Mbar a linear extrapolation (dashed line) to a maximum loading force of 7000 N produced a central pressure of 2.8 Mbar. This method is similar but superior to conventional methods of ultrahigh-pressure calibration because it incorporates supporting data at radial distances.

In the second method the maximum pressure was determined from the pressure distribution by calculation of the ratio of the pressure at a radial distance of 30 μm to the pressure at the center of the flat region. An important factor associated with using the ratio in these calculations is that the flat diamond surface did not undergo plastic deformation. This ratio was constant for anvil set 2 at several central pressures up to 1.8 Mbar (Fig. 2b). The resulting maximum pressure in the central area of anvil set 2 was thus determined independently of the loading force to be 2.8 Mbar when pressure at the 30-μm radius was 1.8 Mbar.

The design of anvil set 2 was found to have exceptional properties. Pressure could be raised smoothly to above 2 Mbar after alignment procedures. The conditions were unusually stable; the pressure in the sample region did not vary perceptibly during the 40-day period of the experiment. Unloading the pressure slowly did not produce as favorable stress distributions as loading the pressure, but the diamond anvils were reclaimed after the experiment. These results with a composite of stainless steel and ruby crystals suggest that it should be possible to pressurize any other gasketed materials stably for study at 2 to 3 Mbar with this apparatus, including solids normally in the gaseous state at 1 bar such as hydrogen.

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Cyclophilin: A Specific Cytosolic Binding Protein for Cyclosporin A

Abstract. *Cyclophilin, a specific cytosolic binding protein responsible for the concentration of the immunosuppressant cyclosporin A by lymphoid cells, was purified to homogeneity from bovine thymocytes. Cation-exchange high-performance liquid chromatography resolved a major and minor cyclophilin species that bind cyclosporin A with a dissociation constant of about 2 × 10^{-7} moles per liter and specific activities of 77 and 67 micrograms per milligram of protein, respectively. Both cyclophilin species have an apparent molecular weight of 15,000, an isoelectric point of 9.6, and nearly identical amino acid compositions. A portion of the NH_2-terminal amino acid sequence of the major species was determined. The cyclosporin A–binding activity of cyclophilin is sulfhydryl dependent, unstable at 56°C and at pH 4 or 9.5, and sensitive to trypsin but not to chymotrypsin digestion. Cyclophilin specifically binds a series of cyclosporin analogs in proportion to their activity in a mixed lymphocyte reaction. Isolation of cyclophilin from the cytosol of thymocytes suggests that the immunosuppressive activity of cyclosporin A is mediated by an intracellular mechanism, not by a membrane-associated mechanism.*

Cyclosporin A, a cyclic undecapeptide of fungal origin is a potent immunosuppressant with low myelotoxicity (*1*). It is used to prevent rejection of kidney and liver transplants (*2*) and in graft versus host disease (*3*). Additional studies suggest applications for cyclosporin A in treatment of autoimmune diseases (*4*), and schistosomiasis (*5*). Cyclosporin A also provides a unique probe of the biochemical factors involved in regulation of the immune and possibly other physiological responses.

Cyclosporin A appears to act on the immune system by inhibiting the initial steps of T-lymphocyte activation. It diminishes the responsiveness of helper-inducer T lymphocytes to interleukin-1 (IL-1) (*6*, *7*). Cyclosporin A also inhibits the production of interleukin-2 (IL-2) by alloantigen- and lectin-stimulated T lymphocytes (*7–10*) and prevents the expression of receptors for IL-2 by precursor cytolytic T lymphocytes (*9*, *11*). However, cyclosporin A has minimal effects on the activation and proliferation of suppressor T lymphocytes (*12*) or on the response of primed T lymphocytes to exogenous IL-2 (*10*, *13*). In addition, cyclosporin A is reported to inhibit γ-interferon production by lymphocytes (*14*) and to suppress delayed-type hypersensitivity reactions and inhibit production of lymphokines that affect macrophage behavior (*15*). However, the specific biochemical mechanism by which cyclosporin A inhibits T-lymphocyte activation and lymphokine production has not been established.

