all types of synapses, regardless of potential differences between the postsynaptic neurons.

Endocrine experiments suggest that although the tuberal hypothalamus is essential for the tonic control of the basal secretion of gonadotrophins, the cyclic trigger for the preovulatory surge, which is present in the adult female but not in the male, requires the integrity of the preoptic area (*1*). The preoptic area is also important for both male and female sexual behavior (*10*). There is also evidence that the amygdala may have effects on gonadotrophin control, and that the stria terminalis is a crucial pathway for these effects (*5*). Our anatomical findings show that the axons of the stria terminalis differ in their mode of termination in the preoptic area and in the tuberal hypothalamus. In addition, the neuropil of the preoptic area (but not of the ventromedial nucleus) is different in the male rat from that in the female. This difference is seen, however, in the terminals of fibers which do not originate in the amygdala, and whose origins are at present unknown. An ovulatory surge of gonadotrophins can be elicited by stimulation of the preoptic area in the male rat or in the female rat treated with androgens as a neonate; neither possesses a spontaneous trigger for the induction of ovulation (*11*). This therefore raises the possibility that the functional differences between the male and the female preoptic areas are due to some difference in the neural connections rather than some intrinsic property of the neurons of the preoptic area, a conclusion which is in agreement with our anatomical demonstration of sexual dimorphism in the mode of termination of the afferent fibers to the preoptic area.

The fact that sexual dimorphism occurs in a part of the brain does not, of course, prove that such dimorphism is related to sexually differentiated functions such as the ability to produce an ovulatory surge of gonadotrophins or sexual behavior. However, the location of the anatomical difference in the preoptic area, which has been shown to be essential for these functions, is persuasive circumstantial evidence.

GEOFFREY RAISMAN
PAULINE M. FIELD
Department of Human Anatomy, University of Oxford, Oxford OX1 3QX, England

References and Notes

1. G. W. Harris and H. J. Campbell, in *The Pituitary Gland*, G. W. Harris and B. T. Donovan, Eds. (Butterworths, London, 1966), p. 99; C. A. Barraclough, in *Neuroendocrinology*, L. Martini and W. F. Ganong, Eds. (Academic Press, New York, 1967), vol. 2, p. 61.
2. Köves and B. Halász, *Neuroendocrinology* **6**, 180 (1970).
3. W. J. H. Nauta, *Physiol. Rev.* **40**, 102 (1960).
4. ———, *J. Anat. (London)* **95**, 515 (1961); W. M. Cowan, G. Raisman, T. P. S. Powell, *J. Neurol. Neurosurg. Psychiat.* **28**, 137 (1965).
5. M. E. Velasco and S. Taleisnik, *Endocrinology* **84**, 132 (1969).
6. I. E. Lawton and C. H. Sawyer, *Amer. J. Physiol.* **218**, 622 (1970).
7. B. V. Critchlow and M. E. Bar-Sela, in *Neuroendocrinology*, L. Martini and W. F. Ganong, Eds. (Academic Press, New York, 1967), vol. 2, p. 101.
8. G. Raisman, W. M. Cowan, T. P. S. Powell, *Brain* **89**, 83 (1966).
9. L. Heimer and W. J. H. Nauta, *Brain Res.* **13**, 284 (1969).
10. R. D. Lisk, in *Neuroendocrinology*, L. Martini and W. F. Ganong, Eds. (Academic Press, New York, 1967), vol. 2, p. 197.
11. J. W. Everett, *Annu. Rev. Physiol.* **31**, 383 (1969).

12. We thank Dr. K. Brown-Grant for advice, and the Medical Research Council (G 970/-668/B) and the Foundations Fund for Research in Psychiatry (grant 70-472) for financial assistance.

