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ACTIVITY-STRUCTURE RELATIONSHIPS IN THE REACTIVATION OF DIETHYLPHOSPHORYL ACETYLCHOLINESTERASE BY PHENYL-1-METHYL PYRIDINIUM KETOXIMES*

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Abstract—The effect of structure on the activity of phenyl-1-methyl pyridinium ketoximes and bis quaternary derivatives in reactivating diethylphosphoryl acetylcholinesterase was investigated.

The inhibited enzyme was reactivated in the presence of acetylcholine, with a pH stat to maintain the pH and record the amount of ester hydrolyzed vs. time during the reactivation. A graphical technique was devised to obtain the pseudo first-order rate constant from this record.

The configurations of the 4-derivatives were assigned on the basis of the Beckmann rearrangement.

The *anti*-2-derivative is 20 times more active than the *syn*, whereas the *anti*-4-derivative is 6 times less active than the *syn*. The remarkable increase in activity that occurs in the bis-4-quaternary compounds occurs with both configurations.

Compared to the aldoximes, introducing a phenyl group has a modest negative effect on the activity in the 4-derivatives, but the effect is more pronounced in the 2-derivatives.

THE reaction of acetylcholinesterase with certain organophosphate compounds such as diethyl phosphofluoridate yields an enzymically inactive diethylphosphoryl enzyme derivative which can be restored to activity by numerous nucleophilic agents.[1,2] The reactivation of diethylphosphoryl acetylcholinesterase by quaternary pyridine oximes[3,4] is especially rapid and is an interesting reaction to study with regard to the relationship between the activity of the reactivator and its structure.

This reaction has some practical importance because it is the basis for an effective treatment of poisoning caused by organophosphorus insecticides. The ion N-methyl pyridinium-2-aldoxime (2PAM) is the best known of these reactivators and is remarkable, potent both as a reactivator *in vitro* of inhibited enzyme and as an antidote *in vivo* for organophosphate poisons in animals and in man.[5–8] Bis quaternary derivatives of the 4-isomer (TMB4) and of the 2-isomer are even more potent.[9–11]

Counting *syn* and *anti* configurations for each of three ring positions, six isomers are possible. Earlier thoughts that all isomers had been synthesized[12] were shown to be wrong[13] and only four isomers are now known: the *syn* and *anti* N-methyl pyridinium-4-aldoximes and the 2- and 3-aldoximes of unknown configuration.

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A detailed explanation offered several years ago[14] for the structure–activity relationships of the three ring positional isomers then known and based upon the assumption that the configurations were *anti*, was shown to be at least partly wrong when the geometric isomer of the existing 4-derivative was synthesized.[15]

Because the set of six tertiary phenylpyridyl ketoximes had been described, we decided to prepare the quaternary derivatives and study the activity–structure relationship in this closely related series. The configurations of the 2- and 3-derivatives had been assigned; we made the assignments for the 4-derivatives on the basis of the products obtained in the Beckmann rearrangement.

EXPERIMENTAL

Organic Compounds

Phenyl-2-pyridyl ketoximes[16–18]

Anti-oxime. Oximation of the ketone in methanol under acidic conditions with two equivalents of hydroxylamine hydrochloride, only half of which was neutralized with potassium hydroxide, yielded the *anti* compound when the solution was concentrated. The solid was washed with water and recrystallized twice from acetone; yield 20%, m.p. 165–167°; reported 165–167°.

Syn-oxime. The ketone was oximated in highly alkaline methanol. The acidified solution was concentrated, and the oxime that precipitated was washed with water. The product was recrystallized from methanol; yield 55%, m.p. 153–154°; reported 151–153°.

Phenyl-3-pyridyl ketoximes[19] *and phenyl-4-pyridyl ketoximes*[20]

Different conditions of alkalinity during oximation of the ketones did not appear to affect the ratio of the *syn* and *anti* isomers; roughly equal amounts were formed.

Anti-oximes. The *anti*-oximes were separated from the *syn*-oximes by taking advantage of their lesser solubility in aqueous alkali. Repeated recrystallizations from this medium followed by recrystallization from methanol gave the 3-derivative in 30% yield, m.p. 172–173°; reported 163–164°; and the 4-derivative in 40% yield, m.p. 195–197°; reported 176–177°.

