

Kinetic Characterization of Acetylcholinesterase of Muscle and Rat Brain

Although similar synaptic events seem to occur at peripheral and central nervous system junctions, certain physiological, ultrastructural and metabolic differences (see ¹ and ², for details) are known to exist between brain and muscle synapses, at least in mammals.

The question may, therefore, be raised as to whether acetylcholinesterase (AChE), so decisive an enzyme for synaptic transmission in cholinergic systems, will analogously show distinctive properties in the 2 tissues. We report here kinetic studies which reveal that this is indeed the case.

Material and methods. Brains and muscles taken from decapitated adult rats were washed in chilled 0.9% NaCl to remove the blood. Blood vessels from the brain surface were carefully dissected out as well. 1:3 saline (0.9% NaCl) homogenates of both tissues were prepared and centrifuged at 90,000 *g* for 60 min at 2°C. The supernatant fractions were collected and the pellets were washed with 0.9% NaCl, rehomogenized and recentrifuged. Again, the supernatants of this second centrifugation were collected and the pellets were homogenized with 1% Triton X-100 buffered to pH 7.0 in 0.1 *M* *tris*-HCl. The same speed, time and temperature (see above) were used in all 3 centrifugations, and the volume of the eluent was also the same. This procedure is a slight modification of the method used by BARRON and BERNISOHN³.

AChE present in the saline and Triton extracts will be referred to as soluble and insoluble, respectively. The enzyme was assayed at 25°C by the technique of ELLMAN

et al.⁴ and the reactions were followed for 3-5 min. Proteins were determined according to the method of LOWRY et al.⁵ with bovine serum albumine as standard.

The enzymatic activity was estimated as μM of acetylthiocholine (ATC) hydrolyzed per ml of extract (or g wet wt. of tissue) per min. Activity (Ac) per mg of protein will be referred to as specific activity (SA).

Acetylthiocholine iodide (Fluka), butyrylthiocholine iodide (Fluka), dithio-bis-2-nitrobenzoic acid (Fluka), 284C51 (Wellcome), D-tubocurarine chloride (Nourypharma), hexamethonium bromide (Bayer) and phenyltrimethylammonium chloride (Eastman) were the reagents used in the kinetic observations and were of analytical grade. The latter 2 drugs were kindly supplied by Dr. KEROE (Paris). Triton X-100 was purchased from Sigma.

Results. The detergent extracts from both brain and muscle yielded larger amounts of AChE than the corresponding saline preparations. Since in the second centrifugation, used to wash actual remaining soluble enzyme, the activity was much lower than in the first one, it is reasonable to conclude that the higher ($\times 6-10$) values of the Triton extracts represent enzyme that was attached to membranes. Although an activating effect of Triton X-100 upon AChE has been reported^{6,7}, we were able to establish that it cannot account for the present values. In fact, the latter indicate that in both brain and muscle the enzyme occurs mainly in the bound form. That AChE is an enzyme chiefly firmly bound to membranes has been often reported and it seems to be generally so in mammals⁸. Things might be different for torpedo and electric eel plaques, however, since it has been claimed that the bulk of the enzyme in these fishes can be extracted in saline media⁹ (cf. also ⁸).

Higher values in brain than in muscle were found for both the soluble (brain: Ac-1,049 nM ATC/min per ml; SA-181 nM ATC/min per mg prot; muscle: Ac-126 nM ATC/min per ml; SA-11 nM ATC/min per mg) and insoluble enzyme (brain: Ac-7,749 nM ATC/min per ml; SA-1,045 nM ATC/min per mg; muscle: Ac-545 nM ATC/min per ml; SA-164 nM ATC/min per mg).

In the present study, some kinetic differences (K_m , K_i for instance) were found to exist between the soluble and the insoluble enzyme from both organs of rat, which could not be attributed to the different extraction media used. Since the bound enzyme is supposed to be the form implicated in transmission (cf.¹⁰), we shall deal below only with insoluble AChE. It was tested for K_m , temperature of inactivation, pH optima, reactivity towards inhibitors and saturation curves. The kinetic properties of the soluble enzyme will be reported in a subsequent paper.

