Kinetics of Acetylcholine Synthesis and Hydrolysis in Myasthenia Gravis

Abstract. *Kinetic studies for choline-O-acetyltransferase (CAT) (E.C. 2.3.1.6) and acetylcholinesterase (E.C. 3.1.1.7) were performed on serum, skin fibroblasts in culture, and biopsied sartorius muscle from normal and myasthenic subjects. There was a significant decrease of CAT activity per milligram of protein in myasthenic muscle compared to normal muscle, and there was no difference in acetylcholinesterase activity per milligram of protein in the same muscle homogenates. Substrate concentration curves for acetyl coenzyme A and CAT also showed a significant reduction in the maximum rate of product formation (V_{max}) per milligram of protein between myasthenic and normal muscle. It is postulated that binding of substrate to CAT is being inhibited by an inhibitor present in muscle.*

It has been suggested that the weakness characterizing the myasthenic patient is related to a defect in neuromuscular transmission (*1*). Up to now no systematic evaluation of the metabolism of acetylcholine in myasthenic tissue has been performed in order to define a biochemical basis for the postulated reduction in acetylcholine released per nerve depolarization. Symptoms of the disease could result from a deficiency in acetylcholine synthesis, increased hydrolysis of acetylcholine, binding inactivation of acetylcholine prior to its action on the cholinergic receptor, blockade of the receptor site for acetylcholine, or the synthesis of a false transmitter (*1*). The presence of antibody to muscle and antibody to thyroid, as well as the coexistence of collagen diseases, has been shown more often in patients with myasthenia gravis than in the general population (*2–4*). The relation between the associated autoimmune phenomena and the myasthenic symptoms remains unclear (*4*).

We have performed enzyme kinetic studies on choline-*O*-acetyltransferase (E.C. 2.3.1.6) (CAT) and acetylcholinesterase (E.C. 3.1.1.7) (AchE) in serum, in skin fibroblast cultures, and in biopsied sartorius muscle from normal and myasthenic subjects. In order to detect the possible production of a false acetylated transmitter, the product of the CAT reaction was identified.

Choline-*O*-acetyltransferase was assayed by a modification of the radioisotope assay of Schrier and Schuster (*5*). A solution was prepared containing $1.09 \times 10^{-3}M$ [1-^{14}C]acetyl coenzyme A, $1.25 \times 10^{-2}M$ choline iodide, $5 \times 10^{-4}M$ neostigmine methyl sulfate, 0.20*M* NaCl, 0.5 percent Triton X-100 (Packard), 0.05*M* K_2HPO_4-KH_2PO_4, and 0.001*M* EDTA at *p*H 6.8. Portions (10 μl) of this solution were incubated for 20 minutes at 37°C with four concentrations of enzyme homogenate in a volume of 50 μl. The reaction was terminated by the addition of 1.0 ml of ice-cold (3°C) water. The mixture was then passed through a Dowex-1 (chloride; 100 to 200 mesh) ion-exchange chromatographic column (0.5 by 5 cm) and followed by 2 ml of 3°C water, and the radioactivity in the eluate was counted in a Beckman liquid scintillation counter in 15 ml of counting fluid [Triton, 250 ml; toluene, 750 ml; and PPO, 940 g (Sigma)]. For identification of the product, the reaction mixture was lyophylized, suspended in 20 μl of water and placed on Whatman No. 1 chromatographic paper; an ascending chromatogram was developed in butanol, acetic acid, and water (4 : 1 : 1) over a 7-hour period with [^{3}H]acetylcholine as standard. The R_F of the radioactive product formed was measured with a Packard radioisotope strip scanner.

