The Physical Chemical Characterization of the Products, Equilibria, and Kinetics of the Complex Transformations of the Antibiotic Porfiromycin

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A physicochemical characterization of the solution transformations of porfiromycin has been completed. Twelve different specific products of acidic and alkaline solution conditions have been identified spectrophotometrically, the kinetics of their transformation quantified, and many of their dissociation constants and apparent equivalent weights determined. Conditions have been established for optimum yields of the discrete products for isolation. Differences in the biological activities of the degradation products have been observed. In light of recent structural assignments for porfiromycin,[14] the postulated fused ring aziridine[14] appears mandatory for biological activity. This structure in porfiromycin resists alkaline attack and maintains biological activity through various alkali-induced structure modifications. However, this group is highly susceptible to acid-catalyzed solvolysis with a concomitant loss of biological activity.

The studies on the stability of the new broad spectrum antibiotic, porfiromycin,[2] in solution were initially designed on the patterns for the other antibiotics studied in this laboratory: fumagillin,[3] streptovaricin,[4] filipin,[5] psicofuranine,[6] streptozotocin,[7] and actinospectacin.[8] The purposes were to determine the stability of porfiromycin as a function of pH and buffer, compare physicochemical and biological assay procedures, and to predict the possible nature of the solution degradation and the conditions for maximum stability.

Fortunately, porfiromycin is intensely colored in solution and has an elegant ultraviolet spectrum[2b] (Fig. 1) which preliminary studies had shown to be affected by acidic and basic conditions.[2b] These spectral changes provide an optimum means of study when changes in structures of molecules of unknown structure must be known to establish the pharmaceutically necessary conditions for formulation and stabilization.[4]

However, this study of porfiromycin became more unique and extensive than anticipated. The spectra changed according to the classical kinetic laws to new spectra, which changed again to others in finite discrete steps. The acid degradation sequences gave different spectrophotometrically characterized intermediates in solution than the alkaline degradation sequences and alternation of treatments introduced even further variations.

The spectra of solutions varied with pH and permitted pK_a assignments to intermediates. A complicated and elegant scheme of transformations as a function of pH could be constructed where quantitative conditions could be devised for isolation of discrete products with maximum yield.

Some materials obtained as discrete solution degradation products were paper-chromatographed *in situ*, their homogeneities were established as different from porfiromycin, and they were observed to have differing biological activities.

Isolation,[9] based on the observed kinetics, yielded new compounds that in several instances had uniquely modified biological activity with respect to the parent compound, a phenomenon as fascinating to the microbiologist as to the physical chemist. The kinetic treatment of the rates of spectral transformations as a function of pH and acid and base concentrations permitted assignment of functional groups to the discrete products of the degradations, even prior to analytical identification of these groups.

Identification of the similarity of products obtained by different degradative routes was possible from the observed coincidence of spectra and pK_a characterization and was considered confirmed by the coincidence of further rates of degradation and their dependence on acid and base concentrations.

In addition to stability evaluation, this particular study most clearly demonstrates the efficacy of the kinetic approach in modifying chemotherapeutic agents by degradative procedures, in providing information for structure determination, and in delineating specific details for optimum yield of discrete degradation products.

Porfiromycin is active *in vitro* and *in vivo* against a variety of Gram-positive and Gram-negative bacteria and mammalian tumor cells.[2] Analytical results on crystalline material indicate that the antibiotic is a neutral substance, slightly soluble in water, moderately soluble in polar organic solvents, essentially insoluble in hydrocarbon solvents, and best fits the empirical formula $C_{16}H_{20}N_4O_5$, molecular weight 348.[2]

In this paper the products of porfiromycin degradation were 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 or A in the

(1) (a) College of Pharmacy, University of Florida, Gainesville, Florida; (b) Presented in part at the Medicinal Chemistry Section, National Meeting of the American Chemical Society, Los Angeles, California, April, 1963.

(2) (a) C. De Boer, A. Dietz, N. E. Lummis, and G. E. Savage, *Antimicrobial Agents Ann.*, 17 (1960); (b) R. R. Herr, M. E. Bergy, T. E. Eble, and H. K. Jahnke, *ibid.*, 23; (c) C. Lewis, H. W. Clapp, L. E. Rhuland, and H. R. Reanies, *ibid.*, 27; (d) L. J. Hanka, *ibid.*, 37; (e) J. S. Evans, E. A. Musser, and J. Gray, *Antibiot. Chemotherapy*, **11**, 445 (1961).

(3) (a) E. R. Garrett and T. E. Eble, *J. Pharm. Sci.*, **43**, 385 (1954); (b) T. E. Eble and E. R. Garrett, *ibid.*, **43**, 536 (1954); (c) E. R. Garrett, *ibid.*, **43**, 539 (1954).

(4) E. R. Garrett, *ibid.*, **48**, 169 (1959).

(5) J. E. Tingstad and E. R. Garrett, *ibid.*, **49**, 352 (1960).

(6) (a) E. R. Garrett and L. J. Hanka, *ibid.*, **49**, 526 (1960); (b) E. R. Garrett, *J. Am. Chem. Soc.*, **82**, 827 (1960).

(7) E. R. Garrett, *J. Pharm. Sci.*, **49**, 767 (1960).

(8) E. R. Garrett and G. R. Umbreit, *ibid.*, **51**, 436 (1962).

(9) W. Schroeder, to be published.

TABLE I

RATE CONSTANTS FOR THE ACID HYDROLYSIS OF PORFIROMYCIN[a]

$[\alpha_A \xrightarrow{H^+} \beta_A(A) \xrightarrow{H^+} \gamma_A(A,A)]$ AS A FUNCTION OF HYDROCHLORIC ACID MOLARITY AT 30°

Run	[HCl]	Temp., °C.	10^5k (sec.$^{-1}$) for $\alpha \rightarrow \beta$	10^5k (sec.$^{-1}$) for $\beta \rightarrow \gamma$	pH Obsd.[b]	pH Calcd.[c]
22	0.005	29.5	1.95	1.51	2.35	2.33
23	.005	28.6	2.28		2.27	2.33
24	.010	29.2	2.73	2.95	2.03	2.04
25	.010	29.5	2.49	2.98	2.01	2.04
26	.015	28.6	3.35		1.85	1.88
27	.020	28.6	3.43		1.74	1.76
28	.030	29.2	3.48	6.87	1.63	1.59
29	.040	29.2	3.83	8.21	1.46	1.47
30	.050	29.5	3.56	13.8	1.45	1.38
31	.100	29.5	4.03	25.3	1.13	0.99

[a] Concentration *ca.* 15 γ/ml. [b] Averaged from pH values of aliquots. [c] Calculated pH values are determined from the mean activity coefficients, f_{HCl}, of HCl in water at 30° by pH = $-\log f$[HCl].[11a]

TABLE II

RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS OF β(A)[a] AT 30.0° AS A FUNCTION OF THE SODIUM HYDROXIDE

CONCENTRATION, $\beta_B(A) \xrightarrow{OH^-} \gamma_B(A,B)$

Run	[NaOH]	10^5k (sec.$^{-1}$)	Calcd. pH
32	0.05	1.04	12.44
33	.10	2.13	12.72
34	.20	3.56	12.99
35	.40	4.95	13.28
36	.75	6.43	13.54
37	.95	8.03	13.64

[a] Concentration 7.65 γ/ml. as based on the original porfiromycin concentration. [b] Calculated from pH = pK_w − pOH; where pK_w = 13.83 at 30°, pOH = log f[NaOH], [NaOH] is the experimental, and f is the mean activity coefficient for NaOH at 30°.[11b]

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, first, acid, *i.e.*, β(A); then, second, basic γ(A,B); and a final degradation in basic solution, δ(A,B,B).

Experimental

The isolation and characterization of the porfiromycin used in these studies has been reported by Herr, *et. al.*[2b]

Kinetic Studies.—For the studies on the acid hydrolysis, porfiromycin (α) was weighed into tared volumetric flasks, dissolved in a few drops of methanol, and diluted to volume with H_2O so the resultant concentration was approximately 30 γ/ml. This solution was then diluted 1:1 with varying concentrations of HCl and the resultant molarities of HCl are recorded in Table I. The porfiromycin in acid solution (α_A) was transferred immediately to stoppered cells and the decrease at the 363 mμ maximum ($\alpha_A \rightarrow \beta_A$) was followed on the Beckman DU spectrophotometer using the temperature control device to maintain the solutions at *ca.* 30°. The transformation of $\beta_A(A) \rightarrow \gamma(A,A)$ was followed by the loss of absorbance at the 310 mμ maximum, and the rate constants for varying molarities of HCl are recorded in Table I.

When the $\alpha_A \rightarrow \beta_A(A)$ reaction was complete for the fourth run of the Table I part of the solution was diluted 1:1 with varying molarities of NaOH and the rates of $\beta_B(A) \rightarrow \gamma(A,B)$ were calculated from the loss of absorbance at the 255 mμ maximum. The molarities of NaOH for the resultant solutions are recorded in Table II as are the rate constants.

When the $\alpha_A \rightarrow \beta(A) \rightarrow \gamma(A,A)$ reaction was complete (30 γ/mL of porfiromycin at 30° in 0.100 M HCl for 17 hr.), aliquots of the solution were diluted 1:1 with varying molarities of NaOH and the rates of $\gamma_B(A,A) \rightarrow \delta(A,A,B)$ were calculated from the loss of absorbance at the 255 mμ maximum. The molarities of NaOH for the resultant solution are recorded in Table III as are the rate constants.

The experimental procedures for the study of the alkaline-induced transformations of porfiromycin (α) and their further acidic and alkaline degradations were similar. However, as some of the intermediates, *viz.*, β(B) (triethylamine salt) and the γ(B,A), became available on isolation and purification,[9] these compounds were used to complete the degradation sequence rather than the intermediates derived from the porfiromycin *in situ*. The molarities of NaOH and HCl and the observed rate constants are recorded in Tables IV, V, and VI.

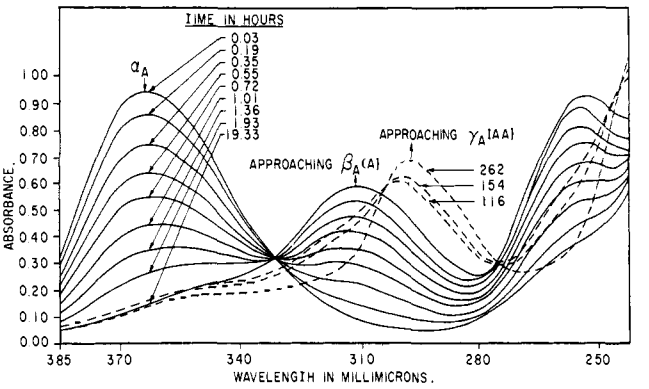


Fig. 1.—Typical curves of the spectral changes during the acid transformation of porfiromycin. The solution was at 30°, pH, 3.6, 0.2 M acetate buffer with an initial concentration of porfiromycin of 30 γ/ml. The spectra were run on concentrations of 15 γ/ml. after 1:1 dilution with the same acetate buffer. The solid curves represent the spectral transformation $\alpha_A \rightarrow \beta_A(A)$. The dashed curves represent the spectral transformation $\beta_A(A) \rightarrow \gamma_A(A,A)$. Each curve is labeled as to the number of hr. after the start of degradation.

