BIOCHEMICAL AND GENETIC STUDIES OF PERIPHERAL MYELINATION IN NORMAL DEVELOPMENT AND IN THE MOUSE MUTANT TREMBLER

Thesis by

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ABSTRACT

I developed a radioimmunoassay for P_0, the major peripheral myelin protein, and adapted an existing radioimmunoassay for myelin basic protein. Results from these assays showed that Schwann cells do not make either protein before myelination begins, and accumulate P_0 and myelin basic protein with the same time course during development. Schwann cells cultured in the absence of neurons do not express detectable levels of either protein, but they do continue to synthesize sulfatide, a myelin sulfolipid, apparently indefinitely, as shown by biosynthetic labeling.

The trembler (Tr/+) mouse mutant has a pronounced reduction in peripheral myelin, while central myelin and peripheral unmyelinated nerves appear normal. This myelin deficiency is caused by an autonomous Schwann cell defect. Since the Trembler phenotype is expressed in heterozygotes, most previous studies were confined to Tr/+ mice. Using two alleles, I show here that the six possible genotypes can be ordered as follows: +/+ > Tr^j/+ > Tr/+ > Tr^j/Tr^j > Tr^j/Tr > Tr/Tr, based on myelin basic protein radioimmunoassays of sciatic nerve extracts. The amount of compact myelin in each genotype, as shown by electron microscopy, is in good agreement with the radioimmunoassay results, with two important provisos. First, Tr/Tr mice have 1% of wild-type myelin basic protein levels but essentially no compact myelin. Secondly, the first steps in the above genetic series reduce both the

average myelin sheath area and the number of myelin sheaths by similar amounts, while the last steps primarily reduce the number of myelin sheaths. These results suggest that, if the activity of the $\underline{Tr}^{+}$ gene product is reduced below a certain point, myelin protein synthesis can be induced without forming a myelin sheath. Visualization of P_0 by indirect immunofluorescence shows that Schwann cells without compact myelin express much lower levels of P_0 than adjacent Schwann cells, associated with the same axon, that do form compact myelin. Finally, although Trembler Schwann cells proliferate excessively *in vivo*, their proliferation is not affected by *Tr* gene dose. *In vitro* $\underline{Tr^{j}/Tr^{j}}$ Schwann cell proliferation is apparently normal. Thus, it is likely that the function of the $\underline{Tr}^{+}$ gene product is not directly concerned with Schwann cell proliferation.

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INTRODUCTION

Many cellular interactions occur only between physically adjacent cells; examples include synapse formation, the formation of gap junctions, and myelination. We are only beginning to learn the molecular details of these interactions, but it seems likely that some of the details will ultimately prove to be of general significance. I have chosen to study the interaction between the axon and the Schwann cell that results in myelination. The dramatic biochemical consequences of this interaction, and the variety of available mutants, are important practical advantages. Moreover, this interaction has two alternative outcomes: the axon may be myelinated or unmyelinated. The axon is known to determine this outcome. These points are discussed in more detail below.

Vertebrate peripheral nerve is composed of two types of nerve axons: rapidly conducting myelinated axons and slowly conducting unmyelinated axons. Myelinated axons are surrounded by a compact, multilamellate myelin sheath, which is formed by a Schwann cell (SC). Groups of unmyelinated axons are enfolded by a layer of SC cytoplasm (1,5).

Myelin functions primarily as an electrical insulator; the presence of this insulation greatly increases the velocity of axonal impulse propagation (2). The synthesis of the myelin sheath results in an enormous and metabolically costly increase in the membrane surface area of the SC (3). Perhaps for this reason, myelination is limited to those axons whose signals must be transmitted rapidly (i.e., motor axons innervating

voluntary muscles, or sensory axons mediating the sensation of "sharp" pain), while less urgent signals (i.e., "dull" pain, or commands to involuntary muscles) are generally carried by unmyelinated axons (4).

Peripheral unmyelinated axons are enfolded by a layer of SC cytoplasm. The functional significance of this relationship is unknown but is usually assumed to be protective or nutritive (6). In contrast, unmyelinated axons in the central nervous system are only occasionally wrapped by astrocytes, and never by oligodendrocytes [the myelin-forming glial cells of the central nervous system; see (6)]. More precisely, a given SC in adult peripheral nerve is associated with either a single myelinated axon, or several unmyelinated axons, but not both (1). Oligodendrocytes are associated only with myelinated axons in the central nervous system (6). Thus, oligodendrocytes may in some sense be committed to form myelin while SCs are not (7). The difference in behavior between SCs and oligodendrocytes *in vivo* has clear molecular consequences *in vitro* (8), as discussed in Chapters 1 and 2 of this thesis.

In the developing nerve, all axon-SC relationships are initially of the unmyelinated type. If an axon is destined to be myelinated, it will become separately enfolded by a SC, which then wraps many times around the axon. The SC membranes in this sheath come into very close and stereotyped apposition [*ca.* 12 nm repeat distance in fixed peripheral myelin; see (5)], to form the mature, compact myelin sheath. It is

possible to surgically confront SCs from one nerve with axons from another nerve, without ambiguity [i.e., without significant amounts of SC migration, proliferation, or ingrowth of axons from other nerves--see (9-12)]. Elegant experiments of this sort have shown that the percentage of axons that are myelinated depends only on the source of the axons, and not on the source of the SCs (9-12). That is, any SC can form (and maintain) myelin but will do so only if continuously induced by the appropriate sort of axon.

Myelin is very plentiful in both the central and peripheral nervous systems. Moreover, the myelin membrane has the highest lipid/protein ratio, and hence the lowest buoyant density, of any membrane in the nervous system (3). These facts allow one to isolate the myelin membrane in high yield and purity by conventional subcellular isolation techniques (3). The protein composition of the myelin membrane, illustrated in Figure 1, is rather simple. The major proteins are found only in myelin. Some of the myelin-specific proteins are shared between central and peripheral myelin (for example, the large and small myelin basic proteins), while others are not (for example, P_0). Specific antisera directed against many of these proteins are available. Those that concern us here are anti-P_0 (13) and anti-myelin basic protein. The latter antibody is often raised against a mixture of large and small myelin basic proteins, as they show almost complete immunological crossreactivity (21). These reagents have been

extensively characterized by indirect immunofluorescence and immunoautoradiography of sodium dodecylsulfate ($NaDodSO_4$)-polyacrylamide gels (13). Using radioimmunoassays based on these sera, I was able to show quantitatively that Schwann cells do not make either protein before myelination begins, and accumulate P_0 and myelin basic protein with the same time course during development (Chapter 2). The levels of both myelin basic protein and P_0 are reduced in the Trembler mutant mouse, a major topic of this thesis. In many experiments I have measured only one or the other of these proteins, however, for technical reasons. Anti-P_0 gives the brightest immunofluorescent labeling, presumably because it is the most abundant peripheral myelin protein (Figure 1) and/or is more accessible to antibody. Anti-myelin basic protein provides the most sensitive radioimmunoassay, perhaps because it is much more antigenic than P_0 (22) and produces higher titer antisera.

Myelin also contains some unusual lipids. One of these, sulfatide, is particularly easy to identify, since it is one of the few lipids that can be biosynthetically labeled with ^{35}S-sulfate. I found that SC cultured in the absence of neurons continue to synthesize sulfatide (Chapter 1). This was the first example of a characteristic myelin component that continues to be expressed by purified SCs in culture.

SCs in the Trembler (Tr/+) mouse have a specific genetic defect in the process of peripheral myelination. The Tr/+ phenotype includes peripheral hypomyelination, reduced axonal

diameter and conduction velocity, and increased SC proliferation (14). Normally unmyelinated nerves, such as the superior cervical trunk, appear completely normal, as does myelin in the central nervous system (14). Surgical experiments, in which SCs of one genotype are confronted with axons of another genotype, have shown that the myelin deficiency in Tr/+ nerve is caused by a defect in the SC and not in the axon (15,16). This conclusion is also supported by tissue culture experiments, in which SCs and neurons of different genotypes are confronted with each other in the absence of fibroblasts (17), as well as by studies of Trembler/wild-type chimeric mice (14).

Although normally unmyelinated nerves in Tr/+ mice appear identical to their wild-type counterparts, if one confronts SCs from these Tr/+ nerves with competent axons (from normally myelinated nerves), the complete Tr/+ phenotype is produced (18). Since these abnormalities are uncovered only if SCs are challenged to myelinate by competent axons, and since the viability of Tr SCs is essentially normal, the Tr defect is apparently concerned in some way with myelination itself (14). It is not known at what stage of myelination the Tr gene acts, although the histological evidence indicates that Tr/+ SCs do wrap competent axons individually and are then blocked, apparently at an earlier stage than other known SC autonomous myelination mutants (14). Nor was it known if the Tr gene product is essential for myelination or not, or, for example,

what the phenotype of a homozygous null mutation at the Tr locus would be. Gene dose experiments should help to answer these questions; Tr homozygotes had not previously been studied at the cellular or molecular levels. In preliminary breeding experiments, I found that the Tr^j/Tr^j genotype is actually lethal. Encouraged by such a clear-cut gene dose effect, I studied this problem more closely, and found that Tr^j/Tr^j mice do live to at least 12 days of age and can be identified at this age by gross nerve appearance. Similar criteria of nerve appearance also apply to the Tr allele. These results allowed me to do detailed gene dose experiments, reported in Chapter 3, which suggest that the Tr gene product is essential for myelination and is not directly concerned with SC proliferation. Immunofluorescence and electron microscopic observations, reported in Chapter 4, support these conclusions and also show an interesting heterogeneity in myelination by Trembler SCs.

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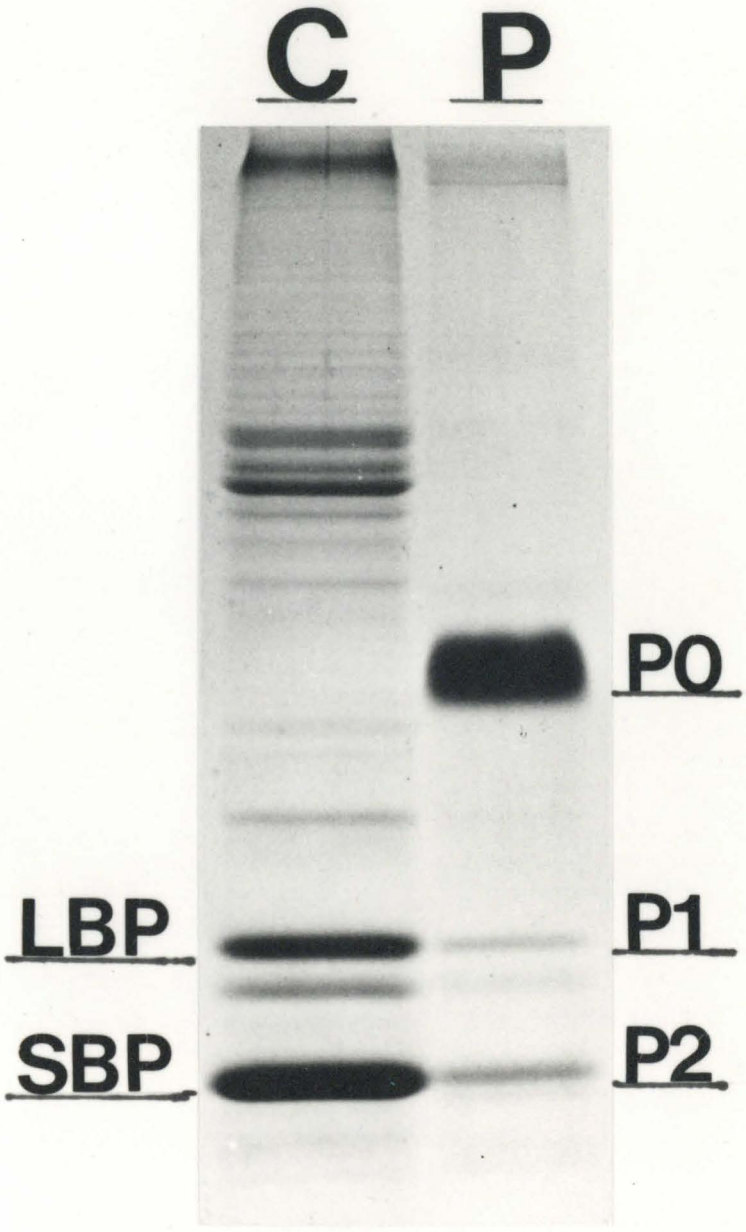
Figure 1. The protein content of purified rat central myelin (C), and rat peripheral myelin (P). Myelin was purified and delipidated by conventional techniques (3), separated by $NaDodSO_4$ polyacrylamide gel electrophoresis, and stained with Coomassie Blue. LBP, large basic protein of central myelin; SBP, small basic protein of central myelin. SBP is related to LBP by an internal deletion of 40 amino acids (3). The basic proteins of peripheral myelin have a more complicated nomenclature. This figure shows a peripheral myelin protein band corresponding exactly in molecular weight to SBP, as well as an extremely faint band of slightly lower molecular weight. The label, "P_2," actually applies only to the latter band (19-21). P_2 represents only about 1% of the protein in rat or mouse peripheral myelin, but is 10% in rabbit peripheral myelin and almost 20% in bovine peripheral myelin (23). P_2 has a basic pI, but has no known structural or antigenic relation to LBP or SBP (3,23). Rat and mouse peripheral myelin also contain two additional basic proteins, however: P_1 and P_r (the latter is shown but not labeled as such), which are apparently identical to LBP and SBP, respectively (3,19). The term "myelin basic protein," as used in the literature and in this thesis, does not refer to all of the basic proteins of myelin, but rather to the subset that react with anti-LBP; namely LBP, SBP, P_1 and P_r. P_0 and P_2 are not found in central myelin. Photo courtesy of J. P. Brockes.

RAT C&P MYELIN



CHAPTER ONE

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Short Communication

Synthesis of Sulfatide by Cultured Rat Schwann Cells

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Abstract: The ^{35}S sulfolipids synthesized by purified cultures of rat Schwann cells, fibroblasts, and a rat cell line (RN2) were studied. Schwann cell ^{35}S sulfolipids were almost entirely [^{35}S]sulfatide, as shown by TLC in two different solvent systems with unlabeled authentic sulfatide run in the same track. RN2 and fibroblasts did not synthesize significant amounts of sulfatide, by the same criteria. Previous studies failed to detect any characteristic myelin components, including sulfatide, on Schwann cells after several days in culture (Brockes et al., 1980*a*; Mirsky et al., 1980). My results show that Schwann cells continue to synthesize some sulfatide in the absence of neurons. **Key Words:** Sulfatide—Schwann cells—Sulfolipids. **Fryxell K. J.** Synthesis of sulfatide by cultured rat Schwann cells. *J. Neurochem.* **35**, 1461–1464 (1980).

Galactosylceramide 3-*O*-sulfate (sulfatide) is considered to be a "myelin-typical" lipid, because it is found in relatively high concentrations in purified myelin, but is probably not specific to myelin (Norton, 1977) or even to the nervous system (Green and Robinson, 1960). The synthesis and accumulation of sulfatide have been correlated with myelination, both *in vitro* and *in vivo* (Silberberg et al., 1972; Norton and Poduslo, 1973; Fry et al., 1972, 1974; Wiggins et al., 1975; Tennekoon et al., 1977), and may become a useful assay for *in vitro* myelination.

Among cultured primary central nervous system (CNS) cells, only oligodendrocytes are sulfatide-positive by indirect immunofluorescence criteria (Raff et al., 1979). In dissociated cultures of the dorsal root ganglion or sciatic nerve, only the Schwann cells (SC) are sulfatide-positive. Not all of the SC are sulfatide-positive, however, and all become sulfatide-negative within a period of 4 days in culture, as determined by immunofluorescence (Raff et al., 1979; Mirsky et al., 1980). [^{35}S]Sulfatide synthesis has been observed in cultures of partially purified oligodendrocytes (Poduslo et al., 1978) and in some clonal cell lines derived from the CNS (Dawson et al., 1977), but not by the fibroblasts, astrocytomas, or neuroblastomas so far examined (Dawson et al., 1972, 1977).

It seems very likely that SC synthesize the sulfatide that is found in peripheral myelin, although this has not been demonstrated directly. Sulfatide may be synthesized (in varying amounts) by all SC. Alternatively, sulfatide synthesis by SC may occur only after interaction with an appropriate class of axon, as seems to be the case for several peripheral myelin proteins (Brockes et al., 1980*a*; Mirsky et al., 1980). I have exploited recently developed techniques for the purification and growth of SC in culture (Brockes et al., 1979) to distinguish these alternatives by studying the sulfolipids synthesized by rigorously purified cultures of SC.

Cultures of RN2 cells and fibroblasts were also studied. RN2 is a clonal cell line derived from an ethylnitroso urea-induced tumor of rat cervical spinal nerve root (Pfeiffer and Wechsler, 1972). It is thought to be a Schwannoma, because of its origin and its synthesis of 2′:3′-cyclic-nucleotide 3′-phosphodiesterase (EC 3.1.4.37) and S100 protein (Pfeiffer et al., 1977). RN2 was included in this study because the question of whether or not it synthesizes sulfatide, a third potential SC characteristic, is presently controversial (Dawson et al., 1977; Lucas et al., 1977). Fibroblasts were included as a control, since present evidence indicates that fibroblasts do not synthesize detectable levels of sulfatide (Dawson et al., 1972).

MATERIALS AND METHODS

Dissociated SC were prepared from neonatal rat sciatic nerve. Such cultures are composed of only two cell types,

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Abbreviations used: CM, chloroform:methanol (proportions by volume); MEM, Modified Eagle's medium; PBS, Dulbecco's phosphate buffered saline without Ca and Mg; SC, Schwann cells. Sulfatide is used in this paper to denote galactosylceramide 3-*O*-sulfate.

SC and fibroblasts (Brockes et al., 1977). Fibroblasts were eliminated by treatments with cytosine arabinoside and monoclonal anti-Thy 1.1 plus complement, essentially as described (Brockes et al., 1979; 1980*a*). This technique produces cultures that are routinely >99.5% SC (Brockes et al., 1979). The SC used in these experiments were cultured for at least 14 days, and passaged 1–3 times. The culture medium was supplemented with a partially purified bovine pituitary factor (Brockes et al., 1979; 1980*b*) in order to stimulate division. This factor was washed out of the medium at the time of isotope addition, or 4 days before isotope addition.

RN2 cells (originally obtained from Dr. S. Pfeiffer) were used at passages 11–17. Fibroblasts were obtained from thigh muscle of neonatal rats, and were used at passages 4–7. All cells were maintained in plastic petri dishes (Falcon), and grown in 10% fetal calf serum in Dulbecco's MEM (Gibco) with added penicillin and streptomycin, in a humidified atmosphere of 10% CO_2 at 36°C.

Confluent cultures were incubated with added Na_2-$^{35}SO_4$ (700–800 Ci/mol; New England Nuclear) for 48 h, washed 3–5 times with HEPES buffered MEM, once with PBS, and removed by gentle rinsing with PBS plus EDTA (5×10^{-4} mol/liter). The cells were collected by centrifugation, resuspended in PBS (0.2–1 ml), and extracted with 20 vol. of CM (1:1). Aliquots for protein analysis were removed either before or after the addition of CM (1:1), stored at 4°C, and assayed essentially by the method of Lowry et al. (1951). The remainder was centrifuged to remove insoluble material, and the pellet was washed once with CM (1:1). Lipids were extracted from the combined supernatants essentially by the method of Folch et al. (1951), except that 0.43% K_2SO_4 was used in place of H_2O, as described by others (Silberberg et al., 1972; Fry et al., 1974; Wiggins et al., 1975). Following the initial solvent partition, the lower phase was re-extracted twice with pure upper phase, and then transferred to a clean test tube and evaporated to dryness under a stream of N_2 gas in a 37°C water bath. The residue was redissolved in CM (2:1). An aliquot of this solution (0.1-ml) was either diluted directly into Aquasol-2 (15 ml; New England Nuclear), or evaporated to dryness and redissolved in Aquasol, and counted in the ^{14}C channel of a scintillation counter.

Another aliquot of the sample was used for TLC. Silica G plates (250 μm; Analtech) were used, in either of two solvent systems: $CHCl_3$-CH_3OH-H_2O (70:30:4 by vol., solvent I), or $CHCl_3$-CH_3OH-15 mol/liter NH_4OH (14:6:1 by vol., solvent II). Solvents were from Mallinckrodt or Matheson Coleman and Bell, and were all reagent grade or better. After solvent development, the plates were marked with radioactive ink and exposed to Kodak X-ray film at −70°C with a DuPont "Cronex" intensifying screen, to detect [^{35}S]sulfolipids. To detect total lipids, the plates were then sprayed with 50% H_2SO_4, charred on a hot plate, and photographed. Preliminary experiments (not shown) demonstrated that the small amount of cellular lipids used here produced negligible charring in the vicinity of the sulfatide bands, and that the sulfatide standard alone produced no image on the autoradiograph. Thus, the standard and unknown could be detected separately even when they were mixed together before spotting, allowing precise comparison of mobilities. Therefore, authentic sulfatide from bovine brain (P-L Biochemicals Inc., Milwaukee, Wisconsin) in CM (2:1) was added to each sample before it was spotted onto the TLC plate.

RESULTS

Both SC and RN2 incorporate significant amounts of ^{35}S into lipid, whereas fibroblasts do not (<2% of SC incorporation per microgram of protein; see Table 1). Considering the ^{35}S specific activity used (Table 1), the normalized ^{35}S incorporation by SC is roughly comparable to the [^{35}S]sulfatide synthesis previously reported in cultures of oligodendrocytes (Poduslo et al., 1978) or cell lines (Dawson et al., 1977), and about an order of magnitude lower than in myelinating spinal cord explants (Fry et al., 1972). The much lower counts consistently obtained in the fibroblast samples show that inorganic ^{35}S contamination of the lipid fractions was negligible, and strongly suggest that the occasional fibroblast contaminating the SC cultures did not affect the results.

