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INHIBITION OF ACETYLCHOLINESTERASE IN DIFFERENT PARTS OF THE RAT BRAIN BY ISOPROPYL METHYLPHOSPHONOFLUORIDATE; *IN VITRO* AND *IN VIVO* EXPERIMENTS

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Abstract—Inhibition of AChE in the cerebellum, pons, frontal cortex and basal ganglia of the rat brain by isopropyl methylphosphonofluoridate *in vitro* was studied and constants characterizing this inhibition (I_{50} and bimolecular rate constant, k_a) were determined. They were practically identical for all parts of the brain studied. In experiments *in vivo*, the dependence of AChE inhibition on the dose of organophosphate, and the time course of AChE inhibition following dose of 0·25 mg/kg i.m. were studied. The dose required for 50 per cent inhibition of AChE (I_{50} dose) was the highest for the basal ganglia and the lowest for the frontal cortex and the cerebellum. The rate of AChE inhibition *in vivo* was most rapid in the frontal cortex and slowest in the basal ganglia. A correlation between AChE activity in different parts of the brain and I_{50} dose of these parts was demonstrated. The differences in inhibition produced in the various areas of the brain are probably caused by different AChE concentration in these structures.

ISOPROPYL methylphosphonofluoridate (IMPF; sarin) belongs to the group of organophosphates, which are potent inhibitors of acetylcholinesterase (AChE, EC 3.1.1.7) and other hydrolytic enzymes. The toxic action of these compounds is due to the inhibition of AChE in different parts of the organism. Many factors will be important for the toxic action of IMPF: for example, its detoxication, the duration of AChE inhibition, its binding to other proteins, its penetration into vital centres, and the affinity of the AChE of these centres for it.

The distribution of IMPF *in vivo* and the influence of some drugs on it have been studied by radioisotopic techniques.[1–3] In these experiments it is difficult to differentiate the toxic compound from its metabolic products. It is known from experiments *in vitro* that the inhibition of AChE by IMPF is greater than that of other cholinesterases,[4–9] and inhibition of all of these enzymes is rapid and irreversible. Therefore, it may be possible to estimate from the AChE inhibition obtained *in vivo* after IMPF administration the concentration of the organophosphate in some organs.[10] However, it is not known whether the rate of reaction of IMPF with AChE from different parts of the brain is similar or different. The bimolecular rate constants for inhibition of AChE from *Electrophorus electricus* and human erythrocytes range from 1 to 6×10^7 $M^{-1} \cdot min^{-1}$.[5,6] In this paper the *in vitro* and *in vivo* rates of reaction of IMPF with AChE from four different parts of the rat brain are compared.

MATERIALS AND METHODS

Inhibition of AChE in vitro

Female Wistar rats (Mezno), weighing 150–200 g, were killed by bleeding from a carotid artery and the brains were removed and frozen at −35°. Cerebellum, pons, frontal cortex and basal ganglia were isolated and homogenized in 10–30 parts (according to the level of AChE activity) of 0·2 M tris–HCl buffer, pH 7·6 using an Ultra-Turrax (Janke & Kunkel, Germany). The homogenates were incubated with various concentrations of IMPF for different periods and the AChE activity was measured. Normal AChE activity was considered as 100 per cent and AChE activity after treatment with IMPF was expressed either as percentage of normal activity or as per cent inhibition.

Inhibition of AChE in vivo

Experiment A. Female Wistar rats (Mezno), weighing 150–200 g, were randomly divided into five groups of six animals each. The control group was injected i.m. with saline, the experimental groups were injected i.m. with IMPF, in doses of 0·05, 0·10, 0·14 or 0·25 mg/kg, i.e. 0·355, 0·71, 1·0 or 1·78 μmoles/kg respectively. The animals were killed by bleeding from a carotid artery 9 min after the injection and homogenates were prepared as described.

Experiment B. Female Wistar rats (Mezno), weighing 150–200 g, were randomly divided into five groups of six animals each. The control group was injected i.m. with saline and the other animals were injected i.m. with 0·25 mg IMPF/kg. The animals were killed by bleeding from a carotid artery at various times after the injection and homogenates were prepared as described.

