cause (i) unstimulated sensitive cell cultures produce no supernatant activity, (ii) none of the active material in the supernatant is sedimentable at 100,000*g*, (iii) the amount of direct stimulation of nonsensitive cells by a supernatant is decreased greatly in experiments where residual antigen is decreased even though the sensitive cells themselves undergo a vigorous proliferative response, and (iv) the active substance causes nonsensitive cells to respond to additional tuberculin.

It is possible that some of the different biological activities which have been detected in the media of sensitive leukocytes exposed to antigen in vitro may be due to the same material. However, in addition to its property of activating nonsensitive lymphocytes to respond to antigen, the heat lability and nondialyzability of our material distinguish it from many of these activities. For example, the inhibition of the migration of guinea pig peritoneal macrophages, an in vitro correlate of delayed hypersensitivity, is mediated by a heat-stable factor produced by lymphocytes upon exposure to specific antigen (*8*). The media of human lymphocyte cultures stimulated by phytohemagglutinin is cytotoxic for many tissue culture lines; the activity is heat-stable at 56°C and labile at 100°C (*9*). Cytotoxic activity is also found in the media of sensitive lymphocytes sitmulated by PPD (*10*). The physical properties of our lymphocyte-activating material differ from the heat-stable, dialyzable polynucleotides released from sensitive spleen cells incubated with specific antigen. These polynucleotides, when subsequently injected in vivo as an adjuvant with the specific or an indifferent antigen, cause transiently larger numbers of mouse spleen plaque-forming cells during the first few days after antigen injection (*11*).

Our studies parallel the earlier experiments in vivo which demonstrated that supernatants prepared from sensitive leukocytes incubated with specific antigen will transfer delayed hypersensitivity to previously nonsensitive individuals (*4*). Only 1/500th the number of cells per milliliter in the studies in vivo were used in our system in vitro for the preparation of supernatants. The supernatants were active in vivo after 1 hour of incubation of cells with antigen (*4*); in our system this smaller number of cells must be incubated with antigen for 36 hours for the production of optimum activity in the supernatant. The biochemical relation between the transfer factor released by antigen from sensitive cells and the dialyzable transfer factor prepared by the mechanical disruption of sensitive leukocytes is still unknown (*12*). Dialyzable transfer factor has been shown to induce nonsensitive cells to respond to tuberculin to a much smaller degree than that obtained with active supernatants (*13*).

Although the production of activity in the supernatant is immunologically specific and although in the presence of such activity nonsensitive lymphocytes will respond to additional antigen, it is not yet known whether the action on the nonsensitive cells in vitro is also immunologically specific.

