acetate (19.90 g., 0.2 mole) and O,O-diethyl phosphorochloridothioate (37.8 g., 0.2 mole) were stirred at 75° for 5 hours. The mixture was cooled to room temperature and filtered. Evaporation of the solvent left 42.4 g. of yellow oil, which was distilled at 0.75 mm., to give 8.20 g. of recovered O,O-diethyl phosphorochloridothioate, b.p. 73–78°. The pot residue thickened to a viscous, undistillable tar during the distillation.

[CONTRIBUTION FROM THE DEPARTMENT OF MICROBIOLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, NEW YORK, NEW YORK]

Studies on the Reactivation of Diethylphosphorylchymotrypsin[1]

BY WILLIAM COHEN AND BERNARD F. ERLANGER

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The reactivation of a lyophilized preparation of diethylphosphoryl (DEP) chymotrypsin by approximately forty nucleophilic agents (oximes and hydroxamic acids) was examined, using a rapid colorimetric assay for chymotrypsin. The hydroxamic acids tested were synthesized by the usual methods as well as by two new synthetic techniques. With the exception of three compounds, all of the nucleophilic agents possessed activities which were related to their dissociation constants. The three exceptions, N-phenylbenzohydroxamic acid, N-phenylnicotinohydroxamic acid and pyridine 4-aldoxime dodeciodide, showed enhanced activities. The effect of *p*H on the reactivation process was also studied as well as the stability of DEP-chymotrypsin relative to chymotrypsin. Kinetic data were calculated on the basis of the catenary reaction DEP-chymotrypsin → chymotrypsin → denatured chymotrypsin. Preparations of DEP-chymotrypsin could be completely reactivated even after standing several months.

Introduction

α-Chymotrypsin reacts stoichiometrically with certain organic phosphates,[2,3] such as tetraethylpyrophosphate (TEPP) and diisopropyl fluorophosphate (DFP), with a resulting loss of enzymic activity. Considerable experimental evidence points to a phosphorylation of the hydroxyl group of a serine residue located at the active site of the enzyme.[4] A number of other esterases[5,6] and phosphoglucomutase[7] are similarly affected. In the case of DFP-inactivated cholinesterase, Wilson and others[8,9] were able to design effective nucleophilic reactivators as a result of a careful study of the specificity of the enzyme. Wilson also recently reported that pyridine aldoximes could slowly reactivate chymotrypsin inhibited by TEPP.[10] Earlier work by Cunningham has established that chymotrypsin, inactivated by diethyl *p*-nitrophenylphosphate,[11] can be partially reactivated by hydroxylamine, and while this paper was in preparation, Green and Nicholls reported the reactivation of Sarin-inactivated chymotrypsin by several oximes and hydroxamic acids.[12]

We are reporting the results of a study of the reactivation of lyophilized preparations of TEPP-inactivated chymotrypsin by a series of oximes and hydroxamic acids. In order to facilitate this study, a rapid assay method for chymotrypsin has been developed, which has made possible the screening of more than 40 compounds. Furthermore, two new methods have been devised for the synthesis of hydroxamic acids directly from carboxylic acids using, in one case, phosphorus pentoxide as the condensing agent, and, in the other, a mixed anhydride intermediate for the preparation of hexano- and N-phenylnicotinohydroxamic acids, respectively.

Experimental

Enzymes.—Three times crystallized α-chymotrypsin which had been dialyzed salt-free and lyophilized was purchased from Worthington Biochemical Co., Freehold, New Jersey.

Diethylphosphoryl-α-chymotrypsin (DEP-chymotrypsin) was prepared by the method of Jansen, *et al.*[2] The dialyzed and lyophilized material retained 0.1–0.7% of the initial activity.

Nucleophilic Reagents.—A number of oximes and hydroxamic acids were purchased from Distillation Products Industries, Rochester 3, New York: acetone oxime, benzaldoxime, cyclohexanaldoxime, methoxyamine, N-methyl-N-nitroso-aniline, N-phenylbenzohydroxamic acid, phenylglyoxaldoxime, 1-phenyl-1,2-propanedione dioxime, 1-phenyl-1,2-propanedione-2-oxime.

We are indebted to Drs. I. B. Wilson and S. Ginsburg of the Department of Biochemistry for the following compounds: nicotinohydroxamic acid methiodide, pyridine-4-aldoxime dodeciodide, 4-pyridine-1,2-ethanedione-2-oxime methiodide, 3-pyridine-1,2-ethanedione-2-oxime methiodide, pyridine-2-aldoxime heptiodide, pyridine-2-aldoxime methiodide, pyridine-2-aldoxime heptiodide, pyridine-2-aldoxime methiodide, pyridine-3-aldoxime methiodide, pyridine-4-aldoxime methiodide, pyridine-4-aldoxime pentiodide.

The following compounds were prepared in this Laboratory according to methods described in the literature: acetohydroxamic acid,[13] *o*-aminobenzohydroxamic acid,[14] *p*-aminobenzohydroxamic acid,[15] benzohydroxamic acid,[16]

(1) This work was supported in part by research grant E-1672 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health and a contract with the Office of Naval Research (NOnr 266(44)). A preliminary account appears in the Abstracts of the 136th American Chemical Society Meeting, Atlantic City, N. J., September 1959, p. 59-C.

