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In Vitro Inhibition of Goat Brain Acetylcholinesterase by Pure and Commercial Anticholinesterase Pesticides

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In vitro inhibition of goat cerebellar acetylcholinesterase by pure and commercial anticholinesterase pesticides clearly indicates a remarkably high inhibitory effect of commercial carbamate and organophosphate pesticides containing a lower percentage of the respective active ingredients comparable to that of the known anticholinesterase agents such as DFP and physostigmine. It may be presumed that injudicious use of commercial formulations conduce severe toxicity in the nontarget mammalian species, namely, goat, and this response of the brain acetylcholinesterase may be utilized as a reliable bioindicator of pesticidal contamination of the terrestrial environment.

INTRODUCTION

Organophosphate and carbamate compounds are widely used in agriculture for proper management and control of pests. There is abundant evidence that both groups of pesticides produce their acute toxic actions by inhibiting acetylcholinesterase. Five organophosphates (El-Sebae *et al.*, 1977) and two carbamates (Chin *et al.*, 1980) were found to significantly inhibit *in vitro* brain acetylcholinesterase (AChE) activity in rat. On the other hand, Chabrier and Jacob (1980) found methyl-1-(*S*-methylphosphoryl-3) imidazolium to be equipotent with DFP in inhibiting brain cholinesterase activity in mice. Blood AChE activity was found to decrease in male rats treated with carbaryl (Dikshith *et al.*, 1976). Carbaryl given to pregnant rats by gastric intubation caused inhibition of AChE in the fetuses (Cambon *et al.*, 1978). The same authors worked in 1979 on pregnant rats and their fetuses with carbamate derivatives, viz. carbofuran, primicarb, and aldicarb. They found mortality of the rat at the higher dose while the lower dosage group had decreased enzyme activity in blood and other tissues.

In India, phenthoate and carbofuran are extensively used by farmers, and cattle, including goat, are found to graze freely near the agricultural fields. Thus, nonjudicious application may lead to accumulation of the pesticides in the goat, thereby causing undesirable neurotoxic effects. Unfortunately, there is no information available on the effect of phenthoate and carbofuran in the goat. This lacuna led these researchers to investigate the neurotoxic potential of these pesticides in terms of inhibition of AChE. The *in vitro* inhibition technique has been adopted because the study of this biochemical lesion is of fundamental importance in assessing the toxicity of the pesticides with anticholinesterase properties.

MATERIALS AND METHODS

Heads of freshly sacrificed goats were collected from the local market under freezing conditions and brought to the laboratory for immediate processing. The cerebel-

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lum was dissected out and homogenized at 0°C with double glass distilled water by means of a Potter–Elvehjem homogenizer having a Teflon-coated pestle to obtain a 5% homogenate because this region was found to possess maximum AChE activity (Guhathakurta and Bhattacharya, 1984). The homogenate was subjected to three purification steps such as solubilization by Triton X-100, neutral salt fractionation, and dialysis. The final partially purified enzyme used for *in vitro* inhibition was obtained by gel filtration through Sephadex G-200 (Pharmacia) (Guhathakurta, 1986).

AChE activity was measured in the partially purified enzyme from goat cerebellum following the method of Ellman *et al.* (1961) in a Beckman 25 DB spectrophotometer with recorder using acetylthiocholiniodide (Sigma) as the substrate and 5,5′-dithiobis-2-nitrobenzoate (Sigma) as the thiol indicator. Before the *in vitro* inhibition studies were set up, conditions for optimal enzyme activity like pH, temperature, substrate, and activator concentrations were determined. Enzyme activity was calculated in terms of nanomoles thiocholine liberated per minute per milligram protein. Protein was estimated (Lowry *et al.*, 1951) using bovine serum albumin as the standard.

In Vitro Inhibition Studies

In order to assess the toxic effects of widely used commercial pesticides, Furadan (Rallis India Ltd.) containing 75% active ingredient carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranil *N*-methylcarbamate) and Elsan (Motilal Pesticides) containing 50% phenthoate (*O,O*-dimethyl-*S*-(a-ethoxy carbonyl benzyl phosphorodithioate) were selected. Assays were also done using 99% pure and known AChE inhibitors such as diisopropyl fluorophosphate, DFP (Sigma), and physostimine (Sigma). For the *in vitro* inhibition assay, an appropriate amount of enzyme solution was preincubated with the inhibitors of varying concentrations separately at 40°C under continuous stirring. An aliquot of this complex was taken at intervals ranging from 0 to 20 min added in a cuvette containing sodium–potassium phosphate buffer with activator indicator and the substrate solution making a total volume of 3 ml. (Bhattacharya *et al.*, 1981).

Results were expressed in terms of time taken for 50% inhibition of the enzyme ($t_{0.5}$) and the biomolecular rate constant (k_2) was calculated according to the equation $k_2 = \ln 2/t_{0.5}[I]$ (Aldridge and Davison, 1952) where k_2 denotes the bimolecular rate constant, and $[I]$ the inhibitor concentration which again reflects the effect of inhibitor concentration at a fixed concentration of the enzyme.

