Prediction of Stability in Pharmaceutical Preparations XIII

Stability, Spectrophotometric, and Biological Assay of the Antibiotic Porfiromycin in Pharmaceutically Useful pH Ranges

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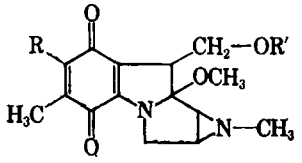
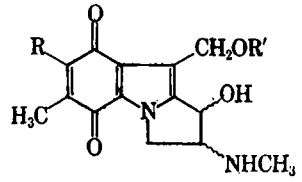
The kinetics of solvolysis of the antibiotic porfiromycin are quantified in the pharmaceutically useful pH ranges in acetate, phosphate, and borate buffers. The devised ultraviolet spectrophotometric assays of solution degraded antibiotic are correlated with the plate-disk assay against *S. aureus* and the paper chromatographic assay against *S. lutea*. General acid catalyzed solvolysis is indicated as well as specific acid-base. In acetate buffers, the primary degradative mechanism is the hydrolysis of the fused ring aziridine group, whereas mild alkali substitutes a hydroxyl for the quinoid ring amine. In the intermediate pH ranges characterized by phosphate buffers reaction at both sites is implied and maximum stability at pH 8 at 30° can be predicted. Conditions for isolation of solvolytic products and their characterizations are given.

THE NEW BROAD SPECTRUM antibiotic porfiromycin has been assigned the fused ring aziridine structure, α, by Webb, *et al.* (1–3).



	R	R′
α	$-NH_2$	$-CONH_2$
β(B)	$-OH$	$-CONH_2$
γ(B,B)	$-OH$	$-COOH$
δ(B,B,B)	$-OH$	$-H$



	R	R′
β(A)	$-NH_2$	$-CONH_2$
γ(A,A) γ(B,A)	$-OH$	$-CONH_2$
γ(A,B) δ(A,A,B)	$-OH$	$-H$

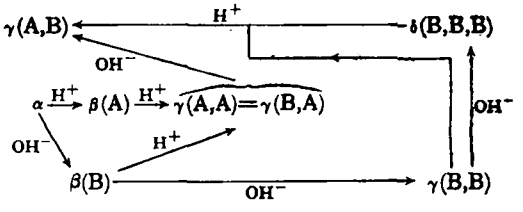
Properties, biological activities and assay methods have been studied (4–8). The kinetics of the acidic and basic catalyzed transformations of porfiromycin in solution have been evaluated and quantitative expressions have been established for their kinetics (9). The products of acidic and alkaline catalyzed solvolysis have been spectrophotometrically identified and their dissociation constants and apparent equivalent weights determined. In addition, these physicochemical and kinetic data have been correlated (9) with structural transformations of the proposed structure for porfiromycin, α (1, 2).

The purpose of this paper is to consider the correlation of biological and spectrophotometric assays of porfiromycin, to evaluate the antibiotic's stability in the pharmaceutically useful pH ranges and to provide additional physicochemical data for characterization.

In this paper the products of porfiromycin degradation are identified by an operational nomenclature. In this scheme porfiromycin is α and distinct spectrophotometric identities resulting from acid or basic solution degradations are represented as β, γ, and δ in their chronological order of appearance. A subscript represents the solution (A = acid, B = basic) of the distinct product. The order of B's or A's in the subsequent parentheses represents the sequence of basic or acid degradation.

For example, δ_B(A,B,B) indicates that this is the third distinct product of porfiromycin degradation in basic solution from firstly, acid, *i.e.*, β(A); then, secondly, basic γ(A,B); and a final degradation in basic solution, δ(A,B,B).

As has been shown previously (9) the possible acid and alkaline transformations of porfiromycin, α, with their isolable intermediates, are



Received October 14, 1963, from the College of Pharmacy, University of Florida, Gainesville.
Accepted for publication December 26, 1963.
This investigation was done at the Research Division, The Upjohn Company, Kalamazoo, Mich.
The authors are greatly indebted to Mrs. Lillian G. Snyder and Mr. Dennis J. Weber for excellent technical assistance and to Dr. G. B. Whitfield, Mr. C. DeBoer and associates for microbiological assays.

TABLE I.—CONDITIONS AND OBSERVED FIRST-ORDER RATE CONSTANTS (k IN SEC.$^{-1}$) FOR THE HYDROLYSIS OF PORFIROMYCIN IN AQUEOUS BUFFERS

Run	° C.	Observed pH at 30° C.	Porfiromycin (mcg./ml.)	Buffer Composition		$10^5 k$ (sec.$^{-1}$)
				[CH_3COOH]	[CH_3COO^-]	
1	30.2	3.60	30	0.1756	0.0122	28.4[a]
2	30.2	4.53	26	0.05	0.05	3.03[a]
3	30.2	4.54	24	0.10	0.10	3.24[a]
4	30.2	4.51	26	0.20	0.20	4.26[a]
5	30.2	4.55	25	0.40	0.40	5.00[a]
				[KH_2PO_4]	[NaOH]	
6	30.2	5.91	16	0.05	0.0057	0.158[a]
7	30.2	6.90	15	0.05	0.02963	0.0216[a]
8	60.0	5.91	28	0.05	0.0057	3.24[a]
9	60.0	6.96	32	0.05	0.02963	0.484[a]
10	60.0	7.91	27	0.05	0.0468	0.179[a]
11	69.7	6.93	26	0.05	0.02963	0.883[b]
12	69.7	6.97	25	0.025	0.01482	0.695[b]
				[H_3BO_3]	[NaOH]	
13	60.0	8.95	28	0.05	0.0213	0.185[c]
14	60.7	8.95	500	0.05	0.0213	0.229[c]
15	60.7	8.95	500	0.05	0.0213	0.946[d]
16	60.0	9.89	29	0.05	0.0439	0.250[c]
17	60.0	9.89	536	0.05	0.0439	0.300[c]
18	60.0	9.89	536	0.05	0.0439	6.16[e]
19	60.0	9.89	536	0.05	0.0439	3.21[f]
20	30.2	...	30	0.00	0.10	81.9[g]
21	30.2	...	30	0.00	0.10	0.352[h]