TABLE III

RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS OF γ(A,A)[a] AT 30° AS A FUNCTION OF THE SODIUM HYDROXIDE

CONCENTRATION, $\gamma_B(A,A) \xrightarrow{OH^-} \delta(A,A,B)$

Run	[NaOH]	10^5k (sec.$^{-1}$)	Calcd. pH[b]
38	0.10	2.43	12.72
39	.20	3.83	12.99
40	.40	5.72	13.28
41	.70	7.34	13.51

[a] Concentration 14 γ/ml. as based on the original porfiromycin concentration. [b] Calculated from pH = pK_w − pOH; where pK_w = 13.83 at 30°, pOH = $-\log f$[NaOH], [NaOH] is the experimental molarity, and f is the mean activity coefficient for NaOH at 30°.[11b]

TABLE IV

RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS OF THE TRIETHYLAMINE SALT OF β(B)[a] AT 30.1° AS A FUNCTION OF THE SODIUM HYDROXIDE CONCENTRATION,

$\beta_B(B) \xrightarrow{OH^-} \gamma_B(B,B) \xrightarrow{OH^-} \delta(B,B,B)$

Run	[NaOH]	Calcd. pH[b]	10^5k (sec.$^{-1}$) $\beta_B(B) \xrightarrow{OH^-} \gamma_B(B,B)$	10^5k (sec.$^{-1}$) $\gamma_B(B,B) \xrightarrow{OH^-} \delta(B,B,B)$
42	0.10	12.72	2.99	0.513
43	.20	12.99	6.64	.601
44	.40	13.28	14.9	.899
45	.60	13.45	25.4	1.05
46	1.00	13.67	57.8	1.75

[a] Studies conducted with 15 γ/ml. of triethylamine salt of β(B).[14] [b] Calculated from pH = pK_w − pOH; where pK_w = 13.83 at 30° and pOH = $-\log f$[NaOH], and [NaOH] is the experimental, and f the mean activity coefficient for NaOH at 30°.[11b]

TABLE V

RATE CONSTANTS FOR THE ACID HYDROLYSIS OF THE TRIMETHYLAMINE SALT OF β(B)[a] AT 29.5° AS A FUNCTION OF THE HYDROCHLORIC ACID CONCENTRATION, $\beta_A(B) \xrightarrow{H^+} \gamma(B,A)$

Run	[HCl]	Calcd. pH[b]	10^5k (sec.$^{-1}$) Exptl.	10^5k (sec.$^{-1}$) Calcd.[c]
47	0.005	2.33	2.05	2.10
48	.01	2.04	3.19	3.09
49	.02	1.76	3.72	3.70
50	.04	1.47	4.31	4.07
51	.08	1.19	4.56	4.47
52	.20	0.82	4.58	4.92

[a] Studies conducted with 14 γ/ml. of triethylamine salt of β(B). [b] Calcd. pH values are determined from the mean activity coefficients, f_{HCl}, of HCl in water at 30° by pH = $-\log f$[HCl].[11a] [c] Calcd. from $k = k_{H^+}$[HCl] $\times \alpha'$ where $k_{H^+} = 1.20$ l./mole/sec., [HCl] is as given and α' is determined from pH = pK_a' + log $\alpha'/(1 - \alpha')$ where pK_a' = 2.5.

TABLE VI

RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS OF γ(B,A)[a] AT 30° AS A FUNCTION OF THE SODIUM HYDROXIDE CONCENTRATION, $\gamma_B(B,A) \xrightarrow{OH^-} \delta(B,A,B)$

Run	[NaOH]	Calcd. pH[b]	10^5k (sec.$^{-1}$)
53	0.10	12.72	2.35
54	.20	12.99	3.23
55	.40	13.28	5.62
56	.70	13.51	8.20
57	1.00	13.67	9.53

[a] Studies conducted with 15 γ/ml. of γ(B,A). [b] Calculated from pH = pK_w − pOH; where pK_w = 13.83 at 30° and pOH = $-\log f$[NaOH], and [NaOH] is the experimental, and the mean activity coefficient for NaOH at 30°.[11b]

Potentiometric and spectrophotometric titrations were performed with the use of glass-calomel electrodes. The pK_a values from the former were estimated by the pH of half-neutralization. The estimation of the pK_a values by the latter method is discussed in more detail in the section on calculations.

Biological Activity of Porfiromycin and its Degradation Products.—Porfiromycin is active *in vitro* and *in vivo* against a variety of both Gram-negative and Gram-positive bacteria and mammalian tumor cells.[2a] It has a high degree of toxicity.[2e] On the basis of papergram bioautographs against *Sarcina lutea*, β(B) and γ(B,B) prepared and evaluated *in situ* still possess biological activity. The isolated triethylamine salt of β(B)[9] preserved the Gram-negative activity but drastically reduced the Gram-positive by 10 times from the original porfiromycin with no significant change in toxicity.[10] However, antitumor cell activity was reduced. The γ(B,B) evaluated *in situ* retained biological activity. The acid degraded porfiromycin products, *e.g.*, β(A) and γ(A,A) = γ(B,A) showed little biological activity.[10]

Additional experimental data and physicochemical characterizations are given in the next section.

Calculations and Results. Initial Acid Degradation of Porfiromycin. Porfiromycin has an ultraviolet spectrum which is not modified with pH except as a function of time. The ultraviolet spectrum of porfiromycin has a maximum at 363 mμ with an apparent absorptivity a = 65.6, absorbance per g. of porfiromycin per l. The absorbance at 365 mμ maximum decreases under neutral or acidic conditions with new maxima appearing at 311 (a = 39.4) and 250 mμ (a = 61.6) at first. This compound, the first acid degradation product of α_A (porfiromycin in acidic solution), is referred to as β_A(A), *i.e.*, in acid solution (as per subscript) and from acid degradation (as represented by the parenthesized letter). Typical curves of the transformations of ultraviolet spectra for the $\alpha_A \rightarrow \beta_A$(A) are given in Fig. 1.

A further acidic degradation occurs as is represented by the dashed lines in Fig. 1. The maxima at 311 and 250 mμ for β_A(A) decrease and new maxima appear at 295 (a = 55.2) and 238 mμ (a = 71.3) for λ_A(A,A).

(10) C. DeBoer, personal communication.

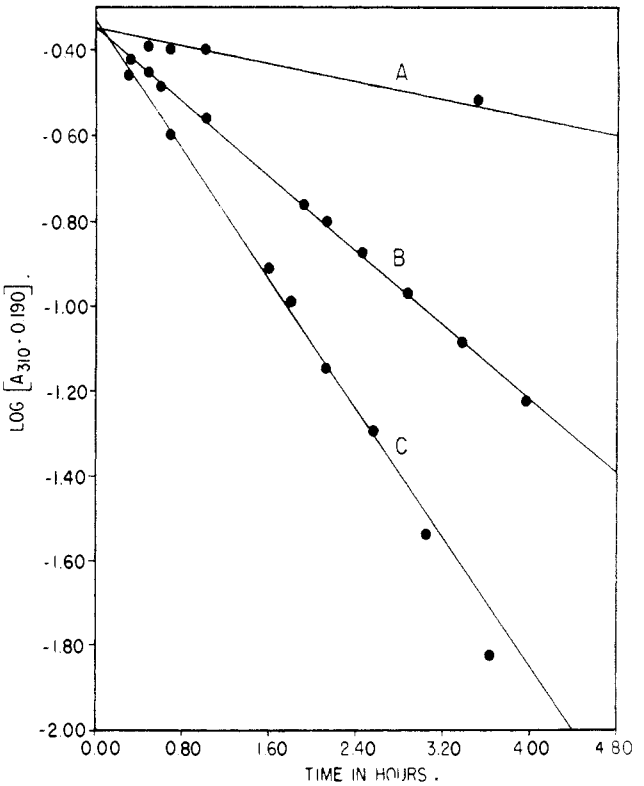


Fig. 2.—Typical apparent first order plots for the transformation of $\beta_A(A) \rightarrow \gamma_A(A,A)$ in acid at 30° as measured by the loss of the 310 mμ chromophore. The initial concentration of porfiromycin was 15 γ/ml. The HCl for the various curves is: 0.010 M, A; 0.050 M, B; and 0.100 M, C.

The acid transformation $\alpha_A \rightarrow \beta_A$(A) is pseudo first order. The apparent first order rate constants and the conditions under which they were obtained are given in Table I. Similarly, the acid transformation $\beta_A(A) \rightarrow \gamma_A(A,A)$ is pseudo first order and typical plots based on the disappearance of the β_A(A) absorbances at the 310 mμ maximum are given in Fig. 2. These curves are characteristic of all the first order plots of changes in spectrophotometric absorbance. The apparent first order rate constants and the conditions under which they were obtained are also given in Table I.

The expression for the estimation of the rate constant k (in sec.$^{-1}$) is

$$\log (A - A_\infty) = -kt/2.303 + \text{constant} \quad (1)$$

where A is the observed absorbance at time, t, and A_∞ is the asymptotic absorbance.

The rate constant, k, can be plotted as a function of hydrogen ion molarity for both transformations. An alternative procedure is to plot log k *vs.* pH as in Fig. 3 and the following empirical relations can be derived

For $\alpha_A \rightarrow \beta_A$(A) at 30°

$$k = 0.168[HCl] + 1.00 \times 10^{-3};\ 0.005 < [HCl] < 0.014 \quad (2)$$

$$k = 7.5 \times 10^{-3}[HCl] + 3.27 \times 10^{-3};\ 0.014 < [HCl] < 0.100 \quad (3)$$

$$\log k = -0.100\text{pH} - 2.294,\ 1.0 < \text{pH} < 2.1 \quad (4)$$

where pH = $-\log f$ [HCl] and f is the mean activity coefficient for HCl in water.[11a]

For $\beta_A \rightarrow \gamma_A$ (A, A) at 30°

$$k = 2.57 \times 10^{-3}[HCl] \quad (5)$$

$$\log k = -\text{pH} - 2.54 \quad (6)$$

where pH = $-\log f$ [HCl] and log k *vs.* pH with a slope of nega-

(11) (a) H. W. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd Ed., Reinhold Publishing Co., New York, N. Y., 1958, p. 716; (b) *ibid.*, p. 729.

tive unity for the latter plot clearly shows that the rate of $\beta \rightarrow \gamma$ is first order in both β and hydrogen ion concentration.

This is not so with the α to β acid transformation; the apparent hydrogen ion dependence varies as a function of the hydrogen ion concentration. Thus, the most rational mechanism is that the hydrogen ion catalysis of the protonated porfiromycin, $\alpha_A - H^+$, has a smaller specific rate constant than that on the nonprotonated as

$$k[\alpha_A] = k_1[H^+][\alpha_A] + k_2[H^+][\alpha_A - H^+] \quad (7)$$

where

$$k_1 > k_2 \text{ and } \alpha_A + H^+ \overset{K_a}{\rightleftharpoons} \alpha_A - H^+ \quad (8)$$

A full discussion of the derivation and use of equations for rates as a function of hydrogen ions (or hydroxyl ions) and the dissociation of a charged species is given in the literature.[6b,7,12]

In general, if the rate of total concentration change of a species, *e. g.*

$$d[\alpha_A]_T/dt = d([\alpha_A] + [\alpha_A - H^+])/dt \quad (9a)$$

$$= -k_1[H^+][\alpha_A] - k_2[H^+][\alpha_A - H^+] \quad (9b)$$

$$= -k_{H^+}[H^+]\{[\alpha_A] + [\alpha - H^+]\} \quad (9c)$$

$$= -k_{H^+}[H^+][\alpha_A]_T = -k[\alpha_A]_T \quad (9d)$$

where eq. 7 was deduced.

It can be shown[6b,7,12] that

$$k/[H^+] = k_{H^+} = k_1/(1 + [H^+]/K_a) + k_2/(1 + K_a/[H^+]) \quad (9e)$$

so that the apparent first order rate constants, k, at any $[H^+]$ can be calculated as functions of the derived bimolecular rate constants k_1, k_2; of the dissociation constant K_a; and of the hydrogen ion concentration $[H^+]$ where the latter is used in this paper either as $f[HCl]$ or 10^{-pH}.

The pK_a of the protonated group in porfiromycin may be estimated from the solid curve for the α to β transformation in Fig. 3 as *ca.* 1.5. The $\log k_1 = -0.40$ may be estimated from the intercept of the tangent with slope of unity to the data at higher pH values

$$\log k = -pH + \log k_1 \quad (10)$$

so that $k_1 \sim 0.40$ l./mole/sec. Similarly, from the data at lower pH values it can be estimated that $k_2 \sim 0.14$ l./mole/sec.

Alkaline Degradation of the First Acid Degradation Product of Porfiromycin. $\alpha \xrightarrow{H^+} \beta(A) \xrightarrow{OH^-} (A,B)$.—The first acid transformation product $\beta(A)$ has maxima at 310 mμ and 250 mμ and an apparent pK_a by spectrophotometry of 7.0 if only one group is present. However, the spectral shifts from acid to base, although reversible and real, are not too pronounced.

Potentiometric titration of $\beta_A(A)$ with alkali shows inflections at 5.5 and 8.0 pH and an estimated pK_a of 7.0 with an estimated equivalent weight of 314 based on the initial porfiromycin. A repeat shows an apparent pK_a of 7.3 and an equivalent weight of 307 with an indication of a possible other group of pK_a 5.1 and equivalent weight of 643. There is uncertainty about this latter group.

On treatment with alkali, $\beta(A)$ is transformed to a new chromophore $\gamma(A,B)$ with a maximum of 298 mμ (a = 49.5) and with loss of the 310 and 250 mμ chromophores. Typical curves of the transformations of ultraviolet spectra for the $\beta_B(A) \rightarrow \gamma_B(A,B)$ are given in Fig. 4. The apparent first order rate constants for the loss of the 255 mμ chromophore in alkali and the conditions under which they were obtained are given in Table II.

