two separate precipitin lines. The lines were located in the region of the immunoelectrophoretograms where the precipitin arcs of γ-serum globulins are found (Figure 8b,c).

It has been demonstrated in earlier studies (Yo and MacPherson, 1972; MacPherson and Armstrong, 1976; Weir and MacPherson, 1978) that when analyzed by immunoelectrophoresis in 1.2% agar containing 0.05 M barbital buffer, pH 8.6, bovine, rat and human SCP had electrophoretic mobilities corresponding to that of a β-serum globulin. An immunoelectrophoretogram of human SCP-PN run in agar at pH 8.6 is presented in Figure 8a. The precipitin lines developed as two double arcs. Weir and MacPherson (1978) concluded that there were two antigenic forms of human SCP, each possessing two distinct configurations with different surface charges.

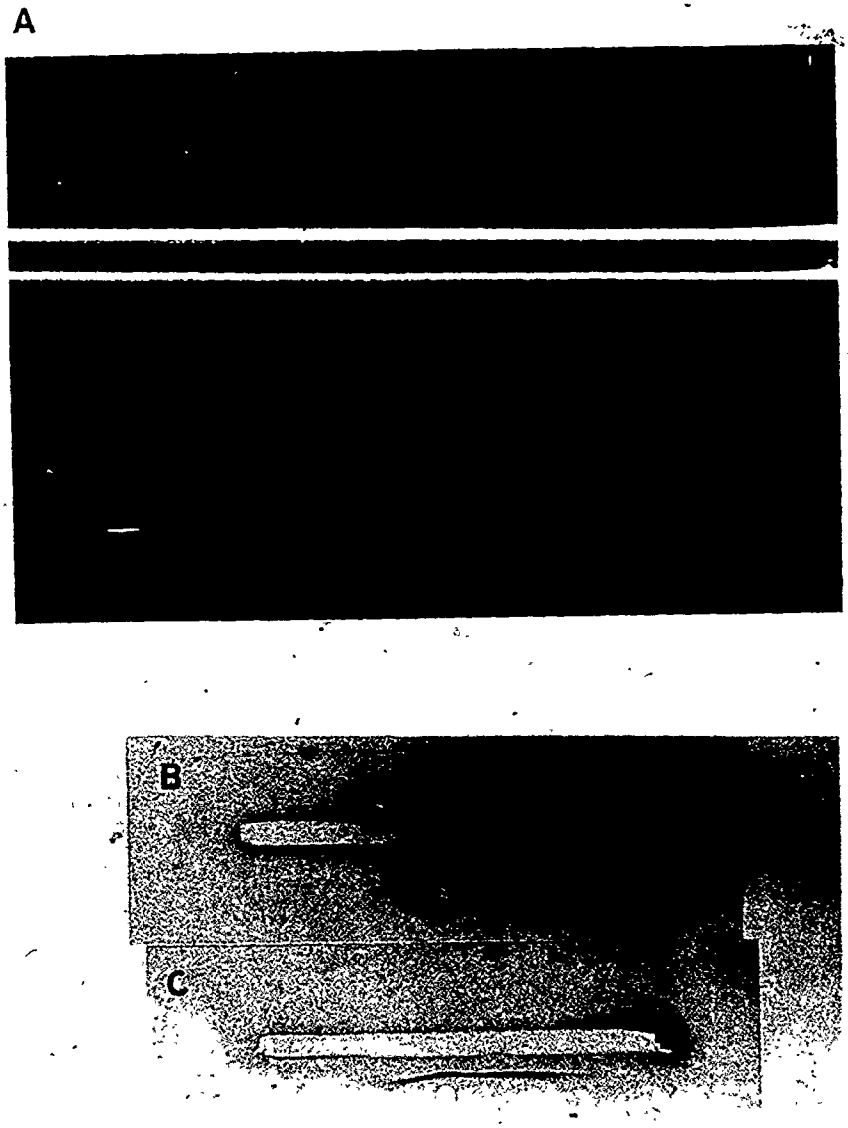
A more plausible explanation for the formation of the double arc in agar immunoelectrophoretograms involves electrostatic interactions between SCP and the agar that appear to

Figure 8. Immunoelectrophoretograms of human SCP in agar and of human SCP-PN and bovine SCP in agarose. Analyses were carried out in either 1.2% or in 1.3% agar containing 0.05 M barbiturate buffer, pH 8.6, at a constant current of 15 mA/slide for 45 min. The extract of human spinal cord (HSCE) contained 30 mg of protein/ml and the purified antigens were run at a concentration of 1 mg/ml.

A. HSCE and human SCP analyzed in agar and developed with an anti-human SCP serum.

B. Bovine SCP analyzed in agarose and developed with an anti-bovine SCP serum.

C. Human SCP-PN analyzed in agarose and developed with an anti-bovine SCP serum.


A
B
C

be strong enough to cause the migration of the protein to be retarded, thereby producing an elongated migration zone. Thus, immunoelectrophoresis of human SCP in agar gives one the impression that SCP has the mobility of a β-serum globulin and a γ-serum globulin. However, in agarose human SCP migrates in a shorter zone because agarose, unlike agar, lacks the large number of acidic sulfate groups which appear to interfere with the migration of SCP.

The relative amounts of human SCP in saline extracts of human nervous tissues as measured by quantitative single radial immunodiffusion were found to be 1:6:60 for brain, spinal cord and spinal nerves, respectively.

3.2 Purity and Yield of Myelin

Electron microscopic examination revealed that bovine peripheral myelin was composed of the characteristic lamellar fragments (Figure 9). Subcellular organelles such as mitochondria and nuclei were absent.

An average of 15 mg of myelin was obtained per gram of peripheral nerve (wet weight). Purified myelin contained 30% of protein.

3.3 Quantitative Analyses

When 0.3 M NaCl extracts of bovine spinal roots were analyzed by quantitative single radial immunodiffusion it was determined that an average of 1.31 mg of SCP-PN was extracted per gram of whole tissue. Delipidation of the tissue prior to NaCl extraction did not change the amount of SCP-PN solubilized. Therefore, it appears that all of the

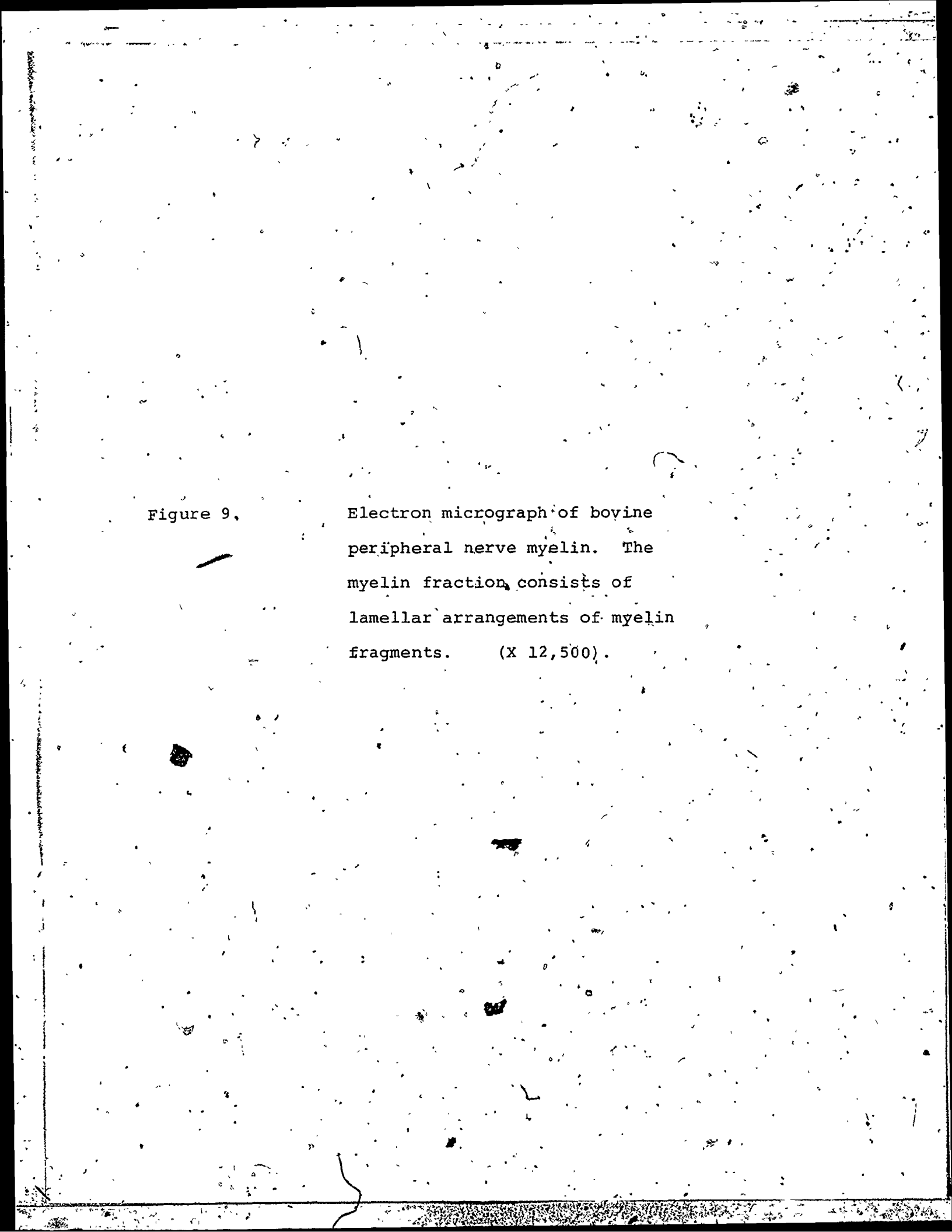


Figure 9. Electron micrograph of bovine peripheral nerve myelin. The myelin fraction consists of lamellar arrangements of myelin fragments. (X 12,500).

immunoreactive SCP-PN in spinal nerves was present in the soluble fraction of the NaCl extracts.

Bovine SCP-PN was detected in the soluble fraction of the initial 0.8 M sucrose homogenate of spinal nerves. The subsequent supernatants obtained in the course of the myelin purification did not contain any immunoreactive SCP-PN. The amount of SCP-PN in the 0.8 M sucrose supernatant was estimated to be 0.33 mg/g of whole tissue.

