R93-35

*Chem.-Biol. Interactions*, 87 (1993) 109–116
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# BIOPOLYMER METAL CATALYSTS FOR THE HYDROLYSIS OF NERVE GASES

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SUMMARY

Glucosamine oligomers — monomer through tetramer — form complexes with $Cu^{2+}$ that catalyse the hydrolysis of the 'nerve gas' 1,2,2-trimethylpropylmethylphosphonofluoridate (soman) by cleaving the P-F bond. A 1/1 glucosamine/$Cu^{2+}$ ratio whether as glucosamine or glucosamine units, gives the highest hydrolytic rate over the 11.5/1 to 1/1 range. This trend also appears to hold for a glucosamine polymer, chitosan, which, when complexed with $Cu^{2+}$ also hydrolyzes soman. The relatively low rate of hydrolysis by this polymer-$Cu^{2+}$ complex, while not yet explainable, is consistent with an extrapolation of the monomer-through-tetramer series. The question may be raised as to whether these biopolymer metal complexes provide any clues to the involvement of $Mn^{2+}$ in the functioning of one class of P-F cleaving enzymes.

*Key words:* Chelate — Chitosan — $Cu^{2+}$ — DFP — Dimebu — Glucosamine — $Mn^{2+}$ — OPA Anhydrase — Soman — 1,2,2-Trimethylpropyl methylphosphonofluoridate

## INTRODUCTION

Over thirty years ago the hydrolysis of isopropylmethylphosphonofluoridate (sarin) and diisopropylphosphorofluoridate (DFP) by $Cu^{2+}$-diamine chelates was explored by Martell and associates [1]. There is now a considerable interest in divalent metal ion-chitosan complexes, especially $Cu^{2+}$-chitosan, probably with a view to the removal of these hazardous ions from the environment [2–6]. From these several perspectives we have begun to examine the hydrolysis of 1,2,2-trimethylpropylmethylphosphonofluoridate (soman) by $Cu^{2+}$-chitosan films

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0009-2797/93/$06.00
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Printed and Published in Ireland

made directly from acetic acid and cupric acetate. The results have been encouraging (see Fig. 1 reproduced from a recent symposium [7]) but variable, probably reflecting sometimes a leaching out of $Cu^{2+}$, difficulty in reproducing the percentage of $Cu^{2+}$ incorporation, varying film thicknesses, and in general the complexities of applying solution kinetics to a heterogeneous system. To simplify these problems we have turned to the first four oligomers of the chitosan series — monomer (glucosamine itself) through tetramer — and compared the results to those obtained with a set of $Cu^{2+}$-chitosan films of controlled $Cu^{2+}$ content and film thickness.

The enzymatic hydrolysis of these toxic organophosphorus compounds, and the involvement of divalent cations, is also an area of continuing research [8]. For some of these OPA anhydrases — Mazur type in our terminology — $Mn^{2+}$ appears to be essential at the low levels reflected by the association constants for $Mn^{2+}$ and 1,10-phenanthroline or 8-hydroxyquinoline-5-sulfonate [8,9], and highly stimulatory at concentrations in the millimolar range [8]. Except for $Co^{2+}$ [10], the other members of this first transition group do not provide stimulation of the enzyme, and neither $Co^{2+}$-chitosan nor $Mn^{2+}$-chitosan promotes the hydrolysis of soman (F.C.G. Hoskin et al., unpublished observations). Thus while it appears unwarranted to view the $M^{2+}$-glucosamine complexes as models for the Mazur type OPA anhydrases, the $Cu^{2+}$ series may be viewed as synthetic enzymes (the term 'synzyme' has been used for a somewhat similar complex [11]) and results from their use are presented here.

