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HYDROLYSIS OF *S*-2-(3-AMINOPROPYLAMINO)ETHYL-PHOSPHOROTHIOATE (WR-2721)

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Abstract—The hydrolysis reaction of *S*-2-(3-aminopropylamino)ethylphosphorothioate (WR-2721), a radioprotective agent currently undergoing clinical trials, was studied under a variety of experimental conditions in order to provide more complete data and to reconcile significant differences found between two previous studies. ^{31}P NMR spectroscopy was primarily used to follow the reaction, but comparable results were also obtained in parallel studies using a spectrophotometric technique and a technique involving liquid chromatography with electrochemical detection, in which the free sulfhydryl product, 2-(3-aminopropylamino)ethanethiol (WR-1065), was measured. Upon hydrolysis, inorganic phosphate and the free sulfhydryl group were formed by cleavage of the P–S bond. The reaction rate versus pH profile at 30° in 42.5 mM buffer, μ = 127.5 mM, showed primarily hydrolysis of the monoanion, with an acid-catalyzed reaction below pH 1.5 to 2.0 involving the neutral species of the ester. The energy of activation at pH 4.0 in 42.5 mM acetate buffer was 25.7 kcal/mole (23.1 kcal/mole by liquid chromatography with electrochemical detection). The entropy of activation at pH 4.0, 36° was positive, and there was a deuterium isotope effect on the reaction. A small buffer effect on the rate of the reaction at pH 4.0 and pH 5.0 was found to include contributions from both general acid and general base catalysis. These data are consistent with a mechanism for hydrolysis of the monoanion involving a partially rate-determining proton transfer to the sulfur atom and the formation of metaphosphate ion, which is rapidly hydrolyzed to inorganic phosphate.

S-2-(3-Aminopropylamino)ethylphosphorothioate (WR-2721) is now undergoing clinical trials as a radiation protective and chemoprotective agent for normal tissue cells [1–3]. Recently, this compound has also been shown to have a clinically significant hypocalcemic effect [4]. Presumably, the protective effect at the cellular level is derived from the dephosphorylated free sulfhydryl metabolite [5], which is believed to be the product of a phosphatase enzyme-catalyzed reaction [6]. Current evidence suggests that the phosphatase may be an alkaline phosphatase [7]. After oral administration of the compound, a significant amount of the radioprotective activity of the compound is quickly lost, perhaps due to an acid-catalyzed hydrolysis of the ester bond in the stomach prior to absorption [6]. It is obviously important to understand the chemical reactions of a substance in clinical trials so that the appropriate delivery mode and protocols may be chosen in order to derive the maximum benefit from the compound. For WR-2721, there are two published studies on the hydrolysis reaction [6, 8]; however, both papers are devoid of critical experimental details and do not report essential data that are necessary for a full understanding of the reaction. Due to the lack of specifics, it is impossible to reconcile two major differences that exist between the two reports: (1) an approximately 4- to 6-fold difference in rate constants, and (2) the form of the pH-rate profile below pH 3, where the maximum rate constants reportedly occur at different pH values and the shapes of the two pH-rate profiles are significantly different. In light of its possible clinical significance, we felt that the hydrolysis reaction for WR-2721 should be studied again with the intention to report a complete set of data and to reconcile the major discrepancies between the two previous studies. Whereas spectrophotometric techniques were used to analyze the reaction in both prior studies, we have attempted to conduct as nearly a definitive study as possible. To this end, we have utilized three independent methods to follow the reaction. ^{31}P NMR† spectroscopy was used to the greatest extent in the present study. In addition, the independent techniques of LCEC analysis and a spectrophotometric assay based on DTNB were also used.

METHODS

S-2-(3-Aminopropylamino)ethylphosphorothioate (WR-2721), 2-(3-aminopropylamino)ethanethiol (WR-1065), and 3-(4-aminobutylamino)pro-

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† Abbreviations: NMR, nuclear magnetic resonance; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); LCEC, liquid chromatography with electrochemical detection; FID, free induction decay; E_a, Arrhenius energy of activation; $\Delta S^{\ddagger}$, entropy of activation.

panethiol (WR-251833) were supplied by Dr. Lawrence Fleckenstein of the United States Army Medical Research and Development Command at the Walter Reed Army Institute of Research. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB; Ellman's reagent) was from the Sigma Chemical Co. Double-distilled, deionized water was used to prepare all solutions. Deuterium oxide (99.75 atom% ^2H, Baker) was used, and all other chemicals were analytical grade.

