A Kinetic Study of the Reaction between 1,1′-Trimethylenebis(4-hydroximinomethylpyridinium) Dibromide and Diisopropyl Phosphorofluoridate[1]

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The SN2 reaction in aqueous solutions, between the oxime 1,1′-trimethylenebis(4-hydroximinomethylpyridinium) dibromide (TMB_4) and diisopropyl phosphorofluoridate (DFP), has been studied by measuring the rate of appearance of fluoride ion, in the pH range 7.4–9.1. The dioximate species reacts only 1.6 times as fast as the monooximate, and its contribution in the over-all reaction at pH 7.4 does not exceed 8.0%.

1,1′-Trimethylenebis(4-hydroximinomethylpyridinium) dibromide (TMB_4) is a powerful reactivator of cholinesterase that had been inhibited with organophosphorus esters.[2] Reactivation with this and related oximes is a composite process. It partly arises from the ability of the reactivator molecule to associate reversibly with the inhibited enzyme. It also depends on the nucleophilicity of the oximate ion which must eventually displace the phosphoryl residue by an SN2 type reaction. With few exceptions,[3] reactivation rates, which form the basis for comparison between existing and potential reactivators, have been given for the over-all reaction. This approach, especially in the case of TMB_4 which embodies two nucleophilic centers, is not helpful in understanding the separate contribution of each of these groups.

We wish to present now a new approach to the study of the reaction between TMB_4 and a convenient substrate, diisopropyl phosphorofluoridate (DFP). This reaction is essentially analogous to the second step of the reactivation process. Two new elements have been introduced in the present study. (a) The sole criterion for measuring the rate of reaction between the oxime and DFP is based on the rate of appearance of fluoride ion. In all earlier studies, the rate of acid formation was taken as the rate of the reaction. However, there is no reason to assume that the breakdown of the phosphorylated oxime derivatives (*e.g.*, C_1, C_2, and C_3, below) with concomitant liberation of acid is a fast reaction. On the contrary, studies with related compounds[4] put into evidence the relative stability of such intermediates which, under slightly basic conditions, lose the phosphoryl group by a Beckmann elimination mechanism, yielding nitriles such as C_4, C_5, and C_6.[5] (b) TMB_4 undergoes two anionic dissociations corresponding to $pK_a = 7.99 \pm 0.02$ and 8.68 ± 0.02. These values have been obtained by careful potentiometric titration of the oxime and are confirmed by the recent report of Bieger and Wasserman.[6] The conclusions reached in earlier studies, based on a single pK_a of 8.2,[7] are, therefore, questionable.

Experimental Section

TMB_4 was prepared according to Poziomek[7] and purified by recrystallization from EtOH–H_2O, mp 240–242°. DFP was prepared according to Chapman[8] and purified by fractional distillation.

pK_a determinations were made by potentiometric titration of TMB_4 (21.2 mg) in KCl solution (25 ml, total ionic strength 0.2) at 25°. The titrant, 0.084 *N* KOH solution, was delivered stepwise from a microburet and the change in pH was read on the expanded scale of a Radiometer, Model 25 pH meter. The theoretical end point was calculated according to the nonlogarithmic method of Benet and Goyan.[9] pK_a values were then calculated according to Noyes[10] as given by Albert and Serjeant.[11] The results of a typical run are given in Table I.

TABLE I
SEPARATION OF OVERLAPPING pK_a VALUES OF TMB_4[a]

0.084 *N* KOH, ml	pH	pK_{a_1}	pK_{a_2}
0.5	8.195	7.98	8.67
0.675	8.47		
0.45	8.12	7.99	8.66
0.725	8.55		
0.4	8.035	7.99	8.68
0.775	8.64		
0.3	7.84	7.97	8.68
0.875	8.83		
0.225	7.66	7.96	8.67
0.95	8.99		

[a] 21.2 mg of TMB_4 in 25.0 ml of 0.2 *N* KCl at 25°.

The rate of fluoride ion formation was followed potentiometrically by direct reading of fluoride ion concentration in the reaction mixture. The potential established between a Model 94-09 fluoride ion activity electrode (Orion Research, Inc.), and the reaction solution was read on the expanded scale of a Radiometer Model 25 pH meter with reference to a standard calomel electrode. The potentiometric readings, in millivolts, were then translated into F^- concentration by using a calibration curve which had been previously prepared for solutions of simular pH and ionic strength, and containing a known fluoride ion concentration.

