Moore, S. (1963) *J. Biol. Chem. 238*, 235.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819.
O'Brien, R. D., Timpone, C. A., & Gibson, R. E. (1978) *Anal. Biochem. 86*, 602.
Schroeder, D. D., Allison, N. J., & Buchanan, J. M. (1969) *J. Biol. Chem. 244*, 5856.
Shen, W.-C., & Colman, R. E. (1975) *J. Biol. Chem. 250*, 2973.
Simpson, R. J., Neuberger, M. R., & Lin, T.-Y. (1976) *J. Biol. Chem. 251*, 1936.
Sluyterman, L. A. E. (1967) *Biochim. Biophys. Acta 139*, 439.
Taussig, A. (1960) *Biochim. Biophys. Acta 44*, 510.
Taussig, A. (1965) *Can. J. Biochem. 43*, 1063.
Thorsland, A., & Lindskog, S. (1967) *Eur. J. Biochem. 3*, 117.
Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26*, 3.

Interaction of Dansylated Peptidyl Chloromethanes with Trypsin, Chymotrypsin, Elastase, and Thrombin†

Glenn S. Penny and Douglas F. Dyckes*

ABSTRACT: A series of N^{α}-1-(dimethylamino)-5-naphthalenesulfonyl (dansyl) derivatives of peptidyl chloromethanes (chloromethyl ketones) were synthesized and employed to introduce the fluorescent dansyl moiety specifically into the active sites of proteinases via affinity labeling. Dansylalanyllysylchloromethane (DALCM) was utilized to inactivate and fluorescently label trypsin and the trypsin-like enzyme thrombin. Dansylleucylphenylalanylchloromethane (DLPCM) was synthesized and selectively employed as an inhibitor of chymotrypsin. The di-, tri-, and tetrapeptides—dansylprolylalanylchloromethane (DPACM), dansylalanylprolylalanylchloromethane (DAPACM), and dansylprolylalanylprolylalanylchloromethane (DPAPACM)—were synthesized and their interaction with elastase was evaluated. The compounds DALCM, DLPCM, and DAPACM all proved to be effective, fast-acting proteinase inhibitors. Studies of energy transfer in the enzyme–inhibitor conjugates led to results entirely consistent with the proposed conformational homology of thrombin with the other serine proteinases studied. The fluorescent affinity labels are believed to possess enormous potential for the localization, isolation, and characterization of enzymes.

Site-specific affinity labeling of serine proteinases with amino acid and peptidyl chloromethanes has been a valuable tool in the study of the structure and function of proteolytic enzymes. For example, by incorporating radioactive labels into the amino acid chloromethanes Tos-Lys-CH_2Cl (TLCK)[1] and Tos-Phe-CH_2Cl (TPCK), it was first possible to identify the histidine residues at the active sites of trypsin (Ong et al., 1964), chymotrypsin (Shaw & Springhorn, 1967), and thrombin (Glover & Shaw, 1971). Similar affinity labels have become probes of the microenviroment of enzyme active sites by replacing the tosyl group with a spin-label (Kosman, 1972) or a fluorescent molecule (Schoellmann, 1972; Vaz & Schoellmann, 1976). Peptidyl chloromethanes have also been found useful in mapping the three-dimensional structure of substrate binding sites (Segal et al., 1971; Shotton et al., 1972).

Many biological effects of amino acid chloromethanes have been documented. TLCK is thought to interact with acrosin and thus prevent fertilization (Zaneveld et al., 1970). It further irreversibly inhibits the translation of mRNA into protein in rabbit reticulocyte (Freedman et al., 1973) and *Escherichia coli* (Rossman et al., 1974), and into polio-virus specific proteins in infected HeLa cells (Summers et al., 1972). Proteolytic activity associated with the rampant growth of tumor and cancer cells has been curbed by the action of TLCK and TPCK, and their application has resulted in the inhibition of the adhesion of Ehrlich ascites tumor cells to plastic (Whur et al., 1974; Powers, 1977). In each of these cited instances, the effective use of TLCK and TPCK as inhibitors is thought to be evidence for the involvement of proteolytic enzymes in the indicated biological processes. In most cases, however, the isolation of the enzymes responsible has not been accomplished.

The dansylated peptidyl chloromethanes described in this work were designed to combine the advantages for visualization and isolation of a fluorescent label and the enhancement of reactivity afforded by peptidyl chloromethanes. Peptide derivatives of phenylalanyl-, lysyl-, and alanylchloromethanes have been found to be as much as 150 times more effective than their tosyl derivatives in the inhibition of chymotrypsin (Powers & Wilcox, 1970; Morihara & Oka, 1970; Kurachi et al., 1973), trypsin (Coggins et al., 1974; Kettner et al., 1978), and elastase (Powers & Tuhy, 1973; Thompson & Blout, 1973; Thompson & Dennis, 1973). The inclusion of a dansyl moiety permits ready visualization of these affinity

† From the Department of Chemistry, University of Houston, Houston, Texas 77004. *Received August 8, 1979.* This research was supported by the U.S. Public Health Service (Grant No. HL 19514).

[1] Abbreviations used: Ac, acyl; BAPA, N^{α}-benzoyl-L-arginine-*p*-nitroanilide; Boc, tBoc, *tert*-butyloxycarbonylurethane protecting group; BPTI, basic pancreatic trypsin inhibitor; dansyl, Dns, 1-(dimethylamino)-5-naphthalenesulfonyl; DALCM, N^{α}-dansyl-L-alanyl-L-lysylchloromethane; DALM-thrombin, Dns-Ala-Lys-CH_2-thrombin, N^{α}-dansyl-L-lysylthrobino(His-43)methane; DALM-trypsin, Dns-Ala-Lys-CH_2-trypsin, N^{α}-dansyl-L-alanyl-L-lysyltrypsino(His-46)methane; DAPACM, N^{α}-dansyl-L-prolyl-L-alanylchloromethane; DAPAM-elastase, Dns-Ala-Pro-Ala-CH_2-elastase, N^{α}-dansyl-L-alanyl-L-prolyl-L-alanylelastino(His-45)methane; Diazald, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide; DLPCM, N^{α}-dansyl-L-leucyl-L-phenylalanylchloromethane; DLPM-chymotrypsin, Dns-Leu-Phe-CH_2-chymotrypsin, N^{α}-dansyl-L-lysyl-L-phenylalanylchymotrypsino(His-57)methane; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; DPACM, N^{α}-dansyl-L-prolyl-L-alanylchloromethane; Et_3N, triethylamine; M.A., mixed anhydride; NMR, nuclear magnetic resonance; PNGB, *p*-nitrophenyl-*p*-guanidinobenzoate; THF, tetrahydrofuran; TLC, thin-layer chromatography; TLCK, N^{α}-tosyl-L-lysylchloromethane; tosyl, Tos, *p*-toluenesulfonyl; TPCK, Tos-Phe-CH_2Cl, N^{α}-tosyl-L-phenylalanylchloromethane or L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; Z, (benzyloxycarbonyl)urethane protecting group.