We reported earlier (*16*) that uptake

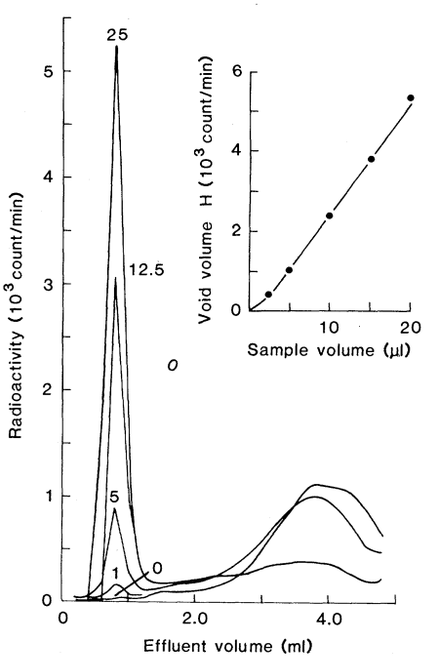

Fig. 1. Sephadex LH-20 column assay for cyclophilin activity. Minicolumns (1.8 ml) of Sephadex LH-20 resin (Pharmacia) were preequilibrated in tris buffer (20 m*M*, *p*H 7.2) containing 2-mercaptoethanol (5 m*M*) and sodium azide (0.02 percent). Samples for assay were diluted to 90 μl with tris buffer containing 7.5 percent newborn calf serum (Gibco) in small glass test tubes. After the addition of 10 μl of [^{3}H]cyclosporin A (50 μg/ml; 0.05 μCi/ml) in 40 percent ethanol, tubes were agitated gently; 50 μl of the sample was applied to the column, and 20 μl was assayed for radioactivity in 5 ml of Liquiscint (New England Nuclear). Columns were eluted with tris buffer, and the fractions were assayed for radioactivity. Data reflect [^{3}H]cyclosporin A elution resulting from complex formation with 0, 2.5, 5.0, 12.5, and 25 μl of a solution of purified cyclophilin (0.7 mg of protein per milliliter). The inset shows that elution of [^{3}H]cyclosporin A through LH-20 columns is proportional to the amount of cyclophilin in the assay. Only the 0.5- to 1.0-ml elutate was assayed for radioactivity in routine assays.

and concentration of [^{3}H]cyclosporin A by cultures of a thymic lymphoma (BW5147) occurs largely in the cytosol and not the cell membrane. Preliminary characterization indicated that this unfractionated binding component was of low molecular weight and displayed isoelectric points of 6.8 and 8.6 (*16*). We now report the purification to homogeneity and the properties of a comparable cytosolic cyclosporin A–binding protein, which we term "cyclophilin."

To quantitate cyclophilin activity during purification, we developed a Sephadex LH-20 column assay that measures the capacity of crude or purified binding protein to cause a proportional amount of [^{3}H]cyclosporin A to elute in the protein-containing void volume of this weakly hydrophobic matrix (Fig. 1); elution of free [^{3}H]cyclosporin A is significantly retarded in LH-20 columns. [^{3}H]Cyclosporin A binding was linear for a more than 90-fold concentration range of cyclophilin (Fig. 1) and was not affected by the presence of 7.5 percent serum required to minimize nonspecific adsorption of cyclosporin A to surfaces.

For preparative isolation of cyclophilin, 200 to 500 g of calf thymus glands were homogenized for 45 seconds (1:4 weight to volume) in 10 m*M* tris buffer (*p*H 7.2) containing 0.15*M* KCl and 1 m*M* phenylmethanesulfonyl fluoride; 2-mercaptoethanol was added to a final concentration of 5 m*M*, and the mixture was homogenized for an additional 45 seconds. After centrifugation at 8000*g* for 20 minutes at 4°C, the supernatant was clarified by filtration through a 0.2-μm Acroflux Capsule (Gelman) at 4°C. As the filtrate was collected, the volume of the retentate was maintained by slow addition of 2 liters of tris buffer (10 m*M*, *p*H 7.2) containing 5 m*M* 2-mercaptoethanol and 0.02 percent sodium azide. Essentially all of the [^{3}H]cyclosporin A–binding activity in the cytosol was associated with a low molecular weight component as determined by size-exclusion high-performance liquid chromatography (HPLC) (Fig. 2A). The clarified cytosol (4 liters) was filtered through a 5-square-foot 100-kD sulfone exclusion membrane (PTHK00001) in a Pellicon cassette system (Millipore) by recirculation with addition of 4 liters of tris buffer and then concentrated to 60 ml in the same apparatus with a 5-square-foot 10-kD exclusion membrane (PTG6-0005). All fractionation steps were performed at 4°C and all solutions, including dialysis media, contained 5 m*M* 2-mercaptoethanol and 0.02 percent sodium azide.

