The Reactivation of Diethylphosphoryltrypsin*

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The process of reactivation of diethylphosphoryltrypsin (DEP-trypsin) was studied in order to determine its relationship to the deacylation step of the catalytic mechanism of trypsin. It was found that DEP-trypsin could be completely reactivated by hydroxylamine and by formohydroxamic acid even after storage for 1 year. In both processes the data indicated the participation of a functional group on the enzyme with a pK_a near neutrality. Other hydroxamic acids and oximes were also tested as reactivators, enhanced activities being shown by L-tyrosinehydroxamic acid, phenylaceto-hydroxamic acid, and *anti*-phenylglyoxaldoxime. DEP-trypsin slowly regained activity in the absence of added reactivator, the rate of this spontaneous reactivation increasing with increasing *p*H. The rate of reactivation, whether spontaneous or mediated by hydroxylamine or formohydroxamic acid, was not increased by the addition of the competitive inhibitor D-lysine methyl ester hydrochloride. Furthermore, the rate of reactivation of DEP-chymotrypsin, whether spontaneous or in the presence of nucleophilic agents, was not influenced by the presence of indole. It was concluded that, although many elements of similarity exist between dephosphorylation and the deacylation step of the enzyme mechanism, many discrepancies remain to be explained.

Trypsin, like a number of other esterases, can be inactivated by certain organophosphorous compounds (Jansen *et al.*, 1949) which have been shown to cause phosphorylation of a single serine residue present at the active center of the enzyme (Oosterbaan *et al.*, 1955). Cholinesterase behaves similarly and, moreover, the studies of Wilson (1959) and of Green and Smith (1958) have shown that nucleophilic agents such as oximes and hydroxamic acids can dephosphorylate and thereby reactivate the enzyme. Wilson (1959) was able to demonstrate a correlation between the structure of an efficient nucleophilic agent and the conformation of the active surface of the enzyme. Experiments performed in this laboratory (Cohen and Erlanger, 1960) indicated that this relationship also applied to the reactivation of diethylphosphorylchymotrypsin.

This paper reports an investigation of the reactivation of diethylphosphoryltrypsin (DEP-trypsin) by a number of nucleophilic agents. It will be shown that DEP-trypsin is capable of complete reactivation and that the process is more rapid than that of DEP-chymotrypsin under comparable conditions. The data will be discussed with a view toward elucidating certain aspects of the catalytic action of trypsin.

EXPERIMENTAL

The trypsin used in this work was Worthington crystalline trypsin, lyophilized.

Preparation of DEP-trypsin.—One-tenth ml of tetraethylpyrophosphate (TEPP) (50% pure) dissolved in 10 ml of 0.05 M Tris-maleate buffer, *p*H 8.0, containing 0.05 M $CaCl_2$, was added in one portion, with swirling, to 1 g of trypsin in 50 ml of the same buffer. The resulting clear solution was allowed to stand at room temperature for 2 hours. At the end of this interval, after some particulate matter was removed by filtration, the filtrate was dialyzed for 48 hours at 4° against deionized water that had been adjusted with 2 N HCl to *p*H 3.5. Assay of the lyophilized product (see below) showed that only 3% of the initial activity remained.

Assay for Trypsin Activity.—The chromogenic substrate benzoyl DL-arginine *p*-nitroanilide hydrochloride (DL-BAPA), the preparation and properties of which are described in another paper (Erlanger *et al.*, 1961), was employed in all trypsin assays. The following procedure was used: 0.1 to 1.0 ml of the solution to be assayed (corresponding to ca. 30 μg of trypsin) was added to a solution containing 5 ml 0.001 M DL-BAPA (prepared from a stock solution of 435 mg DL-BAPA in 10 ml of dimethylsulfoxide by diluting 1 ml up to 100 ml with 0.05 M Tris buffer, *p*H 8.2, containing 0.02 M $CaCl_2$) and, in addition, enough water to produce a total volume of 6 ml. After a 10-minute period of incubation at 25°, the reaction was terminated by the addition of 1 ml of 30% acetic acid and the *p*-nitroaniline released was estimated by absorbance measurements at 410 mμ with a Bausch and Lomb Spectronic 20.

Reactivation with Nucleophilic Agents.—Four parts of a solution of reactivator in water, adjusted to the proper *p*H, was added to one part of 0.15 M Tris-maleate buffer containing 0.03 M $CaCl_2$. On occasion the *p*H of this mixture had to be readjusted slightly. One part of a solution

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of DEP-trypsin in 0.001 M HCl (0.9 mg of enzyme per ml) was added. An aliquot was immediately withdrawn for assay and the reaction mixture rechecked to make certain that no change in *p*H had occurred. Samples were assayed at appropriate time intervals. Controls containing reactivator and trypsin were always set up at the same time and assayed with the reactivation mixtures.

