The combined hexane washings were placed in a centrifuge tube and cooled in Dry Ice. The tube was then quickly centrifuged and the liquid was poured off. The solid in the tube was twice washed with hexane, cooled, and centrifuged. The combined solids were dissolved in a small amount of isopropyl alcohol, 10% sodium iodide solution was added, and the iodine was liberated by the addition of 0.1 N H_2SO_4. The amount of iodine thus formed was estimated in the usual fashion by standard thiosulfate titration.

Acknowledgment.—Support of this work by the National Science Foundation under Grant No. GP-1177 is gratefully acknowledged.

Cycloheximide Transformations. I. Kinetics and Mechanisms in Aqueous Acid[1,2]

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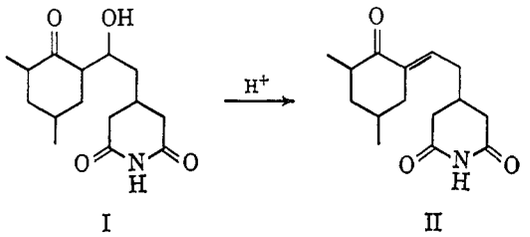
The antibiotic, cycloheximide, undergoes hydrogen ion catalyzed dehydration to anhydrocycloheximide. The mechanism is through dehydration with stereospecific rehydration. The isolated major product of rehydration is a new stereoisomer of cycloheximide and may be either α-epi-Naramycin B or α-epicycloheximide with the former assignment preferred. Hydrolyses of the imides of each of the reaction components occur simultaneously with the above mechanism but at a much slower rate. The rate-determining step in the solvolytic sequence is the hydrolysis to the acid amide. The complex equilibria among the imide, acid amide, and dicarboxylic acid forms of these three components have been quantified as functions of temperature and acidity so that the concentration of any component can be defined as a function of time with the aid of the analog computer.

The antifungal antibiotic cycloheximide (I) is unstable in acid[4] and alkali[5-8] and mild structural changes markedly alter the biological activity.[9] However, the kinetics and mechanisms of such chemical changes have not been quantified. The possible chemical transformations in aqueous solution are dehydration to anhydrocycloheximide (II), imide hydrolysis, dealdolization, and oxidation.[5] In addition, the possibility of stereochemical changes increases the complexity of the system.[6] This paper reports on a complete investigation of the kinetics and mechanisms of the transformations of cycloheximide in aqueous acid.

Results

Spectral Transformations of Cycloheximide in Acidic Solutions.—The absorbance at 245 mμ, due to the formation of the α,β-unsaturated ketone II from the dehydration of cycloheximide (I), increased as a function of time and approached a maximum (Figure 1, upper right). The average yield of anhydrocycloheximide, at this maximum, for the various temperatures was 68%, 40.0°; 70%, 50.0°; 71%, 61.8°; 72%, 70.0°; and 73%, 74.6°. The per cent yields for various hydrochloric acid concentrations were not significantly different at a given temperature. Analysis of the variance showed that this increase of yield with temperature is statistically significant ($P > 0.99$). Subsequently, the absorbance decreased at a slower rate to a minimum value.



I II (1)

First-order plots for the rate of approach to the maximum absorbance obtained at 245 mμ, A_m, were linear for two to three half-lives. Typical plots are given in Figure 2. The apparent first-order rate constants (Table I) were calculated from the slopes based on eq. 2, where A_t is the absorbance at time, t.

$$\ln(A_m - A_t) = -kt + \ln A_m \quad (2)$$

The apparent first-order rate constants were directly proportional to hydrogen ion activity in agreement

$$k = k_{H^+}(a_{H^+}) \quad (3)$$

with eq. 3, where the activities of the hydrogen ion, a_{H^+}, were calculated from the HCl concentrations (Table I) and the activity coefficients, f, in the literature where $a_{H^+} = f[HCl]$.[10] The catalytic rate constants, k_{H^+}, were calculated from the slopes of the plots of k *vs.* f[HCl] (Figure 3 and Table II). An Arrhenius plot of the hydrogen ion catalytic rate constants is given in Figure 4 where

$$\log k_{H^+} = -\Delta H_a/2.303RT + \log A \quad (4)$$

The value of ΔH_a is 19.0 kcal./mole and log A is 13.4.

Apparent first-order rate constants for the subsequent loss of the absorbance, A_t, at 245 mμ (Figure 1, upper), were calculated from the slopes based on eq. 5,

$$\ln(A_t - A_\infty) = -k't + \text{constant} \quad (5)$$

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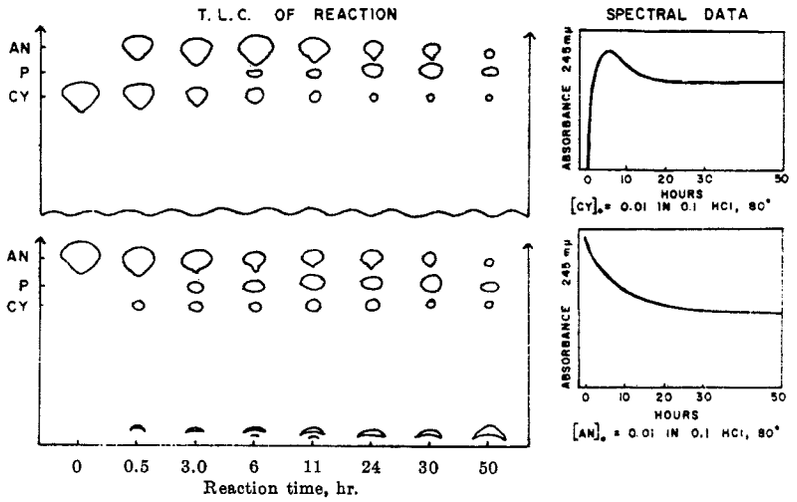


Figure 1.—Examples of spectral changes of cycloheximide, CY (upper right), and anhydrocycloheximide, AN (lower right), with time in aqueous acid. Typical reaction monitoring by thin layer chromatography is given to the left. The spots at the origin are due to generated acid derivatives and a unique product, P, appears with time.

TABLE I

APPARENT FIRST-ORDER RATE CONSTANTS (k IN SEC.$^{-1}$) FOR THE ACID-CATALYZED DEHYDRATION OF CYCLOHEXIMIDE[a] TO ANHYDROCYCLOHEXIMIDE BASED ON MEASUREMENTS OF THE 245-Mμ ABSORBANCE OF ANHYDROCYCLOHEXIMIDE[b]

$$CY \xrightarrow{k} AN$$

[HCl]	10^4k	[HCl]	10^4k	[HCl]	10^4k
40.0°		50.0°		61.8°	
0.016	0.0161	0.0413	0.108	0.0413	0.263
0.024	0.0236	0.180	0.538	0.120	0.769
0.036	0.0333	0.360	1.01	0.180	1.19
0.045	0.0390	0.450	1.31	0.270	1.88
0.054	0.0485	0.540	1.63	0.360	2.38
0.064	0.0579	0.720	2.10	0.400	2.69
0.072	0.0637	0.900	2.69	0.450	3.05
0.080	0.0728	...	...	0.540	3.61
0.090	0.0818	...	...	0.640	4.27
0.400	0.464				
0.540	0.589	70.0°		74.6°	
0.640	0.810	0.0413	0.534	0.0413	0.867
0.720	0.878	0.090	1.17	0.090	1.75
0.800	1.01	0.135	1.75	0.135	2.46
0.900	1.13	0.180	2.36	0.160	3.17
		0.270	3.25	0.180	3.48
		0.360	4.72	0.240	4.43
		0.450	5.68	0.320	5.96

[a] The initial concentration of cycloheximide were either 14.2×10^{-5} or 7.11×10^{-5} M. The apparent first-order rate constants were independent of these initial concentrations. [b] Apparent first-order rate constants as $10^7k'$, in sec.$^{-1}$, for the subsequent loss of the absorbance of anhydrocycloheximide (AN → P), and [HCl], respectively, are given. At 40.0°: 5.90, 0.054; 7.09, 0.080; 8.04, 0.090. At 50.0°: 49.4, 0.180; 112, 0.360; 216, 0.720. At 61.8°: 561, 0.640. At 70.0°: 165, 0.135; 239, 0.180; 519, 0.360; 611, 0.450.

where A_∞ is the final minimum absorbance. The calculated k' (sec.$^{-1}$) values are footnoted in Table I. The hydrogen ion catalytic rate constants, k_{H^+}' (eq. 3), are given in Table II. The value of ΔH_a is 20.6 kcal./mole and log A is 13.5.