0006-2960/80/0419-2888$01.00/0

labels. Thus, they may be used as tools for the localization, isolation, and characterization of proteolytic enzymes. However, in one case a further enhancement reactivity has been observed to result from dansylation of a peptidyl chloromethane. Dns-Ala-Lys-CH_2Cl (DALCM) (Penny et al., 1979) was found to inactivate trypsin 125 times faster than Lys-Ala-Lys-CH_2Cl, the most potent trypsin inhibitor of this class previously reported (Coggins et al., 1974). Whether the phenomenon of increased effectiveness upon dansylation is common for the peptidyl chloromethane inhibitors of serine proteinases was tested through the synthesis and evaluation of the compounds described below.

The heightened reactivity of peptidyl chloromethanes with α-chymotrypsin has been attributed, on the basis of X-ray crystallographic studies (Segal et al., 1971), to an increase in the number of productive contacts between these inhibitors and the enzyme. In a careful study of the α-chymotrypsin active site requirements based on the kinetics of the inhibition this enzyme by various peptidyl chloromethanes, Kurachi et al. (1973) found that the occurrence of a Leu residue in the P_2 position of such an inhibitor made it highly effective. [The designation P_2 follows the convention of Schechter & Berger (1967).] This is consistent with similar results based on the rates of hydrolysis of peptidyl tyrosine amides catalyzed by α-chymotrypsin (Yamashita, 1960; Yamamoto & Izumiya, 1966). Based on the conclusions listed above, we chose to synthesize Dns-Leu-Phe-CH_2Cl (DLPCM) for use in our studies.

Similar experiments with peptidyl alanylchloromethanes, designed to probe the active site requirements of elastase, have indicated that, in general, tripeptides are 2–3 orders of magnitude more reactive than dipeptides and tetrapeptides are up to 7 times more reactive than the corresponding tripeptides in inhibiting that enzyme (Powers & Tuhy, 1973). According to Thompson and Blout (Blackburn, 1976), the increased rate of inactivation of elastase with tetrapeptides is a function of an S_4–P_4-induced rearrangement of the S_1 and S_1' subsite and is thought of in terms of the induced-fit model of Koshland (Koshland & Neet, 1968). The argument for an S_4–P_4-induced rearrangement is strongly supported by X-ray crystallographic investigations of the interactions of elastase with irreversible inhibitors in enzyme–inhibitor conjugates (Shotton et al., 1972). The series of dansylated peptidyl chloromethanes reported upon here, Dns-Pro-Ala-CH_2Cl (DPACM), Dns-Ala-Pro-Ala-CH_2Cl (DAPACM), and Dns-Pro-Ala-Pro-Ala-Ch_2Cl (DPAPACM), incorporate the residues believed to be most effective (Thompson & Blout, 1973) at each position of the lengthened peptide chain and were designed to test the effect of increasing chain length upon reactivity for these inhibitors.

For inactivation studies using thrombin, it was deduced that the trypsin inhibitor, DALCM, is also compatible in every way with thrombin subsites. Thrombin requires a positively charged P_1 residue, as does trypsin, and the B chain of the thrombin substrate, fibrinogen, does in fact possess an alanine residue at its P_2 position (Doolittle, 1973). In addition to the inactivation studies, measurements of energy transfer between the thrombin tryptophanyl residues and the dansyl group of the labeled thrombin molecule (DALM-thrombin) have been carried out. These results are compared with those of similar measurements on the analogous α-chymotrypsin and elastase derivatives.

Materials and Methods

Three times crystallized, lyophilized bovine trypsin, lot no. TRL 35D942, and twice crystallized, lyophilized bovine chymotrypsin, lot no. 38J814 and 35A630, were purchased from Worthington Biochemical Corp., Freehold, NJ. Bovine trypsinogen, one time crystallized, lot no. TGHSC823, was also a Worthington product. Lyophilized porcine elastase (EC 3.4.21.11), lot no. 48C-8095, bovine thrombin (1000 NIH units/mL in 0.05 M phosphate, pH 7.0), lot no. 43C-8040-1 and 47C-8030, and fibrinogen, lot no. 87C-0380, were products of Sigma, St. Louis, MO. Trypsin and chymotrypsin were further purified as described by Johnson & Travis (1976). All other preparations were used as received from the supplier.

Chymotrypsin substrate N^{α}-benzoyl-L-tyrosine-*p*-nitroanilide and the protected L-amino acids Boc-Ala, Boc-Leu, Boc-Pro, and Boc-Phe were purchased from Peninsula Laboratories, San Carlos, CA. The elastase substrate *N*-succinyl-$(L\text{-Ala})_3$-*p*-nitroanilide was a product of Calbiochem, San Diego, CA. Diazald and ethyl chloroformate were purchased from Aldrich Chemical Co., Milwaukee, WI. Triethylamine and dansyl chloride were purchased from Pierce, Rockford, IL. Solvents and all other reagents used were either reagent grade or the best grade available.

Sephadex G-25 and Sephadex G-50 were products of Pharmacia, Piscataway, NJ. Materials for a soybean trypsin inhibitor affinity column used in the purification of trypsin and chymotrypsin were kindly provided by Dr. B. Daniel Burleigh. Packing for poured silica gel columns, MN Kiesel Gel 60, and prepacked silica gel columns and silica gel 60 HPTLC plates used for thin-layer chromatography (TLC) were purchased from Brinkman, Westbury, NY.

Ultraviolet spectra were recorded and rate studies were conducted on either a Cary 118 double-beam recording instrument in the auto mode with variable slit width or a Beckman Model 25 double-beam recording spectrophotometer. Column elutions were routinely monitored at 280 nm with an LKB UV monitor and recorder. NMR spectra were obtained on a Varian XL-100 or T-60 NMR spectrometer.