Cyclophilin activity was adsorbed to a 2.5 by 30 cm affinity matrix (matrix gel Blue A; Amicon), washed with phosphate buffer (0.02*M*, *p*H 7.2), and eluted with 300 ml of a 0.1*M* to 0.3*M* phosphate gradient at 1 ml/min. Cyclophilin activity eluted at approximately 0.2*M* and was electrofocused at 15 W or 1600 V for 22 hours on a 110-ml isoelectric focusing column (LKB) with 1 percent ampholytes (*p*H 8 to 10.5) prepared in a 0 to 65 percent sucrose gradient; a single peak of cyclophilin activity was recovered at *p*H 9.5 to 9.7. Ampholytes were removed by increasing the ionic strength to 1.0*M* phosphate, followed by adsorption onto a 1 by 30 cm phenyl Sepharose CL-4B column (Pharmacia). Cyclophilin activity was eluted with a 200-ml phosphate gradient decreasing from 1.0*M* to 0.1*M*. Final purification to homogeneity was achieved by weak cation exchange HPLC and resolved a major and minor species (Fig. 2B) with [^{3}H]cyclosporin A–binding specific activities of 77 and 67 μg per milligram of protein, respectively. This corresponds to 0.96 mol of cyclosporin A bound per mole of the major cyclophilin species. The major and minor cyclophilin species have nearly identical amino acid compositions, with single tyrosine, tryptophan, and methionine residues. The protein is especially rich in lysine, phenylalanine, and glycine. The major cyclophilin species yielded a single NH_2-terminal sequence with a high-coupling yield (NH_2-Val-Asn-Pro-Thr-Val-) on automated sequence analysis of the intact protein, indicating a single homogeneous polypeptide chain. The unknown molecular difference between the major and minor components appears to be a relatively minor structural difference that does not seem to affect cyclosporin A–binding activity. The first 46 residues of a total of approximately 117 residues have been unambiguously identified (data not given). This sequence was searched in 25-residue segments against the protein sequence database from the National Biomedical Research Foundation (January 1984 version; 2511 protein sequences); for this

