The Inactivation of Chymotrypsin by Diphenylcarbamyl Chloride and Its Reactivation by Nucleophilic Agents*

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ABSTRACT: Diphenylcarbamyl chloride (DPCC) was found to inactivate chymotrypsin and trypsin by means of a 1:1 stoichiometric reaction. The reaction was relatively specific for chymotrypsin, which was inactivated 80 times faster than trypsin. K_m for the reaction with chymotrypsin was found to be $0.6 \pm 0.2 \times 10^{-4}$ M at pH 8.0. The inactivation of chymotrypsin could be competitively inhibited by indole. DPCC was unreactive toward chymotrypsinogen, diethylphosphorylchymotrypsin, and pepsin. The pH–rate profile of the reaction of DPCC with chymotrypsin was bell shaped, with an optimum at 7.8. Its proton release curve was complex; only in the region near pH 5.0–5.5 was 1 equiv of H^+ released.

The inactive product, DPC-chymotrypsin, could be reactivated by a number of nucleophilic agents including hydroxylamine, isonitrosoacetone, and acyl hydroxamic acids. The experimental data are interpreted with respect to their contribution toward an understanding of the enzymic mechanism of chymotrypsin.

Diphenylcarbamyl chloride (DPCC)[1] has been reported to be a specific inactivator of chymotrypsin (Erlanger and Cohen, 1963; Erlanger *et al.*, 1963). Its reaction with chymotrypsin showed the following characteristics: (a) One mole of DPCC could inactivate 1 mole of enzyme. (b) The inactivation could be inhibited by indole. (c) The reaction with chymotrypsin was relatively specific since its rate was about 80 times faster than the inactivation of trypsin. (d) DPCC was unreactive toward chymotrypsinogen, DEP-chymotrypsin, and pepsin. (e) The pH–rate profile of the reaction of DPCC with chymotrypsin was bell shaped, with an optimum at 7.8. (f) The proton release curve of the reaction was complex; only in the region near pH 5.0–5.5 was 1 equiv of H^+ released. (g) K_M for the reaction of DPCC with chymotrypsin was found to be $0.6 \pm 0.2 \times 10^{-4}$ M at pH 8.0. (h) The inactive product, DPC-chymotrypsin, could be reactivated by a number of nucleophilic agents including hydroxylamine, isonitrosoacetone, and acyl hydroxamic acids.

This paper contains details of the experiments that led to the findings cited above. The experimental data are interpreted with respect to their contribution toward an understanding of the enzymic mechanism of chymotrypsin.

Experimental Section

The chymotrypsin used was three times crystallized α-chymotrypsin obtained from Worthington Biochemicals Corp. The trypsin was Worthington crystalline trypsin, lyophilized. Diphenylcarbamyl chloride was a product of Distillation Products, Inc., recrystallized three times from methanol.

Chymotrypsin was assayed by two different procedures depending upon the experiment performed. In one assay, acetyl-DL-phenylalanine β-naphthyl ester was the substrate, used according to a procedure previously described (Cohen and Erlanger, 1960). However, since a typographical error appeared in the paper (later corrected on p. 6431 of the same volume), the details will be given again:

The substrate solution was prepared by dissolving acetyl-DL-phenylalanine β-naphthyl ester in dimethylformamide, followed by dilution with buffer containing methanol so that the final concentrations were: 0.1 mg/ml acetyl-DL-phenylalanine β-naphthyl ester, 10% dimethylformamide, 20% methanol, 0.05 M Tris–chloride buffer. Enzyme action was stopped by the addition of the coupling reagent, a solution containing 1.5% Duponol ME Dry (E. I. du Pont de Nemours & Co.) and 0.075% Fast Scarlet Salt GGN (General Dyestuff Co.) in distilled water. The latter couples with the liberated β-naphthol while the Duponol (detergent) and a subsequent addition of 5 ml of acetone keep the dye in solution. Although the pH optimum of α-chymotrypsin is 7.8, a buffer with a pH of 7.0 was employed in order to minimize nonenzymic hydrolysis of the substrate. The substrate solution must be prepared immediately prior to use in order to avoid high blanks. The coupling reagent should also be freshly prepared.