Excitation and emission spectra were obtained on a Perkin-Elmer Model 43a spectrofluorometer with variable slit widths, a cell temperature regulating unit, and an attached recorder. Spectra were obtained in cells thermostated to 22 °C, with emission and excitation bandwidths set to 10 nm. The instrument was calibrated as recommended by the Subcommittee on Fluorescence Spectroscopy (Udenfried, 1969). Spectra were corrected by using the method of Parker & Rees (1960).

Studies of the inactivation of α-chymotrypsin were carried out at room temperature and pH 5.8 to allow direct comparison with the work of Kurachi et al. (1973). Stock inhibitor solutions were prepared by dissolving a weighed amount of DLPCM in a known volume of acetonitrile. Portions corresponding to the desired concentration of inhibitor (0.18–0.54 mM) were measured out by syringe, diluted to 1.5 mL with acetonitrile, and added to 5 mg of chymotrypsin (by UV $E_{280}^{1\%}$ = 20.4) dissolved in 4.5 mL of pH 5.8, 0.05 M phosphate buffer (40 μM enzyme). At various time intervals, aliquots of 50 μL were removed from the inhibition reaction mixture, diluted to 2 mL with pH 7.0, 0.05 M phosphate buffer, and immediately assayed for residual enzyme activity by using N^{α}-benzoyl-L-tyrosine-*p*-nitroanilide as a substrate. The assay was carried out by adding 0.25 mL of the substrate solution (0.44 mg/mL in CH_3CN) to the diluted reaction aliquot and spectrophotometrically monitoring the initial rate of liberation of *p*-nitroaniline at 405 nm.

A stock solution of 1 mg/mL elastase (by UV $E_{280}^{1\%}$ = 18.5) in 1 mM HCl was used throughout for the elastase inactivation studies. Reaction mixtures typically consisted of 0.05 mL of

Scheme I

$$E + I \overset{K_I}{\rightleftharpoons} E \cdot I \xrightarrow{k_2} E\text{-}I$$

the stock elastase solution, 3 mL of pH 6.5, 0.05 M phosphate buffer, and 1–3 mL of 50% MeOH (MeOH–H_2O, 63:50 v/v), this last to render the inhibitors soluble. The activity of elastase was found to be unchanged for periods up to 2 h under these conditions. Known quantities of inhibitor in methanol were added in microliter amounts to the buffered enzyme solution. Aliquots (0.5 mL) of the inhibition reaction were taken at various time intervals, diluted to 2 mL with pH 7.0, 0.1 M phosphate buffer, and assayed by using *N*-succinyl-$(Ala)_3$-*p*-nitroanilide as a substrate (0.1 mL of a 1 mg/mL solution in MeOH) by monitoring the release of *p*-nitroaniline at 405 nm [modified procedure of Thompson & Dennis (1973) and Visser & Blout (1972)].

The most consistent assay of thrombin activity proved to be the clotting assay. A stock solution of thrombin was prepared by diluting 50 μL of a 1000 NIH units/mL solution (in 0.05 M phosphate, pH 7.0) to 15 mL with pH 7.0, 0.01 M phosphate plus 0.1 M NaCl. A 0.5% solution of fibrinogen was prepared by dissolving 60 mg of fibrinogen in 10 mL of pH 7.0, 0.01 M phosphate plus 0.1 M NaCl. To obtain standard clotting times, we combined thrombin (0.1 mL of stock) and fibrinogen (0.2 mL) and timed the reaction until the solution cleared due to coagulation (Baughman, 1970). For inactivation studies, inhibitors in 1 mM HCl were added in microliter quantities to 1 mL of the thrombin stock solution, and aliquots (0.1 mL) of the inhibition reaction were taken at various time intervals and assayed vs. fibrinogen as outlined above.

Details of the inactivation studies on trypsin have been previously reported (Penny et al., 1979).

Kinetic data were analyzed by the method of Kitz & Wilson (1962) in accordance with Scheme I, where E·I represents the noncovalent enzyme–inhibitor complex and E–I represents the irreversible enzyme–inhibitor conjugate. It is assumed that the complex E·I dissociates when samples of an enzyme–inhibitor reaction mixture are diluted immediately before they are assayed. Thus, losses of enzymic activity reflect the formation of E–I only.

Values of k_{obsd} and subsequently k_2 and K_I were calculated from the equation $\log (v/v_0) = -k_{obsd}t$ using $\log (v/v_0)$ and t as inputs into a Hewlett-Packard 9830A computer operating on a least-squares program. The data were plotted with a Hewlett-Packard 9862A plotter. The initial rate of hydrolysis of a substrate at time zero is v_0, and v is the rate of hydrolysis of an aliquot taken at time t. When $[I] \gg [E]$, then $k_{obsd} = k_2/(1 + K_I/[I])$, and a plot of $1/k_{obsd}$ vs. $1/[I]$ has a slope of K_I/k_2 and an intercept of $1/k_2$ (Kitz & Wilson, 1962).

The synthesis and characterization of the undansylated peptidyl derivatives of phenylalanyl- and alanylchloromethanes have been described (Kurachi et al., 1973; Powers & Tuhy, 1973; Fittkau, 1973).

Dansylation of the N terminus of the inhibitors was accomplished by dissolving the hydrochloride/hydrobromide salt of the deprotected peptidyl chloromethane in dimethylformamide (−20 °C) and treating it with an equivalent of triethylamine, followed immediately by a twofold excess of dansyl chloride. The reaction was generally complete within 15 min. The solvent was then evaporated, and the residue was dissolved in ethyl acetate, washed with 0.1 M HCl and saturated $NaHCO_3$, dried ($MgSO_4$), and evaporated. The products were purified on silica gel by washing off the excess dansyl chloride with chloroform and eluting the desired products with chloroform–acetone, 7:1 v/v (for spectral properties, see Table I).

Table I: Spectral Properties of Inhibitors and Enzyme-Inhibitor Conjugates in pH 7.0, 0.01 M Tris Buffer

compd	dansyl λ_{max} (nm)	$E \times 10^{-6}$ (cm^2 $mmol^{-1}$)	dansyl emission (nm)
Dns-Ala-Lys-CH_2Cl[a]	335	4.0	550
Dns-Leu-Phe-CH_2Cl[b]	330	4.2	530
Dns-Pro-Ala-CH_2Cl[b]	332	4.0	560
Dns-Ala-Pro-Ala-CH_2Cl[b]	332	4.1	560
Dns-Pro-Ala-Pro-Ala-CH_2Cl[b]	330	4.2	560
DALM-trypsin	336	4.0	500
DLPM-chymotrypsin	340	4.2	550
DAPAM-elastase	340	4.1	539
DALM-thrombin	336	4.0	530

[a] Penny et al. (1979). [b] 10% methanol.

