SUMMARY AND CONCLUSIONS

Factors affecting the stability of the hydroxamic acid-iron complex have been studied. It has been shown that the iron concentration is the most important factor for complex stability.

Conditions for a stable complex have been developed and found to be superior to those utilizing H_2O_2.

An assay for succinimide has been designed and found to be reproducible on consecutive days.

The hydroxyaminolysis reaction has been successfully carried out in an aqueous medium. Therefore the assay is potentially useful for pharmaceutical systems.

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Hydroxamic Acids II: Kinetics and Mechanisms of Hydroxyaminolysis of Succinimide

ROBERT E. NOTARI

Abstract □ In alkaline pH at 40° succinimide simultaneously hydrolyzes to form succinamic acid and reacts with NH_2OH to form *N*-hydroxysuccinamide which undergoes ring closure to form *N*-hydroxysuccinimide. At pH 9.5 *N*-hydroxysuccinimide is the major product from hydroxyaminolysis of succinimide at 40°. At pH 12, *N*-hydroxysuccinimide undergoes further hydrolysis to form *N*-hydroxysuccinamic acid. Pseudo first-order rate constants for the loss of succinimide as a function of pH and NH_2OH concentration were established by colorimetric determination of the hydroxamic acid-iron complex or UV spectrophotometric determination of the *N*-hydroxysuccinimide. Reaction conditions favoring *N*-hydroxysuccinimide formation were employed to develop a new UV assay for succinimide. This assay is six times more sensitive than the assay based on formation of the hydroxamic acid with subsequent colorimetric determination of its iron complex.

Keyphrases □ Succinimide—hydroxyaminolysis □ Hydroxyaminolysis, succinimide—mechanism □ Kinetics—hydroxamic acid formation, succinimide □ TLC—separation, identity □ Colorimetric analysis—spectrophotometer □ UV spectrophotometry—analysis

Hydroxamic acids form highly colored ferric hydroxamates which can be conveniently detected by spectrophotometric methods. A previous paper has reviewed the potential utility of the hydroxyaminolysis reaction in the quantitative determination of imides, amides, esters, anhydrides, and acid chlorides (1). That report was concerned primarily with the establishment of optimum conditions for the stability of the hydroxamic acid-iron complex. The effect of each variable upon the yield and stability of the complex was determined and conditions were described wherein the complex was stable for more than 48 hr. An assay method was developed for succinimide based on these data. That method has been applied in this kinetic study.

There are three general considerations in the development of optimum conditions for analysis by hydroxamic acid formation. They may be briefly outlined as follows: (*a*) proper reaction conditions for maximum yield (or minimum side reactions such as hydrolysis); (*b*) reaction conditions which are sufficiently fast to achieve the A_∞ reading in a convenient assay time (reaching the plateau of the *A versus t* curve would provide high and constant readings; see Fig. 1); and (*c*) optimum complexing conditions for the most stable complex in highest possible yield. An additional consideration exists in the case of the assay of imides by this method. Since the hydrolysis of imides results in the formation of amides it is necessary to choose conditions which will selectively assay an imide in the presence of its hydrolysis product. The development of an assay which is both selective as well as sensitive requires a knowledge of the kinetics and mechanisms for the hydroxyaminolysis reaction. Since there are a number of drugs with cyclic imide structures, succinimide was chosen as a model compound for kinetic studies. This study serves as an excellent illustration of the validity of the above premise since the appropriate control of reaction conditions has resulted in a new assay with sixfold increase in sensitivity.

EXPERIMENTAL

Kinetics of Hydroxamic Acid Formation from Succinamide—Hydroxyaminolysis of succinimide was carried out under pseudo first-order conditions with pH and temperature maintained constant by use of a pH-stat (Sargent Recording) equipped with a jacketed beaker serviced by a constant temperature bath and circulating pump. The beaker was maintained closed to the atmosphere and samples were withdrawn by syringe through a rubber gasket. The NH_2OH was maintained in sufficient excess of the succinimide concentration to remain essentially constant through-

Table I—Experimental Conditions and Apparent First-Order Rate Constants for Hydroxyaminolysis of 0.016 *M* Succinimide at 40°

Conditions		$10^2 k_1$, min.$^{-1}$	
	$[NH_2OH]$	Method	
pH	*M*	Colorimetric[a]	Ultraviolet[b]
9.50	0.00	— (0.434)[c]	—
	0.14	1.31	1.42
	0.28	2.43	2.31
	0.55	3.78	3.78
	0.82	5.45	5.12
	1.08	6.88	6.78
10.50	0.14	2.16	—
	0.28	2.61	—
	0.55	4.88	—
	0.82	6.61	—
	1.09	8.37	—

[a] Iron complex absorption at 515 mμ. [b] *N*-Hydroxysuccinimide absorption at 260 mμ. [c] Determined by pH-stat.