Determination of AChE

AChE activity was measured colorimetrically by a modification of the method of Ellman *et al.*,[11,12] using acetylthiocholine iodide (Lachema, Brno, Czechoslovakia) as substrate (1 mM) and 5,5′-dithiobis-2-nitrobenzoic acid (Serva, Heidelberg, Germany) as chromogen. Measurements were carried out on Vitatron scanner (Sci. Instr., Holland) at 412 nm.

Statistical evaluation

The rate constants from *in vitro* measurements were calculated using a MINSK 22 computer.[13,14] The homogeneity of experimental groups for experiments *in vivo* was tested by Bartletts' test and the significance of differences between the groups were tested by regression analysis.[15]

RESULTS AND DISCUSSION

Inhibition of AChE in vitro

The inhibition of AChE by IMPF is very rapid and shows first order characteristics (Fig. 1). From these results the rate constants characterizing inhibition by IMPF of AChE from different brain parts were calculated using the following equation.[16,17]

$$k_a = \frac{2{\cdot}303}{t \cdot [I]} \times \log \frac{v_0}{v_0 - v_i}$$

where k_a is the bimolecular rate constant ($M^{-1} \cdot min^{-1}$), v_0 the velocity of the uninhibited reaction, v_i the velocity of the inhibited reaction, t the duration of exposure of enzyme to inhibitor in the absence of substrate in minutes and $[I]$ the molar concentration of inhibitor. Values of the I_{50}, the molar concentration necessary to produce 50 per cent inhibition after incubation for 9 min, were calculated from the following equation

$$I_{50} = \frac{0 \cdot 69}{k_a \cdot t}.$$

The inhibition constants for the various parts of the rat brain are summarized in Table 1. The differences between the values of the constants for AChE of different parts of the brain are negligible, as shown by the confidence limits.

Inhibition of AChE in vivo

Dependence of AChE inhibition on the dose of IMPF—experiment A. The decrease in AChE activity produced by IMPF in 9 min was dependent on the dose of IMPF. A plot of log % AChE activity vs. dose of IMPF gave a straight line for the AChE of the pons only (Fig. 2). A plot of % AChE inhibition vs. dose of IMPF, using probit-log transformation, gave straight lines for inhibition of the AChE in all parts of the brain studied. From these data, I_{50} doses were calculated, i.e. doses which caused 50 per cent AChE inhibition 9 min after the injection of IMPF. The dose required for 50 per cent inhibition of AChE was the highest for the basal ganglia and lowest for the frontal cortex and the cerebellum as shown in Table 1. The results do not allow to decide about the importance of inhibition of AChE in a particular area of the brain in the mechanism of death but it has been demonstrated that in mice

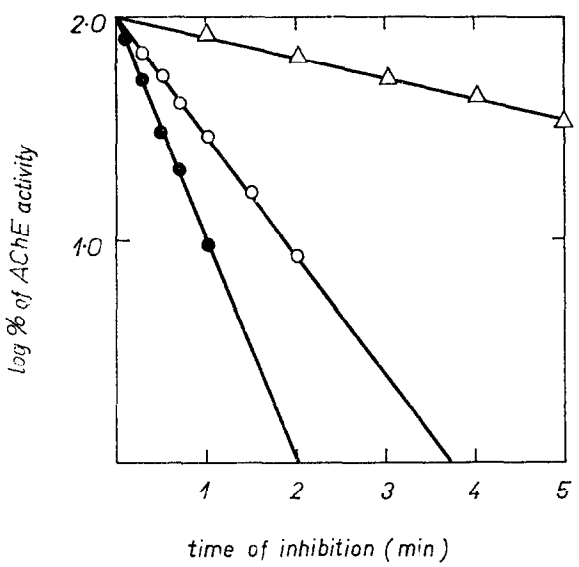

FIG. 1. The time course of inhibition of AChE of basal ganglia homogenates by IMPF *in vitro*. Concentrations of IMPF: △ 1×10^{-8}M, ○ 5×10^{-8}M, ● 1×10^{-7}M.

