Hydroxamic Acids III: Kinetics of Acetohydroxamic Acid Formation from Ethyl Acetate

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Abstract □ At pH 9.5, 40°, ethyl acetate simultaneously undergoes hydrolysis and hydroxyaminolysis to yield acetic acid and acetohydroxamic acid (*N*-hydroxyacetamide), respectively. The pseudo first-order rate constants for the overall reaction have been calculated from data based on the colorimetric determination of the hydroxamic acid-iron complex and by the pH-stat method. Yields of both products have been determined. Both *O*- and *N*-attack by NH_2OH on ethyl acetate has been postulated to explain the observed reaction yields. In the case of the *O*-attack the resulting *O*-acetylhydroxylamine is a reactive intermediate which undergoes further hydrolysis and hydroxyaminolysis to yield acetic acid and acetohydroxamic acid, respectively.

Keyphrases □ Acetohydroxamic acid formation—kinetics, mechanism □ Kinetics—acetohydroxamic acid formation, ethyacetate □ Ethyl acetate—hydrolysis, hydroxyaminolysis □ pH-stat method—rate constants □ Colorimetric analysis—spectrophotometer

Previous papers have reported on the stability of the hydroxamic acid-iron complex (1) and the hydroxyaminolysis of succinimide (2). The reaction between NH_2OH and succinimide was found to be unusual in that it produced *N*-hydroxysuccinimide at pH 9.5, 40°, instead of the expected hydroxamic acid, *N*-hydroxysuccinamide.

The current study was undertaken to examine the kinetics and mechanisms of hydroxyaminolysis of a simple ester, ethyl acetate, in order to determine the factors involved in the production of the hydroxamic acid employed in the assay method for the ester. A method commonly used for esters is that of Goddu *et al.* (3) which employs 2 *N* NH_2OH and an excess of 1 *N* NaOH. Since esters undergo hydrolysis in competition with hydroxyaminolysis it seemed likely that a large excess of hydroxide could decrease the yield of acetohydroxamic acid. In addition, there is evidence that one concentration of hydroxide may not be optimum for the analysis of all compounds. Jencks (4) found the optimum pH for hydroxyaminolysis of formamide to be about 6.2. At that pH succinimide was found to be nearly unreactive (2). At pH 9.5 the reverse was found to be true; formamide is unreactive in comparison to succinimide. Ethyl acetate is reported here to react readily with NH_2OH at pH 9.5, 40°. An analysis of the yield as a function of NH_2OH concentration indicates that 0.75 *M* NH_2OH is sufficient to achieve a maximum yield. Since increasing the NH_2OH concentration has been shown to decrease the absorbance of the iron complex (1) this reduction (compared to 2 *N* mentioned earlier) is a significant consideration. A kinetic scheme which accounts for the relative yields of acetic acid and acetohydroxamic acid is proposed involving the formation of *O*-acetylhydroxylamine as an intermediate.

EXPERIMENTAL

Kinetics of N-Hydroxyacetamide Formation from Ethyl Acetate—Hydroxylaminolysis of ethyl acetate to form the hydroxamic acid was carried out at 40°, pH 9.5, in an excess of NH_2OH (Table I). Reactions were carried out in a constant temperature cell, sealed to the atmosphere, with the pH maintained constant by a pH-stat (Sargent Recording). Samples were removed by inserting the needle of a syringe through a self-sealing rubber gasket. Aliquots of the reaction mixtures were added to ferric perchlorate reagent (usually 1–20 ml.) according to the procedure previously outlined (1). Solutions were stored in the dark and the absorbance of the iron complex was determined at 515 mμ within 24 hr. after mixing and redetermined within 48 to 72 hr. after mixing (Fig. 1). Ethyl acetate (Baker Analyzed reagent) was distilled before use and all reagents were treated in accordance with the procedure previously described (1).

