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Stability of the experimental anticancer agent [[(4-methoxyphenyl)sulfonyl]hydrazono]acetic acid (NSC-267213). I. Kinetics and mechanism of degradation

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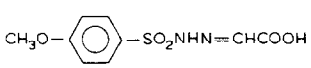
Summary

The degradation of [[(4-methoxyphenyl)sulfonyl]hydrazono]acetic acid (NSC-267213) was studied in buffered aqueous solution at pH 1.0–9.0. Degradation occurred rapidly with $t_{90} < 10$ min at all pH values studied. Apparent first-order kinetics were observed at pH $\geq$ 6.0 while at pH $\leq$ 5.0 the loss appeared biphasic. The major degradation products observed at pH $\geq$ 6.0 were 4-methoxybenzenesulfinic acid and glycolic acid. In addition to these products, at pH $\leq$ 5.0, 4-methoxybenzenesulfonylhydrazide, glyoxylic acid and the isomer of the starting compound were also detected. These products and the kinetic behavior were accounted for by a degradation mechanism involving hydrolysis and isomerization equilibria and elimination of sulfinate from the hydrazone. At pH $\geq$ 6.0, decomposition occurred only via the elimination mechanism. At pH $\leq$ 5.0, hydrolysis and isomerization equilibria affected levels of the hydrazone, however, its irreversible loss was attributable to the elimination mechanism.

Introduction

The investigational anticancer agent [[(4-methoxyphenyl)sulfonyl]hydrazono]acetic acid (*I*) is an arylsulfonylhydrazone:



Arylsulfonylhydrazones are a relatively new class of anticancer agents which have demonstrated promising activity in model tumor systems (Sartorelli et al., 1976; May and Sartorelli, 1978; Grange et al., 1980; Shyam et al., 1985).

Problems exist in the development of *I* as an anticancer agent and these problems can be defined in terms of two characteristics of arylsulfonylhydrazones which are: inadequate aqueous solubility and aqueous instability. In general, an intravenous dosage form is desired for in vivo evaluation of investigational agents to permit activity assessment and dosage evaluation while circumventing most bioavailability considerations. Intravenous dosage forms require adequate aqueous solubility and stability of the drug in order to formulate the desired dose in a biocompatible solution. A $t_{90} \geq 2$ year in aqueous media would allow formulation as a solution while a $t_{90} \geq 24$ h

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might be sufficient for a lyophilized form of the drug for reconstitution immediately prior to administration. Neither of these criteria were met by *I* as evidenced by the $t_{90} \simeq 5$–10 min reported by Cradock (personal communication). The solubility necessary for formulation as an intravenous product depends on the dose to be administered and the rate of administration. The water solubility of *I* was reported to be < 0.1 mg/ml at 25 °C (Cradock, personal communication). The solubility of *I* was low, but adequate to conduct stability studies. However, the stability was inadequate for evaluation of solubilization methods. For this reason, the stability of *I* was of foremost concern in approaching the overall formulation problem. In order to evaluate methods of stabilizing *I*, a thorough understanding of its degradation was necessary. Thus, the present study of the kinetics and mechanism of degradation of *I* was undertaken.

Materials and Methods

Chemicals

All chemicals were analytical grade and used as received from the commercial suppliers unless noted otherwise. Both *I* and 4-methoxybenzenesulfonylhydrazide (NSC-267215, *II*) were obtained from the NCI. Methanol was Fisher HPLC grade. Water for buffers and HPLC mobile phase was deionized and distilled from glass.

Analysis

For UV and visible absorbance measurements a Perkin-Elmer Lambda 1 UV/VIS spectrophotometer equipped with a Perkin-Elmer Model CP331600 Super Sipper or a Perkin-Elmer Model 555 spectrophotometer with an auto-transport/5-cell programmer (Model C618-0330) and temperature controller (Model 5700701) was used. Solutions were prepared in or transferred to quartz cuvettes (1 cm path length and 3.0 ml capacity, Perkin-Elmer C-030-0302).

High-pressure liquid chromatography (HPLC) analyses were done on a system consisting of a Waters Associates Model 6000A pump, a U6K variable volume injector and a Model 440 fixed wavelength detector at 254 nm. Peak areas were quantitated using a Varian CDS 111 integrator. Columns (150 mm × 4.6 mm i.d.) were packed in-house according to a published procedure (Bristow et al., 1977) using the same lot of ODS-Hypersil packing material (5 μm, Shandon lot 5/1165). The mobile phase used for all studies consisted of 85% 0.01 M $(NH_4)H_2PO_4$ (pH 4.0) and 15% methanol. All analyses were done at ambient temperature.

Measurements of pH were made using a Corning Digital Model 112 pH meter equipped with a combination pH electrode (Cole-Palmer).

pK_a determinations

The pK_a of the amide nitrogen of *I* was determined spectrophotometrically. Because the instability of *I* prevented the use of a stock solution, the following procedure was used. The spectrophotometer was zeroed to empty cuvettes in the sample and reference cells. Solid *I* was weighed into the sample cuvette, then 2.0 ml of the appropriate buffer (0.05 M; pH 4.0–6.0 acetate; pH 6.5–8.0, phosphate; pH 9.0, borate; $\mu = 0.2$ adjusted with NaCl) was added to each cuvette. The sample cuvette was shaken to dissolve the solid and the absorbance was read at 240 and 265 nm. The temperature in the cells was controlled at 25.0 °C. Absorbance data were analyzed according to equations described by Albert and Serjeant (Albert and Serjeant, 1971), and the pK_a value of the amide nitrogen, pK_2, was determined to be 6.68 ± 0.41.

Kinetic studies

Solutions for kinetic studies of *I* were prepared by weighing solid *I* into volumetric flasks and adding the appropriate buffer (pH 1.0, 0.1 M HCl; pH 2.0–3.1, phosphate; pH 3.3–6.0, acetate; pH 6.5–8.0, phosphate; pH 9.0, borate; 0.05 M; $\mu = 0.2$, adjusted with NaCl). The resulting concentration was usually $\simeq 6 \times 10^{-5}$ M. The solutions were placed in a constant temperature water bath (Haake, Model D2) at 25.0 °C unless noted otherwise. Samples were taken at timed intervals and analyzed by HPLC. Molar concentrations were calculated from the slope of a standard curve determined over the concentration range of interest. Resulting data were plotted as log M vs time

and rate constants determined from the slope of the linear portion of such plots over at least two half-lives.