^{31}P NMR spectroscopy. Buffer solutions of ionic strength $\mu = 127.5$ mM with sodium chloride were prepared containing 10% deuterium oxide for instrumental lock. pH meter readings were not corrected for the presence of 10% deuterium oxide, but the pD of solutions prepared in 100% deuterium oxide were corrected. The buffer solutions were equilibrated at the desired temperature (± 1°) for at least 30 min. An NTC-200 NMR spectrometer operating at 80.99 MHz and fitted with a 20-mm probe was equilibrated at the same temperature (± 1°) for at least 30 min. To 5.0 ml of the buffer solution in a 12-mm tube was added 6 mg of WR-2721 to give a 5 mM solution; a vortex plug was inserted. A ± 1000-Hz sweep width (quadrature phase detection), a 90° pulse angle, and a 16K data block were used; protons were broad-band decoupled and a line broadening factor was applied to the accumulated FID. The areas under the peaks were calculated and the pseudo-first-order rate constant for the hydrolysis reaction was obtained from the slope of the plot of the natural logarithm of the peak area for the ester as a function of time. Based on least squares standard error analysis, the precision in the pseudo-first-order rate constant is ± 5%.

DTNB assay. Buffer solutions containing 42.5 mM sodium acetate, $\mu = 127.5$ mM with sodium chloride, 10% D_2O, pH 4.0, were equilibrated in a water bath at the desired temperature (± 0.5°) for at least 30 min prior to the addition of WR-2721 to a final concentration of 5 mM. At regular intervals, a 15-μl aliquot was removed and the reaction was quenched in 1.785 ml of 50 mM phosphate buffer, pH 7.0, at room temperature. The assay for the free sulfhydryl group was done with DTNB [9]. To the 1.8-ml quenched solution was added 0.2 ml of 10 mM DTNB in 50 mM phosphate buffer, pH 7.0. The reaction is immediate, but at least 5 min were allowed to pass before spectrophotometric measurements were taken. In addition, although the color is stable for hours, the molar ratio of DTNB to WR-2721 in the final solution must be 16- to 80-fold to ensure reproducible results. The absorbance was read at 412 nm. A linear least squares fit to a plot of $\ln(A_\infty - A_t)$ versus time gave the pseudo-first-order rate constant, k, as the negative value of the slope.

LCEC assay. Buffer solutions were prepared as described above for ^{31}P NMR spectroscopy except that no deuterium oxide was used. To 49 ml of buffer solution was added 1 ml of 10 mmoles/liter WR-2721, and the solution was placed in a water bath set at the desired temperature. At time intervals an aliquot was removed from the reaction mixture and immediately brought to 0° in an ice bath in order to stop the reaction. Aliquots (20 μl) were then injected into the LCEC chromatograph. The Bioanalytical Systems LC-154 liquid chromatograph included a dual piston pump operated at 3000 psi, and a single mercury/gold detector was used [7]. The electrode potential was set at + 0.15 V with respect to an Ag/AgCl reference electrode. The 5-μm octadecylsilane column (250 × 4.6 mm) was maintained at 25° with a constant temperature jacket. The elution of WR-1065 and the internal standard WR-251833 was achieved isocratically using a methanol/water (40:60) mobile phase containing 0.1 M monochloroacetic acid and 1.5 mM sodium octylsulfate, pH 3.0, at a flow rate of 1.3 ml/min [10].