27 May 1971

Stimulation-Dependent Alterations in Peroxidase Uptake at Lobster Neuromuscular Junctions

Abstract. *The uptake of cytochemically demonstrable horseradish peroxidase into small vesicles within nerve endings in lobster stretcher muscles can be enhanced by electrical stimulation of transmitter release by the endings. This is observed particularly if stimulation is interrupted periodically and the nerves permitted to rest.*

It is widely, though not universally, assumed that neurotransmitters are released from nerve endings by processes resembling *exocytosis*, that is, by the fusion of synaptic vesicles with the plasma membrane delimiting the axon. However, as dramatically illustrated by recent work of Bittner and Kennedy (*1*), such a process should add considerable amounts of membrane to the axon surface during periods of sustained rapid firing. In gland cells and some other tissues, the addition of membrane to the surface through exocytosis appears to be compensated for by processes resembling *pinocytosis*, that is, by the budding back into the cell of small vesicles and tubules (see *2*).

Pinocytosis by nerve cells, including by axon endings, has been shown to occur in a wide variety of experimental systems (*3–6*) by use of several macromolecular tracers, including Thorotrast, ferritin, and horseradish peroxidase. But it has proved difficult to demonstrate that tracer uptake rates respond to increases in rates of transmitter release, as might be expected if pinocytosis-like formation of tubules and vesicles were a compensatory or membrane retrieval mechanism. Thus, Birks (*4*) reports that in frog neuromuscular junctions there is little or no change in the frequency of Thorotrast-containing vesicles when the endings are stimulated to prolonged repetitive firing by raising the K^+ concentration of the medium. Among other conceivable explanations, Birks advances the possibility that the tracer-containing tubules and vesicles are artifacts, derived during fixation by alteration of membrane systems that are actually continuous with the plasma membrane of the living cell. Elsewhere (*6*) we have outlined evidence, from studies with lanthanum on axon endings in the rat adrenal medulla, indicating that such an artifactitious origin is unlikely to explain the presence of macromolecular tracers in more than a very few of the vesicles in a given ending. Below we report evidence that there are experimental conditions under which the number of peroxidase-containing

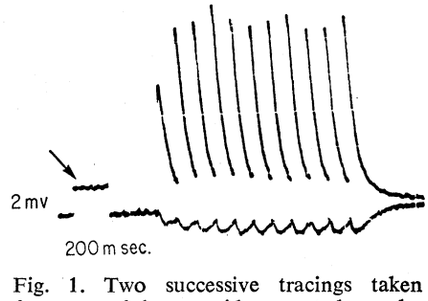

Fig. 1. Two successive tracings taken from one of the peroxidase-treated muscles that was given three 10-minute periods of stimulation alternating with 10-minute rest periods. The arrow indicates a calibrator pulse with the parameters noted (2 mv, 200 msec). In the upper trace, the excitor nerve was stimulated, and in the lower, the inhibitor nerve was stimulated. Corresponding EPSP's and IPSP's are seen (*7*).

vesicles responds markedly to alterations in rates of transmitter release.

The excitatory and inhibitory nerves to the stretcher muscle of lobster walking limbs were prepared for external stimulation as described previously (*7*). The muscle itself was exposed through a hole cut in the carapace and bathed in an artificial seawater (*8*) containing, per liter, 465 meq of Na^+, 10 of K^+, 50 of Ca^{2+}, and 525 of Cl^-. Horseradish peroxidase (1.5 to 6 percent) (Sigma Biochemicals, type II) was usually added to this medium. Synaptic potentials were recorded from the muscle cells by using KCl-filled micropipettes and standard electrical equipment. Figure 1 shows two successive oscilloscope sweeps demonstrating the presence of excitatory postsynaptic potentials (EPSP's) and inhibitory postsynaptic potentials (IPSP's) in one of the peroxidase preparations. Synaptic potentials were monitored throughout all stimulation experiments; we have noted no appreciable effect of peroxidase on the electrical behavior of the neuromuscular junctions.