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TABLE I

HYDROXAMIC ACIDS

Compound	Formula	M.p., °C.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found	Yield, %	Solvent for crystallization
p-Chlorophenoxyacetohydroxamic acid	$C_8H_8O_3NCl$	142–144	47.7	47.7	4.0	4.1	7.0	7.0	44	Methanol–ethyl acetate
Cyclohexanohydroxamic acid[a,b,c]	$C_7H_{12}O_2N$	131–132	59.1	58.8	8.5	9.1	9.8	9.3	70	Methanol
Indol-3-acetohydroxamic acid[a,c,d]	$C_{10}H_{10}O_2N_2$	126–127	63.1	63.1	5.3	5.1	14.7	14.5	63	Water
L-Lysine hydroxamic acid hydrochloride[e,f,g]	$C_6H_{16}O_2N_3Cl\cdot H_2O$	178–179	33.4	33.9	7.5	8.7	19.5	19.7	89	Water–methanol
D-Lysine hydroxamic acid hydrochloride[e,f,h]	$C_6H_{16}O_2N_2Cl\cdot H_2O$	178–179	33.4	33.7	7.5	8.0	19.5	19.3	75	Water–methanol
Hexanohydroxamic acid	$C_6H_{13}O_2N$	65–66	54.9	55.0	10.0	9.6	10.7	10.5	75	Benzene
N-Phenylnicotinohydroxamic acid	$C_{12}H_{16}O_2N_2$	132–135	67.2	67.4	4.7	4.6	13.0	12.9	29	Ether–petroleum ether

[a] Ethyl ester employed as starting material. [b] 2 moles of hydroxylamine employed. [c] 1 additional mole of potassium hydroxide employed. [d] Metallic sodium was employed for preparation of free hydroxylamine. [e] 3 moles of hydroxylamine employed. [f] Reaction mixture contained 7% water to keep lysine derivative in solution. [g] $[\alpha]^{25}D$ (1% in 2 *N* HCl) = +41.3. [h] $[\alpha]^{25}D$ (1% in 2*N* HCl) = −42.9.

chloroacetohydroxamic acid,[17] N,N-diethylhydroxylamine oxalate,[18] ethoxyformohydroxamic acid,[19] formohydroxamic acid,[20] glycine hydroxamic acid,[21] benzoquinone monoxime,[22] *p*-hydroxybenzaldoxime,[23] L-lactohydroxamic acid,[24] *p*-methoxybenzohydroxamic acid,[15] nicotinohydroxamic acid,[15] *p*-nitrobenzohydroxamic acid,[15] phenylacetohydroxamic acid,[25] picolinohydroxamic acid,[15] phthalohydroxamic acid hydroxylamine salt,[14] propionohydroxamic acid,[24] D- and L-tyrosinehydroxamic acid.[26]

The remaining hydroxamic acids are reported here for the first time. Except for the differences noted in Table I, cyclohexanohydroxamic acid, indole-3-acetohydroxamic acid and D- and L-lysinehydroxamic acids were prepared by procedures exemplified by that employed for *p*-chlorophenoxyacetohydroxamic acid. Hexanohydroxamic acid and N-phenylnicotinohydroxamic acid were each prepared by different procedures, as described below.

p-Chlorophenoxyacetohydroxamic Acid.—A one molar solution of hydroxylamine was prepared by mixing equimolar amounts of potassium hydroxide and hydroxylamine hydrochloride in methanol. The potassium chloride precipitate was removed by filtration after cooling the solution to 0°.

To 17 ml. of 1 *M* hydroxylamine (0.017 mole) was added 3.4 g. of *p*-chlorophenoxyacetic acid methyl ester (0.017 mole). After standing at room temperature for 24 hr., the solution was concentrated *in vacuo*. The hydroxamic acid was crystallized by the addition of ethyl acetate and then recrystallized from methanol–ethyl acetate.

Hexanohydroxamic Acid.—The synthesis of hexanohydroxamic acid was accomplished by means of a new procedure: 4.68 ml. (0.06 mole) of pyridine in 5.32 ml. of diethylphosphite were added to 10 ml. of a diethylphosphite solution containing 2.84 g. (0.02 mole) of phosphorus pentoxide and 2.5 ml. (0.02 mole) of hexanoic acid. 2.78 g. (0.04 mole) of solid hydroxylamine hydrochloride was then immediately added and the solution was stirred for 15 minutes at room temperature. After filtration, 8.88 g. (0.08 mole) of calcium chloride, dissolved in 100 ml. of distilled water, was added and a small amount of precipitate removed by filtration. The *p*H of the solution then was adjusted to about 11.5 and the precipitated calcium salt of the hydroxamic acid recovered by filtration. It was resuspended in 50 ml. of water and the suspension caused to dissolve by adjustment of the *p*H to 2.0 using 2 *N* HCl. The acidic solution then was extracted with ethyl acetate and the organic layer distilled *in vacuo* to a residual oil which was dried by repeated distillation using a benzene–methanol mixture. See Table I.