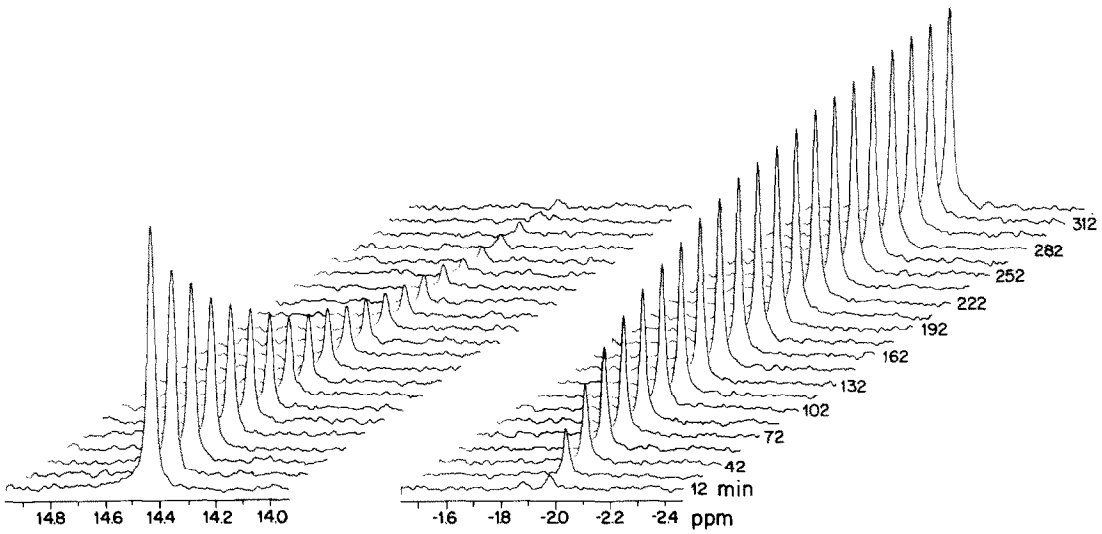

Fig. 1. Hydrolysis of the *S*-phosphorothioate monoester WR-2721 (left) and the formation of inorganic phosphate (right) followed by ^{31}P NMR spectroscopy. WR-2721 (5 mM) was dissolved in 42.5 mM malonate, $\mu = 127.5$ mM with sodium chloride, 10% D_2O, pH 3.5, and equilibrated at 30°. The pseudo-first-order rate constant for the reaction was 180×10^{-6} sec^{-1}.

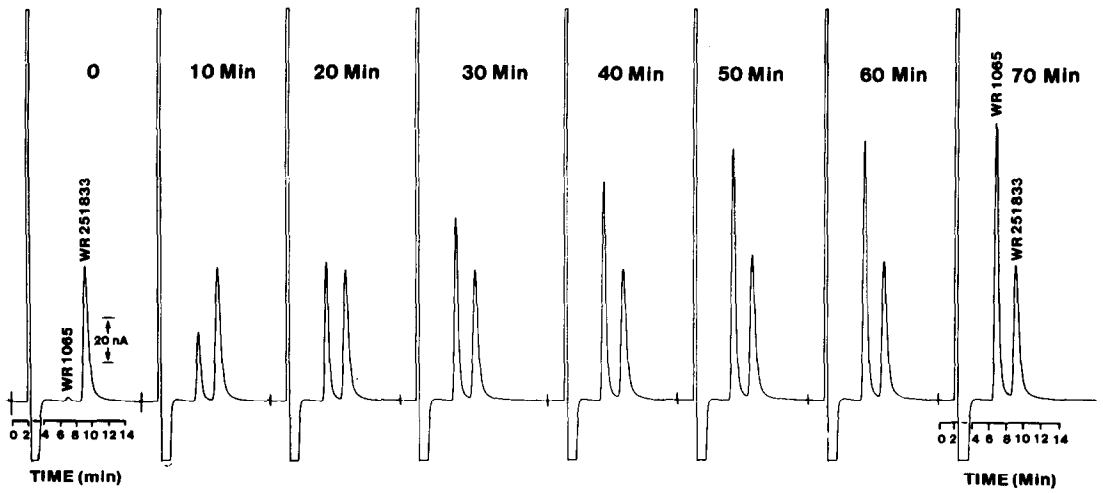

Fig. 2. Formation of WR-1065 resulting upon hydrolysis of WR-2721. WR-2721 was dissolved in 42.5 mM HCl, μ = 127.5 mM with sodium chloride, pH 1.5, at 37°and the reaction was followed by LCEC using a mercury/gold electrode detector. The pseudo-first-order rate constant for the reaction was 512 × 10^{-6} sec^{-1}. The internal standard, WR-251833, was added to aliquots of the reaction mixture to a final concentration of 100 μM at the indicated incubation times.