This report is based on study of several dozen axon endings in 16 walking limb preparations; six of the muscles were soaked in peroxidase-containing medium for 45 to 70 minutes but not stimulated, eight were stimulated in the presence of peroxidase, and two were stimulated in the absence of peroxidase. All experiments were carried out at room temperature (22°C). The stimulation experiments lasted 45 to 70 minutes. Supermaximal stimuli were delivered, usually in bursts of 1.5 seconds duration separated by 0.5-second intervals, at rates of 10 to 30 per second. The total numbers of stimuli delivered to a given preparation ranged from 15,000 to 30,000. As far as can be judged from electrophysiological evidence, essentially all the neuromuscular endings in a preparation maintained good responsiveness during the experiments, and postsynaptic potential frequency faithfully followed stimulus frequency. It should be borne in mind that the postsynaptic potentials recorded often represent the summed effects of several neighboring axon endings.

In some experiments, stimulation was continuous, up to the time of tissue fixation for microscopic examination. In others, stimulation was stopped 10 minutes before fixation, and in still others, three 10-minute periods of stimulation were alternated with 10-minute rest periods. Tissue preparation was by our standard procedures (*9*).

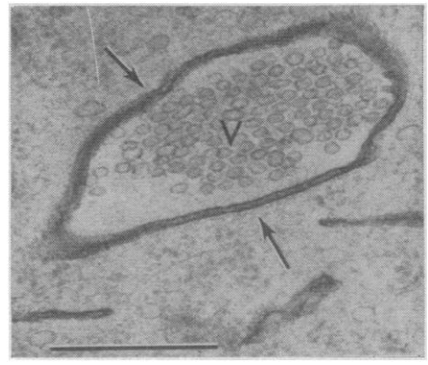

Fig. 2. From a preparation exposed (45 minutes) to peroxidase without electrical stimulation. The thin section was not stained with heavy metals. The electron density in the region indicated by the arrows is due to peroxidase reaction product that has accumulated in the space between muscle and an axon ending. No reaction product is seen in the vesicles (*V*) within the ending. As in this figure, individual thin sections of axon endings often show the axon apparently surrounded completely by muscle; from our observations we would expect to find portions of Schwann cells adjacent to regions of the surface of this ending in serial sections or in a section cut at a different angle. Bar length is 0.5 μm.

In brief, tissue was fixed with a dilute modification of Karnovsky's paraformaldehyde-glutaraldehyde mixture in phosphate buffer or in 2 percent glutaraldehyde in cacodylate buffer, rinsed in fixative-free buffer, frozen on the head of a freezing microtome, incubated (50 minutes, room temperature) in the medium of Graham and Karnovsky (*9*) to demonstrate sites of peroxidase activity, and then postfixed in osmium tetroxide and embedded in Epon. Thin sections were examined in an RCA EMU 3F microscope, usually without heavy-metal staining. Since neuromuscular endings are relatively sparsely distributed in the tissue, the sections used were very large, and each embedded block was sampled at several levels to maximize chances of encountering an ending. Muscle not exposed to peroxidase, and peroxidase-exposed material incubated in peroxide-free medium, served as incubation controls; such preparations contained no reaction product.

Figure 2 shows a nerve ending from a preparation that was not stimulated. Considerable reaction product, indicative of the presence of peroxidase, is seen in the space between axon and muscle. There do not appear to be major barriers preventing penetration of protein to the axon surface (*6, 9, 10*), although there probably are differences in the rates at which peroxidase reaches the surfaces of given endings depending on factors such as the distances of the endings from the outside of the muscle mass. The outlining of endings by reaction product has aided in the search for neuromuscular junctions in the thin sections.

None of the vesicles within the nerve ending shown in Fig. 2 contain demonstrable peroxidase. In some unstimulated endings, we do see vesicles with reaction product, but the number of such vesicles rarely exceeds four or five of the one hundred to several hundred "synaptic" vesicles seen in a given thin section. The situation is markedly different in stimulated preparations. Consonant with Birk's finding, the changes we have thus far observed have been least impressive if stimulation was continuous up to the time of fixation (*4, 10*), although we do find some endings with appreciable numbers of vesicles containing peroxidase. However, when rest periods are provided, the number of vesicles with peroxidase reaction product is often considerably greater than in unstimulated endings. Our greatest success so far has been with the neuromuscular junctions in preparations given alternating periods of stimulation and rest. In these, as many as 10 to 20 percent (30 to 60 vesicles) or more of the vesicles in a given thin section show reaction product (Fig. 3). These findings are consistent with the observations of Jones and Kwanbunbumpen (*11*), who have reported transient depletion of the vesicle population of stimulated nerves fixed immediately after stimulation, but recovery of the population if the endings are permitted to rest for a few minutes before fixation.