Spectral Transformations of Anhydrocycloheximide in Acidic Solutions.—The absorbance of pure anhydrocycloheximide at 245 mμ decreased with time to an asymptotic value (Figure 1, lower), A_∞. The apparent first-order rate constants, k'', calculated from plots in accordance with eq. 5 are listed in Table III.

TABLE II

CATALYTIC RATE CONSTANTS FOR THE HYDROGEN ION CATALYZED DEHYDRATION OF CYCLOHEXIMIDE TO ANHYDROCYCLOHEXIMIDE (k), THE SUBSEQUENT LOSS OF ANHYDROCYCLOHEXIMIDE (k'), AND THE LOSS OF PURE ANHYDROCYCLOHEXIMIDE (k'')

Temp., °C.	$10^3k_{H^+}$[a]	$10^3k_{H^+}'$	$10^3k_{H^+}''$
40.0	0.164	0.0118	0.0147
50.0	0.389	0.0397	0.0497
61.8	0.928	0.120	0.157
70.0	1.74	0.180	0.343
74.6	2.45	...	...

[a] $k_{H^+} = k_{obsd}/a_{H^+}$ (k_{obsd} in sec.$^{-1}$).

TABLE III

APPARENT FIRST-ORDER RATE CONSTANTS (k'' IN SEC.$^{-1}$) FOR THE ACID-CATALYZED LOSS OF THE 245-Mμ ABSORBANCE OF ANHYDROCYCLOHEXIMIDE[a]

[HCl]	$10^4k''$	[HCl]	$10^4k''$
40.0°		50.0°	
0.495	0.0544	0.248	0.0819
0.743	0.0861	0.405	0.147
0.900	0.102	0.495	0.178
...	...	0.619	0.219
...	...	0.743	0.256
...	...	0.810	0.289
61.8°		70.0°	
0.248	0.286	0.090	0.250
0.248	0.270	0.248	0.628
0.306	0.292	0.248	0.656
0.405	0.417	0.306	0.711
0.412	0.450	0.412	0.961
0.495	0.556	0.405	1.00
0.495	0.594	0.495	1.30
0.619	0.672	0.619	1.49
0.743	0.900	0.619	1.58
0.743	0.872	0.810	2.00
0.825	0.914	0.825	2.00
0.900	1.11	...	...

[a] The initial concentrations of anhydrocycloheximide were either 9.11×10^{-5} or 4.55×10^{-5} M. The apparent first-order rate constants were independent of these initial concentrations.

Plots of k'' *vs.* a_{H^+} were linear, passing through the origin in agreement with eq. 3. The catalytic rate constants, k_{H^+}, were calculated from the slopes of plots

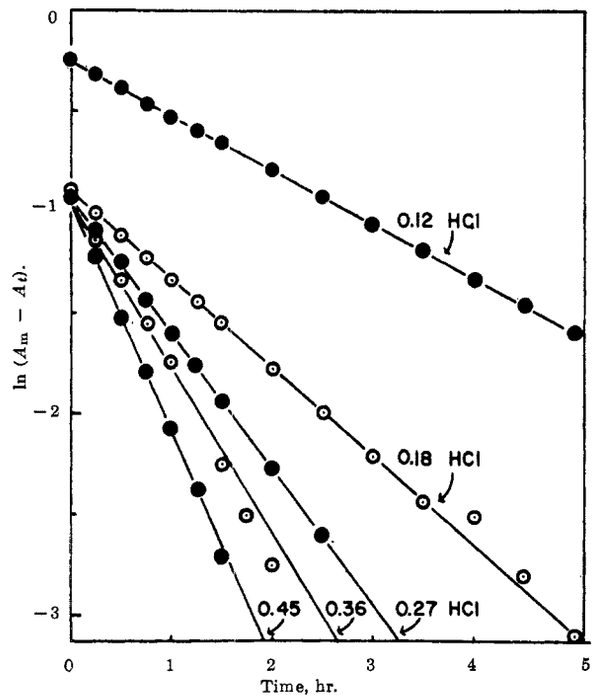


Figure 2.—First-order plots for the increase in absorbance (A_t) at 245 mμ to the maximum absorbance value (A_m) from the acidic dehydration of cycloheximide at 61.8°.

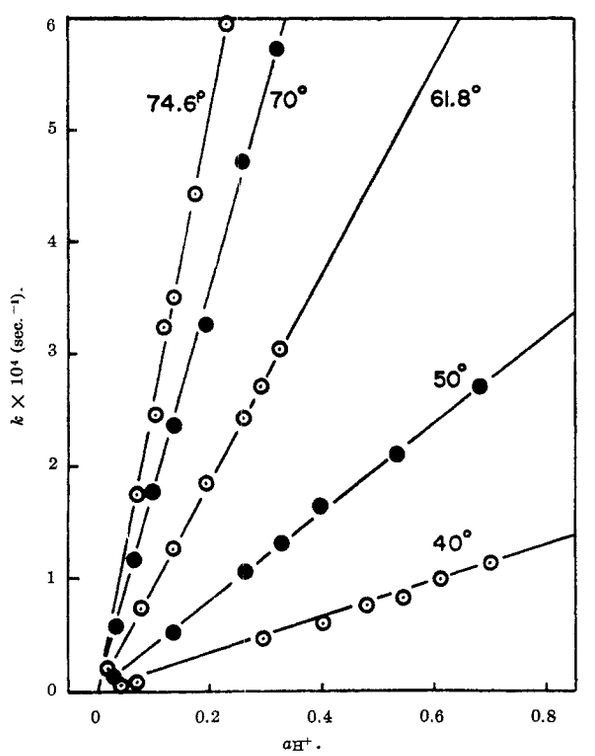


Figure 3.—Apparent first-order rate constants, k, for the hydrogen ion catalyzed dehydration of cycloheximide *vs.* the hydrogen ion activity, a_{H^+}.

similar to Figure 3 and are given in Table II. The value of ΔH_a is 20.6 kcal./mole and log A is 13.6.

The final average per cent yield of anhydrocycloheximide remaining at A_∞ was the same for cycloheximide dehydration as it was for loss of pure anhydrocycloheximide. The values at the various temperatures were 34%, 40.0°; 39%, 50.0°; 48%, 61.8°; and 52%, 70.0°.

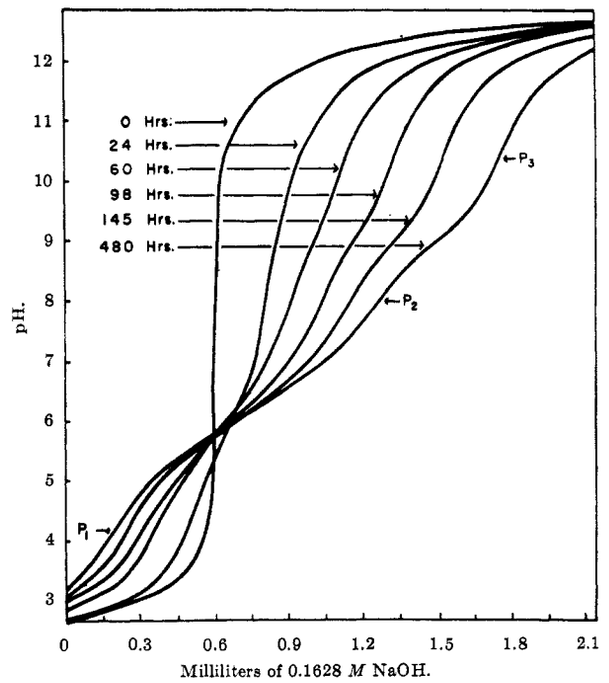
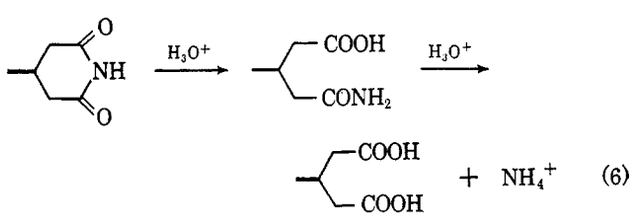


Figure 4.—Potentiometric titrations of aliquots of 0.01 M cycloheximide in 0.1 M HCl maintained at 80.0° as a function of time. The excess HCl has been partially preneutralized. The first end point, P_1, is associated with the remaining HCl, P_2 is associated with the carboxyl groups formed, and P_3 relates to the released ammonium ion.