RESULTS

Typical hydrolysis reactions of WR-2721 followed by ^{31}P NMR and by LCEC are illustrated in Figs. 1 and 2. The pseudo-first-order rate constants for the hydrolysis reaction analyzed by ^{31}P NMR spectroscopy at a given pH (pD), temperature, buffer, and buffer concentration are listed in Table 1. The pseudo-first-order rate constants for the reactions analyzed spectrophotometrically and by LCEC are listed in Table 2. The pH-rate profile for the reaction at 30° in 42.5 mM buffer is shown in Fig. 3. The Arrhenius energy of activation, E_a, for the reaction at pH 4.0 in 42.5 mM acetate buffer from the ^{31}P NMR data was 25.7 kcal/mole. (ln k vs T^{-1}; 5 points, slope 12934, y-intercept 47.6, correlation coefficient 0.997) and from the LCEC data was 23.1 kcal/mole (4 points, slope 11613, y-intercept 33.5, correlation coefficient 0.998). The entropy of activation, $\Delta S\ddagger$, was +7 e.u. at pH 4.0, 36°. There was a deuterium isotope effect on the reaction: $k_{(10\% D_2O)}/k_{(100\% D_2O)}$ at pH 1.8 was 1.7_2 and at pH 4.0 it was 1.4_0. There was a small buffer effect on the rate of the reaction at 30° at pH 4.0 and at pH 5.0. From plots of $k_{(observed)}$ versus total buffer concentration, the slope was the apparent rate constant of the buffer-catalyzed reaction (k_2') and the y-intercept was the rate constant for the reaction not catalyzed by the buffer component (k_0); at pH 4.0 $k_2' = 26 \times 10^{-6}\ M^{-1}sec^{-1}$ and $k_0 = 114 \times 10^{-6}\ sec^{-1}$, and at pH 5.0 $k_2' = 30 \times 10^{-6} M^{-1}\ sec^{-1}$ and $k_0 = 21 \times 10^{-6}\ sec^{-1}$. From a plot of k_2' against the fraction of free base in the buffer, the rate constant for catalysis by the basic component of the buffer (k_B) is found at the intercept of the fraction of free base = 1.0, and the rate constant for the catalysis by the acidic component of the buffer (k_{BH^+}) is found at the intercept where the fraction of free base equals zero. For the hydrolysis reaction at 30° in acetate buffer, $k_B = 32 \times 10^{-6}\ M^{-1}\ sec^{-1}$ and $k_{BH^+} = 25 \times 10^{-6}\ M^{-1}\ sec^{-1}$.

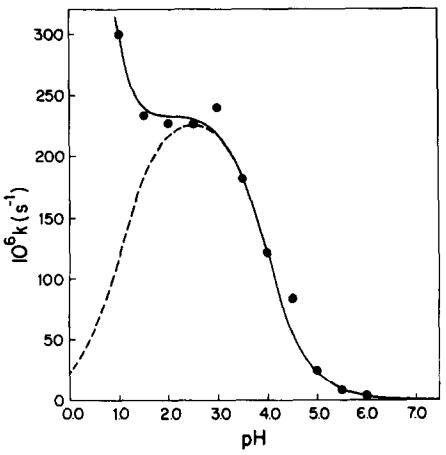

Fig. 3. pH-rate profile for the hydrolysis reaction of WR-2721 followed by ^{31}P NMR at 30° in 42.5 mM buffer, μ = 127.5 mM. The theoretical curve (solid line) was calculated assuming an acid-catalyzed hydrolysis involving the neutral ester species as well as the hydrolysis of the monoanion. The dashed line is the theoretical curve (below pH 3) assuming hydrolysis of the monoanionic species only.

DISCUSSION

The hydrolysis reaction of WR-2721 proceeds by cleavage of the P–S bond to yield a thiol and inorganic phosphate.

$$H_2NCH_2CH_2CH_2NHCH_2CH_2SPO_3H_2 + H_2O \rightarrow H_2NCH_2CH_2CH_2NHCH_2CH_2SH + H_3PO_4$$

^{31}P NMR spectroscopy provides a very easy, direct way to follow this reaction. The hydrolysis of 5 mM WR-2721 in malonate buffer at pH 3.5 that is illus-

Table 1. Pseudo-first-order rate constants for hydrolysis of WR-2721 by ^{31}P NMR