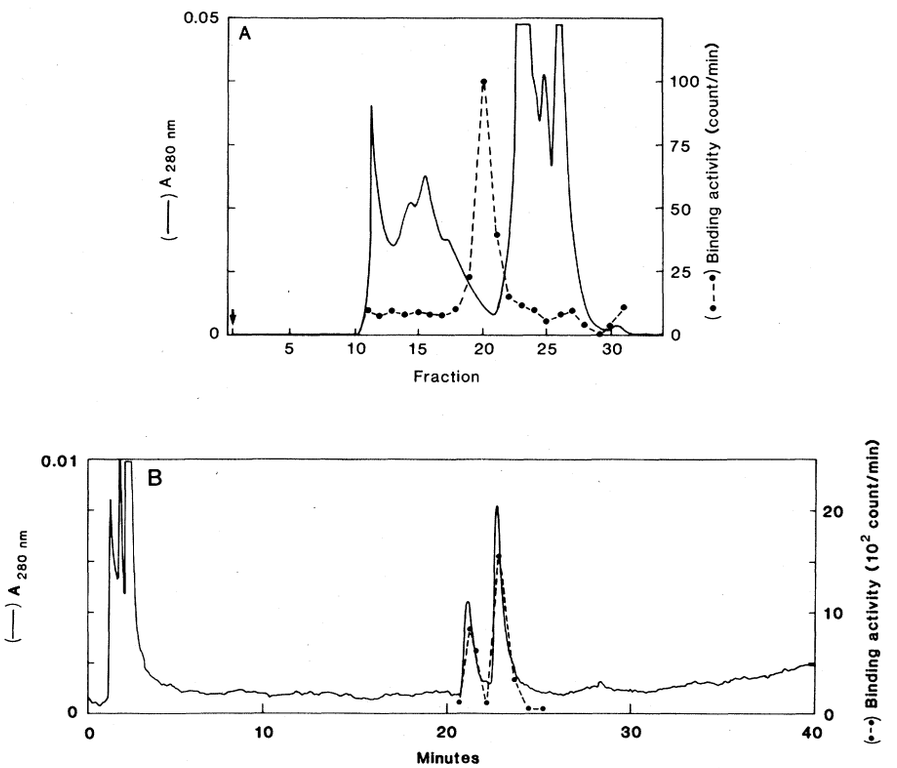

Fig. 2. Chromatography of (A) crude and (B) purified cyclophilin. (A) Size-exclusion HPLC profile of cyclophilin activity in crude thymocyte cytosol. A 100-μl sample of a 100,000*g* supernatant from the calf thymus homogenate was injected into a size-exclusion HPLC column (Spherogel TSK 2000/G; 0.75 by 60 cm; Altex) and eluted with 0.2*M* KH_2PO_4 (*p*H 7.2) at a flow rate 1 ml/min. (B) Weak cation-exchange HPLC resolution of major and minor cyclophilin species. The cyclophilin fraction (100 μg of protein in 200 μl) eluted from phenyl Sepharose was injected onto a weak cation-exchange HPLC column (Synchropak CM 300; 4.1 by 250 mm; pore size 300 Å; Synchron) preequilibrated with 5 m*M* KH_2PO_4 (*p*H 7.2). The column was eluted with a concave gradient of 5 m*M* KH_2PO_4 to 0.5*M* NaCl in 5 m*M* KH_2PO_4 (total volume 50 ml; 1 ml/min). In both chromatography systems, protein elution was monitored by absorbance at 280 nm, and cyclophilin activity was determined by LH-20 column assays of 82-μl portions of 1.0-ml eluant fractions.

Table 1. Specificity of cyclophilin for cyclosporin analogs. Cyclophilin affinity for the analogs was quantitated by enhancement of intrinsic tryptophan fluorescence after the addition of cyclosporin analog (0.25 or 1.0 μg/ml) to 2.5 ml of 5 m*M* KH_2PO_4 buffer (*p*H 7.2) containing cyclophilin (5 μg/ml) and 5 m*M* 2-mercaptoethanol. Retention of analog relative to cyclosporin A was determined on a μBondapak phenyl column (3.9 by 300 mm; 100-Å pore size; Waters Associates) monitored at 210 nm with isocratic elution with 60 percent acetonitrile at 1 ml/min as a measure of hydrophobicity. Data on the inhibition of mixed lymphocyte reactions was provided by J. F. Borel. CsA, cyclosporin A.

Cyclosporin analog	Immunosuppressive activity in mixed lymphocyte reaction	Cyclophilin affinity		Retention time of analog relative to CsA
		0.25 μg/ml	1.0 μg/ml	
CsA	+++	0.34	0.90	1.00
CsC	+++	0.31	0.69	0.70
Dihydro-CsC	+++	0.33	0.70	0.79
CsC ester	+++	0.00	0.04	1.40
CsG	++	0.30	0.87	1.14
Dihyro-CsD	+	0.19	0.72	1.28
8-Acido-dihydro-CsA	+	0.12	0.53	1.00
CsD	±	0.21	0.69	1.20
CsH	−	−0.01	0.02	0.98
O-Acetyl-CsA	−	−0.02	−0.03	1.69

purpose, we used the computer program SEARCH and a mutational data matrix (*17*). No apparent sequence relationship was found between cyclophilin and any protein in the database. Thus cyclophilin appears to represent a new class of proteins unrelated to any known sequences.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (*18*) indicates an apparent molecular size of 15-kD for both cyclophilin species. The [^{3}H]cyclosporin A–binding capability of cyclophilin is lost after exposure for 10 minutes to 56°C, to *p*H 4 at 25°C, or to *p*H 9.5 in the absence of 2-mercaptoethanol or dithiothreitol. Also, the cyclosporin A–binding capacity of cyclophilin is sensitive to digestion by matrix-bound trypsin, but not chymotrypsin.

A strong enhancement of intrinsic tryptophan fluorescence of both cytophilin species was associated with cyclosporin A binding (Fig. 3) and permitted an independent measurement of the equilibrium (dissociation constant, $2 \times 10^{-7} M$). This value is consistent with the uptake of [^{3}H]cyclosporin A by BW5147 lymphoma cells (*16*) and human peripheral blood lymphocytes (*19*), as well as with data from the LH-20 assay. This marked enhancement indicates that the tryptophan residue is in a more hydrophobic environment consequent to cyclosporin A binding.

