ally disintegrate over the next several days. By 2 weeks, the poisoned neurons will be gone.

To demonstrate that SdT is transported to the appropriate neurons, cryostat sections dried in a vacuum desicator at room temperature can be processed for indirect peroxidase immunohistochemistry using the rabbit antiserum (see Fig. 1B). The steps include the following:

1. Presoak for 30 min in 1% normal goat serum in PBS containing 1% Triton X-100
2. Overnight incubation in humidified Petri dishes at 4° with rabbit anti-SdT antiserum at 1 : 5000–1 : 20,000 dilution in PBS
3. Two 5-min washes in PBS
4. Incubation for 60 min at room temperature in biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA)
5. Wash twice in PBS, 5 min each
6. Incubate for 60 min in Avidin-DH (Vector) in PBS
7. Wash twice in PBS, 5 min each
8. Incubate in peroxidase reaction mixture containing 0.5 mg/ml 3,3′-diaminobenzidine and 0.01% H_2O_2 in 0.05 *M* Tris–HCl, pH 7.2, for 10 min
9. Wash in distilled water for 2–3 min, then dehydrate through graded alcohols; clear, mount, and coverslip

In sections processed using the above protocol, SdT-poisoned neurons will contain numerous brown cytoplasmic granules.

[37] Measurement of Neurotoxic Actions on Mammalian Nerve Impulse Conduction

By JONATHAN J. LIPMAN

Neurotoxins act to impair the proper ionic and electrical activity of, and communication between, the excitable tissues of nerve and muscle. The sites at which neurotoxins act differ for each toxin and most toxins act at more than one site. Certain toxins act on neuronal membranes or those of their support cells, while others act on axonal translocation processes. Still others act on the nerve terminal processes responsible for neurotransmitter release. Measurement of the conduction properties of nerve does not supply information on synaptic or postsynaptic toxicity. On the other hand, measurement of the postsynaptic response properties of muscle following nerve stimulation identifies toxicity at the nerve and

the neuromuscular junction in addition to identifying myotoxic effects. Neuromuscular junction preparations may thus be used to screen for neurotoxic effect of microbial and other neurotoxins. Data obtained from such preparations require further examination in other assay systems to identify the specific locus of toxicity. These other assay systems include intracellular recording, which is used to identify toxicity at the nerve axon membrane, and extracellular recording, which provides information on changes in conduction velocity in isolated nerves and in nerve bundles.

For the purpose of this chapter, the use of intracellular recording techniques is first described to illustrate the information which can be gleaned regarding the actions of toxins on nerve membrane processes. Extracellular recording techniques are then described with regard to the measurement of toxic effects on conduction velocity in isolated nerves and nerve bundles. Finally, neuromuscular junction preparations are described, to show how these identify toxic actions at perisynaptic motoneuron and effector tissue sites. Since a working understanding of the physiological processes deranged by toxin action is required, cursory descriptions of key aspects of these processes are given.

Toxicity at Axon Membrane Sites of Action Potential Propagation

The essential properties of the nerve cell membrane are common in principle and mechanism to all excitable membranes, including those of muscle. The intracellular microelectrode technique is used to assess toxicity at this level of organization. This uses a glass microelectrode with which the isolated nerve is impaled.[1] These microelectrodes are 50 to 0.5 μm at the tip and are filled with conducting solution, usually 3 *M* KCl. The electrical potential difference (PD) across the membrane can then be measured between the intracellular electrode and the bathing solution. In the resting state, this PD across the membrane is due to selective segregation of ionic charges largely due to Na^+, K^+, Cl^-, and large organic anions. This segregation, maintained against the ionic concentration gradients by active processes, is illustrated in general terms in Fig. 1. The electrostatic PD across the membrane (E_m) is approximately predicted from the Nernst equation, stated as:

$$E_m = \frac{RT}{Fn} \ln \frac{[\text{external concentration of permeating ion}]}{[\text{internal concentration of permeating ion}]}$$

where R is the thermodynamic gas constant (8.3 $JK^{-1} \cdot mol^{-1}$); T, the absolute temperature; F, the Faraday constant (96,500 C/mol); and n, the

[1] J. Del Castillo and B. Katz, *J. Physiol. (London)* **124,** 560 (1954).

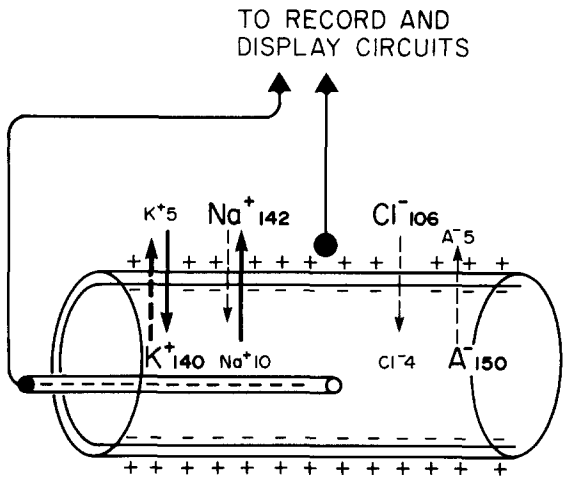

FIG. 1. The ionic basis of the resting membrane potential ionic concentrations is given (mmol/liter). Broken lines indicate the direction of ionic concentration gradients. Solid lines indicate directions of active transport. The membrane is impermeable to large intracellular organic anions (A^-) which contribute to the potential difference. Intracellular recording (as shown) would measure a membrane potential of about −85 mV (inside negative with respect to outside). Modified from W. C. Bowman and M. J. Rand, "Textbook of Pharmacology," 2nd Ed. Blackwell, London, 1980.