After TLC, SC lipids show three bands of ^{35}S sulfolipids. The two major species comigrate precisely with the two bands of authentic sulfatide, in the same track, and in each of two different solvent systems (Fig. 1). The identification of these species as sulfatide rests not only on their TLC mobilities, but also on the fact that they are sulfolipids and are synthesized by SC but not by fibroblasts or RN2. Further structural studies to confirm this identification would be of interest.

RN2 lipids show only one band of ^{35}S incorporation, which does not comigrate with either band of the sulfatide standard (Fig. 1).

TABLE 1. *Incorporation of $^{35}SO_4$ into extractable lipids by various cultured cells*

Sample	Total radioactivity incorporated (c.p.m.)	Protein (mg)	Normalized incorporation (c.p.m./mg)
SC culture 1	302	0.33	915
SC culture 2	267	0.32	834
RN2 culture 1	489	1.52	322
RN2 culture 2	439	1.52	289
Fibroblast culture 1	19	1.18	16

Cultures were incubated with 0.4 mCi of $^{35}SO_4$ per 10 cm petri dish (estimated final specific activity in the media = 60 Ci/mol). Cellular lipids were extracted and counted as described in Materials and Methods. Each culture represents two petri dishes. The total radioactivity incorporated is corrected for background.

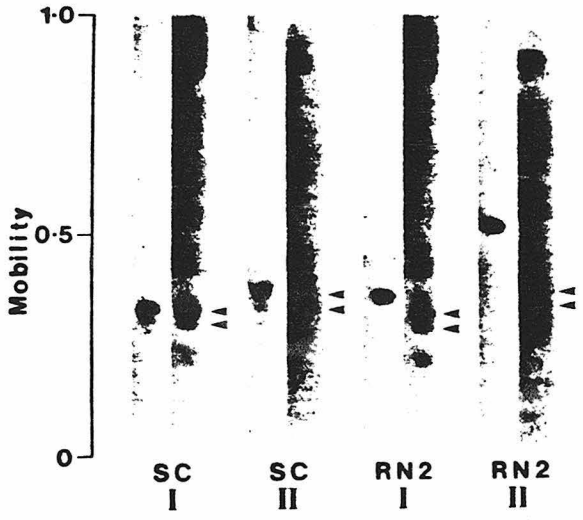

FIG. 1. Thin layer chromatography of sulfolipids from cultured SC and RN2. Cultures were labeled with 2 mCi of $^{35}SO_4$ per 6-cm petri dish and extracted as described in Materials and Methods. Approximately 475 c.p.m. of cellular lipids were mixed with 15 μg of nonradioactive, authentic sulfatide, applied to the TLC plates, chromatographed, exposed to X-ray film for 13 days, and charred as described in the text. From **left** to **right**: SC lipids, developed in solvent I; SC lipids, developed in solvent II; RN2 lipids, solvent I; RN2 lipids, solvent II. Within each column, the autoradiograph (i.e., ^{35}S sulfolipids) is shown on the left, and the same track of the TLC plate after charring (i.e., total lipids) on the right. Arrows show the authentic sulfatide bands on the charred plate. The absolute mobility of the sulfatide bands has varied between experiments (upper band R_f of 0.32–0.37 in solvent I; 0.36–0.36 in solvent II). Thus, the difference in the absolute mobility of sulfatide between the two solvents shown above may not be significant. This does not affect the interpretation of the results, which are based solely on relative mobilities within a given track.

DISCUSSION

In previous studies, Dawson et al. (1977) found that solid tumors of RN2 (or its subclone RN22) did not synthesize significant amounts of sulfatide, and RN22 grown in monolayer culture did not incorporate $^{35}SO_4$ into the sulfatide spots on their TLC system. The general question of whether or not RN2 synthesizes any sulfolipid(s) was apparently not investigated. Lucas et al. (1977) found that RN2 did synthesize detectable amounts of a sulfolipid, which appeared to comigrate with sulfatide in their TLC system. Under somewhat similar conditions, I found that the mobilities were difficult to distinguish unless the standard and unknown were run in the same track. In another solvent system (II), however, the difference in mobility is much greater (Fig. 1). My results thus appear consistent with both earlier reports, and suggest that RN2 makes a single major species of sulfolipid, which is not identical to sulfatide.

Mirsky et al. (1980) found that purified SC lose the ability to be labeled by antibody to sulfatide after a few days in culture. My results show, however, that SC continue to synthesize measurable amounts of sulfatide after more than 2 weeks in culture. Whether the rate of sulfatide synthesis changes with the age of the culture, or some other change is responsible for the immunofluorescence results, is unclear.

Sulfatide synthesis appears to be a long-lasting property of oligodendrocytes in culture as well as SC (Raff et al., 1979; Mirsky et al., 1980). This suggests the need for caution in the interpretation of sulfatide synthesis data from myelinating cultures, as factors other than myelination—e.g., glial cell proliferation—may affect the net rate of sulfatide synthesis.

A continuing interaction with a myelin-inducing axon seems to be required for SC to synthesize detectable amounts of the peripheral myelin proteins P_0, P_1, and P_2 (Brockes et al., 1980*a*; Mirsky et al., 1980). Sulfatide, in contrast, is a characteristic component of the myelin membrane that is synthesized in detectable amounts by SC in the absence of neurons.

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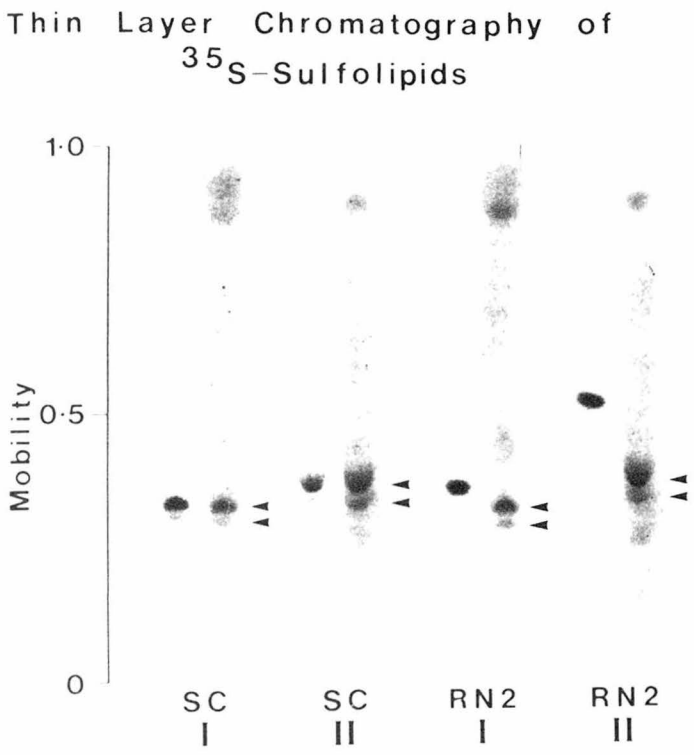

Figure 1. For legend, see page 15.

CHAPTER TWO

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Development and Applications of a Solid-Phase Radioimmunoassay for the P_0 Protein of Peripheral Myelin

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Abstract: This is the first report of a quantitative radioimmunoassay for P_0. The assay uses antigen-coated plastic microwells, with antibody binding detected by ^{125}I-labeled protein A. Either peripheral myelin proteins or purified P_0 may be used as the antigen. Optimal extraction of tissue samples for P_0 immunoassay requires careful attention to the sodium dodecyl sulfate-to-protein ratio. Sodium dodecyl sulfate interference with antibody binding can be minimized by adding an excess of nonionic detergent and carrier protein to the incubation buffer. This method allows the detection of 0.8 ng of P_0 (20 ng/ml). Results from this assay showed little or no immunoreactivity in extracts of brain, central myelin, liver, purified myelin basic proteins, cultured, purified secondary Schwann cells, or membrane preparations from these cells. P_0 was clearly detectable in Schwann cell cultures from 3- to 4-day-old rats at 12–18 h after dissociation (4% of the level in adult sciatic nerve) and in extracts of one-day-old rat sciatic nerve (2% of the level in adult nerve). Myelin basic protein radioimmunoassays showed that the ratio of P_0 to myelin basic protein is essentially constant in extracts of sciatic nerve from one-day-old, four-day-old, and young adult rats. Another result was that P_0 levels are reduced in the trembler mouse sciatic nerve.
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P_0 is the major protein of peripheral myelin (Greenfield et al., 1973; Wood and Dawson, 1973). It accounts for 50–60% of the protein in mammalian peripheral myelin (Greenfield et al., 1973), but has not been detected in central myelin (Braun and Brostoff, 1977; Brockes et al., 1980). P_0 is a glycoprotein with an apparent molecular weight of 28,000–30,000 by sodium dodecyl sulfate (SDS) gel electrophoresis (Brostoff et al., 1975; Benjamins and Morell, 1978; Roomi et al., 1978; Eylar et al., 1979). It is soluble in aqueous solutions of detergents such as SDS or in acidic organic solvents, but not in simple aqueous solutions or in neutral organic solvents (Greenfield et al., 1973; Brostoff et al., 1975).

Previous measurements of P_0 levels have relied on densitometry of SDS-polyacrylamide gels stained with Fast Green (Greenfield et al., 1973; Wiggins et al., 1975) or immunoautoradiography of SDS gels (Brockes et al., 1980). These methods are time consuming, and the former is particularly limited in sensitivity and specificity. So far as we are aware, no quantitative radioimmunoassay (RIA) for P_0 has been described.

Rabbit antisera have been produced against P_0 purified from bovine (Trapp et al., 1979) and rat (Brockes et al., 1980) peripheral myelin. The use of such antisera for quantitative measurement of P_0 levels would be of interest for several reasons. Unlike myelin basic protein assays (Schmid et al., 1974; Cohen et al., 1975; Delassalle et al., 1980; Groome, 1980), which provide a quantitative index of both central and peripheral myelination (Cohen and Guarnieri, 1976), a P_0 assay would be specific for peripheral myelin. An assay for P_0 might be a more sensitive way of detecting peripheral myelin: for example rat peripheral myelin contains roughly 10-fold more P_0 than basic protein[1] (Greenfield et

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Abbreviations used: BSA, Bovine serum albumin; HMEM, HEPES-buffered Eagle's modified minimum essential medium; PBS, Phosphate-buffered saline; PBS/TW, Phosphate-buffered saline plus 0.5 mg/ml Tween 20; RIA, Radioimmunoassay; SDS, Sodium dodecyl sulfate.

[1] The term *myelin basic protein*, as used here, includes the large and small basic proteins of rodent central myelin, as well as the immunologically related basic proteins of rodent peripheral myelin (P_1, P_R, and P_B—see Greenfield et al., 1980; Milek et al., 1981). It does not include the P_2 protein of peripheral myelin, which is basic, but appears to be immunologically unrelated to the above (Milek et al., 1981) and is only a minor component of rat and mouse peripheral myelin (Greenfield et al., 1980).

al., 1973, 1980). The issue of sensitivity is particularly important to us, since we are interested in measuring the production of myelin by cultured rat Schwann cells in the presence and absence of neurons. Finally, measurement of both P_0 and myelin basic protein in the same sample may be useful in some cases.

We therefore set out to define the conditions under which our rabbit antiserum (Brockes et al., 1980) could be used in a quantitative immunoassay. In this report we describe a RIA for P_0, as well as assay results from developing nerve, cultured cells, and the mouse mutant trembler.

MATERIALS AND METHODS

Bovine serum albumin (BSA), essentially globulin-free, polyoxyethylene sorbitan monolaurate (Tween 20), octyl phenoxy polyethoxyethanol (Triton X-100), and gelatin, type I from swine skin, were from Sigma. SDS was from Bio-Rad or Matheson, Coleman and Bell. Phosphate-buffered saline (PBS) was prepared as 0.05 mol/L sodium phosphate pH 7.3, plus 9 mg/ml NaCl and 1 mg/ml NaN_3. PBS plus Tween 20 (PBS/TW) contained 0.5 mg/ml Tween 20. Wistar rats were obtained from Hilltop Lab Animals (Chatsworth, CA), and were bred in this laboratory. Trembler mice (Rex Trembler^J/++ in the C57BL/6J strain) and wild-type controls (C57BL/6J) were obtained from the Jackson Laboratory (Bar Harbor, Maine) and bred in this laboratory.

Myelin basic proteins, purified from rat brain, were a gift from Dr. D. McFarlin. Anti-P_0 was prepared by immunizing rabbits with the P_0 band from SDS gels of rat peripheral myelin proteins and was the same reagent as previously described (Brockes et al., 1980, 1981; Mirsky et al., 1980).

Protein A iodination

Staphylococcus aureus protein A (Pharmacia) was iodinated by the chloramine T method essentially as described (Brockes et al., 1980), except that the ^{125}I-labeled protein A was desalted by centrifugation (Neal and Florini, 1973) through a small column of Sephadex G-25 medium beads (50–150 μm, Sigma). The specific activity of the ^{125}I-labeled protein A was determined by precipitation of an aliquot of the iodination mixture (prior to desalting) with 10% ice-cold trichloroacetic acid. The specific activity was typically 300–350 Ci/mmol. In more recent experiments, protein A has been iodinated to a higher specific activity (2000–3500 Ci/mmol), by iodinating tenfold less protein A (5 μg) with slightly more chloramine T and ^{125}I as described (Bolton, 1977). The reaction was quenched with tyrosine and desalted by centrifugation as described above. Both preparations of ^{125}I-labeled protein A were 50–60% retained by rabbit immunoglobulin G coupled to Sepharose 4B.

Myelin preparation

Peripheral myelin was prepared from rat sciatic nerves (Pel-Freez Biologicals, Rogers, Arkansas) by a shortened version of the method of Everly et al. (1973); two discontinuous sucrose gradient steps and two osmotic shock steps were generally used. The myelin was lyophilized, delipidated with 2:3 CH_3CH_2OH:$(CH_3CH_2)_2O$ (vol/vol) and $(CH_3CH_2)_2O$ (Everly et al., 1973), dried with a stream of N_2 gas or brief lyophilization, and suspended in 2.5 mg/ml SDS. The SDS solution was then immersed in a boiling water bath for 60 s and centrifuged to remove insoluble material (Eppendorf microfuge; 15,600 g × 1 min). The supernatant was stored at −20°C until needed.

Solubilized peripheral myelin proteins were boiled for 4 min in sample buffer and electrophoresed in discontinuous, Tris-buffered 15% polyacrylamide SDS slab gels essentially as described (Hubbard and Lazarides, 1979). The gels were stained with Fast Green and scanned at 580 nm (Greenfield et al., 1973) for determination of the proportion of P_0. No attempt was made to correct for differences in dye-binding capacities between proteins. A shoulder presumably representing Y was sometimes observed and was included in the P_0 peak when we quantitated these gel-scans, as was also done by Greenfield et al. (1973). Cammer et al. (1980) recently found that the Y band, which has a slightly lower apparent molecular weight than P_0 (Benjamins and Morell, 1978), represents unreduced P_0.

Peripheral myelin proteins, solubilized and assayed as described, typically contained 2.5–7.5 mg/ml protein, of which about 30% was P_0. These myelin proteins can be used as a source of P_0 antigen for antibody competition and for coating plastic microwells (see below). A preliminary experiment indicated that repeated freeze-thawing of solubilized peripheral myelin did not reduce its ability to compete for anti-P_0 binding. Therefore, all solubilized samples were stored at −20°C between assays.

Central myelin was prepared from adult rat brain (dissected as described under Sample preparation) by the method of Norton and Poduslo (1973). The central myelin was then lyophilized, delipidated, and solubilized as described above for peripheral myelin, except that 1, rather than 2.5, mg/ml SDS was used.

Purified P_0 preparation

Solubilized rat peripheral myelin protein (2 mg) was electrophoresed on a preparative scale (13 cm wide × 7 cm × 0.5 cm), 15% polyacrylamide, SDS gel. The P_0 band could be visualized directly; its location was checked by staining a side track. The band was cut out, electroeluted, and concentrated (Amicon B15). The purified P_0 showed a single major band by analytical SDS-gel electrophoresis. The only visible minor bands, at approximately 60,000 and 90,000 daltons, presumably represented aggregates of P_0. Densitometry of these gels showed that 63% of the Fast Green stain was well localized to the P_0 band.

Sample preparation

Adult rats and mice were anesthetized with ether and killed by cervical dislocation. Various tissues were dissected out and stored at −70°C. When dissecting out rat brain, a slice of only the dorsal-most cerebral cortex (white and grey matter) was taken so that there would be no contamination with the peripheral myelin of cranial nerves.

After thawing, the tissues were homogenized with a motor-driven glass-on-glass homogenizer (Kontes) in 40 volumes (40 ml/g wet weight) of 1 mg/ml SDS. The homogenates were then immersed in a boiling water bath for 60 s and centrifuged to remove insoluble material (15,600 g × 1 min). The supernatant was stored at −20°C until needed.

Dissociated Schwann cells were prepared from

neonatal rat sciatic nerve. Sciatic nerve cultures are composed of only two cell types, Schwann cells and fibroblasts (Brockes et al., 1977). The fibroblasts were eliminated by treatments with cytosine arabinoside and monoclonal anti/Thy 1.1 plus complement, essentially as described (Brockes et al., 1979, 1980). We used nerves from 3- to 4-day-old rats, as they yield more Schwann cells than those of 1- to 2-day-old rats. Some of the nerves used in this study were stored in liquid N_2 before dissociation for cell culture. This maneuver selects against fibroblasts.

Cultured Schwann cells were rinsed 3 times in PBS without Ca and Mg (GIBCO), incubated for 10 min at room temperature in PBS without Ca and Mg plus 5×10^{-4} mol/L EDTA (Sigma), and removed by gentle rinsing. The cells were collected by centrifugation, sonicated for 5 s (Virtis sonicator model no. 16-850) in PBS plus 1 mg/ml SDS, and stored at −20°C. After thawing, the extracts were boiled and centrifuged as described above for tissue samples.

Schwann cell membranes were prepared by the Tween 40 method of Standring and Williams (1978) except that the cells, which were removed with EDTA as above, were suspended in Tris saline buffer (Standring and Williams, 1978) at 3×10^6 cells/ml. The final membrane pellet was dissolved by boiling in 1 mg/ml SDS as described above.

Total protein was determined with a BSA standard (Sigma) by the method of Lowry et al. (1951).

Myelin basic protein RIA

Myelin basic protein was assayed essentially as described (Cohen et al., 1975; Delassalle et al., 1980), with minor modifications as described below. The incubation buffer was 0.2 mol/L Tris (pH 7.3) + 0.5 mg/ml histone (from calf thymus, Sigma) + 0.5 mg/ml Tween 20 + 1 mg/ml NaN_3. All tubes in a given assay contained identical SDS concentrations. Bound antigen was separated using heat-killed, formalin-fixed *Staphylococcus aureus* bacteria (Bethesda Research Labs). After the usual antibody incubation, the bacteria were added for 1 h at 4°C, pelleted and washed by centrifugation, and the pellet was counted in a gamma counter.

P_0 Radioimmunoassay

We used two kinds of round-bottomed microtiter plates in the RIA—rigid polystyrene plates (Flow Labs. No. 76-213-05), which require NaOH for removal of the adsorbed protein (see below), or flexible acrylic plates (Flow Labs. No. 76-362-05), which may simply be cut up and counted directly in a gamma counter. During all incubations, the microwells were covered and placed inside a humidified plastic box to minimize evaporation.

Microwells were coated with antigen by diluting peripheral myelin protein in PBS and incubating 30 μl per well (approximately 25 ng of P_0) for 2 h at 37°C. Competing, soluble antigen and antibody were preincubated together for 1.5 h at room temperature in uncoated microwells. The preincubation solution contained the following: anti-P_0 serum (×533), Triton X-100 (1 mg/ml), BSA (1 mg/ml), SDS (0.1 mg/ml), and competitor in a final volume of 40 μl of PBS/TW. The final SDS concentration, including the SDS present in the competitor supernatant, was always constant within a given assay. Each competitor dilution was usually done in triplicate.

The coated microwells were washed three times with PBS/TW, and 30 μl from each preincubated antibody-competitor microwell was transferred to a corresponding coated microwell. These were incubated for 18 h at 4°C. They were then washed three times with PBS/TW for removal of unbound antibody, and 100,000 cpm of ^{125}I-labeled *S. aureus* protein A (300–350 Ci/mmol, about 10^{-8} mol of protein A monomer per L) was added to each well in 30 μl of PBS/TW plus 2.5 mg/ml gelatin. A preliminary experiment indicated that omitting the gelatin significantly increased the amount of nonspecifically bound protein A. After incubation for 1 h at 37°C, the microwells were washed three times with PBS/TW for removal of unbound protein A. Bound protein A was solubilized with an excess (125 μl) of 2 mol/L NaOH. After a few minutes (5–10) at room temperature, the NaOH solution was transferred quantitatively to plastic tubes and counted in a gamma counter. Alternatively, when flexible microtiter plates were used, the wells were simply cut out and counted directly.

Another P_0 RIA protocol was used in recent experiments to increase sensitivity. Anti-P_0 was used at a final dilution of ×21,333 and was preincubated with competitor (and the usual detergents, etc.) for 6 h at 4°C. Coating and overnight incubation were the same as previously. 100,000 c.p.m. of ^{125}I-labeled protein A (2000–3500 Ci/mmol, about 10^{-9} mol/L) was incubated in each microwell in 30 μl of PBS/TW plus gelatin for 2 h at room temperature, and the bound ^{125}I-labeled protein A radioactivity was determined as above.

RESULTS

Assay methods

Anti-P_0 binding to antigen-coated microwells and its dependence on antibody concentration are shown in Fig. 1. Maximal binding was reached at an antibody dilution of about ×5 (earlier experiments showed a more abrupt plateau than Fig. 1). Binding was reduced twofold at an antibody dilution of approximately ×100 and fourfold at approximately the antibody dilution (×533) that was routinely used for RIA. Binding by normal rabbit serum was negligible (see, for example, Fig. 2). Anti-P_0 binding to uncoated wells or to wells coated with BSA or myelin basic protein was less than or equal to normal serum binding (not shown).