pH (pD)	Temperature (°C)	Buffer	[Buffer] (mM)	10^6 × Pseudo-first-order rate constant (sec^{-1})	Correlation coefficient	$T_{\frac{1}{2}}$ (min)
1.0	30	Oxalate	42.5	299	0.990_0	39
1.5	30	Oxalate	42.5	232	0.997_8	50
1.8	30			211	0.997_0	55
1.8*	30			123	0.999_6	94
2.0	30	Oxalate	42.5	226	0.999_5	51
2.5	30	Malonate	42.5	225	0.999_7	51
3.0	30	Malonate	42.5	239	0.999_0	48
3.5	30	Malonate	42.5	180	0.998_8	64
4.0	12	Acetate	42.5	10	0.999_0	1181
4.0	18	Acetate	42.5	25	0.999_8	472
4.0	24	Acetate	42.5	63	0.999_2	184
4.0	30	Acetate	42.5	120	0.997_2	96
4.0*	30	Acetate	42.5	86	0.997_3	135
4.0	30	Acetate	85.0	130	0.991_0	89
4.0	30	Acetate	127.5	135	0.994_8	86
4.0	36	Acetate	42.5	362	0.998_6	32
4.5	30	Acetate	42.5	83	0.999_4	139
5.0	30	Acetate	42.5	24	0.998_8	486
5.0	30	Acetate	85.0	24	0.998_3	486
5.0	30	Acetate	127.5	28	0.999_8	417
5.5	30	Acetate	42.5	7	0.996_9	1625
6.0	30	Succinate	42.5	3	0.994_6	3736
9.1	30	Tris	42.5	(<5% in 4 days)		
10.3	30	2-Amino-2-Methyl-1-Propanol	42.5	(<5% in 4 days)		

* pD.

trated in Fig. 1 is representative of a typical reaction. The chemical shifts of the ^{31}P resonance signals for the *S*-phosphorothioate and for the inorganic phosphate product were separated by approximately 14 ppm. Only these two ^{31}P NMR signals were observed during the course of the reaction, indicating that the hydrolysis reaction leading to the formation of inorganic phosphate was unaccompanied by the production of a stable intermediate, such as an *N*-phosphoramidate. The ^{31}P spectra are easily quantitated, and the calculation of the pseudo-first-order rate constant is straightforward. The rate constants, and the experimental conditions under which they were obtained, are listed in Table 1, and the pH-rate profile for the reaction in 42.5 mM buffer, μ = 127.5 mM at 30° is shown in Fig. 3. The data obtained from the spectrophotometric analysis with DTNB (Table 2) are comparable to the data obtained by ^{31}P NMR. Using LCEC, it was possible to study the rate of appearance of the free sulfhydryl product of the hydrolysis reaction, WR-1065. The chromatograms in Fig. 2 show the LCEC measurement of WR-1065 that resulted from the hydrolysis of 0.2 mM WR-2721 in 42.5 mM HCl, pH 1.5, 37°. After an initial buffer peak, the mercaptan product (WR-1065) of the hydrolysis reaction eluted from the column, while the internal standard (WR-251833) was retained slightly longer. This allows for good separation of the two signals under the conditions used here, and thus simplifies the analysis of the chromatograms for qualitative detection and quantitative measurements. The chromatograms were scaled to the constant concentration of the internal standard, which allows one to follow the hydrolysis reaction

Table 2. Pseudo-first-order rate constants for hydrolysis of WR-2721 as measured by a DTNB assay and by LCEC

pH	Temperature (°C)	Buffer	[Buffer] (mM)	10^6 × Pseudo-first-order rate constant (sec^{-1})	Correlation coefficient	$T_{\frac{1}{2}}$ (min)
DTNB						
4.0	24	Acetate	42.5	57	0.992_8	203
4.0	30	Acetate	42.5	123	0.994_6	94
4.0	36	Acetate	42.5	326	0.997_5	35
LCEC						
1.5	37	HCl	42.5	512	0.998_1	23
2.4	37	HCl	4.25	457	0.967_0	25
4.0	19	Acetate	42.5	29	0.991_9	399
4.0	25	Acetate	42.5	74	0.993_8	155
4.0	31	Acetate	42.5	140	0.995_2	82
4.0	37	Acetate	42.5	305	0.998_7	38
4.0	37	HCl	0.10	56	0.946_9	206
9.0	37	Tris	42.5	0.948	0.972_0	293 hr

quite easily by following the appearance of WR-1065 with time. Since there was such good agreement between ^{31}P NMR spectroscopy, the DTNB data and the LCEC measurements of hydrolysis rates, we conclude that, at least under the experimental conditions used, the free sulfhydryl product WR-1065 was sufficiently stable as to allow its direct and specific measurement. These data will serve as an important basis for future studies of the metabolism of WR-2721 and WR-1065 in biological systems.