N-Phenylnicotinohydroxamic Acid.—Tri-*n*-butylamine (1.93 ml., 0.00812 mole) was added to 1.0 g. of nicotinic acid (0.00812 mole) dissolved in 10 ml. of methylene chloride and cooled to 0°. 1.11 ml. of isobutyl chlorocarbonate (0.00812 mole) was added and the solution kept at 0° for 30 minutes to insure complete reaction. After the addition of 0.33 g. of N-phenylhydroxylamine (0.0122 mole) which was dissolved in 10 ml. of methylene chloride, the solution was kept cold for 24 hr. A small precipitate which gave a negative $FeCl_3$ test was removed and the solution taken down *in vacuo*. The residue was dissolved in water and exhaustively extracted with ether. After drying over magnesium sulfate, the ether extract was distilled *in vacuo* and the residue crystallized. See Table I.

Chymotrypsin Assay.—A rapid and reliable chymotrypsin assay was devised in order to allow screening of a large number of nucleophilic reagents. The protein digestion methods which involve measurements of absorption in the ultraviolet were not applicable because of the ultraviolet absorption of many of the organic compounds employed for reactivation of DEP-chymotrypsin. The method of Ravin, *et al.*,[27] modified to eliminate the need for solvent extraction procedures, was used. The assay method depends upon the measurement of the amount of β-naphthol released by the attack of chymotrypsin on the substrate acetyl DL-phenylalanine β-naphthyl ester. The coupling of β-naphthol with a diazonium salt leads to the formation of a dye which is kept in solution by the addition of a water miscible solvent mixture and a detergent.

The substrate solution was prepared by dissolving acetyl-DL-phenylanine 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* THAM (tris-(hydroxymethyl)-aminomethane). Enzyme action was stopped by the addition of a solution containing 0.75% Duponol ME Dry (E.I. du Pont de Nemours & Co.) and 1.5% Fast Scarlet Salt GGN (General Dyestuff Co.) in distilled water. The latter couples with the liberated β-naphthol while the Duponol (detergent) and acetone keep the dye in solution. Although the *p*H optimum of α-chymotrypsin is 7.8, a buffer with a *p*H of 7.0 was employed in order to minimize non-enzymic 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 distilled water were mixed in a test-tube and equilibrated at 25° in a

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water-bath, after which 0.1 ml. of a chymotrypsin solution was added. After 10 minutes, 1 ml. of the coupling reagent, freshly prepared, was pipetted into the test-tube, followed, after a ten-minute 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. Figure 1 is a standard

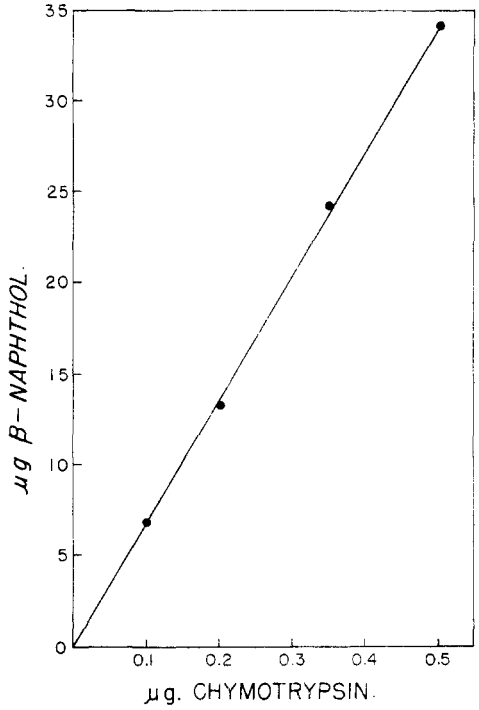


Fig. 1.—Effect of enzyme concentration on activity at 25° in 0.05 *M* THAM–hydrochloric acid buffer, 0.01 *M* $CaCl_2$, *p*H 7.0; $[S]_0 = 2.3 \times 10^{-4}$ *M*, $t = 10$ minutes. The indicated amount of enzyme is dissolved in a final volume of 6 ml. (see text for details).

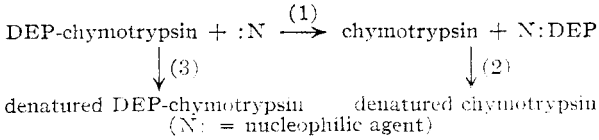
curve relating the quantity of β-naphthol released to micrograms of chymotrypsin.

Reactivation of DEP-Chymotrypsin—A DEP-chymotrypsin solution was prepared by dissolving 10 mg. of the lyophilized material in 1 ml. of 0.3 *M* THAM buffer containing 0.02 *M* calcium chloride which had been adjusted to the desired *p*H with reference to Beckman *p*H 7.0 buffer by means of a glass electrode and a Beckman G *p*H meter. The solutions of nucleophilic agents at the desired concentration were made up in 0.1 *M* THAM buffer containing 20% methanol when required. Solutions were adjusted to the same *p*H as that of the enzyme. Equal volumes of enzyme and reactivator were mixed, the test-tube sealed with Parafilm and incubated at 25° for the desired length of time.

To measure the extent of reactivation, 0.1 ml. of incubated solution was diluted with 0.001 *M* HCl so that 0.1 ml. could be employed for assay of chymotrypsin activity. A chymotrypsin control which had been treated in an identical manner was run concurrently. The method by which the extent of reactivation was calculated is described in the text.