Experiments on the time course of antibody binding to coated microwells (not shown) indicated that, at an antibody dilution of ×21,333, binding was complete within about 2 h at 4°C. We used an 18-h incubation at 4°C in the experiments described in this paper, however, partly for convenience.

SDS-solubilized peripheral myelin proteins were generally used to compete with the myelin protein-coated microwells for anti-P_0 binding. The P_0 content of the myelin protein was measured by densitometry of Fast Green-stained gels. The myelin protein was also compared with purified P_0 in the RIA (see below). Our standard assay protocol gives a working range of about 1 to 100 ng of P_0 (Fig. 2A). To determine the threshold sensitivity, we com-

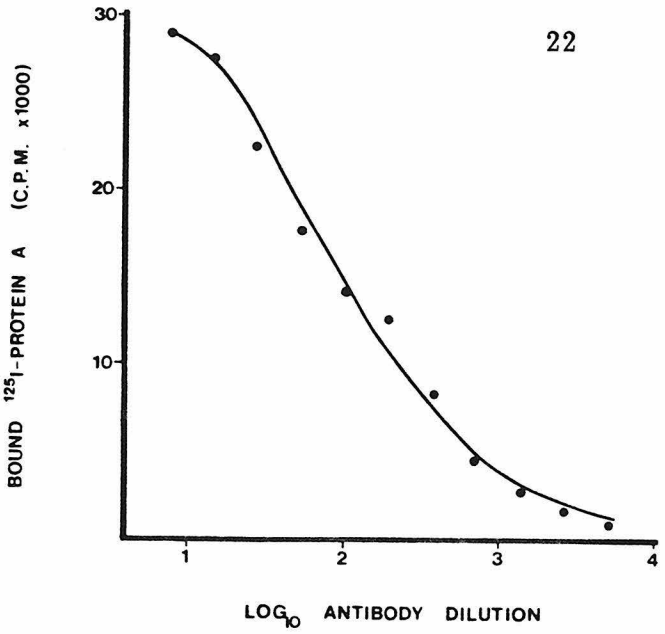
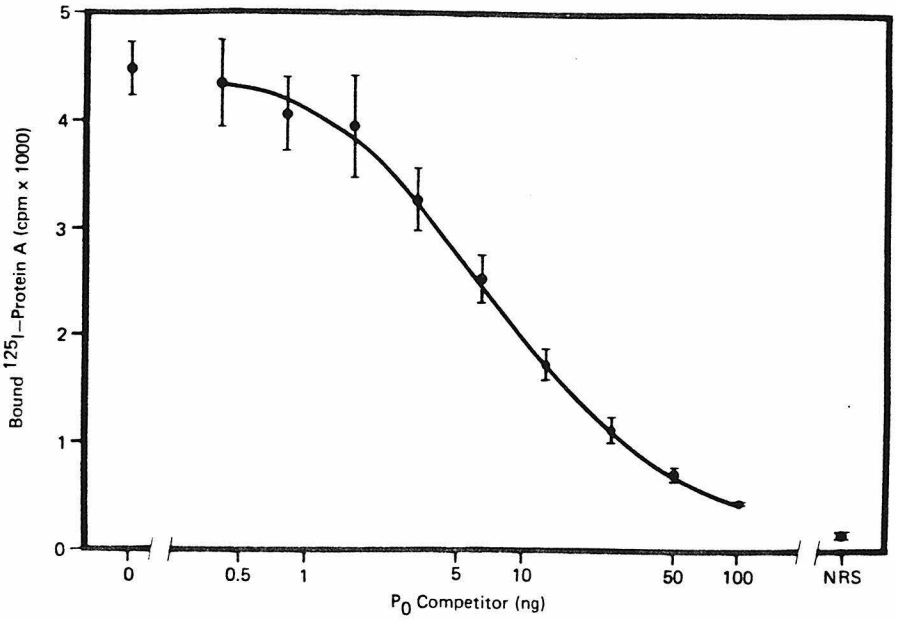


FIG. 1. Anti-P_0 titration. Various dilutions of antibody were preincubated without competitor (but with detergents) and then incubated in coated microwells for 18 h at 4°C as described in Materials and Methods. After washing to remove unbound antibody, each microwell was incubated with 317,000 cpm of ^{125}I-labeled protein A (about 3×10^{-8} mol/L) for 1 h at 37°C and processed as described in Materials and Methods. Preliminary experiments (not shown) indicated that this amount of protein A was not limiting at the highest antibody concentration used. Each point represents the mean of three determinations; standard deviations averaged 4% of the mean and were all less than 10% of the mean.



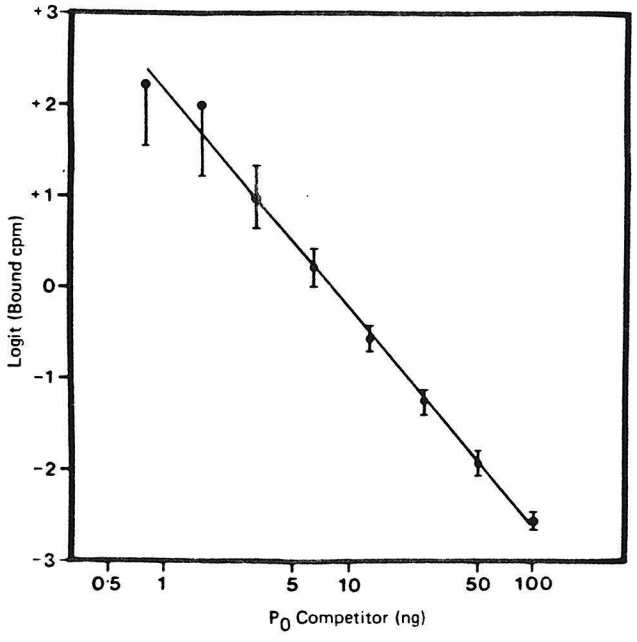


FIG. 2. Standard competition curve. Anti-P_0 was preincubated with various dilutions of peripheral myelin proteins containing the indicated amount of P_0. Samples were subsequently incubated and processed as described in Materials and Methods. (**A,** above) Raw data showing the cpm of ^{125}I-labeled protein A bound versus the amount of soluble P_0 competitor on a log scale. Each point represents the mean ± SD of 12 determinations. NRS, normal rabbit serum (rather than anti-P_0). (**B,** left) The same data linearized by applying the logit transform to the bound cpm Logit (B) = ln(B/1-B), where B = (cpm of sample − cpm normal serum)/(cpm of 0 competitor − cpm normal serum). Each point represents logit (mean cpm); the error bars indicate logit (mean cpm ± SD). These error bars are not symmetrical because of the mathematics of the logit transform.

pared several dilutions of peripheral myelin competitor with wells incubated without competitor. P_0 at 1.6 or 0.8 ng was significantly different from no competitor, by Student's t test ($p < 0.01$, $n = 12$ for each dilution), but P_0 at 0.4 ng was not ($p > 0.3$, $n = 12$). A second RIA protocol, with a much higher antibody dilution and a longer preincubation at lower temperature (see Materials and Methods), gave slightly less than twofold extra sensitivity based on 3–4 standard curves from each protocol.

Although the standard curves were linear throughout much of their range on plots of specifically bound ^{125}I-labeled protein A versus log competitor, the logit transform (Rodbard et al., 1968) provided a somewhat better fit to the data and was used for all data reduction (Fig. 2B). A desk-top computer (Hewlett-Packard 9820A) was programmed to perform a linear regression fit to the logit-transformed standard curve, plot the results, and print out the estimated P_0 content of the unknowns.

Myelin proteins and purified P_0 were compared as soluble competitors by use of myelin-coated wells and gave parallel straight lines (Fig. 3). Wells coated with purified P_0 gave similar results. Thus none of the myelin components remaining after delipidation interfere with antibody binding to P_0.

Anti-P_0 binding was strongly inhibited by 0.1 mg/ml or more SDS. When additional nonionic detergent (Triton X-100) and carrier protein (BSA) were added, inhibition by SDS was reduced (Fig. 4). It seemed desirable to be able to assay weakly reactive samples at the lowest possible dilution and, hence, the highest possible SDS concentration. Therefore, we routinely performed the RIA with 0.1 mg/ml SDS, 1 mg/ml Triton X-100, and 1 mg/ml BSA.

Some detergents do not interfere with antigen-antibody binding to the same extent as SDS. Preliminary experiments showed that, even at a concentration of 40 mg/ml, sodium deoxycholate interfered only slightly (77% of control c.p.m.) and Triton X-100 not at all (103%). On the other hand, lithium diiodosalicylate interfered strongly (51% of control c.p.m. at 1 mg/ml). Therefore, it was of interest to see if high concentrations of nonionic detergents or sodium deoxycholate would extract P_0 quantitatively from tissue samples. Rat sciatic nerves (Pel-Freez) were homogenized in 12 volumes of H_2O, and aliquots of the homogenate were centrifuged after being boiled for 1 min in 2% of one of the following detergents: SDS, sodium deoxycholate, NP40, Triton X-100, or Tween 20. After P_0 RIAs (run separately in SDS, deoxycholate, or Tween 20 with controls for detergent interference), it was found that SDS extracted two- to fourfold more P_0 immunoreactivity than Tween 20, Triton X-100, or sodium deoxycholate, and about 10-fold more than NP40.

When rat sciatic nerve homogenates were extracted with several concentrations of SDS and assayed by SDS gel electrophoresis and densitometry, maximal amounts of P_0 were extracted at an SDS/total protein ratio of 1.5 to 2.0 or more. Maximal

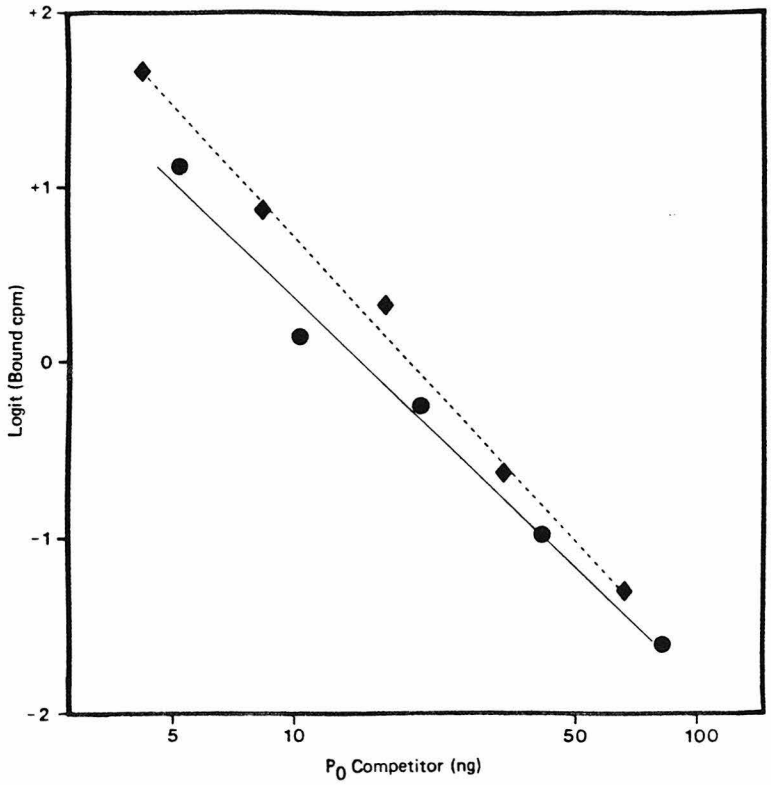


FIG. 3. Comparison of peripheral myelin proteins (●) and purified P_0 (◆) as soluble competitor in the RIA. Samples were prepared and assayed (in peripheral myelin protein-coated wells) as described in Materials and Methods. The P_0 content (abscissa) was measured by densitometry of SDS gels. Data points represent the mean of two separate experiments, each run in triplicate. Lines calculated by the method of least squares are shown for both myelin proteins and purified P_0. Neither slope was significantly different from the theoretical value of −1 ($p > 0.1$ by Student's t test). The purified P_0 showed only 75% of the expected immunoreactivity, presumably due to the unavoidable exposure to excess SDS during electroelution (see text). An experiment using wells coated with purified P_0 gave similar results (not shown).

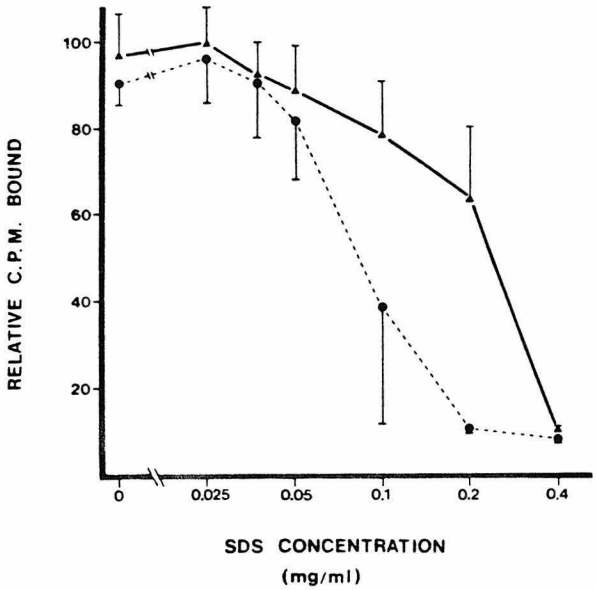


FIG. 4. Detergent effects on anti-P_0 binding. Anti-P_0 was preincubated with the indicated concentration of SDS in the absence of competitor either with (▲) or without (●) 1 mg/ml Triton X-100 and 1 mg/ml BSA. Other details of incubation and processing were as described in Materials and Methods. Each point represents the mean ± S.D. of a total of 12 determinations from two experiments. The experiments were combined by arbitrarily scaling the mean cpm bound at 25 μg/ml SDS, with Triton X-100 and BSA, to 100.

amounts of immunoreactivity, assayed by P_0 RIA, were extracted at a lower SDS/protein ratio (0.4 to 0.8). These observations suggested a compromise extraction protocol aimed at achieving an SDS/total protein ratio of 0.4 to 0.8.

Assay results

Assays of central myelin or brain extracts (Table 1) gave less than 0.1% of the P_0 level in peripheral myelin (Greenfield et al., 1973), providing further evidence that P_0 is confined to the peripheral nervous system (Trapp et al., 1979; Brockes et al., 1980). Liver extracts or purified myelin basic protein showed little or no P_0 immunoreactivity.

Schwann cells cultured for more than one week or membrane preparations from these cells gave P_0 RIA results similar to the above negative controls (Table 1), confirming that Schwann cells do not synthesize detectable amounts of P_0 under standard culture conditions (Brockes et al., 1980; Mirsky et al., 1980). P_0 was clearly detectable in Schwann cells cultured for only 12 to 18 h, however, as expected from the fluorescence results of Mirsky et al. (1980).

Extracts of sciatic nerves from 1-day-old, 4-day-old, or young adult rats gave P_0 levels (as a percent of total protein) of 0.2%, 2%, and 9%, respectively (Table 1). The assay was linear within the range of nerve extract dilutions examined (Fig. 5). Myelin basic protein RIAs revealed that the ratio of P_0 to basic protein did not change significantly with developmental age (Table 1).

In preliminary RIAs of adult mouse sciatic nerve, P_0 levels were 24 ± 4% of total protein in wild-type nerve and 4 ± 0.4% in trembler nerve (mean ± SEM, n = 2 for each genotype) based on the usual rat myelin standard. These mouse nerve extracts were also assayed directly by densitometry of SDS gels (see Materials and Methods), which gave 23 ± 1% P_0 for wild-type and 7 ± 0.4% for trembler. The cross-reaction of our antiserum with mouse P_0 is apparently nearly complete.

DISCUSSION

The specificity of our rabbit anti-rat P_0 antiserum has already been extensively characterized by immunofluorescence as well as immunoautoradiography of SDS gels (Brockes et al., 1980; Mirsky et al., 1980). Immunoreactivity on SDS gels was limited to the P_0 band[2]. In the sciatic nerve, immunoreactivity increased dramatically during development. In the cervical sympathetic trunk, only occasional P_0-positive fibers were observed. No immunoreactivity was detected in brain by either technique.

Results in this report provide additional confirmation of the specificity of our anti-P_0 serum (Table 1). Anti-P_0 binding to uncoated wells or to wells coated with BSA or myelin basic protein was negligible. RIAs showed negligible levels of P_0 immunoreactivity in brain, central myelin, liver, and purified myelin basic protein.

In preliminary experiments, it proved difficult to purify large amounts of P_0 from the rat peripheral nervous system. We therefore found it more convenient to use purified peripheral myelin as a source of P_0 for coating the wells and for soluble competitor. The use of impure antigen for these purposes is justified only if the antiserum is monospecific for P_0, but the results in this and the preceding report (Brockes et al., 1980) support this assumption. Further, the use of purified P_0 rather than myelin proteins does not alter the standard curve significantly (Fig. 3).

Initial experiments with ^{125}I-labeled P_0 (specific activity about 3 μCi/μg) did not show a significant amount of radioactivity specifically bound by anti-P_0, under any of several conditions. The reason for this is unclear. Solid phase assays do avoid the possibility of iodination damage to the antigen, however, and the use of excess solid phase antigen may increase the signal-to-noise ratio in cases of low antibody titer (see also Lessard et al., 1979). Several solid phase assays gave better results with

[2] Another very minor band with a molecular weight slightly lower than P_0 was also labeled. It may be a breakdown product of P_0 (Eylar and Roomi, 1978; Brockes et al., 1980) or unreduced P_0 (Cammer et al., 1980).

TABLE 1. *P_0 immunoreactivity in extracts of rat tissues and cultured rat Schwann cells*

Sample	P_0 immunoreactivity (μg/mg protein extracted)	P_0/MBP immunoreactivity (μg/μg)	Number of samples	Total protein extracted (mg/ml)
Young adult sciatic nerve	87 ± 4	4.7 ± 0.8	4	1.3 ± 0.1
4-day postnatal sciatic nerve	19 ± 2	4.4 ± 0.2	3	1.5 ± 0.04
1-day postnatal sciatic nerve	1.8 ± 0.6	4.7 ± 0.8	3	1.6 ± 0.1
Schwann cells, cultured 12 to 18 h	3.7 ± 0.6		4	1.0 ± 0.3
Schwann cells, cultured 8 days	<0.6[a]		3	2.6 ± 0.3
Schwann cells, cultured >7 days, membrane preparation	<0.6[a]		2	0.28 ± 0.05
Brain	<0.6[a]		3	2.0 ± 0.2
Central myelin	<0.6[a]		3	0.48 ± 0.10
Liver	<0.6[a]		3	3.8 ± 0.3
Myelin basic proteins	0		1	2.5

Samples were solubilized and assayed as described in Materials and Methods. The results are expressed as means ± SEM for the indicated number of samples. For young adult sciatic nerve, we used one nerve per sample from 2 300–330 g female rats. For 1-day and 4-day sciatic nerves, we pooled nerves from 6–7 animals per sample. Schwann cells were obtained from 3- to 4-day-old rat sciatic nerve as described in Materials and Methods. Total protein was measured by the method of Lowry et al. (1951), except for myelin basic proteins, which were measured gravimetrically. MBP, myelin basic protein.

[a] Mean values were less than 0.6 μg/mg, but not necessarily significantly different from 0.6 μg/mg. The actual values were: Schwann cells, 0.3 ± 0.1; Schwann cell membranes, 0.5 ± 0.2; brain, 0.2 ± 0.002; central myelin, 0.3 ± 0.01; liver, 0.1 ± 0.02.

antigen covalently linked to diazo paper or Sepharose beads, noncovalently bound to concanavalin A-conjugated Sepharose beads, or passively coated onto plastic microwells. The passive-coating method was chosen because of its convenience.

The extraction of P_0 with SDS involves an apparent conflict between solubilization and retention of antigenicity. Loss of antigenicity after extraction with higher amounts of SDS is unlikely to be due to proteolysis, since the amount of P_0 observed by SDS-gel electrophoresis and densitometry is gener-

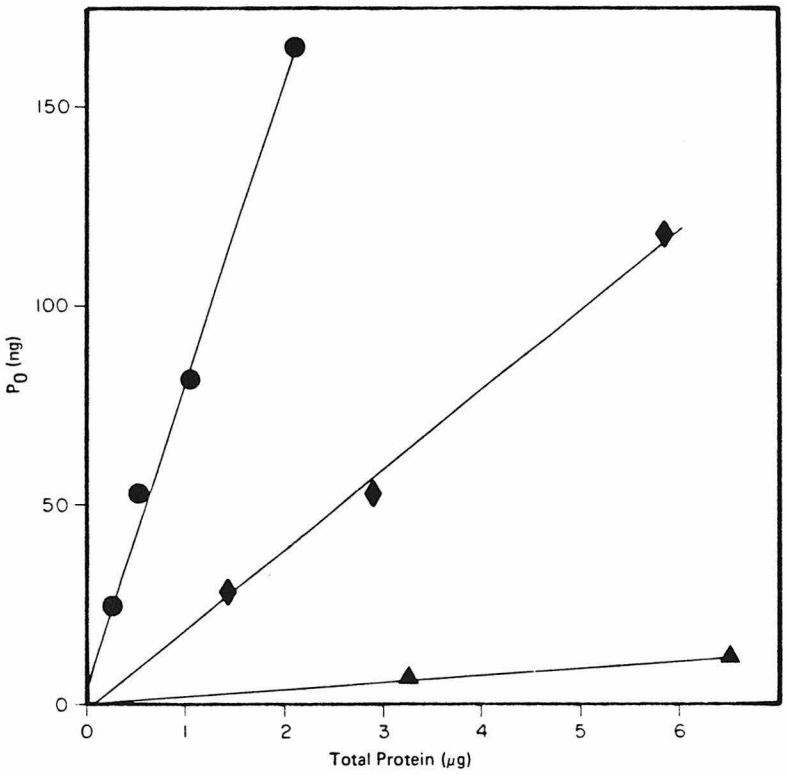


FIG. 5. The linearity of P_0 RIA values from nerve extracts. Samples were prepared and assayed as described in Materials and Methods. Each point represents the mean of determinations on 3 or 4 samples. Lines calculated by the method of least squares are shown for each set of points. ●, young adult rat sciatic nerve; ◆, 4-day rat sciatic nerve; ▲, 1-day rat sciatic nerve.

ally *increased* (to 12–20% of total protein) under these conditions. Direct interference by SDS with the antigen-antibody reaction can be ruled out, since the SDS concentration was standardized within the assay, and in any case additional SDS interference would *increase* the apparent P_0 immunoreactivity. The remaining possibilities include interference by the large amount of extracted lipids and denaturation of P_0 during extraction.