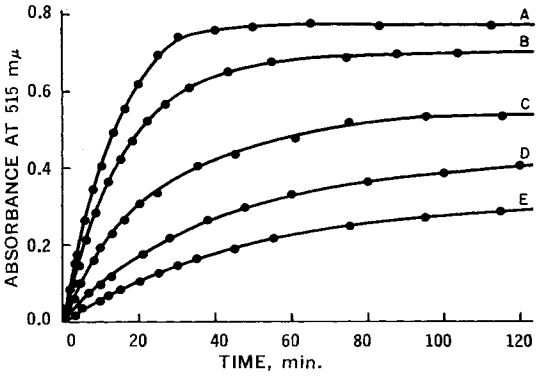


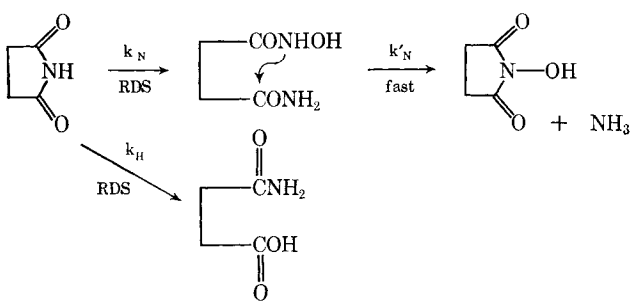
Figure 1—*Increase in absorbance at 515 mμ as a function of reaction time due to the formation of the hydroxamic acid-iron complex formed by dilution of aliquots from reactions containing 0.016* M *succinimide and* $[NH_2OH]$ *equal to:* A, *1.09* M; B, *0.82;* C, *0.55;* D, *0.28; and* E, *0.14 at pH 10.50, 40.0°.*

out a given reaction. The rate of formation of hydroxamic acid was determined by measuring the 515-mμ absorption due to its iron complex using a spectrophotometer (Beckman DU). The complex was formed by addition of an aliquot of the reaction mixture to ferric perchlorate reagent solution in accordance with the procedure previously reported (1). Usually 0.5 ml. of the reaction mixture was added to 10 ml. of ferric perchlorate solution. The absorbance at 515 mμ was determined 24 hr. after mixing the aliquot with the iron solution and again 48 to 72 hr. later to check on color stability. Typical curves showing the increase in the 515-mμ absorbance as a function of reaction time are shown in Fig. 1 and experimental conditions are listed in Table I.

Kinetics of Hydrolysis and Hydroxyaminolysis of Compounds in Table II—The method described in the previous section was employed in the determination of all rate constants involving reactions with NH_2OH reported in this paper. All rate constants for hydrolyses were calculated from the volume of standard NaOH delivered as a function of time by the pH-stat (Sargent Recording) at constant temperature in the absence of NH_2OH. In the case of *N*-hydroxysuccinimide the rates of hydrolysis and hydroxyaminolysis were determined by following the loss of its 260-mμ absorption maximum in addition to using the pH-stat method to measure hydrolysis.

Kinetics of N-Hydroxysuccinimide Formation from Hydroxyaminolysis of Succinimide—Succinimide 0.016 *M* was reacted with various concentrations of NH_2OH under pseudo first-order conditions at pH 9.5, 40°, as previously described. Aliquots were removed as a function of time and diluted with aqueous NaOH at pH 9.5. The absorbance at 260 mμ was determined immediately after dilution. Reaction conditions are listed in Table I.

Determination of the Reaction Products from Hydroxyaminolysis of Succinimide—Hydroxyaminolysis of succinimide at pH 9.5, 40°, gives rise to three products during the time required to expend the succinimide; they are succinamic acid, *N*-hydroxysuccinamide, and *N*-hydroxysuccinimide (see Scheme I). Succinamic acid and *N*-hydroxysuccinimide undergo further transformations at rates much slower than the reaction of succinimide (Table II).