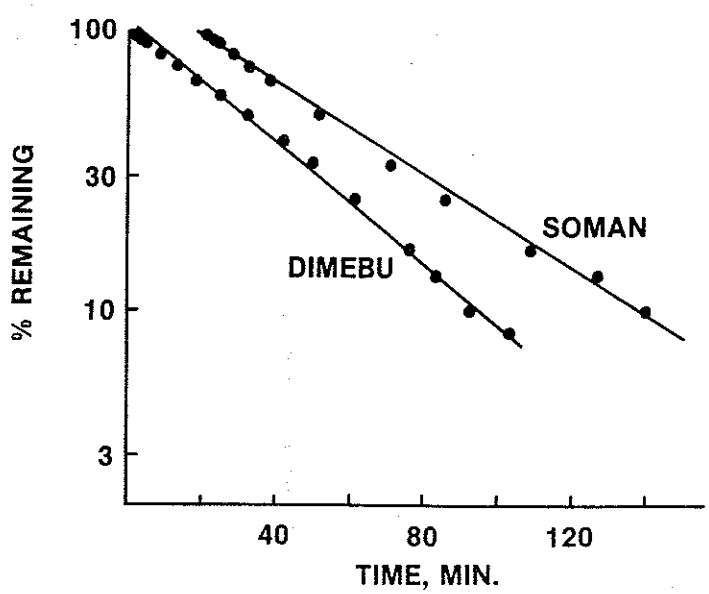


Fig. 1. Semi-log plot of the hydrolysis of soman and dimebu by a $Cu^{2+}$-chitosan film (reproduced, with permission [7]). Amounts of film were chosen to give comparable initial rates. Thus four times as much film was used with soman as with Dimebu. Single reactions appear to be involved with $t_{1/2}$ values of 34 and 31 min.

## METHODS

*Homogeneous 'normal' assay*

One typical assay in this category is described. In a 10-ml beaker were placed 2.45 ml buffer (390 mM KCl, 40 mM NaCl, 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), pH 7), 1.5 ml 0.01 M soman, and a magnetic stirring bar. The hydrolysis of soman was followed with a fluoride-sensitive electrode and the usual recording equipment for 10 – 15 min. Then 1.0 ml buffer containing 0.47 mg glucosamine trimer was added. Over the next 10 – 15 min no change in the rate of hydrolysis was observed. These two rates — actually a single slope are termed the uncatalyzed rate and this is later subtracted. Finally, 0.05 ml water containing 0.26 mg $CuCl_2 \cdot 2H_2O$ was added. There was an immediate increase in the rate of fluoride release, which remained constant during the ensuing measurement period (usually another 10 – 15 min). The concentrations in this determination were 3 mM in soman, $3 \times 10^{-4}$ M in $Cu^{2+}$, and $4.6 \times 10^{-4}$ N in terms of glucosamine units. This is a glucosamine/Cu ratio of 1.5/1 on a normal basis.

*Homogeneous 'molar' assay*

Here all the steps were identical to those described in the previous paragraph, except that the 1.0 ml buffer contained 1.4 mg of the trimer. Here the concentrations were again 3 mM soman and $3 \times 10^{-4}$ M $Cu^{2+}$, but now $4.6 \times 10^{-4}$ M in terms of the trimer. This is an oligo/Cu ratio of 1.5/1 on a molar basis.

*Other homogeneous considerations.* Since the oligomers were supplied as the hydrochlorides (K-I Chemicals, Brisbane, CA), pHs were adjusted in advance of the additions. The pH of the reaction solution was not greater than 7.05 at the start, nor less than 6.85 at the end. The normal and molar ratios were 11.5/1, 1.5/1, and 1/1. The determinations with $Cu^{2+}$ alone required the addition of 1.0 ml buffer without oligomer (ratio = 0/1). All reaction vessels were observed closely, with no evidence of precipitation, cloudiness, or color change.

*Heterogeneous assay.* For these reactions, five chitosan films with varying amounts of $Cu^{2+}$ were employed. The films, cut in a single piece as close to 30 mg as possible, were weighed exactly and placed in 3.5 ml buffer with a stirring bar. The fluoride-sensitive electrode was lowered into the stirred system, and 30 min later 1.5 ml 0.01 M soman was added. After the ensuing hydrolysis had been followed for 10 – 15 min, the film was lifted from the reaction vessel with forceps while the electrode and recording apparatus were left in place and operating. A determination of the uncatalyzed rate of soman hydrolysis was also made. The $Cu^{2+}$-chitosan films were made from a cupric acetate-acetic acid-chitosan sol by a homogeneous variant of the heterogeneous method [2]. The washed and dried films had an average thickness of 0.074 mm ± 0.02 mm (S.D., $n$ = 10) with no obvious trends.

## RESULTS

Figure 2 shows that a 1/1 glucosamine/$Cu^{2+}$ combination gives the highest net rate of soman hydrolysis, whether as glucosamine itself or as oligomeric