The following procedure was routinely employed: 5 ml of freshly prepared substrate solution and 0.9 ml of

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[1] Abbreviations used in this work: DPCC, diphenylcarbamyl chloride; DPC, diphenylcarbamyl; GPANA, glutaryl L-phenylalanine *p*-nitroanilide; DEP, diethylphosphoryl; TPBK, α-*N*-*p*-toluenesulfonyl-β-phenylalanylbromomethane; PMSF, phenylmethanesulfonyl fluoride; DIP, diisopropylphosphoryl.

distilled water were mixed in a test tube and equilibrated at 25° in a water bath, after which 0.1 ml of a chymotrypsin solution was added. After 10 min, 1 ml of the coupling reagent, freshly prepared, was pipetted into the test tube, followed, after a 10-min interval, by 5.0 ml of acetone. The intensity of color was measured using a Bausch & Lomb Spectronic 20 at 485 mμ, the absorption maximum of the dye. The color remained stable for at least 1 hr. Controls without enzyme were run routinely.

The other assay procedure for chymotrypsin used glutaryl-L-phenylalanine *p*-nitroanilide (GPANA) as substrate as previously described (Erlanger *et al.*, 1964).

Inactivation Experiments. Stock solutions of DPCC were usually prepared in acetone. Methanol is also satisfactory if the solution is not kept more than 1 day since some decomposition occurs after longer periods of time even under refrigeration. Dimethyl sulfoxide was used as a solvent early in the investigations but is unsatisfactory because considerable decomposition of DPCC occurs in a few hours.

In a typical inactivation study, the stock DPCC solution was diluted with buffer to the required concentration. Concentrations as high as 4×10^{-3} M DPCC could be obtained in this way. At zero time, measured quantities of the diluted inactivator were added to enzyme in the same buffer. After suitable time periods aliquots were drawn for assay. The choice of assay procedure depended upon the quantity of enzyme in the aliquot. Since assay with GPANA was more convenient, attempts were made to design experiments so that this assay could be used. However, when extremely low concentrations of enzyme (*e.g.*, 10^{-6} M) were required in the experiment, the assay procedure using acetyl-DL-phenylalanine β-naphthyl ester was applied. The latter assay can be used for concentrations of chymotrypsin as low as 0.1 μg/ml; assay with GPANA requires at least 10 μg/ml. All buffers used, except when specified, were 0.01 M Tris–0.01 M maleic acid containing 0.01 M $CaCl_2$. When aliquots of a reaction mixture were diluted, the diluent used was 0.0015 M acetic acid containing 0.01 M $CaCl_2$. Chymotrypsin was exceptionally stable in this solution, the pH of which was 3.5.

Effect of Indole. In these experiments, which were run at pH 7, the concentrations of DPCC and chymotrypsin were 4×10^{-6} and 2×10^{-6} M, respectively. The concentrations of indole were varied: 1.0×10^{-3}, 2.0×10^{-3}, and 3.0×10^{-3} M. Stock solutions of indole were in 20% methanol–buffer solutions. The reaction mixture was made up by adding 1 ml each of stock solutions of DPCC, chymotrypsin, and indole to 17 ml of buffer. Controls without indole and without DPCC were also set up. Incubation was at 25°. At zero time and various other time intervals, 0.2 ml was withdrawn and assayed with acetyl-DL-phenylalanine β-naphthyl ester. Curves were drawn and relative rates were determined by noting the time for 20% inactivation.

Reactivation Experiments. In the reactivation experiments, inactivation was carried out first by incubation with an equimolar quantity of DPCC. Then reactivator was added and the course of reactivation was followed. The following is a typical experiment.

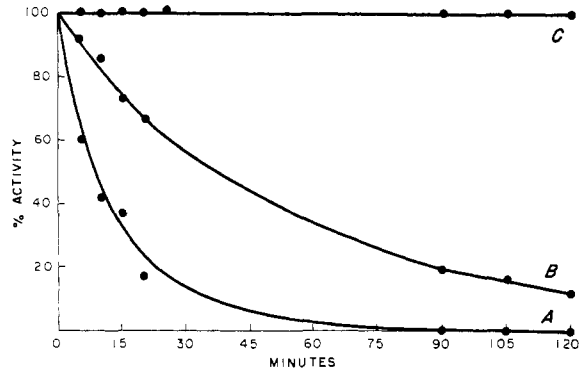

FIGURE 1: Effect of indole on the inhibition of chymotrypsin by diphenylcarbamyl chloride at pH 7.8, 25°. The reaction mixtures contained 1×10^{-6} M chymotrypsin, 2×10^{-6} M diphenylcarbamyl chloride, 9.5×10^{-3} M tris(hydroxymethyl)aminomethane–maleic acid–calcium chloride, 5% methanol. Curve A: 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.