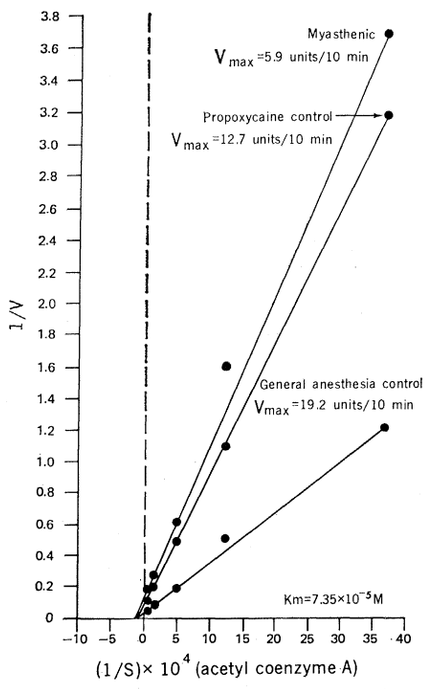

Fig. 1. Substrate concentration curves for acetyl coenzyme A and choline-*O*-acetyltransferase when the choline iodide concentration was kept at $2.5 \times 10^{-3}M$. Each point is the average of duplicate assays, and there was no overlap. The slope of each line and its *Y*-axis intercept were obtained by the least-squares method and the equation $Y = \overline{Y} + b(X - \overline{X})$; *S*, substrate; *V*, nanomoles of ^{14}C product per milligram of protein per 10 minutes at *p*H 6.8 and 37°C.

Acetylcholinesterase was measured by a modification of the method of Reed *et al.* (*6*). A solution was prepared containing $28.94 \times 10^{-3}M$ [1-C^{14}]-acetylcholine, 0.20*M* NaCl, 0.5 percent Triton X-100, 0.05*M* K_2HPO_4-KH_2PO_4, and 0.001*M* EDTA, *p*H 6.8. Portions (10 μl) of this substrate solution were incubated for 10 minutes at 37°C with four concentrations of enzyme homogenate made up to 50 μl with buffer and then passed through a disposable column (0.5 by 5 cm) of Bio-Rad AG 50 × 8 (Na+ form washed with water, 100 to 200 mesh) and washed with 2.0 ml of 3°C water. The activities of acetylcholinesterase and pseudocholinesterase in each muscle homogenate were assayed in the presence and absence of an inhibitor of acetylcholinesterase, 1,5-bis(4-allyldimethylammoniumphenyl)pentane-1,3 dibromide ($4 \times 10^{-4}M$; Burroughs Wellcome BW 284 C51). The radioactivity of the eluate was counted as described for CAT. The rate of reaction was proportional to enzyme concentration in all cases, and the reproductibility of activity determinations was ± 15 percent.

Protein was determined by a modification of the method of Lowry *et al.* (*7*). Homogenates were solubilized for protein assay by stirring in a mixture of 2.5*M* urea and 1 percent sodium dodecyl sulfate for 24 hours and then diluted 1 : 100 in 0.1*N* NaOH.

Patients were biopsied 12 hours after their last dose of pyridostigmine. Skin was obtained from the upper arm by punch biopsy. Skin fibroblast cultures were grown (Falcon plastic flasks) in GIBCO medium F 10 with 10 percent fetal calf serum at 37°C and in an atmosphere of 10 percent CO_2 and 90 percent air. The sartorius muscle was biopsied after anesthetizing the skin and muscle with 4.0 ml of propoxycaine hydrochloride (0.5 percent; Breon, New York). In addition, two

Table 1. Kinetics of acetylcholine synthesis and hydrolysis in sartorius muscle in normals and myasthenics (nanomoles of ^{14}C product formed per milligram of protein per 20 minutes, *p*H 6.8, 37°C, that is, units of activity).

	CAT		AchE	
	Units of activity	Mean ± S.D.	Units of activity	Mean ± S.D.
General anesthesia controls $n = 2$	22.6 ± 1.9 21.3 ± 1.2	22.0 ± 2.3	182 ± 23 180 ± 11	181 ± 24
Propoxycaine controls $n = 4$	12.3 ± 2.2 11.7 ± 0.8 11.2 ± 0.8 11.4 ± 1.5	11.6 ± 0.4	195 ± 7 228 ± 1 253 ± 23 161 ± 9	209 ± 42
Myasthenics* $n = 3$	9.7 ± 1.8 8.2 ± 1.3 8.0 ± 0.1	8.6 ± 0.8	229 ± 9 245 ± 18 330 ± 32	268 ± 55

* $P < .005$ between propoxycaine controls and myasthenics for CAT specific activity.

sartorius muscle biopsies were obtained from nonmyasthenic patients undergoing hip surgery under general anesthesia.