Kinetics of Total Acid Formation from Ethyl Acetate—Ethyl acetate at pH 9.4, 40°, and in the presence of an excess of NH_2OH, hydrolyzes to yield acetic acid and also reacts with NH_2OH to yield *N*-hydroxyacetamide (acetohydroxamic acid). The rate of formation of total acid was followed by use of a pH-stat (Sargent Recording) with standard NaOH as the titer. The concentrations were controlled so that dilution by addition of titer was roughly 1% and did not require any volume correction factor in the calculation of the rate constants. The rate of hydrolysis was established using the pH-stat

Table I—Experimental Conditions, Apparent First-Order Rate Constants, and Analysis of Yields for Hydroxyaminolysis of Ethyl Acetate 0.016 *M*, pH 9.5, 40°

NH_2OH *M*	$10^2 k_1$, min^{-1}		Yields, %					
			Exptl.[a]			Calcd.[b]		
	Colorimetric	pH-Stat	*H*	*A*	Total	*A*	Total	Unacct. For
0.00	—	0.248	00	100	100	—	—	—
0.15	0.543	0.547	28	68	96	45	73	27
0.25	0.793	0.822	47	52	99	31	78	22
0.35	1.06	1.04	64	42	106	24	88	12
0.50	1.46	1.44	75	26	101	17	92	8
0.65	1.79	1.73	75	25	100	14	89	11
0.75	2.23	2.19	80	23	103	11	91	9
0.83	2.31	2.28	86	20	106	11	97	3
1.12	2.98	2.98	84	13	97	8	92	8

[a] Hydroxamic acid (*H*) determined by 515 mμ absorbance of iron complex. Acetic acid (*A*) determined from the total volume of NaOH added by the pH-stat and the volume required to neutralize *H*. [b] According to Scheme I, percent $A = 100\, k_H/k_1 = 0.248/k_1$.

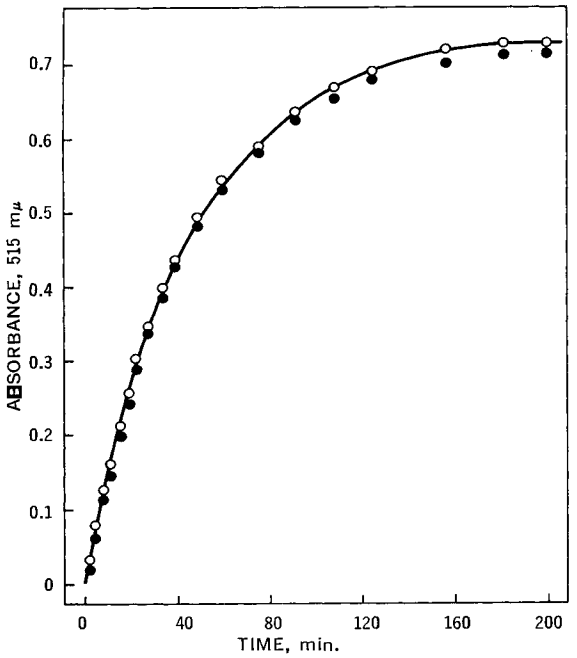


Figure 1—*The absorbance due to the acetohydroxamic acid-iron complex 48 hr.* (○) *and 72 hr.* (●) *after addition of an aliquot of the reaction between 0.016* M *ethyl acetate and 0.75* M NH_2OH, *40°, pH 9.50, to ferric perchlorate as a function of reaction time.*

in the absence of NH_2OH. See Table I for experimental conditions and Fig. 2 for typical data.

Determination of the Yield of Hydroxamic Acid—The final absorbance at 515 mμ, A_∞, achieved in the reaction of ethyl acetate with NH_2OH, is seen to increase with increasing NH_2OH concentration (Fig. 3). It has previously been reported that the color intensity due to the iron complex of *N*-hydroxysuccinamide decreases in the presence of excess NH_2OH (1). Figure 4 shows that this is also the case for *N*-hydroxyacetamide. In order to calculate the final yields of hydroxamic acid from the various reactions containing different NH_2OH concentrations it was necessary to adjust all of the final mixtures in ferric perchlorate reagent to the same set of conditions. A Beer's law plot for *N*-hydroxyacetamide in ferric perchlorate reagent containing 0.0357 *M* NH_2OH was reported to have a slope of 1180 (1). The reactions were allowed to progress until a constant A_∞ was obtained and the final absorbance was redetermined under the conditions used for the Beer's law plot. The concentration of *N*-hydroxyacetamide produced during the reaction was then calculated using the absorptivity of 1180.