A plot of the apparent first order rate constant against molarity in NaOH can be characterized by two empirical equations

$$\beta_B(A) \xrightarrow{k} \gamma_B(A,B) \quad (11a)$$

$$k = 1.88 \times 10^{-5}[NaOH]; \ [NaOH] < 0.20\ M \quad (11b)$$

$$k = 5.85 \times 10^{-6}[NaOH] + 3.0 \times 10^{-6}; \ 0.30\ M < [NaOH] \quad (11c)$$

where k is in sec.$^{-1}$.

However, the nonlinearity indicates a change in the degree of protonation of $\beta(A)$ as a function of alkali concentration.[6b,7,12]

$$k\beta(A)_T = k_1[(OH^-)][\beta(A)] + k_2[OH^-][\beta(A)^-] \quad (12)$$

(12) L. J. Edwards, *Trans. Faraday Soc.*, **46**, 729 (1950).

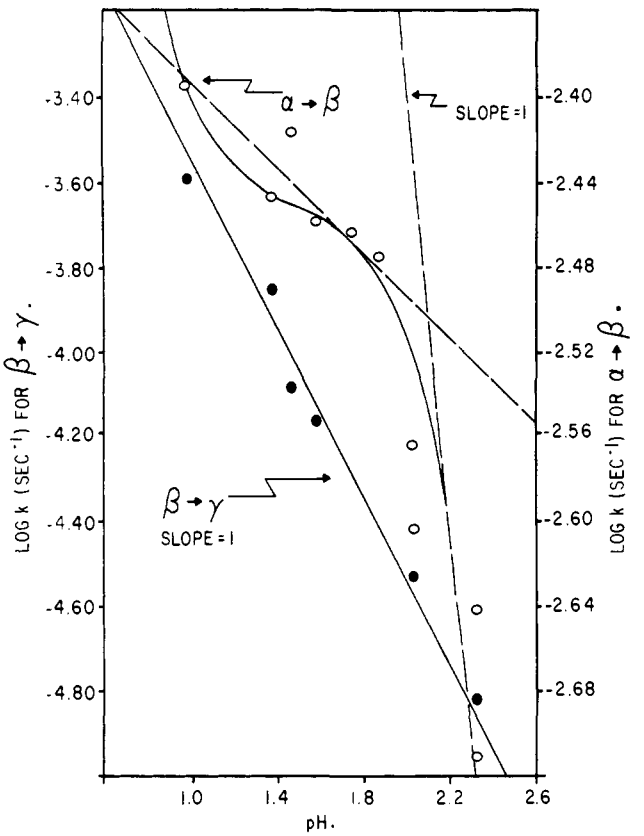


Fig. 3.—Logarithm of rate constants at 30° for the acid transformations of porfiromycin, $\alpha \rightarrow \beta$ and $\beta \rightarrow \gamma$, plotted as a function of pH.

where

$$[\beta(A)] \overset{K_a}{\rightleftharpoons} [\beta(A)^-] + H^+ \quad (13)$$

where a development analogous to eq. 9a–e and 10, the specific hydrogen ion catalyzed reactions of a dissociating acid, may be made. In this case

$$d[\beta(A)]_T/dt = d([\beta(A)] + [\beta(A)^-]/dt \quad (14a)$$

$$= -k_1[OH^-][\beta(A)] - k_2[OH^-][\beta(A)^-] \quad (14b)$$

$$= -k_{OH^-}[OH^-]\{[\beta(A)] + [\beta(A)^-]\} \quad (14c)$$

$$= -k_{OH^-}[OH^-][\beta(A)]_T = -k[\beta(A)]_T \quad (14d)$$

where $[\beta(A)]_T$ is the total concentration of both uncharged and anionic forms and whence eq. 12 is deduced.

It can be shown[6,7,12] that

$$k/[OH^-] = k_{OH^-} = k_1(1 + K_a/[H^+]) + k_2/(1 + [H^+]/K_a) \quad (14e)$$

so that the apparent first order rate constants k at any $[OH^-]$ can be calculated as functions of the derived bimolecular rate constants k_1, k_2; of the dissociation constant K_a; and of the hydrogen ion concentration $[H^+]$ where the latter is used in this paper either as $K_w/f[NaOH]$ or $10^{-pH} = 10^{-(pK_w - pOH)}$.

A plot of $\log k$ *vs.* pH is given in Fig. 5 where $pH = pK_w - pOH$ (Table II) and $pOH = -\log f$ NaOH where f is the mean activity coefficient for [NaOH],[11] and $pK_w = 13.83$ at 30°.[11] Empirically

$$\log k = pH - 18.40; \ pH < 13.0 \quad (15a)$$

$$\log k = pH - 18.74; \ pH > 13.5 \quad (15b)$$

The pK_a of $\beta(A)$ is estimated as 13.35 at 30° from Fig. 5 where the dashed lines represent slopes of unity. Since

$$\log k = \log k_i - pK_w + pH \quad (15c)$$

where equations (15a) and (15b) are of this form, then $k_1 = 2.69 \times 10^{-5}$ and $k_2 = 1.23 \times 10^{-5}$ l/mole/sec.

Alkaline Degradation of the Second Acid Degradation Product of Porfiromycin. $\alpha \xrightarrow{H^+} \beta(A) \xrightarrow{H^+} \gamma(A,A) \xrightarrow{OH^-} \delta(A,A,B)$.—The

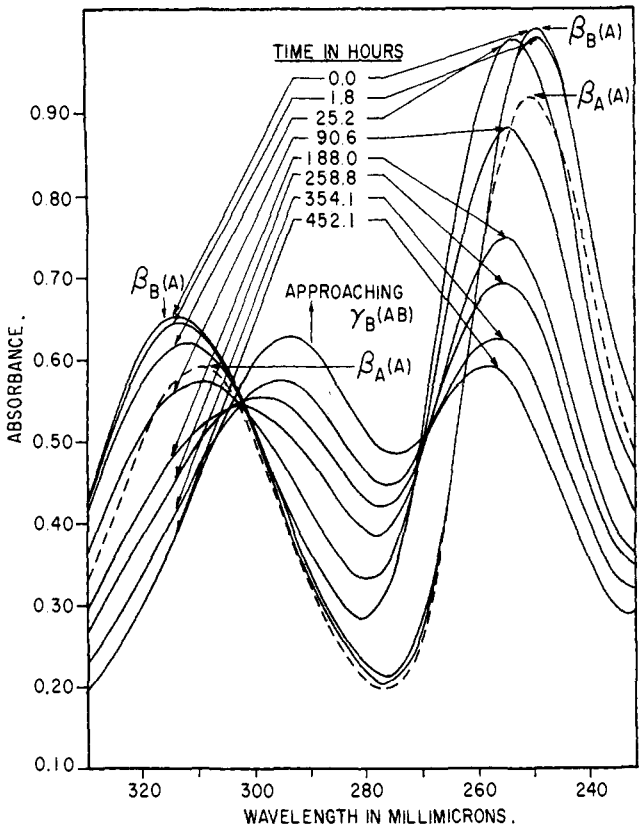


Fig. 4.—Typical curves of the spectral changes of β(A) on subjection to 0.05 *M* NaOH. The solution was at 30° and initially 15 γ/ml. in porfiromycin. The solid curves represent the spectral transformation $\beta_B(A) \rightarrow \gamma_B(A,B)$. The dashed curve represents the spectra of $\beta_A(A)$, *i.e.*, β(A) in acid solution. Each curve is labeled as to the number of hours after the start of degradation.

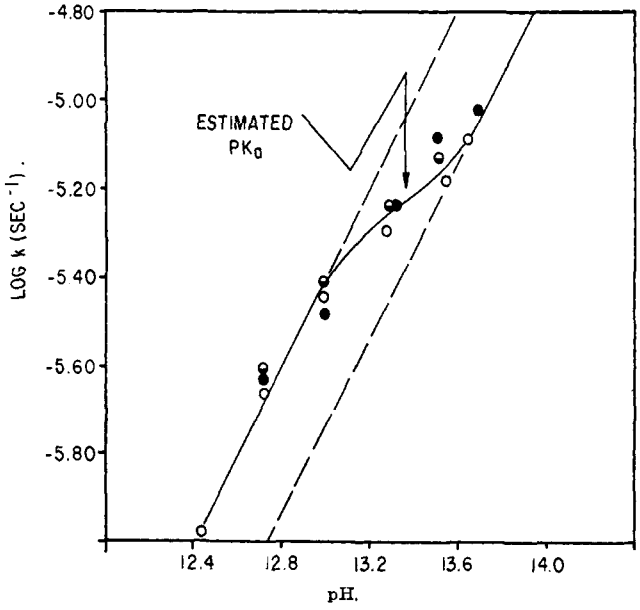


Fig. 5.—Logarithms of rate constants at 30° plotted as a function of pH = $pK_w - \log f$ [NaOH]. The dashed lines have slopes of unity. The symbols and respective transformations are ○, $\beta_B(A) \rightarrow \gamma_B(A,B)$; ◒, $\gamma_B(A,A) \rightarrow \delta_B(A,A,B)$; ●, $\gamma_B(B,A) \rightarrow \delta_B(B,A,B)$.

γ(A,A) ultraviolet spectrum in acid is characterized by maxima at 295 mμ (a = 55.2, a repeat gave 54.1) and 238 mμ (a = 71.3, a repeat gave 68.1) (see Fig. 1 and 6). On the addition of alkali, this spectrum is transformed to one characterized by 255 mμ (a = 70.6, a repeat gave 68.1) and 313 mμ (a = 40.2, a repeat gave 42.1) maxima.

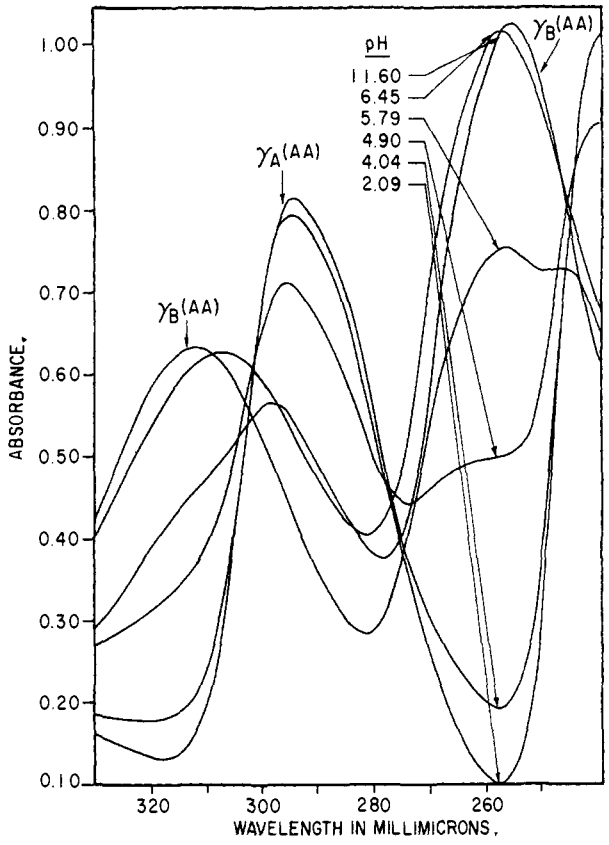


Fig. 6.—Effect of pH on the spectra of a porfiromycin degradation product, γ(A,A), at an original porfiromycin concentration of 15 γ/ml.

Variation of the pH of the solution of γ(AA) resulted in a change in spectra between the absorptivities given above for the alkaline and acid solution forms (Fig. 6). This is a reversible phenomenon. The γ_A(A,A) after adjustment to pH 11.6 and reversion to pH 2.2 demonstrated the same ultraviolet spectrum as it had initially. A plot of the logarithmic function

$$\log [A_{H^+} - A]/[A - A_{OH^-}] = pH + pK_a \qquad (16)$$

against pH where A is the 255 mμ absorbance of the solution at any pH and A_{H^+} and A_{OH^-} are the asymptotic values of the absorbances at extreme acid and alkaline conditions respectively, is given in Fig. 7. The significance of eq. 16 and such a plot is discussed in the literature.[12,13] The slope of the logarithmic function *vs.* pH approximates unity, indicating that only one functional group's dissociation or protonation affects the chromophore. The intercept, *i.e.*, when the logarithmic function is zero, is equal to the pK_a and is 5.0.

