Å away.[15] Dreiding models indicate that IIb could readily intercalate so that these groups are opposite C-2 and C-3, respectively. Assuming that the conformation of GSH in solution is similar to that in the crystal, the large negative $\Delta S_{IIb}^{\ddagger}$ may be due to the restriction of vibrational and rotational freedom of these groups in the transition state.[16] This path appears to be more difficult for the more angular Ib.

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Reagents for the Cross-Linking of Proteins by Equilibrium Transfer Alkylation

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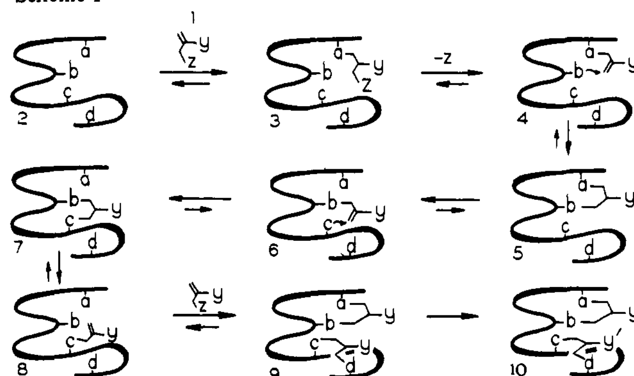
Abstract: Reagents (**11** and **12**) have been synthesized which can interact with nucleophilic groups on proteins and biopolymers by a sequence of consecutive Michael reactions yielding cross-linked and multi-cross-linked structures. Consecutive Michael reactions occur because Michael addition to the reagent then allows elimination of the trimethylammonium or mercaptonitrobenzoate function unmasking the latent double bond. A second Michael is then possible. These consecutive Michael reactions potentially allow the cross-link(s) to circumambulate the protein framework until the most thermodynamically stable cross-link equilibrium is established. Intermolecular cross-links can be established between protein subunits and multienzyme complexes. These reagents are designed so that addition and sequential cross-linking may be monitored by spectroscopy, and the cross-link may be subsequently fixed. This fixing process may be accomplished at variable times so as to provide a spectrum of cross-linked derivatives representing the sequence of steps. The chemistry of the process was established using cysteine, acetylcysteine, lysine, alkyl mercaptans, and alkylamines as models of protein residues. The intra- and intermolecular cross-linking character of these reagents was demonstrated using ribonuclease. Multiple cross-links could be introduced. In one of the modified ribonucleases, two links were introduced—one link was established between lysine residues 7 and 37; the other is probably between lysine residues 31 and 41. These links were determined by tryptic peptide mapping. A modified ribonuclease having three cross-links and intermolecularly cross-linked ribonuclease dimers and trimers were also produced.

Introduction

Chemical techniques for establishing the tertiary and subunit structures of enzymes, proteins, and biopolymers have traditionally depended upon cross-linking reagents with two essentially noninteracting alkylating or acylating groups.[1-5] Usually, chemical reagents used for the purpose of modification and cross-linking studies have not been differentiated from one another as to their ability to form equilibrium or nonequilibrium attachments during the reaction process.[6] Equilibrium cross-linking has been studied primarily within the framework of exchanges and recombinations of disulfide links of cystine residues,[7] and the concepts have not been extrapolated to reagent cross-linking. However, the nature of the bonding functions of most cross-links can be classified into two mechanistically distinct groups: kinetic (nonreversible) and equilibrium controlled reaction attachments. Our proposal is that these mechanistically different reagent characteristics can potentially alter the sites of residue interlinking. We wish to introduce compounds with generalized structure **1** (z = a leaving group, y = an electron-withdrawing substituent) having

Scheme I



a unique association of functionality[7-11] which allows the study of equilibrium established cross-linked derivatives of proteins and biopolymers by consecutive Michael reactions.[12]

The process may be illustrated by considering the interaction of **1** with a diagrammatic protein **2** having several nucleophilic sites (a, b, c, etc., Scheme I). Michael addition[13-16] to an ex-

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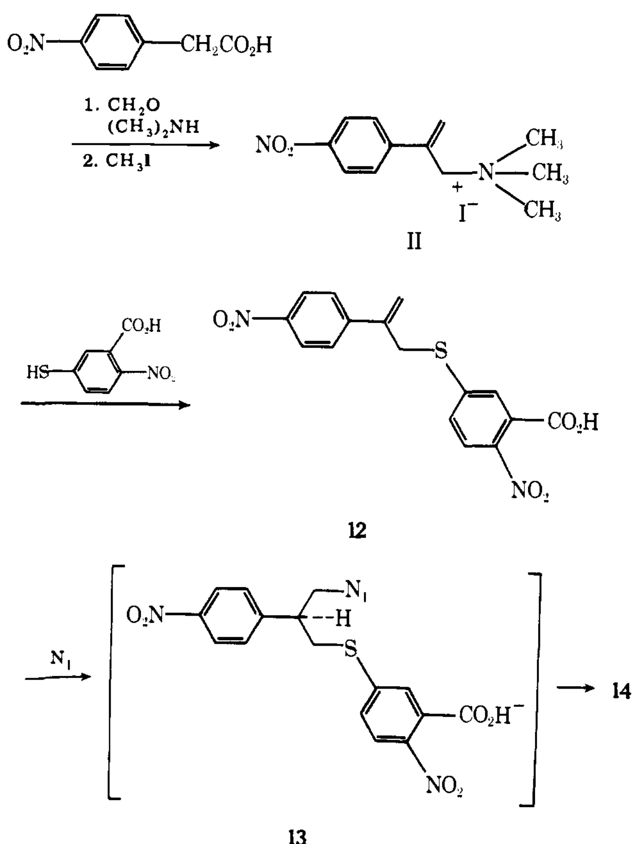


posed nucleophilic residue a on the protein would be followed by retro-Michael of the leaving group (overall equivalent to an S_N2' process) with the unmasking of the latent double bond ($3 \rightarrow 4$).[7] The covalently attached species **4** can now undergo an intramolecular Michael reaction yielding a cross-linked derivative **5**. Since the Michael reactions are reversible, derivative **5** is not only in equilibrium with **4** but also with **6**. Thus, retro-Michael from residue a leaves the reagent moiety attached to residue b, giving the potential for a second intramolecular Michael to residue c affording a new cross-link **7**. The sequence of Michael and retro-Michael reactions could potentially continue to available sites (b-c $\rightleftarrows$ c-d, etc.) allowing the cross-link to circumambulate the protein framework until the lowest energy cross-link equilibrium was established. Since, after conversion of **5** to **6**, residue a would be available to interact with a second reagent molecule, multiple cross-links are possible (**9**). Over a period of time, the cross-linking moiety can "walk" to positions on the protein chain inaccessible to ordinary cross-linking agents, because under normal conditions the sequential alkylating character of the reagent is never lost. In the same fashion, equilibrium intermolecular cross-links could be established between protein subunits and multiprotein or enzyme complexes by a cascade of consecutive Michael reactions.