Spontaneous Reactivation.—One part of a DEP-trypsin solution (0.9 mg DEP-trypsin per ml of 0.006 M borate-maleate-acetate buffer containing 0.004 M $CaCl_2$, *p*H 7) was added to five parts of 0.06 M borate-maleate-acetate buffer, containing 0.04 M $CaCl_2$, adjusted to the proper *p*H. Nitrogen was passed slowly through the solutions for about 30 seconds, after which the tubes were tightly stoppered. Incubation was at 25°. Separate tubes were set up for each time. Trypsin controls were run simultaneously.

Nucleophilic Reagents.—The following compounds were purchased from Distillation Products Industries, Rochester 3, N.Y.: *N*-phenylbenzohydroxamic acid, *anti*-phenylglyoxaldoxime, and 1-phenyl-1,2-propanedione-2-oxime.

We are indebted to Drs. I. B. Wilson and S. Ginsburg for pyridine-2-aldoxime methiodide and pyridine-2-aldoxime.

The following compounds were prepared in this laboratory by established methods: benzohydroxamic acid (Blatt, 1943), nicotinohydroxamic acid (Hackley *et al.*, 1955), formohydroxamic acid (Hickenbottom, 1948), phenylacetohydroxamic acid (Jones, 1912), L-tyrosinehydroxamic acid (Foster *et al.*, 1954), cyclohexanohydroxamic acid (Cohen and Erlanger, 1960), D- and L-lysinehydroxamic acid (Cohen and Erlanger, 1960), hexanohydroxamic acid (Cohen and Erlanger, 1960), and propionohydroxamic acid (Jones and Neuffer, 1917).

β-Alaninehydroxamic acid is a new compound and was prepared in the following manner: 10 g (0.11 mole) of β-alanine was suspended in 200 ml of methanol. After the suspension was cooled in an ice bath, hydrogen chloride gas was passed in until saturation was effected. The methanol was removed *in vacuo* and the residual oil was treated with methanol and hydrogen chloride again. Removal of the solvent *in vacuo* yielded an oil that could be crystallized by the addition of dry ether. Recrystallization from methanol-ether yielded 11.1 g (72%); m.p. 95–96° (Lengfield and Stieglitz, 1893, report 94–95°).

The above ester (5 g, 0.036 mole) was dissolved in 20 ml of methanol containing 0.83 g (0.036 mole) of sodium. This solution was added to an equal volume of a methanol solution of hydroxylamine (prepared by adding 2.5 g (0.036 mole) of hydroxylamine hydrochloride to 20 ml of methanol in which 0.83 g (0.03 mole) of sodium was dissolved). Crystallization of β-alanine hydroxamic acid occurred after 24 hours at 4°. The crystals were recovered by filtration and recrystallized from methanol; yield 2.6 g (69%), m.p. 135–136°. (Found: C, 34.3; H, 8.0; N, 27.0. $C_3H_8O_2N_2$ requires C, 34.6; H, 7.7; N, 26.9.)

Results

As demonstrated previously (Cohen and Erlanger, 1960) the rate constant of the reactivation process cannot be determined directly because of the occurrence of a number of side-reactions, the most important ones being as shown in Scheme I, where N: represents the nucleophilic agent.

$$\text{DEP-enzyme} + \text{N:} \xrightarrow{(1)} \text{enzyme} + \text{N:DEP}$$

$$\downarrow (3) \qquad\qquad \downarrow (2)$$

denatured DEP-enzyme — denatured enzyme

Scheme I

Reaction (1) is the reactivation process. In order to determine the rate constant for the reactivation of DEP-chymotrypsin, it was necessary first to evaluate that of reaction (2). Reaction (3), on the other hand, could be minimized by a suitable adjustment of the experimental conditions (Cohen and Erlanger, 1960). Preliminary experiments designed to study the reactivation of DEP-trypsin indicated that it could be reactivated by formohydroxamic acid more than three times faster than DEP-chymotrypsin under comparable conditions, and, furthermore, that it was possible to find conditions under which reaction (2), the denaturation of trypsin, as well as reaction (3), proceeded at a negligible rate (see footnote *n* of Table I).

The results of a typical experiment are shown in Figure 1. It can be seen that DEP-trypsin could be reactivated to about 60% of maximal activity in approximately 240 minutes with 0.2 M formohydroxamic acid at *p*H 8. During this incubation period, a trypsin control, under identical conditions, lost approximately 10% of its activity (Fig. 1). Since the denaturation of trypsin follows second-order kinetics at this *p*H (Northrup *et al.*, 1948) and therefore is influenced by the concentration of active enzyme, it follows that the DEP-trypsin–reactivator mixture denatured to an even lesser extent. For our purposes, therefore, it was not necessary to correct for reaction (2). Controls containing reactivator and trypsin were always run, however, to eliminate the possibility that a particular nucleophilic agent might increase the rate of denaturation. This was found to be the case for benzohydroxamic acid, only.