On treatment with alkali, the chromophore of λ_B(A,A) is transformed to a new chromophore δ_A(A,A,B) with a maximum at 298 mμ (a = *ca.* 52) (Fig. 8). On acidification δ_A (A,A,B) has a maximum at 285 mμ (a = *ca.* 48).

Plots of the loss of the 255 mμ chromophore in alkali are apparent first order and the apparent first order rate constants and the conditions under which they were obtained are given in Table III.

A plot of the apparent first order rate constant against molarity in NaOH for $\gamma_B(A,A) \xrightarrow{k} \delta_B(A,A,B)$ does not pass through the origin and indicates a change in the degree of protonation of γ(A,A) as a function of alkali concentration, *i.e.*

$$(Ak[\gamma(A,A)]_T = k_1[OH^-][\gamma(A,A)] + k_2[OH^-][\gamma(A,A)^-] \qquad (17)$$

where

$$[\gamma(A,A)] \underset{}{\overset{K_a}{\rightleftharpoons}} [\gamma(A,A)^-] + [H^+] \qquad (18)$$

This is clearly shown in Fig. 5, a plot of log k *vs.* pH where pH has been calculated from pOH = $-\log f$ [NaOH] and pH = pK_w − pOH (Table III). The dashed lines represent the slopes of

(13) E. R. Garrett, *J. Am. Chem. Soc.*, **79**, 3401 (1957).

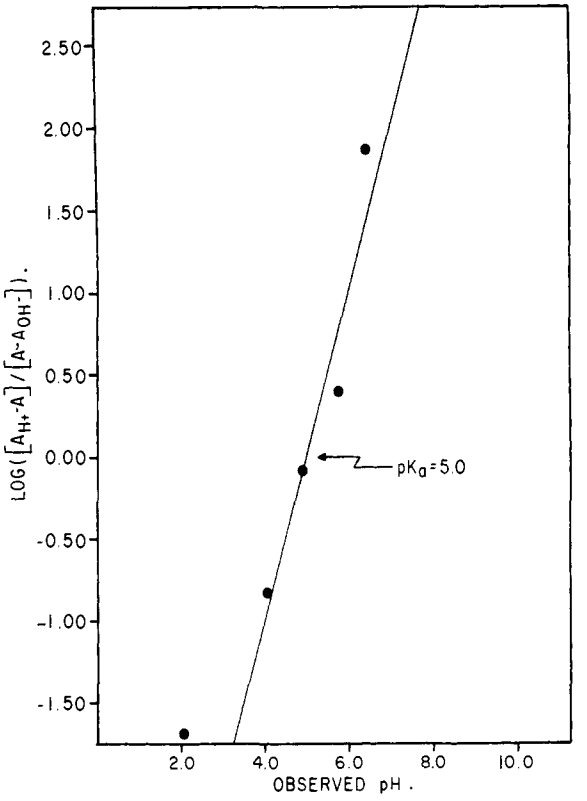


Fig. 7.—Determination of the pK_a of γ(A,A) by spectrophotometry at 255 mμ.

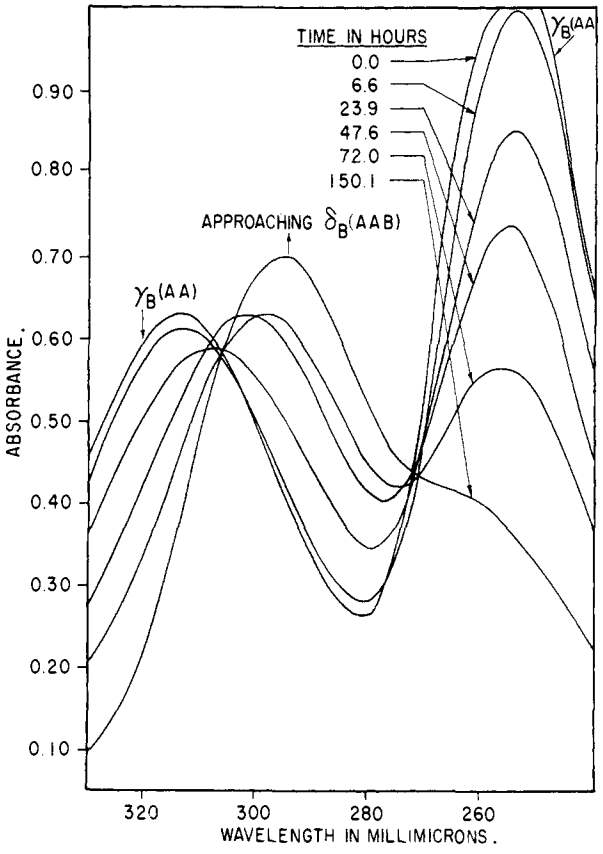


Fig. 8.—Typical curves of the spectral changes representing the transformation γ_B(A,A) → δ_B(A,A,B) in 0.2 *M* NaOH at 30° at an original porfiromycin concentration of 15 γ/ml.

unity and the resultant expressions are the same as for β_B(A) → γ_B(A,B) as given by eq. 15a, 15b, and 15c where the additional

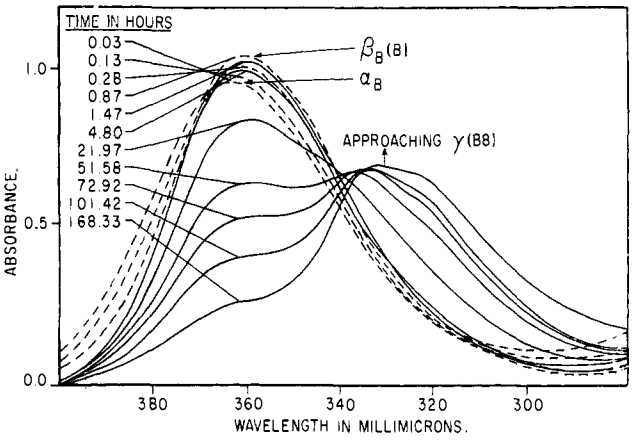


Fig. 9.—Typical curves of the spectral changes during the initial alkaline transformations of porfiromycin. The solution was at 30°, 0.1 *M* NaOH, with an initial concentration of porfiromycin of 15 γ/ml. The dashed curves represent the spectral transformations of α_B → β_B(B). The solid curves represent the spectral transformation of β_B → γ_B(B,B). Each curve is labeled as to the number of hr. after the start of degradation.

pK_a of γ(A,A) is thus estimated as 13.35 at 30°. Equations of the form of (14) are also applicable.

The estimated k_1 and k_2 for the specific hydroxyl ion catalyzed degradations of the non anionic and anionic moieties of both β(A) and γ(A,A) are the same within experimental error.

This indicates that the β(A) group of pK_a 13.35 is retained by γ(A,A) after acid hydrolysis of the former; that in all probability the same group is alkali degraded in γ(A,A) as in β(A); and that the modification of β(A) by acid to γ(A,A) does not change or modify the rate of attack by the hydroxyl ion. The γ(A,A) appears to be acid stable as no significant change of spectra beyond that assigned to γ(A,A) was observed.

Alkaline Degradation of Porfiromycin and the Appearance of Intermediates.—In alkaline solution the porfiromycin absorbance at 363 mμ dramatically decreased with time and a new chromophore appeared with a λ_{max} at 333 mμ (Fig. 9). The half-life of the porfiromycin chromophore was 55 hr. at 30° in 0.1 *M* NaOH. However, when an attempt was made to correlate the half-life of the porfiromycin biological activity by plate–disk assay,[2d] the biological activity was found to disappear at a much faster rate under these conditions (half-life 14 min.) than did the porfiromycin 365 mμ chromophore (see Fig. 9).

When this apparent inconsistency was observed, the changes in spectra were followed carefully for the first 30 min. and a slight but significant change in chromophore was determined (Fig. 9). The 363 mμ (a = 63.8) maximum shifted to 360 mμ (a = 67.1) and the absorbance increased. The coincidence of the slopes and thus rates of this chromophoric enhancement with loss in biological activity was conclusively demonstrated. This is considered as the transformation of porfiromycin α_B → β_B(B).

Initial Alkaline Degradation of Porfiromycin. α_B → β_B(B).—The rate constant for α_B → β_B(B) is 8.2 × 10^{-4} sec.$^{-1}$ at 30° in 0.10 *M* NaOH.

The change in spectra of β(B) with pH is not quite a reversible phenomenon due to the fast acid degradation of this material. The maximum in alkali (λ_{max} = 360 mμ, a = 67.1 at pH 12.4) shifts to a new maximum in acid (est. λ_{max} = 333 mμ, a = 36 at pH 2.1) which is rapidly lost with time.

The pH at which the greatest change in absorbance, A, occurs, *i.e.*, dA/dpH is a maximum, permits an estimate of the pK_a of β(B), *ca.* 4.3. A plot of log $[(a_{HA}C - A)/(A - A_{A^-}C)]$ *vs.* pH[12,13] should have a slope of unity if only one disssociable group affects the chromophore and the pK_a should be the intercept of such a plot. The C is the concentration, a_{HA} and a_{A^-} are the asymptotic absorptivities at a given wave length in acid and alkaline solutions, respectively, and A is the absorbance at this wave length at any pH. Thus $A_{A^-}C$ and $a_{HA}C$ are the asymptotic absorbances A_{OH^-} and A_{H^+} in alkaline and acid solutions, respectively. Such a plot for the absorbance at the 360 mμ wave length gives a reasonably unit slope with an intercept of 4.1 which is the estimated pK_a of β(B). Repeat spectrophotometric titrations gave pK_a estimates of 4.43 and 4.45.

Potentiometric titration with standard acid of the β(B) triethylamine salt isolated by Schroeder[9] according to the hy-

TABLE VII

PREPARATION, KINETICS, AND CHARACTERIZATION OF PRODUCTS OF PORFIROMYCIN, α, DEGRADATION IN ACID AND BASE

Compound	pK_a[a]	Method of detn.	Possible nature of group	Est. mol. wt.	λ_{max} (mμ)	a[b]	pH
α_B	1.5	Kinetic	Amide		363	65.6	12.4
	ca. 1	Potentiometric[d]		334, 344, 334			
		X-Ray (2)		344			
β_B(B)	4.3[c]	Spectral			360	67.1	12.4
$H^+ \downarrow \uparrow OH^-$	4.44[g]	Spectral	Uncharged				
	4.38[g]	Potentiometric	acid	375			
β_A(B)	5.5[g]						
		Potentiometric (50% ethanol)		328	330	36.4	2.1
	2.5	Kinetic					
		Potentiometric (nonaqueous)[g]	Amide	360			
γ_B(B,B)	7[c]	Spectral			333	51.7	12.4
$H^+ \downarrow \uparrow OH^-$	11	Kinetic	Uncharged acid				
γ_A(B,B)					320	24	5.0
δ_B(B,B,B)	8–11[e]	Spectral			305–320	16.3	12.2
$H^+ \downarrow \uparrow OH^-$	2–6[c]	Spectral					
δ_A(B,B,B)					Indeterminate		7.85
ϵ_B(B,B,B,B)					290	16	12.4
ϵ_A(B,B,B,A)					290	16	2.0
$OH^- \downarrow \uparrow H^+$	*ca.* 5.5	Spectral					
ϵ_B(B,B,B,A)					300	16	12.0
α_A	1.5	Kinetic	Amide		363	65.6	2.03
	ca. 1	Potentiometric[d]		334, 344, 334			
		X-Ray (2)		344			
β_A(A)					311	39.4	2.07
$OH^- \downarrow \uparrow H^+$	13.3	Kinetic	Uncharged acid		250	61.6	2.07
β_B(A)							
	7.0[c]	Spectral			314	43.5	12.0
	7.0[e]	Potentiometric	Amine	314[f]	250	67.3	12.0
	7.3[e]	Potentiometric		307[f]			
γ_A(A,A) = γ_A(B,A)	13.7	Kinetic	Uncharged acid		295	55.2, 54.1	2.2
					238	71.3, 68.1	2.2