For most protein residues, the assignment of the positions of such mobile cross-links by chemical degradative techniques would require the reagents to have activating functions for the Michael reaction which may be removed or modified ($9 \rightarrow 10$), so that manipulation of the material would not then change the mobile cross-link position. Such modification would "fix" the cross-link in place. We suggest the acronym ETAC for these types of *e*quilibrium *t*ransfer *a*lkylating *c*ross-link reagents and processes.

We have designed, synthesized, and developed the application of two reagents containing the elements of **1**, which combine certain spectral and functional group advantages for protein studies. (Their synthesis is outlined in Scheme II and detailed in the Experimental Section.) Though cationic reagent **11** (ETAC-I) is the more reactive, reagent **12** (ETAC-II) is the more water soluble at pH values above 5 and allows as well the spectral monitoring of the first Michael and elimination step by the release of mercaptonitrobenzoate anion (reduced Ellman's reagent, λ_{max} 412 nm).[17] In both reagents **11** and **12**, the second Michael (cross-link steps $4 \rightarrow 5 \rightarrow 7$, etc.) may be monitored by the decrease of the *p*-nitrostyrene absorption at 320 nm[18,19] (see Figure 1). Aside from the equilibrium character of these reagents, such features as water solubility combined with the ability to monitor the separate sequential cross-linking steps have not been available within the structures of previous cross-linking agents. Furthermore, for many situations, the reagents may be used in stoichiometric quantities. Loss of reagent by hydrolysis, even at intermediate steps in the sequence, is not a problem because structures such as **14** (N_1 = OH) (Scheme III) can continue to establish the cross-link equilibrium.

The *p*-nitro function on the cross-linking moiety(ies) can be reduced to an amino function by dithionite extremely rapidly.[20] With the removal of this function which allows for the Michael-retro-Michael reaction, the cross-link becomes positionally fixed ($9 \rightarrow 10$). This "quenching" process may be carried out even at times prior to the establishment of the final equilibrium, so that if there is a buildup of an intermediate (such as **7**) the sequence of steps might be observed. The usual mapping techniques can then be accomplished without the cross-link(s) dissociating from the amino acid or peptide fragments during protein hydrolysis.[15] A further advantage of using this reduced cross-linked moiety is that amino acid residues and peptide fragments containing this function can be exclusively identified by diazotization with HNO_2 and coupling with β-naphthol to yield an azo dye (UV max 500 nm).

The equilibrium Michael and retro-Michael processes which result in exchange processes were first confirmed in intermolecular reactions using model thiols (pH ~8) and amine substrates (pH ~10). The intra- and intermolecular cross-linking character of these reagents for proteins was then evaluated in a preliminary fashion by reaction with the well-characterized enzyme ribonuclease.[21-23]

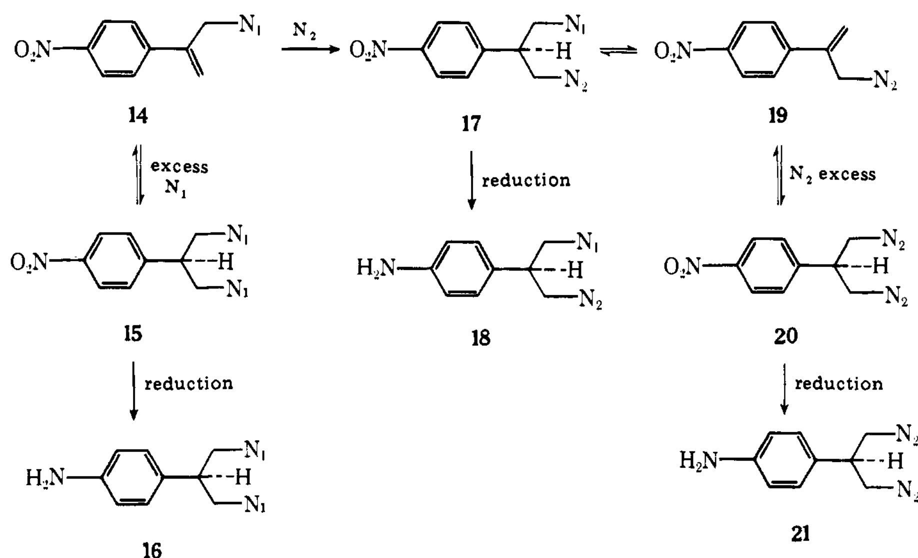
Experimental Procedures

Preparation of Reagents. ETAC-I [2-(*p*-Nitrophenyl)allyltrimethylammonium Iodide] (11).[24] *p*-Nitrophenylacetic acid (30.2 g, 0.166 mol) was dissolved in 210 mL (1.7 mol) of 33% aqueous dimethylamine solution, the mixture was cooled to 10 °C by an ice bath, and immediately the dropwise addition of 150 mL of 38% aqueous formaldehyde solution was started. The atmosphere of nitrogen was maintained and the temperature of the reaction controlled at 15-20 °C. When the addition of formaldehyde was complete, the reaction mixture was stirred for 0.5 h at 20 °C, for an additional 0.5 h with warming to 60 °C (carbon dioxide evolved during this time period), and finally at room temperature for 10 h. The light yellow oil which separated from solution was taken up in three 30-mL portions of chloroform. The chloroform layer was back-extracted with three 30-mL portions of 3 N HCl, the acid layer neutralized with saturated $KHCO_3$ solution, and the separated oil reextracted with chloroform (3 × 30 mL). The chloroform extract was washed with an equal volume of saturated sodium chloride solution and dried over Na_2SO_4. Evaporation of the chloroform yielded 31.5 g (93%) of crude α-(*N,N*-dimethylaminomethyl)-*p*-nitrostyrene. A small portion of this material was purified by distillation under reduced pressure: bp 60 °C (1.5 mm); NMR ($CDCl_3$) δ 2.20 (6 H, s), 3.30 (2 H, s), 5.40 (1 H, s), 5.63 (1 H, s), 7.66 (2 H, d), 8.13 (2 H, d); MS *m/e* 206 (M^+); UV λ_{max} 300 nm. Anal. Calcd for $C_{11}H_{14}N_2O_4$: C, 64.04; H, 6.85; N, 13.59. Found: C, 63.96; H, 6.94; N, 13.51. **Caution:** this material has vesicant properties.

