**Diacetylmonoxime Reactivation of Acetylcholinesterase and Butyrylcholinesterase Inhibited by Dichlorvos in Central and Peripheral Nervous System of Rat**

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**Abstract.** The *in vivo* AChE and BuChE inhibition in different tissues of the male albino rats by an organophosphorus compound dichlorvos (DDVP; 47 mg/kg) was examined. The AChE percent inhibition in the brain regions was as follows: striatum>hippocampus>medulla>pons>cortex>thalamus>cerebellum>spinal (65.6>55.3>52.4>45.6>41.6>35>22.5>16.6). In the other target organs the AChE inhibition was in the order of whole brain>liver>kidney>testis>serum (40> 39.9>38>37>30>29.2). *In vivo* inhibition of serum BuChE was more severe than inhibition of its AChE. In the kidney AChE activity was more inhibited in comparison to its BuChE activity. The *in vivo* effect of pretreatment of DAM (100 mg/kg) on DDVP-induced AChE inhibition was also studied. DAM provided significant protection (p <0.01) against DDVP inhibited AChE in almost all tissues. DAM protected 90 % (P <0.01) against DDVP inhibition in pons and spinal, about 75 % in hippocampus, medulla and cortex. DAM did not show significant protection against serum AChE inhibition but provided significant (p<0.01) protection against serum BuChE inhibition.

**Keywords:** AChE, BuChE, Dichlorvos, DDVP, DAM, Inhibition, Reactivation, Rat, Brain.

### 1. Introduction

Despite the use of new selective insecticides organophosphorus (OPs) remains an important class of pesticides. Dichlorvos, an organophosphorus compound (DDVP: 2.2-dichlorovinyl dimethyl phosphate) is a contact and stomach-acting insecticide with fumigant and penetrant action. It is widely used as crop protection against sucking and chewing insects. Non-target species including mammals are frequently injured by OPs (1). Neurotoxicity from OP exposure is generally related to inhibition of acetylcholinesterase (AChE; EC 3.1.1.7), (2). Which subsequently result in the accumulation of Acetylcholine (ACh) at neuroeffector sites (3). Inhibition of brain AChE is generally regarded as a useful marker and most sensitive measure of organophosphorus toxicity (4). Regarding the neurochemical mechanism of OPs effects, a study on discrete brain regions (cortex, cerebellum, striatum, hippocampus, stem, thalamus, pons, and optic chiasma) serum, kidney, and testis showed a considerable quantitative difference of inhibition of AChE (5). All OPs elicit their primary effects by phosphorylating or phosphonylating the serine hydroxyl at the active site of the enzyme AChE, causing accumulation of excess ACh at various cholinergic sites causing toxic manifestations (6). The inhibited enzyme can be reactivated by certain drugs (cholinesterase reactivators) such as hydroxylamine, hydroximic acids and oximes. (7). Among the oximes pralidoxime (PAM), obidoxime and trimedoxime (TMB-4) are well known cholinesterase reactivators (8,9). Recently H-oxime H16 and HLÖ7 are proved to be most effective oxime against toxic nerve agents (10,11). Only a few studies conducted recently have offered some information regarding the neurochemical mechanism of reactivation of the effect of OPs by oximes in brain regions and other tissues. The present study was undertaken to evaluate the ability of Diacetylmonoxime (DAM) to reactivate Rat AChE inhibited by DDVP in discrete regions of the brain, Serum, liver, kidney, testis and whole brain. Butyrylcholinesterase (BuChE; EC 3.1.1.8)
is present in the central nervous system in appreciable amount but appears to be associated mainly with supportive elements such as glia or vascular tissue, without direct implications in neurotransmission (12). Effect of DAM on DDVP-induced BuChE inhibition in serum and kidney was also studied.

