Although the result of its reaction with the enzyme is inactivation, I can be considered to be one of a new class of chymotrypsin substrates (cf. ref. 11). Diphenylcarbamyl p-nitrophenyl ester (II) also has been prepared and found to react with chymotrypsin, the second order rate constant being 3.4 l. mole^{-1} sec.$^{-1}$ at 37°, pH 8.0 (0.0875 M Tris, 0.00875 M CaCl$_2$, 5% acetone, $E_0 = S_0 = 3.5 \times 10^{-5} M$). Since no turnover occurs, it should be possible, under suitable conditions, to use II or a related compound as a reagent for the titration of chymotrypsin.[12]

Other phenylcarbamyl derivatives are being prepared and a study of their reactivity with chymotrypsin may lead to a clearer understanding of the mechanism and specificity of this enzyme.

Acknowledgment.—We wish to acknowledge the excellent technical assistance of Miss J. Graubard, Mrs. A. Cooper and Mr. M. Lache as well as the support of The U.S. Public Health Service (AI-01672–05) and the Office of Naval Research (Nonr-266-73).

(10) The specificity apparent in the acylation reaction appears to be less evident in the deacylation process. This phenomenon was also reported for the reaction of N-*trans*-cinnamoylimidazole with trypsin and chymotrypsin (M. L. Bender and E. T. Kaiser, *J. Am. Chem. Soc.*, **84**, 2556 (1962)) as well as their reaction with p-nitrophenylacetate (J. A. Stewart and L. Ouellet, *Can. J. Chem.*, **39**, 751 (1959)). It was also reflected in the data reported for the reactivation of the diethylphosphoryl derivatives of these enzymes (ref. 1 and 7). Rate differences, however, do exist: DEP and N-*trans*-cinnamoyltrypsin deacylate faster than their respective chymotrypsin derivatives, the former deacylating three times more rapidly; acetyl and DPC-chymotrypsin deacylate more rapidly than their respective trypsin derivatives.

(11) I. B. Wilson, M. A. Harrison and S. Ginsburg, *J. Biol. Chem.*, **236**, 1498 (1961).

(12) G. R. Schonbaum, B. Zerner and M. L. Bender, *ibid.*, **236**, 2930 (1961).

DEPARTMENT OF MICROBIOLOGY
COLUMBIA UNIVERSITY   BERNARD F. ERLANGER
630 WEST 168TH STREET   WILLIAM COHEN
NEW YORK 32, N. Y.

ON THE PROBLEM OF THE SERINE–HISTIDINE HYDROGEN BOND IN THE ACTIVE SITE OF α-CHYMOTRYPSIN[†]

Sir:

We have measured the liberation of hydrogen ions during the reaction of phenylmethanesulfonyl fluoride (PMSF) with α-chymotrypsin as a function of pH. It was found in this study that differences in proton-binding properties of the free enzyme and its sulfonyl derivative are negligible in the pH region 7 to 8. This finding is of general interest, since it indicates that current discussions of the mechanism of α-chymotrypsin-catalyzed hydrolyses exaggerate the possibility that the active-site serine hydroxyl group is hydrogen bonded to an imidazole group in the free enzyme.[1]

Evidence that the sulfonylation of the active site of chymotrypsin by PMSF leads to an inactive enzyme containing a single serine sulfonate residue will be described in detail in a separate paper. Two remarks are pertinent to the considerations presented below: (1) the 1:1 stoichiometry of the reaction was established by measuring the loss of esterase activity upon titration of chymotrypsin with PMSF and by measuring the extent of reaction of excess C^{14}-labeled PMSF with chymotrypsin and (2) the sulfonyl enzyme is stable indefinitely in aqueous solution at 25° between pH 3 and 9.

