LOCALIZATION OF A BASIC PROTEIN IN THE MYELIN OF VARIOUS SPECIES WITH THE AID OF FLUORESCENCE AND ELECTRON MICROSCOPY

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ABSTRACT

In this study, alanine was shown to be the N-terminal amino acid of a basic protein of low molecular weight that was isolated from either human or guinea pig brain. Antibodies prepared against the guinea pig protein were labeled with either fluorescein or ferritin. Studies with the labeled antibodies showed that an immunohistochemically similar protein is found in the myelin sheaths of central and peripheral nervous tissues of chicken and frog and a variety of mammalian species. Loss of integrity of the myelin during processing was shown to enhance markedly the antigen-antibody reaction.

INTRODUCTION

In a previous study (1), a basic protein of low molecular weight was isolated from guinea pig brain. Antigenic properties of this protein have been studied by radioautographic methods in presensitized lymph node cells. The response of these presensitized cells to this protein were carried out using either tritiated uridine or tritiated leucine as the radioactive metabolic precursor (2). Cells of the size of medium lymphocytes rapidly synthesized ribonucleic acid. Subsequently, rapid protein synthesis occurred in the small lymphocytes (2). Whereas the initial studies were concerned with the response of the lymph node cells to the cationic protein, it was then of interest to characterize the protein further and to find its location in the brain.

METHODS

Determination of the N-terminal Amino Acid

Two techniques were used for this determination.

1. Phenyl thiohydantoin derivative: Two milliliters of a pyridine-triethylamine-phenylisothiocyanate solution (10:3:1) were added to 3.75 mg of the cationic protein isolated from guinea pig brain. The solution was incubated at 40°C for 90 minutes. The remainder of the procedure was carried out as described by Edman (3). The derivative was dissolved in 0.1 ml of 90 per cent acetic acid and chromatographed in solvents II and III as described by Sjöquist (4).

2. Quantitative measure of the dinitrofluorobenzene (DNP) derivative of the cationic protein: A modification (5) of the original Sanger method (6)

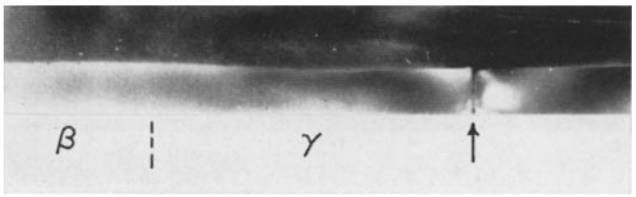


FIGURE 1 Immunoelectrophoretic pattern of serum from a rabbit sensitized to the cationic guinea pig brain protein. The anode is to the left. The arrow indicates the electrophoretic origin, and the dashed line indicates the approximate boundary between the rapidly migrating γ-globulins and the slowly migrating β-globulins. The antigen was layered over the agar after electrophoresis. The precipitin line can be seen as a faint line above the rapidly migrating γ-globulins.

was used to obtain the DNP–amino acid derivative from the protein. To 4.5 mg of the cationic protein from either human or guinea pig brain, dissolved in 3 ml of 0.1 N KCl, was added 0.1 ml of dinitrofluorobenzene. The solution was incubated at 40°C and maintained at pH 9.0 with the aid of an automatic pH titrimeter (1 N KOH). Hydrolysis of the derivatized protein was carried out in 6 N HCl at 120°C for 18 hours in sealed ampules. The dinitrophenyl derivatives of alanine, aspartic acid, and phenylalanine were prepared in the same manner. Ascending chromatography was performed on Whatman No. 3 paper with a butanol–acetic acid–water solvent (4:1:5). The spots containing the amino acid derivatives were cut from the paper and eluted, and the optical densities of the solutions were read in a Beckman spectrophotometer (560 mμ).

Preparation and Immunoelectrophoresis of the Anti Brain Protein Serum

Two female albino rabbits, each weighing 4 pounds, were injected in each hind footpad with 0.2 ml of a Freund adjuvant–guinea pig brain protein emulsion containing 3.0 mg of protein. The same rabbits were then injected intravenously twice weekly for 6 weeks with 3.0 mg of the cationic brain protein. The rabbits were then bled and the serum was collected. The serum was tested for the presence of antibodies by the double diffusion method of Ouchterlony (7). A strong precipitin line was seen.

