From a consideration of the sedimentation constant (3.5 S) and NH_2-terminal amino acid data (1 mole of DNP-alanine per 47,000 g of protein), it seems reasonable that the moleculyr weight of prorennin is between 40,000 and 50,000. The molecular weight of a sample of crystalline rennin has been estimated to be 40,000 (Schwander *et al.*, 1952), and Jirgensons *et al.* (1958) have found glycine to be NH_2-termical in a sample of purified rennin. Thus it seems that as much as 10–15% of the prorennin molcule may be released as peptide(s) during the formation of rennin. The sigmoidal shape of the activation curve in Figure 6, which shows the release of peptide material during activation, is suggestive of an autocatalytic process. However this sigmoidal shape may also be interpreted as indicative of the occurrence of sequential reactions during the activation:

$$\text{prorennin} \longrightarrow \text{intermediate} \longrightarrow \text{rennin}$$

Aronson (1962) has interpreted the activation curves for prothrombin as indicative of the formation of an intermediate in the conversion of prothrombin to thrombin.

In the fragmentation during activation, prorennin appears to resemble pepsinogen. Pepsinogen (mw 42,000) is converted to pepsin (mw 34,000) with the release of peptide fragments, one of which is a pepsin inhibitor. The NH_2-terminal sequence in pepsinogen, pepsin, and the inhibitor is leu-ileu (leu), ileu-gly, and leu-glu, respectively (Van Vunakis and Herriott, 1957). Since the C-terminal group in both pepsinogen and pepsin is alanine, pepsin is derived from the C-terminal portion of pepsinogen while the peptides released are derived from the NH_2-terminal portion of the chain. The nature of the peptide(s) released during the activation of prorennin has not yet been determined, however it is apparent that at least a part of this peptide material is released from the NH_2-terminal portion of the proenzyme.

The further characterization of prorennin, the nature of the peptide material released during activation, and the mechanism of activation are under investigation at the present time.

ACKNOWLEDGMENT

We are indebted to Dr. J. W. Mehl, University of Southern California, for the use of his analytical ultracentrifuge.

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Evidence for an Electrophilic Mechanism in Catalysis by Hydrolytic Enzymes*

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Received February 3, 1964

Diphenylcarbamyl chloride, diphenylcarbamyl fluoride, methylphenylcarbamyl chloride, and methylphenylcarbamyl fluoride were studied as acid-transferring inhibitors of chymotrypsin, trypsin, acetylcholinesterase, and serum cholinesterase. The fluorides were the better inhibitors in all cases. They are quite potent inhibitors and are the best of the known inhibitors of chymotrypsin and trypsin. The greater activity of the fluorides is evidence that there is an electrophilic component in the enzymic mechanism.

Certain compounds in reaction with proteases and esterases transfer an acid group to the active center yielding an inactive enzyme derivative. These acid-transferring inhibitors include a large number of organophosphates, carbamates, and sulfonates, of which diisopropylfluorophosphate (DFP) is perhaps the best known (Jansen *et al.*, 1949). Dimethylcarbamyl fluoride (Wilson *et al.*, 1961) and methanesulfonyl fluoride (Kitz and Wilson, 1962; Fahrney and Gold, 1963) are examples of fluorine derivatives belonging to the other two classes of compounds.

Acid-transferring inhibitors react with enzymes in a manner analogous to the reactions of substrates (Wilson, 1951). Although there is some disagreement it is generally held that substrates react to form an acyl-enzyme as a reaction intermediate (Wilson *et al.*, 1950). The acyl-enzyme is then rapidly hydrolyzed to complete the catalysis

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

where E is the enzyme, S the substrate, P_1 the alcohol product, P_2 the acid product, and E′ the acyl-enzyme. The reactions of acid-transferring inhibitors differ in

* This work was supported by the Division of Research Grants and Fellowships of the National Institutes of Health (grant NB 573 [16]), by a U. S. Public Health Service and Research Career Award (GM K3-15,012), and by a National Science Foundation grant (18926).

