Prediction of Stability in Pharmaceutical Preparations IX

Solution Degradation and Chemical Assay of the Antibiotic Actinospectacin

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The kinetics of degradation of the antibiotic actinospectacin[1] in solution have demonstrated that the material is unstable in the alkaline region and that its degradation is catalyzed by buffer anions such as borate, phosphate, and carbonate. Equations have been obtained to characterize degradation as a function of hydroxyl ion concentration and temperature. A chemical assay has been devised based on the formation of the copper complex of a derived dithiocarbamic acid which is extractable and spectrophotometrically measurable in organic solvent. Optimum conditions are described to measure the unique secondary amine functions of the active antibiotic and the change in solubility characteristics of the copper complex of the derived dithiocarbamic acids. Bioassay has been correlated with this chemical assay and optimum conditions defined for its use.

ACTINOSPECTACIN sulfate (1) is a new, therapeutically effective antibiotic, active *in vitro* and *in vivo* against a variety of Gram-positive and Gram-negative organisms (2). It is a material of apparent molecular formula $C_{14}H_{26}N_2O_7$ which is isolated as the hydrated sulfate, and in that form has an apparent molecular weight of 506 (3). It has two secondary amine groups and the two pKa values assigned to these groups are 6.95 and 8.70 (3). Its high water solubility and low organic solubility indicate a hydroxylated compound.

This paper reports on studies designed to establish a chemical assay based on the presence of secondary amine groups, to determine the stability of actinospectacin as a function of pH and temperature, to compare chemical and biological assay procedures, and to predict the possible nature of the solution degradation and the conditions for maximum stability.

EXPERIMENTAL

General Procedure.—The isolation and characterization of the actinospectacin sulfate used has been reported (1–3). Kinetic studies were conducted principally at 30 and 60° in 0.1 *M* hydrochloric acid, acetate buffers (pH 4.65); phosphate, bicarbonate, and borate buffers (pH 7–11); and in varying concentrations of sodium hydroxide. No significant degradation of actinospectacin was observed by chemical or biological assay in 0.1 *M* hydrochloric acid and at pH 4.65 for over 43 days at 30°. Alkaline degradation, however, was significant and studied in considerable detail.

Aliquots were taken at recorded intervals from the actinospectacin solutions maintained in constant temperature baths at ±0.05°.

The aliquots were immediately neutralized and then diluted 1:4 with 0.1 *M* pH 7.85 phosphate buffer for microbiological assay, or diluted 1:4 with 0.2 *M* sodium hydroxide – 0.4 *M* acetic acid buffer for chemical assay, except for the phosphate and acetate buffered solutions which were assayed without dilution. The resultant solution submitted for microbiological assay was 0.08 *M* in pH 7.85 phosphate buffer and contained 450 mcg./ml. as based on undegraded actinospectacin. The resultant solution assayed chemically contained 400 mcg./ml. as based on undegraded actinospectacin.

Estimates of heats of activation of alkaline degradation were obtained by kinetic studies in 0.1 *M* sodium hydroxide at several temperatures.

The complete conditions are given in Table I for the kinetic runs and the estimated apparent first-order rate constants.

Chemical Assay Procedure.—The basis of the chemical assay was the reaction of the secondary amine function of undegraded actinospectacin with carbon disulfide to form dithiocarbamic acid. Addition of a cupric salt gave a complex extractable into organic solvents which could be measured spectrophotometrically (4–6).

The actinospectacin aliquot (1 ml. of an aqueous solution containing 50 to 500 mcg.) was pipetted into a 15-ml. glass-stoppered test tube and 4 ml. of a solution of carbon disulfide, pyridine, and isopropyl alcohol in the ratio 35:25:65 was added. Then, 2 ml. of an 0.0025 *M* cupric chloride solution in water-pyridine, 1:1, was added. The tube was swirled to mix the reactants and allowed to stand at room temperature for 30 ± 5 minutes. Some of the carbon disulfide settled to the bottom.

Subsequently, 3 ml. of water and 3 ml. of benzene were added and the mixture was shaken and inverted to extract the actinospectacin-dithiocarbamate-copper complex. The liquid phases were allowed to separate and 5 ml. of the organic layer was with-

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[1] The trade name of The Upjohn Co. for the antibiotic actinospectacin is Trobicin.

TABLE I.—CONDITIONS AND RATE CONSTANTS (k in sec.$^{-1}$) FOR THE APPARENT FIRST-ORDER DEGRADATION OF ACTINOSPECTACIN SULFATE AS DETERMINED BY CHEMICAL ASSAY

Kinetic Run No.	°C.	Buffer Composition after Correction for Neutralization of Actinospectacin Salt		Molarity of Actinospectacin in Degrading Solution[a]	Average Observed pH for Run[b]	10^4k sec.$^{-1}$
		(NaOH)				
1	30.2	0.002 *M*	...	0.0040	10.14	1.34
2	30.2	0.012	...	0.0040	11.68	7.90
3	30.2	0.017	...	0.0040	...	10.1
4	30.2	0.042	...	0.0042	...	13.5
5	30.2	0.092	...	0.0040	...	16.2
6	30.2	0.142	...	0.0041	...	19.7
7	30.2	0.192	...	0.0040	...	23.2
8	30.2	0.242	...	0.0040	...	24.6
9	30.2	0.292	...	0.0042	...	26.7
10	40.2	0.092	...	0.0040	...	47.6
11	49.9	0.092	...	0.0040	...	116.0
		(K_2CO_3)	($KHCO_3$)			
12	60.0	0.092 *M*	0.008 *M*	0.0040	10.61	120.0
13	60.0	0.060	0.040	0.0040	9.84	39.5
14	60.0	0.026	0.074	0.0040	9.23	13.6
		(NaH_2BO_3)	(H_3BO_3)			
15	60.0	0.0372	0.0128	0.0040	9.20	1.13
16	60.0	0.0263	0.0237	0.0040	8.82	0.596
17	60.0	0.0120	0.0380	0.0039	8.16	0.285
18	60.0	0.0042	0.0458	0.0040	6.88[c]	0.113
		($NaKHPO_4$)	(KH_2PO_4)			
19	60.0	0.0427	0.0073	0.0040	7.22	0.447
20	60.0	0.0284	0.0216	0.0040	6.71	0.129
21	30.2	0.0973	0.0062	0.0020	8.13	0.007[d]