valency of the permeant ion. A sophistication of the intracellular recording technique, the voltage clamp method developed by Hodgkin and Huxley,[2] allows the toxicologist to change or control the membrane potential and to prevent the all-or-nothing action potential from occurring. By using this method to separately examine sodium and potassium currents, the actions of toxins affecting one or the other may be studied. The voltage clamp technique necessitates the introduction of two electrodes into the axon, one of which monitors the membrane potential in the usual way; the other being connected to the output of a feedback amplifier producing just sufficient current to hold the potential at the desired value.

The sites of toxin action on nerve membrane excitability and function are explicable with reference to the ionic basis of nerve membrane electrical events. At rest, active ionic charge separation gives the nerve membrane a resting potential of about 40 to 60 mV measured between the intracellular electrode and the external bathing fluid. The inside is negative with reference to the outside (Fig. 2). The nerve axon may be stimulated experimentally by producing a localized reduction in membrane PD of about 15 mV smaller than the resting potential (termed the *depolariza-*

[2] A. L. Hodgkin and A. F. Huxley, *J. Physiol. (London)* **117**, 500 (1952).

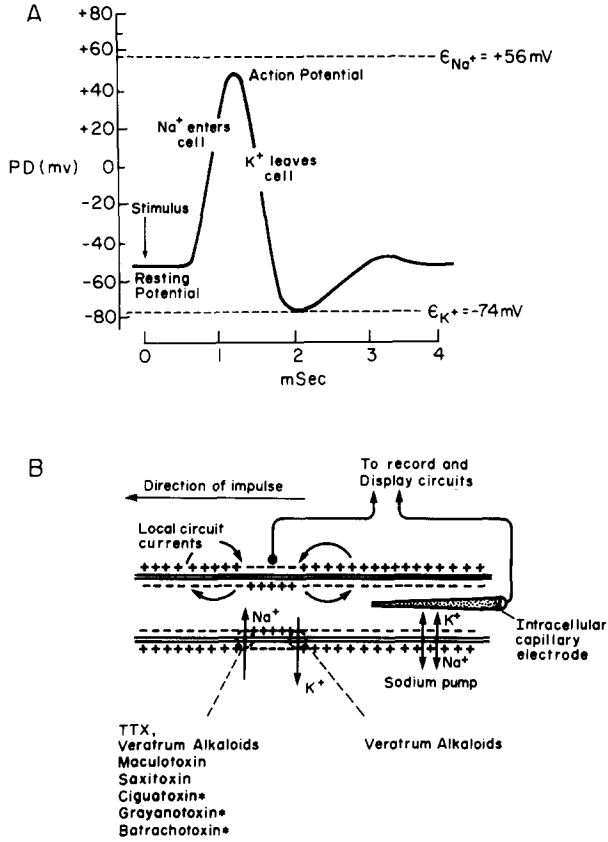

FIG. 2. Intracellular recording. (A) Illustrating the changes in membrane potential (PD, mV) measured between an intracellular capillary electrode and an electrode outside the membrane, as would be observed during the generation and propagation of an action potential illustrated in (B). The equilibrium potentials for sodium (E_{Na^+}) and potassium (E_{K^+}), as calculated from the Nernst equation, are shown. Data are idealized findings from the squid giant axon. (B) Showing the ionic and electrical events occurring during the generation of an action potential. Dotted lines indicate sites of neurotoxic action of some toxins. TTX, Tetrodotoxin. Those toxins shown block sodium channel activation, except where marked with asterisks (*), which depolarize by impairing inactivation.

tion threshold) which triggers an action potential (AP). Although a sequence of subthreshold depolarizations may summate to trigger an action potential, these must occur within a brief interval, known as the period of latent addition. Experimentally, the depolarization is conveniently initiated by a suprathreshold pulse of current passed between closely adjacent electrodes on the outside of the nerve. The voltage drop across the membrane resistance under the cathode locally depolarizes the membrane to