Method of preparation[a]	Degradation and rate dependence:[c] $d[X]/dt = -k[X]$	Kinetics of degradation[a,c]
$\alpha + NaOH \rightleftharpoons \alpha_B$ Instantaneous	$\alpha_B \xrightarrow{NaOH} \beta_B(B)$, $k[\alpha_B] = k_{OH^-}[OH^-][\alpha_B]$	$k = 8.2 \times 10^{-4}$ in 0.1 M NaOH at 30° $= 6.16 \times 10^{-5}$ in borate buffer at 60° $= 9.46 \times 10^{-6}$ in borate buffer at 60.6°
$\alpha_B + NaOH \rightarrow \beta_B(B)$, $t_{1/2} = 0.23$ hr. in 0.1 M NaOH (pH 12.4)	$\beta_B(B) \xrightarrow{NaOH} \gamma_B(B,B)$, $k[\beta_B(B)] = k_{OH^-}[OH^-][\beta_B(B)]$	$k = 3.4 \times 10^{-5}$[NaOH] or $\log k = pH - 18.1$ where $pH = -\log f$[NaOH] and $pH = pK_w - pOH$
$\beta_B(B) + HCl \rightleftharpoons \beta_A(B)$ Instantaneous	$\beta_A(B) \xrightarrow{HCl} \gamma_A(B,A)$, $k[\beta_A(B)]_T = \alpha' k' \cdot [\beta(B)]_T = k_{H^+}[H^+]\alpha'[\beta_A(B)]_T = k_{H^+} \cdot [H^+][\beta_A(B)]$ where α' is the degree of disassociation of $\beta_A(B)H^+ \rightleftharpoons \beta_A(B) + H^+$; $pK_a = 2.5$ as calcd. from $pH = pK_a + \log \frac{\alpha'}{1-\alpha'}$, and $\beta(B)_T = \beta(B) + \beta(B)H^+$	$k = 1.20\alpha'$[HCl] where α' is from $pH = pK_a + \log \frac{\alpha'}{1-\alpha'}$; $pK_a = 2.5$
$\beta_B(B) + NaOH \rightarrow \gamma_B(B,B)$, $t_{1/2} = 54.6$ hr. in 0.1 M NaOH (pH 12.4)	$\gamma_B(B,B) \xrightarrow{NaOH} \delta_B(B,B,B)$, $k[\gamma_B(B,B)] = k_1[OH^-][\gamma(B,B)] + k_2[OH^-][\gamma(B,B)^-]$ where $\gamma(B,B) \rightleftharpoons \gamma(B,B)^- + H^+$	$k = 1.1 \times 10^{-6}$[NaOH]; [NaOH] > 0.10 or $\log k = pH - 19.4$, pOH > 13.4 where $pOH = -\log f$[NaOH] and $pH = pK_w - pOH$, $k_1 = 2.7 \times 10^{-6}$, k_2 was undetermined
$\gamma_B(B,B) + HCl \rightleftharpoons \gamma_A(B,B)$ Instantaneous with almost simultaneous degradation of $\gamma_A(B,B)$	$\gamma_A(B,B) \xrightarrow{HCl} \delta_A(B,B,A)$,[h] $k[\gamma_A(B,B)] = k_{H^+}[H^+][\gamma_A(B,B)]$	k and k_{H^+} were of too high magnitude to determine; the irreversible degradation of $\gamma_A(B,B)$ is almost instantaneous with acidification of $\gamma_B(B,B)$[h]
$\gamma_B(B,B) + NaOH \rightarrow \delta_B(B,B,B)$, $t_{1/2} = 375$ hr. in 0.1 M NaOH (pH 12.4)	$\delta_B(B,B,B) \xrightarrow{NaOH} \epsilon_B(B,B,B,B)$	60 days in 0.1 M NaOH
$\delta_B(B,B,B) + HCl \rightleftharpoons \delta_A(B,B,B)$ Instantaneous with almost simultaneous degradation of $\delta_A(B,B,B)$	$\delta_A(B,B,B) \xrightarrow{HCl} \epsilon_A(B,B,B,A)$	Essentially instantaneous
$\delta_B(B,B,B) + NaOH \rightarrow \epsilon_B(B,B,B,B)$ Significant change noted after 60 days	Possible end product	
$\delta_A(B,B,B) + HCl \rightarrow \epsilon_A(B,B,B,A)$ Instantaneous	Possible end product	
$\epsilon_A(B,B,B,A) + NaOH \rightleftharpoons \epsilon_B(B,B,B,A)$ Instantaneous	Possible end product	
$\alpha + HCl \rightleftharpoons \alpha_A$ Instantaneous	$\alpha_A \xrightarrow{HCl} \beta_A(A)$, $k[\alpha] = k_1[H^+][\alpha_A] + k_2[H^+][\alpha_A H^+]$ where $\alpha_A + [H^+] \overset{K_a}{\rightleftharpoons} [\alpha_A H^+]$	$k = 0.168$[HCl] $+ 1.00 \times 10^{-3}$; $0.005 <$ [HCl] < 0.014 $k = 7.5 \times 10^{-3}$[HCl] $+ 3.27 \times 10^{-3}$; $0.014 <$ [HCl] < 0.100 or $\log k = -0.100pH - 2.29$ where $pH = -\log f$[HCl]; $1.0 < pH < 2.1$; $K_a \sim 1.5$; $k_1 \sim 0.40$; $k_2 \sim 0.14$
$\alpha_A + HCl \rightarrow \beta_A(A)$, $t_{1/2} = 0.1$ hr. in 0.01 M HCl (pH 2.03);	$\beta_A(A) \xrightarrow{HCl} \gamma_A(A,A)$, $k[\beta_A(A)] = k_{H^+}[H^+] \cdot [\beta_A(A)]$	$k = 2.57 \times 10^{-3}$[HCl] or $\log k = -pH - 2.54$ where $pH = -\log f$[HCl]
$\beta_A(A) + NaOH \rightleftharpoons \beta_B(A)$ Instantaneous	$\beta_B(A) \xrightarrow{NaOH} \gamma_B(A,B)$, $k[\beta(A)] = k_1[OH^-] \cdot [\beta(A)] + k_2[OH^-][\beta(A)^-]$ where $\beta(A) \overset{K_a}{\rightleftharpoons} \beta(A)^- + H^+$	$k = 1.88 \times 10^{-5}$[NaOH]; [NaOH] < 0.20 M $k = 5.85 \times 10^{-6}$[NaOH] $+ 2.45 \times 10^{-6}$; $0.20 <$ NaOH or $\log k = pH - 18.4$, $pH < 13.0$; $\log k = pH - 18.74$, $pH > 13.5$ where $pOH = -\log f$[NaOH] and $pH = pK_w - pOH$; $K_a \sim 13.3$; $k_1 = 2.69 \times 10^{-5}$, $k_2 = 1.23 \times 10^{-5}$
$\beta_A(A) + HCl \rightarrow \gamma_A(A,A)$, $t_{1/2} = 6.5$ hr. in 0.01 M HCl (pH 2)	Stable in acid	

TABLE VII (*Continued*)

Compound	pK_a[a]	Method of detn.	Possible nature of group	Est. mol. wt.	λ_{max} (mμ)	a[b]	pH
$\gamma_A(B,A)$	5.0[c]	Spectral	Amine				
$OH^-\downarrow \uparrow H^+$	*ca.* 5[c]	Potentiometric					
$\gamma_B(A,A) = \gamma_B(B,A)$	*ca.* 8[c]	Potentiometric	Carboxyl				
					255	70.6, 68.1	11.6
					313	40.2, 42.1	11.6
					255	66.6	11.5
	13.7	Kinetic	Uncharged acid		312	41.9	11.5
$OH^-\downarrow \uparrow H^+$	5.15[d]	Spectral					
	5.0[e]	Spectral	Amine				
	4.95[f]	Potentiometric					
	7.6[d]	Potentiometric	Carboxyl	320	295	54.5	2.07
					238	68.8	2.07
$\gamma_A(B,A) = \gamma_A(A,A)$							
$\delta_A(B,A,B) = \delta_A(A,A,B)$							
$OH^-\downarrow \uparrow H^+$							
$\delta_{\delta_B(B,A,B)} =$							
$\delta_B(A,A,B) = \gamma_B(A,B)$	*ca.* 12.2[c]	Spectral			295	45.2[j], 52[j]	12.5
$H^+\downarrow \uparrow OH^-$	3–5[c]	Spectral			290	45[j]	11.9
					285	43.3[j], 48[j]	2.37
$\gamma_A(A,B)$							

[a] All temperatures at 30° unless specifically stated. [b] In general, based on the absorbance/g. of original undegraded porfiromycin/liter. [c] All k values without subscripts refer to the pseudo first order rate of change of the porfiromycin intermediates in sec.$^{-1}$; all k_i with subscripts are second order rate constants in l./mole/sec. units; [X] is concentration of product; f = activity coefficient; and pK_w at 30° = 13.83. [d] Titration with perchloric acid in dioxane. [e] Potentiometric and spectral titration of the reaction product

drolysis conditions described herein, gave a pK_a of 4.38 and an equivalent weight of 476, and thus the free acid should have an equivalent weight of 375. A pK_a of 5.5 was observed in 50% ethanol. This increase in pK_a with ethanol concentration confirms the fact that the β(B) is uncharged in the acid form since pK_a values of uncharged acid generally increase with lowering of the dielectric.

Second Alkaline Transformation, β_B(B) → γ_B(B,B).—The subsequent relatively slow decrease of the 360 mμ maximum (a = 67.1 at pH 12.4) in alkali with the appearance of a 330 mμ maximum (a = 51.7 at pH 12.4) is attributed to $\beta_B(B) \xrightarrow{k} \gamma_B$-(B,B) (Fig. 9). The pertinent rate data for the apparent first order loss of the 360 mμ chromophore under the influence of alkali for the triethylamine salt of β(B) are summarized in Table IV.

A plot of the apparent first order rate constant, k in sec.$^{-1}$, against sodium hydroxide concentration passes through the origin and indicates that no dissociating group modified the binolecularity of the hydroxyl ion attack on β(B), *i.e.*

$$d[\beta(B)]/dt = k[\beta(B)] = k_{NaOH}[\beta(B)][NaOH] \quad (19)$$

where $k = 3.44 \times 10^{-5}$[NaOH] and $k_{NaOH} = 3.44 \times 10^{-5}$ l./mole/sec.

An alternative estimate of the bimolecular rate constant, k_{OH} can be obtained from a plot of log k *vs.* pH where pH has been calculated from pOH = $-\log f[OH^-]$ and pH = pK_w − pOH (see Table IV). The slope is close to unity and

$$\log k = pH - 18.1 = pH + \log k_{OH} - pK_w \quad (20)$$

and since pK_w = 13.83 at 30°, $k_{OH} = 3.2 \times 10^{-5}$ l./mole/sec., which agrees well with the value calculated for k_{NaOH}.

Variation of pH has a decided effect on the spectrum of γ(B,B) as is shown in Fig. 10. Adjustment of pH below 10 gives a spectrum that changes rapidly with time so that a spectrophotometric estimate of a possible pK_a is difficult to obtain. However, if spectra are run as a function of time at a given pH and absorbance values linearly extrapolated to time zero, a series of curves are produced that should approximate the undegraded γ(B,B) at the several pH values. These extrapolated spectra are plotted in Fig. 10. At the lower pH values of 5.08 and 4.27, the error in this technique is most noticeable in that the maximum attributed to δ_A(B,B,A) at 295 mμ starts to appear. However, a pK_a can be estimated as *ca.* 7.0 where (d absorbance)/(dpH) is an apparent maximum.

Third Alkaline Transformation, γ_B(B,B) → δ_B(B,B,B).—The 330 mμ maximum of γ_B(B,B), a = 51.7 at pH 12.4, slowly decreases and shifts to 320 mμ, a = 16.3 at pH 12.4 (Fig. 11). The pertinent rate data for the apparent first order transformation $\gamma_B(B,B) \xrightarrow{k} \delta_B$(B,B,B) on the basis of the loss of 330 mμ maximum are summarized in Table III.

The plot of the apparent first order rate constants, k in sec.$^{-1}$, against sodium hydroxide concentration does not pass through the origin. This differs from the alkaline hydrolysis of β(B) and indicates that the rates of alkaline hydrolysis of γ(B,B) are affected by a dissociating group. An empirical equation showing the sodium hydroxide concentration dependence is

$$k = 1.1 \times 10^{-6}[NaOH]; \quad [NaOH] > 0.10 \quad (21)$$

The plot of log k *vs.* calculated pH for the alkaline hydrolysis of γ(B,B) varies widely from a slope of unity and thus implies that γ(B,B) has a pK_a *ca.* 11 which may be attributed to an uncharged acid which does not significantly affect the chromophore on dissociation so that

$$k[\gamma(B,B)] = k_1[OH^-][\gamma(B,B)] + k_2[OH^-][\gamma(B,B)^-] \quad (22)$$

where

$$[\gamma(B,B)] \overset{K_a}{\rightleftharpoons} [\gamma(B,B)^-] + H^+ \quad (23)$$

and

$$\log k = pH - 19.4; \quad pOH > 13.4 \quad (24)$$

so that $k_2 \sim 2.7 \times 10^{-5}$. The data are inadequate for the estimation of log k at pOH < 13.4 and thus k_1 is undetermined.

