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Inactivation of sarin and soman by cyclodextrins in vitro

B. Désiré and S. Saint-André

Centre de Recherches du Service de Santé des Armées, Division de Chimie et Pharmacologie, 108 boulevard Pinel, F-69275 Lyon Cedex 03 (France), 4 August 1986

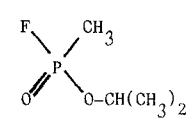
Summary. Cyclodextrins catalyzed the inactivation of sarin and soman but did not inactivate tabun and VX. Furthermore, sarin and soman showed greater affinity for β-cyclodextrin than for α- or γ-cyclodextrins. Thus β-cyclodextrin appears to be an attractive starting material for the preparation of a catalyst able to inactivate sarin and soman more effectively. Such a catalyst might contribute to improving the therapy of poisoning caused by these two nerve agents.
Key words. Nerve agents; sarin; soman; tabun; VX; cyclodextrin.

Cyclodextrins (cycloamyloses, CD) are toroidal oligosaccharides composed of α-(1,4)-linkages of a number of D(+)-glucopyranose units produced from starch by *Bacillus macerans*[1]. This report deals with the inactivating effect of α-cyclodextrin (cyclohexamylose), β-cyclodextrin (cycloheptaamylose) and γ-cyclodextrin (cyclooctaamylose) on the following potent anticholinesteratic nerve agents: sarin, soman, tabun and VX.

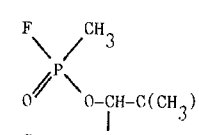
It is a well-known fact that cyclodextrins can bind large organic molecules (substrates) and then attack them within the complex through their secondary hydroxyl group (s) or through the corresponding alkoxide ion(s)[2]. Thus cyclodextrins are able to catalyze the hydrolysis of certain organophosphorus compounds: diaryl pyrophosphates[3], isopropyl 4-nitrophenyl methylphosphonate[4], diaryl methylphosphonates[5] and dimethyl 4-nitrophenylphosphate[6]. In particular, it has been shown that 1) soman is a good substrate for β-cyclodextrin at pH 7.40[7,8] 2) sarin is a rather poor substrate for α-cyclodextrin at pH 7.40[8] and 9.00[9,10] and for β-cyclodextrin under the same conditions[8] but that 3) tabun is not a substrate for γ- and β-cyclodextrin[8]. However, dissociation constants of the cyclodextrin-substrate complexes and phosphonylation constants (see below) were measured only when using α-cyclodextrin/sarin[9,10] and β-cyclodextrin/soman[7,8] systems. It appeared interesting therefore to obtain the constant values, not yet determined, which characterize the interaction between each cyclodextrin and each nerve agent considered. The aim of this determination was to find out whether one of the cyclodextrins had more affinity with these four organophosphorus compounds than the others. The cyclodextrin which most efficiently forms a complex with the nerve agents and which could, in addition, be covalently modified in order to improve its nucleophilic attack on these within the complex, might be a useful component in the therapy of poisoning by these compounds.

Material and methods. Sarin, soman, tabun and VX obtained from the Centre d'Etudes du Bouchet, Vert-le-Petit, France, were racemic compounds at least 98% pure. Stock solutions of nerve agents (2 mg/ml) in absolute ethanol were kept at −40 °C. Cyclodextrins and acetylcholinesterase (type VI-S) were purchased from Sigma, St-Louis, Mo. All other reagents were of analytical grade.

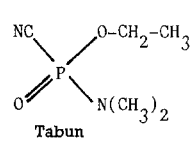
The mechanism consistent with covalent catalysis of nerve agents PX by cyclodextrins is given in the scheme of figure 1. Dissociation constant K_d and rate constants k_2 and k_3 correspond to the various processes indicated, k_o being the rate constant for spontaneous hydrolysis of the nerve agent. This mechanism is identical with the enzymatic Michaelis-Menten mechanism. If $|CD|_o$ and $|PX|_o$ are the initial concentrations of cyclo-



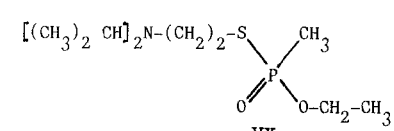
Sarin



Soman



Tabun



VX

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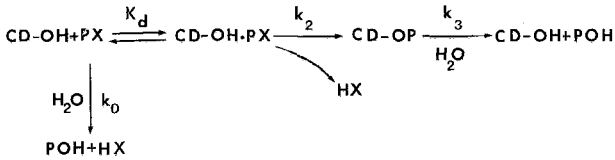