(1) (a) L. W. Cunningham, *Science*, **125**, 1145 (1957); (b) G. H. Dixon, H. Neurath and J.-F. Pechère, *Ann. Rev. Biochem.*, **27**, 489 (1958); (c) T. Spencer and J. M. Sturtevant, *J. Am. Chem. Soc.*, **81**, 1874 (1959); (d) T. C. Bruice, *Proc. Natl. Acad. Sci. U.S.*, **47**, 1924 (1961); (e) F. H. Westheimer, *Advan. Enzymology*, **24**, 460 (1962).
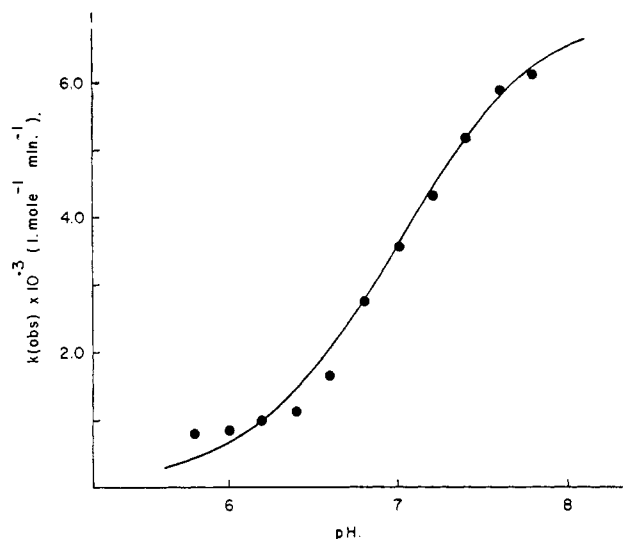

Fig. 1.—The effect of pH on the observed second-order rate constant: curve calculated from equation 1 (see text) using $pK_a = 7.0$.

Figure 1 shows the effect of pH on the observed second-order rate constant for the reaction of PMSF with chymotrypsin. The solid line is based on the equation

$$k(\text{obs}) = k/(1 + a_H/K_a) \tag{1}$$

using $pK_a = 7.0$.[2] The data do not fit equation 1 exactly at low pH; similar deviations were reported by Bender, et al.[3] Rates were measured at 10° in 0.1 M KCl by following the release of HF with a Radiometer Titrigraph pH-stat.

Two procedures were used to establish the amount of hydrogen ion liberated upon sulfonylation. The first employs PMSF as a primary standard. About 6 μmoles of enzyme in 10 ml. of KCl solution was allowed to attain equilibrium at 15°[4] at a given pH in the pH-stat. A precisely known quantity of PMSF (ca. 4 μmoles) in 0.100 ml. of 2-propanol was then introduced using a λ-pipet, and the liberation of acid was followed by automatic addition of 0.016 M NaOH until equilibrium was re-established. The NaOH solution, λ-pipet, and buret of the pH-stat were calibrated concurrently by saponifying PMSF at pH 9.0, 25°. The precision (and accuracy) of the experiment was well within 1%; e.g., six runs at pH 7.00, 0.1 M KCl, gave 0.983 ± 0.003 equivalent of hydrogen ion released per mole of PMSF. With this method a series of experiments was conducted over the pH region 6.2 to 7.8 and at ionic strengths ranging from 0.1 to 1 M KCl. The results are summarized in Fig. 2. Control experiments showed that the acid-binding capacity of the protein is not affected by 1% 2-propanol and that the sulfonyl fluoride is stable in the absence of enzyme.

Alternatively, the enzyme may be allowed to react with an excess of PMSF. This method is convenient at low pH where rates of reaction are low and was used to obtain those points below pH 6.2 in Fig. 2. The operational molarity of the enzyme solutions was estimated by titration of esterase activity with PMSF, by treatment with excess PMSF-C^{14}, and by measuring proton release at pH 7.4, using excess PMSF. Under the latter conditions it has been established that proton release is exactly equivalent to the amount of PMSF

(2) It is assumed that K_M for the reaction does not vary over the pH range 6 to 8 (see ref. 3).

(3) M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1962).