Our data demonstrate a significant partial decrease in CAT activity per milligram of protein in myasthenic skeletal muscle as compared to controls. No significant differences in AchE activity were found between normal and myasthenic skeletal muscle (Table 1). Serum and skin fibroblasts grown in cell culture were assayed for CAT, but no significant differences were noted between normal and myasthenic tissues.

Each muscle homogenate was assayed for CAT activity at four concentrations of protein in duplicate. There was an average 26 percent reduction in CAT specific activity in myasthenic homogenates as compared to propoxycaine control homogenates ($P < .005$).

The Michaelis constant K_m (acetyl coenzyme A) for a propoxycaine control, a general anesthesia control, and for two myasthenic homogenates was $7.35 \times 10^{-5} M$ when the choline iodide concentration was kept at $2.5 \times 10^{-3} M$. Substrate concentration curves indicated that noncompetitive inhibition seemed to be occurring in a myasthenic homogenate compared with a normal homogenate for the maximum rate of product formed (V_{max}). There was a 54 percent reduction in myasthenic activity, that is, 5.9 units (a unit is the number of nanomoles of [1-^{14}C]acetylcholine formed per milligram of protein per 10 minutes at *p*H 6.8 and 37°C) compared to 12.7 units for a propoxycaine control, and 19.2 units for a general anesthesia control (Fig. 1). A mixing experiment with equal volumes of myasthenic and normal muscle homogenates produced intermediate levels of activity. Product identification in all samples showed [1-^{14}C]acetylcholine.

The enzyme activities in muscle reported here most likely reflect metabolism at the nerve terminal. Sartorius is a muscle in which it is thought that end plates occur diffusely throughout its length and depth (*8*). There is no cholinergic innervated structure within skeletal muscle in the lower extremity other than the muscle fiber itself at the end plate. The parasympathetic nervous system does not innervate smooth muscle of blood vessels of the leg (*9*).

It is postulated that a noncompetitive endogenous inhibitor might be present in myasthenic muscle, as is suggested by the substrate concentration curves. If this is true, the autoimmune findings in myasthenia gravis may be related to the defect in neuromuscular transmission. The impairment in acetylcholine synthesis with a normal rate of hydrolysis, however, represents only one factor causing the clinical symptoms in this disease. Hypoplastic motor end plates with a reduced nerve terminal surface area, antibodies to muscle, and postjunctional membrane insensitivity to acetylcholine must also be considered (*3, 4, 10*).

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Neuronal Circuit Mediating Escape Responses in Crayfish

Abstract. *The neuronal circuit underlying rapid abdominal flexion in response to phasic tactile stimulation comprises identified afferents, interneurons of two orders, a decision unit, and several motor neurons. The circuit is organized hierarchically as a "cascade" in which electrical synapses predominate at higher levels. Behavioral habituation results from lability at chemical junctions early in the pathway.*

One objective of work with the restricted nervous systems found in invertebrates (*1*) is to construct circuits of identified cells that are responsible for a specific behavioral event. Such circuits logically begin with sensory elements and end with muscles. Those described most fully to date include segmental shortening reflexes in annelids (*2*) and withdrawal responses in mollusks (*3*). We report here the elucidation of a more complex response of exteroceptive origin. It comprises a population of sensory fibers, three layers of central interneurons, and a duplex motor pathway; a variety of synaptic mechanisms are employed at the different kinds of junctions involved.

The behavior is a simple one. When crayfish are given a phasic mechanical stimulus to an abdominal segment, they often respond with a sudden contraction of the abdominal flexor muscles. Such a contraction, which propels the animal rapidly backward, is brought about by activity in nine large motor neurons located in each abdominal half-segment. One, the motor giant cell, is excited by the central giant fibers at an electrotonic junction located near its