Figure 1. The catalytic mechanism which describes the interaction of an organophosphonate PX with cyclodextrin. CD-OH stands for unionized cyclodextrin and displays one of the secondary hydroxyl groups. Three steps are considered: formation of an initial complex CD-OH.PX, phosphonylation of cyclodextrin to give phosphonylated cyclodextrin CD-OP, and dephosphonylation leading to hydrolysed organophosphonate P-OH.

dextrins and substrates respectively and $|CD|_o \gg |PX|_o$, the scheme leads to Eq. 1:

$$k_{CD} - k_o = (k_2 - k_o)|CD|_o/(|CD|_o + K_d) \quad (1)$$

where k_{CD} stands for the rate constant observed for the hydrolysis of PX in the presence of cyclodextrin.

Procedures used in the measurement of the inactivation of nerve agents by cyclodextrins were as described previously[7,8]. Briefly, the activity of the nerve agents was measured by monitoring simultaneously the irreversible inhibition of acetylcholinesterase from *Electrophorus electricus* by these organophosphorus compounds and the hydrolysis of 0.5 mM acetylthiocholine, using the spectrophotometric method of Ellman et al.[11]. The activity of a nerve agent, $k_i|PX|$, is given by Eq. 2[12]:

$$Ln(A_\infty - A_t) = -k_i|PX|t + Ln(A_\infty - A_o) \quad (2)$$

where A is the absorbance observed at the time denoted by the subscript and k_i the second-order rate-constant for the irreversible inhibition of acetylcholinesterase in the presence of 0.5 mM acetylthiocholine. Cyclodextrin solutions in buffer were incubated with appropriate concentrations of nerve agents at 25 °C for varying lengths of time. Small aliquots (5–20 µl) were added to 3 ml of the spectrophotometric assay and the change in absorbance at 412 nm was recorded. At each incubation time, $k_i|PX|$ was given by the slope of the straight line obtained by fitting Eq. 2 to a set of Ln $(A_\infty - A_t)$, t data by least squares criteria. When $|CD|_o \gg |PX|_o$ a replot of Ln $(k_i|PX|)$ versus incubation times was also a straight line (see below) with slope k_{CD}. If $k_{CD} > k_o$, separate determinations of k_{CD} at different concentrations of cyclodextrin allowed k_2 and K_d to be calculated according to Eq. 1 by the method of Wilkinson[13]. Furthermore, assuming that cyclodextrins have a pK_a of 12.1[1,2,6], the ratio of monoionized to unionized cyclodextrins at any pH is $r = 10^{-12.1}/|H^+|$. Therefore the limiting value k_2^{max} of k_2 corresponding to complete monoionization of cyclodextrin will be k_2/r.

Results and discussion. In the presence of 50 mM α- or γ-cyclodextrin or 10 mM β-cyclodextrin, the inactivation rates of tabun and VX were not significantly different from the spontaneous hydrolysis rates, i.e. $k_{CD} = k_o$, even at pH as high as 9.0 and 11.0 respectively. The reason for the inefficacy of cyclodextrins against tabun and VX (no complex formation or no acylation step if a complex is formed) was not actually determined. The inactivation of sarin and soman was however accelerated by α-, β- and γ-cyclodextrins. First order kinetics were observed in the plots of Ln $(k_i|PX|)$ deduced from acetylcholinesterase inhibition versus varying duration of time of inactivation of both nerve agents by all cyclodextrins, as illustrated in figure 2 for the β-cyclodextrin/sarin system. Sarin and soman present a center of asymmetry at the phosphorus atom. Soman exhibits a second asymmetric center in the pinacolyl moiety. Consequently, the racemic forms are composed of (−)- and (+)-sarin or of P(−)C(−)-, P(−)C(+)-, P(+)C(−)- and P(+)C(+)-soman. It has been previously stressed that, under our experimental conditions, only (−)-sarin and P(−)-soman isomers which are alone inhibitors of acetylcholinesterase[14,15] are considered. This explains the first-order kinetics observed in the plots of Ln $(k_i|PX|)$ versus time (fig. 2) assuming moreover that in the case of soman the inactivation rates of P(−)C(−)- and P(−)C(+)-soman due to a given cyclodextrin are close enough.

