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"Titration" of acetylcholinesterase with soman

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PREVIOUSLY described methods[1] for estimation of the number of active sites in acetylcholinesterase-preparations (AChE, EC 3.1.1.7) seem to be rather troublesome and often expensive with respect to working time and the required amount of test material. The applicability of a more recently reported analytical procedure, described by Bender *et al.*[2] is confined to preparations of relative high enzymatic activity. We should like to report on a considerably less expensive method in which only about 10^{-6} moles of acetylcholine are split per min per mg of enzyme; the active site concentration is in the range of 10^{-12} moles/mg enzyme, which is far below the required concentration for any direct (e.g. optical) detection method.*

The simple analytical procedure used in our laboratory is based on the measurement of the enzymic activity of AChE after partial inhibition by the phosphonylating agent soman [*O*-(1-methyl-2.2-dimethylpropyl)-methyl-fluorophosphonate].

Due to the presence of two asymmetric centers (the P-atom and the C-1-atom of the 1-methyl-2.2-dimethylpropyl-residue), soman consists of four stereoisomers. Keijer and Wolring[4] found in 1969 that two of them (with the same configuration around the P-atom) react very fast with AChE (inhibition rate constants $k_i = 2{\cdot}8 \times 10^7$ and $1{\cdot}2 \times 10^8$ M^{-1} min^{-1} respectively), whereas the other two are relatively poor inhibitors ($k_i \leqslant 10^4$ M^{-1} min^{-1}). Concerning the stereospecificity of chymotrypsin towards soman, Ooms and van Dijk[5] reported a very similar observation: one pair of diastereomers (with identical configurations at the P-atoms) reacts fast ($k_i = 2{\cdot}0 \times 10^5$ for both), the other slowly ($k_i < 10^3$). The method reported here is based on these findings.

(a) *Determination of concentration of the soman stock solution*

The solvent used for all stock solutions and experiments was distilled water containing 0·1 M NaCl and 0·02 M $MgCl_2$. In order to determine accurately the concentration of fast-reacting soman in stock solutions, the inhibition of chymotrypsin was measured.

* When this work was in progress, M. Nenner reported another method for the determination of active sites, based upon a special kinetical treatment of the bimolecular reaction between AChE and an organophosphorous inhibitor.[3]

(1) An analysis of the chymotrypsin preparation used yielded a content of $2{\cdot}9 \times 10^{-8}$ moles of active sites per mg of enzyme (determined according to Bender,[2, 6] as well as by determination of the *p*-nitrophenol evolved in the reaction with *O,O*-diethyl-*O*-*p*-nitrophenyl-phosphate).

(2) To 20·0 mg ($5{\cdot}8 \times 10^{-7}$ moles) of chymotrypsin in 100 ml tris-buffer (5×10^{-2} M, pH 7·60; 20°) 1 ml soman stock solution (0·01%, w/v, in absolute acetonitrile) was added. At suitable intervals aliquots were withdrawn and diluted 2×10^3-fold for determination of the enzymic activity by pH-stat titration with *N*-acetyl tyrosine ethylester as substrate. After about 140 min the activity falls off to a constant level corresponding to 47·0 per cent inhibition.

Using the activity vs. time curves, and considering only the fast-reacting components, k_i was calculated to be $1{\cdot}4 \times 10^5$ M^{-1} min^{-1}. This value found at 20° is to be compared with that of Ooms and van Dijk[5] determined at 25° ($2{\cdot}0 \times 10^5$ M^{-1} min^{-1} in veronal buffer, pH 7·7).

Assuming that the soman sample contains 25 per cent of each of the four enantiomers, the result indicates that 1 ml of the 0·01% stock solution contains $2 \times 2{\cdot}7 \times 10^{-7} = 5{\cdot}4 \times 10^{-7}$ moles of total soman.

(b) *Inhibition of AChE*

The soman stock solution was diluted to $1{\cdot}0 \times 10^{-4}$ (w/v, in absolute ethanol). In a typical experiment 10 μl of soman solution ($5{\cdot}4 \times 10^{-11}$ moles total soman) was added with a calibrated Eppendorf-Mikroliter-Pipette to 10·0 mg AChE (Serva, Heidelberg, 1000 EU/mg) in 1·0 ml tris-buffer (5×10^{-3} M, pH 7·60; 37°). Activity was measured at various times by diluting 5×10^2-fold and carrying out pH-stat titrations with acetylcholine as substrate. Activity decreased to a constant level within the first 5 min. Comparison with the original activity (as determined in control runs with 1% ethanol instead of soman) yielded the percentage of AChE inhibited (51·9 ± 5 per cent).