Syn-oximes. The alkaline solution remaining after the separation of crude *anti*-oxime was acidified and crude *syn*-oxime precipitated. This product was dissolved in a minimal volume of hot aqueous alkali. On cooling, more crude *anti*-oxime separated, and acidification yielded a better crude *syn*-oxime. This process was repeated and, after two recrystalizations from methanol, pure compounds were obtained: the 3-derivative in 30% yield, m.p. 142–143°; reported 141–143°; and the 4-derivative in 15% yield, m.p. 155–157°; reported 152–153°.

1-*Methyl pyridinium iodides* (quaternary ketoximes). The 1-methyl pyridinium iodides were obtained in >90% yield by the reaction of the tertiary phenyl pyridyl ketoximes with methyl iodide in hot acetone.

Bis quaternary ketoximes. Symmetrical bis quaternary compounds containing a pentamethylene bridge linking the nitrogen atoms were prepared from the *syn* and *anti* 3-isomers and quaternary compounds containing a trimethylene bridge from the *syn* and *anti* 4-isomers, by the same procedure used for aldoxime derivatives[11] (data in Table 1). Mixed melting points were depressed.

TABLE 1. QUATERNARY COMPOUNDS

Compound	Color	m.p.°C	Analysis			
			C	H	N	I
Phenyl-1-methylpyridinium ketoximes, iodides						
2-*anti*	white	184–5	49·97	4·17	7·97	37·1
2-*syn*	yellow	212–3	46·36	4·20	8·04	37·8
3-*anti*	sl. yellow	242–3				37·0
3-*syn*	sl. yellow	232–4				37·4
4-*anti*	white	216–7	46·01	4·06	7·96	36·8
4-*syn*	yellow	209–10	45·79	3·89	8·08	37·5
calculated			45·90	3·85	8·24	37·3
Bis-quaternary phenyl-1-methyl-pyridinium ketoximes, iodides						
Penta methylene bridge						
3-*anti*	sl. yellow	239				35·8
3-*syn*	sl. yellow	249				35·1
calculated						35·3
Trimethylene bridge						
4-*anti*	sl. yellow	242–3				37·0
4-*syn*	sl. yellow	244				36·1
calculated						36·7

Beckmann rearrangement of phenyl-4-pyridyl ketoximes

One gram of the ketoxime was warmed for 15 min with 3 g phosphorus pentachloride. The reaction was vigorous. Ice was added to the syrupy mass, and after the reaction subsided the mixture was extracted with chloroform. When the aqueous solution was neutralized, a precipitate formed which was recrystallized from ethanol.

The product from the higher melting oxime was obtained in 80% yield. It melted at 170–171° and did not depress the melting point of an authentic sample of isonicotinoyl anilide prepared from methyl isonicotinate and aniline.

The product from the lower-melting isomer was obtained in 90% yield and melted at 202–203°. The melting point corresponds to 4-benzaminopyridine, m.p. 202°.

Enzyme

Acetylcholinesterase [acetylcholine, acetylhydrolase (3·1·1·7)] from the electric organ of *Electrophorus electricus* was partially purified by column chromatography.[22] It had a specific activity of 100 mmoles acetylcholine hydrolyzed per hour/mg protein.

Inhibition

A concentrated solution of enzyme, about 5×10^{-6} N, was reacted with 100% excess diethylphosphoryl thiocholine iodide at pH 7·0, 25°.