RESULTS

The purification steps employed in the present study yielded an enzyme with 46-fold purification which was a stable product and kinetic properties comparable to AChE purified from other sources. The optimum conditions were as follows: pH, 7.6; temperature, 40°C; substrate concentration, 4×10^{-4} *M*; K_m, 3.0×10^{-5} *M*; activator concentrations $MgCl_2$, 5 m*M*, and NaCl, 30 m*M*.

Table 1 depicts the rate of inhibition of AChE pretreated with the different test compounds. It clearly indicates that AChE inhibition is concentration dependent and thus the time taken for 50% inhibition of the enzyme is directly related to the potency of the inhibitory compound. It is interesting to note that inhibitor concentrations as low as 10^{-10} *M* DFP and 10^{-11} *M* physostigmine are capable of 50% inhibition within 20 min. Furthermore, 5×10^{-6} *M* DFP produced complete inhibition of the enzyme

TABLE 1

INTERRELATIONSHIP OF INHIBITOR CONCENTRATION, $t_{(0.5)}$, AND BIMOLECULAR RATE CONSTANT IN *IN VITRO* INHIBITION OF GOAT CEREBELLAR AChE

Inhibitor	Concentration (M)	$t_{0.5}$ (min)	k_2 ($M^{-1} \times min^{-1}$)
Elsan	3×10^{-6}	14	0.17×10^5
(50% phenthoate)	3×10^{-7}	20	1.15×10^5
	5×10^{-7}	2	0.07×10^7
DFP	5×10^{-9}	11	1.30×10^7
(99%)	5×10^{-10}	15	9.3×10^7
Furadan	5×10^{-6}	15	0.09×10^5
(75% carbofuran)	5×10^{-7}	20	0.70×10^5
	6×10^{-9}	10	0.12×10^8
Physostigmine	6×10^{-10}	13	0.90×10^8
(99%)	6×10^{-11}	21	5.50×10^8

within 1 min and a concentration as low as 5×10^{-10} M inhibited the enzyme by 50% within 15 min. Elsan containing 50% phenthoate also inhibits the enzyme by 50% within 20 min at a concentration of 3×10^{-7} M, while Furadan (75% carbofuran) demonstrates a similar rate of inhibition at a concentration of 5×10^{-7} M. Higher concentrations of all the compounds tested caused a higher rate of inhibition as evidenced by lower $t_{0.5}$ (min) values.

DISCUSSION

The enzyme characteristics reveal that goat AChE has a lower K_m value than the K_m values in bovine erythrocyte (Reiner and Simeon, 1977; Grossmann and Liefländer, 1979), electric organ of *Electrophorus electricus* (Bhattacharya *et al.*, 1981), and teleost brain (Jash, 1982). This indicates that the AChE of goat purified in the present investigation has a higher affinity toward the substrate. It is well known that enzymes are usually inactivated by heat treatment. However, the goat AChE has its optimum activity at a temperature as high as 40°C as reported for human blood cholinesterase (Reiner *et al.*, 1974). Thus it may be surmised that goat AChE is a heat-stable enzyme and therefore suitable for *in vitro* inhibition which entails prolonged incubation periods.

Inhibition of this enzyme by DFP and physostigmine is comparable to that in earlier reports using AChE from the electric organ of eel (Michel and Krop, 1951; Brossi *et al.*, 1986). DFP is one of the most potent organophosphates known for its fast inactivation and aging of AChE (Michel and Krop, 1951) and the same effect was noted in the present study. However, the goat enzyme is much more sensitive to DFP than rat (Chabrier and Jacob, 1980). In goat 5×10^{-6} M DFP produced complete inhibition of the enzyme within 1 min and a concentration as low as 5×10^{-10} M inhibited the enzyme by 50% within 15 min. This demonstrates that the goat may be much more adversely affected than other experimental mammals under field conditions.

The bimolecular rate constants are considered to be a better index of the inhibitory power of an anticholinesterase agent than IC_{50} values (Aldridge and Davison, 1952).

Comparison of the commercial and pure inhibitors in the present study reveals that DFP and physostigmine have more inhibitory power than Elsan and Furadan, although physostigmine is more inhibitory (higher k_2) than DFP. Elsan, an organophosphate compound, is more inhibitory than Furadan (a carbamate compound). Interestingly, Furadan is a comparatively purer product than Elsan. From the comparison of the k_2 values, it can therefore be concluded that the higher rate of inhibition by Elsan is probably due to the impurities in the commercial compound. A similar investigation was carried out by Aldridge and Davison (1952) to prove that in the case of AChE inhibitors impurities in the insecticide may add to the inhibitory effect.

CONCLUSIONS

It is concluded that, in general, organophosphates are more toxic than carbamates, and commercial compounds containing a lesser percentage of the active ingredient have a higher degree of inhibition of AChE.

Thus, the results signify that the commercial organophosphates and carbamates used as pesticides may be more deleterious than the pure compounds to the nontarget species. Furthermore, the extreme specificity of the biochemical lesion caused by the pesticides may be considered a reliable indicator of the anticholinesterase properties of such widely used pesticides.

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