Although direct comparisons of our data with the data in the two previous studies on the hydrolysis reaction of WR-2721 [6, 8] are limited by the lack of published experimental details in the previous publications, where comparisons can be made, we find that the data of Grachev *et al.* [8] and our data are similar for the magnitude of the pseudo-first-order rate constants, but that the form of the pH-rate profile given by Swynnerton *et al.* [6] more closely resembles the one that we observed. Furthermore, Grachev *et al.* [8] report a maximum rate of hydrolysis for the monoanion of the ester at pH 2.0, a pK of 4.4 to 4.5 for the second dissociation constant of the ester, and an E_a (at pH $\approx$ 2.0) of 24.8 kcal/mole. In contrast, Swynnerton *et al.* [6] report a maximum rate of hydrolysis at pH 3 and a pK of about 4 for the second dissociation constant of the ester, as estimated from the pH-rate profile. From the more extensive ^{31}P NMR data, we found a maximum rate of hydrolysis at pH 3.0, a pK of $\approx$ 4.0 for the second dissociation constant of the ester from the pH-rate profile, and an E_a (pH 4.0 in 42.5 mM acetate, μ = 127.5 mM) of 25.7 kcal/mole. Thus, we found similarities to, and differences with, both studies. In addition, we found that the entropy of activation at pH 4.0, 36°, was +7 e.u., there was a kinetic deuterium isotope effect ($k_{(10\%\ D_2O)}/k_{(100\%\ D_2O)}$) at pH 1.8 ($1.7_2$) and at pH 4.0 ($1.4_0$), and there was a small buffer effect on the rate of reaction at 30° at pH 4.0 and at pH 5.0.

Whereas the hydrolysis reaction of *O*-phosphate monoesters has been studied extensively [11], much less work has been published on the hydrolysis reactions of *N*-phosphoramidate monoesters [12] and *S*-phosphorothioate monoesters [13–16]. In general, the pH-rate profiles observed for the hydrolysis of *S*-phosphorothioate monesters are bell-shaped with a maximum rate of hydrolysis between pH 2 and 4, and may be understood by assuming that the monoanion of the ester is the only ionic species responsible for the observed hydrolysis. No acid-catalyzed reaction is found, and the rate constants are similar in magnitude and independent of the nature of the alkyl or aryl ester [8, 13–16]. The pH-rate profile for WR-2721 that Swynnerton *et al.* [6] and we observed indicates that, in addition to the reaction of the monoanion, there is an acid-catalyzed reaction. The profile is similar to that observed for the hydrolysis of methyl phosphate [17] and can be described in terms of the neutral species, acid concentration, and the monoanion species (equation 1).

$$k = (k_N C_H+) \times \frac{C_N}{C_P} + k_M \frac{C_M}{C_P} \qquad (1)$$

Here, k is the observed rate constant, k_N is the second-order rate constant for the neutral species, C_H+ is the concentration of the acid, C_N is the concentration of the neutral phosphate ester, k_M is the first-order rate constant for the phosphate ester monoanion, C_M is the concentration of the phosphate ester monoanion, and $C_P = C_N + C_M + C_D$ where C_D is the concentration of the phosphate ester dianion. Using equation 1, the theoretical pH-rate profile for the hydrolysis reaction of WR-2721 was generated using the parameters $k_M = 239\ sec^{-1}$, $pK_1 = 1$, and $pK_2 = 4.0$, for the *S*-phosphorothioate dissociation constants, and plotted in Fig. 3 (solid line). The curve is partly empirical because the data were insufficient to evaluate the term ($k_N C_H+$) accurately, but otherwise there is a very good agreement between the theoretical curve and the experimental data. The theoretical curve for the hydrolysis of the monoanion is bell-shaped and is shown in Fig. 3 (dashed line below pH 3). It is clear that the experimental data cannot be fitted assuming participation by this form alone.

The E_a for the hydrolysis reaction of *S*-phosphorothioate monoesters ranges between 22 and 25 kcal/mole [8, 14–16] and $\Delta S\ddagger$ is positive for the monoanion [14–16]. Kinetic deuterium isotope effects of 1.2 to 2.1 have been reported [14–16]. Our data are consistent with these observations. Although the absence of any buffer catalysis of the *S*-phosphorothioate monoester hydrolysis has been reported [15, 16], we did find a small buffer effect at 30° at pH 4.0 and pH 5.0, with the effect being somewhat more pronounced at pH 4.0 than at pH 5.0. However, the general acid-general base catalyzed contribution to the hydrolysis reaction of WR-2721 that was observed by us was small and the evidence is not definitive. Additional studies regarding this aspect of the reaction are indicated.