Acidic Hydrolyses of Cycloheximide and Anhydrocycloheximide.—Solutions of cycloheximide or anhydrocycloheximide (0.1 M HCl at 80.0°) were titrated as a function of time and produced curves with three end points (Figure 4). The first end point, P_1, represents the volume of NaOH consumed by the excess HCl. The second end point, P_2, is attributed to the sum of the carboxylic acids. The third end point, P_3, is ascribed to the ammonium ion formed from hydrolysis of the amide. The pK_a associated with P_2 is 5.8 and that of P_3 is 8.8 in 65% ethanol.



The per cent yield of carboxylic acid, A, with respect to the initial concentration of imide, was calculated from the titer consumed between end points P_1 and P_2 and the normality of the standard alkali. The per cent yield of ammonium ion, B, was calculated similarly from the titer consumed between end points P_2 and P_3. These values as a function of time are given in Figure 5. The amounts of monocarboxylic acid amide, C, at any time can be calculated from $C = A - 2B$ and is also given in Figure 5. The yields of carboxylic acid, A, and ammonium ion, B, approached the theoretical maxima of 200 and 100%, respectively. The rates of carboxylic acid and ammonium ion formation were the same starting with either cycloheximide or anhydrocycloheximide in aqueous acid.

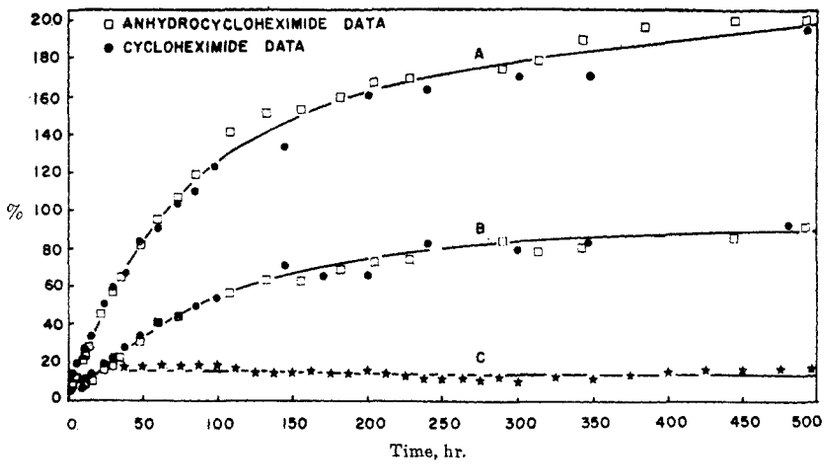


Figure 5.—The generation of carboxylic acid groups and free ammonium ions from the acid solvolyses of cycloheximide and anhydrocycloheximide (0.01 *M*) in 0.1 *M* HCl at 80.0°. Curve A is per cent yield of carboxyl group per mole of initial imide, and curve B is the per cent yield of ammonium ion per mole of initial imide. Curve C represents the difference between the values of curve A and twice the values of curve B and may be considered to represent the per cent yield of the monocarboxylic acid amide at any time.

First-order plots for the appearance of the ammonium ion were made on the basis of eq. 7.

$$\ln(100 - B) = -kt \qquad (7)$$

The apparent first-order rate constant for the hydrolysis of the imide to the dicarboxylic acid was $k = 2.02 \times 10^{-6}$ sec.$^{-1}$ for both cycloheximide and anhydrocycloheximide in 0.1 *M* HCl at 80.0°. The apparent yields of the acid amide were small and relatively constant with time so that the first step in solvolysis of the imide to the acid amide may be considered rate determining and significantly slower than the subsequent hydrolysis of the acid amide.

Thin Layer Chromatographic Monitoring.—Thin layer chromatograms of cycloheximide or anhydrocycloheximide reactions in 0.1 *M* HCl at 80.0°, as a function of time, produced three common spots when developed with ethyl acetate (Figure 1). The anhydrocycloheximide spot (R_f 0.61) was visible under ultraviolet light or on reaction with 2,4-dinitrophenylhydrazine (DNPH). The spot corresponding to cycloheximide (R_f 0.47) was visualized by DNPH. The third spot (R_f 0.53) was not visible under ultraviolet light nor by DNPH spray, but was detected by charring with H_2SO_4 and heat (Figure 1).

Characterization of the Isolated Reaction Components.—The ultraviolet spectra of the isolated anhydrocycloheximide and the isolated cycloheximide were identical with their respective standards. The infrared spectrum for isolated cycloheximide was in agreement with that of pure cycloheximide and thus with many of its isomers.

An infrared spectrum of the isolated reaction product (R_f 0.53) indicated the presence of all the original functional groups of cycloheximide. Elemental analysis, molecular weight determination, and ultraviolet spectrum were the same as those of cycloheximide. The infrared spectrum agreed with that reported for α-epiisocycloheximide[11] but this does not necessarily signify that P is this compound.

The isolated product, P, was compared with samples of known isomers of cycloheximide. The product, P, was separable from and therefore not identical with α-epiisocycloheximide, isocycloheximide, neocycloheximide, Naramycin B, and cycloheximide, using ethyl acetate as the developing solvent. Naramycin B and neocycloheximide gave visible spots with DNPH reagent at room temperature, whereas the product, P, did not.

Computer Fitting of Spectral Transformations.—The absorbance data were expressed in terms of mole fraction of the initial concentration of the reactant. The resultant data for the dehydration of cycloheximide (CY) and for the loss of pure anhydrocycloheximide (AN), under identical conditions of temperature and HCl concentration, were programmed on the PACE TR-10 analog computer according to eq. 8. The initial

$$\mathrm{CY} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{AN} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{P} \qquad (8)$$

conditions were changed to represent a mole fraction of unity for the reactant (either cycloheximide or anhydrocycloheximide) and zero for the products. The computer was thus made to follow both sets of data for a given temperature, HCl concentration, and set of rate constants. The four values giving the best fit to the data were recorded in each case and the results are given in Table IV. The boundary conditions were such that the solution of four rate constants was a unique solution for each case. A typical example of such analog computer fitting to anhydrocycloheximide curves obtained from solutions of anhydrocycloheximide and cycloheximide are given in Figure 6.

The individual rate constants, k_i, were linear with hydrogen ion activity in agreement with eq. 3. The hydrogen ion catalytic constants, k_{H^+}, calculated from the slopes of the plots in Figure 7, are given in Table IV. The heat of activation associated with each individual rate constant, ΔH_{a_i}, was calculated from the various k_{H^+} values at 70.0 and 61.8°, and the results are given in Table IV.

The rate constants for each temperature and HCl concentration were set into the analog computer program. The computer generated curves for anhydrocycloheximide, cycloheximide, and product, as a function of time, starting with each one of the three as the reactant. Curves generated in this way for the condi-

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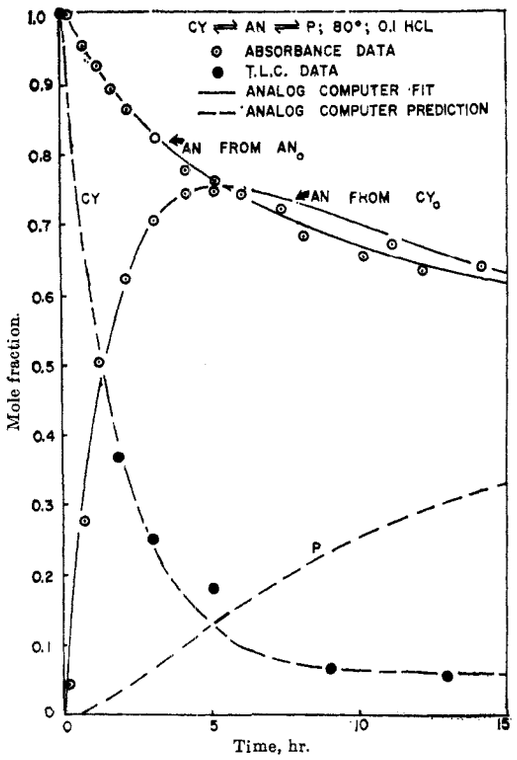


Figure 6.—Plots showing the agreement between the analog computer predicted curve for cycloheximide (CY) *vs.* time, based on the fitting of spectrophotometric absorbance data from anhydrocycloheximide and cycloheximide solutions with the experimental points from a quantitative thin layer assay for CY.