initiate subsequent events. A large and sudden increase in the sodium permeability (termed *activation*) follows (see Fig. 2), and the membrane potential momentarily becomes reversed as sodium flows down its concentration gradient. This depolarization overshoots and reverses in polarity, approaching but not reaching the equilibrium potential for Na^+ before the Na^+ channels close (*inactivation*). Tetrodotoxin (TTX) obtained from the ovaries of the Japanese puffer fish (suborder Tetradontiae) has a cationic guanidium ion at physiological pH. This substitutes for Na^+, blocks the increase in Na^+ conductance (blocks activation), and arrests AP recruitment at this stage. Local anesthetic agents likewise prevent Na^+ activation by a membrane-stabilizing action. TTX also blocks conduction in skeletal muscle cells where the inward depolarizing current is carried by sodium ions, but not in smooth muscle where this is carried by other ions. A related toxin, saxitoxin, is produced by unicellular dinoflagellates (*Gonyaulax catanella*) which are ingested by a variety of shellfish. It was first isolated from the Alaskan butterclam (*Saxidomus giganteus*). The saxitoxin molecule possesses two guanidium ions and blocks neuromuscular transmission (see below) as well as nerve conduction. It is clinically responsible for "paralytic shellfish poisoning," a distinct clinical entity whose symptoms resemble tetrodotoxin poisoning. The veratrum alkaloids (obtained from *Veratrum alba*) exert a part of their toxic effect by delaying activation. Batrachotoxin (from the skin of the frog *Phallobates aurotaenia*), conversely, produces irreversible depolarization by increasing the resting Na^+ permeability and opening Na^+ conduction gates, blocking inactivation. Ciguatoxin, from ciguateric moray eels and grayanotoxin, from rhododendron leaves, exert a similar neurotoxic action to batrachotoxin. Their effect is antagonized, therefore, by tetrodotoxin. As the positive overshoot peaks and sodium channels inactivate, the restoration of the resting membrane potential is facilitated by a secondary increase in potassium permeability along its concentration gradient. A component of the neurotoxic actions of the veratrum alkaloids is exerted by inhibition of these potassium channels. Following these ionic exchanges which constitute the action potential, the resting state is recovered by an increase in the activity of the Na^+/K^+ pump, during which time the nerve is refractory to further impulse transmission. A band of refractoriness therefore allows the AP as it propagates along the nerve, the local circuit current generated by the AP triggering further depolarization of the resting region ahead in the direction of propagation.

Toxic Effects on Conduction

The significance of the local circuit current in initiating the AP is critical as we consider the properties of myelinated neurons. The velocity

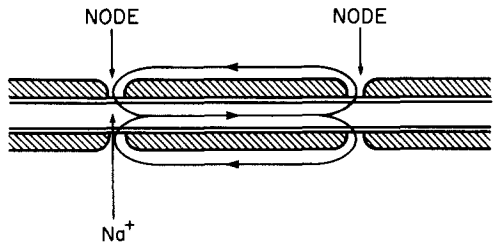

FIG. 3. Local circuit currents in myelinated nerve emanating from a node of Ranvier at which an action potential occurs travel to the next node to initiate an impulse there. Conduction along the nerve as a whole is termed saltatory since it jumps from one node to another. The myelin sheath is shown shaded.

of an AP's travel along a nerve is invariate for a particular fiber; it is determined by fiber size (which also determines the excitability or threshold for depolarization) and the presence or absence of myelination (see Table I). Myelination is a biological strategy for increasing the conduction velocity (CV) of a nerve. Nerve fibers are functionally classified on the basis of CV and fiber size. Class A fibers are the fastest conducting of the myelinated fibers. B fibers are also myelinated but are of smaller diameter than A, and hence slower in conduction. C fibers are nonmyelinated and hence are the slowest. The myelin sheath of a myelinated nerve develops from the surface membranes of the Schwann cells which envelope the developing nerve. The sheath is electrically of high resistance, and is discontinuous along the length of the nerve, gaps interrupting successive Schwann cell-derived lengths of myelin. Those gaps, termed nodes of Ranvier, expose the underlying axon (Fig. 3). Nerve conduction velocity in myelinated fibers is so much faster than in nonmyelinated axons because APs occur only at the nodes. Local circuit currents generated by the AP pass at the speed of electrical conduction through the axoplasm and extracellular fluid to the next available node, where depolarization is triggered. The greater the diameter of the axon the lower is the axoplasmic resistance to local circuit current and the faster is the electrotonic spread of current from one node to the next. This type of conduction, where the impulse jumps from one node to the next, is termed saltatory conduction.

Neurotoxic demyelination such as that produced by diphtheria toxin (from *Corynebacterium diphtheriae*) can thus be detected in myelinated nerve as a reduction in CV. This has been observed in a variety of preparations.[3-5] In isolated nerves, Rasminsky and Sears[6] have demonstrated

[3] B. H. Waksman, R. D. Adams, and H. C. Mansmann, *J. Exp. Med.* **105,** 591 (1957).
[4] H. E. Kaeser and E. H. Lambert, *Electroencephalogr. Clin. Neurophysiol. Suppl.* **22,** 29 (1962).

