hours at room temperature, water (5.5 ml.) was added and the clear solution thus obtained was acidified with N hydrochloric acid (congo red). The cloudy suspension was kept overnight in the refrigerator, and then it was centrifuged, the supernatant was decanted and the residue dried *in vacuo*. The amorphous powder (157 mg.) was dissolved in hot methanol from which it crystallized after standing in the refrigerator for 2 days. The crystalline protected decapeptide acid thus obtained (90 mg., m.p. 165–170°, sintering at 160°) was hydrogenated for 48 hours at normal pressure in a 1:1 mixture of acetic acid and water (20 ml.) in the presence of 10% palladium-on-charcoal (three portions of 100 mg. of catalyst were added at equal intervals during the reduction). After removal of the catalyst by filtration the solution was freeze-dried. The free decapeptide (50 mg.), $[\alpha]^{22}D$ −70.7° (c 0.99, N acetic acid), was homogeneous by paper chromatography (butanol–acetic acid–water, 4:1:5, R_f 0.50) and by paper electrophoresis (pyridine acetate buffer pH 4.6 and triethanolammonium acetate buffer pH 6.3) when developed with ninhydrin and Sakaguchi reagents. The quantitative amino acid analysis gave the ratios: lysine:arginine:proline:glycine:phenylalanine, 1.0:2.0:3.4:2.0:1.8.

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Sulfonyl Fluorides as Inhibitors of Esterases. I. Rates of Reaction with Acetylcholinesterase, α-Chymotrypsin, and Trypsin[1a]

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The second-order rate constants for inactivation of acetylcholinesterase, α-chymotrypsin, and trypsin by a series of sulfonyl fluorides have been determined. These compounds are most reactive toward chymotrypsin, but the same order of relative reactivities holds for both chymotrypsin and trypsin: phenylmethane- > 2-phenylethane-1- > benzene- > 2-methylpropane-1- > methanesulfonyl fluoride. This order, except for methanesulfonyl fluoride, is rationalized on the basis of steric and polar factors: the data can be fitted to the general Taft relationship. Methanesulfonyl fluoride, however, is about one-ten thousandth as reactive toward chymotrypsin as would be predicted. With acetylcholinesterase only the methane and benzene derivatives react at measurable rates, and limits are set upon the values of the rate constants for the other compounds. A hypothesis compatible with the data is that a reversible complex between the sulfonyl fluoride and enzyme forms before sulfonylation. At present, the evidence that binding markedly increases reactivity is suggestive, but not conclusive.

Introduction

Twenty four years ago Schrader[2] reported that methanesulfonyl fluoride is a potent insecticide. Recently this finding was extended by Myers and Kemp,[3] who found that methanesulfonyl fluoride has a lethal dose for rats comparable to that of diisopropyl phosphorofluoridate (DFP). In these studies it was observed that the symptoms of acute poisoning are similar to those produced by toxic alkyl phosphates. Moreover, they demonstrated that methanesulfonyl fluoride is an inhibitor of rat-brain cholinesterase *in vitro* and suggested that inhibition occurs by sulfonylation of a group in the active site of the enzyme. A year later Hartley and co-workers[4] were able to show that 1-dimethylaminonaphthalene-5-sulfonyl chloride can sulfonylate the active site of chymotrypsin.

Although ever since these investigations the potentiality of sulfonyl fluorides as reagents for characterization of functional groups within the active site was recognized and this information passed on through reviews,[5] no new studies have come to light concerning inhibition of hydrolytic enzymes by these reagents. The work presented here consists primarily of comparative rate studies of a series of sulfonyl fluorides with acetylcholinesterase, α-chymotrypsin, and trypsin. These studies were undertaken in order to lay the groundwork for a chemical investigation of the mechanism of inhibition.

Experimental

Diisopropyl phosphorofluoridate was purchased from Aldrich Chemical Co. Methane-, 2-methylpropane-1-, benzene-, phenylmethane-, and 2-phenylethane-1-sulfonyl fluorides were prepared from the corresponding chlorides by the general method of Tullock and Coffman.[6] Two sulfonyl fluorides are reported here for the first time.