[a] Rate constant calculated from loss of absorbance at 363 mμ ($\alpha \rightarrow \beta$). [b] Aliquots were submitted for plate-disk assay (7) at two different phosphate concentrations for both runs 11 and 12 to test the effect of phosphate concentration on the sensitivity of the assay. It had no apparent effect. Rate constants for $\alpha \rightarrow \beta$ were calculated from U.V. spectra. [c] Rate constant calculated from loss of absorbance at 360 mμ ($\beta \rightarrow \gamma$). [d] Same run as No. 14, but rate constant calculated from loss of potency by plate-disk assay ($\alpha \rightarrow \beta$) (7). [e] Same run as No. 17, but rate constant calculated from loss of potency by *S. lutea* papergram ($\alpha \rightarrow \beta$) (4). [f] Same run as No. 17, but rate constant calculated from loss of absorbance at 367.5 mμ for time interval during which aliquots showed potency by bioassays ($\alpha \rightarrow \beta$). [g] Rate constant calculated from loss of potency by plate-disk assay ($\alpha \rightarrow \beta$) (7). [h] Same run as No. 20, but rate constant calculated from loss of absorbance at 360 mμ ($\beta \rightarrow \gamma$).

EXPERIMENTAL

The isolation and characterization of the porfiromycin used in these studies has been reported by Herr, *et al.* (5).

Kinetic Studies.—The appropriate amount of porfiromycin or isolated degradation product was weighed into a tared volumetric flask, a few drops of methanol were added to dissolve the sample, and the solution was diluted up to volume with the appropriate aqueous buffer. The buffer solutions had been previously equilibrated at the temperatures of the degradation studies. The flasks were covered with aluminum foil to prevent possible photolytic degradation, put in a constant temperature bath, and at recorded time intervals a spectrum was run on a Cary model 11 or 14 recording spectrophotometer after appropriate dilution, usually to 12 mcg./ml.

The conditions for the degradation of porfiromycin in acetate buffers at 30° are given in Table I, runs 1–5. The pH was measured by glass-saturated calomel electrodes by a Cambridge pH meter and recorded. The average is reported. Aliquots of these solutions were taken at various times. The aliquots were diluted 1:1 with sufficient alkali to adjust to pH 7 and the spectra of the resulting 12–15 mcg./ml. solutions were recorded on the Cary recording spectrophotometer. Run 1 was also diluted 1:1 with the same acetate buffer and the spectra observed. Aliquots of the same acetic acid–acetate buffered solution, runs 2–5, Table I, were taken at the same intervals and neutralized 1:1 with 0.1 *M* phosphate buffer, pH 7.8, which also included sufficient alkali to neutralize the excess acetic acid. These samples were assayed by the plate-disk method against *S. aureus* (7).

The conditions for the degradation of porfiromycin in phosphate and borate buffers are given in Table I, runs 6–12 and runs 13–21, respectively. The procedure for spectrophotometric assay was the same for runs 6–12, 13, 16, and 20, Table I, as that given for the acetate buffers except that the 1:1 dilution before reading on the spectrophotometer was made with the same buffer used in the kinetic study.

Runs 11 and 12 were also used to study the effect of different phosphate buffer concentrations on the biological assay.

Simultaneously with the removal and treatment of aliquots from runs 11 and 12 for spectrophotometric assay, two other aliquots were removed from each run for biological assay by plate-disk against *S. aureus* (7), each to be assayed under different phosphate buffer concentrations so that the effect of this variable could be evaluated. Also, solutions of porfiromycin of 500 mcg./ml. were prepared in borate buffers, maintained at desired temperature, and aliquots removed at timed intervals (see runs 14, 15, 17, 18, and 19, Table I). For plate-disk assay against *S. aureus* (7), 0.1-ml. aliquots were diluted to 5 ml. with 0.1 *M* phosphate buffer, pH 7.9, and assayed directly. For spectrophotometric assay, 0.2-ml. aliquots were diluted to 10 ml. with buffer. For paper chromatographic assay (4) by ultraviolet scanning and against *S. lutea* 1-ml. aliquots were adjusted to the appropriate pH with concentrated reagents so that no significant change in concentration occurred.

Studies were conducted in borate buffers at 60° and at pH values 9 (runs 14, and 15) and 10 (runs 17–19). The aliquots of the pH 10 study (run 18) were adjusted to pH 7 and submitted for papergram assay by both ultraviolet scanning and against *S. lutea* (4).

The aliquots of the pH 9 study (runs 14 and 15) were adjusted to pH 8 and 9 and both adjusted samples were submitted for papergram assay by ultraviolet and against *S. lutea* (4).

