HYDRATES OF QUATERNARY AMMONIUM ALDEHYDES AS POTENTIAL REACTIVATORS OF SARIN-INHIBITED ACETYLCHOLINESTERASE

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Synthesis of 2- (**5a**), 3- (**5b**) and 4-formyl-1-methylpyridinium iodides (**5c**) and (2,2-dihydroxyethyl)trimethylammonium chloride (**6a**) is described. In aqueous solution aldehydes **5** exist predominantly as hydrates – geminal diols **7**. The ability of the geminal diols **6a** and **7** to reactivate acetylcholinesterase inhibited by isopropyl methylfluorophosphonate (Sarin) was tested *in vitro*. Although compounds **6a** and **7** exhibit an affinity towards the acetylcholinesterase bonding site comparable with that of the natural substrate acetylcholine, their reactivation ability was negligible. Second-order rate constants for cleavage of 4-nitrophenyl diphenyl phosphate (PNPDPP) with anions of the studied geminal diols were measured in order to determine their nucleophilicity.

Key words: Acetylcholinesterase; Organophosphorus inhibitors; Reactivators of phosphonylated acetylcholinesterase; Pyridinium salts; Sarin; Phosphonates.

Many derivatives of organic phosphorus acids, particularly some alkyl phosphates and alkyl phosphonates, are highly active irreversible inhibitors of acetylcholinesterase[1] (ACE). In addition to the generally known poisonous compounds such as isopropyl fluoromethylphosphonate (Sarin), Soman, Tabun, VX, *etc*., many compounds of this group, *e.g.* paraoxon, parathion, dimefox, metrifonat, are used as insecticides and veterinary preparations. Their inhibitory effect is based on phosphonylation (or phosphorylation) of the hydroxy group in serine at the so-called esteratic site of the active center of the enzyme[2] (as exemplified in Scheme 1 by inhibition of ACE with Sarin). Acetylcholinesterase plays an important physiological role in the cholinergic nervous system and therefore its inhibition is a life-endangering factor. For this reason, there is a constant quest for ways of reactivating

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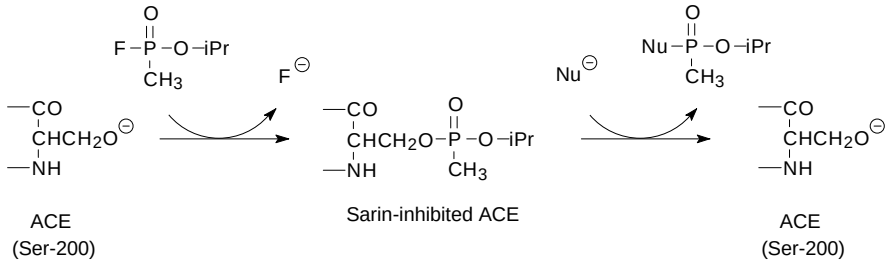
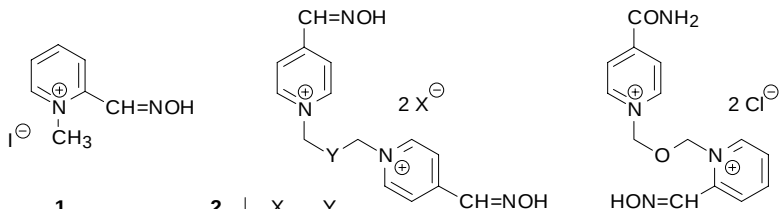
the phosphonylated (or phosphorylated) ACE and thus restoring its physiological function. This can be done by use of suitable nucleophilic reagents (Scheme 1).



SCHEME 1

Very soon after discovery of toxic effects of organophosphates, the reactivation ability of quaternary pyridinium aldoximes has been found[3] and these compounds still represent the leading structure of all currently used ACE reactivators, such as 2-PAM (pralidoxim) **1**, TMB-4 (trimedoxim) **2a**, toxogonin (obidoxim) **2b**, and HI-6 **3** (ref.[4]).

