Colorimetric Determination of Amides as Hydroxamic Acids

FELIX BERGMANN, *The Hebrew University, Hadassah Medical School, Jerusalem, Israel*

IN 1889 Hoffmann (*5*) showed that a concentrated aqueous solution of acetamide and hydroxylamine hydrochloride gave an almost quantitative yield of acethydroxamic acid.

$$RCONH_2 + NH_2OH \longrightarrow RCONHOH + NH_3$$

This reaction is analogous to the conversion of esters or anhydrides into hydroxamic acids. Therefore, it should be possible to determine amides by the same colorimetric method, which has been so successfully applied to these two groups of compounds (*4, 7*)—viz., the formation of a colored complex between hydroxamic acid and ferric chloride. In a few sporadic cases this has been done—e.g., for the determination of the phenylacetamido group of penicillin G (*1, 2*). Hestrin (*4*), however, reported that amides failed to react under the conditions which he used for the determination of esters (reaction time 1 minute at room temperature). The author has now studied more thoroughly the conditions which permit the analytical determination of amides as their corresponding hydroxamic acids. This method is of special interest in view of recent reports on the enzymatic conversion of amides (*3*) and peptides (*6*) into hydroxamic acids.

METHODS

The reagents used differed but slightly from those described by Hestrin (*4*).

Solution 1. Hydroxylamine sulfate, 2 *N*
2. Sodium hydroxide, 3.5 *N*
3. Hydrochloric acid, 3.5 *N*
4. Ferric chloride, 0.74 *M* in 0.1 *N* hydrochloric acid solution

All amides used in this investigation were dissolved in water at concentrations of 5 or 10 $\times$ 10^{-3} *M*. The alkaline hydroxylamine reagent (2 ml.), prepared by mixing equal volumes of solutions 1 and 2, and graded volumes of the amide solution, with the addition of enough water to give a total volume of 3 ml., was kept at various temperatures for different periods of time. The reaction mixture was then rapidly cooled to room temperature, 1 ml. each of solutions 3 and 4 were added, and the extinction determined in a Klett-Summerson photoelectric colorimeter, using filter No. 54 (spectral range 500 to 570 mμ). A No. 50 filter (spectral range 470 to 530 mμ) was used for fluoroacetamide, because fluoroacethydroxamic acid has its maximum of absorption near 500 mμ. Readings were carried out within 5 minutes. The color of the complexes fades slowly, showing a decrease of 15 to 20 Klett units per μmole per hour. In the case of fluoroacethydroxamic acid, fading progressed much faster, about 50 Klett units per micromole per hour. Each experiment comprised five different concentrations of amide in order to test the linear relationship between extinction and concentration. This relationship was found to hold in all cases.

In preliminary experiments the reaction with hydroxylamine was carried out at various pH values. For both acetamide and nicotinamide the rate of reaction increased with increasing pH, but above pH 13 the hydrolysis by hydroxyl ion became faster than the interaction with hydroxylamine. Therefore, the standard mixture described above with a pH of about 13.8 was used for all determinations.

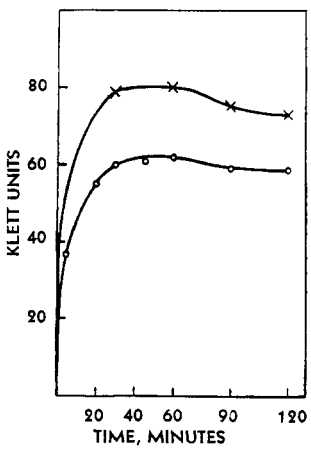


Figure 2. Reaction with Hydroxylamine at 26° C.

-x-x-x- Formamide
-o-o-o- Fluoroacetamide

RESULTS

The reaction of hydroxylamine with amides was studied at three different temperatures—viz., 26°, 60°, and 100° C. It was found that the conditions for the maximum colorimetric value were different for each compound. In Figures 1 to 4 are shown a number of representative curves. Pure acethydroxamic acid gave a value of 105 under standard conditions. Acetamide reached this value after interaction for 8 hours at 26° C., but at 60° C. the maximum reached after 2 hours was only 90, and at 100° C., 65 (reached after 10 minutes). Furthermore, the curves showed that at room temperature the maximum persisted over a period of several hours, at 60° C. only for 30 minutes, and at 100° C. less than 5 minutes. Since other amides behaved in a similar fashion, the following rules can be established:

The rate of reaction between amides and hydroxylamine increases with temperature, but a competitive reaction—viz., the hydrolysis of the amide — becomes more and more preponderant, thus depressing the yield of hydroxamic acid.