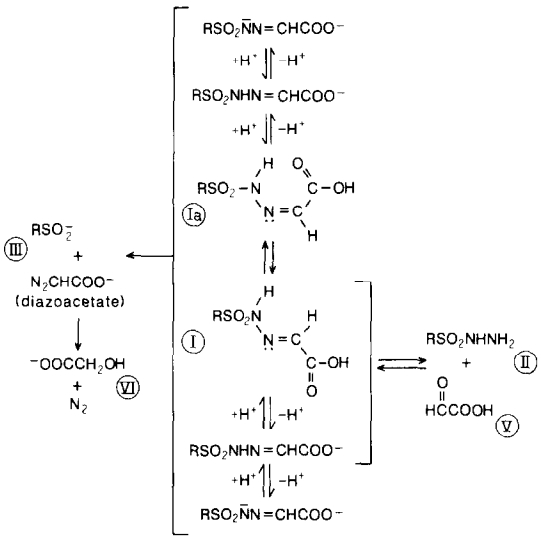
The effect of buffer concentration on the loss of *I* was assessed at pH 2.0 (0.05–0.15 M), pH 4.5 (0.01–0.2 M) and pH 7.0 and 9.0 (0.01–0.15 M) and no significant effects were noted. The ionic strength was varied (using NaCl) at pH 4.5 (0.02–0.2) and pH 7.0 (0.01–0.2) and shown not to affect the rate of loss of *I*.

The following degradation products were analyzed during kinetic studies of *I* by HPLC: the isomer of *I* (*Ia*), 4-methoxybenzenesulfonylhydrazide (*II*), 4-methoxybenzenesulfinic acid (*III*) and 4-methoxybenzenesulfonic acid (*IV*). Rate constants for the loss of *Ia* and *II* were determined from plots of log (peak area) vs time for *Ia* and log molar concentration (calculated from standard curves) vs time for *II*. The molar concentration of *III* was determined from peak area data using the slope of standard curves and the rate constants for the appearance of *III* were calculated from plots of log $([III]_\infty - [III]_t)$ vs time. The plots were linear providing degradation of *III* was not significant. In some cases, depending on reaction time and pH, degradation of *III* to *IV* occurred and significant curvature of semilog plots for the appearance of *III* resulted. Correction for the degradation of *III* was made by summing the concentrations of *IV* (estimated from a standard curve of 4-toluenesulfonic acid) and *III*. This correction linearized plots for appearance of *III* in most cases.

Glyoxylic acid (*V*) was analyzed by colorimetric assay (Kramer et al., 1959; McFadden and Howes, 1960) after degradation of *I* was complete. (*I* demonstrated significant positive interference in the assay.) Glycolic acid (*VI*) was also analyzed by a colorimetric assay (Niederwieser et al., 1978). This assay required high starting concentrations of *I* which resulted in suspensions until most of *I* had degraded to more soluble products. Thus, analysis of *VI* was done only after degradation of *I* was complete.

Kinetic studies of *II* were done in a manner similar to those for *I* at pH 2.0 and 4.5, $T = 25.0\,^\circ C$, $C_0 \simeq 1 \times 10^{-4}$ M. For kinetic studies of *Ia* under basic conditions it was necessary to isolate *Ia* from *I*. This was accomplished by repeated collection of the HPLC eluent corresponding to *Ia* following injections of degrading *I*. The collected eluent was adjusted to pH ≥ 7 to prevent equilibration of *Ia* to *I* and loss of *Ia* was monitored by HPLC.

Scheme 1. Proposed mechanism for degradation of I in aqueous media. (R=4-methoxyphenyl)



Results and Discussion

Stability in aqueous solution

At pH ≤ 5.0, *I* degraded with a rapid initial loss followed by a slower terminal phase as shown for reactions at pH 2.0 and 4.5 in Fig. 1. The initial phase of the reaction was complete in $\simeq 40$ min to 4 h (dashed lines in Fig. 1) depending on the pH of the solution. As the pH decreased, the initial rate of loss of *I* increased. Because non-linearity of the semilog plot was encountered after only two or three initial data points, no rate constants were determined for the early portion of the reaction.

During the slower terminal phase of the reaction, the degradation of *I* appeared to follow log-linear behavior. Rate constants are listed in Table 1. In general, slower terminal loss of *I* was observed at lower pH values, however, a slight

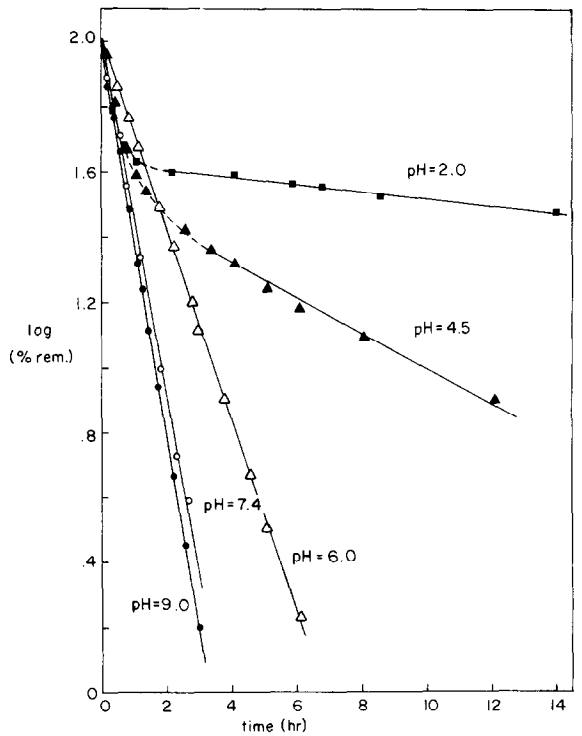


Fig. 1. Log-linear plot for the loss of *I* as a function of time at different pH values. Reaction conditions: $C_0 \simeq 6\times10^{-5}$ M, 25 °C, 0.05 M buffer, $\mu = 0.2$. Degradation of *I* was monitored by HPLC (see Materials and Methods). Solid lines were determined by linear regression analysis. Dashed lines connect data points but represent no data analysis.

