Sulfonyl Fluorides as Inhibitors of Esterases. III. Identification of Serine as the Site of Sulfonylation in Phenylmethanesulfonyl α-Chymotrypsin*

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ABSTRACT: Phenylmethanesulfonyl chymotrypsin and diisopropylphosphoryl chymotrypsin react with 2-mercaptoethylamine in 8 M urea solution at *p*H 8.0 to give a product containing 0.3–0.5 residue of *S*-aminoethylcysteine per molecule of protein. This is considered proof that the site of sulfonylation in the sulfonyl enzyme is the hydroxyl group of a specific serine residue. The reaction appears to involve direct displacement of the sulfonate anion by the mercaptide anion in competition with intramolecular elimination of the sulfonate group.

S-Aminoethylcysteine is also obtained when 1-dimethylaminonaphthalene-5-sulfonyl chymotrypsin is treated as described. This indicates that the corresponding sulfonyl chloride inhibits chymotrypsin by sulfonylating the hydroxyl group of the serine residue in the active site, rather than the side chain of a histidine residue as has been suggested in the literature.

Inhibition of α-chymotrypsin by sulfonyl fluorides has been shown to occur by sulfonylation of a group in the active site of the enzyme (Gold and Fahrney, 1964). This reaction appears to parallel the well-known reaction of organophosphate esters, such as diisopropylphosphorofluoridate (DFP),[1] with chymotrypsin and a number of other enzymes (Koshland, 1960). However, while the site of phosphorylation of chymotrypsin is known to be the hydroxyl group of a specific serine residue through degradation of the inhibited enzyme and isolation of phosphorylated serine peptides, the site of sulfonylation has been deduced indirectly by consideration of the properties and reactions of sulfonyl chymotrypsins. The weight of this evidence favors the presence of an *O*-sulfonylserine residue in sulfonyl chymotrypsin (Strumeyer *et al.*, 1963; Gold and Fahrney, 1964), although other evidence points to the presence of a sulfonylhistidine residue (Hartley and Massey, 1956; Gundlach *et al.*, 1962). It is possible that both conclusions are correct, since the various investigations were conducted with different sulfonyl chymotrypsins; Gold and Fahrney used phenylmethanesulfonyl fluoride as the sulfonylating agent and Strumeyer *et al.* used *p*-toluenesulfonyl chloride, while Hartley and Massey and Gundlach *et al.* used DANS chloride.

A direct investigation of the nature of the sulfonylated group in the sulfonyl enzyme by degradation and isolation of a sulfonyl amino acid or peptide cannot be expected to succeed in view of the fact that the sulfonyl group is rapidly eliminated in the form of sulfonate anion when the protein is denatured in solution (Gold and Fahrney, 1964). This elimination has been interpreted as cyclization of an *O*-sulfonylserine residue to an oxazoline (Figure 1, reaction 3a), which may occur whenever the peptide chain has achieved sufficient freedom of rotation to form the planar ring structure. Degradation can be successful only in the case where the aspartylserine peptide bond in the active site is hydrolyzed before cyclization can occur.

In the present study a successful attempt was made to detect *O*-sulfonylserine groups in chymotrypsin derivatives by adding a powerful nucleophile, 2-mercaptoethylamine, to the protein in 8 M urea solution. If direct displacement of the sulfonate group by the mercaptide ion (Figure 1, reaction 1) can compete with intramolecular elimination a fraction of the sulfonylserine residues will be converted to residues of *S*-aminoethylcysteine. The latter amino acid can be easily determined in protein hydrolysates by means of the automatic amino acid analyzer of Spackman *et al.* (1958). In this way a sulfonylserine group was detected in phenylmethanesulfonyl chymotrypsin and in DANS chymotrypsin. DIP chymotrypsin, too, yielded *S*-aminoethylcysteine when allowed to react with 2-mercaptoethylamine under standard conditions; however, chymotrypsin, chymotrypsinogen, and *trans*-cinnamoyl chymotrypsin (Schonbaum *et al.*, 1961) gave no *S*-aminoethylcysteine, while diphenylcarbamyl chymo-

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[1] Abbreviations used in this work: DFP, diisopropylphosphorofluoridate; DIP, diisopropylphosphoryl; DANS, 1-dimethylaminonaphthalene-5-sulfonyl.