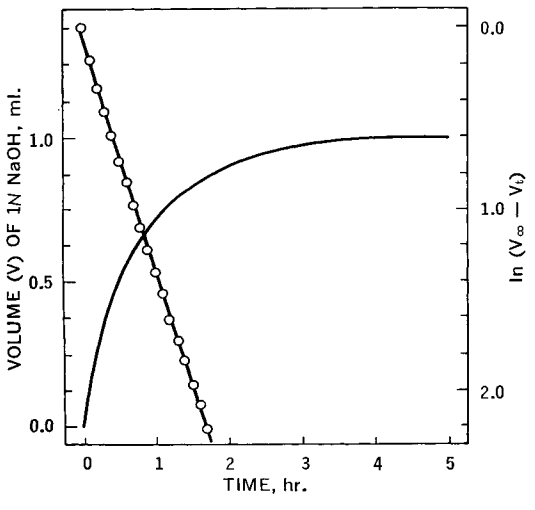
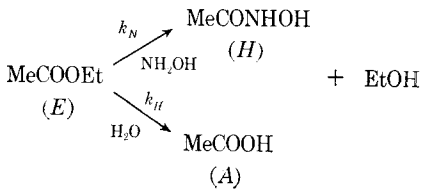


Figure 2—*The pH-stat curve showing volume of titrant* versus *time (left ordinate and solid line) for the reaction between 0.016* M *ethyl acetate and 0.75* M NH_2OH, *40°, pH 9.50. The first-order plot resulting from the same pH-stat data (right ordinate and O).*

Determination of the Yield of Acetic Acid—The final volume of titer added by the pH-stat is a function of the relative amounts of acetic acid and the hydroxamic acid and their corresponding pKa values. Since the pKa of acetic acid is 4.76, it will consume NaOH on a 1:1 molar basis at pH 9.5. The pKa of *N*-hydroxyacetamide was determined by potentiometric titration in a constant-temperature cell at 40° and $\mu = 0.024$ in order to simulate the reaction conditions. The synthesis of *N*-hydroxyacetamide has been previously reported (1). Calculations based on these data are found under results.

RESULTS

Kinetics of Hydroxyaminolysis of Ethyl Acetate—At pH 9.5, 40°, in sufficient excess of NH_2OH, ethyl acetate simultaneously undergoes hydroxyaminolysis and hydrolysis to yield *N*-hydroxyacetamide (acetohydroxamic acid) and acetic acid, respectively (Scheme I).



Scheme I

Under the conditions of constant pH and NH_2OH concentration (see Table I) the overall reaction is pseudo first-order in ethyl acetate. The rate expressions for Scheme I, where *E* represents the ethyl acetate concentration, *H* the hydroxamic acid concentration, and *A* the concentration of acetic acid, are as follows:

$$dE/dt = -(k_N + k_H)E \quad \text{(Eq. 1)}$$

$$dH/dt = k_N E_o e^{-(k_N + k_H)t} \quad \text{(Eq. 2)}$$

$$dA/dt = k_H E_o e^{-(k_N + k_H)t} \quad \text{(Eq. 3)}$$

Solving the differential equations within the limits of $t = 0, \infty$, yields in each case the expression

$$\ln|X_\infty - X_t| = \ln|X_\infty - X_0| - (k_N + k_H)t \quad \text{(Eq. 4)}$$

where $X = E$, H, or A. It can be seen from Eq. 4 that the pseudo-first-order rate constant, k_1, calculated from data representing the concentration of any of the three species, is equal to the sum of $k_N + k_H$ regardless of which of the three components, *E*, *H*, or *A*, is considered.

Pseudo first-order rate constants were determined by two methods. The appearance of *N*-hydroxyacetamide was followed by complexing it with iron and measuring the resulting color spectrophotometrically (Fig. 1). The pseudo first-order rate constants, k_1, were then calculated from plots according to Eq. 5, where X = absorbance at 515 mμ.

$$\ln|X_\infty - X_t| = \ln|X_\infty - X_0| - k_1 t \quad \text{(Eq. 5)}$$

The appearance of acetic acid and acetohydroxamic acid was also followed as total acid titrated by the pH-stat method. Pseudo first-order rate constants were again calculated from plots in accordance with Eq. 5 where X = volume of standard NaOH solution delivered by the pH-stat system (Fig. 2). A comparison of the values obtained by the two methods can be made by examining Table I.