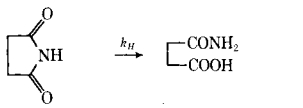
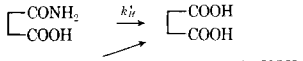
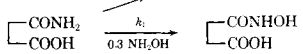
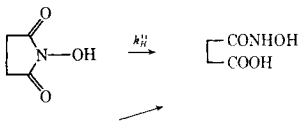
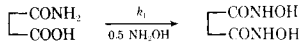
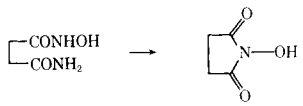
Scheme I

The presence of *N*-hydroxysuccinamide and *N*-hydroxysuccinimide was demonstrated by TLC on the reaction mixture using silica gel support and benzene–methanol–acetic acid (15:5:0.1) as the solvent. The spots were detected using a ferric perchlorate spray and the R_f values compared to standards. The hydroxamic acid appeared as a purple smear at R_f = 0.1 and the *N*-hydroxysuccinimide produced a brown spot at R_f = 0.33. *N*-Hydroxysuccinimide (Aldrich Chemical Co.) was used as a reference standard and the preparation of *N*-hydroxysuccinamide is described in a previous paper (1).

Additional evidence for the formation of *N*-hydroxysuccinimide from hydroxyaminolysis of succinimide was provided by a spectrophotometric titration. Succinimide was reacted with 1.1 *N* NH_2OH at pH 9.5, 40°, for 2 hr. Aliquots were diluted with aqueous HCl or NaOH to adjust the pH and then diluted to a constant volume. The final pH and the absorbance at 260 mμ were immediately determined. A similar procedure was applied to *N*-hydroxysuccinimide.

Comparison of the Succinimide Assay Method Based on Hydroxamic Acid to that Based on N-Hydroxysuccinimide—The application of the hydroxamic acid-iron complex formation to the quantitative analysis of succinimide has been described in an earlier paper (1). The following method was used to compare the sensitivity of the hydroxamic acid-iron complex method to a new method based

Table II—Comparison of Observed First-Order Rate Constants in Presence and in Absence of NH_2OH at pH 9.5, 40°

$k_1 = k_N + k_H$, Succinimide[a]	$10^2 k$, min.$^{-1}$ 1 to 6
	0.43
	N.R.
	≪0.02
	0.023
	0.08
	Too fast To measure

[a] See Table I for experimental conditions.

Table III—Analysis of the Yields from the Hydroxyaminolysis of 0.016 *M* Succinimide at pH 9.5, 40°

NH_2OH *M*	Yield, %: *N*-Hydroxysuccinimide obs.[a]	Succinamic Acid calcd.[b]	*N*-Hydroxysuccinamic Acid calc.[c]
0.00	0.0	—	—
0.14	46.3	31.4	17.5
0.28	66.5	18.2	9.7
0.55	78.7	11.4	7.6
0.82	82.5	8.1	12.3
1.08	85.0	6.3	27.0

[a] A_∞/a at 260 mμ. [b] Percent = 100 k_H/k_1. [c] Based on total absorbance at 515 mμ, $(A_\infty - [\text{N—OH—}SI]a_{\text{N-OH-}SI} - [SA]a_{SA})/a_{\text{N-OH-}SA} = [\text{N—OH—}SA]$.

upon the UV absorption of *N*-hydroxysuccinimide, which is the major product at pH 9.5, 40° (Table III).

Five solutions containing different succinimide concentrations (0.0034 to 0.017 *M*) and 0.6 *N* NH_2OH were adjusted to a pH of 8.3 and allowed to react overnight at 40°. One milliliter of each solution was added to 250 ml. of NaOH to give a final pH of 9 and the absorbance was determined at 260 mμ. The same reaction mixtures were also assayed by formation of the colored iron complex and determination of its 515 mμ absorbance as previously described (1). A similar set of experiments was conducted using an initial pH of 11.7 instead of 8.3.