The serum was subjected to electrophoresis in a vertical starch gel (borate buffer, 0.026 M, pH 8.8), according to the method of Smithies (8). Electrophoresis was carried out at room temperature (16 hours at 15 v/cm). The gel was then cut in half and overlaid with 1.5 per cent agar in saline. The agar was covered with cationic protein solution, and the agar layer was subsequently examined for the appearance of precipitin lines.

Preparation of the Fluorescent Gamma Globulin and Reaction with Sections of Nervous Tissue

For the preparation of either the fluorescent or the ferritin-labeled antibody, the γ-globulin was precipitated by the addition of ammonium sulfate to the normal or immune rabbit serum at 0°C to a final concentration of 33 per cent saturation. Fluorescent γ-globulin was prepared according to the method of Riggs *et al.* (9).

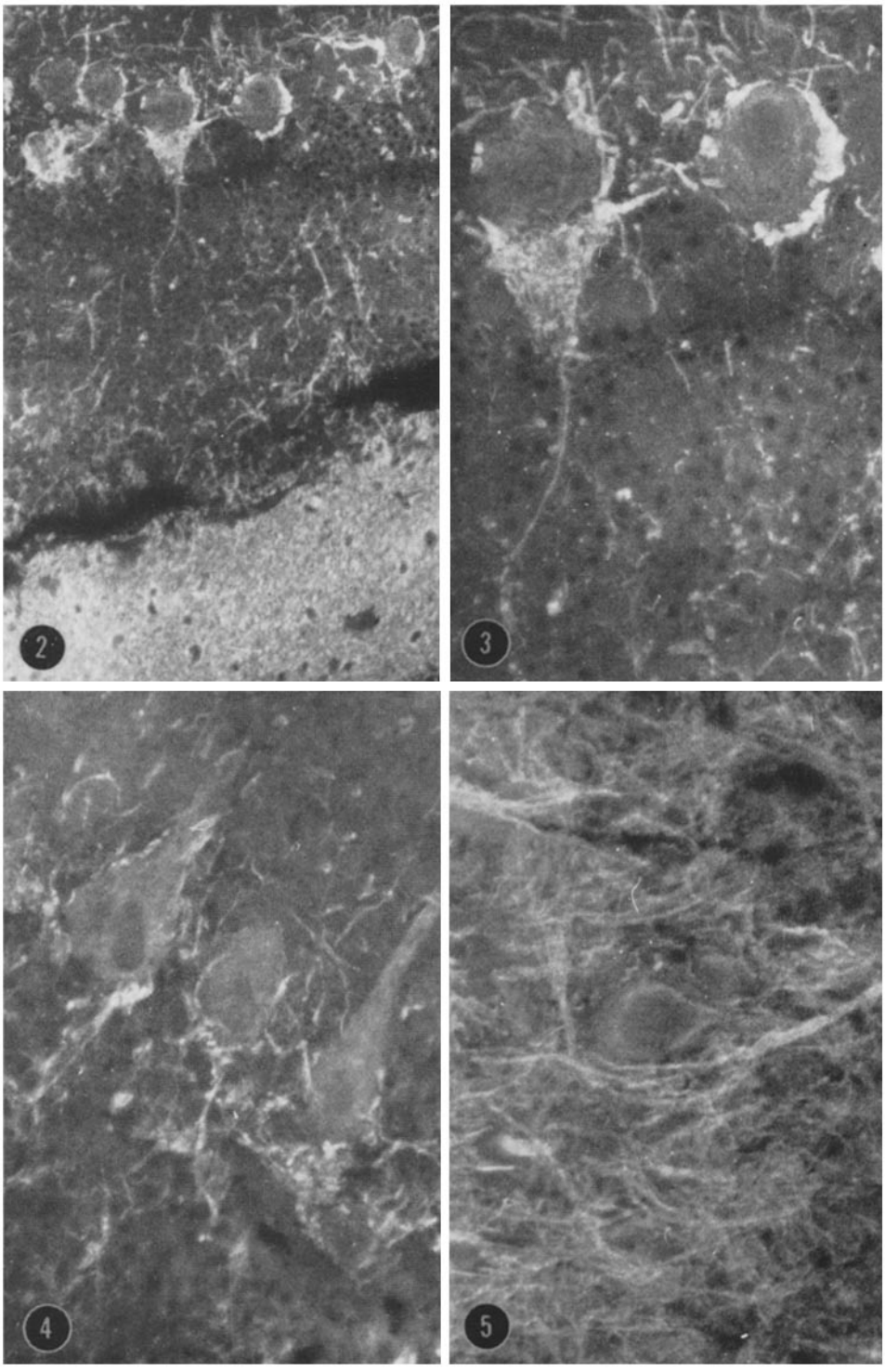
The γ-globulin fraction (120 mg protein) from serum of immunized or normal animals was dissolved in 8 ml of bicarbonate buffer (0.5 M, pH 9.0) and dialyzed against a fresh portion of the same buffer for 12 hours. To this dialyzed protein solution was

FIGURE 2 A section of guinea pig cerebellum exposed to the fluorescent anticationic protein antibody. The medullary layer of the cerebellum has intense fluorescence, as do some fibers of the Purkinje cell. × 240.