Unlike the situation in some related systems (*2, 3, 5, 6, 9*), only a few of the vesicles involved in tracer uptake in the present study are "coated." Like the other vesicles in the nerve endings, most are about 40 to 60 nm in maximum diameter and lack obvious coats; a few are somewhat larger. We have not found sufficient numbers of coated vesicles to be able to evaluate their responses to stimulation.

Crustacean neuromuscular junctions of the type we are studying are thought to transmit by glutamate or γ-aminobutyric acid (*12*). Transmission appears to be by release of "quanta," as in cholinergic and adrenergic endings, and it seems reasonable to assume that the small vesicles found in the nerve

endings are responsible for quantal transmitter release (*1, 12*). In crayfish neuromuscular junctions, the rate of electrically stimulated transmitter release has been estimated as on the order of one quantum per impulse per ending (*1*). If this holds true for lobster junctions, the present results suggest that processes resembling pinocytosis could account for retrieval of a large fraction of the membrane that might be added to the axon surface during transmitter release. The geometry of the lobster endings is fairly complex and we have not studied serial sections extensively. However, in individual thin sections, single endings often have maximal dimensions of more than 5 μm. Since the thin sections we use are approximately 50 to 100 nm thick (*13*), stimulated endings such as the one shown in Fig. 3 probably contain a total of at least several thousand vesicles that have acquired tracer during the course of the experiment. Our information is not adequate to evaluate differences, if any, among endings of differing types (excitatory, inhibitory, and so forth).

Several additional points are worth noting. First, our results suggest, but obviously do not demonstrate, that transmitter release is by exocytosis. Second, the findings do not necessarily imply that most "synaptic" vesicles derive initially from the plasma membrane. There is growing evidence that at least some of the vesicles found in axon endings are transported there from elsewhere; possible sources of such vesicles (*11, 14*) include the perikarya and the axonal agranular reticulum (*14*).

In addition, we cannot tell, from present techniques, whether the bit of membrane that delimits a peroxidase-containing vesicle is the same bit of membrane that originally surrounded a transmitter-containing vesicle or whether exogenous molecules taken up *within* the vesicles are potentially important for neuronal metabolism or for cellular interactions.

Related to this is the question of the fate of tracer-containing vesicles. In many situations, pinocytosis vesicles fuse with multivesicular bodies and other types of lysosomes. The contents of the vesicles are presumed to be digested, and the modes of formation of multivesicular bodies [via cuplike bodies (*5, 6*) and other intermediate structures (*15*)] might conceivably permit the vesicle membrane to be digested as well, by eventual incorporation within the lysosome (*3, 5, 6, 15*); lysosomal digestion of Schwann cell plasma membrane derivatives is known to occur in Wallerian degeneration (*16*). Multivesicular bodies that accumulate exogenous tracers are found at some nerve endings, and we have noted a few in the lobster material. However, it would be very premature to conclude that the peroxidase-containing vesicles in the lobster endings are all fated to be rapidly degraded; the possibility that many are reused for transmission is not ruled out. Nevertheless, the involvement of lysosomes in sequestering exogenous material in all regions of neurons (*3, 5*) may well be accompanied by lysosome-mediated degradation of plasma membrane and related intracellular membranes; this could have important implications for normal turnover and for some of the storage diseases (*5*).

Finally, we are not claiming to have demonstrated either an obligatory one-to-one correspondence between numbers of transmitter quanta released and numbers of peroxidase-containing vesicles formed or that preceding transmitter release is an invariable requirement for *endocytosis* (pinocytosis and related processes) at axon endings. In two unstimulated endings in our preparations, the numbers of vesicles with tracer reached roughly 8 percent, and in several stimulated and rested endings, 20 to 50 percent or more of the vesicles showed demonstrable peroxidase. These figures approximate those expected for a one-to-one relationship if one makes reasonable assumptions about rates of spontaneous and induced release of quanta. However, the preparations were too variable for reliable extrapolation from such observations, and the complexities of the experimental system inhibit us from making elaborate calculations. For example, the axons are cut as part of the preparative procedure, and they are bathed in a medium containing a high concentration of foreign protein; the behavior of the endings may thus be abnormal in some respects.