If the reaction time is extended beyond the optimal period required for maximum colorimetric yield, a gradual decomposition of the hydroxamic acid already formed is observed. Therefore, at elevated temperatures, the optimal period is considerably shortened. This is due to the fact that the energy of the car-

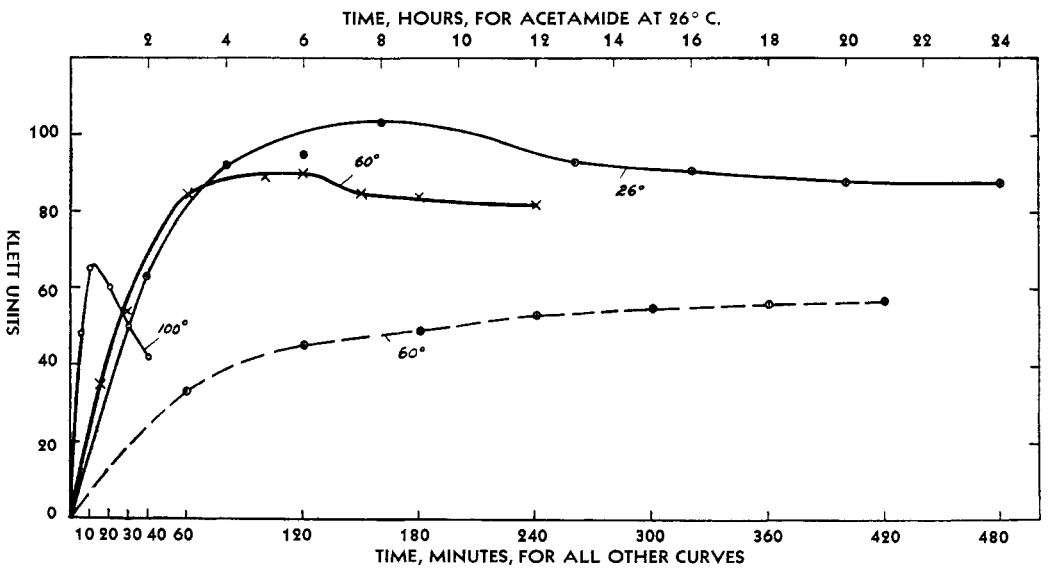


Figure 1. Rate of Reaction of Acetamide (Full Curves) and of *N*-Methylacetamide (Dashed Curve) with Hydroxylamine at Various Temperatures

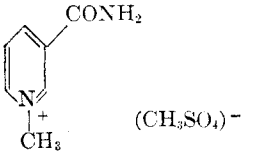
(Note that for this reason the initial slope for the acetamide curves at 26° and at 60° C. is not identical)

Table I. Optimal Conditions for Conversion of Amides into Hydroxamic Acids

Name of Compound	Temperature, ° C.	Reaction Time, Min.	Klett Reading, Units per Micromole
Acetamide	60	120	90
	26	480	103
N-Methylacetamide	60	420	57
Acetanilide	60	180	70
N^4-Acetylsulfanilamide	60	240	70
Acetylglycine	60	240	35
Fluoroacetamide	26	60	62
Formamide	26	60	80
	60	10	75
Dimethylformamide	26	240	45
Succinimide	60	120	85
Caprolactam	60	420	41
Asparagine	60	180	38
Glutamine	60	180	35
Glutathione	60	120	48
Glycylglycine	60	120	25
Nicotinamide	26	480	45
N^1-Methylnicotinamide methosulfate (I)	26	360	45
Nicotinic acid methylamide	60	240	30
Coramine (nicotinic acid diethylamide)	60	480	6
Pantothenic acid, calcium salt	26	300	80
Barbitone	100	45	1.7
Pentobarbitone	60	300	1.5
Phenobarbitone	100	120	7.5
Evipan, sodium	100	30	9

bon-nitrogen bond in amides and hydroxamic acids is nearly the same and conditions which lead to faster hydrolysis of the former will also produce more rapid decomposition of the latter.

The results obtained with about 20 amides are represented in Table I. We can now relate, in many cases, the rate of reaction to the specific structure of an amide. For example, formamide reached its maximum at 26° C. in less than 1 hour, acetamide after 8 hours. Substitution of amide hydrogen reduced the speed of reaction considerably. *N*-Methylacetamide (Figure 1) reached its maximum after 7 hours (60° C.) and 24 hours (26° C.), respectively, as compared to 2 and 8 hours for acetamide itself. The corresponding figures for formamide were 10 and 40 minutes, for dimethyl formamide, 40 and 300 minutes, respectively. In accordance with this observation acetylglycine and peptides gave a slow reaction and low colorimetric values. A similar relationship was found among the derivatives of nicotinamide. Nicotinamide itself reached its maximum value of 52 per μmole per ml. after reaction for 8 hours at 26° C., whereas its *N*,*N*-diethyl derivative (coramine) gave a maximum value of 6 Klett units per μ mole after 8 hours at 60° C. On the other hand, the quaternary pyridinium salt (I) behaved like the parent compound.