A stock solution of chymotrypsin consisted of 20 mg in 10 ml of 0.1 M Tris–chloride buffer containing 0.1 M $CaCl_2$, pH 8.0. The stock solution of DPCC contained 5.6 mg in a solution consisting of 10 ml of acetone diluted to 30 ml with 0.1 M Tris–chloride buffer containing 0.1 M $CaCl_2$, pH 8.0. Two milliliters of the chymotrypsin solution was incubated at 25° for 40 min with 2 ml of the DPCC solution. Both substances were at a concentration of 4×10^{-5} M assuming a molecular weight of 25,000 for chymotrypsin. Assay showed complete inactivation. To 1.6 ml of 1 M isonitrosoacetone in the above pH 8.0 buffer was added 0.4 ml of the inactivation solution. After various periods of time aliquots were withdrawn, diluted with the pH 3.5 solution described above, and assayed.

Results

Stoichiometry of the Reaction. Curve A of Figure 1 shows the time course of the reaction between DPCC (2×10^{-6} M) and chymotrypsin (1×10^{-6} M) at pH 7.8, 25°. The reaction was found to follow second-order kinetics at the concentrations specified with 50% inhibition requiring about 8 min. Calculation of the second-order rate constants for the reaction gave the figures shown in Table I, which also contains rate constants for the reaction of chymotrypsin with DFP and TPBK. DPCC can inactivate trypsin too, but at a rate almost two magnitudes lower than the rate of inactivation of chymotrypsin (Table I). No reaction occurred with pepsin, chymotrypsinogen, or DEP-chymotrypsin. This was determined by incubation with DPCC for 1 hr, followed by addition of chymotrypsin, further incuba-

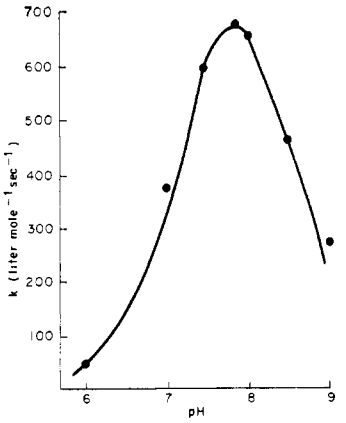

FIGURE 2: Effect of pH on rate of reaction of α-chymotrypsin (2×10^{-6} M) with DPCC (4×10^{-6} M). Buffer, 0.06 M boric acid–maleic acid–sodium acetate, 0.036 M $CaCl_2$, 25°. Controls include a determination of the decomposition of DPCC at various hydrogen ion concentrations. Up to pH 9.0, decomposition is negligible under experimental conditions.

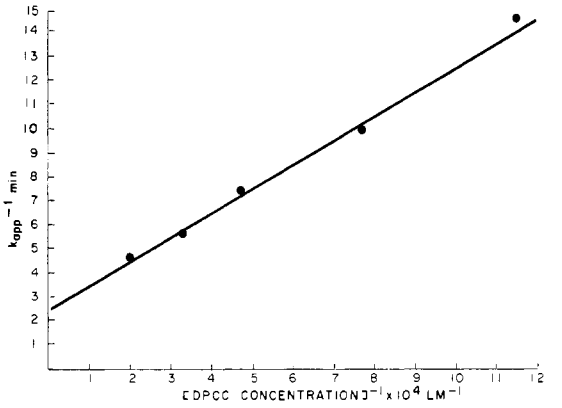

FIGURE 3: Dependence of k_{app} on concentration of DPCC.

TABLE I: Specific Rate Constants of Inactivation.[a]

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

[a] Liters mole^{-1} second^{-1}. [b] 0.01 M Tris-maleate, $CaCl_2$; 0.04% acetone, pH 7.8, 25°. [c] 0.1 M phosphate, pH 7.7, 25° (Ooms, 1961). [d] 0.1 M phosphate, pH 6.0, 37°. [e] This value was calculated using the data for 100-min exposure as presented in Table I of Schoellman and Shaw (1962).

TABLE II: Stoichiometry of Reaction.[a]

Enzyme (M $\times 10^4$)	DPCC (M $\times 10^4$)	$OD_{410m\mu}$
1.2	1.2	0.002
1.2		0.437
1.2	0.6	0.194

[a] Substrate: GPANA.

tion for 1 hr, and assay for chymotryptic activity. In the case of chymotrypsinogen, activation by trypsin was carried out as well.