An appropriate plot of the data in Figure 1 indicated that the reactivation process in the presence of a large excess of formohydroxamic acid followed first-order kinetics. The apparent rate constant can therefore be calculated from formula (1) (*cf.* Green and Nicholls, 1959), where A is the concentration of the reactivator and t is the time of incubation. Calculation yielded an apparent rate constant for the reactivation of DEP-

TABLE I

RATES OF REACTIVATION OF DEP-TRYPSIN BY VARIOUS NUCLEOPHILIC AGENTS[a]

Compound	Concentration (moles/liter)	pK_a[b]	$k_1 \times 10^4$[c]	$k_{A^-} \times 10^2$[c]
	Hydroxamic Acids			
Glycine hydroxamic acid	0.037	7.70[d]	45.8	2.75
L-Lysine hydroxamic acid	0.012	7.93[d]	43.2	4.12
D-Lysine hydroxamic acid	0.070	7.93[d]	11.5	1.10
Nicotinohydroxamic acid	0.048	8.30[e]	56.4	11.82
Formohydroxamic acid	0.048	8.50[f]	46.7	14.85[n]
Benzohydroxamic acid	0.054	8.75[g]	41.1	22.50
N-Phenylbenzohydroxamic acid	0.007	9.15[d]	36.5	51.63
Phenylacetohydroxamic acid	0.021	9.18[h]	86.0	131.30
L-Tyrosinehydroxamic acid	0.016	9.20[i]	53.1	84.80
Propionohydroxamic acid	0.131	9.45[h]	11.6	32.78
Cyclohexanohydroxamic acid	0.042	9.75[d]	49.6	279.60
Hexanohydroxamic acid	0.034	9.75	45.4	246.00
β-Alanine hydroxamic acid	0.100	9.88	50.1	380.50
	Oximes			
Pyridine-2-aldoxime methiodide	0.118	8.00[j]	8.96	0.98
Isonitrosoacetone	0.348	8.30[k]	9.58	1.99
anti-Phenylglyoxaldoxime	0.027	8.30	18.98	3.98
Pyridine-2-aldoxime	0.100	10.40[j]	11.26	283.00
Formaldoxime	0.429	10.48[l]	4.41	133.20
Hydroxylamine	0.601	6.20[m]	4.69	0.054[n]

[a] DEP-trypsin was reactivated at 25° in 0.025 M Tris-maleate buffer, 0.005 M $CaCl_2$, *p*H 7.0. E = 0.15 mg/ml. Concentration of reactivator indicated in main body of table. [b] Dissociation constants not previously reported were determined with a Radiometer Type SBR2C/SBU1a/TTT1b; 0.15 mmole of acid in 10 ml of 0.2 M NaCl was titrated with 0.4 N NaOH under an atmosphere of nitrogen. [c] Liter mole^{-1} min^{-1}. [d] Cohen and Erlanger (1960). [e] Hackley *et al.* (1955). [f] This value is more accurate than value reported by Cohen and Erlanger (1960). [g] Green *et al.* (1958). [h] Wise and Brandt (1955). [i] Foster *et al.* (1954). [j] Wilson *et al.* (1958). [k] Green and Smith (1958a). [l] Green and Smith (1958b). [m] Davies and Green (1956). [n] Erlanger and Cohen (1960) reported a k_1 (k_{A^-}) of 0.04 for the reactivation of DEP-chymotrypsin by hydroxylamine. Under the conditions used at that time considerable denaturation of enzyme occurred which introduced a large error into the calculations. Subsequently, the reactivation of DEP-chymotrypsin by hydroxylamine was re-run under conditions similar to that described in footnote *a* of this table. The new, more accurate value of k_{A^-} of reactivation of DEP-chymotrypsin by hydroxylamine is 0.015 and by formohydroxamic acid, 3.94.

trypsin by formohydroxamic acid at *p*H 8.0 of 1.25×10^{-2} liter/mole·minute.

$$k_1 = \frac{2.3}{At} \log \frac{[\text{DEP-trypsin}]}{[\text{DEP-trypsin} - \text{trypsin}]} \qquad (1)$$

The Effect of Concentration of Formohydroxamic Acid.—The extent of reactivation of DEP-trypsin (0.3 mg/ml) in the presence of increasing concentrations of formohydroxamic acid (0.025 M Tris-maleate; 0.00125 M $CaCl_2$; *p*H 8.0; 25°; incubation time, 1 hour) is shown in Figure 2. The rate increases linearly up to a concentration of 0.60 M formohydroxamic acid. At higher concentrations an apparent deviation from linearity was observed owing to a significant decrease in the concentration of DEP-trypsin during the incubation period. Use of equation (1) to calculate the rate constant for the reactivation process at each of the formohydroxamic acid concentrations resulted in a value of 1.25×10^{-2} liter/mole·minute, which is in agreement with that calculated from the data in Figure 1.