3.4 Myelin-Associated SCP-PN

Purified myelin, solubilized in 1% SDS, was analyzed by immunodiffusion and SDS-polyacrylamide gel electrophoresis. The electrophoretic pattern of myelin proteins is shown in Figure 10. It can be seen from gel 2 that myelin contains at least one protein which has the same electrophoretic mobility as SCP-PN. Immunodiffusion studies revealed that immunoreactive SCP-PN was present in purified myelin.

3.5 Myelin Extractions

Following the extraction of myelin with 0.3 M NaCl, the density of the stained bovine SCP-PN band in the myelin electrophoretograms decreased considerably. This is evident in Figure 10, gel 3, as well as from Figures 11a and 11b, which contain densitometric scans of gels of myelin and NaCl-extracted myelin, respectively. SCP-PN was found in NaCl extracts of myelin by immunodiffusion analyses, but was not detected in SDS-solubilized NaCl-extracted myelin.

Figure 10. SDS-polyacrylamide gel electrophoretograms of myelin proteins, bovine SCP-PN, lysozyme and pepsin. Electrophoresis was performed at pH 7.0 in 10% polyacrylamide gels. Each gel contained 20 to 30 µg of myelin proteins or 5 to 10 µg of standard proteins. The gels are: 1, bovine SCP-PN; 2, myelin; 3, NaCl-extracted myelin; 4, 0.3 M NaCl extract of myelin; 5, NaCl-extracted myelin homogenized in the presence of SCP-PN; 6, lysozyme; 7, NaCl-extracted myelin homogenized in the presence of lysozyme; 8, pepsin; 9, NaCl-extracted myelin homogenized in the presence of pepsin.


1 2 3 4 5 6 7 8 9

After electrophoresis some of the slab-gels containing proteins of myelin and NaCl-extracted myelin were stained with the periodic acid-Schiff's reagent. Two bands characteristic of glycoproteins appeared; one at the position of the P_0 protein and one at the position of the PAS-II protein. The PAS-II glycoprotein has the same electrophoretic mobility as SCP-PN in SDS-polyacrylamide gels (Kitamura, Suzuki and Uyemura (1976). However, these authors have demonstrated that SCP-PN and PAS-II are distinct proteins that do not cross-react immunologically and that have different amino acid compositions. Hence, the band containing SCP-PN and PAS-II in SDS-polyacrylamide gels will hereinafter be referred to as the SCP-PN + PAS-II band.

The protein compositions of myelin, extracted myelin and the myelin extracts were calculated from the densitometric profiles (Figure 11) of the SDS-polyacrylamide gels and are presented in Table 2. It is evident that after NaCl or HCl extraction of myelin the percentage of total myelin protein in the SCP-PN + PAS-II peak decreased from 25% to 8%. Subsequent extractions of myelin with NaCl or HCl did not lower the amount of protein in the SCP-PN + PAS-II peak.

After extraction, percentages of total myelin proteins, of P_1, P_0, protein X, protein Y and the high molecular weight proteins, either increased or remained relatively constant. It was calculated from scan C in Figure 11 that 81% of the protein in the first NaCl extract was extracted from the SCP-PN + PAS-II peak.

Figure 11. Densitometric profiles of SDS-polyacrylamide gel electrophoretograms. Electrophoresis was carried out in 10% polyacrylamide gels at pH 7.0. Each gel contained 6 to 8 µg of protein.

A. Unextracted myelin

B. NaCl-extracted myelin

C. The first NaCl extract of myelin

The proteins P_0, X, Y and P_1 are labeled according to the nomenclature of Greenfield *et al.*, (1973). HMW, high molecular weight proteins.

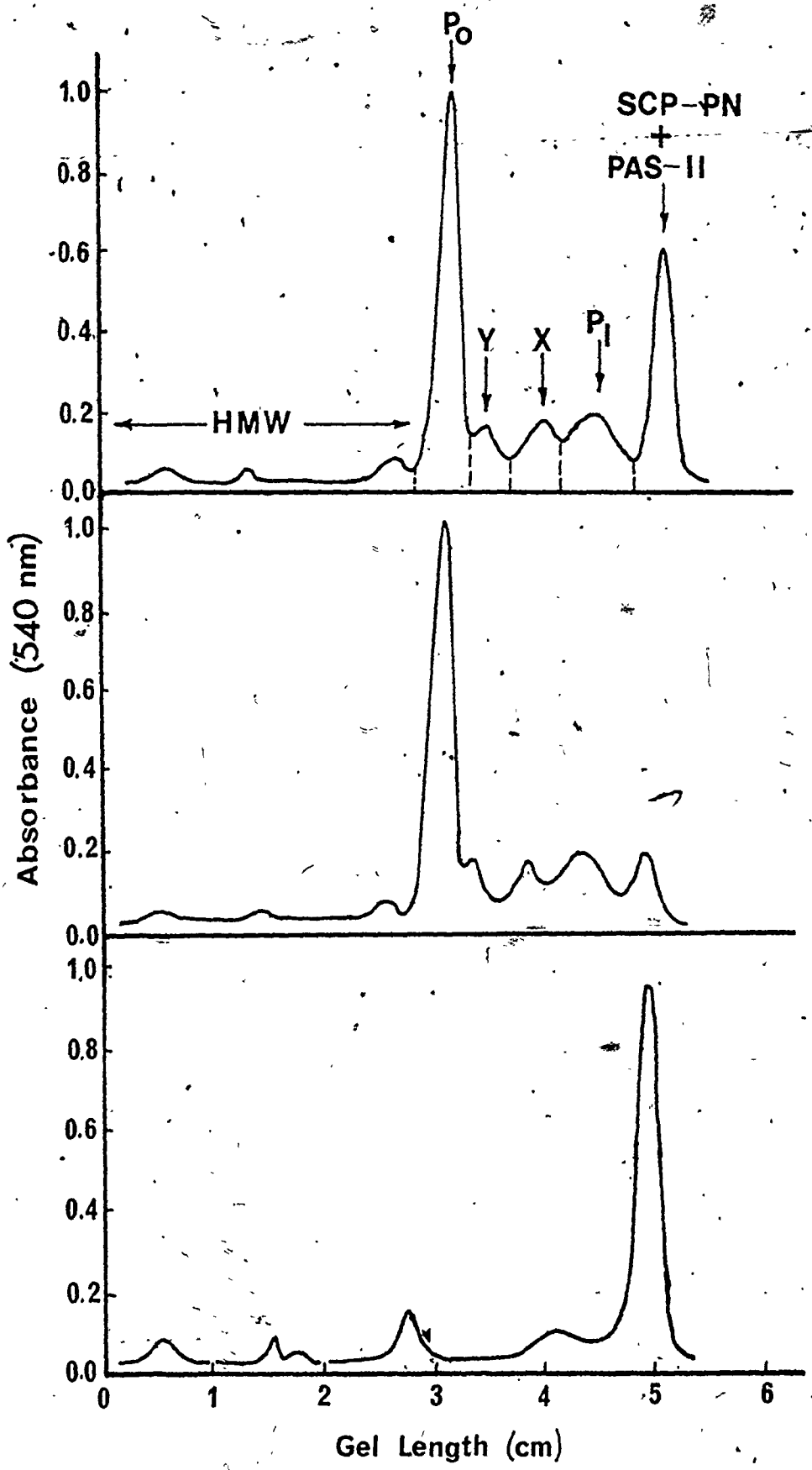

P0
SCP-PN
+
PAS-II
Y
X
PI
HMW
Absorbance (540 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Gel Length (cm)

TABLE 2: Protein composition of bovine peripheral nerve myelin before and after extraction with 0.3 M NaCl and 0.05 M HCl.

(n.d., not detected. The values are averages of duplicate determinations on two different samples and are expressed as percent total protein).

Treatment of myelin	% Total Protein				
	SCP-PN + PAS-II	P_1	X + Y	Protein P_0	High molecular weight
Unextracted	25	14	10	43	8
Extracted 1 X with NaCl	8	14	13	59	6
Extracted 2 X with NaCl	8	13	13	55	11
Extracted 3 X with NaCl	10	15	11	56	8
First NaCl extract	81	6[a]	n.d.	6[b]	7
Extracted 1 X with HCl	10	13	12	58	7
Extracted 2 X with HCl	8	13	12	57	10
Extracted 3 X with HCl	8	13	11	53	15
First HCl extract	73	8[a]	12	7	n.d.

[a] Unidentified NaCl soluble protein that co-migrates with P_1 in 10% polyacrylamide gels but migrates just in front of P_1 in 20% polyacrylamide slab-gels (Fig. 12).

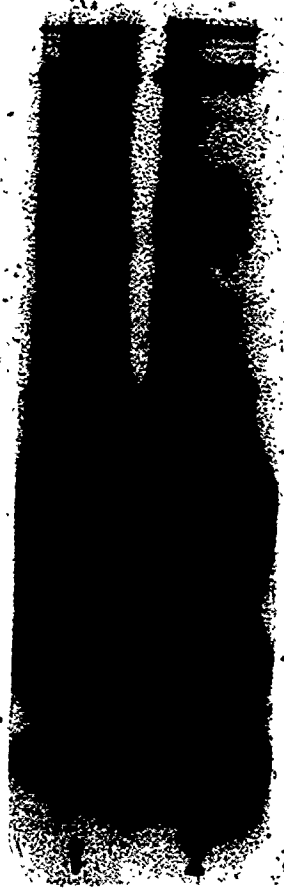
[b] Unidentified NaCl soluble protein that co-migrates with protein P_0 in 10% polyacrylamide gels (Fig. 10, gel 4).

The P_0 protein is the major protein of the peripheral myelin membrane and is considered to be an integral membrane protein (Everly, Brady and Quarles, 1973; Wood and Dawson, 1974; Brostoff *et al.*, 1975b). Since P_0 was not detected in the NaCl extracts of myelin, it is apparent that all of the myelin membranes were removed from the extracts by centrifugation at 170,000 x g.

Other proteins present in the extracts included two unidentified proteins, each of which comprised approximately 6% of the total myelin protein. It is evident from Figure 10, gel 4, and Figure 11, scan B, that the larger unidentified protein had an electrophoretic mobility slightly slower than that of P_0 protein, while the electrophoretic mobility of the smaller unidentified protein was similar to that of P_1. Thus, the large and small unidentified proteins were measured as P_0 and P_1, respectively, in Table 2. In 20% polyacrylamide slab-gels, the P_1 band was resolved into two bands. The electrophoretic patterns of myelin and NaCl-extracted myelin are presented in Figure 12. It is evident that some of the protein in the leading band was extracted from myelin by 0.3 M NaCl.