2-Methylpropane-1-sulfonyl Fluoride.—2-Methylpropane-1-sulfonyl chloride[7] (8.4 g., 0.05 mole) was added to a stirred suspension of powdered sodium fluoride (10.2 g., 0.25 mole) in 6 ml. of tetramethylenesulfone. The mixture was heated at 110° for 3 hours, then cooled. After filtration the product was distilled; b.p. 47° (12 mm.), 5.06 g., 72%.

Anal.[8] Calcd. for $C_4H_9FO_2S$: C, 34.27; H, 6.47; S, 22.88. Found: C, 34.46; H, 6.72; S, 23.19.

2-Phenylethane-1-sulfonyl Fluoride.—2-Phenylethane-1-sulfonyl chloride[7] (10.25 g., 0.05 mole), NaF (10.2 g., 0.25 mole), and 10 ml. of dimethylformamide were stirred for 4 hours at 110°. Forty ml. of water and 25 ml. of methylene chloride were added; the organic layer was washed with water, dried, and the solvent evaporated. The residue was dissolved in 5 ml. of chloroform and 10 ml. of petroleum ether (30–60°), treated with charcoal, and filtered. The product crystallized on cooling and was recrystallized twice from chloroform–petroleum ether; 5.83 g., 62%, m.p. 26.5–27°.

Anal. Calcd. for $C_8H_9FO_2S$: C, 51.05; H, 4.82; S, 17.04. Found: C, 51.05; H, 5.23; S, 17.10.

Phenylmethanesulfonyl fluoride, m.p. 91–92°, reported[9] 90–91°; benzenesulfonyl fluoride, b.p. 80.0° (9 mm.), saponification equivalent 80.2 (theor. 80.1); and methanesulfonyl fluoride, b.p. 123°, reported[9] 124°, sapon. equiv. 49.4 (theor. 49.0). The latter two compounds were homogeneous by vapor phase chromatography.

Enzymes.—A purified preparation of acetylcholinesterase from the electric organ of *Electrophorus electricus* was used.[10] It had an activity of 1.4 mmoles of substrate hydrolyzed per min. per mg. of protein when assayed as described below. Twice recrystallized α-chymotrypsin and three times recrystallized trypsin were purchased from Worthington Biochemical Corporation.

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(1) (a) This work was supported by a National Science Foundation Predoctoral Fellowship (D.E.F.), a Grant-in-aid from the Muscular Dystrophy Associations of America, Inc., and Special Fellowship BT-484 (A.M.G.) and Neurochemistry Training Grant 2B-5216 from the National Institute of Neurological Diseases and Blindness. (b) To whom correspondence concerning this paper should be sent.

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(4) (a) V. Massey, W. F. Harrington and B. S. Hartley, *Discussions Faraday Soc.*, **20**, 24 (1955); (b) B. S. Hartley and V. Massey, *Biochim. et Biophys. Acta*, **21**, 58 (1956).

(5) (a) E. A. Barnard and W. D. Stein, in "Advances in Enzymology," ed. by F. F. Nord, Vol. XX, Interscience Publishers, Inc., New York, N. Y., 1958, p. 92; (b) D. K. Myers, in "The Enzymes," ed. by P. D. Boyer, H. Lardy and K. Myrbäck, Vol. 4, 2nd Ed., Academic Press, Inc., New York, N. Y., 1960, Part A, p. 476.

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(8) Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Melting and boiling points are uncorrected.

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Enzyme Assays.—Acetylcholinesterase was assayed by measuring the disappearance of acetylcholine with a modification of Hestrin's[11] ferric–hydroxamic acid method. A one-ml. beaker containing 1.00 ml. of enzyme solution (1–5 μg. of protein) was dropped into 5 ml. of substrate solution. The final composition of the reaction mixture was 1.67 mM acetylcholine bromide, 0.1 M NaCl, 0.02 M phosphate (Na), pH 7.0. After two min. at 25° the reaction was stopped by the rapid addition of 4 ml. of ice-cold hydroxylamine solution (freshly prepared by adding 3.5 g. of $NH_2OH \cdot HCl$ to 50 ml. of 1.75 M NaOH). Then 2 ml. of 2.8 M HCl and 3 ml. of a 10% $FeCl_3 \cdot 6H_2O$ solution in 0.1 M HCl were added. The resulting ferric–hydroxamic acid complex was measured at 540 mμ with a Beckman Model DU spectrophotometer.