The pseudo first-order rate constant, k_1, may be further defined as

$$k_1 = k_N + k_H = k_2[NH_2OH] + k_H \quad \text{(Eq. 6)}$$

where k_H represents the rate constant for hydrolysis at pH 9.5, 40°, and k_N is observed rate constant for the formation of *N*-hydroxyacetamide. A plot of k_1 *versus* $[NH_2OH]$ would then be linear with slope k_2 and intercept k_H. Figure 3 shows good agreement with this equation where the intercept value was determined experimentally.

Determination of the Yield of Hydroxamic Acid—The final absorbance at 515 mμ, due to the iron complex of *N*-hydroxyacetamide (acetohydroxamic acid) formed from ethyl acetate, is seen to increase with increasing NH_2OH concentration (Fig. 3).

The concentration of hydroxamic acid present at the end of each reaction was calculated from the absorptivity of acetohydroxamic acid under identical complexing conditions. The results are given in Table I.

Determination of the Yield of Acetic Acid—The yield of acetic acid was calculated from the final volume of NaOH added by the pH-stat. Since the hydroxamic acid was determined experimentally, it was assumed that the remainder of the ethyl acetate was converted to acetic acid. The final volume of titer added by the pH-stat should then be equal to that neutralized by the two components. The pKa of *N*-hydroxyacetamide was determined to be 9.18 at 40°, $\mu = 0.024$. The total volume, V_T, of N normal NaOH is defined

$$V_T = (0.676 f_H + f_A)E_0V/N \qquad \text{(Eq. 7)}$$

where 0.676 is the fraction of *N*-hydroxyacetamide neutralized at pH 9.5, 40°, $f_H = H_\infty/E_0$, $f_A = 1 - f_H$, and V = reaction volume. The yield of acetic acid present at the end of the reaction can be calculated from

$$\%A = 100NV_T/VE_0 - 0.676\,(\%H) \qquad \text{(Eq. 8)}$$

which is derived from Eq. 7. The data for f_H can be obtained from the percent yields (H) listed in Table I which were calculated from the 515-mμ absorbance of the iron complex as described in the previous section. The results of applying Eq. 8 to these data are also given in Table I. The total of the percent yields for two reaction components is, within the experimental error, equal to the theoretical value of 100%.

DISCUSSION

Under first-order conditions of constant pH 9.5, 40° and an excess of NH_2OH, ethyl acetate undergoes simultaneous reactions to form acetic acid and the hydroxamic acid, *N*-hydroxyacetamide. The simplest illustration of this process is shown in Scheme I. Figure 3, which shows good agreement between the observed rate constants and Eq. 6, gives no indication of the inadequacy of Scheme I. However, this scheme is not sufficient to describe the ethyl acetate-NH_2OH reaction. This can be illustrated by considering the percent yields of *N*-hydroxyacetamide and acetic acid. It can be shown from Eqs. 3 and 6 that the yields of acetic acid, percent A, can be calculated in accordance with Scheme I by the equation

$$\%A = 100\,k_H/k_1 \qquad \text{(Eq. 9)}$$

where the rate constant for hydrolysis of ethyl acetate is 0.248 (10^2 k_H in min.$^{-1}$) and k_1 is the observed first-order rate constant in the presence of NH_2OH. The results of such calculations are listed under the title of calculated yields in Table I. It can readily be seen that the percent yields of acetic acid, calculated in this manner, do not agree with the experimentally determined yields of acetic acid which are listed in the same table.

It is instructive to examine the manner in which the percent yields of acetic acid, calculated according to Scheme I, differ from the experimental values. It is immediately obvious that the actual yields are always larger than the calculated yields under the same conditions. It is also evident that the largest discrepancy occurs at the lowest NH_2OH concentrations.

In the calculations based upon Scheme I it is assumed that the production of acetic acid occurs only by hydrolysis of ethyl acetate and that its rate constant, k_H, is therefore independent of NH_2OH concentration. While this appears to be very nearly true at 0.75 to 1.12 *M* NH_2OH, it is not the case at 0.15 to 0.25 *M* NH_2OH. This is illustrated by the fact that 27% of the initial concentration of ethyl acetate remains unaccounted for by Scheme I calculations whereas the experimental material balance is within 4% at 0.15 *M* NH_2OH (Table I). It is therefore necessary to introduce a reaction scheme which will produce much more acetic acid at low NH_2OH concentration (relative to Scheme I) and only slightly more than that expected from Scheme I at high NH_2OH concentration. This cannot be explained by assuming general-base catalysis by NH_2OH or general acid-base catalysis by *N*-hydroxyacetamide since the concentrations of both of these components are increasing as the percent unaccounted for is decreasing.

