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pH Effects on the Kinetics of the Cystine–Cyanide Reaction

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Investigation of the kinetic dependency on pH of the cystine–cyanide reaction at 28° and over the pH range 7.5 to 12.5 yields bimolecular rate constants (mole^{-1} l. min.$^{-1}$) of 60, 1.65, and 0.30 for the reaction of the respective cystine species, cystine$^{\pm\pm}$, cystine$^{\pm-}$, and cystine^{--}, with cyanide anion. Comparison of these rate constants with the corresponding constants for the cystine–sulfite reaction indicates sulfite to be more S-nucleophilic than cyanide toward cystine and cystine derivatives. In both the cyanide and sulfite reactions the magnitude of the rate constant increases markedly with protonation of cystine and it is suggested that, in addition to electrostatic effects, intramolecular catalysis by hydrogen ion may be responsible for this effect.

It was deemed of interest to undertake an investigation of the kinetic dependency on pH of the cystine–cyanide reaction for its intrinsic value in elucidating the effect of charge on the cystine molecule on the reaction rate and as a preliminary basis for the understanding of differential reactivity of protein disulfide bonds[1] toward cyanide. Such a study has not heretofore been undertaken despite the obvious number of pH dependent species which might participate in the reaction. The kinetics of the cystine–cyanide reaction have previously been demonstrated[2] to be bimolecular and preliminary investigation of pH effects on reaction rates indicated a complex dependence of rates on pH, and that at room temperature the reaction of hydrogen cyanide, compared to cyanide anion, with cystine is negligibly slow.[2,3] The work reported herein was accordingly carried out over the pH range 7.5 to 12.5. The kinetic runs were carried out at constant ionic strength in the presence of a tenfold excess of total cyanide, adjustment of pH and buffering being attained by variation of the cyanide anion to hydrocyanic acid concentration.

As expected on theoretical grounds and by analogy with the cystine–sulfite reaction[4] rate dependency of the cystine–cyanide reaction on pH is satisfactorily interpreted over the pH range studied on the basis of cyanide anion as the attacking species, and RSSR$^{\pm\pm}$, RSSR$^{\pm}$, and RSSR^{--} as the reactive cystine species.[5] As further expected, the individual bimolecular rate constants increase with increasing positive charge on the cystine molecule.

Experimental

Materials.—Fisher A.R. potassium cyanide from freshly opened bottles was used. Fresh solutions were prepared prior to each run and were assayed with silver nitrate.[6] The reagent assayed 98.5% potassium cyanide.

Reagent grade L-cystine from Nutritionals Biochemical Corp. was dried *in vacuo* over P_2O_5 and used as such. Assay by amperometric titration[7] indicated 99.6% purity.

Kinetic Procedure.—For each run the following stock solutions were made and equilibrated at 28°: 0.012 *M* cystine in water with the addition of 2 equiv. of potassium hydroxide; 0.120 *M* potassium cyanide; dilute (0.012 to 0.150 *M*) nitric acid solution. Prior to a run, 5.00 ml. of a given nitric acid solution was added to 5.00 ml. of the cyanide solution and immediately followed (zero time) by 5.00 ml. of the cystine solution. The flask was then stoppered and, at intervals of 5 min., 1 ml. aliquots were removed over the first 25 min. of the reaction. The aliquots were immediately plunged into 50 ml. of 0.01 *N* sulfuric acid, effectively stopping the reaction, and subsequently titrated amperometrically, as given below, for thiol content. The pH of each run was measured in a duplicate tube and did not vary over the course of a run, the excess cyanide effectively buffering the reaction. The nitric acid solutions indicated above covered the pH range 7.6 to 10.85. The run at pH 11.43 was carried out in the absence of added nitric acid and that at pH 12.5 was carried out in 0.04 *M* potassium hydroxide. The ionic strength μ = 0.052, was constant for all runs except that at pH 12.5. At pH 12.5 the ionic strength was 0.092.