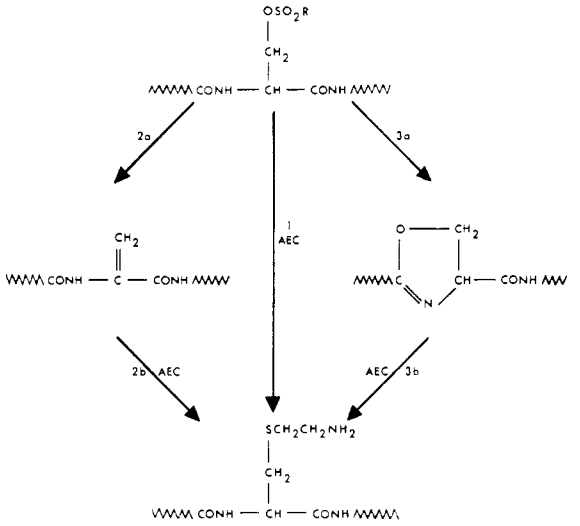

FIGURE 1: Reaction scheme for sulfonyl chymotrypsin. AEC is 2-mercaptoethylamine.

trypsin (Erlanger and Cohen, 1963) gave only a small amount.

Experimental

Materials. α-Chymotrypsin and chymotrypsinogen A were three-times-recrystallized, salt-free preparations obtained from Worthington Biochemical Corp. Phenylmethanesulfonyl fluoride was prepared in this laboratory as described earlier (Fahrney and Gold, 1963). DFP was obtained from Merck and Co., Inc., and DANS chloride, *N-trans*-cinnamoylimidazole, and 2-mercaptoethylamine hydrochloride were obtained from Nutritional Biochemicals Corp. Diphenylcarbamyl chloride and *N*-glutaryl-L-phenylalanine *p*-nitroanilide were kindly provided by Dr. B. F. Erlanger. S-Aminoethylcysteine hydrochloride was a gift from Dr. H. Lindley.

Standard Conditions for Reaction of Proteins with 2-Mercaptoethylamine. Protein (20–50 mg) in 2.0 ml of water was added to 8.0 ml of a solution of urea and 2-mercaptoethylamine hydrochloride, previously adjusted to *p*H 8.0 with NaOH. The *p*H was readjusted to 8.0 at 40° with 6 N NaOH using a glass-electrode *p*H meter, and the solution was incubated at 40° for 5–6 hours. The final concentration of urea was 8 M and that of 2-mercaptoethylamine was 2 M. These conditions were modified as described in various experiments. After incubation the solution was acidified to *p*H 3 with 6 N HCl and dialyzed exhaustively against 1 mM HCl.

Hydrolysis of Proteins. A volume of dialyzed protein solution containing *ca.* 5 mg of protein was evaporated to dryness in a 18 × 150-mm test tube in a vacuum desiccator containing $CaCl_2$. The residue was treated with 1.0 ml of constant-boiling HCl (distilled in glass), frozen in a dry-ice bath, evacuated to a pressure of 50 μ, and sealed. The tube was heated at 112° for 22 hours and opened, and the acid was evaporated to dryness *in vacuo* over solid NaOH.

Amino acid analyses were carried out with a Spinco Model 120 automatic amino acid analyzer. *S*-Aminoethylcysteine is well resolved on the 50-cm column with 0.38 N citrate buffer, *p*H 4.26. The chromatogram is carried out at a flow rate of 30 ml/hr at 30°, changing to 50° at 11 hours 20 minutes. The *S*-aminoethylcysteine peak appears 29 ml ahead of the ammonia peak. Partial resolution can be obtained with the 15-cm column using 0.35 N citrate buffer, *p*H 5.28, at 50°. Under these conditions *S*-aminoethylcysteine emerges 5 ml after lysine. All quantitative measurements were carried out with the 50-cm column.