Also shown in Figure 2 is a point (open square) representing the extent of reactivation after 3 hours' incubation in the presence of 0.75 M formohydroxamic acid, and one (solid square) representing the activity of the control after 3 hours. Eighty-three per cent of the initial control activity was obtained. During this period of time, the activity of the trypsin-formohydroxamic acid control dropped to 76% of its initial level. The reactivation, therefore, is essentially complete. The apparent 109% reactivation is not due to experimental error but is the result of the following factors: (*a*) DEP-trypsin is more stable than trypsin,[1] and (*b*) denaturation, since its rate depends upon the concentration of active enzyme, occurs more rapidly in the trypsin control.

It was possible to obtain complete reactivation of DEP-trypsin even after storage of lyophilized preparations for 1 year. In this respect, therefore, DEP-trypsin resembled DEP-chymotrypsin (Cohen and Erlanger, 1960).

Effect of pH on Reactivation by Formohydroxamic Acid.—The effect of *p*H upon the rate of reactivation of DEP-trypsin by 0.1 M formohydroxamic acid is shown in Figure 3. The curve is bell shaped, with an optimum at *p*H 8.15, and is similar to that found for the reactivation of DEP-

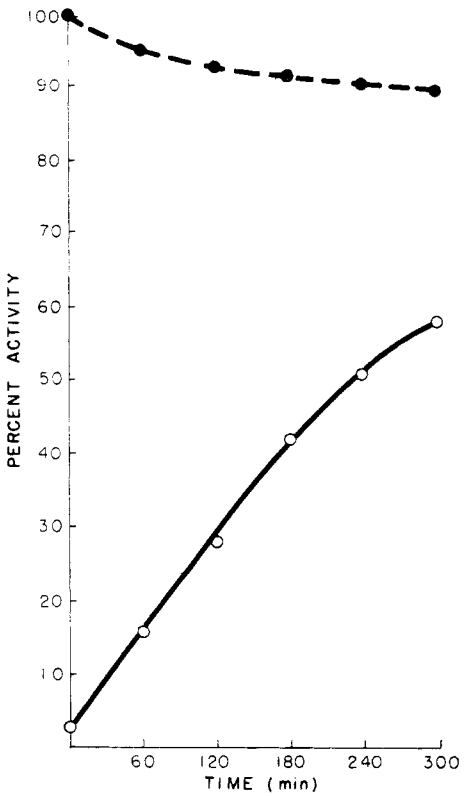


FIG. 1.—Time course of reactivation of DEP-trypsin (0.3 mg/ml) by 0.2 M formohydroxamic acid in 0.025 M Tris-maleate buffer, *p*H 8.0, containing 0.00125 M $CaCl_2$; temp., 25°. O—O, DEP-trypsin; ●---●, trypsin.

chymotrypsin (Cohen and Erlanger, 1960). The points represent the actual values obtained experimentally. They are superimposed upon a curve derived from a calculation of the rate constants for a reaction between the anion of formohydroxamic (pK_a 8.50) and a protonated group on the enzyme (pK_a 7.72). It should be noted that the same curve would apply to a reaction involving the un-ionized hydroxamic acid and an unprotonated group on the enzyme (see Discussion).

Reactivation of DEP-trypsin by Hydroxylamine.

[1] In our earlier studies on DEP-chymotrypsin (Cohen and Erlanger, 1960), the phosphorylated enzyme was also found to be more stable than the active enzyme. We proposed that phosphorylation of the serine hydroxyl group caused a conformational change at the active center, resulting in an inherently more stable configuration. We proposed, furthermore, that this conformational change should be considered to be the normal consequence of a reaction between chymotrypsin and a substrate. We believe the same to be true for trypsin. A recent paper (Havsteen and Hess, 1962) has confirmed our earlier findings and, in addition, reports changes in optical rotatory dispersion as a result of phosphorylation of chymotrypsin. The authors conclude, as we did, that a conformational change has occurred at the active center of chymotrypsin.

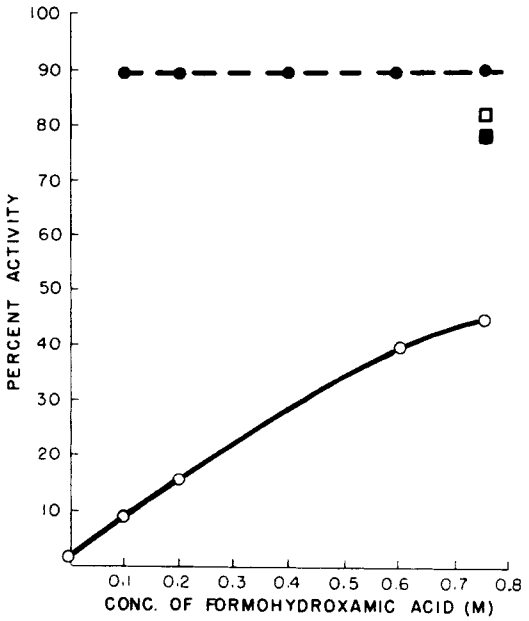


FIG. 2.—Effect of formohydroxamic acid concentration upon rate of reactivation. Conditions as described in legend to Figure 1. Time, 1 hour. O—O, DEP-trypsin; ●---●, trypsin; □, DEP-trypsin, 3 hours; ■, trypsin, 3 hours (see text).