The specificity of cyclophilin for binding cyclosporin A is shown by data in Table 1. Ten natural and synthetic derivatives of cyclosporin A were examined for their ability to enhance intrinsic cyclophilin fluorescence (Table 1) or compete with [^{3}H]cyclosporin A for binding cyclophilin in LH-20 column assays (not shown). In both test systems, only those compounds that inhibited mixed lymphocyte reactions were capable of binding to cyclophilin (Table 1). An exception, however, was an ester of cyclosporin C that may be metabolized by macrophage nonspecific esterase in the mixed lymphocyte reaction to yield free cyclosporin C. Further evidence for the specificity of cyclophilin for cyclosporin A is apparent in the lack of correlation between the hydrophobic nature of cyclosporin derivatives, determined by hydrophobic-interaction HPLC, and their affinity for cyclophilin (Table 1).

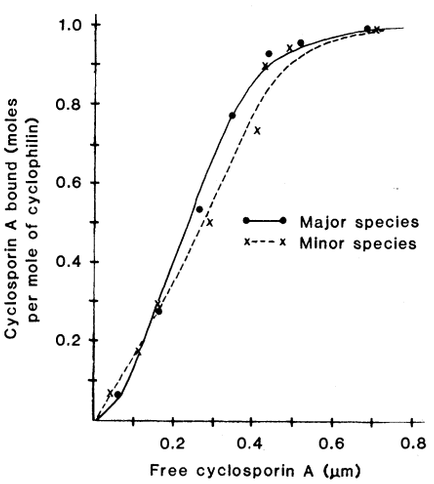

Fig. 3. Saturation and dissociation characteristics of cyclophilin species. The [^{3}H]cyclosporin A–binding characteristics of the major and minor cyclophilin species were determined by quantitation of cyclosporin A–induced enhancement of intrinsic tryptophan fluorescence with a spectrofluorimeter (model 4800, SLM Instruments). Portions of [^{3}H]cyclosporin A (25 μg/ml; 88,000 cpm/μg) in 5 m*M* KH_2PO_4 (*p*H 7.2) were added to purified cyclophilin (5 μg/ml) in 2.5 ml of KH_2PO_4 (5 m*M*, *p*H 7.2) containing 2-mercaptoethanol (5 m*M*). Fluorescence enhancement was recorded in the ratio mode with excitation at 289 nm and emission at 340 nm, the maximum established for cyclophilin. After each addition of [^{3}H]cyclosporin A, portions of the cyclophilin solution were withdrawn to quantitate the total concentration of cyclosporin A.

We have also detected equivalent concentrations of cyclophilin activity of the same molecular size, but differing isoelectric points, in cytosol extracts of murine and human thymus and mature T cells. In addition, cyclophilin activity has been detected in nonlymphoid tissues, with high concentrations present in brain and kidney, organs subject to toxic side effects during cyclosporin A therapy in humans (*20, 21*).

Characterization of the functional activities associated with cyclophilin promises a novel biochemical dissection of the mechanism for antigen and IL-1–triggered T-lymphocyte activation. Furthermore, the differential inhibitory effect of cyclosporin A on IL-2 production (*7–10*) and expression of IL-2 receptors by T lymphocytes (*9, 11*) versus the inability of cyclosporin A to block IL-2–driven proliferation of activated T lymphocytes (*10, 13*) implies a different role for cyclophilin in regulation of these T-lymphocyte activities. Of significant interest is the occurrence of cyclophilin activity in nonlymphoid tissues, suggesting that as with other growth and differentiation hormones, natural ligands may exist that exert various biological effects, dependent on the species and physiologic state of target cells.