2. Materials and Methods

2.1. Chemicals

Acetylthiocholine iodide (ATCI), Butyrylthiocholine iodide (BuTCI), 5,5’-Dithio-bis 2-nitrobenzoic acid (DTNB) and bovine serum albumin were purchased from Sigma chemicals CO. (St. Louis M.O.). Technical grade dichlorvos, (DDVP; 2,2-dichlorovinyl dimethyl phosphate) and Diacetylmonoxime (DAM) were obtained from All India Medical Corporation (Mumbai India). All other chemicals used were of analytical grade.

2.1.1 Animals & Treatment

Male albino Rats (CFT-Wistar strain) weighing between 225-250 g were used for these investigations. Rats were maintained in individual plastic cages in a temperature-controlled room under standard laboratory conditions with free access of water and commercial feed ad lib. Rats were divided into four groups of five each. Experimental rats were fasted overnight. Each group receiving different treatment(s): Group I: Control animals; oil (1 ml/kg). Group II: single dose DDVP (47mg/kg; 1/3 LD50), Group III: DAM (100mg/kg) as solution in isotonic saline i.m. Injection volume was 1 ml/kg. Group IV: DAM (100 mg/kg) i.m. pretreatment, 30 min. before, DDVP (47mg/kg) administration. Rats in groups III and IV received daily DAM (100mg/kg) for three days. Stock solution of DDVP (16mg/ml) was prepared in 100% pure peanut oil. Stock solution of DAM (100mg/ml) was prepared in normal saline. Stock solutions were made freshly just before the use.

2.1.1.1 Sample preparation & Enzyme assay

Rats (group of five) were anaesthetized by Diethyl ether 16 hr. after administration of DDVP and their abdominal cavities surgically opened. For serum sample blood was collected by cardiac puncture. Blood was allowed to clot for 5 min. followed by centrifugation at 1000 x g at 4°C for 10 minute. Brains were then quickly removed, washed in ice-cold saline and blotted dry. Each brain was placed on ice bag and different regions viz. cortex, striatum, cerebellum, pons, thalamus, hippocampus, medulla and spinal cord were isolated according to Zeman and Innes (13). Liver, kidney and testis were also taken out immediately. A 10 % (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) using potter-Elvehjem type homogenizer followed by centrifugation at 2000 x g at 4°C for 10 min. in a sorwell high speed refrigerated centrifuge. All homogenates were kept at −70°C immediately after preparation and analyzed on the same day for the activity of AChE and BuChE. AChE activity was determined spectrophotometrically by measuring the rate of hydrolysis of the substrate, ATCI/BuTCI according to the method of Ellman et al., (14). Aliquots of the homogenates were diluted with 2.6 ml 0.1 M phosphate buffer (pH 7.4) to a total volume of 3 ml, which contained 100 mM DTNB and 75 mM ATCI or BuTCI. The enzyme activity was calculated by extinction co-efficient (E412= 1.36 x 10^4 M^{-1} cm^{-1}) for the yellow anion 5-thio-2 nitrobenzoic acid. The AChE and BuChE activity was expressed as nmole of ATCI / BuTCI hydrolyzed per min per milligram of protein and for serum ATCI/BuTCI hydrolyzed/ml serum/min. All spectrophotometric measurements were done in duplicate. Proteins were determined according to Lowry et al. (15) using bovine serum albumin as standard.

2.1.1.1.1 Statistical analysis

LD50 values were calculated by probit regression analysis of death occurring within 24hr. after administration of DDVP at five different doses with six Rats per dose (16).Mean values and standard deviation were calculated for each test group on the basis of value obtained for individual tissue from Rats. Data were analyzed by one-way ANOVA to determine treatment effects. In all cases p>0.05 or p>0.01 was considered statistically significant.