(c) *Calculation of the number of active sites per mg of AChE*

According to the findings of Keijer and Wolring[4] the inhibition can be produced only by half the soman introduced into the reaction mixture, because only the two fast-reacting enantiomers are able to inhibit the enzyme completely within 5 min. Using the previously determined soman concentration (and accounting for dilutions), the number of moles of active sites per mg of AChE was calculated to be $5{\cdot}2 \pm 0{\cdot}5 \times 10^{-12}$ for the example given above.

The results presented by Ooms[5] and Keijer[4] indicate that both chymotrypsin and AChE are very sensitive to asymmetry around the P-atom but much less sensitive to asymmetry around the C-1-atom of the side chain of soman. The question arises as to whether the fast reacting diastereomers are the same in the cases of both enzymes. This was clarified here by a simple additional experiment. After appropriate dilution the reaction mixture described in a2 was examined with respect to its inhibitory potency against AChE: no measurable inhibition of AChE occurred within 90 min. This result means that the fast inhibition of AChE is due to the same pair of stereoisomers as is the inhibition of chymotrypsin.

In connection with our reactivation studies on soman-inhibited AChE,[7] another problem was of importance. After inhibition of AChE by an equimolar amount of fast-reacting soman, the reaction mixture should still contain the slow reacting component. The reactivity of this component towards oximes such as the reactivator TMB 4* is unknown. Also unknown is whether a phosphonyl-oxime (a possible inhibitor of AChE) is produced from TMB 4. In order to clarify this matter, the reaction mixture B was mixed with TMB 4 (5×10^{-2} M). No additional inhibition occurred within 4 hr (reactivation was impossible because of rapid "aging" of the phosphonylenzyme[3]), indicating that the presence of the slow inhibiting soman enantiomers would not disturb the reactivation process of non-aged phosphonyl-AChE.

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* TMB 4 = 1,3-bis-(4-hydroxyiminomethyl-pyridinium-(1))-propandibromide.

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Metabolism of diazepam and its metabolites by guinea pig liver microsomes

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LIVER microsomal preparations of various animal species metabolize diazepam added *in vitro*. This drug is predominantly hydroxylated in C_3 position and only slightly *N*-demethylated by rat liver[1] while it is more *N*-demethylated than hydroxylated in mice.[1] Previous studies *in vivo* indicated that guinea pigs are also able to metabolize diazepam.[2] This note summarizes the findings obtained by adding diazepam and some of its known metabolites (see Fig. 1) to guinea pig liver microsomes.

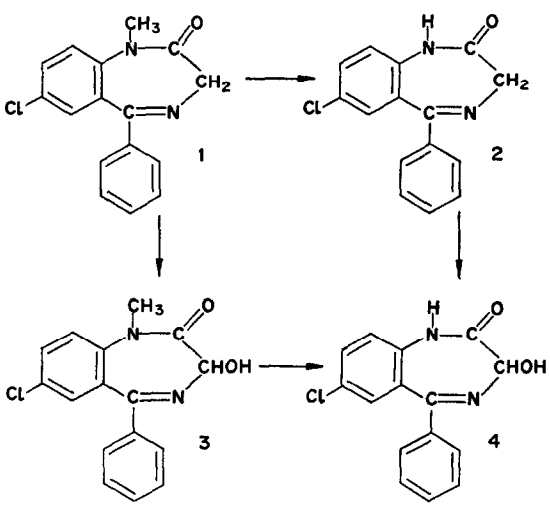

FIG. 1. (1) Diazepam. (2) *N*-demethyldiazepam. (3) *N*-methyloxazepam. (4) Oxazepam.

Materials and Methods

(1) *Animals.* Male Albino guinea pigs (body weight 300–350 g) fed with Alcom-Alal pellets, were used in all experiments.

(2) *Microsome preparation.* Animals were killed and the livers were immediately removed and homogenized in ice-cold 1·15% KCl solution (1:4 w/v) with a teflon glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min and then the supernatant fraction was again centrifuged at 105,000 *g* for 1 hr (rotor 40′—Beckman Model L ultracentrifuge). The liver microsomes were suspended in 1·15% KCl solution.

(3) *Incubation* in vitro. The incubation mixture contained substrate, enzyme source, pyridine nucleotides and an NADPH-generating system according to that described by Kato *et al.*[3] The incubation volume of 5 ml consisted of 2·5 ml of microsomal suspension equivalent to 1 g of liver,