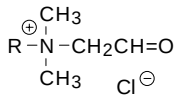
The high effectivity of the mentioned compounds is due to several factors: (i) the presence of easily ionizable hydroxyimino group ($7 < pK_a < 8$) ensuring a high concentration of the nucleophilic oximate anion even at the physiological pH, (ii) the high nucleophilicity of the oximate anion (compared with *O*-nucleophiles of comparable basicity), caused by the so-called α-effect of the hydroxyimino nitrogen lone electron pair[4a,5], and (iii) high affinity of pyridinium salts towards ACE as a result of Coulombic attraction between the positively charged quaternary nitrogen of the reactivator and the so-called anionic center of the enzyme[2].



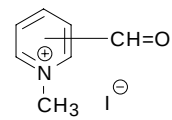
2	X	Y
a	Br	CH_2
b	Cl	O

In spite of the existence of the ACE reactivators, many attempts to find new and more effective compounds have been made[4b,6], however, the search consists mostly in structural variations of the leading structure, quaternary pyridinium aldoximes. In the present study, we decided to investigate the reactivation ability of (2-oxoethyl)tri-

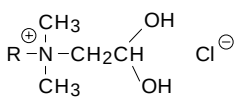
methylammonium chloride (**4a**), 2-, 3-, and 4-formylpyridinium iodides (**5a**–**5c**), and their hydrates **6a** and **7**. Although these compounds have already been described[7,8], they were not tested as ACE reactivators. We have been inspired by studies of Menger *et al.*[9] published in the eighties. They found[9a] that organophosphates are rapidly hydrolysed in homomicelles of a cationic tenside (2-oxoethyl)dimethyltetradecylammonium



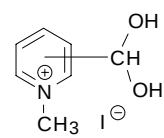
4	R
a	CH_3
b	$CH_3(CH_2)_{13}$



5	Position
a	2
b	3
c	4



6	R
a	CH_3
b	$CH_3(CH_2)_{13}$

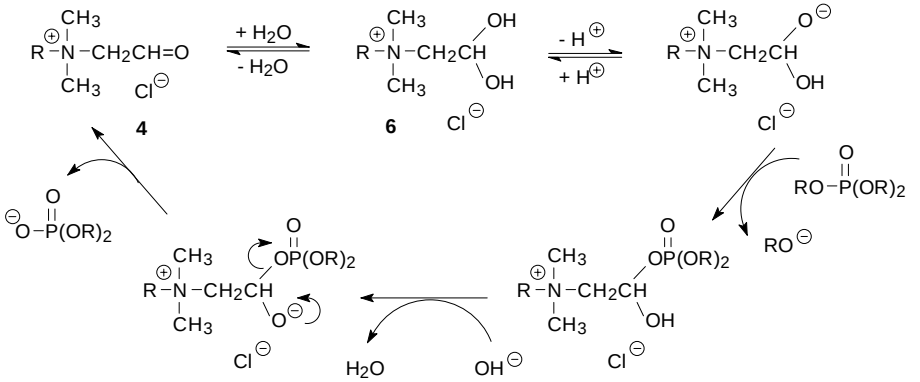


7	Position
a	2
b	3
c	4

chloride (**4b**). The organophosphate phosphorus atom is attacked by the nucleophilic anion which is formed by deprotonation of hydroxy group in geminal diol **6b** arising by addition of water molecule to the carbonyl group of aldehyde **4b**, activated by the electron-accepting quaternary ammonium group. The nucleophilic group in tenside **4b** is recovered in a catalytic cycle (Scheme 2).