[a] Calculated from assumed molecular weight, 505.87. [b] Maximum range of 0.1 pH unit for duration of study. pH was read at the temperature of the run. [c] Was prepared to be 7.7 pH. [d] Because of the few points available for this run, this represents only the magnitude of the rate.

drawn, dried over 0.3 Gm. of anhydrous sodium sulfate, and transferred to a cell. Spectrophotometric measurement at 470 mμ with a Beckman model B spectrophotometer was made against a reagent blank prepared in the same manner but without antibiotic. A typical absorption band for the copper-dithiocarbamate complex of actinospectacin is given in Fig. 1. A standard curve of absorbance of organic phase extracted antibiotic *vs.* actinospectacin sulfate concentration was prepared in the same manner and followed Beer's law (see Fig. 2).

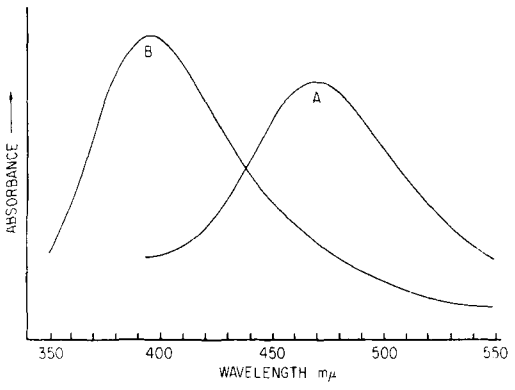


Fig. 1.—Typical visible absorption bands for the copper-dithiocarbamate complex of actinospectacin extracted in the upper organic phase (A) and for the copper dithiocarbamate complex of degraded actinospectacin unextracted from the lower aqueous phase (B).

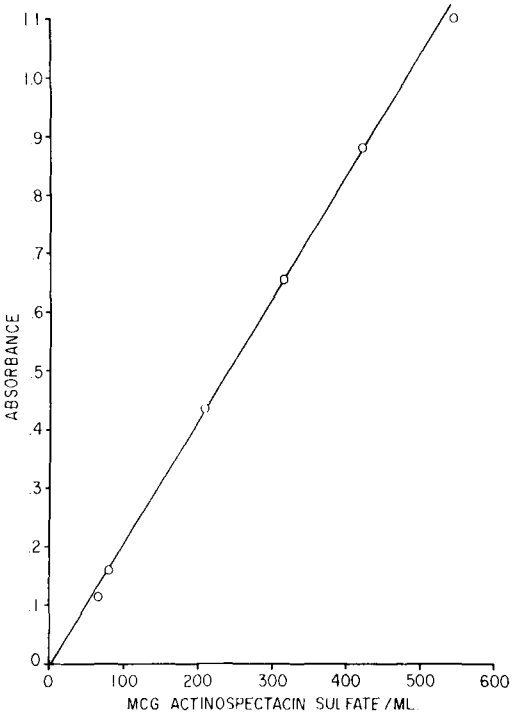


Fig. 2.—Standard curve for the 470 mμ absorbance of the complex, actinospectacin-dithiocarbamate-Cu^{++}, extracted into the upper organic phase against the initial concentration of actinospectacin-sulfate in aqueous solution.

As the alkaline degradation proceeded, color attributable to complex increased in the aqueous phase. At the beginning of a reaction, colored complex was totally soluble in the organic layer; at the end of a reaction, colored complex was totally soluble in the aqueous layer. The reaction could also be followed by the increasing appearance by spectrophotometry of colored reaction complex remaining in the aqueous phase after extraction (see Fig. 1).

Effect of Conditions on Absorbance of Colored Complexes.—A phenomenon occurred with several of the kinetic runs that warranted further investigation. The absorbance of the actinospectacin-dithiocarbamate-copper complex extracted in the organic phase at zero time for several of the kinetic runs did not agree with the values expected from the standard curve (see Fig. 2). In several instances the apparent absorbances were significantly less.

Of course, this did not affect the estimation of the apparent first-order rate constants for the decomposition of actinospectacin but it did raise the question as to the validity of the assay. The assay's reliability was checked by its excellent replication under the same conditions.

To investigate this phenomenon, aliquots of aqueous actinospectacin solutions of the same antibiotic concentration with various amounts of other materials were chemically assayed. The resultant apparent absorbances of the complex extracted into the organic phases were plotted as a function of the concentrations of these other materials present. These addends were similar to the components of the buffers used in the kinetic runs, i. e., acetic acid, sodium acetate, hydrochloric acid, and potassium chloride (see Fig. 3).

Chloride and acetate ions had no effect when the source of these ions was a neutral salt, but the corresponding acids gave a definite decrease in the color intensity of the actinospectacin-dithiocarbamate-copper complex extracted in the organic phase. In contrast, no decrease in the intensity of color from the complex with di-*n*-butylamine was noted when excess acetic or hydrochloric acid was added.

Characterization of Actinospectacin-Dithiocarbamate-Copper Complex.—An 0.7701-Gm. sample of actinospectacin sulfate was dissolved in 50 ml. pyridine, 25 ml. water, and 50 ml. isopropyl alcohol. This was titrated by the method of Critchfield and Johnson (4) with 31.15 ml. 0.100 *M* sodium hydroxide to phenolphthalein end point. Then 5 ml. of carbon disulfide was added and 30.40 ml. 0.100 *M* sodium hydroxide was necessary to reach the phenolphthalein end point again. A simultaneously prepared blank took 0.2 ml. titer to neutralize and another 0.2 ml. subsequent to the addition of carbon disulfide. Thus, 3.09 meq. of sulfuric acid and 3.02 meq. of dithiocarbamic acid (R_1R_2N—CS—SH) were found which calculates to 1.023 sulfuric acid per molecule and is consistent with the characterization of the original actinospectacin sulfate used, $C_{14}H_{26}N_2O_7 \cdot 1.12H_2SO_4 \cdot 3.43H_2O$. mol. wt. 505 (3). The pKa's were 7.0 and 8.8. It also demonstrates the formation of two dithiocarbamic acid groups per molecule.