Data obtained relating to inactivation of sarin and soman by α-, β- and γ-cyclodextrins are summarized in the table. For the γ-cyclodextrin/sarin system the values of k_2 and K_d could not be determined because the plot of $k_{CD} - k_o$ versus $|\gamma\text{-CD}|_o$ was a straight line in the concentration range of γ-cyclodextrin used ($\leqslant 50$ mM). Kinetic constants for the α-cyclodextrin/(−)-sarin system have been determined[9,10] by measurement of the hydrolysis rate of racemic sarin, and optically active forms of sarin with an autotitrator (pH stat). The values obtained for k_2^{max} and K_d were 1.2×10^3 min^{-1} and 40 mM respectively; these agree favorably with the values that we report in the table for this system. From the data in the table it appears that k_2^{max} values are of the same order of magnitude ($2–3 \times 10^3$ min^{-1}) for the α-cyclodextrin/sarin, γ-cyclodextrin/soman and β-cyclodextrin/soman systems but approximately one order of magnitude lower for the β-cyclodextrin/sarin and γ-cyclodextrin/soman systems. The values of dissociation constants range over roughly two orders of magnitude. It is to be noted that β-cyclodextrin binds sarin and soman much more tightly than α- and γ-cyclodextrins. Assuming that cyclodextrins and monoionized cyclodextrins have the same K_d values[2,10], we can determine the second-order rate-constants k_2^{max}/K_d for the reaction of sarin and soman with monoanions of cyclodextrins. These values may be compared with the corresponding values for the inactivation of sarin and soman by hydroxide ion which are close to 2×10^3 M^{-1} min^{-1}[8,9]. According to the cyclodextrin and the nerve agent considered, inactivation by monoanions of cyclodextrins is 10–2500-fold faster than inactivation by hydroxide ion (table).

The results of this study show that β-cyclodextrin appears to be an attractive starting material for the preparation of a better catalyst of soman and sarin inactivation. Appropriate covalent modifications of β-cyclodextrin at one of the secondary hydroxyls are used to improve catalytic efficacy, properly speaking, rather than affinity[1]. Thus, suitable substitution(s) at secondary hydroxyl(s) by nucleophilic group(s) with pK_a much lower than 12.1 might accelerate the acylation step and possibly achieve an improvement in the therapy of poisoning caused both by sarin and by soman.

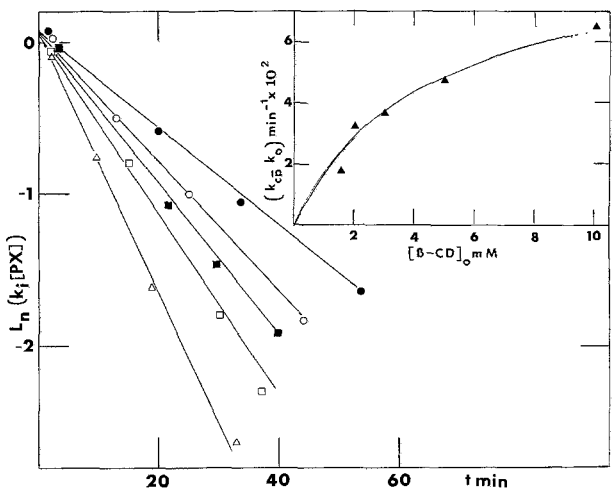

Figure 2. Inactivation of sarin by β-cyclodextrin. One series of plots of Ln $(k_i|PX|)$, $(k_i|PX|min^{-1})$ versus time for initial sarin concentration of 92 µM and following β-cyclodextrin concentrations: 1.5 mM (●), 2 mM (○), 3 mM (■), 5 mM (□) and 10 mM (△). Inset: Corresponding plots of $k_{CD} - k_o$ versus $|\beta\text{-CD}|_o$. Each point (▲) is the mean of three separate determinations. Medium: 10 mM Tris pH 9.0, ionic strength 0.155, 25 °C.

Constant values which characterize the inactivation of racemic sarin and of racemic soman by cyclodextrins under the conditions indicated[a]