Under the conditions of the assay the relation between extent of hydrolysis and amount of enzyme is non-linear. This is mainly because choline, which is a product of hydrolysis, is a competitive inhibitor. It is practical, therefore, to correct for choline inhibition ($K_I = 1.5 \times 10^{-4}$ M) by use of the integrated rate expression. This procedure gives a straight line relation between the observed extent of hydrolysis and the relative enzyme concentration.

The assay of chymotrypsin was based on the procedure given by Schwert and Takenaka.[12] The reaction mixture was 0.05 M phosphate (Na), pH 7.0, 1 mM N-acetyl-L-tyrosine ethyl ester, 0.1% gelatin (to improve linearity), and chymotrypsin (0.2–1.0 μg./ml.). The reaction was started by adding 2 ml. of chymotrypsin solution containing 0.075 M phosphate, pH 7.0, to 1 ml. of unbuffered substrate–gelatin solution in a cuvette, and the optical density decrease at 237 mμ was recorded as a function of time.

The esterase activity of trypsin, with N-benzoyl-L-arginine ethyl ester as substrate, was also determined by the method of Schwert and Takenaka,[12] except that the pH of the reaction mixture was adjusted to 7.2 instead of 8.0 (to slow non-enzymic substrate hydrolysis). The assay medium was 0.05 M Tris–HCl, pH 7.2, 1 mM $CaCl_2$, 1 mM N-benzoyl-L-arginine ethyl ester, and trypsin (1–4 μg./ml.). The reaction was started by the addition of 2 ml. of trypsin in $CaCl_2$–Tris buffer to 1 ml. of unbuffered substrate solution. The hydrolysis of substrate was followed by the increase in optical density at 253 mμ.

Rates with chymotrypsin and trypsin were linear for the first few minutes and were proportional to enzyme concentration in the ranges used; precision was ±2% for chymotrypsin and ±1% for trypsin.

Kinetics of Inhibition.—Each enzyme was allowed to react with a series of sulfonyl fluorides under carefully controlled conditions. The reactions were set up so that the inhibitor was in great excess over the enzyme; *e.g.*, the concentration of chymotrypsin was routinely 3 μg./ml. (*ca.* 1.1×10^{-7} M) with inhibitor concentrations from 4×10^{-6} to 2×10^{-2} M. At appropriate time intervals, aliquots were withdrawn and the enzyme activity determined. For each inhibitor a series of single runs was performed over a 4 to 8-fold range of inhibitor concentrations. Five to 6 points per run were taken covering *ca.* 50 to 80% of the complete reaction.

The inhibitor solutions were prepared by dissolving the sulfonyl fluoride in 2-propanol, followed by dilution with distilled water so that the concentration of 2-propanol was 10%. Ten ml. of freshly prepared inhibitor solution and 10 ml. of enzyme solution were mixed in a Lusteroid vessel thermostated in a water-bath at 25° (all solutions were thermally equilibrated at this temperature prior to addition). The first aliquot was removed *ca.* 1 min. after mixing. The final compositions of the reaction mixtures routinely employed with each enzyme were: (1) *acetylcholinesterase*, 5 μg. protein/ml., 0.1 M NaCl, 0.02 M phosphate (Na), pH 7.0, 0.05% gelatin, 10^{-4} M Versene; (2) *chymotrypsin*, 3 μg./ml., 0.075 M phosphate (Na), pH 7.0; and (3) *trypsin*, 6 μg./ml., 1.5 mM $CaCl_2$, 0.075 M Tris–HCl, pH 7.2. All reaction mixtures contained 5% 2-propanol.

In some runs, particularly the reaction of chymotrypsin with highly reactive inhibitors, the assay procedure did not completely quench the reaction at higher inhibitor concentrations; here the initial slope of the assay plot was used to obtain the enzyme concentration. For check runs under second-order conditions, the initial concentrations of both chymotrypsin and inhibitor were set equal using a mol. wt. of 27,300 for chymotrypsin[13] and the reaction was quenched by an 80 to 100-fold dilution prior to assaying.

Several inhibitors do not react at measurable rates with acetylcholinesterase at the highest concentrations attainable in 5% 2-propanol at 25°. The concentrations used and the longest intervals tested were: 1×10^{-3} M phenylmethanesulfonyl fluoride, 150 min.; 8×10^{-3} M phenylethanesulfonyl fluoride, 310 min.; 1×10^{-2} M 2-methylpropane-1-sulfonyl fluoride, 240 min.