TABLE IV

APPARENT FIRST-ORDER RATE CONSTANTS (SEC.$^{-1}$), CATALYTIC RATE CONSTANTS (k_{H^+} IN L. MOLE^{-1} SEC.$^{-1}$), AND HEATS OF ACTIVATION FOR k_{H^+} (ΔH_a IN KCAL./MOLE)[a]

Temp., °C.	a_{H^+}[b]	10^4k k_1	k_{-1}	k_2	k_{-2}
61.8	0.190	1.01	0.090	0.122	0.112
	0.216	1.15	0.100	0.138	0.131
	0.292	1.53	0.137	0.190	0.169
	0.466	2.48	0.216	0.297	0.278
	k_{H^+}	5.29	0.463	0.637	0.588
70.0	0.0704	0.736	0.075	0.0708	0.0750
	0.189	2.00	0.204	0.191	0.202
	0.203	2.10	0.222	0.203	0.217
	0.240	2.50	0.264	0.242	0.257
	k_{H^+}	10.4	1.04	1.01	1.07
80.0	0.0723	1.45	0.104	0.143	0.184
	k_{H^+}	19.7	1.44	2.11	2.57
	ΔH_a	15.4	17.8	9.84	12.6

[a] Calculated from analog computer fittings of anhydrocycloheximide (AN) concentration *vs.* time, based on eq. 8. [b] As calculated from f[HCl], where the activity coefficients were obtained from the literature.[10]

tions of 0.1*M* HCl at 80.0° were experimentally verified in two ways. The concentration of cycloheximide, calculated as a function of time by quantitative thin layer chromatography, was in agreement with the corresponding curve predicted by the analog computer (Figure 6). The computer curve predicting the production of anhydrocycloheximide as a function of time, from the product, P, was used to calculate the apparent first-order rate constant, k, according to eq. 9, where $[AN]_\infty$ is the final anhydrocycloheximide concentration and $[AN]_t$ is the concentration at any time, t. The isolated product, P, was then dissolved in 0.1 *N* HCl at 80.0° and ultraviolet spectra were obtained as a function of time. The rate constant, k, for the increase in absorbance at 245 mμ was calculated from eq. 9 where $[AN] = A_{245}$. In both cases the values for k were 2.9×10^{-5} sec.$^{-1}$.

$$\ln([AN]_\infty - [AN]_t) = -kt \quad (9)$$

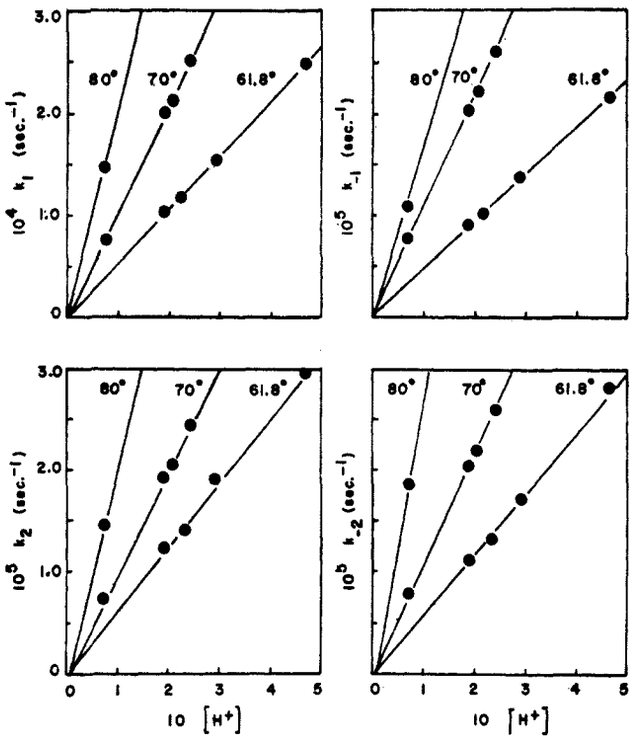


Figure 7.—Dependence of individual apparent first-order rate constants upon hydrogen ion activity, [H$^+$], at various temperatures. Rate constants were obtained by analog computer fittings to the mechanism given in eq. 8.

Discussion

Spectral Transformations.—Plots for the formation of anhydrocycloheximide, as a function of time, from the acid dehydration of cycloheximide passed through a maximum (Figure 1, upper). This maximum was *ca.* 70% and significantly increased with temperature. The subsequent decrease of anhydrocycloheximide, at about one-tenth the rate of its production (Table I), implies a loss of either cycloheximide or anhydrocycloheximide to another product, P. The final mole fraction of anhydrocycloheximide remaining after the dehydration of cycloheximide was the same as that remaining after decrease of pure anhydrocycloheximide under similar conditions. This suggests that common equilibria are ultimately achieved in both cases and possible representations are

$$AN \rightleftharpoons CY \rightleftharpoons P \quad (10)$$

$$CY \rightleftharpoons AN \rightleftharpoons P \quad (11)$$

$$CY \rightleftharpoons AN \rightleftharpoons P \quad (12)$$
$$\uparrow\downarrow$$
$$\text{"CY"}$$

where the initial cycloheximide, CY, or its chromatographically indistinguishable isomer(s), "CY," are ultimately in small yield.

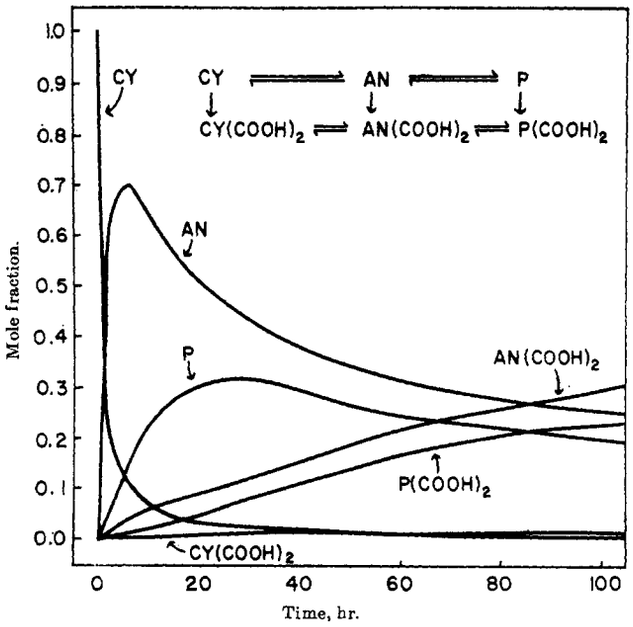
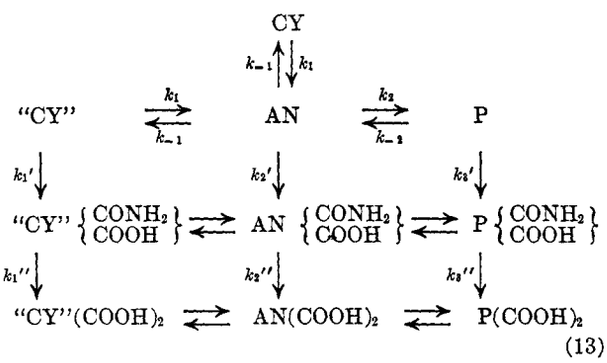


Figure 8.—Plots of the mole fraction *vs.* time for each of the products of cycloheximide reaction in 0.1 *M* HCl at 80.0° as predicted by the analog computer.