The reactivation of ACE is also based on cleavage of a phosphate or phosphonate and one can therefore assume that geminal diols of the type **6a** and **7** could reactivate ACE. Whereas classical reactivators of the aldoxime type (such as **1**) dephosphorylate ACE in the stoichiometric ratio, geminal diols **6a** and **7** should act in a catalytic process (Scheme 2). A necessary condition for reactivators is their sufficient affinity to ACE. The structure of hydrate **6a** resembles that of choline, a natural product arising by action of ACE on acetylcholine, and geminal diols **7** have some features similar to those of "classical" reactivators, aldoximes **1**–**3**. Therefore, we assumed that the studied compounds could exhibit sufficient affinity to ACE. The only weakness of the geminal diols was the pK_a value published[9a] for diol **6b** (10.9). However, in spite of this high

value we decided to study the reactivation ability of compounds **6a** and **7** towards ACE inhibited by phosphonylation.



SCHEME 2

EXPERIMENTAL

Melting points were measured on a Boetius block and are uncorrected. ^{1}H NMR spectra were taken on a Varian Gemini 300 (300 MHz) and a Bruker AM 400 (400 MHz) instruments. Chemical shifts are given in ppm and are referenced to tetramethysilane, coupling constants *J* are in Hz.

Chemicals

Chloroacetaldehyde diethyl acetal (Aldrich), 2-, 3-, and 4-pyridinecarbaldehyde (Merck), acetylcholine iodide (ACI) (Lachema), isopropyl methylfluorophosphonate (VOZ 072 Zemianske Kostol'any), 3-cyclohexylaminopropane-1-sulfonic acid (Sigma). 4-Nitrophenyl diphenyl phosphate (PNPDPP) was prepared and purified by a described procedure[10]. Solvents were purified and dried by usual procedures[11]. The enzyme was obtained from brains of male white laboratory Wistar rats (Velaz, Prague). The animals in light ether narcosis were killed by cutting the cervical artery. After opening the calva, the brain was taken out, washed with cold physiological solution, and subjected to three freeze (to –35 °C)–thaw cycles. Homogenization in distilled water using a Ultra-Turrax instrument (Janke–Kunkel, Germany) at 20 000 r.p.m. for 1 min yielded a 10% (w/v) homogenate. Aliquots (2 ml) of the homogenate were placed into small test tubes and stored at –35 °C in a freezer and defrozen immediately prior the use.

Formyl-1-methylpyridinium Iodides **5a**–**5c** (General Procedure)

A mixture of pyridinecarbaldehyde (1.0 g, 9.8 mmol), methyl iodide (4.1 g, 28 mmol) and acetone (2.4 ml) was heated at 60 °C for 8 h. After cooling, the separated crystals were collected, washed with dry ether (20 ml) and dried *in vacuo* over phosphorus pentoxide.

2-Formyl-1-methylpyridinium Iodide (**5a**)

Yield 1.6 g (68%), m.p. 181–182 °C (ref.[8] gives m.p. 180–183 °C). ^{1}H NMR spectrum ($(CD_3)_2SO$): 4.66 s, 3 H (CH_3); 8.35 m, 1 H (H-5); 8.53 d, 1 H, *J*(3,4) = 7.8 (H-3); 8.79 t, 1 H, *J*(4,3) = *J*(4,5) = 7.8 (H-4); 9.15 d, 1 H, *J*(6,5) = 5.9 (H-6); 10.29 s, 1 H (CH=O). ^{1}H NMR spectrum (D_2O): 4.33 s, 3 H (CH_3); 6.33 s, 1 H ($CH(OD)_2$); 7.97 m, 1 H (H-5); 8.26 d, 1 H, *J*(3,4) = 7.9 (H-3); 8.50 dd, 1 H, *J*(4,3) = 7.8, *J*(4,5) = 7.9 (H-4); 8.70 d, 1 H, *J*(6,5) = 6.0 (H-6).