In 0.1 *M* NaOH solution the porfiromycin absorbance at 363 mμ dramatically decreased with time and a new chromophore appeared with a $\lambda_{max.}$ at 333 mμ. The half-life of the porfiromycin chromophore was 55 hours at 30° (run 21). However, when an attempt was made to correlate the half-life of the porfiromycin biological activity by plate-disk assay (7), the biological activity was found to disappear at a much faster rate (half-life of 14 minutes) under these conditions (run 20) than did the porfiromycin 365-mμ chromophore.

When this apparent inconsistency was observed, the changes in spectra were followed carefully for the first half-hour and a slight but significant change in chromophore was determined. The 363 mμ (a = 63.8) maximum shifted to 360 mμ (a = 67.1) and the absorbance *increased.*

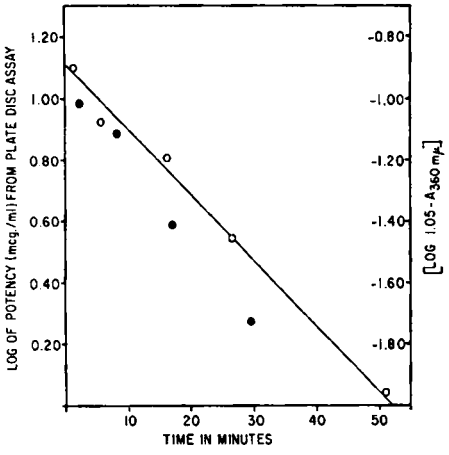


Fig. 1.—Correlation of rate of loss of biological potency by plate-disk assay with rate of increase of porfiromycin absorbance at 360 mμ in 0.10 *M* NaOH at 30°. Key: O, logarithm of potency by plate-disk assay plotted against time; ●, logarithm of the difference between 1.05 and the absorbance at 360 mμ plotted against time for a 15 mcg./ml. solution of porfiromycin.

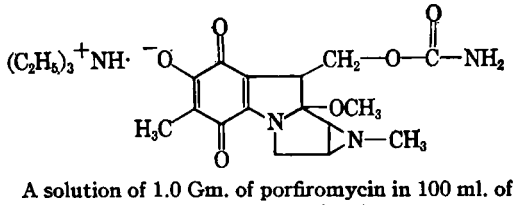
Even considering the small changes in absorbance which would introduce relatively large error into a first-order plot, the coincidence of the slopes of such plots for absorbance changes and bioassay values was apparent (Fig. 1). Thus, the correlation of the rates of this chromophoric enhancement with loss in biological activity was conclusively demonstrated. This was considered as the transformation of porfiromycin $\alpha_B \rightarrow \beta_B(B)$ (9).

Visible Spectra of Porfiromycin and Its Acid and Alkaline Degradation Products.—The visible spectra of porfiromycin and its products of acid and alkaline degradation were also run on the Cary 11 spectrophotometer at several pH values ranging from acid to alkaline solutions. The changes in color of these products as a result of changes in pH are noted in Table II.

The conditions for their preparation were determined from the kinetics described previously (9). The β(B), γ(B,B), and δ(B,B,A) were all prepared *in situ*, starting with the triethylamine salt of β(B). The γ(B,A) and δ(B,A,B) were prepared *in situ*, starting with isolated γ(B,A). All other materials were prepared from porfiromycin in solution as previously specified (9) and were analyzed spectrophotometrically *in situ*.

Isolation and Characterization of Solvolytic Products of Porfiromycin.

Preparation of β (B) Triethylamine Salt



A solution of 1.0 Gm. of porfiromycin in 100 ml. of 0.1 *N* sodium hydroxide was stirred at room temperature for 75 minutes and then allowed to stand at 5° for an additional 45 minutes. Cold methylene chloride (100 ml.) was then added and the mixture acidified with 12 ml. of 1 *N* sulfuric acid. The cold mixture was shaken and the organic layer quickly

TABLE II.—VISIBLE PHENOMENA OF PORFIROMYCIN AND ITS ACID-BASE DEGRADATION PRODUCTS

Compound	In Alkaline Solution: $\lambda_{max.}$	In Alkaline Solution: a[a] at $\lambda_{max.}$	In Alkaline Solution: Color of Solution	In Acid Solution: $\lambda_{max.}$	In Acid Solution: a at $\lambda_{max.}$	In Acid Solution: Color of Solution	Method of Preparation
Porfiromycin	572	0.78	Purple	Degrades rapidly to β(A)			
β(B)	575	0.47	Purple	Degrades rapidly to γ(B,A)			Dissolution of Triethylamine salt of β(B)
γ(B,B)	530	0.53	Slight pink	Degrades rapidly to δ(B,B,A)			Alkaline Degradation of β(B), Triethylamine Salt
β(A)	548	3.61	Red-purple	532	3.17	Red-purple	Acid Degradation of Porfiromycin
δ(B,B,A)	556[b]	1.45	Yellow and turbid	430	2.75	Yellow and turbid	Acid Degradation of γ(B,B)
γ(B,A)	556	3.61	Purple	457	1.52	Yellow	Dissolution of Isolated γ(B,A)
γ(A,A)	556	4.03	Purple	457	1.70	Yellow	Acid Degradation of β(A)
δ(A,A,B)	537	0.104	Light pink	475[b]	0.069	Light pink	Alkaline Degradation of γ(A,A)
γ(A,B)	537	0.11	Pale yellow	475[b]	0.092	Pale yellow	Alkaline Degradation of β(A)
δ(B,A,B)	537	0.041	Yellow	475[b]	0.034	Pale yellow	Alkaline Degradation of γ(B,A)