† Public Health Service Predoctoral Research Fellow. This report is from a dissertation to be submitted by H. Peter Metzger in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

that the enzyme derivative which is formed is hydrolyzed slowly or not at all.

Although the ease of hydroxide ion–catalyzed hydrolysis of an acid-transferring inhibitor is an important factor determining its potency as an enzyme inhibitor (Aldrich, 1954) and on this basis chlorides ought to be far more active than fluorides, the contrary might be true if the enzymic process involves an electrophilic as well as a nucleophilic mechanistic component. Proposals of this kind have, of course, been considered, but at the present time there is no real evidence that an electrophilic mechanism exists. A comparison of the activity of fluorides and chlorides might therefore yield valuable information concerning the enzymic mechanism. Unfortunately, nearly all acid chlorides are hydrolyzed rapidly in water and therefore are not suitable for study. The recent finding by Erlanger (1963) that the relatively stable diphenylcarbamyl chloride is a potent inhibitor of chymotrypsin enabled us to compare fluorides with chlorides. We describe here our work with diphenylcarbamyl chloride, diphenylcarbamyl fluoride, methylphenylcarbamyl chloride, and methylphenylcarbamyl fluoride in the inhibition of chymotrypsin, trypsin, acetylcholinesterase, and serum cholinesterase. The fluorides proved to be very good inhibitors indeed and in all cases they are much more potent than the corresponding chlorides.

Experimental

Inhibitors

Diphenylcarbamyl chloride (Distillation Products Industries) was recrystallized twice from methanol.

Diphenylcarbamyl fluoride was prepared from the chloride by reaction with 50% excess SbF_3 in hot xylene for 1 hour. Antimony halides were precipitated with ether and the solution was decolorized with charcoal, filtered, and evaporated to dryness. The solid was recrystallized twice with decolorization from methanol, yielding white crystals, mp 81–82°, yield 60%.

Anal. Calcd. for $C_{13}H_{10}NFO$: C, 72.54; H, 4.68; N, 6.51. Found: C, 72.70; H, 4.63; N, 6.45.

Methylphenylcarbamyl chloride was synthesized by the method of Weygand and Mitgau (1955) with some changes. *N*-Methylaniline in ethyl acetate was added dropwise with stirring over 1 hour to a saturated solution of phosgene in benzene at room temperature, and the reaction mixture was stirred for another hour. The solvents were evaporated and the solid product was recrystallized from benzene, mp 85–86°, reported mp 85–86°.

Methylphenylcarbamyl Fluoride.—This compound was previously synthesized using carbonyl fluoride (Emelius and Wood, 1948) but it was easier for us to start with the chloride (above). The chloride was melted on a steam bath, mixed with 50% excess SbF_3, and stirred for 1 hour. The mixture was left overnight at room temperature and the liquid product was separated by filtration through glass wool. The product was distilled at 68°/0.05 mm; yield, 55%; density, 1.169 at 25.0°.

Enzymes

Acetylcholinesterase was purified from electric organ tissue of *Electrophorus electricus* by the method of Kremzner and Wilson (1963). The assay used for this enzyme depended upon the decrease in acetylcholine concentration as measured colorimetrically by the hydroxamic acid method (Hestrin, 1949). The assay medium was 1.0 ml of 3×10^{-3} M acetylcholine, 0.1 M NaCl, 0.02 M $MgCl_2$, 0.01% gelatin, and 0.02 M sodium phosphate at *p*H 7.0, 25.0°. The assay was started by the addition of 0.100 ml of enzyme solution of suitable dilution and was stopped after 2 minutes by adding 2.0 ml of alkaline hydroxylamine. One minute later HCl and $FeCl_3$ were added to develop color and the optical density was read with a Leitz Photrometer using a 545-mμ filter. The purified enzyme preparation used in this work had an activity of 2.5 mmoles of acetylcholine hydrolyzed per minute per ml, and contained 0.75 mg protein per ml.

