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Enzymatic Hydrolysis of Organophosphorus Compounds

VIII. Effect of Anions

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Molybdate and tungstate are markedly active in increasing the spontaneous hydrolysis of tabun. Among the 25 anions tested, chromate, metavanadate, perborate and selenite are also active in this respect, but less so than molybdate and tungstate. None of the anions influence the enzymatic hydrolysis of tabun by phosphorylphosphatase (prepared from human serum), except fluoride which is an inhibitor. The degradation products formed after treatment of tabun with molybdate do not inactivate cholinesterase. This enzyme is protected from inactivation by tabun when mixed with molybdate prior to addition of tabun.

The effect of metallic ions on phosphorylphosphatases was reported in a recent paper of this series [1]. The present report deals with the results obtained in studies of the effect of a variety of anions on both the enzymatic and spontaneous hydrolysis of dimethylamido-ethoxy-phosphoryl cyanide (tabun).

METHODS AND MATERIAL

The determination of hydrolysis rates, spontaneous as well as enzymatic, was carried out in a 0.04 M $NaHCO_3-CO_2$ buffer by the Warburg manometric technique at 25°C and pH 7.6 [2]. Hydrolysis rates were expressed in μl CO_2 evolved (b_{30}) or μmoles of substrate hydrolysed in 30 min. Tabun was used as substrate, and its concentration in the reaction mixture (total volume, 2.00 ml) was 5.3×10^{-3} M (*i. e.* 10.6 μmoles of tabun present).

The phosphorylphosphatase preparation was prepared from human serum, dissolved in the bicarbonate buffer, and dialysed free from other ions. The cholinesterase preparation was purified from human serum and was free from phosphorylphosphatase activity. Cholinesterase activity was determined in a Ringer bicarbonate buffer by the Warburg technique with acetylcholine iodide (3.3×10^{-3} M) as substrate.

In testing the effects of various anions, sodium and ammonium salts were used in most cases; the salt concentration in the reaction mixture during hydrolysis rate determinations was 1.0×10^{-3} M. The phosphorylphosphatase was incubated 50 min with the ions before the substrate (tabun) was added. The same incubation time was employed in iactivation experiments with cholinesterase, except as otherwise stated.

RESULTS

Effect of anions on the spontaneous hydrolysis of tabun. The effects of a number of anions on the rate of spontaneous hydrolysis of tabun are illustrated in Table 1. Most anions did not influence the hydrolysis rate in the concentration range tested. Perborate, selenite and thiocyanate increased the rate of hydrolysis, and still more active were chromate and metavanadate. Especially active in increasing the rate of spontaneous hydrolysis were tungstate and molybdate. The effect of molybdate ($Na_2MoO_4.2H_2O$) was such that the time required for 50 % hydrolysis of 10.6 μmoles of tabun under the experimental conditions used was decreased from 3h 40 min to 15 min by 20 μmoles of molybdate, and to 2 min by 100 μmoles.

Fig. 1 illustrates the effect of molybdate on tabun hydrolysis as a function of molybdate concentration, and Fig. 2 the graphical evaluation of first order reaction constants for the reactions in the absence and presence respectively of molybdate.

Table 1. Effect of anions (1.0 mM) on the spontaneous (non-enzymatic) and enzymatic hydrolysis of tabun. Enzyme: purified phosphorylphosphatase of human serum. Sodium salts used, unless otherwise stated.

Ion	% Activation (a) or Inhibition (i)	
	Non-enzymatic	Enzymatic
$HCOO^-$	0	10 a
$C_2O_4^{2-}$	0	0
CN^-	0	0
NO_3^-	0	0
$H_2PO_2^-$	0	0
$P_2O_7^{4-}-HP_2O_7^{3-}$	0	5 a
$BO_2^- - H_2BO_3^-$	0	0
BO_3^- *	20 a	0
$B_4O_7^{2-}$	0	12 a
SO_3^{2-}	0	0
SO_4^{2-}	0	0
$S_2O_3^{2-}$	0	0
$S_2O_8^{2-}$	0	8 a
SH^-	0	12 i
SCN^-	11 a	8 i
SeO_3^{2-}	20 a	0
AsO_3^{3-}	0	5 a
VO_3^- **	81 a	0
CrO_4^{2-}	52 a	0
MoO_4^{2-}	175 a	0
MoO_4^{2-} **	175 a	0
$[Mo(MoO_4)_6]^{6-}$ **	1 190 a	0
WO_4^{2-}	159 a	0
F^-	100 i	54 i
ClO^-	< 10 a	0
ClO_3^-	0	0
ClO_4^- (10^{-4} M) ***	0	0

* active ion, probably HO_2^-; ** NH_4-salt; *** Ca-salt.

