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THERMODYNAMIC INVESTIGATION OF CAMEL RETINA ACETYLCHOLINESTERASE INHIBITION BY CYCLOPHOSPHAMIDE

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The present work addresses the estimation of energy parameters such as Gibb's free energy change (ΔG), enthalpy change (ΔH), entropy change (ΔS) and activation energy (E_a) of acetylcholinesterase (AChE, EC 3.1.1.7) from camel retina in the absence and presence of the antineoplastic drug cyclophosphamide (CP). A spectrophotometric method was used for the determination of AChE activity, which was the basis for determination of these parameters. The PZ factor (number of sterically and energetically favorable collisions occurring between CP and AChE) have also been studied in this investigation. The energy parameters obtained in the present investigation were compared with the values reported elsewhere.

Keywords: Acetylcholinesterase; energy; inhibition; cyclophosphamide; retina.

INTRODUCTION

In the central nervous system (CNS), acetylcholinesterase (AChE, EC 3.1.1.7) fulfills a vital role at cholinergic synapses by rapidly hydrolyzing the neurotransmitter acetylcholine. One of the factors for efficiency is an extremely high catalytic turnover rate. This enzyme which is one of the fastest known[1], is capable of eliminating the substrate acetylcholine after its release at cholinergic synapses, thus allowing precise temporal control of muscle contraction. AChE is found in several non-neuronal cells such as muscle[2], sarcoplasmic reticulum[3] erythrocytes, lymphocytes, platelet, bone marrow[4] and placenta[5] of various species where its biological function is not clear. It has been extensively documented that the vertebrate retina, which is embryologically derived from the CNS, is rich in ACh (neurotransmitter) and cholinesterases.[6–9]

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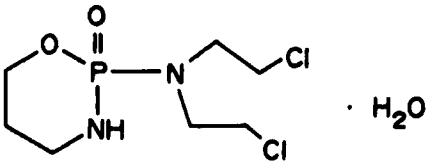

FIGURE 1 Chemical structure of cyclophosphamide monohydrate [2-{Bis(2-chloroethyl)amino}tetrahydro-2H-1,3,2-Oxaza-phosphorine 2-oxide].

There is a wealth of data demonstrating the effect of cholinergic drugs and anticholinesterases on the function of the retina.[10] Inhibition of AChE has been reported to either hasten or reduce the process of dark adaptation.[11] Thus, although decreases in visual activity have often been noted following exposure to anticholinesterases and muscarinic cholinergic agonists which may cause an increase in the flicker fusion frequency[12], these effects have largely been ascribed to changes in accommodative ability.[13]

Cyclophosphamide monohydrate (CP; Figure 1) is one of the anticancer drugs of the alkylating group which is used in the treatment of tumors[14–16] and in the treatment of rheumatoid arthritis.[17] It is also considered one of the most active single agents in the treatment of retina pathology, such as iridocyclitis, retinitis, keratitis, scleritis and conjunctivitis.[18–20] It is considered one of the most active single agents in the treatment of retinoblastoma.[21]

It has been reported[22–25] that CP has the ability to inhibit AChE activity *in vitro*. There are no such reports however on the effect of CP on the energetic parameters of the AChE-catalyzed reaction. The various energy parameters that have been studied in detail include: (1) energetics of enzyme catalysis[26], (2) energy characteristics of an ATP-hydrolase reaction catalyzed by solubilized Ca^{2+}, Mg^{2+}-ATPase from smooth muscle cell membrane[27], (3) energetics of ligand and inhibitor interactions with AChE[28] and substrate-ligand interactions with AChE and energetics of binding[29], (4) isothermal titration calorimetric studies of *Saccharomyces cerevisiae* myristoyl-CoA: protein N-myristoyltransferase for determinants of binding energy and catalytic discrimination among acyl-CoA and peptide ligands[30], (5) characterization of dinucleotide substrate "UpA" binding to RNase including a computer modelling and energetics approach[31], (6) thermodynamic study of dihydrofolate reductase inhibitor selectivity[32], (7) role of phospholipid in the calcium-dependent ATPase of the sarcoplasmic reticulum[33] and (8) the interaction of purified AChE from pig brain with liposomes.[34]

The activation energy (E_a) is useful as a quantitative measure of the thermodynamic barrier overcome in the course of catalysis. In the special case of enzymatic

catalysis, it has been used in relating conformational changes during catalytic events and in comparing homologous enzymes of diverse phylogenetic origin. Thus, E_a would appear to be a parameter of general interest to enzymologist.