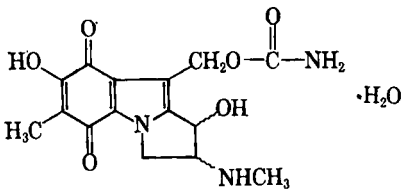
[a] Absorptivity is in Absorbance/Gm./L. calculated on the basis of a concentration of the original undegraded material of 0.5 mg./ml. [b] This was not a distinct maximum.

separated. The aqueous phase was re-extracted with another 50-ml. portion of methylene chloride. The methylene chloride extracts were combined and washed with ice water. After drying over magnesium sulfate for 10 minutes, the methylene chloride solution was concentrated to a purple gum on the Rinco rotating evaporator at 20°. The gum was dissolved in 175 ml. of ether and stirred at room temperature while 2 ml. of triethylamine were added. The crystals which separated were collected and washed with ether. The yield of air dried material was 830 mg. Recrystallization could be effected by adding ether containing some triethylamine to a chloroform solution of the crude product. The salt lost birefringence at 180–185° but did not melt below 320°.

Anal.—Calcd. for $C_{22}H_{24}N_4O_6$: C, 58.7; H, 7.55; N, 12.4; O, 21.3; OCH_3, 6.9. Found: C, 58.48; H, 7.59; N, 12.21; O, 21.12; OCH_3, 6.17.

I.R. spectra: $\lambda_{max.}$ at 3570, 3320, 3270, 3170, 2600, 2450, 1715, 1620, 1580, 1567, 1510 sh, 1500 sh, 1485, 1340, 1327, 1320, 1220, 1102, 1050, 695 cm.$^{-1}$.

Preparation of $\gamma(B,A)$

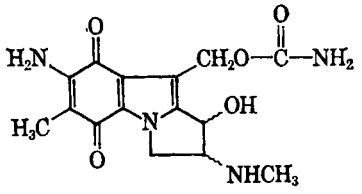


A solution of 1.0 Gm. of porfiromycin in 100 ml. of 0.1 *N* sodium hydroxide was stirred at room temperature for 1 hour. The resulting solution of β(B) was then acidified with 25 ml. of 1 *N* hydrochloric acid and allowed to stand at room temperature overnight. The color of the solution changed from blue through brown to orange. Neutralization of the orange solution with ammonia deposited deep blue crystals of the desired product. The yield was 600 mg. Recrystallization from wet dimethylformamide-methanol afforded the analytical sample. The somewhat hygroscopic material analyzed best for a monohydrate.

Anal.—Calcd. for $C_{15}H_{17}N_3O_6 \cdot H_2O$: C, 51.0; H, 5.4; N, 11.9. Found: C, 51.65; H, 5.78; N, 11.82; OCH_3, none.

I.R. Spectra: $\lambda_{max.}$ at 3600, 3500 sh, 3300, 3160, 2730, 2440, 1717, 1660, 1580, 1535, 1493 sh, 1485, 1335, 1102, 1085, 780, 745 cm.$^{-1}$.

Preparation of $\beta(A)$



Two grams of porfiromycin were dissolved in a small amount of methanol and diluted to a liter volume with 0.01 *N* HCl. The pH of the solution (2.65) was lowered to 2.0 with sufficient 2 *N* HCl and was allowed to stand at room temperature for 20 minutes to allow time for completion of $\alpha_A \rightarrow \beta$(A) reaction. The pH of the solution was adjusted to 10.5 with 1.0 *N* NaOH, and saturated with anhydrous Na_2SO_4. A heavy precipitate appeared and was filtered through a fine glass frit under suction and the residue washed with acetone until most of the β(A) had been extracted. (I.R. analysis of the residue showed over 90% Na_2SO_4.) The acetone solution was taken to dryness by evaporation at room temperature. The filtrate from the original solution was resaturated with Na_2SO_4 and extracted once with acetone in a separator. Most of the color left the water phase after one extraction, so only one was deemed necessary. The acetone extract was combined with the acetone washings of the filter residue and taken to dryness by evaporation at room temperature. The β(A) precipitated out as a hard, glassy plate on the bottom of the evaporating dish and was broken up and bottled for use.

Anal.—Calcd. for $C_{15}H_{18}N_4O_5$: C, 53.9; H, 5.41. Found on correction for ash: C, 53.2, 54.2; H, 5.35, 5.68.

I.R. spectra $\lambda_{max.}$ at 3420, 3320, 3270 OH/NH; 1710, 1665 (weak) C=O; 1600, 1495 C=C/C=N/N—H deformation; and 1120, 1085, 1050 cm.$^{-1}$ C—O/C—N.

Preparation of $\gamma(A,A)$.—Further subjection of the porfiromycin to strong acid resulted in a material which precipitated at pH 5. This material was filtered off, washed, and considered γ(A,A). Its infrared and titration curves were the same as for γ(B,A).

RESULTS AND DISCUSSION

Effect of Buffers on the Degradation of Porfiromycin. $\alpha \rightarrow \beta$**(A).**—The rates of degradation of porfiromycin in acetate buffers were studied by the loss of absorbance at 363 mμ of initially 24–30 mcg./ml. solutions at 30°. The rate constants and conditions were listed as runs 1 through 5 in Table I.