Thin Layer Chromatography.—Both cycloheximide and anhydrocycloheximide in 0.1 *M* HCl at 80° produced three major spots on thin layer chromatograms which were developed with ethyl acetate (Figure 1). The similarity of the R_f values for P in four different solvent systems implies that both cycloheximide and anhydrocycloheximide gave rise to the same product in agreement with eq. 10–12. The spot corresponding to the product, P, became visible sooner in the reaction started with anhydrocycloheximide than it did in the reaction started with cycloheximide. This suggests that the product, P, arises from anhydrocycloheximide rather than from cycloheximide, and that eq. 11 or 12 is more appropriate.

Transformations of Cycloheximide.—A summary scheme for the transformations of cycloheximide would appear to be eq. 13, where "CY" may or may not be CY.



(13)

The k_t and k_{-t} rate constants characterizing dehydration and possible rehydration can be assumed to be the same for intact imides and the mono or dicarboxylic acids. This assumption is well supported by the similarity in absorptivity and wave length of maximum absorbance of AN and AN(COOH)$_2$ (8250 and 9000 at 241 mμ)[4,5] and the fact that the ultraviolet absorption remains constant after *ca.* 20 hr. (Figure 1), whereas the imide hydrolysis still continues for 500 hr. (Figure 6).

When the additional approximation is made that chromatographically indistinguishable cycloheximide isomers "CY" have rate constants, k_1 and k_{-1} similar in magnitude to that of cycloheximide, CY, the eq. 13 can be fitted by an analog computer program for eq. 11. It was necessary to assume some finite value for k_{-1} for a best fit.

These approximations permitted satisfactory analog computer fits to the spectral data observed with time (Figure 6) and were supported by the facts that quantitative thin layer chromatography agreed with the total "CY" predicted (Figure 6).

If the rate constants for CY and "CY" in eq. 12 did differ slightly, it would have had negligible effect on the fitting since the "CY" products ultimately comprise less than 10% of the total yield. It was established that the individual rate constants, k_t and k_{-t}, were subject to hydrogen ion catalysis (Figure 7).

The total monocarboxylic acid amide concentration is small at all times (Figure 5) and can be considered negligible since $k'' >> k' = 2.02 \times 10^{-6}$ sec.$^{-1}$ at 80.0°, 0.1 *M* HCl.

The rate of imide hydrolysis to the corresponding dicarboxylic acid was the same starting with cycloheximide as it was starting with anhydrocycloheximide (Figure 5). It can be reasonably assumed that the rate of imide hydrolysis is the same for the product, P, as for AN.

An over-all plot generated by the analog computer to show the mole fraction of each significant reaction component in eq. 13 as a function of time that resulted from the reaction of cycloheximide in 0.1 *M* HCl at 80.0° was constructed in this way (Figure 8).

This over-all picture is consistent with experimental observations. The spot corresponding to the product, P, was at its maximum size at 28 hr. (Figure 1). The quantitative thin layer chromatographic assay for cycloheximide agreed with the analog computer prediction for "CY" (Figure 6).

Characterization of the Products.—Elemental analysis, molecular weight determination, infrared spectrum, and ultraviolet spectrum indicated that the product, P, was an isomer of cycloheximide. It was separable from the known isomers, α-epiisocycloheximide, isocycloheximide, "neocycloheximide,"[12] Naramycin B, and cycloheximide, by thin layer chromatography. The product, P, could not be visualized by dinitrophenyl hydrazine spray on the plates which was similar to α-epiisocycloheximide but contrary to cycloheximide, anhydrocycloheximide, Naramycin B, and "neocycloheximide."

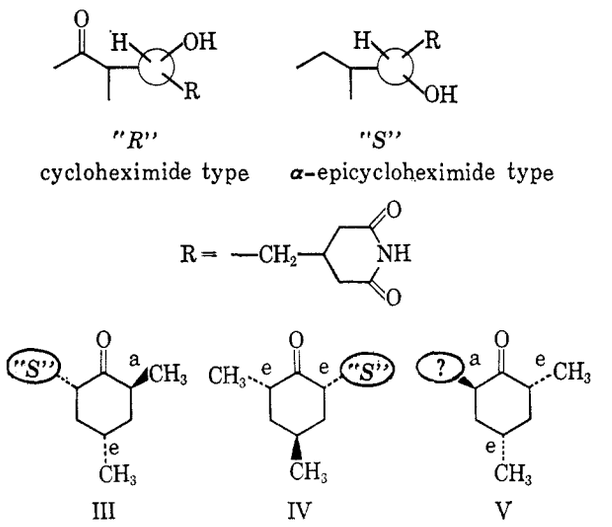
It follows that the product, P, must be one of three unreported isomers of cycloheximide that are given in Chart I.

Since previous cycloheximide isomerizations[7b] failed to produce the present isomer P, it is implied that its formation here is associated with the mechanism which employs the anhydrocycloheximide intermediate.

(12) The "neocycloheximide" used for comparison purposes on the thin layer chromatograms had not been assigned a configuration of the asymmetric α-carbon atom by F. Johnson, W. D. Gurowitz, and N. A. Starkovsky, *Tetrahedron Letters*, 1167 (1962). Thus, V in Chart I, one of the three unreported isomers of cycloheximide which must be P, may be either α-epineocycloheximide with the *S* configuration or it may be neocycloheximide with the *R* configuration on the α-carbon, whichever one is of the opposite α-carbon configuration of the reference "neocycloheximide" obtained from Dr. Johnson.

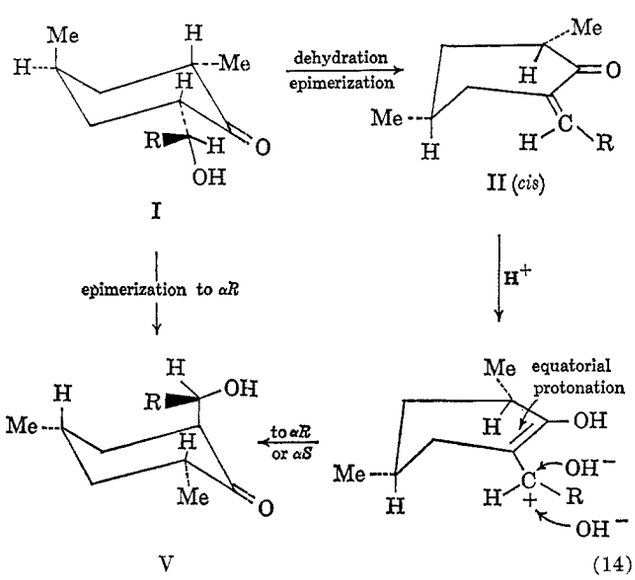
CHART I

CONFIGURATION OF α-CARBON



There have been conflicting conclusions in the literature as to whether the methyl groups in anhydrocycloheximide are *cis* or *trans*.[13,14] Schaeffer and Jain[15] have recently concluded that anhydrocycloheximide exists as a mixture of the *cis* and *trans* forms. However, none of the previous studies employed the mild HCl conditions of the present study nor was anhydrocycloheximide prepared under our more temperate conditions.

The formation of V, whether it be neocycloheximide ($2R$:$4S$:$6S$:αR) or α-epineocycloheximide ($2R$:$4S$:$6S$:-αS), would require *cis*-anhydrocycloheximide as the intermediate. Formation of *cis*-anhydrocycloheximide from cycloheximide requires dehydration with epimerization of the methyl at C-2 as demonstrated in eq. 14.



(14)

Previous experimental evidence for a variety of keto steroids has established that protonation of the enol occurs preferentially from the axial position.[16] Thus, the formation of V from *cis*-anhydrocycloheximide II is unlikely, since it requires equatorial addition of the proton to the enol. Also, axial addition of the proton to the enol would result in isocycloheximide configurations, ($2S$:$4R$:$6S$) and these known compounds are separable from P by thin layer chromatography. The only manner in which V can result from axial protonation is if the *cis*-anhydrocycloheximide, II, hydrates in the alternate conformer where both methyls are axial, and this is energetically unlikely.

This is consistent with the fact that Okuda[7b] was able to isomerize cycloheximide ($2S$:$4S$:$6S$:αR) to isocycloheximide ($2S$:$4R$:$6S$:αR) and to Naramycin B ($2S$:$4S$:$6R$:αR). He also isomerized Naramycin B to isocycloheximide. These isomerizations can proceed by epimerization with axial protonation. He was not able to isomerize isocycloheximide or α-epiisocycloheximide ($2S$:$4R$:$6S$:αS). The latter two isomers are 2e, 4e, 6e, and would therefore require equatorial protonation to achieve epimerization. The compound "neocycloheximide" ($2R$:$4S$:$6S$:α?) has been obtained only by direct synthesis[12] and never from cycloheximide.