Preliminary experiments with delipidation and/or other detergents, however, were not promising. Therefore, we decided to use intermediate levels of SDS for P_0 extraction. The amount of P_0 immunoreactivity extracted from adult rat sciatic nerves by this method (87 μg/mg; see Table 1) is only 62% of the value reported by Wiggins et al. (1975), who used SDS-gel electrophoresis and densitometry. Our own observations on nerves extracted with high levels of SDS are similar to those of Wiggins et al. (1975). This suggests that, at the moderate levels of SDS optimal for retention of antigenicity, extraction of P_0 from rat nerve is incomplete. The extraction of P_0 is quantitatively reproducible, however (RIA standard deviations between samples were typically 10% of the mean for adult mouse and rat nerve). Preliminary experiments with mouse nerve, using our standard extraction protocol (see Materials and Methods), gave apparently complete extraction of P_0 and complete retention of antigenicity (see Results). This suggests that the difficulty in extracting P_0 may reflect difficulty in adequately homogenizing the larger, tougher rat nerves. In any case, when this assay is used to study a single problem, standardization of the extracted protein concentrations would be desirable.

The presence of SDS in the RIA buffer interferes with antibody binding (Fig. 2). We were able to minimize this interference by adding Triton X-100 and BSA to the RIA incubation buffer. Both the inhibition of antigen-antibody binding by SDS and the reduction of this inhibition by Triton X-100 have been observed previously (Dimitriadis, 1979) and are consistent with other observations (Clarke, 1975; Robinson et al., 1980). This mixture of ionic and nonionic detergents seems to be quite effective in solubilizing proteins (Dimitriadis, 1979) and presumably is sufficient to keep P_0 solubilized.

Our standard RIA protocol is capable of detecting 0.8 ng of P_0 (20 ng/ml), given sufficient replicates. Routine assays with only three data points at each competitor dilution, however, typically gave detection limits of about 1.5 ng P_0. Both our standard protocol and a modified protocol that was roughly twofold more sensitive (see Results) could detect P_0 clearly in extracts of rat sciatic nerve at one day after birth, when myelination only just begins. Variation of readings between assays was usually less than 10% of the mean if the unknowns could be measured at several dilutions within each assay.

The dependence of P_0 levels in rat sciatic nerve on age (Table 1) is similar to that reported previously by others (Wiggins et al., 1975; Brockes et al., 1980). The ratio of P_0 to myelin basic protein did not change with developmental age (Table 1). This indicates that the synthesis of these proteins may be initiated at the same early stage of myelination, contrary to previous suggestions (Mirsky et al., 1980).

P_0 was clearly detectable in Schwann cells after 12 to 18 h in culture, but not after 8 days in culture (Table 1). These results support previous reports (Brockes et al., 1980; Mirsky et al., 1980) that rat Schwann cells synthesize myelin proteins only when they are specifically signaled to do so by the appropriate class of axon (Brockes et al., 1981), although we cannot yet rule out the possibility that Schwann cells stop synthesizing myelin proteins artifactually as a result of exposure to standard cell culture conditions. This RIA should prove to be useful for studying the induction of P_0 synthesis in cultured Schwann cells.

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CHAPTER THREE

THE TREMBLER LOCUS OF THE MOUSE: TWO SEMIDOMINANT ALLELES DETERMINE PERIPHERAL MYELINATION WITHOUT A DIRECT EFFECT ON SCHWANN CELL PROLIFERATION

ABSTRACT

Trembler mice show a pronounced reduction in peripheral myelin, while central myelin and peripheral unmyelinated nerves appear normal. This myelin deficiency is caused by an autonomous Schwann cell defect. Falconer found in 1951 that Tr/+ and Tr/Tr mice could not be distinguished by behavior or fertility; more recent studies have used Tr/+ mice. Gene dose studies at the cellular and molecular levels, however, should be very informative. I show here that Tr/Tr mice have a 10-fold lower level of myelin basic protein (in the sciatic nerve at 12 days of age, as measured by radioimmunoassay) than Tr/+ mice, and 100-fold lower than +/+ mice. A similar result was obtained with a second allele, Tr^j, whose effects are 2-3 fold weaker in each case. These two mutations do not complement each other. That is, the six possible genotypes can be ordered as follows: +/+ > Tr^j/+ > Tr/+ > Tr^j/Tr^j > Tr^j/Tr > Tr/Tr, allowing a detailed titration of the extent of myelination over a 100-fold range. Although Schwann cell proliferation is increased in Trembler nerves, there is little or no difference in this respect between Tr^j/+ and Tr^j/Tr^j nerves. These results indicate that the Trembler gene product is essential for peripheral myelination and is not directly concerned with Schwann cell proliferation. Curiously, Tr^j/Tr^j mice die at 12-19 days of age, while Tr/Tr mice have apparently normal viability.

INTRODUCTION

Vertebrate peripheral nerve is composed of two types of nerve axons: rapidly conducting "myelinated" axons and slowly conducting "unmyelinated" axons. Myelinated axons are surrounded by a compact, multilamellate myelin sheath, which is formed by a Schwann cell (SC). Groups of unmyelinated axons are enfolded by a layer of SC cytoplasm. In the mature nerve, any given SC is associated with either a single myelinated axon, or several unmyelinated axons, but not both. In the developing nerve, all axon-SC relationships are initially of the unmyelinated type. If an axon is destined to be myelinated, it will become separately enfolded by a SC, which then wraps many times around the axon and synthesizes myelin-specific proteins such as P_0 and P_1 (1-7). It is possible to surgically confront SCs from one nerve with axons from another nerve, without ambiguity [i.e., without significant amounts of SC migration, proliferation, or ingrowth of axons from other nerves--(8-11)]. These elegant experiments have shown that the percentage of axons that are subsequently myelinated depend only on the source of the axons, and not on the source of the SCs (8-11). That is, any SC can form (and maintain) myelin but will do so only if continuously induced by the appropriate sort of axon.

SCs in the Trembler (Tr/+) mouse have a specific genetic defect in this process of peripheral myelination. The Tr/+ phenotype includes peripheral hypomyelination, reduced axonal

diameter and conduction velocity, and increased SC proliferation (15). Normally unmyelinated nerves, such as the superior cervical trunk, appear completely normal, as does myelin in the central nervous system (15). Surgical experiments similar to those discussed above have shown that the myelin deficiency in Tr/+ nerve is caused by a defect in the SC and not in the axon (12-13). This conclusion is also supported by tissue culture experiments, in which SCs and neurons of different genotypes are confronted with each other in the absence of fibroblasts (14), as well as by studies of Trembler/wild-type chimeric mice (15).

Although normally unmyelinated nerves in Tr/+ mice appear identical to their wild-type counterparts, if one confronts SCs from these Tr/+ nerves with competent axons (from normally myelinated nerves), the complete Tr/+ phenotype is produced (16). Since these abnormalities are uncovered only if SCs are challenged to myelinate by competent axons, and since the viability of Tr SCs is essentially normal, the Tr defect is apparently concerned in some way with myelination itself (15). It is not known at what stage of myelination the Tr gene acts, although the histological evidence indicates that Tr/+ SCs do wrap competent axons individually and are then blocked, apparently at an earlier stage than other known SC autonomous myelination mutants (15). Nor is it known if the Tr gene product is essential for myelination or not, or, for example, what the phenotype of a homozygous null mutation at the Tr

locus would be. Gene dose experiments should help to answer these questions, but the only criteria that are readily available to distinguish Tr/+ from Tr/Tr mice are breeding experiments (which are hampered by the low reproductive success of Tr males (17)). In preliminary breeding experiments, I found that the Tr^j/Tr^j genotype is actually lethal. Encouraged by such a clear-cut gene dose effect, I studied this problem more closely, and found that Tr^j/Tr^j mice do live to at least 12 days of age and can be identified at this age by gross nerve appearance. Similar criteria of nerve appearance also apply to the Tr allele. These results allowed me to do detailed gene dose experiments, reported below, which suggest that the function of the Tr^+ gene product is essential for myelination and is not directly concerned with SC proliferation (an early step in the axon-SC interaction).

MATERIALS AND METHODS

Genetics. The Tr mutation arose spontaneously in an outbred stock of mice (17) and was later maintained in a partially inbred, triple mutant stock (Tr, Re, and nu; D. S. Falconer, personal communication). I obtained Tr/+ mice from this stock (Institute of Animal Genetics, University of Edinburgh, U. K.), and maintained them by crosses to the C57BL/6J strain as described in the legend to Table 3. The Tr^j mutation arose spontaneously in the DBA/2J strain and was then repeatedly backcrossed to the C57BL/6J strain (18,19). I

obtained Re Tr^j/++ mice (back-crossed to C57BL/6J for a total of 9 generations) and control C57BL/6J mice, from the Jackson Laboratory (Bar Harbor, Maine), and maintained them by interbreeding as described in the text.

Myelin basic protein (MBP) radioimmunoassay (RIA). MBP from rat brain (a generous gift from D. McFarlin) was iodinated with chloramine T to a specific activity of about 50 µCi/ug, using the buffers described by Cohen et al. (20), and then desalted by two centrifugation steps (21) through Sephadex G-25 medium beads (50-150 µm, Sigma). Mouse nerves were homogenized in 1 mg/ml $NaDodSO_4$ / 100 mM LiCl / 0.1 mg/ml NaN_3, and boiled for 4 min. Diluted tissue homogenates were preincubated with antibody (rabbit anti-rat MBP diluted 1:1000) for 6-12 hr in 0.1 ml of the following buffer: 0.2 M Tris pH 7.3 / 2.5 mg/ml calf thymus histone (Sigma) / 0.05% Tween 20 (w/v) / 1 mg/ml NaN_3. All wells within an assay contained the same amount of $NaDodSO_4$; this and all subsequent steps were done at 4°C. Two thousand c.p.m. of ^{125}I-labeled MBP was added to each tube and incubated for an additional 12-24 hr. Ten µl of 5% (v/v) fixed Staphylococcus aureus bacteria (Bethesda Research Labs) was added to each tube, vortexed briefly, incubated for 1 hr, layered over 4 ml of 24% sucrose (in incubation buffer without histones), centrifuged at 1200 g for 30 min, the top 1 ml of solution aspirated, and the pellet (plus remaining sucrose solution) counted in a gamma counter. Residual unbound ^{125}I-labeled MBP was thus left above the sucrose solution, outside

the counting window of the gamma counter. The standard curve used known amounts of unlabeled rat MBP as competitor, and normal rabbit serum to measure non-specific binding of ^{125}I-labeled MBP. The results were processed using an unweighted logit transform, as described (6). The competition of mouse nerve extracts against ^{125}I-labeled rat MBP was linear over the range from 0.3 to 7 ng MBP/ml; all nerve extract data points were confined to the range from 0.3 to 3.5 ng/ml.

In vivo proliferation assay. Sciatic nerves were removed using sterile technique (22) and placed in 0.5 ml of modified Eagle's medium buffered with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) in one well (per animal) of a 24 well plate (Linbro). The nerves were briefly examined under a dissecting microscope, in order to remove fragments of muscle or fat and to record the nerve appearance (see Results). The medium was then changed to 1 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum and 2 µCi of ^{125}I-deoxyuridine (^{125}IUDR; New England Nuclear). The nerves were incubated for 6 hr at 37°C in a humidified atmosphere of 10% CO_2. The nerves were then rinsed three times with phosphate-buffered saline and stored in liquid N_2. After thawing, the nerves were homogenized vigorously, with a motor-driven Teflon pestle, in ice-cold 20 mM ATP. Aliquots of this homogenate were used to measure total DNA (see below). To the remainder of the homogenate were added an equal volume of ice-cold 20% trichloroacetic acid (w/v), and 10 µl of 10 mg/ml bovine serum

albumin in dH_2O. This solution was centrifuged for 2.5 min x 15,000 g at 4°C, and the pellet counted in a gamma counter. The precipitable radioactivity was reduced 90% when 1 mM hydroxyurea, a specific inhibitor of ribonucleotide reductase [and hence of DNA synthesis (23)], was present throughout the 6 hr incubation.

Total DNA assay. DNA levels were measured with Hoechst 33258 (Calbiochem) in a high salt buffer as described (24), with controls for the background fluorescence due to the ATP homogenization solution.

RESULTS

Tr^j/Tr^j mice die before 19 days of age. Tr^j/Tr^j mice were produced in conjuction with the linked genetic marker rex (Re), which produces a wavy coat (25), in the following breeding scheme:

(i) Re Tr^j/++ x Re Tr^j/++ → Re Tr^j/??

(ii) Re Tr^j/?? x ++/++ → Re Tr^j/++ and ??/++

Re Tr^j/++ males and females were mated to each other in cross (i), and the resulting progeny were selected for further breeding experiments only if they showed both Re and Tr^j phenotypes*. The exact genotype of these Re Tr^j/?? progeny was deduced by mating them to wild-type mice in cross (ii). Since both Re and Tr^j are (at least incompletely) dominant, Re Tr^j parents that are homozygous for either mutant gene will yield 100% progeny of the corresponding phenotype; heterozygotes,

50%. Of 61 Re Tr^j/?? parents scored in this way, none were Tr^j/Tr^j, although 22 Tr^j/Tr^j were expected. This difference is highly significant (Table 1). It is also suprising, since Tr/Tr mice are viable and fertile (17). In control matings, Re +/Re + C57BL/6J mice showed apparently normal viability and fertility (not shown), consistent with previous reports (25). Thus the phenotype of Tr^j/Tr^j mice prevented them in some way from completing the above breeding experiment. One would like a stricter accounting, however, of exactly what happened to the Tr^j/Tr^j mice--whether they died, were sterile, or had an unrecognized phenotype that caused them to be not selected for use in cross (ii).

Several lines of evidence indicate that the lack of verified Tr^j/Tr^j adults is not due to sterility. One argument concerns the sex distribution of the Re Tr^j/?? mice that failed to produce scorable progeny. Tr is an autosomal locus. Tr/+ males, but not females, have markedly reduced reproductive success (17), as found in other peripheral neuropathies (38). Thus we would expect many Re Tr^j/?? male parents not to produce scorable progeny in any case. Of the 51 Re Tr^j/?? females tested, however, only three failed to produce scorable progeny. Of these three female parents, two produced apparently normal litters that were cannibalized. Sciatic nerves from one of the latter adult Re Tr^j/?? females were examined histologically and showed a typical Tr^j/+ phenotype (see below). Secondly, a more direct approach is to examine

the phenotypic ratios of all of the progeny of cross (i). If $\underline{Tr^j/Tr^j}$ mice survive, then one would expect the classic 3:1 ratio of Rex mice vs. mice with wild-type fur, while if $\underline{Tr^j/Tr^j}$ die, one would expect a 2:1 ratio (actually 2.048 : 0.952 because of crossing over). This experiment is not difficult if the mice are scored at the age of weaning (19 days or more). The observed ratio at this age (313:160) is in reasonable agreement with a "2:1" ratio and rules out a 3:1 ratio ($P \ll 0.001$ by the chi^2-test).

Similarly, an unexpected phenotype of $\underline{Tr^j/Tr^j}$ mice can be ruled out. If these mice survived but were scored as non-Trembler, then a 3:1 ratio of Rex to non-Rex would be expected. If the $\underline{Tr^j/Tr^j}$ mice were scored as not Rex, then a "2:2" ratio would be expected (the exact value depending on other assumptions), which can also be ruled out. Taken together, these results show that $\underline{Tr^j/Tr^j}$ mice die before 19 days of age.

The results in Table 1 also suggest that the $\underline{Re\ Tr^j/Re}$ + genotype may be partially lethal, and thus that the $\underline{Tr^j}$ allele is associated with one or more recessive lethal mutations lying between $\underline{Re}$ and $\underline{Tr}$ on the genetic map. This effect is at the border of statistical significance, however, and cannot be regarded as well established.

$\underline{Tr^j/Tr^j}$ mice survive to at least 12 days of age. Among the 12 to 16-day-old progeny of cross (i), there is a distinct subset of young with a severe, rigid spastic paralysis, quite

distinct from $\underline{Tr^j}$/+ behavior (which can barely be recognized at this age). These spastic young usually do have the Re/Re phenotype (25) while their littermates usually do not, as expected if the spastic phenotype represents $\underline{Tr^j/Tr^j}$ mice. At 12 days of age, their body weight averages 25% less than that of their littermates. Whole sciatic nerves from these spastic Re/Re mice appear translucent and light yellow-brown when viewed by diffuse transmitted light, while their littermates' nerves are completely opaque and grey to black (Figure 1). Further experiments showed that the "clear nerve" phenotype occurred with the frequency expected of $\underline{Tr^j/Tr^j}$ mice, among the 12- to 13-day-old progeny of many different kinds of crosses (Table 2). This indicates both that there is a 1:1 correspondence between the "clear nerve" phenotype and the $\underline{Tr^j/Tr^j}$ genotype, and that $\underline{Tr^j/Tr^j}$ mice generally live to at least 12 days of age.

All Tr genotypes may be uniquely identified at 12 days of age by behavior and nerve appearance. C57BL/6J progeny of cross (i), or the progeny of Tr/+ hybrids crossed to each other, fall into three non-overlapping classes in terms of sciatic nerve levels of MBP (Table 3). In all cases tested in this way (11/11 mice for $\underline{Tr^j}$; 27/27 for Tr), the class to which each mouse belonged was correctly predicted, based on behavior and nerve appearance.

$\underline{Tr^j}$/+ sciatic nerves appear light to medium grey at 12 days of age, while +/+ nerves appear dark grey to black, when

viewed by diffuse transmitted light. Although this is a subtle difference, it was reinforced by the Re phenotype and by behavior. Tr^j/+ mice show a steady increase in tension of the leg muscles after decapitation, while +/+ mice show only rapid and chaotic kicking. The increase in leg tension is much more pronounced in Tr^j/Tr^j mice, whose nerve appearance also makes them easy to identify (Figure 1). At younger ages (3 or 6 days), the differences in nerve appearance are more obvious, but behavioral differences are not apparent. Deduced genotypes at these ages were verified by histology in 6/6 cases (not shown).

By the same method, Tr/+ sciatic nerves appear much lighter than Tr^j/+ nerves, consistent with their lower myelin content (Table 3). Tr/+ sciatic nerves appear light grey to yellow and are only slightly opaque at 12 days of age. Tr/+ nerves are easy to distinguish from +/+ nerves by appearance alone. Tr/Tr nerves are more transparent than Tr/+, and do not have any grey coloration by transmitted light. This is a subtle distinction, and it is not accompanied by any behavioral differences. With practice, it can be reliably scored, however (see above).

Two arguments suggest that the difference in MBP content between Tr^j and Tr (Table 3) is not an artifact of genetic background. First, +/+ mice from these two genetic backgrounds have similar MBP levels (Table 3). Secondly, the Tr allele was measured on a hybrid genetic background that should be more vigorous than C57BL/6J, and yet showed lower MBP levels.

The allelic difference in MBP content does not explain the death of $\underline{Tr^j}/\underline{Tr^j}$ mice. $\underline{Tr^j}/\underline{Tr^j}$ mice have *more* MBP than $\underline{Tr}/\underline{Tr}$, but $\underline{Tr^j}/\underline{Tr^j}$ die before weaning, while $\underline{Tr}/\underline{Tr}$ mice survive at least to the age of weaning (in the $\underline{Tr}/+$ x $\underline{Tr}/+$ cross described in Table 3, 106/137 surviving progeny had the $\underline{Tr}$ phenotype). Thus the death of $\underline{Tr^j}/\underline{Tr^j}$ mice is not simply due to a lack of peripheral myelin. It is also not due to a lack of central myelin; cerebellar MBP levels in $\underline{Tr^j}/\underline{Tr^j}$ mice are essentially normal (not shown). The possibility that the differences in survival between $\underline{Tr^j}/\underline{Tr^j}$ and $\underline{Tr}/\underline{Tr}$ are due to the different genetic backgrounds has not yet been ruled out.

Schwann cell proliferation. $\underline{Tr}/+$ mice show abnormal SC proliferation which extends into adulthood (40). If the Trembler myelination deficit is caused by abnormal SC proliferation, then $\underline{Tr^j}/\underline{Tr^j}$ mice should have a much higher rate of SC proliferation than $\underline{Tr^j}/+$ mice do. The identification of $\underline{Tr}$ genotype by nerve appearance allows me to use much younger mice than previous studies that relied on behavior to identify genotypes (13). Therefore, I chose relatively short labeling times, to facilitate the measurement of the change in proliferation rates with age. Preliminary experiments showed that intact nerves could be labeled with ^{125}IUDR to an easily detectable level (*ca.* 10,000 cpm per animal) after 6 hr *in vitro*. The rate of incorporation declined somewhat during the 6 hr incubation period (Figure 2A), which presumably reflects the limitations of our culture system in maintaining

intact nerves. Nevertheless, this assay provides a simple, semiquantitative measurement of cellular proliferation.

The rate of proliferation decreased steeply from 3 to 12 days of age, as previously reported (41). At 3 days of age, the high variability between litters (presumably due to variation in developmental age) did not justify any more definite conclusion. In older litters, $\underline{Tr^j}/+$ and +/+ mice differed significantly, particularly in ^{125}IUDR incorporation at 6 days of age and DNA levels at 12 days of age (Figure 2B, 2C). $\underline{Tr^j}/+$ and $\underline{Tr^j}/\underline{Tr^j}$ were not significantly different in either respect.