Results

The reactivation of DEP-chymotrypsin by a nucleophilic agent is accompanied by a number of side reactions which complicate the task of measuring the extent of reactivation. Some of these reactions are



Unless the reactivation process is exceedingly rapid, these side reactions cannot be ignored. It follows, then, that measurement of chymotryptic activity at any particular time is not a true measure of the extent of reaction 1, since reactions 2 and 3 are occurring simultaneously. Furthermore, one cannot correct for reaction 2 by direct comparison with a chymotrypsin control. The denaturation of chymotrypsin in the presence and absence of nucleophilic agents was studied and was found to follow first order kinetics with respect to enzyme concentration.[28a] A chymotrypsin control will therefore denature most rapidly during the early part of the experiment, whereas the rate of reaction 2 will be highest toward the end of the experimental run. As a result, at alkaline *p*H, where denaturation is rapid, a direct comparison of reactivated samples with a chymotrypsin control can result in an apparent reactivation which is in excess of 100%.

In order to obtain a true measure of the effectiveness of a nucleophilic agent, a method must be devised to determine the rate constant of reaction 1. Earlier work carried out in this Laboratory[28b] had indicated that DEP-chymotrypsin was considerably more stable than chymotrypsin. If this could be confirmed, it might be possible to omit consideration of reaction 3 and thus simplify the task of measuring the rate of reaction 1. The stability of DEP-chymotrypsin relative to that of chymotrypsin was examined by means of two experiments. In the first, the effect of incubation at alkaline *p*H was determined. Both α-chymotrypsin and DEP-chymotrypsin at a concentration of 5 mg./ml. were incubated to 0.1 *M* THAM buffer, *p*H 9.0 for 24 hr. at 25°. These solutions along with controls of freshly prepared solutions of chymotrypsin and DEP-chymotrypsin were subsequently incubated with 1 *M* formohydroxamic acid at *p*H 7.0 for 24 hr. at 25° (to reactivate DEP-chymotrypsin) and assayed for enzymic activity. The results showed that DEP-chymotrypsin which had been incubated at *p*H 9.0 lost only 15% of its potential activity, while, on the other hand, α-chymotrypsin under similar conditions had lost 52% of its activity.

In the second experiment, separate solutions of α-chymotrypsin and DEP-chymotrypsin at a concentration of 5 mg./ml. in 0.1 *M* THAM buffer at *p*H 7.0 were heated at 56° for various time intervals. Aliquots were incubated with 1 *M* formohydroxamic acid at *p*H 7.0 for 24 hr. at 25° and assayed. In this manner the half-life of chymotrypsin at 56° was found to be three minutes, while that of DEP-chymotrypsin was ten minutes, or more three times as long as that of the active enzyme. These data show conclusively that phosphorylation of chymotrypsin increases its stability to a considerable extent. As a result of these experiments it was decided to disregard reaction 3.

The rate of reaction 1 was estimated by assuming the simplified system

(28) (a) The data indicated that the nucleophilic agents participated in the denaturation process, but, since they were in great excess, their concentrations remained essentially constant. (b) B. F. Erlanger, *Biochim. Biophys Acta*, **27**, 646 (1958).

$$\text{DEP-chymotrypsin} + :\text{N} \xrightarrow[(1)]{k_1} \text{chymotrypsin} + \text{DEP:N}$$

$$(2)\downarrow k_2$$

denatured chymotrypsin

Reaction 1 is a second order reaction but since the nucleophile was in large excess in all experiments, its concentration could be assumed to remain constant. k_1 therefore is equal to $'k_1/C_{\text{nucleophile}}$ where $'k_1$ is the first order rate constant for reaction 1. k_2 is the first order reaction constant for reaction 2.

$'k_1$ can now be evaluated from the equation

$$C_{\text{chymotrypsin}} = C_{\text{DEP-chymotrypsin}} \times \frac{'k_1}{k_2 - 'k_1} (e^{-'k_1 t} - e^{-k_2 t})$$

where

$C_{\text{chymotrypsin}}$ = concentration of chymotrypsin at time t as measured by assay against acetyl-DL-phenylalanine β-naphthyl ester;

$C_{\text{DEP-chymotrypsin}}$ = concentration of DEP-chymotrypsin at zero time.

k_2 was determined by measurement of the rate of denaturation of chymotrypsin in the presence of the various reactivators at the appropriate hydrogen ion concentration.

The above equation is modeled after the general equation describing the catenary reaction A → B → C where both steps are first order.[29] Solution was by means of successive approximations.

An additional fact must be considered: our findings (see below) and those of other laboratories indicate that the anionic form of the reactivator is the nucleophile. The concentration of the anion can, of course, be determined from the dissociation constant of the nucleophilic agent. In those cases where the data were not available, these values were determined from titration curves.

Table II lists the values of $'k_1$, k_1 and the dissociation constants of a number of oximes and hydroxamic acids. The compounds are arranged in order of decreasing dissociation constants. These experiments were carried out at *p*H 7.0 employing nucleophilic agents at a concentration of 0.005 *M*. Within a factor of two, the k_1 values for a majority of the compounds increased proportionally with increasing basicity of reactivator, *i.e.*, with increasing nucleophilic character. Figure 2, a plot of pK_a *vs.* log of reactivation rate constants, also illustrates this fact. There are several exceptions: pyridine-4-aldoxime, dodeciodide, N-phenylbenzohydroxamic acid, N-phenylnicotinohydroxamic acid were about ten times more effective than would have been predicted from their dissociation constants. Phthalohydroxamic acid and *p*-nitrobenzohydroxamic acid were less active.