For calculation of kinetic constants the data for a given run were treated according to the first-order rate law since the reactions go to completion[2] and were carried out at a tenfold ratio of total cyanide to cystine. The pseudo-first-order rate constants, k', for each run were obtained from the slope of the usual plot (Fig. 1) or by calculation of k' at each experimental point.

Amperometric Titration.[8,9]—The titration of thiol in 0.01 *N* sulfuric acid was carried out with the Sargent Ampot at an applied voltage of −0.4 v. utilizing a rotating platinum electrode and a Beckman saturated calomel electrode. The titrant was standard silver nitrate, 0.001 *N*, and was standardized against cysteine. In 0.01 *N* sulfuric acid cyanide does not titrate with silver. Under these conditions, thiocyanate is titrated. However, as previously observed,[2] thiocyanate is not liberated during the reaction. Between titrations the rotating platinum electrode was stored in concentrated nitric acid.

Results

Treatment of the kinetic data obtained is based on the warranted assumption that at 28° and over the pH range 7.5 to 12.5 cyanide anion reacts with each of the three charge forms of cystine present in this pH interval. Accordingly, three separate rate equations may be written

$$v_1 = k_1(\mathrm{RSSR}^{\pm\pm})(\mathrm{CN}^-) \quad (1)$$

$$v_2 = k_2(\mathrm{RSSR}^{\pm-})(\mathrm{CN}^-) \quad (2)$$

$$v_3 = k_3(\mathrm{RSSR}^{--})(\mathrm{CN}^-) \quad (3)$$

and these may be combined into the over-all rate equation

$$v = [k_1(\mathrm{RSSR}^{\pm\pm}) + k_2(\mathrm{RSSR}^{\pm-}) + k_3(\mathrm{RSSR}^{--})](\mathrm{CN}^-) \quad (4)$$

Considering the over-all initial rate, eq. 4 becomes

$$v_0 = [k_1(\mathrm{RSSR}^{\pm\pm})_0 + k_2(\mathrm{RSSR}^{\pm-})_0 + k_3(\mathrm{RSSR}^{--})_0](\mathrm{CN}^-) \quad (5)$$

(1) For example, at pH 12.5, only one of the four disulfide bonds of ribonuclease is attacked at a cyanide to ribonuclease molar ratio of 2.8: O. Gawron and R. Windisch, unpublished work.

(2) O. Gawron and J. Fernando, *J. Am. Chem. Soc.*, **83**, 2906 (1961).

(3) H. Fraenkel-Conrat, *ibid.*, **63**, 2533 (1941).

(4) For a general discussion of the effect of charge on this reaction, see R. Cecil and J. R. McPhee, "Advances in Protein Chemistry," Vol. XIV, Academic Press, Inc., New York, N. Y., 1959, p. 303.

(5) RSSR$^{\pm\pm}$, RSSR$^{\pm-}$ and RSSR^{--} represent cystine zwitterion, cystine with a negative charge of unity, and cystine with a negative charge of two, respectively.

(6) I. M. Kolthoff and E. B. Sandell, "Textbook of Quantitative Inorganic Analysis," The Macmillan Co., New York, N. Y., 1952, pp. 458–460.

(7) I. M. Kolthoff and W. Stricks, *J. Am. Chem. Soc.*, **72**, 1952 (1950).

(8) O. Gawron, J. Fernando, and M. McMennamin, unpublished work.

(9) M. McMennamin, M.S. Thesis, Duquesne University, June, 1962.