The determination of K_m and V_{max} at different temperatures by using different substrate concentration (5–7 concentration range) is a tedious and time-consuming process. We wish to describe a short cut procedure which provides all the necessary data for the estimation of all energetic parameters of enzyme catalyzed reaction either in the presence or absence of inhibitors. In this method, each determination can be completed within 8 min. The basis of this method is to assay the enzyme activity at various temperatures using two different substrate concentrations i.e. assay with low and high substrate concentration.[35] This two substrate system has already been applied to the kinetic study of the inhibition of AChE from human erythrocyte by cisplatin.[36] The simplicity of the method developed in our laboratory suggests that it may be of more general utility and prompts this report.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade. Acetylthiocholine iodide (used as substrate, ASCh), 5,5′-dithiobis-(2-nitro) benzoic acid (DTNB) and CP were purchased from Sigma Chemical Co. (USA). Bovine serum albumin was obtained from Fluka Chemika-BioChemika (Switzerland).

Enzyme Preparation

Retinas of young camels (*Camelus dromedarius*) were obtained from the local abattoir. The whole retina from each eye was rapidly removed, rinsed in pre-cooled 0.9% saline solution, blotted and weighed. In each batch of enzyme preparation twenty whole retinas were prepared by homogenizing them in pre-cooled 50 mM sodium phosphate buffer (pH 7.4). The homogenization and centrifugation processes for the extraction of retina membrane-bound AChE by 0.5% Triton X-100 were followed as described in a previous report.[37]

Enzyme Assay

AChE activity was determined by the spectrophotometric method of Ellman *et al.*[38] A 4 min incubation time was selected for the enzyme assay.[39] The assay mixture (1 ml) contained 0.05 M sodium phosphate buffer, pH 7.4, 0.5 mM ASCh, 0.25 mM

DTNB and 18 μg of retinal protein as enzyme source. To study the effect of CP, the enzyme was preincubated with the drug at 25°C for 4 min prior to addition of substrate.

Estimation of Kinetic Parameters

Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) have been determined by using the two substrate concentrations design (lower and higher concentration) according to the following equations.[35]

$$K_m = [\{(S_1/V_1)(S_2 - S_1)\}/\{(S_2/V_2) - (S_1/V_1)\}] - S_1$$

$$V_{max} = \{(S_2/V_2) - (S_1/V_1)/(S_2 - S_1)\}$$

In the above equations, S_1 is the lower (0.03125 mM) and S_2 is the higher (0.50 mM) substrate concentration, and V_1 and V_2 respectively are the initial velocities at the respective substrate concentration. The selection of these two substrate concentration (i.e. 0.03125 mM and 0.50 mM) was based on the conditions reported for the precise construction of the two substrates design.[35]

Estimation of Energy Parameters

Energy parameters (ΔG, ΔH and ΔS) have been determined by applying the following equations:[40]

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

$$\Delta H = 2.3\ R(\text{slope of Log } K_{eq} \text{ versus } 1/T) \quad (2)$$

$$\Delta S = 2.3\ R(\text{intercept of Log } K_{eq} \text{ versus } 1/T) \quad (3)$$

Where K_{eq} and T denote the equilibrium constant and temperature respectively.

The activation energy (E_a) was calculated according to the Arrhenius equation;

$$E_a = 2.3\ R(\text{slope of Log } V_{max} \text{ versus } 1/T) \quad (4)$$

The activation energy (E_a) for the hydrolysis of ASCh by camel retina AChE was determined by plotting, log V_{max} for AChE activity versus $1/T$ (Arrhenius plot). The value of the negative slope of this plot was inserted in Equation (4) to give E_a, where R is the Boltzman constant and is equal to 1.98.