Several authors have reported on *O- versus N*-attack of NH_2OH on esters and thioesters. An excellent review can be found in "Bioorganic Mechanisms" by Bruice and Benkovic (5). Jencks (6) obtained *O*-acylhydroxylamines as intermediates in the hydroxyaminolysis of several esters. He obtained *O*-acetylhydroxylamine in solution from the reaction of *p*-nitrophenyl acetate (PNPA) with NH_2OH at neutral pH. He was not able to crystallize the compound due to its instability. The consideration of both *O*- and *N*-attack by NH_2OH on ethyl acetate leads to Scheme II. *O*-Acetylhydroxyl-

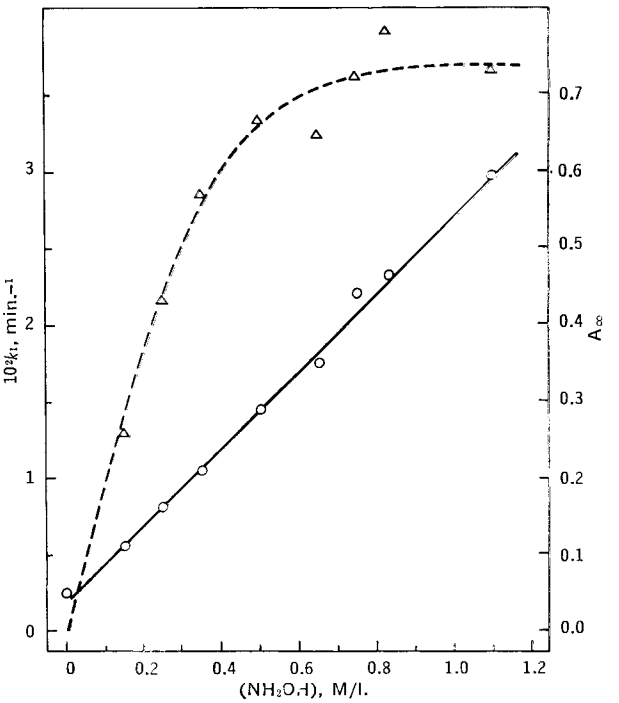


Figure 3—*First-order rate constants,* k_1, *for 0.016* M *ethyl acetate at pH 9.50, 40°, as a function of* NH_2OH *concentration (left ordinate, O). Final absorbance values at 515 mμ* (A_∞) *for the colored complex of the acetohydroxamic acid formed from 0.016* M *ethyl acetate at pH 9.50, 40°, as a function of* NH_2OH *concentration (right ordinate, △).*

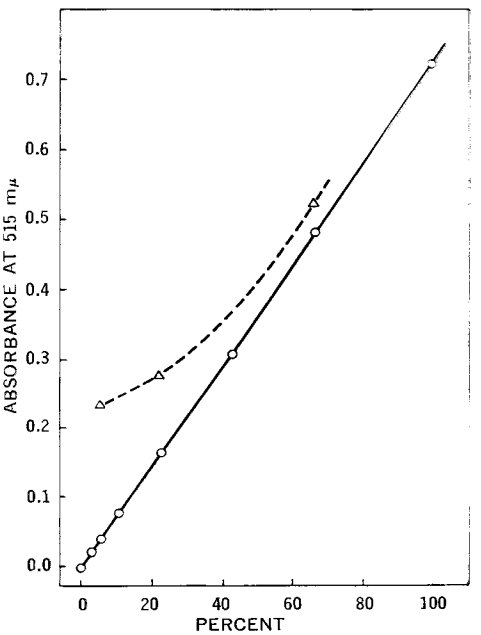
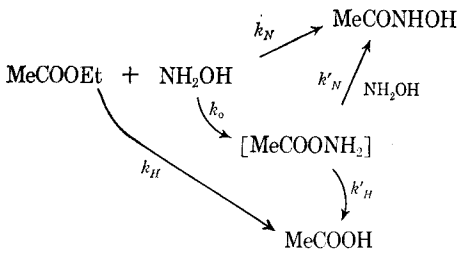