What we have shown is that there are conditions of potential physiological importance under which the rates of vesicle formation from the plasma membrane are quite high. It could still turn out that the normal relations between transmitter release and endocytosis are indirect, that release occurs at different loci from endocytosis, that transmitter vesicles or pinocytosis vesicles are heterogeneous in origin and

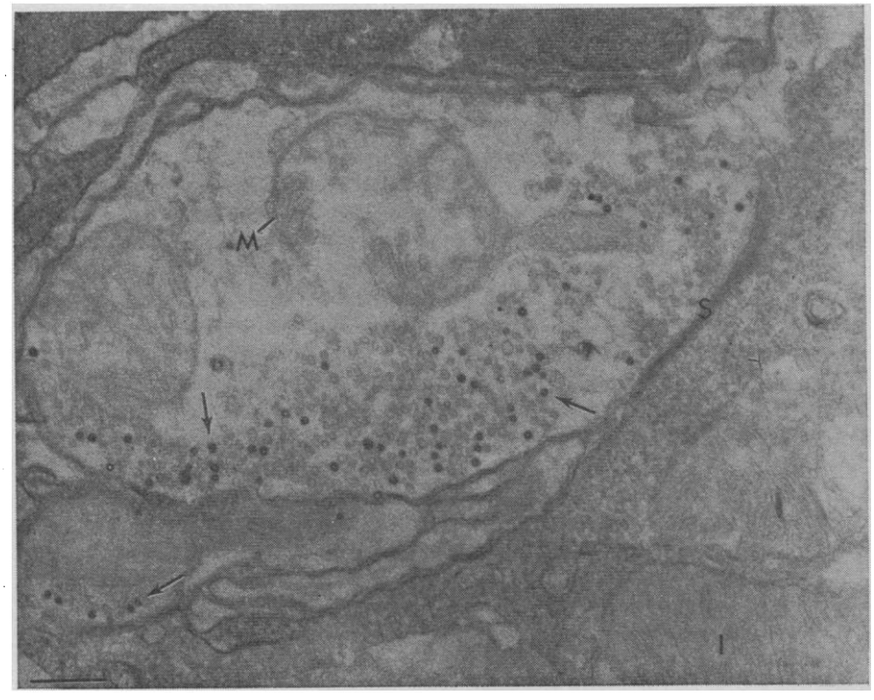

Fig. 3. From a preparation exposed to peroxidase and stimulated for three periods of 10 minutes each, alternating with 10-minute rests. The stimuli were delivered at 10 to 20 per second and the total number given was approximately 16,000. The thin section was not stained with heavy metals. Peroxidase reaction product is present within many small vesicles in an axon ending; a few of the vesicles are indicated by arrows. *M* designates axonal mitochondria; *S*, the space between the axon and muscle; and *I*, muscle mitochondria. As in other tissues (*17*), occasional mitochondrial cristae show reaction product. Bar length is 0.5 μm.

behavior, that endings vary depending on the types of transmitters they contain or the rates at which they are functioning, or that presently unknown mechanisms operate along with endocytosis to diminish the surface area of axons.

ERIC HOLTZMAN
Department of Biological Sciences, Columbia University, New York 10027

ALAN R. FREEMAN*
Department of Physiology, Rutgers University Medical School, New Brunswick, New Jersey