Variation of pH has a decided effect on the spectrum of δ_B-(B,B,B). In alkaline solution, δ_B(B,B,B) has a broad maximum at pH 12.16 at 305–320 mμ, a = 16.3. The shoulder at 330 mμ may be due to γ_B(B,B) impurity. Addition of acid to pH 2 and immediate readjustment to pH 12 gives a spectrum with a loss in absorbance at the 320–360 mμ region; the maximum is now sharp.

Method of preparation[a]	Degradation and rate dependence[c] d[X]/dt = $-k$[X]	Kinetics of degradation[a,c]
$\gamma_A(A,A) + NaOH \rightleftharpoons \gamma_B(A,A)$ Instantaneous $\gamma_A(B,A) + NaOH \rightleftharpoons \gamma_B(B,A)$ Instantaneous	$\gamma_B(A,A) \xrightarrow{NaOH} \delta_B(A,A,B)$ $k[\gamma_B(A,A)] = k_1[OH^-][\gamma(A,A)] + k_2[OH^-][\gamma(A,A^-)]$ where $\gamma(A,A) \overset{K_a}{\rightleftarrows} \gamma(A,A^-) + H^+$ where $\gamma(A,A) = \gamma(B,A)$; $\delta(A,A,B) = \delta(B,A,B)$	$\log k = pH - 18.4$; $pH < 13.6$ $\log k = pH - 18.83$; $pH > 13.5$ where $pOH = -\log f\,NaOH$ and $pH = pK_w - pOH$; $K_a \sim 13.3$; $k_1 = 2.69 \times 10^{-5}$; $k_2 = 1.23 \times 10^{-5}$
$\beta(B) + HCl \rightarrow \gamma_A(B,A)$, $t_{1/2} = 0.1$ hr. in 0.01 M HCl	Stable in acid	
$\delta_B(B,A,B)$ (= $\delta_B(A,A,B)$) + HCl $\rightleftharpoons$ $\delta_A(B,A,B)$ (= $\delta_A(A,A,B)$) Instantaneous $\gamma_A(B,A)$ (= $\gamma_A(A,A)$) + NaOH $\rightarrow$ $\delta_B(B,A,B)$ (= $\delta_B(A,A,B)$) $t_{1/2}$ = 82 hr. in 0.1 M NaOH $\beta(A) + NaOH \rightarrow \gamma(A,B)$ $t_{1/2}$ = 91 hr. in 0.1 M NaOH $\gamma_B(A,B) + HCl \rightleftharpoons \gamma_A(A,B)$ Instantaneous	$\gamma_A(A,B) = \delta_A(B,A,B) \xrightarrow{HCl} \delta_A(A,A,B) = \epsilon_A(B,A,B,A) = \epsilon_A(A,A,B,A)$ Instantaneous < pH 4	

in the reaction media, *i.e.*, *in situ*. [f] As based on the original weight of the porfiromycin degraded. [g] Potentiometric and spectral titrations of the isolated reaction product. [h] $\delta(B,B,A)$ is produced almost immediately by the acidification of $\gamma(B,B)$ with 295 mμ (a = 40.5) and 238 mμ (a = 45.1) maxima at pH 2.4. The $\delta_B(B,B,A)$ maxima are 312 mμ (a = 36.7) and 258 mμ (a = 47.8) in alkali with spectral pK_a *ca.* 5. [i] $\delta(B,A,B)$. [j] $\delta(A,A,B)$.

at 300 mμ, a = 16. Adjustment to power pH values, *i.e.*, 7 and 5, gives spectra that change with time. The speed of spectral change increases with acidity so that an $\epsilon(B,B,B,A)$ may be postulated with a maximum at 290 mμ in acid solution, a = 16. Inspection of the curves indicates that $\delta(B,B,B)$ may have a pK_a between 8 and 11 and another between 2 and 6. A pK_a for $\epsilon(B,B,B,A)$ may be estimated as *ca.* 5.5.

$\delta(B,B,B)$ may also be transformed to an $\epsilon(B,B,B,B)$ since some of this former material showed a slow change in maximum with time to 290 mμ.

Acid Degradation of Intermediates Appearing from the Alkaline Degradation of Porfiromycin: The First Acid Degradation of the Initial Alkaline Degradation Project, $\beta_A(B) \overset{k}{\rightarrow} \gamma_A(B,A)$.—The alkaline degradation product of porfiromycin, $\beta(B)$, has an apparent pK_a of 4.4 and is most probably uncharged in the acid form since the apparent pK_a increases in 50% ethanol to 5.5. In mild acid the spectra of $\beta_A(B)$ with a maximum at 330 mμ, a = 36.4, readily changes to one, $\gamma_A(B,A)$ with maxima at 295 mμ, a = 54.5, and 238 mμ, a = 68.8. This transformation of spectra in 0.01 M HCl at 30° is clearly shown in Fig. 12. Apparent first order rate constants for this transformation as based on the increase of the 295 mμ maximum are given in Table V.

The nonlinearity of the plot of the apparent first order rate constants, k in sec.$^{-1}$, against HCl concentration clearly indicates that a dissociating group modifies the simple bimolecularity of the hydrogen ion attack on $\beta(B)$[6b,7,12] The decrease in an apparent bimolecular rate constant, *i.e.*, k(sec.$^{-1}$)/[HCl] with increasing [HCl] and the apparent achievement of a constant k indicates that

$$\frac{-d[\beta(B)]}{dt} = k_1[H^+][\beta(B)] + k_2[H^+][\beta(B)H^+] \quad (25)$$

where

$$\beta(B)H^+ \overset{K_a}{\rightleftharpoons} \beta(B) + H^+ \quad (26)$$

where

$$[\beta(B)]_t = [\beta(B)] + [\beta(B)H^+] \text{ and } k_2 < k_1 \quad (27)$$

If $k_2 = 0$, then

$$-d[\beta(B)]_t/dt = k[\beta(B)_t] = \alpha' k_{H^+}[H^+][\beta(B)]_t = k_{H^+}[H^+][\beta(B)] \quad (28)$$

where α' is the degree of dissociation of $\beta(B)H^+$ and can be calculated from

$$pH = pK_a + \log \frac{\alpha'}{1 - \alpha'} \quad (29)$$

When a pK_a of 2.5 is chosen and α' is derived from the calculated pH values given in Table V, then a k_{H+} = 1.20 l./mole/sec. gives consistent k values with the experimental when the [HCl] concentrations of Table V are used in the calculations as equivalent to the hydrogen ion concentrations. The calculated and experimental k values are given in Table V and show good agreement.

The plot of log k *vs.* pH also graphically demonstrates the fact that a dissociating group modified the simple bimolecularity of the hydrogen ion attack on $\beta(B)$ since it is nonlinear and deviates from a slope of unity.

The Coincidence of $\gamma(B,A)$ and $\gamma(A,A)$.—The acid degradation of the first product of the alkaline degraded porfiromycin results in a material that has the same ultraviolet absorption spectra at the same pH values as the second acid degradation product of porfiromycin, $\gamma(A,A)$, *i.e.*, maxima at 295 mμ (a = 54.5) and 238 mμ (a = 68.8) at pH 2.07 and maxima at 312 mμ (a = 41.9) and 255 mμ (a = 66.6) at pH 11.5 (see Fig. 12, 13, and 14).

Spectrophotometric titration of $\gamma(B,A)$ (see Fig. 13) gave an estimated pK_a' of 5.0. Repeat spectrophotometric titration gave 5.15 with a possible indication of one at *ca.* pH 8.0. This latter was based on only slight change in absorbance and is useful only in confirming the potentiometric titrations.

Potentiometric titrations of $\gamma(B,A)$ as isolated by Schroeder[9] showed that the material is soluble in acid and alkali and precipitates in the neutral region. Estimate of the lower pK_a on alkaline titration was 4.95; of the higher pK_a on acid titration was 7.6. On acid titration of alkaline dissolved $\gamma(B,A)$, the pH rose momentarily as precipitation started at pH 7.6.

On alkaline titration of acid dissolved $\gamma(B,A)$, the pH dropped momentarily as precipitation started at pH 4.9. The titer assigned to both groups gave an apparent equivalent weight of 159.5. Thus since two groups are most probably represented, the best estimate of the molecular weight of $\gamma(B,A)$ is 320. Titration of isolate $\gamma(A,A)$ gave results consistent with the values

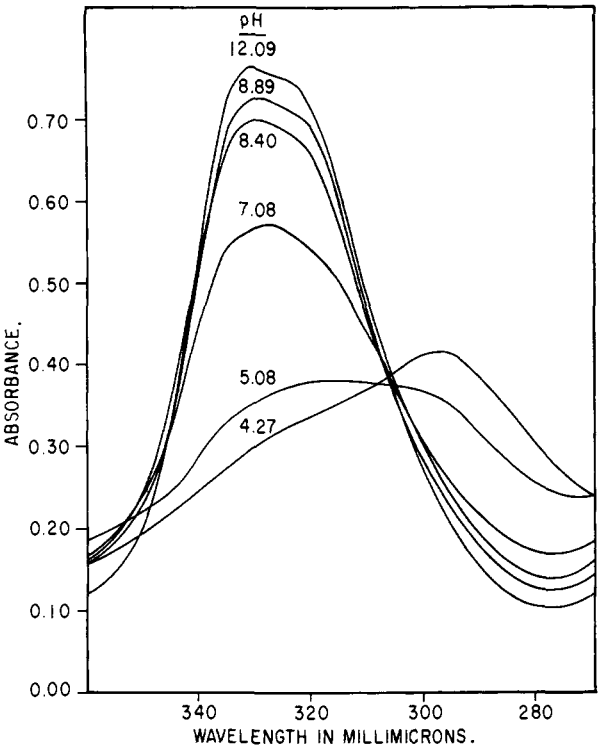


Fig. 10.—Effect of pH change on the instantaneous spectra of the second alkaline degradation product of porfiromycin, γ(B,B) at 30°. The concentration based on the original triethylamine salt of β(B) was 16 γ/ml. Each spectral curve is labeled with the pH of the solution.

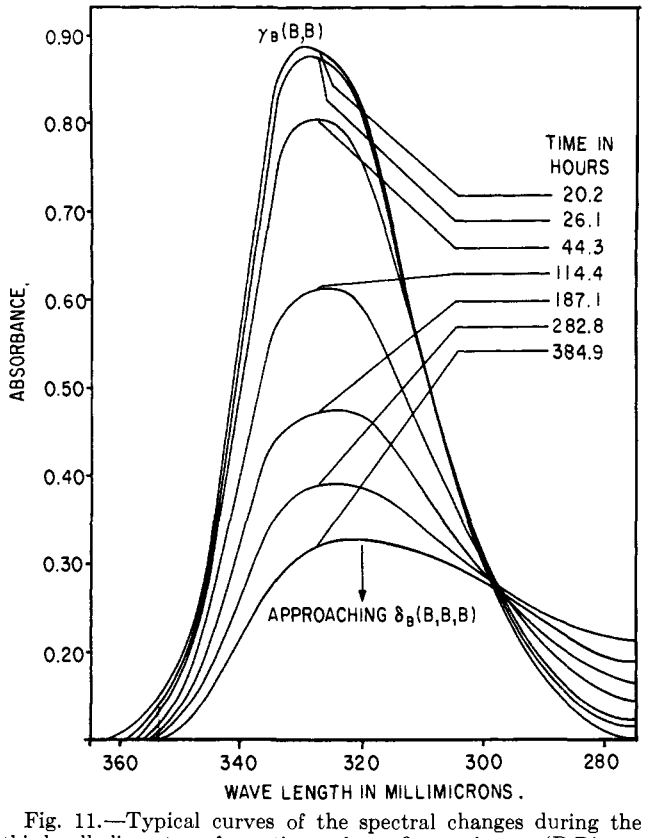


Fig. 11.—Typical curves of the spectral changes during the third alkaline transformation of porfiromycin, γ_B(B,B) → δ_B(B,B,B). The solution was at 30°, 0.1 *M* NaOH with an initial concentration of the triethylamine salt of β(B) of 15 γ/ml. Each curve is labeled as to the number of hr. after the start of the degradation.