The actinospectacin-dithiocarbamate-copper complex in the organic phase was isolated from the separated organic layer by evaporation of the solvent at room temperature. The infrared spectrum of this material was nearly identical with that of the original actinospectacin sulfate and thus indicated that no apparent degradation had occurred in this isolation procedure.

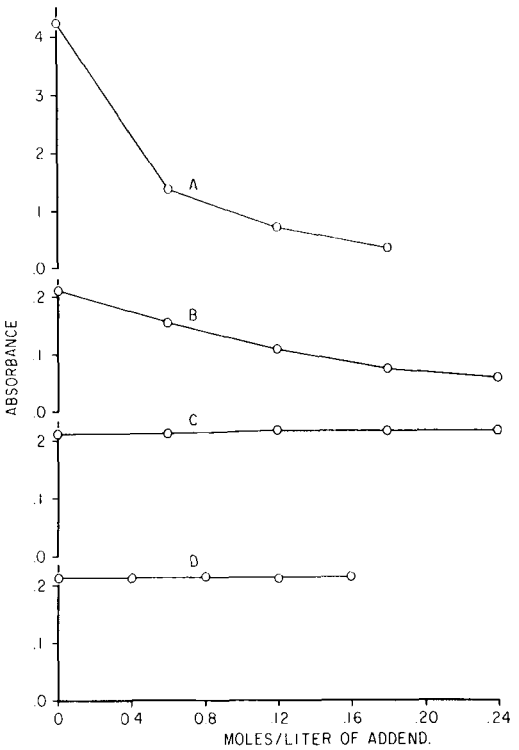


Fig. 3.—Effect of addends in original aqueous solution on 470 mμ absorbance of actinospectacin-dithiocarbamate-Cu^{++} complex extracted into upper organic phase

Curve	Initial Amount of Actinospectacin (mcg.)	Addend
A	208	HCl
B	104	$HC_2H_3O_2$
C	104	$NaC_2H_3O_2$
D	104	KCl

This material had 11.68% ash. If it is assumed that the nonhydrated form of the actinospectacin-dithiocarbamate-copper complex, $C_{14}H_{24}N_2O_7(CS_2)_2$-Cu, mol. wt. 547, is extracted in the organic phase, then the theoretical per cent copper for a copper to complex ratio to be 1:1 would be 11.5% as copper. If the ash were copper oxide, the theoretical for a 1:1 ratio would be 14.4% on the assumption of a nonsolvated sample. The experimentally obtained value is inconsistent with a 2:1 ratio where a 25.9% ash would be expected and thus confirms a 1:1 ratio of copper to partially solvated complex.

Samples of 0.304 mg. actinospectacin sulfate were treated in the manner described by the chemical assay procedure with slight modifications. The concentration of cupric chloride was varied. The absorbances of the colored actinospectacin-dithiocarbamate-copper complex in the organic layer attained the following values for a stated copper: actinospectacin sulfate molar ratio: 0.214, 1:1;

0.409, 2:1; 0.427, 3:1; 0.427, 5:1; 0.410, 7:1; 0.385, 10:1; 0.061, 20:1; and 0.055, 50:1.

The colored complex extracted into the organic phase achieved a maximum at *ca.* 4:1 ratio of reactants, copper to actinospectacin. With additional copper concentrations, the amount of 1:1 colored complex drastically decreased, proving the formation of a noncolored actinospectacin-dithiocarbamate-copper reaction product of a copper:actinospectacin ratio of 2:1 or more.

Characterizations of Degraded Actinospectacin and Its Dithiocarbamate Complex.—A 25-mg. sample of actinospectacin sulfate was degraded in 25 ml. 0.1 *M* sodium hydroxide at 25° for 24 hours, which is greatly in excess of the time necessary for complete loss of biological activity and identification as actinospectacin by the chemical assay. The resultant solution was neutralized so that the original material was at a 1:1 dilution with water. A series of aliquots of x ml., varying from 0.000 to 1.000 ml. by 0.050-ml. increments, were transferred to test tubes. A solution of cupric chloride of a molarity equivalent to the neutralized, diluted reaction solution was prepared. Then, $(1 - x)$ ml. of this cupric chloride solution was added to each test tube containing x ml. of the degraded actinospectacin solution according to the method of continuous variations (7). The chemical assay reagents and solvents were added as in the manner previously described. The absorbances were read in the Cary recording spectrophotometer, model 11, from 350–500 mμ using the tungsten lamp. The observed maximum was 462.5 mμ in the aqueous phase. The organic layer was colorless. A plot of the absorbance at this wavelength maximum against the mole fraction of degraded actinospectacin in degraded actinospectacin + copper gave a clear maximum at 0.5 mole fraction. This demonstrates that the 462.5 mμ maximum is defined by a 1:1 ratio of copper to the dithiocarbamate of degraded actinospectacin (7). When the ratio of copper to degraded actinospectacin was 4:1 or greater, a 390 mμ maximum started to appear. When additional copper was added so that the ratio was 10:1 in favor of the copper, the wavelength of the maximum absorbance in the aqueous layer shifted to 391 mμ (Fig. 1) and the 463 mμ maximum disappeared. This clearly indicates that a 2:1 complex of copper to the dithiocarbamate of degraded actinospectacin is formed at higher copper concentrations.

Infrared spectroscopy could not differentiate between actinospectacin and its degradation product. Nuclear magnetic resonance indicated that a doublet assigned to $-\underset{|}{CH}-CH_3$ in actinospectacin has been transformed to two sets of doublets in degraded actinospectacin; a new $-\underset{|}{CH}-CH_3$ has appeared. The area of the resultant two $-\underset{|}{CH}-CH_3$ groups is equivalent to the area attributable to one in the original actinospectacin. The same functional groups exist in actinospectacin and degraded actinospectacin except that all peak values are shifted about 10 cycles higher in the latter case. However, the peaks are superimposable when shifted, thus indicating slight changes in geometry.