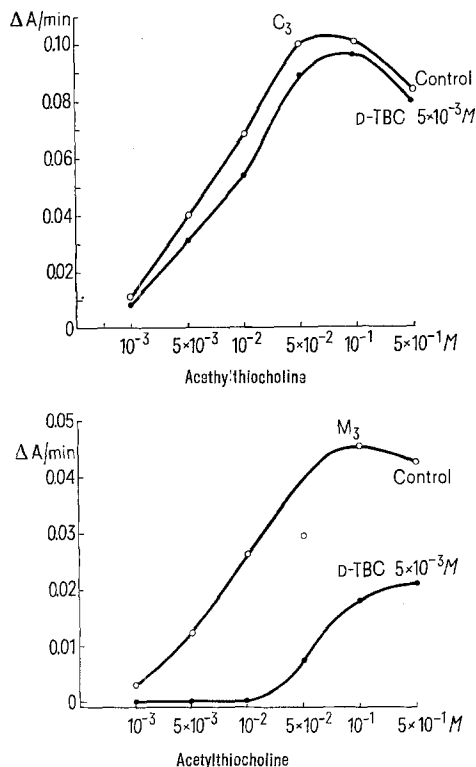


Fig. 1. Actions of cholinergic receptor effectors on AChE from brain (C_3) and muscle (M_3) of rat. AChE activities, expressed as $\Delta A/\text{min}$ (ordinates), are plotted as a function of ATC concentrations (abscissas), with and without the effectors (control curves). D-tubocurarine (D-TBC) shows a slight action on AChE from brain (a), while it markedly inhibits muscle AChE (b). Note that, in the latter case, inhibition by excess of substrate is removed by D-TBC.

¹ B. KATZ, *Nerve, Muscle and Synapse* (McGraw-Hill, New York 1966), p. 143.

² J. R. WILLIAMSON, J. B. CLARK, W. J. NICKLAS and B. SAFER, *Ion Homeostasis and Brain* (Eds. B. K. SIESJÖ and S. C. SØRENSEN; Academic Press, New York 1971), p. 381.

³ K. D. BARRON and J. BERNISOHN, *J. Neurochem.* **15**, 273 (1968).

⁴ G. L. ELLMAN, K. D. COURTNEY, V. ANDRES JR. and R. M. FEATHERSTONE, *Biochem. Pharmacol.* **7**, 88 (1961).

⁵ O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).

⁶ R. L. JACKSON and M. A. APRISON, *J. Neurochem.* **13**, 1367 (1966).

⁷ H. D. CRONE, *J. Neurochem.* **18**, 489 (1971).

⁸ K. HO and G. L. ELLMAN, *J. Neurochem.* **16**, 1505 (1969).

⁹ J. MASSOULIÉ and F. RIEGER, *Eur. J. Biochem.* **14**, 430 (1970).

¹⁰ D. NACHMANSOHN, *Science*, **168**, 1059 (1970).

Muscle AChE showed a higher K_m than brain AChE. It can even be 2 orders of magnitude higher (muscle: $17 \times 10^{-3} M$; brain: $0.17 \times 10^{-3} M$). The apparent K_m values were calculated from double reciprocal plots, using a regression analysis program on an IBM 1130 digital computer.

When the extracts were incubated at $55^\circ C$ and aliquots taken each 10 min during 30 min, brain AChE was found to be more resistant to inactivation than was AChE from muscle. Both were inactivated, however, by heating for 30 min at $70^\circ C$. The pH curves showed small but consistent differences: the optima for brain and muscle are 7.6 and 7.8, respectively.

Incubation of brain enzyme with 284C51 ($10^{-4} M$), the classical inhibitor of AChE, for 40 min, resulted in an almost complete loss of activity (99.2%), while for the enzyme from muscle, incubated in similar conditions, the loss of activity amounted only to 50%.