(11) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

(12) G. W. Schwert and Y. Takenaka, *Biochim. et Biophys. Acta*, **16**, 570 (1955).

(13) A. M. Gold and D. E. Fahrney, manuscript in preparation.

Results

If sulfonyl fluoride is in large excess over enzyme, the rate of inactivation of the enzyme is proportional to the concentration of the sulfonyl fluoride. The data on inhibition gave linear plots of $-\log E$ against time, where E is the enzyme activity at a given time. Pseudo-first-order rate constants were obtained from the slopes of these plots. The second-order rate constant, k_{obsd}, was calculated by dividing each pseudo-first-order constant by the sulfonyl fluoride concentration at which it was obtained. The results obtained from the reaction of acetylcholinesterase, chymotrypsin, and trypsin with a series of sulfonyl fluorides are shown in Table I.

TABLE I

SECOND-ORDER RATE CONSTANTS[a] (L. MOLE^{-1} MIN.$^{-1}$) FOR INACTIVATION OF ENZYMES BY SULFONYL FLUORIDES

	Enzyme		
Inhibitor (RSO_2F) R	Chymotrypsin pH 7.0	Trypsin pH 7.2	Acetylcholinesterase pH 7.0
Phenylmethane-	1.49×10^4	2.71×10^2	$<6.1 \times 10^{-2}$[b]
2-Phenylethane-1	6.78×10^3	1.20×10^2	$<3.6 \times 10^{-3}$[b]
Benzene-	2.38×10^2	14.7	4.11
2-Methylpropane-1-	72	4.6	$<3.8 \times 10^{-3}$[b]
Methane-	1.3	0.75	1.49×10^2
DFP	2.7×10^3	3.0×10^2	1.3×10^4[c]

[a] Average deviations are within 3–5%, except 8% for methanesulfonyl fluoride with chymotrypsin. [b] Estimated upper limit for rate constant. See text. [c] Calculated from data of Aldridge (W. N. Aldridge, *Biochem. J.*, **53**, 62 (1953)) for erythrocyte acetylcholinesterase, 37°, 0.0357 M $NaHCO_3$.

An important advantage of the use of pseudo-first-order conditions is that the rate constants are independent of enzyme concentrations, which are difficult to establish. The values listed in Table I for chymotrypsin were reproducible to within their limits of precision with different batches of enzyme estimated to be 80 to 91% pure. Check runs with chymotrypsin performed under second-order conditions gave essentially the same second-order rate constants for phenylmethanesulfonyl fluoride and DFP as derived from pseudo-first-order data (see Table II). The results obtained under second-order conditions are estimated to be accurate to only 10% owing to the uncertainty in the concentration of active chymotrypsin. For comparison, the rate constant for DFP was measured under conditions used by Ooms[14]; the values are in good agreement.

TABLE II

COMPARISON OF PSEUDO-FIRST-ORDER AND SECOND-ORDER RESULTS WITH CHYMOTRYPSIN

		Rate constant (l. mole^{-1} min.$^{-1}$)	
Inhibitor	Conditions[a]	Pseudo-first-order kinetics	Second-order kinetics
Phenylmethanesulfonyl fluoride	pH 7.0 5% 2-propanol	1.5×10^4	1.2×10^4
	pH 7.8		1.9×10^4
DFP	pH 7.0 5% 2-propanol	2.7×10^3	2.5×10^3
	pH 7.8		2.0×10^4[b]

[a] Phosphate (Na) concentrations: pH 7.0, 0.075 M; pH 7.8, 0.1 M. [b] Ooms[14b] reports a value of 1.9×10^4 mole^{-1} min.$^{-1}$ under similar conditions.

Three of the sulfonyl fluorides react too slowly with acetylcholinesterase to show measurable inhibition at the highest concentrations attainable in 5% 2-propanol. In these cases maximum values for the second-order rate constants were estimated by as-

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

suming 1% reaction in the interval observed. The constants for inhibition of acetylcholinesterase by methanesulfonyl fluoride[14a] and benzenesulfonyl fluoride are both an order of magnitude lower than the rate constants calculated from the I_{50} values of Myers and Kemp[3]; the limiting value of the rate constant for phenylmethanesulfonyl fluoride is several orders of magnitude smaller. These discrepancies may arise from the fact that Myers and Kemp used a crude brain homogenate as their enzyme source, whereas our preparation was a highly purified fraction of *Electrophorus* electric organ.