In a typical run, a freshly prepared mixture of 0.10 *M* phosphate buffer (pH 7.4–9.1) (10 ml), KCl (1 *M*, 2.0 ml), NaF (1.26×10^{-3} *M*, 0.25 ml), a solution of DFP in *i*-PrOH (3.66×10^{-2} *M*, 1.0 ml) and distilled H_2O (6.75 ml) was stirred and left to equilibrate with the electrode for 3 min. A solution of TMB_4 (4.6×10^{-2} *M*) in buffer of the same pH (1.0 ml) was then added; this is t_0. Millivolt readings were then taken at 1-min intervals. The results of a run at pH 7.4 are given in Figure 1. Plots at pH

(1) This investigation is part III of a series; part II: Y. Ashani and S. Cohen, *Israel J. Chem.*, **5**, 59 (1967).

(2) F. Hobbiger, D. G. O'Sullivan, and P. W. Sadler, *Nature*, **182**, 1488 (1958).

(3) A. L. Green and H. J. Smith, *Biochem. J.*, **68**, 28, 32 (1958).

(4) J. C. Lamb, G. M. Steinberg, S. Solomon, and B. E. Hackley, *Biochemistry*, **4**, 2475 (1965).

(5) G. M. Steinberg and S. Solomon, *ibid.*, **5**, 3142 (1966).

(6) D. Bieger and O. Wasserman, *J. Pharm. Pharmacol.*, **19**, 844 (1967).

(7) E. J. Poziomek, B. E. Hackley, and G. M. Steinberg, *J. Org. Chem.*, **23**, 714 (1958).

(8) N. B. Chapman and B. C. Saunders, *J. Chem. Soc.*, 1010 (1948).

(9) L. Z. Benet and J. E. Goyan, *J. Pharm. Sci.*, **54**, 1179 (1965).

(10) A. A. Noyes, *Z. Physik. Chem.*, **11**, 495 (1893).

(11) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen and Co. Ltd., London, 1962, pp 51–56.

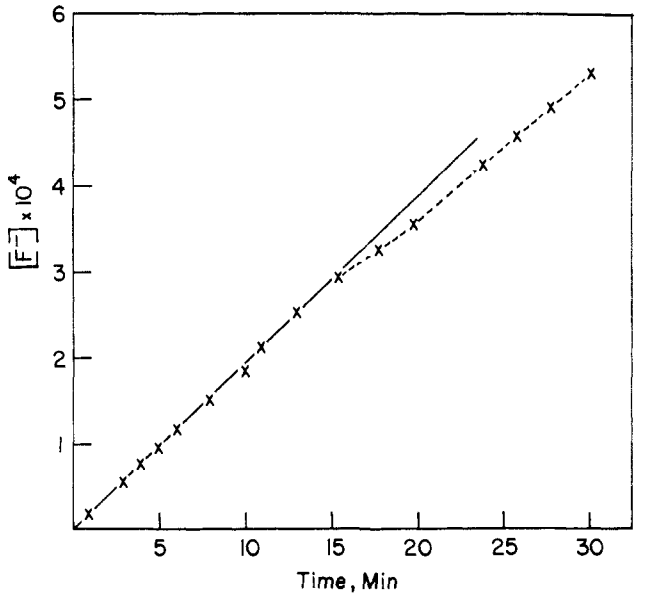
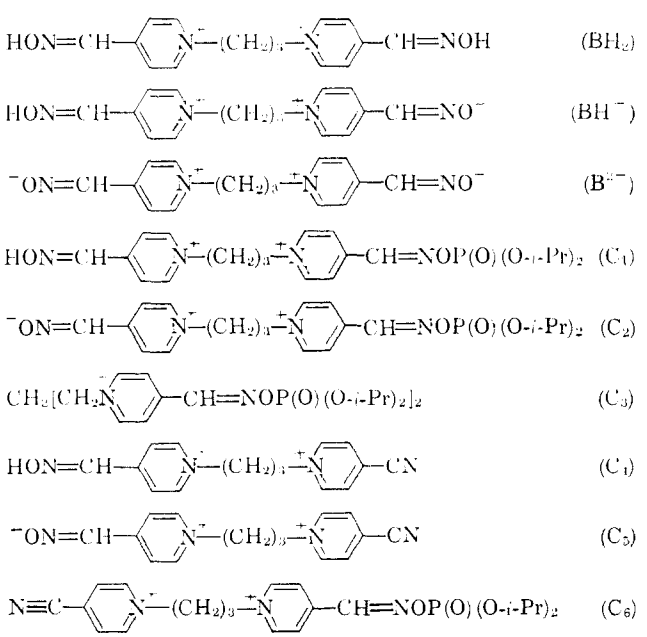


Figure 1.—Rate of fluoride ion formation at 25°; pH 7.4 0.05 M phosphate buffer, 0.1 N KCl, $[TMB_4] = 23.1 \times 10^{-4}$ M, $[DFP] = 18.3 \times 10^{-4}$ M.

values 7.4–9.1 have the same shape. In all cases, the theoretical quantity of F^- was eventually evolved. A plot of the initial velocity at pH 7.4 against the concentration of TMB_4 is given in Figure 2.