The remainder was used for the preparation of the salt without purification.

The crude α-(*N,N*-dimethylaminomethyl)-4-nitrostyrene was

Scheme III



dissolved in ether (150 mL) and cooled to 0 °C. To the ether solution was added 30 mL of iodomethane cautiously. The flask was fitted with a $CaSO_4$ drying tube, and the reaction mixture stirred at room temperature for 4 h. During this time quaternary ammonium iodide (**11**) precipitated as a yellow solid. Recrystallization from hot water yielded 2-(*p*-nitrophenyl)allyltrimethylammonium iodide (ETAC-I): 48.6 g (91.3%); mp 203–204 °C; NMR (Me_2SO-d_6) δ 3.03 (9 H, s), 4.65 (2 H, s), 6.03 (1 H, s), 6.12 (1 H, s), 7.91 (2 H, d), 8.28 (2 H, d); MS *m/e* 162, 142, 128, 127, 58 (base peak); IR $\nu_{max}^{KBr\ pellet}$ (cm^{-1}) 3000, 1595, 1500, 1400, 1330; UV λ_{max} 295 nm (ϵ 13 000). Anal. Calcd for $C_{12}H_{17}N_2O_2I$: C, 41.39; H, 4.92; N, 8.05. Found: C, 41.33; H, 4.88; N, 8.04.

ETAC-II [2-(*p*-Nitrophenyl)allyl-4-nitro-3-carboxyphenyl Sulfide] (12). 2-Nitro-5-chlorobenzoic acid (6.0 g, 30 mmol) was suspended in 150 mL of water and a few drops of 25% NaOH solution added to it to bring the pH to ~7.5. To this mixture was added a solution of 7.9 g (33 mmol) of sodium sulfide in 50 mL of water. The resulting solution was heated under nitrogen at 45–50 °C for 2 h, cooled in an ice bath, and neutralized to pH 3 with 6 N HCl. The yellow precipitate was filtered, washed with cold water, and dried. The yield was 5.05 g (83%) of 5-mercapto-2-nitrobenzoic acid, mp 135–137 °C.[17]

To a solution of 9.6 g (27.5 mmol) of ETAC-I (**11**) in 500 mL of hot water, a solution of 5.0 g of 5-mercapto-2-nitrobenzoic acid (25.0 mmol) in 40 mL of 1.25 M sodium carbonate (5.3 g, 50 mmol) solution was added in 10 portions under a nitrogen atmosphere. The reaction mixture was heated to 60 °C for 16 h. The resulting orange-red solution was cooled in an ice bath and neutralized with 6 N HCl. The gummy yellow material which separated out was extracted with ethyl acetate (3 × 20 mL) and dried ($MgSO_4$), and the solvent removed by rotoevaporation. On drying under vacuum the oily material gradually solidified. Recrystallization from ethanol-water mixture gave 6.5 g (64%) of ETAC-II [2-(*p*-nitrophenyl)allyl-4-nitro-3-carboxyphenyl sulfide] (**12**): mp 170–171 °C; NMR (acetone-d_6) δ 4.43 (2 H, s), 5.59 (1 H, s), 5.70 (1 H, s), 7.66 (5 H, m), 8.10 (2 H, d); IR $\nu_{max}^{KBr\ pellet}$ (cm^{-1}) 3000, 1700, 1590, 1570, 1510, 1415, 1340, 1300; UV λ_{max} 320 nm (ϵ 16 000); MS *m/e* 360 (M^+). Anal. Calcd for $C_{16}H_{12}N_2O_6S$: C, 53.33; H, 3.36; N, 7.77; S, 8.90. Found: C, 53.41; H, 3.41; N, 7.72; S, 8.87.

The compound is available from Pierce Chemical Co., Rockford, Ill.

Chemistry of ETAC Reagents. Preparation of a Monoadduct of a Thiol (Example: *n*-Butanethiol). To a solution of 1.74 g (5 mmol) of ETAC-I in 100 mL of hot water, 0.55 mL (0.45 g, 5 mmol) of *n*-butanethiol dissolved in 15 mL of methanol and 1.6 mL of 25% NaOH solution were added. The reaction mixture was heated under nitrogen for 15 h at 75 °C. Upon concentrating the solution to half the volume, a brown oil separated which was extracted with three 10-mL portions of chloroform, washed once each with equal volumes of 2 N HCl, saturated $KHCO_3$ solution, and with saturated NaCl solution, and dried (Na_2SO_4). Removal of solvent gave 1.023 g (81%) of the desired adduct (**14**, N_1 = SnC_4H_8), α-(*n*-butylthiomethyl)-4-nitrostyrene. The compound was purified by distillation at reduced pressure: bp 135 °C (1.0 mm); NMR ($CDCl_3$) δ 0.90 (3 H, t), 1.50 (4 H, m), 2.52 (2 H, t), 3.62 (2 H, s), 5.40 (1 H, s), 5.60 (1 H, s), 7.62 (2 H, d), 8.15 (2 H, d); IR $\nu_{max}^{CHCl_3}$ (cm^{-1}) 3050, 2970, 2890, 1605, 1525, 1350, 1120, 870; MS *m/e* 354 (M^+). Anal. Calcd for $C_{13}H_{17}NO_2S$: C, 62.12; H, 7.03; N, 5.45; S, 12.84. Found: C, 61.95; H, 6.82; N, 5.57, 5.53; S, 17.72, 12.70.

Reaction with *N,N*-Dimethylaminoethanethiol. Preparation of α-[2-(*N,N*-Dimethylaminoethyl)thiomethyl]-4-nitrostyrene. To a solution of 1.74 g (0.005 mol) of ETAC-I (**11**) in 125 mL of hot water was added a solution of 0.71 g (0.005 mol) of *N,N*-dimethylaminoethanethiol hydrochloride dissolved in 6 mL of water. The pH of the resulting solution was adjusted to 8 by adding ammonia solution. The reaction mixture was then heated at 60 °C for 14 h under nitrogen. The mixture was then cooled and the light brown oil which separated out was extracted with three 30-mL portions of chloroform. The chloroform solution was extracted with three 30-mL portions of 2 N HCl; the acid layer was neutralized with saturated $KHCO_3$ solution and then back-extracted into three 30-mL portions of chloroform. The chloroform solution was washed with three 30-mL portions of saturated NaCl solution and dried (Na_2SO_4), and the solvent was removed by rotary evaporation. On drying under vacuum, 0.724 g (54%) of **19** was obtained. An 89% yield could be obtained using sodium bicarbonate rather than ammonia to adjust pH: NMR ($CDCl_3$) δ 2.18 (6 H, s), 2.46 (4 H, s), 3.56 (2 H, s), 5.31 (1 H, s), 5.50 (1 H, s), 7.56 (2 H, d), 8.12 (2 H, d); MS *m/e* 266 (M^+). The compound was further characterized by conversion to its quaternary ammonium iodide by reaction with iodomethane in ether followed by recrystallization from hot water, mp 182–183 °C. Anal. Calcd for $C_{14}H_{21}N_2SO_2I$: C, 41.18; H, 5.18; N, 6.86; S, 7.85; I, 31.08. Found: C, 41.22; H, 5.14; N, 6.90; S, 7.96; I, 31.13.