Reactivation

The reactivation scheme is

$$E' + R \underset{}{\overset{K}{\rightleftharpoons}} E' \cdot R \overset{k'}{\rightarrow} E + \text{product}$$

where E' is the diethylphosphoryl enzyme, R is the reactivator, $E' \cdot R$ is a complex that may form, and the product is assumed to be the O-phosphoryl oxime. Under pseudo first-order condition, $(R) \gg (E')$, the measured constant is given by

$$k = \frac{k'}{1 + \frac{K}{(R)}}$$

If $(R) \ll K$, $k = (k'/K)(R) = k_R(R)$ where k_R is the second-order rate constant. In the presence of 0·001 M acetylcholine in a medium consisting of 25 ml of 0·03 M NaCl, 0·02 M $MgCl_2$, 5×10^{-5} M EDTA, and 0·01% gelatine, k was measured, starting with completely inhibited enzyme diluted to a concentration of about 2×10^{-11} N. Acetylcholine was hydrolyzed at an increasing rate as reactivation progressed. The pH was maintained constant at 7·4, 37°, with a Radiometer pH stat and the amount of ester hydrolyzed, $-\Delta S$, was recorded as a function of time.

A graphical method was devised for obtaining k from the record of $-\Delta S$ vs. time (Fig. 1). For a first-order reactivation reaction the amount of active enzyme is given by $E = E^\circ(1 - e^{-kt})$ where E^0 is the amount of total enzyme. Since the velocity of hydrolysis, v, is proportional to E,

$$v = v^0(1 - e^{kt})$$

In our experiments, v^0 is constant; therefore

$$-\Delta S = \int_0^t v\,dt = v^0 t + \frac{v^0}{k}(e^{-kt} - 1)$$

At $t = 1/k \equiv t^0$

$$-\Delta S = 0{\cdot}368\, v^0 t^0$$

The quantity $v^0 t^0$ is the amount of ester that is hydrolyzed with uninhibited enzyme in time t^0. Therefore, a line originating at the start of the reactivation, drawn with a slope 0·368 times the slope of the record made with uninhibited enzyme, intercepts the curve at t^0. This method is illustrated in Fig. 1.

If lines are drawn with slopes 0·116, 0·215, or 0·295 of the value of the slope of the line obtained with uninhibited enzyme, the intercepts with the reactivation curve will occur at $0{\cdot}25t^0$, $0{\cdot}50t^0$, or $0{\cdot}75t^0$. Once t^0 is obtained, the correspondence of the reactivation curve with the theoretical curve can be judged with the aid of the following table.

t/t^0	$-\Delta S/v^0 t^0$
0·2	0·019
0·4	0·070
0·6	0·149
0·8	0·249
1·0	0·368
1·2	0·501
1·4	0·647

In those cases where the reactivator acted as a reversible inhibitor of the enzyme during the reactivation, the reference slope was determined by assaying the uninhibited enzyme in the presence of the reactivator.

RESULTS

Organic. The terms *syn* and *anti* refer to the relationship of the hydroxyl group and the phenyl group in the phenyl-pyridylketoxime series.

