# Determination of Cycloheximide and Its Degradation Products Alone and in Mixtures

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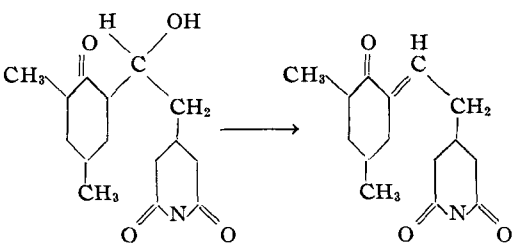
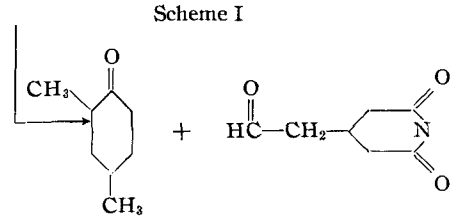
**Procedures for determining cycloheximide and its degradation products, anhydrocycloheximide, 2,4-dimethylcyclohexanone, and glutarimide β-acetaldehyde, separately and in mixtures are devised. They are based on the relative oximation reactivities of the carbonyl compounds under specified conditions. The method is useful for aqueous and methanolic solutions. Simultaneous spectrophotometric assay of anhydrocycloheximide is necessary for assay of mixtures. The use of potentiometry and imide analytical methods expands the utility of the method to cases where imide solvolysis may have occurred or where the degradation products are derived from an unknown amount of cycloheximide precursor.**

TWO SPECTROPHOTOMETRIC methods for assaying cycloheximide have been developed. One is based upon the reaction of the imide with alkaline hydroxylamine to produce a hydroxamic acid which is then converted to the colored ferric hydroxamate (1). The other is a color reaction with resorcinol, which occurs with compounds capable of forming an α,β-unsaturated ketone when heated in HCl (2).



Cycloheximide Anhydrocycloheximide

Scheme I



2,4-Dimethylcyclohexanone Glutarimide β-acetaldehyde

Scheme II

Neither method can distinguish between cycloheximide and its dehydration product, anhydrocycloheximide (Scheme I), an α,β-unsaturated ketone (3). The first method is not capable of differentiating among imides, one of which could be glutarimide β-acetaldehyde, a dealdolization product (3, 4), as per Scheme II.

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The methods described here were developed to assay for cycloheximide in the presence of any or all of the carbonyl compounds resulting from its degradation. The analysis was adapted from an established carbonyl assay based on the formation of the oxime (5).

The basic principle of the operational method developed in this paper is to take advantage of varying reaction rates for the formation of the respective oximes which are assayed titrimetrically. Recent review articles cover the recent theoretical and practical aspects and applications of these kinetic methods (6, 7).

## EXPERIMENTAL

### Reagents

**Hydroxylamine Hydrochloride, 1.0 M ($NH_2OH$).**—Dissolve 69.5 Gm. of reagent grade hydroxylamine hydrochloride in a mixture of 100 ml. reagent grade absolute methanol and 900 ml. of methyl cellosolve.

**2-Dimethylaminoethanol, 0.08 M (DMAE).**—Dilute 8 ml. of Eastman white label DMAE to 1 L. with methyl cellosolve.

**Standard Perchloric Acid, 0.02 M ($HClO_4$).**—Dilute 3.6 ml. of reagent grade perchloric acid (70–72%) to 2000 ml. with reagent grade absolute methanol.

**2 - Amino - 2 - (hydroxymethyl) - 1,3 - propanediol (TRIS).**—Eastman white label TRIS is the acidimetric standard for the standardization of the perchloric acid solutions.

### Carbonyl Compounds

Cycloheximide was obtained from Dr. G. Boyack, The Upjohn Co., and purified (4). Anhydrocycloheximide was prepared as described in the literature (8). The characterization of these materials has been reported (9). Glutarimide β-acetaldehyde was supplied by Lawes (4). The method of Kornfeld *et al.* (3) was used to prepare 2,4-dimethylcyclohexanone by alkaline degradation of cycloheximide. 2,4-Dimethylcyclohexanone: I. R. spectrum (8), $\bar{\nu}$ in cm.$^{-1}$ (pure): 2850 (CH), 1710 (C=O), 1450 (—$CH_2$—), 1370 (—$CH_3$); $\eta_D^{25}$ 1.4436 (corrected); literature value 1.4435 (3).