RESULTS AND CALCULATIONS

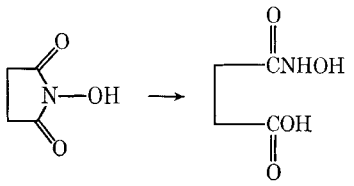
Kinetics of Hydroxyaminolysis of Succinimide—Reaction mixtures containing succinimide and excess NH_2OH at constant pH and temperature were sampled as a function of time and aliquots were added to ferric perchlorate reagent solution. Typical plots illustrating the increase in absorbance at 515 mμ, due to the hydroxamic acid-iron complex, as a function of the reaction time are shown in Fig. 1. Pseudo first-order rate constants, k_1, were calculated from the slopes of plots based on

$$\ln (A_\infty - A_t) = -k_1 t + \ln (A_\infty - A_0) \qquad \text{(Eq. 1)}$$

where A is the absorbance at 515 mμ. Typical plots are shown in Fig. 2 and the rate constants are given in Table I.

Reactions at pH 9.5, 40°, were also followed by diluting aliquots with NaOH solution and measuring the absorbance at the 260-mμ maximum due to *N*-hydroxysuccinimide anion. Plots of ln $(A_\infty - A_t)$ *versus* t were linear after a short lag period and the rate constants calculated from the slopes were similar to those calculated from the colorimetric data (see Table I).

The rate constants calculated from the pH-stat data for succinimide were much smaller than those calculated from the corresponding absorption data. This is due to the fact that succinimide has a pKa of 9.40 at 40° (2). Under the present experimental conditions succinimide exists primarily as the anion and the formation of the hydroxamate did not require a significant addition of titer by the pH-stat. Rate constants calculated by the pH-stat method must represent the formation of the dianion of *N*-hydroxysuccinamic acid (Scheme II).



Scheme II

Kinetics of Hydrolysis and Hydroxyaminolysis of Compounds in Table II—Rate constants for hydrolysis were calculated from

$$\ln (V_\infty - V_t) = -k_H t + \ln (V_\infty - V_0) \qquad \text{Eq. (2)}$$

where V is the volume of standard NaOH solution added by the pH-stat. The rate constant for hydrolysis of *N*-hydroxysuccinimide was also calculated by simultaneously measuring the decrease in the 260-mμ absorption band as a function of time. Results were the same with either method. The rate of loss of *N*-hydroxysuccinimide in the presence of NH_2OH was also determined by following the loss of the 260-mμ absorption band.

In all other cases the reaction with NH_2OH was studied by complexation with iron and applying Eq. 1. Results are found in Table II and in the *Discusssion* section. The rate of hydroxamic acid formation from succinamic acid was too slow to study with the pH-stat. The pseudo first-order rate constant calculated from absorbance data was $k_1 = 2.15 \times 10^{-4}$ (min.$^{-1}$) in the presence of 0.296 *N* NH_2OH and 0.03 *N* NaOH at 40°.

Determination of Reaction Products from Hydroxyaminolysis of Succinimide—Hydroxyaminolysis of succinimide was found to give rise to a product(s) which produced a colored complex with iron (Fig. 1) and a product having an UV absorption maximum at 260 mμ. The 260-mμ absorption band was attributed to *N*-hydroxysuccinimide. The presence of *N*-hydroxysuccinimide, in reactions at pH 9.5, 40°, was demonstrated in two ways. A spectrophotometric titration of the reaction mixture resulting from the hydroxyaminolysis of succinimide was compared with that of a known sample. The results, shown in Fig. 3, indicate that both the reaction product and *N*-hydroxysuccinimide possess a pKa of 5.9 and that the absorptions of the unprotonated forms are equal. Reaction mixtures subjected to TLC also gave a spot corresponding to that of *N*-hydroxysuccinimide in both R_f value and color with ferric perchlorate reagent.

N-Hydroxysuccinamide was shown to give to *N*-hydroxysuccinimide by performing TLC (previously described) on the hydroxamic acid before and after dissolution at pH 9.5. A prominent spot for *N*-hydroxysuccinimide could be visualized only after dissolution in alkaline pH. The reaction was too fast to calculate a rate constant at pH 9.5, 40°. At room temperature, pH 6, 21% is converted to *N*-Hydroxysuccinimide during the time required for dissolution. At pH 9.5, 40°, 76% was converted in less than 10 min. It is apparent that *N*-hydroxysuccinimide arises from *N*-hydroxysuccinamide and that the rate is sufficiently fast for *N*-hydroxysuccinamide to be considered in the steady state at pH 9.5, 40°.