TABLE I
PROPERTIES OF PERIPHERAL MAMMALIAN NERVE

Property	A			B	C	
	Fiber group				Fiber group	
	α	β	γ		Sympathetic	Dorsal root
	Motor to striated muscle and sensory afferents	Sensory afferents	Muscle spindle efferents and sensory afferents	Preganglionic autonomic efferents	Postganglionic sympathetic	Sensory afferents
Fiber diameter (μm)	← 1–25 →			3	← 1–1.3 →	
Conduction velocity (m/sec)	100–200	60	40	3–14	← 1–2.01 →	
Spike (AP) duration (m/sec)	← 0.4–0.5 →			1.2	← 2.0 →	
Absolute refractory period duration (m/sec)	← 0.4–0.5 →			1.2	← 2.0 →	

increased internodal conduction times of 500 μsec in ventral nerve root fibers demyelinated by diphtheria toxin compared with 19.7 μsec in control fibers.

Conduction velocity is most conveniently measured by extracellular recording techniques. Nerves occur *in vivo* in mixed bundles containing both myelinated and nonmyelinated axon embedded in a ground substance, the endoneurium. The bundles themselves are organized into nerve trunks bounded by perineural connective tissue. When extracellular recording electrodes are placed on the surface of a nerve trunk at some distance from the stimulating electrodes, a monophasic record of the compound action potential (CAP) of the whole trunk is obtained.[7] Extracellular recordings of this type differ in meaning and appearance from the intracellular recordings referred to previously. The monophasic record indicates the passage of AP depolarization under the recording electrode relative either to a widely separated or indifferent electrode. The CAP is the summated current due to individual APs of conducting nerves within the nerve trunk. Since most nerve trunks contain fibers of several different types and each type conducts at a different velocity (Table I), the impulses arrive temporally separated to some measurable degree at the recording electrode, and the shape and form of the CAP reflects these individual contributions. Each potential wave in the CAP reflects the population response of a single fiber type. The AP travelling along the fast A_α fibers are detected first, followed by A_β, A_γ, B, and finally C fiber potentials. The stimulation thresholds for the thicker fibers are lower than for the finer ones, so that it is possible to stimulate the fast conducting fibers preferentially, although the converse is not the case; any stimulus adequate to trigger slow fibers is necessarily suprathreshold for faster fibers. The process of selective stimulation of the fibers in a nerve bundle is illustrated in Fig. 4. In practice, the CVs of a number of nerves of a single fiber type vary with a distribution that is typical for the bundle. Several factors conspire to complicate the interpretation of CAPs of a nerve bundle. These factors include a rather complex recovery cycle attending the passage of each AP as it is generated during the sequence of repetitive impulse trains.[8] The CAP of nerve bundles may nevertheless be analyzed in terms of the distribution of its component conduction velocities (DCV). A formal analysis of the factors responsible for and the analy-

[5] J. A. Morgan-Hughes, *J. Neurol. Sci.* **7,** 157 (1968).
[6] M. Rasminsky and T. A. Sears, *J. Physiol. (London)* **227,** 323 (1972).
[7] H. S. Gasser and J. Erlanger, *Am. J. Physiol.* **62,** 496 (1922).
[8] S. G. Waxman, *in* "Conduction Velocity Distributions" (L. J. Dorfman, K. L. Cummins, and L. J. Leifer, eds.). Liss, New York, 1981.

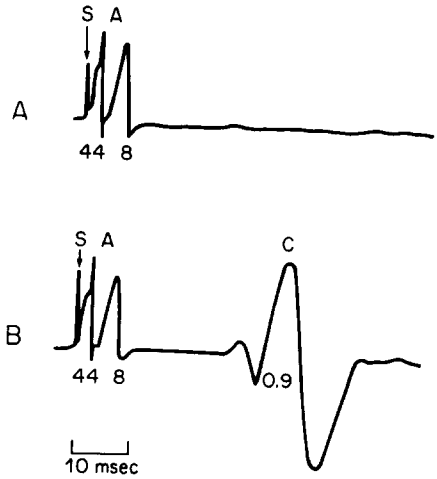

FIG. 4. The compound action potential (CAP) measured from rabbit saphenous nerve, antidromically stimulated 4 cm from the measuring electrodes. S, Stimulus artifact. (A) Following rectangular pulse stimulation of 100-μsec duration, two groups of fast and slow A fiber potentials conducting at 44 and 8 m/sec. (B) When the stimulus amplitude was doubled using 500-μsec duration, the higher threshold "C" fibers are also excited, conducting at 0.9 m/sec. The rabbit saphenous nerve does not contain B fibers, classified on the basis of threshold. Adapted from P. J. Watson, *Eur. J. Pharmacol.* **1,** 407 (1967).

sis of these distributions is reviewed by Dorfman *et al.*[9] Using the DCV technique, Hernández-Cruz and Munoz-Martinez[10] find that Tullidora toxin (from buckthorn, *Karwinskia humboldtiana*) preferentially affects larger rather than smaller diameter fibers. These authors provide a plausible explanation, therefore, for the ability of the toxin to produce clinical quadraplegia with intact sensorium via preferential motoneuron blockade.

