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# IONIC STRENGTH DEPENDENCE OF THE INHIBITION OF ACETYLCHOLINESTERASE ACTIVITY BY $Al^{3+}$

Thomas R. SHARP * and Terrone L. ROSENBERRY

*Department of Pharmacology, Case Western Reserve University School of Medicine, 2119 Abington Road, Cleveland, OH 44106, U.S.A.*

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Inhibition of acetylcholinesterase activity by $Al^{3+}$ has been examined by initial velocity kinetics and by a first-order kinetic method. Both methods yield an inhibition constant of approx. 1.7 mM at 0.1 M ionic strength. The initial velocity study indicates a noncompetitive mechanism of inhibition by $Al^{3+}$. Inhibition at 10 mM ionic strength shows a $K_i$ of 0.03 mM. Evaluation of the ionic strength dependence concurs with the results of Nolte et al. (Biochemistry 19 (1980) 3705). An effective charge in the binding site of $-9$ predicts the ratio of inhibition constants at high and low ionic strength. Extrapolation to zero ionic strength gives a $K_i^0 = 0.34\ \mu M$.

## 1. Introduction

The inhibition of acetylcholinesterase (AChE; EC 3.1.1.7, acetylcholine acetylhydrolase) activity by $Al^{3+}$, among other trivalent cations, has been reported [1–3]. Pharmacological and toxicological data regarding the effects of $Al^{3+}$ on biological systems are of particular importance, considering the widespread exposure of the human population to aluminum used in building materials, food packaging products, cosmetics, etc. Correlations have been made between elevated aluminum content of tissues and certain types of neurological disorders [4,5]. AChE is therefore a logical target enzyme to examine for effects by $Al^{3+}$, considering its key role in cholinergic neural transmission in the peripheral and central nervous systems.

Effects of $Al^{3+}$ on AChE activity have been reported, but the details are unclear. Patocka [3] reports activation of bovine erythrocyte AChE at approx. 0.17 mM $Al^{3+}$, followed by inhibition at higher concentrations, the magnitude of activation and inhibition being dependent on ionic strength. Horse plasma cholinesterase [3] also shows activation (at 0.4 mM $Al^{3+}$), then inhibition, but without any ionic strength dependence. Recent data presented by Rosenberry and colleagues (personal communications) [15] have demonstrated that the AChE from human erythrocytes is a markedly different protein from that localized in the neuromuscular junction. Nonspecific cholinesterase is also known to be a rather different enzyme from AChE. The data of Marquis and Lerrick [1] show that $Al^{3+}$ acts as a noncompetitive inhibitor of *Electrophorus* AChE by virtue of the fact that $Al^{3+}$ does not change the apparent $K_m$ of AChE for its substrate. The concentration dependence of inhibition by $Al^{3+}$, however, is confusing. Their data do not allow a reasonably precise single estimate of the inhibition constant. It is unclear

* To whom correspondence should be addressed at (present address): Department of Chemistry, The University of Utah, Salt Lake City, UT 84112, U.S.A.

from their report whether the source of $Al^{3+}$, their handling of it, their methods of performing the experiments, or some other factor, is responsible for the uncertainty.

In an earlier report [9], we reexamined the kinetic mechanism of inhibition by $Al^{3+}$, and examine here the ionic strength dependence of inhibition of AChE activity by $Al^{3+}$ using a pseudo-first-order kinetic method [9].

## 2. Materials and methods

AChE was prepared from the electric organ of the electric eel, *Electrophorus electricus*, by the affinity chromatography procedure of Rosenberry et al. [6] and Chen et al. [7]. The 11 S form of AChE was routinely used.

Acetylcholine bromide and acetylthiocholine iodide were purchased from Eastman. 10 mM $Na^+$-Hepes buffer, pH 7.5, was used in all of the measurements. Buffers were prepared with Hepes-free acid (Sigma) and adjusted to the desired pH with NaOH. 0.1 M NaCl was included in reaction mixtures which are described as at high ionic strength. Measurements at low ionic strength were performed in a buffer of 10 mM $Na^+$-Hepes and 5 mM NaCl, pH 7.5. Aluminum salts used were $AlCl_3 \cdot 6H_2O$ (Alfa Inorganics), $Al_2(SO_4)_3 \cdot (14\text{–}18)H_2O$ (Matheson), and $AlK(SO_4)_2 \cdot 12H_2O$ (Fisher). Stock solutions of the $Al^{3+}$ salts were prepared fresh daily in distilled $H_2O$.