3. Results

3.1. Control Enzyme Activity
Baseline enzyme level for AChE is shown in Table –1 and –2. Result shows differential AChE activity in discrete regions of the brain. Among the brain regions the highest AChE activity in striatum was 3.673±1.2 nmole/min/mg protein and the lowest in the hippocampus and thalamus was 1.857±6.2 and 1.880±0.9 nmole/min/mg proteins respectively. The discrete regions of control rat brain showed differences in the AChE activities in the order of striatum >medulla>pons>cortex>cerebellum>hippocampus>thalamus>spinal. In the other organs AChE level was in the order of serum>brain>kidney=testis>liver. The serum BuChE activity (12.92±4.9 nmole/min) was almost four times more than its AChE activity (3.792±4.3 nmole/min). Similarly in the kidney also the level of BuChE was much higher i.e. 4.741±7.2 nmole/min/mg protein in comparison to its AChE activity which was 1.591±2.7 n mole/min/mg protein.

3.1.1 In vivo inhibition of AChE and BuChE by DDVP

Single dose of DDVP (47mg/kg) elicit differential toxicity in the brain region. The in vivo inhibition of AChE in the discrete regions of rat brain ranged from 65.5% (in striatum; p<0.01) to 22.5% (in cerebellum; p <0.05) (Fig- 1). DDVP elicit significant percent inhibition in AChE activity of the brain regions in the order of striatum>hippocampus>medulla>pons>cortex>thalamus>cerebellum>spinal (65.6>55.3>52.4>45.6 >41.6>35>22.5>16.6). AChE inhibition was also significant (p<0.05) in other organs due to DDVP and was in the order of liver>kidney>whole brain>testis>serum (39.9>38>37>30>29.2; Fig-2). In the serum DDVP-induced BuChE inhibition (38.9 %) was more pronounced than was its AChE inhibition (29.2 %). However in the kidney AChE activity was more inhibited than was its BuChE activity.

3.1.1.1 In vivo effect of DAM on AChE

Administration of DAM alone (100 mg/kg) to rats for three days did not produce any significant inhibition of AChE in the brain regions and the other target organs hence efficacy of DAM on DDVP-induced AChE inhibition was calculated from control values (Fig-3 & 4).

3.1.1.1.1 Effect of pretreatment of DAM against DDVP-induced AChE and BuChE inhibition

The Data in Table-1 indicate that the pretreatment of DAM (100 mg/kg) for three days prior to the single dose of DDVP (47 mg/kg) to the male rats provided significant protection (p<0.01) against DDVP inhibited AChE in almost all regions of the brain. Results from the study revealed that DAM offered maximum protection with more than 90% (p<0.01) reactivation in pons and spinal. Although it provided more than 75% protection against DDVP inhibited AChE in the hippocampus, medulla and cortex. However, DAM produced 50% protection in the cerebellum and thalamus and 40% protection in the striatum against DDVP inhibited AChE (Fig- 3). DAM did not show any significant protection against AChE inhibition in the serum but provided highly significant (p<0.01) protection against inhibition of serum BuChE (Table-2). Highest protective action of DAM was found significantly (p<0.01) in reactivating AChE inhibition in the testis, which was reactivated to normal level of AChE. In case of liver AChE inhibition DAM also showed considerable protection but unable to produce any protection in the kidney (Table-2 & Fig-4).