One would expect that the solubilization of protein from the SCP-PN + PAS II band would result in an increase in the percentage of proteins remaining in the extracted myelin. However, the P_1 content of extracted myelin was the same as that of unextracted myelin because some of the unidentified protein in the P_1 band was solubilized in the 0.3 M NaCl extract.

Figure 12. SDS-polyacrylamide slab-gel electrophoretograms of myelin and NaCl-extracted myelin. Analyses were carried out in a discontinuous 20% polyacrylamide slab-gel system. Lanes 1 and 2 contained 75 µg of protein from NaCl-extracted and unextracted myelin, respectively.


P0
P1
SCP-PN+
PAS-11

When peripheral nerves were homogenized in 0.8 M sucrose containing 0.15 M NaCl, the protein in the SCP-PN + PAS-II band accounted for only 17% of the total myelin protein. This indicates that the presence of 0.15 M NaCl in the homogenizing medium reduced the amount of SCP-PN bound to myelin by approximately 50%.

3.6 Bovine SCP-PN in NaCl-Extracted Myelin

Bovine SCP-PN was not detected when NaCl-extracted myelin was examined by immunodiffusion analysis. However, this result did not preclude the possibility that the amount of SCP-PN in NaCl-extracted myelin was insufficient for identification by immunodiffusion analysis. Thus, the amount of SCP-PN in the total protein extracted from the SCP-PN + PAS-II band of SDS-polyacrylamide gels was investigated. Following acetone precipitation of the extracted protein, the SCP-PN in the saline-soluble fraction of the precipitate was measured by quantitative single radial immunodiffusion. It was determined that 16 µg of SCP-PN was present in the 211 µg of extracted protein. Thus, SCP-PN comprised approximately 8% of the protein in the SCP-PN + PAS-II band. If SCP-PN + PAS-II comprise 8% of the total myelin protein in NaCl-extracted myelin, then it is evident that SCP-PN accounts for less than 1% of the total protein in NaCl-extracted myelin.

3.7 Absorption of Proteins on Myelin

The protein compositions of myelin and NaCl-extracted myelin following the addition of varibus amounts of exogenous

proteins to myelin suspensions are given in Table 3. It is evident from Figure 10, gel 5, and from Table 3, that when 0.25 mg of bovine SCP-PN was added to a suspension of NaCl-extracted myelin in 0.8 M sucrose, the protein in the SCP-PN + PAS-II band of the electrophoretogram of the water-washed myelin pellet accounted for 36% of the total myelin protein. The addition of 0.25 mg of SCP-PN to unextracted myelin resulted in an increase of protein in the SCP-PN + PAS-II peak from 25% to 44% of the total myelin protein. Thus, it is apparent that NaCl-extracted myelin is capable of rebinding SCP-PN and that myelin can bind more SCP-PN than what is normally found in purified myelin.

However, the data in Table 3 indicate that the amount of protein that was bound to myelin was greater than the amount that was added. For example, the amount of protein in the 5 mg of myelin was approximately 1.5 mg. Approximately 25% of this amount, or 0.38 mg was SCP-PN + PAS-II. If one assumes that all of the 0.25 mg of exogenous SCP-PN was bound to the myelin, then 0.63 mg of 1.75 mg of myelin protein would be SCP-PN + PAS-II. This value is 36% of the total protein. An explanation for this discrepancy involves the method used to estimate the percentage of total protein. The amount of dye that a protein binds and/or the amount of light that a stained band transmits are not linearly correlated with the amount of protein. For example, it was determined that as the amount of purified SCP-PN in SDS-polyacrylamide gels increased, the weight of the densitometric scans of the peaks increased exponentially. Thus, some of the

TABLE 3: Protein composition of bovine peripheral nerve myelin and NaCl-extracted myelin homogenized in the presence of SCP-PN, lysozyme or pepsin. (The values are averages of duplicate determinations on two different samples. HMW, High molecular weight; n.a., not applicable; n.d., not detected).

Treatment of myelin	Protein added to homogenate	Amount (mg)	Percentage total protein						
			Lysozyme	SCP-PN + PAS-II	P_1	X+Y	Protein P_0	HMW	Pepsin
Unextracted	None	None	n.a.	25	13	12	45	5	n.a.
	SCP-PN	0.05	n.a.	30	10	12	46	2	n.a.
	SCP-PN	0.25	n.a.	44	9	9	36	2	n.a.
	Lysozyme	0.25	20	22	9	9	36	4	n.a.
	Pepsin	0.25	n.a.	26	11	16	42	5	n.d.
NaCl-Extracted	—	—	n.a.	6	13	13	64	4	n.a.
	SCP-PN	0.05	n.a.	14	11	13	58	4	n.a.
	SCP-PN	0.25	n.a.	36	8	14	39	3	n.a.
	Lysozyme	0.25	25	8	12	13	38	4	n.a.
	Pepsin	0.25	n.a.	8	17	21	50	4	n.d.

values in Table 3 indicate that more protein had bound to myelin than the amount that had been added.

It will be seen from Table 3 and Figure 10, gel 6, that lysozyme, a basic protein which does not occur in myelin, bound to myelin and NaCl-extracted myelin under the same conditions as described above. Pepsin, an acidic protein isolated from gastric secretions, did not bind to either myelin or NaCl-extracted myelin.

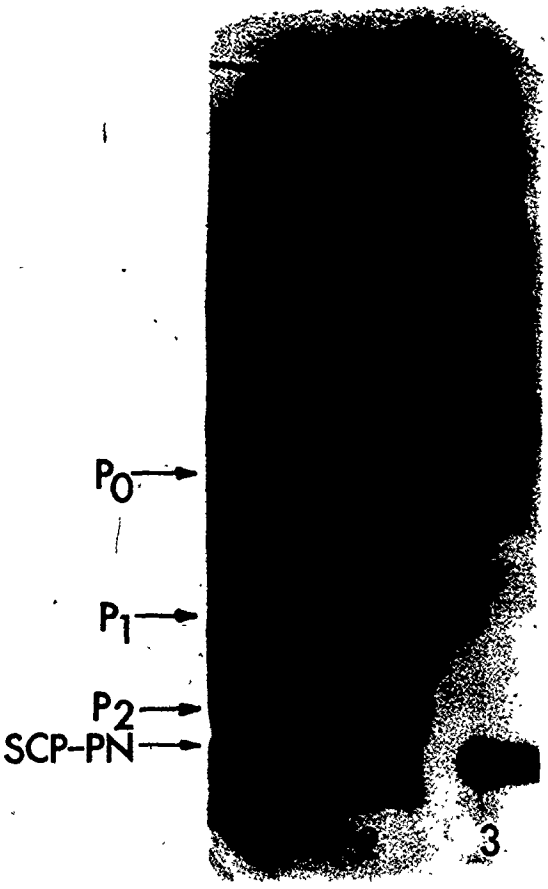
3.8 Fixative-Extraction of Tissues

Bovine spinal nerves were homogenized with either acetone or 95% ethanol/ether. The insoluble fraction was extracted with 0.15 M NaCl and analyzed to ascertain whether bovine SCP-PN was rendered insoluble by either of the organic solvents. Immunodiffusion studies on the NaCl extracts revealed that neither acetone nor 95% ethanol/ether could prevent the solubilization of SCP-PN by 0.15 M NaCl.

3.9 Rat SCP-PN and Peripheral Myelin

Examination of slab-gel electrophoretograms (Figure 13) containing purified rat SCP-PN and rat peripheral myelin proteins clearly revealed that there was no band in the electrophoretogram of myelin proteins at the position to which rat SCP-PN migrated. Thus, as one might expect, rat SCP-PN was not detected by immunodiffusion analyses in rat peripheral myelin solubilized in SDS. These findings indicate that rat SCP-PN is not a component of peripheral myelin.

Figure 13. SDS-polyacrylamide slab-gel electrophoretograms of rat SCP-PN and rat peripheral nerve myelin proteins. Analyses were carried out in a discontinuous, 20% polyacrylamide slab-gel system. The lanes contain: 1, a mixture of delipidated rat peripheral nerve myelin (65 µg) and SCP-PN (12 µg); 2, delipidated rat peripheral myelin (65 µg); 3, SCP-PN (12 µg). The bands containing P_0, P_1 and P_2 were determined by comparing the electrophoretogram to that of Singh, Silberlicht and Singh (1978).


P0
P1
P2
SCP-PN
3

MacPherson (personal communication) has determined that the molecular weight of rat SCP-PN is approximately 11,300 daltons. However, the P_2 protein of rat peripheral myelin has a molecular weight of 13,500 daltons according to Singh, Silberlicht and Singh (1978). Therefore, rat SCP-PN is not equivalent to the P_2 protein of rat peripheral myelin with respect to molecular size or immunochemical reactivity.