FIGURE 3 A detail of Fig. 2. × 600.

FIGURE 4 Another section of guinea pig cerebellum exposed to the fluorescent antibody, showing the dendritic tree of the Purkinje cell. × 600.

FIGURE 5 A section of the guinea pig cerebrum showing the uptake of the fluorescent antiserum by the myelinated fibers and perinuclear region of the cell. × 600.



added 10 ml of saline, 3 ml of the bicarbonate buffer, and 2 ml of acetone. This was cooled in a dry ice acetone bath until ice crystals began to form. At this point 1.5 ml of acetone, containing 6 mg of fluorescein isothiocyanate, was added to the above solution and stirred overnight at 4°C. The solution was then passed through a column containing Sephadex G-25 (1.5 × 30 cm) suspended in phosphate buffer (0.01 M, pH 7.2). The fluorescent protein emerged with the front and was separated from the unreacted fluorescein isothiocyanate, which remained near the top of the column. The fluorescent γ-globulin was mixed with pig kidney acetone powder and centrifuged to remove any non-specifically reacting material. The fluorescent protein remaining in solution was passed through a 0.35-μ Millipore filter to give a clear solution.

Fresh samples of various tissues were rapidly frozen in a cryostat and sections 6 μ thick were cut from the frozen block. The sections were immediately placed in 95 per cent ethanol at room temperature for 10 minutes and then were air-dried. A drop of the fluorescent immune or normal γ-globulin solution was placed on each brain section and the sections were incubated in a moist chamber at room temperature for 30 minutes. The sections were washed in 0.01 M phosphate buffer (pH 7.5) and mounted with glycerol containing 10 per cent of the phosphate buffer.

Preparation of the Ferritin–Gamma Globulin and Reaction with Sections of Cerebellum

The method of Rifkind *et al.* (10) was used. The γ-globulin fraction from normal or immune serum was dissolved in borate buffer (0.1 M, pH 9.6) and dialyzed against the same borate buffer for 12 hours.

To 3 ml of phosphate buffer (0.05 M, pH 7.5) containing 78 mg of ferritin was added 0.1 ml of toluene diisocyanate. This was kept at room temperature for 8 minutes and then held at 4°C for an additional 45 minutes. The solution was centrifuged at 4000 *g* for 30 minutes. The supernatant solution was placed in the cold for another hour. Eight ml of the borate buffer (0.1 M, pH 9.5) containing 75 mg of γ-globulin were added to the ferritin isocyanate solution and the mixture was stirred at 37°C for 1 hour.

The solution containing the ferritin–γ-globulin complex was first dialyzed against 0.1 M ammonium carbonate for 17 hours at 4°C, and then for 2 hours against phosphate buffer (0.06 M, pH 7.5). The solution was centrifuged at 4000 *g* for 30 minutes. The supernatant solution was centrifuged for 2 hours at 106,000 *g* and the pellet containing the ferritin–γ-globulin complex was resuspended in the phosphate buffer (0.06 M, pH 7.5). The resulting suspension was centrifuged at 4000 *g* for 30 minutes and the clear solution was passed through a sterile 0.35 μ Millipore filter into a sterile container.

Fresh cerebellum was obtained from anesthetized guinea pigs and rapidly placed in phosphate-buffered (0.1 M, pH 7.5) 10 per cent formalin. The tissue was kept in this solution for 1 hour and then placed in the phosphate buffer (0.06 M, pH 7.5) containing 10 per cent dimethylsulfoxide for an additional hour. Fifty-μ frozen sections were cut and immersed in the ferritin-antibody solution for 45 minutes. The tissue sections were washed three times, for 20-minute periods each, with phosphate buffer (0.02 M, pH 7.5), and were then processed for embedding in Epon 812. Unstained, thin sections, cut on a Porter-Blum MT-3 ultramicrotome, were examined in an RCA EMU-3E microscope. Typical areas were photographed at initial magnifications of 14,400 to 20,200 and subsequently enlarged.