The sulfate salt of the isolated degraded actinospectacin, dried in the same manner as actinospectacin sulfate at high vacuum and room temperature, had an equivalent weight of 252, so that its molecular weight of 504 is the same as actinospectacin sulfate. Its pKa's were 7.1 and 8.9.

Optical Activity and Degradation.—A solution of actinospectacin was prepared so that it was 0.05 *M* in actinospectacin free base and 0.10 *M* in sodium hydroxide. The optical rotation was read in a 1-dcm. tube at intervals of time at 400 mμ. The initial reading of +0.020 changed with time and asymptotically approached −1.700. The logarithm of the difference of the optical rotation at any time, t, and the asymptotic value was plotted against the time, t, and was a straight line. The resultant rate constant for the degradation as followed by this stereochemical transformation at 25° in 0.10 *M* sodium hydroxide was 9.73×10^{-4} sec.$^{-1}$, a half life of 11.8 minutes. The extrapolated initial rotation was +0.0188.

It will later be shown (from Eq. 13) that the degradation at 25° in 0.092 *M* sodium hydroxide by chemical assay is 9.55×10^{-4} sec.$^{-1}$.

Biological Assay Procedure.—The biological assay was a plate-disk assay against *Klebsiella pneumoniae* (8) and was run simultaneously with the chemical assay on aliquots of degrading actinospectacin. When anomalies resulted in bioassay dependent on whether or not the aliquot was diluted with phosphate buffer, a special study on the effect of pH 7.85 phosphate buffer concentration on apparent antibiotic potency was undertaken.

Actinospectacin (125.4 mg.) was weighed into a 50-ml. volumetric flask, diluted to volume with 0.15 *M* sodium hydroxide from the 30° bath, maintained at 30° and assayed at recorded times by the following procedures: *Bioassay Samples.*—Five-milliliter aliquots were pipetted into 10-ml. volumetric flasks containing 0.4 ml. 1.904 *M* hydrochloric acid to "stop" the reaction. One milliliter of neutralized solution was then pipetted into each of five 5-ml. volumetric flasks and diluted to volume with the appropriate concentration of pH 7.85 phosphate buffer to make the resultant solutions 0.16, 0.10, 0.05, 0.025, and 0.01 *M* in phosphate buffer. One milliliter of each of the five resultant solutions was transferred to an assay bottle and submitted for microbiological assay without further dilution. The concentration of the final solution prior to alkali degradation was 465 mcg./ml.

Chemical Assay Samples.—Aliquots (0.8 ml.) were pipetted into a 5-ml. volumetric flask containing *ca.* 4 ml. of an equal mixture of 0.2 *M* sodium hydroxide and 0.4 *M* acetic acid, and diluted up to 5 ml. with the same buffer mixture. One milliliter of the resultant solution was assayed by the chemical assay procedure. The concentration of the final solution prior to alkali degradation was 402 mcg./ml.

The results showed that the molarity of 7.85 pH phosphate buffer significantly affected the estimate of the apparent antibiotic concentration from microbiological assay as compared with the concentration from chemical assay (see Fig. 4). It was clearly shown that if the 7.85 pH buffer concentration was maintained above 0.05 *M* in the solution to be microbiologically assayed, a 1:1 correspondence with the chemical assay resulted for material degraded in 0.15 *M* sodium hydroxide at

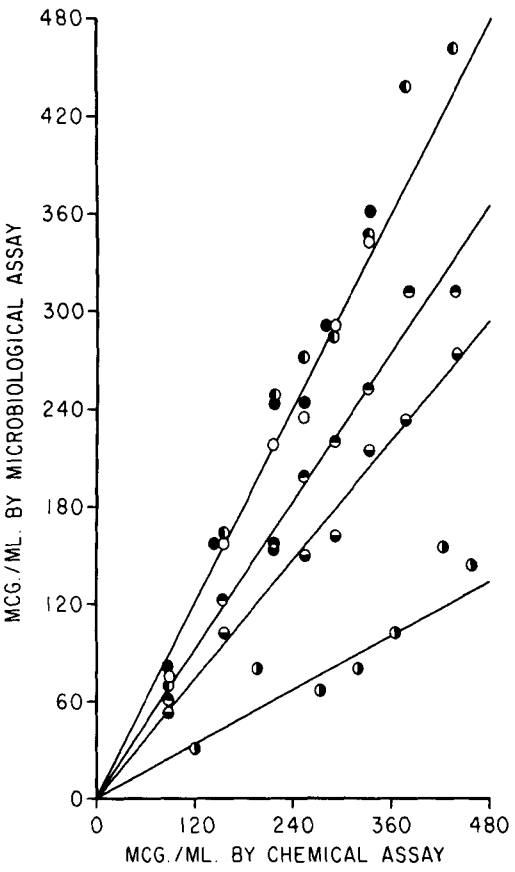


Fig. 4.—Effect of pH 7.85 phosphate buffer concentration on the microbiological assay of actinospectacin. The aliquots assayed were sampled at various times from an actinospectacin solution degrading in 0.15 *M* sodium hydroxide at 30°. ○, 0.160; ●, 0.100; ◐, 0.050; ◓, 0.025; ◒, 0.010; ◑, 0.000 *M* of phosphate buffer.