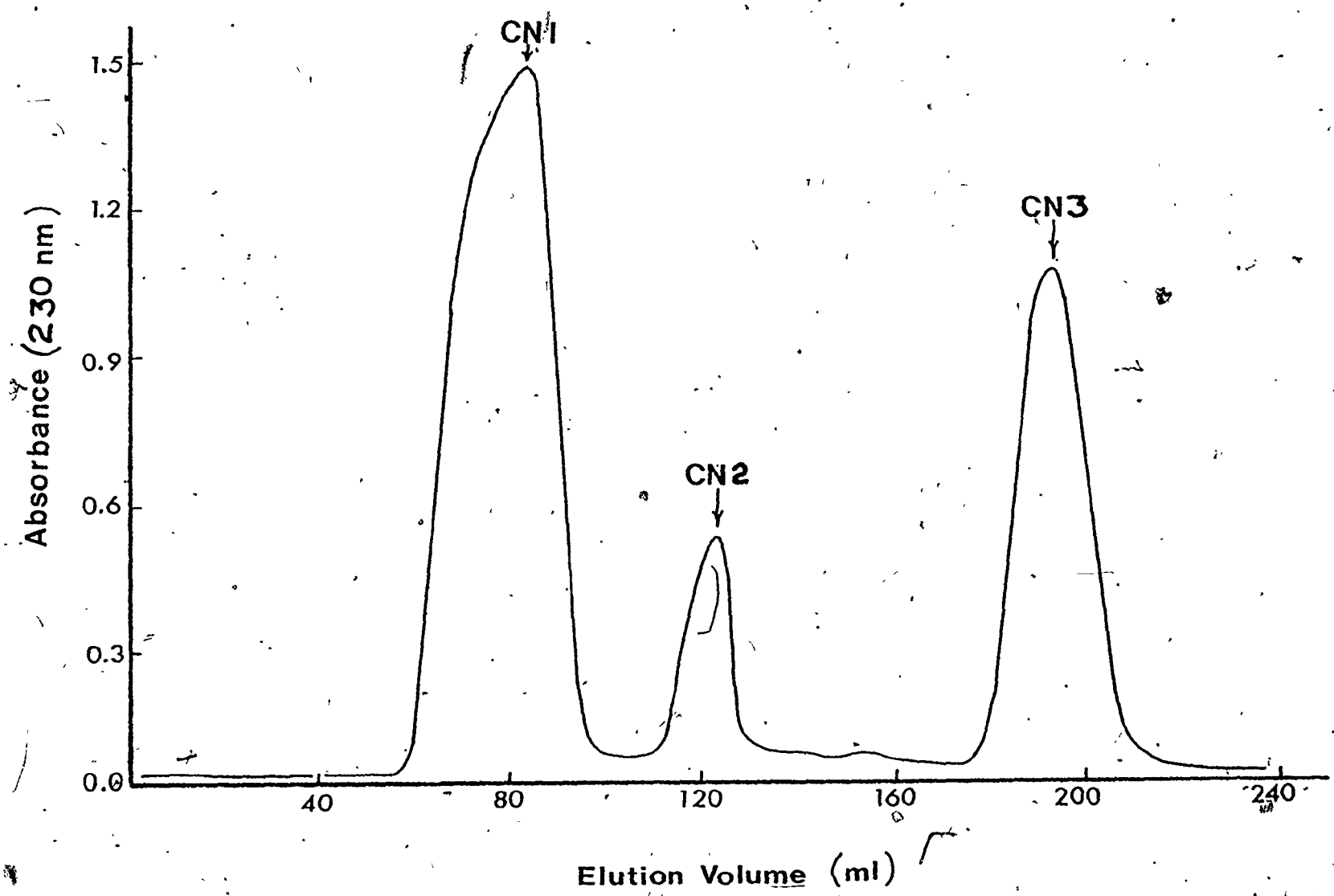
3.10 CNBr-Derived Peptides

Attempts were made to purify each of the CNBr-derived peptides of bovine SCP. The elution pattern of the peptides from Bio-Gel P6 is illustrated in Figure 14. The three major peaks were designated CN1, CN2 and CN3 with respect to the elution order from the column. The purity of each peptide peak was analyzed by three different electrophoretic methods.

A photograph of the SDS-polyacrylamide gel electrophoretograms of bovine SCP, CN2 and CN3 is shown in Figure 15. SCP, CN2 and CN3 each formed one band in the SDS-gels. CN1 contained uncleaved SCP and partially cleaved SCP. In polyacrylamide gel electrophoresis at pH 2.7 all of the samples of CN1 formed one band indicating that all of the components had the same mobility as SCP. CN2 and CN3 each formed one band. CN2 had the fastest mobility of the three peptides.

During high voltage paper electrophoresis CN1 did not migrate from the origin. Nor did CN1 migrate in the second

Figure 14. Elution pattern of CNBr-derived peptides of bovine SCP on Bio-Gel P6. Approximately 30 mg of lyophilized material was applied in 1.2 ml to a 1.8 x 90 cm chromatography column. Fractions containing 2 ml were collected on elution with 0.1 N acetic acid at a flow rate of 15 ml/h.


CN1
CN2
CN3
Absorbance (230 nm)
1.5
1.2
0.9
0.6
0.3
0.0
40
80
120
160
200
240
Elution Volume (ml)

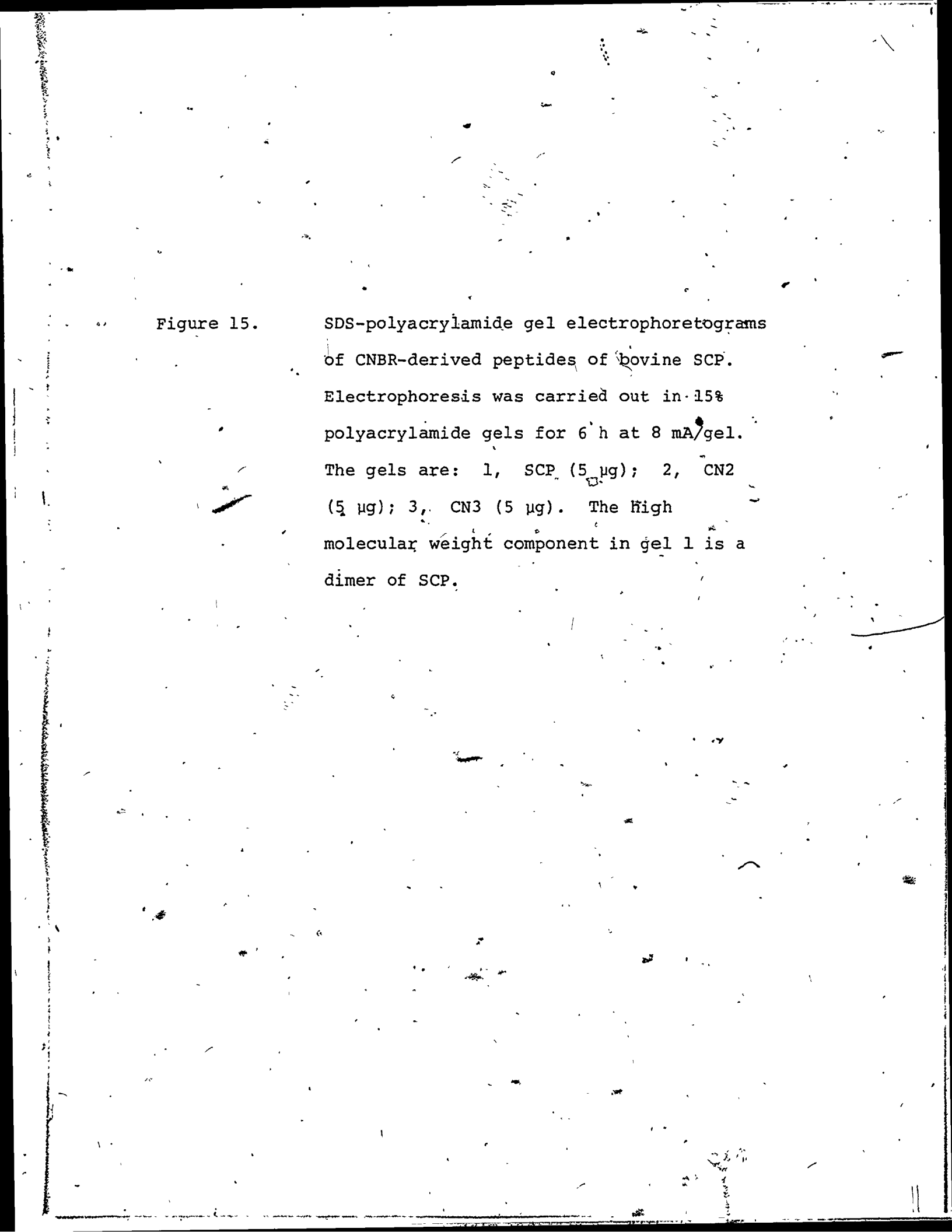


Figure 15. SDS-polyacrylamide gel electrophoretograms of CNBR-derived peptides of bovine SCP. Electrophoresis was carried out in 15% polyacrylamide gels for 6 h at 8 mA/gel. The gels are: 1, SCP (5 µg); 2, CN2 (5 µg); 3, CN3 (5 µg). The high molecular weight component in gel 1 is a dimer of SCP.

dimension during ascending chromatography. It is apparent that under the conditions of electrophoresis and chromatography the components of CN1 were insoluble. CN2 and CN3 each had one component that migrated towards the anode. However, when CN2 was treated with 2-mercaptoethanol prior to electrophoresis, two distinct peptides were discerned.

The amino acid compositions of CN2 and CN3 are given in Table 4. Homoserine was not detected because several times the usual amount of sample is required to enable homoserine content to be accurately determined. The two peptides differ notably in that CN2 contains five valine residues and two half-cystine residues. CN3 does not contain half-cystine and has 1 valine residue.

The amino acid sequence of protein P_2 (SCP-PN) proposed by Kitamura *et al.* (1980) is given in Figure 16. It can be predicted from the position of the methionine residues that the action of CNBr on SCP-PN should produce four peptides: two small peptides at the COOH-terminal (CB3, CB4), one peptide at the NH_2-terminal (CB1) and a larger centrally-located peptide (CB2). The amino acid compositions of CB1, CB3 and CB4 are compared to the amino acid compositions of CN2 and CN3 in Table 5. The similarities suggest that CN3 is derived from the NH_2-terminal end of SCP (CB1) while CN3 appears to be equivalent to the two small peptides at the COOH-terminal end of SCP-PN (CB3 and CB4). Thus, there are three lines of evidence which strongly suggest that CN2 is composed of two peptides joined by a disulfide bond. First, two peptides

TABLE 4: Amino acid compositions of CNBr-derived peptides of bovine SCP.

(Results are the averages obtained from two analyses).

Amino acid	composition (mol/100 mol amino acid)	
	CN2	CN3
Lysine	22.5	12.5
Histidine		
Arginine	6.7	
Aspartic Acid	5.3	15.4
Threonine	4.6	5.6
Serine		12.3
Glutamic Acid	12.8	13.6
Proline		
Glycine		8.1
Alanine		
Half cystine	10.5	
Valine	28.7	7.7
Isoleucine	4.5	
Leucine		9.6
Tyrosine	4.4	5.4
Phenylalanine		9.8

Figure 16. The complete amino acid sequence of bovine P_2 (SCP-PN). The sequence is from the report of Kitamura *et al.* (1980). The sequence of SCP-PN determined by MacPherson and Bradshaw (personal communication) is identical to that present here..