The PZ factor is determined by applying the equation:

$$\ln PZ = (E_a/RT) + \ln K_{eq} \tag{5}$$

where Z is the number of collisions occurring between reacting molecules and AChE in unit volume per unit time, (E_a/RT) is the frequency factor which measures the probability that any molecule will have sufficient energy to react, and P is the steric factor giving the fraction of energetically favorable collisions that are also sterically favorable for inhibition to take place.[41]

Heat of activation (ΔH^*) was calculated from the following equation:[41]

$$E_a = \Delta H^* + RT \tag{6}$$

The temperature coefficient, Q_{10} (which is the factor by which the rate constant is increased by raising the temperature 10°C) was calculated from the following equation:

$$E_a = (2.3\ R\ T_1 T_2 \log Q_{10})/10 \tag{7}$$

(where T_1 and T_2 are the two temperatures).

Estimation of Protein

The protein content of the enzyme preparation was estimated according to the method of Lowry *et al.*[42], using bovine serum albumin as standard. The detergent, Triton X-100, interfered with this estimation but this was overcome by centrifuging the precipitate at 1000 × g for 4 min at 25°C as described in our recent publication.[43]

Graphics

The graphs were plotted by using Grafitter program[44], performed on a PC-486 Compaq; prolinea-466.

RESULTS AND DISCUSSION

For the determination of enthalpy (ΔH) and entropy (ΔS), $\log K_{eq}$ for AChE reaction in the absence and presence of CP (0.5–1.5 mM) at each temperature

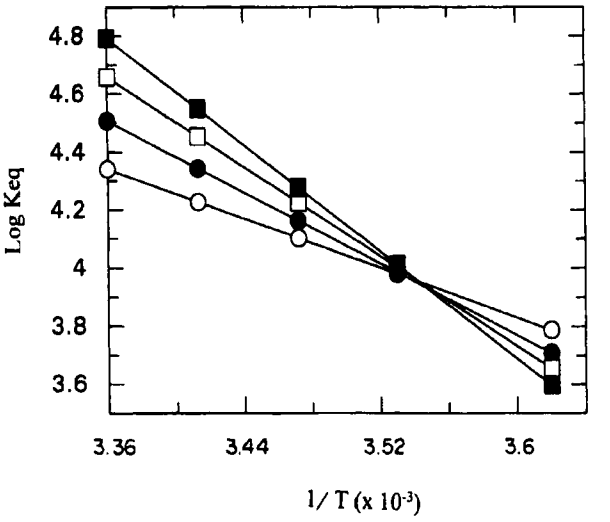

FIGURE 2 Plot of log K_{eq} versus reciprocal of temperature (K°) for the AChE reaction in the absence (○) and presence of 0.50 mM (●), 1.0 mM (□) and 1.5 mM (■) CP concentration. Each point is the mean value of three separate determinations (correlation coefficient were 0.952, 0.974, 0.950 and 0.953 for the lines for ○, ●, □ and ■ respectively).

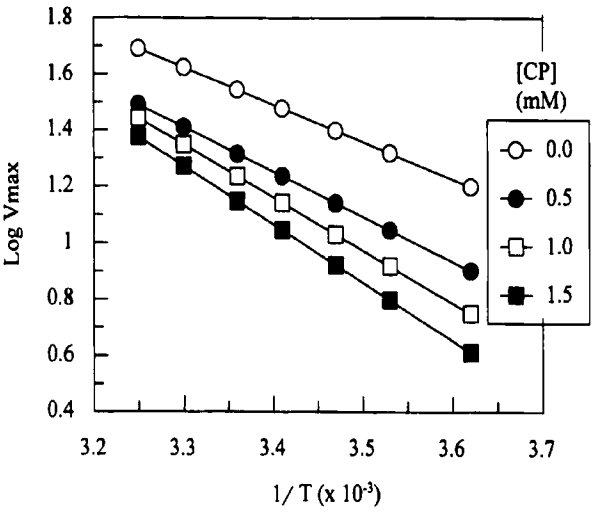

FIGURE 3 Plot of log V_{max} versus reciprocal of temperature (K°) for the AChE reaction in the absence (○) and presence of 0.05 mM (●), 1.0 mM (□) and 1.5 mM (■) CP concentration. Each point is the mean value of three separate determinations (correlation coefficient were 0.991, 0.972, 0.986 and 0.982 for the lines for ○, ●, □ and ■ respectively).