DISCUSSION

The $\underline{Tr^j}$ mutation was named as an allele of $\underline{Tr}$ based on genetic mapping and histopathologic features (19), but there was previously no basis for testing complementation at this locus. Among the progeny of $\underline{Tr^j}/+$ x $\underline{Tr}/+$ mice, I found a distinct subset of progeny having the "clear" nerve appearance and correspondingly low MBP levels (Table 3). Thus $\underline{Tr^j}$ and $\underline{Tr}$ do not complement each other and are confirmed as being mutations in the same gene, although lack of complementation between these two semidominant alleles could have other interpretations.

The $\underline{Tr^j}$ mutation has several unusual advantages for study in cell culture. Many other cell autonomous, developmental mutants have been identified as such partly because they cause

the degeneration of the cell type in which they are expressed [as in pcd or rd, see (26)] or cause a relatively well-understood molecular deficit [as in shi or twi, see (15,27)]. In contrast, Tr^j/Tr^j SCs are viable, and the molecular basis of their lesion is unknown, so cell culture experiments should be interesting. Powerful methods are available for the purification and maintenance of dissociated SCs in tissue culture (22,28,39). The present study shows that Tr^j/Tr^j nerves may be identified as such before being dissociated or homogenized, so that "genetically pure" cultures of dissociated Tr^j/Tr^j SCs could be grown. Actually, the yield of viable SCs from Tr^j/Tr^j nerves is higher that from wild-type nerves (K. J. Fryxell & J. P. Brockes, unpublished).

The 100-fold deficit of myelination in Tr/Tr mice, shown in this study, strengthens the already interesting parallels between the Tr mutation (on chromosome 11, affects only peripheral myelin) and the jp mutation (on the X chromosome, affects only central myelin). Of the five best-characterized myelin-specific genetic loci in the mouse (15), only these two respect the border between the central and peripheral nervous systems, and it is now clear that mutations at these two loci also have by far the most potent, and the earliest, effects on myelination. All five of these loci are expressed in the glial† cell rather than the axon (15,27,29,30). Moreover, recent work suggests that jp is associated with increased proliferation of oligodendrocytes (the glial cell that

generates myelin in the central nervous system) and roughly normal numbers of oligodendrocytes (31), somewhat similar to Tr. Both jp alleles were described as being recessive (26), and both Tr alleles as being dominant (17,19). It is now clear, however, that Tr is semidominant at the cellular level, while jp is sex-linked and cell autonomous (29). Thus only one copy of the jp gene is active in any given cell, so its dominance at the cellular level is not known and may not reflect any fundamental difference between jp and Tr. In any case, these considerations tend to suggest that many of the glial genes controlling the early stages of myelination are central/peripheral specific, while the glial genes involved in the later steps are not. Such speculation may not be premature, since most of these loci are fairly well characterized and have two mutant alleles with similar phenotypes. Moreover, the only comparably potent dysmyelinating mutation in the rat is specific to central myelin (32). Given the feasibility of the molecular cloning of myelin protein structural genes, these issues should be clarified in the next few years.

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FOOTNOTES

*Since + $\underline{Tr^j}$/+ $\underline{Tr^j}$ progeny will arise only by double crossing over, they should be rare. Given 22% recombination between *Re* and $\underline{Tr^j}$ (33,34), 95% of the $\underline{Tr^j}/\underline{Tr^j}$ mice will also have the Rex phenotype. Thus, the search for $\underline{Tr^j}/\underline{Tr^j}$ progeny was restricted to *Re* progeny of convenience.

†Precisely which kind of glial cell is directly affected by the *jp* mutation is not yet clear (30,35,36).

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Figure 1. Comparison of whole sciatic nerve appearance from a 12-day-old $\underline{Tr^j}/\underline{Tr^j}$ mouse (left) and +/+ littermate (right), as viewed by diffuse transmitted light.

100 µm

Figure 2. Specific DNA synthesis rates and total DNA levels in sciatic nerves from C57BL/6J mice of various ages and genotypes. ^{125}IUDR incorporation into DNA, and total DNA content, were measured as described in Materials and Methods. The genotypes of the mice were identified as described under Results. Each point represents the mean ±SEM of individual determinations on 3-7 mice, with a few exceptions as noted below.

(A) In vitro time course of ^{125}IUDR incorporation. Wild-type nerves were incubated in ^{125}IUDR-containing medium, with or without hydroxyurea, for varying times. Two litters were used; one litter was 6 days old and the other was 8 days old. The data were combined by scaling the maximal ^{125}IUDR incorporation within each litter to 100%. Each point represents 2-3 mice. ▲, no added hydroxyurea; ●, 1 mM hydroxyurea.

(B) Total DNA content as a function of age. Two litters of each age (from cross (i), see Results) were assayed. ▲, +/+; ■, $\underline{Tr^j}/+$; ●, $\underline{Tr^j}/\underline{Tr^j}$.

(C) Specific DNA synthesis rates as a function of age. The mice used were the same as in (B) above, except that for 12-day-old $\underline{Tr^j}/\underline{Tr^j}$ mice, ^{125}IUDR incorporation could only be measured in 2 mice, while total DNA was measured in 3 mice. Variation between litters in ^{125}IUDR incorporation was significant at 3 days of age. To avoid introducing a bias into this limited amount of data, the 3-day-old litters were weighed equally (that is, mean and SEM were calculated between litters rather than between individuals). ▲, +/+; ■, $\underline{Tr^j}/+$; ●, $\underline{Tr^j}/\underline{Tr^j}$.

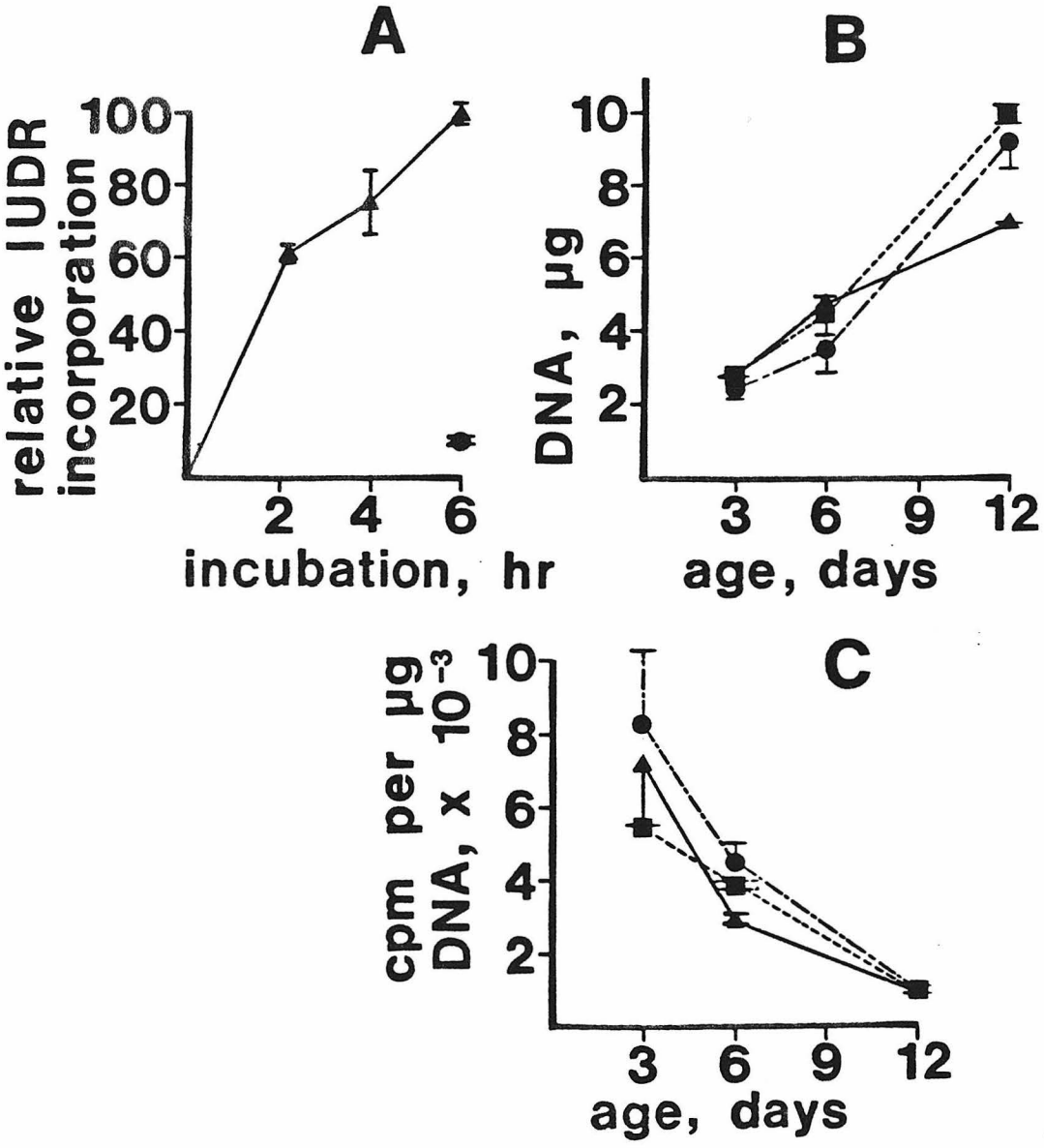
A
relative IUDR incorporation
100
80
60
40
20
2
4
6
incubation, hr
B
DNA, µg
10
8
6
4
2
3
6
9
12
age, days
C
cpm per µg DNA, x 10⁻³
10
8
6
4
2
3
6
9
12
age, days

Table 1. Deduced genotypes of Re Tr^j/?? mice and comparison with theoretical expectations.

	Expected			Observed
	if all survive	if Tr^j/Tr^j die	if Tr^j/Tr^j and 1/2 of Re Tr^j/Re + die	
Re Tr^j/++	28.5	44.8	50.0	55
Re Tr^j/Re Tr^j	14.2	0.0	0.0	0
Re Tr^j/Re +	8.0	12.6	7.0	3
Re Tr^j/+ Tr^j	8.0	0.0	0.0	0
Re +/+ Tr^j	2.3	3.6	4.0	3
P (chi^2 test)	<<0.001	<0.05	NS	

Re Tr^j/?? C57BL/6J mice were produced from cross (i) and their genotype deduced from cross (ii) as described under Results. Seventy-four Re Tr^j/?? mice were tested, of which 61 produced scorable progeny (13/23 male parents and 48/51 female parents). The deduced genotypes of the 61 Re Tr^j/?? parents are listed under the heading "Observed." The comparable numbers of animals listed under "Expected" were calculated by assuming 22% recombination between Re and Tr^j (33,34). Tr^j and Tr do have approximately the same genetic map location. For example, using the appropriate subset of the above breeding data,* I found a recombination frequency of 203/1008, or 20.1%. The mean number of progeny scored

per Re Tr^j/?? parent was 18 (range 2-46). The data from each Re Tr^j/?? parent was compared individually to the next most likely genotype, by the chi^2 test. In 55 cases, $P < 0.001$; in 2 cases, $0.001 < P < 0.01$; in 1 case, $0.01 < P < 0.05$; and in 3 cases, $P > 0.05$. The latter marginal cases are included in order to emphasize that all of the Re Tr^j/?? parents with marginal reproductive success did produce non-Trembler offspring. Omission of these cases, however, would not affect any of the other conclusions. NS, not significant.

*Data for measuring recombination frequency were limited to those Re Tr^j/?? parents for which: (1) the complete genotype was known at the $P < 0.001$ level of significance, (2) recombinant progeny could have been identified (i.e., parental genotype of Re Tr^j/++ or Re +/+ Tr^j), and (3) at least 5 progeny were scored.

Table 2. Observed frequency of the "clear nerve" phenotype among the progeny of various crosses.

parental genotypes			% having clear nerves	number of progeny scored
Re Tr^j/++	x	Re Tr^j/++	28%	79
+ Tr^j/++	x	+ Tr^j/++	24%	51
++/++	x	Re Tr^j/++*	0%	31
Re +/++	x	Re +/++†	0%	34

Sciatic nerves were dissected from 12- to 13-day-old C57BL/6J mice, and viewed with a x3 objective, using diffuse transmitted illumination. The nerves were scored as having a "clear" or "opaque" appearance as described under Results.

*One of the parents in this group was actually Re Tr^j/Re +.

†Some of the parents in this category were actually Re +/Re +.

Table 3. Myelin basic protein immunoreactivity in sciatic nerve extracts from 12- to 13-day-old mice.

genotype	genetic background	MBP (μg/mg protein mean ± SEM)*	% of wild-type
+/+	C57BL/6J	16.3 ± 0.9	100%
$\underline{Tr^j}$/+	C57BL/6J	4.9 ± 0.2	30%
$\underline{Tr^j}/\underline{Tr^j}$	C57BL/6J	0.33 ± 0.06	2%
+/+	CE x CE	16.9 ± 0.9	100%
$\underline{Tr}$/+	CE x CE	1.65 ± 0.04	10%
$\underline{Tr}/\underline{Tr}$	CE x CE	0.10 ± 0.02	1%
$\underline{Tr^j}/\underline{Tr}$	CE x C57BL/6J	0.11 ± 0.02	1%

The genotypes of mice were identified as described under Results, and their nerves assayed for MBP content as described in Materials and Methods. 3-5 animals were assayed in each category. CE denotes the F_1 progeny of a cross between C57BL/6J mice and mice from the Edinburgh colony. The different genetic backgrounds did not affect the MBP levels in wild-type mice ($P > 0.9$, Student's t-test).

*The results are stated in terms of the μg of rat MBP required to give equivalent competition. Total protein was measured essentially by the method of Lowry et al. (37).

CHAPTER FOUR

DISCONTINUOUS MYELINATION IN THE TREMBLER MOUSE: GENE DOSE DEPENDENCE SUGGESTS THE EXISTENCE OF A THRESHOLD IN PERIPHERAL MYELINATION

ABSTRACT

The dependence of myelination in Trembler mice on gene dose, demonstrated in Chapter 3, allows one to do a detailed titration of the extent of myelination over a 100-fold range. This range has been studied by indirect immunofluorescent staining for the myelin protein P_0 in frozen sections of nerve and whole mounts of cornea, as well as by electron microscopy. In frozen sections, the Schwann cells (SCs) of $\underline{Tr^j/Tr^j}$ nerve show a remarkable heterogeneity in staining: a few SCs are brightly stained, while most of the SCs are only very faintly, but significantly, stained. Longitudinal frozen sections suggest that these brightly stained regions are generally only one SC in length. A similar result was obtained from whole mounts of $\underline{Tr/+}$ cornea. Thus, the heterogeneity in P_0 content is not due to differences between axons. Electron micrographs show that, as one progresses from the most myelinated genotype to the least myelinated, the first steps affect both average myelin sheath cross-sectional area and myelin sheath number to a similar extent, while the last steps affect primarily myelin sheath number. This is not easily explained by the difficulty in recognizing very thin myelin sheaths. SC numbers remain essentially constant after the first step in this genetic series. The last step, the $\underline{Tr/Tr}$ genotype, had no recognizable compact myelin. I conclude that all Trembler SCs respond to an axonal signal to initiate

myelination by synthesizing small amounts of P_0. A minority of SCs in Tr/+ or Tr^j/Tr^j mice express much larger amounts of P_0, which is assembled into compact myelin.

INTRODUCTION

As decribed more fully in the previous chapter, the Tr/+ phenotype includes not only hypomyelination, but also excess Schwann cell (SC) proliferation, reduced axonal diameter, and other symptoms. A detailed study of the gene dose dependence of these various aspects of the Tr phenotype may provide insights into the function of the Tr^+ gene product. Moreover, the ability to reduce the amount of peripheral myelin over five distinct steps may reveal other aspects of myelination, apart from the function of the Tr^+ gene product itself.

I have chosen several complementary methods to approach this problem. Immunofluorescence on frozen sections provides a sensitive way of localizing myelin-specific proteins such as P_0, regardless of whether or not they are assembled into myelin. It is almost impossible, however, to follow a particular axon over long distances in longitudinal sections. Immunofluorescent staining of whole mounts of the cornea reveals a few myelinated sensory axons (1), which can be followed over relatively long distances. These preparations are much thicker (ca. 150 μm) than conventional frozen sections, however, and have a correspondingly higher level of background fluorescence. Finally, electron microscopy provides a clear view of morphological details, such as the frequency of compact myelin, and the extent to which axons are individually wrapped by SCs.

MATERIALS AND METHODS

Immunofluorescence on 5-6 μm frozen sections was performed essentially as previously described (2). Briefly, the sections were fixed with ice-cold 95% ethanol / 5% acetic acid for 10 min, neutralized, incubated with primary antibody (rabbit anti-P_0 at a dilution of x40) for 35 min at room temperature, washed, incubated with secondary antibody [rhodamine-labeled goat anti-rabbit IgG, $F(ab')_2$ fragment (Cappel), adsorbed with bovine liver powder and used at a dilution of x40] for 35 min at 20°C, washed, mounted, and viewed with Zeiss epifluorescence optics. In some cases, the nerves were also prefixed (before sectioning) with 0.1% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) without calcium or magnesium (Gibco) for 30 min at 20°C.

Corneas for whole mount staining were removed immediately after sacrifice with fine scissors and placed in PBS. Contaminating tissues (lens, iris, etc.) were removed with watchmaker's forceps. The corneas were fixed for 30 min in ice-cold 95% ethanol / 5% acetic acid, and neutralized by washing in HEPES-buffered MEM overnight at 4°C, with many changes. The primary antibody (anti-P_0, x25) and secondary antibody (goat anti-rabbit, x40) were the same reagents used above. The buffer used for antibody dilution and washing was 50 mM sodium phosphate pH 7.0 / 9 mg/ml NaCl / 1 mg/ml NaN_3 / 1 mg/ml bovine serum albumin (IgG free, Sigma) / 0.05% Tween 20. After each antibody incubation, the corneas were

washed at least 4 times, at least 2 hr per wash, by rocking gently at 4°C.

Electron microscopy. The breeding and identification of Tr mice was described in Chapter 3. Sciatic nerves from 12- to 14-day-old mice were exposed in situ by carefully slitting the overlying muscle with a scalpel. The exposed nerve was bathed in primary fixative for 10-15 minutes at room temperature. The following primary fixative was used: 1.5% glutaraldehyde / 0.5% paraformaldehyde / 0.1 M cacodylate pH 7.4 / 0.002% $CaCl_2$. In a later series of experiments, 0.1 M sucrose and 0.05% $CaCl_2$ were added to the primary fixative (3). The area and number of myelin sheaths were similar in wild-type nerves fixed under these two conditions, and so these data were combined. The volume of SC cytoplasm was reduced, however, when nerves were fixed in the presence of sucrose. These results are in agreement with those of Dyck and colleagues (4).

After fixation in situ, the nerves were gently removed and primary fixation continued for 1-4 hr at room temperature. The nerves were postfixed in 2% osmium tetroxide in the same buffer for 1-4 hr at 4°C, washed, dehydrated in ethanol, embedded in Epon, and thin sections (silver to light gold) cut with a diamond knife. The sections were stained with uranyl acetate and lead citrate, and examined on a Philips 201 or 301 electron microscope. Photographic prints were made at a final enlargement of x7933 or x8354, and the cross-sectional area of compact myelin measured with a digitizing tablet (Tektronix), under a magnifying glass. Two nerves (from two animals) of

each genotype except +/+ were examined. Four +/+ nerves (from four animals, two of each genetic background) were examined.

RESULTS

By indirect immunofluorescence, Trembler nerves show much less staining for P_0 than do wild-type nerves (Figure 1). A few bright "hot spots" of fluorescence are present, however (Figures 1, 3 and 4), which are sometimes as brightly labeled as a wild-type myelin sheath (Figures 2 and 3). Immunofluorescent staining for myelin basic protein (MBP) indicates a qualitatively similar reduction and heterogeneity in MBP *vs*. P_0 staining in $\underline{Tr^j}/\underline{Tr^j}$ (not shown). Radioimmunoassays on extracts of $\underline{Tr^j}/+$ mouse sciatic nerves also indicate that P_0 and MBP are both reduced 3-5 fold in this genotype, depending presumably on age (see Chapters 2 and 3).

Although only a few SCs are brightly labeled for P_0 in transverse sections of $\underline{Tr^j}/\underline{Tr^j}$ sciatic nerve, most of the rest of the cells are faintly labeled (Figure 4). In order to show that this faint labeling is specific, we also stained the SCs that enfold unmyelinated axons in the cervical sympathetic trunk of the +/+ mouse (Figures 5 and 6). These SCs were not labeled by anti-P_0 (Figure 5), although occasional myelinated axons in this section were very brightly labeled (Figure 6). Incubation with normal rabbit serum instead of rabbit anti-P_0 produced staining indistinguishable from that in Figure 5.

Two arguments suggest that the brightly stained profiles seen by immunofluorescence in $\underline{Tr^j}/\underline{Tr^j}$ correspond to myelin

sheaths. First, in transverse sections at high magnification, the brightly stained profiles generally appear as thin-walled cylinders, while the lightly stained cells do not. The halo of fluorescence around the brightest "hot spot" in Figure 1 is due to the vertical orientation of the cylinder; most of the fluorescent cylinder is not in the plane of focus. Figure 4 is too overexposed to demonstrate this point. Secondly, estimates of the number of these brightly stained profiles (1 per 1000 μm^2, based on two complete $\underline{Tr^j/Tr^j}$ sciatic nerve transverse sections) are in rough agreement with the number of myelin sheaths seen in electron micrographs (2 per 1000 μm^2, see Table 1).