### Method and Principles

Partially convert the hydroxylamine salt to the free base by a measured aliquot of the strong base,

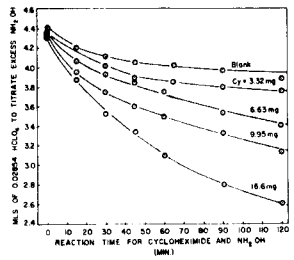


Fig. 1.—Titer of $HClO_4$ consumed by excess $NH_2OH$ as a function of oximation time at 22° for various amounts of cycloheximide.

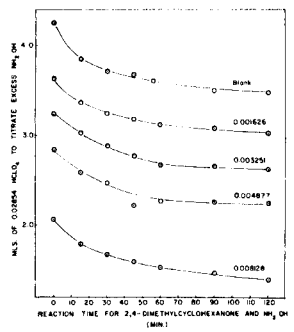


Fig. 2.—Titer of $HClO_4$ consumed by excess $NH_2OH$ as a function of oximation time at 22° for various gram amounts of 2,4-dimethylcyclohexanone.

DMAE. Add an amount of DMAE so as to give at least a 2:1 molar ratio of $NH_2OH$ to carbonyl compound in the mixture reacted at constant temperature in a media as nonaqueous as possible. At the desired time, titrate the unreacted excess hydroxylamine in nonaqueous media with $HClO_4$. The reactions involved in the method are:

Free $NH_2OH$ formation: $NH_3OH^+Cl^- + DMAE \rightarrow (DMAE)H^+Cl^- + NH_2OH$ Reaction *1*

Oximation: $NH_2OH + \begin{matrix}\diagdown \\ \diagup\end{matrix}C{=}O \rightleftarrows \begin{matrix}\diagdown \\ \diagup\end{matrix}C{=}NOH + H_2O$ Reaction *2*

Titration: (excess) $NH_2OH + H^+ \rightarrow NH_3OH^+$ Reaction *3*

## Procedure

Maintain all reagents and solutions at 22°. Transfer a 1-ml. aliquot containing 0.01 to 0.06 meq. of carbonyl compound in methanolic solution to a 30-ml. beaker. Add 2 ml. of $NH_2OH$ to the mixture with constant stirring at time zero. Titrate the excess $NH_2OH$ to the predetermined pH of the end point after the desired time of reaction. A 15-min. reaction period is satisfactory. (The apparent end point pH on the Radiometer titrator is 3.2. A syringe microburet is used.)

Prepare a simultaneous blank in the same manner except use 1 ml. of methanol instead of the methanolic solution of the carbonyl compound.

## Preparation of Carbonyl Compound Calibration Curves

Plot the titer consumed by the excess $NH_2OH$ as per Reaction *3* against oximation time for the reaction of Reaction *2* for various sample weights, as in Figs. 1 and 2. Plot the volume of titer consumed at a given time against the weight of the carbonyl compound, as in Figs. 3 and 4. An alternative is to plot the difference between this volume of titer and that of the blank.

## Determination of Unknown Amounts of Single Carbonyl Compounds

Determine $HClO_4$ titer consumption for the unknown amount of the carbonyl compound to be assayed and subtract the $HClO_4$ titer consumption for a pertinent blank for the respective oximation time. From this difference, use the calibration curve to determine the amount of carbonyl compound. The stated procedure is for about 0–16 mg. of the cited carbonyl compounds. For other amounts, calibrate so that the DMAE gives at least a 2:1 molar ratio of free $NH_2OH$ to the carbonyl compound.

Assay aqueous solutions of the carbonyl compound by taking 1 ml. of aqueous solution containing the specified range of amounts of carbonyl compound. Preneutralize any excess strong acids. Substitute 1 ml. of methanol for the stated 1 ml. of $H_2O$ that was added to the mixture in the standard procedure.

## Preparation of Calibration Curves for Determination of Cycloheximide and Its Degradation Products in Mixtures

Prepare a series of mixtures of cycloheximide (CY), anhydrocycloheximide (AN), 2,4-dimethylcyclohexanone (2,4-D), and glutarimide β-acetaldehyde (GBA) so that the constancy of the following expression is satisfied for the 1-ml. aliquot to be assayed:

$$\text{meq. CY} + \text{meq. AN} + \tfrac{1}{2}(\text{meq. GBA} + \text{meq. 2,4-D}) = \text{meq.}_0 \quad \text{(Eq. 1)}$$

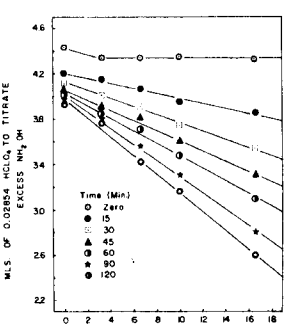


Fig. 3.—Plots of the volume of $HClO_4$ titer consumption upon the sample weight of cycloheximide for various oximation times at 22°.