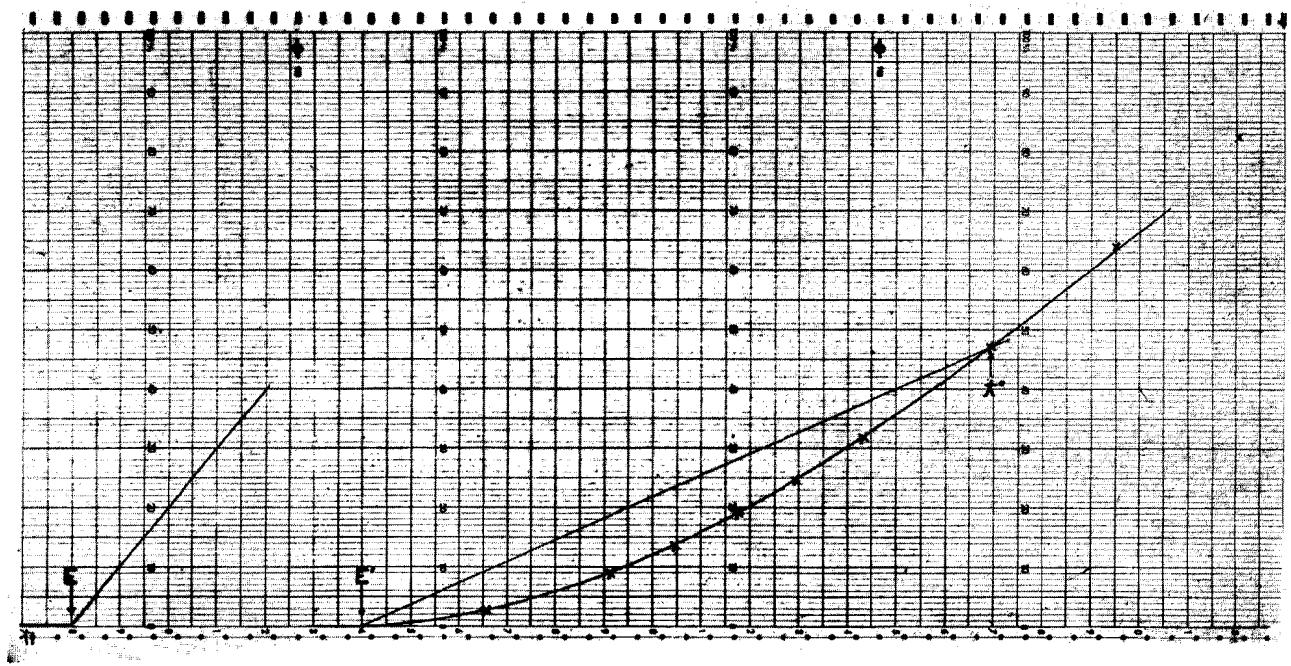

FIG. 1. A reproduction of the experimental record obtained with the Radiometer automatic titrator. Each major division on the ordinate represents 0·375 μmole NaOH; each line on the abscissa is equivalent to 1 min.

Measurements were made with 4×10^{-7}M *syn*-bis-phenylketoxime (compound 11), at pH 7·4, 37°, in a medium consisting of 1×10^{-3} M acetylcholine bromide, 0·03 M NaCl, 0·02 M $MgCl_2$, 0·01% gelatin, and 5×10^{-5} M EDTA, with a "CO_2-free" technique. Uninhibited enzyme is added at point E and the rate of enzymic hydrolysis of acetylcholine obtained. In a separate experiment completely inhibited enzyme is added at point E′ and the time course of the hydrolysis of acetylcholine is recorded. A line starting at E′ was drawn with a slope 0·368 times the slope of the line obtained with uninhibited enzyme. The intercept with the reactivation record occurs at $t^\circ = 1/k$. The x's are points calculated from t° (see text).

In the Beckmann rearrangement, the higher melting isomer of phenyl-4-pyridyl ketoxime yielded isonicotinoyl anilide, whereas the lower melting isomer yielded 4-benzaminopyridine. The former is therefore assigned the *anti* configuration and the latter the *syn*.

Because the *syn* and *anti* isomers yield different methiodides, we can safely assume that the configuration is retained during N-methylation. The alternative assumption requires the reversal of the configuration in each case, even though the alkylation occurs remote from the oxime functional group. The isomers of pyridine-4-aldoxime yielded different methiodides which from their nuclear magnetic resonance spectra were judged to have retained the parent configuration.[15]

Method. Our method for obtaining k, the pseudo first-order rate constant, in the reactivation of diethylphosphoryl enzyme is illustrated in Fig. 1 with phenyl-1, 1′-trimethylene-bis-pyridinium-4-diketoxime, compound 11. The suitability of the method is attested to by the correspondence between the recorded curve and the points calculated from this value of k.

Since the reactivation rate constant was the same in the presence of 0·005 M and 0·001 M acetylcholine, we know that 0·001 M acetylcholine, the standard concentration, does not interfere with the reactivation.

Reactivation. Since the enzyme concentrations, approximately 10^{-11} normal, were in all cases very much less than the reactivator concentrations, first-order conditions prevailed. The first-order constants we obtained were proportional to the concentra-