It follows that if V results from I it would more likely come from direct epimerization at C-2 (eq. 14) of cycloheximide than through an anhydrocycloheximide intermediate. Such a direct epimerization to give neocycloheximide, V ($2R$:$4S$:$6S$:αR), is highly improbable under our conditions and from the fact that P appears more readily from anhydrocycloheximide than from cycloheximide.

The two remaining isomers that may be P, *i.e.*, α-epi-Naramycin B (III) ($2S$:$4S$:$6R$:αS) and α-epicycloheximide (IV) ($2S$:$4S$:$6S$:αS), could arise from cycloheximide through a *trans*-anhydrocycloheximide intermediate. The hydroxyl must be removed and then replaced in the opposite α-carbon configuration, *i.e.*, *S*. The possible mechanisms for the formation of cycloheximide and its isomers III, IV, and VI (Naramycin B) may be illustrated as in eq. 15.

It can be expected that *trans*-anhydrocycloheximide and its enol form would have some conformational mobility, II'a and II'b. Rehydration by axial protonation without stereospecific hydroxyl addition would give the racemates I, III, IV, and VI of which I and VI have been shown to be indistinguishable by thin layer chromatography. Thus, either IV or III could be the product P. Infrared spectra of the extracted thin layer spot retained throughout the reaction by cycloheximide I did not indicate the presence of discernible amounts of Naramycin B (VI).

However, it is possible that the cycloheximide spot may contain two or three of the potential four isomers.

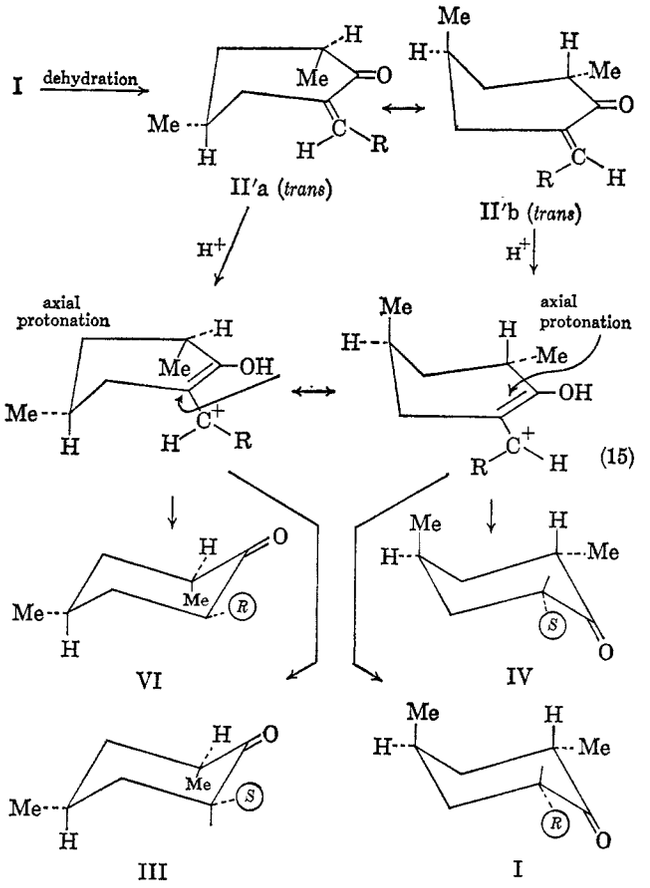
It can be argued that the simplest explanation is dehydration to anhydrocycloheximide with retention of ring configuration with nonstereospecific rehydration of anhydrocycloheximide. Hydroxyl ion addition to one

(13) (a) H. J. Schaeffer and V. K. Jain, *J. Pharm. Sci.* **52**, 639 (1963); (b) N. A. Starkovsky, F. Johnson, and A. A. Carson, *Tetrahedron Letters*, 1015 (1964).

(14) Dr. Francis Johnson of the Dow Chemical Co. will shortly publish his definitive findings that anhydrocycloheximide, m.p. 136°, has *cis*-methyl groups and that it equilibrates to a mixture of *cis* and *trans* in acid with *ca.* 18% of the latter form. He also states that at high temperatures and with higher concentrations of acid than were used in the present studies, anhydrocycloheximide isomerizes to epianhydrocycloheximide. Epianhydrocycloheximide is completely separable from anhydrocycloheximide by thin layer chromatography and was not observed under the milder conditions of these studies.

(15) H. J. Schaeffer and V. K. Jain, *J. Pharm. Sci.* **53**, 144 (1964).

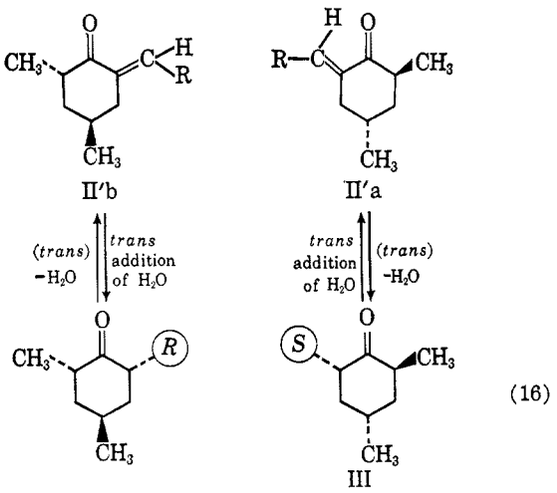
(16) E. L. Eliel, "Stereochemistry of Carbon Compounds," McGraw-Hill Book Co., New York, N. Y., 1962, p. 241.



side of the carbonium ion results in cycloheximide or Naramycin B formation from the respective conformations. Addition to the opposite side of the conformation forms α-epi-Naramycin B (III) or α-epicycloheximide (IV). The formation of all four can go through the preferred axial protonation of the enol as shown in eq. 15.

An alternative explanation considers stereospecific *trans* elimination and stereospecific addition of water through the *trans*-anhydrocycloheximide conformations.[17]

The scheme of eq. 16 shows that the stereospecific



(16)

trans addition of water to *trans*-anhydrocycloheximide formed by *trans* elimination of water from its cycloheximide-type precursors will give cycloheximide (I) from one ring conformation and α-epi-Naramycin B from the other ring conformation. However, since the CY spot retained throughout the reaction does not maintain the biological activity of cycloheximide, it is unlikely that hydration of the conformer II'b by this mechanism occurs readily.

Although the yield after 28 hr. in 0.1 *M* HCl at 80.0° at the cycloheximide spot (Figure 1) is less than 5% of the total possible, a significant amount of material could be isolated by quantitative thin layer chromatography. The products which are not separable from cycloheximide which may result from axial protonation of the *trans*-anhydrocycloheximide forms II'a and II'b (eq. 15) are cycloheximide (I) and Naramycin B (VI). Since P is most probably either α-epi-Naramycin B (III) or α-epicycloheximide (IV), the alternate product may be included in the retained cycloheximide spot. The formation of *cis*-anhydrocycloheximide as a major or partial intermediate could produce the isocycloheximide configurations (2*S*:4*R*:6*S*). Thus, the retained cycloheximide spot could contain cycloheximide (2e,4a,6e) (I), Naramycin B (2a,4e,6e) (VI), the isocycloheximides (2e,4e,6e), and *either* α-epi-Naramycin B (2a,4e,6e) (III) or α-epicycloheximide (2e,4a,6e) (IV).