Since one product of hydrolysis, choline, is a competitive inhibitor of acetylcholinesterase, only an approximately linear relation exists between the amount of active enzyme and the amount of enzyme hydrolyzed. Thus, in an assay of a partially inhibited enzyme, a given diminution of the extent of hydrolysis only approximates the diminution of enzyme activity. To correct this discrepancy, a standard curve of substrate hydrolyzed against time was prepared by hydrolyzing a substrate preparation to within about 75% completion. An amount of substrate hydrolyzed in a given time could then be correlated with a quantity of active enzyme and so used to determine the true percentage of inhibition.

In a typical inhibition experiment, 0.100 ml of a 50-fold dilution of the purified enzyme preparation (in the above buffer solution) was added to 0.890 ml of buffer at 25.0°. The inhibition was started by adding 10 μl of a methanolic solution of the inhibitor. At suitable time intervals 0.100-ml portions were withdrawn and assayed as described.

Serum cholinesterase (human) was a commercial product made by Cutter Laboratories and sold under the trade name Cholase. A stock solution was made containing 2.4 mg of the preparation per ml of buffer. The buffer was the same as used for acetylcholinesterase. In a typical inhibition experiment, 0.500 ml of the enzyme solution was added to 0.490 ml of buffer at 25.0° and the reaction was started by the addition of 10 μl of a methanolic solution of the inhibitor. At suitable times 0.100-ml portions were withdrawn and assayed in the same way as acetylcholinesterase.

α-Chymotrypsin was a crystalline product obtained from Worthington Biochemical Corp., Freehold, N. J. A stock solution was prepared containing 23 mg/ml of water (10^{-3} M). The assay method depends upon the formation of β-naphthol by the hydrolysis of acetyl-DL-phenylalanine β-naphthyl ester (Cohen and Erlanger, 1960). One-half ml of a 10^{-3} M stock substrate solution in dimethylformamide was added to 5.0 ml of 0.05 M Tris, *p*H 8.0, in 20% (v/v) methanol and used immediately. To start the reaction, 0.100 ml of an enzyme solution of appropriate dilution was added and the reaction was allowed to continue for 2 minutes at 25.0°. The reaction was stopped by the addition of 1.0 ml of 1.5% Duponol M E Dry (E.I. du Pont de Nemours & Co.) and 0.075% Fast Scarlet Salt GGN (General Dyestuff Co.). The diazonium salt coupled with the β-naphthol producing a colored solution and the detergent stopped the enzyme reaction and helped keep the dye in solution. One minute later, 5.0 ml of acetone was added and the optical density was read. The absorption maximum of the dye is at 485 mμ. For convenience we used the Leitz Photrometer. The "445-mμ" filter gave the closest agreement with Beer's law but a calibration curve was still desirable.

In a typical experiment, stock enzyme solution was diluted 20 times into a 0.01 M Tris/HCl buffer, *p*H 7.5, containing 0.1 M NaCl. A 10-μl portion of this diluted enzyme solution was added to 1.0 ml of the stock buffer and thermostated at 25.0°. The reaction was started by the addition of 10 μl of a methanolic

solution of the inhibitor. At appropriate times, 0.100-ml portions were withdrawn and assayed as described above.

Trypsin was a crystalline product obtained from Worthington Biochemical Corp. A stock solution was prepared containing 24 mg of enzyme per ml of water (10^{-3} M). The assay method used depends upon the formation of *p*-nitroaniline by the hydrolysis of *N*-benzoyl-DL-arginine-*p*-nitroanilide (Erlanger *et al.*, 1961). A stock solution of 0.050 M substrate in dimethylformamide was prepared and used daily to make an assay medium by diluting it 50 times with 0.050 M Tris, *p*H 8.2, 0.02 M $CaCl_2$ in 10% dimethylformamide. The assay was started by adding 0.200 ml of the enzyme solution to 5.00 ml of the assay medium at 25.0°. After 6 minutes the reaction was stopped by adding 1.00 ml of 30% (v/v) acetic acid and the optical density was read at 410 mμ (Beckman DU).

In a typical experiment 10 μl of a methanolic solution of inhibitor was added to 5.00 ml of trypsin solution (10^{-5} M) at 25.0°, made by diluting the stock enzyme solution 100 times in 0.04 M Tris, *p*H 7.0, 0.02 M $CaCl_2$. At suitable times 0.200-ml portions were withdrawn and assayed as described.