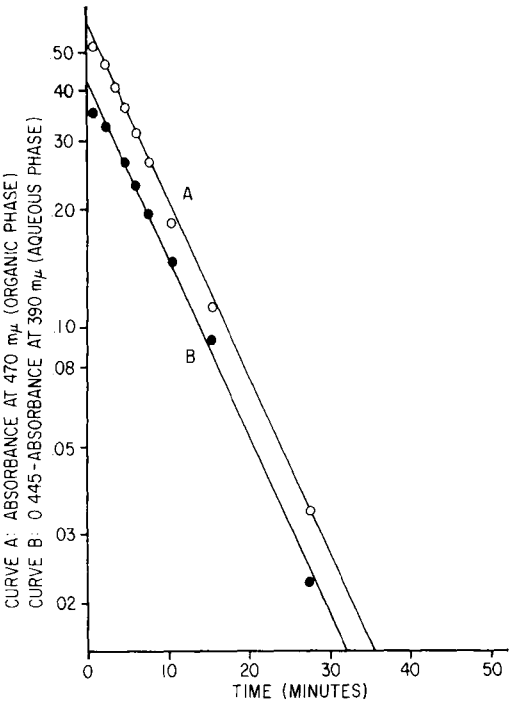


Fig. 5.—Apparent first-order rate plots for the degradation of actinospectacin in 0.15 *M* sodium hydroxide aqueous solution at 30°. Curve A is derived from the absorbance at 470 mμ of the actinospectacin-dithiocarbamate-Cu^{++} complex extracted in the upper organic phase and is a measure of the disappearance of the antibiotic. Curve B is derived from the absorbance at 390 mμ of the dithiocarbamate-Cu^{++} complex of degraded actinospectacin and is a measure of the appearance of the product of degradation.

30°. Thus, in all attempted correlations of bioassay with color assay, this minimum phosphate buffer concentration was maintained. The attempted correlations were conducted on the series of alkali-degrading actinospectacin solutions where the statistics of regression are given in Table II.

RESULTS AND DISCUSSION

Apparent First-Order Rates of Alkaline Degradation.—The alkaline degradation of actinospectacin in solution is the major route of antibiotic decomposition. At a given pH or hydroxyl ion concentration, it is apparently first order

$$\log A = \frac{-kt}{2.303} + \log A_0 \qquad \text{(Eq. 1)}$$

where A is the absorbance at 470 mμ of the organic phase complex (with Cu^{++} and CS_2) at any time, t, and A_0 is the absorbance at initial time, t_0. Figure 5 demonstrates the linearity of such semilogarithmic plots. The extraordinary phenomenon of the Cu^{++} CS_2 complex of the degraded actinospectacin being completely water soluble is demonstrated by curve B in Fig. 5, a line parallel to curve A for the decrease of actinospectacin complex absorbance in the organic phase. The rate of degraded actinospectacin complex appearance in the aqueous phase completely parallels the rate of actinospectacin complex disappearance from the organic phase. This former plot is based on the expression

$$\log (A'_\infty - A') = \frac{-kt}{2.303} + \log A'_\infty \qquad \text{(Eq. 2)}$$

where A' is the aqueous phase absorbance at 390 mμ (the $\lambda_{max.}$ of the degraded complex absorbance in the water phase) at any time, t, and A'_∞ is the final or asymptotic value of this absorbance, 0.445.

Formation of Dithiocarbamates and the Possible Nature of the Complexes.—The presumed reactions (4, 5) in the formation of dithiocarbamates and their copper complexes are

$$R_1R_2NH + CS_2 \rightleftharpoons R_1R_2N{-}CS{-}SH \qquad \text{(Eq. 3)}$$

$$R_1R_2N{-}CS{-}SH + Cu^{++} \rightleftharpoons R_1R_2N{-}CS{-}SCu^{+} + H^{+} \qquad \text{(Eq. 4)}$$

$$R_1R_2N{-}CS{-}SCu^{+} + R_1R_2N{-}CS{-}SH \rightleftharpoons (R_1R_2N{-}CS{-}S)_2Cu + H^{+} \qquad \text{(Eq. 5)}$$

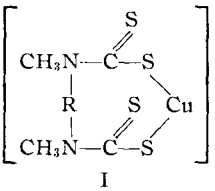
TABLE II.—STATISTICS OF REGRESSIONS OF BIOASSAYS (y) ON CHEMICAL ASSAYS (x) FOR ACTINOSPECTACIN DEGRADING IN ALKALINE SOLUTION AT 30.2°

Molarity of NaOH	No. of Points Used	Line Forced through Origin; $y=mx$				Line of Best Fit; $y=mx+b$						
		m	σm	σy	$t\sigma m$	m	σm	b	σb	σy	$t\sigma m$	$t\sigma b$
0.02	7	1.05	0.039	27.7	0.095	1.22	0.059	−48.7	15.8	17.8	0.151	40.6
0.044	6	0.898	0.038	25.0	0.099	0.846	0.099	14.9	26.2	26.8	0.274	72.6
0.047	7	0.816	0.046	36.0	0.112	0.776	0.116	13.1	34.4	38.8	0.298	88.5
0.082	7	0.854	0.031	26.6	0.075	0.934	0.091	28.1	29.9	26.9	0.235	76.9
0.115	6	1.05	0.038	22.0	0.098	1.08	0.119	− 9.50	28.2	24.3	0.331	78.4
0.190	8	0.891	0.048	35.8	0.113	0.821	0.120	20.4	31.7	37.4	0.293	77.5
0.200	8	0.852	0.021	15.9	0.049	0.959	0.031	−31.4	8.46	9.45	0.077	20.7

$$(R_1R_2N{-}CS{-}S)_2Cu + Cu^{++} \rightleftharpoons 2R_1R_2N{-}CS{-}SCu^{+} \quad \text{(Eq. 6)}$$

The introduction of two CS_2 per molecule was demonstrated experimentally by the titration method of Critchfield and Johnson (4).

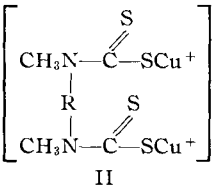
When these series of reactions and the previously described procedures were used to follow the rates of degradation of actinospectacin there was, initially, no colored complex in the aqueous phase. Finally, after degradation, there was no colored complex in the organic phase. It thus follows that this degradation of actinospectacin modified the amine groups in a manner that changes an organic phase-soluble complex to a water-soluble one. A possible explanation is an alkaline induced variation of a stereospecific relation of the two nitrogens in actinospectacin. In the case of the organic phase-soluble actinospectacin complex, both dithiocarbamic acid groups in the molecule may be complexed by one cupric ion as per Eqs. 3–5, giving I.