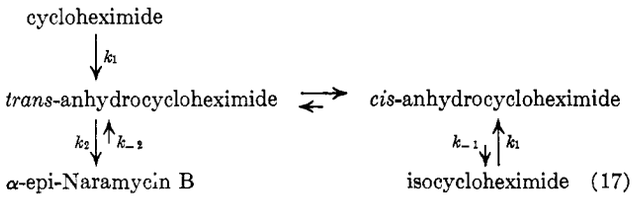
The n.m.r. data and the lack of significant biological activity of the isolates from the CY spot indicate that cycloheximide is not a major component. The presence of a doublet at ~59 c.p.s. with a splitting constant of 5.9 c.p.s. confirms the presence of an equatorial methyl group.[18] The singlet peak at a lower field, ~84 c.p.s., is lower than that for any previously observed axial methyl group, and the lack of any discernible splitting makes it difficult to correlate with any known n.m.r. spectra of cycloheximides. However, this information, with the lack of a ~75-c.p.s. doublet, does tend to exclude cycloheximide and Naramycin B as major components of the CY spot. The indicated positive Cotton-effect curves for the CY spot on optical rotatory dispersion give evidence[7] for a 4–6 *cis* conformation, *i.e.*, 4e,6e, which points to the isocycloheximide and α-epi-Naramycin B as candidates for the CY spot. The former is a ready and stable product of cycloheximide isomerization.[7]

The product P shows two doublets in the n.m.r. spectrum, one at high field, ~57 c.p.s., with a splitting constant of ~5.1 c.p.s. to indicate an equatorial methyl group,[18] the other doublet at a lower field, ~74 c.p.s., having a splitting constant of ~8.4 c.p.s. assignable to an axial methyl group.[18] The n.m.r. spectrum thus validates a structure of *trans* methyls. This is consistent with either α-epi-Naramycin B (2a,4e,6e) (III) or α-epicycloheximide (2e,4a,6e) (IV) as being P. The indicated positive Cotton-effect curves for the P spot on optical rotatory dispersion give evidence[7] for a 4–6 *cis* conformation, *i.e.*, 4e,6e, which points to α-epi-Naramycin B as P.

From the extant information and if the indicated nature of the positive Cotton curves is accepted, the following transformations (eq. 17) are plausible, where

(17) This suggestion was made by Dr. T. Okuda and is intriguing in its simplicity. Dr. Okuda also states that the correlation between infrared spectra of α-epi-Naramycin B (III) and α-epiisocycloheximide (ν_{OH} 3442 cm.$^{-1}$) is expected since for Naramycin B, ν_{OH} 3226 cm.$^{-1}$, and for isocycloheximide, ν_{OH} 3240 cm.$^{-1}$, whereas for cycloheximide, ν_{OH} 3509 cm.$^{-1}$ and the product P has ν_{OH} 3400 cm.$^{-1}$.

(18) F. Johnson and N. A. Starkovsky, *Tetrahedron Letters*, 1173 (1962).



(17)

the relative magnitude of the arrows implies the extent of the equilibria and the proposed isocycloheximide is in small yield.

Experimental Section

The elemental analyses in this investigation were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and Huffman Laboratories, Inc. Wheatridge, Colo.

Purification of Cycloheximide.—Cycloheximide was purified by recrystallization[6]: m.p. 114–116°, lit.[6] m.p. 115–116°; ν^{film}, cm.$^{-1}$, 3430 (OH), 3150, 3020 (NH), 1710, 1675 (C=O).[5]

Anal. Calcd. for $C_{15}H_{23}NO_4$: C, 64.03; H, 8.24; N, 4.98. Found: C, 64.22; H, 8.36; N, 4.87.

Preparation of Anhydrocycloheximide.—Anhydrocycloheximide was made from cycloheximide by the following modification of the procedure of Rao.[4] Ten grams of cycloheximide was dissolved in 150 ml. of 6 *N* HCl. The solution was warmed slowly until a flocculant precipitate formed at 40–50°. This was cooled immediately and allowed to crystallize. The compound was recrystallized twice from aqueous methanol: m.p. 135–136°, lit.[5] m.p. 134–135°; λ_{max}^{EtOH} 240 mμ (a_m 8100[5,12]); ν^{film}, cm.$^{-1}$, 3110, 3050 (NH), 1710, 1675 (C=O).[12]

Anal. Calcd. for $C_{15}H_{21}NO_3$: C, 68.41; H, 8.04; N, 5.32. Found: C, 68.26; H, 7.82; N, 5.62.

Spectral Transformations of Cycloheximide and Anhydrocycloheximide in Acidic Solutions.—Aliquots of aqueous stock solutions of the compound under study were diluted to appropriate volumes with standard HCl solutions previously equilibrated in constant-temperature baths. All solutions were prepared from nitrogen-purged water. Samples were removed as a function of time and cooled immediately, and the absorbance were read on a Beckman DU spectrophotometer at 245 mμ or complete spectra were obtained with a Cary Model 15 recording spectrophotometer. (See Tables I and II for experimental conditions.)

Acidic Hydrolysis of Cycloheximide and Anhydrocycloheximide.—Aliquots of a solution of 0.01 *N* cycloheximide in 0.1 *M* HCl at 80° were removed as a function of time and diluted with ethanol, and the excess HCl was partially neutralized. The sample was then titrated with the Radiometer titrator. The first end point was the remaining HCl. The acidic hydrolysis of a 0.01 *M* anhydrocycloheximide was studied in the same manner.

Thin Layer Chromatographic Evaluation of the Reaction Course.—The reactions described above were followed simultaneously by spectrophotometry and by thin layer chromatography.

Plates, 20 × 20 cm., were coated with a 0.4-mm. layer of silica gel G with phosphor indicator (Catalog No. 8071, Research Specialties Co.). Reactions were sampled as a function of time. Samples of 20 μl. were spotted on each of two plates at a distance of 3 cm. from the base of the plates. Standard solutions of cycloheximide, anhydrocycloheximide, and glutarimide β-acetaldehyde[5] were also applied. The solvent front was allowed to advance 10 cm.

Both plates were examined under ultraviolet light at 245 mμ and the results were recorded. One plate was sprayed with concentrated H_2SO_4 and heated for 10 min. at 120°. The second plate was sprayed with 2,4-dinitrophenylhydrazine (DNPH) carbonyl reagent.[19] Results were recorded for each treatment.

Quantitative Thin Layer Chromatography of Cycloheximide.—A calibration curve for cycloheximide in 0.1 *M* HCl was prepared by applying known quantities to six different plates and developing with ethyl acetate as described above. Detection was by H_2SO_4 with heat. The diameter was measured in both a horizontal and vertical direction and the mean value was used to calculate the area of the spot. A calibration plot of the square root of the spot area against the logarithm of the weight of the substance[20] was linear over a suitable range for experimental reaction concentrations. The concentration of cycloheximide in 0.1 *M* HCl at 80° was determined by measuring the spots while the anhydrocycloheximide produced was determined spectrophotometrically.

Isolation of Reaction Components by Preparative Thin Layer Chromatography.—Cycloheximide was treated in 0.1 *M* HCl at 80° for 28 hr., cooled, and extracted with ethyl acetate. The extract was concentrated by evaporation with a Rinco flash evaporator at room temperature. Aliquots were applied to preparative plates (1-mm. coating) by sliding the plates just beneath the tip of the needle on a syringe microburet while the solution was ejected upon the plate. Separation was achieved by developing 12 cm. with ethyl acetate, air drying the layer, and developing a second time.

The anhydrocycloheximide zone was scraped by visualization under ultraviolet light at 254 mμ. A narrow band was scraped just below the anhydrocycloheximide zone. A guide was established by preparing a second plate and charring it with H_2SO_4 plus heat. The cycloheximide zone was scraped by using this guide. A further check was effected by occasionally scraping half a plate and treating the remaining half with H_2SO_4 plus heat.

The scrapings of the three zones were each extracted with ethyl acetate and the silica gel was removed with a fine sintered-glass filter. The extracts were checked for content by thin layer chromatography using anhydrocycloheximide and cycloheximide as standards. The extracts were dried under vacuum at room temperature. Crystals were obtained for anhydrocycloheximide and the reaction product.

Anhydrocycloheximide was treated in similar manner except that the reaction was run for 10 hr. (Figure 1).

Characterization of the Reaction Components.—Extracts of the three zones from chromatographic separation of cycloheximide and anhydrocycloheximide reactions were compared, using four developing solvents giving a wide range of R_f values. The developing solvents were ether, acetone, ethyl acetate, and chloroform–methanol (1:1). Infrared curves were obtained for the reaction product and for cycloheximide. Ultraviolet curves were obtained for all three zones using methanol as the extracting solvent.