Results

In most cases the rates of reaction of the inhibitor with the enzymes were measured under conditions where pseudo-first-order conditions prevailed, and good first-order plots were obtained. The first-order constants were found to be proportional to the inhibitor concentration indicating that the reactions are second order. In cases where the inhibitor concentrations were as little as twice that of the enzyme, second-order plots were made and the rate constants were calculated from the straight lines which we obtained using several initial inhibitor concentrations. The second-order constants are given in Table I. In all cases the fluorides are the more potent inhibitors, ranging from 5 to 160 times more active than the corresponding chloride.

Diphenylcarbamyl fluoride (k = 1.3 × 10^4 liters/mole sec) is evidently a very potent inhibitor of serum cholinesterase; even so it is distinctly less active than diisopropylfluorophosphate (k = 2.5 × 10^5 liters/mole sec) and tetraethyl pyrophosphate (k = 8.3 × 10^5 liters/mole sec). On the other hand it is the most potent known inhibitor of chymotrypsin and trypsin. Methylphenylcarbamyl fluoride is one of the most potent inhibitors of acetylcholinesterase (k = 1.2 × 10^4 liters/mole sec); it is nearly as potent as tetraethyl pyrophosphate (k = 3.5 × 10^4 liters/mole sec) and more potent than diisopropylfluorophosphate (k = 3.17 × 10^2 liters/mole sec). For further comparisons, second-order rate constants for a number of inhibitors and enzymes are given by Heath (1961) and Jandorf *et al.* (1955).

Table I

Specific Rate Constants for the Reaction between the Enzyme and the Acid-Transferring Inhibitor[a]

	Acetylcholinesterase[b]	Human Serum Cholinesterase[b]	Chymotrypsin[c]	Trypsin[d]
Diphenylcarbamyl fluoride	347	12,900	3790	58
Diphenylcarbamyl chloride	2.2	145	480	7
$k_{fluoride}/k_{chloride}$	160	89	8	8.3
Methylphenylcarbamyl fluoride	12,300	2070	18	0.14
Methylphenylcarbamyl chloride	2300	163	3.5	0
$k_{fluoride}/k_{chloride}$	5.4	13	5	...

[a] In units of liters/mole sec. [b] 0.1 M NaCl, 0.02 M $MgCl_2$, 0.01% gelatin and 0.02 M sodium phosphate at *p*H 7.0, 25°. [c] 0.1 M NaCl, 0.01 M Tris/HCl at *p*H 7.5, 25°. [d] 0.02 M $CaCl_2$, 0.04 M Tris/HCl at *p*H 7.0, 25°.

The reaction between diphenylcarbamyl fluoride and chymotrypsin was carried out in the presence of indole. This compound, a reversible competitive inhibitor of the enzyme, should slow the rate of reaction if the carbamylation occurs at the active site. This was found to be the case and the results appear in Table II.

Ethylammonium ion, a competitive inhibitor of trypsin, was added to the reaction between this enzyme and diphenylcarbamyl fluoride. The reaction rate was found to be slower, as shown by the results appearing in Table II.

Two reversible competitive inhibitors of acetylcholinesterase, tetramethylammonium ion and tetraethylammonium ion, also slowed the rates of reaction between three of the carbamyl halides which we have studied, and these results also appear in Table II. When this enzyme was completely inhibited by diphenylcarbamyl fluoride and then diluted, about one-half of the original activity returned in 1 day. When the experiment was done by dilution into 1.0 M NH_2OH, one-half activity returned in 2 hours.

Discussion

It is interesting to compare the rate of enzyme inhibition by these acid-transferring inhibitors with the rate of acylation of the enzyme during the course of ordinary substrate hydrolysis.