		pH	α-CD	β-CD	γ-CD
Sarin	Concentration range of CD used (mM)		2.5–50	1.5–10	10–50
	k_2 min^{-1}	8.0	$1.6 \pm 0.2 \times 10^{-1}$		
	k_2 min^{-1}	9.0		$9.7 \pm 0.7 \times 10^{-2}$	
	k_2^{max} min^{-1}		$2.0 \pm 0.2 \times 10^{3}$	$1.2 \pm 0.1 \times 10^{2}$	
	K_d mM	8.0	40 ± 10		
	K_d mM	9.0		4.9 ± 0.7	
	k_2/K_d M^{-1} min^{-1}	8.0			1.1 ± 0.1[b]
	k_2^{max}/K_d M^{-1} min^{-1}		$5.0 \pm 1.4 \times 10^{4}$	$2.5 \pm 0.4 \times 10^{4}$	$1.4 \pm 0.1 \times 10^{4}$
Soman[c]	Concentration range of CD used (mM)		7.5–50	0.2–4.0	2.5–50
	k_2 min^{-1}	7.4	$3.9 \pm 0.4 \times 10^{-2}$	$5.9 \pm 0.6 \times 10^{-2}$	
	k_2 min^{-1}	8.0			$2.4 \pm 0.2 \times 10^{-2}$
	k_2^{max} min^{-1}		$2.0 \pm 0.2 \times 10^{3}$	$3.0 \pm 0.3 \times 10^{3}$	$3.0 \pm 0.2 \times 10^{2}$
	K_d mM	7.4	18 ± 5	0.53 ± 0.05	
	K_d mM	8.0			5.5 ± 1.1
	k_2^{max}/K_d M^{-1} min^{-1}		$1.1 \pm 0.3 \times 10^{5}$	$5.6 \pm 0.8 \times 10^{6}$	$5.4 \pm 1.2 \times 10^{4}$

[a] All measurements were made in 10 mM Tris buffer, ionic strength 0.155 and at 25 °C. [b] Slope of the plot of k_{CD}–k_0 against $|\gamma\text{-CD}|_0$ which was a straight line. [c] Values corresponding to inactivation of soman by β-cyclodextrin are taken from[8].

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Relationship of prodigiosin condensing enzyme activity to the biosynthesis of prodigiosin and its precursors in *Serratia marcescens*

L.K.N. Cho, J.A. Lowe+, R.B. Maguire and J.C. Tsang*

Department of Chemistry, Illinois State University, Normal (Illinois 61761, USA), 30 June 1986

Summary. Prodigiosin condensing enzyme (PCE) activities were present in *Serratia marcescens* wild type 08, mutants OF, WF and 9-3-3. Their specific activities exhibited different maxima and at different times during the late log phase or the early stationary phase of cell growth. The levels of prodigiosin and its precursors also showed a significant increase at this period. The results support that prodigiosin and/or its precursors are secondary metabolites. The ubiquity of the PCE activity in mutants deficient in prodigiosin biosynthesis suggest that this particular enzyme may also be present in non-pigmented clinical isolates.
Key words. Serratia marcescens; growth; prodigiosin; prodigiosin condensing enzyme; secondary metabolites.

Prodigiosin is a characteristic red pigment synthesized by *Serratia marcescens*. This pigment has been shown to have some antibiotic properties. Biosynthesis of prodigiosin has been suggested to be mediated by plasmids[1,2] or transferred by a transducing phage[3]. In general, genes containing the genetic information for biosynthesis of secondary metabolites such as antibiotics, are both chromosomal and extrachromosomal[4]. In at least one case, structural genes for antibiotic synthesis have been located on plasmids[5]. Normally the structural genes are chromosomal, whereas regulatory genes controlling the expression of the genetic information appear to be extrachromosomal[4,6,7]. Recently, cloning and expression in *Escherichia coli* of *Serratia marcescens* genes encoding prodigiosin biosynthesis supported the idea that genes responsible for the production of prodigiosin lie mainly on the chromosome and that prodigiosin production is probably not mediated by a plasmid[8].

Many clinical isolates of *S. marcescens* are non-pigmented and multiply resistant to antibiotics. When resistance plasmids of non-pigmented strains were transferred to pigmented strains of *S. marcescens*, spontaneous loss of production occurred. This has been suggested as a possible explanation for the predominance of multiply resistant non-pigmented *Serratia* clinical isolates[9,10]. However, it was suggested that the decrease in pigmentation associated with RP4 carriage resulted from plasmid suppression of growth rate[11]. Therefore, the relationship between the presence of resistance plasmids and pigment production remains unclear. Perhaps prodigiosin serves a survival function in nature where nutrients are limited for microbial growth. On the other hand, in clinical isolates, when adequate nutrients are available for all species present, prodigiosin production may not be necessary.

A bifurcated pathway was proposed for the biosynthesis of prodigiosin[12–14]. The final step in prodigiosin biosynthesis involves the Prodigiosin Condensing Enzyme (PCE) which condenses two substrate precursors: a monopyrrole, 2-methyl-3-amylpyrrole (MAP) and a bipyrrole, 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC). Mutants have been isolated which are blocked in various steps in either the MAP or MBC path-