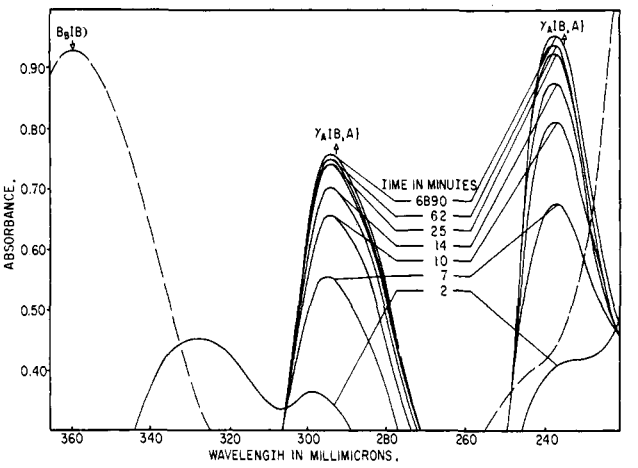


Fig. 12.—Typical curves of the spectral changes of β(B) in 0.01 *M* HCl at 30°, β_B(B) → γ_A(B,A). The initial concentration as porfiromycin was 14 γ/ml. Each curve is labeled as to the number of min. after the start of the degradation. The dashed curve is the spectrum of β_B(B) in alkaline solution.

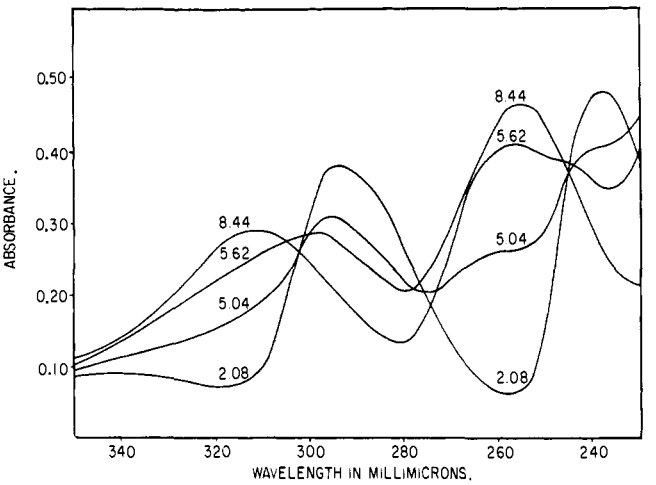


Fig. 13.—Effect of pH change on the spectra of the acid degraded first alkaline degradation product of porfiromycin, γ(B,A). The concentration of this material was 14 γ/ml. Each spectral curve is labeled with the pH of the solution.

from the titration of isolated γ(B,A). There were no differences in the infrared spectra. In alkaline solution γ(B,A) and γ(A,A) are purple; in acid solution they are both yellow. The spectra of γ(B,A) as characterized by maxima at 312 mμ and 255 mμ in alkali are transformed by alkali to a spectrum of γ_B(B,A,B) with a maximum at 298 mμ, a = 45.2 at pH 12.45 (Fig. 14). This spectral transformation is coincident with that of γ_B(A,A) to δ(A,A,B) (Fig. 8). The rate constants and conditions for the γ(B,A) $\xrightarrow{OH^-}$ δ(B,A,B) are given in Table VI for the apparent first order transformation as based on the loss of the 255 mμ chromophore.

The dependence of the log rate constant, k, on pH is shown in Fig. 5. This dependency is coincident with that of γ(A,A) $\xrightarrow{k}$ δ(A,A,B), eq. 15, 17, and 18. It is thus strongly indicated that δ(A,A,B) and δ(B,A,B) are the same compound and thus γ(A,A) and γ(B,A) are the same compound. Also, the γ(A,B) has spectra similar to δ(B,A,B) and δ(A,A,B) so that all three of these products may be considered as similar.

The spectrum of the degradation intermediate δ(B,A,B) = δ(A,A,B) = γ(A,B) shows a shift with pH from 295 mμ (pH 12.5) to 290 mμ (11.9 pH) and a slight shift at pH 2.4 to 285 mμ These phenomena indicate a pK_a *ca.* 12.2 by spectra and possible pK_a *ca.* 3-5. There were indications of irreversible reaction in that neutralization to pH 10-12 of the acidified material did not completely restore the γ_B(B,A,B) spectrum.

Acid Degradation of the Second Alkaline Degradation Product γ(B,B) → δ(B,B,A).—This reaction goes extremely fast so that the γ(B,B) chromophore is irreversibly changed on acidification or

even on adjustment of the solution to neutrality. The γ_B(B,B) maximum of 333 mμ become the δ_A(B,B,A) maxima of 295 mμ (a = 40.5) and 238 mμ (a = 45.1) at pH 2.4. In alkali the spectra of δ_B(B,B,A) shift to maxima at 312 mμ (a = 36.7) and 255 mμ (a = 47.8). The greatest change in absorbance with pH occurs at pH *ca.* 5.0 so that this is a good estimate of pK_a. Although the absorptivities which are based on the concentration of the original porfiromycin are not quite the same for δ(B,B,A) and γ(B,A) = γ(A,A), yet the positions and pH shifts of the maxima are the same. The estimated pK_a are the same. It is possible that these compounds may be very similar in their fundamental chromophore.

The Products of Porfiromycin Degradation.—A complete tabulation of the distinct porducts of porfiromycin degradation in solution is listed in Table VII. This table also gives the reversible equilibria due to dissociation of prosthetic groups, the estimated pK_a of these groups as obtained by spectrophotometric and potentiometric titration of the products isolated or as obtained *in situ*, the estimated molecular weights, and the spectral characteristics of the compounds. In addition, the mode of degradation and its rate dependence are given as well as quantitative empirical and kinetic expressions that permit calculation of first order rates of transformations as functions of pH and catalytic species.

Discussion

Subsequent to these studies and the analysis of the transformations reported in Table VII, the structure, I (Fig. 15) was assigned to porfiromycin, α, $C_{16}H_{20}N_4O_5$ (molecular weight 348)[2b] by Webb, *et al.*[14]

The lack of a spectrophotometrically or potentiometrically observed pK_a in aqueous solution for porfiromycin[2b] implies that the 7-amino group and the 1,2-fused ring aziridine are nontitratable.[15] The mild acid hydrolysis of porfiromycin, $\alpha \xrightarrow{H} \beta$(A), in the light of the assignment of structure I to porfiromycin is reasonably assigned to the transformation of structure I to II, β(A), $C_{15}H_{18}N_4O_5$ (mol. wt. 334). Thus, the fused ring aziridine group 1, 1a, 2 of I is the group titratable in glacial acetic acid and in dioxane (see Table VII) with perchloric acid.[15] Since it is the group reactive to mild acid hydrolysis to II, the kinetically observed pK_a 1.5 (Fig. 3) is readily assignable to the aziridine nitrogen in I.[15]

The shift of the absorption maximum 363 mμ of I to 311 mμ of II (Fig. 1) is equivalent to that given for mitomycin A to *apo*-mitomycin A, from 320 mμ to 285 mμ, on mild acid hydrolysis. This latter transformation has also been assigned to solvolysis of the aziridine ring and the loss of the 9a-methoxyl.[14a] The difference between the wave lengths of the maxima of *apo*-mitomycin A,[14a] 285 mμ and 232 mμ, is similar to the difference between the absorption maxima of β(A), 311 mμ and 250 mμ, and the respective absorptivities of *apo*-mitomycin A and β(A) (Fig. 1 and Table VII) are of the same magnitudes.

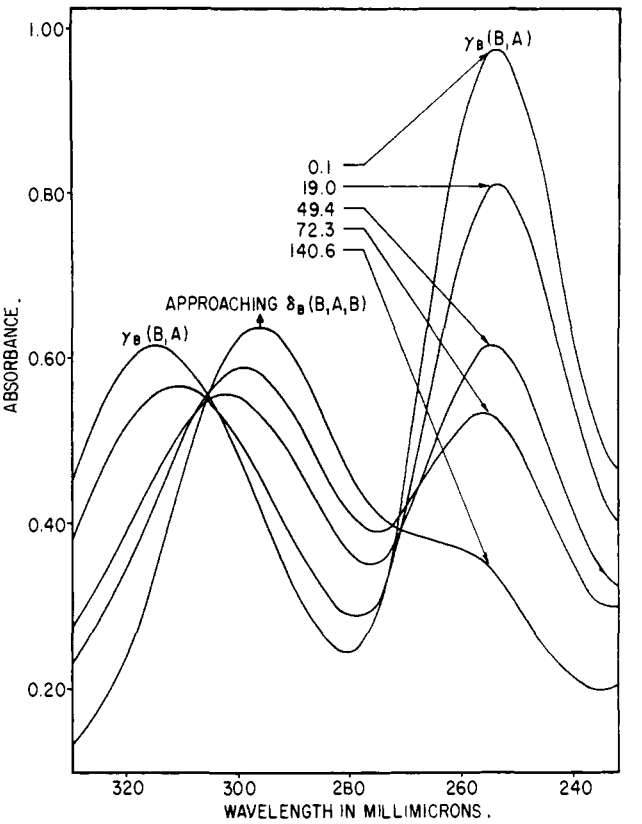


Fig. 14.—Typical curves of the spectral changes of γ(B,A) in 0.20 *M* NaOH at 30°, γ_B(B,A) → δ_B(B,A,B). The initial concentration as γ(B,A) was 15 γ/ml. Each curve is labeled as to the number of hr. after the start of the degradation.

The fact that the potentiometrically titratable group of pK_a 7 of β(A) (II) does not give large spectral changes during the titration (Fig. 4) is consistent with the assignment of the methylamine group at positions 1 or 2 in II and that it is distant from the ultraviolet chromophore.

Subsequent acid hydrolysis of II, β(A) $\xrightarrow{H^+}$ γ(A,A) can result in III. The spectral data, for γ(A,A) = γ(B,A) [λ_{max}^{HCl} 238 mμ (a = 70), 295 mμ (a = 55), λ_{max}^{NaOH} 255 mμ (a = 70), 313 mμ (a = 41)] are the same as those given for IIF, the comparable degradation product of mitomycin A.[14]

Compound γ(A,A) = γ(B,A) possesses an acidic and basic group of pK_a values 5 and 8. Spectrophotometric titration of γ(A,A) showed that the chromophore was greatly affected by the ionic form of the functionality of pK_a 5 (Fig. 6 and 7). This would be expected if this assignment was to the phenolic hydroxyl as the acidic function at the 7-position of III. The titration of the pK_a 8 had only minor effect on the chromophore, indicated by a slight displacement of the isosbestic points (Fig. 6), and this pK_a can be assigned to the methylamine group at the 2-position of III. This implies that III can exist as a zwitterion in aqueous solution.[16]

The slow transformation of the chromophore of β(A) by alkali to γ(A,B) can be reconciled to the postulated structure II by assuming that alkaline solvolysis of II results in the hydrolysis of the carbamate group at

(14) (a) J. S. Webb, D. B. Cosulich, J. H. Mowat, J. B. Patrick, R. W. Broschard, W. E. Meyer, R. P. Williams, C. F. Wolf, W. Fulmor, C. Pidacks, and J. E. Lancaster, *J. Am. Chem. Soc.*, **84**, 3185 (1962); (b) J. S. Webb, D. B. Cosulich, J. H. Mowat, J. B. Patrick, R. W. Broschard, W. E. Meyer, R. P. Williams, C. F. Wolf, W. Fulmor, C. Pidacks, and J. E. Lancaster, *ibid.*, **84**, 3187 (1962); (c) A. Tulinsky, *ibid.*, **84**, 3188 (1962).

(15) The lack of a titrable pK_a in the range 8–9, characteristic of the ethyl enimine function [C. E. O'Rourke, L. B. Clapp and J. O. Edwards, *ibid.*, **78**, 2159 (1956)] would argue *a priori* against the presence of an aziridine group in porfiromycin. The stated half-lives of 16 hr. at 25° and 3.5 hr. at 35° for the acid hydrolysis of 2,2-dimethylethyleneimine in 1 *M* HCl [V. B. Schatz and L. B. Clapp, *ibid.*, **77**, 5113 (1955)] would certainly not favor the prediction of a half-life of 0.1 hr. in 0.01 *M* HCl at 30° for the acid hydrolysis of an aziridine which was observed for porfiromycin. Of course, it can be argued that the nature of the ring system fused to the aziridine in porfiromycin lowered the pK_a of the cyclic nitrogen functionality to the pK_a *ca.* 1.5. This pK_a was originally considered as possibly being an amide. However, this decreased basicity should act against, rather than for the observed increased rate of acid-catalyzed solvolysis of the ethyleneimine in I to the substituted ethanolamine of II. A possible explanation for the discrepancies in stability of the aziridine ring in contrast to the models may lie in the participation of the methoxyl *trans* to the aziridine ring in I. Notwithstanding these facts, the discussion of the text is an attempt to reconcile the data of this paper with the proposed structure (I) of porfiromycin.