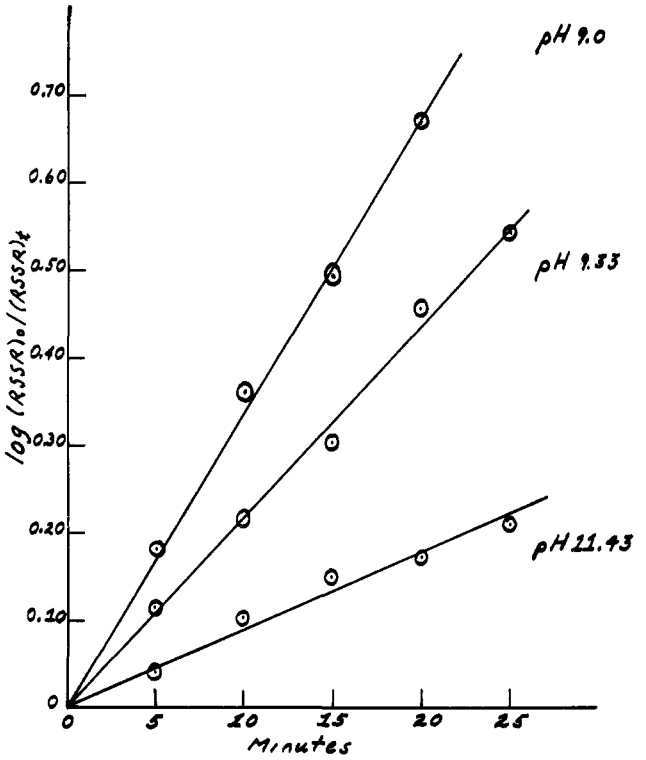


Fig. 1.—First-order plots for several runs at 28° and different pH values: total cystine concentration, 0.004 M; total cyanide concentration, 0.040 M.

Since each kinetic run follows first order kinetics for the disappearance of cystine by virtue of its having been carried out with excess total cyanide, the initial rate is also obtained from the relationship

$$v_0 = k'(\mathrm{RSSR_T})_0$$

where k' is the experimental first-order rate constant and $(\mathrm{RSSR_T})_0$ represents total initial cystine concentration.

Equation 5 may then be written as

$$k'(\mathrm{RSSR_T})_0 = [k_1(\mathrm{RSSR^{\pm\pm}})_0 + k_2(\mathrm{RSSR^{\pm -}})_0 + k_3(\mathrm{RSSR^{--}})_0](\mathrm{CN^-})_0 \quad (6)$$

and by substitution from the appropriate equilibrium expression

$$K_{\mathrm{HCN}} = \frac{(\mathrm{H^+})(\mathrm{CN^-})}{(\mathrm{HCN})} \quad (7)$$

$$K_3 = \frac{(\mathrm{H^+})(\mathrm{RSSR^{\pm -}})}{(\mathrm{RSSR^{\pm\pm}})} \quad (8)$$

$$K_4 = \frac{(\mathrm{H^+})(\mathrm{RSSR^{--}})}{(\mathrm{RSSR^{\pm -}})} \quad (9)$$

terms involving $(\mathrm{RSSR_T})_0$, $(\mathrm{H^+})$, $(\mathrm{CN_T})_0$, and ionization constants for the terms on the right side of eq. 6,

$$k' = \left[\frac{k_1}{1 + \frac{K_3}{(\mathrm{H^+})} + \frac{K_3K_4}{(\mathrm{H^+})^2}} + \frac{k_2}{1 + \frac{(\mathrm{H^+})}{K_3} + \frac{K_4}{(\mathrm{H^+})}} + \frac{k_3}{1 + \frac{(\mathrm{H^+})}{K_4} + \frac{(\mathrm{H^+})^2}{K_3K_4}}\right]\frac{(\mathrm{CN_T})_0K_{\mathrm{HCN}}}{(\mathrm{H^+}) + K_{\mathrm{HCN}}} \quad (10)$$

eq. 10 results, $(\mathrm{CN_T})_0$ being the total added cyanide concentration.