Figure 2 is a plot of the rate constant *vs.* acetate ion concentration or acetic acid concentration for runs 2 through 5 maintained at constant pH, where $[C_2H_3O_2^-] = [HC_2H_3O_2]$, but where ionic strength was not maintained constant. The relationship is given by the equation

$$k = k_{HC_2H_3O_2}[HC_2H_3O_2] + k_0 \quad \text{(Eq. 1}a\text{)}$$

$$= 5.69 \times 10^{-5}[HC_2H_3O_2] + 2.75 \times 10^{-5} \quad \text{(Eq. 1}b\text{)}$$

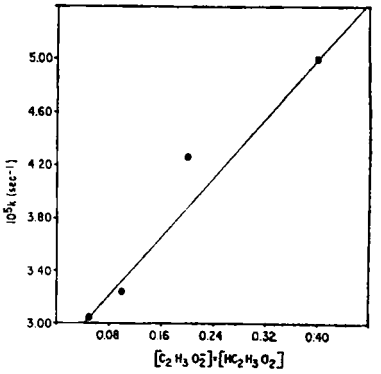


Fig. 2.—Dependence of rate of porfiromycin degradation at 30° and pH 4.54 on acetate ion and/or acetic acid concentration.

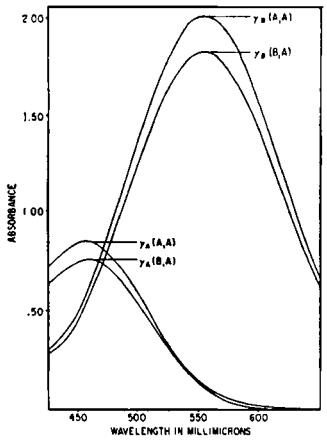


Fig. 3.—Visible spectra of γ(A,A) and γ(B,A) in acid (pH <3), subscript A, and base (pH >9), subscript B. The γ(B,A) was 0.5 mg./ml. The γ(A,A) was derived *in situ* from porfiromycin at 0.5 mg./ml.

at 30° and at pH 4.55, k in sec.$^{-1}$. Of course, [$k_{HC_2H_3O_2}$] is kinetically equivalent to $k_{C_2H_3O_2^-}$[$C_2H_3O_2^-$] so that whether rate acceleration by general base or general acid catalysis is due to acetate ions or undissociated acetic acid molecules, respectively, cannot be concluded on kinetic grounds. However, since the product of solvolysis in these acetate buffers can be spectrophotometrically identified as β(A) (9) which is associated with the opening of the fused aziridine ring of porfiromycin Iα (1, 2, 9) and since the aziridine ring is not attacked by even strong nucleophile hydroxyl ion (9), the most plausible catalytic species is the undissociated acetic acid and general acid catalysis is implicated in the transformation $\alpha \rightarrow \beta$(A).

If in Eq. 1, $k_0 = k_{H^+}$ [H^+] and [H^+] = 10^{-pH} = $10^{-4.55}$ = 2.8 × 10^{-5}, then k_{H^+} at 30° is 1.0 L./mole/sec. This is consistent with k(sec.$^{-1}$) at pH 3.60 at 30° of 2.84 × 10^{-4} (run No. 1, Table I) where [H^+] = 2.51 × 10^{-4}. Thus $k_{H^+} \sim 1$ L./mole/sec. is confirmed. This, of course, is the bimolecular rate constant for the hydrogen ion catalyzed solvolysis of undissociated porfiromycin, α, where $k[\alpha] = k_{H^+}[\alpha][H^+]$.

Run 6, Table I, in phosphate buffers has a $k_{5.9pH}$ = 1.58 × 10^{-6} sec.$^{-1}$ based on the loss of the 363 mμ band at 30°; the assumed route is $\alpha \rightarrow \beta$(A). Thus a calculated value where the rate constant depends solely on hydrogen ion catalyzed solvolysis could be $k = k_{H^+}[10^{-pH}] = k_{H^+}[H^+] = 1 \times 1.4 \times 10^{-6} = 1.4 \times 10^{-6}$ sec.$^{-1}$ which is reasonably consistent with the observed value. Similarly, for run 7, Table I, $k_{8.9pH}$ = 2.16 × 10^{-7} at 30°. The calculated rate constant would be $k = k_{H^+}[10^{-pH}] = 1.4 \times 10^{-7}$.

Although in both of these cases the k(sec.$^{-1}$) values calculated on the basis of hydrogen ion attack on uncharged porfiromycin are of the proper order of magnitude as the observed value, it is indicated that the observed value exceeds the calculated. This could be due to two possible phenomena, the possible catalytic effect of $H_2PO_4^-$ ion and the superimposition of an additional degradation sequence, *i.e.*, $\alpha \rightarrow \beta$(B) $\rightarrow \gamma$(B,B) which is enhanced with the higher pH and which gives an apparent faster change in the spectra used to evaluate rate constants (9).

A plot of log k (k in sec.$^{-1}$) *vs.* pH for studies in phosphate buffers at 60° for runs 8, 9, and 10 (see Table I) is not truly linear with a slope of unity. The slope of unity would be expected if $k = k_{H^+}[H^+]$ and log $k = -pH + \log k_{H^+}$. Neither is the plot of k *vs.* [$H_2PO_4^-$] completely linear. Thus, since porfiromycin has no titratable groups in this region it must be concluded that porfiromycin degradation in the intermediate pH ranges, pH 5.9 to 8, is catalyzed by hydrogen ion [$\alpha \xrightarrow{H^+} \beta$(A)], may be catalyzed by $H_2PO_4^-$ concentration and may be catalyzed by hydroxyl ion [probably $\alpha \xrightarrow{OH^-} \beta$(B) $\xrightarrow{OH^-} \gamma$(B,B)] (9).

