Sulfonyl Fluorides as Inhibitors of Esterases. II. Formation and Reactions of Phenylmethanesulfonyl α-Chymotrypsin*

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Phenylmethanesulfonyl fluoride reacts stoichiometrically with α-chymotrypsin, producing an inactive monosulfonyl enzyme and one equivalent of free acid. Titration of the enzyme with the inhibitor results in nearly complete inactivation when the equivalent amount of inhibitor is present, and treatment of enzyme with a large excess of [7-^{14}C]phenylmethanesulfonyl fluoride leads to incorporation of the expected amount of ^{14}C. Irreversibly inhibited chymotrypsin and chymotrypsinogen do not react significantly with the radioactive inhibitor. The release of acid in the reaction was measured over a range of *p*H; in the *p*H region 7–8 nearly one equivalent of acid is released. The reaction rate is a function of *p*H, apparently being dependent upon a group of pK_a 7.0 in the nonprotonated form. A Michaelis constant of 5.6×10^{-3} M and k_2 of 3.1 min^{-1} were determined at *p*H 6.0, 10°. The reaction is slowed in the presence of β-phenylpropionate ion. It is concluded that the reaction is highly specific for the active site and that the mechanism is analogous to that for acylation. The stability of the sulfonyl group in [7-^{14}C]-phenylmethanesulfonyl chymotrypsin was investigated. At 40° the sulfonyl group is lost above *p*H 8.5 and below *p*H 3 but is stable at intermediate *p*H. In 8 M urea solution the protein loses the sulfonyl group at a constant rate from *p*H 2 to 8.5 but shows basic catalysis above *p*H 8.5. When the sulfonyl enzyme is desulfonylated at *p*H 2, 40°, the product has no enzymic activity; however, when this product is allowed to stand at *p*H 7 it spontaneously regains esterase activity. The reactivated enzyme has 60–70% of the esterase activity of α-chymotrypsin when activities are compared in a standard assay and based upon equal numbers of active sites. The position of the sulfonyl group in phenylmethanesulfonyl chymotrypsin is probably the hydroxyl group of the serine residue at the active site. When the sulfonyl enzyme is unfolded at *p*H 2, 40° the sulfonyl group is lost in an intramolecular cyclization reaction leading to an oxazoline ring. The latter opens rapidly in the acidic solution yielding a product having an *O*-aspartylserine linkage in the active site. On standing at *p*H 7 the protein rearranges via an *O,N*-acyl shift to an active enzyme. The fact that dinitrophenylation of "ester" protein followed by acid hydrolysis leads to isolation of dinitrophenylserine supports this hypothesis. Similar treatment of the enzymically active product leads to no trace of dinitrophenylserine.

Phenylmethanesulfonyl fluoride (PMSF)[1] irreversibly inhibits α-chymotrypsin at a rate comparable to that of diisopropylphosphorofluoridate (Fahrney and Gold, 1963a). Kinetic measurements, however, did not provide information concerning the chemistry of the reaction between PMSF and chymotrypsin; the questions of whether a sulfonyl enzyme is formed and the number and positions of the sulfonyl groups remained unanswered. An analogy with the well-characterized reaction of DFP would suggest that a single sulfonyl group is introduced and the hydroxyl group of the serine residue at the active site is the site of sulfonylation. Contrary evidence has been presented in the case of the reaction of chymotrypsin with 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride); Hartley and Massey (1956) have suggested that the side chain of a histidine residue in the active site is sulfonylated. Gundlach *et al.* (1962) have supported this conclusion by an independent method. In the case of acetylcholinesterase, Alexander *et al.* (1963) have shown indirectly that a sulfonyl enzyme is formed in reactions with certain esters of methanesulfonic acid.

This paper presents evidence indicating that the comparison between DFP and PMSF is valid. PMSF is a selective reagent which sulfonylates a serine residue in the active site without significant side reactions. Some of the properties of the phenylmethanesulfonyl enzyme differ from those of the diisopropylphosphoryl enzyme, and the former has been found to undergo a series of chemical transformations which result in nearly complete reactivation.

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[1] Abbreviations used in this work: PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropylphosphorofluoridate; ATEE, acetyl-L-tyrosine ethyl ester; PMS, phenylmethanesulfonyl; DIP, diisopropylphosphoryl; FDNB, 1-fluoro-2,4-dinitrobenzene; CM, carboxymethyl; DNP, dinitrophenyl.

EXPERIMENTAL

Materials and Methods

α-Chymotrypsin and chymotrypsinogen (each recrystallized three times) were purchased from Worthington Biochemical Corp. [7-^{14}C]Benzyl chloride (specific activity, 1.20 mc/mmole) was obtained from New

England Nuclear Corp. DFP was a product of Aldrich Chemical Co. and *N-trans*-cinnamoylimidazole was purchased from Nutritional Biochemicals Co.

Enzymic activity measurements were made as described earlier (Fahrney and Gold, 1963a), using the spectrophotometric procedure of Schwert and Takenaka (1955) with *N*-acetyl-L-tyrosine ethyl ester (Parks and Plaut, 1953) as substrate. The concentration of protein was calculated from the optical density at 282 mμ; it was assumed that the extinction coefficients for the PMS, DIP, and desulfonylated chymotrypsin are the same as that of α-chymotrypsin: 2.07 ml mg^{-1} cm^{-1} (Schwert and Kaufman, 1951).

[7-^{14}C]Phenylmethanesulfonyl Fluoride.—[^{14}C]PMSF was prepared in accordance with published procedures (Johnson and Sprague, 1936; Tullock and Coffman, 1960) for unlabeled materials. One ml of methanol and 70 mg of thiourea (0.92 mmole) were added to 105 mg of [7-^{14}C]benzyl chloride (0.83 mmole) (specific activity, 1.20 mc/mmole). The mixture was refluxed for 30 minutes and the methanol was evaporated in a stream of nitrogen. A small magnetic stirring bar and 4 ml of water were added, the mixture was cooled to 5°, and chlorine gas was passed in with stirring. After 5 minutes the resulting phenylmethanesulfonyl chloride was collected on a sintered-glass filter, washed with ice water, and dried overnight *in vacuo* over sodium hydroxide pellets. The chloride was then washed from the filter with alcohol-free chloroform into a 5-ml round-bottom flask and the chloroform was evaporated. A magnetic stirring bar, 0.5 ml of acetonitrile,[2] and 167 mg of dry, powdered sodium fluoride were added, and the mixture was heated to 90° for 6 hours. Two ml of alcohol-free chloroform was added, the mixture was filtered into a sublimation apparatus, and the solvent was removed in a stream of nitrogen. The product sublimed (45°, 0.3 mm) rapidly; 65.3 mg (0.375 mmole), mp 86–90° uncorrected, 45% based on benzyl chloride. A purer product had been expected: five preliminary experiments, using nonradioactive benzyl chloride, gave 48 ± 1%, mp 90–92° (reported 90–91°, Davies and Dick, 1932).