In order to determine the stoichiometry of the reaction of DPCC with chymotrypsin, a known quantity of reagent was incubated in one run with an equimolar and, in another run, with an excess of chymotrypsin until inactivation was complete. Excess chymotryptic activity was then determined. The results of a typical experiment are shown in Table II. In one case equal volumes of 1.2×10^{-4} M DPCC and 1.2×10^{-4} M chymotrypsin (on a weight basis, assuming a molecular weight of 25,000) were incubated at 25° until the reaction was complete (30 min). Similarly, 1.2×10^{-4} M enzyme was incubated with 0.6×10^{-4} M DPCC. For both experiments, a control lacking DPCC was run. As can be seen from Table II, equimolar quantities caused complete inactivation. It should be noted, however, that at a ratio of 0.5 mole of DPCC to 1.0 mole of chymotrypsin inhibition exceeded 50%. This is not due to an error in the method but is a reflection of the fact that the chymotrypsin preparation was not 100% pure. The data in Table II can be used to calculate the purity of the preparation by means of the equation: % of active enzyme = $(A \times 100)/[2(A - A_{0.5I})]$ where A = activity of enzyme in absence of inhibitor, $A_{0.5I}$ = residual activity after incubation with 0.5 mole of inhibitor. Thus the preparation used in the experiments leading to the data in Table II was 91% active enzyme, the remainder of the material being inert protein and moisture. A more direct means of titrating active enzyme using a chromogenic reagent was described in a previous paper (Erlanger and Edel, 1964).

pH Dependence of the Reaction. Shown in Figure 2 is the effect of pH upon the reaction of DPCC with chymotrypsin. The curve is bell shaped, with an optimum near pH 7.8. This would suggest that two functional groups on the enzyme are involved in the acylation step of enzyme catalysis. In this case one is apparently a conjugate acid with a pK_a near 8.7; the other a base, pK_a *ca.* 7.0.

Effect of Indole. As can be seen in curves B and C of Figure 1, indole inhibits the inactivation of chymotrypsin by DPCC. If the inhibition were competitive, the following relationship would hold (Metzger and Wilson,

1964): $k/k_1 = 1 + ([I]/K_I)$, where k = rate of inactivation in absence of indole, k_1 = rate of inactivation in presence of indole, [I] = concentration of indole, and K_I = dissociation constant of the chymotrypsin–indole complex which is 0.7×10^{-4} M (Huang and Niemann, 1953). Shown in Table III are the results of a series of experiments in which the rate of inactivation was determined at constant enzyme and DPCC concentrations but in the presence of three different concentrations of indole. The agreement between the calculated and observed values of k/k_1 is quite good, indicating that DPCC and indole are competing for the same site on the enzyme.

TABLE III: Competitive Inhibition by Indole[a]

Indole (M)	k/k_1 (Calcd)	k/k_1 (Found)
1.0×10^{-3}	2.4	2.6
2.0×10^{-3}	3.9	4.2
3.0×10^{-3}	5.3	5.5

[a] [DPCC], 4×10^{-6} M; [E], 2×10^{-6} M; buffer, 0.01 M Tris–0.01 M maleic acid, 0.01 M $CaCl_2$, pH 7.0, 25°. Details in Experimental Section.

K_m of the Reaction of DPCC with Chymotrypsin.[2] The results of the experiments with indole provide evidence for the reaction of DPCC with the active site of chymotrypsin. Its ability to inactivate chymotrypsin more rapidly than it inactivates trypsin indicates that DPCC is a specific reagent, *i.e.*, it forms a Michaelis–Menton complex with chymotrypsin as follows:

$$E + DPCC \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}DPCC \xrightarrow{k_3} DPC\text{-}E + H^+ + Cl^-$$

with E–DPCC representing the Michaelis–Menton complex. Therefore $K_m = (k_2 + k_3)/k_1$.

Kitz and Wilson (1962) have shown that K_m of an inactivator can be obtained from a plot of the expression

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_m}{k_3}\frac{1}{[I]}$$

if the inactivator concentration is much greater than the initial enzyme concentration and if the mixture is diluted extensively before assay for residual activity. Shown in Figure 3 is a plot of such an experiment carried out at pH 8. K_m, calculated from the slope and the intercept of the ordinate, was found to be $0.6 \pm 0.2 \times 10^{-4}$ M.

Reactivation of DPC-chymotrypsin. Like organophosphorous-inhibited chymotrypsin (Cohen and Erlanger, 1960), DPC-chymotrypsin can be reactivated by nucleophilic reagents. The data in Table IV show that hydroxylamine and isonitrosoacetone can reactivate DPC-chymotrypsin completely, isonitrosoacetone being more effective. Formohydroxamic acid also can cause reactivation, but it was not certain whether complete reactivation was possible because of extensive denaturation of the enzyme during the required period of incubation.

