MAXIMAL INHIBITION OF CHOLINESTERASE IN THE CENTRAL NERVOUS SYSTEM

BY

W. SCHAUMANN

From the Pharmakologisches Institut der Universität Frankfurt/M., Germany

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In experiments on mice treated with pralidoxime iodide (pyridine-2-aldoxime methiodide; PAM) and atropine, the cholinesterase activity in the brain was assayed after poisoning with very high doses of organophosphorous anticholinesterases. Acetylcholine was added to the buffer solution in which the brains were homogenized. This precaution reduced the combination between free inhibitor present in the tissue and active enzyme, and the cholinesterase activity found was below 0.5% of controls. When the experimental data were corrected for spontaneous reactivation *in vitro* during incubation, the calculated activities *in vivo* were even less. It is concluded that mice can survive complete inactivation of the cholinesterase in the central nervous system, if enough atropine is given to protect the animals against the toxic effects of the accumulating acetylcholine.

Death after poisoning with anticholinesterases which penetrate into the central nervous system is due to both central and peripheral inhibition of cholinesterase. Pralidoxime iodide (pyridine-2-aldoxime methiodide; PAM) effectively protects the peripheral cholinesterase or reactivates it after inhibition by organophosphates (Kewitz and Nachmansohn, 1957), whereas atropine protects against the actions of the accumulating acetylcholine in the central nervous system. In combination with pralidoxime iodide, atropine increases the LD₅₀ about 10 times (Askew, 1957; Hobbiger, 1957; Kewitz, Wilson, and Nachmansohn, 1956; Wills, Kunkel, Brown, and Groblewski, 1957). The question arises whether the brains of mice surviving excessive doses of organophosphates under these conditions contain any active cholinesterase.

Several experimental difficulties must be overcome in order to answer this question:

1. After high doses of organophosphates, the brain contains large amounts of free inhibitor which must be prevented from blocking the remains of active enzyme during and after homogenization. This was done by adding acetylcholine to the buffer before homogenization (Schaumann, 1960a).

2. After poisoning with diethyl phosphates, there is spontaneous reactivation *in vitro* and the experimental values must be corrected accordingly (Schaumann, 1960a).

3. Labile organophosphates like paraoxon are destroyed enzymically in the body so quickly that

reactivation of inhibited cholinesterase *in vivo* very soon prevails over inactivation by free inhibitor (Schaumann and Schiller, 1960a). Therefore, only anticholinesterases with a long half-life were used in the present experiments.

METHODS

Male white mice received 50 mg./kg. pralidoxime iodide + 10 mg./kg. atropine intraperitoneally and at the same time one of the following anticholinesterases subcutaneously: diethyl-dimethylaminothioethyl-phosphate acid oxalate (217-AO), parathion (OO - diethyl - O - p - nitrophenyl - phosphorothionate; E 605), or dyflos. The animals were decapitated 1 or 2 hr. after injection. The brains were dissected at once, weighed and homogenized with 1.9 ml. buffer solution per 100 mg. of brain. The buffer contained 7.5 to 10 mm. acetylcholine, 0.03 M magnesium chloride, and 0.03 M sodium bicarbonate. Two ml. of the homogenate was pipetted into Warburg vessels and at once incubated at 37°. The manometers were gassed with 95% nitrogen + 5% carbon dioxide, and the production of carbon dioxide was measured from 30 to 90 and from 90 to 150 min. after inserting the manometers. The concentration of acetylcholine in the homogenates was determined colorimetrically according to the method of Hestrin (1949) at the beginning of the incubation and after 3 hr. The carbon dioxide production from brain homogenates to which an excessive amount of an anticholinesterase had been added was subtracted as a blank from the manometric values. The colorimetric readings were corrected for the spontaneous hydrolysis of acetylcholine during incubation. Cholinesterase activity

was calculated from the production of carbon dioxide (manometric determination) or the consumption of acetylcholine (colorimetric determination). The homogenate of a normal mouse brain diluted 1:20 served as a control. All determinations were done in duplicate.

RESULTS

Determination of Cholinesterase Activity in vitro

The manometric determination of cholinesterase activity during two successive hours shows a measurable increase in activity after poisoning with 217-AO and parathion (Table Ia). The rate of reactivation is practically linear during this time, and the readings taken between 30 and 90 min. after the beginning of the incubation therefore represent the activity after 60 min. (a_{60}); those of the second period the activity after 120 min. (a_{120}).