Figure 4—*Beer's law plot made by diluting aliquots of the final reaction mixture from 0.016* M *ethyl acetate and 0.75* M NH_2OH *at pH 9.50, 40°, and complexing each dilution with iron. The abscissa refers to the percent of the reaction in each aliquot and the ○ were diluted with 0.75* M NH_2OH *while the △ were diluted with distilled water.*



Scheme II

amine is shown as a reactive intermediate at pH 9.5, 40°. This would be expected from Jencks' data. At room temperature *O*-acetylhydroxylamine is destroyed immediately by 0.01 *N* NaOH. It reacts with NH_2OH to form the hydroxamic acid at 25°, pH 6.5. It should be noted here that Jencks found no evidence for the formation of *O*-acetylhydroxylamine from ethyl acetate and NH_2OH. This is due to the fact that ethyl acetate reacted more slowly with NH_2OH than did *O*-acetylhydroxylamine. Thus the *O*-acetylhydroxylamine was a reactive intermediate and did not accumulate in sufficient quantities for isolation or characterization in this case. This also prohibited the use of differential reaction rates between ester and intermediate as was done for the reactions of nitrophenyl esters with NH_2OH. For example, in the case of PNPA the formation of *p*-nitrophenolate ion occurred relatively fast and served as a means for calculating the amount of PNPA remaining. With no PNPA remaining, the subsequent slow appearance of the hydroxamic acid (as evidenced by the iron complex absorption) was a measure of the conversion of *O*-acetylhydroxylamine to *N*-hydroxyacetamide. A comparison of the yield of hydroxamic acid formed when the PNPA was first consumed to that formed after the reaction had finished indicated the relative ease of *O- versus N*-attack. In the case of ethyl acetate, where the *O*-acetylhydroxylamine is more reactive than the ester itself, it is not possible to observe this two-stage reaction. The present report represents the first indication of both *O*- and *N*-attack of NH_2OH on ethyl acetate.

Scheme II illustrates the mechanism proposing *O*-acetylhydroxylamine as a reactive intermediate. That such would be the case can be seen from its instability to NaOH (previously mentioned) as well as its reactivity to NH_2OH. Jencks (7) has defined k_N' at 25° as

$$k_N' = [NH_2OH](0.7 + 6.0\,[NH_2OH] + 1.7\,[NH_3OH^+]) \quad \text{(Eq. 10)}$$

which reduces to

$$k_N' = [NH_2OH](0.7 + 6.0\,[NH_2OH]) \quad \text{(Eq. 11)}$$

at pH 9.50 since the pKa of NH_3OH^+ is 6.0 (8). The values of k_N' calculated from Eq. 11 for the various NH_2OH concentrations used in the current study are: 0.15 *M*, 0.036; 0.25 *M*, 0.138; 0.35 *M*, 0.34; 0.50 *M*, 0.92; 0.65 *M*, 1.94; 0.75 *M*, 2.92; 0.83 *M*, 3.99; 1.12 *M*, 9.30. These values are already from 7 to 300 times larger than the observed rate constants in Table I at 40°. This factor would become even larger when comparing *O*-acetylhydroxylamine rate constants at 40° since these would be expected to increase at the higher temperature.

Since $(k_N' + k_H') \gg k_0$, the pseudo-first-order rate constant, k_1, would be defined in accordance with Scheme II as

$$k_1 = k_H + k_N + k_0 \quad \text{(Eq. 12)}$$

which can be rewritten

$$k_1 = k_H + k_2'[NH_2OH] \quad \text{(Eq. 13)}$$

where k_2' is the sum of the individual bimolecular rate constants for *N*- and *O*-attack. Thus Fig. 3 has a slope of k_2' and an intercept of k_H according to Eq. 13 and Scheme II.