Recrystallization from benzene-cyclohexane afforded a purer labeled product, mp 90–92°. The infrared spectrum in chloroform solution was identical with that of unlabeled PMSF, except for the presence of an extremely weak band near 6.0 μ (Perkin-Elmer Model 21 infrared spectrophotometer). There was no trace of the bands characteristic of phenylmethanesulfonyl chloride.

Chromatography of PMS and DIP Chymotrypsin.—These proteins were chromatographed on columns of CM-cellulose (0.90 meq/g) (Ellis and Simpson, 1956) to remove impurities containing N-terminal serine. Chymotrypsin (500 mg) was dissolved in 25 ml of 0.02 M sodium citrate buffer, *p*H 6.0, and the solution was treated with either PMSF or DFP as desired (about 1.4 moles/mole enzyme). After standing for 1 hour at 5° the solution was introduced into a column 1.8 × 50 cm which had been equilibrated with 0.02 M citrate buffer at 5°. Elution was effected with a linear gradient of *p*H 6.0 citrate buffer at a flow rate of 20–30 ml/hour. The mixing chamber contained 500 ml of 0.02 M citrate and the reservoir contained 500 ml of 0.05 M citrate.

[7-^{14}C]Phenylmethanesulfonyl α-Chymotrypsin.—[^{14}C]PMS chymotrypsin was prepared routinely by adding 20 μmoles of [^{14}C]PMSF in 2 ml of 2-propanol to about 18 μmoles (500 mg) of chymotrypsin in 50 ml of 0.1 M NaCl. The *p*H was maintained at 7.0 by addition of 0.01 M NaOH. After 30 minutes at room temperature the reaction mixture was dialyzed in the cold for 16 hours against 1 mM HCl with frequent changes and then lyophilized. Such preparations have immeasurably low enzymic activity, are stable indefinitely when stored at −18°, and have specific radioactivities identical to those of samples assayed before lyophilization.

Ester Protein.—Routinely 15 mg of PMS chymotrypsin in 75 ml of 0.04 M NaCl was allowed to stand for 3.5 hours at 40°, *p*H 2.0, then dialyzed in the cold against 1 mM HCl and lyophilized.

Determination of [7-^{14}C]Phenylmethanesulfonyl Residues in Protein Derivatives.—Protein solutions were dialyzed at 4° against 1 mM HCl. After the protein concentration was determined from the optical density at 282 mμ duplicate 1.000-ml aliquots, containing 0.1–0.2 mg of protein, were evaporated in planchets. The elapsed time for 10,000 counts/sample was measured with a Geiger-Mueller counter. The complete removal of excess [^{14}C]PMSF and of [^{14}C]phenylmethanesulfonic acid upon dialysis against 1 mM HCl was confirmed in control experiments.

Dinitrophenylation of Chymotrypsin Derivatives.—Two procedures were used to prepare DNP proteins. (A) Ten mg of protein was allowed to react with fluorodinitrobenzene in ethanol-water buffered with $NaHCO_3$ in accordance with Levy's procedure (Fraenkel-Conrat *et al.*, 1955). (B) The reaction was carried out in a *p*H-stat in the presence of 6 M guanidine hydrochloride, as adapted from procedures of Phillips (1958) and of Smillie and Neurath (1959). Twelve mg of protein was dissolved in 3 ml of 0.04 M KCl, and 4.1 g of guanidine hydrochloride, 1 ml of ethanol, and 0.1 ml of FDNB were added. The *p*H was adjusted to 7.0 with 1 N KOH, and the reaction was allowed to proceed at this *p*H for 40 minutes at 40° (*p*H-stat). The reaction was stopped by lowering the *p*H to 3; the mixture was then transferred to a 50-ml centrifuge tube and four volumes of cold 0.1 N HCl were added. The precipitate was washed repeatedly with 10-ml portions of cold 0.1 N HCl until the supernatant cleared, then twice with acetone. After removal of excess acetone in a stream of nitrogen, the DNP protein was dried *in vacuo* over NaOH pellets.

Ten mg of DNP protein was hydrolyzed in 1 ml of constant-boiling HCl for 12 hours in a sealed, evacuated 18 × 150-mm test tube immersed in refluxing toluene. Subsequent extraction and two-dimensional paper chromatography of the ether-extractable DNP-amino acids were carried out as described by Levy (Fraenkel-Conrat *et al.*, 1955) except that 0.75 M phosphate, *p*H 6.0, was used as solvent for the second dimension. The spots were cut out and the DNP-amino acids were eluted with 4 ml of 1% $NaHCO_3$ solution. DNP-amino acid concentrations were calculated from the optical density at 360 mμ, with extinction coefficients from Fraenkel-Conrat *et al.* (1955). The mw of the DNP protein, based upon the assumption that 22 DNP residues are introduced (Wilcox *et al.*, 1957), is 22 × 166 + 25,000 = 28,600.

Reaction of PMSF with Chymotrypsin

Titration of Chymotrypsin Esterase Activity with PMSF.—Appropriate volumes of 0.200 mM PMSF in 10% 2-propanol were added from a microburet to 5-ml samples containing 29.4 mg of chymotrypsin in 0.1 M phosphate (Na), *p*H 7.0. After standing for 1 hour at 25°, each reaction mixture was diluted to a suitable volume and assayed for total enzyme activity.

[2] Reagent grade acetonitrile (100 ml) was refluxed for 1 hour with 1 g of phenylmethanesulfonyl chloride and distilled before use.

Specificity of PMSF.—Solutions (1×10^{-5} M) of the following proteins were allowed to react with 0.2 mM [^{14}C]PMSF (0.05 M phosphate, *p*H 7.0, 5% 2-propanol): chymotrypsin, chymotrypsinogen, diisopropylphosphoryl chymotrypsin (DIP chymotrypsin), and phenylmethanesulfonyl chymotrypsin (PMS chymotrypsin). The latter two were prepared by allowing 1.4 and 1.1 equivalents of DFP and PMSF, respectively, to react with chymotrypsin for 1 hour. The enzymic activity of each protein was determined before adding [^{14}C]PMSF. After 24 hours at 25° the reaction mixtures were dialyzed against 1 mM HCl in the cold and the specific radioactivity of the protein was determined.

Measurement of Hydrogen-Ion Liberation.—Quantitative measurements of the extent of liberation of hydrogen ion during the reaction of PMSF with chymotrypsin were made at constant *p*H with a Radiometer Titrigraph automatic titrator equipped with a scale-expander. The *p*H-stat was calibrated with Beckman standard buffer solutions. Jacketed reaction vessels were thermostated by means of circulating water.

Stock solutions of freshly prepared PMSF (recrystallized three times from benzene-cyclohexane) in 2-propanol were stored in the cold and checked repeatedly by introducing a volume containing about 100 μmoles of PMSF into 8 ml of 0.1 M KCl at *p*H 7 in the *p*H-stat. In this manner 0.1% hydrolysis or alcoholysis could have been detected; none was found. The same test ruled out the presence of PMS chloride, which is hydrolyzed under these conditions.