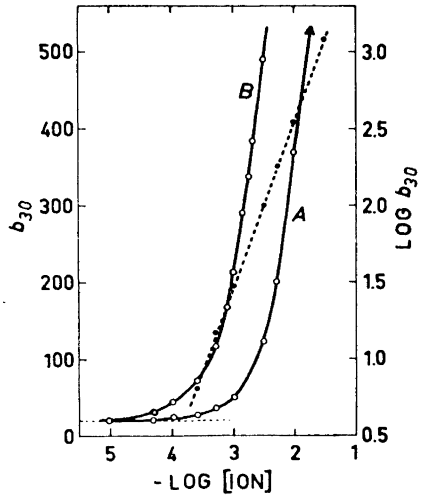

Fig. 1. Effect of molybdate on the spontaneous initial hydrolysis rate of tabun. Molar concentrations of anion are those in the reaction mixture (2.00 ml) during rate determination. A: $Na_2MoO_4 \cdot 2H_2O$; B: $(NH_4)_6[Mo(MoO_4)_6] \cdot 4H_2O$. The log curve (dotted line) refers to the effect of Na_2MoO_4 (log μl CO_2/30 min), corrections made for blank (no molybdate present).

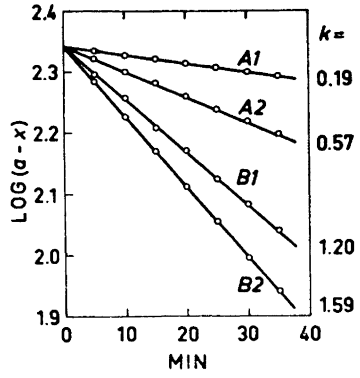

Fig. 2. Graphical evaluation of first order reaction constants for the spontaneous (A) and enzymatic (B) hydrolysis of tabun, in the absence (1) and presence (2) of 1.0 mM Na_2MoO_4. Note that the differences between the rate constants for the reactions taken part in the absence and presence of molybdate are the same (0.38 and 0.39 h^{-1} respectively).

It was found in previous reports that copper(II) salts accelerate the spontaneous hydrolysis of tabun [1] and di*iso*propoxy-phosphoryl fluoride [4]. Under the experimental conditions of the present investigation, 4 μmoles of $CuCl_2$ increased the initial rate of tabun hydrolysis by a factor of 2, in both the absence and presence of molybdate (Table 2). The effects revealed by Cu^{2+} and molybdate are therefore assumed to be purely additive, and a copper complex which might be particularly active, was not formed. Mixtures of $CuCl_2$ and molybdate with other ratios than that used in Table 2 gave similar results.

The spontaneous hydrolysis of tabun was inhibited by fluoride (Table 1). It was of interest to find out whether this effect was reflected in the cholinesterase inactivating effect of a tabun solution "stabilized" with fluoride. The

Table 2. Hydrolysis of tabun in the presence of mo ybdate and copper(II) chloride. Initial reaction rate expressed as μmoles of tabun/10 min.

10.6 μmoles tabun	0.3
+ 15 μmoles Na_2MoO_4	2.6
+ 4 μmoles $CuCl_2$	0.7
+ 15 μmoles Na_2MoO_4 + 4 μmoles $CuCl_2$	5.2
+ 2 μmoles ammonium molybdate	2.7
+ 2 μmoles ammonium molybdate + 4 μmoles $CuCl_2$	5.0

Table 3. Effect of fluoride on the cholinesterase inactivating effect of tabun. Tabun was incubated for 24 h in bicarbonate solutions with and without NaF (1 mM) at room temperature. The inactivating effect of these solutions was then measured and expressed in pI_{50} values [8]. A freshly prepared tabun solution was used as control ("No incubation"). Other experimental conditions, as in Table 4.

Incubation medium	No incubation	HCO_3^-	$HCO_3^- + F^-$
pI_{50}	7.80	6.35	6.95

experimental results of Table 3 show that this is the case. A solution of tabun in a bicarbonate buffer containing NaF was more powerful in inactivating cholinesterase than a bicarbonate solution without fluoride. The stabilization, however, was far from complete in this experiment, but the effect of fluoride in this respect was obvious.

Effect of anions on the enzymatic hydrolysis of tabun. Most of the anions studied did not influence the enzymatic hydrolysis of tabun by a purified phosphorylphosphatase preparation of human serum (Table 1). The results obtained with molybdate are illustrated in Fig. 2. Fluoride was an inhibitor, and weak inhibiting effects were obtained with hydrosulphide and thiocyanate. There was no marked acceleration of the enzymatic reaction in any case when the anions (2 μmoles) were added to the reaction mixture. The weak increase in the rate of enzymatic hydrolysis observed with borate, formate, persulphate, etc. may be due to complex formation with some enzyme inhibitor present in the preparation used.