—In Figure 4 are the results of a series of experiments designed to determine the effect of *p*H upon the reactivation of DEP-trypsin by hydroxylamine. Buffer of an unusually high concentration was used because of the tendency of hydroxylamine to decompose into basic products, in particular NH_4OH. The curve has an optimum and is in accord with a reaction between NH_2OH (pK_a 6.20) and a protonated group on the enzyme with a pK_a near neutrality.

Complete reactivation of DEP-trypsin could be obtained by incubation with 2 M NH_2OH for 18 hours at pH 7.0 at 25° or with 1.25 M NH_2OH under the same conditions for 24 hours. These conditions were chosen arbitrarily and should not be considered minimal. According to Cunningham (1954) and as confirmed in this laboratory hydroxylamine was incapable of completely reactivating DEP-chymotrypsin, the activity levelling off at approximately 40%. We are now investigating this phenomenon.

Reactivation by Various Nucleophilic Agents.—A number of oximes and hydroxamic acids were tested as reactivators of DEP-trypsin. The results of this study (*p*H 7, 25°) are shown in Table I, in which two categories of compounds are listed: hydroxamic acids and oximes (hydroxylamine is listed with the oximes). The compounds are arranged in order of their dissociation constants. Two reactivation rate constants are given: k_A, in which the concentration of the nucleophilic compound is considered, and k_{A^-}, in which the concentration of the anionic form of the reactivator is used in the calculations.

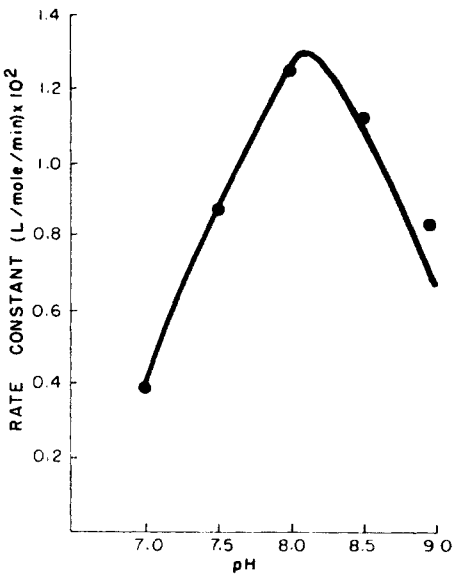


FIG. 3.—Effect of *p*H upon reactivation of DEP-trypsin by 0.1 M formohydroxamic acid. Time, 2 hours; temp., 25°, buffer, same as in Figure 1.

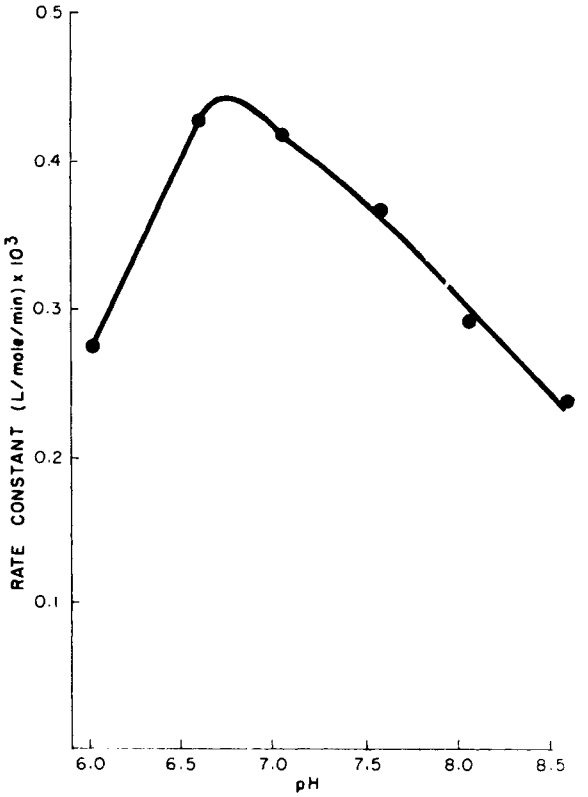


FIG. 4.—Effect of *p*H upon reactivation of DEP-trypsin by 0.25 M hydroxylamine at 25° in 0.25 M Tris–0.05 M maleate buffer containing 0.005 M $CaCl_2$. Temp., 25°. For assay, substrate was dissolved in 0.25 M Tris buffer containing 0.002 M $CaCl_2$, *p*H 8.2.