RESULTS

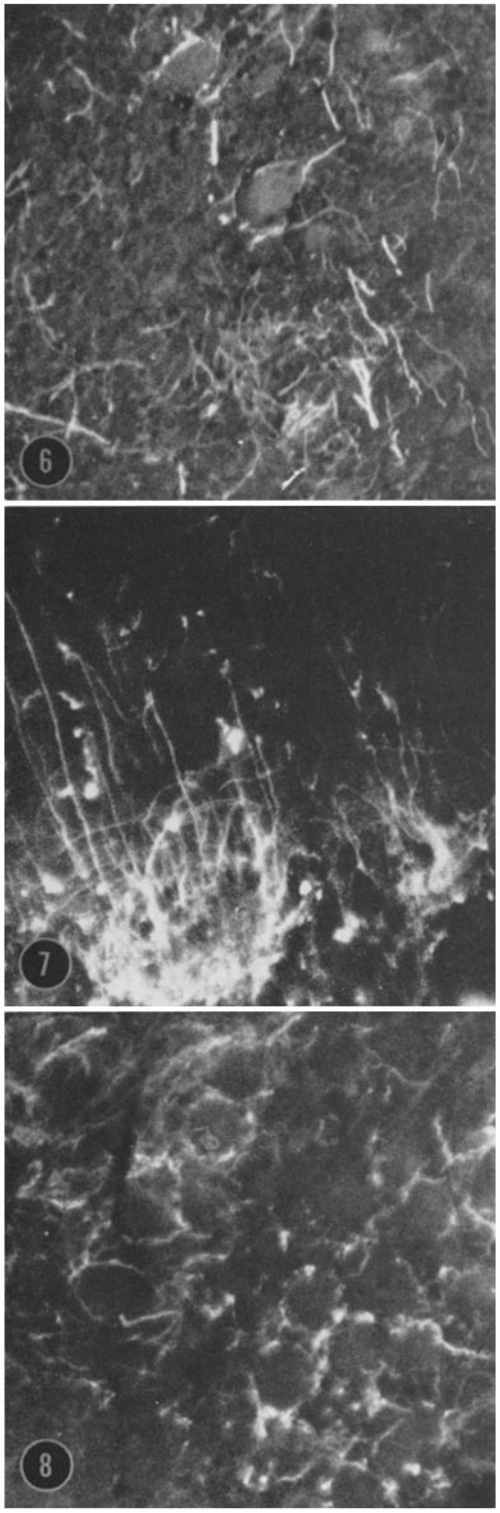
N-Terminal Amino Acid of the Basic Protein

The phenyl thiohydantoin amino acid derivative of the cationic protein isolated from guinea pig white matter migrated in solvent II (Sjöquist, 4) with an R_f of 0.21 and in solvent III with an R_f of 0.89. The authentic phenyl thiohydantoin derivative of alanine migrated with the same mobility as the unknown amino acid derivative in each solvent system.

The dinitrophenyl amino acid derivative of the cationic proteins derived from both human and guinea pig white matter migrated in the solvent system with an R_f of 0.75, as did the authentic DNP-alanine. The molecular weight of the guinea pig cationic protein is 4850 as determined by quantitative measurement of the recovery of known DNP-alanine. This determination was not made on the human material.

Immune Electrophoresis of Rabbit Serum Antibody to the Cationic Guinea Pig Protein

A fine precipitin line formed in the agar layer parallel to the plane of the starch gel. Precipitate was observed only over the region containing the rapidly migrating γ-globulin (Fig. 1). The absence of spurring indicates a single antigenic component in the cationic protein solution. This is consistent with the finding of a single strong precipitin line in the immune diffusion studies.



Fluorescent Antibody Studies

Fluorescein-labeled γ-globulin from normal or immunized rabbits was layered over frozen sections of guinea pig peripheral and central nervous tissue, liver, pancreas, striated muscle, testes, seminal vesicles, spleen, and kidney. The peripheral (sciatic nerve and sympathetic chain ganglion) and central (cerebral cortex, cerebellum, spinal cord) nervous tissue were the only areas giving evidence of specific reaction with the immune γ-globulin. When fluorescent γ-globulin from non-immunized rabbits was layered on these tissues, no such reaction was observed.

The binding of the anticationic protein antibody with myelinated fibers of the guinea pig cerebellum was studied in more detail. From Fig. 2 it can be seen that the fluorescence was most intense in the medullary layer. Fluorescence was also observed on the Purkinje cells and on the fibers in the granular layer coming from the Purkinje cells. Little fluorescence was observed in the molecular layer.