The fraction of initial ethyl acetate concentration (E_0) converted to acetic acid would be defined as

$$A_T/E_0 = (k_H + k_0k_H'/(k_H' + k_N'))/k_1 \quad \text{(Eq. 14)}$$

where A_T is total acetic acid concentration at t_∞ and the fraction of *N*-hydroxyacetamide formed would be defined as

$$H_T/E_0 = (k_N + k_0k_N'/(k_N' + k_H'))/k_1 \quad \text{(Eq. 15)}$$

where H_T is total hydroxamic acid concentration at t_∞. The fraction of acetic acid which is produced through *O*-attack of NH_2OH on ethyl acetate and the subsequent hydrolysis of *O*-acetylhydroxylamine, f_A', is defined by

$$f_A' = (k_0k_H'/(k_N' + k_H'))/k_1 \quad \text{(Eq. 16)}$$

Since k_0 is apparently linear with NH_2OH concentration (Fig. 3) and one of the NH_2OH concentration terms in k_N' is raised to the second power (Eq. 11) the fraction, f_A', would be expected to decrease with increasing $[NH_2OH]$. As f_A' approaches zero the percent yield of acetic acid would approach that defined by Eq. 9 since Eq. 14 can be rewritten

$$\%A = (k_H/k_1 + f_A')100 \quad \text{(Eq. 17)}$$

This is in agreement with the results shown in Table I where the calculated yields of acetic acid approach the experimental values at high $[NH_2OH]$.

As illustrated in Scheme II, both the NH_2OH and OH^- are competing for the intermediate, *O*-acetylhydroxylamine. At low NH_2OH concentrations the OH^- competes favorably and converts additional ethyl acetate to acetic acid by hydrolyzing the *O*-acetylhydroxylamine. At higher NH_2OH concentrations more of the *O*-acetylhydroxylamine is converted to the hydroxamic acid. At sufficiently high NH_2OH concentration the formation of *O*-acetylhydroxylamine simply represents an indirect route for the hydroxamic acid since OH^- attack becomes insignificant. The production of acetic acid in this case would be due to hydrolysis of ethyl acetate which has a rate constant of k_H (Table I). Under these conditions the kinetic scheme is equivalent to that in Scheme I. Thus the acetic acid yields calculated according to Scheme I (Table I) approach the experimentally determined values at the higher NH_2OH concentrations.

SUMMARY

1. The kinetics of hydroxyaminolysis and hydrolysis of ethyl acetate have been studied under pseudo first-order rate conditions at pH 9.5, 40°.

2. Pseudo first-order rate constants, k_1, were calculated by the pH-stat method which measures formation of both acetic acid and acetohydroxamic acid and from colorimetric data obtained by complexing the hydroxamic acid with iron. Both methods gave similar values for the rate constants.

3. The acetohydroxamic acid yields were determined from the absorbance of the iron complex. The acetic acid yields were calculated from the final volume of standard NaOH solution added by the pH-stat and a knowledge of the volume neutralized by the hydroxamic acid.

4. The percent yields of acetic acid determined in this manner were higher than the values calculated by assuming that hydrolysis of ethyl acetate represents the only reaction which produces acetic acid. As the NH_2OH concentration was increased this difference between experimental and calculated yields decreased.

5. A mechanism which is consistent with the kinetic data and the relative yields of hydroxamic acid and acetic acid has been proposed. This mechanism includes both *O*- and *N*-attack by NH_2OH on ethyl acetate. The product of *N*-attack is acetohydroxamic acid. The product of *O*-attack is the reactive intermediate, *O*-acetylhydroxylamine. This intermediate does not accumulate but instead reacts immediately either with NH_2OH to form acetohydroxamic acid or with hydroxide to form acetic acid. The relative yields of these two acids is therefore a function of the relative concentrations of NH_2OH and OH^-.

6. The pseudo first-order rate constant, k_1, is thus defined as $k_1 = k_N + k_0 + k_H$, where k_N and k_0 are the pseudo first-order rate constants for *N*- and *O*-attack by NH_2OH and k_H is the rate constant for hydrolysis in the absence of NH_2OH. A plot of k_1 *versus* NH_2OH was found to be linear with intercept equal to the experimentally determined value for k_H and slope equal to the sum of the bimolecular rate constants for *N*- and *O*-attack.