3-Formyl-1-methylpyridinium Iodide (**5b**)

Yield 2.0 g (83%), m.p. 172–176 °C (ref.[8] gives m.p. 173–175 °C). ^{1}H NMR spectrum ($(CD_3)_2SO$): 4.50 s, 3 H (CH_3); 8.35 dd, 1 H, *J*(5,4) = 7.8, *J*(5,6) = 6.2 (H-5); 9.00 d, 1 H, *J*(4,5) = 8.0 (H-4); 9.22 d, 1 H, *J*(6,5) = 6.0 (H-6); 9.55 s, 1 H (H-2); 10.20 s, 1 H (CH=O). ^{1}H NMR spectrum (D_2O): 4.33 s, 2.7 H (CH_3 of **7b**); 4.41 s, 0.3 H (CH_3 of **5b**); 6.14 s, 0.9 H ($CH(OD)_2$); 7.97 dd, 0.9 H, *J*(5,4) = 7.7, *J*(5,6) = 6.4 (H-5 of **7b**); 8.18 t, 0.1 H, *J*(5,4) = 5.4, *J*(5,6) = 6.9 (H-5 of **5b**); 8.53 d, 0.9 H, *J*(4,5) = 8.02 (H-4 of **7b**); 8.68 d, 0.9 H, *J*(6,5) = 6.0 (H-6 of **7b**); 8.84 s, 0.9 H (H-2 of **7b**); 8.91 d, 0.1 H, *J*(4,5) = 7.4 (H-4 of **5b**); 8.95 d, 0.1 H, *J*(6,5) = 5.9 (H-6 of **5b**); 9.30 s, 0.1 H (H-2 of **5b**); 10.07 s, 0.1 H (CH=O).

4-Formyl-1-methylpyridinium Iodide (**5c**)

Yield 2.3 g (94%), m.p. 106–109 °C (ref.[8] gives m.p. 105–106 °C). ^{1}H NMR spectrum ($(CD_3)_2SO$): 4.45 s, 3 H (CH_3); 8.49 d, 2 H, *J*(3,2) = *J*(5,6) = 5.4 (H-3,5); 9.25 d, 2 H, *J*(2,3) = *J*(6,5) = 5.9 (H-2,6); 10.24 s, 1 H (CH=O). ^{1}H NMR spectrum (D_2O): 4.30 s, 3 H (CH_3); 6.12 s, 1 H ($CH(OD)_2$); 8.03 d, 2 H, *J*(3,2) = *J*(5,6) = 6.41 (H-3,5); 8.70 d, 2 H, *J*(2,3) = *J*(6,5) = 6.6 (H-2,6).

(2,2-Diethoxyethyl)trimethylammonium Chloride (**8**)

A mixture of chloroacetaldehyde diethyl acetal (12.0 g, 78.7 mmol), anhydrous trimethylamine (9.2 g, 155 mmol) and benzene (14.7 g) was heated in a sealed tube at 100 °C for 51 h. The unreacted trimethylamine was distilled off and the residue was neutralized with HCl (1 : 1). The solvent was evaporated and the crude product was crystallized from acetone. Yield 0.40 g (2.4%), m.p. 131–142 °C. ^{1}H NMR spectrum ($(CD_3)_2SO$): 1.16 t, 6 H, *J*(2′,1′) = 6.9 (CH_3); 3.14 s, 9 H (CH_3N); 3.50 d, 2 H, *J*(1,2) = 4.9 (CH_2N); 3.63 m, 4 H (CH_2O); 5.02 t, 1 H, *J*(2,1) = 5.2 (CH).

(2,2-Dihydroxyethyl)trimethylammonium Chloride (**6a**)

Acetal **8** (0.40 g, 1.9 mmol) was refluxed with 5.6% HCl (3.2 ml) under argon for 20 min. After evaporation of the solvent, the product was washed with acetone and dried *in vacuo* over phosphorus pentoxide. Yield 0.28 g (96%), m.p. 133–140 °C (ref.[7] gives m.p. 123–124 °C; ref.[12] gives m.p. 142–144 °C). ^{1}H NMR spectrum ($(CD_3)_2SO$): 3.14 s, 9 H (CH_3); 3.27 d, 2 H, *J*(1,2) = 5.0 (CH_2); 5.26 t, 1 H, *J*(2,1) = 5.2 (CH); 6.75 s, 2 H (OH).