Conduction velocity studies thus provide information on a nerve's ability to generate and propagate an AP. Factors affecting generation, such as sodium conduction blockade, and propagation, such as demyelination, interfere with the DCV measured from single nerves or bundles. Clinically, these principles form the basis of the practice of electromyography.[11] CV studies do not directly provide information on toxic effects mediated peri- or postsynaptically, however, for which nerve–muscle preparations must be used.

[9] L. J. Dorfman, K. L. Cummins, and L. J. Leiffer (eds.), "Conduction Velocity Distributions: A Population Approach to Electrophysiology of Nerve." Liss, New York, 1981.

[10] A. Hernández-Cruz and E. J. Munoz-Martinez, *Neuropathol. Appl. Neurobiol.* **10,** 11 (1984).

[11] M. Cherington and R. D. Snyder, *N. Engl. J. Med.* **278,** 95 (1968).

Presynaptic Toxic Sites

The intracellular microtubular system of the neuron is in active use and turnover, translocating cytoplasmic components to and from nerve cell body to presynaptic terminal. Toxins that inhibit the metaphase of mitotic division also disrupt this microtubular integrity and block nerve function. Examples of such toxins include colchicine (from the autumn crocus), the vinca alkaloids (vinblastine and vincristine from *Vinca rosea,* the periwinkle plant), podophyllotoxin (from *Podophyllum* resin), and griseofulvin (from the molds, *Penicillium griseofulvum* Dierckx and *P. janczewskii*). The neurotoxic effect of these toxins is to disrupt microtubular translocation processes and hence to block neurotransmitter delivery and availability at presynaptic nerve endings, both central[12,13] and peripheral.[14]

The AP on arrival at the nerve terminus evokes the release of neurotransmitter substances which transit the cleft between presynaptic membrane and effector cell. Acetylcholine (ACh) is the neurotransmitter at the neuromuscular junction striated muscle, often used to assay for these neurotoxic effects. A large number of neuromuscular junction preparations are available to the toxicologist, prominent among these being the rat (or mouse) phrenic nerve diaphragm which has been used, for instance, to assay β-bungarotoxin[15,16] and the rat hemidiaphragm preparation. This has been used for assaying the effects of lophotoxin.[17] The most widely used system is probably the frog nerve–sartorius muscle preparation described below.

At the resting neuromuscular junction prior to the arrival of an AP, a steady, nonstimulated, low-frequency release of quanta of ACh molecules occurs. These quanta, representing the entire contents of single vesicles, transit the synaptic cleft and provoke miniature end-plate potentials (MEPPs) in the muscle. Individually these are inadequate to induce a muscle membrane depolarization. On receipt of an AP at the nerve terminus, concerted exocytotic release of many ACh quanta occurs. Unlike the AP itself, this quantal release is not blocked by tetrodotoxin, indicating that sodium conductance is not critical for this presynaptic process. Present evidence suggests that vesicle migration and ACh release require Ca^{2+} conductance changes and, indeed, Ca^{2+}-binding agents (chelating

[12] P. R. Howe, P. F. Rogers, and W. W. Blessing, *Neurosci. Lett.* **52,** 287 (1984).
[13] R. E. Davis, B. E. Schlumpf, and P. D. Klinger, *Toxicol. Appl. Pharmacol.* **80,** 308 (1985).
[14] E. M. Volkov and V. N. Frosin, *Neirofiziologia* **16,** 231 (1984).
[15] R. B. Kelly and F. R. Brown, *J. Neurobiol.* **5,** 135 (1974).
[16] C. C. Chang and M. C. Huang, *Navnyn-Schmiedeberg's Arch. Pharmacol.* **282,** 129 (1974).
[17] P. Culver and R. S. Jacobs, *Toxicon* **19,** 825 (1981).

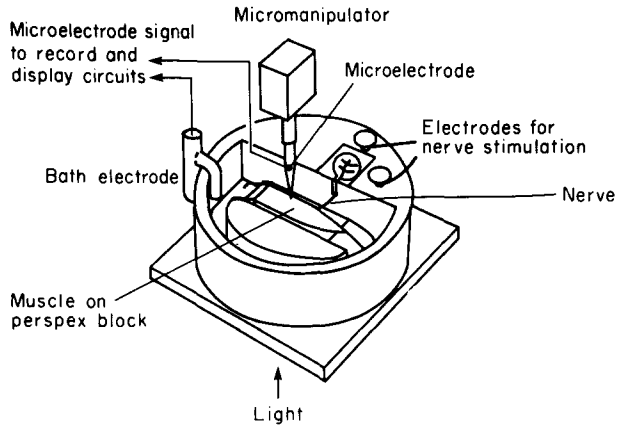

FIG. 5. A nerve–muscle chamber suitable for toxicity studies on the frog nerve–sartorius muscle preparation, after Fatt and Katz.[18] Muscle cells in the end-plate region are impaled by the intracellular microelectrode for examination of end-plate potentials arising from nerve stimulation. In use, the bath is fluid filled to about 3 mm above the muscle. See text for details.