The KOH solution used in the syringe buret, the micropipet, and syringe buret itself were calibrated concurrently by saponifying PMSF at *p*H 9.0, 25°. Six ml of 0.1 M KCl was placed in the reaction vessel and the *p*H was adjusted to 9.0. A precisely known quantity of PMSF (about 4 μmoles) in 0.100 ml of 2-propanol was then introduced using a micropipet (the same micropipet was employed throughout all the experiments). The reaction, which leads to the release of two equivalents of hydrogen ion, was complete in less than 2 minutes. Since CO_2 absorption by the reaction system could be detected only over a 10–15 minute interval, alkali uptake due to CO_2 absorption during saponification was negligible.

The stability of PMSF under the conditions used below was determined in the *p*H-stat by raising the *p*H of a 0.4 mM solution in 0.1 M KCl, 15°, stepwise to *p*H 8.0. The possible effect of 1% 2-propanol on proton-binding properties of chymotrypsin was checked by adding 2-propanol to enzyme solutions equilibrated in the *p*H-stat.

Two titration procedures have been used: (A) Enzyme in excess over PMSF and (B) PMSF in excess over enzyme. Procedure A employs PMSF as a primary standard. About 6 μmoles of enzyme in 10 ml of KCl solution (0.1, 0.3, and 1 M) was allowed to attain equilibrium at 15° at a given *p*H in the *p*H-stat, 1 M KOH being used initially to adjust the *p*H. At low ionic strengths the attainment of equilibrium prior to sulfonylation is slow (5–40 minutes, depending on the *p*H). Autolysis is negligible at 15° or lower. A precisely known quantity of PMSF (*ca.* 4 μmoles) in 0.100 ml of 2-propanol was then introduced and the liberation of acid was followed by automatic addition of 0.016 M KOH until equilibrium was re-established. Many of the reactions proceed, initially, at a rate somewhat greater than the response rate of the *p*H-stat so that the *p*H decreased momentarily as much as 0.2 unit in runs at *p*H values above 7. The total base consumed in the reaction is, however, accurately recorded.

In method B, 4 μmoles of enzyme was allowed to react with 6–20 μmoles of PMSF as described above. The operational molarity of the enzyme solution was estimated by measuring proton release (using excess PMSF) at *p*H 7.4, where it has been established that proton release is exactly equivalent of the amount of PMSF reacting.

Kinetic Studies.—Rates were measured by automatic titration of the acid produced in the course of the reaction of PMSF with chymotrypsin. Care was exercised to maintain the *p*H of the reaction system constant to within 0.02 unit.

Runs for a *p*H-rate profile were conducted at low PMSF concentration (4 μmoles enzyme and 4 μmoles PMSF in 60 ml 0.1 M KCl, 10°) and the initial velocities were determined from the slope of the early (linear) part of the recorder trace. The apparent second-order rate constant was calculated from the initial velocity. Since the stoichiometry of hydrogen-ion liberation is not 1:1 over the *p*H range covered, initial velocities were corrected according to proton release values given (see Fig. 2). For the determination of the Michaelis constant PMSF was present in great excess over enzyme; accordingly, pseudo-first-order rate constants were calculated from the slopes of plots of $-\log(a - x)$ against time, where a is the total volume of base added at $t = \infty$ and x is that added at time t from an initial time chosen about 5–10 seconds after introduction of PMSF.

Reactions of PMS Chymotrypsin

Desulfonylation in Aqueous Solution.—Solutions of [^{14}C]PMS chymotrypsin (2 mg/ml) in 0.05 M buffers were incubated at 25 or 40°. After the appropriate time interval, the extent of desulfonylation was estimated by determining the amount of protein-bound radioactivity as described above. Under conditions where rapid desulfonylation was observed, the reaction was quenched by dilution with ice-cold phosphate buffer (*p*H 7.0) prior to dialysis. The following buffers were used: HCl–NaCl, *p*H 1 and 2; citrate (Na), *p*H 3, 4, and 5; phosphate (Na), *p*H 7.0; Tris-HCl, *p*H 8.0; and carbonate (Na), *p*H 9.5.

Desulfonylation in 8 M Urea Solution.—The rates were measured in 8 M urea, 0.1 M buffer, at 10°. The ionic strength was adjusted to 0.3 in all cases by addition of NaCl; calculations were based on literature pK_a values for aqueous solutions at 10°. Measurements of *p*H in the presence of urea were recorded as measured at 10° with a glass electrode (Beckman Model G *p*H meter). To start a reaction, 0.4 ml of a freshly prepared [7-^{14}C]PMS chymotrypsin solution (about 40 mg/ml in 1 mM HCl) was pipetted into 3.6 ml of urea-buffer-NaCl solution in a test tube thermostated in a water-bath (all solutions were thermally equilibrated at the bath temperature prior to mixing). At appropriate time intervals, 0.4-ml aliquots were withdrawn and diluted 8-fold with ice-cold 0.1 M citrate buffer, *p*H 3; after dialysis against 1 mM HCl (4°) the specific radioactivity of the protein was determined. The first aliquot was removed about 1 minute after mixing. Buffers used were HCl, *p*H 2; acetate, *p*H 4; phosphate, *p*H 6 and 8; carbonate, *p*H 8.5–10.8.

Reactivation.—Freshly prepared solutions of nonradioactive PMS chymotrypsin (about 1 mg/ml) in 0.05 M carbonate (Na), *p*H 9.5, were incubated at 40°. Five-ml samples were removed at 10, 20, and 30 minutes, brought to *p*H 7.0 by addition of 0.1 N HCl, and diluted to 25 ml prior to assaying for enzyme activity. A control solution of uninhibited chymo-

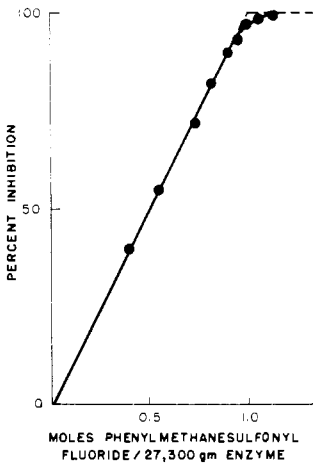

FIG. 1.—Titration of chymotrypsin esterase activity with PMSF. Enzyme is 2×10^{-4} M in 0.05 M phosphate buffer, *p*H 7.0. Incubated at 25° for 1 hour prior to assay.