I

Substitution in the acyl residue also exerted a pronounced influence. This could be demonstrated in two cases:

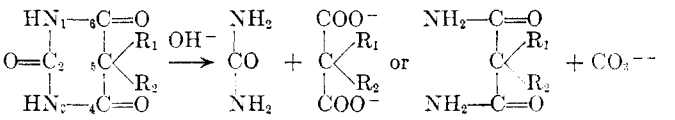
Fluoroacetamide showed the fastest reaction of all acetamides, reaching the speed of reaction of formamide (Figure 2).

Pantothenic acid, although it is substituted in the amide group, gave a comparatively fast reaction at room temperature, reaching a high maximum of 90 Klett units per μmole. At 100° C. it reacted instantaneously (45 seconds). This was undoubtedly due to the α-hydroxy group in the pantoic acid moiety.

Acetanilide (Figure 3), which was used as representative of *N*-arylamides, has been reported by Hoffmann (5) to react sluggishly in alcoholic solution. In aqueous solution there was rapid reaction with hydroxylamine, which reached its maximum at 60° C. after 3 hours. The reaction of N^4-acetylsulfanilamide was similar, but slower.

Succinimide, in agreement with theoretical predictions, gave only one equivalent of hydroxamic acid—i.e., the maximum value of 85 was comparable to the value obtained for ethyl hydrogen succinate (90). This the author ascribes to the fact that the alkaline reagent converts the amide into the sodium salt of the monoamido acid, which then reacts with hydroxylamine.

An interesting behavior was found in the barbiturate series, where the applicability of the reaction depended on the substituents in the 5-position. Thus, barbitone and pentobarbitone gave readings of 1 to 2 Klett units per μmole per ml. At 100° C. phenobarbitone reached a maximum of 7.5 after 2 hours and evipan a maximum of 9 after 0.5 hour. The analytically useful range of concentration in this series was thus between 5 and 50 μmoles per ml. *In view of the inability of urea or guanidine salts to react with hydroxylamine under standard conditions, it was assumed* that two different courses of ring opening were possible in alkaline media. The main reaction occurred at the linkages N^1—C^6 and N^3—C^4, thus producing free carboxyls which were not converted into hydroxamic acids. However, depending on the substituent at C^5, the bonds N^1—C^2 and N^3—C^2 may be broken first in a certain percentage of the molecules, thus producing the reactive amide grouping, as shown in the following scheme:



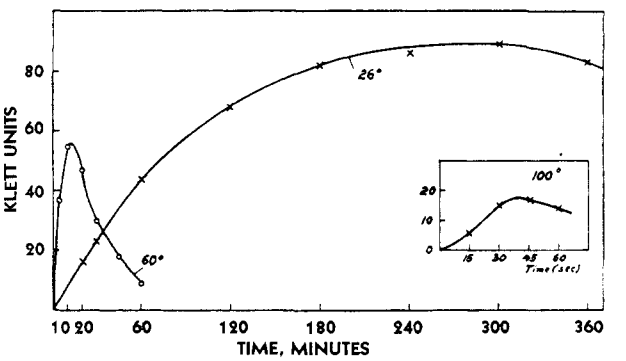


Figure 3. Reaction of Pantothenic Acid with Hydroxylamine at Various Temperatures

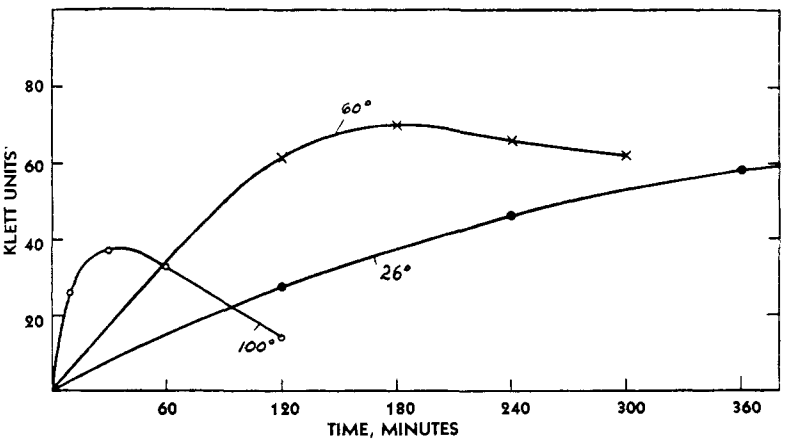


Figure 4. Reaction of Acetanilide (in Aqueous Solution) with Hydroxylamine

This hypothesis requires a study of the course of the alkaline hydrolysis of barbiturates, as influenced by various substituents, which is now being undertaken.