I

Actinospectacin-2CS_2-Cu Complex
Organic Phase Soluble; $\lambda_{max.}$ = 470 mμ

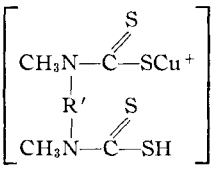
The 1:1 = Cu:actinospectacin nature of this complex, I, was shown experimentally by the agreement of the per cent ash as CuO with the theoretical for 1:1. Addition of excess cupric ion, 20:1 = Cu:actinospectacin, dramatically decreased the absorbance extracted into the organic phase, proving the formation of a new complex consistent with Eq. 6 so that we have II



II

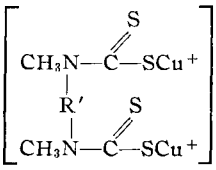
Actinospectacin-$(CS_2Cu^{+})_2$ Complex

For degraded actinospectacin a chelated copper ion may not be sterically possible as in I so that a charged water-soluble 1:1 complex would be III, a 2:1 complex would be IV.



III

Degraded Actinospectacin-2CS_2-Cu^{+} Complex
Aqueous Phase Soluble; $\lambda_{max.}$ = 462.5 mμ



IV

Degraded Actinospectacin-$(CS_2Cu^{+})_2$ Complex
Aqueous Phase Soluble; $\lambda_{max.}$ = 390 mμ

By the method of continuous variations (7), III was shown to be a 1:1 = Cu:degraded actinospectacin complex. The new 390 mμ maximum characteristic of IV appeared when excess cupric ion was added in a 10:1 ratio to the degraded actinospectacin.

Infrared and nuclear magnetic resonance spectroscopy could only indicate steric transformation in the alkaline degradation of actinospectacin; they could not identify changes in functional groups.

Comparison of the equivalent weights and pKa's of actinospectacin sulfate and isolated degraded actinospectacin sulfate also could not demonstrate any change in the molecule on degradation.

In addition, the change in optical activity in 0.1 *M* sodium hydroxide confirmed the models proposed. Alkaline degradation of actinospectacin involved a stereochemical transformation and the rates so calculated were identical to the rates by chemical assay.[2]

The variation in the absorbance of the actinospectacin-dithiocarbamate-copper complex extracted into the organic phase when acids were added needs explanation.

The pyridine which is used as part of the solvent mixture for the analytical reaction may be implicated. It partially neutralizes the salt of the secondary amine and helps solubilize the carbon

[2] These experimental facts may not be considered conclusive evidence to negate functional group changes. It is possible that degraded actinospectacin may have enhanced aqueous solubility and that its dithiocarbamate-copper complex may have low organic solvent solubility.

disulfide in the mixed aqueous system. Pyridine has an aqueous pKa = 5.36 (9) so that the resultant apparent pH of the pyridine–isopropyl alcohol–water–carbon disulfide–cupric chloride solution prior to extraction is significantly decreased by the addition of acids (see Fig. 6). The shape of the pH *vs.* molarity of acid curve (Fig. 6) is very similar to the shape of the curve, absorbance *vs.* molarity of acid (Fig. 3).

This implies that the equilibria of Eqs. 4 and 5 are forced to the left by increased hydrogen ion concentration and that the dithiocarbamate-Cu^+ complex is water soluble and does not contribute to the color in the organic layer.

Rate of Degradation as a Function of Alkalinity.—Figure 7 plots the apparent first-order rate constant, k in $sec.^{-1}$, as a function of the [NaOH] solution prepared for degradation. Actually, since the actinospectacin has an apparent molecular weight of 506 and has two amine groups in the salt form, twice the molar concentration of the actinospectacin should be subtracted from the abscissa values to give the true $[OH^-]$ concentration. Generally, actinospectacin = 0.0040 and thus, the given $[NaOH]_{apparent} = [NaOH]_{true} + 0.0080$ in Fig. 7.

The two lines in Fig. 7 represent linear estimates of the variation of apparent first-order rate constants, k in $sec.^{-1}$ at 30.2°, with hydroxyl ion concentration and may be given as

$$k = k_{OH}[OH^-] = 0.0533\ [OH^-] \quad \text{(Eq. 6)}$$

$$[OH^-] < 0.020$$

$$k = k'_{OH}[OH^-] + K = 5.33 \times 10^{-3}\ [OH^-] + 11.6 \times 10^{-4} \quad \text{(Eq. 7)}$$

$$0.020 < [OH^-]$$

The fact that this apparent hydroxyl ion catalysis constant varies as a function of the magnitude of the $[OH^-]$ is a strong indication of a change in the nature of the actinospectacin with hydroxyl ion. The most natural explanation is that the sugar-like antibiotic has one or more ionizable protons of high pKa, *ca.* 12–13, and the anionic form resists hydroxyl ion attack more readily than the neutral molecule.

An alternative method of correlating the apparent first-order rate constant, k in $sec.^{-1}$, for alkaline attack and hydroxyl ion concentration is by a log k *vs.* pOH plot. See Fig. 8 where pOH is calculated from

$$pOH = \log f[NaOH]_{true} \quad \text{(Eq. 8)}$$

where the activity coefficient, f, at 30° is obtained from Harned and Owen (10).