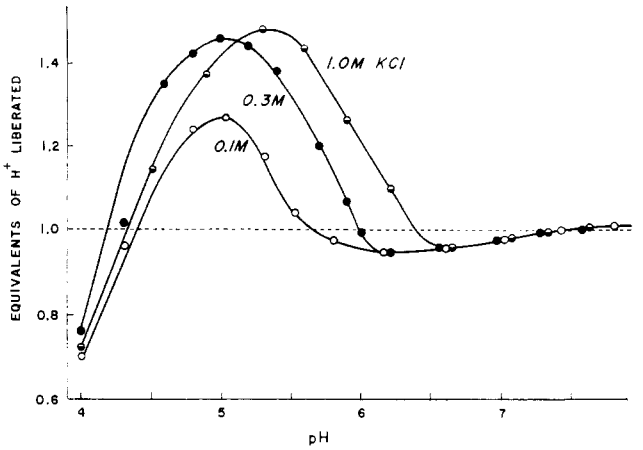

FIG. 2.—The liberation of hydrogen ion in the reaction of PMSF with chymotrypsin. Solutions contain 1% 2-propanol; 15°.

trypsin was treated in the same way, except that a 2000-fold dilution preceded the assay.

Solutions of PMS chymotrypsin (about 0.1 mg/ml; 4 mg/ml) were incubated in 0.04 M NaCl at 40°, *p*H 2. Two-ml aliquots were removed at appropriate times and diluted 5-fold with ice-cold water. Immediately after adjustment of the *p*H to 7 the volume was brought quantitatively to 25, 50, 100, or 200 ml; enzymic activity was determined at once and again after about 4 hours (preliminary experiments indicated that activity returns rapidly at *p*H 7 and that equilibrium is reached within 4 hours). In control experiments with native chymotrypsin diluted solutions were assayed immediately, at which time full activity was present.

In another experiment, 15 mg of chymotrypsin was dissolved in 25 ml of 0.1 M NaCl solution. A portion, 2.5 ml, of the chymotrypsin solution was diluted to 10 ml with 1 mM HCl to determine the specific esterase activity (against ATEE) and equivalent weight. The equivalent-weight determination was carried out by adding 0.5 ml of 0.2 M phosphate buffer (*p*H 7.0) and 0.2 ml of 2 mM [^{14}C]PMSF in 2-propanol to 3.0 ml of diluted chymotrypsin solution. After 10 minutes at room temperature, the reaction mixture was centrifuged and dialyzed against two 4-liter portions of cold 1 mM HCl (4–6 hours each dialysis). The specific radioactivity of the protein was then determined as described above. PMS chymotrypsin was prepared by allowing the remaining 22.5 ml of chymotrypsin solution to react with 0.6 μmole of unlabeled PMSF in 0.3 ml of 2-propanol for 1 hour at *p*H 7, 25°. After dialysis against 1 mM HCl, the solution of PMS chymotrypsin was diluted to 0.15 mg/ml in 0.04 M NaCl and acidified to *p*H 2.0 with HCl. Desulfonylation was then carried out at 40° for 3.5 hours. The solution was cooled in an ice bath and the *p*H was adjusted to 7 with NaOH. If the solution is not cooled before raising the *p*H to 7 the protein tends to precipitate. After the solution had stood for 4 hours at *p*H 7, 25°, both ATEE-esterase and equivalent-weight determinations were performed as before. For the latter, 3 ml of protein solution was used, the final protein concentration being the same as previously.

RESULTS

Stoichiometry.—The result of reaction of identical aliquots of α-chymotrypsin with varying amounts of PMSF under standard conditions is shown in Figure 1. Inhibition is proportional to the amount of inhibitor up to 95% and is complete (extrapolated) when 1 mole of inhibitor is added to 27,300 g of enzyme. Since this is close to the estimated molecular weight of the protein the reaction appears to have 1:1 stoichiometry. The slowness with which the sulfonylation reaction goes to completion with equimolar reactants may account for the slight curvature near the equivalence point. An apparent molecular weight of 27,300 is reasonable because the enzyme preparation was probably impure; if the molecular weight is taken as 25,000 (Wilcox *et al.*, 1957), the purity is 92%.

The specificity of the reaction was investigated further by measuring the extent of reaction of excess ^{14}C-labeled PMSF with chymotrypsin. Only 1 mole of protein-bound inhibitor per mole protein can be detected after allowing chymotrypsin to stand 24 hours with an excess of inhibitor (2×10^{-4} M:1×10^{-5} M). In the absence of enzyme the inhibitor is stable for many hours under the conditions of the experiment. The radioactive inhibitor does not react to a significant extent with chymotrypsinogen, PMS chymotrypsin, or DIP chymotrypsin; the degrees of labeling are presented in Table I. These data are convincing evidence that PMSF can sulfonylate only the active site of chymotrypsin under the conditions used. This result is confirmed and extended by the observation that, even at a concentration of 1×10^{-3} M, nonradioactive PMSF does not react at a measurable rate with chymotrypsin in 8 M urea (proton release followed).

The liberation of hydrogen ion during the reaction of PMSF with chymotrypsin has been described in a preliminary communication (Fahrney and Gold, 1963b);

TABLE I
EXTENT OF REACTION OF EXCESS [^{14}C]PMSF WITH CHYMOTRYPSIN AND DERIVATIVES[a]

Protein	Specific Activity (dpm/mg)	Moles PMSF Bound/Mole Protein[b]
Chymotrypsin	97,500	1.00
Chymotrypsinogen	1,020	0.000[c]
PMS chymotrypsin	556	0.006
DIP chymotrypsin	583	0.006

[a] Conditions were: 1×10^{-5} M protein, 0.2 mM [^{14}C]PMSF, 0.05 M phosphate, *p*H 7.0, 5% 2-propanol, 25°, 24 hours. [b] Specific activity of [^{14}C]PMSF was 1.20 mc/mmole; mw of chymotrypsin taken as 27,300. [c] Corrected for 1.05% chymotryptic activity present in chymotrypsinogen.

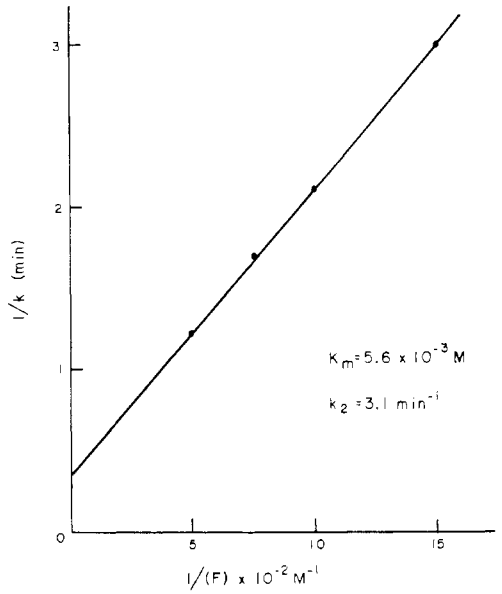

FIG. 3.—Determination of the Michaelis constant for the PMSF chymotrypsin reaction. Reaction rates were determined with 80 ml of 5 × 10^{-5} M enzyme in 0.10 M KCl, 2.4% 2-propanol at *p*H 6.0, 10°.

the results are shown in Figure 2. The data above *p*H 5.8 were obtained under conditions where enzyme is in excess over inhibitor. In these experiments the inhibitor serves as primary standard, and the concentration of enzyme need not be known exactly. Control experiments showed that the acid-binding capacity of the protein is not affected by 1% 2-propanol and that the sulfonyl fluoride is stable in the absence of enzyme. The precision (and accuracy) of the experiment was well within 1%; e.g., six runs at *p*H 7.00, 0.1 M KCl, gave 0.983 ± 0.003 equivalent of hydrogen ion released per mole PMSF. Similar values were obtained in 0.3 and 1 M KCl, indicating that the release of one proton in the neutral range of *p*H is not a fortuitous event due to electrostatic effects, etc. Since sulfonylation of an imidazole or amino group having a pK_a near 7 would have led to the release of 1.5 protons at *p*H 7, the results suggest that a hydroxyl group is sulfonylated, i.e., the serine residue at the active site.