Determination of pK_a (ref.[13])

Acid constants pK_a of compounds **6a** and **7a** were determined by potentiometric titration of their 5.0 . 10^{-4} M aqueous solutions with 0.01 M sodium hydroxide at 25 °C at constant ionic strength $I \approx 0.25$ mol kg^{-1} rendered with potassium chloride. The titrations were performed using an RTS 822 (RADIOMETER, Kobenhaven) titrator. The acid constants were calculated using the Henderson–Hasselbach equation (*1*)

$$pK_a = pH - \log ([A^-]/[HA]) , \quad (1)$$

where $[A^-]$ is the concentration of the geminal diol anion and [HA] is the concentration of undissociated geminal diol.

The pK_a values of compounds **7b** and **7c** were determined spectrophotometrically by measuring the dependence of absorbance A on the pH of their $1.0 \cdot 10^{-4}$ M aqueous solutions at 25 °C at the absorption maxima of the dissociated and undissociated forms. The measurements were carried out on a Specord M42 (Carl Zeiss, Jena) instrument. The pH values were adjusted with 0.1 M phosphate buffers. The pK_a values were calculated by Eq. (*2*).

$$pK_a = pH + \log [(A_{max} - A_{obs})/(A_{obs} - A_{min})] \quad (2)$$

Determination of Reactivation Effectivity of Geminal Diols

The reactivation effectivity was calculated from the measured activities of the intact enzyme (A_0), Sarin-inhibited enzyme (A_I), and reactivated enzyme (A_R), using Eq. (*3*).

$$\% R = [1 - (A_0 - A_R)/(A_0 - A_I)] \cdot 100 \quad (3)$$

Determination of Activity of Intact ACE (A_0)

Rat brain homogenate (0.5 ml) was added to a 0.3 M sodium chloride solution (20 ml). Then a 0.02 M solution of ACI (2.0 ml) was added and the system was adjusted with 0.3 M sodium chloride solution to a total volume of 25.0 ml. The liberated acetic acid was titrated with 0.01 M sodium hydroxide on an RTS 822 titrator in the pH-stat mode (pH 8.0). The ionic strength of the solution was rendered with sodium chloride at constant value $I \approx 0.25$ mol l^{-1}. The slope of the linear part of the time dependence of the sodium hydroxide used represents the activity (A_0) of the intact enzyme (in fact, the initial rate v_0 of the enzymatic reaction).

Determination of Activity of Inhibited ACE (A_I)

Rat brain homogenate (0.5 ml) was treated with $5 \cdot 10^{-8}$ M aqueous solution of Sarin (0.5 ml) for 30 min, which resulted in about 85% inhibition of the enzyme. A 0.02 M solution of ACI (2.0 ml) was added and the mixture was adjusted to 25.0 ml with 0.3 M sodium chloride solution. The activity of the inhibited enzyme (A_I) was immediately determined analogously as described in the preceding experiment.

Determination of Activity of Reactivated ACE (A_R)

Reactivation of the enzyme inhibited in the above experiment was performed immediately after the inhibition. A solution (1.0 ml) of the reactivator was added to the enzyme, the resulting reactivator concentration being $1.0 \cdot 10^{-3}$ mol l^{-1}. After 10 min of reactivation at 25 °C, a 0.02 M solution of ACI (2.0 ml) was added. The mixture was adjusted to 25.0 ml with 0.3 M sodium chloride solution and

immediately afterwards the activity of the reactivated enzyme (A_R) was determined analogously as described in the preceding experiments.