Calibration constants, the area under the curve corresponding to 1 μmole of sample, were determined for *S*-aminoethylcysteine, lysine, and histidine. The ratio of the constant for *S*-aminoethylcysteine to that for lysine is 0.96, while the ratio of constants for *S*-aminoethylcysteine and histidine is 1.05. Assuming that α-chymotrypsin contains 13 residues of lysine and 2 residues of histidine (Wilcox *et al.*, 1957) the yield of *S*-aminoethylcysteine is given by the equations:

$$y = 13A_{AEC}/0.96A_{Lys}$$

and

$$y = 2A_{AEC}/1.05A_{His}$$

where y is the yield of *S*-aminoethylcysteine, in residues per molecule of protein, and A_{AEC}, A_{Lys}, and A_{His} are the areas under the peaks corresponding to *S*-aminoethylcysteine, lysine, and histidine, respectively. The yields calculated on the basis of lysine and histidine usually agreed well and the former is reported in all cases.

Assay of enzyme activity was carried out by a modification of the method of Erlanger.[2] One ml of water or 1 mM HCl containing up to 0.10 mg of active chymotrypsin was added to 2.00 ml of 0.0020 M *N*-glutaryl-L-phenylalanine *p*-nitroanilide in 0.03 M $CaCl_2$, 0.075 M Tris-HCl, *p*H 7.6. The solution was incubated at 25° for 30 minutes and then treated with 0.10 ml of 0.5% phenylmethanesulfonyl fluoride in 2-propanol. The liberated *p*-nitroaniline was estimated by measuring its absorption at 410 mμ with a Bausch and Lomb Spectronic 20 colorimeter. Blanks were carried through by excluding enzyme or, where appropriate, adding phenylmethanesulfonyl fluoride just prior to addition of enzyme.

Protein concentrations were calculated from the absorbance at 282 mμ using the extinction coefficient 2.07 ml/mg for a 1-cm light path (Schwert and Kaufman, 1951) or from the dry weight of protein. Concentration of DANS proteins was always based on dry weight.

Phenylmethanesulfonyl chymotrypsin was prepared by adding 1.2 molar equivalents of phenylmethanesul-

[2] B. F. Erlanger, private communication.

fonyl fluoride in 2-propanol (7 mg/ml) to a 2% solution of α-chymotrypsin in 0.1 M NaCl at 25°. The *p*H was maintained at 7.0 by automatic addition of dilute NaOH. When alkali consumption was complete the *p*H was adjusted to 3 with HCl and the solution was dialyzed exhaustively against 1 mM HCl and lyophilized. Remaining enzymic activity was less than 1%.

DIP chymotrypsin was prepared similarly to phenylmethanesulfonyl chymotrypsin using 1.4 molar equivalents of DFP in 2-propanol (6 mg/ml). Remaining enzymic activity was less than 1%.

Diphenylcarbamyl chymotrypsin was prepared similarly to phenylmethanesulfonyl chymotrypsin using 1.2 molar equivalents of diphenylcarbamyl chloride in 2-propanol (12 mg/ml). Remaining enzymic activity was less than 1%.

Cinnamoyl Chymotrypsin. α-Chymotrypsin (170 mg) in 17 ml of distilled water was equilibrated at *p*H 4.0 in a *p*H-stat at 25°. A solution of 1.52 mg of *N-trans*-cinnamoylimidazole in 1.0 ml of acetonitrile was added and 0.0202 N HCl was automatically added to bring the *p*H back to 4.0. The volume of HCl required was 0.36 ml, close to the theoretical amount. The solution was then frozen and lyophilized without delay.

DANS proteins were prepared as described by Gundlach *et al.* (1962). Protein (104 mg) in 16 ml of 0.03 M Na_2HPO_4 was treated at 0° with 4 ml of acetone containing 1.4, 2.8, or 5.6 mg of DANS chloride. After 24 hours the solution was dialyzed exhaustively against 0.01 N acetic acid in the cold and lyophilized.

These preparations differed from those described by Hartley and Massey (1956) in that they had no distinct absorption maximum at 335–340 mμ in 0.10 M Na_2HPO_4 but showed nearly constant absorption from 315 to 340 mμ. The concentration of DANS residues was calculated from the absorption at 325 mμ, using an extinction coefficient of $3.36 \times 10^3\ M^{-1}\ cm^{-1}$.

Mixed Disulfide. A solution of 12 mg of cystine and 5.7 mg of 2-mercaptoethylamine hydrochloride in 10 ml of water was adjusted to *p*H 9.0 with 1 N NaOH and allowed to stand at 25° for 2 hours. The solution was acidified to *p*H 2.0 with 1 N HCl and an aliquot was added to a standard amino acid mixture and analyzed on the 50-cm column.