In longitudinal sections, the entire wild-type nerve is brightly stained (Figure 2), but only occasional $\underline{Tr^j/Tr^j}$ internodes are brightly stained (Figure 3). In our best-oriented $\underline{Tr^j/Tr^j}$ longitudinal sections, the phase contrast image suggested to us that most axons could be followed for at least 75 μm before they left the plane of section. Nevertheless, the brightly stained profiles observed in these $\underline{Tr^j/Tr^j}$ longitudinal sections averaged only 32 μm in length (range: 23 to 45 μm; N = 11), suggesting that the brightly stained profiles each represent single SCs, and that adjacent SCs on the same axon are not brightly stained. The preservation of morphology in frozen sections is not good enough, however, to completely rule out the possibility that the apparent ends of these brightly stained profiles correspond to points at which a myelinated axon leaves the plane of section. This objection

was removed by staining whole mount preparations, in which isolated brightly stained profiles of a similar length were also observed (Figure 7).

The Tr/+ genotype was selected for the cornea whole mount experiment, rather than Tr^j/Tr^j, because electron microscopic observations (see below) suggested that heterogeneity in staining would also be present in this genotype, but with much more frequent "hot spots." Given the small number of axons in the cornea, brightly stained profiles should be difficult to find in Tr^j/Tr^j cornea.

In whole mounts of the +/+ mouse cornea, bright areas of staining are closely apposed (Figure 7B). Bright areas of staining in Tr/+ cornea are generally separated by an unstained gap that is equivalent in length to one or more of the brightly stained profiles (Figure 7A). Although one can find a few longer gaps between myelin internodes in +/+ mouse cornea, as well as a few closely apposed internodes in Tr/+ cornea, these are not the rule. The above observations were made on bundles of 2-4 axons, which run circumferentially around the edge of the cornea, near the cornea/scleral border. Visualization of all axons in the cornea with indirect immunofluorescence, using a monoclonal anti-neurofilament antibody, showed that axons periodically leave these bundles and run radially in towards the center of the cornea (not shown). In the +/+ cornea, the radially oriented axons cease to be myelinated soon after they leave the circumferential bundles. Where more than one radially oriented internode of myelin is present on a given +/+

axon, each internode is shorter than the last. Interestingly, the radially orientated axons do not seem to be myelinated at all in the Tr/+ cornea.

In electron micrographs of +/+ sciatic nerve, quantitation of the cross-sectional area of myelin sheaths showed that their size is broadly distributed from about 1 to 10 μm^2; histograms (not shown) of this distribution did not show any definite peaks. Each step in the genetic series from +/+ to Tr^j/Tr^j mice results in about a 2-fold decrease in the average cross-sectional area per myelin sheath (Table 1). The number of myelin sheaths showed 2-fold decreases in the first steps, but a more than 10-fold decrease from Tr/+ to Tr^j/Tr^j. Myelin profiles were scored as such only when associated with axons. Debris resembling myelin which was clearly not associated with axons (or whose inner diameter was less than 0.4 μm, in which case an axon, if present, might not have been clearly seen), was not counted. The frequency of such debris was roughly similar in Tr/+ and Tr^j/Tr^j nerves. This frequency was qualitatively estimated to be similar to the frequency of myelinated axons in Tr^j/Tr^j (about 2 per 1000 μm^2), although it must be admitted that there is not a sharp dividing line between the smallest debris of this sort and other darkly staining organelles. The periodicity of compact myelin in Tr^j/Tr^j nerves was found to be 14.1 ± 0.8 nm (mean ± SD, N = 11). This is not significantly different from the myelin period in +/+ nerves (13.6 ± 0.8 nm, N = 11; $p > 0.1$ by the Student's t-test).

No myelin sheaths were found in Tr/Tr mice, although two entire sciatic nerve cross sections (from two mice) were scanned in photographic prints. One of these nerves was also completely scanned at a higher magnification directly on the electron microscope, without finding any myelin.

The total amount of myelin, represented by the product of myelin sheath size and myelin sheath number, agrees well with the MBP content of these nerves (compare the last column in Table 1 with Table 3 of Chapter 3). This suggests, as do the fluorescence results, that most of the P_0 and MBP in these nerves is found in compact myelin. Estimates of the amount of compact myelin in Tr^j/Tr^j and Tr/Tr (1% and 0% of wild-type values, respectively), were somewhat lower than the corresponding measurements of MBP levels (2% and 1% of wild-type values, respectively). Along with the fluorescence results, this implies that a significant part of the P_0 and MBP in these nerves is present in lightly staining cells that do not form compact myelin. Several electron microscopic observations suggest that these lightly staining cells have, in general, enfolded a single axon. The total number of "singly enfolded" plus myelinated axons is constant in all genotypes (Table 2), suggesting that this total represents the same population of axons in all genotypes, namely those competent to induce myelination. (Total nerve cross-sectional area was similar in all genotypes.) Secondly, the "singly enfolded" axons, in general, had a larger diameter than axons in the same

section that were wrapped in bundles (see Figure 8) suggesting again that the singly enfolded axons represent the "competent" subpopulation. Some of the "singly enfolded" axons were actually wrapped by a spiral of many layers of SC cytoplasm that had not condensed into compact myelin. This phenomenon appeared to show a gene dose effect, in that such spirals were quite extravagant in $\underline{Tr^j}/+$ nerves (Figure 8B), and rare in $\underline{Tr}/\underline{Tr}$ nerves (Figure 8E).

As expected from the results of Chapter 3, no difference in SC numbers was found between the various Trembler genotypes (Table 1).

DISCUSSION

Two arguments indicate that the small number of myelin sheaths found in $\underline{Tr}$ homozygotes is not due to a failure to recognize very thin myelin sheaths. First, sheaths as small as 0.1 μm^2 were not difficult to recognize, and were, in fact, not infrequent in $\underline{Tr}/+$ and $\underline{Tr^j}/\underline{Tr^j}$ nerves. This corresponds approximately to a sheath of 2 myelin lamellae around an axon 1 μm in diameter. Although sheaths of 0.1 μm^2 could be recognized, the mean sheath size in $\underline{Tr^j}/\underline{Tr^j}$ nerves (0.66 μm^2, see Table 1) was much larger. Secondly, a few examples of "singly enfolded" axons in $\underline{Tr}/+$ and $\underline{Tr^j}/\underline{Tr^j}$ nerves were examined at a higher magnification and showed no compact myelin.

The heterogeneity of myelination in Trembler mouse nerves shows a superficial resemblance to the myelin degradation caused by diphtheria toxin. Waksman et al. (5) showed that the injection of low doses of diphtheria toxin can cause the degradation of isolated internodes of myelin, as well as abnormal SC proliferation, while most myelin internodes and all axons remain intact. This phenomenon was subsequently termed "segmental demyelination" (6), which term was applied to the Trembler phenotype by early workers (7) because of the sparing of axons, the heterogeneity in myelination evident in electron micrographs of Tr/+ nerve, and the relatively short internodes [suggested in Tr/+ nerves by a generalized lipid stain (8,9) and also characteristic of remyelination after diphtheria toxin injection (10)]. It is far from clear, however, that the resemblance is more than superficial. The heterogeneity in demyelination after diptheria toxin injection is presumably simply due to the potency of diphtheria toxin at the molecular level (11).

Two other possible interpretations of the heterogeneity of myelination in Trembler nerves can be ruled out. The heterogeneity is not due to differences between axons, since adjacent SCs on the same axon behave quite differently (Figure 7). It is also notable that myelinated and unmyelinated axons in Tr^j/Tr^j nerves often have a similar diameter (Figure 8D). Secondly, the heterogeneity in myelination is not caused by SC proliferation, since these two

phenomena have a completely different dependence on gene dose (Table 2). The morphological heterogeneity in axonal ensheathment does, however, reflect a corresponding molecular heterogeneity in the expression of myelin proteins. Whether the molecular heterogeneity is due to differences in myelin protein synthesis or degradation is unclear. Indeed, the cause of this heterogeneity could be different from (i.e., subsequent to) the function of the $\underline{Tr^+}$ gene product. It is interesting to note, however, that a somewhat similar heterogeneity in myelination has been observed in the jimpy mouse (12,13), adding yet another parallel between these two genetic loci (Chapter 3). The problem posed by this heterogeneity is better defined in Trembler than in the jimpy mutant. In Trembler, axons that fail to be myelinated not only contact mutant SCs, but also induce the SCs to synthesize myelin-specific proteins such as P_0, which the SCs would not otherwise make. In jimpy, axons that fail to be myelinated may not be contacted by mutant oligodendrocytes.

In conclusion, these results show that Trembler SC are able to perform the first few steps in a normal axon-SC interaction. Trembler SCs wrap competent axons in a 1:1 (one SC to one axon) fashion, and are induced to synthesize detectable levels of the myelin proteins P_0 and MBP. Depending on the exact genotype, most of the SCs are blocked at this stage, while a minority of SCs go on to express much higher levels of myelin proteins, which are assembled into compact myelin.

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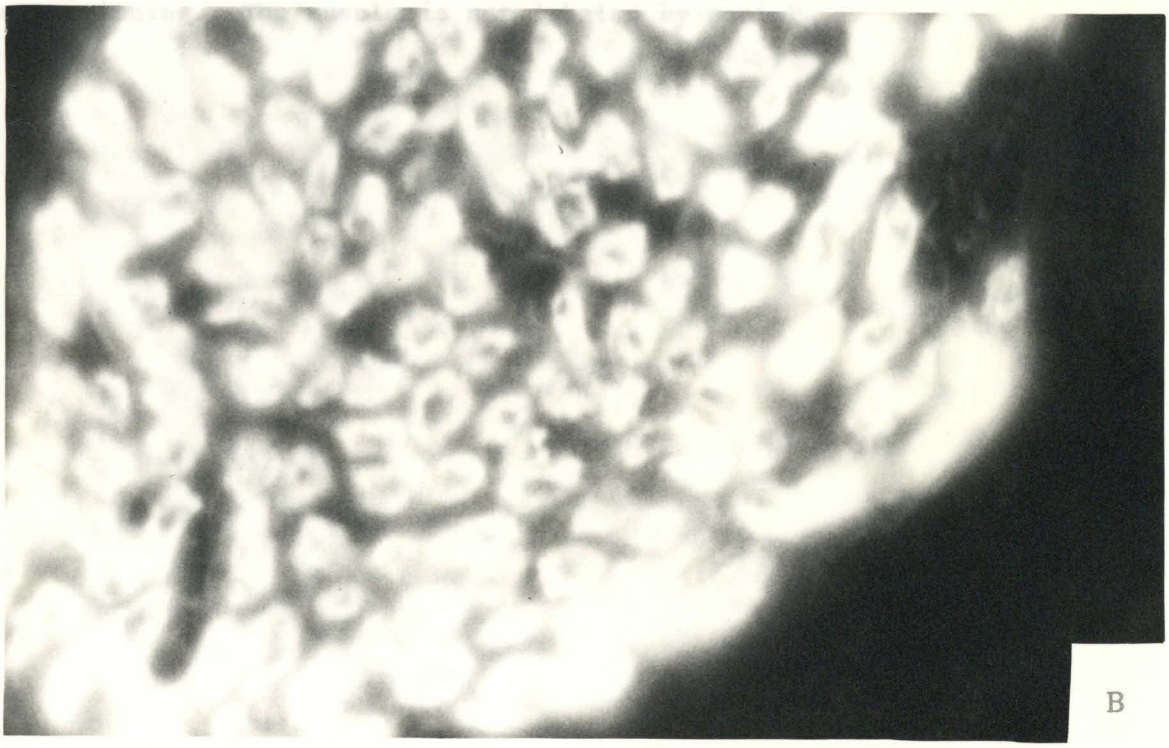
Figure 1. Frozen transverse sections of mouse sciatic nerve, stained with rabbit anti-P_0 followed by rhodamine-labeled goat anti-rabbit IgG, and viewed with rhodamine epi-fluorescence optics. (A) $\underline{Tr^j}/\underline{Tr^j}$. Six "brightly labeled" cells are present, but none in this field of view stain as brightly as +/+ myelin sheaths, printed here at the same exposure. (B) +/+.

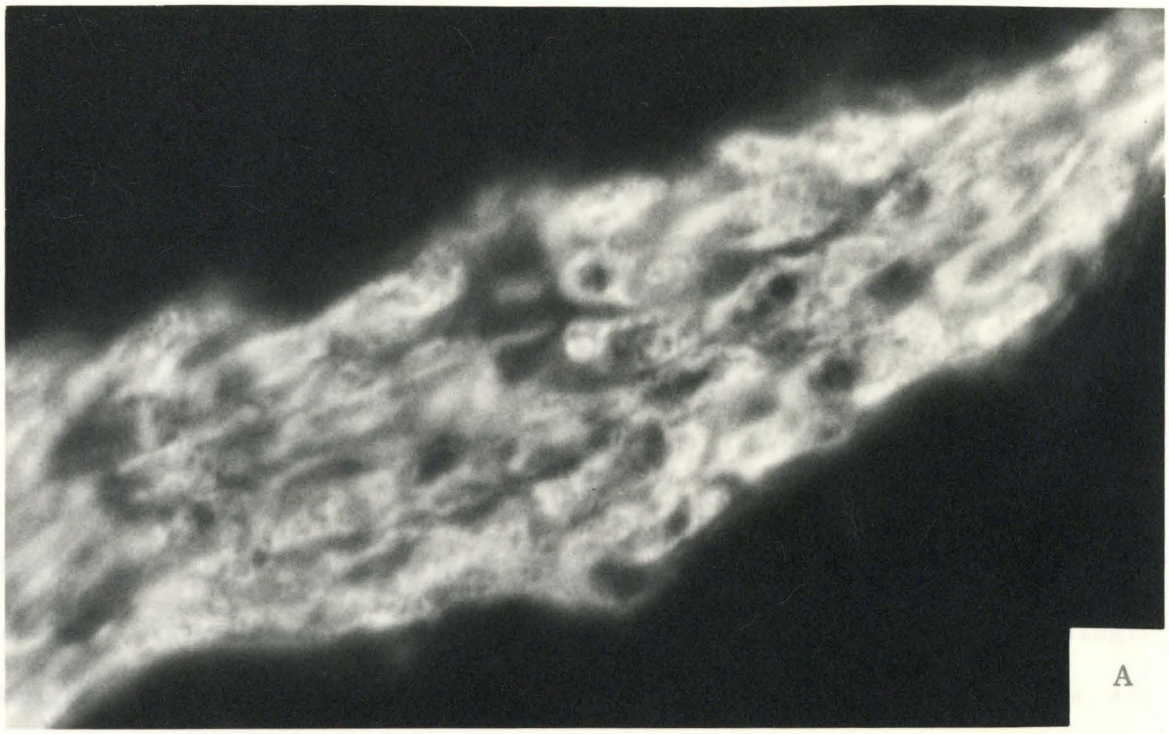
A



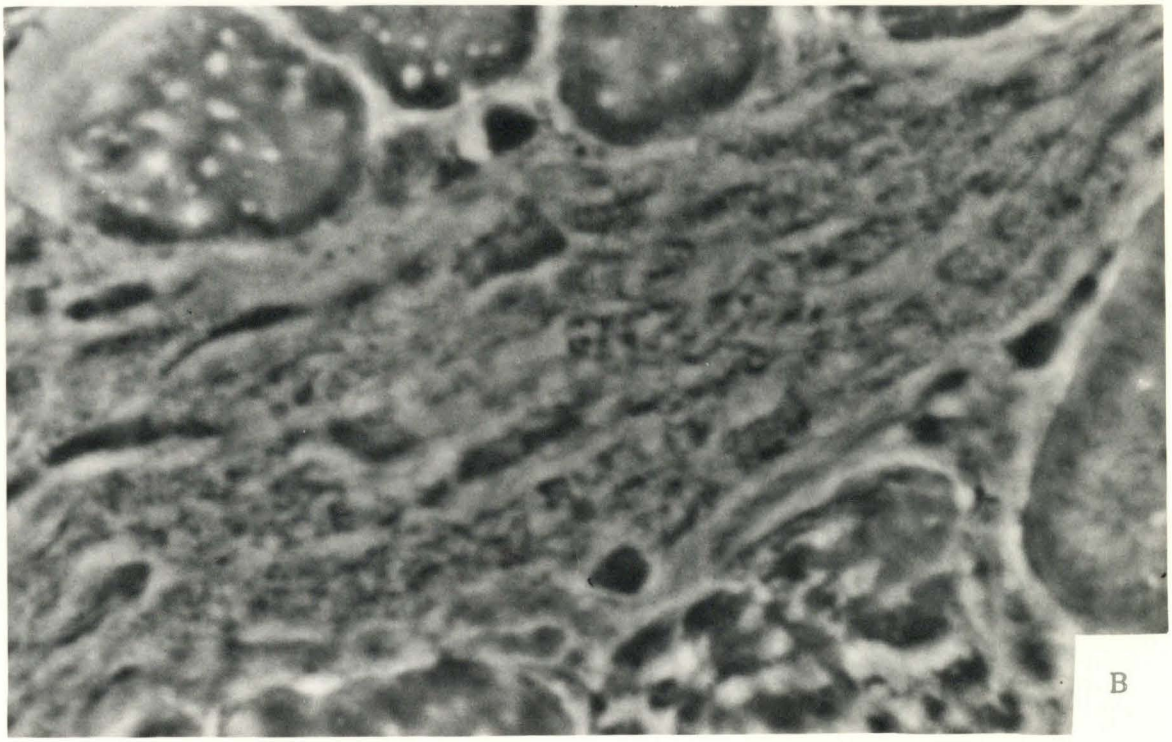
B

10 µm

Figure 2. Frozen longitudinal section of +/+ mouse sciatic nerve, stained with anti-P_0 as before. (A) Rhodamine fluorescence. (B) Phase contrast view of the same field. The nerve was embedded in a piece of muscle, evident here at the upper left and lower right, for convenience in sectioning. The muscle is not labeled by anti-P_0.



A

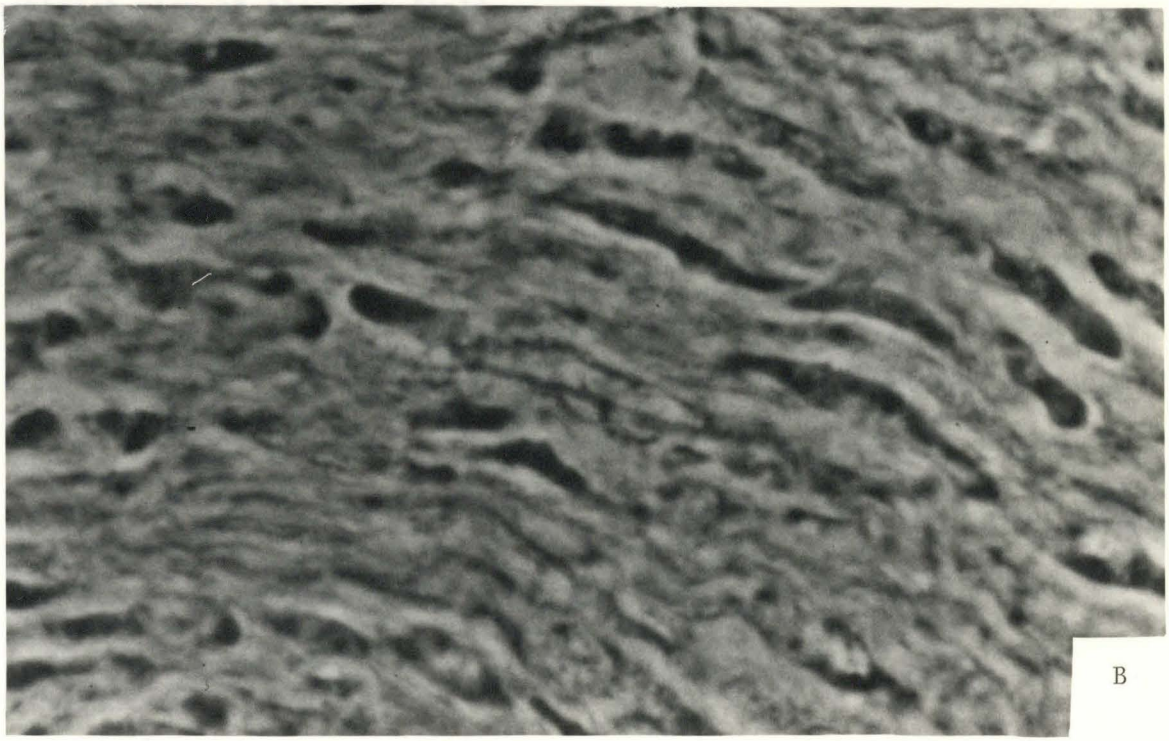


B

10 µm

Figure 3. Frozen longitudinal section of $\underline{Tr^j/Tr^j}$ mouse sciatic nerve, stained with anti-P_0 as before. (A) Rhodamine fluorescence. A single brightly labeled cell is evident, stained roughly as brightly as +/+ myelin sheaths. (B) Phase contrast view of the same field.