Fluorescence at the axon hillock of the Purkinje, cells (Fig. 3) indicated the presence of discrete myelinated fibers that converge at the apex of the hillock. The fibers appeared similar to the axons of the basket cell (Jakob, 11, p. 789). The myelinated axon of the Purkinje cell was fluorescent. Adjacent to the plasmalemma of some perikarya were fluorescent ringlets which appeared similar to the synapse between a Purkinje cell collateral and another Purkinje cell body (11, p. 781).

The nuclei of the Purkinje cells, and of all the cells to be described, appeared black and therefore did not contain the basic protein being studied. The perinuclear areas of the Purkinje cells exposed to the labeled antibodies were fluorescent (Fig. 4), as were the perinuclear areas of some of the cells in the cerebral cortex (Fig. 5). Fig. 4 shows only a few regions of fluorescence in the vicinity of the unmyelinated dendritic tree of the Purkinje cells, and these are probably due to myelinated fibers

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FIGS. 6 to 8 are sections of cerebellum from pig, monkey, and chicken reacted with the fluorescent anti guinea pig protein antibody.

FIGURE 6 Pig cerebellum. × 240.

FIGURE 7 Rhesus monkey cerebellum. × 432.

FIGURE 8 Chicken cerebellum. × 432.

which lie among the dendrites. When the cerebellum of either human, pig, rat, monkey, rabbit, or chicken was exposed to the fluorescent immune globulin, the myelinated fibers in the medullary lamina became intensely fluorescent and the myelinated fibers around the Purkinje cells also were fluorescent. Figs. 6 to 8 show the reaction of the cerebellum of pig, monkey, and chicken with the fluorescent antiserum. The reaction was as intense with these tissues as with the guinea pig cerebellum.

Myelinated fiber tracts in the spinal cord of human and rat also avidly absorbed the immune serum (Figs. 9 and 10). The fluorescence appeared to be localized in the myelin sheath rather than in the axon.

The peripheral nervous system reacted with the fluorescent immune serum, as indicated by the photograph of the monkey sympathetic chain ganglion (Fig. 11). The myelinated part of the sciatic nerve bound the immune serum quite strongly. In sections cut in the plane of the optic chiasm of the rabbit, the pattern resulting from the fluorescent antibody labeling (Fig. 12) was not the same as that seen with the Klüver myelin stain. The pattern seen after treatment with fluorescent antibody was that of a crossing of bundles of fibers, with the crossover point lying in the median sagittal plane of the chiasm. The junctions appeared to be separated by spaces which decreased as one moved caudally from the chiasm. With the Klüver stain, the crossing appeared to be more random, with no evidence that bundles of fibers were involved. Sections from the cerebrum of the frog (Fig. 13) specifically absorbed the fluorescent antiserum, demonstrating the presence of the basic protein in the nervous tissue of amphibia.

Electron Microscope Studies

Further information on the location of the protein was afforded by studies with the electron microscope. Frozen sections of the guinea pig cerebellar medullary lamina, treated with the ferritin–normal globulin complex and studied in the electron microscope, showed precipitation of ferritin particles along the layers of the myelin sheath (Fig. 14). The ferritin–immune γ-globulin reacts with the myelin sheath in the same regions with quantitatively greater uniformity of distribution throughout the tissue and along the membranes, greater concentration, and larger particle size (Fig. 15). The well preserved, closely packed myelin absorbed the antiserum to a much lesser extent than did the unfolded, disrupted part of the myelin sheath (Fig. 15). The reaction of normal γ-globulin with the myelin may be indicative of the presence of antibodies in the serum of rabbits not previously injected with the basic protein.

The fact that antibody was observed to bind the perinuclear region of neurons when the fluorescent technique was used, whereas no ferritin particles were seen intracellularly, may be explained by the difference in experimental procedure. The fluorescent antibody technique, using 6-micron sections, directly exposes the subcellular components to the antibody. The 50-micron-thick blocks of tissue used in the ferritin antibody reaction do not permit direct contact of the antibody with the subcellular structure.