A Cary 210 UV-VIS scanning spectrophotometer, interfaced directly into a North Star Horizon computer with 56 kilobytes of random access memory and 360 kilobytes of on-line disk storage, was used for these kinetic measurements. The computer serves as a direct data-logging and analysis device. A program has been developed which acquires data directly from the spectrophotometer, stores the collected data on disk, and performs selected analyses of the data. Details of the working system can be found elsewhere [8,9].

Initial velocity studies were performed using the spectrophotometric procedure of Ellman et al. [10], in which the hydrolysis of acetylthiocholine is coupled to production of the thionitrobenzoate dianion ($\lambda_{max}$(412 nm), $e = 14150\ M^{-1}\,cm^{-1}$ [11]) from 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Pseudo-first-order measurements of AChE activity under substrate-limiting conditions were also made using a substrate-limited kinetic procedure, detailed by Sharp and Rosenberry [9]. The latter procedure consists of determining the $k'_{obs}$ for the pseudo-first-order hydrolysis of a small amount of substrate ($[S] \ll K_m$) by a constant amount of AChE as a function of increasing inhibitor concentration. Plots of $k_{obs}/k'_{obs}$ vs. [inhibitor] yield straight lines (eq. 1), with the slope equal to $1/K_i$, where $k_{obs}$ is the first-order rate constant describing the time course of hydrolysis in the absence of inhibitor, and $k'_{obs}$ the rate constant in the presence of a known inhibitor concentration. Fig. 1 shows the data used for determining $K_i$ at 0.1 and 0.01 M ionic strengths, utilizing eq. 1.

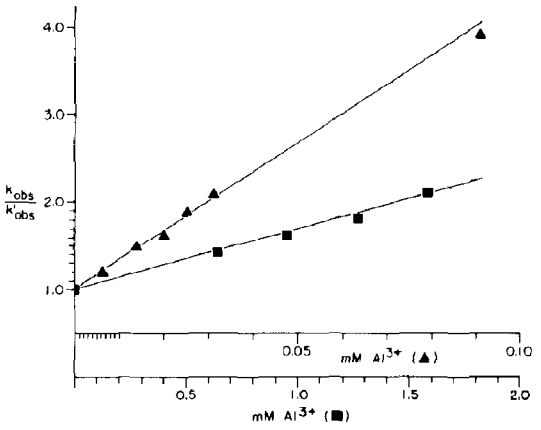


Fig. 1. Dependence of $k_{obs}/k'_{obs}$ on $Al^{3+}$ inhibitor concentration at different ionic strengths, according to eq. 1. (■) Determinations at 0.1 M ionic strength. (▲) Determinations at 0.01 M ionic strength. See section 2 for solution compositions. Note that determinations at different ionic strengths refer to different abscissa scales of inhibitor concentration.

$$\frac{k_{obs}}{k'_{obs}} = \frac{[I]}{K_i} + 1 \qquad (1)$$

## 3. Results and discussion

The inhibition constant $K_i$ and mechanism of inhibition of AChE activity by $Al^{3+}$ have been previously published [9], in which $K_i = 1.7$ mM at

0.1 M ionic strength. $Al^{3+}$ inhibits AChE activity noncompetitively, as indicated by the common intersection of the lines of a double-reciprocal plot on the $x$-axis at $(-1/K_m)$ for the substrate used – in this instance, acetylthiocholine. Measurement of $K_i$ by the substrate-limited pseudo-first-order method [9] confirms the magnitude of $K_i$, giving a value of 1.5 mM at 0.1 M ionic strength (see fig. 1). The noncompetitive mechanism is consistent with an earlier report on $Al^{3+}$ inhibition of AChE activity [1], but our values of the inhibition constant are approx. 50-times higher than the range previously reported [1].