Considering the model discussed in the introduction, the velocity of enzyme-catalyzed hydrolysis of the substrate, v, is

$$v = \frac{k(\mathrm{E}^\circ)}{1 + \frac{K_m}{\mathrm{S}}}$$

where

$$k = k_3 \Big/ \left(1 + \frac{k_3}{k_4}\right)$$

$$K_m = \frac{k_2 + k_3}{k_1} \Big/ \left(1 + \frac{k_3}{k_4}\right)$$

where k_4 includes $[H_2O]$. When $S \ll K_m$ the reaction becomes second order and

$$v = \frac{k}{K_m}(\mathrm{E})(\mathrm{S}) = \left(k_3 \Big/ \frac{k_2 + k_3}{k_1}\right)(\mathrm{E})(\mathrm{S})$$

Evidently the second-order rate constant for the acylation of the enzyme by substrate is given by k/K_m liters/mole sec.

For methyl hippurate and chymotrypsin, k/K_m = 33 liters/mole sec (Bernhard *et al.*, 1960). Thus we see that chymotrypsin reacts far more rapidly with diphenylcarbamyl fluoride, k = 3.8 × 10^3 liters/mole sec, than it does with methyl hippurate, but not nearly as fast as it reacts with better substrates, for example, *N*-acetyltyrosine ethyl ester, where k/K_m = 2.8 × 10^5 liters/mole sec (Cunningham, 1954). Acetylcholinesterase reacts more rapidly with methylphenyl-

TABLE II

THE EFFECT OF REVERSIBLE COMPETITIVE INHIBITORS ON THE RATE OF REACTION BETWEEN THE ENZYME AND THE ACID-TRANSFERRING INHIBITOR

Enzyme	Carbamate Inhibitor	Concn (M)	Reversible Competitive Inhibitor	Concn (M)	Calculated k_i/k'	Found k_i/k'
Acetylcholinesterase[a]	Methylphenylcarbamyl chloride	1.3×10^{-6}	Tetramethylammonium iodide[b]	1.2×10^{-3}	2.0	1.8
Acetylcholinesterase[a]	Methylphenylcarbamyl chloride	1.3×10^{-6}	Tetramethylammonium iodide[b]	2.4×10^{-3}	3.0	2.8
Acetylcholinesterase[a]	Methylphenylcarbamyl fluoride	4.2×10^{-5}	Tetramethylammonium iodide[b]	2.4×10^{-3}	3.0	3.3
Acetylcholinesterase[a]	Diphenylcarbamyl fluoride	1×10^{-6}	Tetramethylammonium iodide[b]	2×10^{-3}	2.6	2.2
Acetylcholinesterase[a]	Diphenylcarbamyl fluoride	1×10^{-6}	Tetraethylammonium iodide[c]	2.5×10^{-4}	2.0	1.8
Trypsin[d]	Diphenylcarbamyl fluoride	2.17×10^{-5}	Ethylamine[e]	4.62×10^{-1}	8.3	8.6
Chymotrypsin[f]	Diphenylcarbamyl fluoride	2.0×10^{-6}	Indole[g]	2×10^{-3}	3.8	3.2

[a] 0.1 M NaCl, 0.02 M $MgCl_2$, 0.01% gelatin and 0.02 M sodium phosphate at pH 7.0, 25.0°. [b] $K_I = 1.2 \times 10^{-3}$ (Wilson and Alexander, 1962). [c] $K_I = 2.5 \times 10^{-4}$ (Bergmann and Shimoni, 1953). [d] 0.02 M $CaCl_2$, 0.04 M Tris/HCl at pH 7.0, 25°. [e] $K_I = 6.2 \times 10^{-2}$ (Inagami and Murachi, 1963). [f] 0.1 M NaCl, 0.01 M Tris/HCl at pH 7.5, 25°. [g] $K_I = 7.2 \times 10^{-4}$ (Huang and Niemann, 1953).