The bimolecular rate constant for the hydroxyl ion catalyzed solvolysis of porfiromycin to β(B) can be estimated from the rate of loss of biological assay (run 20, Table I) as k_{OH^-} = 8.2 × 10^{-3} L./mole/sec. at 30°.

Prediction of Stability.—Estimates of the stability of porfiromycin, α, in unbuffered aqueous solution at 30° may be based on the expression.

$$k(\text{sec.}^{-1}) = 1.0\,[H^+] + 8.2 \times 10^{-3}[OH^-] \quad \text{(Eq. 2}a\text{)}$$

$$= 1.0 \times 10^{-pH} + 8.2 \times 10^{-3}\,10^{-(pK_w - pH)} \quad \text{(Eq. 2}b\text{)}$$

where the first quantity on the right in Eq. 2 estimates the apparent first-order transformation, $\alpha \rightarrow \beta$(A), and the second quantity on the right estimates the apparent first-order transformation, $\alpha \rightarrow \beta$(B). Approximate values for the half-life of porfiromycin are: 0.2 hours at pH 3, 2 hours at pH 4, 20 hours at pH 5, 200 hours at pH 7, 11,000 hours at pH 8, 2300 hours at pH 9, 250 hours at pH 10, 25 hours at pH 11, 2.5 hours at pH 12, and 0.25 hours in 0.1 *M* NaOH. The maximum stability of porfiromycin at 30° is *ca.* pH 8 with an estimated half-life of 1.3 years.

The estimated half-life at pH 3 of 0.21 hours indicates that porfiromycin should be significantly degraded in the stomach on oral ingestion.

Visible Spectra of Porfiromycin and Its Acid and Alkaline Degradation Products.—The similarities of (B,A) and (A,A) have already been pointed out as based on ultraviolet spectroscopy and spectrophotometric and potentiometric titrations (9). The coincidence of these two compounds is very clearly demonstrated by the visible spectra in Fig. 3.

The similarities of γ(A,B), δ(B,A,B), and δ(A,A,B) have also been pointed out as based on ultraviolet spectroscopy (9). The similar shift in visible spectra from acid to basic conditions (see Table II)

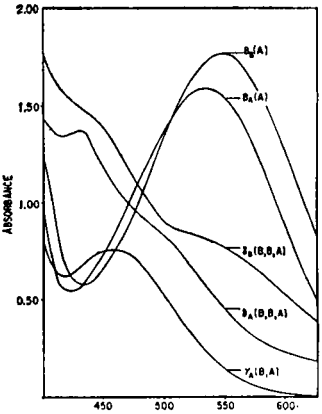


Fig. 4.—Visible spectra of β(A), δ(B,B,A), and γ(B,A) in acid (pH <3), subscript A, and base (pH >8), subscript B. The γ(B,A) was 0.5 mg./ml. The β(A) was derived *in situ* from porfiromycin at 0.5 mg./ml. The δ(B,B,A) was derived *in situ* from β(B), triethylamine salt at 0.5 mg./ml.

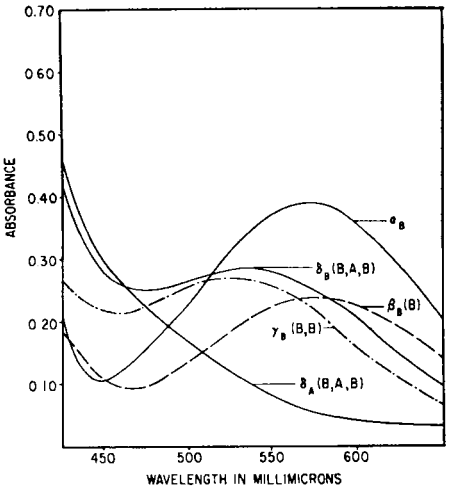


Fig. 5.—Visible spectra of porfiromycin, α, β(B), γ(B,B), and δ(B,A,B) in acid, subscript A, and basic solutions, subscript B. The δ(B,A,B) was derived *in situ* from γ(B,A) at 6.7 mg./ml. The porfiromycin and β(B) triethylamine salt were at 0.5 mg./ml. and the γ(B,B) was derived *in situ* from β(B) triethylamine salt.

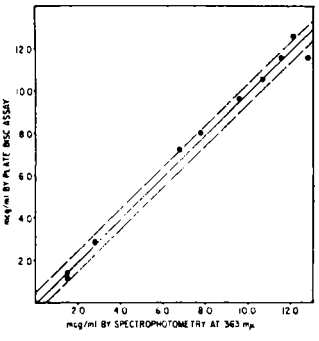


Fig. 6.—Correlation of bioassay, plate disk *vs. S. aureus*, and spectrophotometric assay at 363 mμ for porfiromycin degrading at 30° in 0.05 *M* acetic acid–0.05 *M* sodium acetate. Key: — — —, standard deviation in bioassay; ———, regression fitted by least squares.

tends to confirm these similarities. Several anomalies do exist although some explanation may be made on the fact that the apparent absorptivities are calculated on the basis of the weight of the starting materials, which materials as stated above, differed in the several cases. The δ(B,A,B) has 0.37 the absorbance at the maxima of γ(A,B) in the acid and alkaline regions. However, the ratios are maintained under both conditions as are the qualitative spectral shifts. Typical examples of the visible spectra of these products of porfiromycin solvolysis are given in Figs. 4 and 5.