The reactivation follows the equation:

$$\ln \frac{100 - a_0}{100 - a_t} = K_r \cdot t \dots (1)$$

a_0 , a_t = % activity after 0 and t min. of incubation; t = duration of incubation in min., K_r = first order rate constant for the reactivation.

When the average activities for a_{60} and a_{120} from Table I are inserted together with $t=60$ in equation (1), K_r can be calculated. K_r was lower after 60 mg./kg. of parathion—i.e., the rate of reactivation was slower than after 217-AO. Apparently, the high concentrations of free inhibitor found after parathion (Schaumann and Schiller, 1960b) reduced the speed of reactivation in spite of the addition of acetylcholine.

The cholinesterase inhibited by dyflos is not reactivated (Hobbiger, 1957; Davison, 1953). It was, therefore, sufficient to determine the enzyme activity once. The activities found are valid for the whole incubation period (Table II).

The colorimetric determinations give an average of the cholinesterase activity during the whole incubation period. The rate of reactivation being practically linear, the figures can be compared with the activities measured manometrically at the middle of the incubation period, i.e., after 90 min. (a_{90}). It follows from equation (1) that a_{90} is the geometric mean between $100 - a_{60}$ and $100 - a_{120}$. As shown in Tables I and II, there is a good agreement between the results obtained with both methods. In spite of low activities, the manometric determination gave just as dependable results as the colorimetric determination under the present experimental conditions.

Calculation of Cholinesterase Activity in vivo

No more than 3 min. elapsed between killing the animals and inserting the manometers. Therefore, the activity at the beginning of the

incubation period, a_0 , represents the cholinesterase activity *in vivo*. For 217-AO and parathion, a_0 can be calculated by inserting into equation (1) $t=90$, a_{90} from Table I for a_t and the respective values for K_r . No correction is necessary with dyflos. The results are summarized in Table III. When the mice were killed 1 hr. after the injection of 10 mg./kg. of 217-AO, a cholinesterase activity of 3.5–3.9% was found after 90 min. of incubation. From these values an activity *in vivo* of 0.04 to 0.3% of normal can be

TABLE I
MEASURED CHOLINESTERASE ACTIVITY OF BRAIN

The mice were killed (a) 1 hr. after 10 mg./kg. 217-AO subcutaneously, and (b) 2 hr. after 60 mg./kg. parathion subcutaneously; in both substances 50 mg./kg. pralidoxine iodide + 10 mg./kg. atropine was also given intraperitoneally. All values are % of control activity.

(a)

Exp. No.	Cholinesterase Activity			
	Manometrically			Colorimetrically
	a_{60}	a_{120}	a_{90}	a_{90}
3	0.9	2.6	1.75	2.10
	2.6	5.3	3.95	3.60
	2.6	5.4	4.00	3.60
	2.2	4.5	3.35	3.30
4	3.1	5.4	4.25	3.70
	4.2	6.1	5.15	4.20
	3.2	5.8	4.50	4.00
	2.8	5.0	3.90	3.50
Mean:	2.7	5.0	3.86 ± 0.35	3.50 ± 0.22
	$K_r = 0.0004$			

(b)

5	1.6	3.4	2.50	2.6
	1.7	3.3	2.50	2.8
	2.1	4.0	3.05	3.1
	2.2	4.4	3.30	3.4
6	1.5	3.1	2.30	2.1
	2.5	4.2	3.35	2.9
	2.0	3.2	2.60	2.3
Mean:	1.94	3.67	2.80 ± 0.16	2.74 ± 0.22
	$K_r = 0.000296$			

TABLE II

MEASURED CHOLINESTERASE ACTIVITY OF BRAIN

The mice were killed 1 hr. after injection of 80 mg./kg. dyflos subcutaneously and 50 mg./kg. pralidoxime iodide + 10 mg./kg. atropine intraperitoneally. All values are % of control activity.

Exp. No.	Cholinesterase Activity	
	Manometrically	Colorimetrically
1	0.45	0.45
	0.40	0.50
	0.20	0.45
	0.45	0.55
2	0.75	0.85
	0.70	0.95
	0.53	0.55
	0.55	0.50
Mean:	0.50 ±0.06	0.60 ±0.07

TABLE III

CALCULATED CHOLINESTERASE ACTIVITY OF BRAIN *IN VIVO*

mmt.=manometric; col.=colorimetric determination. All values are % of control activity.