The following procedure was used for preparing the conjugates of α-chymotrypsin and elastase with their specific peptidyl fluorescent inhibitors. The enzyme (5–10 mg) was dissolved in 3.0 mL of 0.05 M sodium phosphate buffer (pH 7.0), and 0.5 mL of 50% methanol containing a 10-fold excess of inhibitor was added. The solution, which was slightly cloudy, was maintained at 40 °C for 2 h (Schoellmann, 1972). An assay for residual amidase activity with N^{α}-benzoyl-L-tyrosine-*p*-nitroanilide for chymotrypsin and N^{α}-succinyl-$(Ala)_3$-*p*-nitroanilide for elastase showed the remaining enzymatic activity in each case to be less than 1%. The fluorescent enzyme–inhibitor conjugates were purified on Sephadex G-50 by using 1% acetic acid as the eluant. The appropriate (fluorescent) fractions were pooled and lyophilized, and the conjugate was obtained as a fluffy white fluorescent powder in ~85% yield.

The thrombin conjugate, DNS-Ala-Lys-CH_2-thrombin (DALM-thrombin), was prepared by dissolving thrombin (~5 mg) in pH 7.0, 0.01 M phosphate and treating it with a 50-fold excess of DALCM in 1 mM HCl. The pH of the solution was adjusted to 7.0 with 0.1 M phosphate, and the reaction was maintained at 40 °C overnight. The conjugate was purified as described above. Lyophilization produced a highly fluorescent white fluffy powder (yield, ~75%). The preparation showed no clotting activity.

The synthesis of DALM-trypsin has been described in a previous publication (Penny et al., 1979).

Results and Discussion

Dansylation of Peptidyl Chloromethanes. Dansylation via deprotection of the N terminus and addition of Dns-Cl was found to be the only successful means of introducing the dansyl group into peptidyl chloromethanes. Various attempts were made to couple the dansyl amino acids, Dns-Ala and Dns-Leu, to Lys(Z)-CH_2Cl and Phe-CH_2Cl in this and earlier work (Penny et al., 1979) without a great deal of success. Methods of activating the C terminus of dansyl amino acids included the use of DCC, formation of the acid chloride with thionyl chloride, formation of the mixed anhydride with ethyl or *tert*-butyl chloroformate, and *p*-nitrophenyl ester activation. Monitoring each of the activation reactions by TLC showed that numerous fluorescent side products were produced in all cases. Addition of the amino acid chloromethane to the "activated" dansyl amino acid provided only marginal yields of the desired dansylated peptidyl chloromethanes. Side reactions involving the decomposition of the dansyl group which may also account for the observed fluorescent side products in coupling dansylated amino acids have been reported by Neadle & Pollitt (1965) and Narita (1970). Similar decompositions have been reported for N^{α}-tosyl amino acid chlorides

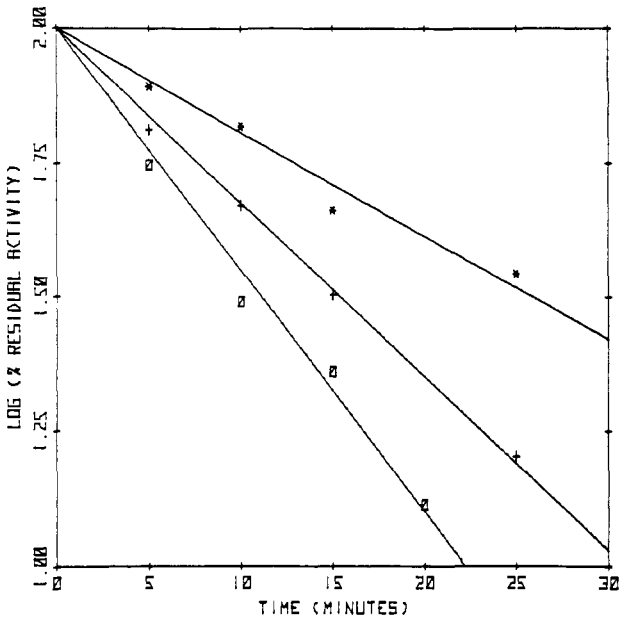

FIGURE 1: Inactivation of bovine thrombin by various concentrations of DALCM. Plot shows the percentage of remaining clotting activity vs. time in pH 7.0, 0.01 M phosphate plus 0.1 M NaCl. Enzyme concentration = 0.5 μM. DALCM concentrations are (*) 5, (+) 10, and (0) 15 μM.

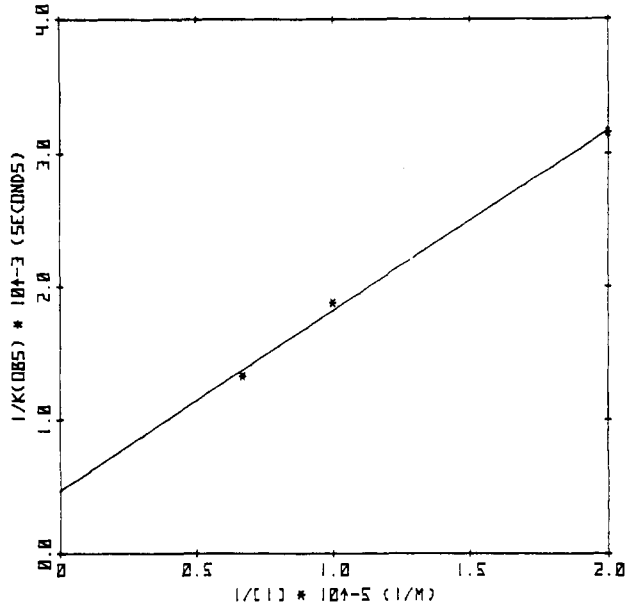

FIGURE 2: Saturation kinetics for the inactivation of bovine thrombin by DALCM. The plot is for data obtained from clotting assays at 23 °C in 0.01 M phosphate buffer, pH 7.0. Enzyme concentration was 0.5 μM.

in alkaline solutions (Beecham, 1957).