N-Hydroxysuccinimide undergoes hydrolysis to yield a product having pKa values of 4.4 and 9.1 by potentiometric titration. This product does not move from the origin during the TLC procedure previously described and it does give a violet color with ferric perchlorate. It does not give rise to an absorption band at 260 mμ under the conditions in this paper.

N-Hydroxysuccinamic acid was prepared from succinic anhydride by the method previously described (1). It does not appear

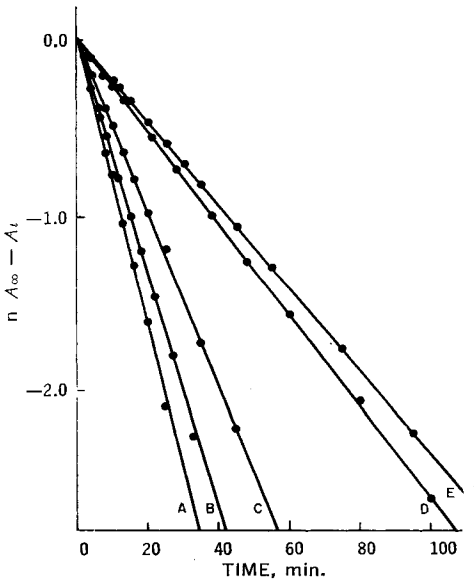


Figure 2—*First-order plots for the increase in absorbance at 515 mμ due to the hydroxamic acid-iron complex formed from aliquots of reactions containing 0.016* M *succinimide and* [NH_2OH] *equal to* A, *1.09* M; B, *0.82;* C, *9.55;* D, *0.28; and* E, *0.14 at pH 10.50, 40°.*

to cyclize to form *N*-hydroxysuccinimide under the present conditions as indicated by the lack of 260-mμ absorbance.

Comparison of the Succinimide Assay Method Based on Hydroxamic Acid to that Based on N-Hydroxysuccinimide—The conditions for obtaining the maximum yield and stability of the iron complex of the hydroxamic acid produced from succinimide are defined in a previous paper (1). This study compares the assay of succinimide under reaction and complexation conditions giving the highest yield of absorption at 515 mμ with the assay giving the highest yield of 260-mμ absorption.

In order to determine reaction conditions for achieving high absorbance by the iron complex, the hydroxyaminolysis of succinimide was carried out at fixed concentrations of reactants and varying pH. Optimum complexing conditions (1) were employed in each case and the final absorbance value was determined at each pH. Figure 4 shows that the highest color intensity is achieved between pH 11 and 12. A pH of 11.6 was chosen for the analysis of succinimide by formation of the hydroxamic acid-iron complex.

Conversely, the yield of *N*-hydroxysuccinimide, as evidenced by the 260-mμ absorption band, decreases as the reaction conditions are held constant and the pH is increased (Fig. 4). A pH of 8.3 was therefore chosen for the assay of succinimide by the formation of *N*-hydroxysuccinimide. Figure 5 illustrates the increase in yield of *N*-hydroxysuccinimide with increasing NH_2OH at constant pH. The yield increases sharply from 0 to 0.6 *N* NH_2OH but does not significantly increase from 0.6 to 1.1 *N*. The reaction was carried out with 0.6 *N* NH_2OH on this basis since NH_2OH has been implicated in color instability (1).

Figure 6 compares the result of assaying succinimide by the hydroxamic acid-iron complex method to that of the *N*-hydroxysuccinimide method. The absorbance due to the iron complex (515 mμ) had to be corrected to correspond to the concentrations shown in Fig. 5 due to the difference in dilution between the two techniques (see *Experimental*). A comparison of the slopes of the lines shows more than a sixfold increase in sensitivity using the UV absorption method instead of the colorimetric.

