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Inhibition of Sheep Brain Acetylcholinesterase by Hexachlorophene

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Hexachlorophene (HCP) is a broad spectrum fungicide and bacteriocide (Nakaue *et al.* 1972) used as an anthelmintic agent in sheep (Hall and Reid 1972). HCP is neurotoxic and its neuropathology includes paralysis associated with edema and spongy degeneration of cerebral white matter (Lockhart 1972; Shuman *et al.* 1973). Since HCP is an established neurotoxicant, it is possible that neurotransmission could also be affected. As a majority of neurons in sheep brain are cholinergic in action, an attempt has been made in this study to assess the response of acetylcholinesterase (AChE) to HCP treatment *in vitro*.

MATERIALS AND METHODS

Sheep brains were procured from a local slaughter house after an animal's decapitation and stored immediately in a dry beaker in a freezing mixture. The meningi were removed and the brains were repeatedly washed with mammalian Ringer solution. The cerebral cortex free from capillaries was removed and homogenized in a Remi homogenizer at 500 strokes per minute with 10% (W/V) 0.25 M ice-cold sucrose solution. The crude homogenate was employed as an enzyme source. All these steps were carried out at temperature below 0° C unless otherwise indicated.

AChE activity was assayed by the method of Metcalf (1951). The assay mixture contained 100 μM of sodium phosphate buffer (pH 7.4), various concentrations of Acetylcholine (ACh; 0.4–4.0 mM), 0.2 mg of enzyme source in a total volume of 2.5 ml. It was incubated at 37° C for 30 minutes. The reaction was stopped with 2 ml of alkaline hydroxylamine hydrochloride followed by 1 ml of 1:1 HCl. The reaction mixture was centrifuged at 2000g for 10 min. Aliquots were checked for ACh concentration spectrophotometrically using ferric chloride. The protein content of the enzyme source was analyzed by the method of Lowry *et al.* (1951).

AChE activity was assayed as described earlier in the presence of various concentrations (0.04 to 2.8 mM) of HCP to determine dose/response profiles and to determine the I_{50} after the method of Wang and Buhler (1978). AChE was assayed for its dependency on ACh in the presence of HCP I_{50} concentration (0.28 mM). V_{max} and K_m were determined using least squares as the best fit. The inhibitory constants K'_i and K_i in the presence of HCP were calculated as suggested by Dixon and Webb (1979).

Temperature versus rate profiles were studied from 25° C to 50° C with an interval of

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5° C. Energy of activation (Δ E) was calculated from the Arrhenius equation (Δ E= 4.576 X (T_1 -T_2) X log (K_2 - K_1)/(T_2 - T_1) cals/mole) as given by Dixon and Webb (1979).

RESULTS AND DISCUSSION

Variation in AChE activity as a function of HCP concentration is summarized in Table 1. The HCP concentration versus AChE response relationship yielded a characteristic inhibition curve with the 50% inhibition of enzyme activity at 0.28 mM HCP. Enzyme activity was sharply inhibited up to 0.4 mM of HCP and very high concentrations of HCP were required to exert further inhibition.

Table 1. Effect of HCP Concentration on AChE Activity in Sheep Brain Homogenates

Concentration of HCP in mM	Activity of AChE	% change over control
0.00	9.86 ± 0.36	——
0.04	9.53 ± 0.52	-3.35
0.08	9.08 ± 0.50*	-7.91
0.12	8.03 ± 0.29*	-18.50
0.16	7.53 ± 0.26*	-23.63
0.20	6.45 ± 0.36*	-34.60
0.24	5.82 ± 0.22*	-40.97
0.28	4.90 ± 0.31*	-50.30
0.32	4.06 ± 0.24*	-58.82
0.36	3.52 ± 0.26*	-64.30
0.40	3.09 ± 0.34*	-68.66
0.80	2.79 ± 0.54*	-71.70
1.20	2.62 ± 0.08*	-73.40
1.60	2.12 ± 0.30*	-78.49
2.00	1.12 ± 0.19*	-88.58
2.40	0.72 ± 0.13*	-92.67
2.80	nil	nil

AChE activity levels are represented in μM of ACh/mg protein/hr.
All values are means, ± S.D. of 8 samples.
* Significantly different from control (0.0 mM HCP) $p<0.001$.