For evaluation of the individual bimolecular rate constants, k_1, k_2, and k_3, eq. 10 was rearranged to eq. 11

$$k'\left[\frac{(\mathrm{H^+}) + K_{\mathrm{HCN}}}{(\mathrm{CN_T})_0K_{\mathrm{HCN}}}\right] = \frac{k_1}{1 + \frac{K_3}{(\mathrm{H^+})} + \frac{K_3K_4}{(\mathrm{H^+})^2}} + \frac{k_2}{1 + \frac{(\mathrm{H^+})}{K_3} + \frac{K_4}{(\mathrm{H^+})}} + \frac{k_3}{1 + \frac{(\mathrm{H^+})}{K_4} + \frac{(\mathrm{H^+})^2}{K_3K_4}} \quad (11)$$

and the term on the left side of eq. 11 was plotted against pH. For calculation of the left-hand term the experimental value,[10,11] 5.01×10^{-10} ($pK = 9.30$), for the dissociation of hydrocyanic acid was used. Two such plots were constructed, one covering the pH 7.5 to 9.5 range, and the other, the pH 9.0 to 12.5 range. For calculation of the individual rate constants pK_3 and pK_4 values[12] of 8.00 and 10.25, respectively, were used and it is to be noted that unless the numerical values of k_1 and k_2 are extremely high all terms but that involving k_3 on the right side of eq. 11 become negligible at pH 12.5. Accordingly k_3 can be calculated directly from the experimental value of k' at pH 12.5 and a value of 0.30 $\mathrm{mole^{-1}}$ l. $\mathrm{min.^{-1}}$ is thus found. In a similar way the term involving k_1 is negligible in the pH 11 region and k_2 was calculated from eq. 11 using the value previously obtained for k_3, and corresponding $k'[((\mathrm{H^+}) + K_{\mathrm{HCN}})/(\mathrm{CN_T})_0K_{\mathrm{HCN}}]$ and pH values over the pH range 10 to 11 selected from the plot covering the pH range 9.0 to 12.5. A value of 1.6 ± 0.1 $\mathrm{mole^{-1}}$ l. $\mathrm{min.^{-1}}$ for k_2 was found. For the calculation of k_1, corresponding values of $k'[((\mathrm{H^+}) + K_{\mathrm{HCN}})/(\mathrm{CN_T})_0K_{\mathrm{HCN}}]$ and pH were selected over the pH range 8.5 to 9.5 from the plot covering the pH range 7.5 to 9.5. Utilizing these values and the previously obtained k_3 and k_2 values, eq. 11 was solved for k_1 to give a value of 60 ± 5 $\mathrm{mole^{-1}}$ l. $\mathrm{min.^{-1}}$.

The values of k_1, k_2, and k_3 obtained as described above were used to construct the theoretical (solid curves, calculated from eq. 11 and 10) plots presented in Fig. 2 and 3. Figure 2 is a plot of $k'[((\mathrm{H^+}) + K_{\mathrm{HCN}})/(\mathrm{CN_T})_0K_{\mathrm{HCN}}]$ *vs.* pH, the $k'[((\mathrm{H^+}) + K_{\mathrm{HCN}})/(\mathrm{CN_T})_0K_{\mathrm{HCN}}]$ values being calculated from eq. 11 at appropriate pH intervals and Fig. 3 is a plot of k' *vs.* pH, the k' values being calculated from eq. 10 at appropriate pH intervals. It is to be noted that in both plots the experimental points agree reasonably well with the calculated curve.

Discussion

Table I presents the kinetic constants obtained at 28° in this investigation and also the comparable constants for the reaction of sulfite and bisulfite ions with the several ionic species of cystine.[13,14] From Table I it is to be noted that the relative effect of cystine charge is the same for reaction with cyanide ion and for reaction with sulfite ion. In both instances, relative

(10) Determined at 28° by potentiometric titration of potassium cyanide with standard nitric acid and obtained from the plot of pH *vs.* log (HCN)/($\mathrm{CN^-}$); O. Gawron and S. Mahboob, unpublished.

(11) H. T. S. Britton and R. A. Robinson, *Trans. Faraday Soc.*, **28**, 535 (1932), report a pK of 9.32 at 25° as determined by potentiometric titration.