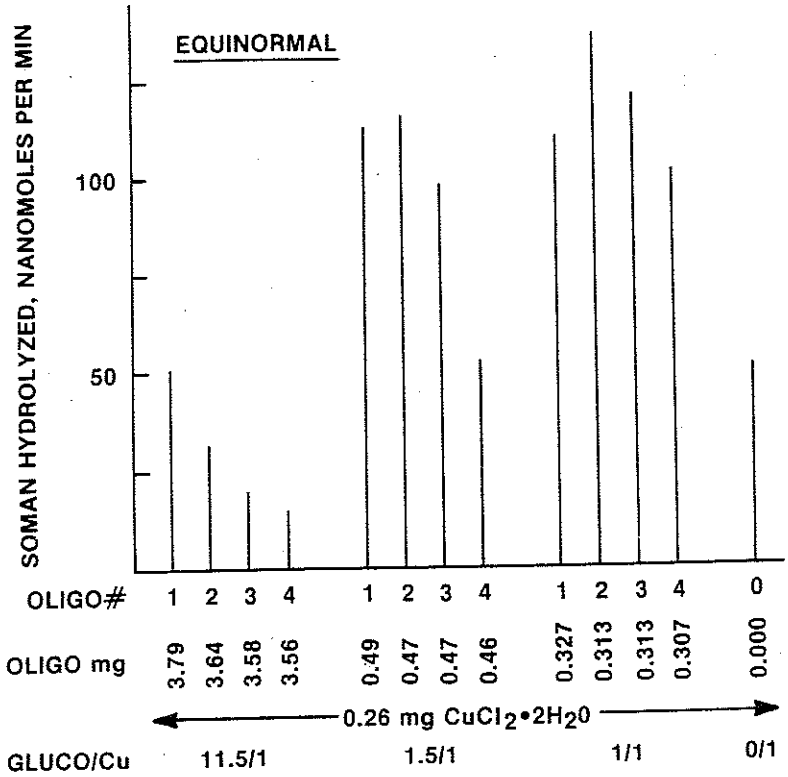


Fig. 2. The hydrolysis of soman catalyzed by combinations of glucosamine oligomers (monomer through tetramer) with $Cu^{2+}$, or by $Cu^{2+}$ alone (right hand bar). The uncatalyzed rate has been subtracted. As a reference point, 0.47 mg of oligo no. 3 in the reaction volume of 5.0 ml is $4.6 \times 10^{-4}$ N in terms of glucosamine ($1.53 \times 10^{-4}$ M in terms of the trimer), and $3 \times 10^{-4}$ M in $Cu^{2+}$. For that set (glucosamine/Cu = 1.5/1), each of the four reactions is equinormal with respect to glucosamine.

glucosamines. Almost any increase in the glucosamine/$Cu^{2+}$ ratio reduces the hydrolytic rate, and for the 11.5/1 ratio, well below the rate for the same amount of $Cu^{2+}$ alone. For the 1.5/1 ratio the $Cu^{2+}$ dimer-through-tetramer series suggests a trend of falling activity; this is clearly seen for the entire monomer-through-tetramer, 11.5/1 ratio, series. Figure 3 shows the results of sets of experiments in which the oligomer/$Cu^{2+}$ ratios are equimolar within each set with respect to the oligomer. Here again the trend of falling activity is reinforced, perhaps not unexpectedly since, for the dimer-through-tetramer, the glucosamine/$Cu^{2+}$ ratios are 2×, 3×, and 4× on a normal basis those shown in Fig. 3 on a molar basis. Although the results are not shown, exactly the same trends were found with DFP and 3,3-dimethylbutylmethylphosphonofluoridate (dimebu).

Figure 4 shows the primary data obtained with the $Cu^{2+}$-chitosan films. First,