TABLE I Effect of CP on activation energy (E_a) of AChE catalyzed reaction

[CP] (mM)	*Slope*	*E_a (kcal/mole)*
0.0	−1.3308	6.061
0.5	−1.591	7.245
1.0	−1.8732	8.531
1.5	−2.0655	9.406

Slope = log V_{max}/Temperature

TABLE II Effect of CP on energy parameters of camel retina AChE

Energy Parameter	*0.0 mM CP*	*0.5 mM CP*	*1.0 mM CP*	*1.5 mM CP*
ΔH (kcal/mole)	9.37	12.76	17.61	21.01
ΔH^* (kcal/mole)	5.47	6.65	7.94	8.81
ΔS (e.u)	51.30	63.39	80.44	92.50
ΔG (kcal/mole)	−5.91	−6.13	−6.37	−6.55
$PZ[\times 10^4$ M]	2.35	3.86	4.53	6.34
Q_{10}	1.43	1.53	1.65	1.74

ΔH^* represents heat of activation

was plotted against reciprocal of temperature in K° (Figure 2). Enthalpy (ΔH) and entropy (ΔS) were calculated from this plot as (Equations (2) and (3)) while Gibb's free energy change (ΔG) was calculated by using the values of these two parameters in Equation (1). Activation energy (E_a) for the control and for CP (0.5–1.5 mM) treated reactions was calculated from Equation (4) and the plot is presented in Figure 3. The Arrhenius plots for AChE in the absence and in the presence of CP were linear; the values of their slope and the activation energy is summarized in Table I. The PZ factor for control and for CP treated reactions was calculated by using Equation (5). The values of all these energy parameters (ΔH, ΔH^*, ΔS, ΔG, Q_{10}) and PZ factor are presented in Table II.

Since E_a and slope increased in equal proportions (20–55%) as the CP concentration increased so the thermodynamic barrier for AChE–ASCh complex to overcome in the formation of products increases. A negative value of ΔG for CP (Table II), confirmed the inhibitory role of CP with AChE activity. Entropy values (ΔS) indicated that CP did not inactivate the AChE by denaturation. However, an increase in the PZ factor for CP, indicates that the main factor controlling the

potency of the CP is the favorable orientation and distribution of potential energy among colliding molecules that is required for inhibition to take place.

The values of the E_a obtained by the present method are in good agreement with those obtained by the more laborious conventional procedure such as 2.7–5.9 (kcal·mole^{-1}) for above transition temperature and 4.8–9.6 (kcal·mole^{-1}) for below transition temperature in case of non-linear, Arrhenius plots of AChE from various origins while 3.8–7.1 (kcal·mole^{-1}) in case of linear Arrhenius plots of control system.[45–50] In the inhibition system 3.3–4.6 (kcal·mole^{-1}) for above transition temperature and 8.9–16.3 (kcal·mole^{-1}) for below transition temperature in case of non-linear Arrhenius plots of AChE from various origins while 9.2–11.4 (kcal·mole^{-1}) in case of the linear Arrhenius plots have been cited in the literature.[47–53]

As indicated in Table II, the values of ΔH, ΔH^*, ΔS, ΔG, PZ factor and Q_{10} increased in different proportions i.e. 124.2, 61.1, 80.3, 10.8, 169.8, 21.7% respectively as the CP concentration increased. Similar to E_a, the values of Q_{10} and ΔH^* are in good agreement with reported values such as the Q_{10} for *Walterinnesia aegyptia* venom AChE[45], *Bungarus fasciatus* venom AChE[46] and chicken brain AChE[47] which were reported as 1.45, 1.38 and 1.59 (below transition temperature) and 1.23 (above transition temperature) respectively. The ΔH^* for *W. aegyptia* venom AChE[45] and *B. fasciatus* AChE[46] has been reported as 5.944 kcal/mole at 25°C and 5.110 kcal/mole respectively. In the present study a 61.06% increase in ΔH^* by 1.5 mM CP, indicating that a CP-induced conformational change occurred in camel retina AChE.