TABLE IV: Reactivation of DPC-Chymotrypsin.

Reagent	Concn (M)	pH	Time for 50% Reactivation	Time for Complete Reactivation[a]
Isonitrosoacetone	0.8	8.0	28.5 min	18 hr
Hydroxylamine	2.0	7.5	12 hr	48 hr
Formohydroxamic acid	1.0	7.0	25 hr	Undetermined

[a] These times were chosen for convenience. They do not represent the time necessary for complete reactivation but indicate only that complete reactivation had occurred within the time interval.

Effect of pH on the Reactivation Process. The effect of pH on the reactivation of DPC-chymotrypsin by isonitrosoacetone is shown in Figure 4. The points coincide well with the calculated curve for a reaction involving a group on the enzyme having a pK_a of 7.1 and un-ionized isonitrosoacetone (pK_a = 8.5).

The pH–rate profile for the reactivation of DPC-chymotrypsin by hydroxylamine is given in Figure 5. The solid curve represents a reaction in which the enzyme is utilizing a group with a pK_a of 7.7. Hydroxylamine is taken to have a pK_a of 6.2 (Davies and Green, 1956).

Discussion

As demonstrated by Wilson (1959) and by work in

[2] K_m is used instead of K_I, even though inactivation of the enzyme occurs, in order to avoid confusion with the conventional use of K_I for the dissociation constant of an enzyme with a reversible competitive inhibitor. In the latter case the formation of the acyl enzyme does not take place; therefore $K_I = k_2/k_1$.

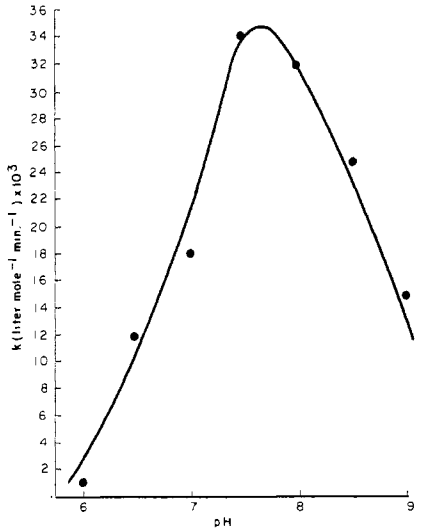

FIGURE 4: Effect of pH on reactivation of DPC-chymotrypsin by isonitrosoacetone. DPC-chymotrypsin was prepared by addition to 23 mg (0.1 mmole) of DPCC in 2 ml of methanol to 40 ml of a solution of 350 mg (0.014 mmole) of α-chymotrypsin in 40 ml of 0.05 M Tris–chloride buffer, pH 7.7, containing 0.01 M $CaCl_2$. One-hour incubation at 25° (negligible activity remained after 10 min) was followed by 24-hr dialysis and lyophilization. Conditions of reactivation: buffers, 0.1 M Tris–0.1 M maleic acid–0.1 M $CaCl_2$, pH 6.0, 6.5, 7.0, 7.5, 8.0; 0.06 M borate–0.06 M maleic acid–0.06 M acetate–0.1 M $CaCl_2$, pH 8.0, 8.5, 9.0; [DPC-chymotrypsin], 2.4 $\times$ 10^{-5} M; [isonitrosoacetone], 0.3 M. GPANA used for assay.

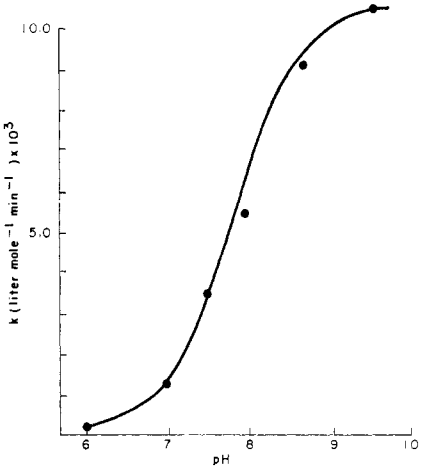

FIGURE 5: Effect of pH on reactivation of DPC-chymotrypsin by hydroxylamine. DPC-chymotrypsin preparation same as described in legend to Figure 4. Conditions of reactivation: buffer, 0.125 M Tris–0.125 M maleate–0.005 M $CaCl_2$ in all H^+ concentrations. [DPC-chymotrypsin], 2.4 $\times$ 10^{-5} M; [NH_2OH], 0.5 M.