agents) exert neurotoxic action at this locus. Postsynaptic receptor activation, in response to an excitatory neurotransmitter, simultaneously increases the membrane permeability to all small ions, including Na^+, K^+, and Ca^{2+}, causing localized depolarization of an extent and degree proportional to the rate of neurotransmitter–receptor interaction. The isolated frog nerve–sartorius muscle preparation of Fatt and Katz[18] has been used to examine these miniature end-plate potentials and excitatory postsynaptic potentials (EPSPs). In this preparation (Fig. 5), an intracellular recording electrode impales the muscle cells of the end-plate region. A buffer containing an elevated Ca^{2+} concentration (3.6 m*M* instead of 1.8 m*M*) is used to raise the threshold of the muscle fiber and to raise the end-plate potential by about 25%. Measurement of the end-plate potential in this preparation is facilitated by treating the muscle with tubocurarine (explained below).[19,20] The effect of this treatment is to reduce the amplitude of the EPP below the threshold of the muscle fiber so that no muscle impulse arises and a local subthreshold potential change remains. Kao and Nishiyama[21] have used this preparation to explore saxitoxin action and Abe *et al.*[22] have used it to assay β-bungarotoxin.

[18] P. Fatt and B. Katz, *J. Physiol. (London)* **115,** 320 (1951).
[19] J. C. Eccles, B. Katz, and S. W. Kuffler, *J. Neurophysiol.* **4,** 362 (1941).
[20] S. W. Kuffler, *J. Neurophysiol.* **5,** 18 (1942).
[21] C. Y. Kao and A. Nishiyama, *J. Physiol. (London)* **180,** 50 (1965).
[22] T. Abe, A. R. Limbrick, and R. Miledi, *Proc. R. Soc. London, Ser. B* **194,** 545 (1976).

The excitatory postsynaptic potential (EPSP) differs from the AP in four ways. First, it is a simple depolarization rather than a reversal in polarity; second, it is a graded response depending on the amount of neurotransmitter released; third, it has no refractory period such that successive responses may summate; and fourth, it is nonpropagated and attenuates rapidly with distance from the receptor. Summation of EPSPs to a critical value triggers the postsynaptic event. α-Latrotoxin (from black widow spider venom) is believed to inactivate the presynaptic membrane by forming ion channels in the membrane which permit the passage of Na^+, K^+, Ca^{2+}, and Mg^{2+}.[23-25] At the neuromuscular junction, this provokes spontaneous release of ACh quanta, thereby increasing the frequency of postsynaptic MEPPS to 500–1000 times normal. This is followed by a gradual decline over 30–60 min to zero and to a condition of presynaptic neurotransmitter depletion and inactivation. Observation of this sequence of events has been made for the concentrated venom itself.[26] In contrast to α-latrotoxin, botulinum toxin initially produces a flaccid paralysis when administered to the whole animal. This appears to be due to the inhibition of evoked release of ACh by combination with membrane release sites. Postsynaptically reduced MEPP frequency is observed, eventually leading to complete inhibition.[27,28] β-Bungarotoxin from the Taiwan banded krait, and Australian red–black spider venom are believed to disrupt synaptic vesicles and deplete the nerve endings of acetylcholine. The effect of tetanus toxin, in contrast to that of botulinus toxin, is to produce a spastic paralysis by preventing the release of ACh and other neurotransmitters selectively in spinal inhibitory processes of the central nervous system (CNS).[29] However, at the neuromuscular junction, tetanus toxin inhibits ACh release in a botulinus toxin-like manner.[30,31] Tetanus toxin is not delivered to the CNS via the circulation but by internalization within and retrograde transport by the peripheral nervous system in sensory, motor, and autonomic axons.[32,33]

[23] W. P. Hurlbut and B. Ceccarelli, *Adv. Cytopharmacol.* **3,** 87 (1979).
[24] H. E. Longenecker, Jr., W. P. Hurlbut, A. Mauro, and A. W. Clark, *Nature (London)* **225,** 701 (1970).
[25] A. Finkelstein, L. L. Rubin, and M. C. Tzeng, *Science* **193,** 1009 (1976).
[26] J. E. Smith, A. W. Clark, and T. A. Kuster, *J. Neurocytol.* **6,** 519 (1977).
[27] E. J. Schantz and H. Sugiyama, *Essays Toxicol.* **5,** 99 (1974).
[28] A. S. V. Bergen, F. Dickens, and L. J. Zatman, *J. Physiol. (London)* **109,** 10 (1949).
[29] V. B. Brooks, D. R. Curtis, and J. C. Eccles, *J. Physiol. (London)* **135,** 655 (1957).
[30] G. N. Kryzhanovsky, *Navnyn-Schmiedeberg's Arch. Pharmacol.* **276,** 247 (1973).
[31] N. Ambache, R. S. Morgan, and G. P. Wright, *J. Physiol. (London)* **107,** 45 (1948).
[32] K. Stöckel, M. Schwab, and H. Thoenen, *Brain Res.* **99,** 1 (1975).
[33] W. Dimpfel and E. Habermann, *Navnyn-Schmiedeberg's Arch. Pharmacol.* **280,** 177 (1973).