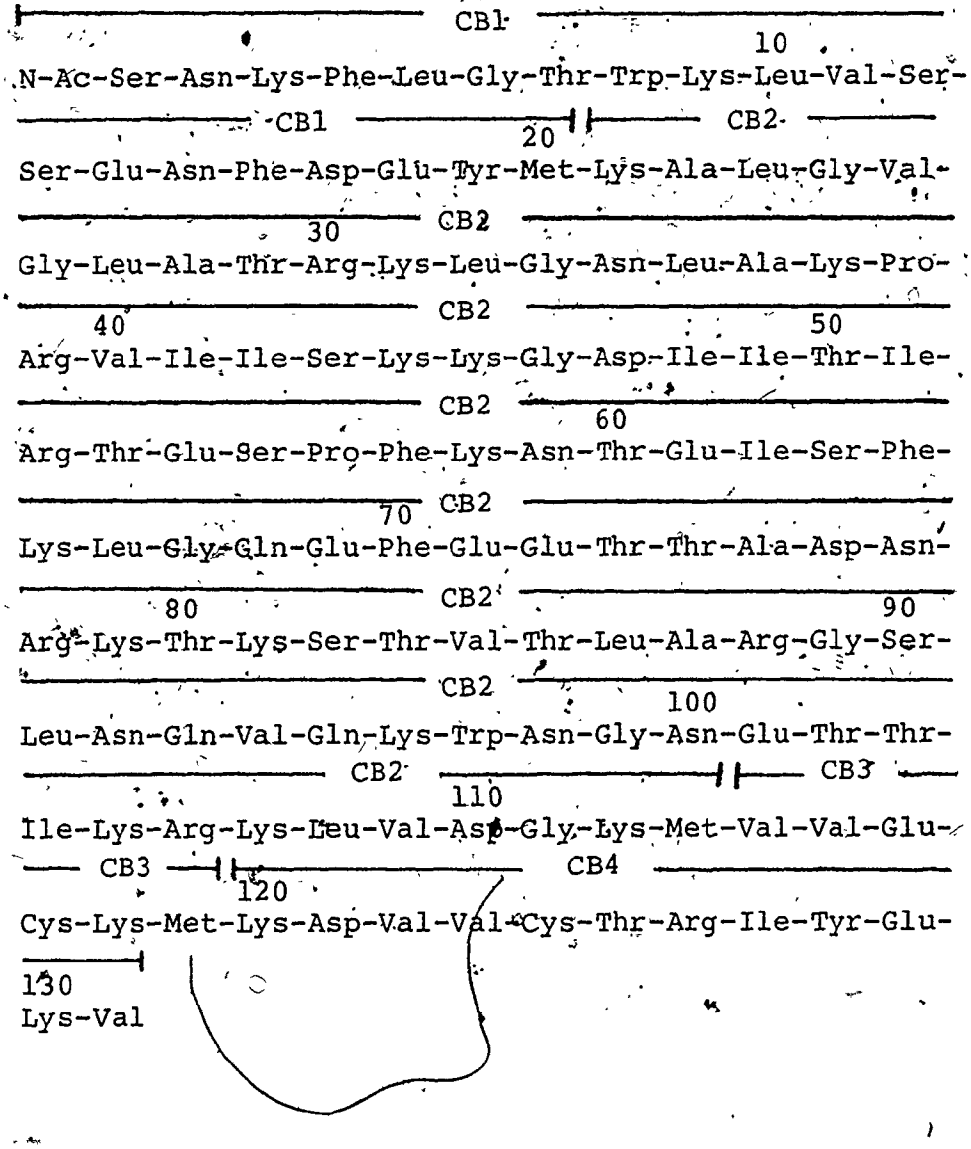

CB1
10
N-Ac-Ser-Asn-Lys-Phe-Leu-Gly-Thr-Trp-Lys-Leu-Val-Ser-
CB1
20
CB2
Ser-Glu-Asn-Phe-Asp-Glu-Tyr-Met-Lys-Ala-Leu-Gly-Val-
30
CB2
Gly-Leu-Ala-Thr-Arg-Lys-Leu-Gly-Asn-Leu-Ala-Lys-Pro-
40
CB2
50
Arg-Val-Ile-Ile-Ser-Lys-Lys-Gly-Asp-Ile-Ile-Thr-Ile-
CB2
60
Arg-Thr-Glu-Ser-Pro-Phe-Lys-Asn-Thr-Glu-Ile-Ser-Phe-
70
CB2
Lys-Leu-Gly-Gln-Glu-Phe-Glu-Glu-Thr-Thr-Ala-Asp-Asn-
80
CB2
90
Arg-Lys-Thr-Lys-Ser-Thr-Val-Thr-Leu-Ala-Arg-Gly-Ser-
CB2
100
Leu-Asn-Gln-Val-Gln-Lys-Trp-Asn-Gly-Asn-Glu-Thr-Thr-
CB2
110
CB3
Ile-Lys-Arg-Lys-Leu-Val-Asp-Gly-Lys-Met-Val-Val-Glu-
CB3
120
CB4
Cys-Lys-Met-Lys-Asp-Val-Val-Cys-Thr-Arg-Ile-Tyr-Glu-
130
Lys-Val

TABLE 5: Comparison of the amino acid compositions of CNBr-derived peptides of bovine SCP and bovine P_2 (SCP-PN)[a].

Amino acid[b]	Composition (minimum number of residues)			
	SCP		P_2	
	CN2	CN3	CB(3 + 4)	CB1
Lysine	3	2	3	2
Histidine				
Arginine	1		1	
Aspartic Acid	1	3	1	3
Threonine	1	1	1	1
Serine		2		3
Glutamic Acid	2	2	2	2
Proline				
Glycine		1		1
Alanine				
Half Cystine	2		2	
Valine	5	1	5	1
Isoleucine	1		1	
Leucine		2		2
Tyrosine	1	1	1	1
Phenylalanine		2		2

[a]Data of Kitamura *et al.* (1980)

[b]The value for homoserine was not estimated.

were detected in the paper electrophoretograms of reduced CN2. Secondly, CN2 contains two half-cystine residues. Thirdly, the amino acid composition of CN2 is very similar to that of CB(3 and 4) of SCP-PN.[1] This sequence contains two half-cystine residues and a methionine residue.

The ability of each peptide to form immunoprecipitates with an anti-bovine SCP-PN serum was tested by immunodiffusion analyses. Peptide concentrations ranging from 0.2 to 3.0 mg/ml were used. However, none of the peptides formed precipitin lines.

CHAPTER 4: DISCUSSION

4.1 Human SCP and SCP-PN

4.1.1 Purification and Characterization

During the early studies on SCP, Yo and MacPherson (1972) had identified a component in 0.1 M NaCl extracts of human spinal cord that formed two precipitin lines when allowed to react with an anti-bovine SCP serum in immunodiffusion tests. Thus, 0.15 M NaCl was used to effectively extract human SCP and SCP-PN from the tissue.

The purification procedure for human SCP and SCP-PN study is very similar to the method used to purify bovine SCP (MacPherson and Armstrong, 1976). Moreover, the chromatographic properties of human SCP or SCP-PN on CM-52 cellulose are similar to those of bovine SCP or SCP-PN. For example, an increase in the concentration from 0.1 M to 0.2 M of NaCl is required for the complete removal of the human or bovine SCP from the ion exchanger.

When basic proteins bind to cellulosic ion exchangers, strong bonds are formed. This can result in poor resolution and unsatisfactory chromatographic results (Khym, 1974). For example, the effect of linear and step gradients of NaCl on the chromatographic behavior of polylysine peptides has been examined by Smith and Stahmann (1969). Interestingly, it was found that an 0.1 M increase in the salt

concentration of the eluting buffer was required from the time that the migration of the peptides was first detected until they moved freely down the column. The need to use two buffers to completely elute human SCP may reflect strong bonding between SCP and the ion exchanger. However, this chromatographic property of SCP may be indicative of chemical differences in the SCP eluted in E_1 and E_2. For example, the immunodiffusion analyses indicate that human SCP and SCP-PN each contains two distinct populations of molecules. Hence further work is needed to clarify this problem.

Contaminating proteins were not detected in preparations of human SCP and SCP-PN by SDS-polyacrylamide gel electrophoresis or in the NH_2-terminal amino acid analyses. These results indicate a high degree of homogeneity in the preparations of SCP and SCP-PN.

Although human SCP and SCP-PN have similar chemical and immunochemical properties, they are not identical. Human SCP-PN has a slower mobility in SDS-polyacrylamide gels and contains less glutamic acid.

The acidic amino acid content of SCP and SCP-PN is approximately 24% while the basic amino acid content is approximately 19%. However, the isoelectric point of SCP-PN, 9.9, indicates that SCP-PN is a basic protein. The similarity between the elution properties of SCP and SCP-PN from CM-52 cellulose indicates that the isoelectric point of SCP is similar to that of SCP-PN. Thus, it appears that a number of the acidic residues of SCP and SCP-PN are amidated.

The amidation of these residues would decrease the contribution of the acidic residues to the net charge of the proteins thereby producing a basic protein.

The amino acid composition of human SCP indicates that there is no half-cystine in the protein. However, there is reason to suggest that this result should not be regarded as conclusive. Due to a shortage of material, only two preparations could be analyzed. Thus, a few comments regarding the possible presence of half-cystine in SCP are in order. It is evident that bovine SCP contains two half-cystine residues because bovine SCP can form polymers in SDS-polyacrylamide gels in the absence of 2-mercaptoethanol. Sequence studies (Kitamura *et al.*, 1980; Weise *et al.*, 1980b) have established that bovine SCP-PN (P_2) also contains two half-cystine residues. Yet the amino acid composition of bovine SCP-PN (P_2) also contains two half-cystine residues. Yet, the amino acid composition of bovine SCP published by MacPherson and Armstrong (1976) indicated that SCP contained only 0.5 moles of half-cystine per 100 moles of amino acid. Moreover, in earlier studies of the amino acid composition of bovine P_2, no half-cystine was detected (Brostoff *et al.*, 1974). Furthermore, it was observed that human SCP-PN occasionally formed polymers during gel filtration chromatography. Therefore, the possibility that human SCP contains half-cystine can not be excluded until further studies are conducted.

