NON-COMPETITIVE INHIBITION OF SERUM ACETYLCHOLINESTERASE (E.C.3.1.1.7) BY VACOR (N-3-PYRIDYL METHYL-N'-P-NITROPHENYL UREA) IN BANDICOOT RATS

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Summary: Single dose (1 *mg*/rat) administration of VACOR (N-3-pyridyl methyl-N'-p-nitrophenyl urea) produced significant paralytic symptoms in rats. Such rats which lived for one week were sacrificed and serum AChE was extracted and purified by Sephadex column chromatography. The serum enzyme from the normal rats showed the existence of two isozymes on polyacrylamide gels which could be resolved through column chromatography. Both the isozymes showed non-competitive inhibition with VACOR *in vitro;* but to a varied degree. The ACh concentration increased by 70-75% in serum and 95-100% in erythrocytes of the poisoned rats. VACOR thus affects ACh hydrolysis thereby causing an accumulation of ACh in the serum of poisoned rats.

Key words: single dose rodenticide, VACOR (N'-3-pyridylmethyl-N'-p-nitrophenyl urea), non-competitive inhibition, serum acetylcholinesterase, AChE isozymes, *Bandicota bengalensis*

INTRODUCTION

N-3-pyridyl methyl-N'-p-nitrophenyl urea (trade name VACOR), is a selective single dose (acute) rodenticide (14). Of late, it is gaining popularity in India to control the common commensal rodents. The toxicological studies of this rodenticide have been restricted to its efficacies and dosage requirements (3,13,14). Little is known about its effect in sub-lethal doses on the physiology of rats. Such studies would help unravelling the basic mechanism of action of this rodenticide in a wholistic or general physiological point of view. In the present work, the effect of sub-lethal dose of VACOR on AChE activity, and *in vitro* inhibition of AChE by VACOR are described.

MATERIALS AND METHODS

The lesser bandicoot rats *Bandicota bengalensis bengalensis* Gray were captured by burrow excavations in paddy fields around Bangalore, and stocked in metal cages (30x30x 30 cm). Prior to experimentation, the rats were adjusted to laboratory conditions for a week. During this period, they were maintained on rat feed (Hindustan Lever Ltd.,

India) with adequate supply of water. After conditioning, a single sublethal dose of VACOR (1 *mg*/individual) was mixed in 1*g* bait (Rice flour + Ground nut oil + Ground nut kernel + Garlic; 79 : 20 : 0.5 : 0.5 by weight) and administered to the rats to ensure that all the poison mixed was ingested. This bait formulation and dose requirement were fixed, based on the LD_{50} studies (15). Subsequently the rats were allowed to feed *ad lib* on regular ration for one week before they were sacrificed. AChE (E.C.3.1.1.7) activity and ACh content were assayed in serum and erythrocytes colorimetrically (9). Protein was estimated according to Lowry *et al.* (11). Serum AChE was purified through ammonium sulfate fractionation according to Habibulla *et al.* (8). The purified enzyme was subjected to polyacrylamide gel electrophoresis (4). To resolve isozymes, the gels were stained with N-methyl indoxyl acetate (7). For the assay of isozyme activity, the purified enzyme was further fractionated by Sephadex column chromatography. The partially purified enzyme containing about 5 *mg* protein was applied on a Sephadex G 200 column (260 x 8 cm). 5 *ml* fractions of the enzyme were eluted with 0.01M, pH 7.6 phosphate buffer. The protein of the eluates was estimated colorimetrically (11). Two proteins were eluted one at 55 to 60 *ml* and the other at 95 to 100 *ml*. These two hydrolysed the substrate, showing correspondence to two isozymes observed on the polyacrylamide gels. The rates of enzymic activity of these isozymic eluates, were studied (9) *in vitro*, with different concentrations of substrate and VACOR (0.5 *mg* in 0.1 *ml* phosphate buffer). With these results the Michaelis-Menten kinetics of the enzyme were discussed.

RESULTS AND DISCUSSION

Fig. 1 illustrates the fractionation of two isozymes of AChE in serum of normal and poison administered rats. Two enzyme proteins were detected on acrylamide zymograms. They showed relative mobilities of 0.7, and 0.8 respectively.