Determination of Dissociation Constants K_{dis} of Enzyme–Reactivator Complexes

Appropriate volume of 0.01 M solution of the geminal diol was added to rat brain homogenate (0.5 ml) so as to achieve the desired diol concentration (C_D) in the interval $5.0 . 10^{-5}$ to $5.0 . 10^{-3}$ mol l^{-1}. Then a 0.02 M solution of ACI (2.0 ml) was added, the mixture was adjusted to 25.0 ml with 0.3 M sodium chloride and the enzyme activity (A_{INH}) was immediately determined analogously as in the preceding experiments. The dissociation constant (K_{dis}) was obtained by nonlinear regression[14] from the dependence of A_{INH} on C_D (Eq. (*4*), see ref.[15]).

$$A_{INH} = A_{max} C_S/[C_S + K_M(1 + C_D/K_{dis})], \qquad (4)$$

where, in addition to the above-defined symbols, A_{max} denotes the maximum (limiting) rate of the enzymatic reaction, C_S is substrate concentration and K_M is the Michaelis constant for hydrolysis of acetylcholine with ACE ($1.9 . 10^{-4}$ mol l^{-1}).

Kinetics of Cleavage of PNPDPP

Solutions of the geminal diols of the desired concentration were prepared in 0.05 M CAPS buffer (pH 10.4). The reaction was followed at 25.0 ± 0.1 °C using an HP 8452A spectrophotometer (diode array, Hewlett–Packard) equipped with a thermostatted multicell transport cell holder HP89075C. The reaction was initiated by addition of $5.0 . 10^{-4}$ M solution of the substrate in acetonitrile (20 μl) into the spectrophotometric cell containing a buffered solution (1.98 ml) of the geminal diol; in all cases, the resulting concentration of the substrate was $5.0 . 10^{-6}$ mol l^{-1}. The concentration of the formed 4-nitrophenoxide ion was monitored at 400 nm. The reactions followed the first-order konetics up to 100% conversion. The rate constants were obtained by nonlinear regression of the dependence of absorbance on time using program Enzfitter (R. J. Leatherbarrow, Elsevier–Biosoft, Amsterdam 1987), the error of the interpolation being not higher than 0.6%.

RESULTS AND DISCUSSION

Synthesis of Reactivators

(2,2-Dihydroxyethyl)trimethylammonium chloride (**6a**) was prepared by alkylation of trimethylamine with chloroacetaldehyde diethyl acetal and subsequent hydrolysis of the obtained (2,2-diethoxyethyl)trimethylammonium chloride (**8**). The synthesis was analogous to that of the corresponding bromide[7]. The respective quaternary pyridinium carbaldehydes **5a**–**5c** were obtained by alkylation of 2-, 3-, and 4-pyridinecarbaldehydes with methyl iodide[8]. The obtained salts were identified by their melting points and ^{1}H NMR spectra. The assumption that in aqueous solution aldehydes **5** exist predominantly in the form of hydrates was confirmed by their ^{1}H NMR spectra in deuterium oxide. The spectra of compounds **5a** and **5c** exhibited no aldehyde proton signals but, instead, they contained signals at 6.33 and 6.12 ppm due to $–CH(OD)_2$ groups in the respective hy-

drates **7a** and **7c**. In aqueous solution of salt **5b**, the ratio aldehyde : hydrate was approximately 10 : 90, as follows from the integrated signals of the groups –CH=O (10.07 ppm) and $-CH(OD)_2$ (6.14 ppm).

Affinity of Geminal Diols to ACE and Their Ability to Reactivate the Sarin-Inhibited Enzyme

The affinity of the prepared compounds to ACE and their ability to reactivate the phosphonylated enzyme was studied by *in vitro* experiments with rat brain ACE, using isopropyl methylfluorophosphonate (Sarin). The activity of the geminal diols towards intact ACE was expressed by values of dissociation constants of the ACE–diol complexes (K_{dis}). The values of K_{dis} were derived from the assumption that geminal diols behave as competitive inhibitors of cleavage of the natural substrate acetylcholine by ACE. The relation between the dissociation constant K_{dis} and the experimentally accessible quantities is given by Eq. (*4*). The obtained values of K_{dis} are shown in Table I. It is obvious that the affinity of the geminal diols to ACE is comparable with that of the natural substrate acetylcoline; in some cases (**6a** and **7b**) is even higher.