It has been established that bovine SCP and SCP-PN (P_2) do not contain histidine (Brostoff *et al*; MacPherson, Armstrong and Tan, 1976; Kitamura *et al.*, 1980; MacPherson and Bradshaw, personal communication). Thus, the histidine content of SCP-PN preparations has been used as an indicator of contaminating proteins or peptides. It is evident from the amino acid analyses that preparations of human SCP and SCP-PN contain histidine. Further work will be required to establish whether histidine is present as a contaminant or is an amino acid residue of human SCP and SCP-PN. The results of Uyemura, Suzuki and Kitamura (1978) indicate that human P_2 (SCP-PN) contains 1.5 moles of histidine per 100 moles of amino acid. Therefore, at the present time it would be incorrect to attribute the presence of histidine in human SCP and SCP-PN to contaminating proteins or peptides.

The amino acid compositions of human SCP and bovine SCP and human SCP-PN and bovine SCP-PN are similar except that a small amount of histidine was detected in both of the human proteins. The NH_2-terminal amino acids of human SCP and bovine SCP-PN are blocked. Human SCP and SCP-PN are immunochemically identical to bovine SCP by the criterion of immunodiffusion analysis. It is not surprising that the chemical and physical properties of human and bovine SCP were found to be similar. The distribution of bovine and human SCP in the nervous system is also the same. Thus, it is evident that human SCP and SCP-PN are more similar in

chemical, physical and immunochemical properties to the basic bovine proteins than to the acidic rat proteins.

The extraction of SCP and SCP-PN from whole tissue with 0.15 M NaCl suggests that the human SCP is a cytoplasmic protein or a peripheral membrane protein. However, in 1978, Uyemura, Suzuki and Kitamura purified and characterized a protein named BF-P_2 from human peripheral nerve myelin. Human BF-P_2 was shown to be immunochemically identical to bovine BF (SCP-PN) by the criterion of immunodiffusion analysis. These observations suggest that human SCP-PN is identical to the human BF-P_2.

4.1.2 Immunochemical Characterization

When saline extracts of human, monkey or rabbit spinal cords were allowed to react with an anti-bovine SCP serum in immunodiffusion analyses, two precipitin lines appeared opposite the wells. These precipitin bands fused, without spurring, with the single line formed by the homologous bovine SCP (MacPherson and Armstrong, 1976). This result indicated that human, rabbit or monkey SCP was composed of two immunochemically distinct antigens both of which cross-reacted extensively with bovine SCP. To explain this phenomenon, Weir and MacPherson (1978) hypothesized that bovine SCP contained two different immunogenic amino acid sequences and that human SCP contained two different populations of molecules, each of which contained only one of the two immunogenic regions at present in bovine SCP. When the

antigens were compared by immunodiffusion analyses using an anti-human SCP-PN serum or an anti-bovine SCP serum, bovine SCP formed one line with the homologous serum and two lines with the heterologous serum while human SCP formed two lines with both sera.

Human SCP and SCP-PN each formed one band in SDS-polyacrylamide gel electrophoretic analyses. SCP-PN formed one narrow peak in isoelectrofocusing analyses. Thus, the two antigenic forms could not be separated on the basis of molecular weight or charge. Therefore, the differences that exist between the two immunogenic amino acid sequences may involve minor substitutions or deletions that cannot be detected by SDS-polyacrylamide gel electrophoresis or isoelectric focusing.

Weir and MacPherson (1978) have noted that immunoelectrophoretic profiles of human SCP and SCP-PN contained two continuous parallel lines in the form of a double arc. It was suggested that SCP and SCP-PN each contained two molecular forms that had the electrophoretic mobilities in agar at pH 8.6 of a β-serum globulin and a γ-serum globulin. This study suggests that the extended precipitin lines observed in immunoelectrophoretograms performed in agar slides are due to interactions between SCP and the acidic sulfate groups of agar. It appears that the electrostatic interactions between the basic SCP and the negatively charged agar support are strong enough to interfere with the migration of

protein. Hence, a trail of SCP is left behind as it migrates towards the cathode.

The suggestion by Weir and MacPherson (1978) that the polypeptide chains of SCP can be realigned in such a way as to yield two differently charged configurations appears to be improbable. Only one form of human and bovine SCP has been detected in SDS-polyacrylamide gel electrophoretic analyses and in isoelectric focusing analyses. Uyemura, Kato-Yamanaka and Kitamura (1978) determined from circular dichroic spectra that bovine SCP-PN has a β-conformation with virtually no α-helical structure. Thus, it appears unlikely that a small protein of mostly β-conformation could rearrange its polypeptide chain in such a way as to internally mask or expose charged residues, thereby changing the overall charge of the protein from that of a basic protein to that of a neutral protein.

4.1.3 Future Studies

The characterization of human SCP and SCP-PN was limited in this study by the amount of purified protein that could be isolated from the human nervous tissue. The questions raised in this report, such as the presence of histidine and cystine in the human proteins deservce further investigation.

Perhaps, the most important reason for continuing the study of human SCP is to investigate its biological activity. The similarities between bovine and human SCP suggest that an anti-encephalitogenic amino acid sequence might also

occur in human SCP. Therefore, it is necessary to determine whether SCP can prevent or suppress EAE induced by human myelin basic protein.

4.2 Cellular Location of SCP-PN

The results of earlier studies (Yo and MacPherson, 1973; MacPherson and Armstrong, 1976; MacPherson, 1978) indicate that all of the immunoreactive bovine SCP-PN could be solubilized by homogenizing tissue with 0.1 M NaCl. In the present report, the SCP-PN content of bovine spinal roots was estimated by single radial immunodiffusion to be 1.3 mg/g of tissue. However, the soluble fraction of 0.8 M sucrose homogenates of spinal roots contained approximately 0.33 mg of SCP-PN per g tissue. Thus, the amount of SCP-PN in the sucrose-insoluble fraction was likely to be the difference between the former and latter values, 0.98 mg of SCP-PN/g of tissue.

Alternatively, the SCP-PN content of the sucrose-insoluble fraction can be compared to the estimated amount of SCP-PN in purified myelin. The amount of SCP-PN + PAS-II decreased from 25% to 8% of the total myelin protein when myelin was extracted with 0.3 ml M NaCl. Since SCP-PN accounts for less than 1% of the protein in NaCl-extracted myelin, SCP-PN and PAS-II comprised approximately 19% and 6% of the total myelin protein in purified myelin. The yield of myelin protein obtained was 4.5 mg/g of tissue. Therefore, 0.86 mg of SCP-PN/g of tissue was present in the myelin fraction. Because the yield of myelin is lower than the amount of

myelin in the tissue, the value of 0.86 mg of SCP-PN/g of tissue closely agrees with the value of 0.98 mg of SCP-PN/g of tissue.

The problem of determining whether a protein is integrally or peripherally associated with a membrane is that there are exceptions to the criteria that have been proposed to distinguish between integral and peripheral membrane proteins. Singer (1974) has proposed the following criteria to distinguish between peripheral and integral membrane proteins: (1) Peripheral membrane proteins can be removed from membranes under mild conditions that do not disrupt the lipid bilayer. Such conditions include low concentration of electrolytes or weak acids and the presence of metal chelating agents. (2) Solubilized peripheral membrane proteins are free of lipid, whereas integral membrane proteins are lipid-associated when solubilized. (3) Peripheral membrane proteins are soluble and molecularly dispersed in neutral aqueous buffers. Integral membrane proteins are solubel in neutral aqueous buffers.

The first criterion can not be used as an all-inclusive means by which membrane proteins can be classified. For example, the mitochondrial ATPase (Pullman *et al.*, 1960) and calsequestrin from sarcoplasmic reticulum (MacLennan and Wong, 1971) are two peripheral membrane proteins that require detergents or chaotropic salts to release them from their membranes.

There are also exceptions to the second and third criteria. Tennenbaum and Folch-Pi (1963) were able to convert

the proteolipid protein of central myelin to a water-soluble lipid-free form by removing the organic solvent by dialysis.

Although the properties of bovine SCP-PN fall within the three criteria cited for peripheral membrane protein, one may still argue that a portion of the polypeptide could be embedded in the myelin membrane. However, this possibility appears to be remote, if not due to a lack of evidence, then because of the ease by which SCP-PN is released from whole tissue and purified myelin, not only by salt, but also by sucrose. SCP-PN can be completely extracted from myelin, except for a small amount that is possibly trapped in myelin vessicles.

The SCP-PN content of the soluble fraction of 0.8 M sucrose homogenates of whole tissue was 25% of the SCP-PN content of whole tissue. The presence of SCP-PN in the 0.8 M sucrose may reflect the cellular location of SCP-PN. During the homogenization of spinal nerve roots in 0.8 M sucrose, cytoplasmic components are released into the sucrose upon the disruption of plasma membranes. On the other hand, until the myelin sheath is osmotically shocked in water, it remains in a compact lamellar form (Spohn and Davison, 1972). If SCP-PN is a cytoplasmic protein then its presence in the soluble fraction may reflect the inability of myelin to bind all of the SCP-PN by electrostatic attraction. Alternatively. it may reflect the ability of the tissue-derived electrolyte to limit the amount of SCP-PN that binds to myelin.

If SCP-PN is loosely-bound to myelin *in vivo*, then the SCP-PN in the soluble fraction could simply be the amount of protein released from myelin membranes that are exposed to the tissue-derived electrolyte in the 0.8 M sucrose. Therefore, the presence of SCP-PN in the soluble fraction could reflect either a cytoplasmic or myelin origin of SCP-PN.

The results of the myelin-binding experiments indicate that the basic proteins lysozyme and SCP-PN can bind to peripheral myelin membranes. The binding of lysozyme suggests that the binding is non-specific because lysozyme is not a myelin protein. In fact, the lysozyme used in this study was isolated from the egg white of chicken eggs. If SCP-PN is a cytoplasmic protein that binds non-specifically to myelin, then the presence of SCP-PN in purified myelin appears to be the result of two factors.

First, SCP-PN is a basic protein with an isoelectric point of 9.9. Myelin, according to the results of Hulcher (1963), behaves as an anion in aqueous suspensions due to its phospholipid content. Secondly, 0.8 M sucrose, the solvent used to homogenize peripheral nerve, contains a very low concentration of electrolyte. Thus, the ionic strength is very low. Thus, in the homogenate of whole tissue, most of the cationic SCP-PN could bind to the anionic myelin membranes and remain bound throughout the purification procedure. The high ionic strength of 0.3 M NaCl favors the dissociation of electrostatic bonds. Therefore, almost all of the SCP-PN remains in the soluble fraction.