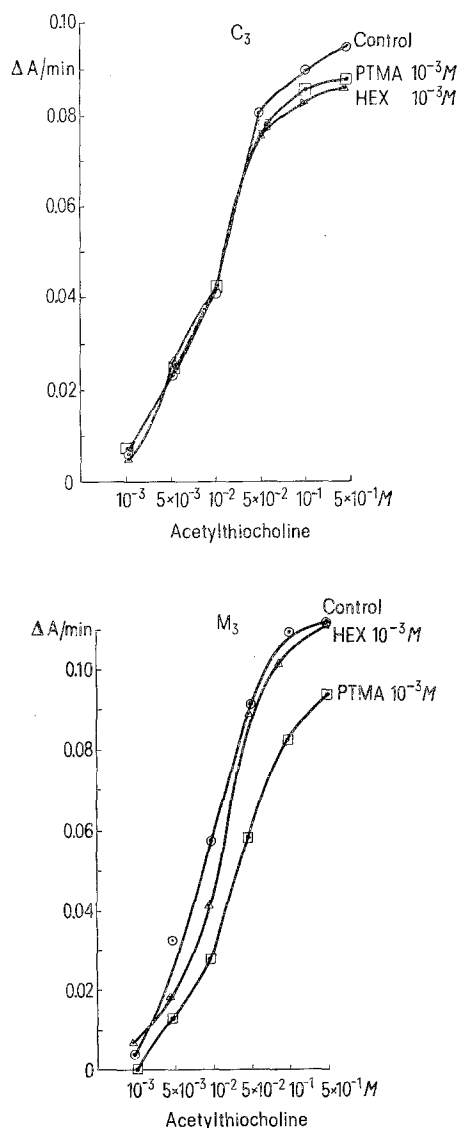


Fig. 2. (a) PTMA (phenyltrimethylammonium) and HEX (hexamethonium) have no action upon brain enzyme, except for high concentrations of the substrate (10^{-1} – $5 \cdot 10^{-1} M$) at which a small inhibitory effect is found. (b) Muscle AChE is significantly inhibited by PTMA. The inhibition is not competitive. The weak effect induced by HEX is partially competitive.

Hexamethonium (HEX), D-tubocurarine (D-TBC) and trimethylphenylammonium (PTMA), which are considered to act on the cholinergic receptors, were tested in view of the fact that AChE possess other sites besides the catalytic ones^{11,12}. In Figures 1 and 2, it can be seen that the enzyme from the 2 sources reacts differently towards these drugs. Muscle AChE was strongly inhibited, while the activity of the brain enzyme was slightly, or not at all, modified. PTMA and D-TBC induced a marked, non-competitive inhibition of muscle AChE (Figures 1b and 2b), indicating the existence of regulatory sites on the enzyme, while HEX displays a weak, partially competitive effect (Figure 2b), suggesting an affinity of this drug for both regulatory and catalytic sites.

All the substrate-velocity plots yielded sigmoidal curves (Figures 1 and 2). No hyperbolic curves were depicted in any study done thus far. In some instances, inhibition of AChE by excess of substrate (Figures 1 and 2), which has often been described, could not be observed (Figure 1b), as has been pointed out by CHANGEUX¹¹. This was frequently so with the muscle enzyme.

Discussion. The observation that muscle enzyme has a higher K_m than brain enzyme deserves comment. In fact, this would mean that the affinity of the former for the substrate is lower and, therefore, acetylcholine will tend to accumulate in muscle. Accordingly, synthesis of the mediator is likely to occur in lower rate in the latter. Both circumstances may contribute, to some extent, to the nongraded character of the electrical response in muscle.

The fact that D-TBC and PTMA markedly inhibit the muscle enzyme is also interesting, since they are generally found to induce blockade and stimulation, respectively, of the nicotinic neuromuscular receptors, whereas HEX is said to act mainly on the nicotinic receptors of the autonomic ganglia, a finding which may explain its almost complete lack of activity in our preparation.