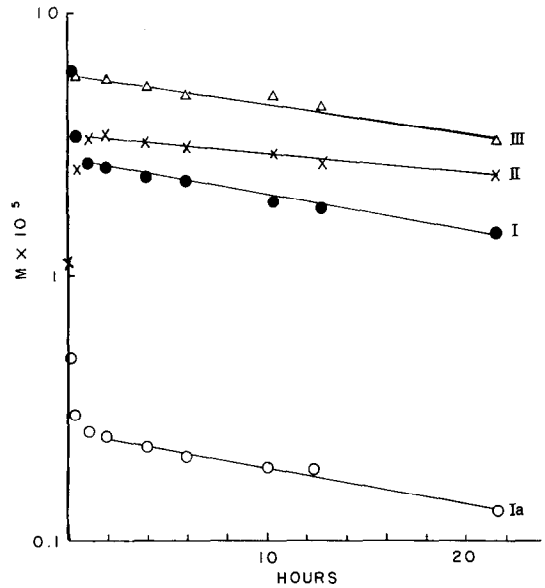
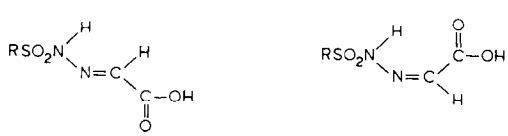


Fig. 2. Log-linear plot of loss of *I* ($C_0 \simeq 6\times10^{-5}$ M) and appearance and loss of degradation products *Ia* (concentration estimated according to a standard curve determined for *I*), *II* and *III* as a function of time for a reaction at pH 2.0 (0.05 M phosphate, $\mu = 0.2$) at 25.0 °C. The reaction was followed by HPLC as described in Materials and Methods. Solid lines were determined by linear regression analysis. Rate constants are reported in Table 1.

decrease in k_{obs} was noted as the pH increased from 4.5 to 5.0.

HPLC analysis of solutions of *I* at pH ≤ 5.0 yielded peaks corresponding to the isomer of *I* (*Ia*), 4-methoxybenzenesulfonylhydrazide (*II*) and 4-methoxybenzenesulfinic acid (*III*). The capacity factors (k') for the products were 7.8, 6.0 and 1.2, respectively, compared to 3.8 for *I*. Profiles of *I* and these degradation products over time at pH 2.0 and 4.5 are shown as log-linear plots in Figs. 2 and 3. At late reaction times, an additional peak, *IV* (4-methoxybenzenesulfonic acid), eluted just prior to *III* ($k' = 0.9$). The mass balance of the components of the reaction (*I*, *Ia* (levels estimated from standard curves for *I*), *II*, *III* and *IV* (levels estimated from a standard curve for 4-toluenesulfonic acid)) was generally within 10% of theoretical.

The designation of *Ia* as the isomer of *I* about the carbon-nitrogen double bond (proposed structures shown below)



I
E form
R = 4–CH3OC6H4–

Ia
Z form

was inferred from the observed equilibrium between *I* and *Ia* and was based on the known occurrence of such geometric isomers for hydrazones (Karabatsos and Krumel, 1967; Kitaev et al., 1970; Smith, 1983; Ishibashi et al., 1986). Immediate reinjection of the collected eluent corresponding to the peak for *Ia* afforded peaks for both *I* and *Ia*. The configurations of the isomers could not be determined experimentally due to the

TABLE 1

Observed first-order rate constants [a] for loss of I, Ia, II and appearance of III at pH 1.0 to 9.0 [b]

pH	k_{obs} (h^{-1}) [c]				
	$I(\times 10^2)$	$Ia(\times 10^2)$	$II(\times 10^2)$	$III(\times 10^2)$	Number of determinations
1.0	0.99 ± 0.08	–	0.56 ± 0.09	0.92 ± 0.26	3
1.7	2.10	2.50	1.20	1.91	1
2.0	2.85 ± 0.05	3.20 ± 0.50	1.60 ± 0.50	2.50 ± 0.50	2
2.5	5.03 ± 0.06	5.25 ± 0.24	2.52 ± 0.04	5.48 ± 0.24	3
3.0	7.26 ± 0.19	7.56 ± 0.39	4.40 [d]	8.34 ± 0.06	2
3.3	8.62 ± 0.05	8.89 ± 0.10	4.34 ± 0.09	8.84 ± 0.08	3
3.7	9.80	10.50	4.50	–	1
4.1	10.91 ± 0.96	10.98 ± 0.81	4.90 ± 0.21	10.88 ± 0.08	3
4.5	12.40 ± 1.30	10.30 ± 0.97	5.30 ± 0.38	10.82 ± 0.78	8
4.7	10.41 ± 0.21	10.00 ± 0.27	4.83 ± 0.18	10.13 ± 0.00	3
5.0	9.03 ± 0.30	8.18 ± 0.20	4.90 ± 0.20	9.86 ± 0.30 [e]	4
5.5	–	5.20	5.30	23	1
6.0	69	–	–	55	1
7.0	100 ± 1	0.35 [d,g]	–	107 ± 6	4
7.4	125 ± 2	–	–	–	4
8.0 [f]	137 ± 4	–	–	141 ± 11	5
9.0	137 ± 6	0.54 [d,g]	–	143 ± 5	5

[a] The k_{obs} values for *I*, *Ia* and *II* represent the terminal loss of these species at pH ≤ 5.5. At pH ≥ 6.0, the loss of *I* and *Ia* was monophasic and k_{obs} was determined over the entire reaction time. For *III*, k_{obs} represents its appearance during degradation of *I*.
[b] Reaction conditions: $C_0(I) \simeq 6 \times 10^{-5}$ M, 25.0 °C, 0.05 M buffer (pH 1.0, 0.1 N HCl, $\mu = 0.2$; pH 2.0–3.3, phosphate, $\mu = 0.2$; pH 3.7–6.0, acetate, $\mu = 0.2$; pH 7.0–8.0, phosphate, $\mu = 0.2$; pH 7.4, phosphate, μ uncontrolled; pH 9.0, borate, $\mu = 0.2$).
[c] The k_{obs} listed are means ± S.D. of the number of assays reported in the last column of the table.
[d] One determination.
[e] Two determinations.
[f] These reactions were run at room temperature (≃ 26 °C) and C_0 varied from 7.9×10^{-6} to 3.8×10^{-4} M.
[g] $C_0(I) \simeq 3 \times 10^{-4}$ M.

low solubility and stability of the isomers in aqueous acidic solution where the isomerization was proposed to occur. Additionally, crystals amenable to X-ray analysis could not be obtained. However, the configuration of *I* was proposed to be the E form (known to be more thermodynamically stable (Karabatsos et al., 1964; Kitaev et al., 1970; Katsuki et al., 1972)) due to higher levels of *I* at pseudo-equilibrium. The proposed structural assignment was consistent with relative reactivities at high pH values as discussed below.

During degradation of *I* at pH < 3, *Ia* was present at the time of first sampling and its subsequent loss paralleled the loss of *I* (i.e., rapid initial loss followed by slower terminal loss, Fig. 2). At pH ≥ 3, levels of *Ia* rapidly increased initially, then underwent a slower loss which again paralleled the loss of *I* (see Fig. 3). Maximum levels of *Ia* were observed at pH 3.0 (≃ 16% of the initial peak area of *I*). Rate constants for the terminal loss of *Ia* (Table 1) were statistically indistinguishable from rate constants for the loss of *I*.