Exchange Reaction [14, N_1 = SnC_4H_9 → 17 → 19, N_2 = $SCH_2CH_2N(CH_3)_2$]. The monoadduct of *n*-butanethiol (1.25 g, 5 mmol) was dissolved in 20 mL of MeOH. To this solution 0.53 g (5 mmol) of *N,N*-dimethylaminoethanethiol (freshly generated from its hydrochloride) was added, and the mixture heated at reflux for 24 h. At the end of this period, the methanol was removed under vacuo and the remaining oil dissolved in 20 mL of $CHCl_3$. It was then extracted with three 10-mL portions of 4 N HCl; the acid extract was neutralized (saturated $KHCO_3$ solution) and then extracted with three 10-mL portions of chloroform. This chloroform layer was washed with an equal volume of saturated NaCl and dried (Na_2SO_4) and the solvent removed to give 0.620 g (42%) of the *N,N*-dimethylaminoethanethiol adduct: NMR ($CDCl_3$) δ 2.18 (6 H, s), 2.46 (4 H, s), 3.56 (2 H, s), 5.31 (1 H, s), 5.50 (1 H, s), 7.56 (2 H, d), 8.12 (2 H, d); MS *m/e* 266. This compound was again characterized by conversion to its quaternary ammonium iodide by reaction with iodomethane in ether followed by recrystallization from hot water, mp 182–183.3 °C. The chloroform layer obtained after extracting with acid was washed with saturated $KHCO_3$ and saturated NaCl solutions and dried, and the solvent removed to give 0.83 g (58%) of the *n*-butanethiol mo-

noadduct identified by comparison with starting material by NMR and thin-layer chromatography.

Equilibrium Formation and Trapping of Mixed Diadduct of *N,N*-Dimethylaminoethanethiol and *n*-Butanethiol. Preparation of 1-*n*-Butylthiol-3-[2-(*N,N*-dimethylaminoethyl)thio]-2-*p*-aminophenylpropane (18). To 0.266 g (0.001 mol) of α-[2-(*N,N*-dimethylaminoethyl)thiomethyl]-4-nitrostyrene [**14**, N_1 = $SCH_2CH_2N(CH_3)_2$] were added 2.2 mL (1.80 g, 0.02 mol) of *n*-butanethiol and 2.75 mL (2.02 g, 0.02 mol) of triethylamine. A small portion of this mixture was transferred to an NMR tube and to it a few drops of MeOH-d_4 with 1% Me_4Si were added. To the remaining reaction mixture was added 20 mL of MeOH. Both the NMR tube and the bulk of the reaction mixture were heated at reflux. The progress of the reaction was followed by observing the decrease in the NMR signals due to the vinyl protons of **14** at δ 5.40 and 5.60. After about 1.5 h these signals had completely disappeared. To the reaction mixture 0.522 g (0.003 mol) of $Na_2S_2O_4$ dissolved in 20 mL of EtOH-water solution was added. A few drops of 4 N NH_3 solution were added to obtain a homogeneous solution. The reaction mixture was heated under nitrogen on a steam bath for 2 h and then left to stir at room temperature for a further period of 2 h. The solvents were then removed on a rotary evaporator. To the residue 15 mL of 2 N HCl was added and then extracted with three 10-mL portions of $CHCl_3$. The aqueous acidic solution was neutralized with saturated $KHCO_3$ solution and evaporated to dryness in vacuo, and the residue was extracted with 10 mL of MeOH. The methanol solution was concentrated to a small volume and applied to a preparative TLC silica gel plate. Development with $CHCl_3$-MeOH (4:1) solvent mixture gave 0.100 g (30%) of **18** (N_1 = $SCH_2CH_2NMe_2$; N_2 = SnC_4H_9) as a light yellow viscous oil. Attempts to further purify this compound were unsuccessful. When the above reaction was allowed to proceed for 10 h prior to dithionite reduction, the products isolated by TLC were 3% **18** and 30% **21**. The latter compound was identified by NMR as the diadduct of *n*-butanethiol. The spectral characteristics of **18** [N_1 = $SCH_2CH_2NMe_2$; N_2 = SnC_4H_9] are as follows: NMR (MeOH-d_4) δ 0.90 (3 H, t), 1.50 (4 H, m), 2.32 (6 H, s), 2.46 (2 H, t), 2.58 (4 H, s), 2.86 (5 H, m, br), 6.70 (2 H, d), 7.05 (2 H, d); MS *m/e* 237 (M^+ − S*n*-Bu), 119, 58 (base peak).

Equilibrium Formation and Trapping of Diadduct with *n*-Butanethiol [α,α-Bis(*n*-butylthiomethyl)-*p*-toluidine]. To 0.225 g (1 mmol) of the monoadduct of *n*-BuSH, a solution of 2.2 mL of *n*-BuSH (20 mmol) and 2.5 mL of Et_3N (20 mmol) was added. A small portion of this material was pipetted out into an NMR tube and to it a few drops of MeOH-d_4 (with 1% Me_4Si) were added. To the rest of the reaction mixture 15 mL of MeOH was added. Both the NMR tube and the bulk of the reaction mixture were heated at reflux under nitrogen. The progress of the reaction was followed by observing the decrease in the intensity of the vinyl peaks. When the complete disappearance of the vinyl peaks was noted (8 h), the reaction equilibrium was "frozen" by reducing with 0.52 g (3 mmol) of sodium dithionite in 1:1 EtOH-water and a few drops of ammonia solution. The initially yellow solution rapidly turned milky white. The reaction mixture was heated under nitrogen for a further period of 2 h on a steam bath to ensure complete reduction. The solution was then evaporated to dryness under reduced pressure and the residue extracted with chloroform. Addition of pentane to the chloroform solution caused precipitation of the diadduct. After filtration, the material was recrystallized from chloroform-benzene-pentane mixture yielding 0.150 g (50%) of the diadduct **16** (N_1 = SnC_4H_9): mp 171-173 °C dec; NMR ($CDCl_3$) δ 0.90 (6 H, t), 1.52 (8 H, m), 2.52 (4 H, m), 2.90 (5 H, m), 7.0 (4 H, s, br); MS *m/e* 311 (M^+). Anal. Calcd for $C_{17}H_{29}NS_2$: C, 65.54; H, 9.38; N, 4.50; S, 20.58. Found: C, 65.49; H, 9.25; N, 4.58; S, 20.54.