LEONARD A. KASHNER
Columbia University, New York

References and Notes

1. G. D. Bittner and D. Kennedy, *J. Cell Biol.* **47**, 585 (1970).
2. A. I. Amsterdam, I. Ohad, M. Schramm, *ibid.* **41**, 753 (1969); S. K. Masur, E. Holtzman, I. L. Schwartz, R. Walter, *ibid.* **49**, 582 (1971); E. Holtzman, *Exocytosis and Endocytosis* (report of workshop at the 1971 Gatlinburg, Tennessee, Conference on Membranes (Wistar Institute Press, Philadelphia, in press); E. Holtzman and R. Dominitz [*J. Histochem. Cytochem.* **16**, 320 (1968)] and S. Abrahams and E. Holtzman [see (*6*) and *J. Cell Biol.*, abstract in press] report that such processes occur in the adrenal medulla, a tissue closely related to nervous tissue.
3. J. Rosenbluth and S. L. Wissig, *J. Cell Biol.* **23**, 307 (1964); G. M. Villegas and J. Fernandez, *Exp. Neurol.* **15**, 18 (1966); M. W. Brightman, *Progr. Brain Res.* **29**, 19 (1968); N. H. Becker, A. Hirano, H. M. Zimmerman, *J. Neuropathol. Exp. Neurol.* **27**, 439 (1968); A. H. Bunt, *J. Ultrastruct. Res.* **28**, 411 (1969); S. L. Zacks and A. Saito, *J. Histochem. Cytochem.* **17**, 161 (1969); U. Smith, *Tissue and Cell* **2**, 427 (1970).
4. R. I. Birks, *Ann. N.Y. Acad. Sci.* **135**, 8 (1966).
5. E. Holtzman and E. R. Peterson, *J. Cell Biol.* **40**, 863 (1969); E. Holtzman, in *Lysosomes in Biology and Pathology*, J. T. Dingle and H. B. Fell, Eds. (North-Holland, Amsterdam, 1969), vol. 1, p. 191.
6. E. Holtzman, *Philos. Trans. R. Soc. Lond. Ser. B* **261**, 407 (1971). The key lanthanum experiment involved intravenous administration of peroxidase to the adrenal medulla followed by perfusion fixation with lanthanum-containing aldehyde fixatives. In such preparations, endings incubated in the full medium for demonstration of peroxidase activity show many vesicles with peroxidase reaction product, whereas identical material incubated in the absence of substrate (H_2O_2) shows lanthanum at the axon surfaces but only in a very few tubules or vesicles within the endings.
7. H. Grundfest, J. P. Reuben, W. H. Rickles, *J. Gen. Physiol.* **42**, 1301 (1959).
8. A. R. Freeman, J. P. Reuben, P. W. Brandt, H. Grundfest, *ibid.* **50**, 423 (1969).
9. E. Holtzman, A. R. Freeman, L. A. Kashner, *J. Cell Biol.* **44**, 438 (1970); M. J. Karnovsky, *ibid.* **27**, 137A (1965); R. C. Graham and M. J. Karnovsky, *J. Histochem. Cytochem.* **14**, 291 (1966).
10. E. Holtzman, A. R. Freeman, L. A. Kashner, *Fed. Proc.* **30**, 216 (1971).
11. S. F. Jones and S. Kwanbunbumpen, *J. Physiol. (Lond.)* **207**, 31, 51 (1970). For review, see also A. Peters, S. L. Palay, H. de F. Webster, *The Fine Structure of the Nervous System* (Hoeber, New York, 1970).
12. For discussion and further references, see: H. L. Atwood and H. S. Johnston, *J. Exp. Zool.* **167**, 457 (1968); H. L. Atwood and W. A. Morin, *J. Ultrastruct. Res.* **32**, 35 (1970); Z. W. Hall, M. W. Bownds, E. A. Kravitz, *J. Cell Biol.* **46**, 290 (1970); P. M. Orkund and E. A. Kravitz, *ibid.* **49**, 75 (1971).
13. L. D. Peachey, in *Thin Sectioning and Associated Techniques* (Sorvall, Norwalk, Conn., 1967).
14. For review, see F. E. Bloom, L. L. Iversen, F. O. Schmitt, Eds., *Macromolecules in Synaptic Function*, Neurosciences Research Bulletin, vol. 8, No. 4 (1970). See also D. S. Smith, U. Jarlfors, R. Beranek, *J. Cell Biol.* **46**, 199 (1970), for axonal transport of vesicles; S. Teichberg, E. Holtzman, J. Abbott (*ibid.*, abstract in press) and E. Holtzman (*6*) discuss roles of the axonal agranular reticulum in formation of dense-cored vesicles.
15. See (*5*) for review, and D. S. Friend and M. G. Farquhar, *J. Cell Biol.* **35**, 357 (1967).
16. E. Holtzman and A. B. Novikoff, *ibid.* **27**, 651 (1965).
17. A. B. Novikoff and S. Goldfischer, *J. Histochem. Cytochem.* **17**, 675 (1969).
18. Support for this research was provided by NIH grant NS-09475 NEUB to E.H.
* Present address: Department of Psychiatry, Indiana University Medical Center, Indianapolis.