References

[1] Quinn, D.M. (1987). *Chem. Rev.*, **87**, 955–979.
[2] Cabezas-Herrera, J., Moral-Naranjo, M.T., Campoy, F.J. and Vidal, C.J. (1994). *Biochim. Biophys. Acta*, **1225**, 283–288.
[3] Campoy, F.J., Cabezas-Herrera, J. and Vidal, C.J. (1992). *J. Neurosci. Res.*, **33**, 568–578.
[4] Al-Jafari, A.A. and Kamal, M.A. (1994). *Cell Biochem. Funct.*, **12**, 29–35.
[5] Simone, C., Derewlany, L.O., Oskamp, M., Johnson, D., Knie, B. and Koren, G. (1994). *J. Lab. Clin. Med.*, **123**, 400–406.
[6] Weikert, T., Rathjen, F.G. and Layer, P.G. (1994). *J. Neurochem.*, **62**, 1570.
[7] Criswell, M.H. and Brandon, C. (1993). *Vision Res.*, **33**, 1747–1753.
[8] Criswell, M.H. and Brandon, C. (1992). *Brain Res.*, **577**, 101–111.
[9] Jen, L.S., Tsang, D., Chau, R.M.W. and Shen, W.Z. (1990). *Brain Res.*, **523**, 156–160.
[10] Rengstorff, R. and Royston, M. (1976). *J. Optom. Physiol. Opt.*, **53**, 70–80.
[11] Trussov, M.S. (1962). *Oftalmol. Zh.*, **17**, 366–371. [Translation of Rengstorff, R.H. and Gurvis, V. (1975). *Am. J. Optom. Physiol. Optics.*, **52**, 438–440].
[12] Alpern, M. and Jampel, R.S. (1959). *Am. J. Opthalmol.*, **47**, 464–476.
[13] Hutchins, J.B., Bernanke, J.M. and Jefferson, V.E. (1995). *Exp. Eye Res.*, **60**, 113–125.
[14] Deppe, G., Maiviya, V.K., Boike, G. and Young, J. (1991). *Eur. J. Gynaecol. Oncol.*, **12**, 93–97.
[15] Shiu, W., Teo, P. and Wong, K. (1990). *Br. J. Clin. Pract.*, **44**, 519–520.