Reaction of ETAC-I with Amines. Preparation of α-(*N*-Benzylamino)methyl-4-nitrostyrene (14, N_1 = $NHCH_2C_6H_5$). To a solution of 1.74 g (0.005 mol) of ETAC-I in 100 mL of hot water was added 2.75 mL (2.67 g, 0.025 mol) of freshly distilled benzylamine dissolved in 20 mL of methanol. The reaction mixture was heated under nitrogen at 65 °C for 10 h. The solvents were then removed on a rotary evaporator and the product taken up in 25 mL of chloroform. The chloroform solution was extracted with three 10-mL portions of 3 N HCl, the acid layer neutralized with 10% Na_2CO_3 solution, and the separated oil reextracted with three 10-mL portions of chloroform. After washing with saturated sodium chloride solution (3 × 10 mL) and drying over Na_2SO_4, the chloroform was removed on a rotary evaporator to yield 0.96 g (72%) of α-(*N*-benzylamino)methyl-4-nitrostyrene (**14**, N_1 = $NHCH_2C_6H_5$). It was purified by distillation under reduced pressure: bp 135 °C (1.25 mm); NMR ($CDCl_3$) δ 1.56 (1 H, s), 3.63 (2 H, s), 3.92 (2 H, s), 5.43 (1 H, s), 5.56 (1 H, s), 7.26 (2 H, s), 7.54 (2 H, d), 8.10 (2 H, d); IR $\nu_{max}^{CHCl_3}$ (cm^{-1}) 3230, 3000, 1640, 1595, 1510, 1340, 870; MS *m/e* 268 (M^+). Anal. Calcd for $C_{16}H_{16}N_2O_2$: C, 71.60; H, 6.10; N, 10.45. Found: C, 71.64; H, 6.13; N, 10.43.

Exchange Reactions with Amines. (i) To a solution of 0.134 g (0.005 mol) of α-(*N*-benzylamino)methyl-4-nitrostyrene (**14**) (N_1 = $NHCH_2C_6H_5$) in 10 mL of MeOH was added 0.06 mL (0.04 g, 0.5 mmol) of freshly distilled diethylamine. The reaction mixture was refluxed under nitrogen for 20 h. At the end of this period, the solution was concentrated to about 2 mL on a rotary evaporator. Preparative thin-layer chromatography of the reaction mixture on silica gel plate using CH_2Cl_2 with 1% MeOH as developing solvent yielded 38 mg (53%) of α-(*N,N*-diethylaminomethyl)-4-nitrostyrene (**19**, N_2 = NEt_2) and 50 mg (47%) of starting α-(*N*-benzylaminomethyl)-4-nitrostyrene.

The former compound, α-(*N,N*-diethylaminomethyl)-4-nitrostyrene, was purified by distillation under reduced pressure: bp 98 °C (1.25 mm); NMR ($CDCl_3$) δ 0.95 (6 H, t), 2.54 (4 H, q), 3.41 (2 H, s), 5.40 (1 H, s), 5.54 (1 H, s), 7.65 (2 H, d), 8.10 (2 H, d); IR $\nu_{max}^{CHCl_3}$ (cm^{-1}) 3000, 2960, 2810, 1595, 1515, 1340, 870; MS *m/e* 234 (M^+). Anal. Calcd for $C_{13}H_{18}N_2O_2$: C, 66.64; H, 7.74; N, 11.95. Found: C, 66.60; H, 7.69; N, 11.92. The latter compound, α-*N*-benzylaminomethyl)-4-nitrostyrene, was characterized as described in its preparation.

(ii) **Exchange Reaction of Diethylamine with α-(*N,N*-Dimethylaminomethyl)-4-nitrostyrene.** To 0.206 g (0.001 mol) of α-(*N,N*-dimethylaminomethyl)-4-nitrostyrene (**14**, N_1 = NMe_2) was added 2.0 mL (1.46 g, 0.020 mol) of diethylamine. A small portion of the mixture was transferred to an NMR tube and to it a few drops of MeOH-d_4 in 1% Me_4Si were added. To the remainder of the mixture 20 mL of MeOH was added. Both the contents of the sealed NMR tube and the bulk of the reaction mixture were heated at reflux. The progress of the reaction was monitored by observing the change in the signals due to vinyl protons between δ 5.0 and 6.0. In addition to the vinyl proton peaks at δ 5.40 and 5.63 for the starting dimethylamino compound, there was a new vinyl peak which began to appear at δ 5.54 corresponding to the diethylamino compound. Under these conditions, the ratio of peak heights was δ 5.40:5.54:5.64 (10:6:4), from which the ratio of products was determined to be 40:60.

Exchange Reaction of Dimethylamine with α-(*N,N*-Diethylaminomethyl)-4-nitrostyrene. Dimethylamine gas was passed through a tower of KOH pellets and then introduced into a solution of 0.023 g (1×10^{-4} mol) of α-(*N,N*-diethylaminomethyl)-4-nitrostyrene in MeOH-*d* contained in an NMR tube which was cooled in an ice-water bath. The dimethylamine was bubbled in a steady stream for about 20 min. An NMR spectrum of this reaction mixture showed vinyl signals at δ 5.40 and 5.63 corresponding only to dimethylaminomethylnitrostyrene. The NMR tube was then heated at 60 °C for 10 min and the spectra were recorded. Signals were seen at δ 5.40, 5.54, and 5.63 indicating reconversion to the diethylamino compound. The tube was cooled in an ice-water bath and dimethylamine gas bubbled in for 20 min. The NMR spectra again showed vinyl proton signals at δ 5.40 and 5.63 only indicating the complete conversion to the dimethylamino compound.