If the assumption is made that, in the case of the hydroxamic acids, the anionic form is the active nucleophilic agent, one should expect a uniformity in the values of k_A of reactivation in the absence of a specific interaction with DEP-trypsin. This is a result of the fact that, within a series of compounds, the relative nucleophilic activities are reflected in the dissociation constants of the compounds. The anions of the more powerful nucleophiles, therefore, are present in proportionately lower concentrations than the weaker nucleophilic agents. The data in Table I confirm this expectation of uniformity. For those compounds with higher k_A values we can assume a specific interaction between the enzyme and the reactivator on the basis of the previous results with DEP-chymotrypsin (Cohen and Erlanger, 1960) in which enzyme reactivator interactions were substantiated by competitive studies.

A more graphic demonstration of this phenomenon is shown in Figure 5, in which k_A - of each reactivator is plotted against its dissociation constant. All points that fall above (or below) the straight line represent compounds that specifically interact with DEP-trypsin. They include L-tyrosine hydroxamic acid, phenylacetohydroxamic acid, D-lysine hydroxamic acid, propionohydroxamic acid, and (see Table I) *anti*-phenylglyoxaldoxime. It is interesting to note that, contrary to its interaction with DEP–chymotrypsin (Cohen and Erlanger, 1960), *N*-phenylbenzohydroxamic acid shows no specificity for DEP-trypsin.

Effect of pH upon Spontaneous Reactivation.—Green and Nicholls (1959) showed that Sarin-inactivated chymotrypsin slowly regained activity when incubated in the absence of added reactivator. They found, moreover, that the rate of spontaneous reactivation became faster as the solution was made more acidic. This surprising result (see Discussion) prompted us to examine the spontaneous reactivation of DEP-trypsin.

As shown in Figure 6, the rate of spontaneous reactivation of DEP-trypsin increased with increasing *p*H. Our results differ, therefore, from those of Green and Nicholls (1959).

DISCUSSION

The hydrolysis of a substrate RCO-X by trypsin can be considered to occur *via* the two-step mechanism presented schematically in Figure 7. In the first step, the substrate reacts with the reactive serine of trypsin, represented by E-OH, to yield an acylated enzyme. Deacylation of the enzyme follows, yielding free enzyme and the hydrolyzed product. It has been suggested (*cf.* Green and Nicholls, 1959) that phosphorylation and dephosphorylation of the enzyme might occur *via* a similar mechanism which can be represented in a simplified form as shown in the lower portion of Figure 7, the important aspect of this reaction being the slow rate at which dephosphorylation and, therefore, regeneration of the enzyme occur.

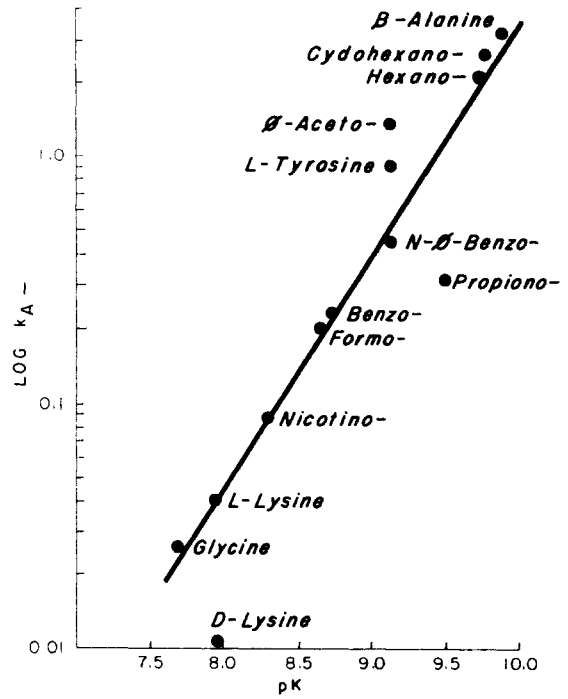


FIG. 5.—Relationship between k_A- and pK_a of reactivator (see footnotes to Table I).