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References and Notes

1. G. Pearmain, R. R. Lycette, P. H. Fitzgerald, *Lancet* **1963-I**, 637 (1963); R. Schrek, *Amer. Rev. Resp. Dis.* **87**, 734 (1963); N. R. Ling, *Lymphocyte Stimulation* (North-Holland, Amsterdam, 1968), pp. 147–158.
2. W. H. Marshall, F. T. Valentine, H. S. Lawrence, in *Leukocyte Culture Conference*, W. O. Rieke, Ed. (Appleton-Century-Crofts, New York, 1969), p. 475; *J. Exp. Med.*, in press.
3. J. A. Mills, *J. Immunol.* **97**, 239 (1966); J. J. Oppenheim, R. A. Wolstencroft, P. G. H. Gell, *Immunology* **12**, 89 (1967); W. McFarland and D. H. Heilman, *Amer. Rev. Resp. Dis.* **93**, 742 (1966).
4. H. S. Lawrence and A. M. Pappenheimer, Jr., *J. Clin. Invest.* **36**, 908 (1957); *J. Exp. Med.* **104**, 321 (1956).
5. S. Kasakura and L. Lowenstein, *Nature* **208**, 794 (1965); S. Kasakura and L. Lowenstein, *Transplantation* **5**, 459 (1967).
6. B. D. Kahan and R. A. Reisfeld, *Transpl. Proc.* **1**, 483 (1969).
7. B. D. Kahan, R. Reisfeld, L. Epstein, J. G. Southworth, in *Histocompatibility Testing*, E. S. Curtoni, P. L. Mattiuz, R. M. Tosi, Eds. (Munksgaard, Copenhagen, 1967), p. 295.
8. B. R. Bloom and B. Bennett, *Science* **153**, 80 (1966); J. R. David, *Proc. Nat. Acad. Sci. U.S.* **56**, 72 (1966).
9. W. P. Kolb and G. A. Granger, *Proc. Nat. Acad. Sci. U.S.* **61**, 1250 (1968).
10. N. H. Ruddle and B. H. Waksman, *J. Exp. Med.* **128**, 1267 (1968); A. S. Lebowitz and H. S. Lawrence, *Fed. Proc.* **28**, 630 (1969).
11. M. Nakano and W. Braun, *J. Immunol.* **99**, 570 (1967); W. Braun, M. Nakano, L. Jaraskova, Y. Yajima, L. Jimenez, in *Nucleic Acids in Immunology*, O. J. Plescia and W. Braun, Eds. (Springer-Verlag, New York, 1968), p. 347.
12. H. S. Lawrence, S. Al-Askari, J. R. David, E. C. Franklin, B. Zweiman, *Trans. Ass. Amer. Physicians* **76**, 84 (1963).
13. P. M. Fireman, M. Boesman, Z. Haddad, D. Gitlin, *Science* **155**, 337 (1967); H. S. Lawrence and F. T. Valentine, *Proc. N.Y. Acad. Sci.*, in press.
14. Supported by PHS grant AI-01254-14 and in part by the Streptococcal and Staphylococcal Commission of the Armed Forces Epidemiology Board. F.T.V. supported by PHS training grant AI-00005-11 and by the John Polachek Foundation for Medical Research. We thank Miss Toby Nathan for technical assistance.

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Neutron Diffraction of Cell Membranes (Myelin)

Abstract. *Small-angle neutron diffraction (wavelength 4.05 angstroms) of human and rabbit sciatic nerve has been carried out by means of the Brookhaven high flux beam reactor with an automated slit camera. Most of the free water of the nerves was substituted in order to minimize incoherent scatter of hydrogen atoms. The differences in amplitude and phase shifts between neutrons and x-rays resulted in a neutron diffraction pattern that was completely different from the x-ray pattern. The neutron pattern consisted of a single peak of about 89-angstrom spacing in the region examined (up to 6-angstrom spacing). The strong third, fourth, and fifth order reflections (about 60, 45, and 36 angstroms) seen in the x-ray pattern were suppressed. The neutron data indicated a strong scattering from one portion of the membrane.*

The recent availability of high flux neutron reactor sources makes it practical to examine biological specimens only 1 to 2 mm thick. X-ray diffraction estimates the electron density distribution of the specimen, whereas neutron diffraction measures the distribution of those atomic nuclei that have a large cross section for elastic coherent scattering (*1–3*). Combination of x-ray and neutron diffraction data can be expected to aid considerably in the structure analysis of biological materials.

The very low level of radiation damage associated with neutron diffraction (as a result of the low quantum energy of each neutron) is an important advantage for biological work. However, a severe disadvantage is the high level of incoherent neutron scattering of hydrogen. In many cases, this will require that D_2O be substituted for the free water of the biological specimen.

We have studied the neutron diffraction of human and rabbit sciatic nerve, using the high flux beam reactor of the Brookhaven National Laboratory and a small-angle neutron diffraction camera designed and built by Dr. Nathans. The general arrangement is shown in Fig. 1. Neutrons from the reactor were

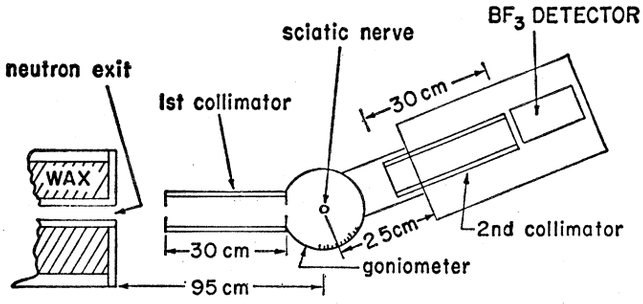


Fig. 1 (above). Plan view of the neutron diffractometer of Dr. R. Nathans, Brookhaven National Laboratory. The neutrons exit from the steel pipe on the left and are collimated by a pair of 2.5-mm cadmium slits. The sciatic nerve is supported vertically, parallel to the slits, and is kept moist by dripping 0.18*M* NaCl in D_2O over it. The second collimator also consists of a pair of 2.5-mm vertical cadmium slits. The neutrons are detected by a BF_3 proportional detector. The goniometer and counting procedures are automated and under computer control.