On the basis of this line of reasoning, if the ionic strength of the homogenizing medium were to be increased, then one would expect the amount of SCP-PN in purified myelin to decrease. Indeed when peripheral nerves were homogenized in 0.8 M sucrose containing 0.15 M NaCl, the amount of protein in the SCP-PN + PAS-II peak decreased by approximately 33%. Because PAS-II is insoluble in acid and saline (Kitamura, Suzuki and Uyemura, 1976) the decrease in the amount of protein in the SCP-PN + PAS-II peak was likely caused entirely by the extraction of SCP-PN. A similar effect of electrolyte was reported by Greenfield *et al.* (1973), who found that the P_2 content of rabbit sciatic nerve myelin purified by homogenization in 0.32 M sucrose was 25% greater than the amount of P_2 in myelin purified by the cesium chloride method.

The binding of SCP-PN and lysozyme to myelin only suggests that the binding is non-specific and is due to electrostatic interactions. It does not prove that SCP-PN is a cytoplasmic protein, but rather provides a plausible mechanism by which the presence of SCP-PN in myelin can be explained.

Although, pepsin was not able to bind to myelin under the conditions of the myelin-binding experiment, the binding of other acidic proteins to myelin can not be excluded. However, the mechanisms by which acidic proteins bind to myelin deserve further consideration.

Interestingly, serum albumin not only binds to peripheral myelin, but is also present as a contaminant in purified peripheral myelin (Roomi *et al.*, 1978). Albumin is the principal protein component of plasma. One of the functions of

albumin is to transport various lipids and hormones. The means by which albumin binds to myelin is not known. In view of the lipid binding properties of albumin, it is possible that the albumin binds specifically to particular lipids in the myelin membrane.

The presence of albumin in purified peripheral nerve myelin indicates that the protein composition of purified myelin does not reflect the composition as it occurs *in vivo*. Therefore, it is possible that myelin contains other proteins that are artifactually redistributed into the myelin fraction during purification. Several investigators have questioned whether proteins derived from non-myelin membranes contribute to the protein composition of purified myelin. For example, DeVries (1976) isolated a membrane fraction from the bovine brain that was enriched in axonal membranes. It was found that the electrophoretic profiles of the high molecular weight proteins of this fraction and of the myelin fraction were similar. Although the evidence is circumstantial, it is possible that some of the proteins of either of these two membrane fractions originate from the other.

The enzyme 2', 3'-cyclic nucleotide-3'-phosphohydrolase, has been used as a marker enzyme of central myelin for many years (Kurihara and Tsukada, 1968). Shapira *et al.* (1978) have assayed various membrane fractions of rabbit brain for the activity of this enzyme. The lightest membrane fraction that contained multilamellar myelin had the lowest enzyme activity. The heaviest membrane fraction contained single membrane fragments and had the highest enzyme activity. The

authors concluded that 2', 3'-cyclic nucleotide-3'-phosphohydrolase may be absent in multilamellar myelin and may be associated with a denser membrane such as glial plasma membrane.

The contamination of non-myelin membrane fractions with cytoplasmic proteins has been established. For example, MacPherson and Kleine (1978) demonstrated that rat synaptic plasma membranes contain many components of the soluble fraction derived from rat brain.

To determine the cellular and subcellular location of a particular protein, a specific method is required that can clearly and unambiguously reveal the localization of the protein. It is not surprising that the investigators of SCP-PN and P_2 have used an immunohistological technique to localize this protein (MacPherson and Wallace, 1980; Eylar *et al.*, 1980). However, the results of the two studies are contradictory.

The multiple layer or sandwich technique introduced by Weller and Coons (1954) employs an anti-gamma globulin serum conjugated to a fluorescent dye to locate antigens *in situ*. The conjugate binds to antigen-specific antibodies that have previously combined with the antigens in a tissue section. This technique combines specificity and sensitivity of antibodies with the precision of microscopy.

The localization of bovine SCP-PN and rabbit P_2 was accomplished by using antibodies that were specific for

their respective proteins. However, the fixation and washing of the sections differed in the two studies. MacPherson and Wallace (1980) used acetone to fix cryostat sections of bovine spinal nerves which were then dried under an air dryer. Eylar *et al.* (1980) fixed the sections in 95% ethanol/ether without drying and then washed the sections with saline for an unspecified period of time.

Before discussing the potential effects of the differences between the two fixation methods, a few comments regarding the purpose and mechanisms of tissue fixation in immunocytochemistry are in order. According to Nairn (1969) the main objective of fixation is to obtain a preparation that contains the antigen *in situ* in a sufficiently insoluble form. The antigen must not be modified or denatured in such a way that it will no longer react with the specific antibody. Furthermore, fixation must occur with minimum morphological disturbance.

The mechanisms by which fixatives reduce the solubility of protein include: (1) covalent modification as with glutaraldehyde, (2) chelation of proteins with heavy metals, (3) precipitation due to charge reduction and increases in hydrophobicity as with picric acid, (4) precipitation because of water removal as with ethanol or acetone and (5) denaturation as with heat (Sternberger, 1974). Among these fixatives, ethanol and acetone are the two most commonly used fixatives for the fluorescent antibody technique.

The extraction of SCP from bovine spinal roots with 0.1 M NaCl after the tissue had been homogenized in acetone or ethanol/ether clearly demonstrated that neither fixative rendered SCP-PN (P_2) insoluble in saline. This result indicates that washing crysotat sections after fixation would favor the solubilization of P_2 from its cellular location in a tissue section.

The problem of fixing highly soluble proteins has been encountered in other studies. Morikawa *et al.* (1968) found that ribonuclease was localized within the confines of lysosomes in alcohol-fixed paraffin sections of mammalian pancreas. However, if the tissue sections were fixed in phosphate-buffered formalin, then an indiscriminant cytoplasmic fluorescence was observed. Similarly, Engelhardt *et al.* (1971) was able to localize α-fetoprotein in liver cells only when the tissue sections were fixed with ethanol/acetic acid and embedded in paraffin. The α-fetoprotein was not found in cryostat sections that had been fixed in ethanol or ethanol/acetic acid and was not clearly visualized in acetone-fixed sections that were not paraffin-embedded.

Therefore, the question arises as to whether SCP-PN (P_2) was subject to solubilization and re-distribution during the fixation process in either or both of the localization studies. It is doubtful that displacement of SCP-PN from myelin to axons could occur during acetone fixation because SCP-PN is completely insoluble in acetone. Furthermore, it

is difficult to explain why SCP-PN would leave myelin, a membrane to which SCP-PN readily binds. On the other hand, it is quite likely that if P_2 is present in cytoplasm and axoplasm, then, after fixation, the P_2 would diffuse out of the section during the saline wash and be redistributed onto myelin.

Wachsmith *et al.* (1975) have demonstrated that 70% of the aldolase, a cytoplasmic enzyme, can diffuse out of an acetone-fixed kidney section in one minute. However, when an anti-aldolase serum is applied to the fixed sections, the aldolase is fixed *in situ* by precipitation with the antibody.

The results of the tissue extraction experiment suggest that the fixation and treatment of tissue sections containing soluble antigens, such as SCP-PN, deserve more attention. It is obvious from the results of Eylar *et al.* (1980) and MacPherson and Wallace (1980) that the localization of SCP-PN (P_2) has not been conclusively established.

However, in view of the problems that are inherent with the fixation of soluble antigens, it appears that SCP-PN is more likely to be localized in cytoplasm. The results of this study have formed a plausible mechanism which can account for the presence of SCP-PN in purified myelin.

To conclusively establish the localization of SCP-PN, the fixation of tissue sections will have to receive more attention in future studies. It is important to determine whether omitting the saline wash of the fixed tissue sections affects the localization of P_2.

4.3 Rat SCP-PN and Rat Myelin

The results of this investigation clearly indicate that rat SCP-PN, unlike bovine SCP-PN, is not present in purified rat peripheral nerve myelin. A plausible explanation for the absence of rat SCP-PN in myelin is that rat SCP-PN is an acidic protein (MacPherson, Armstrong and Yu, 1981). Thus, one would not expect rat SCP-PN to bind electrostatically to myelin during whole tissue disruption. The significance of this result dispells the widespread belief held by myelin chemists that the three main proteins of peripheral nerve myelin are the P_0, P_1 and P_2 proteins (Kitamura, Suzuki and Uyemura, 1976; Roomi *et al.*, 1977; Quarles *et al.*, 1978; Singh, Silberlicht and Singh, 1978; Smith and Curtis, 1979; Eylar *et al.*, 1980; Weise *et al.*, 1980a). Unfortunately, the nomenclature has been used indiscriminately for all species. The 13,500 dalton component of the peripheral myelin of the various species studied by SDS-polyacrylamide gel electrophoresis has been assumed to be equivalent to the bovine P_2 protein solely on the basis of molecular weight. However, the results in this report have demonstrated that rat SCP-PN distinctly differs from rat P_2 in molecular size and in immunochemical reactivity.

Moreover, Greenfield, Brostoff and Hogan (1980), reported that the alleged rat P_2 protein exhibited cross-reactivity in a radioimmunoassay for MyBP but not in a radio-immunoassay for bovine P_2. These authors concluded that the

rat P_2 protein of peripheral nervous system myelin was analogous to the small basic protein of the central nervous system.

It appears that the assumption that SCP-PN is identical to P_2 in all species has led to the misinterpretation of results of previous studies.

For example, Trapp *et al.* (1979) used the peroxidase-antiperoxidase method to localize the P_0, P_1 and P_2 proteins in the rat nervous system. However, the antisera used in this study were directed towards the proteins from the bovine nervous system. The anti-bovine P_2 serum stained only selective myelin sheaths, the staining being concentrated in the Schmidt-Lanterman incisures. A Schmidt-Lanterman incisure is a split in the major dense line of myelin that contains Schwann cell cytoplasm (Hall and Williams, 1970). The authors concluded that the rat P_2 protein is not present in all myelin sheaths and suggested that the synthesis of P_2 has to be triggered or that only a select population of Schwann cells are capable of synthesizing P_2.

It is evident that the anti-bovine P_2 antibodies were not localizing the alleged rat P_2 protein which now appears to be related to the rat small MyBP. In fact, the protein that was localized in the Schmidt-Lanterman incisures was probably that protein present in low concentration in extracts of rat peripheral nerve that cross-reacts with bovine SCP-PN (MacPherson, Armstrong and Yu, 1981). The staining that was

seen in the Schmidt-Lanterman incisures does not necessarily mean that this is a myelin protein. There is no evidence in the report of Trapp *et al.* (1979) to suggest that the staining was not due to the presence of the protein in the Schwann cell cytoplasm contained in the clefts.