this laboratory (Cohen and Erlanger, 1960; Cohen *et al.*, 1962; Erlanger and Cohen, 1963; Erlanger *et al.*, 1963) studies of the reaction of substances specific for the active center of enzymes can yield information concerning mechanisms of catalysis, in particular if the reagents cause inactivation of the enzyme. The utility of these reagents rests mainly upon the fact that, in reacting with the enzyme, they make use of at least part of the mechanism by which the enzyme normally attacks substrates. The reaction of chymotrypsin with a substrate can be represented as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{-}S \xrightarrow{k_3} E{-}S \xrightarrow[H_2O]{k_4} E + P_2$$
$$\Big\downarrow +$$
$$P_1$$

where E—S is the Michaelis complex and E-S is the acylated enzyme. In the case of the reaction of chymotrypsin with an inactivator such as DFP or DPCC, k_4 is extremely slow; hence the enzyme is inactivated. If a strong nucleophile such as hydroxylamine is substituted for water, k_4 can be greatly increased so that active enzyme can be recovered. Thus, with inactivators, we have a means of isolating the acylation or the deacylation step so that each can be studied in detail.

The organophosphorous inactivators were successfully utilized in this way but they suffer from one disadvantage: there is no evidence that the binding or specificity site of the enzyme is participating in the phosphorylation step. The characteristics of the inactivation of chymotrypsin by DPCC enumerated in the introduction of this paper constitute good evidence that this inactivation is truly specific in that the dissociation content of its Michaelis complex is greatly in favor of the complex. Hence it is like a true specific substrate of chymotrypsin. In addition, like the usual substrates, its electrophilic group is a carbon rather than a phosphorus. It is more likely then that a study of its deacylation would be relevant to the deacylation step of chymotrypsin catalysis.

In the context of the above remarks, we can conclude the following from the experimental data:

(a) A minimum of two functional groups in chymotrypsin participate in the acylation step, one with a pK_a near 7 and the other with a pK_a near 8.7. The data do not exclude the possible participation of another more basic functional group, as was suggested by some other findings made in this laboratory (Erlanger *et al.*, 1965). The work of Labouesse and his associates (1964) indicated that the group of pK_a = 8.7 is the N-terminal isoleucine of chymotrypsin. Its function in the catalytic mechanism is as yet unknown although it was suggested that it played a part in maintaining the enzymes' proper (active) conformation.

(b) The experiments dealing with the reactivation of DPC-chymotrypsin allow us to conclude that a functional group on the enzyme with a pK_a near 7 is a participant. Figure 5, which shows the pH–rate profile

for the reactivation of DPC-chymotrypsin by hydroxylamine, clearly indicates an interaction of NH_2OH with an unprotonated group in the enzyme having a pK_a of 7.7. The curve for the reactivation by isonitrosoacetone, on the other hand (Figure 4), implicates unionized reactivator and a group on the enzyme with a pK_a near 7.1. The differences in the calculated pK_a of the group on the enzyme taking part in the two reactivation processes is not a serious point of concern since high concentrations of reactivator may have some effect upon the apparent dissociation constant. Furthermore, Bruice and Schmir (1959) have shown the pitfalls inherent in deriving exact pK_a values from pH–rate profiles.

It is interesting to note that the data in Figure 5 represent the first case of reactivation by hydroxylamine in which a sigmoid curve was obtained. Cunningham (1954) found a bell-shaped curve for the reactivation of DEP-chymotrypsin by hydroxylamine. We confirmed his finding (Cohen and Erlanger, 1960) and extended it to DEP-trypsin (Cohen *et al.*, 1962). There, too, a bell-shaped curve was found. We concluded that the mechanism of reactivation by hydroxylamine of organophosphorous-inhibited enzymes was not strictly analogous to the deacylation step of the catalytic mechanism of chymotrypsin and trypsin (Cohen *et al.*, 1962). It is possible that the dephosphorylation step has an added feature, general acid catalysis, which is the case, for example, in the hydroxylaminolysis of amides (Jencks and Gilchrist, 1964). Thus the characteristics of the inactivation and reactivation processes, when diphenylcarbonyl chloride is the inactivator, closely resemble the proposed acylation and deacylation steps that are believed to make up the catalytic mechanism of chymotrypsin.