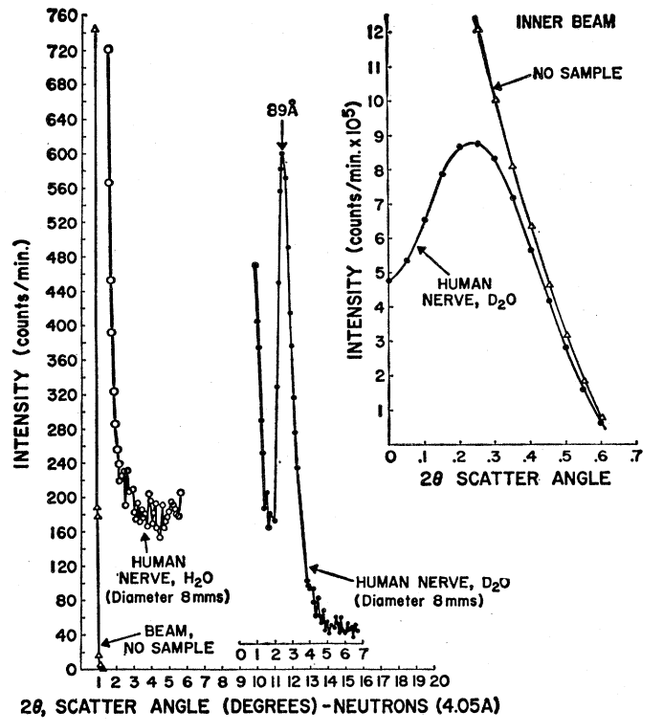
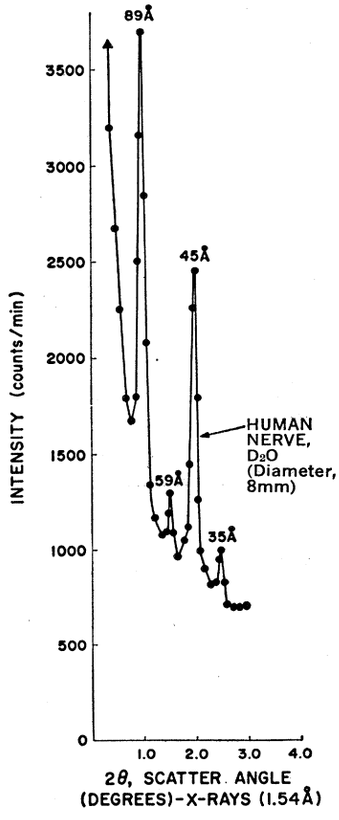


Fig. 2 (right). Neutron diffraction from a 9-mm specimen of human nerve with and without partial substitution of the free water by D_2O. The incoherent hydrogen scatter completely suppresses the diffraction peak (89 Å) without substitution of D_2O. (Inset) Interparticle interference produces a minimum in the central beam. The deuterated sample shows a single strong peak at 89 Å.

monochromatized by passage through a slightly curved, long, polished steel pipe to give a narrow band of wavelengths (4.05 ± 0.41 Å). The beam was collimated by vertical cadmium slits. The detector was a BF_3 neutron counter. The goniometer and the counter could be operated manually or under computer control, and the total counts per chosen time increment were printed out. The direct beam had a total angular width of 0.5° at half height, a divergence of 0.17°, and an intensity (collimated) of 2×10^6 neutrons per minute. The background count, without a sample, was less than 10 neutrons per minute at 2°. Human sciatic nerve (9 mm thick) and rabbit sciatic nerve (3 mm thick) were examined with and without prior exchange of the free water for D_2O by overnight soaking at 4°C in D_2O containing 0.18*M* NaCl. The nerve was supported vertically, parallel to the slits, and kept under tension by a weight of about 50 g. A water or D_2O solution of 0.18*M* NaCl was run slowly down over the nerve at room temperature.