TABLE 1. CONSTANTS CHARACTERIZING AChE INHIBITION OF DIFFERENT PARTS OF THE RAT BRAIN BY IMPF *in vitro* AND *in vivo*

	In vitro			*In vivo*	
Part of the brain	AChE activity (μmoles/min/g wet wt.)	$10^{-7} \times k_a$ ($M^{-1}min^{-1}$)	$10^{+9} \times I_{50}$ (M) for 9 min inhibition	*$t_{0.5}$ (min)	$10^{+7} \times I_{50}$ dose (moles/kg)
Cerebellum	7·5 ± 1·41	2·12 (1·54–2·39)	3·62 (3·21–4·98)	1·2 (0·8–1·6)	2·3 (1·75–3·0)
Pons	15·5 ± 3·54	2·05 (1·54–2·42)	3·74 (3·17–4·98)	1·08 (0·8–1·4)	5·6 (4·1–7·7) †5·1 (4·8–5·5)
Frontal cortex	6·0 ± 2·12	2·16 (1·47–2·86)	3·55 (2·68–5·22)	0·86 (0·6–1·2)	2·3 (1·7–3·1)
Basal ganglia	42·5 ± 7·78	2·35 (1·82–3·77)	3·26 (2·03–4·21)	1·91 (1·5–2·4)	13·0 (8·0–21·0)

The results are means ± S.E.M. (AChE activity) or means with their 95 per cent confidence limits.
* Time for 50 per cent inhibition after 0·25 mg IMPF/kg i.m.
† I_{50} dose was obtained for a plot of log % AChE activity vs. dose of IMPF (cf. Fig. 2).

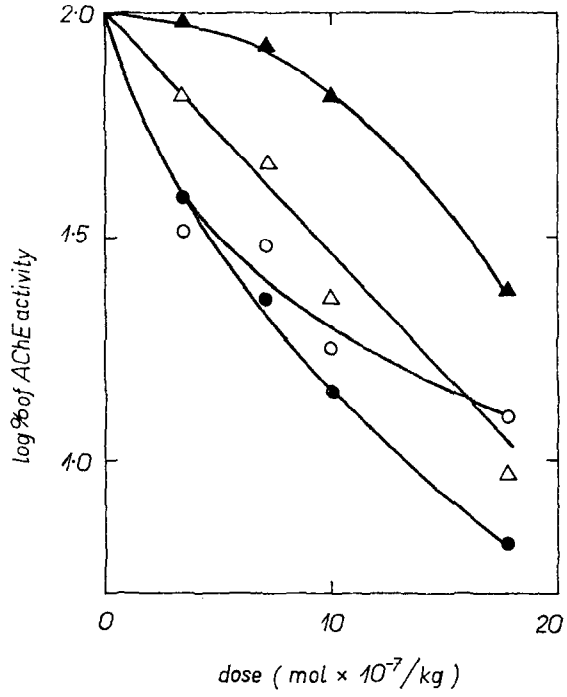

FIG. 2. Activity of AChE in four parts of the rat brain 9 min after the injection of different doses of IMPF. ○ cerebellum, △ pons, ● frontal cortex, ▲ basal ganglia.

AChE inhibition in the pons and medulla oblongata by IMPF *in vivo* was correlated with mortality whereas for other parts of the brain (mesencephalon, diencephalon and basal ganglia) this correlation was not used.[18]

The time course of AChE inhibition—experiment B. AChE activity after injection of IMPF decreased with time. The lowest activity was obtained in the frontal cortex. In the basal ganglia, AChE activity at the time of death was approximately 20 per cent, which corresponds with results obtained in experiment A. Complete inhibition of AChE was not obtained in any part of the brain. The rate of decrease in AChE activity shows that the reaction was not following first order kinetics (Fig. 3).

From the decrease in AChE activity in the areas of brain studied after injection of IMPF the half-time ($t_{0.5}$) for AChE inhibition *in vivo* was calculated. The $t_{0.5}$ values were obtained by computer analysis and are also summarized in Table 1. It appears from these results that the inhibition of AChE was most rapid in the frontal cortex and slowest in the basal ganglia.

Comparison of AChE inhibition in vitro *and* in vivo

For the inhibition of AChE *in vivo* (I_{50} dose) an approximately 100–600 times higher dose of IMPF was necessary than for inhibition *in vitro* (I_{50}). Affinity of IMPF for AChE in different parts of the brain *in vitro* was the same. The high doses required *in vivo* might be explained by differences in the rate of penetration of IMPF to different sites. The abnormal kinetics may be caused by removal of the inhibitor. For this the following possibilities exist:

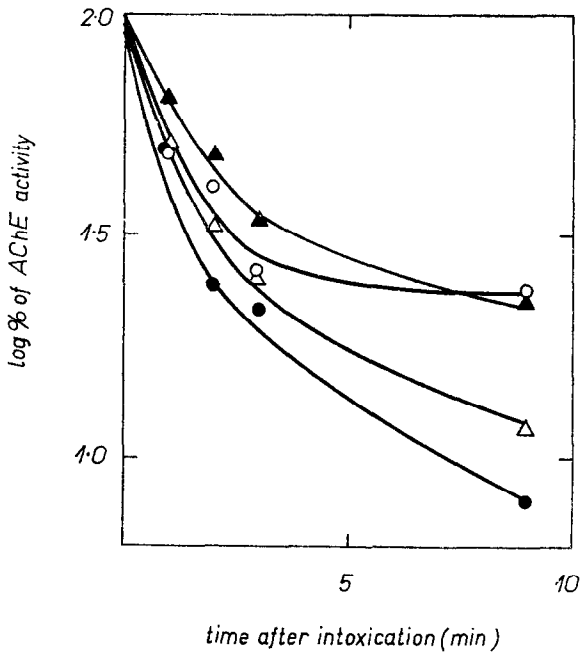

FIG. 3. Inhibition of AChE in four parts of the rat brain *in vivo* after injection of 0·25 mg/kg of IMPF. ○ cerebellum, ● frontal cortex, △ pons, ▲ basal ganglia.

(a) removal of IMPF by water, A-esterases or phosphorylphosphatases. The rate of this reaction, in comparison with the reaction of AChE with IMPF, will be low;

(b) removal of IMPF by reaction with cholinesterases and other B-esterases. By these reactions a large amount of the injected dose of IMPF might be removed;

(c) inhibition of AChE in various parts of the brain is dependent on the AChE concentration in these parts. The local concentration of AChE in the organized structures of the brain might be as high or higher than the inhibitor concentration presented to it. This can result in different inhibition in different areas;

(d) the rate of supply of inhibitor to AChE differs, because blood flow differs between areas.

The interplay between these possibilities will be complex: The first two possibilities (a) and (b) are mainly external to the central nervous system and should affect all areas of the brain equally. This is not so and therefore (c) and (d) must be considered in addition. The rate of supply of inhibitor to the brain (d) should be proportional to the vascularity of the different areas of the brain. There may be some changes in the permeability of the blood–brain barrier, and vascularity of the areas of the brain after treatment with organophosphorus compounds.[19] The concentration of AChE (c) does seem to be important for there is a correlation between control AChE activity of the different areas of the brain and I_{50} dose (Fig. 4).

From these results it may be concluded that although factors (a) and (b) probably are very important in influencing the large difference between constants I_{50} *in vitro* and I_{50} dose *in vivo*, they are unlikely to be a major influence on the differences in inhibition produced in the various areas of the brain. The direct relationship between

I_{50} dose and AChE activity in different areas of the brain (Fig. 4) suggest that the latter is a major determinant. Thus one important factor influencing the inhibition of AChE following injection of IMPF, which varies between organs, as demonstrated by Hobbiger,[20] is the AChE concentration, which varies between parts of the same organ.

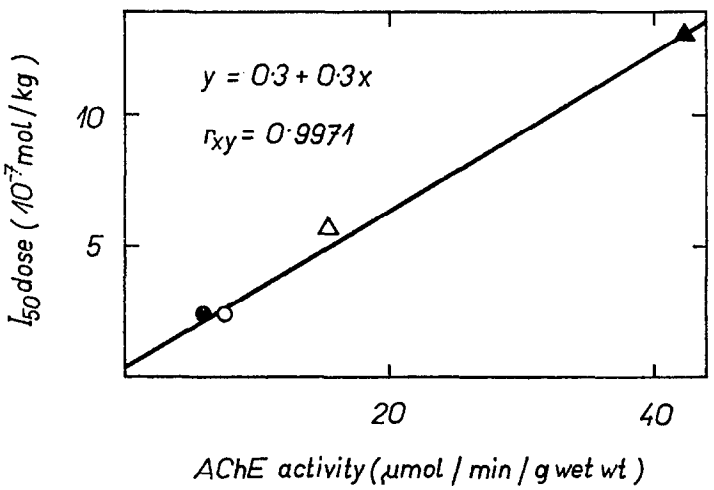

FIG. 4. Correlation between I_{50} dose of IMPF and AChE activity in different parts of the rat brain. ○ cerebellum, ● frontal cortex, △ pons, ▲ basal ganglia.

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