A difference in the extent of purification of the enzyme was noticed in poisoned rats. During the first stage of purification (ammonium sulfate fractionation), there was 121-fold purification of enzyme in poisoned animals and a 57-fold in normal animals. In the second stage of purification (Sephadex column), the enzyme of poisoned animals was further purified by 152 fold with reference to isozyme I and 2689 fold with reference to isozyme II (Fig.1). In the normal animals, the isozyme I was purified by 36-fold and the isozyme II was purified by 641-fold.

There was a significant decrease in the AChE activity of serum and erythrocytes due to poisoning. On poisoning there was 78% inhibition of AChE activity of serum in the crude sample, 55% inhibition after the first stage of purification (ammonium sulfate fractionation), and 10% after the second stage of purification (Sephadex column) (Table I). On the other hand, ACh content of serum and erythrocytes increased significantly on poison-

ing (Table II). Velocity maximum and Michaelis-Menten constants of the enzyme of normal serum, poisoned serum, VACOR added normal and poisoned serum (*in vitro*) are shown in Table III. All enzymes showed substrate inhibition (Above 4 mM concentration of ACh).

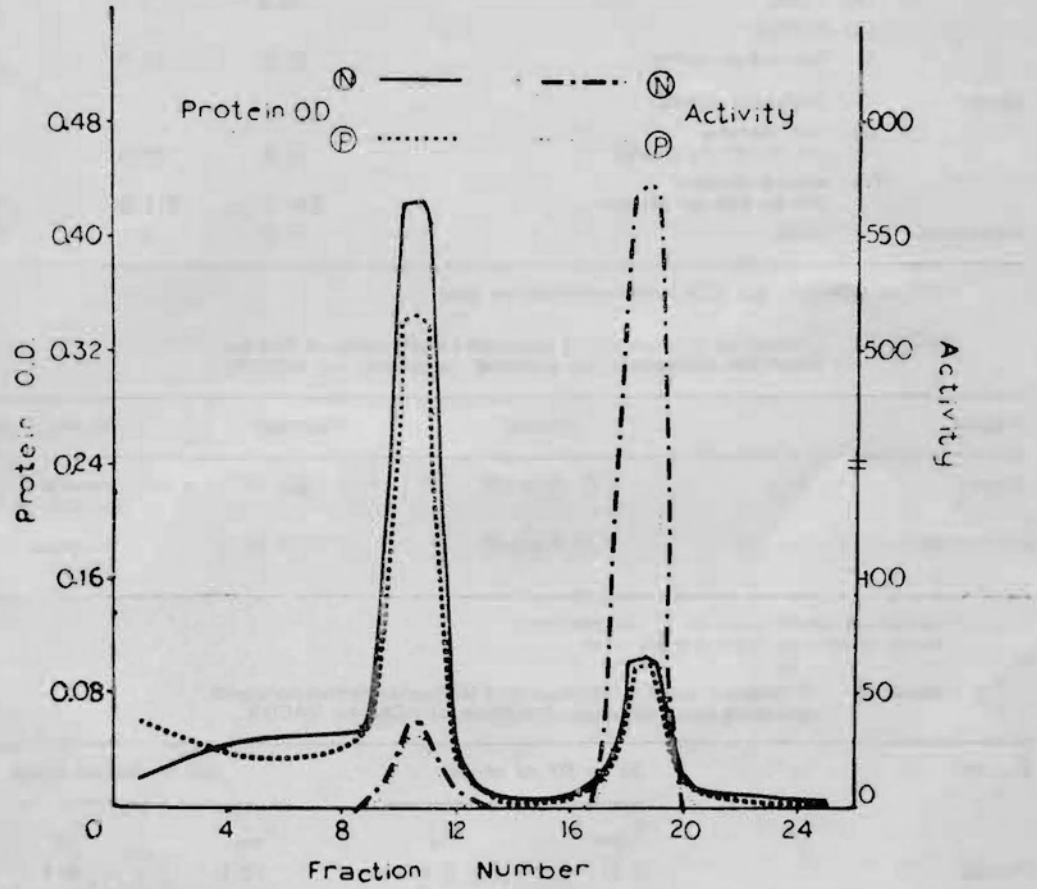

Fig.1: Sephadex fractionation of serum AChE. N represents serum enzyme of normal rats and P that of poison administered rats. Enzymic activity is represented in terms of μg Ach hydrolysed/*min*/*mg* protein.