The presence of the hydroxyl band in the infrared spectrum of the isolated reaction product inferred that it was an isomer of cycloheximide: ν^{film}, cm.$^{-1}$, 3400 (OH), 3170, 3050 (NH), 1710, 1675, 1230 (sh) (C=O). The elemental analysis and molecular weight determination for the product, P, were compared with those of cycloheximide.

Anal. Calcd. for $C_{15}H_{23}NO_4$: C, 64.03; H, 8.24; N, 4.98; mol. wt., 281.35. Found: C, 63.89; H, 8.10; N, 4.81; mol. wt., 309 (Rast camphor).

One or more samples of isocycloheximide, α-epiisocycloheximide, neocycloheximide, and Naramycin B were obtained from Johnson[13,16,17,21] and Okuda.[7,11] Aliquots of each were placed singly and in combination with the reaction product on two thin layer plates. The plates were developed 12 cm. with ethyl acetate. One plate was treated with DNPH spray and the other was treated with H_2SO_4 plus heat.

Cycloheximide was treated for 28 hr. in 0.1 *M* HCl at 80.0° and chromatographed on thin layer as previously described. The CY and P bands were extracted as described previously. The dried extracts were assayed by n.m.r. spectroscopy.[18] The material from the CY spot showed a doublet at ~59 c.p.s. with a splitting constant of 5.9 c.p.s. and a singlet peak at ~84 c.p.s. The material from the P spot showed two doublets, one at ~57 c.p.s. with a splitting constant of 5.1 c.p.s., whereas the other doublet was at ~74 c.p.s. with a splitting constant of 8.5 c.p.s.

Although both samples were contaminated by colored impurities so that the wave length and amplitude of optical rotatory dispersion curves were not quantifiable, both samples gave positive Cotton-effect curves in methyl alcohol.

The spot materials were tested as toxicants against *saccharomyces pastorianus*[22] where the observed inhibition is at 0.020

(19) R. L. Shriner, R. C. Fuson, and D. Y. Curtin, "The Systematic Identification of Organic Compounds," 4th Ed., John Wiley and Sons, Inc., New York, N. Y., 1962, p. 111.

(20) S. J. Purdy and E. V. Truter, *Analyst*, **87**, 802 (1962).

(21) N. A. Starkovsky and F. Johnson, *Tetrahedron Letters*, 919 (1964).

(22) M. R. Siegel and H. D. Sisler, *Biochim. Biophys. Acta* **87**, 70, 83 (1964).

p.p.m. for cycloheximide. Material from the P spot gave no inhibition at 100 p.p.m. whereas material from the CY spot inhibited at 60 p.p.m.

Acknowledgment.—We are indebted to Dr. Francis Johnson of the Dow Chemical Company, Dr. Gerald A. Boyack of the Upjohn Company, Dr. Bernard C. Lawes of Mead Johnson and Company, Dr. Tomoharu Okuda of the Tokyo Research Laboratories, Tanabe Seiyaku Company, Ltd., and Dr. Howard J. Schaeffer of the State University of New York at Buffalo for their kindness in providing compounds for comparison and evaluation. In addition, the comments and information of Drs. Francis Johnson and T. Okuda should be expressly acknowledged. The n.m.r. information was obtained by Dr. Johnson, the O.R.D. by Dr. Okuda, and the biological activities by Dr. M. R. Siegel of the University of Maryland. The technical assistance of Mr. John Toomey is most appreciated.

Stereochemical Course of the Robinson Annelation Reaction. *cis*-9-Hydroxy-10-methyldecalin-2,5-dione

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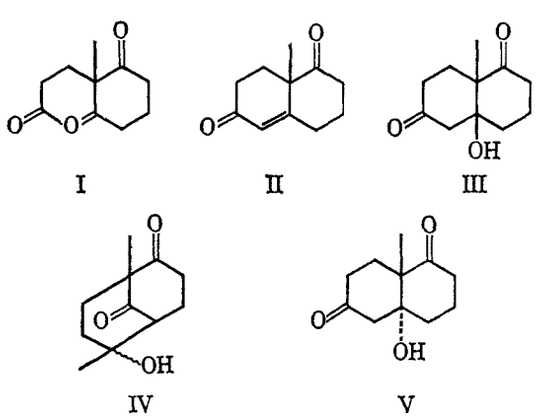
cis-9-Hydroxy-10-methyldecalin-2,5-dione (III) has been prepared by careful pyrrolidine-catalyzed cyclization of 2-methyl-2-(3-oxobutyl)cyclohexane-1,3-dione (I). Piperidine-catalyzed cyclization of I afforded a mixture of III and bridged ketol IV. Conversion of the known β-epoxide VI to III defined the stereochemistry of the ketol, which is *cis*, as expected.

Our interest in the details of the intramolecular aldol condensation which constitutes the second step of the Robinson annelation sequence[2] led us to explore further the well-known[3] cyclization of 2-methyl-2-(3-oxobutyl)cyclohexane-1,3-dione (I) to 10-methyl-$\Delta^{1,9}$-octalin-2,5-dione (II). Isolation of a crystalline ketol by careful amine-catalyzed cyclization of the oily Michael adduct I was the primary objective. It was found that, when I was treated briefly with pyrrolidine at 0°,[4] followed by chromatography, a trace of crystalline material other than II could be isolated. Numerous experiments to raise the yield of this substance indicated the importance of very rapid chromatography at low temperatures. Thus, when I was treated with 1 equiv. of pyrrolidine in ether at 0° for 12 min., followed by chromatography on Florisil at −30°, a 10% yield of solid, m.p. 141–142°, was obtained. With 1 equiv. of pyrrolidine, 0.3 equiv. of acetic acid, a reaction time of 6 min. at 0°, and similar chromatography, the yield was 20%. On the basis of evidence presented in this and the following paper,[5] this 141–142° compound has been shown to be *cis*-9-hydroxy-10-methyldecalin-2,5-dione (III).

When the less effective cyclization catalyst[6] piperidine was used for a 5-hr. period at room temperature, a 33% yield of a nicely crystalline product, m.p. 99–100°, was obtained. This material was shown to be a mixture of III and a bridged ketol IV, m.p. 115–116°. This bridged ketol could be isolated by treating the 99–100° material with pyrrolidine, which rapidly effected dehydration of II, while leaving IV unchanged. Recrystallization of a 1:1 mixture of III and IV produced the 99–100° material.

The n.m.r. spectra of III and IV served to establish their respective carbon skeletons: III exhibited one methyl resonance (δ = 1.32 p.p.m.); IV exhibited two (δ = 1.15 and 1.40 p.p.m.). The configuration of IV at the carbon atom bearing the methyl and hydroxyl groups has not been determined. However, in view of our interest in the stereochemical course of the Robinson annelation reaction, we undertook to establish unequivocally whether the stereochemistry of the 141–142° hydronaphthalenic ketol was *cis*, as in III, or *trans*, as in V.



Westen[7] has separated and carefully characterized the two stereoisomeric epoxides, VI (originally prepared by Wharton,[8] m.p. 158–158.5°) and VII, derived from the benzoate VIII. We prepared the crystalline β-

(1) To whom correspondence should be addressed.

(2) T. A. Spencer, K. K. Schmiegel, and K. L. Williamson, *J. Am. Chem. Soc.*, **85**, 3785 (1963).

(3) S. Ramachandran and M. S. Newman, *Org. Syn.*, **41**, 38 (1961), and references cited therein.

(4) The amine-catalyzed cyclization of I is much faster than that of 2-acetoxy-2-(3-oxobutyl)cyclohexane-1,3-dione (XII).[2]

(5) K. L. Williamson, L. R. Sloan, T. Howell, and T. A. Spencer, *J. Org. Chem.*, **31**, 436 (1966).

(6) A preliminary account of a study of the relative effectiveness of a variety of amines as catalysts for the conversions I → II, I → III, and III → II has been published: T. A. Spencer, H. S. Neel, T. W. Flechtner, and R. A. Zayle, *Tetrahedron Letters*, 3889 (1965).

(7) H. H. Westen, *Helv. Chim. Acta*, **47**, 575 (1964).

(8) P. S. Wharton, *J. Org. Chem.*, **26**, 4781 (1961).