Complete reactivation was achieved using 1.0 *M* formohydroxamic acid at *p*H 7.0, t = 48 hours. The ability of lyophilized preparations of DEP-chymotrypsin to be reactivated did not decrease even after storage for several months.

Effect of *p*H.—The rate of reactivation at 25° of DEP-chymotrypsin by 0.05 *M* phenylacetohydroxamic acid (pK_a 9.2) and 0.05 *M* glycine hydroxamic acid (pK_a = 7.7) was investigated between *p*H 6.0 and 9.0. The activities were measured after 24 hr. and the relative reactivation rates recorded in Fig. 3. The curves for both nucleophilic agents possessed optima which were related to their dissociation constants. Phenylacetohydroxamic acid showed optimal activity at *p*H 8.0, while the more acidic glycine hydroxamic was most active at *p*H 7.4. Moreover, the entire *p*H-activity curve of the latter was shifted toward the more acid region.

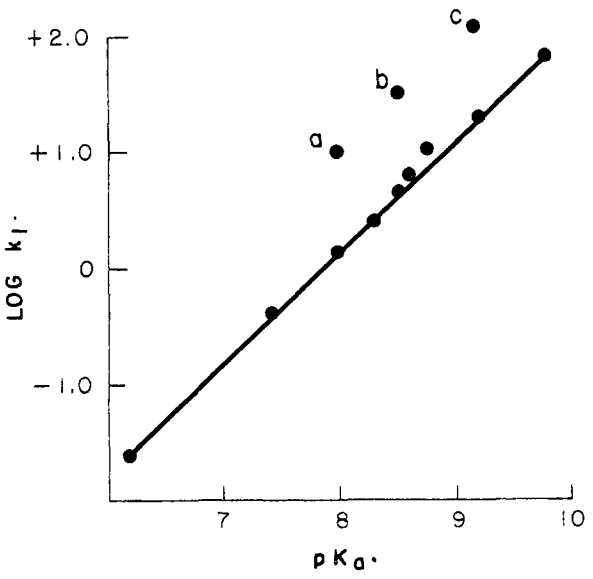


Fig. 2.—Relationship between dissociation constant of reactivator and rate of reactivation of DEP-chymotrypsin at 25° in 0.2 *M* THAM–hydrochloric acid buffer, 0.01 *M* $CaCl_2$, *p*H 7.0; $[E]_0$ = 5 mg./ml.; reactivator = 0.005 *M*. A representative number of points are included: a, N-phenylnicotinohydroxamic acid; b, pyridine-4-aldoxime dodeciodide; c, N-phenylbenzohydroxamic acid.

Discussion

The studies of Wilson[8] had shown that the most potent reactivators of DEP-inactivated acetylcholinesterase were nucleophilic compounds whose structures were complementary to parts of the active site of the enzyme. Expressed in another way, the efficiency of a reactivator depended upon two factors: the nucleophilic character of the compound and its structural complementariness relative to the active site of the enzyme. In the case of chymotrypsin, the latter property (*i.e.*, complementariness) has been examined to a considerable extent by means of kinetic studies of competitive inhibitors of the enzyme.[30] The results of these studies were expected to serve as a guide in the design of effective reactivators of DEP-chymotrypsin. Unfortunately, they did not. For example, indole-3-acetic acid is eight times as effective a competitive inhibitor as benzoic acid, while L-tyrosine and phenylacetic acid are twice as effective. Yet, as reactivators, the activities of the respective hydroxamic acids are of the same order, any differences being attributable to their nucleophilicities

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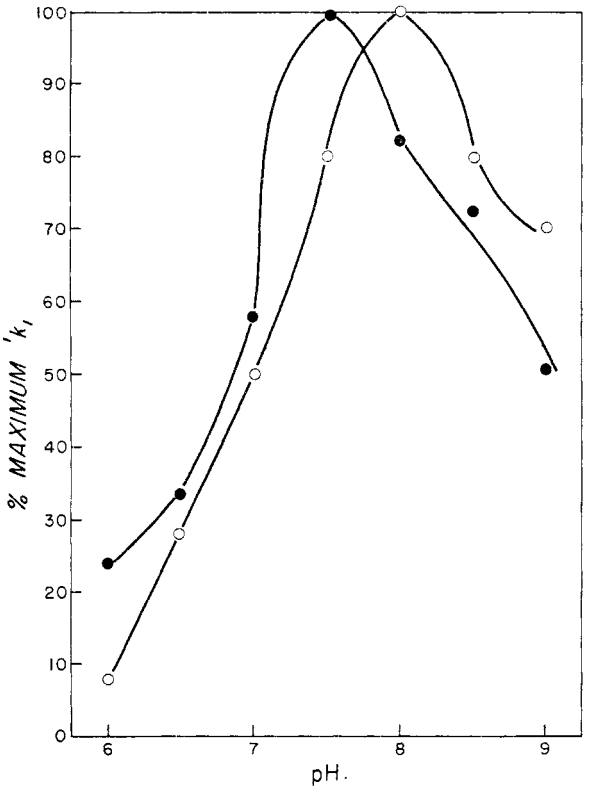