Performic Acid Oxidation. Performic acid was prepared by allowing a mixture of 0.50 ml of 30% H_2O_2 and 9.5 ml of 98% formic acid to stand at room temperature for 2 hours. The dry hydrolysate from *ca.* 5 mg of protein was dissolved in 1.0 ml of the HCO_3H solution and allowed to stand at 25° for 30 minutes. The solution was then evaporated to dryness *in vacuo* over NaOH and analyzed as usual.

Results

When phenylmethanesulfonyl chymotrypsin was allowed to react with 1 M 2-mercaptoethylamine in 8 M urea, *p*H 8.0, at 40° for 5 hours a yield of 0.35 residue of *S*-aminoethylcysteine was obtained. Temperature appears to have little effect on the yield, since modifying the conditions to 0° for 21 hours resulted in 0.28 residue. A temperature of 40° was chosen for all subsequent work; experiments with phenylmethanesulfonyl[7^{14}C] chymotrypsin (Gold and Fahrney, 1964) indicated that desulfonylation has a half-time of *ca.* 20 minutes under these conditions in the absence of 2-mercaptoethylamine.

Increasing the concentration of 2-mercaptoethylamine to 2 M had some effect, raising the yield to 0.40–0.50 residue. This proved to be the most satisfactory set of conditions, although a concentration of 1 M was used for some purposes.

Yields of *S*-aminoethylcysteine as a function of *p*H are shown in Table I. The reaction conditions were standard, except that 1 M mercaptan was used. Native chymotrypsin was included as a control at each *p*H except 6. The yield of *S*-aminoethylcysteine from phenylmethanesulfonyl chymotrypsin at *p*H 8 is lower than usual; however the increased yield at higher *p*H is partly accounted for by the increased blanks. Apparently cystine residues of the native enzyme partially undergo β-elimination at high *p*H producing dehydroalanine residues to which the mercaptan can then add (Figure 1, reaction 2b).

TABLE I: Yields of *S*-Aminoethylcysteine as a Function of *p*H.[a]

	Yield[b]	
*p*H	Phenylmethanesulfonyl α-Chymotrypsin	α-Chymotrypsin
6.0	0.06	
8.0	0.27	Trace
10.0	0.57	0.09
12.0	0.70	0.16

[a] Conditions of reaction were: 0.2–0.4% protein, 8 M urea, 1 M 2-mercaptoethylamine, 40°, 3–5 hours. [b] Residues per molecule of protein.

S-Aminoethylcysteine was identified and estimated in all hydrolysates by chromatography on the 50-cm column of the automatic amino acid analyzer as described. It is well resolved from other materials and accurate quantitative measurements can be made. The identity of the *S*-aminoethylcysteine was confirmed in many hydrolysates by chromatography on the 15-cm column, where the order of elution is different. Although quantitative measurements cannot be made because of incomplete resolution, appearance of a peak in the characteristic position for *S*-aminoethylcysteine was taken as proof of identity.

All hydrolysates of proteins that had been treated with 2-mercaptoethylamine at *p*H 8 showed significant

amounts of a component which emerged from the 50-cm column about 70 ml after histidine. A substance emerging in the same position was found after incubating cystine with 2-mercaptoethylamine at *p*H 9; consequently, the unknown material is tentatively identified as the mixed disulfide of cysteine and 2-mercaptoethylamine. When a hydrolysate of mercaptan-treated phenylmethanesulfonyl chymotrypsin was allowed to react with performic acid neither *S*-aminoethylcysteine nor the mixed disulfide could be found.

The mechanism of the reaction was investigated by delaying the addition of the mercaptan until desulfonylation was complete. A solution of 40 mg of phenylmethanesulfonyl chymotrypsin in 10 ml of 8 M urea containing 0.01 M Tris, *p*H 8.0, was incubated at 40° for 3 hours. Solid 2-mercaptoethylamine hydrochloride was then added to give a final concentration of 1 M, the *p*H was readjusted to 8.0 with 50% NaOH, and the solution was incubated at 40° for 5 hours. The usual process of dialysis and hydrolysis was carried out and a yield of only 0.03 residue of *S*-aminoethylcysteine was found. When the procedure was modified so that the mercaptan was added immediately, rather than waiting for 3 hours, and incubation was then carried out for 5 hours at 40°, the yield of *S*-aminoethylcysteine was 0.30 residue. A control experiment with native chymotrypsin resulted in formation of 0.02 residue of *S*-aminoethylcysteine by the first procedure.