Discussion

In the case of chymotrypsin and trypsin an explanation based on the intrinsic reactivity of the sulfonyl fluorides accounts for the relative reaction velocities, except for those of methanesulfonyl fluoride. The rates of reaction with these enzymes are related to steric and polar properties of the sulfonyl fluorides as predicted by the general Taft[15] relationship

$$\log k = \rho^*\sigma^* + \delta E_s + \text{constant} \qquad (1)$$

where k is the second-order rate constant for inactivation by RSO_2F. E_s and σ^* are steric and polar substituent constants, respectively, and are independent of the nature of the reaction. The adjustable parameters, ρ^* and δ, are reaction constants measuring the susceptibility of the reaction series to polar and steric effects, respectively. As shown in Fig. 1, plots of ($\log k - \delta E_s$) *versus* σ^* yield straight lines. Values of δ for chymotrypsin and trypsin (1.5 and 1.2, respectively) were selected by trial and error so that the slope, ρ^*, would be the same in both cases, *i.e.*, about 3.2. This correlation is only suggestive and has little statistical rigor. Methanesulfonyl fluoride, however, does not fall in the series; it reacts with chymotrypsin at one-ten thousandth the predicted rate, a fact which presumably points to the intervention of a different mechanism.

A remote possibility[16] exists that methanesulfonyl fluoride reacts with a "sulfonyl fluoride-acceptor" group adjacent to the active site at a rate comparable to that of the other sulfonyl fluorides, but that this reaction is undetected because the smaller bulk of the methyl group does not affect substrate hydrolysis. This would imply that inhibition observed at high concentrations (*ca.* 2×10^{-2} *M*) is due to extensive sulfonylation leading to partial denaturation of the protein. Trypsin was incubated with 8×10^{-4} *M* methanesulfonyl fluoride under the standard conditions for 30 minutes; during this interval 2% inhibition was observed. Phenylmethanesulfonyl fluoride was then added and a rate constant of 2.76×10^2 l. mole^{-1} min.$^{-1}$ obtained. This result establishes clearly that methanesulfonyl fluoride does not sulfonylate a reactive group near the active site without destroying enzymic activity.

Two fundamentally different pathways for inhibition by a sulfonyl fluoride are possible, each involving nucleophilic attack on the sulfur atom by a group in the active site: the familiar SN2 and enzyme-substrate complex mechanisms.[17] If the reaction proceeds by Path I, the sulfonyl halide is acting as an ordinary sulfonylating agent and its reactivity should be predictable from principles of organic chemistry. If inhibition proceeds by Path II there are two important factors determining the effectiveness of a sulfonyl fluoride as an inhibitor. First, there is the affinity of the compound for the active site; secondly, there is the readiness with which the compound, once bound at the site, can sulfonylate it.

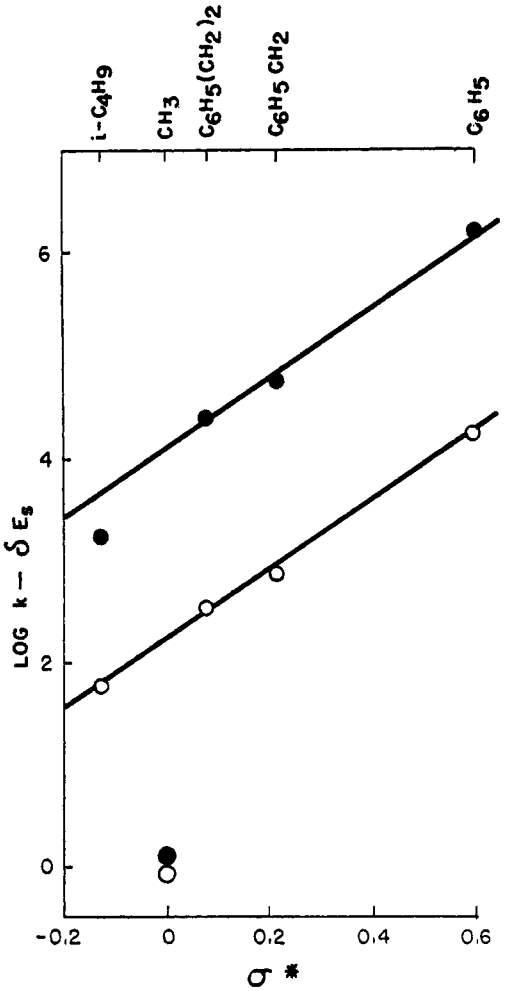

Fig. 1.—Plot of ($\log k - \delta E_s$) *versus* σ^*: chymotrypsin (δ = 1.5), ●; trypsin (δ = 1.2), ○; E_s for C_6H_5 taken as −2.55.