The incoherent scatter of water hydrogen was found to completely mask the diffraction pattern (Fig. 2). Deuteration experiments with frog sciatic nerve indicated that the free water of nerves could be largely substituted by D_2O without significant structural change (as indicated by x-ray diffraction) of the myelin structure. Overnight exchange of water of the human sciatic nerve by 0.18*M* NaCl in D_2O reduced background scatter counts from 180 to 45 per minute with the appearance of a sharp peak at 89 Å. The inner beam profile showed a pronounced intensity dip (inset of Fig. 2). This was interpreted as resulting from interparticle interference of the axon-myelin cylinders. The same deuterated piece of nerve was examined by x-ray diffraction ($CuK\alpha - \lambda = 1.54$ Å) by means of an automated Kratky small-angle slit-camera arrangement (Fig. 3). The x-ray diffraction pattern showed a



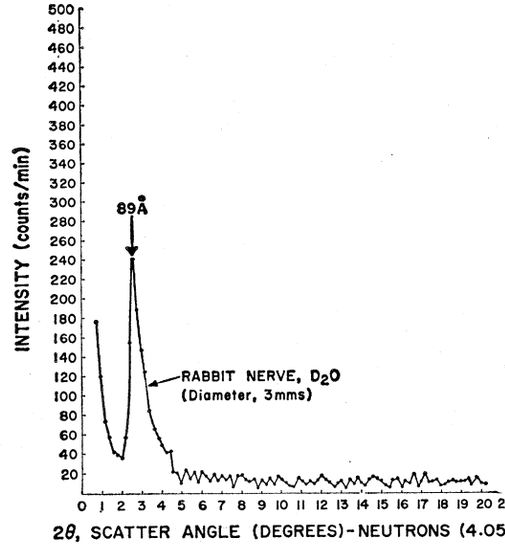


Fig. 3 (left). X-ray diffraction ($CuK\alpha$) of the same deuterated piece of human sciatic nerve as previously used for neutron diffraction experiment. The third, fourth, and fifth orders of a fundamental periodicity of 178 Å are present.

Fig. 4 (above). Neutron diffraction of deuterated rabbit nerve. The pattern resembles the one from human nerve. The third, fourth, and fifth order reflections given by x-ray diffraction are suppressed.

nearly normal spectrum of fundamental periodicity of 178 Å, but with a somewhat reduced 300 reflection. Mammalian peripheral nerves are reported to have a fundamental periodicity near to 184 Å (*4*). The neutron diffraction pattern involved a remarkable suppression of the 300, 400, and 500 reflections of the human myelin x-ray pattern. The suppression of the third, fourth, and fifth orders of the x-ray diffraction pattern was confirmed by examination of deuterated rabbit sciatic nerves (Fig. 4).

The most direct interpretation of the observed neutron diffraction pattern is that the neutrons see a sinusoidal distribution of scattering material. This could arise if neutrons diffracted either from the protein portion only, or the lipid portion only, of the membrane. Alternatively, the third, fourth, and fifth reflections may be suppressed because they contain a large contribution of neutron scattering from hydrogen (which is 180° out of phase with the carbon scattering). However, it seems remarkable that the antiphase contribution would exactly equal the in-phase contribution. A detailed quantitative interpretation of the neutron and x-ray data is being prepared.

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References and Notes

1. G. E. Bacon, *Neutron Diffraction* (Oxford Univ. Press, Oxford, ed. 2, 1962).
2. P. M. Harris and R. A. Erickson, in *Molecular Physics*, D. Williams, Ed. (Academic Press, New York, 1962), vol. 3, p. 348.
3. I. Waller, in *Advanced Methods of Crystallography*, G. N. Ramachandran, Ed. (Academic Press, New York, 1964), p. 157.
4. F. O. Schmitt, R. S. Bear, K. J. Palmer, *J. Comp. Cell. Physiol.* **18**, 31 (1941).
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Cyclic Adenosine Monophosphate: Possible Mediator for Norepinephrine Effects on Cerebellar Purkinje Cells