DISCUSSION

Hydroxyaminolysis of Succinimide—At pH 9.5, 40°, succinimide simultaneously reacts with NH_2OH to form the hydroxamic acid, *N*-hydroxysuccinamide, and undergoes hydrolysis to form succinamic acid. *N*-Hydroxysuccinamide undergoes rapid ring closure to form *N*-hydroxysuccinimide (see Scheme I). This ring closure is much more rapid than the hydroxyaminolysis reaction. Pseudo first-order rate constants calculated from the appearance of the 260-mμ absorption maximum of *N*-hydroxysuccinimide are in agreement with those calculated from the appearance of color

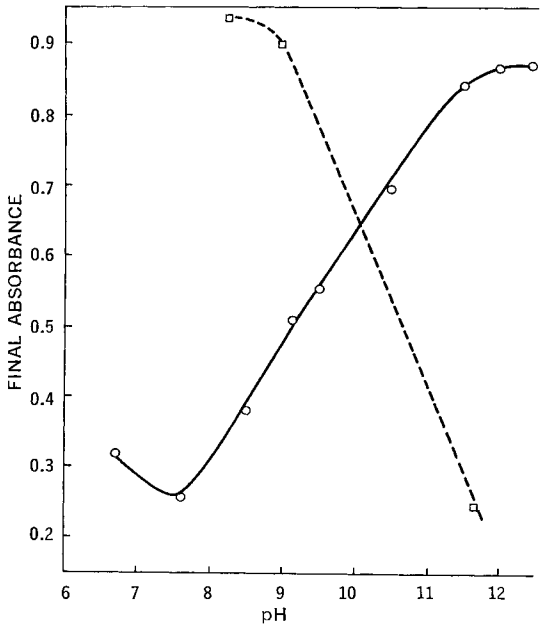


Figure 4—*The final absorbance values at 515 mμ due to formation of the hydroxamic acid-iron complex* (○) *and the final absorbance values at 260 mμ due to formation of* N*-hydroxysuccinimide* (□) *from reactions containing fixed concentrations of succinimide and* NH_2OH *and varying pH at 40°.*

due to the iron complexes of the hydroxamic acid and *N*-hydroxysuccinimide (Table I). The rate constant, k_1, represents the sum of the rate constants for hydrolysis, k_H, and hydroxyaminolysis, k_N. The bimolecular rate constant, k_2, for the formation of the hydroxamic acid can be calculated from the slope of k_1 *versus* [NH_2OH] where the intercept is k_H (Fig. 5). The value of k_2 is approximately the same at pH 9.5 and 10.5 as can be seen from the fact that the slopes are similar in Fig. 5. The increase in rate constant which accompanies this increase in pH is therefore due to the increase in hydrolysis rather than hydroxyaminolysis. Since more of the succinimide would hydrolyze at the higher pH value, the yield of hydroxamic acid would be expected to decrease with increasing pH. One would expect from these data that the final absorbance due to the color complex would decrease at higher pH values due to the increased loss of succinimide through the competing hydrolysis reaction.

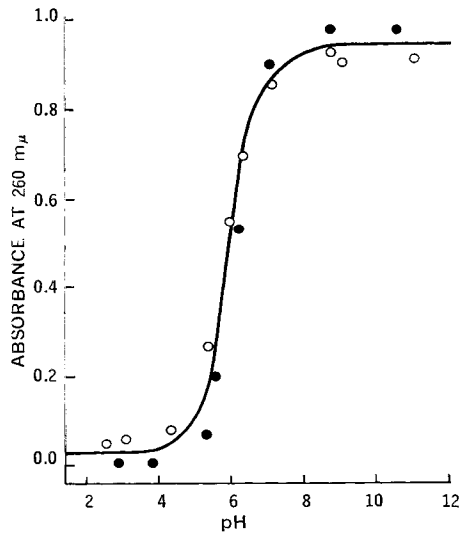


Figure 3—*Absorbance at 260 mμ as a function of pH for solutions containing* N*-hydroxysuccinimide* (●) *and solutions containing aliquots of a 2-hr. reaction containing 0.016 succinimide and 1.1* N NH_2OH *at pH 9.5, 40°.*