Fig. 3.—Effect of *p*H on reactivation of DEP-chymotrypsin at 25° in 0.2 *M* THAM–hydrochloric acid buffer, 0.01 *M* $CaCl_2$; $[E]_0$ = 5 mg./ml.; ●, 0.05 *M* glycine hydroxamic acid; ○, 0.05 *M* phenylacetohydroxamic acid.

as manifested by their dissociation constants. Furthermore, in the series cyclohexanone oxime, benzohydroxamic acid and phenylacetohydroxamic acid, in which the distance between the nucleophilic groups and the cyclic nucleus increases one carbon at a time, no marked variation in effectiveness as reactivators was observed. In fact, none of the above compounds was more active than acetohydroxamic acid, propionohydroxamic acid or hexanohydroxamic acid, compounds which are not known to bind specifically to chymotrypsin. Thus it appears that the two characteristics required for effective competitive inhibition of chymotrypsin (*i.e.*, complementariness and proper spacing in the inhibitor, of the functional groups which make contact with the enzyme surface) are not sufficient to insure an effective reactivator. One can infer that a reactivator, in addition, must possess a rigidity which serves to properly orient the nucleophilic group for an effective attack on the phosphorus atom of the phosphorylated enzyme.

Only in the case of three of the reagents studied, N-phenylbenzohydroxamic acid, N-phenylnicotinohydroxamic acid and pyridine-4-aldoxime dodeciodide, were the activities several fold greater than could be predicted from their dissociation constants. With respect to the two hydroxamic acids, substrate specificity studies[30] had indicated that the introduction of a second aromatic group (*i.e.*, in our case N-phenyl) increased binding and at the same time might have contributed to a

TABLE II

RATE OF REACTIVATION[a] BY VARIOUS OXIMES AND HYDROXAMIC ACIDS OF DEP-CHYMOTRYPSIN

Compound	pk_a[b]	$'k_1$, min.$^{-1}$ × 10^{-5}	k_1, l./mole min. × 10^{-2}
Methoxylamine	4.70[c]	0.09	0.02
Hydroxylamine	6.20[d]	0.18[m]	.04
Benzoquinone monoxime	6.25	1.13	.28
Nicotinohydroxamic acid methiodide	6.50[d]	0.66	.17
4-Pyridine-1,2-ethanedione-2-oxime methiodide	7.10	.66	.30
3-Pyridine-1,2-ethanedione-2-oxime methiodide	7.20	.50	.27
Glycine hydroxamic acid	7.70	.96	.77
D-Lysine hydroxamic acid	7.93	.60	1.13
L-Lysine hydroxamic acid	7.93	.42	0.80
N-Phenylnicotinohydroxamic acid	8.00	7.66	18.85
p-Nitrobenzohydroxamic acid	8.01[e]	0.96	2.15
Pyridine-2-aldoxime methiodide	8.00[f]	0.78	1.72
Pyridine-2-aldoxime heptiodide	8.00[f]	1.80	4.04
Nicotinohydroxamic acid	8.30[e]	0.96	4.02
Phenylglyoxaldoxime	8.30[g]	1.20	5.33
Chloroacetohydroxamic acid	8.40	0.30[m]	1.22
Pyridine-4-aldoxime methiodide	8.50[f]	1.20	7.87
Pyridine-4-aldoxime dodeciodide	8.50[f]	9.00	58.90
Pyridine-4-aldoxime pentiodide	8.50[f]	1.02	6.66
Picolinohydroxamic acid	8.60[e]	0.60	4.90
Formohydroxamic acid	8.65	1.20	10.90
p-Chlorophenoxyacetohydroxamic acid	8.75	0.84	9.40
Benzohydroxamic acid	8.75[h]	1.14	19.22
p-Hydroxybenzohydroxamic acid	8.93	0.30[m]	5.16
p-Methoxybenzohydroxamic acid	9.00[e]	0.84	17.20
N-Phenylbenzohydroxamic acid	9.15	15.00[l]	428.00
Phenylacetohydroxamic acid	9.18[i]	0.83	29.50
o-Aminobenzohydroxamic acid	9.17[j]	.16[m]	21.65
D-Tyrosine hydroxamic acid	9.20[k]	.83	26.50
L-Tyrosine hydroxamic acid	9.20[k]	.92	29.70
Pyridine-3-aldoxime methiodide	9.20[f]	.90	29.20
p-Aminobenzohydroxamic acid	9.32	.89	35.20
L-Lactohydroxamic acid	9.35	.42	18.85
Acetohydroxamic acid	9.40[i]	.72	36.40
Propionohydroxamic acid	9.45[i]	.90	52.50
Phthalohydroxamic acid	9.48	.11[m]	7.31
Indole-3-acetohydroxamic acid	9.58	.76	45.80
Cyclohexanohydroxamic acid	9.75	.48	54.00
Hexanohydroxamic acid	9.75	1.08	121.50
Benzaldoxime		0.35	
Ethoxyformohydroxamic acid		.90	
N,N-Diethylhydroxylamine		.06	
Cyclohexanaldoxime		.32	
1-Phenyl-1,2-propanedione-2-oxime		.60	
Acetone oxime		.05[m]	