The identity of *II* as 4-methoxybenzenesulfonylhydrazide was established by coelution with an authentic sample on the HPLC system used. Levels of *II* during degradation of *I* increased rapidly to a maximum, then decreased slowly. The initial rapid increase of *II* occurred over the same time as the initial rapid loss of *I*. The slower terminal loss of *II* followed apparent first-order kinetics. Rate constants (Table 1) for loss of *II* were half of the value of rate constants for the terminal loss of *I*.

4-Methoxybenzenesulfinic acid, *III*, was identified on the HPLC system by coelution with an authentic sample and by mass spectral analysis of

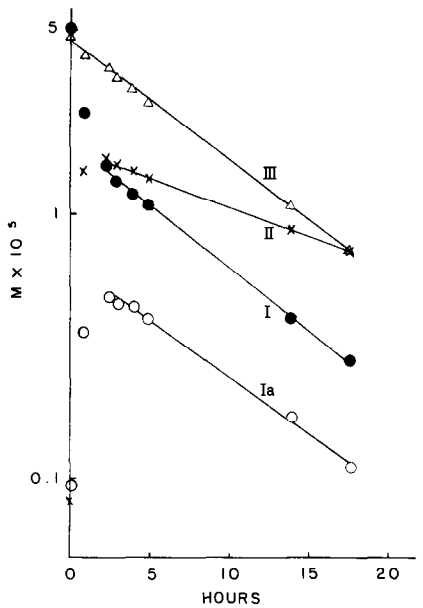


Fig. 3. Log-linear plot of loss of *I* ($C_0 \simeq 6\times10^{-5}$ M) and appearance and loss of degradation products *Ia* (concentration estimated according to a standard curve determined for *I*), *II* and *III* as a function of time for a reaction at pH 4.5 (0.05 M acetate, $\mu = 0.2$) at 25.0 °C. The reaction was followed by HPLC as described in Materials and Methods. Solid lines were determined by linear regression analysis. Rate constants are reported in Table 1.

TABLE 2

Levels of glyoxylic acid (V) and glycolic acid (VI) after degradation of I[a,b] at pH 2.0 to 9.0 (as % of initial concentration of I)

pH	V	VI
2.0 (phosphate buffer)	16 [c,d]	– [e]
	16 [c,d]	47 [c]
3.0 (water)	3 [cf]	80 [c]
4.5 (acetate buffer)	2 [cf]	– [e]
	8 [cf]	– [e]
	4 [cf]	66 [c]
7.0	2 [c]	– [e]
	1 [c]	– [e]
	1.5 ± 0.4 [g]	59.1 ± 6.9 [g]
9.0	3 [c]	– [e]
	1.15 [h]	109.2 [h]

[a] Reaction conditions: C_0 (*I*) ranged from 7×10^{-5} to 1.5×10^{-3} M; room temperature; pH 2.0, 0.05 M phosphate buffer, $\mu = 0.2$; the pH of the water solution was 3.0 as measured after degradation of > 99% of *I*; pH 4.5, 0.05 M acetate buffer, $\mu = 0.2$; pH 7.0, 0.05 M phosphate buffer, $\mu = 0.2$; pH 9.0, 0.05 M borate buffer, $\mu = 0.2$.

[b] *I* was monitored by HPLC and *V* and *VI* analyzed when < 1% of the initial concentration of *I* remained.

[c] One determination.

[d] Levels of *II* ≃ 10% of the initial concentration of *I*.

[e] *VI* was not determined.

[f] Levels of *II* < 5% of the initial concentration of *I*.

[g] Mean ± S.D. of 7 determinations.

[h] Average of two determinations.

a lyophilized solution of degraded *I* in 0.1 N NaOH (which showed only peak *III* by HPLC analysis). During degradation of *I*, the production of *III* occurred in an apparent first-order manner with rate constants approximately equal to those for loss of *I* (see Table 1). Concentrations of *III* after complete loss of *I* and *II* were approximately equal to the starting concentration of *I* (provided further degradation to *IV* was insignificant).

After extensive degradation of *I*, HPLC analysis also yielded *IV*, proposed to be 4-methoxybenzenesulfonic acid. Formation of *IV* was observed following treatment of *III* with concentrated nitric acid, consistent with the known disproportionation reaction of sulfinic acids to form sulfonic acids under acidic conditions (Shiner et al., 1980). The appearance of *IV* during degradation of *I* was attributed solely to the disproportionation of *III*. Rate constants were not determined because *IV* occurred late in the degradation reaction of *I* and did not affect the rates of loss of *I*, *Ia* or *II*.

Analysis for glyoxylic acid (*V*) showed levels similar to *II* after complete loss of *I* and insignificant levels of *V* after complete loss of *II* (see Table 2). Glycolic acid (*VI*) was shown to be a major degradation product of *I* at all pH values (Table 2).

At pH ≥ 6.0, the degradation of *I* occurred in an apparent first-order manner (k_{obs} reported in Table 1). As the pH of the solution increased, the stability of *I* decreased. The major degradation products of *I* at pH ≥ 6.0 were *III* and *VI* (Table 2). The quantitative production of *III* followed apparent first-order kinetics and the observed rate constants were approximately equal to those for the loss of *I*. Disproportionation of *III* to *IV* was not significant at these pH values.

Only low levels of *II* were noted during de-

gradation of *I* at pH 6.0 (at maximum ≃ 3% of the initial concentration of *I*), and *II* was not observed during degradation of *I* at pH > 6.0. In addition, levels of *V* were found to be insignificant after loss of *I* at pH 7.0 and 9.0 (Table 2).

The isomer, *Ia*, was present at low levels (≃ 1–2% of the initial peak area of *I*) and was constant throughout the degradation of *I* at pH > 6.0. Evaluation of the rate constant for loss of *Ia* showed that *Ia* was much more stable than *I* under alkaline conditions (see Table 1).

A change in reaction mechanism appeared to occur at pH 5.0–6.0. The levels and kinetic behavior of *II*, *V* and *VI* observed at pH 5.5 were similar to reactions at pH ≤ 5.0. However, the kinetic behavior of *I* (smooth curve on a log–linear plot) was intermediate to the observed biphasic behavior at lower pH values (pH ≤ 5.0) and the monophasic behavior noted at higher pH values (pH ≥ 6.0). In addition, the rate constants for appearance of *III* and for the terminal loss of *Ia* were intermediate to the values for rate constants determined at pH 5 and 6 (see Table 1).

Mechanism of degradation

Loss of *I* from aqueous solution was found to be due to a combination of hydrolysis, isomerization and elimination reactions as shown in Scheme 1. The hydrolysis and isomerization reactions involved equilibria and were primarily important at pH ≤ 5.0. The elimination reaction (i.e., loss of sulfinate) occurred throughout the pH range 1–9, increasing in rate as the pH increased. At pH ≥ 6.0, the elimination mechanism was the only significant route by which *I* was lost. At pH ≤ 5.0, elimination of sulfinate accounted for the irreversible loss of *I* and the eventual loss of the equilibrium products, *Ia*, *II* and *V*.