Another virtue of this inactivator is its apparent specificity for chymotrypsin, as witnessed by its respectable K_m and its higher rate of reaction with that enzyme relative to its reaction with trypsin or acetylcholinesterase (Metzger and Wilson, 1964). Interestingly enough, however, deacylation of DPC-chymotrypsin by isonitroacetone was not so strikingly more rapid than deacylation of DPC-trypsin: a half-time of 28.5 min for DPC-chymotrypsin, 63 min for DPC-trypsin, using, in both cases, 0.8 M isonitroacetone at pH 8.0 (Erlanger and Cohen, 1963). This would imply that specificity plays more of a part in the acylation step of the reaction of chymotrypsin with a substrate. Bender and Kaiser (1962) came to a similar conclusion as a result of their studies on the deacylation of *trans*-cinnamoyltrypsin and *trans*-cinnamoylchymotrypsin. On the other hand, within a range of chymotrypsin substrates they found that specificity played an important part in the deacylation process (Bender *et al.*, 1964).

The specificity and high reactivity of diphenylcarbamyl chloride support the suggestion of Cohen and Erlanger (1960) and Wilson and Erlanger (1960) that chymotrypsin has two sites capable of binding cyclic structures. Additional support comes from the report of Metzger and Wilson (1964) that methylphenylcarbamyl chloride is a poorer inactivator of chymotrypsin than DPCC, by a factor of approximately 150, and from the finding that *N*-phenylacetohydroxamic acid is as poor a reactivator of DEP-chymotrypsin as benzo- or acetohydroxamic acid (Cohen and Erlanger, unpublished). On the other hand, *N*-phenylbenzohydroxamic acid, which has two aromatic rings, is highly active (Cohen and Erlanger, 1960). Experiments are now in progress using highly constrained aromatic carbamyl chlorides to ascertain the relative positions in space of the ring-binding portions of the chymotrypsin molecule.

In summary, the reaction of DPCC with chymotrypsin can be characterized as follows: First there occurs noncovalent binding of the inactivator to the active site of the enzyme, the evidence suggesting that both aromatic rings participate in the binding. This is followed by carbamylation of the enzyme, presumably on the active serine residue (Brown and Hartley, 1964), with the participation of at least two functional groups on the enzyme. Metzger and Wilson (1964) suggest an additional electrophilic assistance in the acylation reaction. In the deacylation process, as in the deacylation of other acyl-chymotrypsins, only one group in the enzyme (pK *ca.* 7) is implicated by the data. However, the evidence does not allow us to eliminate the possible function of a group with a pK_a higher than 12.

A number of laboratories have observed that the stoichiometry of the acylation of chymotrypsin is unusual with respect to the release of H^+. We have found this to be the case for DPCC, as well. Theoretically, if DPCC reacts with the hydroxyl group of serine, 1 mole of H^+ should be released per mole of inactivated product. Instead a rather complex pattern has been observed (Erlanger *et al.*, 1963).[3] At pH 7.8, for example, only about 0.5 mole of H^+ is released. Furthermore, the titration curve of DPC-chymotrypsin differs from that of chymotrypsin: groups with an apparent pK_a of 8.7 and 5.5 in the latter change to $pK_a > 10.6$ and 3.5, respectively, in the former (Erlanger *et al.*, 1963). To dismiss these findings as fortuitous and unrelated to the catalytic process is to be guilty of over-

[3] Gutfreund and Sturtevant (1956) reported the release of only 0.6 mole of H^+/mole of acetylated chymotrypsin using *p*-nitrophenylacetate at pH 6.6. According to Fahrney and Gold (1963), 1 mole of H^+ is released for each mole of phenylmethanesulfonylchymotrypsin formed at pH 7, but more than 1 mole is released at higher H^+ concentrations and less at lower H^+ concentrations. The stoichiometry of the reaction of DFP with chymotrypsin as reported by Labouesse and co-workers (1964) showed approximately 1 mole of H^+ at pH 7 and below, but less under conditions of higher pH. We found slightly different yields of H^+ (Castleman and Erlanger, unpublished): at pH 7.0, 0.87 mole; at pH 8.0, 0.87 mole; at pH 10, 0.4 mole; and at pH 5.5, 1.5 moles. The stoichiometry of the reaction between DFP and chymotrypsin appears to combine characteristics of the reactions with PMSF and with DPCC in that the stoichiometry is similar to that of PMSF at pH 7 and above, but similar to DPCC at lower pH. We are not able to explain the results of Marini and Behr (1964), who reported that the titration curves of DIP-chymotrypsin and of chymotrypsin are identical, results that would lead to the release of 1 mole of H^+ at all hydrogen ion concentrations. His results also contradict the findings of Labouesse *et al.* (1964). The titration curves of the various acylchymotrypsins will be presented in detail in a forthcoming paper.