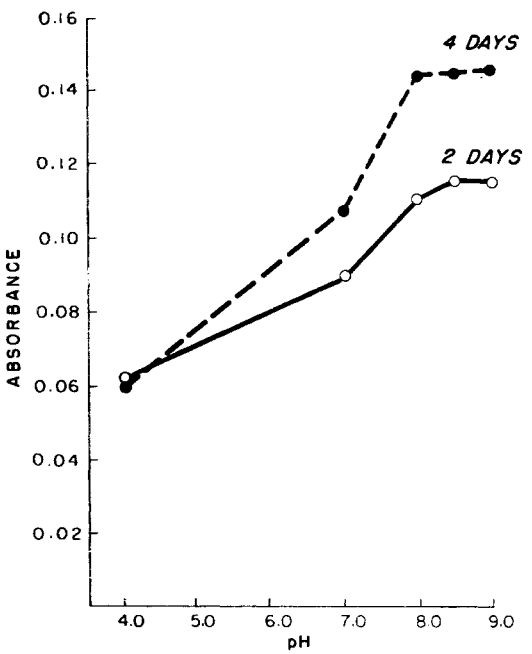


FIG. 6.—Effect of *p*H upon spontaneous reactivation of DEP-trypsin (0.15 mg/ml) in 0.05 M borate–maleate–acetate buffer, 0.03 M $CaCl_2$, temp., 25°.

If it is true that dephosphorylation and deacylation are mechanistically similar, the findings reported herein should be in accord with the facts known about the deacylation process. We have found, however, that although a number of similarities exist, agreement is by no means complete.

Let us consider our findings.

(*a*) The rate of spontaneous reactivation of DEP-trypsin was found to increase as the *p*H was increased, the curve being *S*-shaped, with a midpoint near *p*H 7 (Fig. 6). This is in accord with the data reported by Stewart and Ouellet (1959) for the deacetylation of monoacetyltrypsin and also agrees with the findings of Bender *et al.* (1961) for the deacylation of cinnamoyl-α-chymotrypsin. On the other hand, the rate of spontaneous reactivation of Sarin-inactivated chymotrypsin was reported to occur more rapidly as the *p*H was lowered (Green and Nicholls, 1959). In order to determine whether this discrepancy was due to the presence of a different organophosphorous functional group, an attempt was made to ascertain the effect of *p*H upon the reactivation of DEP-chymotrypsin. Unfortunately, the reactivation rate was too low to yield precise data. The question therefore remains unresolved. It might be pointed out, however, that, unlike the studies reported in this paper, the results of Green and Nicholls (1959) were obtained from experiments in which various types of buffers were used to cover the entire *p*H range. The discontinuous character of their data clearly indicates that the nature of the buffer influences the rate of spontaneous reactivation. A repetition of their experiments with Sarin-inactivated chymotrypsin in a buffer system similar to that used in our laboratory might be in order.

(*b*) The reactivation of DEP-trypsin by formohydroxamic acid appears to involve a group on the enzyme with a *pK* of 7.72 (see Fig. 3). Stewart and Ouellet (1959) found that the deacetylation of monoacetyltrypsin depended upon an unprotonated functional group with a *pK* of 6.9. The discrepancy in dissociation constants need not disturb us, since, as shown by Bruice and Schmir (1959), the apparent value of the dissociation constant can depend upon the kinetics of the reaction. However, the state of the functional group (*i.e.*, whether it is or is not protonated) during the reactivation process cannot be ascertained, since the data will fit an interaction between an unprotonated group on the enzyme and the un-ionized hydroxamic acid as well as one between a protonated group on the enzyme and the hydroxamate anion. Swindler and Steinberg (1956) have shown that the hydroxamate anion participates in the hydrolysis of simple organophosphorus compounds. Extrapolation to our system would lead to the conclusion that the group on the enzyme must be protonated. An alternative scheme, however, might be a base-catalyzed attack of the un-ionized hydroxamic acid similar to that postulated by Anderson *et al.* (1961) for the deacylation of the hydroxyl of a serine residue, *viz.* Scheme II, where B represents the unprotonated group with a *pK* near neutrality. Scheme II might be favored if a direct attack by hydroxamate anion is prevented by the existence of a negatively