The results of this study and of Greenfield, Brostoff and Hogan (1980) indicate that it is incorrect to use the mobility of proteins in SDS-polyacrylamide gels as the sole means of identifying proteins. A more specific method, such as immunochemical reactivity is required.

4.4 CNBr-Derived Peptides of Bovine SCP

According to Gross (1967), CNBr reacts only with methionine under acidic conditions and these conditions are mild enough to avoid non-specific cleavage. Thus, CNBr was used to cleave bovine SCP into three peptides.

The two smaller peptides, CN2 and CN3, were successfully purified in this study. Preparations of CN3 contained one component. However, CN2 was shown to contain two peptides after reduction with 2-mercaptoethanol. Under non-reducing conditions, high voltage paper electrophoresis of CN2 revealed the presence of one component that was equivalent to two peptides linked by a disulfide bond.

The amino acid compositions of the small CNBr-derived peptides of bovine SCP are very similar to certain amino acid sequences of bovine SCP-PN. Earlier studies by MacPherson, Armstrong and Tan (1976) revealed that only

minor differences exist between the amino acid compositions of SCP and SCP-PN. Furthermore, the two forms of the protein were shown to be identical by the criterion of immunodiffusion analyses. Thus, both proteins contain closely related or identical antibody binding sites. The similarity of other physical and chemical properties suggests that the amino acid sequences of SCP and SCP-PN are very similar. If one assumes that the sequences are similar, then it appears that CN2 and CN3 are derived from the COOH-terminal and the NH_2-terminal ends of SCP respectively.

CN1 appears to be a large peptide that contains the majority of the amino acid residues of SCP. In the present report it was not possible to compare CN1 and the sequence of SCP-PN between residues 21 and 113 as CN1 contained uncleaved and partially cleaved SCP and could not be separated from SCP by gel filtration on Sephadex G-50 superfine or Bio-Gel P6. It is interesting to note that Weise *et al.* (1980) purified all of the CNBr peptides of P_2 using only one gel filtration step. However, the purity of the peptide preparations was not revealed.

The presence of uncleaved and partially cleaved SCP in the reaction mixture presented a problem when attempts were made to purify CN1. Purified CN1 can be obtained by gel filtration only after SCP has been completely cleaved by CNBr or when CN1 can be separated from the unreacted material by another method. Cleavage at methionine residues may be limited by the formation of methionine sulfoxide which is

resistant to the reaction of CNBr. However, the amount of uncleaved and partially cleaved SCP could possibly be reduced by determining the optimum conditions for CNBr cleavage. Some of the conditions that should be investigated include the solvent, the ratio of CNBr to methionine, the temperature and the duration of the reaction. Despite the presence of uncleaved material in CN1 preparations, it may be possible to purify CN1 by other procedures such as isoelectric focusing or ion exchange chromatography.

CNBr reacts with methionine residues to form a mixture of homoserine and homoserine lactone. However, if the methionyl side chains are oxidized to the sulfoxide or sulfone, then the reaction with CNBr will not occur. Thus, the incomplete cleavage of bovine SCP may have been the result of the formation of sulfoxide or sulfone groups. The use of gel filtration to purify CN1 will be successful only when the conditions of the reaction are such that SCP is completely cleaved.

It is interesting to note that when rabbit P_2 was reacted with CNBr, the protein was not completely degraded (Ishaque *et al.*, 1980). Rabbit CN1 contained uncleaved and partially cleaved P_2. However, purified rabbit CN1 was obtained following gel filtration on Sephadex G-75 fine using 0.2 M acetic acid as an eluting buffer. A 70% yield of purified CN1 was recovered.

The gel filtration pattern of the CNBr peptides is unusual with respect to the elution order of CN2 and CN3. If it is assumed that the CN2 and CN3 are identical to the equivalent peptides of SCP-PN, then the molecular weights of CN2 and CN3 would be approximately 2,100 and 2,400 daltons, respectively. Thus, one would expect CN3 to be eluted before CN2. However, CN2 was eluted before CN3. In the report of Weise *et al.* (1980), it was shown that CN2 derived from P_2 contained more amino acid residues than CN3 and that CN2 was eluted from Sephadex G-50 before CN3. There are two factors that may influence the elution order of CN2 and CN3. First, molecular interactions may occur between CN3 and the gel matrix that could retard the movement of CN3 through the molecular sieve. Secondly, despite an assumed difference in molecular weights, the conformations of CN2 and CN3 may be such that the apparent molecular size of CN2 is greater than that of CN3.

In molecular sieve chromatography, ideal chromatographic behaviour requires that the concentration of solute in the solvent of the void volume is equal to the concentration of the solute within the stationary phase (Morris and Morris, 1976). However, when non-ideal chromatographic behaviour occurs, the solute concentration within the stationary phase is greater than the solute concentration outside. Thus, the solute is being artifactually concentrated in the stationary phase. Gelotte (1960) suggested three types of interaction that could give rise to non-ideal chromatographic behaviour:

aromatic adsorption, ion exchange or ion exclusion. Ion exchange effects have been noted on Sephadex which contains a low carboxyl content while ion exclusion occurs mostly with acidic solutes. Upon the examination of the aromatic amino acid content of CN2 and CN3 (data of Kitamura *et al.*, 1980) it is evident that CN3 contains 3 aromatic residues while there is only 1 aromatic residue in CN2. Thus, it appears that CN3 exhibits non-ideal chromatographic behavior on Bio-Gel P6 possibly due to interaction of its aromatic residues with the gel.

It is evident from the gel filtration pattern of the CNBr peptides that the area of the CN3 peak is considerably greater than that of CN2. CN3 contains two phenylalanine residues whereas CN2 does not contain phenylalanine. The absorption spectrum indicates that the phenylalanine absorbs light at 230 nm. Thus, the difference in the area of the CN2 and CN3 peaks could reflect the increased absorption due to the presence of the phenylalanine residues in CN3.

CHAPTER 5: CONCLUSIONS

(1) Human SCP and SCP-PN were purified from spinal cords and spinal nerves, respectively, by extraction with 0.15 M NaCl, ion exchange chromatography and gel filtration on Sephadex G-50 superfine. Human SCP is closely related to the anti-encephalitogenic bovine SCP with respect to immunochemical reactivity and amino acid composition. Human SCP is distributed in the brain, spinal cord and spinal nerves in the proportions of 1:6:60 respectively.

(2) Purified human SCP and SCP-PN formed one band in SDS-polyacrylamide electrophoretograms and had estimated molecular weights of 13,700 ± 1,370 and 14,700 ± 1,470 daltons, respectively. The isoelectric point of human and bovine SCP-PN was 9.9. The amino acid compositions of human SCP and SCP-PN were similar except that SCP contained 11.9% of glutamic acid, whereas SCP-PN had 16% of glutamic acid.

(3) Immunodiffusion analyses using an anti-human SCP-PN serum or an anti-bovine SCP serum revealed that human SCP and SCP-PN each contain two distinct antigenic forms. The major forms and the minor forms of SCP

and SCP-PN are immunochemically identical to each other. Immunoelectrophoretic analyses indicated that human SCP and SCP-PN and bovine SCP have the electrophoretic mobility of a γ-serum globulin in agarose at pH 8.6.

(4) Bovine SCP-PN (P_2) was detected in the soluble fraction of the 0.8 M sucrose homogenate of bovine peripheral nerves and in purified peripheral nerve myelin by SDS-polyacrylamide gel electrophoresis and by immunodiffusion analyses. The SCP-PN content of the soluble fraction of the 0.8 M sucrose homogenate and of the 0.3 M NaCl homogenate of peripheral nerves was 0.33 and 1.33 mg/g of tissue, respectively.

(5) When myelin was extracted with 0.3 M NaCl the bovine SCP-PN content decreased from approximately 19% to less than 1% of the total myelin protein. The protein which comigrated with SCP-PN in SDS-polyacrylamide gel electrophoretograms was identified as the PAS-II glycoprotein.

(6) The basic proteins SCP-PN and lysozyme were shown to bind to suspensions of myelin and NaCl-extracted myelin in 0.8 M sucrose. Pepsin, an acidic protein, did not bind to myelin or NaCl-extracted myelin under the same conditions.

(7) Acetone and 95% ethanol/ether, 1:1, were found to be poor histological fixatives in as much as they were ineffective in preventing the subsequent saline-solubilization of bovine SCP-PN from whole tissue which had been homogenized in these fixatives.

(8) The results suggest that bovine SCP-PN (P_2) is a cytoplasmic protein, and during the homogenization of peripheral nerves in 0.8 M sucrose, most of the positively charged SCP-PN released from the cytoplasm binds to negatively charged myelin and remains associated with myelin throughout the purification procedure. This interpretation is consistent with previous immunohistological studies in this laboratory in which SCP-PN was localized in axons and, possibly, also in Schwann cell cytoplasm. In these studies the tissue sections were not washed with saline after acetone fixation but were first incubated with anti-bovine SCP serum.

(9) Rat SCP-PN was not detected in rat peripheral nerve myelin and was shown to be distinct from the rat P_2 protein on the basis of molecular size and immunochemical reactivity.

(10) Cleavage of bovine SCP with cyanogen bromide produced three peptides. Following gel filtration on Bio-Gel P6, peptides CN2 and CN3 were found to be pure by SDS-polyacrylamide gel electrophoresis, discontinuous polyacrylamide gel electrophoresis at pH 2.7 and high voltage electrophoresis. Preparations of peptide CN1 contained uncleaved and partially cleaved SCP. Most of the preparations were estiamted to contain approximately 90% CN1.

(11) A comparison of the amino acid compositions of two of the CNBr peptides of bovine SCP with those published by Kitamura *et al.* (1980) for bovine SCP-PN (P_2) suggests that the primary structure of the two proteins is very similar. CN3 contains approximately 18 amino acid residues and appears to be derived from the NH_2-terminal end of SCP. CN2 is derived from the COOH-terminal end and contains approximately 19 amino acid residues. CN2 is composed of two peptides joined by a disulfide bond. CN1 is a large peptide which has an estimated molecular weight of 9,000 ± 900 daltons.

(12) None of the peptides formed precipitates with an anti-bovine SCP serum in immunodiffusion analyses.

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