It is evident from the substrate dependent kinetic studies that enzyme activity followed first order kinetics upto 3.2 mM of ACh in the control, whereas it was only 2.8 mM in the presence of HCP suggesting certain enzyme active sites were masked or some active sites were made inaccessible for E–S catalysis. From a Lineweaver–Burk double reciprocal plot a slight increase in K_m and marked decrease in V_{max} were observed. Since the changes in V_{max} (–46 %) are more pronounced than K_m (+11%) in the presence of HCP, the nature of inhibition may be categorized as mixed type tending towards non–competitive inhibition (Table 2; Fig 1).

The inhibitory constant in this mixed type of inhibition was derived according to the equations of Dixon and Webb (1979). The noncompetitive inhibitory constant (K'_i) is the dissociation constant of the EIS complex and competitive inhibitory constant (K_i) is that of EI complex. $K'_i < K_i$ (Table 2) denoted that the AChE inhibition might be mainly due to the reduction in the active site density of the enzyme rather than decreased E–S affinity.

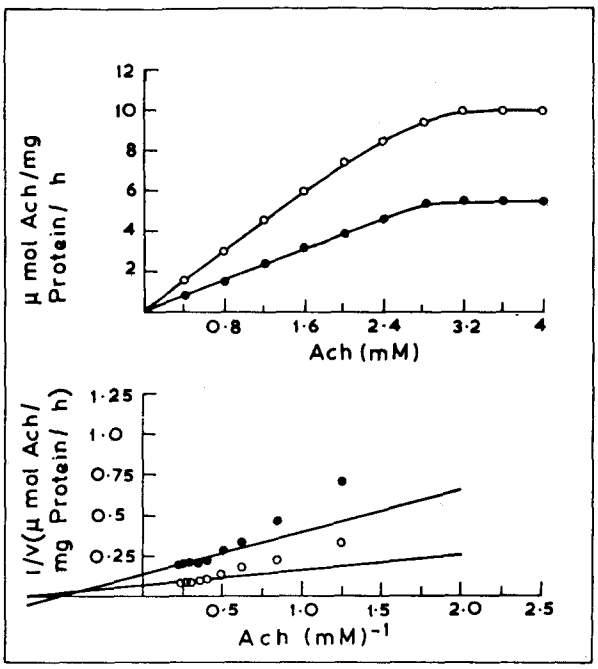

Figure 1. Lineweaver-Burk plot for the inhibition of Sheep brain acetylcholinesterase by HCP at various substrate concentrations. Each point is the mean velocity of six experiments.

Table 2. Changes in Kinetic Parameters of AChE in The Presence of I_{50} Concentration of HCP

Sample	Kinetic parameters			
	V_{max}	K_m	K_i	K'_i
Control	10.0± 0.39	1.35± 0.02	—	—
0.28 mM of HCP	5.4± 0.26*	1.50± 0.03*	2.546	0.822
% Change	-46.0	+11.1		

All values are means, ± S.D. of 8 samples. * Significantly different from control $p<0.001$.

Temperature dependent velocity studies suggested that the maximal velocity was reached at 40° C in both control and experimental assays. Data on temperature versus rate profiles were fitted to Arrhenius equation to calculate the energy of activation (Δ E). In the presence of HCP, Δ E values were elevated suggesting that the enzyme demanded a higher than the normal energy of activation which might account for the declining catalytic efficiency of the enzyme (Table 3).

The observed changes in the kinetic parameters and energy of activation show that HCP strongly inhibits AChE activity *in vitro*. Since it is well established that the HCP is accumulated in the brains of intoxicated animals (Kimbrough 1973; Towfighi and Gonatas 1973; Towfighi *et al.* 1974; Ulsamer *et al.* 1975), it may be speculated that the

Table 3. Effect of HCP on Activation Energy Values of Sheep Brain AChE

Temperature range in °C	Control	Experimental	% Change
25–30	21950± 320	23530± 455**	+ 7.2
30–35	13370± 979	16780± 1040**	+ 25.5
35–40	3070± 450	3698± 387*	+ 20.46

All values are means, ± S.D. of 8 samples.
Significantly different from control * $p<0.01$; ** $p<0.001$.

accumulated HCP may inhibit AChE *in vivo.* Studies showing inhibition of several neural enzymes by HCP affecting the energy metabolism have been reported (De Lucia *et al.* 1978). However whether such an inhibition manifests and contributes to the HCP neurotoxicity *in vivo,* especially with reference to AChE remains to be established.

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