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Quantitative Determination of Hyoscyamine and Scopolamine by Direct Photodensitometry of Thin-Layer Chromatograms

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Abstract □ A rapid and simple method for the quantitative evaluation of thin-layer chromatograms of hyoscyamine and scopolamine by direct photodensitometry was investigated. Excellent separation with discrete spots for the two alkaloids was quickly obtained using absolute methanol–ammonia T.S. (200:1 v/v) as the developing solvent. Spots were made visible by spraying with Munier and Macheboeuf's modification of Dragendorff's reagent. Linear standard curves were obtained for each alkaloid when the integrated function of the size and density of the spot was correlated with micrograms of alkaloid applied. The upper and lower limits of the standard curves were 4 to 65 mcg. for hyoscyamine, and 7 to 60 mcg. for scopolamine. The optimal range for both alkaloids was approximately 20 to 30 mcg. Pure alkaloids and plant extracts were determined by photodensitometric and spectrophotometric methods, and results were compared.

Keyphrases □ Hyoscyamine, scopolamine—separation, determination □ TLC—separation □ Dragendorff's reagent—color development □ Photodensitometry, direct—quantitative analysis

The qualitative resolution of complex mixtures by thin-layer chromatography (TLC), introduced by Stahl (1) in 1956, has been extensively investigated. The quantitation of these resolved compounds has been less extensively studied. Quantitative TLC is in essence based upon either elution of the compounds contained in the spot or measurement directly on the plate.

The quantitative method of relating spot size to the quantity of compound on thin-layer chromatograms was thoroughly investigated by Purdy and Truter (2, 3). They explored three general methods of analysis based upon spot size and found a linear relationship between the square root of the area and the logarithm of the weight.

Privett and Blank (4, 5) described a method for the quantitation of lipids separated by TLC by applying direct densitometry to thin-layer chromatograms sprayed with a saturated solution of potassium dichromate in 80% (by weight) sulfuric acid. The plate was subsequently heated at 180° for 25 min. in order to char the spots. The area under the densitometer curves was directly proportional to the amount of sample in the spot. The direct scanning procedures for TLC plates have been evaluated critically by Klaus (6). Optical densitometry of charred spots has been extensively applied to determinations of lipids and glycerides but does not appear to have been used widely before with the studies of alkaloids. Genest *et al.* (7, 8) reported quantitative TLC data by direct densitometry on certain alkaloids of morning glory seeds and opium. Shellard and Alam (9) described a TLC densitometric method for the quantitation of some *Mitragyna* oxindole alkaloids.

The present work was undertaken to develop the technique of direct densitometric measurement of individual TLC spots of hyoscyamine and scopolamine as free bases. This densitometric method of area measurement is simple to perform and compares favorably with spectrophotometric techniques. Furthermore, in using a photoelectric device, the process of measurement is rapid and convenient.

EXPERIMENTAL

Equipment—Chromoscan recording and integrating densitometer equipped with TLC attachment (Joyce, Loebl and Company, Ltd., Gateshead-on-Tyne, Great Britain), spectrophotometer (Beckman model DU), pH meter (Beckman zeromatic), centrifuge, flash evaporator, adjustable TLC applicator (model SII Desaga/Brinkmann Instruments, Inc.), standard glass carrier plates (20 × 20 cm.), glass developing tanks (Desaga) lined with solvent-saturated filter paper, lambda pipets.

Materials—*Hyoscyamus niger* L., field-grown, oven-dried at 50°, ground to 40 mesh, served as the standard plant powder. All chemicals used were analytical reagent grade. Hyoscyamine (New York Quinine and Chemical Works, Inc.) and scopolamine (Aldrich Chemical Co.) were used. Each alkaloid standard produced only one spot on a chromatogram.

Operating Conditions for Densitometer—Chromoscan: filter 490 mμ, aperture 10 × 0.5 mm., cam A, gain 5, optical wedge 0–0.5 o.d.; thin-layer attachment: filter 490 mμ, aperture 5 × 0.5 mm., specimen expansion ratio 1:2. Light source for both units was a 12 v., 100 w. standard tungsten projection lamp. Both units were operated by reflectance.

Preparation of the Plates—Silica Gel G was purified by refluxing with anhydrous methanol for 8 hr. in a continuous extraction apparatus. Thirty-three grams of purified Silica Gel G was mixed with 67 ml. of distilled water and shaken vigorously for 45 sec. in a glass-stoppered conical flask. The slurry was spread on 20 × 20-cm. standard glass carrier plates to a thickness of 500μ. The coated