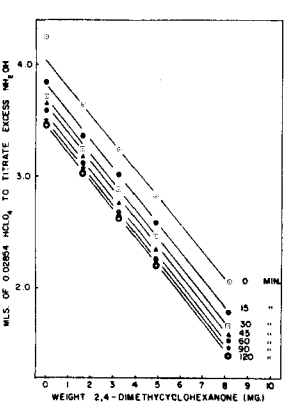


Fig. 4.—Plots of the volume of $HClO_4$ titer consumption upon the sample weight of 2,4-dimethylcyclohexanone for various oximation times at 22°.

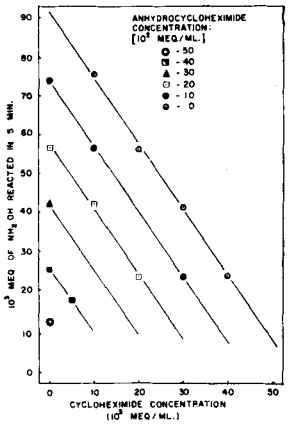


Fig. 5.—Calibration curves for the analysis of a mixture of carbonyls resulting from the degradation of cycloheximide (0.050 meq./ml.). From the spectrophotometric determination of anhydrocycloheximide (appropriate line) and the milliequivalents of $NH_2OH$ reacted in 5 min. of oximation at 22° (ordinate), the cycloheximide concentration can be obtained (abscissa).

For the stated procedures, $meq._0 = 0.050$. An equivalent amount of 2,4-D may be substituted for the GBA if this compound is difficult to obtain. A satisfactory series of mixtures was used in Fig. 5, where the difference between 0.050 meq./ml. and the pertinent CY and AN concentrations are equal to one-half the sum of the equimolar concentrations of GBA and 2,4-D added to a mixture. This difference could also be one-half the necessary amount of 2,4-D alone added to a synthetic mixture.

Assay the synthetic mixture by the given procedure for the single carbonyl compound, except that the oximation time for the reaction of Reaction *2* is 5 min. Correct for the titration of the blank. Determine either the milliliters of $HClO_4$ necessary to titrate the excess $NH_2OH$ or the derived milliequivalents of $NH_2OH$ reacted in that interval. Plot against the cycloheximide concentration of the aliquot for a constant anhydrocycloheximide concentration, as in Fig. 5. The series of lines are the calibration curves for the mixtures.

### Determination of Cycloheximide in the Presence of Its Degradation Products

This method is directly applicable to the assay of known concentrations of cycloheximide solutions that degrade by the reactions of Schemes I and II. Start with a solution that is 0.050 *N* in the sum of cycloheximide and its degradation products. Determine the anhydrocycloheximide concentration of the solution to be analyzed by spectrophotometry (9). The absorptivity of the 245 mμ $\lambda_{max.}$ of anhydrocycloheximide is 8100 in a Beckman model DU spectrophotometer at 0.1-mm. slit width.

Determine the milliliters of $HClO_4$ titer or the milliequivalents of $NH_2OH$ reacted by the procedure given for the single carbonyl compound assay using a 1.00-ml. aliquot. The modification for aqueous solutions can be made as previously cited. From these data on the ordinate and line for the pertinent anhydrocycloheximide concentration, read on the abscissa of Fig. 5 for the cycloheximide concentration. Double the difference between 0.050 and the sum of the determined anhydrocycloheximide and cycloheximide concentrations to determine the sum of the equimolar glutarimide β-acetaldehyde and 2,4-dimethylcyclohexanone concentrations.

If the total milliequivalents of a solution or in a sample are unknown with respect to cycloheximide and its degradation products, use the procedure of Forist and Theal (1) to determine the total imide. Then prepare a solution of 0.050 *N* in imide and analyze as above.

## RESULTS AND DISCUSSION

The ease of oximation of the studied carbonyl compounds followed the expectation that the increased positivity of the carbonyl carbon increases the rate of oximation (10). It follows that oximation of aldehydes proceeds rapidly, alkyl substitution at the α-carbon of a ketone hinders oximation, and electron withdrawing groups such as the vinyl group increase the rate of oximation.

The glutarimide β-acetaldehyde and the least substituted cyclohexanone, 2,4-dimethylcyclohexanone, were oximated completely within 5 min., as shown in Fig. 6 by the slope of unity for these compounds. Neither anhydrocycloheximide nor cycloheximide was oximated completely even after 2 hr. under the conditions used. Figure 1 demonstrates the increasing consumption of $NH_2OH$ by cycloheximide over the blank for periods beyond this time; this is demonstrated also by the nonparallels in the plots of Fig. 3. The plots for anhydrocycloheximide were similar. A 50% conversion to the oxime took 40 min. for anhydrocycloheximide and 80 min. for cycloheximide. The desired sensitivity can be obtained by the choice of an appropriate oximation time. The plots for the oximation of 2,4-dimethylcyclohexanone and glutarimide β-acetaldehyde were parallel and indicate an almost instantaneous oximation. (See Figs. 2 and 4.)