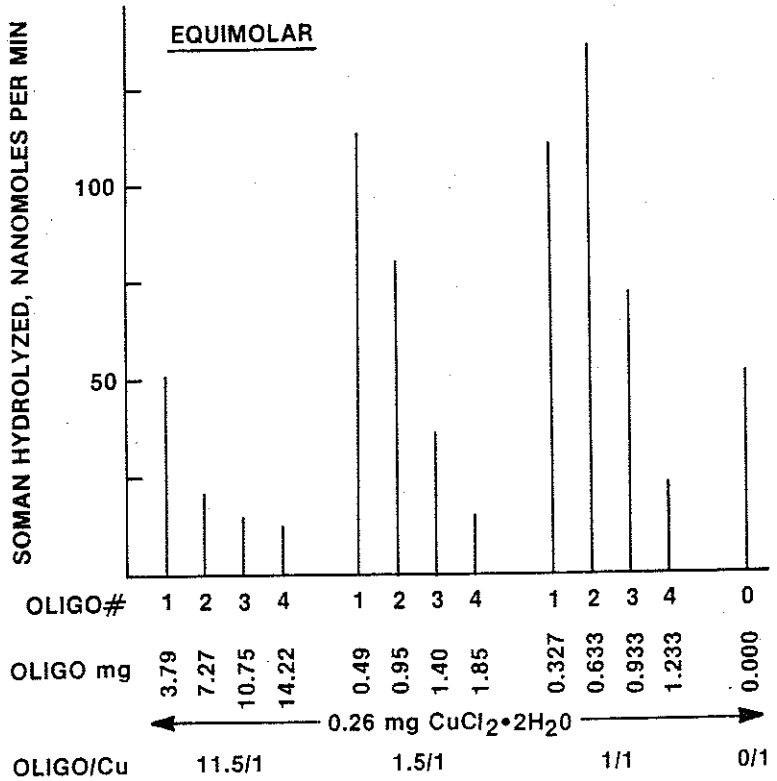


Fig. 3. The hydrolysis of soman catalyzed by combinations of glucosamine oligomers (monomer through tetramer) with $Cu^{2+}$, or by $Cu^{2+}$ alone (right hand bar). The uncatalyzed rate has been subtracted. As a reference point, 1.40 mg of oligo no. 3 is $4.6 \times 10^{-4}$ M in terms of the trimer. For that set (oligo/Cu = 1.5/1), each of the four reactions is equimolar with respect to the oligomer.

it is evident that increasing amounts of $Cu^{2+}$ in the films give increasing rates of soman hydrolysis. Second, removal of the film from the reaction vessel causes an immediate decrease in the hydrolysis rate. Third, the rate after removal of the film does not return completely to the uncatalyzed rate.

In Fig. 5 the primary data have been normalized to a 'per gram' basis, the uncatalyzed rate has been subtracted both from the film-catalyzed rate and from the rate after removal of the film, and the glucosamine/$Cu^{2+}$ ratios have been inserted beneath each set of points. This is comparable to the glucosamine/$Cu^{2+}$ ratios on a normal basis shown in Fig. 2. Again, increasing amounts of $Cu^{2+}$ in the film give increasing rates of soman hydrolysis. It also appears that the amount of activity after removal of the film (to be equated with the amount of $Cu^{2+}$ leached out) remains fairly constant.

## DISCUSSION

The results show that a one-to-one association of glucosamine unit and $Cu^{2+}$ is the most active in the catalytic hydrolysis of soman. Martell's earlier work [1]

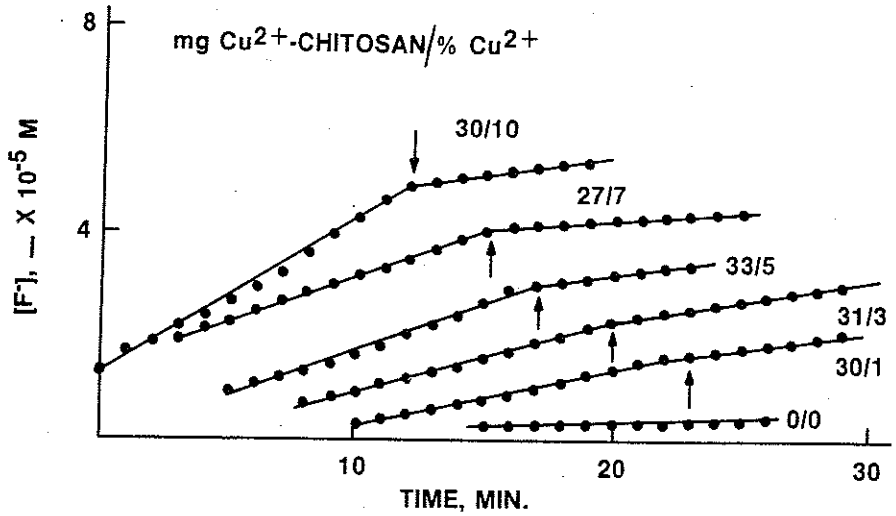


Fig. 4. Hydrolysis of soman by $Cu^{2+}$-chitosan films. The notation 30/10 is for a 30-mg film containing 10% $Cu^{2+}$. Arrows show points at which films were removed from reaction vessels. Aqueous (uncatalyzed) soman hydrolysis is marked 0/0. Curves offset for clarity.