Thus, we may conclude that the hydrolysis reaction of WR-2721 above pH 1.5 to 2.0 proceeds mainly via the monoanion, with no contribution from the dianion; in more acidic media, contributions from the neutral species (or the conjugate acid) become important. The positive entropy of activation was consistent with a unimolecular, dissociative rate-determining step. The deuterium isotope effect implies that a proton-transfer step is partially rate-determining. A mechanism for the hydrolysis of WR-2721 that is consistent with these facts may be formulated. Proton-transfer to the sulfur atom, which is nearly complete in the transition state, is partially rate-determining in the slow decomposition of the monoanion to the sulfhydryl compound and metaphosphate ion, which is in turn rapidly hydrolyzed to the observed product, inorganic phosphate. Although speculative, hydrolysis of the neutral species might occur by nucleophilic attack of water in conjunction with a rate-determining proton transfer to the sulfur atom. These proposals are consistent with the other studies of *S*-phosphorothioate monoester hydrolysis [8, 13–16], as well as the hydrolysis of *N*-phosphoramidate monoesters [12]. Similar observations and proposals were presented from early studies on the hydrolysis of *O*-phosphate monoesters; however, upon further investigation, modifications to the mechanism have been made in

order to accommodate the new data such that now the dissociative mechanistic pathway of metaphosphate ion formation appears to be precluded, and a "preassociative" mechanistic pathway has been proposed [11]. It is reasonable to believe that additional studies on the hydrolysis reactions of *S*-phosphorothioate monoesters and *N*-phosphoramidate monoesters will yield evidence that the *O*-, *S*-, and *N*-phosphate monoester hydrolysis reaction mechanisms exhibit many mechanistic parallels.

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REFERENCES

1. Y. Tanakia and T. Sugahara, in *Radiation Sensitizers, Their Use in the Clinical Management of Cancer* (Ed. L. W. Brady), p. 421. Masson, New York (1980).
2. D. Glover, J. H. Glick, C. Weiler, J. Yuhas and M. M. Kligerman, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1781 (1984).
3. M. M. Kligerman, D. J. Glover, A. T. Turrisi, A. L. Norfleet, J. M. Yuhas, L. R. Coia, C. Simone, J. H. Glick and R. L. Goodman, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1773 (1984).
4. D. J. Glover, L. Riley, K. Carmichael, B. Spar, J. Glick, M. Kligerman, Z. Agus, E. Slatopulsky, M. Atie and S. Goldfarb, *New Engl. J. Med.* **309**, 1137 (1983).
5. J. F. Utley, N. Seaver, G. L. Newton and R. C. Fahey, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1525 (1984).
6. N. F. Swynnerton, E. P. McGovern, D. J. Mangold, J. A. Nino, E. M. Gause and L. Fleckenstein, *J. Liquid Chromat.* **6**, 1523 (1983).
7. L. M. Shaw, H. Bonner, A. Turrisi, A. L. Norfleet and D. J. Glover, *J. Liquid Chromat.* **7**, 2447 (1984).
8. S. A. Grachev, E. V. Kropachev and G. I. Litvyakova, *Zh. obshch. Khim.* **45**, 1461 (1975).
9. G. L. Ellman, *Archs Biochem. Biophys.* **82**, 70 (1959).
10. L. M. Shaw, H. S. Bonner, A. T. Turrisi, A. L. Norfleet and M. Kligerman, *J. Liquid Chromat.* in press.
11. J. E. Parente, J. M. Risley and R. L. Van Etten, *J. Am. chem. Soc.* **106**, 8156 (1984).
12. S. J. Benkovic and E. J. Sampson, *J. Am. chem. Soc.* **93**, 4009 (1971).
13. S. Akerfeldt, *Acta chem. scand.* **17**, 319 (1963).
14. D. C. Dittmer, O. B. Ramsay and R. E. Spalding, *J. org. Chem.* **28**, 1273 (1963).
15. S. Milstien and T. H. Fife, *J. Am. chem. Soc.* **89**, 5820 (1967).
16. T. H. Fife and S. Milstien, *J. org. Chem.* **34**, 4007 (1969).
17. C. A. Bunton, D. R. Llewellyn, K. G. Oldham and C. A. Vernon, *J. chem. Soc.* 3574 (1958).