A number of polypeptide neurotoxins derived from snake venoms and snails act at the neuromuscular junction. So-called alpha-type venom toxin, e.g., α-bungarotoxin and α-conotoxin,[34] act postsynaptically to bind the ACh receptor (see below); others act presynaptically to prevent ACh release. Both effects are measurable in isolated nerve–muscle preparations. Examples of these presynaptic toxins include the aforementioned β-bungarotoxin (from the banded krait, *Bungarus multicinctus*), crotoxin (from the South American rattlesnake, *Crotalus terrificus*), notexin (from the Australian tiger snake, *Notechis scutatus scutatus*), and taipoxin (from *Oxyuranus scutellatus scutellatus,* the Australian taipan).[35] The mechanisms of neurotoxic action have been fairly well explored, particularly for β-bungarotoxin, which disrupts presynaptic neurotransmitter vesicles. The neuromuscular blockade produced by this agent occurs in three stages.[36,37] Initially, a slight reduction in the stimulated EPP is observed, followed by an increase which is attributed to a toxic influx of Ca^{2+} lasting 30–60 min. During the third stage, MEPP frequency diminishes to complete neuromuscular blockade. Atraxotoxin from the Australian funnel-web spider (*Atrax robustus*) similarly evokes an initial increase of ACh release followed by depletion of ACh from somatic motor nerve endings. Unlike black widow spider venom, atratoxin does not affect adrenergic neurons, however. A different toxic effect at this site, induced by preferentially inactivating Ca^{2+} entry into the nerve terminal, is responsible for the toxicity of the polypeptide ω-conotoxins from fish-hunting cone snails of the genus *Conus*.[34]

Intrasynaptic Sites of Toxicity

Between the presynaptic membrane and the receptors of the effector cells lies the synaptic cleft, which neurotransmitters must cross to evoke receptor and postreceptor responses. Neurotransmitter synthesis, storage and inactivation by either reuptake, metabolism, or both, each provide vulnerable sites for neurotoxic action. A large variety of neurotransmitter and neuromodulator substances are used by the nervous system subserving various functions in different types of synapses. Although a review of such substances and their susceptibility to toxic derangement lies outside the scope of the present chapter, the cholinergic synapse at the striated neuromuscular junction may usefully be considered. Acetylcholinester-

[34] B. M. Olivera, W. R. Gray, R. Zeikus, J. M. McIntosh, J. Varga, J. Rivier, V. DeSantos, and L. J. Cruz, *Science* **230,** 1338 (1985).
[35] B. D. Howard and C. B. Gundersen, Jr., *Annu. Rev. Pharmacol. Toxicol.* **20,** 307 (1980).
[36] T. Abe, S. Alema, and R. Miledi, *Eur. J. Biochem.* **80,** 1 (1977).
[37] R. B. Kelly, R. J. von Wedel, and P. N. Strong, *Adv. Cytopharmacol.* **3,** 77 (1979).

ase (AChE) is the principal enzyme responsible for terminating the action of acetylcholine (ACh) by hydrolysis at this site. AChE is located both pre- and postjunctionally. Prejunctionally, it is formed in the cholinergic cell body and transported by axonal translocation processes to the presynaptic nerve terminus. Postjunctionally, AChE is formed in muscle but its formation arises as a consequence of presynaptic ACh release and effect. Neurotoxic inhibitors of AChE include physostigmine from calabar beans, the seeds of the vine *Physostigma venenosum,* and galantamine, an alkaloid extracted from the bulbs of the caucasian snowdrop *Galanthus woronowii.* The effects of intrasynaptic toxin actions are best revealed by neuromuscular junction assay systems, as described above. AChE inhibition at the neuromuscular junction greatly enhances postsynaptic cholinergic receptor stimulation, evoking spontaneous muscle fasciculation and enhanced and prolonged muscle contraction. Certain central nervous system (medullary) synapses subserving respiratory movements are cholinergic, and central AChE toxicity is usually manifest, therefore, as respiratory paralysis.

Postsynaptic Sites of Toxicity

Neurotoxins which act at the postsynaptic ACh receptor at the neuromuscular junction block this receptor by either a depolarizing or nondepolarizing action. They therefore have a muscle relaxing effect. The "α-type" toxins exert such postsynaptic ACh receptor blockade either directly at the ACh-binding site or at functionally linked membrane structures. Included among these are polypeptide constituents of the venoms of certain snakes in the family Elapidae (cobras, kraits, corals, mambas, tiger snakes, death adders, black snakes, and taipans) and the sea snakes, Hydrophiidae. These polypeptide α-toxins bind strongly, specifically, and irreversibly to the ACh receptor. Curare, a generic term for various alkaloids extracted from plants of the species *Strichnos* and *Chondrodendon,* is a nondepolarizing blocker of the receptor. Presently, the most important of these alkaloids is (+)-tubocurarine isolated from *Chondrodendon tomentosum* by King in 1935.[38] It is so named from the practice of preparing this drug as a blowgun arrow poison by packing it into tubes of bamboo by certain Amazonian aboriginals from the rain forests of Peru's eastern border. Another curare-type neurotoxin is C-toxiferine, isolated from the bark of *Strychnos toxifera.* The prefix C indicates the practice of the aboriginals, who isolated this, of packing the prepared toxin in calabashes (or gourds). C-Toxiferine is about 25 times more potent than tubocurarine and has a longer duration of action. Nondepolarizing blockade is

[38] H. King, *Nature (London)* **135,** 469 (1935).