The interaction of AChE with a number of cationic inhibitors, and its substrates, shows a very marked ionic strength dependence [12–14], and that inhibition is quite complex [13]. A detailed examination of the ionic strength dependence of the interaction of AChE with *N*-methylacridinium [14] has shown that the product of effective charge numbers for the enzyme inhibitor complex is quite high, such that the binding site contains from 6 to 9 negative charges. $K_i$ was determined for $Al^{3+}$ inhibition of AChE at 10 mM ionic strength, using the pseudo-first-order method, to be 0.03 mM – an approx. 50-fold increase in the affinity of the enzyme for its inhibitor (see fig. 1). By using eq. 7 of Nolte et al. [14],

$$\log K_i = \log K_i^0 + \frac{2AZ_E Z_L I^{1/2}}{1 + B\bar{a}I^{1/2}} \qquad (2)$$

where $K_i$ is the inhibition constant measured at ionic strength $I$, and $K_i^0$ would be the inhibition constant at zero ionic strength. The constants in the second term on the right-hand side of eq. 2 are defined as in ref. 14. The constant $\bar{a}$ is the mean distance of closest approach between the two interacting species, and has been assigned the value of 1.3 nm [14].

By substituting the values for $K_i$ and ionic strength into eq. 2 for each of our measurements, and then subtracting one equation from the other, the following is obtained;

$$\log \frac{K_{i(high)}}{K_{i(low)}} = X_{high} - X_{low} \qquad (3)$$

where $K_{i(high)}$ and $K_{i(low)}$ are the $Al^{3+}$ inhibition constants at high and low ionic strength, respectively, and $X_{high}$ and $X_{low}$ the evaluations of the second term on the right-hand side of eq. 2 for the ionic strengths used in the experiments. Using a $Z_L$ for $Al^{3+}$ of +3 and a $Z_E$ of −9 for the enzyme, as determined by Nolte et al. [14], an expected ratio of the inhibition constants is calculated from eq. 3 to be 59, in comparison with the ratio of observed inhibition constants of 1.6 mM/0.03 mM = 53. This agreement is quite good, considering the fact that it is based upon only two experimentally determined points, and also considering the uncertainty of the value, as well as the physical significance, of $\bar{a}$ [14]. These observations are in reasonable quantitative, as well as qualitative, agreement with the conclusions of Nolte et al. [14] that the ionic strength dependence of AChE interactions with its inhibitors and substrates involves a multiply charged binding site on the enzyme. Taking the extrapolation one step further by substituting either inhibition constant and its corresponding ionic strength back into eq. 2, a $K_i^0$ of approx. 0.34 $\mu$M at zero ionic strength is obtained.

Physical localization of AChE in the basal laminar sheath of the neuromuscular junction suggests that AChE would experience the relatively constant ionic strength of serum, approx. 0.15 M as in physiological saline. Alterations in ionic strength would not be expected to play any physiologically significant role in regulating enzyme activity. The significance of our measurements and calculations is, rather, in supporting the hypothesis of Nolte et al. [14]. The extreme efficiency which AChE exhibits in binding its substrate and inhibitors resides in the large effective charge density around the active site of AChE, providing a surface area larger and more effective in 'trapping' ligands than the active site itself. This is a quite reasonable property of an enzyme which plays a key role in the very rapid physiological recovery of the vertebrate neuromuscular junction.

The question of the exact nature of the solution species of aluminum which interacts with AChE is still open. An extensive literature exists on the solution chemistry of aluminum [16]. It is known that $Al^{3+}$ forms larger aggregates in solution, but that this process appears to be a slow one, and

dependent on a number of other factors. Indeed, we have observed that inhibition constants determined using old $AlCl_3$ solutions were significantly different from those with the freshly prepared solutions. We took the precaution of only reporting results determined using freshly prepared $AlCl_3$ solutions. Therefore, our calculations assuming an effective charge of +3 for the inhibitory aluminum ion are reasonable, given the circumstances. The exact nature of the inhibitory species in old $AlCl_3$ solutions, as well as the mechanism of inhibition, remains to be determined.

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