[a] DEP-chymotrypsin was reactivated at 25° in 0.2 *M* THAM buffer, 0.01 *M* $CaCl_2$, *p*H 7.0, $[E]_0$ = 5 mg./ml., reactivator = 0.005 *M*. [b] The dissociation constants of compounds which have not been previously reported were determined by potentiometric titration at μ = 0.2 (NaCl). [c] T. C. Bissot, R. W. Parry and D. H. Campbell, THIS JOURNAL, **79**, 796 (1957). [d] Ref. 33. [e] Ref. 15. [f] Ref. 10. [g] Ref. 9. [h] A. L. Green, G. L. Sainsbury, B. Saville and M. Stansfield, *J. Chem. Soc.*, 1583 (1958). [i] Ref. 13. [j] Ref. 31. [k] Ref. 26. [l] Data are extradolated from 0.0025 *M* concentration. [m] Data calculated from experiments using 0.05 *M* reactivator.

more optimal orientation of the substrate or competitive inhibitor relative to the enzyme surface. These inferences derive additional support from our finding that N-phenylbenzohydroxamic acid is a powerful competitive inhibitor of chymotrypsin, having an activity twice as great as that of indole, under the same experimental conditions.

However, the reason for the enhanced activity of pyridine-4-aldoxime dodeciodide is not as readily apparent. If a model of the molecule is constructed, it can be seen that the terminal six carbons of the dodecyl group can be made to assume a ring-like structure a short distance away from the nucleophilic aldoxime group. Models of the other nitrogen substituted pyridine aldoximes tested cannot be manipulated in this way. The lack of rigidity of this structure, however, detracts somewhat from a mechanism of this kind.

The kinetic data in this paper were calculated on the basis of the participation of the anionic form of the nucleophilic reagent in the reactivation process. This is an assumption which also was made by other workers[8,9] and is not an unreasonable one especially since Stolberg and Mosher[31] have shown that hydroxamic acid anions can hydrolyse organophosphorus compounds *via* an unstable intermediate which decomposes into an isocyanate and an ester of phosphoric acid. It was thought that further insight into the mechanism of reactivation might result from a study of the effect of *p*H on the extent of reactivation using two hydroxamic acids with different dissociation constants. Phenylacetohydroxamic acid (pK_a 9.2) and glycine hydroxamic acid (pK_a 7.7) were chosen. As shown in Fig. 3, two *p*H optima were obtained, the positions of which were influenced by the dissociation constants of the nucleophilic agent. The shapes of the curves implicate at least two functional groups as participants in the reactivation process. One of them is most certainly the hydroxamic acid group; the other is on the enzyme and has a pK_a of approximately 7.0. These findings are in general agreement with those of Cunningham[11] and of Green.[12] The curves, however, do not establish whether the hydroxamic acid or its anion makes the nucleophilic attack. Though the latter is favored by the evidence cited above,[31] the data would then require that the functional group on the enzyme be protonated. These facts would be inconsistent with the view that the reactivation process is analogous to the last step in the normal enzyme–substrate interaction, wherein the group with $pK \sim 7$ is unprotonated. Green[12] has attempted to deal with this paradox as well as with the observed difference in behavior between sarin-inactivated chymotrypsin and acetylchymotrypsin, the rate of spontaneous reactivation of the former being more rapid at acid *p*H[12] while that of the latter increases with increasing *p*H.[32]

Another point which needs to be clarified is the finding of Cunningham[11] that the participating group on the enzyme possesses a pK_a of 8.0. Our data and those of Green lead to pK_a value of approximately 7.0. One can, perhaps, conclude from the above inconsistencies that the mechanism of reactivation is more complicated than has been suggested thus far.

Attempts to reactivate freshly prepared sarin-inactivated chymotrypsin using N-phenylbenzohydroxamic acid were unsuccessful. This was rather surprising considering that the above compound was the most efficient reactivator of DEP–chymotrypsin. It is possible that the isopropyl group of sarin (isopropoxymethyl fluorophosphonate) sterically hinders the approach of N-phenylbenzohydroxamic acid. It is more likely, however, that this phenomenon is related to the finding of Davies and Green[33] that the relative rates at which a number of nucleophilic agents reactivate phosphorylated acetylcholinesterase depended upon the phosphorus derivative used to inactivate the enzyme. Although the cause of this phenomenon has not been elucidated, steric hindrance cannot be the sole factor.

Unlike both phosphorylated pseudocholinesterase[34] and acetylcholinesterase,[35] lyophilized preparations of DEP-chymotrypsin can be completely reactivated even after storage for several months. It was shown in the case of the first two enzymes that spontaneous hydrolysis of one of the alkoxy groups linked to the phosphorus occurred, yielding a non-reactivatable product.[36] Obviously, this does not occur with DEP-chymotrypsin.