Recently, it has been convincingly demonstrated that ACh receptor and AChE are distinct proteins (see refs. in ¹³). Yet, the present evidence, as similar results previously reported, of the existence of sites for ACh antagonists and agonists on the enzyme molecules, appears to invite consideration of the involvement of AChE as well in the mechanisms underlying the influences of the so-called ACh receptor effectors upon synaptic responses. As a matter of fact, the effects of these and related drugs on transmission are still poorly understood. Some are said to show biphasic (inhibitory and excitatory) actions; others seem to act oppositely in different receptors¹⁴. This appears to be the case, for instance, for PTMA, which has been found to have a stimulating action on nicotinic neuromuscular receptors and an inhibitory effect on some nicotinic receptors of *Aplysia* neurons¹⁵. It seems reasonable to assume that its stimulating action on myoneural junctions is related, at least in part, to its inhibitory action on AChE shown here. Likewise, one should expect AChE inhibition by D-TBC to produce some stimulation of the neuromuscular synapses, but to our knowledge such effect has not been described. Probably, the mechanism of action of curare is more complex than that of PTMA.

¹¹ J. P. CHANGEUX, *Molec. Pharmac.* 2, 369 (1966).

¹² G. KATO, E. TAN and J. YUNG, *Nature, Lond.* 236 NB, 185 (1972).

¹³ R. W. OLSEN, J. C. MEUNIER and J. P. CHANGEUX, *Febs Lett.* 28, 96 (1972).

¹⁴ G. KOELLE, *The Pharmacological Basis of Therapeutics* (Eds. L. S. GOODMAN and A. GILMAN; MacMillan, New York 1970), p. 603.

¹⁵ J. KEHOE, *J. Physiol., Lond.* 225, 115 (1972).

In conclusion, it can be said that whatever may be the nature of these allosteric sites on AChE, it is clear from the present data that brain and muscle enzymes are endowed with different sets of them

Résumé. La présente étude a rélevé chez le rat des différences entre l'AChE du cerveau et celle du muscle, en ce qui concerne les pH optima, la température d'inactivation, le K_m , les courbes de saturation et la réponse

aux inhibiteurs. L'existence de sites allostériques différents dans l'enzyme des deux organes a aussi été observée. L'auteur suggère que ces propriétés cinétiques distinctes tiennent, en partie du moins, à des différences histophysiologiques entre les synapses de ces deux tissus.

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Untersuchungen zur Thermodynamik des peripheren Sehprozesses

Nach Belichtung des Netzhautgewebes laufen physikalisch-chemische Prozesse ab, die mit der Quantenabsorption beginnen und zu einer transretinalen Potentialänderung (ERG) führen. Bei den potentialbildenden Vorgängen an den Zellmembranen handelt es sich um räumlich und zeitlich ineinandergreifende, untereinander gekoppelte Reaktionen. Selbst wenn eine grosse Zahl von Einzelreaktionen innerhalb und ausserhalb der Zellen abläuft, so lässt sich oft durch das Experiment für die Summe von Vorgängen eine Brutto-Reaktionskonstante angeben, deren Temperaturabhängigkeit Aufschluss über die Aktivierungsenergie des Gesamtvorganges gibt¹. Unter diesem Gesichtspunkt haben wir den Temperatureinfluss auf das Belichtungspotential der isolierten Froschretina näher untersucht.

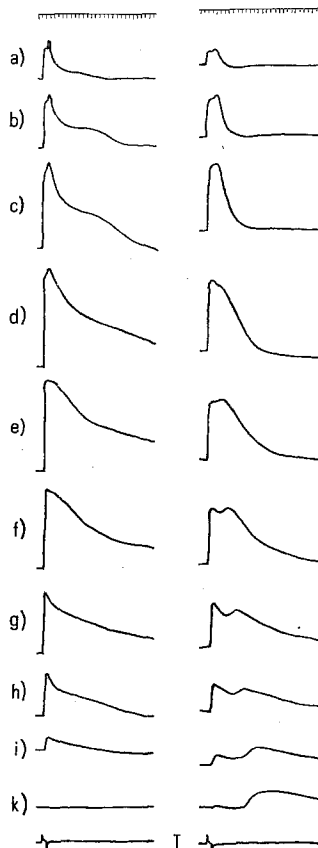


Fig. 1. Temperaturabhängigkeit des Belichtungspotentials einer dunkeladaptierten Froschretina. Die Temperaturen betragen (°C): a) 29,6; b) 27,3; c) 25; d) 20,3; e) 15,7; f) 13,1; g) 10,7; h) 8,8; i) 6,9 und k) 6. Beleuchtungsstärke: linke Reihe 35 Lux, rechte Reihe 0,031 Lux. Oberste Zeile: Zeitmarkierung 1 sec; unterste Zeile: Reizmarke 1 sec, Eichsignal 200 μ V.