(12) H. Borsook, E. L. Ellis, and H. M. Huffman, *J. Biol. Chem.*, **117**, 281 (1937); thermodynamic ionization constants at 25°.

(13) R. Cecil and J. R. McPhee, *Biochem. J.*, **60**, 496 (1955).

(14) J. R. McPhee, *ibid.*, **64**, 22 (1956).

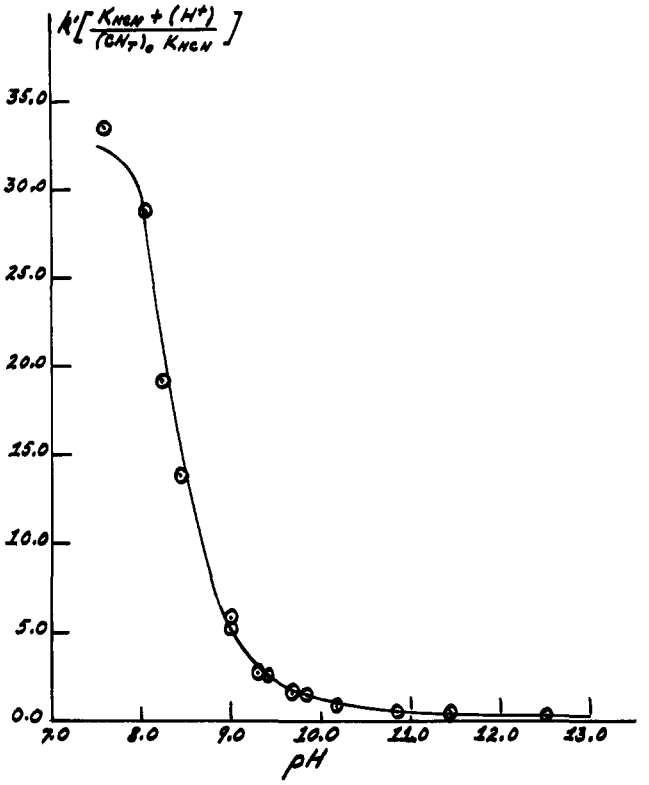


Fig. 2.—Plot of k', the pseudo-first-order rate constant, divided by the initial cyanide ion concentration (calculated from the measured pH) *vs.* pH: open circles, experimental points; smooth curve, calculated as described in the text; k' in units of min.$^{-1}$; cyanide ion concentration in moles/l.

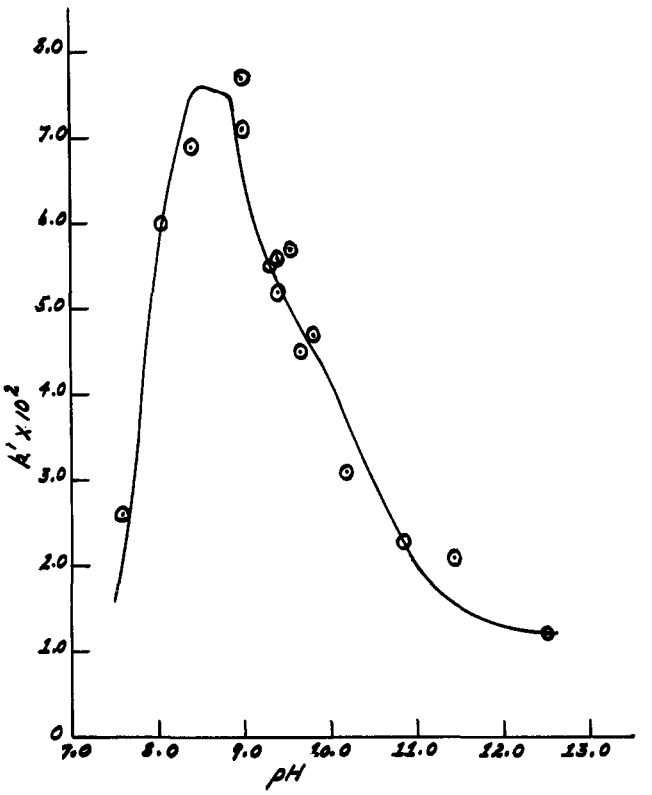


Fig. 3.—Plot of k', the pseudo-first-order rate constant, *vs.* pH: open circles, experimental points; smooth curve calculated as described in the text; k' in units of min.$^{-1}$.