Titrations were also carried out with PMSF in excess. This procedure was used over the entire range but was especially convenient at low *p*H where the rate of reaction is low. Results from the two methods agreed closely at all points.

An accurate determination of the operational molarity of a chymotrypsin solution can be made by titration of the acid released on treatment with excess PMSF. The operational molarity is defined as the molar concentration of active sites and does not depend on the molecular weight and purity of the enzyme. For comparison, the apparent molecular weight of chymotrypsin was determined by four different methods: by partial inhibition of esterase activity with PMSF, by treatment with excess [^{14}C]PMSF, by measurement of proton release at *p*H 7.4 using excess PMSF, and by titration with *N-trans*-cinnamoylimidazole. For the latter method, developed by Schonbaum *et al.* (1961), the relevant extinction coefficients were not redetermined. The results are summarized in Table II.

Kinetics.—The reaction is assumed to follow the course shown in equation (1), in which E is chymotrypsin, F is the sulfonyl fluoride, and E · F is the intermediate Michaelis complex; the products P are sulfonyl chymotrypsin and hydrogen and fluoride ions. The kinetic equations for the sulfonylation reaction in the case where the sulfonyl fluoride is in large excess are

$$\mathrm{E} + \mathrm{F} \underset{-1}{\overset{1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{F} \xrightarrow{2} \mathrm{P} \qquad (1)$$

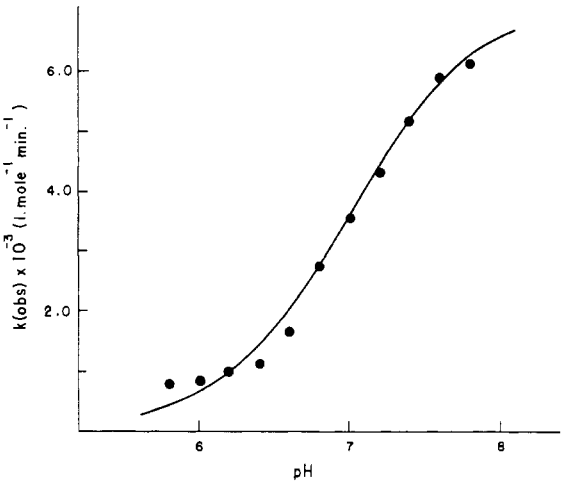

FIG. 4.—The effect of *p*H on the observed second-order rate constant for the PMSF chymotrypsin reaction: curve calculated from equation (6) using pK_a 7.0.

TABLE II
COMPARISON OF MOLECULAR WEIGHT DETERMINATIONS

Method	Apparent Molecular Weight[a] × 10^{-3} Lot A	Lot B
Titration of esterase activity	27.3	
Degree of ^{14}C labeling[b]	27.3	27.4
Proton release	27.2	27.4
TCI[c] assay		28.2

[a] Precision roughly 2% except 4% for value with TCI assay. [b] Protein was allowed to react with 1.2 equivalents of [^{14}C]PMSF; degree of labeling assumed to be 1.00. [c] TCI is *N-trans*-cinnamoylimidazole.

$$\frac{-d(\mathrm{E})}{dt} = \frac{k_2(\mathrm{E})(\mathrm{F})}{K_m + (\mathrm{F})} \qquad (2)$$

$$\log\left[\frac{(\mathrm{E})_T - (\mathrm{P})}{(\mathrm{E})_T}\right] = \frac{-kt}{2.3} \qquad (3)$$

$$k = \frac{k_2(\mathrm{F})}{K_m + (\mathrm{F})} \qquad (4)$$

where $(\mathrm{E})_T$ is the total concentration of chymotrypsin added, K_m is the Michaelis constant $(k_{-1} + k_2)/k_1$, and k is the observed first-order rate constant. Equation (4) may be rewritten in the linear form

$$\frac{1}{k} = \frac{1}{k_2} + \frac{K_m}{k_2(\mathrm{F})} \qquad (5)$$

A plot of $1/k$ against $1/(\mathrm{F})$ is shown in Figure 3; a straight line is obtained with the slope K_m/k_2 and the intercept $1/k_2$. Values of the kinetic parameters for the PMSF chymotrypsin system are: K_m = 5.6 × 10^{-3} M and k_2 = 3.1 min^{-1}. The data plotted in Figure 3 were obtained at *p*H 6.0, 10°, in 0.10 M KCl, 2.4% 2-propanol; under these conditions the reaction is about one-thirtieth as rapid as at *p*H 7.0, 25°, and may be followed accurately in the *p*H-stat at high concentrations of PMSF.

Figure 4 shows the effect of *p*H on the observed second-order rate constant k_2/K_m for the reaction of low concentrations of PMSF with chymotrypsin. Conditions were the same as above. This *p*H dependence implies that sulfonylation of the enzyme re-

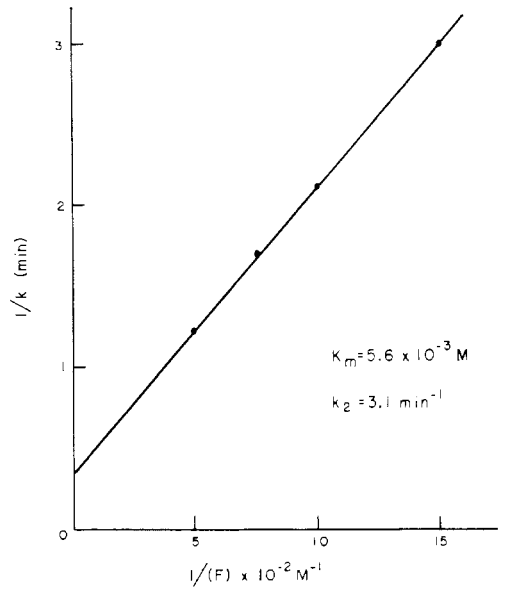

FIG. 3.—Determination of the Michaelis constant for the PMSF chymotrypsin reaction. Reaction rates were determined with 80 ml of 5×10^{-5} M enzyme in 0.10 M KCl, 2.4% 2-propanol at *p*H 6.0, 10°.