	Cholinesterase Activity after Subcutaneous Injection of:		
	217-AO 10 mg./kg.	Parathion 60 mg./kg.	Dyflos 80 mg./kg.
<i>In vitro</i> (a_{90})			
mmt. ..	3.86	2.80	0.50
col. ..	3.50	2.74	0.60
<i>In vivo</i> (a_0)			
mmt. ..	0.33	0.17	0.50
col. ..	0.04	0.11	0.60

calculated. Two hr. after 60 mg./kg. of parathion the figures were even lower and from the activities found after 90 min. incubation and the rate constant of reactivation given in Table Ib, a cholinesterase activity *in vivo* of 0.1 to 0.2% of normal can be calculated. After injection of 40 mg./kg. of dyflos, Kewitz and Nachmansohn (1957) found 1.4% cholinesterase activity in the brain. In the present experiments, 80 mg./kg. was tolerated, and the activity was reduced to 0.5 to 0.6% of normal.

DISCUSSION

Using a histochemical method, McIsaac and Koelle (1959) found that physiologically liberated acetylcholine is hydrolysed only by the so-called functional cholinesterase on the cell surface and not by the reserve cholinesterase within the cells. Pharmacologically equipotent doses of two organophosphates inhibited the functional enzyme to the same degree, but only one of them penetrated into the cells; the other one left the reserve cholinesterase practically intact. In consequence, the tissue homogenates showed a considerably higher cholinesterase activity after the latter compound.

The cholinesterase activity in the brains of mice poisoned with equitoxic doses of different organophosphates decreases inversely with the stability of the respective compound in the body. In other words, the longer the half-life of a substance in the brain, the lower are the cholinesterase values found after a lethal dose (Schaumann, 1960b). This is confirmed by the present results (Table III). The free inhibitor found in mouse brain after injection of dyflos has a half-life of about 15 min. after 217-AO of about 40 min., whereas after parathion there is no measurable decrease in free inhibitor during 10 hr. (Schaumann and Schiller, 1960b). The mice were still alive 2 hr. after the injection of 60 mg./kg. of parathion, but they died 1 to 3 hr. later, when the therapeutic effects of pralidoxime iodide and atropine had worn off. A stable inhibitor will have more time to penetrate into the cell than one which is quickly metabolized. It is therefore most likely that the activities remaining after dyflos and 217-AO are due to inner or reserve cholinesterase.

Even if one assumes that the minute enzyme activities *in vitro* were due to external, functional cholinesterase, they do not constitute the absolute minimum necessary for the maintenance of life. In spite of pralidoxime iodide treatment, the animals showed severe muscular fasciculations and fibrillations, symptoms characteristic for peripheral inactivation of cholinesterase. It has previously been shown that in mice pretreated with pralidoxime iodide and atropine, the injection of a lethal dose of 217-AO reduced the cholinesterase activity in the diaphragm to the critical value for a lethal neuromuscular block in spite of the reactivator. When artificial respiration was given in addition to pralidoxime iodide and atropine, practically unlimited amounts of paraoxon and 217-AO could be given to rats without affecting the respiratory centre (Schaumann, 1959; 1960b).

When the lethal dose of an organophosphate was injected after pretreatment with pralidoxime iodide, death was due to the central inhibition of cholinesterase alone. Under these conditions, the enzyme activity found in the brains of mice was 1 to 5.5% of the control level, depending on the compound used (Schaumann, 1960b). This is the minimum amount of cholinesterase necessary to prevent the accumulation of toxic concentrations of acetylcholine in the central nervous system. However, if enough atropine is given to prevent the toxic effects of acetylcholine, the cholinesterase in the brains of rats and mice can apparently be inactivated completely without causing death.

It should perhaps be pointed out that this statement is not inconsistent with the assumption of cholinergic transmission within the central nervous system. Only the acetylcholine receptor, and not the cholinesterase, is primarily concerned in the process of transmission. The liberated acetylcholine which accumulates after the inhibition of

the cholinesterase can be removed by diffusion (Ogston, 1955) and can be hydrolysed in the periphery which, due to the protective action of pralidoxime iodide, contains active enzyme.

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