(4) At low ionic strengths the attainment of equilibrium prior to sulfonylation is slow (5–40 min., depending on the pH). Autolysis is negligible at 15° or lower.
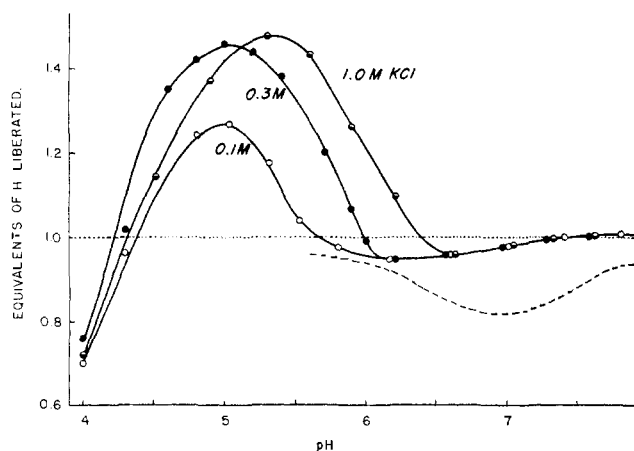
Fig. 2.—The liberation of hydrogen ions during the reaction of PMSF with α-chymotrypsin: dashed line calculated for a change in pK_a from 6.8 to 7.1.

reacting. The values determined by these methods agreed within 1%.

The acylation and deacylation steps in chymotrypsin-catalyzed hydrolyses are pH controlled. It is generally agreed that the simplest explanation requires that the basic form of a dissociable group (assumed to be imidazole) participates directly in the rate-controlling step. A number of investigators[5] have attempted to evaluate the pK_a of this essential group by kinetic means with a variety of acylating agents; they agree on values of 6.6–7.0 for acylation and 6.8–7.4 for deacylation. In all cases the apparent pK_a for deacylation is at least 0.1 pK_a unit greater than that for the acylation step. A change in pK_a from 6.8 to 7.1[3] (cf. dashed line in Fig. 2) would have led to the liberation of only 0.82 equivalent of hydrogen ion at pH 7.0, whereas in fact 0.98 equivalent is released. The method used here, actually a difference titration, is capable of an accuracy within 1% and leads to the conclusion that no group in chymotrypsin having an apparent pK_a near 7.0 undergoes a change of more than 0.03 pK_a unit upon sulfonylation of the enzyme.[6] This fact is inconsistent with the postulate that the active-site serine hydroxyl is hydrogen-bonded to an imidazole group in the free enzyme.

The release of "extra" protons below pH 6 (see Fig. 2)[7] shows that the titration curve of the derivative is different from that of chymotrypsin in that pH region and suggests different conformations. If phenylmethanesulfonyl chymotrypsin has a conformation at pH 7–8 differing from that of the native enzyme, this is not reflected in the titration data.

(5) For pertinent references, see ref. 3.

(6) It must be noted, however, that the values above pH 6.2 are roughly consistent with a change in pK_a from 6.0 to 6.1.

(7) The results are not corrected for uptake of hydrogen ions by fluoride at low pH.

(8) (a) National Science Foundation predoctoral fellow; (b) Research Career Development Award grantee of the National Institutes of Health.

(9) To whom inquiries should be addressed.

DEPARTMENT OF BIOCHEMISTRY
COLLEGE OF PHYSICIANS AND SURGEONS
COLUMBIA UNIVERSITY   DAVID E. FAHRNEY[8a]
NEW YORK 32, N. Y.   ALLEN M. GOLD[8b,9]
RECEIVED NOVEMBER 15, 1962

INTRAMOLECULAR BIFUNCTIONAL GENERAL BASE–GENERAL ACID-CATALYSIS OF ESTER SOLVOLYSIS[1]

Sir:

Earlier studies have provided support for the view that the ready methanolysis of the C-16 acetate ester grouping in ceveratrum alkaloid derivatives is a base-