Abstract. *Microelectrophoretic application of norepinephrine or cyclic adenosine monophosphate reduces the discharge frequency of Purkinje cells in the rat cerebellum. In contrast, other nucleotides accelerate the discharge rate of most units. Parenterally administered theophylline, which inhibits the hydrolysis of cyclic adenosine monophosphate enhances the effects of norepinephrine and cyclic adenosine monophosphate. Therefore, norepinephrine may be able to regulate Purkinje cells functionally by metabolic stimulation of cyclic adenosine monophosphate synthesis.*

Despite the wealth of information about the neuronal connections to cerebellar cortex (*1*), the synaptic transmitters operating within this structure have not yet been defined. Histochemical (*2*) and biochemical (*3*) studies, confirming the presence of norepinephrine-containing nerve endings and high turnover rates of norepinephrine (NE) in the cerebellar cortex, prompted us to test the responsiveness of Purkinje cells to this drug when administered electrophoretically from 5-barrel micropipettes. We now report that almost all Purkinje cells exhibit reproducible reductions in spontaneous discharge rate in response to NE (*4*) and suggest that this response may be mediated by 3′,5′-adenosine monophosphate (cyclic-AMP).

Adult albino rats were decerebrated or anesthetized with chloral hydrate (350 mg/kg). Routine techniques of single-unit recording and microelectrophoretic drug administration were used (*5*); standard electrical methods prevented both polarization of the electrode tip during drug ejection and the undesirable diffusion of drugs from the pipette.

Spontaneously active nerve cells were identified as Purkinje cells on the basis of the so-called "inactivation potentials" or "climbing fiber responses" (*6*), namely, high-frequency (300 to 500/sec) bursts of two to five spikes, superimposed on a slow wave, seen in capacitance-coupled recordings. In our study, neurons exhibiting such bursts also showed a rapid irregular rate of single spike spontaneous discharge (usually 50 to 100 per second).

Nearly all (98 percent) of the 143 Purkinje cells studied responded to NE, by a reduction in their spontaneous discharge rate (*7*). Although the mean discharge rate decreased markedly during the administration of NE (Fig. 1A), Purkinje cell discharge tended to occur at the same preferred interspike intervals observed during the control period. This is reflected in the interspike interval histograms shown in Fig. 1, B and C. Furthermore, "climbing fiber" responses were rarely affected by microelectrophoresis of NE (Fig. 3). A characteristic feature of the response to NE was its slow onset and a persistence for many seconds after termination of the ejection current.

The question arises as to how NE reduces firing rate. In many peripheral sympathetically innervated tissues, the influence of NE may be mediated metabolically by cyclic-AMP. Norepinephrine is thought to increase the synthesis of this nucleotide by stimulating the activity of adenyl cyclase (*8*). This enzyme, which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic-AMP, has a high specific activity within the cerebellum (*9*) and is still further activated by NE in this structure in vitro (*10*). Therefore, we tested the responsiveness of Purkinje cells to cyclic-AMP administered by microelectrophoresis. Cyclic-AMP (Fig. 1) also reduced mean discharge rates of Purkinje cells with the same minimum effects upon the most probable interspike interval and climbing fiber bursts as seen with NE. Of 59 Purkinje cells studied, 44 (75 percent) responded to cyclic-AMP application. Within this responsive group of cells, 73 percent (32 cells) exhibited a reduction in discharge rate and 18 percent (8 cells), an elevation. Nine percent of the responsive cells showed a biphasic or reversible type of response, consisting of a sequential acceleration and reduction of rate during or shortly after drug application, or of a complete reversal of the direction of response upon subsequent testing. In contrast to the delayed onset and termination of the response to NE, more rapid effects were observed with microelectrophoresis of cyclic-AMP. The unresponsiveness of 25 percent of the cells tested with cyclic-AMP may be accounted for by the presence of phosphodiesterase, a soluble enzyme that hydrolyzes cyclic-AMP, in the cerebellar cortex (*9*). Although the dibutyryl derivative of cyclic-AMP is generally more potent in peripheral tissues (*11*), we found no ob-