Preparation of *S*-(2-*p*-Nitrophenyl)allylcysteine. To a solution of 3.48 g (10 mmol) of ETAC-I in 225 mL of hot water was added a 1.58-g sample (10 mmol) of cysteine hydrochloride dissolved in 25 mL of water. The pH of the reaction mixture was adjusted to 8.0 by adding 6 N ammonia solution. The reaction mixture was heated at 50-55 °C for 17 h under nitrogen atmosphere. The solution was then cooled to room temperature and the pH decreased to 6.5 by adding 2 N HCl. The reaction mixture was allowed to stand in an ice bath for 1 h, during which time the desired *S*-(2-*p*-nitrophenyl)allylcysteine precipitated. The precipitate was filtered and washed several times with cold water, ethanol, and acetone. A second crop of product could be obtained upon concentrating the method liquor, adjusting the pH to 6.5, and chilling: yield, 2.1 g (74.5%); mp 195-197 °C dec; NMR (CF_3CO_2H) δ 3.3 (2 H, m), 3.84 (2 H, s), 4.56 (1 H, m), 5.56 (1 H, s), 5.76 (1 H, s), 7.72 (2 H, d), 8.25 (2 H, d); MS *m/e* 282 (parent), 208, 91, 74 (base); IR $\nu_{max}^{KBr\ pellet}$ (cm^{-1}) 3300, 3040, 1610, 1580, 1500, 1400, 1330. Anal. Calcd for $C_{12}H_{14}N_2O_4S$: C, 51.08; H, 4.99; N, 9.94; S, 11.36. Found: C, 51.10; H, 5.00; N, 9.89; S, 11.37.

Preparation of *N*-(2-*p*-Nitrophenyl)allyllysine (14, N_1 = ε-Lysine).

To 0.35 g (1 mmol) of ETAC-I dissolved in 25 mL of hot water, a solution of 0.16 g (1.1 mmol) of lysine monohydrochloride in 10 mL of water was added. The pH of the reaction mixture was adjusted to 10.5 by the addition of aqueous trimethylamine. The mixture was heated at 60 °C for 20 h under nitrogen. The solution was then evaporated to dryness on a rotary evaporator, the residue redissolved in 15 mL of water, and the solution evaporated again. The last traces of trimethylamine were removed on a vacuum pump. The residue was then taken up in 5 mL of water and 2.5 mL of the solution was applied to each of two cellulose preparative thin-layer chromatography plates (20 × 20 cm; 1000 μm). Development of the plates in BuOH-HOAc-H_2O (3:1:1, v/v) solvent mixture followed by extraction with water yielded 0.15 g (30%) of the desired lysine monoadduct. It was recrystallized from a mixture of water and acetone, mp 212 °C dec; NMR (D_2O/DSS) δ 1.80 (6 H, m), 3.05 (2 H, t), 3.80 (1 H, t), 4.25 (2 H, s), 5.83 (1 H, s), 6.0 (1 H, s), 7.78 (2 H, d), 8.30 (2 H, d). Anal. Calcd for $C_{15}H_{21}N_3O_4$: C, 58.62; H, 6.89; N, 13.67; O, 20.82. Found: C, 58.51; H, 6.78; N, 13.65.

Preparation of 1,3-Bis-*S*-cysteinyl-2-*p*-aminophenylpropane (16, N_1 = S-Cys). To 0.348 g (1 mmol) of ETAC-I dissolved in 25 mL of hot water a solution of 1.57 g (10 mmol) of cysteine hydrochloride in 10 mL of water was added. The pH of the solution was adjusted to 8.0 by adding 6 N NH_3 solution. The solution was then cooled to room temperature and to it a mixture of 0.142 g (1 mmol) of Na_2SO_4, 20 mL of 1 N ammonia solution, and 0.522 g (3 mmol) of sodium dithionite was added. The reaction mixture was stirred at room temperature for 0.5 h and then boiled gently for an additional 0.5-h period. The small amount of insoluble material that separated upon cooling was removed by filtration and the filtrate acidified with 5 mL of concentrated HCl. It was then evaporated to dryness on a rotary evaporator. The residue was repeatedly evaporated with water to remove excess hydrochloric acid and finally taken up in the minimum amount of boiling 95% ethanol. The mixture was filtered by gravity and the filtrate treated with 50% solution of pyridine in 95% ethanol until the solution was just basic to wet Congo red paper. After chilling for several hours, light yellow crystals appeared. The crystals were filtered off and dissolved in a minimum amount of hot water, and 10 vol of absolute ethanol was added. Upon chilling, the monohydrochloride of 1,3-bis-*S*-cysteinyl-2-*p*-aminophenylpropane crystallized. The crystals were filtered, washed with cold absolute ethanol, and dried. The yield was 0.10 g (28%): mp 132 °C dec; NMR (D_2O/DSS) δ 7.50 (4 H, s), 4.40 (3 H, m), 3.80 (2 H, t), 3.22 (2 H, m). Anal. Calcd for $C_{15}H_{24}N_3O_4S_2$: C, 43.94; H, 5.90; N, 10.25; S, 15.64. Found: C, 43.88; H, 5.84; N, 10.28; S, 15.59. The compound elutes at 246 min on amino acid analysis columns (as described later).

Kinetic Studies. Kinetic studies of the reaction of thiols and amines with ETAC-II reagent were followed by the measurement of the increase in absorption at 412 nm and the decrease in absorption at 320 nm. Absorption measurements were carried out using a Beckman DU2 spectrophotometer. Solvents, dilutions, etc., are given in the figure captions. The UV spectra of the reactions were recorded with a Cary-14 UV spectrophotometer. ORD spectra of the protein samples were taken in water using a JACSO Model ORD/UV-5 instrument.

The products of the exchange and trapping experiments on ETAC-II followed by UV could be compared with each of the products prepared preparatively from ETAC-I in the previous section using TLC on precoated silicic acid (fluorescent indicator) and cellulose plates [solvents, benzene-methanol-acetic acid (11:2:1) and 1-butanol-acetic acid-water (3:1:1), respectively]. Visualization was accomplished with UV quenching (silicic acid) or ninhydrin (cellulose).

Protein Studies. Reduction of Ribonuclease. (a) By Mercaptoethanol.[25-27] To 2 mL of an 8 M solution of urea (deionized and recrystallized, 1.0 g, 0.016 mol) in water taken in a 15-mL centrifuge tube were added 20 mg (1.46 μmol) of bovine pancreatic ribonuclease (type XII, purchased from Sigma Chemical Co.) and 60 μL (840 μmol) of freshly distilled β-mercaptoethanol. The pH of the solution was adjusted to 8.5 by adding a few drops of aqueous methylamine. Alternatively, the urea, ribonuclease, and mercaptoethanol were dissolved in Tris-HCl buffer of pH 8.5. The reaction mixture was flushed for 15 min with nitrogen and then allowed to stand at 37 °C for 5 h or at room temperature for 24 h under a stream of N_2. The reaction mixture was then cooled to −5 °C in an ice-salt bath and 10 mL of cold solution of 1 N HCl-absolute EtOH (1:39, v/v) was added. On cooling to −5 °C, a white fluffy precipitate of the protein appeared. The precipitate was centrifuged and washed three times with the HCl-EtOH solution and twice with 10 mL of cold ether. All precipitation and washing operations were performed under nitrogen atmosphere. The washed precipitate was dissolved in 1 mL of deionized water and then lyophilized. Estimations of the thiol groups present were carried out using the procedure of Ellman.[17,28] The best results obtained by this method afforded ribonuclease having only six thiol groups. The usual preparation had only four thiols per mol of ribonuclease.