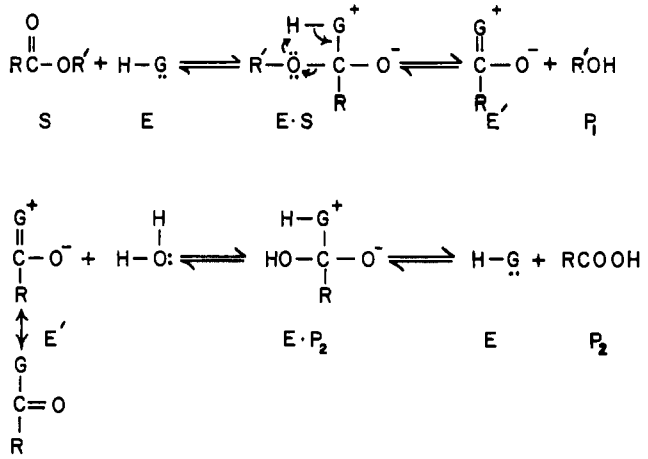
carbamyl fluoride, $k = 1.2 \times 10^4$ liters/mole sec, than with ethyl acetate, $k/K_m = 3 \times 10^3$ liters/mole sec (Wilson, 1952), but very much less rapidly than with acetylcholine, $k/K_m = 1.3 \times 10^7$ liters/mole sec (Wilson and Harrison, 1961). Trypsin reacts with tosyl-arginine methyl ester, $k/K_m = 3 \times 10^5$ liters/mole sec, very much faster than it does with diphenylcarbamyl fluoride, $k = 38$ liters/mole sec, but its rate of reaction with benzoyl-L-arginine amide, $k/K_m = 86$ (Erlanger *et al.*, 1961), is hardly more rapid. Evidently then it is the slowness of the deacylation step which makes these substances inhibitors rather than substrates.

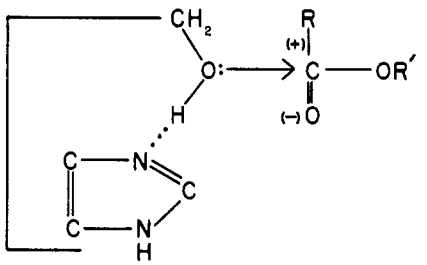
We have tacitly assumed that the site of carbamylation is the active site of the enzyme. In those cases where the reaction rate is very high, this alone suggests that the active site is involved, especially when the inhibitor is a fluoride. Other evidence bearing on the question of whether these acid-transferring inhibitors react at the active site comes from studies using reversible competitive inhibitors. In the presence of a reversible competitive inhibitor, some of the active sites become occupied and the rate of a reaction which occurs at the active site then becomes proportional to the concentration of the free sites. The ratio of rate constants is $k/k' = 1 + [(I)/K_I]$, where k' is the rate constant in the presence of (I), k in its absence, (I) is the reversible competitive inhibitor, and K_I is the equilibrium constant for the dissociation of the enzyme inhibitor complex. The value of K_I is known from the reversible competitive inhibition of substrate hydrolysis.

Previous work (Wilson *et al.*, 1961) has shown that in the case of acetylcholinesterase, carbamyl enzymes were formed by reaction with carbamic acid, *N*-methylcarbamic acid, and *N*,*N*-dimethylcarbamic acid derivatives, and the inhibited enzyme could be reactivated with nucleophilic reagents. When these inhibition reactions were carried out in the presence of a reversible competitive inhibitor the reaction rates were slowed to the expected extent, indicating that the inhibition was occurring at the active site. The same tests were applied in this work to determine whether carbamylation occurs at the active site. As we indicated in the previous section and Table II, the rate of reaction between three of the carbamates and acetylcholinesterase is slowed in the presence of two reversible competitive inhibitors, and the inhibited enzyme could be reactivated by hydroxylamine. Similarly, in the case of chymotrypsin, the reaction with diphenylcarbamyl chloride was slowed in the presence of indole, and the inhibited enzyme could be reactivated (Erlanger, 1963). We find that indole slows the diphenylcarbamyl fluoride inhibition as well (Table II). Trypsin conforms to this pattern also as the acid transferring inhibitor reacts more slowly with the enzyme in the presence of a reversible competitive inhibitor (Table II). It seems, therefore, quite certain that the active site is carbamylated in at least the faster reactions, i.e., in all cases except perhaps trypsin and chymotrypsin with the phenylmethylcarbamyl halides, and trypsin and acetylcholinesterase with diphenylcarbamyl chloride.