The study was broadened with phosphorylphosphatases of other sources (swine kidney and liver). Anions did not have any significant effect on these enzymes either, except for calcium perchlorate which was a strong inhibitor of the kidney enzyme (93 % inhibition by 10^{-4} M $Ca(ClO_4)_2$).

Effect of molybdate on the cholinesterase inactivation by tabun. With the observation of strong increase in the spontaneous hydrolysis rate of tabun by molybdate, the effect of this and some other anions on the cholinesterase inactivation by tabun was studied. The results obtained are summarized in Table 4. Molybdate, in the concentrations used, did not influence the cholinesterase activity (Column 4). When cholinesterase was inactivated 85—95 % by tabun and molybdate was then added, no reactivation of enzyme activity was observed (Column 3). A tabun solution which had been treated for 30 min with molybdate prior to adding to the enzyme solution, did not inhibit cholinesterase (Column 6). It was also observed that the enzyme could be "protected" from inactivation by tabun when the enzyme was mixed with molybdate prior to addition of tabun (Column 5); compared with controls, complete protection was not achieved in the experimental series of Table 4. Complete protection, however, was obtained by using higher molybdate concentration. Similar results were obtained with ammonium molybdate. Sodium perborate which showed an accelerating effect on tabun hydrolysis (Table 1),

Table 4. Effect of molybdate and perborate on the cholinesterase (ChE) inactivation by tabun. Inactivator (tabun), 1.0×10^{-8} M (during incubation of ChE). Concentration values refer to the reaction mixture during enzyme activity determination, unless otherwise stated. Incubation time 30 min for reagents in brackets, followed by another 50 min incubation with salt or/and inactivator before addition of substrate. Activity expressed in b_{30} values.

		1	2	3	4	5	6
Salt	M	Control	(ChE + Tabun)	(ChE + Tabun) + Salt	(ChE + Salt)	(ChE + Salt) + Tabun	(ChE + (Tabun + Salt)
$Na_2MoO_4 \cdot 2H_2O$	1.0×10^{-3}	134	11.5	10.5	136.5	66	135
	5.0×10^{-3}	156	20.5	27.5	151.5	132.5	156.5
$(NH_4)_6[Mo(MoO_4)_6] \cdot 4H_2O$	7.5×10^{-4}	150.5	26	23	146.5	81	150
$NaBO_2$	1.0×10^{-4}	150	2.5	3	146	—	67

but less so than molybdate, also gave similar results vis-à-vis cholinesterase inactivation.

Effect of anions on the hydrolysis of sarin. Similar studies of spontaneous hydrolysis as those described for tabun were performed by Larsson [5] with sarin (methyl-*iso*propoxy-phosphoryl fluoride). Among the salts tested as catalysts with the gasometric technique, sodium perborate, sodium molybdate and calcium hypochlorite markedly accelerated the hydrolysis of sarin. Calcium hypochlorite was also active on some sarin analogoues and on parathion. The effect of calcium hypochlorite on the tabun hydrolysis was much lower. Generally, various organophosphorus compounds show differences as to the catalytic effect of various inorganic agents, cations as well as anions, on spontaneous hydrolysis.

DISCUSSION

An interpretation of the catalysing effect of a salt on the spontaneous hydrolysis of tabun (and other organophosphorus compounds as well) may be the formation of a labile complex with the organophosphorus compound. This likely is the explanation of the activation of hydrolysis rates caused by molybdate and tungstate, the complex formed being of unknown nature. The formation of such complexes with molybdate and other organophosphorus compounds [7] has been discussed.

The hydrolysis products of tabun do not inhibit cholinesterase [6,8] when present in concentrations lower than 10^{-3} M. The products formed in the molybdate catalysed reaction are also inactive as cholinesterase inhibitors, as demonstrated above. The fact that cholinesterase mixed with molybdate is protected from inactivation by tabun, the capacity of protection being dependent on the molybdate concentration, makes it likely that molybdate reacts directly with the phosphorus atom which is the site of the inhibiting compound that reacts with the active group of the enzyme molecule. The more this

atom is in the cationic state, dependent on the electrophilic strenght of the acid constituent of the molecule (CN in the case of tabun), the higher affinity does it have for the enzyme and also for molybdate. It is reasonable to regard the formation of an organophosphorusmolybdate complex as a sort of "model" reaction for that of the substrate-enzyme complex (*cf.* Ref.[4]).

In contrast to certain metallic ions [1], anions do not influence the enzymatic hydrolysis of tabun by phosphorylphosphatase. The enzyme activity is probably not dependent on the presence of SH-groups, for those agents which react with these groups do not inhibit the activity. The inhibiting effect observed with fluoride is probably due to a shift in equilibrium of the reactions in which cyanide is set free, the base-catalysed spontaneous reaction as well as the enzymatic hydrolysis reaction.

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