R. J. K. gratefully acknowledges the support of a National Science Foundation grant.

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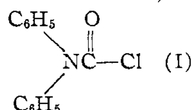
SPECIFIC INACTIVATION OF CHYMOTRYPSIN BY DIPHENYLCARBAMYL CHLORIDE

Sir:

We wish to report the inactivation of α-chymotrypsin by a reagent of high specificity: diphenylcarbamyl chloride (I). The inactivation, which occurs as a



result of a mole for mole reaction between I and the enzyme, is retarded by indole and can be reversed by nucleophilic agents.

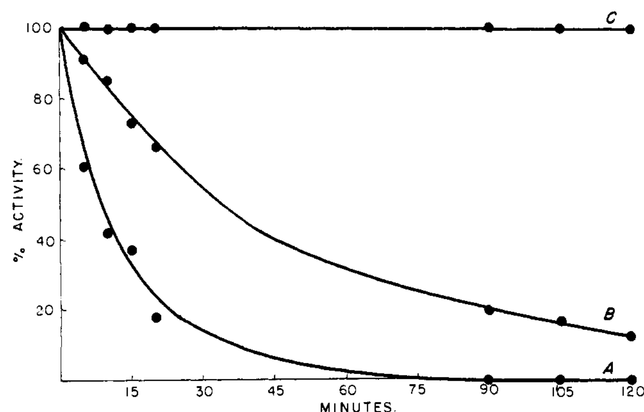


Fig. 1.—Effect of indole on the inhibition of chymotrypsin by diphenylcarbamyl chloride at pH 7.0, 25°. The reaction mixtures contained 1×10^{-6} *M* chymotrypsin, 2×10^{-6} *M* diphenylcarbamyl chloride, 9.5×10^{-3} *M* tris-(hydroxymethyl)-aminomethane–maleic acid–calcium chloride, 5% methanol. Curve A: in the absence of indole (the points are superimposed on a curve calculated from the second order rate constant); curve B, 2×10^{-3} *M* indole; curve C, 1×10^{-2} *M* indole.

In the experiments to be described, chymotryptic activity was determined using acetyl DL-phenylalanine β-naphthyl ester according to the procedure previously described[1]; trypsin assays used the chromogenic substrate benzoyl DL-arginine *p*-nitroanilide hydrochloride.[2]

Curve A of Fig. 1 shows the progress of the inactivation of a 10^{-6} *M* solution of α-chymotrypsin by a 2×10^{-6} *M* concentration of I (Distillation Products Industries, recrystallized from ethanol; m.p. 85°; lit.[3] 85°) at pH 7.0, 25°. The interaction under all conditions studied followed second order kinetics, its rate constant at pH 7.0 being shown in Table I. Also included in Table I are the rate constants for the inactivation of α-chymotrypsin by two other reagents, diisopropyl fluorophosphate(DFP)[4] and α-N-*p*-toluenesulfonyl-β-phenylalanylbromomethane (TBPK), the latter having been reported recently by Schoellmann and Shaw.[5] Even after making allowances for differences in the experimental conditions, I appears to be the most reactive of the three.

(1) W. Cohen and B. F. Erlanger, *J. Am. Chem. Soc.*, **82**, 3928 (1960); also correction, 6431 (1960).
(2) B. F. Erlanger, N. Kokowsky and W. Cohen, *Arch. Biochem. Biophys.*, **95**, 271 (1961).
(3) H. Erdmann and P. Huth, *J. prakt. Chem.*, [II] **56**, 7 (1897).
(4) A. J. J. Ooms, *Nature*, **190**, 533 (1961).
(5) G. Schoellmann and E. Shaw, *Biochem. Biophys. Res. Commun.*, **7**, 36 (1962).

TABLE I

SPECIFIC RATE CONSTANTS OF INACTIVATION[a]

Inactivator	Chymotrypsin	Trypsin
I[b]	610	8.2
DFP[c]	317	...
TPBK[d]	2.37[e]	...

[a] l. mole^{-1} sec.$^{-1}$. [b] 0.01 *M* Tris-maleate, $CaCl_2$; 0.04 percentage acetone; pH 7.0; 25°. [c] 0.1 *M* phosphate, pH 7.7, 25°. [d] 0.1 *M* phosphate pH 6.0, 37°. [e] This value was calculated using the data for 100 minutes exposure as presented in Table I of ref. 5.

The inactivation of chymotrypsin by I was inhibited by indole as shown in curves B and C of Fig. I. Since indole is a competitive inhibitor of chymotrypsin,[6] its effect upon the inactivation process is good evidence for the reaction of I with a segment of the active center of α-chymotrypsin.