(b) Using Dithioerythritol.[29] To 2 mL of an 8 M solution of urea (UltraPure grade, 1.0 g, 0.016 mol) in deionized quartz-distilled water was added 20 mg (1.46 μmol) of ribonuclease (type XII, Sigma Chemical Co.), 462 mg (0.003 mol) of dithioerythritol, and 4 mg of the disodium salt of EDTA. The pH of the solution was adjusted to 8.51 by adding 10% $MeNH_2$ solution. The reaction mixture was flushed with a rapid stream of nitrogen for 15 min using a syringe needle coated with a plastic sleeve. The reaction was then allowed to stand at 37 °C for 4.5 h under nitrogen atmosphere. The rest of the workup procedure was usually as described for the mercaptoethanol reduction. The protein was alternatively precipitated with a 20% solution of trichloroacetic acid in water. The washing procedure was similar to the one employed for the HCl/EtOH precipitation method. Complete reduction of RNase to produce eight SH groups was obtained by this reduction procedure.

Reaction of ETAC-I with Partially Reduced Ribonuclease. Partially reduced RNase A, which was obtained by reduction of RNase A with mercaptoethanol, was assayed by Ellman's procedure and found to contain four SH groups. A 300-μL sample (20.1 nmol) of a 6.7×10^{-5} M solution of this partially reduced RNase was taken in an ampule and to it were added consecutively 300 μL of ammonia solution of pH 8 and 12 μL (60 nmol) of a 5×10^{-3} M solution of ETAC-I. In another ampule, 300 μL of ammonia solution and 300 μL of the same RNase solution were taken. Both ampules were evacuated briefly using a vacuum pump and then sealed. The sealed ampules were heated at 57 °C for 20 h. At the end of this time, the ampules were broken open and to each 36 μL (180 nmol) of a 5×10^{-3} M solution of sodium dithionite was added. The ampules were heated for 5 min at 57 °C. Their contents were transferred to ignition tubes and lyophilized. Each of the residues was heated with 6 N HCl containing Me_2SO in evacuated sealed tubes for 20 h at 110 °C, and then subjected to amino acid analysis. The results of the analysis are given in Table II. In this analysis the cysteine disulfide bonds were oxidized to cysteic acid during hydrolysis. A loss of two lysine and four cysteic acid residues was apparent.

Reduction of Reduced Ribonuclease (8 SH) with ETAC-II. A 15.0-mg sample of ribonuclease A (1.12 μmol) reduced by dithioerythritol and estimated to contain 8 SH residues per mol of enzyme was dissolved in 2.5 mL of 0.1 M phosphate buffer of pH 8 containing 1.62 mg (4.5 mol) of ETAC-II. The reaction mixture was stirred under nitrogen at room temperature. Aliquots of 50 μL were diluted to 3.0 mL for UV measurements. The reaction was attended with progressive increase in the absorbance at 412 nm and decrease in that at 320 nm. Completion of the cross-linking reaction was observed in about 8 h. Reductive fixing was carried out with 2.23 mg (13.5 μmol) of $Na_2S_2O_4$ for 5 min. At the end of the reduction most of the protein had precipitated. The protein was centrifuged, the supernatant discarded, and the residue washed twice with 5-mL portions of distilled water. A small portion of the protein was dissolved in glacial HOAc and water added and "diazo" color reaction carried out as described later. A 0.1-mg portion of the cross-linked protein was incubated with Tris-acetate-sodium dodecyl sulfate ($NaDodSO_4$) solution and subjected to gel electrophoresis as outlined elsewhere in this work.

Reaction of ETAC-II with Ribonuclease (Phosphate Buffer). A 12.5-mg sample of ETAC-II reagent was dissolved in 25 mL of 0.1 M Na_2HPO_4 solution. A 60-mg sample (4.38 μmol) of RNase A (bovine pancreatic, type XII-A, purchased from Sigma Chemical Co.) was dissolved in 6.3 mL of the above solution (3.2 mg of ETAC-II, 8.76 μmol), and the pH was adjusted to 10.5 using 0.1 M NaOH. The final volume of the solution was 10.0 mL. The reaction was stirred under nitrogen at 37 °C. The progress of the reaction was again monitored by the appearance of the peak at 412 nm due to 5-mercapto-2-nitrobenzoate, and the disappearance of the *p*-nitrostyryl peak at 320 nm (Figure 2). When the reaction was complete (36 h), it was quenched with 4.6 mg (26.28 mmol) of sodium dithionite for 5 min under N_2 at 40 °C. The reaction mixture was then desalted by passage through a 0.8 × 24 cm column of Sephadex G-25 and lyophilization of the void volume fractions 1 and 2.

Reaction of ETAC-II with Ribonuclease (Tris Buffer). To a solution of 0.64 mg of ETAC-II (1.75 mmol) dissolved in 1 mL of 0.1 M Tris base was added 12 mg of RNase A (0.876 mmol), and the pH was adjusted to 10.5 by adding 0.1 M NaOH solution. The total volume was adjusted to 2.0 mL. The reaction was heated at 37 °C. The progress of the reaction was followed taking the UV of the solution. Aliquots of 50 μL were withdrawn at appropriate time intervals and diluted to 3.0 mL for UV measurement. The reaction was complete within 2 h as evidenced by complete disappearance of the 320-nm peak. The reaction was then quenched (1.0 mg of $Na_2S_2O_4$), desalted, lyophilized, and subjected to $NaDodSO_4$ gel electrophoresis in the usual way. The most prominent band was due to trimeric species (a double band) with only a faint band for dimer. Only a trace of monomer band was present.

$NaDodSO_4$-polyacrylamide gel electrophoresis was carried out using essentially the procedure of Weber and Osborn.[30] A 0.1-0.3-mg sample of protein mixture (previously desalted and lyophilized) was dissolved in 100 μL of 0.04 M Tris-acetate buffer (pH 6.4) containing 0.04 M dithioerythritol, 0.001 M EDTA, and 1% $NaDodSO_4$ and was incubated for 0.5 h at 65 °C under nitrogen. Then 20 μL of Biophore marker dye solution (Bio-Rad, containing 0.5% bromophenol blue and 30% sucrose solution in fivefold dilution) was added. A 30-μL sample of this mixture was applied to each gel tube (Biophore, 12%, $NaDodSO_4$ buffer introduced).