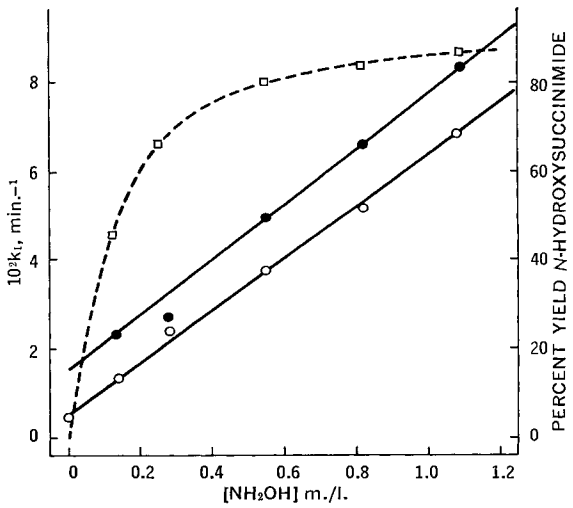


Figure 5—*Pseudo first-order rate constants,* k_1 (*left ordinate*), *as a function of* [NH_2OH] *for 0.016* M *succinimide at 40°, pH 9.50* (○) *and pH 10.5* (●). *The percent yield of* N*-hydroxysuccinimide* (*right ordinate*, □) *as a function of* [NH_2OH] *at 40°, pH 9.50.*

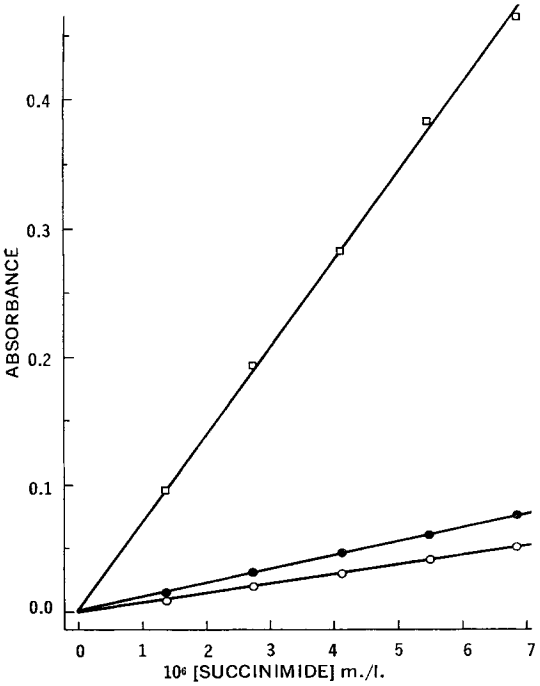


Figure 6—*Comparison of assay methods for succinimide by reacting with NH_2OH at 40°, pH 8.3 and measuring the* N-*hydroxysuccinimide at 260 mμ* (□) *or the hydroxamic acid-iron complex at 515 mμ* (○) *and at pH 11.7 measuring the iron complex at 515 mμ* (●).

The succinamic acid formed by hydrolysis of succinimide was found to have an overall rate constant, k_1, for hydroxyaminolysis plus hydrolysis, of only 1/100th the value of succinimide and would not therefore contribute significantly to the color formation. Figure 4 shows the final absorbance at 515 mμ for the color complex resulting from 0.016 *M* succinimide and 0.825 *M* NH_2OH at 40° from pH 6 to 13. The absorbance is seen to increase between pH 8 and 11 instead of decreasing as would be expected with increased succinimide hydrolysis. The reason for this increase is due to a change in the reaction scheme. Although the hydrolysis of succinimide is increasing over this range, the hydrolysis of *N*-hydroxysuccinimide is also increasing. The rate constant (in $min.^{-1}$) for *N*-hydroxysuccinimide hydrolysis at pH 9.5, 40°, is 2.3×10^{-4} and at 10.5 is 29.1×10^{-4}. Thus, as the pH is increasing, the yield of *N*-hydroxysuccinamic acid is also increasing (see Scheme II). A reaction carried out at pH 11.6 was found to give a high yield of absorbance at 515 mμ without any significant absorbance at 260 mμ.

The absorptivity of the color complex of *N*-hydroxysuccinimide is 528 under the conditions of this study. Figure 4 indicates an approximate increase in 515 mμ absorbance of threefold in going from pH 6 to 12. This absorbance at pH 12 agrees with the estimated value for the *N*-hydroxysuccinamic acid complex which would be expected to be about 1400 as was determined for the hydroxamic acid prepared from succinimide (1). The major product at pH 9.5 in the presence of 0.55 to 1.09 *N* NH_2OH, 40°, is *N*-hydroxysuccinimide (Fig. 5) whereas the major product at pH 12 under the same conditions is *N*-hydroxysuccinamic acid (Fig. 4).