the results are shown in Figure 2. The data above *p*H 5.8 were obtained under conditions where enzyme is in excess over inhibitor. In these experiments the inhibitor serves as primary standard, and the concentration of enzyme need not be known exactly. Control experiments showed that the acid-binding capacity of the protein is not affected by 1% 2-propanol and that the sulfonyl fluoride is stable in the absence of enzyme. The precision (and accuracy) of the experiment was well within 1%; e.g., six runs at *p*H 7.00, 0.1 M KCl, gave 0.983 ± 0.003 equivalent of hydrogen ion released per mole PMSF. Similar values were obtained in 0.3 and 1 M KCl, indicating that the release of one proton in the neutral range of *p*H is not a fortuitous event due to electrostatic effects, etc. Since sulfonylation of an imidazole or amino group having a pK_a near 7 would have led to the release of 1.5 protons at *p*H 7, the results suggest that a hydroxyl group is sulfonylated, i.e., the serine residue at the active site.

Titrations were also carried out with PMSF in excess. This procedure was used over the entire range but was especially convenient at low *p*H where the rate of reaction is low. Results from the two methods agreed closely at all points.

An accurate determination of the operational molarity of a chymotrypsin solution can be made by titration of the acid released on treatment with excess PMSF. The operational molarity is defined as the molar concentration of active sites and does not depend on the molecular weight and purity of the enzyme. For comparison, the apparent molecular weight of chymotrypsin was determined by four different methods: by partial inhibition of esterase activity with PMSF, by treatment with excess [^{14}C]PMSF, by measurement of proton release at *p*H 7.4 using excess PMSF, and by titration with *N-trans*-cinnamoylimidazole. For the latter method, developed by Schonbaum *et al.* (1961), the relevant extinction coefficients were not redetermined. The results are summarized in Table II.

Kinetics.—The reaction is assumed to follow the course shown in equation (1), in which E is chymotrypsin, F is the sulfonyl fluoride, and E · F is the intermediate Michaelis complex; the products P are sulfonyl chymotrypsin and hydrogen and fluoride ions. The kinetic equations for the sulfonylation reaction in the case where the sulfonyl fluoride is in large excess are

$$\mathrm{E} + \mathrm{F} \underset{-1}{\overset{1}{\rightleftharpoons}} \mathrm{E} \cdot \mathrm{F} \xrightarrow{2} \mathrm{P} \qquad (1)$$

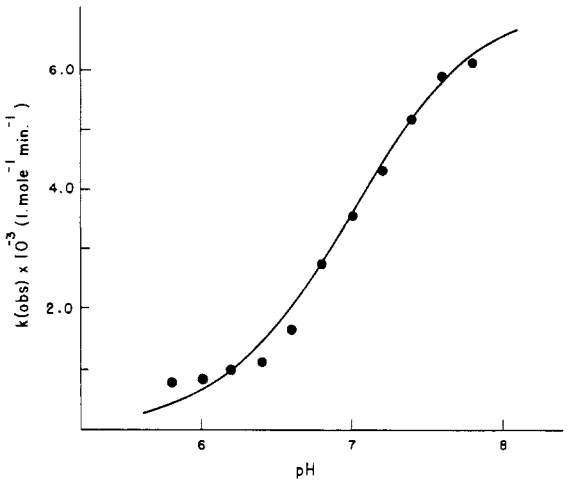

FIG. 4.—The effect of *p*H on the observed second-order rate constant for the PMSF chymotrypsin reaction: curve calculated from equation (6) using pK_a 7.0.

TABLE II
COMPARISON OF MOLECULAR WEIGHT DETERMINATIONS

Method	Apparent Molecular Weight[a] × 10^{-3} Lot A	Lot B
Titration of esterase activity	27.3	
Degree of ^{14}C labeling[b]	27.3	27.4
Proton release	27.2	27.4
TCI[c] assay		28.2

[a] Precision roughly 2% except 4% for value with TCI assay. [b] Protein was allowed to react with 1.2 equivalents of [^{14}C]PMSF; degree of labeling assumed to be 1.00. [c] TCI is *N-trans*-cinnamoylimidazole.

$$-\frac{d(\mathrm{E})}{dt} = \frac{k_2(\mathrm{E})(\mathrm{F})}{K_m + (\mathrm{F})} \qquad (2)$$

$$\log\left[\frac{(\mathrm{E})_T - (\mathrm{P})}{(\mathrm{E})_T}\right] = \frac{-kt}{2.3} \qquad (3)$$

$$k = \frac{k_2(\mathrm{F})}{K_m + (\mathrm{F})} \qquad (4)$$

where $(\mathrm{E})_T$ is the total concentration of chymotrypsin added, K_m is the Michaelis constant $(k_{-1} + k_2)/k_1$, and k is the observed first-order rate constant. Equation (4) may be rewritten in the linear form

$$\frac{1}{k} = \frac{1}{k_2} + \frac{K_m}{k_2(\mathrm{F})} \qquad (5)$$

A plot of $1/k$ against $1/(\mathrm{F})$ is shown in Figure 3; a straight line is obtained with the slope K_m/k_2 and the intercept $1/k_2$. Values of the kinetic parameters for the PMSF chymotrypsin system are: $K_m = 5.6 \times 10^{-3}$ M and $k_2 = 3.1$ min^{-1}. The data plotted in Figure 3 were obtained at *p*H 6.0, 10°, in 0.10 M KCl, 2.4% 2-propanol; under these conditions the reaction is about one-thirtieth as rapid as at *p*H 7.0, 25°, and may be followed accurately in the *p*H-stat at high concentrations of PMSF.

Figure 4 shows the effect of *p*H on the observed second-order rate constant k_2/K_m for the reaction of low concentrations of PMSF with chymotrypsin. Conditions were the same as above. This *p*H dependence implies that sulfonylation of the enzyme re-

TABLE III
YIELDS OF DNP-AMINO ACIDS FROM CHYMOTRYPSIN DERIVATIVES[a]

Protein	Treatment	DNP-Ala (%)	DNP-Ileu (%)	DNP-Ser (%)
PMS chymotrypsin	Desulfonylated at *p*H 2, 40°, 3.5 hr	54[b]	46[b]	22[b]
PMS chymotrypsin	Desulfonylated at *p*H 2, then allowed to stand at *p*H 7, 25°, 4 hr in 6 M guanidine HCl	56	44	1.5
DIP chymotrypsin	None	54	46	0
DIP chymotrypsin	*p*H 2, 40°, 3.5 hr	56	42	0

[a] Yields are uncorrected. [b] Average of three experiments; other values are average of duplicate experiments. Average deviations do not exceed 10% of stated values.

for appearance of potential activity at 40° in 0.04 M NaCl, *p*H 2; enzyme assays were carried out after allowing the samples to stand at *p*H 7, 25°, for 4 hours.[4] Control experiments with native chymotrypsin show that the enzyme is perfectly stable at 40°, *p*H 2 (0.04 M NaCl) over the time required for these experiments and that full activity appears immediately. If desulfonylation is conducted with concentrated (4 mg/ml) solutions of PMS chymotrypsin, the solutions become turbid and no reactivation is observed.