rates decrease markedly as the net charge on the disulfide becomes more negative, and this decrease in rate is presumably attributable to a lessened electrostatic attraction for the attacking anion. In this connection, it is also of interest to note that all three bimolecular rate constants for reaction with sulfide ion are higher than the corresponding rate constants for cyanide attack. Similarly, the bimolecular rate constant,[13] 9.0 mole^{-1} l. min.$^{-1}$ at 25° for the reaction of sulfite with N,N′-diformylcystine dianion, is higher than the bimolecular rate constant,[2] 1.5 mole^{-1} l. min.$^{-1}$ at 35°, for the corresponding reaction with cyanide. It is thus apparent that in the above reactions and in a kinetic sense sulfite is more S-nucleophilic than cyanide albeit, because of diplacement of SO_3^{-2} from RS-SO_3^- by CN^-, cyanide has been considered more nucleophilic.[15,16] While the faster rate of sulfite, as compared to cyanide, with cystine$^{\pm\pm}$ and cystine$^{\pm-}$ might be expected on the basis of greater electrostatic attraction for sulfite than for cyanide by cystine species bearing positive charge, independent of net charge, the faster rate of sulfite with cystine^{--}[17] and with N,N′-diformylcystine dianion is not explicable on this basis. Indeed, in these latter instances, it might be expected, because of increased electrostatic repulsion, that cyanide would react faster. A possible explanation for the above observations may reside in the fact that divalent anions of oxygen acids interact to a greater degree than do monovalent anions with cystine.

(15) A. J. Parker and N. Kharasch, *Chem. Rev.*, **59**, 583 (1959).
(16) J. M. Swan, *Australian J. Chem.*, **14**, 69 (1961).
(17) The rate constants for the reaction of cyanide with cystine^{--} and for the reaction of sulfite with cystine^{--} are not strictly comparable since different pK_3 and pK_4 values were used for the calculations.

Sulfate ion, for example, has a marked salting-out effect on the solubility of cystine[18] and carbonate and phosphate inhibit[13,14] the cystine–sulfite and cystamine–sulfite reactions. The nature of such an interaction is, of course, problematical.

TABLE I
EFFECT OF CHARGE ON RATE CONSTANT

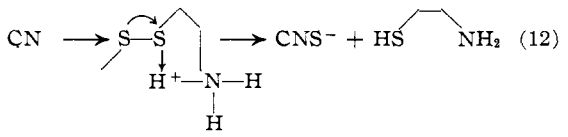
Species	Reactant	Rate constant[c]	Reactant[b]	Rate constant[a]	Reactant[a]	Rate constant[b]
Cystine$^{\pm\pm}$	CN^-	60	HSO_3^-	Negligible	SO_3^{-2}	1100
Cystine$^{\pm-}$	CN^-	1.65	HSO_3^-	Negligible	SO_3^{-2}	5–70
Cystine^{--}	CN^-	0.30	HSO_3^-	Negligible	SO_3^{-2}	3[c]

[a] R. Cecil and J. R. McPhee, *Biochem. J.*, **60**, 496 (1955); J. R. McPhee, *ibid.*, **64**, 22 (1956). [b] At 25°, mole^{-1} l. min.$^{-1}$. pK_3 and pK_4 values[d] 7.7 and 9.2, respectively, were used for calculation of constants. [c] In carbonate buffer. Reaction is inhibited by carbonate ions. [d] R. K. Cannan and C. J. G. Knight, *Biochem. J.*, **21**, 1384 (1927). [e] At 28°, mole^{-1} l. min.$^{-1}$.