[16] Siodlak, M.Z., Stell, P.M., Wilson, J.A., Green, J.A., Allison, R.S., Adler, D. and Squadrellisaraceno, M. (1990). *J. Laryngol. Otol.*, **104**, 631–638.
[17] Campe, P.C. and Harvey, R.A. (1993). In Lippincott's illustrated reviews: Pharmacology, (Mucel, M.J., Gertner, S.B. and Perper, M. (eds) p. 350. J.B. Lippincott Company: New York.
[18] Carreras, B., Griffin, D.E. and Silverstein, A.M. (1982). *Invest. Opthalmol. Vis. Sci.*, **22**, 571–578.
[19] Mangouritsas, G. and Ulbig, M. (1994). *Ger. J. Ophthalmol.*, **3**, 68–70.
[20] Stroup, W.G. and Banks, M.C. (1994). *Acta Neuropathol. Berl.*, **87**, 14–22.
[21] White, L. (1983). *Retina*, **3**, 194–199.
[22] Al-Jafari, A.A. (1993). *Toxicol. Lett.*, **66**, 125–131.
[23] Al-Jafari, A.A., Duhaiman, A.S. and Kamal, M.A. (1995). *Toxicology*, **96**, 1–6.
[24] Kamal, M.A., Nasim, F.H. and Al-Jafari, A.A. (1996). *Biochem. Mol. Biol. Int.*, **159**, 47–53.
[25] Al-Khwtyer, F., Kamal, M.A. and Al-Jafari, A.A. (1996). *Toxicol. Lett.*, **87**, 69–76.
[26] Albery, W.J. and Knowles, J.R. (1987). *J. Theor. Biol.*, **21**, 173–189.
[27] Kosterin, S.A., Slinchenko, N.N. and Gergalova, G.L. (1994). *Biokhimiya*, **59**, 889–904.
[28] Das, Y.T., Brown, H.D. and Chattopadhyay, S.K. (1987). *Biochem. Cell Biol.*, **65**, 798–802.
[29] Das, Y.T., Brown, H.D. and Chattopadhyay, S.K. (1986). *Gen. Pharmacol.*, **17**, 715–720.
[30] Bhatnagar, R.S., Jackson-Machelski, E., McWherter, C.A. and Gordon, J.I. (1994). *J. Biol. Chem.*, **269**, 11045–11053.
[31] Seshadri, K., Rao, V.S. and Vishveshwara, S. (1994). *J. Biomol. Struct. Dyn.*, **12**, 581–603.
[32] Sasso, S.P., Gilli, R.M., Sari, J.C., Rimet, O.S. and Briand, C.M. (1994). *Biochim. Biophys. Acta*, **1207**, 74–79.
[33] Hidalgo, C., Ikemoto, N. and Gergely, J. (1976). *J. Biol. Chem.*, **251**, 4224–4232.
[34] Reavill, C.A., Wooster, M.S. and Plummer, D.T. (1978). *Biochem. J.*, **173**, 851–856.
[35] Mabood, S.F., Khawja, P., Dougles, J.B. and Akhtar, M.A. (1984). *Pak. J. Biochem.*, **17**, 31–38.
[36] Al-Jafari, A.A. (1995). *Int. J. Biochem. Cell Biol.*, **27**, 965–970.
[37] Al-Jafari, A.A. (1996). *J. Ocul. Pharmacol. Ther.* (in press).
[38] Ellman, G.L., Courtney, D., Andres, V. and Featherston, R.M. (1961). *Biochem. Pharmacol.*, **7**, 88–95.
[39] Al-Jafari, A.A., Al-Khwyter, F., Kamal, M.A. and Alhomaida, A.S. (1996). *Jpn. J. Pharmacol.*, **72**, 49–55.
[40] Segel, I.H. (1975). *Biochemical Calculations*, pp. 145–207. New York: John Wiley and Sons.
[41] Abdel-Aal, Y.A.I. (1983). *J. Pesticide Sci.*, **8**, 99–103.
[42] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1953). *J. Biol. Chem.*, **193**, 265–275.
[43] Al-Jafari, A.A. and Kamal, M.A. (1996). *Biochem. Mol. Biol. Int.*, **38**, 577–586.
[44] Leatherbarrow, R.J. (1992). GraFit. Version 3.0. Enthacus Software Ltd., Staines, U.K.
[45] Al-Jafari, A.A., Kamal, M.A., Duhaiman, A.S. and Alhomida, A.S. (1995). *Mol. Cell Biochem.*, **151**, 21–26.
[46] Kumar, V. and Elliot, W.B. (1975). *Comp. Biochem. Physiol.*, **51C**, 255–258.
[47] Al-Jafari, A.A. (1992). *Drug Chem. Toxicol.*, **15**, 295–312.
[48] Al-Jafari, A.A. (1993). *Int. J. Biochem.*, **25**, 319–324.
[49] Meisami, E. (1984). *J. Neurochem.*, **42**, 883–886.
[50] Barton, P.L., Futerman, A.H. and Silman, I. (1985). *Biochem. J.*, **231**, 237–240.
[51] Haque, S.J. and Poddar, M.K. (1983). *Biochem. Pharmacol.*, **32**, 3443–3446.
[52] Aldridge, W.N. (1953). *Biochem. J.*, **54**, 442.
[53] Cheema, A.A., Shah, F.H. and Shakoori, A.R. (1985). *Pak. J. Zool.*, **17**(4) 373–379.