TABLE IV
RECOVERY OF POTENTIAL ENZYME ACTIVITY AT *p*H 2, 40°

Time (min)	Specific Activity[a] (%)	Desulfonylation[b] (%)	Activity (%) × 100 / Desulfonylation (%)
0	0.8		
10	15	25	60
20	31	46	68
30	37	60	62
40	48	70	69
120	64	98	65

[a] After standing at *p*H 7, 25°, for about 4 hours. Activity based on equal weight of native chymotrypsin. [b] Calculated.

Since its specific activity is significantly less than that of native α-chymotrypsin, the reactivated product is either partially inactive or has reduced enzymic activity toward ATEE in the standard assay. That the latter possibility holds was shown by allowing a completely desulfonylated and reactivated sample of enzyme to react with excess [7-^{14}C]PMSF. The amount of protein-bound ^{14}C indicated an apparent equivalent weight of 104% that of the chymotrypsin which had been used in this series of experiments; consequently, the yield of active sites is 96%. If the enzymic activity of the protein is expressed in terms of relative molar activity,[5] the reactivated enzyme has an activity of 67%. This reduced activity may be a result of changes in V_{max}, K_m, or both.

Second-order rate constants for the reaction of the reactivated enzyme with benzenesulfonyl fluoride and PMSF were determined as described previously (Fahrney and Gold, 1963a) by following loss of enzyme activity. The rate constant for benzenesulfonyl fluoride (2.40 ± 0.06 × 10^2 liters/mole min) is the same as that obtained with the native enzyme, while the rate constant for PMSF (1.28 ± 0.02 × 10^4 liters/mole min) is 14% lower.

[4] PMS trypsin may be reactivated by a similar process.

[5] Relative molar activity =

$$\frac{\text{OD/min mole}^{-1}\text{ active site} \times 100}{\text{OD/min mole}^{-1}\text{ active site of native chymotrypsin}}$$

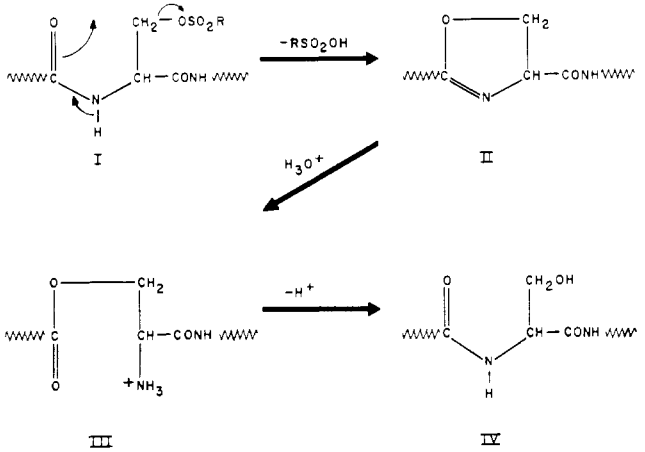

FIG. 7.—The mechanism of reactivation of PMS chymotrypsin.

Samples of the inactive ester protein were prepared in dilute solution as described above and isolated by dialysis and lyophilization. This material, which could regain the usual level of enzymic activity at *p*H 7, was subjected to dinitrophenylation as a test for the presence of a free amino group of serine. When the reaction with FDNB was conducted in aqueous alcoholic bicarbonate solution, variable amounts of DNP-serine could be isolated after hydrolysis and paper chromatography. The yields amounted to 22, 17, 5, and 9%. Dinitrophenylation in 6 M guanidine hydrochloride solution likewise gave low, variable yields of DNP-serine (20, 16, 5, and 4%). However, when the ester protein was allowed to reactivate at *p*H 7 prior to dialysis and lyophilization, it gave no trace of DNP-serine under the latter experimental conditions.

DISCUSSION

The reaction of PMSF with α-chymotrypsin clearly has one-to-one stoichiometry over a wide range of concentration when followed by a variety of methods. There is negligible nonspecific sulfonylation over long periods of time at high inhibitor concentrations. The dependence of reaction rate on PMSF concentration is consistent with formation of an intermediate, dissociable complex between sulfonyl fluoride and enzyme and indicates that the reaction takes place at the active site via an enzymic mechanism. Such concentration dependence has not been observed with DFP but is well-established in the case of *p*-nitrophenyl acetate (Gutfreund and Sturtevant, 1956). The reduced rate of reaction in the presence of β-phenylpropionate, a competitive inhibitor of chymotrypsin-catalyzed reactions, is also consistent with the hypoth-

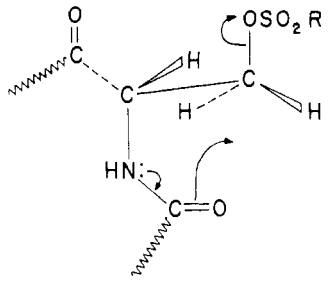

FIG. 8.—Perspective diagram of conformation required for oxazoline formation.

esis. The *p*H-dependence of the reaction rate resembles that for acylation by substrates (Bender *et al.*, 1962), which presumably requires direct participation of the nonprotonated form of a basic group with an apparent pK_a of 6.6–7.0. Finally, the lack of reactivity of PMSF toward chymotrypsinogen and urea-denatured chymotrypsin provides further evidence for the enzymic nature of the reaction.

Although the rate of sulfonylation was found to depend upon the ionization of a group with an apparent pK_a of 7.0, data on the release of protons rule out this group as the site of sulfonylation. If that were the case, about 1.5 protons would be released at *p*H 7.0, and the proton-release curve would have a steep negative slope. Instead, sulfonylation causes release of one proton as does phosphorylation with DFP (Jansen *et al.*, 1950).[6] The simplest and most direct conclusion is that a hydroxyl group is sulfonylated; other conclusions that may be drawn from this data are discussed in a previous communication (Fahrney and Gold, 1963b).

The facile loss of sulfonate anion suggests that the site of sulfonylation is not the imidazole group of a histidine residue. Studies with *N*-benzenesulfonyl imidazole (Staab and Wendel, 1960) indicate that this substance is stable indefinitely in neutral aqueous solution at room temperature but is 30% hydrolyzed in 1 hour in 0.5 N HCl at 22°. In contrast, the sulfonyl enzyme, in 8 M urea, is half desulfonylated in 6.3 hours at 10° and the rate is independent of *p*H between *p*H 2 and 8.5.[7] On the other hand, ordinary esters of sulfonic acids are hydrolyzed slowly relative to desulfonylation of the protein. In 8 M urea the rate of desulfonylation exceeds the rate of hydrolysis of *n*-propyl methanesulfonate (Barnard and Robertson, 1961) in water by a factor of 130 at *p*H 9.5, 10°.