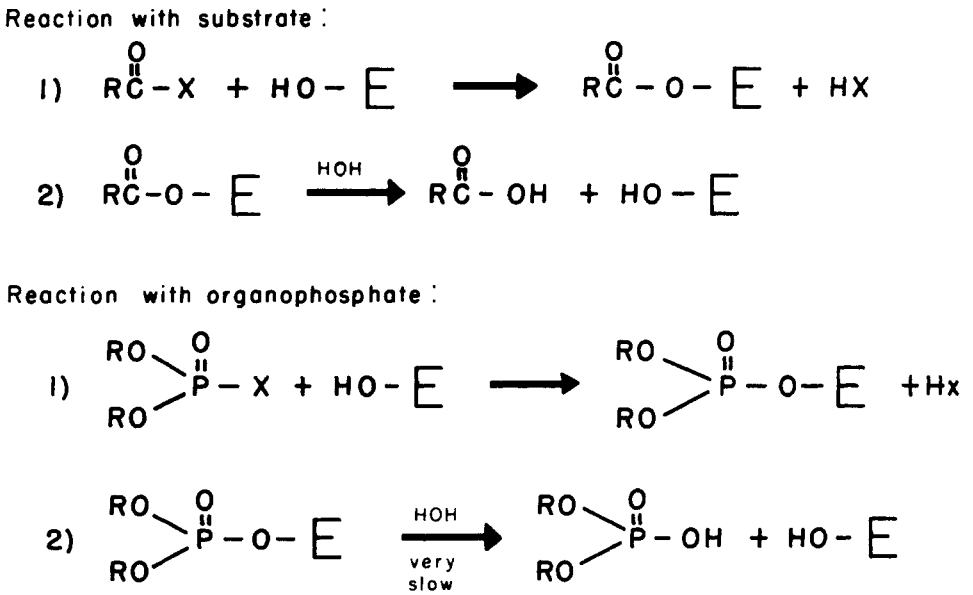
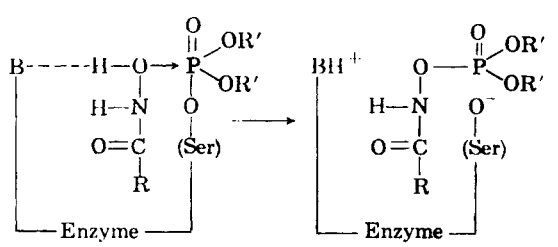


FIG. 7. Schematic representation of mechanism of enzyme acylation-deacylation and phosphorylation-dephosphorylation processes.

charged group near the active site, *e.g.*, an aspartyl residue. The data previously obtained for the reactivation of DEP-chymotrypsin (Cohen and Erlanger, 1960) will also fit the above scheme.

(*c*) Reactivation by hydroxylamine also involves a functional group on the enzyme with a dissociation constant near 10^{-7} (Fig. 4). The data, however, will fit *only* if we assume that this group must be protonated to be effective. Here, therefore, we are forced to accept a mechanism completely unlike the deacetylation mechanism of Stewart and Ouellet (1959). This does not come as a complete surprise, since the occurrence of a *p*H optimum for the hydroxylamine-mediated reactivation (Fig. 4) is not in accord with the data obtained for the hydroxylaminolysis of acetylated enzymes (*cf.* Bernhard *et al.*, 1960). The mechanism of reactivation by hydroxylamine, therefore, cannot be analogous to the mechanism of enzyme deacylation.

We can conclude from the above that (*a*) spontaneous reactivation probably occurs by means of a mechanism similar to the deacylation step in the ordinary enzyme-substrate reaction, (*b*) reactivation by hydroxamic acids *might* proceed in a similar way, but (*c*) hydroxylamine-mediated reactivation does not.



Scheme II

A discovery that might eventually reveal certain important aspects of the deacylation process is that of Foster (1961), who reported that deacetylation of monoacetylchymotrypsin is promoted by the competitive inhibitor indole. Our finding that the most effective reactivator of DEP-chymotrypsin was also a potent competitive inhibitor (Erlanger and Cohen, 1960) is somewhat analogous, although it must be pointed out that in the case of the reactivator, the nucleophilic and the binding groups are on the same molecule. It seemed of interest, therefore, to examine the effect of indole upon the reactivation process. This was investigated and it was found that indole, at a concentration of 10^{-3} M, did not influence the reactivation of DEP-chymotrypsin, whether spontaneous or mediated by hydroxylamine or by formohydroxamic acid. Furthermore, D-lysine methyl ester, an effective competitive inhibitor of trypsin ($Ki = 5 \times 10^{-4}$, unpublished) at a concentration of 7.5×10^{-4} M had no effect upon the reactivation of DEP-trypsin at *p*H 7 whether reactivator was present or not. If, as Foster suggests, the indole promotional effect can be viewed as another expression of enzyme specificity, and if dephosphorylation and deacylation can occur *via* similar mechanisms, it is difficult to understand why indole did not promote dephosphorylation. Either dephosphorylation and deacetylation are not similar processes or the effect of indole is not as visualized by Foster. The latter point deserves consideration, since, of all the competitive inhibitors studied by Foster, only indole produced a significant increase in the rate of deacylation.

With regard to the study of the effect of various hydroxamic acids upon the reactivation process (Table I), we can only conclude that none of the reactivators tested is highly specific. The some-

what greater activities of phenylacetohydroxamic acid, L-tyrosinehydroxamic acid, and *anti*-phenylglyoxaldoxime can be taken to indicate that trypsin possesses a binding site with an affinity for ring structures as well as the site that is specific for basic functional groups. The unusually low reactivity of D-lysinehydroxamic acid is an interesting finding and can be explained if we assume that D-lysinehydroxamic acid is bound specifically to the enzyme with its nucleophilic group unfavorably situated. When bound in this manner it serves to interfere with other hydroxamic acid molecules attempting to make a nonspecific attack. Further attempts to prepare more efficient reactivators of DEP-trypsin are now in progress.

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