In considering the relative values of the several rate constants for the three cystine forms reacting with a given reagent, cyanide or sulfite, it is also possible that intramolecular catalysis occasioned by intramolecular hydrogen bonding plays a role in determing the relative magnitude of the rate constant for a given cystine species. Models[19] of cystine can be easily constructed with a dihedral angle of 90° between the two carbon–sulfur bonds,[20] and hydrogen bonds between α-amino hydrogens and proximal sulfur atoms, and with both sulfur atoms free of steric hindrance to backside attack, the preferred direction of attack.[21] On the

(18) T. L. McMeekin, E. J. Cohn, and M. H. Blanchard, *J. Am. Chem. Soc.*, **59**, 2717 (1937).
(19) Constructed with Fisher–Hirschfelder–Taylor atom models.
(20) M. Calvin, *Federation Proc.*, **13**, 697 (1954).
(21) A. Fava and A. Iliceto, *J. Am. Chem. Soc.*, **80**, 3478 (1958).

basis of such a model, the reaction (12) may be catalyzed by hydrogen ion, acting intramolecularly.



Hydrogen ion catalysis of disulfide exchange, a related reaction, has been demonstrated[22,23] to occur with several cystine derivatives, and thus intramolecular hydrogen ion catalysis of cyanide and sulfite displacement on sulfur may also be operative, and the relative values of the rate constants, 200:5.5:1, for the reaction of cyanide with cystine$^{\pm\pm}$, cystine$^{\pm-}$, and cystine^{--}, respectively, may be due to both electrostatic and intramolecular catalytic effects. If such intramolecular catalytic effects occur, it is also of interest to note that ions such as carbonate and phosphate may exert their inhibitory effects by interfering with such intramolecular hydrogen ion catalysis.

Table I also indicates the negligible rate of reaction of bisulfite ion with cystine$^{\pm\pm}$ and, presumably, also with cystine$^{\pm-}$ and cystine^{--}. The negligible activity of bisulfite ion as an S-nucleophile in this reaction may be correlated with the weakness of bisulfite ion as a base, pK_a 1.9 for the conjugate acid, since, in general, the greater the base strength the greater is the degree of S-nucleophilicity.[11]

Acknowledgment.—Support of this work by Grant G-24212 from the National Science Foundation is gratefully acknowledged.

(22) A. P. Ryle and F. Sanger, *Biochem. J.*, **60**, 535 (1955).
(23) R. E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, **80**, 1666 (1958).

COMMUNICATIONS TO THE EDITOR

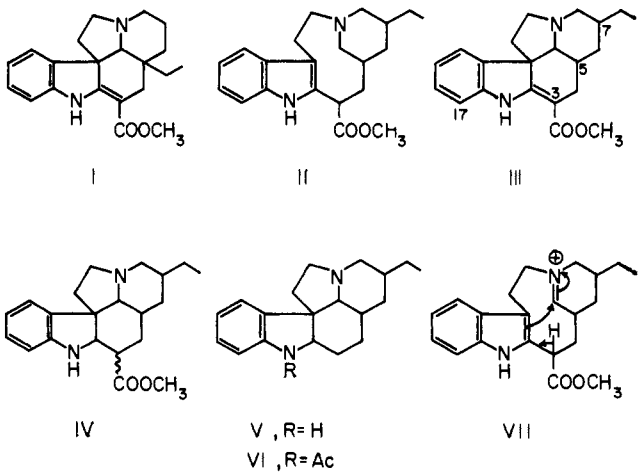
The Synthesis of a Vincadifformine-Type Skeleton *via* a Novel Transannular Cyclization Reaction

Sir:

The Aspidosperma alkaloids have received considerable attention for a long time and, particularly in recent years, a large number of these alkaloids have been isolated. One of the interesting variations of the Aspidosperma type skeleton is exemplified by the Vinca alkaloid, vincadifformine (I), recently elucidated largely by means of mass spectrometry.[1] This alkaloid and the structurally related alkaloids such as tabersonine,[2] minovincine,[3] minovincinine,[3] and vindolinine[4] are of considerable biogenetic interest since they may provide the connecting links between the aspidospermine- and akuammicine-type bases.[5] We now wish to present the first laboratory synthesis of a vincadifformine-type skeleton *via* a transannular cyclization reaction similar to the type proposed in Wenkert's biosynthetic hypothesis.[5]

In a previous communication[6] we were able to demonstrate the transannular cyclization of an ionic intermediate to an Aspidosperma skeleton and had suggested that an entry into the vincadifformine type of system could probably be realized by utilization of the appropriate carbomethoxydihydrocleavamine derivative. The results presented here provide experimental evidence in support of this claim.

Carbomethoxydihydrocleavamine (II),[7] on reaction with mercuric acetate in acetic acid at room temperature followed by reflux, provided a crude mixture which, after chromatographic separation, yielded a major product obtained as an amorphous powder and two other alkaloids in smaller amounts. These latter two substances are discussed in the accompanying communication[8] while evidence is presented here which establishes structure III for the amorphous product. This amorphous material possessed the molecular formula[9] $C_{21}H_{26}O_2N_2$, $[\alpha]^{26}D$ −503° (EtOH), and showed the following spectral properties. λ_{max}^{MeOH} 226, 298, and 326 mμ (log ϵ 4.07, 4.12, and 4.24); $\lambda_{max}^{CCl_4}$ 6.0 and 6.25 μ; n.m.r. signals[10]: 6.7–7.6 p.p.m., area = 4H; 8.95 p.p.m., area = 1H; 3.77 p.p.m., area = 3H, in good agreement with vincadifformine.[1] Chemical evidence in support of III was provided by zinc–sulfuric acid reduction[1,11] of the latter to yield two isomeric dihydro derivatives. The major product,[12] isolated as a white



(1) C. Djerassi, H. Budzikiewicz, J. M. Wilson, J. Gosset, J. Le Men, and M. M. Janot, *Tetrahedron Letters*, 235 (1962).
(2) M. Plat, J. Le Men, M. M. Janot, J. M. Wilson, H. Budzikiewicz, L. J. Durham, Y. Nakagawa, and C. Djerassi, *ibid.*, 271 (1962).
(3) M. Plat, J. Le Men, M. M. Janot, H. Budzikiewicz, J. M. Wilson, L. J. Durham, and C. Djerassi, *Bull. soc. chem. France*, 2237 (1962).
(4) C. Djerassi, S. E. Flores, H. Budzikiewicz, J. M. Wilson, L. J. Durham, J. Le Men, M. M. Janot, M. Plat, M. Gorman, and N. Neuss, *Proc. Natl. Acad. Sci.*, **48**, 113 (1962).
(5) E. Wenkert, *J. Am. Chem. Soc.*, **84**, 98 (1962).
(6) J. P. Kutney and E. Piers, *ibid.*, **86**, 953 (1964).
(7) We are very grateful to Dr. M. Gorman and Dr. N. Neuss, Eli Lilly Laboratories, for providing the experimental procedure for preparing this compound prior to publication. This compound was first prepared by Professor G. Büchi, Massachusetts Institute of Technology.
(8) J. P. Kutney, R. T. Brown, and E. Piers, *J. Am. Chem. Soc.*, **86**, 2287 (1964).
(9) Satisfactory analyses were obtained for all substances reported.
(10) All n.m.r. spectra were measured in deuteriochloroform with tetramethylsilane as the internal standard using a Varian A60 spectrometer. All signals are reported as (c.p.s./60) units in p.p.m.
(11) P. N. Edwards and G. F. Smith, *J. Chem. Soc.*, 152 (1961).
(12) A more detailed description of the dihydro derivatives and the various interconversions will be presented in our full paper.