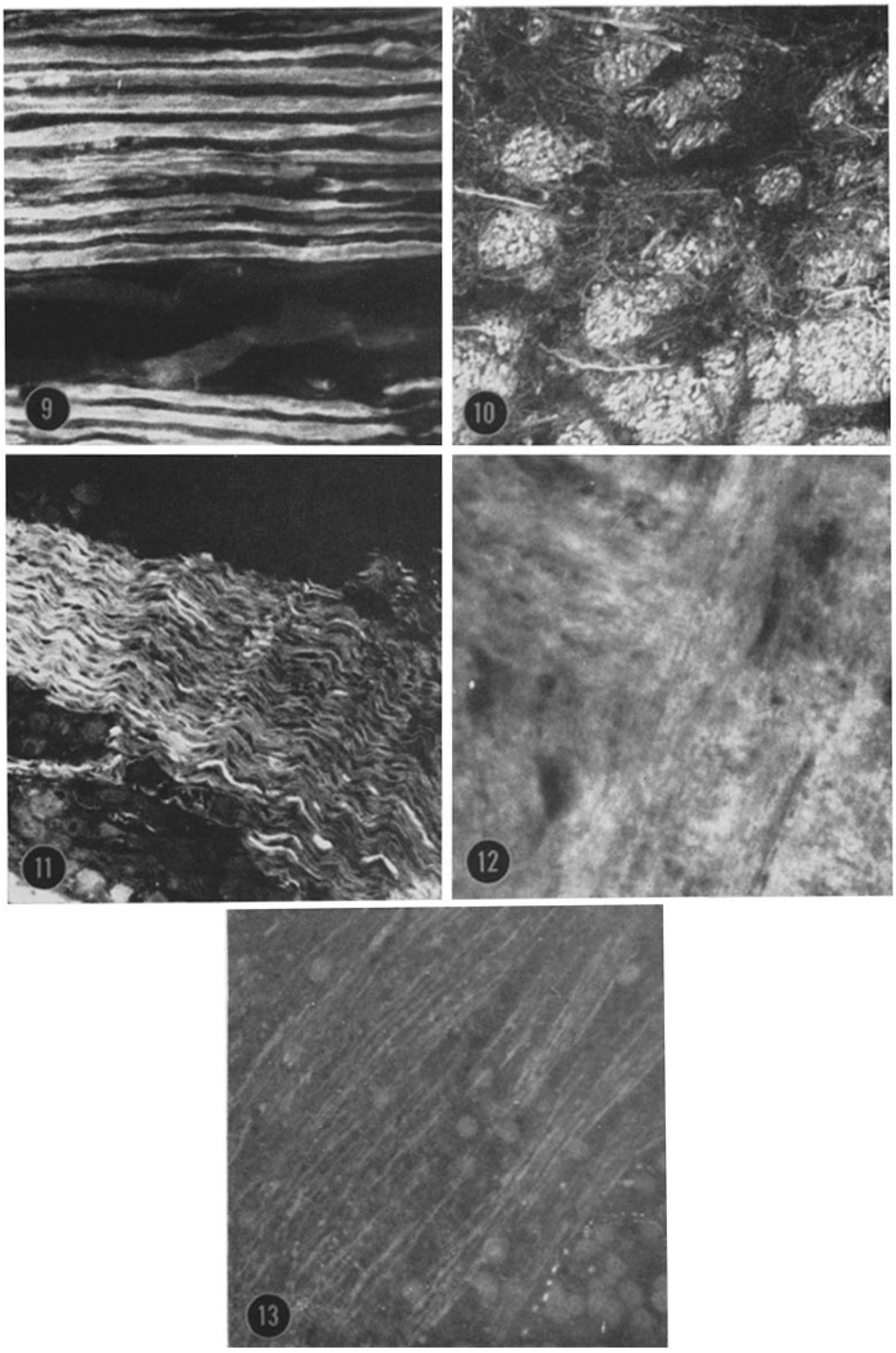
FIGURE 9 Ventral root from human spinal cord (biopsy specimen) exposed to the fluorescent immune γ-globulin. The fluorescence is concentrated at the myelinated membrane. × 600.

FIGURE 10 Ventromedial section of the rat cervical spinal cord exposed to the fluorescent immune γ-globulin. Ascending and descending myelinated fiber tracts are intensely fluorescent. × 240.

FIGURE 11 Sympathetic chain ganglion of monkey exposed to the fluorescent immune γ-globulin. × 172.

FIGURE 12 Section of the rabbit optic chiasm reacted with the fluorescent immune γ-globulin. Note the crossing pattern. × 600.

FIGURE 13 Section of frog cerebrum exposed to the fluorescent immune γ-globulin. × 240.