Our position, then, is that the active sites of the enzymes are carbamylated (with the previous reservations) at a speed equal to or greater than the rate at which they are acylated by relatively poor substrates. We conclude therefore that the enzymic mechanism is involved in the reaction.

All current proposals for the mechanism of these hydrolytic enzymes (and hydrolytic enzymes in general) involve an attack on the carbonyl carbon atom of the substrate by an enzymic nucleophile, leading to the formation of an acyl enzyme intermediate. A typical proposal (Wilson *et al.*, 1950) and, in fact, the first of this kind is where G is a structure containing an unidentified nucleophile represented by the electron pair and an unidentified acid is represented by H. This is a general acid-nucleophile mechanism. Evidence that the hydroxyl group of serine and the imidazole group of histidine are involved has led to proposals identifying the nucleophile as a serine hydroxyl group whose activity has been enhanced by the imidazole acting as a general base (Cunningham, 1957).





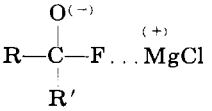
Numerous proposals of a similar nature have been made and some of these have been discussed by Bender (1962).

This work bears on the question of whether a tetrahedral intermediate or an electrophilic mechanism (or both) are involved in the catalysis. We believe that the greater activity of the acid fluorides as compared to the chlorides as acid-transferring inhibitors is strong evidence that an electrophilic mechanism is involved.

The usual order of reactivity of carbon-halogen compounds and phosphoryl and sulfonyl halides is I > Br > Cl > F. Substitution reactions with alkyl halides (Cooper and Huges, 1937; Chapman and Levy, 1952a), aqueous hydrolysis of acyl and sulfonyl halides (Swain and Scott, 1953), hydrolysis of chloro- and fluorophosphonates as well as their reaction with aniline (Saunders and Stacey, 1948) all conform to this order. It has been observed previously (Emelius and Wood, 1948), as well as during the course of this research, that, similarly to other acyl halides, carbamyl chloride derivatives are more readily hydrolyzed than the fluorides. This is the expected order if the rate-controlling step involves the rupture of a halogen-carbon (sulfur, phosphorus) bond.

In a few special circumstances such as the reaction of ammonia (Reinheimer *et al.*, 1961) and some amines (Bunnett, 1958) with 2,4-dinitrohalobenzenes and the reactions of benzoyl halides with a Grignard reagent (Entemann and Johnson, 1933), the order is reversed. The strong electron-withdrawing character of the nitro groups in the first case and the highly nucleophilic property of the carbanion in the second case especially favor the formation of tetrahedral complexes. These results have been explained by assuming that a complex is formed as an intermediate; the smaller size and high electronegativity of fluorine as compared to chlorine favor the formation of such a complex. Even though the expulsion of fluoride from the complex is more difficult than the expulsion of chloride, the overall rate can be faster for the fluoride if either the rate of formation of the complex is rate controlling or if a greater concentration of the fluoride complex more than compensates for the greater difficulty in expelling fluoride. Thus the formation of a tetrahedral intermediate does not necessarily lead to a reversed order.

In the case of the reaction of the Grignard reagent with the acid fluoride, it is possible, even probable, that an ion such as $MgCl^+$ is involved in an electrophilic catalysis:



and this may be the reason why the fluoride reacts faster.

The greater electronegativity of fluorine, its greater basicity, and its ability to form hydrogen bonds suggest that appropriate reactions of fluorides are more readily subject to acid catalysis than are those of chlorides. This is borne out in the solvolysis of alkyl fluorides, which, although less reactive than the corresponding chlorides in basic solution, are subject to acid catalysis (Miller and Bernstein, 1948; Chapman and Levy, 1952b). Especially significant for interpreting our findings is the fact that the hydrolysis of benzoyl fluoride is subject to a very lively acid catalysis while the hydrolysis of benzoyl chloride is not (Bevan and Hudson, 1953). The logical interpretation made by all these authors is that the acid catalyzes these reactions by electrophilic assistance in the removal of the fluorine atom. These, then, are cases of electrophilic catalysis in a nucleophilic substitution reaction.