4. Discussion

The present investigations demonstrate protective efficacy of Diacetylemonoxime against AChE inhibition produced by DDVP in discrete regions of the brain in addition to whole brain, liver, kidney, testis and serum. The regional difference in the level of AChE of brain areas of untreated rat was significant (p<0.05) in the present investigation. Highest AChE activity was found in the striatum and lowest in the hippocampus and thalamus. However, lowest level of AChE was reported in the cerebellum (17). Striatum is the area rich in cholinergic neurons and AChE activity was higher in this area (18). The differential AChE activity in the brain regions of untreated rats was, in the present study, in confirmation (5) except we found lower AChE activity in the thalamus than in the cortex and cerebellum. AChE activity was comparatively found to be lower in serum, kidney and testis. It has been reported that in Kidney, liver and serum AChE activity is low (19). BuChE activity was found to be the predominant than AChE activity in serum and kidney of untreated rats. This may account for higher specificity to the substrate BuTC compared to ATC (20). BuChE in serum samples of mammals including rat showed more preference to BuTC compared to ATC (21). Upon inhibition of AChE by organophosphorus in the nervous system, the neurotransmitter acetylcholine accumulates in synapses, causing excessive stimulation of cholinergic receptors on
postsynaptic cells, leading to cholinergic toxicity. It is known that feedback inhibition of acetylcholine release can occur through activation of muscarinic acetylcholine receptors located on presynaptic terminals (22, 23). Presynaptic muscarinic receptors diminish further Acetylcholine release and thereby may reduce the excessive stimulation of postsynaptic cholinergic receptors following AChE inhibition (24). Present study reveals that DDVP produce maximum toxicity in brain regions and whole brain. AChE inhibition was 65% in striatum, 55% in hippocampus, 52% in medulla, 45% in pons, 41% in cortex, 35% in thalamus, 22% in cerebellum and 16% in spinal. These findings were similar since soman as previously reported to produce maximum inhibition of AChE in the striatum of rat (25). Regional differences in AChE inhibition by organophosphorus depends on their adaptive responses to pesticide exposure could therefore influence sensitivity to some anticholinesterase (26). It has been stated that brain is the first organ affected by organophosphorus poisoning (27) and an early inhibition of AChE takes place in this organ (28). The spinal cord is often neglected portion of CNS. Yet we have shown that DDVP affects AChE activity to the same extent here as in the major brain regions (29). Excellent correlation between brain regional and spinal cord AChE activity was reported (30, 31). AChE inhibition in either in nervous tissues or muscles has fairly been accepted as a toxic effect because activity in these target tissues is involved in neurotransmission (32). But AChE inhibition by OPs on other tissues or organs (such as liver, kidney and testis) different from them has seldom been studied. Results of this investigation of DDVP poisoning in rat AChE activity was inhibited significantly (p<0.05) i.e. 40% in liver, 38% in kidney and 30% in testis. The differential inhibition of AChE activities in these tissues may be due to presence of isoenzyme with different affinity for the substrate and the inhibitor. Further pesticide itself may be present in different amount in different tissues producing differential inhibition or inhibitor may be metabolized at different rates (33). Regarding the differential inhibition of serum AChE and BuChE in DDVP treated rats BuChE appeared to be slightly more inhibited then was AChE. The inhibition of BuChE can serve as protective mechanism against DDVP toxicity by reducing the amount available for AChE inhibition (18).

The result of the present investigation demonstrates a role for the direct action of DAM in reactivating AChE inhibition after exposure of rats to single dose of DDVP. Pretreatment of DAM provided protection against DDVP-induced AChE inhibition in all of the brain areas examined; however each area was not affected to the same degree. DAM exhibited excellent protection against the toxic effect of DDVP (demonstrating the AChE reactivation) produced nearly equivalent levels of protection in hippocampus, pons and medulla. The amount of reactivation of phosphorylated brain enzyme that can be achieved by TMB-4 or toxogonin differ in different areas of brain, due to regional differences in the amount of functional (i.e. more accessible, extra cellular) enzyme and non-functional (i.e. less accessible, intracellular) enzyme (34). Kuca and co-workers (35) reported that there were significant differences in reactivation potency of all tested oximes. The oxime TO205 seems to be the most efficacious followed by TO046, HI-6, HS-6, K027, obidoxime, MMC and 2-PAM. In addition, the findings of their study revealed that the reactivation potency of the tested reactivators depends on many factors such as the number of pyridinium rings, the number of oxime groups and their position, as well as the length and the shape of Linkage Bridge between two pyridinium rings.