An investigation of K_m and V_{max} of the enzyme showed that both parameters were affected by the poison. Both K_m and V_{max} values of the isozymes of AChE decreased on poisoning (Table III). In the presence of VACOR both the isozymes showed considerable reduction in V_{max} and K_m values. Decrease in K_m value on poisoning indicates an increase in the affinity of the enzyme towards the substrate, since high K_m values represent low affinity (5,12).

TABLE I : Inhibition of AChE activity in serum and erythrocyte of field rat *Bandicota bengalensis* at different stages of purification after sub-lethal administration of VACOR.

Sample	*Fraction*	*Normal**	*Poisoned**	*Percent inhibition*
	(A) Crude	0.9	0.2	78
	(B) Purified			
	1. Ammonium sulfate	51.0	23.0	55
Serum	2. Sephadex column			
	(i) First isozyme (55 to 60 *ml* eluate)	32.0	29.0	10
	(ii) second isozyme (95 to 100 *ml* eluate)	571.0	511.0	10
Erythrocyte	Crude	1.0	0.1	90

*Values represent *µg* ACh hydrolysed/*min*/*mg* prot.

TABLE II : Changes in ACh content of serum and erythrocytes of field rat *Bandicota bengalensis* on sublethal poisoning of VACOR.

Sample	*Normal**	*Poisoned**	*Incidence of change*
Serum	1.2±0 07	2.1±0.18	Increase $P<0.01$
Erythrocytes	0.6±0.03	1.2±0.04	Increase $P<0.01$

*Values are Mean±S.E. of 12 observations.
Mean values represent mg ACh/*ml*.

TABLE III : Changes in velocity maximum and Michaelis-Menten constants indicating non-competitive inhibition of AChE by VACOR.

Enzyme	*55 to 60 ml eluate*		*95 to 100 ml eluate*	
	V_{max}	K_m	V_{max}	K_m
Normal	0.6	8.0	10.5	9.1
Normal + Vacor	0 3	5.0	6.9	7.4
Poisoned	0.4	7.0	4.5	3.0
Poisoned + Vacor	0.2	3 8	3.1	2.6

V_{max} in mM ACh hydrolysed/*mg* prot/min.
K_m in mM Ach.
V_{max} and K_m calculated from Lineweaver-Burk plots (6).
*All enzymes showed substrate (above 4 mM) inhibition.

It appears that there is a non-competitive inhibition of the enzyme by VACOR as the velocity maximum and Michaelis-Menten constants are different.

The cause of death on VACOR poisoning is only known to be due to the blockade of niacinamide metabolism and pulmonary arrest (13). The pulmonary arrest may be due to an increase in ACh content (present results) which leads to stiffening of respiratory muscles. Such paresis of the respiratory muscles may be largely responsible for dyspnea and inadequate breathing. Depression of brain AChE in vertebrates is known to cause physiological and behavioural manifestations that reduce the animal survival ability (1,6). Inhibition of AChE in the brain of rats and mice on pesticide poisoning is known (2). Involvement of ACh-AChE system in functions other than synaptic transmission is not fully understood. The membrane-bound AChE plays an important role in health and disease (10). Kutty *et al.* have discussed at length the possible role of AChE in the maintenance of stability, structure and function of cellular membranes (10).

It is clear from these results that besides inhibiting the nicotinamide metabolism, VACOR inhibits the AChE isozyme activities to a varied degree non-competitively. it could be envisaged that the impaired AChE activity in poisoned rats may contribute more towards the functional unstability of excited membranes, which cumulatively may culminate in the cessation of mobility and life. Increased ACh contents corroborates these views further as the disturbed enzymic activity slowens the rate of its hydrolysis.

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