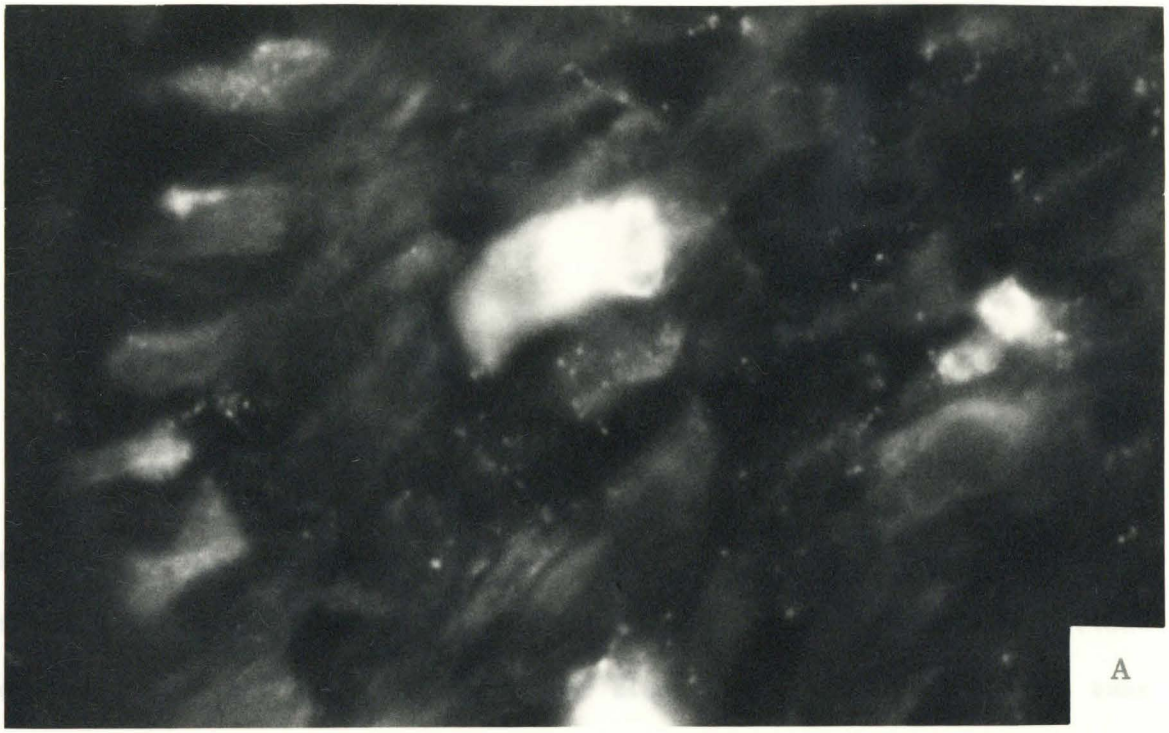
A



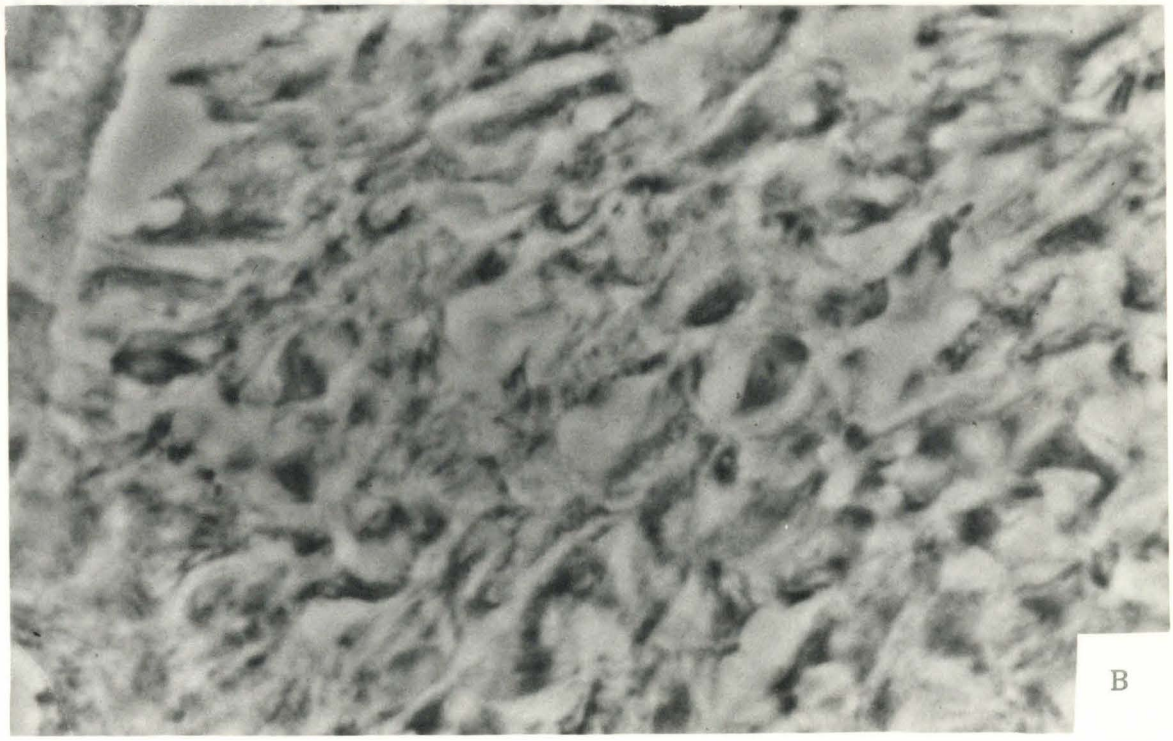
B

10 µm

Figure 4. Frozen transverse section of $\underline{Tr^j/Tr^j}$ mouse sciatic nerve. The nerve was prefixed with 0.1% paraformaldehyde, sectioned, and stained with anti-P_0 as described in Materials and Methods. (A) Rhodamine fluorescence, printed after a longer exposure than Figures 1-3, to more clearly show the faintly labeled cells. (B) Phase contrast view of the same field.



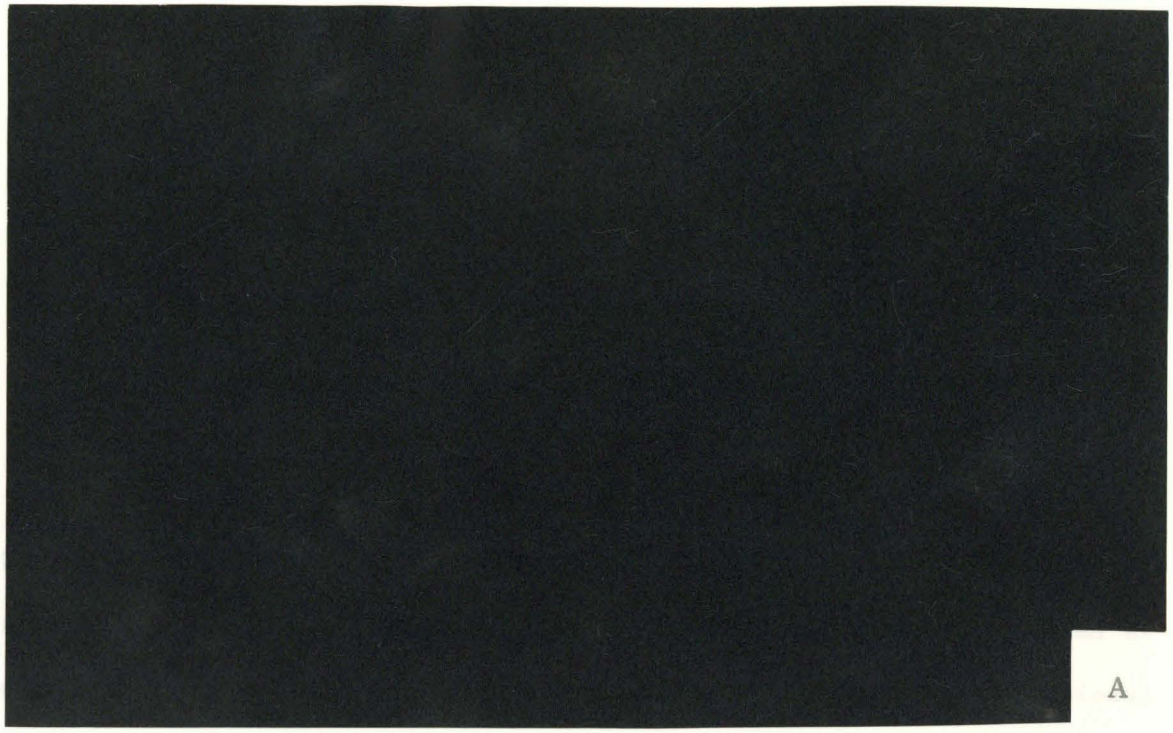
A



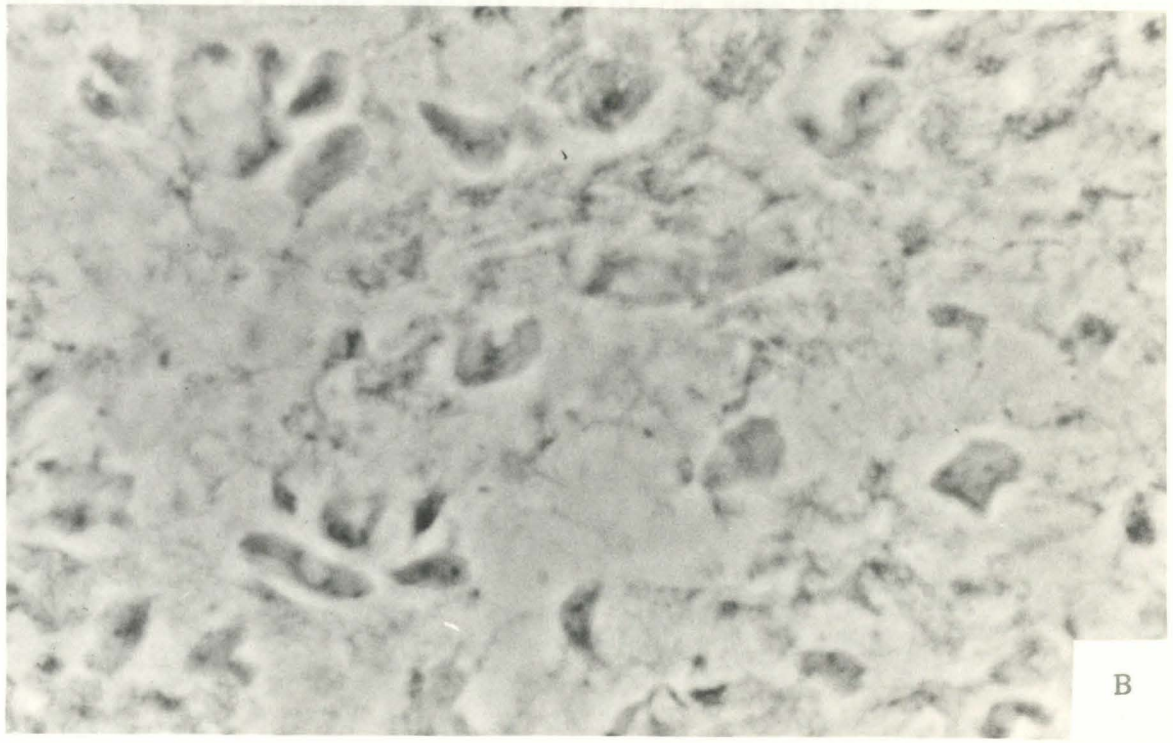
B

10 µm

Figure 5. Frozen transverse section of +/+ mouse cervical sympathetic trunk. The nerve was prefixed with 0.1% paraformaldehyde, and sections stained with anti-P_0. (A) Rhodamine fluorescence, printed at the same exposure as Figure 4A. Faintly labeled cells are not present. (B) Phase constrast view of the same field.



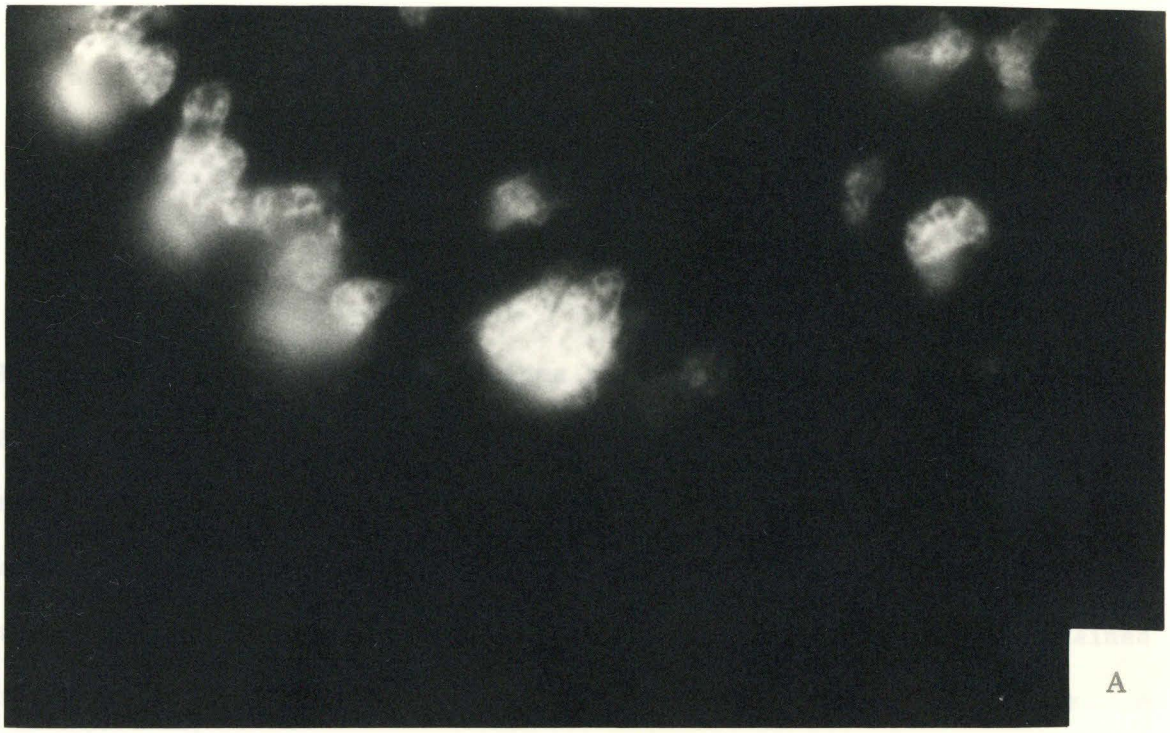
A



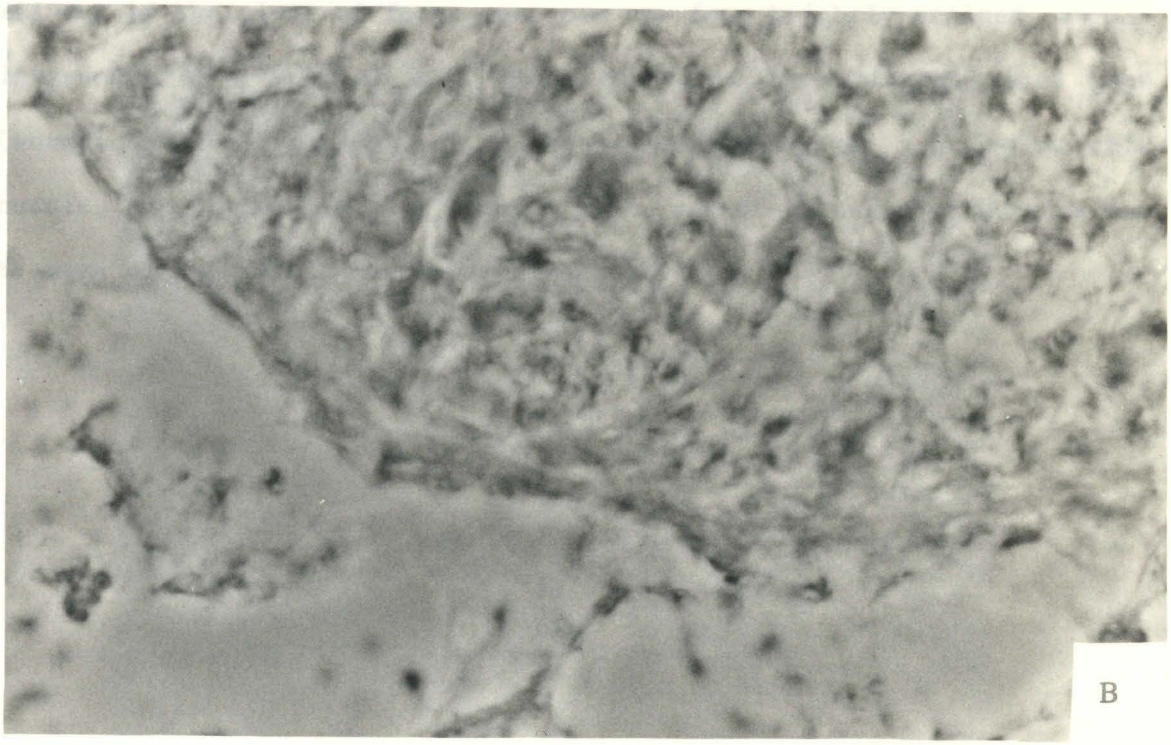
B

10 µm

Figure 6. Frozen transverse section of +/+ mouse cervical sympathetic trunk, processed as in Figure 5. (A) Rhodamine fluorescence, printed at the same exposure at the same exposure as Figures 4A and 5A. Occasional myelinated axons are very strongly labeled. Lightly labeled cells are not present. (B) Phase contrast view of the same field.



A

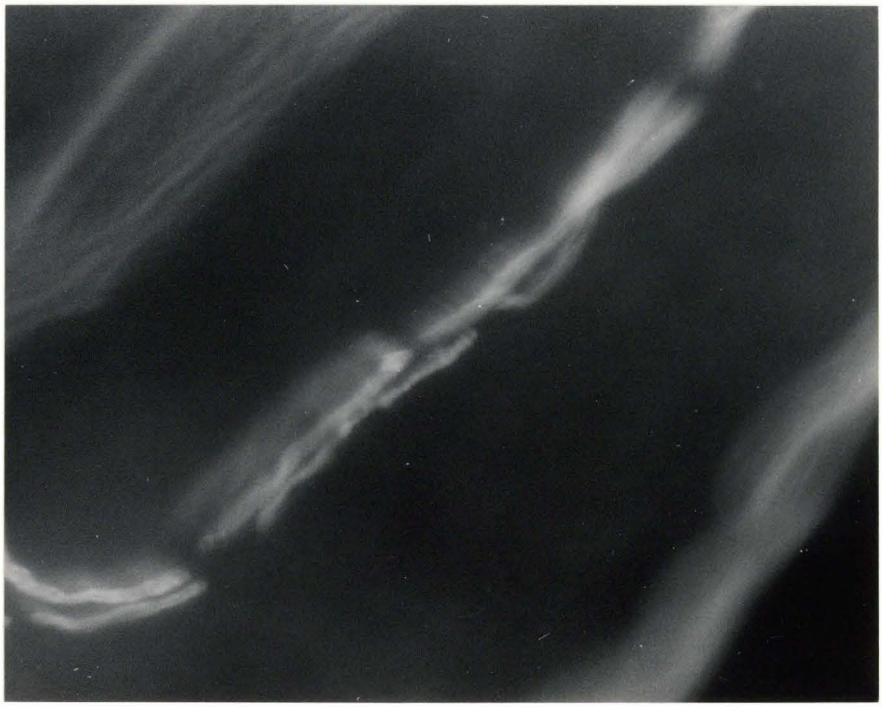


B

10 µm

Figure 7. Whole mount preparations of mouse cornea, stained with anti-P_0 as described in Materials and Methods. (A) Tr/+ cornea, viewed by rhodamine fluorescence optics. A bundle of two or more (probably three) axons is shown. Brightly stained patches on any given axon are separated by unstained regions as long as, or longer than the stained regions. (B) +/+ cornea, viewed by rhodamine fluorescence optics. A bundle of two myelinated axons is shown. Although the axons cross in and out of the plane of focus, one can see four brightly stained, normal internodes of myelin on each axon, separated by small unstained gaps (normal nodes of Ranvier). A third myelinated axon runs below this bundle, out of the plane of focus. Other out-of-focus nerve bundles can be seen at upper left and lower right.

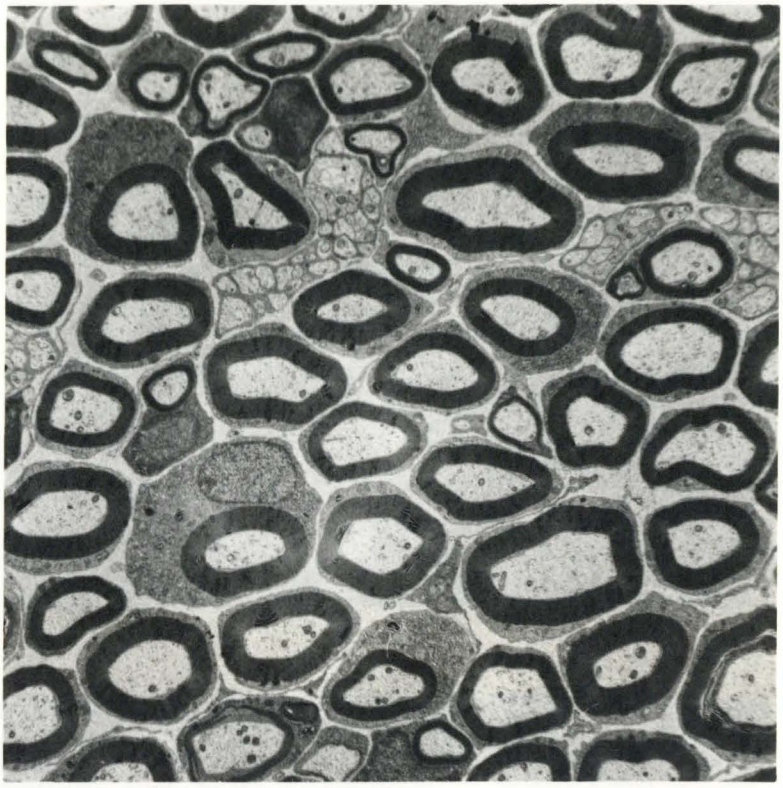
A



10 μm

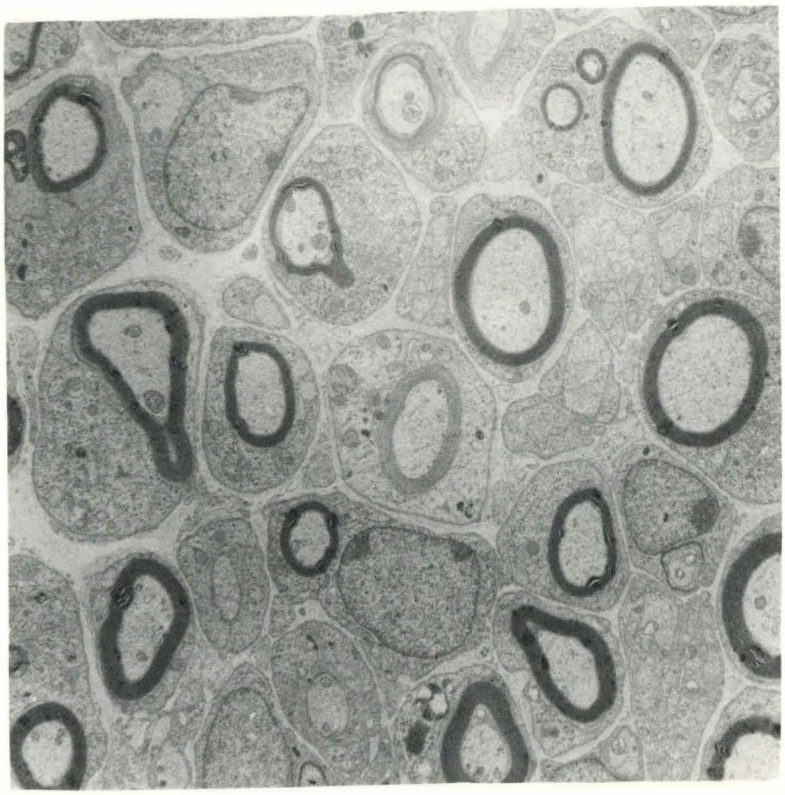
B

Figure 8. Electron micrographs of transverse sections of sciatic nerves from mice of various genotypes. (A) +/+. (B) $\underline{Tr^j}/+$. (C) $\underline{Tr}/+$. (D) $\underline{Tr^j}/\underline{Tr^j}$. (E) $\underline{Tr}/\underline{Tr}$.