The most important contribution to the loss of *I* in aqueous media at pH 1–9 was the elimination of sulfinate with the associated production of diazoacetate. Subsequent rapid degradation of the diazo product would yield N_2 and glycolate (at pH 6.0, $t_{1/2} < 3$ min; at pH 9.0, $t_{1/2} \simeq 90$ min (Kreevoy and Konasewich, 1970)). The degradation products *III* and *VI*, expected from the elimination reaction, were observed during the decomposition of *I* over the pH range 1.0–9.0. The quantitative formation of *III* and equal rate constants for loss of *I* and appearance of *III* were consistent with direct formation of *III* from *I*. The quantitative production of *III* and the detection of *VI* in solutions of degraded *I* indicated that diazoacetate was formed during degradation of *I*. The diazo species would not be expected to be detectable during degradation of *I* in acidic to slightly alkaline solution due to its instability under these conditions. In addition, diazoacetate was not observable by UV spectroscopy (λ_{max} 258 nm, ϵ = 7,000–10,000 AU cm^{-1} M^{-1} (Kreevoy and Konasewich, 1970; Ben-Efraim, 1978)) during decomposition of *I* at ≃ pH 12 where its stability should be adequate for detection ($t_{1/2} \simeq 38$ h in aqueous NaOH at pH 12 (Kreevoy and Konasewich, 1970)). The lack of detection of diazoacetate might be due to an accelerated loss of this species in solutions of *I* since without such an acceleration, accumulation of the diazo species should be observed. Specific reasons for this proposed enhanced degradation of diazoacetate were not investigated.

Levels of glycolic acid (*VI*), the hydrolysis product of diazoacetate, after complete degradation of *I* were quantitative at pH 9.0 but less than quantitative at pH ≤ 7.0 (Table 2). Less than quantitative levels of *VI* were attributed to incomplete formation from diazoacetate. It is known that diazoacetate can undergo a variety of reactions in aqueous solution in addition to hydrolysis (King and Bolinger, 1936; Kreevoy and Konasewich, 1970).

TABLE 3

Rate constants for the loss of I at various temperatures at pH 9.0 and data for Eyring plot [a]

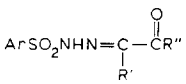
T(°C)	$k \times 10^4$ (s^{-1}) [b]	$1/T \times 10^3$ (K)	$\ln(k/T)$ (s^{-1} K^{-1})
19.5	1.994	3.419	−14.20
25.0	3.728	3.356	−13.59
30.0	7.019	3.300	−12.98
35.0	13.40	3.247	−12.35

[a] For Eyring plot of above data: slope = -1.079×10^4; intercept = 22.65.

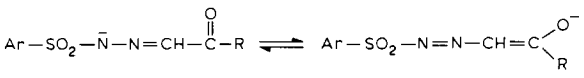
[b] Rate constants are the mean of 3 determinations which varied by less than ±2.5%.

The degradation of *I* via elimination of sulfinate was proposed to be a unimolecular reaction. Analysis of data from reactions of *I* at pH 9.0 at 4 temperatures over the range of 19.5 to 35.0 °C (Table 3) by the Eyring equation resulted in a value for $\Delta S^{\ddagger} = -1.1 \pm 2.3$ eu at 25 °C which was consistent with the entropy of activation expected for a unimolecular reaction (Jencks, 1969).

The unimolecular elimination proposed to account for the loss of *I* is analogous to the Bamford-Stevens reaction in which tosylhydrazones are treated with excess base to form alkenes from the aldehyde or ketone portion of the hydrazone (Bamford and Stevens, 1952; Powell and Whiting, 1959). For the Bamford–Stevens reaction, ionization of the NH group is the first step of the reaction and the rate-determining step is elimination from the anion (Powell and Whiting, 1959; Shapiro, 1976; Liu et al., 1977; Makhova et al., 1978). The usual conditions for the Bamford–Stevens reaction involve non-aqueous solvents, high temperatures (refluxing solvent) and strongly basic media (base generated by metallic sodium addition to the solvent) (Shapiro, 1976).



However, arylsulfonylhydrazones containing an α-carbonyl group (α to the methine carbon of the hydrazone bond, general structure shown below) were less stable toward the elimination reaction than other arylsulfonylhydrazones as evidenced by the milder conditions necessary to effect their decomposition (Cava et al., 1958; Regitz, 1978; Shiba et al., 1983). It may be speculated that the instability associated with the presence of the α-carbonyl substituent in some arylsulfonylhydrazones is due to the electron delocalization shown in Eqn. 1.



(1)

The negative charge on the ionized arylsulfonylhydrazone may reside partially on the oxygen of the carbonyl group (the "azo" form) in addition to the nitrogen (the hydrazone form). In the resonance form containing the negatively charged oxygen, the SO_2–N bond may be weakened due to the introduction of the N–N π-bond. A weakening of the sulfonyl-nitrogen bond would facilitate the elimination reaction. Reports of the synthesis of diazo compounds from *p*-toluenesulfonylhydrazones of *o*-quinones (Cava et al., 1958; Ried and Dietrich, 1961) showed similar azo resonance structures in the reaction sequences, but did not discuss the contribution of the resonance forms to the reactivity of the tosylhydrazones.

The elimination reaction of *I* was proposed to occur in aqueous solutions at room temperature. Since the elimination reaction of *I* occurred under such mild conditions, its reactivity was clearly comparable to other arylsulfonylhydrazones containing an α-carbonyl group.

At pH $\geq$ 6.0, degradation of *I* was attributed only to the elimination mechanism. The major degradation products were those expected from the elimination, i.e., *III* and *VI*. The hydrolysis products, *II* and *V*, were not observed at significant levels and only very low levels of *Ia* were present. The apparent first order loss was consistent with similar observations for the Bamford-Stevens reaction (Powell and Whiting, 1959; Liu et al., 1977). As the pH increased in the range of pK_2 (the pK_a of the amide nitrogen, $pK_2 = 6.68$), the observed rate constant increased. When essentially all of *I* was ionized at the NH group, further increases in pH did not change the rate of reaction indicating that the rate-determining step was elimination of sulfinate from ionized *I*.