Results and Discussion

As a working hypothesis, the following species are assumed to be involved in the reaction between TMB_4 and DFP in dilute, slightly basic aqueous solutions.



The uncharged form of TMB_4 does not react with DFP under similar conditions.[12]

(12) C. B. Koelle, "Cholinesterases and Anticholinesterase Agents," Springer-Verlag, Berlin, 1963, p 930.

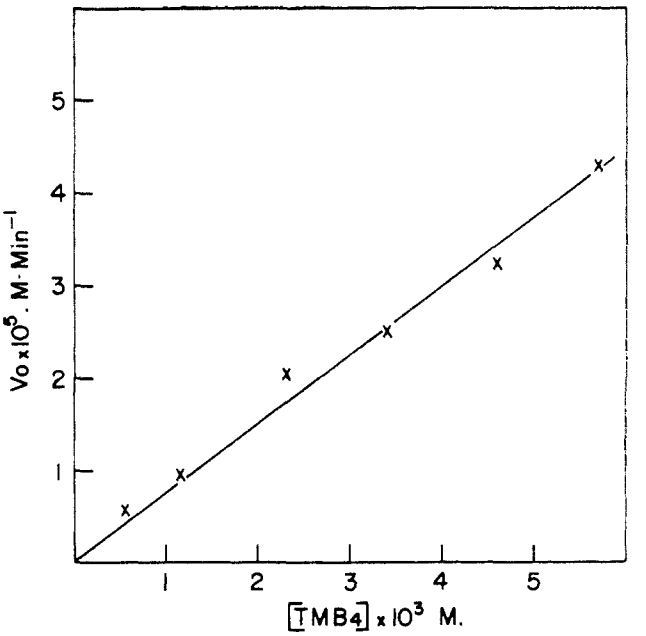


Figure 2.—Plot of the initial rate of fluoride ion formation against TMB_4 concentration at 25°; pH 7.4 0.05 M phosphate buffer, 0.1 N KCl, $[DFP] = 18.3 \times 10^{-4}$ M.

The steps leading to fluoride ion formation are as follows.

$$BH_2 + OH^- \underset{}{\overset{K_1}{\rightleftharpoons}} BH^- + H_2O \quad (1)$$

$$BH^- + OH^- \overset{K_2}{\rightleftharpoons} B^{2-} + H_2O \quad (2)$$

$$BH^- + DFP \xrightarrow{k_1} C_1 + F^- \quad (3)$$

$$B^{2-} + DFP \xrightarrow{k_2} C_2 + F^- \quad (4)$$

$$C_1 + OH^- \overset{K_3}{\rightleftharpoons} C_2 + H_2O \quad (5)$$

$$C_2 + DFP \xrightarrow{k_3} C_3 + F^- \quad (6)$$

$$C_1 \xrightarrow{k_4} C_4 \quad (7)$$

$$C_2 \xrightarrow{k_5} C_5 \quad (8)$$

$$C_5 + OH^- \overset{K_4}{\rightleftharpoons} C_6 + H_2O \quad (9)$$

$$C_5 + DFP \xrightarrow{k_6} C_6 + F^- \quad (10)$$

Accordingly, the differential equation for the rate of appearance of F^- would then be

$$\frac{d[F^-]}{dt} = k_1[BH^-][DFP] + k_2[B^{2-}][DFP] + k_3[C_2][DFP] + k_6[C_5][DFP] \quad (11)$$

or

$$\frac{d[F^-]}{dt} = [DFP](k_1[BH^-] + k_2[B^{2-}] + k_3[C_2] + k_6[C_5]) \quad (12)$$

The analytical solution of the above equations may not be obtainable. Instead, the differential method[13] was

(13) K. J. Laidler, "Chemical Kinetics," McGraw-Hill Book Co., Inc., New York, N. Y., 1950, pp 15–16.