The relative stability of DEP-chymotrypsin deserves some comment. The presence of a substrate or competitive inhibitor is known to stabilize an enzyme, presumably by protecting the substrate binding site from denaturation.[37] The stability of DEP-chymotrypsin, however, probably is the result of a different mechanism, since, as Wilson has shown using DEP-cholinesterase, the organophosphorus compounds do not combine with that segment of the active center concerned with specificity but react solely with the esteratic site.[8] Undoubtedly, the absence of the process of self-digestion plays a part. The possibility must be considered, however, that the functional groups of the active center might have assumed a different, more stable steric configuration as a result of the reaction with tetraethylpyrophosphate. The arrangement in space of the various parts of the active center is a resultant of forces exerted by a number of hydrogen bonds (as well as other forces). The hydroxyl group of serine is believed to contribute to this hydrogen bonded structure. The approach of the organophosphate and subsequent displacement of the hydrogen from the hydroxyl group undoubtedly disturbs the "native" configuration, producing a new configuration, which may be inherently more stable. Looking at this as an interaction of substrate with enzyme the

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(36) H. S. Jansz, D. Brons and M. G. P. J. Warringa, *Biochim. Biophys. Acta*, **34**, 573 (1959).

(37) For a discussion of this phenomenon, see P. D. Boyer, H. Lardy and K. Myrbäck, "The Enzymes," Vol. I, Academic Press, Inc., New York, N. Y., 1959, pp. 210–212.

image of a flexible active center conforms with the induced fit hypothesis of Koshland.[38] We are suggesting that the approach of the substrate toward the serine hydroxyl triggers the shift in configuration.

(38) *Ibid.*, p. 334.

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[CONTRIBUTION FROM THE RESEARCH DIVISION, BRISTOL LABORATORIES, DIVISION OF BRISTOL-MYERS COMPANY]

Derivatives of 6-Aminopenicillanic Acid. I. Partially Synthetic Penicillins Prepared from α-Aryloxyalkanoic Acids

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The preparation of a number of new racemic α-aryloxyalkanoic acids (I) is reported. These and some previously described racemic α-aryloxyalkanoic acids were used for the N-acylation of 6-aminopenicillanic acid (6-APA) (II). The new diastereoisomeric α-aryloxyalkylpenicillins thus produced (III) were isolated as potassium salts. The two possible diastereoisomers of one of these new penicillins, namely, α-phenoxyethylpenicillin, were prepared in pure form and their distinctive physical properties are recorded.

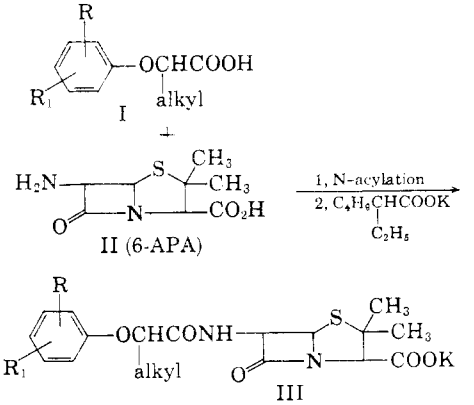
It has been known for some time that new penicillins could be produced by biosynthetic methods, that is by feeding the mold a variety of suitable precursors which could be incorporated into the penicillin molecule. Although numerous penicillins have been prepared in this manner, the method was found to be limited by the structural type of precursors that could be utilized by the mold.[1,2]

An important contribution to the problem of penicillin preparations was achieved recently when Sheehan and Henery-Logan reported the total synthesis of penicillin V.[3] This synthesis has opened a way by which an unlimited number of novel penicillins or analogs may now be prepared. However, at this time the procedure does not readily lend itself to the large scale preparation of penicillins of potential therapeutic value.

The recent disclosure by Batchelor, *et al.*,[4] that 6-aminopenicillanic acid (6-APA) (II) could be obtained by fermentation made available a most useful chemical intermediate which can be used for the practical preparation of a number of new penicillins not amenable to production by biosynthetic methods.

We have undertaken an extensive synthetic program using 6-APA as starting material, hoping to obtain novel penicillins that have one or more of the following advantages: (1) broader antimicrobial spectra, (2) more favorable absorption patterns and (3) reduced undesirable side effects. We wish to report a series of new penicillins prepared by the N-acylation of 6-APA (II) with a variety of α-aryloxyalkanoic acids (I). The intermediate α-aryloxyalkanoic acids used are described in Table I. These acids have been prepared by four different methods which are depicted in Table I as A, B, C and D. It should be noted that method C was found to be the most general and most reliable method especially when steric hindrance was present.

The new penicillins (III) described in Table II have been prepared by condensation of the α-aryloxyalkanoic acids with 6-APA through the acid chloride (method A) or the mixed carboxylic–carbonic anhydride[5] (method B) using either ethyl or isobutyl chloroformate. In all cases the reaction products were isolated as potassium salts by cation interchange with potassium 2-ethylhexanoate.



The infrared spectra (KBr) of the new potassium α-aryloxyalkylpenicillins of Table II showed a strong adsorption at 5.55–5.68 μ which is characteristic of the β-lactam ring. The presence of this four-membered lactam ring was further demonstrated by the quantitative hydroxylamine assay for penicillins.[6]

(1) H. T. Clarke, J. R. Johnson and R. Robinson, Editors, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 657.

(2) J. E. Philip, *et al.*, *J. Biol. Chem.*, **189**, 479 (1951); O. K. Behrens and M. J. Kingkade, *ibid.*, **176**, 1047 (1948). This last paper provides leading references to preceding papers in this series.

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(6) J. H. Ford, *Anal. Chem.*, **19**, 1004 (1947).