Inactivation of Trypsin and Thrombin with DALCM. The inhibition of trypsin with DALCM has already been reported (Penny et al., 1979). At pH 7.0 DALCM is significantly more reactive toward trypsin than similar chloromethane-type inhibitors. Even at concentrations of 1:1 in dilute solutions ([I] = [E] = 0.8 μM), total inactivation resulted within 40 s. At these concentrations it is no longer meaningful to evaluate k_{obsd} since [I] is not significantly greater than [E]. Thus, a direct evaluation of the parameters k_2 and K_I was not possible. By use of a bimolecular approach, a bimolecular rate constant k_2' was calculated and used to approximate k_2/K_I. The calculated k_2' for DALCM reacting with trypsin at pH 7.0 is 2.5×10^5 M^{-1} s^{-1}. This value compares favorably with the k_2/K_I value of 2.0×10^3 M^{-1} s^{-1} for the structurally similar inhibitor Lys-Ala-Lys-CH_2Cl (Coggins et al., 1974).

The inactivation of thrombin by DALCM does follow good first-order kinetics (Figure 1). At inhibitor concentrations of 5–15 μM and a thrombin concentration of 0.5 μM at pH 7.0, k_2 was calculated to be 2.1×10^{-3} s^{-1}, and $K_I = 2.8 \times 10^{-5}$ M for a k_2/K_I value of 75 M^{-1} s^{-1} (Figure 2). This represents a fivefold increase in reactivity over that of the structurally similar tripeptide Phe-Ala-Lys-CH_2Cl (Coggins et al., 1974).

Inactivation of Chymotrypsin by DLPCM. The inactivation of α-chymotrypsin by DLPCM was followed as a function of time at pH 5.8 to allow a direct comparison to similar inhibitors (Kurachi et al., 1973). Good pseudo-first-order kinetics were observed. The k_2/K_I value for DLPCM was calculated to be 2.8 M^{-1} s^{-1}. The structurally similar inhibitor Ac-Leu-Phe-CH_2Cl is reported to have a $k_{obsd}/[I]$ of 1.34 M^{-1} s^{-1} over the same range of concentrations ([E] = 40 μM; [I] = 0.2–0.8 μM) (Kurachi et al., 1973). K_I was determined to be 0.24 mM, and k_2 was determined to be 0.67×10^{-3} s^{-1}.

Inactivation of Elastase. The dipeptidyl chloromethane Dns-Pro-Ala-CH_2Cl was capable of producing only a 5% loss of amidase activity in elastase assays in periods of up to 4 h, even at concentration ratios of [I]/[E] = 100 ($k_{obsd}/[I]$ = 0.06 M^{-1} s^{-1}). This is in line with the reported $k_{obsd}/[I]$ value of 0.05 M^{-1} s^{-1} for Ac-Pro-Ala-CH_2Cl (Thompson & Blout, 1973).

The tripeptidyl chloromethane DAPACM did prove to be an effective inhibitor of elastase. Good pseudo-first-order kinetics were obtained on treating 0.26 μM elastase at [I]/[E] ratios of 10–100. From the plot of $1/k_{obsd}$ vs. 1/[I], a k_2/K_I value of 24 M^{-1} s^{-1} can be calculated, where $k_2 = 4.7 \times 10^{-3}$ s^{-1} and $K_I = 0.2$ mM. As expected, when K_I is much greater than the chosen inhibitor concentration, the double-reciprocal plot approaches the origin and $k_{obsd}/[I]$ becomes a direct measure of k_2/K_I. For a total of 12 determinations at four concentrations, the average $k_{obsd}/[I]$ = 24 M^{-1} s^{-1}. The k_2/K_I values obtained for DAPACM represent a threefold increase over the rate of inactivation of elastase by the structurally similar inhibitor Ac-Ala-Pro-Ala-CH_2Cl (k_2/K_I = 8.3 M^{-1} s^{-1} at pH 6.5; Thompson & Blout, 1973). This rate enhancement by the dansyl group is similar to that seen for the benzyloxycarbonyl group by Thompson & Dennis (1973), who found that Z-$(Ala)_3$-CH_2Cl was 2–3 times more effective in inactivating elastase than Ac-$(Ala)_3$-CH_2Cl under similar conditions.

The tetrapeptide, DPAPACM, was employed vs. elastase as a test of the S_4–P_4-induced rearrangement argument of Shotton and Hartley (Blackburn, 1976). It was expected that the dansylated tetrapeptidyl chloromethane would be ~7 times more effective than the corresponding tripeptide, DAPACM, in accordance with the result for Ac-Pro-Ala-Pro-Ala-CH_2Cl reported by Thompson & Blout (1973). However, it was found that this tetrapeptide was less effective than the tripeptide and was approximately only as effective as the dipeptide. Concentration ratios ([DPAPACM]/[elastase]) of 100:1 produced only 5–10% inactivation of elastase within 2 h. Possible clues to the reason for the lack of reactivity of the tetrapeptide are found in the NMR spectrum of DPAPACM. When the chemical shifts of DPAPACM resonances are compared with the chemical shifts of corresponding resonances in DPACM and DAPACM, the following downfield shifts are noted: (1) an alanine side chain (Ala_β, δ 1.25 to δ 1.40), (2) the chloromethyl group ($COCH_2Cl$, δ 4.1 to δ 4.3), and (3) the dimethylamino group of the dansyl moiety (δ 2.8 to δ 3.0). On

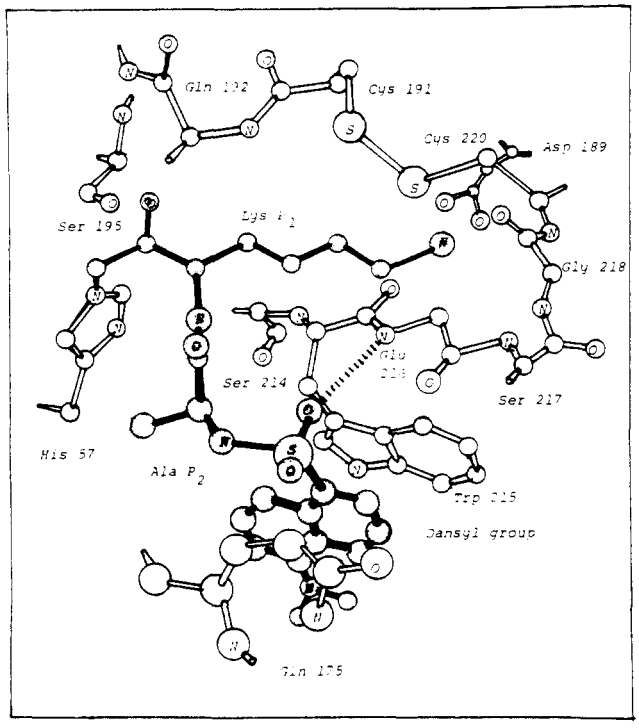

FIGURE 3: Possible hydrophobic cleft on the surface of trypsin occupied by the dansyl group in DALM-trypsin [adapted from Segal et al. (1972)].