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Intraneuronal Substance P Contributes to the Severity of Experimental Arthritis

Abstract. *There is evidence that substance P is a peptide neurotransmitter of some unmyelinated primary afferent nociceptors and that its release from the peripheral terminals of primary afferent fibers mediates neurogenic inflammation. The investigators examined whether substance P also contributes to the severity of adjuvant-induced arthritis, an inflammatory disease in rats. They found that, in the rat, joints that developed more severe arthritis (ankles) were more densely innervated by substance P–containing primary afferent neurons than were joints that developed less severe arthritis (knees). Infusion of substance P into the knee increased the severity of arthritis; injection of a substance P receptor antagonist did not. These results suggest a significant physiological difference between joints that develop mild and severe arthritis and indicate that release of intraneuronal substance P in joints contributes to the severity of the arthritis.*

Recent studies have implicated the peripheral nervous system in the inflammation seen in arthritis. For example, in rats with experimental arthritis, the concentration of the undecapeptide substance P (SP) increases in peripheral nerves that have branches innervating inflamed joints (*1*). Furthermore, if capsaicin, a neurotoxin that is relatively selective for unmyelinated sensory neurons (*2*), is administered to rats before or after the onset of arthritis, paw swelling and tenderness is diminished (*3*). The hypothesis that the nervous system contributes to arthritis is supported by studies demonstrating that the inflammation and tissue destruction seen clinically in rheumatoid arthritis and experimentally in adjuvant- or collagen-induced arthritis more frequently and more severely involve the more densely innervated, distal joints of an extremity (*4*). We report here further evidence that the nervous system contributes to arthritic inflammation, and, more specifically, that the severity of arthritis can be attributed, at least in part, to actions of SP in the affected joint.

Our initial experiments established that the joints most severely affected are indeed the most densely innervated and have the highest SP concentration. It is difficult to directly measure the innervation density of joints. We therefore used two indirect measures, the nociceptive threshold of the joint capsule, which should be inversely correlated with innervation density (*5*), and the magnitude of the spinal projection of the afferent fibers.

Vocalization in response to a noxious stimulus was measured in rats lightly anesthetized with pentobarbital. A pair of stimulating electrodes, separated by 1 mm, were placed against the surgically exposed joint capsule. Increasingly intense 1-second trains of monopolar pulses (100 μsec; 8 Hz) were delivered to evoke vocalization (*6*).

Nociceptive thresholds of the left and right ankle joints of normal rats were similar, as were the thresholds of left and right knee joints (Fig. 1). The threshold of a given ankle or knee joint was also constant throughout the experiment, and thresholds at different points on the same joint capsule were similar. Consistent with its having a less dense innervation, however, the knee joint (n = 8) showed a mean threshold three times higher than that of the ankle joint (n = 8) (3.5 ± 0.5 versus 0.56 ± 0.25 W, respectively) ($P < 0.01$, Student's t-test).

To examine the central projection of joint afferent neurons, we injected, under fluoroscopic guidance, a 1 to 4 percent solution of wheat germ agglutinin coupled to horseradish peroxidase (WGA-HRP; Sigma) into ankle and knee joints on opposite sides. Forty-eight hours later the rats were perfused with a mixed-aldehyde fixative. Frozen sections (50 μm) of the third lumbar to second sacral spinal segments were cut and reacted for anterogradely and transganglionically transported WGA-HRP (*7*).

Injection of 4 percent WGA-HRP revealed that the ankle has a significant afferent projection to the dorsal horn of the spinal cord. No projection from the knee joint capsule could be found. Since it could be argued that HRP is more dilute in the knee than in the ankle because of the larger joint space and that this may contribute to differential uptake of HRP by knee and ankle joint afferent fibers, a series of animals was studied in which increasingly higher concentrations of WGA-HRP were injected into the knee. Five rats were injected in opposite ankle and knee joints in dose ratios as high as 20:1 (25 μl of 4 percent solution in the knee and 5 μl of 18 percent solution in the ankle). Even at the highest dose of WGA-HRP, only minimal reaction product was recorded in the dorsal horn ipsilateral to the knee injection. In all rats, however, a concentrated area of reaction product was seen in sections of the fourth lumbar segment of the spinal cord ipsilateral to the injected ankle. The densest afferent projection was in a wedge shape that covered lamina I and the substantia gelatinosa (Fig. 1). Since many small nociceptive primary afferents terminate in the superficial dorsal horn, it appears that the ankle receives a major nociceptive innervation.

To assess whether SP is found in joint capsules and whether it is derived from joint innervation, we collected joint capsules (including both fibrous capsule and synovial membrane) from normal rats and from rats whose sciatic and femoral nerves had been bilaterally transected 6 days earlier. Substance P was measured by radioimmunoassay (*8*). Immunoreactivity in tissue extract had a retention time identical to that of synthetic SP when separated by high-performance liquid chromatography and measured by