The path of desulfonylation outlined in Figure 7 is consistent with the rate data. Sulfonate esters of *N*-acylethanolamines rapidly yield the corresponding oxazolines under mild conditions (Boyd and Rittner, 1960).[8] The reaction closely resembles the formation of oxazolines from *N*-(2-haloethyl) amides and *N*-(2-hydroxyethyl) amides (Cornforth, 1957). Formation of oxazoline in this reaction undoubtedly involves displacement of the sulfonate group by the oxygen atom of the amide group, since the reaction has been shown to lead to 100% inversion of configuration of the electrophilic carbon atom (Cornforth, 1957). Accordingly, the reaction proceeds via the planar conformation depicted in Figure 8; such stringent steric requirements may explain the fact that there is little or no tendency for the native protein to undergo desulfonylation. Presumably the folded configuration of the sulfonyl enzyme prevents some of the atoms from assuming their necessary positions in the transition state. That desulfonylation is observed in aqueous solution at *p*H 1, 25°, and at *p*H 2, 40°, is remarkable, since the reaction in 8 M urea is *p*H independent below *p*H 8. However, optical rotation studies (Schellman, 1958; Brandts and Lumry, 1961) have shown that α-chymotrypsin undergoes a reversible transition at 32°, *p*H 2, which results in partial unfolding of the protein. The acidic catalysis observed in aqueous solution probably reflects the fact that facile loss of sulfonate requires unfolding of the PMS chymotrypsin molecule.

When desulfonylation is conducted in acidic solution the protein oxazoline (II) must be hydrolyzed rapidly to the corresponding *O*-acylserine derivative (III) (Porter *et al.*, 1960; Martin and Parcell, 1961), which is stable at low *p*H. At neutral or alkaline *p*H, compound III would be expected to undergo the well-established *O,N*-acyl shift to re-form the original peptide structure IV.

Dinitrophenylation of the ester protein (III) to demonstrate the presence of a free serine amino group is complicated by the competing *O,N*-acyl shift, which can be extremely rapid. Elliott (1952), working with *N,O*-rearranged silk fibroin, found that at *p*H 8.5 only 4.5% of the serine residues react with FDNB in 2.5 hours at 20°, whereas at *p*H 5, 23% react in 4 hours at the same temperature. At *p*H 5 the rate of reaction of the exposed amino groups with FDNB is slower, but the *O,N* shift does not occur at a significant rate. With DIP chymotrypsin Elliott's procedure at *p*H 5 gave less than 10% yields of DNP-alanine and DNP-isoleucine, two of the natural end groups, even with the reaction time extended to 8 and 12 hours at room temperature. However, when the reaction was conducted with the ester protein at *p*H 7 in 6 M guanidine hydrochloride, low but satisfactorily reproducible yields of DNP-serine were obtained. If the *O,N*-acyl shift was permitted to go to completion prior to adding FDNB only trace amounts of DNP-serine could be detected. These results constitute the main evidence that PMS chymotrypsin contains a sulfonylserine residue,[9] in agreement with what is known about the structures of DIP and acetyl chymotrypsin (Koshland, 1960).

Suggestions have been made frequently that the initial site of acylation of chymotrypsin is the imidazole group of a histidine residue and that transfer of the acyl (or phosphoryl) group to a serine hydroxyl group follows. Staab and Wendel (1960) have shown that *N*-benzenesulfonyl imidazole does not undergo alcoholysis readily; only 5% of ethyl benzenesulfonate could be isolated after refluxing the imidazolide in absolute ethanol for 4 hours. However, in the presence of catalytic amounts of sodium ethoxide the reaction proceeds rapidly at room temperature. One may conclude either that histidine is not the primary nucleo-

[6] Erlanger *et al.* (1963) have reported a different proton release curve for the reaction of diphenylcarbamyl chloride with α-chymotrypsin.

[7] Recent studies indicate that the rate is also *p*H independent in aqueous solution between *p*H 1.3 and 4 at temperatures high enough to unfold the protein.

[8] Photaki (1963) has shown that *N*-acyl-*O*-sulfonylserine esters readily yield dehydroalanine derivatives under alkaline conditions; however, under modified conditions these compounds will also form oxazolines (Dr. Sara Ginsburg, personal communication).

[9] Direct proof has recently been obtained by treating PMS chymotrypsin with 2-mercaptoethylamine in 8 M urea at *p*H 8. *S*-Aminoethylcysteine can be detected after hydrolysis of the product. An equivalent proof has been published by Strumeyer *et al.* (1963) who showed that a protein containing a dehydroalanine residue is produced when *p*-toluenesulfonyl chymotrypsin is treated with 0.1 N NaOH.

phile in chymotrypsin, or that, if it is, the active-site serine hydroxyl group in the initially formed sulfonyl enzyme must be somehow activated to increase its nucleophilicity. The latter conclusion appears to offer no advantage over assuming direct acylation of an activated serine hydroxyl group.

Reactivation of PMS chymotrypsin follows the steps outlined in Figure 7. The first isolable product in the desulfonylation at *p*H 2, 40°, is the enzymically inactive ester III. This product may be reactivated only if desulfonylation is carried out in dilute solution (about 0.1 mg/ml); otherwise irreversible denaturation results. When the *O,N*-acyl shift is allowed to go to completion by letting this intermediate stand at *p*H 7, an active enzyme is produced. Although dinitrophenylation of isolated samples of the ester protein led to low and variable yields of DNP-serine, the DNP-serine must have been derived from the precursor of the reactivated enzyme, since the latter contained 96% of the active sites of the original α-chymotrypsin used in the experiment. DNP-serine was not found after dinitrophenylation of isolated samples of reactivated enzyme, thus excluding the possibility that N-terminal serine residues were generated by rupture of peptide bonds.

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Studies on Rabbit Muscle Enolase. Chemical Evidence for Two Polypeptide Chains in the Active Enzyme*

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End-group analyses of rabbit muscle enolase have shown that the 85,000 molecular weight active enzyme contains two polypeptide chains. Lysine was identified as the carboxy-terminal residue in both chains by hydrazinolysis and carboxypeptidase digestion. No free amino terminus could be detected in this enzyme. After extensive digestion with pronase, however, 1.2 moles of *N*-acetylalanine per mole of enzyme was isolated, and it was concluded that *N*-acetylalanine is the amino-terminal residue in both peptide chains.

Some chemical and physical properties of rabbit muscle enolase (RME)[1] have been reported by Boser (1959), Czok and Bücher (1960), Holt and Wold (1961), and Malmström (1962). In the early work it was found that the 85,000-mw enzyme gave a single

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[1] Abbreviations used in this work: RME, rabbit muscle enolase; s-RNA, solub'e ribonucleic acid; DFP, diisopropylphosphorofluoridate.