The unimolecular elimination mechanism was also an important route of degradation of *I* at pH $\leq$ 5.0 as evidenced by the presence of *III* and *VI* after degradation of *I*. However, the presence of the hydrolysis products, *II* and *V*, and the isomer, *Ia*, during degradation of *I*, as well as the biphasic kinetics observed, indicated that the decrease in the concentration of *I* was also affected by hydrolysis and isomerization. These are equilibrium reactions and the degradation of *I* via the elimination of sulfinate caused a perturbation in the equilibria and the subsequent complete loss of *II*, *V*, and *Ia* via reformation of *I*.

The elimination reaction at pH $\leq$ 5.0 was pro-

posed to occur primarily from low but reactive concentrations of the species of *I* containing the ionized amide nitrogen ($-SO_2\bar{N}N=CHCOO^-$ and $-SO_2\bar{N}N=CHCOOH$). The two more prevalent species of *I* at pH ≤ 5.0 would not be ionized at the amide nitrogen ($-SO_2NHN=CHCOO^-$ and $-SO_2NHN=CHCOOH$). These species would have extremely low reactivity towards the unimolecular elimination based on the proposal that ionization of the NH group was a destabilizing factor towards the elimination reaction (Powell and Whiting, 1959; Shapiro, 1976; Makhova et al., 1978). One report (Regitz, 1975) did note the formation of diazo compounds without addition of base from quinone tosylhydrazones but it was not determined whether the elimination occurred from the unionized form or from a small but reactive fraction of the ionized form.

A monoanion of *I* containing the protonated carboxylic acid and the ionized amide nitrogen ($-SO_2\bar{N}N=CHCOOH$) would be present at low concentrations in solutions of *I* at pH ≤ 5.0. This monoanion might have an appreciable contribution to the elimination reaction of *I* due to its high reactivity, even though the fraction present would be small. Based on $pK_1 \simeq 3.0$ (for the carboxylic acid, estimated from the pK_a for a similar hydrazone derivative (Pretzer and Repta, 1987)) and $pK_2 = 6.68$, approximately 2% of *I* would exist as this monoanion at pH 5. The reactivity of the monoanion should be similar to that of an ionized ester derivative ($ArSO_2\bar{N}N=CHCOOC_2H_5$, $k = 17\ h^{-1}$) (Pretzer and Repta, 1987). An azo form of the monoanion may also be important to its reactivity (Eqn. 1).

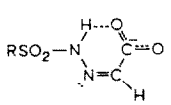
Low but reactive levels of the dianion, $-SO_2\bar{N}N=CHCOO^-$ (proposed to be the species responsible for loss of *I* at pH ≥ 6.0) would also be expected to contribute to the loss of *I* at pH ≤ 5.0 via the elimination mechanism. Approximately 2% of *I* would be present as the dianion at pH 5 based on $pK_2 = 6.68$ and this fraction would decrease as the pH decreased.

Detection of *Ia*, the proposed isomer of *I*, indicated that isomerization of *I* occurred in aqueous solution. Isomerization was proposed to occur via reversible acid-catalyzed addition of water across the C=N bond, allowing free rotation about this bond. Subsequent expulsion of water could result in either isomer. Levels of *I* were consistently much greater than *Ia*, thus, the equilibrium clearly favored *I*.

At pH ≤ 5.0, the loss of *Ia* paralleled the loss of *I*. In this pH range, the loss of *Ia* was proposed to occur through the facile isomerization equilibrium with *I*. Initially, isomerization of *I* to *Ia* rapidly established pseudo-equilibrium levels of the isomers. At pH ≥ 3, the appearance of *Ia* occurred concurrently with the rapid loss of *I* and appearance of *II* (hydrolysis). At pH < 3, maximum levels of *Ia* were present before attainment of maximum levels of *II*. The similar rate constants for the terminal loss of *Ia* and *I* resulted from loss of *Ia* via the reverse reaction of the isomeric equilibrium to reform *I* as *I* degraded by the elimination mechanism.

At pH > 6.0, low levels of *Ia* (1–2% of the initial peak area of *I*) were present and were constant throughout the degradation of *I*. Rate of loss of *Ia* was independent of levels of *I*. Both isomers appeared to degrade by the elimination of sulfinate. (*III* was the only degradation product observed by HPLC in stability studies of *Ia*.) However, at the higher pH values, the stability of *Ia* ($t_{1/2} \simeq 200$ h at pH 7.0) was much greater than *I* ($t_{1/2} < 1$ h at pH 7.0). In addition, *Ia* was present at the first sample taken ($t < 1$ min) during the degradation reaction of *I*. The isomerization reaction (if occurring) would be expected to be slow enough at the higher pH values that the formation of *Ia* from *I* should be observed. These data indicated that interconversion between *I* and *Ia* did not occur at pH ≥ 6.0. The lack of facile equilibrium between the isomers at pH ≥ 6.0 precluded the loss of *Ia* via degradation of *I*. Thus, the 1–2% levels of *Ia* present at these pH values were attributed to an impurity of *Ia* in solid *I*.

The large difference in the reactivity of the isomers was attributed to hydrogen bonding between the NH and the carbonyl oxygen in the Z-form resulting in increased stability in alkaline solution. Hydrogen bonding would be strongest in the ionized form (see below).



Evidence for such hydrogen bonding was not obtained experimentally for *I* or *Ia*. However, numerous examples of hydrogen bonding for similar compounds exist in the literature (Stewart, 1953; Schulte-Frohlinde et al., 1954; Van Duin, 1954; Isherwood and Jones, 1955; Vogel and Matter, 1959; Yao, 1964; Katsuki et al., 1972; Nashima et al., 1977; Cracknell et al., 1983; Ishibashi et al., 1986). The effect of hydrogen bonding in the Z isomer would be to increase the pK_a of the NH group. Similar effects of hydrogen bonding have been reported (Rose and Stuehr, 1971; Handbook of Chemistry and Physics, 1977; Hibbert, 1984). The Z isomer would thus exhibit greater stability towards the elimination reaction. A slower rate of elimination of sulfinate from the Z isomer of a similar arylsulfonylhydrazone was also observed (House and Blankley, 1968). The more stable isomer in alkaline solution (the Z form) was the isomer present at low levels, indicating that *I* (the starting hydrazone) was the E isomer. The configurations assigned according to the relative reactivities in the elimination reaction were consistent with those proposed based on the relative thermodynamic stability of hydrazone isomers (inferred from maximum levels at pseudo-equilibrium).