TABLE II
PRIMARY SITES OF ACTION OF, AND DETECTION METHODS FOR, SOME NEUROTOXINS ON MAMMALIAN PERIPHERAL NERVOUS SYSTEMS

Toxin	Primary site of action	Detection method		
		Intracellular recording from nerve	Extracellular recording from nerve	Neuromuscular junction
Tetrodotoxin	Nerve membrane	★	★	★
Batrachotoxin	Nerve membrane	★	★	★
Ciquatoxin	Nerve membrane	★	★	★
Grayanotoxin	Nerve membrane	★	★	★
Veratrum alkaloids	Nerve membrane	★	★	★
Saxitoxin	Nerve membrane	★	★	★
Vinca alkaloids	Intraneuronal microtubles		★	★
Colchicine	Intraneuronal microtubles		★	★
Tullidora toxin	Nerve fiber		★	★
Diphtheria toxin	Myelin sheath		★	★
β-Bungarotoxin	Presynaptic terminal			★
α-Latrotoxin	Presynaptic terminal			★
Black widow spider venom	Presynaptic terminal			★
Botulinum toxin	Presynaptic terminal			★
Tetanus toxin	Presynaptic terminal			★
Crotoxin	Presynaptic terminal			★
Notexin	Presynaptic terminal			★
Atratoxin	Presynaptic terminal			★
ω-Conotoxin	Presynaptic terminal			★
Physostigmine	Intrasynaptic			★
Galantamine	Intrasynaptic			★
(+)-Tubocurarine	Postsynaptic membrane			★
Venom α-toxins	Postsynaptic membrane			★
C-toxiferine	Postsynaptic membrane			★
Nicotine	Postsynaptic membrane			★
Lobeline	Postsynaptic membrane			★
Coniine	Postsynaptic membrane			★
α-Conotoxin	Postsynaptic membrane			★

competitive, that is reversible, and prevents the response of skeletal muscle to ACh or other nicotinic agonists. The classical studies of Bernard[39] showed that when curare was applied to the neuromuscular junction of the frog, stimulation applied to the nerve failed to evoke a muscular

[39] C. Bernard, *C. R. Seances Soc. Biol. Ses Fil.* **2**, 195 (1851).

response, whereas stimulation applied directly to the muscle evoked muscle twitches.

Recently, lophotoxin, isolated from several species of sea whips (genus *Lophogorgia*) has been shown to exert a curare-like block at the ACh receptors of the rat hemidiaphragm and frog rectus abdominus muscle preparations.[17,40]

Depolarizing blocking agents compete with ACh for the ACh receptor and, since they exert ACh agonistic activity, cause an initial depolarization before blocking further stimulation by endogenous ACh. Depolarization block is thus preceded by muscle fasciculation and augmentation of maximum evoked muscle twitch amplitude. Naturally occurring depolarizing blockers include nicotine (from *Nicotiana,* tobacco), coniine from hemlock (*Conium maculatum*), lobeline (from *Lobelia inflata*), murexine (also called urocanylcholine) from certain mollusks and leptodactyline from the skin of a lizard. Depolarizing agents may be distinguished from other types of blocking drugs by their effects on multiply innervated muscle of frogs and fowl. Thus, the rectus abdominus muscle of the frog and the gastrocnemius and neck muscles of the fowl respond to depolarizing agents with a sustained contracture, whereas other agents produce a flaccid paralysis. Tubocurarine, of course, antagonizes their action.

In summary (see Table II), techniques for the detection of neurotoxicity on isolated nerve and nerve–muscle junction have shown that neurotoxins acting on central and peripheral nervous tissue act at the nerve membrane to block ion conduction and propagation, act axonally to interfere with axonal translocation processes, act presynaptically to derange presynaptic transmitter storage and release, and act intra- and postsynaptically to derange synaptic-effector tissue responsiveness.

Acknowledgment

I wish to acknowledge, with gratitude, the influence of Professor P. S. J. Spencer (UWIST, Wales) and Professor W. C. Bowman (Strathclyde) upon my thoughts and interests. Editorial assistance in the production of this manuscript was given to me by numerous colleagues and I especially thank Drs. P. E. Teschan and Andrew Fingret for their comments.

[40] W. Fenical, R. K. Okuda, M. M. Bandurraga, P. Culver, and R. S. Jacobs, *Science* **212,** 1512 (1981).