Penetration of Acetylcholine into Nerve Fibers Demonstrated by a New Chemical Method. (19761)

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In the course of pharmacodynamic studies on a number of quaternary ammonium compounds it was found that these compounds distributed themselves into the axoplasm of the giant axon of the Squid(1). In view of the existing controversy on the penetration of acetylcholine (ACh) into the interior of nerve fibers, distribution studies on ACh were carried out.

The affirmative evidence for the permeability of nerve fibers to ACh has been indirect. Lissak(2) showed that cholinergic axons, when stimulated, permit the outflow of ACh into the bathing Ringer's solution and Eccles(3) found that soaking the unanesthetized frog spinal cord in weak ACh solutions caused prolonged spontaneous discharges of the motor neurones. On the other hand, Nachmansohn(4) failed to demonstrate the presence of ACh labelled with N^{15} in the axoplasm extruded from Squid axons that had been immersed in 0.1 *M* ACh solution; it was not stated that the giant axons used were cleaned microscopically.

A sensitive chemical method for the determination of ACh(5) as well as improved techniques(6) for extruding uncontaminated axoplasm and for perfusing the axon sheath made it possible for us to demonstrate that ACh penetrates into the interior of the resting axon.

A chemical method for quaternary ammonium compounds has been adapted to the determination of ACh(5,7). To 0.5 cc of a sample containing ACh, 0.5 cc of bromphenol blue (0.08% in 30% K_2HPO_4) and 300 mg each of Na_2CO_3 and K_2HPO_4 were added. The colored dye complex formed was extracted by freshly washed ethylene dichloride (containing 4% iso-amyl alcohol). The ethylene dichloride extract was read in a spectrophotometer at 600 mμ. The lower limit of sensitivity is 1 μg per sample.

Two preparations were used: 1) axons prepared microscopically free of all adjacent connective tissue without injury to the axon; 2) axons prepared grossly free of excessive connective tissue without injury to the axon. In the first type of preparation, stainless steel needles were inserted into the cut ends of the axon and tied securely. The measured section of the axon, thus prepared, was immersed in ACh, 0.01 *M* in buffered artificial sea-water. After varying times of immersion, the axoplasm was extruded from the axon by forcing air and sea-water through the system, without injury to the axon sheath. In the second type of preparation, after immersion in 0.1 *M* ACh, the axoplasm was extruded by the conventional technic with adequate precautions against contamination. All experiments were carried out in the absence of any anticholinesterases. The results of the chemical determinations on the extruded axoplasm are summarized in Table I.

After extrusion of the axoplasm the axon sheath was filled with artificial buffered sea-water and immersed in 0.01 *M* ACh to determine the degree of penetration of ACh

TABLE I. Concentration of ACh in Axoplasm of Giant Axon.

Immersion time (min)	Molarity of ACh outside	Molarity* of ACh in axoplasm	Ratio, M inside / M outside	Type† of preparation
0	0	0	0	1
0	0	0	0	2
120	0	0	0	2
15	.01	.003	.3	1
30	.01	.0075	.75	1
20	.1	.009	.09	2
30	.1	.011	.11	2
60	.1	.023	.23	2
120	.1	.038	.38	2

* Calculated from the estimated volume of the section of axons used in preparation 1 and wt of axoplasm extruded in preparation 2 (calculations by both methods are in close agreement).

† 1—Microscopically cleaned fiber; 2—Grossly cleaned fiber.

through the sheath in the absence of axoplasm. The sea-water inside the axon was extruded after the immersion period and chemical determinations made. The rate of penetration of ACh was much more rapid in this case; equilibrium was reached between 5-10 minutes. At the end of the experiment, the axon sheath was tested for leaks by means of a water soluble dye.

In other experiments microscopically cleaned axons were immersed in 0.01 *M* ACh for 90 minutes and then washed for 10 seconds, 1 minute, and 30 minutes, in fresh aliquots of sea-water. The quantities of ACh determined in the washes were respectively: 4.4, 2.2, and 5.7 x 10^{-3} *M*. The 10 second wash was considered as surface contamination. The amount of ACh in the other washes was considered to represent a major portion of the ACh which had been distributed in the axon during the immersion period (molarities calculated from volume of axons).

Summary. The penetration of acetylcholine into the interior of the resting Squid axon, which had been immersed in ACh solutions, was demonstrated by its appearance in the extruded axoplasm. The outflow of ACh from resting axons previously immersed in ACh solutions was also demonstrated.

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Protein Separations and Interactions on Filter Paper.* (19762)

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Two technics are available whereby proteins may be caused to move on filter paper—chromatography and microelectrophoresis. The former method involves the movement of proteins by capillary ascent. A mixture of proteins diffuses from a spot into a streak by the action of an aqueous developing solvent (1). The latter technic, based on the Tiselius method, makes use of the different mobilities of the various proteins in an electric field(2). A combination of these two methods, chromatography in the first dimension followed by electrophoresis in the second dimension, provides a variation in the present 2-dimensional chromatography and makes it possible to study the separation and interaction of various proteins on filter paper.

Whatman No. 3 MM paper has been used throughout. The dry purified proteins are dissolved in 0.85% saline, and 0.04 ml aliquots are placed about 4 inches from the lower left hand corner of strips of filter paper approximately 8″ x 15″ in size. The spots are dried at room temperature, the papers formed into cylinders and stapled so that the edges do not touch, and then placed in shallow dishes containing the developing solvent. After the papers are thoroughly wetted by capillary action (about 1.5 hours), they are unstapled, rinsed quickly in veronal buffer solution (pH = 8.6), and placed in the electrophoresis apparatus.

The electrophoresis apparatus consists of a pair of plastic troughs (Perspex) 17″ x 4″ x 3¼″ each divided longitudinally into two compartments. Electrical contact between the

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