Neither of the studied compounds exhibited reactivation effects. The measured values of % *R* ranged in the interval 0–2%, *i.e.* within the range of experimental error (Table I). This failure of the geminal diols to reactivate Sarin-inhibited ACE can be explained by their low acidity. Their pK_a values are 11.0–13.8 (Table I) and thus at the physiological pH only a negligible fraction of the diols exists in the ionized form.

Reactivity of Geminal Diols Towards 4-Nitrophenyl Diphenyl Phosphate (PNPDPP)

These experiments were aimed at assessing the nucleophilicity of anions of the geminal diols. For the cleavage of PNPDPP under pseudo-first order reaction conditions, the slope of the plot of the observed rate constant (k_{obs}) *vs* concentration of the geminal diol

TABLE I
Acidity, affinity toward ACE, and reactivation ability of the prepared compounds

Compound	pK_a	$K_{dis} . 10^4$	% *R*
6a	11.2	1.3	0.3
7a	11.0	3.8	<0.1
7b	13.3	1.1	<0.1
7c	13.8	2.6	2.0
Acetylcholine		1.9	

anion (c_{A^-}) represents the second order rate constant (k_2) for this reaction. The rate constant k_2, which is a measure of nucleophilicity of anions of geminal diols, can thus be obtained from the relation k_{obs} *vs* c_{A^-} by linear regression. The c_{A^-} values are calculated from the total concentrations of the geminal diols (c_{HA}) using Eq. (*5*).

$$c_{A^-} = c_{HA} K_a/([H^+] + K_a) \qquad (5)$$

The rate constants k_{obs} were determined at 25 °C and pH 10.4 (0.05 M CAPS buffer). Under the described conditions, compounds **7** decomposed, probably as a result of nucleophilic attack of the pyridine nucleus by hydroxide ion[16]. The dependence of k_{obs} on concentration c_{HA} could be measured only for the geminal diol **6a** (Fig. 1). From this dependence we calculated the reaction rate $k_2 = 5.4 \cdot 10^{-2}$ l mol^{-1} s^{-1} (standard deviation $4.3 \cdot 10^{-3}$ l mol^{-1}s^{-1}).

It appears that the nucleophilicity of the anion of geminal diol **6a** is very low compared with that, *e.g.*, of the hydroxide ion ($k_2 = 0.4$ l mol^{-1} s^{-1}, ref.[17a]), choline anion ($k_2 = 3.1$ l mol^{-1} s^{-1}, ref.[17b]) or oximate anion of **1** ($k_2 = 1.0$ l mol^{-1} s^{-1}, ref.[16]). Thus, the low reactivity of the hydroxylic anion in the geminal diols also obviously contributes to their observed low reactivation ability.

CONCLUSIONS

Hydrates of aldehydes with the aldehyde group activated by the electron-accepting quaternary ammonium group are not suitable for reactivating phosphonylated or phosphorylated ACE. Although they exhibit affinity toward the enzyme comparable to that of the natural substrate, the concentration of nucleophilic species under physiological conditions and their reactivity are low. The surprisingly low reactivity of the geminal diol **6a** toward the model substrate PNPDPP raises the question whether the reported[9] high hydrolytic activity of the homologous compound **6b** is caused by micellar medium rather than by the catalytic cycle suggested by the authors.

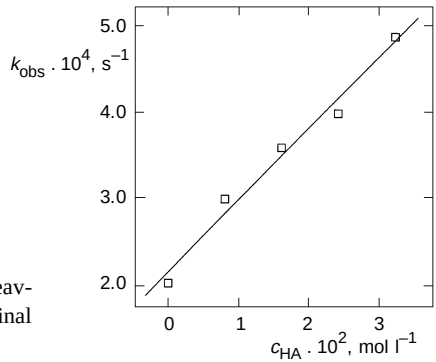


FIG. 1
Dependence of observed rate constant for cleavage of PNPDPP, k_{obs}, on concentration of geminal diol **6a**, c_{HA}

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