It can be seen in Table III that there is very little increase in the percent yield of *N*-hydroxysuccinimide when the NH_2OH concentration is increased from 0.55 to 1.08 *M*. The initial increase in yield in going from 0 to 0.55 *M* NH_2OH is primarily due to increasing the fraction k_N/k_1 by increasing the product $k_2[NH_2OH]$. This is also reflected in the decrease in succinamic acid percent listed in Table III. Thus, increasing the NH_2OH concentration has a significant effect on the yield as long as k_H is significant as compared to k_1. It can be seen from Table III that k_H is 31% of the overall rate constant at 0.14 *M* NH_2OH but only 11, 8, and 6% at 0.55, 0.82, and 1.08 *M* NH_2OH, respectively. In this case there is no great benefit in employing high NH_2OH concentrations, such as 3 *M* as is commonly employed (3) in the analysis of amides and imides. In addition, there are two distinct disadvantages in using high NH_2OH concentrations. It has previously been shown that the NH_2OH is a key factor in both the decrease of color formation from the hydroxamic acid-iron complex as well as in the stability of the color (1). On that basis it is well to use the minimum NH_2OH required to convert the imide to the hydroxamic acid. In addition, it has been found that the decomposition of NH_2OH causes an acidic pH shift during the reaction. At modest NH_2OH concentrations this does not affect the assay but at high concentrations it can cause a problem in reproducibility of yields (Fig. 4).

It should be noted that the hydrolysis of an imide to an amide represents a potential source of error in the hydroxamic acid-iron complex method since both are capable of forming a hydroxamic acid. That is, the assay might lack selectivity. This is not the case for the assay of succinimide in the presence of its hydrolysis product, succinamic acid. At 40°, pH 9.5 and 0.3 *M* NH_2OH, the reaction of succinimide is 100 times faster than that of succinamic acid. At 40°, in the presence of 0.3 *M* NH_2OH and 0.03 *N* NaOH, succinimide reacts to yield a 515-mμ absorbance of 0.475 after 0.5 hr. by the hydroxamic acid-iron complex method. Under similar conditions succinamic acid gives no color at 0.5 hr. and after 21 hr. produces an absorbance value of only 0.060.

Analysis of Succinimide by Reaction with Hydroxylamine—The kinetics of hydroxyaminolysis of succinimide has been discussed in the previous section. This data has been used to develop an assay for succinimide which is sixfold more sensitive than the method employing complexation with iron. The principle involved is the control of reaction conditions so as to produce *N*-hydroxysuccinimide rather than hydroxamic acid (see Schemes I and II). *N*-Hydroxysuccinimide has an absorptivity of 8900 at 260 mμ in alkaline pH. Under the conditions defined previously for optimum color production (1) the hydroxamic acids in Schemes I and II would have absorptivities of roughly 1400 at 515 mμ. The absorptivity of *N*-hydroxysuccinimide is thus more than sixfold greater than that of the hydroxamic acid-iron complexes. A complete change in the reaction product from hydroxamic acid to *N*-hydroxysuccinimide would result in an increase in sensitivity of more than sixfold.

Figure 6 illustrates the increased sensitivity observed in the assay of succinimide by measuring the *N*-hydroxysuccinimide produced upon reaction with NH_2OH. In order to plot these on the same concentration scale, the absorbance readings of the iron solutions were corrected for the difference in dilutions between the methods. The highest absorbance was obtained by measuring the hydroxysuccinimide resulting from the hydroxyaminolysis of succinimide at initial pH 8.28, 40°, in the presence of 0.6 *N* NH_2OH. The same reaction at pH 12 produced no *N*-hydroxysuccinimide and therefore had the maximum yield of hydroxamic acid (Fig. 4). It can be seen that the resulting 515-mμ absorbance plot has less than $^1/_6$ the slope of that for the *N*-hydroxysuccinimide absorbance at 260 mμ.

The method is of potential utility in the assay of other cyclic imides. The author is currently investigating this reaction using several cyclic imide drugs and similar organic compounds. There are two considerations in developing an assay based on the formation of the cyclic *N*-hydroxyimide. Conditions must be found which favor the hydroxyaminolysis of the original cyclic imide. If this ring is too stable to open, the reaction with NH_2OH will be too slow. On the other hand, the conditions must favor the closure of the ring once the hydroxamic acid is formed.

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