Correlation of Spectral and Biological Assays in Acetate Buffers.—The undegraded porfiromycin, α (runs 2–5, Table I), after adjustment to pH 7 was calculated on the basis of the following equations:

$$A_t = a_\alpha[\alpha] + a_\beta[\beta] \qquad \text{(Eq. 3)}$$

where A_t is the absorbance at any time and a_α and $a\beta$ are the absorptivity of porfiromycin, α, and of β(A), respectively, at 363 mμ. The initial absorbance at $t = 0$, is $A_0 = a_\alpha[\alpha]_0$; the final absorbance at $t = \infty$ is $A_\infty = a_\beta[\beta]$.

Thus, the concentration of α, *i.e.*, $[\alpha]$, can be calculated from

$$[\alpha] = \frac{A_t - A_\infty}{A_0 - A_\infty}[\alpha]_0 \qquad \text{(Eq. 4)}$$

where

$$[\alpha]_0 = [\beta]_\infty = [\alpha] + [\beta] \qquad \text{(Eq. 5)}$$

Aliquots of the same acetic acid-acetate buffered solution which were taken at the same intervals and neutralized 1:1 with 0.1 *M* phosphate buffer, pH 7.8, were assayed by the plate-disk method against *S. aureus* (7).

A typical example of the regression of the microbiological plate-disk assay against the estimates obtained by spectrophotometry is given in Fig. 6. The line of best fit by least squares is the solid line whereas the dashed lines represent the plus or minus standard deviations and are estimates of the error in the biological assay.

The statistics of the regression are given in Table III. The average standard deviation of the assay is 10%. Although two out of the four runs are consistent with a 1:1 relation of the assays, *i.e.*, the regression slope is approximately unity and the intercept of regression approximately zero, two show differences from these values. Overall, however, it can be concluded that the spectrophotometric assay is a valid measure of porfiromycin's biological activity in its transformation to β(A) in acetate buffers at pH 4.5.

Correlation of Spectral and Biological Assays in Phosphate Buffers.—The spectrophotometric assay was performed on porfiromycin degrading in phosphate buffered solutions at 70°. These were the

TABLE III.—STATISTICS OF REGRESSION ($y = mx + b$) OF BIOASSAYS[a] (y) ON SPECTROPHOTOMETRIC ASSAYS[b] (x) IN MCG./ML. FOR PORFIROMYCIN IN VARYING ACETATE CONCENTRATION BUFFERS (pH 4.5) AT 30° C. (RUNS 2–5, TABLE I)

Buffer Composition [CH_3COOH]	Buffer Composition [CH_3COO^-]	pH	Run[c]	n[d]	Least Squares Fit	σ_b[e]	σ_m[f]	σ_y[g]	Remarks
0.05	0.05	4.53	2	11	$y = 0.979X + 0.062$	0.262	0.031	0.471	The slope, m, is not significantly different from 1.0; intercept, b, not significantly different than 0.
0.10	0.10	4.54	3	8	$y = 1.332X - 1.372$	0.658	0.076	0.797	Both slope and intercept are significantly different from theoretical values 1.0 and 0.0.
0.20	0.20	4.51	4	7	$y = 1.015X + 0.762$	0.640	0.068	0.503	Slope and intercept not significantly different from theoretical.
0.40	0.40	4.55	5	8	$y = 1.234X - 1.222$	0.496	0.059	0.714	Slope and intercept are significantly different from theoretical.

[a] Plate disk against *S. aureus*. [b] As estimated from the 353 mμ absorbance. [c] From Table I. [d] Number of assays. [e] Standard deviation of intercept, b. [f] Standard deviation of slope, m. [g] Standard deviation about regression, presumably of bioassay, y.

TABLE IV.—STATISTICS OF REGRESSION ($y = mx + b$) OF BIOASSAYS[a] (y) ON SPECTROPHOTOMETRIC ASSAYS[b] (x) IN MCG./ML. FOR PORFIROMYCIN IN VARYING PHOSPHATE CONCENTRATION BUFFERS AT 70° (RUNS 11 AND 12, TABLE I)

Buffer Composition of Solution in 70° Bath		Buffer Composition of Aliquot Submitted for Bioassay							
(KH_2PO_4)	(NaOH)	(KH_2PO_4)	(NaOH)	pH	n[c]	Least Squares Fit	σ_b[d]	σ_m[e]	σ_y[f]
0.050	0.0296	0.0500	0.0296	6.93	12	$y = 0.741X + 0.632$	1.11	0.119	1.55
0.050	0.0296	0.0375	0.0222	6.93	12	$y = 0.819X + 0.151$	1.19	0.127	1.66
0.025	0.0148	0.0250	0.0148	6.97	12	$y = 0.660X + 1.658$	0.997	0.105	1.25
0.025	0.0148	0.0125	0.0074	6.97	12	$y = 0.598X + 2.101$	1.43	0.151	1.80

[a] Plate disk against *S. aureus*. [b] As estimated from the 353 mμ absorbance. [c] Number of assays. [d] Standard deviation of intercept, b. [e] Standard deviation of slope, m. [f] Standard deviation about regression, presumably of bioassay, y.