As expected, betaines—e.g., trigonelline—do not react with hydroxylamine.

DISCUSSION

The reaction of amides with an alkaline hydroxylamine reagent represents a convenient method for the colorimetric determination of amides. The advantages of the method are apparent especially in those cases, where it is necessary to determine for an acid and its amide simultaneously—e.g., nicotinic acid and its amide. On the other hand, the method does not distinguish between nicotinamide and its metabolic derivatives, which still contain the amide group. Another important example is the determination of glutamine in the presence of urea, or the determination of N^4-acetylsulfanilamides in the presence of the free amines. The application of such new possibilities to clinical analysis will be reported elsewhere.

It is evident from the data that amides react much more slowly than the corresponding esters. In many cases this may be used to analyze for both compounds successively by the same method. For example, ethyl acetate reacts completely at room temperature within 2 minutes, whereas acetamide requires 6 hours under these conditions. The formation of hydroxamic acids thus becomes a valuable tool for kinetic studies.

ACKNOWLEDGMENT

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Amperometric Determination of Fluoride

C. R. CASTOR AND J. H. SAYLOR
Duke University, Durham, N. C.

SEVERAL amperometric methods for the determination of fluoride ion have appeared in the literature. Haul and Greiss (*2*) proposed both amperometric and polarographic methods based on the precipitation of lead chlorofluoride. Petrow and Nash (*7*) further developed and improved the amperometric titration of fluoride in a chloride solution with lead. Langer (*4*) and Luzina (*5*) adapted the thorium nitrate determination to an amperometric titration. However, all these methods have some apparent difficulties, particularly with respect to the interference of a number of diverse ions.

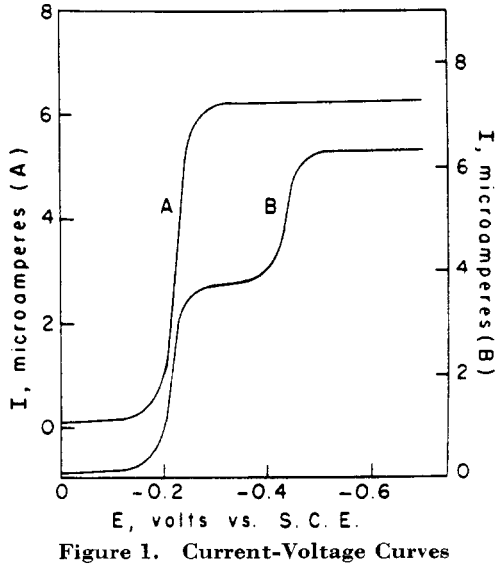

Figure 1. Current-Voltage Curves

A. **Superchrome Garnet Y alone**
B. **Superchrome Garnet Y plus aluminum**
Both solutions buffered at pH 4.6

Willard and Dean (*8*) used di-*o*-hydroxyazo dyes for a polarographic determination of aluminum. The method was based upon the polarographic behavior of Pontachrome Violet SW (Colour Index 169), which in the presence of aluminum forms an aluminum-dye complex ion. They found the diffusion current of the aluminum-dye compound to be proportional to the aluminum concentration. The present investigations showed that the addition of fluoride ion to this complex resulted in the formation of AlF_6^{---} ion, with the resulting decomposition of the complex.

The method described depends upon the decrease in diffusion current of an aluminum-dye complex in the presence of fluoride ion with a corresponding increase in the diffusion current of the free dye. It was found that the low solubility of Pontachrome Violet SW made it impractical for use in the amperometric titrations. However, investigation showed that Superchrome Garnet Y (Colour Index 168), which is the sodium salt of 5-sulfo-2-hydroxybenzeneazoresorcinol, has similar polarographic characteristics and a much greater solubility. The current-voltage curves of this dye alone and in the presence of aluminum are shown in Figure 1. The total diffusion current remains constant, but part of the wave is displaced approximately 0.2 volt more negative than the original dye wave and now appears as a distinct second wave. The height of the first wave is proportional to the concentration of free dye in the solution, while the height of the second wave is proportional to the concentration of the aluminum present as an aluminum-dye complex ion.

EXPERIMENTAL

Apparatus and Reagents. A Sargent manual polarograph, Model III, was used to obtain the titration curves. A modified Fredrickson cell (*1*) which could be used for sample volumes of 30 to 100 ml., was used in all titrations. Water at 25° ± 0.1° C. was circulated through the cell from a thermostat. A saturated calomel electrode was used as a reference electrode with an agar-saturated potassium chloride salt bridge providing contact