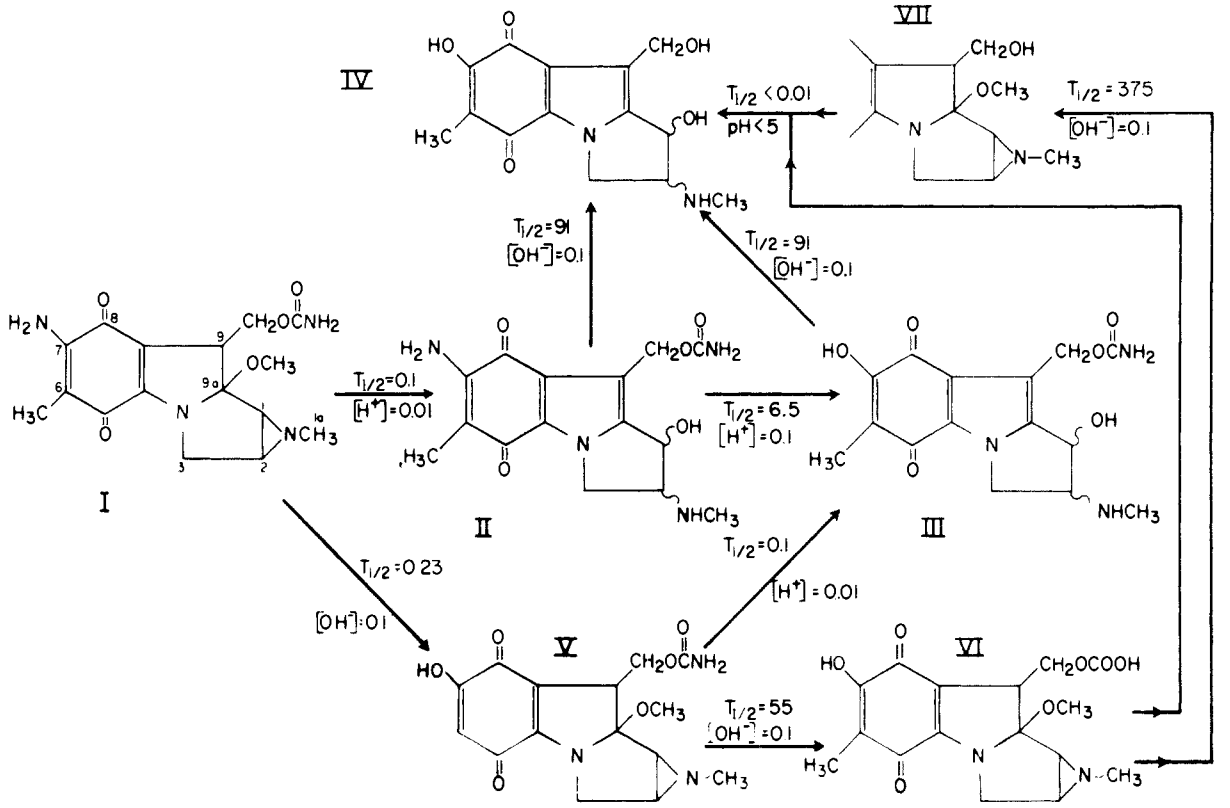
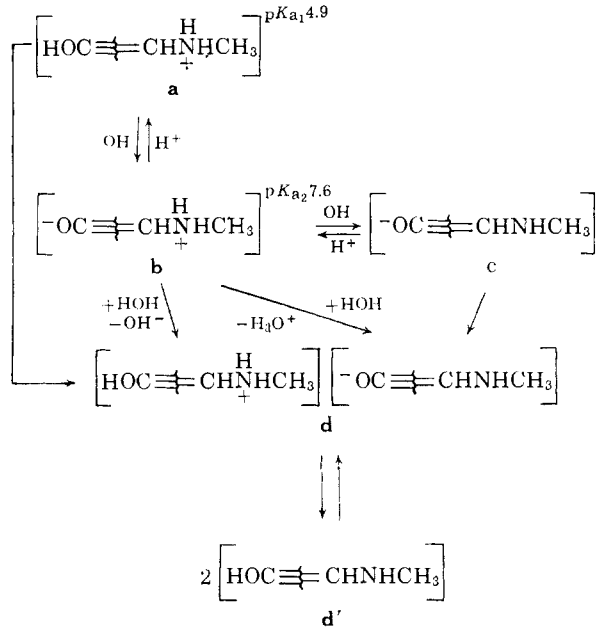


Fig. 15.—Schemes for acidic and basic degradations of porfiromycin at 30°. The half-lives, $t_{\frac{1}{2}}$, are in hours.

position 9 as in the case of *apo*-mitomycin A[14b] so that the resultant γ(A,B) is IV or some rearrangement product thereof.

(16) Since acid titration of alkaline dissolved material, γ(B,A) = γ(A,A), gave a significant pH rise on precipitation and alkaline titration of acid dissolved material gave a significant pH decrease on precipitation, the following models can be postulated to fit the assigned pK_a values and these facts



It can be postulated that on alkaline titration of a to b, since the pK_a values are so close, some c results and the solution could be supersaturated with respect to d. With the incipient precipitation, a and b can coprecipitate to give d and/or d'. Thus hydrogen ions are released and the pH significantly decreased concomitantly. Similarly, on acid titration of c to b, some a results and c and b coprecipitate to give d and/or d'. Thus hydroxyl ions are released and the pH significantly increased concomitant with the incipient precipitation.

The slow transformation of the chromophore of γ(A,A) (III) to δ(A,A,B) by alkali has rates and rate dependencies similar to the β(A) to γ(A,B) transformation and implies that the same rate determining step in the transformation of the chromophore is involved in both. Also the spectra of δ(A,A,B) and γ(A,B), the spectrophotometric pK_a values, and the shifts in spectra on acidification are similar. This information can be rationalized on the basis that β(A) of structure II is readily transformed to III by alkali. However, since the spectra of II and III in alkaline solution are similar, this cannot be observed kinetically. Thus, in the alkaline transformations of both γ(A,A) and β(A) it is actually the kinetics of III and IV that are being followed spectrophotometrically.

This thesis can find further support in the rapid alkaline transformation of porfiromycin, $\alpha \rightarrow \beta$(B) where only minor spectral changes occur at 365 mμ (Fig. 9). The resultant product β(B) is a salt-forming acid and would be consistent with V, the product of I by solvolytic substitution of the amine at the 7-position.

The spectral shifts of the β(B) with pH [λ_{max}^{NaOH} 360 mμ (a = 67), 330 mμ (a = 36), λ_{max}^{HCl} 295 mμ (a = 55), 238 mμ (a = 69)] and the pK_a 4.4 (5.5 in 50% ethanol–water, v./v.) are consistent with structure V. It must be noted however that in alkaline solutions the spectra of V more closely resemble the spectra of porfiromycin (I) than that of III with the similar phenate at the 7-position. However, in acid solutions, the spectra of V and III are nearly equivalent. The very fast acid hydrolysis of β(B) (V) is easily associated with the solvolysis of the aziridine ring to give III [γ(B,A) =

γ(A,A)] (Fig. 12). The subsequent slow alkaline hydrolysis of β(B) could be assigned to the solvolysis of the carbamate at position 10 to give γ(B,B) which could be deduced to be VI, where the resultant spectrophotometric pK_a of *ca.* 7 can still be assigned to the phenolic hydroxyl.

Although the λ_{max}^{NaOH} is shifted from 360 mμ (a = 67) for β(B) (V) to 233 mμ (a = 52) for γ(B,B) (VI) the shapes of the absorption curves and the absorptivities are similar.

The extremely fast hydrolysis of γ(B,B) (VI) to δ(B,B,A) (IV) on acidification of the solution can be assigned on the basis of these postulated structures to the solvolysis of the aziridine ring to give IV (with a spectrophotometric pK_a *ca.* 5 as expected) plus other rearranged products. The actual δ(B,B,A) appears to be a mixture of products.

Further alkaline solvolysis of γ(B,B) to δ(B,B,B) kinetically demonstrates a new pK_a value of *ca.* 11 absent in β(B) and which could be assigned to an uncharged acid. The acid and basic forms due to this pK_a did not significantly affect the chromophore of γ(B,B). This leads to the preference of structure VI for γ(B,B) rather than a hydroxymethyl at the 9-position. δ(B,B,B) also reacted quickly on mild acidification to indicate further that the aziridine group is not readily attacked by alkali. The further alkaline hydrolysis of γ(B,B) so diminished the chromophoric absorptivities that more drastic structural changes should be postulated although VII is a reasonably major product.

Except for γ(B,B) which has been explained on other grounds, the kinetically observed pK_a *ca.* 11–13 of a weak uncharged acid was demonstrated only when these structure assignments were consistent with the presence of the secondary ethanolamine function[14] assigned to positions 1 and 2 as in β(A), γ(A,A), and γ(B,A). It is probable that this group has weak acid character.

Structure and Biological Activity.—The fascinating change of biological activity with the physicochemical transformations of porfiromycin permit definitive assignment of action. Porfiromycin (I) had both Gram-positive and Gram-negative activity. In all cases when the postulated fused ring aziridine[14] may be considered intact as in porfiromycin (I), β(B) (*i.e.*, V), or γ(B,B) (*i.e.*, VI), antibacterial activity was retained. Modification of other portions of the molecule as in V and VI by alkali only modified the kind and degree of biological activity. For example, the replacement of the 7-amino by a hydroxyl group reduced the Gram-positive activity, whereas Gram-negative activity was retained in both β(B) and γ(B,B). The loss of all activities can be considered concomitant with the loss of the aziridine ring on acid solvolysis.

Acknowledgments—The author is greatly indebted to Mrs. Lillian G. Snyder and Mr. Dennis J. Weber for excellent technical assistance, to Dr. G. B. Whitfield and associates for microbiological assays, and to Dr. W. Schroeder and Mr. C. DeBoer for their discussion and interest.

The Preparation and Bacteriostatic Activity of Halogenated Carbanilates

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The preparation and *in vitro* bacteriostatic activity of some halogenated carbanilates against *Staphylococcus aureus* is described. The relationship of chemical structure to specific activity is discussed.

The relationship of bacteriostatic activity to the chemical structure of two series of substituted carbanilides[1] has been reported previously. The effect of substitution on the phenyl rings of the carbanilides was fairly well established and further investigation was directed toward replacing the urea bridge with isoteric and non similar bridges. This paper reports the series of highly active halogenated carbanilates, several of which are active in dilutions of 1–10 million. The physical data for 107 carbanilates are given in Tables I to III which are numbered consecutively for easy cross reference with their bacteriostatic activities. Throughout this paper, the figures given under activity refer to the maximum dilution which will completely inhibit the growth *in vitro* of the test organism *Staphylococcus aureus*. The bacteriostatic test procedure is given in the Experimental part.

As reported previously, maximum activity in the halogenated carbanilides was obtained when chlorine was introduced into the 3- and 4-positions of one phenyl ring and the 3- and/or 4-positions of the second ring (1) but substitution of any *ortho* position reduced drastically or completely suppressed activity. Since the phenyl esters of carbanilic acid may be viewed as being formed by replacing the urea bridge, –NHCONH–, with the carbamate bridge, NHCOO–, the compounds given in Table I show, in most cases, the same specificity as was found in the urea series. The maximum activity was obtained when chlorine was introduced into the 3- and 4-positions of the carbanilic phenyl ring and the 3- and 4-positions of the phenyl ester ring (**91**). However, unlike the carbanilides, activity was lost or lowered when the phenyl ester ring was only monosubstituted in the 3- or 4-positions (**89**–**90**).

At this point the 3,4-dichlorophenyl moiety was retained as an essential element for activity and a series of aliphatic esters was prepared.

Activity appears to improve gradually as the carbon chain increases in length, reaching a maximum and plateauing at C_4 to C_8, but dropping in effectiveness

(1) D. J. Beaver, D. P. Roman, and P. J. Stoffel, *J. Am. Chem. Soc.*, **79**, 1236 (1957); *J. Org. Chem.*, **24**, 1676 (1959).