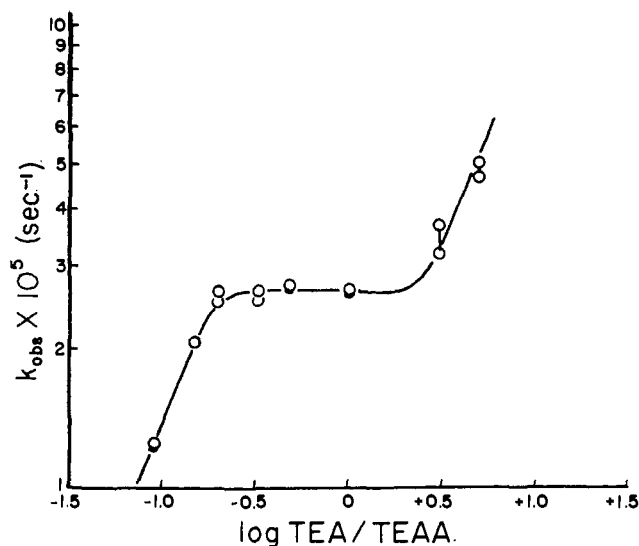(1) This is part VII of a series entitled "Intramolecular Catalysis"; part VI is reference 9.

Fig. 1.—Logarithmic plot of the rate of methanolysis of cevadine D-orthoacetate diacetate (III) against the logarithm of the buffer ratio in 0.06 M triethylamine/triethylammonium acetate buffers at 25° and ionic strength 0.09.

catalyzed solvolysis[2] which is facilitated by a neighboring hydroxyl group bearing a cis-1,3-diaxial relationship to the ester group.[3–8] Furthermore, the solvolysis of 1,3-diaxial hydroxyacetates recently has been shown to be subject to general base–general acid catalysis.[9] Evidence is presented herewith for the argument that the tertiary nitrogen atom of ceveratrum ester alkaloid derivatives participates in an intramolecular basic catalysis of the solvolysis of C-16 esters. The reaction is therefore an instance of *intramolecular bifunctional general base–general acid-catalysis of ester solvolysis*.[10]

Acetate esters were methanolyzed in solutions prepared by dissolving each compound in chloroform (10% of the total volume), adding buffer and tetramethylammonium chloride, and diluting to the required volume with 10% aqueous methanol. The rate of production of methyl acetate, the solvolysis product, was determined by direct gas chromatographic analysis of the reaction mixture, as described earlier.[9]

RATES OF ESTER SOLVOLYSIS AT 1:3 TRIETHYLAMINE:TRIETHYL-
AMMONIUM ACETATE BUFFER (0.12 M) AND IONIC STRENGTH
0.09 AT 25°

Compound	k_{obs} (sec.$^{-1}$) (pseudo-first-order)[11]	Ratio of rates
Dehydrocevadine-D-orthoacetate diacetate (I)	1.2×10^{-8}	1
Formamido-ketone from cevadine D-orthoacetate diacetate (II)	4.8×10^{-7}	40
Cevadine D-orthoacetate diacetate (III)	1.2×10^{-5}	1000

(2) W. J. Rosenfelder, J. Chem. Soc., 2638 (1954).

(3) (a) S. M. Kupchan and W. S. Johnson, J. Am. Chem. Soc., 78, 3864 (1956); (b) S. M. Kupchan, W. S. Johnson and S. Rajagopalan, Tetrahedron, 7, 47 (1959).

(4) S. M. Kupchan and C. R. Narayanan, J. Am. Chem. Soc., 81, 1913 (1959).

(5) S. M. Kupchan, C. I. Ayres, M. Neeman, R. H. Hensler, T. Masamune and S. Rajagopalan, ibid., 82, 2242 (1960).

(6) S. M. Kupchan, N. Gruenfeld and N. Katsui, J. Med. Pharm. Chem., 5, 690 (1962).

(7) Cf. H. B. Henbest and B. J. Lovell, Chemistry and Industry, 278 (1956); J. Chem. Soc., 1965 (1957).

(8) Cf. R. West, J. J. Korst and W. S. Johnson, J. Org. Chem., 25, 1976 (1960).

(9) S. M. Kupchan, S. P. Eriksen and M. Friedman, J. Am. Chem. Soc., 84, 4159 (1962).

(10) Preliminary polarimetric data in accord with the interpretation discussed herein were presented at the 140th Natl. Meeting of the American Chemical Society, Chicago, September, 1961 (S. M. Kupchan, A. Afonso and P. Slade, Abstracts, p. 88-Q).