DISCUSSION

The present study expands the number of chemical similarities that have been found between the cationic protein from brain and other basic proteins. In the report describing the isolation and partial characterization of the cationic protein from guinea pig brain it was stated that there were no free sulfhydryl groups in the protein (1). Other cationic proteins, such as histones and protamines, do not contain cysteine in appreciable concentrations (12). The observation that the protein from the guinea pig or human brain has alanine as the N-terminal amino acid is of interest because of the following reports. Phillips and Johns (13) indicated that the N-terminal amino acid of histones from various tissues is alanine, and Butler *et al.* (14) reported that the N-terminal amino acid of some microsomal cationic proteins is alanine. The absence of free sulfhydryl groups and the occurrence of alanine as the N-terminal amino acid of the protein under study indicate its close relationship with many other basic proteins.

In this study, the cationic protein from guinea pig brain was shown to be immunologically similar to that of the myelin sheaths of both central and peripheral nervous tissues of a variety of mammals, and also of central nervous tissue of chicken and frog. The cationic protein isolated from the brain of a guinea pig was antigenic for rabbits. The antibody formed against the brain protein was a rapidly migrating γ-globulin. With the aid of the fluorescent antibody techniques, it was demonstrated that the cationic protein was localized in the myelin sheath and the perinuclear region of the neuron. The ferritin antibody technique confirmed the localization of the protein in the myelin sheath but yielded no information on the localization within the perikaryon. The rim of fluorescence observed near the nuclei of the Purkinje and the pyramidal cells corresponds to an area of high concentration of membranous organelles (Nissl substance and Golgi region).

Because tissue other than nervous tissue did not specifically bind the fluorescent anticationic protein antibodies, it can be concluded that the reaction is specific for nervous tissue. Its absence in the nuclei of Purkinje cells and other neurons indicates that the cationic protein is not derived from histone associated with DNA. Since uptake of fluorescent antibody was observed in all regions of the nervous system containing myelin, an antigenically similar cationic protein must be synthesized in all these regions. Furthermore, because the central nervous tissue of human, monkey, pig, guinea pig, rabbit, rat, chicken, and frog and the peripheral nerves of the rabbit, monkey, and guinea pig specifically bound the anti-guinea pig cationic protein antibody, it can be concluded that all these animals synthesize an antigenically similar cationic protein that is associated with the myelin. The finding that the N-terminal amino acid of both the human and the guinea pig protein is alanine indicates at least one instance of chemical similarity in the two species. These data are consistent with earlier observations of Kabat *et al.* (15) and subsequent work by other investigators (see reference 16 for review), giving evidence that in the brains of various mammals there exists an immunologically similar protein.

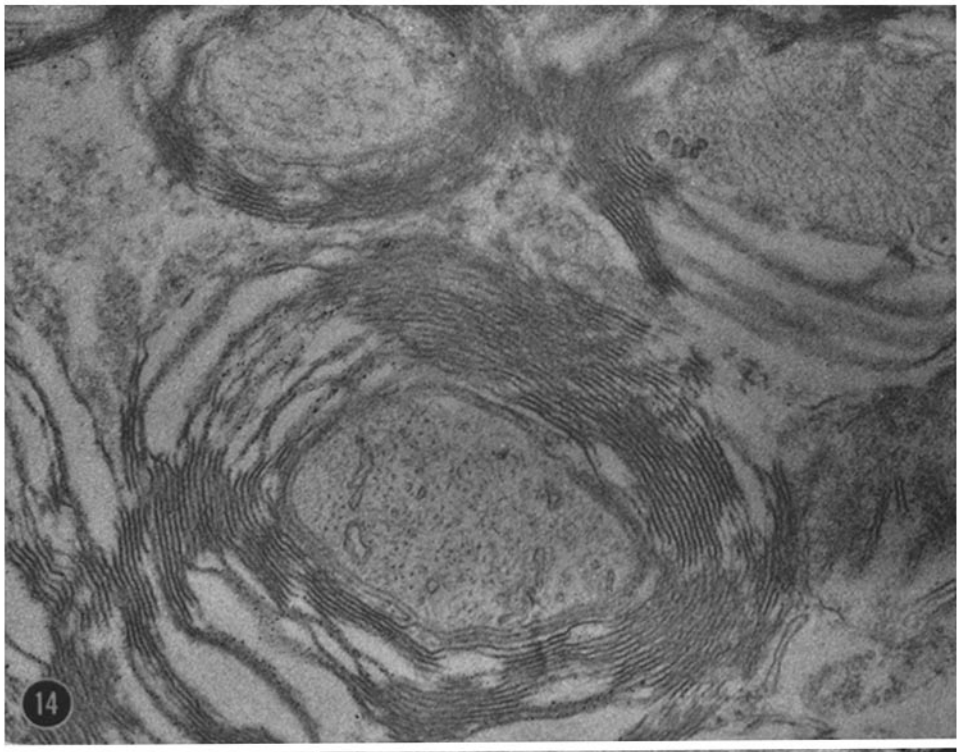
Two conclusions can be drawn from the electron microscope studies. The first is that the basic protein is indeed a component of the myelin sheath, and does not appear to be related to the axolemma. The second is that the protein of the intact myelin has its antigenic groups blocked in some way. Those areas of myelin that were disrupted in the fixation procedure bound the antibody to a greater extent than did the intact areas. The important implication of this is that many of the antigenically reactive groups of the protein may not react with circulating antibody unless some previous damage to the myelin (such as viral or bacterial infection or other traumatic injury) has unmasked these sites.