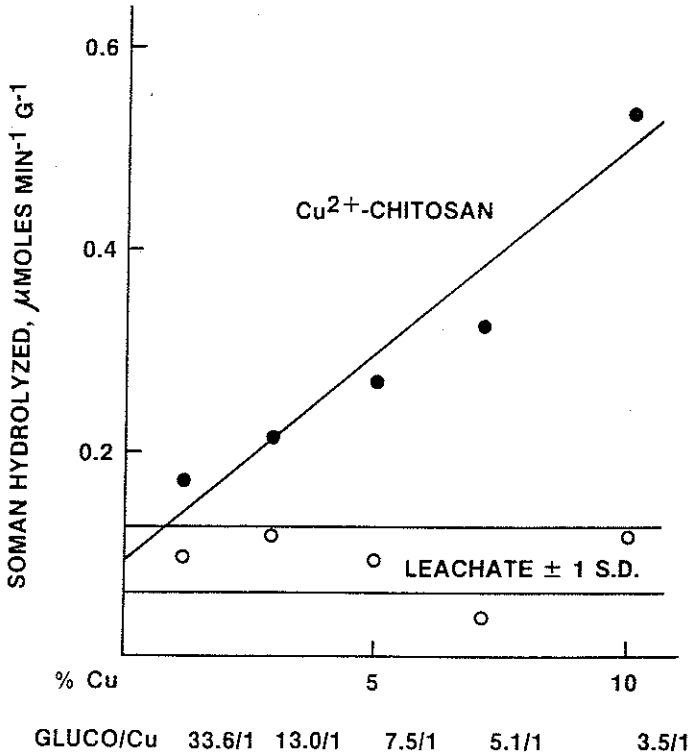


Fig. 5. Net soman hydrolysis (uncatalyzed rate subtracted from $Cu^{2+}$-chitosan and leachate curves) normalized to a 'per gram' basis. Note that abscissa has two scales.

suggested that a bidentate chelation would be the most active. This appears to be so with the oligomeric glucosamines, although potentiometric and circular dichroic measurements suggest that the chelation involves the amine and a nearby hydroxyl [5]. The decrease in activity when the glucosamine/$Cu^{2+}$ ratio increases may be due to a tridentate linkage which Martell had suggested would decrease activity [1]. It is not clear why the activity consistently decreases for the tetramer, or more correctly for the dimer-trimer-tetramer series over the entire range of glucosamine/$Cu^{2+}$ ratios tested on a normal basis, or oligo/$Cu^{2+}$ ratios on a molar basis. This consistency, also seen with DFP and dimebu, suggests perhaps some inter-oligomeric linkage and a consequent hindrance of the approach of the two substrates, organophosphorus compound and water, to the active site, i.e., the hydroxylamine chelated $Cu^{2+}$.

A sharp decrease in activity for the $Cu^{2+}$-chitosan film may be seen compared to the $Cu^{2+}$ oligomers. Allowing for the different units used on the ordinates of Figs. 2 and 5, and extrapolating Fig. 5 to a 1/1 glucosamine/$Cu^{2+}$ ratio, the $Cu^{2+}$-chitosan film is 0.1% to 3% as active as the corresponding $Cu^{2+}$-oligomer combinations. However, this requires some explanation. Recently, we have combined in a single reaction vessel a combination pH electrode attached to a pH-stat and a combination fluoride electrode attached to its recording equipment. We find that the pH-stat reports, on the average, three times as much soman hydrolysis by $Cu^{2+}$-chitosan films as the fluoride-sensitive electrode reports.

A soluble enzyme preparation, squid OPA anhydrase [8], shows the same activity by either method. The tentative conclusion is that a substantial fraction of the fluoride from the hydrolysis of soman is entrapped within the $Cu^{2+}$-chitosan matrix. Allowing for the fluoride retention, and extrapolating to a 1/1 glucosamine/$Cu^{2+}$ ratio, the $Cu^{2+}$-chitosan film would still have only a fraction of the activity of the tetramer. Thus, there is a consistency in the decreasing activity of the series, monomer, dimer, trimer, tetramer,..., polymer.

While problems remain that must be addressed in applying solution kinetics to these membrane systems (and here we refer to structures $10^4$ times thicker than the plasma membranes of cellular systems), the catalyzed hydrolysis of soman provides a chemical response of $M^{2+}$-chitosan and related chelates in addition to the structural-physical measurements such as titration, X-ray diffraction, and circular dichroism. It had been our expectation that DFP and dimebu would prove to be suitable analogues of soman without the restrictions usually imposed on the use of the latter. While dimebu is hydrolyzed about 10 times faster than soman [7], $Cu^{2+}$ alone hydrolyzes dimebu about as fast as the best $Cu^{2+}$ chitosan combination. DFP is hydrolyzed at about a fifth of the soman rate and, again, the $Cu^{2+}$ rate is about as fast. Thus soman appears to be the most useful test agent for the chemical exploration of these $Cu^{2+}$ oligomeric glucosamine chelates. It is less clear whether these bipolymer-metal complexes will provide any clues to the involvement of $Mn^{2+}$ in the functioning of the Mazur type OPA anhydrases.

ACKNOWLEDGMENTS

The parts of this work dealing with DFP and dimebu were carried out under

the auspicies of Battelle; those dealing with soman, under the auspicies of IIT Research Institute.

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