Variation in reactivation of Tabun induced AChE inhibition has been reported by three oximes HI-6, obidoxime, and K048 (36). Patocka (11) showed DAM was about 10 times more effective against sarine poisoning in rat than in mouse, guinea-pig, rabbit and monkey. It has been reported that MINA and DAM were effective in reducing AChE inhibition by organophosphorus pesticides (37). It is known that atropine sulphate is essential part of the treatment of OP poisoning (38). It is considered that HI-6 is more effective than 2-PAM in the therapy of OP toxicity, especially for agents that undergo rapid aging (39). The difference may be attributed, at least in part to higher reactivating potency of HI-6 (40, 41).

In conclusion, prophylactic administration of DAM potentially protected AChE activity in brain regions in addition to serum and other tissues from inhibition by DDVP. One possible explanation for this might be slowing of aging of DDVP-AChE complex. Alternately, DAM might block the binding of DDVP to the esteric site of AChE (12). DAM offers possibility of providing prophylactic benefit against DDVP toxicity. Thus DAM may prove useful therapy of DDVP intoxication.
5. Acknowledgement

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6. References


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Table 1: Effect of pretreatment of Diacetylmonoxime (DAM) on DDVP-induced acetylcholinesterase inhibition in the discrete regions of Rat brain.

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Control</th>
<th>DDVP (100 mg/kg)</th>
<th>DAM (100 mg/kg)</th>
<th>DAM+DDVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum</td>
<td>3.673 ± 1.2 (100)</td>
<td>1.261 ± 3.4** (34.4)</td>
<td>3.432 ± 6.4 (93.4)</td>
<td>2.213 ± 1.7* (60.3)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.857 ± 6.2 (100)</td>
<td>0.839 ± 9.4** (44.6)</td>
<td>1.723 ± 2.6 (93.2)</td>
<td>1.692 ± 7.1** (91.1)</td>
</tr>
<tr>
<td>Medulla</td>
<td>3.538 ± 0.37 (100)</td>
<td>1.685 ± 0.98* (47.6)</td>
<td>3.391 ± 1.3 (95.9)</td>
<td>3.22 ± 4.2** (91.1)</td>
</tr>
<tr>
<td>Pons</td>
<td>2.667 ± 1.1 (100)</td>
<td>1.453 ± 12.4** (54.5)</td>
<td>2.592 ± 2.8 (97.1)</td>
<td>2.745 ± 1.4** (102.9)</td>
</tr>
<tr>
<td>Cortex</td>
<td>2.212 ± 1.9 (100)</td>
<td>1.3 ± 4.3 (58.4)</td>
<td>2.114 ± 8.1 (95.5)</td>
<td>1.966 ± 3.4** (88.8)</td>
</tr>
<tr>
<td>Thalamus</td>
<td>1.88 ± 0.9 (100)</td>
<td>1.225 ± 3.6* (65)</td>
<td>1.886 ± 7.17 (99.1)</td>
<td>1.528 ± 6.9* (81.2)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>2.159 ± 0.15 (100)</td>
<td>1.675 ± 8.1* (77.5)</td>
<td>2.071 ± 9.2 (95.9)</td>
<td>1.973 ± 2.7** (89.8)</td>
</tr>
<tr>
<td>Spinal</td>
<td>1.774 ± 1.8 (100)</td>
<td>1.479 ± 4.4 (83.4)</td>
<td>1.716 ± 0.6 (96.7)</td>
<td>1.667 ± 13.1 (98.4)</td>
</tr>
</tbody>
</table>

Note: Data represent mean ± S.E. (n = 5 groups). Asterisk(s) indicates a significant difference * P<0.05 and ** < 0.01 between treated and control, the numerals in parentheses denote the % change from control.