Replicates of solutions of the carbonyl compounds and blank solution were prepared and randomized. The operator had no knowledge of the contents of the preparations. The maximum variability among replicates for the $HClO_4$ titrations was ±0.01 ml. with the microburet used. The total maximum variability in the estimate of carbonyl compound was 2% if the difference in the $HClO_4$ titer of the unreacted oxime for sample and blank was 1.000 ml. The regressions of Fig. 5 clearly demonstrate that for a known concentration of anhydrocycloheximide, the error in the assay of cycloheximide concentration is also 2%.

The method for the determination of various carbonyl compounds in degrading mixtures is based on the varying oximation rates, as shown in Fig. 6 for the conditions specified. It depends on the assay of a known amount of cycloheximide as the precursor in the aliquot to be assayed and the known stoichiometry of the degradations given in Schemes I and II. Figure 5 is operationally constructed so

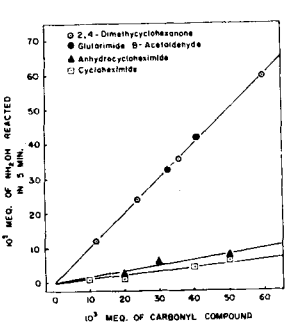


Fig. 6.—Comparative amounts of free $NH_2OH$ consumption by cycloheximide and its carbonyl containing degradation products for a 5-min. oximation period at 22°.

that the rates of dealdolization can be studied by this method. However, in conjunction with imide analysis (1) for choosing the necessary amount of sample to be assayed, the method is applicable in those cases where the stoichiometry of Schemes I and II holds.

The procedures stated can be applied only in the absence of imide hydrolysis. The method depends on titration of excess base by $HClO_4$, and any acidic function produced would decrease the titratable free hydroxylamine. However, the amount of hydrolyzed imide can be compensated by prior potentiometric titration of the sample to be analyzed by methods given previously (9) and equivalent DMAE added. Under these conditions the amount of imide (1) plus the difference between the carboxylic acid content and the liberated ammonia determined by potentiometry (9) would have to be used to determine the amount of mixture to be analyzed.

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# Schiff Base Formation in the Development of a Spectrophotometric Assay for Sulfonamides

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**The reaction between aromatic amines and aromatic aldehydes was investigated as the basis of a spectrophotometric assay for sulfonamides. Those properties of the equilibrium reaction which affect the absorptimetric properties of the system were studied to evaluate the method. The effects of pH, ionic strength, concentration of aldehyde, solvent system, and temperature were determined. The method was applied to the determination of sulfanilamide, sulfadimethoxine, and succinylsulfathiazole, and comparative analyses were performed with the method of Bratton and Marshall. The low absorbance method of precision spectrophotometry was employed in the determination of microgram quantities of sulfanilamide.**

THE OBJECTIVE of this investigation was to evaluate a spectrophotometric method of analysis for sulfonamides based on the formation of chromophoric Schiff bases (*N*-alkyl aldimines). The reaction between various aldehydes and sulfonamides has been reported previously by Butler and Ingle (1) and Castle (2), who prepared crystalline Schiff base derivatives of sulfonamides and utilized the crystalline properties of these derivatives as a means of qualitative identification. An assay procedure based on this method also has been reported for *p*-aminosalicylic acid and isoniazid (3).

In the equilibrium reaction, which occurs in the Schiff base formation (Fig. 1), a carbinolamine intermediate is formed upon the reaction of amine and aldehyde. A second equilibrium reaction subsequently takes place in which the carbinolamine intermediate forms the Schiff base and water. These reactions are known to be dependent upon the hydronium ion concentration, and the effects of pH on the equilibrium and kinetic properties of Schiff base formation have been studied (4).

In this study the effects of temperature, pH, ionic strength, solvent, and concentration of reactants on the spectral properties of Schiff bases were determined to ascertain which factors required careful control. The method was applied to the analysis of several sulfonamides, and the precision, accuracy, and sensitivity were evaluated. A comparative study of this method was made with the method of Bratton and Marshall (5).

## EXPERIMENTAL AND RESULTS

**Reagents.**—Vanillin U.S.P.[1] and salicylaldehyde[2] were employed as the chromogenic reagents.

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