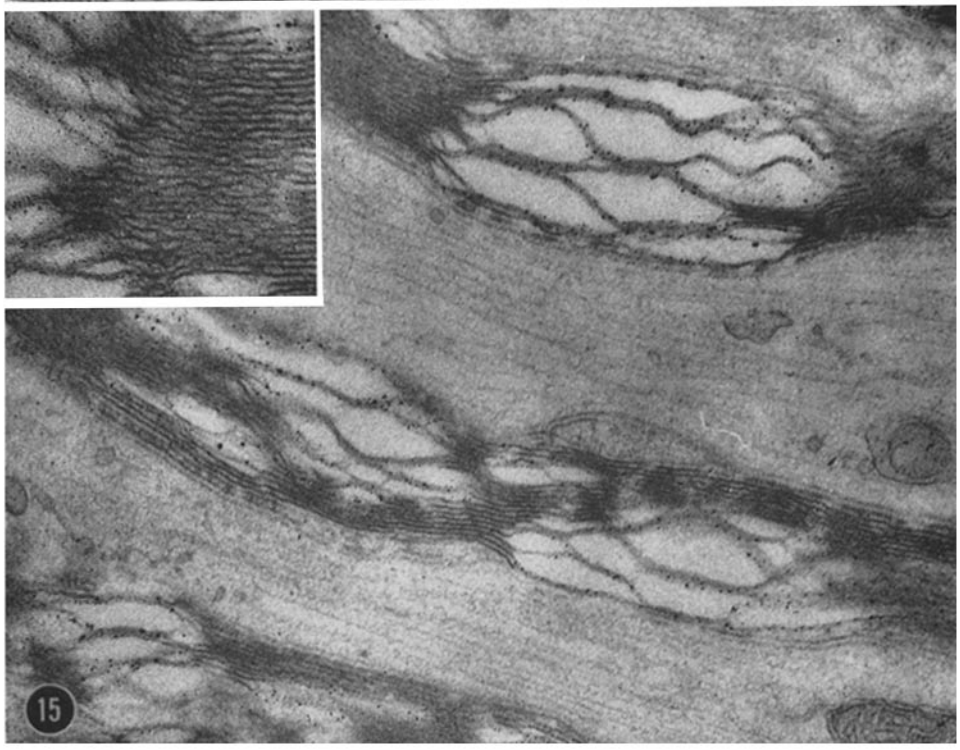
The further elucidation of the antigenic proper-

FIGURE 14 Electron micrograph of guinea pig cerebellum reacted with ferritin–normal γ-globulin, showing ferritin particles along layers of myelin sheath. × 41,500.

FIGURE 15 Electron micrograph of guinea pig cerebellum reacted with ferritin-labeled immune γ-globulin. The concentration of electron-opaque particles indicates the extent of the immune reaction. × 41,500.

Inset shows a region of the cerebellum. × 58,500.





ties of the cationic protein is of interest for the following two reasons. The first is that cationic proteins are involved as structural elements in the mitochondrion (17), the microsome (14, 18), and the myelin sheath (reference 16, p. 293; reference 19). The second is that cationic proteins such as histones have been shown to inhibit protein synthesis rather than accelerate it (20–22). The difference between the response of the immunologically active cell producing antibody against the cationic protein and the response of other cells to this protein might give an indication of the role of such proteins as regulators of protein synthesis. All the foregoing considerations add to the significance of cationic proteins as elements of membranes bounding cellular organelles. The antigenic properties of these membrane proteins are of interest in the study of the origin of the autoimmune response.

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