estimating our knowledge of enzymic catalytic mechanisms. It is not unlikely that conformational changes that cause changes in the reactivity of functional groups may play an important part in catalysis. Thus, for example, a functional group in a hydrophobic environment could, upon a conformational change, become exposed to water molecules. Acylation would be favored in a hydrophobic environment; deacylation would be favored in the presence of water. The pK_a of a functional group undergoing this change of environment would, of course, change. If it were a carboxyl group, its pK_a would be abnormally high in an apolar environment but would decrease to normal values upon exposure to an aqueous environment. This is what we find when we compare the titration curves of DPC-chymotrypsin and chymotrypsin (Erlanger *et al.*, 1963; Castleman and Erlanger, unpublished). The carboxyl group need not be a participant in the catalytic mechanism but could be considered as an indicator of the environment at the active center.

The ability of a protein to supply variable local conditions can also serve another purpose, namely, to decrease the extent of solvation of selected functional groups and thus to increase their activity (*cf.* Parker, 1962). This may be the case for the unusually reactive serine residue of chymotrypsin and related esterases.

The above remarks are admittedly speculative but they could serve to explain why it is apparently necessary for an enzyme to be a macromolecular peptide chain of polar and apolar amino acids and why it has not been possible to design a low molecular weight model system with the efficiency and specificity of an enzyme.

Acknowledgment

We wish to acknowledge the able technical assistance of Miss J. Graubard and Mr. M. Lache.

References

Bender, M. L., and Kaiser, E. T. (1962), *J. Am. Chem. Soc. 84*, 2556.

Bender, M. L., Kézdy, F. J., and Gunter, C. R. (1964), *J. Am. Chem. Soc. 86*, 3714.

Brown, J. R., and Hartley, B. S. (1964), *Abstr. Fed. Europ. Biochem. Soc.*, A-29.

Bruice, T. C., and Schmir, G. L. (1959), *J. Am. Chem. Soc. 81*, 455.

Cohen, W., and Erlanger, B. F. (1960), *J. Am. Chem. Soc. 82*, 3928.

Cohen, W., Lache, M., and Erlanger, B. F. (1962), *Biochemistry 1*, 686.

Cunningham, L. W. (1954), *J. Biol. Chem. 207*, 443.

Davies, D. R., and Green, A. L. (1956), *Biochem. J. 63*, 529.

Erlanger, B. F., Castleman, H., and Cooper, A. G. (1963), *J. Am. Chem. Soc. 85*, 1872.

Erlanger, B. F., and Cohen, W. (1963), *J. Am. Chem. Soc. 85*, 348.

Erlanger, B. F., Cohen, W., Vratsanos, S. M., Castleman, H., and Cooper, A. G. (1965), *Nature 205*, 868.

Erlanger, B. F., Cooper, A. G., and Bendich, A. J. (1964), *Biochemistry 3*, 1880.

Erlanger, B. F., and Edel, F. (1964), *Biochemistry 3*, 364.

Fahrney, D. E., and Gold, A. M. (1963), *J. Am. Chem. Soc. 85*, 349.

Gutfreund, H., and Sturtevant, J. M. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 719.

Huang, H. T., and Niemann, C. (1953), *J. Am. Chem. Soc. 75*, 1395.

Jencks, W. P., and Gilchrist, M. (1964), *J. Am. Chem. Soc. 86*, 5616.

Kitz, R., and Wilson, I. B. (1962), *J. Biol. Chem. 237*, 3245.

Labouesse, B., Oppenheimer, H. L., and Hess, G. P. (1964), *Biochem. Biophys. Res. Commun. 14*, 318.

Marini, M. A., and Behr, F. (1964), *Biochim. Biophys. Acta 89*, 309.

Metzger, H. P., and Wilson, I. B. (1964), *Biochemistry 3*, 926.

Ooms, A. J. J. (1961), *Nature 190*, 533.

Parker, A. J. (1962), *Quart. Rev. (London) 16*, 163.

Schoellman, G., and Shaw, E. (1962), *Biochem. Biophys. Res. Commun. 7*, 36.

Wilson, I. B. (1959), *Federation Proc. 18*, 752.

Wilson, I. B., and Erlanger, B. F. (1960), *J. Am. Chem. Soc. 82*, 6422.