Table 2: Effect of pretreatment of Diacetylmonoxime (DAM) on DDVP-induced AChE and BuChE inhibition in the various tissues of Rat.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Control</th>
<th>DDVP (47 mg/kg)</th>
<th>DAM (100 mg/kg)</th>
<th>DAM+DDVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum^a</td>
<td>3.792 ± 4.3 (100)</td>
<td>2.686 ± 11.2* (70.8)</td>
<td>3.697 ± 17.6 (97.4)</td>
<td>2.945 ± 9.6* (77.6)</td>
</tr>
<tr>
<td>Whole brain</td>
<td>2.164 ± 2.9 (100)</td>
<td>1.36 ± 3.1** (60)</td>
<td>2.084 ± 2.4 (96.3)</td>
<td>1.858 ± 1.5 (85.8)</td>
</tr>
<tr>
<td>Testis</td>
<td>1.695 ± 19 (100)</td>
<td>1.179 ± 8.2* (69.5)</td>
<td>1.973 ± 6.5 (116.4)</td>
<td>1.929 ± 9.2** (113.8)</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.591 ± 2.3 (100)</td>
<td>0.979 ± 6.2* (62)</td>
<td>1.546 ± 12 (96.9)</td>
<td>1.121 ± 14.5 (70)</td>
</tr>
<tr>
<td>Liver</td>
<td>1.17 ± 15 (100)</td>
<td>0.704 ± 9.8* (60)</td>
<td>1.191 ± 3.3 (101.7)</td>
<td>0.989 ± 17 (84.5)</td>
</tr>
<tr>
<td>Serum (BuChE)^b</td>
<td>12.92 ± 6.9 (100)</td>
<td>7.9 ± 14* (61.1)</td>
<td>12.72 ± 17.6 (98.4)</td>
<td>12.58 ± 21.3** (97.3)</td>
</tr>
<tr>
<td>Kidney (BuChE)^b</td>
<td>4.7 ± 12 (100)</td>
<td>3.4 ± 4.9* (73.4)</td>
<td>4.68 ± 8.3 (97.8)</td>
<td>3.588 ± 6.4 (76.6)</td>
</tr>
</tbody>
</table>

Note: * Data represents mean ± S.E. (n = 5 groups). Asterisk(s) indicates a significant difference * P<0.05 and ** < 0.01 between treated and control, the numerals in parentheses denote the % change from control. (a) Activity was expressed as nmole substrate hydrolyzed ml serum⁻¹ min⁻¹. (b) Butyrylcholinesterase activity expressed as nmole BuTCl hydrolyzed/min/mg protein.

Fig. 1: DDVP-induced AChE inhibition in brain regions of Rat. Results are expressed as percentage of inhibition (as compared with untreated rats) ± S.E. (St. striatum; Hi. hippocampus; Me. medulla; Po. Pons; Co. cortex; Th. Thalamus; Ce. Cerebellum; Sp. Spinal.). *Significance difference between treated and control groups * P<0.05 and ** P<0.01.
Fig. 2: DDVP-induced AChE inhibition in various tissues of Rat. Results are expressed as percentage of inhibition (as compared with untreated rats) ± S.E. (Wb. Whole brain; Liv. Liver; Kid. Kidney; Tes. testis; ser. Serum) *Significance difference between treated and control groups * P<0.05 and ** P<0.01.

Fig. 3: Effect of pretreatment of DAM (100mg/kg; im. For three days prior to administration of 47mg/kg DDVP) and DDVP on AChE activity (in Vivo) in discrete regions of rat brain.(St. striatum; Hi. hippocampus; Me. medulla; Po. Pons; Co. cortex; Th. Thalamus; Ce. Cerebellum; Sp. Spinal.). *Significance difference between treated and control groups * P<0.05 and ** P<0.01.

Fig. 4: Effect of pretreatment of DAM (100mg/kg; im. For three days prior to administration of 47mg/kg DDVP) and DDVP on AChE activity (in vivo) various tissues of Rat.(Wb. Whole brain; Liv. Liver; Kid. Kidney. Tes. testis; ser. Serum) *Significance difference between treated and control groups * P<0.05 and ** P<0.01.