$$\text{EnB: } + \text{RSO}_2\text{F} \xrightarrow{k} \text{EnB}^+\text{—SO}_2\text{R} + \text{F}^- \quad \text{Path I}$$

$$\text{EnB: } + \text{RSO}_2\text{F} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{EnB: } \ldots \text{RSO}_2\text{F}$$

$$\text{(E)} \qquad \text{(I)} \qquad\qquad \text{(E·I)} \qquad \text{Path II}$$

$$\downarrow k_2$$

$$\text{EnB}^+ - \text{SO}_2\text{R} + \text{F}^-$$

$$\text{(P)}$$

For Path II the usual steady state approximation that d (E·I)/dt is negligible leads to

$$(\text{E·I}) = \frac{k_1(\text{E})(\text{I})}{k_{-1} + k_2} \qquad (2)$$

A better approximation[18] is obtained by differentiating eq. 2, recalling that (I) >> (E) under the experimental conditions.

$$\frac{\text{d(E·I)}}{\text{d}t} = \frac{k_1(\text{I})}{k_{-1} + k_2} \frac{\text{d(E)}}{\text{d}t} \qquad (3)$$

Since the conservation equation for enzyme, E_T =

(14a) An identical value for the rate constant has been reported by R. Kitz and I. B. Wilson, *J. Biol. Chem.*, **237**, 3245 (1962).

(15) (a) R. W. Taft, Jr., in "Steric Effects in Organic Chemistry," ed. by M. S. Newman, John Wiley and Sons, Inc., New York, N. Y., 1956, Chapter 13; (b) W. A. Pavelich and R. W. Taft, Jr., *J. Am. Chem. Soc.*, **79**, 4935 (1957).

(16) H. Fraenkel-Conrat, in "The Enzymes," ed. by P. D. Boyer, H. Lardy, and K. Myrbäck, Vol. I, 2nd Ed., Academic Press, Inc., New York, N. Y., 1959, p. 592.

(17) α-Chymotrypsin has been shown to react with only one equivalent of phenylmethanesulfonyl fluoride in 24 hr. when excess inhibitor is present at a concentration of 2×10^{-4} *M*. In the absence of enzyme the inhibitor is stable for hours under the conditions of the experiment. See ref. 13 and 18a.

(18) D. H. McDaniel and C. R. Smoot, *J. Phys. Chem.*, **60**, 966 (1956).

E + E·I + P, gives

$$0 = \frac{d(E)}{dt} + \frac{d(E\cdot I)}{dt} + \frac{d(P)}{dt} \quad (4)$$

it follows that

$$0 = \frac{d(E)}{dt} + \frac{k_1(I)}{k_{-1} + k_2}\frac{d(E)}{dt} + k_2(E\cdot I) \quad (5)$$

By substituting eq. 2 into eq. 5 to eliminate (E·I), the steady state solution for the velocity of enzyme disappearance is obtained as

$$\frac{-d(E)}{dt} = \frac{k_2(E)(I)}{K_M + (I)} \quad (6)$$

where K_M is the Michaelis constant, $(k_{-1} + k_2)/k_1$. The rate is not a linear function of (I); in fact, it reaches a limiting value at high concentrations of I corresponding to complete conversion of E to E·I, *i.e.*, saturation of the enzyme. With the assumption that $(I) << K_M$ (which is reasonable at concentrations of I low enough to make measurements by our method) eq. 6 reduces to

$$\frac{-d(E)}{dt} = \frac{k_2(E)(I)}{K_M}, k_{obsd.} = \frac{k_2}{K_M} \quad (7)$$

This rate formula is linear in (I) and is indistinguishable from a second-order rate equation. Nevertheless, this result holds for the formation of a dissociable enzyme–inhibitor complex and emphasizes the role of binding in reactivity.