A number of derivatives of α-chymotrypsin were treated with 2-mercaptoethylamine under the standard conditions. The results are given in Table II. It is noteworthy that DIP chymotrypsin gives nearly as good a yield of *S*-aminoethylcysteine as does phenylmethanesulfonyl chymotrypsin. The cinnamoyl and diphenylcarbamyl derivatives would not be expected to form any *S*-aminoethylcysteine; however the diphenylcarbamyl chymotrypsin does give a small reproducible yield.

TABLE II: Yields of *S*-Aminoethylcysteine from α-Chymotrypsin Derivatives.

Protein	Yield[a]
α-Chymotrypsin	Trace[b,c]
Phenylmethanesulfonyl chymotrypsin	0.47, 0.40, 0.38
DIP chymotrypsin	0.38, 0.33
Diphenylcarbamyl chymotrypsin	0.08, 0.06
Cinnamoyl chymotrypsin	Trace[b]

[a] Residues per molecule of protein. [b] The yield was of the order of 0.01, but could not be accurately estimated. [c] Four experiments.

Several preparations of DANS chymotrypsin and DANS chymotrypsinogen containing varying numbers of DANS residues per molecule of protein were made and subjected to the standard reaction conditions with 2-mercaptoethylamine. The results are given in Table III. It is clear that the DANS chymotrypsins all give approximately the same yield of *S*-aminoethylcysteine as does phenylmethanesulfonyl chymotrypsin. This is strong evidence for the presence of an *O*-DANS-serine residue in each of the samples. The chymotrypsinogen used had less than 1% chymotrypsin activity and could be completely activated with trypsin; the DANS chymotrypsinogen with 2.0 DANS residues per molecule could be activated to the extent of 70% while the material with 4.5 DANS residues could be 60% activated.

Discussion

The procedure described appears to be a general method for the detection of *O*-sulfonylserine residues and possibly *O*-phosphorylserine residues in proteins. However, the appearance of a low yield of *S*-aminoethylcysteine from diphenylcarbamyl chymotrypsin suggests that the reaction is not specific and a variety of *O*-acylserine types may give positive results.

A similar procedure has been reported by Strumeyer *et al.* (1963), who treated *p*-toluenesulfonyl chymotrypsin with 0.1 N NaOH at 0°. These conditions cause the sulfonylserine residue to undergo β-elimination with the formation of a dehydroalanine residue (Figure 1, reaction 2a). Subsequent treatment with mercaptans leads to conjugate addition and finally to the isolation of the appropriately substituted cysteine derivative. Unfortunately, control experiments with native chymotrypsin lead to yields of substituted cysteines which are about one-half as great as the yields from *p*-toluenesulfonyl chymotrypsin. These high blanks are probably owing to β-elimination in cystine or unsubstituted serine residues.

It is possible to conceive of three alternate mechanisms, illustrated in Figure 1, for the formation of *S*-aminoethylcysteine in the present experiments: (1) direct displacement of the sulfonate anion by the mercaptide anion; (2) elimination to form dehydroalanine and readdition of mercaptan; (3) cyclization to an oxazoline followed by ring-opening with the mercaptan. The last reaction has been shown to occur with simple oxazolines (Fry, 1950) and might occur in a denatured protein oxazoline in 8 M urea at *p*H 8. The two latter mechanisms may be excluded by the observation that if addition of 2-mercaptoethylamine is delayed until desulfonylation is complete no *S*-aminoethylcysteine is formed. A dehydroalanine residue in chymotrypsin is stable at high and neutral *p*H in the absence of nucleophiles capable of adding to the double bond, and there is no reason to believe that it would not be stable under the conditions of these experiments. Studies on the kinetics of hydrolysis of 2-methyloxazoline. (Martin and Parcell, 1961) indicate that this substance is stable in aqueous solution above *p*H 7. Consequently the reaction of sulfonyl chymotrypsin with

TABLE III: Yields of *S*-Aminoethylcysteine from DANS Proteins.