TABLE II
RATE OF APPEARANCE OF FLUORIDE ION IN THE REACTION BETWEEN TMB_4 AND DFP AT 25°[a]

Run no.	pH[b]	α[c]	β[d]	$[A]_0 \times 10^4$	$[D]_0 \times 10^4$	$V_0 \times 10^5$, M min^{-1} Obsd[e]	Calcd[f]
1	7.4	0.209	0.011	5.40	18.30	0.57	0.44
2	7.4	0.209	0.011	11.55	18.30	0.96	0.94
3	7.4	0.209	0.011	23.10	18.30	2.06	1.88
4	7.4	0.209	0.011	34.60	18.30	2.50	2.82
5	7.4	0.209	0.011	46.20	18.30	3.20	3.76
6	7.4	0.209	0.011	57.70	18.30	4.30	4.70
7	7.9	0.427	0.071	4.50	26.40	1.60	1.26
8	7.9	0.427	0.071	9.00	26.40	2.80	2.53
9	7.9	0.427	0.071	18.00	26.40	5.50	5.05
10	7.9	0.427	0.071	27.00	26.40	8.00	7.58
11	8.1	0.499	0.131	6.70	26.40	2.70	2.47
12	8.1	0.499	0.131	13.40	26.40	5.30	4.94
13	8.1	0.499	0.131	20.10	26.40	7.35	7.41
14	8.1	0.499	0.131	26.80	26.40	10.60	9.88
15	8.65	0.464	0.434	22.40	19.00	9.10	9.76
16	8.75	0.426	0.500	22.40	19.00	9.70	10.34
17	8.8	0.404	0.533	22.40	19.00	10.60	10.61
18	9.0	0.314	0.655	22.40	19.00	12.40	11.51
19	9.1	0.270	0.709	22.40	19.00	10.50	11.88
20	9.1	0.270	0.709	22.40	19.00	13.60	11.88

[a] All symbols have the same meaning as in eq 14. [b] 0.05 M phosphate buffer. [c] Calculated from the equation $\alpha = K_1[H^+]/(K_1K_2 + K_1[H^+] + [H^+]^2)$. [d] Calculated from the equation $\beta = K_1K_2/(K_1K_2 + K_1[H^+] + [H^+]^2)$. [e] Derived from the initial slope when $t = 0$. [f] Calculated according to the equation $V_0 = (19.6\alpha + 31.9\beta)[A]_0[D]_0$.

used. Thus, when $t = 0$, both C_2 and C_5 vanish and eq 12 may be simplified to

$$V_0 = [D]_0(k_1[BH^-]_0 + k_2[B^{2-}]_0) \quad (13)$$

where the subscript zero refers to initial quantities at $t = 0$, and V_0 is the initial rate of fluoride ion formation.

Equation 13 can be solved for k_1 and k_2 if values for V_0 are known at least at two different pH values. In practice the following modification of eq 13 has been used to determine k_1 and k_2.

$$V_0/\alpha[D]_0[A]_0 = k_1 + k_2\beta/\alpha \quad (14)$$

where α is fraction of TMB_4 present as the monoanion BH^-, β is fraction of TMB_4 present as dianion B^{2-}, and $[A]_0$ is initial concentration of TMB_4.

Twenty kinetic runs were performed at eight different pH values in order to obtain good statistical results (Table II). The constants k_1 and k_2, calculated according to eq 14 by the least-square method, have the values $k_1 = 19.6 \pm 1.8\ M^{-1}$ min^{-1} and $k_2 = 31.9 \pm 2.1\ M^{-1}$ min^{-1} at 25°.

In view of the low ratio of k_2 to k_1 (1.6), one would relate the slightly higher reactivity of the dianion of TMB_4 (with respect to the monoanion) to statistical factors rather than higher order of nucleophilicity in the former species. Theoretically, at least, there is two times the probability for DFP to react with B^{2-}, which is a symmetrical molecule, than with BH^-.

At pH 7.4 which is also the physiological pH, this slight advantage becomes negligible in view of the relatively low concentration of the dianion. Under these conditions it may be shown that the contribution of this species in the over-all reaction with DFP, expressed as $100k_2\beta/(k_1\alpha + k_2\beta)$, does not exceed 8%.

The reactivation of phosphorylated cholinesterase with TMB_4 should be considered in the light of the above findings.

Formally, the displacement of the phosphoryl residue from the inhibited enzyme is an analogous reaction which depends on the nucleophilicity and concentration of the attacking species, its size and shape. Since the nucleophilicity of the dioximate anion is negligible in comparison with that of the monooximate, reactivation could benefit from the former species only if it is present in a high enough concentration. Under fixed physiological conditions, this proposition requires the design of oximes sterically related to TMB_4 but that also enjoy higher dissociation constants.