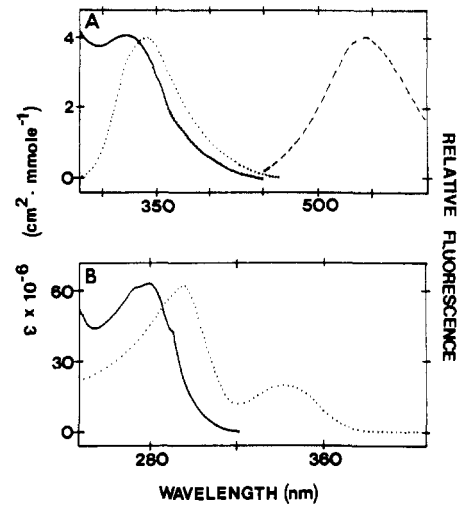

FIGURE 4: Absorption, emission, and excitation spectra of DALM–thrombin. (A) Spectral overlaps of the emission of Trp residues and the absorption of the Dns group: (—) difference absorption spectrum of the dansyl group in DALM–thrombin; (···) emission spectrum of thrombin excited at 295 nm; (---) emission spectrum of the Dns group in DALM–thrombin excited at 340 nm; (shaded area) emission of Trp residues and the dansyl group in DALM–thrombin excited at 295 nm. (B) Excitation and absorption spectra: (···) excitation spectrum of DALM–thrombin at the emission wavelength of the Dns group (530 nm); (—) absorption spectrum of native thrombin.

the basis of the downfield shifts of these protons, it would appear as though the dansyl group occupies a position which is relatively close to the methyl and chloromethyl groups of the P_1 alanine. This apparent proximity relationship could result from an intermolecular dimer association in which the P_1 alanine of one molecule contacts the dansyl group of the other. Alternatively, a chain reversal in the tetrapeptide would bring about an intramolecular P_1 Ala–Dns interaction. Either case would effectively preclude the simultaneous interaction of the P_1–P_4 residues of DPAPACM with the S_1–S_4 subsites of elastase, resulting in low reactivity.

Reasons for the Heightened Reactivity of DALCM with Trypsin. The markedly enhanced reactivity of DALCM with trypsin seems to be peculiar to this inhibitor–enzyme pair and not a characteristic of dansylated peptidyl chloromethanes. An investigation of models representing the interaction of the bound inhibitor with trypsin reveals that the dansyl group may occupy a relatively solvent-shielded cleft on the enzyme surface in the vicinity of the hydrophobic residues Trp-215 and Gln-175 (chymotrypsin numbering system) (Figure 3). In the case of chymotrypsin, this binding mode is not likely since the corresponding chymotrypsin residue, Lys-175, has a positively charged hydrophilic side chain which is expected to repel the hydrophobic dansyl group from entering the cleft. With elastase, it is apparent that the dipeptide DPACM does not possess enough subsite interactions to be recognized. The tripeptide, which is recognized, is unfortunately too long to take advantage of any cleft that might exist in the vicinity of Tyr-215 and Thr-175 (chymotrypsin numbering system). The supposition that the dansyl group occupies a relatively apolar microenvironment in the DALM-trypsin conjugate, but not in the chymotrypsin and elastase conjugates, is supported by the emission spectra of the bound dansyl groups. In DALM-trypsin the dansyl emission (495 nm) shows a marked hyperchromic shift relative to that of DALCM (540 nm) (Penny et al., 1979). Such a shift, characteristic of hydrophobic interactions by the bound dansyl moiety (Vaz & Schoellmann, 1976), is not observed in DLPM-chymotrypsin or in DAPAM-elastase. Similarly, DALM-thrombin has an emission maximum at 530 nm (Figure 4), indicating that the dansyl group is not highly shielded from the aqueous solvent. It is thought that the homologous residue to Lys-175 in chymotrypsin is Arg-175 in thrombin (Magnusson, 1970).

Finally, it is of interest that DALCM, which is so highly active toward trypsin, will not covalently label trypsinogen. Attempts to label trypsinogen under the same conditions used for thrombin, and then at 50 times that concentration, yielded no detectable protein-bound fluorescence. This is in contrast to the results of Bode et al. (1978), who were able to bind basic pancreatic trypsin inhibitor (BPTI) to trypsinogen, and Gertler et al. (1974), who demonstrated acylation of the "active site" serine residue of trypsinogen by *p*-nitrophenyl-*p*-guanidinobenzoate (PNGB). In the first of these examples, however, the inhibiting protein appears to enforce a trypsin-like conformation upon the zymogen through a large number of intermolecular contacts. On the other hand, the *p*-nitrobenzoyl derivative appears still to possess a trypsinogen-like conformation. Selective labeling in this case is ascribed to the probability that the serine in question is activated by the "charge relay" relationship, which exists even in the zymogen. In both cases the reactivity of the inhibitor is substantially lowered, from $K_1 = 2 \times 10^{-13}$ M^{-1} to $K_1 = 5 \times 10^{-5}$ M^{-1} in the case of BPTI (Bode, 1979) and by a second-order rate factor of 10^{-7} in the case of PNGB (Gertler et al., 1974). Our own results indicate that if labeling of the "active site" of trypsinogen with DALCM were possible, the rate must be at least 10^{12} times slower than that of the observed reaction with trypsin.