14 April 1971

Biological Activity and Synthesis of an Encephalitogenic Determinant

Abstract. *A 45-residue fragment of the basic protein of myelin is encephalitogenic in the rabbit and monkey but relatively inactive in the guinea pig. Synthetic peptides containing the sequence of a tryptic peptide of the fragment Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys are moderately encephalitogenic.*

We have previously reported the isolation, purification, and sequence of a fragment of the basic protein of myelin from five species of mammals which is highly encephalitogenic in the rabbit (*1–3*). This fragment is isolated from an acid extract of acetone-ether defatted central nervous system (CNS) tissue and is produced by the action of an acid protease (*2*, *3*). It is the major degradation product of the basic protein under these conditions. The amino acid sequence of the bovine fragment is shown in Fig. 1. Residue numbers indicate the position of the amino acids of the fragment (45 to 90) in the parent molecule (*3*). The peptide bonds of the basic protein cleaved by the acid protease are indicated by the slash marks. These two Phe/Phe sequences are the only aromatic dipeptide sequences in the basic protein.

The encephalitogenic activity of the fragment in the rabbit, guinea pig, and monkey is shown in Table 1. In the rabbit 50 μg of the fragment (bovine) produced experimental allergic encephalomyelitis (EAE) in nine of ten animals with an average day of onset of 14. This is the minimum dose for maximum activity. As little as 15 μg is active. This degree of activity is comparable to that observed with equimolar amounts of basic protein.

In the guinea pig, the fragment is considerably less active. At a dose of 1.0 μg no clinical signs and only mild histological changes were seen in two of five animals. At 5.0 μg the only clinical sign was weight loss of 60 to 90 g in four of five animals between days 17 and 26 after challenge. Mild to moderate histological changes were found in the CNS of these four animals. Maximum encephalitogenic activity is seen with equimolar amounts of basic protein (*4*).

Three adult squirrel monkeys were injected intradermally with a water-in-oil emulsion containing 500 μg of the fragment (human) and 3 mg of heat-killed tubercle bacilli (HRV-37); this dose was given via the four foot pads. Clinical signs developed 8, 10, and 15 days, respectively, after challenge. The animals stopped moving about the cage, sat on their haunches, and refused to eat or drink. Each was killed 2 days after onset because of severe inanition and dehydration. Three animals injected with complete Freund's adjuvant only remained well until the time they were killed, 2 months after challenge. The animal that became ill on day 15 showed typical lesions of the disease, particularly throughout the white matter of both frontal lobes. Multiple sections of the entire CNS of the other

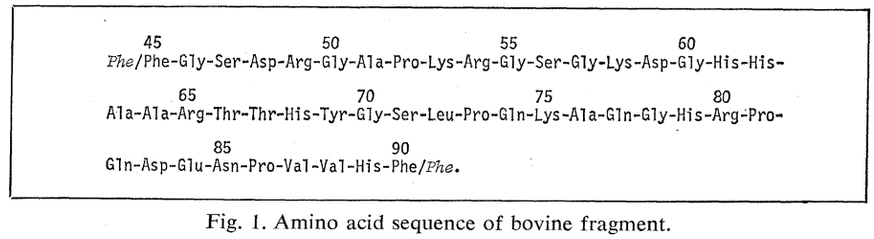

Fig. 1. Amino acid sequence of bovine fragment.