A

5 µm



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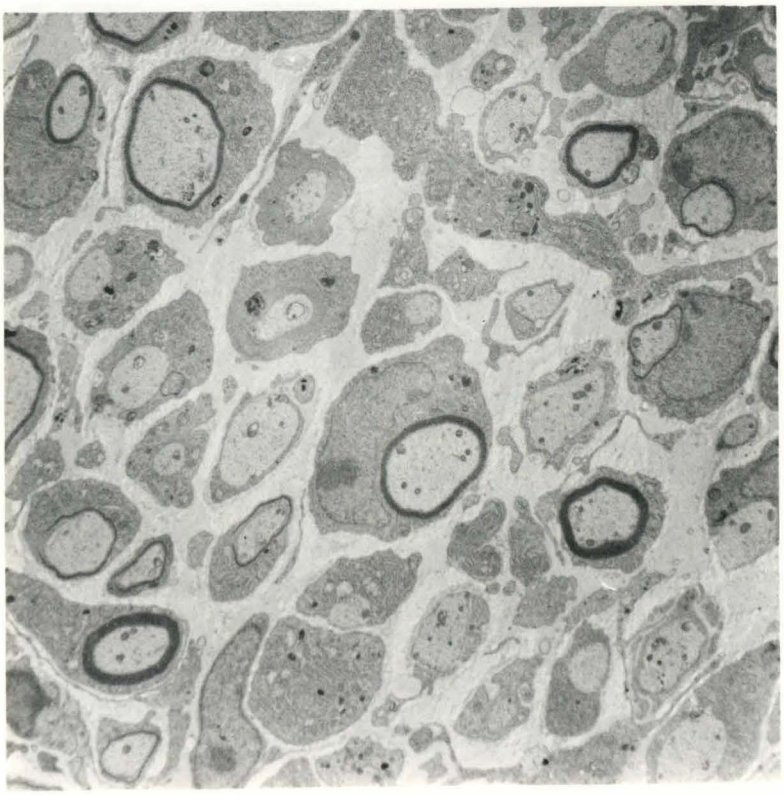
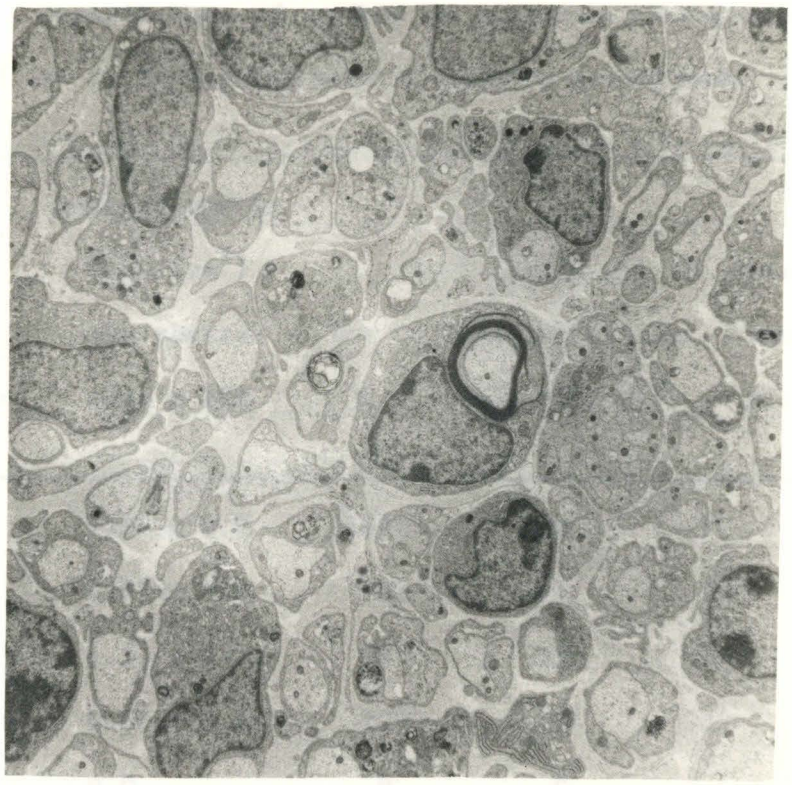
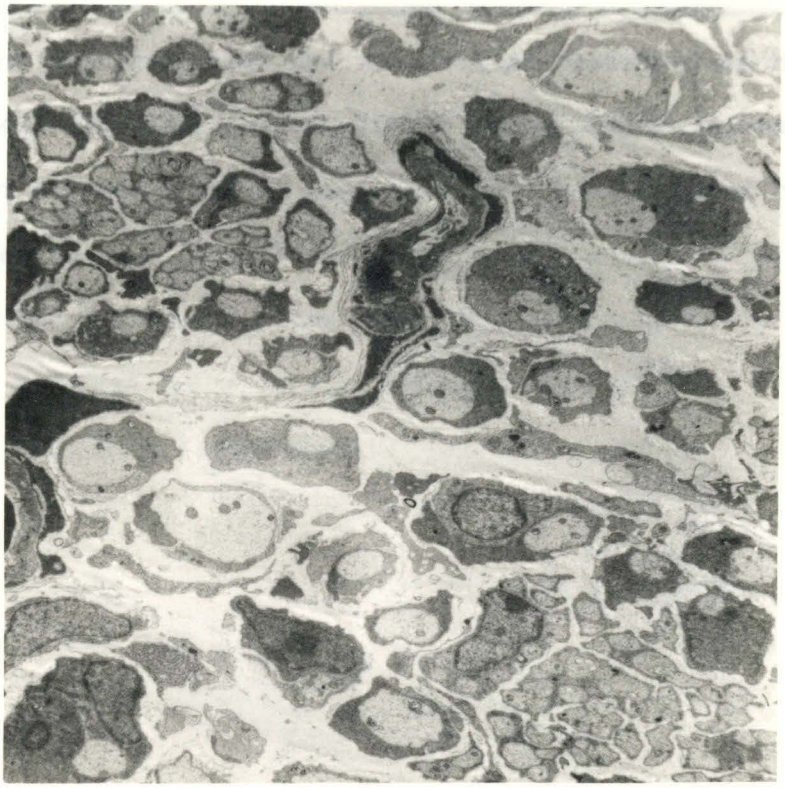


C

5 µm



D



E

5 µm

Table 1. Quantitation of myelin in electron micrographs from sciatic nerves of 12- to 14-day-old mice of various gentotypes.

genotype	average myelin area (μm^2) per sheath mean ± SEM	N =	number of myelin sheaths per 1000 μm^2	total myelin area (% of wild-type)
+/+	4.2 ± 0.2	146	70	100%
$\underline{Tr^j}/+$	2.0 ± 0.2	70	40	27%
$\underline{Tr}/+$	1.1 ± 0.1	50	25	9%
$\underline{Tr^j}/\underline{Tr^j}$	0.66 ± 0.14	22	2	1%
$\underline{Tr}/\underline{Tr}$	0.0	0	0	0%

The nerves were processed and analyzed as described in Materials and Methods. The last column, "total myelin area," represents the product of columns 2 and 4, expressed as a percentage of the wild-type value.

Table 2. Counts of axon and cell numbers in electron micrographs from sciatic nerves of 12- to 14-day-old mice of various genotypes.

genotype	axons* per 1000 μm^2	% axons* myelinated	N =	Schwann cells per 1000 μm^2	N =
+/+	73	96%	313	6.0	26
$\underline{Tr^j}/+$	52	78%	88	18	30
$\underline{Tr}/+$	69	36%	151	16	35
$\underline{Tr^j}/\underline{Tr^j}$	69	3%	254	16	58
$\underline{Tr}/\underline{Tr}$	71	0%	444	17	109

The nerves were processes and analyzed as described in Materials and Methods. "N" referes to the total number of axons or Schwann cells counted, respectively.

*Axons were counted only if they were: individually enfolded (i.e., one axon enfolded by one SC), not enfolded (bare), or myelinated. Bundles of axons enfolded by a single SC were not counted. Bare axons were extremely rare in all genotypes.

APPENDIX

TREMBLER SCHWANN CELLS IN CULTURE

MATERIALS AND METHODS

Mouse Schwann cells (SCs) were purified and cultured by methods similar to those used for rat SCs (1), with minor modifications as described below. $\underline{Tr^j/Tr^j}$ sciatic nerves were identified as described in Chapter 3, slowly cooled in a medium containing 20% fetal calf serum and 8% dimethyl sulfoxide, and stored in liquid nitrogen. This allows one to freeze nerves from single $\underline{Tr^j/Tr^j}$ animals and later use the nerves in larger batches. The nerves were quickly thawed, and dissociated with trypsin and collagenase (1). We found that fibroblasts were preferentially killed, for unknown reasons, by this freeze/thaw procedure. Wild-type nerves were dissected from 3- to 5-day-old C57BL/6J mice and frozen as above, as controls. Although the $\underline{Tr^j/Tr^j}$ nerves were obtained from 12- to 15-day-old animals for ease of identification, 12- to 15-day-old +/+ nerves did not give satisfactory SC yields.

After a few days in culture, fibroblasts were killed by treatment with a monoclonal anti-Thy 1.2 IgM antibody (New England Nuclear) and complement (1). Visualization of Thy-1 positive cells in these cultures by indirect immunofluorescence, using a monoclonal anti-Thy 1.2 IgG antibody (New England Nuclear) showed that, as observed in rat sciatic nerve cultures, all of the Thy-1 positive cells have a flat morphology, but not all of the flat cells are Thy-1 positive.

Mouse SCs were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and partially-purified glial growth factor (2), in a humidified atmosphere containing 10% CO_2, at 37°C. Cellular proliferation was assayed by the incorporation of ^{125}I-labeled deoxyuridine, as described (2).

RESULTS

Wild-type, freshly dissociated SCs contain myelin debris (3,4), which may affect their behavior in vitro. Ultrastructural (3) and immunofluorescence (4) observations have shown that this myelin debris is rapidly eliminated in culture. Therefore, proliferation experiments were confined to SCs that had been cultured for at least one week, and typically two weeks, in order to test both genotypes in the absence of myelin debris.

Both +/+ and $\underline{Tr^j}/\underline{Tr^j}$ mouse SCs show a similar sensitivity to partially purified glial growth factor (Figure 1). Mouse SCs, however, show a plateau response at about a 1000-fold lower concentration of partially purified glial growth factor than rat SCs do (not shown). It has been reported that mouse SCs are stimulated to divide by fibroblast growth factor (5), although rat SCs are not (6). Since the initial step in the purification of fibroblast growth factor from bovine pituitary (7) is similar to the initial step used in the purification of glial growth factor from the same tissue (2), it is likely that this extract also contains substantial amounts of fibroblast growth factor.

$\underline{Tr^j/Tr^j}$ SCs did not differ greatly from +/+ SCs in their background rate of unstimulated proliferation, or in their maximal amount of proliferation (Table 1).

Intriguing differences between genotypes in morphology were often observed. +/+ SCs were generally more elongated, and showed a greater tendency to orient themselves parallel to each other, than did $\underline{Tr^j/Tr^j}$ SCs (Figure 2). The morphology of cells in these cultures is quite variable, however, and no attempt was made to quantify this apparent morphological difference.

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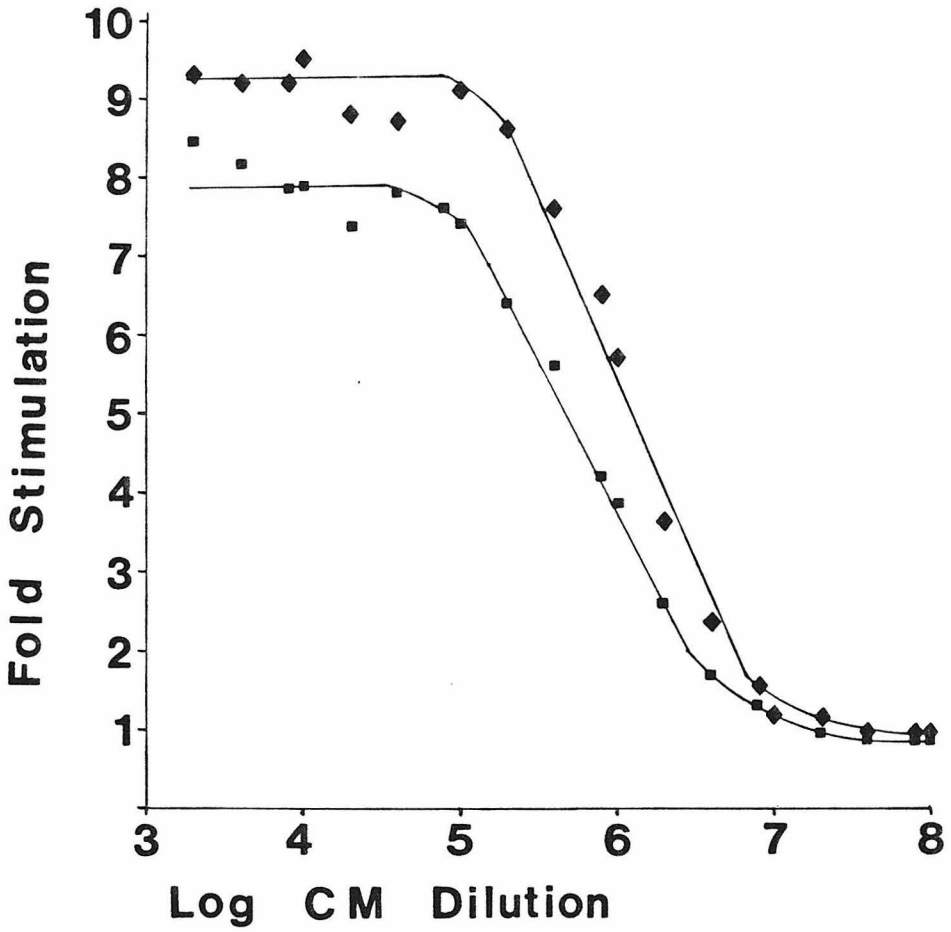
Figure 1. Proliferation of mouse Schwann cells. Proliferation was assayed by ^{125}IUDR incorporation as described. "Fold stimulation" denotes the following ratio: (cpm observed at indicated pituitary extract dilution) / (cpm observed without pituitary extract). ◆, +/+ mouse SC. ■, $\underline{Tr^j}/\underline{Tr^j}$ SC. Each point represents the mean of five determinations.


Fold Stimulation
10
9
8
7
6
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1
3
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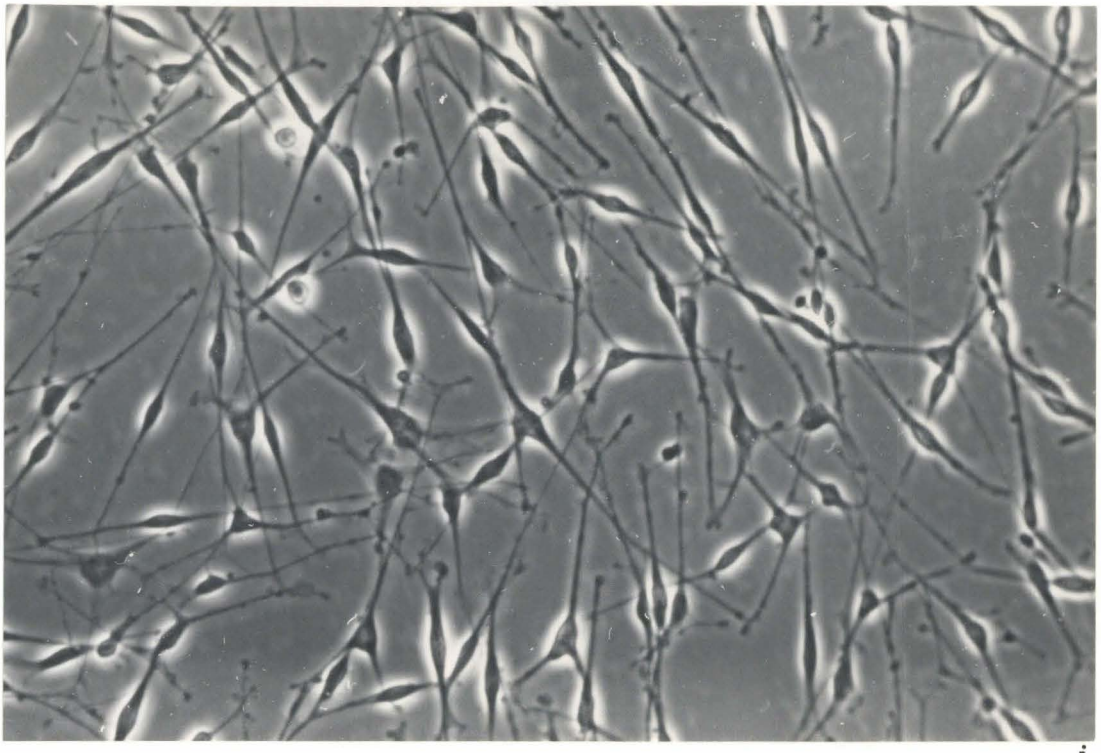
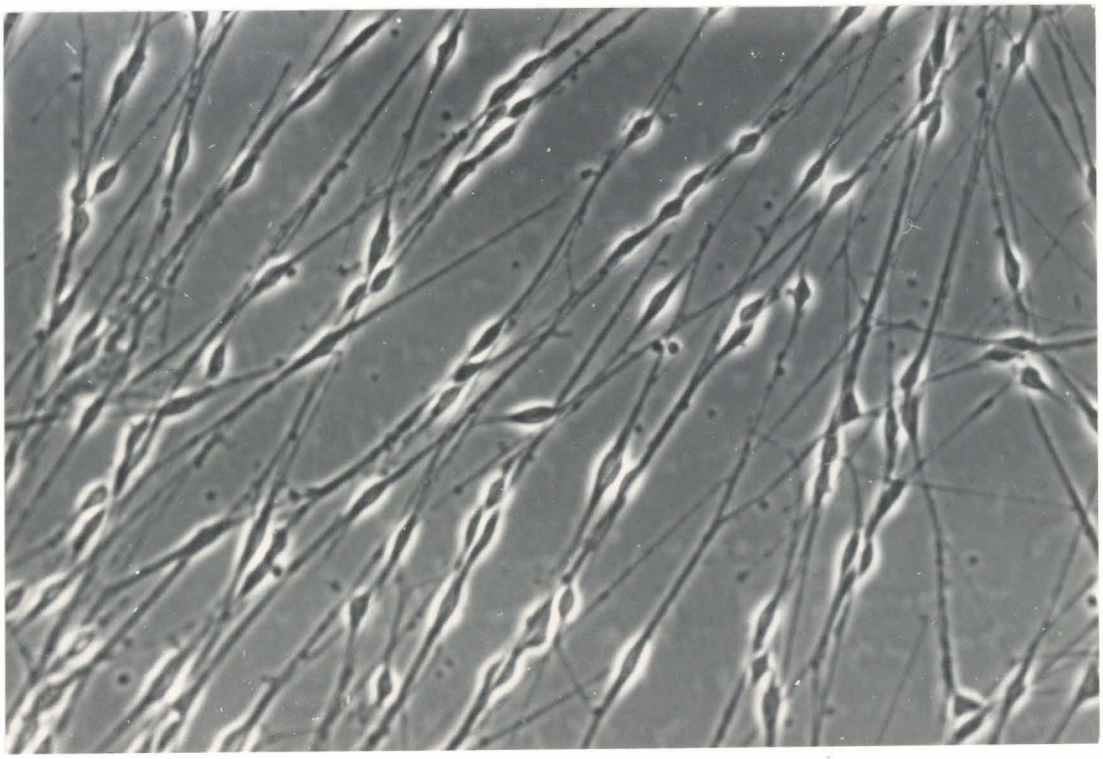
8
Log CM Dilution

Figure 2. Living mouse SC cultures, shown in phase contrast optics.



$\underline{Tr^{j}}/\underline{Tr^{j}}$



100 µm

+ / +

Table 1. Mouse Schwann cell proliferation.

Experiment	$\underline{Tr^j/Tr^j}$ background	+/+ background	$\underline{Tr^j/Tr^j}$ fold stim.	+/+ fold stim.
1	1675	1098	12.3	23.0
2	1100	643	7.9	9.1
3	528	1283	16.4	21.4

SC proliferation was assayed by ^{125}IUDR incorporation as described. "Background" represents the cpm incorporated without added pituitary extract. "Fold stim." represents the following ratio: (cpm at optimal pituitary extract dilution) / (cpm without pituitary extract). Each entry represents the mean of at least three determinations in one experiment.