TABLE 2. BIMOLECULAR RATE CONSTANTS FOR THE REACTIVATION OF DIETHYLPHOSPHORYL ACETYLCHOLINESTERASE

No.	Compound (iodides)	Configuration	k (liters/moles min)	Relative rate
1	1-Methyl pyridinium-2-aldoxime	?	$4{\cdot}6 \times 10^{4}$	1·00
2	1-Methyl pyridinium-4-aldoxime	syn	$6{\cdot}3 \times 10^{2}$	$1{\cdot}4 \times 10^{-2}$
3	1,1′-Trimethylene-bis-pyridinium-4-dialdoxime	syn	$2{\cdot}6 \times 10^{5}$	5·6
4	1,1′-Trimethylene-bis-pyridinium-2-dialdoxime	?	$4{\cdot}5 \times 10^{3}$	$1{\cdot}0 \times 10^{-1}$
5	1,1′-Pentamethylene-bis-pyridinium-2-dialdoxime	?	$1{\cdot}5 \times 10^{5}$	3·2
6	1,1′-Oxydimethylene-bis-pyridinium-4-dialdoxime	syn	$2{\cdot}4 \times 10^{5}$	5·2
7	Phenyl-1-methyl pyridinium-2-ketoxime	syn	$3{\cdot}8 \times 10^{1}$	$8{\cdot}3 \times 10^{-4}$
8	Phenyl-1-methyl pyridinium-2-ketoxime	anti	$8{\cdot}0 \times 10^{2}$	$1{\cdot}7 \times 10^{-2}$
9	Phenyl-1-methyl pyridinium-4-ketoxime	syn	$1{\cdot}7 \times 10^{2}$	$3{\cdot}7 \times 10^{-3}$
10	Phenyl-1-methyl pyridinium-4-ketoxime	anti	$3{\cdot}0 \times 10^{1}$	$6{\cdot}5 \times 10^{-4}$
11	Phenyl 1, 1′-trimethylene-bis-pyridinium-4-diketoxime	syn	$9{\cdot}8 \times 10^{4}$	2·1
12	Phenyl 1, 1′-trimethylene-bis-pyridinium-4-diketoxime	anti	$4{\cdot}7 \times 10^{4}$	1·0

tions of the reactivators, indicating that the reactivations are bimolecular and that the concentrations used were definitely smaller than the dissociation constants for the reactivator-inhibited enzyme complexes.

The second-order rate constants are given in Table 2. Only the measurements with the phenyl-pyridinium ketoximes are original with this work; the other compounds

have been previously studied,[3, 4, 9–11, 23] but the values obtained in this work are included for comparisons.

In preliminary experiments the 3-derivatives proved to be very poor reactivators and we did not study them further.

DISCUSSION

In the few cases where comparisons can be made, there seems to be a correspondence in the activity-structure relationship within the phenylketoxime and aldoxime series. Thus the 3-derivatives of both series are much less active than the other members. Similarly the *syn*-bis-pyridinium-4-dialdoxime containing a trimethylene bridge (TMB4), compound 3, is 360 times more active than the *syn*-monoquaternary methiodide (4PAM), compound 2, while *syn*-phenyl-bis-pyridinium-4-diketoxime containing a trimethylene bridge, compound 11, is 570 times more active than the corresponding monoquaternary reactivator, compound 9. It is interesting that the much greater activity found in the bis-4 compounds occurs with both configurations in the phenylketoxime series. Presumably the same will be found in the aldoxime series.

Again, *syn*-1-methyl pyridinium-4-aldoxime is three times more active than the *anti* isomer[15] and, similarly, *syn*-phenyl-1-methyl pyridinium-4-ketoxime, compound 9, is six times more active than the *anti* isomer, compound 10.

The *anti* isomer of phenyl-1-methyl pyridinium-2-ketoxime, compound 8, is twenty times more active than the *syn* isomer, compound 7. On the basis of the seeming correspondence between the aldoxime and phenylketoxime series, one might hazard the guess that *anti*-2PAM will prove to be more active than *syn*-2PAM. If the presently available 2PAM should have the *syn* configuration, an effort to obtain the *anti* isomer might be rewarding.

It is clear that we cannot extrapolate the effect of the spacial relationships of the oxime function found in the ketoxime series to the aldoxime series because the position of the phenyl group is also involved. Even though Table 2 shows that the phenyl group has only a modest negative effect on the activity of the 4-derivatives, the effect on the 2-derivatives is more pronounced and, in just this case, the position of the phenyl group may obscure the effect of the position of the oxime function.

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