Simple hydrolysis of *I* would be expected to form the corresponding hydrazide (*II*) and glyoxylic acid (*V*). Both products were observed during the degradation of *I* at pH $\leq$ 5.0 but not at pH > 6.0. In cases where analysis of both *II* and *V* was done, these products were present at approximately the same molar concentration (within 5%). The hydrolysis of hydrazones is known to be an equilibrium reaction (Cordes and Jencks, 1962, 1963; Lowry and Richardson, 1981; Smith, 1983; Temerk et al., 1984). The addition of either *II* or *V* to solutions of *I* at pH 2.0 and 4.5 resulted in increased levels of *I*. Furthermore, addition of *V* resulted in decreased levels of *II* [1]. These observations were consistent with the equilibrium nature of the hydrolysis reaction. Additionally, in the pH range 1–5, ionizations of *I*, *II* and *V* may affect the equilibrium. The pK_a for deprotonation of the terminal amine group of *II* was estimated to be 2–3 based on pK_a values of similar compounds (Dzhan-Temirova and Chernykh, 1977). The pK_a of *V* is 3.34 (Merck Index, 1983). The ionized and unionized forms of *II* and *V* would be expected to have different reactivities in the reverse reaction of the equilibrium to form *I*, resulting in changes in the value of K_{eq} with pH. The ionization of the carboxylic acid of *I* ($pK_1 \simeq 3.0$ based on the pK_a of the *N*-methyl derivative of *I* (Pretzer and Repta, 1987) would also be expected to have an effect on the rate of hydrolysis and thus on the value of K_{eq}.

The initial rapid loss of *I* at pH < 5.0 was attributed to the hydrolysis reaction (and to a lesser extent to the isomerization reaction). However, as the pH increased, the contribution of the hydrolytic equilibrium of *I* to the preliminary phase of the reaction decreased. During the initial phase of the reaction at pH 1, the amount of the hydrolysis product, *II*, formed was $\simeq$ 85–90% of the amount of *I* lost. Thus, during this portion of the reaction, the hydrolysis of *I* accounted for all but $\simeq$ 10–15% of its rapid initial loss. As the pH increased, the percentage of *I* which formed *II* decreased. At pH 5, the maximum level of *II* observed was $\simeq$ 25% of the amount of *I* lost during the initial portion of the reaction. The reasons for the decreased importance of the hydrolysis reaction may be a combination of an intrinsically greater hydrolysis rate at lower pH values, increased contribution of the elimination reaction to the loss of *I* as pH increased and the effect of ionizations of *I*, *II* and/or *V* on the hydrolytic equilibrium.

After attainment of pseudo-equilibrium, slower terminal loss of *I* and *II* was observed and their complete degradation eventually occurred. In addition, analysis showed no significant levels of *V* after total loss of *I* and *II*. The terminal loss of the components of the equilibrium (*I*, *II* and *V*) was attributed to a parallel irreversible degradation route for at least one of the components of the equilibrium. *V* was stable in aqueous solution at pH 4.0 over > 2 weeks. *II* degraded in aqueous solution, but at a rate much slower than that observed in solution with *I*. At pH 2.0, the k_{obs} for decomposition of *II* was $\simeq 3.5 \times 10^{-4}$ h^{-1};

[1] While addition of *II* would also be expected to deplete *V*, analysis of *V* was not done in these particular studies.

while in solution with *I*, k_{obs} for terminal loss of *II* was 1.6×10^{-2} h^{-1}. At pH 4.5, k_{obs} for the terminal disappearance of *II* was 5.3×10^{-2} h^{-1} in the presence of *I* and 1.4×10^{-2} h^{-1} in the absence of *I*. In addition, the observed rate constant for the terminal loss of *II* at pH < 5.0 was reproducibly half of the value of rate constants for the terminal loss of *I* and appearance of *III* (see Table 1). This two-fold difference in rate constants can be attributed to the loss of *II* through the bimolecular reverse reaction of the hydrolytic equilibrium. At or near equilibrium, the rate of the forward reaction equaled the rate of the reverse reaction and for every mole of *I* which underwent hydrolysis, one mole each of *II* and *V* was formed. Thus, an expression for the concentration of *II* in terms of *I* was obtained (Eqn. 2).

$$[II] = \left(K_{eq}[I]\right)^{1/2} \tag{2}$$

Near equilibrium, loss of *I* can be described by Eqn. 3:

$$\frac{-d[I]}{dt} = k_{obs}[I] \tag{3}$$

which, when integrated and substituted into Eqn. 2 yields Eqn. 4:

$$[II]_t = \left[K_{eq}\left([I]_0 e^{-k_{obs}t}\right)\right]^{1/2} \tag{4}$$

The apparent first-order loss of *II* in the terminal phase of the reaction can also be described by Eqn. 5 where k_{II} is the observed first-order rate constant for loss of *II*.

$$[II]_t = [II]_0 \, e^{-k_{II}t} \tag{5}$$

Equating Eqns. 4 and 5 results in a relationship for the observed rate constants of *I* and *II* (Eqn. 6).

$$k_{II} = k_{obs}/2 \tag{6}$$

Thus, assuming loss of *II* via the reverse reaction of the hydrolysis equilibrium, *II* would degrade at half the rate of *I*. This conclusion was consistent with the experimental observations.

The overall loss of *I* can be described by Eqn. 7.

$$\frac{-d[I]}{dt} = k_1[H^+][I] - k_{-1}[H^+][II][V] + k_2[I]_{total} + k_3[H^+][I] - k_{-3}[H^+][Ia] \tag{7}$$

In this equation, k_1 and k_{-1}, and k_3 and k_{-3} are rate constants for the forward and reverse reactions of the hydrolysis and isomeric equilibria, respectively. Both are acid-catalyzed and include the hydrogen ion concentration (assumed to be to the first power). The term for the elimination reaction is a composite of four species of *I* in various ionization states (Eqn. 8).

$$k_2 = k'f_{I^{2-}} + k''f_{I_N^-} + k'''\left(f_{I_0} + f_{I_{COO^-}}\right) \tag{8}$$

The constants k', k'' and k''' are intrinsic rate constants for elimination from the dianion (I^{2-}), the amide nitrogen monoanion (I_N^-) and species of *I* containing the unionized amide nitrogen (I_0: unionized *I* and I_{COO^-}: the carboxylate monoanion), respectively. The species I_0 and I_{COO^-} were considered to have approximately the same relatively low reactivity, described by k''', since the loss of the amide hydrogen appeared to be required for substantial reactivity in the elimination reaction (Powell and Whiting, 1959; Shapiro, 1976; Makhova et al., 1978).