An empirical relation valid over the pOH range 0.6 to 2.2, from Fig. 8, is

$$\log k = -0.376\ pOH - 2.324;\ pOH < 2.2 \quad \text{(Eq. 9)}$$

This is most probably an estimate of

$$k\ [\text{actinospectacin}] = k_{OH}[OH^-][\text{uncharged actinospectacin}] + k'_{OH}[OH^-]\ [\text{anionic actinospectacin}] \quad \text{(Eq. 10)}$$

where from the dashed line of Fig. 8

$$\log k = -pOH + \log k_{OH}^-;\ pOH > 2.2 \quad \text{(Eq. 11)}$$

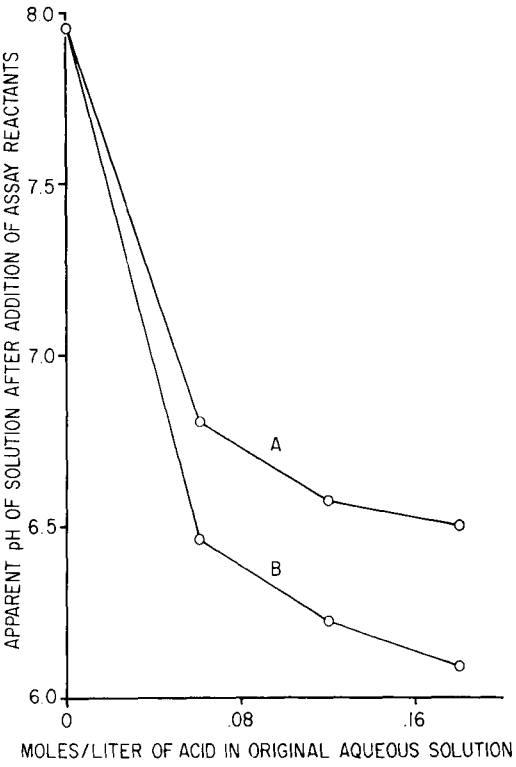


Fig. 6.—Effect of acid added to the original aqueous solution to be assayed for actinospectacin on the apparent pH of the pyridine-isopropyl alcohol-water-carbon disulfide-cupric chloride solution, the composition of the solution just prior to extraction with benzene. Curve A is for acetic acid and Curve B is for hydrochloric acid.

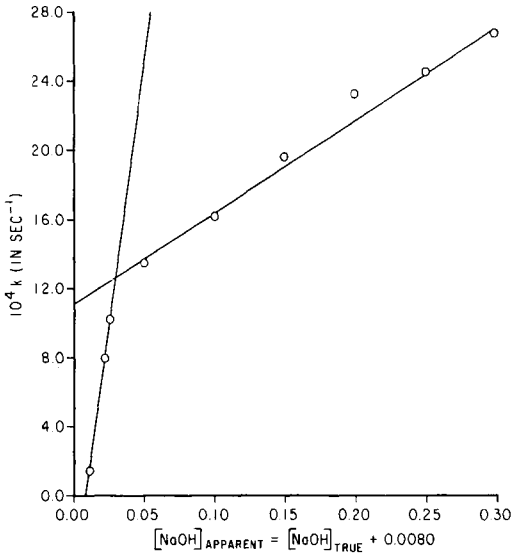


Fig. 7.—Apparent first-order rate constants for the alkaline degradation of actinospectacin as a function of [NaOH] concentration at 30.2°.

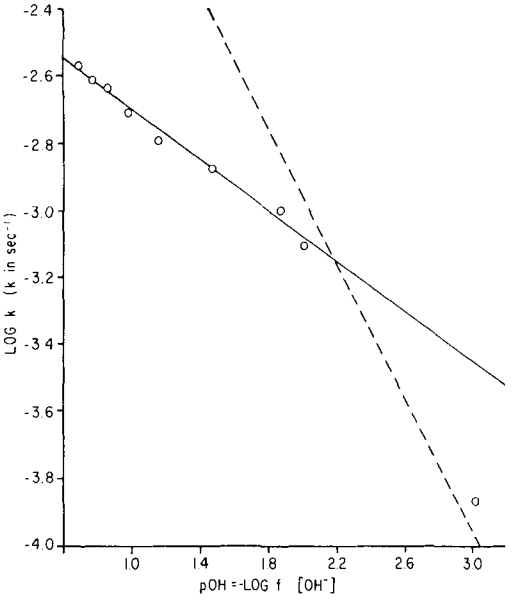


Fig. 8.—Plot of the logarithm of the apparent first-order rate constant for the alkaline degradation of actinospectacin at 30.2° against pOH = log f [OH$^-$] where f is the activity coefficient (9) and [OH$^-$] = [NaOH]$_{True}$. The dashed line has a slope of unity.

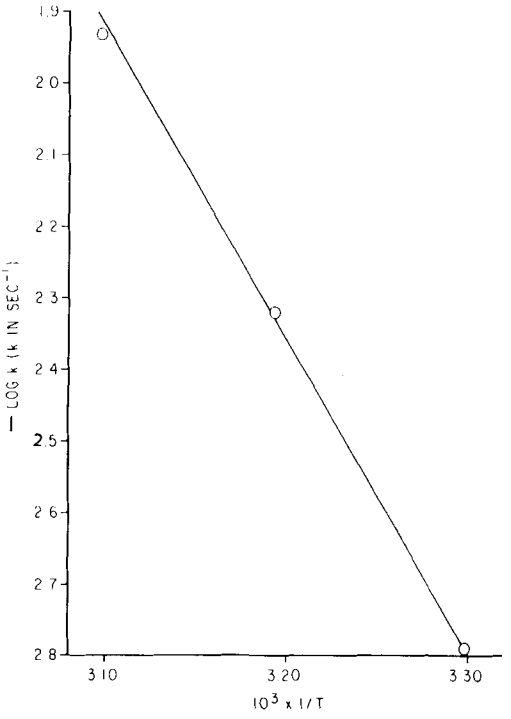


Fig. 9.—Arrhenius plot for the degradation of actinospectacin in 0.092 M sodium hydroxide.

so that log k_{OH^-} = 0.95 or that k_{OH^-} = 0.11 L./M/sec.[3] An estimated pKa of uncharged actinospectacin would be 12.6.[4]

Rate of Degradation as a Function of Buffer Kind.—Since stability in the less alkaline regions had more practical significance and it was indicated that significant degradation did occur at 30° in pH 8 phosphate buffer (half life *ca.* 50 days), an attempt to study degradation in phosphate, borate, and carbonate buffers at 60° was made (see Table I). The rate was not completely dependent on pH but was also a function of the buffer used with carbonate buffers 10 times more catalytic than borate at pH 9 and phosphate *ca.* four times more than borate at pH *ca.* 7.5.