If the formation of a reactive complex occurs to a significant extent ($k_2 << k_{-1} << k_1$), K_M will tend to be small and $k_{obsd.}$ large. On the other hand, if the formation of a reactive complex between inhibitor and enzyme is an unlikely event, ($k_2 >> k_{-1}$, k_1), eq. 6 simplifies to

$$-d(E)/dt = k_1(E)(I), k_{obsd.} = k_1 \quad (8)$$

and the reaction velocity is first order in inhibitor under all experimental conditions.

In the present case the kinetic data alone do not permit a choice between alternatives, since kinetic evidence for the formation of a complex would have to rest on the observation of a "saturation" effect with increasing inhibitor concentration.[18a] The SN2 mechanism is incompatible with the finding that phenylmethanesulfonyl fluoride inhibits chymotrypsin 12,000 times more rapidly than does methanesulfonyl fluoride. This result cannot be accommodated on the basis of simple steric and polar factors, since on these grounds the two compounds should differ only slightly. The anomalously low reactivity of the methane derivative suggests that binding facilitation (reaction *via* Path II) might contribute substantially to the large factor separating the rates for the two inhibitors. However, this explanation is tenable only if it is assumed that each sulfonyl fluoride, except methane-, is bound to approximately the same extent (K_M values are similar). Then relative reaction rates would be governed primarily by k_2 which should be subject to the usual steric and polar effects of the R group.

The same argument holds for trypsin. Although the relative reactivities are lower, the order is identical. Trypsin is highly specific for derivatives of lysine and arginine; however, low, inherent chymotryptic activity has recently been reported for this enzyme.[19] Also, Sturtevant[20] has pointed out that binding of the nonspecific substrate *p*-nitrophenyl acetate by chymotrypsin exceeds that of the specific substrate N-acetyl-L-tyrosine ethyl ester and has suggested that the specificity of the enzyme is expressed primarily in the deacylation step, and not necessarily in formation of the enzyme–substrate complex. It may be significant that methanesulfonyl fluoride reacts at nearly the same rate with chymotrypsin and trypsin. This comparison indicates that this inhibitor may react by Path I with both enzymes, rather than by Path II with poor binding (large K_M).

The results with acetylcholinesterase are in some respects the most interesting. The SN2 mechanism does not appear to provide a basis for explaining the fact that the phenylmethane, phenylethane, and 2-methylpropane derivatives fail to react at measurable rates. A proposal that steric repulsion between these molecules and the enzymic site is severe enough to greatly retard the reaction fails to account for the reactivity of benzenesulfonyl fluoride. Furthermore, relatively bulky organophosphates are often extremely reactive toward acetylcholinesterase; *e.g.*, diisobutyl *p*-nitrophenyl phosphate (second-order rate constant of 7.5×10^5 l. mole^{-1} min.$^{-1}$)[21] is 5,000-fold more reactive than methanesulfonyl fluoride. Clearly the sulfonyl fluorides have a much lower reactivity toward this enzyme; in the case of trypsin and chymotrypsin, however, several sulfonyl fluorides react as rapidly as good organophosphate inhibitors. Apparently for all three enzymes the most prominent feature of their reaction with sulfonyl fluorides is that rates are strongly influenced by binding and proper orientation.

Acknowledgment.—We thank Dr. David Nachmansohn for his support and encouragement in this work.

(18a) The phenylmethanesulfonyl fluoride–chymotrypsin system has been studied recently at high inhibitor concentrations by following proton release with a *p*H-stat; a value for K_M of 7×10^{-4} *M* was obtained at *p*H 6.0, 8°.

(19) (a) S. Maroux, M. Rovery and P. Desnuelle, *Biochim. et Biophys. Acta*, **56**, 202 (1962); (b) T. Inagami and J. M. Sturtevant, *J. Biol. Chem.*, **235**, 1019 (1960).

(20) (a) T. Spencer and J. M. Sturtevant, *J. Am. Chem. Soc.*, **81**, 1882 (1959); (b) J. M. Sturtevant, in "Protein Structure and Function," Brookhaven Symposia in Biology, No. 13, 1960, p. 158.

(21) A. J. J. Ooms, A. Hansen and L. Ginjaar, Proceedings of the IVth International Congress of Biochemistry, Vienna, 1958, Abstract No. 46.