Protein	DANS Residues[a]	Inhibition (%)	*S*-Aminoethylcysteine[b]	*S*-Aminoethylcysteine × 100/% Inhibition
α-Chymotrypsin	0	0	Trace	
DANS chymotrypsin	1.2	52	0.22	0.42
DANS chymotrypsin	1.8	84	0.31	0.37
DANS chymotrypsin	2.3	88	0.41, 0.48	0.47, 0.54
DANS chymotrypsin	4.3	98	0.52, 0.48	0.53, 0.49
Chymotrypsinogen A	0		Trace	
DANS chymotrypsinogen	2.0		0.02	
DANS chymotrypsinogen	4.5		0.06	

[a] Assuming a molecular weight of 25,000. [b] Residues per molecule of protein.

2-mercaptoethylamine is tentatively viewed as a direct displacement of the sulfonate anion by the mercaptide anion competing with the intramolecular formation of oxazoline, which does not lead to formation of *S*-aminoethylcysteine.

The question of the nature of DANS chymotrypsin is complex because of the high reactivity and relatively low specificity of DANS chloride. The reagent appears to act at the active site more rapidly than at any other position, but the difference in rates is not great. When more than a single DANS residue is introduced into the enzyme molecule the excess must be at positions other than the active site. Hartley and Massey (1956) identified histidine as the site of sulfonylation in DANS chymotrypsin on the basis of its fluorescent color but were unable to make a quantitative estimate. More recently Gundlach *et al.* (1962) analyzed the protein for sulfonylhistidine residues by treating it with an excess of a diazonium salt and then hydrolyzing with strong acid. Any sulfonylhistidine present in the protein should fail to react with the diazonium salt, but should undergo hydrolysis to appear as free histidine. Histidine residues that are not sulfonylated should couple with the diazonium salt and not appear as free histidine after hydrolysis. In this way they found 0.3 residue of DANS histidine in a preparation of DANS chymotrypsin. The low content of sulfonylhistidine was explained on the basis of the probable ready hydrolysis of a sulfonylimidazole under the conditions necessary to effect coupling with a diazonium salt; however, it is known from the studies of Staab and Wendel (1960) that *N*-benzenesulfonylimidazole is relatively stable under the conditions of temperature and *p*H used by Gundlach *et al.* (1962) for coupling. It seems likely that only 0.3 residue of sulfonylhistidine was originally present in the protein sample, which was more than 90% inhibited.

In view of these analytical results and the known structures of DIP, acetyl, and phenylmethanesulfonyl chymotrypsins it is most likely that DANS chloride inhibits α-chymotrypsin by sulfonylating the hydroxyl group of a specific serine residue in the active site. The sulfonylhistidine which is certainly present in the DANS chymotrypsin must be the result of nonspecific sulfonylation, which the highly reactive DANS chloride is capable of effecting. The observation that DANS chymotrypsinogen, which may be largely activated with trypsin, contains very little sulfonylserine supports this conclusion.

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References

Erlanger, B. F., and Cohen, W. (1963), *J. Am. Chem. Soc. 85*, 348.
Fahrney, D. E., and Gold, A. M. (1963), *J. Am. Chem. Soc. 85*, 997.
Fry, E. M. (1950), *J. Org. Chem. 15*, 438.
Gold, A. M., and Fahrney, D. (1964), *Biochemistry 3*, 783.
Gundlach, G., Köhne, C., and Turba, F. (1962), *Biochem. Z. 336*, 215.
Hartley, B. S., and Massey, V. (1956), *Biochim. Biophys. Acta 21*, 58.
Koshland, D. E. (1960), *Advan. Enzymol. 22*, 45.
Martin, R. B., and Parcell, A. (1961), *J. Am. Chem. Soc. 83*, 4835.
Schonbaum, G. R., Zerner, B., and Bender, M. L. (1961), *J. Biol. Chem. 236*, 2930.
Schwert, G. W., and Kaufman, S. (1951), *J. Biol. Chem. 190*, 807.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Staab, H. A., and Wendel, K. (1960), *Ber. 93*, 2902.
Strumeyer, D. H., White, W. N., and Koshland, D. E. (1963), *Proc. Natl. Acad. Sci. U.S. 50*, 931.
Wilcox, P. E., Cohen, E., and Tan, W. (1957), *J. Biol. Chem. 228*, 999.