Rate of Alkaline Degradation as a Function of Temperature.—Figure 9 gives the Arrhenius plot for the degradation of actinospectacin at 0.092 M sodium hydroxide. The representative equation is

$$\log k = -4{,}350\,(1/T) + 11.57 \quad \text{(Eq. 12)}$$

where T is absolute temperature and the heat of activation is 20.0 Kcal./mol.

Correlation of Chemical Assay and Microbiological Assay.—During the course of several alkaline degradations, aliquots removed at varying intervals of time were subjected to both microbiological and chemical assays. The statistics of the regression of bioassay *vs.* chemical assay are given in Table II for lines of best fit independent of intercept and for lines of best fit forced through the origin. Of the seven regressions only two, the first and the last listed in Table II, can be stated as having statistically significant nonzero intercepts. Two of the regressions are given in Fig. 10 where the solid lines represent the curve fitted by least squares and the dashed lines are based on adjusted intercepts, $b \pm \sigma_b$, and forced through the mean values, $\bar{y}$ and $\bar{x}$, of the microbiological and chemical assays, respectively. The bracketing of the origin by the intercepts of these dashed curves is apparent. The slope of unit, in general, is demonstrated within the estimated error of the slopes and it can be concluded that a good correspondence exists between the microbiological and the chemical assays.

Prediction of Stability.—Significant solution degradation of actinospectacin occurs in alkaline solution and appears to be a function of hydroxyl ion concentration and the type of buffer anion, *viz.*, carbonate, borate, and phosphate. At 30°, the half life is 50 days in pH 8 phosphate buffer.

On the basis of studies in varying sodium hydroxide concentrations, the half life at 30° in unbuffered aqueous solutions is 1000 days at pH 7.0, 100 days at pH 8.0, and 10 days at pH 9.0.

Experimental studies have shown no significant degradation over two months at pH 4.5 and in 0.1 M hydrochloric acid at 30°.

[3] The reader should not be disturbed at the difference between this value and the one for k_{OH^-} given in Eq. 6. They are defined for different equations. The latter value and [NaOH] are used with Eq. 6 to derive k. The former value and pOH derived from Eq. 8 from consideration of OH$^-$ activity are used with Eq. 11 to derive k. Both methods are valid within experimental error to define k under the conditions specified.

[4] Subsequent titration by D. J. Weber of actinospectacin salt in pyridine with 0.1 N tetra n-butylammonium hydroxide against standard glass-calomel electrodes (methanol saturated with lithium chloride) demonstrated a weakly acidic group that was consistent with this deduced function.

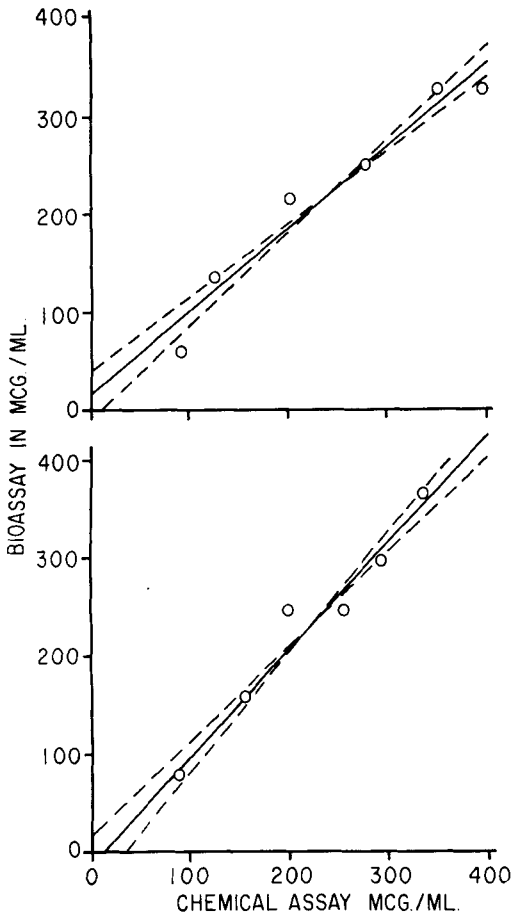


Fig. 10.—Correlation of microbiological plate-disk assay (*Klebsiella pneumoniae*) with chemical assay. The solid line represents the best fit by least squares whereas the dashed lines pass through the mean values and the intercept ± standard deviation of the intercept. The upper curve is based on degradation in 0.044 *M* sodium hydroxide; the lower is based on degradation in 0.115 *M* sodium hydroxide at 30.2°.

SUMMARY

1. Actinospectacin is degraded by specific hydroxyl ion catalysis and the anionic constituents of various buffers: carbonate, phosphate, and borate with decreasing effectiveness in that order. At 30°, the half life is 50 days in pH 8 phosphate buffer, whereas in unbuffered aqueous solution the predicted half life is 1000 days at pH 7.0 and 100 days at pH 8.0 at 30°. Below pH 5, actinospectacin should be stable for all practical purposes.

2. A chemical assay procedure has been devised and applied to the determination of actinospectacin which depends on the unique structure of its secondary amine groups. The formation and extraction of cupric complexed dithiocarbamic acids of actinospectacin permit quantification of undegraded antibiotic by spectrophotometric measurement of visible absorbance in the organic phase. The absorbance of the residual color in the aqueous phase measures degraded antibiotic.

3. The reliability of the bioassay (plate disk *vs. Klebsiella pneumoniae*) was shown to vary with phosphate buffer concentration so that a minimum of 0.05 *M* pH 7.85 phosphate buffer must be present.

4. The bioassay correlates well with the chemical assay.

5. Evidence is presented to demonstrate that the stereospecific relation of the secondary amines in actinospectacin is vital for its biological activity and response to the chemical assay.

6. Complete kinetic expressions have been determined for the alkaline solution degradation of actinospectacin at the various temperatures and a pKa *ca.* 12 for its hydrogen ion dissociation, probably of a sugar-like structure, has been kinetically observed.

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