The disorganization of the enzyme active site, which is characteristic of the zymogen in serine proteinases, occurs in trypsinogen primarily as a loss of the proper substrate binding site (Freer et al., 1970). This difference in the binding site accounts for the lessened reactivities of both BPTI and PNGB. In the case of DALCM, which appears to have a special reactivity toward trypsin and is in any case more selective than BPTI or PNGB (it will not inhibit α-chymotrypsin) (Penny

Table II: A Summary of Energy Transfer Data Obtained for Dansylated Peptidyl Chloromethane Labeled Proteinases

Dns conjugate	Φ_D	$J_{AD} \times 10^{-15}$ (cm^6 mmol^{-1})	R_0 (Å)	E_T (%)	R_{obsd} (Å)	$R_{expected}$ (Å)
DALM-trypsin	0.11	3.4	17.7	37.3[a] 39.5[b]	19.3 19.0	20.5
DLPM-chymotrypsin	0.10	4.0	17.5	38.2[a] 38.9[b]	19.3 19.2	20.2
DAPAM-elastase	0.12	3.5	19.1	42.0[a] 44.3[b]	20.2 19.9	21.0
DALM-thrombin	0.11	3.4	16.4–19.7[c]	33.5[a] 31.4[b]	18.4–22.6[c] 19.1–22.9[c]	20.4–23.0[c]

[a] Evaluated from the corrected excitation spectrum of the acceptor in the presence of the donors. [b] Evaluated by measuring the extent of donor fluorescence quenched in the presence of the acceptor. [c] See supplementary material.

et al., 1979), this difference is even more crucial, and trypsinogen cannot be selectively labeled by this molecule under any practical conditions.

Energy Transfer Measurements. Electronic excitation energy may be transferred from a donor to an acceptor chromophore through an induced resonance if the absorption spectrum of the acceptor overlaps the emission spectrum of the donor. The theoretical basis of this transfer of energy was first derived by Perrin (1925) and was further developed by Förster (1948, 1951). For a rigid system that contains one fluorescent donor and one acceptor, the efficiency of energy transfer, E_T, between the excited state of a donor dipole and the ground state of an acceptor dipole is

$$E_T = \frac{R_0^6}{R_0^6 + R^6} \quad (1)$$

R is the distance between the two chromophores, and R_0 (eq 2) is the distance between the donor and acceptor at which

$$R_0^6 = (8.8 \times 10^{-25})\Phi_D \kappa^2 \eta^{-4} J_{AD} \quad (2)$$

half of the energy of the donor is transferred to the acceptor. R_0 depends on the quantum yield of the donor in the absence of the acceptor (Φ), an orientation factor (κ^2), the refractive index of the medium (η), and the spectral overlap (J_{AD}) of the donor fluorescence (F_D) and acceptor absorption (ϵ_A) (eq 3).

$$J_{AD} = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda}{\int F_D(\lambda) \, d\lambda} \quad (3)$$

Chromophores attached to the ends of fixed rigid systems have been used to establish the validity of the Forster theory (Latt et al., 1965; Stryer & Haugland, 1967; Conrad & Brand, 1968; Eisinger et al., 1969; Haugland et al., 1969; Cantor & Pechukas, 1971). Protein systems containing multiple donors (Trp residues) and a single acceptor (Dns group) offer a challenge in that the efficiency of energy transfer is determined by a composite of donors which cannot be separated.

The inability to separate some of the individual donor effects becomes a factor in calculating R_0. For example, the quantum yield (Φ) of each Trp residue may be influenced by its particular position on the protein (Grinvald & Steinberg, 1976). In this work a steady-state approach is used, in which an average contribution of Trp residues is employed (Teale, 1960; Eisinger et al., 1969). (For further discussion, see paragraph at end of paper regarding supplementary material.) The quantum yields for chymotrypsin, trypsin, elastase, and thrombin used here are 0.10, 0.11, 0.12, and 0.11, respectively, determined by comparison with chymotrypsin.

The orientation factor (κ^2) will in all probability differ between Trp–Dns pairs in a dansylated protein system. Only in the case where the donor–acceptor pair is free to rotate at a rate which is larger than the deexcitation rate of the donor can a value of $\langle\kappa^2\rangle_{av} = 2/3$ be used. This value was employed in our calculations for DAPAM-elastase in which the dansyl group is far enough removed from the point of enzyme-inhibitor attachment to make free rotation of that group likely. For each of the other conjugates, a system of limited rotation was postulated, and crystallographic coordinates were employed to calculate a set of κ^2 values for the individual Trp–Dns pairs and from these the value of R_0. Details are given in the supplementary material.

The spectral overlap integral, J_{AD}, for each conjugate was evaluated as described by Conrad & Brand (1968). Values are given in Table II. The spectral overlap of DALCM and thrombin is provided for an example of spectra used in the calculation of J_{AD} and E_T (Figure 4). The refractive index of the protein medium (η) in all cases was taken to be 1.5 (Eisinger et al., 1969).

The experimental efficiencies of energy transfer, E_T, were determined from the excitation spectra of the Dns conjugates or by measuring the extent of Trp quenching in the presence of the acceptor molecules (Haugland & Stryer, 1967). The values of E_t and R_0 for each conjugate were used in the Förster equation (eq 1) to provide fluorescence-derived R values (R observed). In Table II these values are compared with R values afforded by averaging the theoretical E_T for each Trp–Dns distance obtained from X-ray data (R calculated). This comparison enables an assessment of the application of the Förster equation in predicting transfer efficiencies and weighted mean internuclear distances (R values). The values of R observed and R calculated differ by only ±1.5 Å in the dansylated enzyme–inhibitor conjugates of trypsin, chymotrypsin, and elastase.

If the B chain of thrombin is homologous to chymotrypsin as supposed, its Trp residues will correspond in their spatial coordinates to homologous chymotrypsin residues. The R expected values for DALM-thrombin, calculated from the α-chymotrypsin coordinates (Birktoft & Blow, 1972), range from 20.4 to 23.0 Å; the R observed values range from 18.4 to 22.6 Å and from 19.1 to 22.9 Å in separate evaluations. Thus, the expected and observed R values for DALM-thrombin show the same close correspondence seen for the expected and observed R values of the other serine proteinase–inhibitor conjugates tested. This result is entirely consistent with the proposed conformational homology of thrombin and chymotrypsin.

Acknowledgments

The authors thank Dr. James Bailey and Dr. Shiao-Chun Tu for the use of their fluorescence equipment. The kind assistance of Dr. Stanley Deming and Bill Price in the writing and running of computer programs is gratefully acknowledged. We also thank Dr. B. D. Burleigh for his expert advice and

for his aid in the purification of trypsin and chymotrypsin samples for this work.