At high pH values ($\geq$ 6.0), the isomerization and hydrolysis reactions do not occur to a significant extent. Under these conditions, the elimination reaction can be attributed primarily to the dianion of *I* and Eqn. 7 is reduced to Eqn. 9.

$$\frac{-d[I]}{dt} = k_2[I]_{total} = k'[I^{2-}] \tag{9}$$

In the terminal phase of the degradation of *I* at pH $\leq$ 5.0, attributing the loss of *I* solely to the elimination reaction may also be appropriate. Assuming the rate constants associated with the equilibria (k_1, k_{-1}, k_3 and k_{-3}) were large compared to k_2 (i.e., the equilibria were rapidly attained), the observed rate constant would be due only to the elimination reaction. Because the rate constants for the forward and reverse reactions of the

equilibria could not be determined experimentally, such a quantitative comparison could not be made. However, other experimental observations suggested that k_{obs} for the terminal loss of I at pH ≤ 5.0 could be attributed to the elimination mechanism. The observed first-order kinetics in the terminal phase of the reaction indicated that significant deviation from the equilibrium conditions did not occur. In addition, derivation of the relationship of k_{obs} for I and II (Eqns. 2–6) was based on the assumption that the equilibria did not contribute to the loss of I in the terminal phase. The result of this derivation ($k_{II} = k_{obs}/2$) was consistent with the experimental data, supporting the validity of the assumption.

Assuming that k_{obs} was due only to the elimination reaction during the terminal portion of the reaction at pH 1 – 5 and during the entire reaction at pH ≥ 6.0, theoretical observed rate constants were calculated from Eqn. 8. The results are shown in Fig. 4 where the circles represent the experimental data points and the solid line was calculated according to Eqn. 8 (where $k' = 1.38$ h^{-1} determined by extrapolation of the rate data at high pH values). The fractions, f, of the ionized species of I were determined from Eqns. 10–13.

$$f_{I^{2-}} = \frac{K_{1,app}K_{2,app}}{[H^+]^2 + K_{1,app}[H^+] + K_{1,app}K_{2,app}} \quad (10)$$

$$f_{I_N^-} = \frac{1}{1+K_z} \times \frac{K_{1,app}[H^+]}{[H^+]^2 + K_{1,app}[H^+] + K_{1,app}K_{2,app}} \quad (11)$$

$$f_{I_0} = \frac{[H^+]^2}{[H^+]^2 + K_{1,app}[H^+] + K_{1,app}K_{2,app}} \quad (12)$$

$$f_{I_{COO}^-} = \frac{K_z}{1+K_z} \times \frac{K_{1,app}[H^+]}{[H^+]^2 + K_{1,app}[H^+] + K_{1,app}K_{2,app}} \quad (13)$$

K_z describes the relative fraction of the monoanion species and equals K_1/K_2 where K_1 and K_2 are the micro dissociation constants for the carboxylic acid and NH ionizations, respectively [2] (Albert and Serjeant, 1971). The value of the apparent ionization constant, $K_{1,app}$ (for ionization of –COOH) was 1×10^{-3} (determined from equations described by Albert and Serjeant, 1971). Values for $K_{2,app}$, k'' and k''', determined during the fitting procedure were 2.28×10^{-7}, 4.7 h^{-1} and 0.012 h^{-1}, respectively [3]. The value for k'' (for the nitrogen monoanion) determined from the data analysis (4.7 h^{-1}) was greater than $k' = 1.38$ h^{-1} for the dianion (determined from the maximum rate constant under basic conditions). The higher reactivity of the monoanion was attributed to delocalization of the negative charge on the nitrogen (Eqn. 1) which was not possible for the dianion. The value for k''' of 0.012 h^{-1} was much smaller than k' or k'' as expected based on the proposed lower reactivity of forms of I containing the unionized amide nitrogen.

The most significant deviation of the theoretical line in Fig. 4 from the experimental data occurred at pH 4.5–6.0. The deviation could be attributed to the contributions of the equilibria (hydrolysis and isomerization) to the terminal phase loss of I, thus invalidating the assumption inherent in calculation of the theoretical line. Due to decreased acid catalysis and possibly also to changing contributions of the various ionized species of I, Ia, II and V to the equilibria, values of the rate constants k_1, k_{-1}, k_3 and k_{-3} would decrease as the pH increased. Slower initial loss of I was observed with increasing pH from 1.0 to 5.0 indicating that the rates of equilibration were decreasing. At pH > 6.0, no equilibrium products were observed. Thus, in the pH range 4.5 to 6.0,

[2] K_1 was assumed to be equal to the dissociation constant for the *N*-methyl derivative of I: 8.71×10^{-4} (Pretzer and Repta, 1987). K_2 was assumed to be equal to the K_a for an ester similar in structure to I: 2.24×10^{-5} (Pretzer and Repta, 1987).

[3] If K_z (Eqns. 11 and 13) was also fitted during calculation of theoretical k_{obs} values, the resulting values of the parameters were: $K_{2,app} = 2.28 \times 10^{-7}$, $k'' = 4.5$ h^{-1}, $k''' = 0.012$ h^{-1} and $pK_2 = 4.67$ (similar to the estimation of 4.65 based on the pK_a of the ester used in initial calculations).

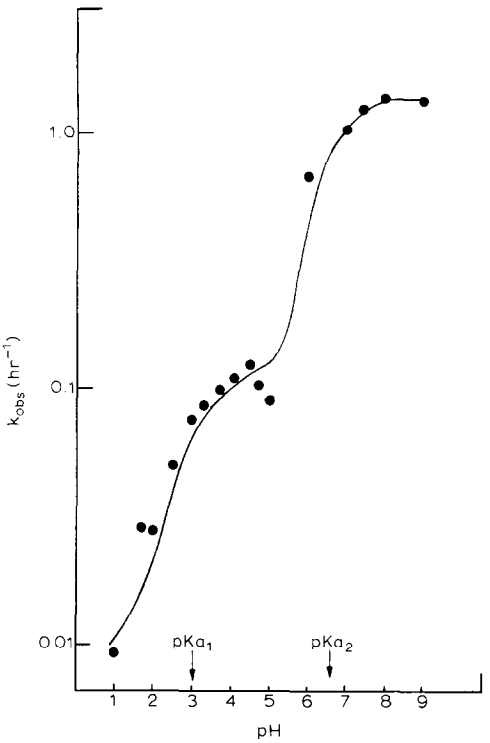


Fig. 4. Plot of log k_{obs} vs pH for loss of *I*. Closed circles are experimental data (Table 1) and the solid line was calculated from the equation: $k_{obs} = k'f_{I^{2-}} + k''f_{I_{N^-}} + k'''(f_{I_0} + f_{I_{COO^-}})$ where $k' = 1.38\ h^{-1}$. (The fractions of the various species were calculated according to Eqns. 10–13 using $K_{1,app} = 1\times10^{-3}$, $K_1 = 8.71\times10^{-4}$, $K_2 = 2.24\times10^{-5}$.)

the values of k_1, k_{-1}, k_3 and k_{-3} may not be sufficiently larger than k_2 to support the assumption that k_{obs} was due only to the elimination reaction and k_{obs} may include kinetic contributions from the hydrolysis and isomerization reactions as well.

Acknowledgements

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