Methode. Wir verwendeten die Technik der isolierten Retina²⁻⁵. Die Netzhäute von Fröschen (*Rana esculenta*) wurden durch Präparation bei Rotlicht vom anhaftenden Pigmentepithel gelöst und auf dem Netz (Monodur) eines Trägers befestigt. Dieser wurde in die Messkammer einer doppelwandigen Glasapparatur⁵ eingeführt, deren Mantelraum von einer thermostatisierten Badflüssigkeit durchströmt wurde. Die Messkammer wurde mit 10 cm³ einer Tyrodelösung gefüllt, durch die gereinigte Luft hindurchperlte. Der pH-Wert betrug 7,7. Alle Versuche wurden bei Dunkeladaptation durchgeführt. Das Belichtungspotential wurde mit Ag/AgCl-Elektroden abgenommen, gleichspannungsgekoppelt verstärkt und auf einem Schreiber sowie Oszillographen registriert. Der Lichtreiz hatte eine Dauer von 1 sec und wurde im Abstand von 3 min ausgelöst. Die Beleuchtungsstärke (J) konnte mit Hilfe von Neutralfiltern im Bereich von 400 bis $1,3 \times 10^{-5}$ Lux verändert werden. Zur Auswertung wurde die Amplitude der b-Welle des Belichtungspotentials (ϕ_b) herangezogen, die vom tiefsten Punkt der a- bis zum Maximum der b-Welle ausgemessen wurde.

Ergebnisse. Der Temperatureinfluss auf das Belichtungspotential der isolierten Froschretina ist in Figur 1 an Hand von Originalelektroretinogrammen veranschaulicht. Bei 20°C sind die Potentialwerte am grössten und nehmen sowohl mit fallender wie auch steigender Temperatur ab. Oberhalb von 25°C zeigt sich eine irreversible Schädigung der Netzhautfunktion, die möglicherweise auf einen erhöhten Abbau von thermolabilen Zwischenprodukten des photochemischen Primärprozesses zurückzuführen ist. Bemerkenswert ist ferner, dass bei 6°C im Bereich niedriger Beleuchtungsstärken nur noch die e-Welle registrierbar ist, die einen späten Aus-Effekt darstellt⁶.

Die Temperaturabhängigkeit der Funktion $\phi_b = f(\log J)$ ist für einen charakteristischen Versuch in Figur 2 wiedergegeben. In einem Bereich von 7 bis 30°C ergibt sich bei der gewählten Darstellungsform für niedrige Beleuchtungsstärken ein linearer Kurvenverlauf. Aus der Figur geht hervor, dass die linearen Kurvenabschnitte für alle Temperaturen einen gemeinsamen Schnittpunkt mit der Abszisse aufweisen. Der zugehörige Extinktionswert beträgt 6,7 und entspricht einer Beleuchtungsstärke von $7,89 \times 10^{-5}$ Lux. Dieser als Schwellenreizstärke zu bezeichnende Wert wird demnach durch die Temperatur

¹ H. NETTER, *Biologische Physikochemie* (Akad. Verlagsgesellschaft Athenaion, Potsdam 1951).

² D. OTTOSON und G. SVAETICHIN, *Acta physiol. scand.* 29, Suppl. 106, 538 (1953).

³ T. FURUKAWA und I. HANAWA, *Jap. J. Physiol.* 5, 289 (1955).

⁴ W. SICKEL, *Neurophysiologie und Psychophysik des visuellen Systems* (Eds. R. JUNG und H. KORNUBER; Springer, Berlin-Göttingen-Heidelberg 1961), p. 80.

⁵ U. BORCHARD, *Diss. Math. Nat.*, Univ. Köln 1969.

⁶ F. CRESCITELLI und E. SICKEL, *Vision Res.* 8, 801 (1968).