Supplementary Material Available

Discussion of the calculation of κ^2 values for the individual Trp–Dns pairs from crystallographic data to obtain $\langle\kappa^2\rangle_{av}$ values for each conjugate (4 pages). Ordering information is given on any current masthead page.

References

Baughman, J. (1970) *Methods Enzymol. 19*, 145–184.
Beecham, A. F. (1957) *J. Am. Chem. Soc. 79*, 3257–3261.
Birktoft, J. J., & Blow, D. M. (1972) *J. Mol. Biol. 68*, 187–240.
Blackburn, S. (1976) *Enzyme Structure and Function*, p 157, Marcel Dekker, New York.
Bode, W. (1979) *J. Mol. Biol. 127*, 357–374.
Bode, W., Schwager, P., & Huber, R. (1978) *J. Mol. Biol. 118*, 99–112.
Cantor, C. R., & Pechukas, P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2099–2101.
Coggins, J. R., Kray, W., & Shaw, E. (1974) *Biochem. J. 138*, 579–585.
Conrad, R. H., & Brand, L. (1968) *Biochemistry 7*, 777–786.
Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 1–109.
Eisinger, J., Feuer, B., & Lamola, A. A. (1969) *Biochemistry 8*, 3908–3915.
Fittkau, V. S. (1973) *J. Prakt. Chem. 315*, 1037–1044.
Förster, T. (1948) *Ann. Phys. (Leipzig) 2 (Ser. 6)*, 55.
Förster, T. (1951) *Flureszenz Organischer Verbindugen*, Van den Hoeck and Ruprecht, Göttingen, West Germany.
Freedman, M. M., Friedburg, D., Macha, J., & Troll, W. (1973) *Biochem. Pharmacol. 22*, 2441–2451.
Freer, S. T., Kraut, J., Robertus, J. D., Wright, M. T., & Xuong, Ng. H. (1970) *Biochemistry 9*, 1997–2009.
Gertler, A., Walsh, K. A., & Neurath, H. (1974) *Biochemistry 13*, 1302–1310.
Glover, G., & Shaw, E. (1971) *J. Biol. Chem. 246*, 4594–4601.
Grinvald, A., & Steinberg, I. Z. (1976) *Biochim. Biophys. Acta 427*, 663–678.
Haugland, R. P., & Stryer, L. (1967) in *Conformation of Biopolymers* (Ramachandran, G. N., Ed.) Vol. 1, pp 321–335, Academic Press, New York.
Haugland, R. P., Yguerabide, J., & Stryer, L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 23–30.
Johnson, D. A., & Travis, J. (1976) *Anal. Biochem. 72*, 573–576.
Kettner, C., Springhorn, S., & Shaw, E. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1183–1191.
Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.
Koshland, D. E., Jr., & Neet, K. E. (1968) *Annu. Rev. Biochem. 37*, 359–410.
Kosman, D. J. (1972) *J. Mol. Biol. 67*, 247–264.
Kurachi, K., Powers, J. C., & Wilcox, P. E. (1973) *Biochemistry 12*, 771–777.
Latt, S. A., Cheung, H. T., & Blout, E. R. (1965) *J. Am. Chem. Soc. 87*, 995–1003.
Magnusson, S. (1970) *Thromb. Diath. Haemorrhag. 38*, 97.
Morihara, K., & Oka, T. (1970) *Arch. Biochem. Biophys. 138*, 526–531.
Narita, K. (1970) in *Protein Sequence Determination* (Needleman, S. B., Ed.) pp 38–49, Springer-Verlag, Heidelberg, West Germany.
Neadle, D. J., & Pollitt, R. J. (1965) *Biochem. J. 97*, 607–608.
Ong, E. B., Shaw, E., & Schoellmann, G. (1964) *J. Am. Chem. Soc. 86*, 1271–1272.
Parker, C. A., & Rees, W. T. (1960) *Analyst 35*, 587–600.
Penny, G. S., Dyckes, D. F., & Burleigh, B. D. (1979) *Int. J. Pept. Protein Res. 13*, 95–101.
Perrin, J. (1925) in *2me Conseil de Chemie, Solvay*, Paris, p 322, Gauthier-Villars, Paris.
Powers, J. C. (1977) *Chem. Biochem. Amino Acids, Pept., Proteins 4*, 65–178.
Powers, J. C., & Wilcox, P. E. (1970) *J. Am. Chem. Soc. 92*, 1782–1783.
Powers, J. C., & Tuhy, P. M. (1973) *Biochemistry 12*, 4767–4774.
Rossman, R., Norris, C., & Tnoll, W. (1974) *J. Biol. Chem. 249*, 3412–3417.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.
Schoellmann, G. (1972) *Int J. Protein Res. 4*, 221–227.
Segal, D. M., Powers, J. C., Cohen, G. H., Davies, R. R., & Wilcox, P. E. (1971) *Biochemistry 10*, 3728–3738.
Shaw, E., & Springhorn, S. (1967) *Biochem. Biophys. Res. Commun. 27*, 391–397.
Shotton, D. M., White, N. J., & Watson, H. C. (1972) *Cold Spring Harbor Symp. Quant. Biol. 36*, 91–105.
Stryer, L., & Haugland, R. P. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 719–726.
Summers, D. F., Shaw, E. N., Stewart, M. L., & Maizel, J. V. (1972) *J. Virol. 10*, 880–884.
Teale, F. W. J. (1960) *Biochem. J. 76*, 381–388.
Thompson, R. C., & Blout, E. R. (1973) *Biochemistry 12*, 44–47.
Thompson, S., & Dennis, I. J. (1973) *Eur. J. Biochem. 38*, 1–5.
Udenfried, S. A. (1969) in *Fluorescence Assay in Biology and Medicine*, Vol. II, pp 594–614, Academic Press, New York.
Vaz, W. L. C., & Schoellmann, G. (1976) *Biochim. Biophys. Acta 439*, 206–218.
Visser, L., & Blout, E. R. (1972) *Biochim. Biophys. Acta 268*, 257–260.
Whur, P., Payne, N. E., & Koppel, H. (1974) *Exp. Cell Res. 86*, 422–425.
Yamamoto, T., & Izumiya, N. (1966) *Arch. Biochem. Biophys. 114*, 459.
Yamashita, T. (1960) *J. Biochem. (Tokyo) 48*, 846–852.
Zaneveld, L., Robertson, R., & Williams, W. (1970) *FEBS Lett. 11*, 345–347.