TABLE V.—STATISTICS OF REGRESSION ($y = mx + b$) OF PAPERGRAMS (y) ON PLATE-DISK ASSAYS[a] (x) IN MCG./ML. FOR PORFIROMYCIN IN pH 9 AND pH 10 BORATE BUFFERS AT 60° (RUNS 14 AND 17, TABLE I)

Buffer Composition		Type of Papergram Analysis	pH of Aliquots Submitted for Papergrams	n[b]	Least Squares Fit	σ_b[c]	σ_m[d]	σ_y[e]	Run[b,f]
[H_3BO_3]	[NaOH]								
0.05	0.0439	U.V. paper strip	*ca.* 7	8	$y = 2.499X - 182.3$	37.2	0.106	44.5	17, 18, 19
0.05	0.0439	*S. lutea*	*ca.* 7	8	$y = 1.326X + 40.08$	29.1	0.083	34.8	17, 18, 19
0.05	0.0213	*S. lutea*	7.85	12	$y = 1.572X - 169.3$	75.4	0.190	88.2	14, 15
0.05	0.0213	U.V. paper strip	7.85	13	$y = 1.335X - 67.7$	36.6	0.0960	51.6	14, 15
0.05	0.0213	*S. lutea*	8.95	4	$y = 2.192X - 328.1$	167.0	0.386	84.0	14, 15
0.05	0.0213	U.V. paper strip	8.95	5	$y = 1.640X - 91.5$	93.7	0.241	88.1	14, 15

[a] Against *S. aureus*. [b] Number of assays. [c] Standard deviation of intercept, b. [d] Standard deviation of slope, m. [e] Standard deviation about regression. [f] See Table I.

same runs as 11 and 12 of Table I. Simultaneously with the removal and treatment of aliquots for spectrophotometric assay, two aliquots were removed for biological assay under two different phosphate buffer concentrations. Variation of phosphate buffer from 0.0125 to 0.05 *M* had no apparent effect on the plate-disk assay against *S. aureus*. The statistics of the regressions of bioassay *vs.* spectrophotometric assay are given in Table IV. Although the intercepts of all four regressions are not significantly different from zero, the slopes are significantly different from and are less than unity. This implies that destruction of spectrophotometric absorbance is correlated with destruction of activity but that absorbance may register a higher value than activity. This is readily explainable if it is assumed that at pH 7 two alternate degradation pathways exist:

$$\beta(B) \leftarrow \alpha \rightarrow \beta(A) \qquad \text{(Eq. 6)}$$

However, $\beta(B)$ has a very similar spectrum to α and thus destruction of the measured biological activity may proceed slightly faster than the diminution of the absorbance.

Correlation of Spectral and Biological Assays by Paper Chromatogram of Porfiromycin in Borate Buffers.—Studies were conducted in borate buffers at 60° and at pH values 9 and 10. The aliquots of the pH 10 study were adjusted to pH 7 and submitted for papergram assay by both ultraviolet scanning and against *S. lutea* (4). In both cases, the correlation of papergram assay with plate-disk (see Table V) was good.

The aliquots of the pH 9 study were adjusted to pH 8 and 9 and both adjusted samples were submitted for papergram assay by ultraviolet and against *S. lutea* (4). Again the correlations of papergram assay with plate-disk assay (7) (see Table V) were good.

However, in almost all cases the papergram assay decreased at a greater rate with time than did the paper-disk assay until at low assay values the former demonstrated no porfiromycin whereas the latter did. This can be explained (*a*) by a negative bias in paper chromatography assay at low concentrations or (*b*) by the fact that the papergram is more specific than the plate-disk assay, that porfiromycin degrades to intermediates which still exhibit plate-disk potency but not papergram activity or ultraviolet absorbance at the spot assigned to porfiromycin.

Both $\beta(B)$ and $\gamma(B,B)$ prepared *in situ* were submitted for bioactivity on papergrams and were shown to have biological activity.

Consistency of Isolated Intermediates with Proposed Scheme for the Solvolytic Transformations of Porfiromycin.—The isolations of $\beta(B)$, $\gamma(B,A)$, $\beta(A)$, and $\gamma(A,A)$ were based on the observed kinetics reported previously (9) and in this paper. The elemental analysis and physicochemical characterizations of these isolates are consistent with the reported structural assignments given in the introduction.

REFERENCES

(1) Webb, J. S., Cosulich, D. B., Mowat, J. H., Patrick, J. B., Broschard, R. W., Meyer, W. E., Williams, R. P., Wolf, C. F., Fulmor, W., Pidacks, C., and Lancaster, J. E., *J. Am. Chem. Soc.*, 84, 3185(1962).
(2) *Ibid.*, 84, 3187(1962).
(3) Tulinsky, A., *ibid.*, 84, 3188(1962).
(4) De Boer, C., Dietz, A., Lummis, N. E., and Savage, G., in Gray, P., Tabenkin, B. and Bradley, S. G., "Antimicrobial Agents Annual 1960," Plenum Press, New York, N. Y., 1961, pp. 17–22.
(5) Herr, R. R., Bergy, M. E., Eble, T. E., and Jahnke, H. K., *ibid.*, pp. 23–26.
(6) Lewis, C., Clapp, H. W., Rhuland, L. E., and Reames, H. R., *ibid.*, pp. 27–36.
(7) Hanka, L. J., *ibid.*, pp. 37–39.
(8) Evans, J. S., Musser, E. A., and Gray, J., *Antibiot. Chemotherapy*, 11, 445(1961).
(9) Garrett, E. R., *J. Med. Chem.*, 6, 488(1963).