Dimethylcarbamyl chloride reacts with nucleophiles in water by a unimolecular ionic mechanism (Hall, 1955). Dilute sodium hydroxide does not increase the rate of hydrolysis but mercuric ion does (electrophilic assistance in the removal of the chloride) (Hall and Lueck, 1963). The reaction of the fluoride has not been studied nor have the reactions of phenylcarbamyl halides. If the reactions of the carbamyl halides with these enzymes should involve an ionic mechanism it must be one in which the enzyme assists the ionization. We know that the reaction with an enzyme does not occur by a unimolecular ionic mechanism in which the enzyme functions as a simple acyl acceptor, because the rate of reaction is very much faster than the rate of hydrolysis and the rate is proportional to the concentration of enzyme. Ionization of the inhibitor in the form of an enzyme complex would account for the dependence of the rate on the enzyme concentration but we should still have to propose an assisted ionization to account for the fact that the rate of enzymic reaction is faster than the rate of hydrolysis. An assisted ionization immediately suggests an electrophilic mechanism; but since it is not known that even the nonenzymic reactions of phenylcarbamates involve an ionic mechanism, it remains doubtful that this mechanism is involved in the enzymic reactions. If the enzymic reaction involves a nucleophilic attack the greater reaction rate of the fluoride can be explained by electrophilic assistance in the removal of the halide, as in the ionic mechanism, or by the formation of a tetrahedral intermediate with the enzyme. In this connection we must note that although in the "neutral" hydrolysis benzoyl chloride reacts 50–100 times as fast as the fluoride, the fluoride reacts 40% faster with hydroxide ion (Swain and Scott, 1952). The change from Cl to F facilitates bond formation, but even with the highly nucleophilic and negatively charged hydroxyl ion the effect, although very large, is able to do little more than compensate for the greater difficulty in bond breaking. The enzymic nucleophile can

hardly be as effective as hydroxyl ion in perturbing the mechanism and even so the effect observed with hydroxyl ion (40%) is too small to account for our results. Our results can be explained by the formation of a tetrahedral intermediate with the enzyme or by the operation of an electrophilic mechanism which facilitates the removal of the halide. The two, of course, are not exclusive and it is our thought that probably both are involved. Had we been able to work at very high inhibitor concentrations and separate our measurements into binding constants and maximum rates we might have been better able to judge the relative importance of the two possible explanations. As it stands, we can in a logical sense claim only that one or the other mechanism is required by the data. The discussion of the reaction of benzoyl chloride and of benzoyl fluoride with hydroxide ion applies also to the formation of a tetrahedral complex so that, of the two, the electrophilic mechanism seems to us to offer the better explanation.

Some of this work bears on another problem. Certain competitive inhibitors of acetylcholinesterase and trypsin accelerate the rate of reaction of these enzymes with certain acid-transferring inhibitors and substrates. The rates of reaction of methanesulfonyl fluoride (Kitz and Wilson, 1963) and dimethylcarbamyl fluoride (Metzger and Wilson, 1963) with acetylcholinesterase are increased 35- and 14-fold, respectively, by tetraethylammonium ion. However as we have already stated, this compound decreases the rate of reaction with diphenylcarbamyl fluoride and methylphenylcarbamyl fluoride. Similarly, the hydrolysis of the poor trypsin substrate, acetylglycine ethyl ester, is accelerated 10-fold by ethylamine (Inagami and Murachi, 1963), but again we find that the rate of reaction of trypsin with diphenylcarbamyl fluoride is decreased by ethylamine. The acceleration of reaction rate does not occur with these acid-transferring inhibitors.

Acknowledgments

The authors wish to acknowledge their indebtedness to Dr. Sara Ginsburg for synthesizing the inhibitors, including the new compound diphenylcarbamyl fluoride. Recrystallized diphenylcarbamyl chloride was a gift from Dr. Erlanger. Purified acetylcholinesterase was kindly supplied by Dr. Kremzner.

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