I was not reactive toward chymotrypsinogen, diethylphosphorylchymotrypsin or pepsin, as shown by subsequent addition of α-chymotrypsin and assay for chymotryptic activity after a suitable incubation period; controls lacking I were run simultaneously. In the case of chymotrypsinogen, following exposure to I, activation by trypsin was carried out as well. The formation of active chymotrypsin occurred exactly as with a chymotrypsinogen control.

I was capable of inactivating trypsin but at a rate that was approximately one-eightieth that of the inactivation of chymotrypsin (see Table I). The inhibition of trypsin was not an unexpected finding since, for example, acetyl L-tyrosine ethyl ester, a specific substrate of chymotrypsin, is also hydrolyzed by trypsin, the *K*m for the latter reaction at pH 8.0, 25°, being thirteen times that of the reaction with chymotrypsin,[7] I therefore exhibits a considerably higher specificity for chymotrypsin relative to trypsin than does acetyl-L-tyrosine ethyl ester and should prove to be useful for the inactivation of the small quantities of chymotrypsin always present in preparations of crystalline trypsin. The inhibition of trypsin by I is, incidently, additional evidence for the presence of a ring binding site at the active center of this enzyme, as was previously indicated by reactivation studies on diethylphosphoryltrypsin.[8]

Trypsinogen and diethylphosphoryl (DEP) trypsin are not affected by I.

The failure of I to react with DEP-chymotrypsin and DEP-trypsin may indicate, but certainly does not prove, that I is specific for the reactive serine present at the active centers of the esterases. This aspect is now under investigation. However, like the organophosphorus-inhibited enzymes in which the participation of the serine residue has been proven,[9] diphenylcarbamyl (DPC) chymotrypsin and DPC-trypsin can be completely reactivated by nucleophilic reagents. Chymotrypsin or trypsin (each at a concentration of 4×10^{-5} *M*) was exposed at 25° to an equimolar concentration of I in 0.1 *M* Tris buffer, pH 8.0, containing 0.1 *M* $CaCl_2$. After 30 minutes and 90 minutes, respectively, more than 99% inactivation of chymotrypsin and trypsin had occurred. To one part of the enzyme solution was added four parts of *M* isonitrosoacetone in the above buffer (final reactivator concentration, 0.8 *M*). Fifty per cent. reactivation of DPC-chymotrypsin occurred in 28.5 minutes; similar reactivation of DPC-trypsin required 63 minutes. After 18 hours both preparations showed complete reactivation.[10]

(6) H. T. Huang and C. Niemann, *J. Am. Chem. Soc.*, **75**, 1395 (1953).
(7) T. Inagami and J. M. Sturtevant, *J. Biol. Chem.*, **235**, 1019 (1960).
(8) W. Cohen, M. Lache and B. F. Erlanger, *Biochem.*, **1**, 686 (1962).
(9) N. K. Schaffer, S. C. May, Jr., and W. H. Summerson, *J. Biol. Chem.*, **202**, 67 (1953).

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).

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RECEIVED OCTOBER 29, 1962

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.

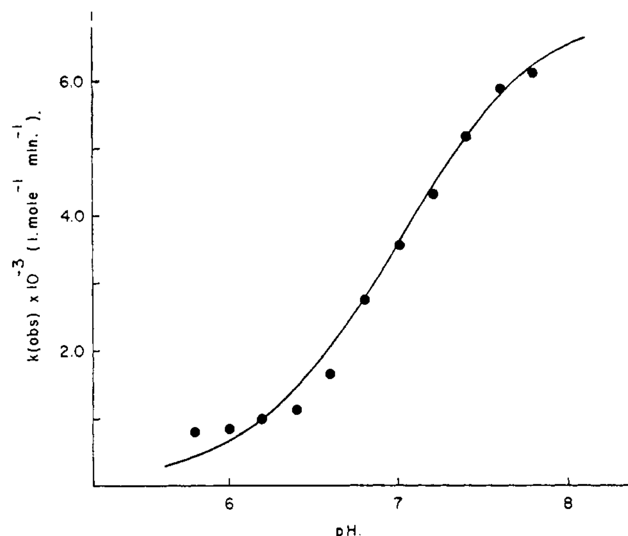

Fig. 1.—The effect of pH on the observed second-order rate constant: curve calculated from equation 1 (see text) using $pK_a = 7.0$.

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

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

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

† This work was supported by the National Science Foundation, Grant No. G-12901, and the Muscular Dystrophy Associations of America, Inc.

(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).

(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.