Electrophoresis was run at constant current employing 5-6 mA per tube and generally required 5-7 h. The protein bands were visualized by staining the gels with 0.025% Coomassie brilliant blue dye for 7-12 h prior to destaining by diffusion in a solution consisting of 75 mL of HOAc-50 mL of MeOH-875 mL of water. A separate fixing step in 2-propanol-HOAc-water (4:1:5) solution prior to destaining gave sharper protein bands, particularly when low concentrations of proteins were used. The gel length before and after staining as well as the distance of bromophenol blue migration were measured to allow calculation of the relative protein mobilities. The plot of mobility vs. log of molecular weight gave a straight line. Densitometer tracings of the gels were performed at 550 nm in 7.5% acetic acid solution on a Beckmann DU spectrophotometer equipped with an automatic gel scanner attachment. The gels were stored at room temperature in 7.5% acetic acid solution in the dark. The gel pattern for cross-linked native RNase (Figure 5) shows the formation of monomer, dimer, trimer, and a trace of tetramer. For cross-linked reduced RNase, the gel pattern (Figure 7) also shows tetramer, pentamer, and higher molecular weight aggregates as well.

Gel Permeation Chromatography of Cross-Linked Ribonuclease. A 60-mg ribonuclease sample which had been cross-linked with 2 mol of ETAC-III in phosphate buffer, reduced, desalted on Sephadex G-25, and then lyophilized was dissolved in 6.0 mL of deionized water. A 3.0-mL sample of this solution was applied to a 2.5 × 90 cm column of Sephadex G-75 (Pharmacia Fine Chemicals), preequilibrated with 0.5 M $(NH_4)_2CO_3$. The column was eluted with 0.05 M $(NH_4)_2CO_3$ at a flow rate of 13.8 mL/h. Fractions of 4.6 mL each were collected. The eluant was monitored by UV at 280 nm. The elution curve is shown in Figure 6. The fractions corresponding to the maximum of each peak of the elution curve were combined and lyophilized. Small portions of these were subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis on Bio-Rad 12% gels to determine their molecular weight. Table I shows the mobilities of a portion of the various fractions and the automated amino acid analysis of a portion of each fraction.

"Diazo Color" Test of Aminophenylpropane-Linked Proteins and Peptides. To 0.5 mL of a solution of sodium nitrite (69.0 mg dissolved in 4.0 mL of water) was added 0.5 mL of ice-cold 2 N HCl solution. This solution was cooled to −5 °C and to it was added 0.4 mg of the desalted protein or peptide. After 10 min, 0.5 mL of a solution of β-naphthol (72 mg in 5 mL of 2 N NaOH) was added. The resulting solution was left to stand at 0 °C. Within 2 h a rose-red color developed which had a UV maxima at 500 nm. Proteins not having a cross-linking residue gave no color.

Amino acid analyses were performed on a modified Beckman Model B automatic amino acid analyzer on a single column using Durrum DC-1A cation exchange resin with Pico (Pierce) system II buffers. Samples to be analyzed were hydrolyzed with 6.2 N HCl in evacuated (50 μm) sealed tubes at 110 °C for 20-40 h. At least two samples of each analysis were run for each protein or peptide. The results were averaged.

Reduction and carboxymethylation of RNase and its derivatives were carried out in the following manner. A 20-mg sample of protein (either bis-cross-linked or native) was dissolved in 2.0 mL of a solution which was 8 M in guanidine·HCl and 3 mM in EDTA. A 5.4-mg (35 μmol) sample of dithioerythritol was dissolved in this solution. The pH was adjusted to 8.5 by adding 40% aqueous methylamine. Nitrogen was bubbled through the reaction mixture for 15 min, and the reduction was allowed to proceed in the dark for 20 h at room temperature. At the end of this period, the pH was decreased to 7.6 (pH meter) by adding 0.1 M HCl. A 0.46-mL sample of a 0.2 M solution of iodoacetamide (17.3 mg) was then added and alkylation allowed to proceed in the dark for 2 h at room temperature. The excess reagent was removed by gel filtration on Sephadex G-25, 0.9 × 25 cm column, in the dark. Elution of the column with deionized water was followed by monitoring by UV at 280 nm. The protein was found in the first two void volume fractions. The protein solution was then lyophilized.

Tryptic Digestion. Each reduced and carboxymethylated protein was digested with Tos-PheCH$_2$Cl-treated trypsin (2% by weight of protein) in 0.1 M $(NH_4)_2CO_3$ buffer adjusted to pH 8.5 with $(NH_4)_2CO_3$ or NH_4OH. Digestion proceeded for 18 h at 37 °C. Two more additions of trypsin were made during this time.

Peptide Mapping. Peptide mapping was performed essentially by the method described by Katz et al.[31] About 0.5 mg of the salt-free tryptic digest of the proteins (dissolved in ~60 μL of buffer) was applied to a 58 × 69 cm^2 sheet of Whatman No. 3MM chromatography paper. Descending chromatography was run for 20 h in the upper layer obtained by partitioning *n*-BuOH-acetic acid-water (4:1:5). After chromatography, the chromatogram was dried at 30 °C for 24 h in a drying oven. High voltage electrophoresis was then carried out in the orthogonal direction (in Savant high-voltage electrophoresis tank) at pH 6.5 (pyridine-acetic acid-water, 100:4:900) for 90 min at 2000 V (current not allowed to exceed 200 mA). After drying the paper at 50 °C for at least 60 min, the spots corresponding to the tryptic peptides were visualized with fluorescamine and then in some cases with ninhydrin-cadmium acetate reagent.

Elution of Peptides from Paper. The peptides were eluted from maps visualized using fluorescamine, first with the buffer used for chromatography and then with 10% HOAc. A trough was set up inside an air-tight chamber with the eluant and a tab from the peptide spot cut out for elution. The eluant descended through the paper into a tube below. After lyophilization, the peptides were hydrolyzed and amino acid composition was determined.

Preparation of Cross-Linked Lysine. To 10 mg of poly-(L-lysine) HBr (Sigma Chemical Co., type II, mol wt 1000-4000) dissolved in 10 mL of 0.1 M Na_2HPO_4 was added a 2.7-mg (7.5 × 10^{-3} mmol) sample of ETAC-II. The pH of the resulting solution was adjusted to 10.5 by adding 0.1 M NaOH. The reaction mixture was heated at 38 °C under nitrogen and the progress of the reaction followed by UV spectroscopy. A 50-μL sample of the reaction mixture was diluted to 3.0 mL with 0.1 N phosphate buffer of pH 8 for UV measurements. The bis addition to ETAC-II was complete after 22 h, as evidenced by the disappearance of the 320-nm peak. At the end of this time, some polymer had precipitated from the reaction mixture. The total reaction mixture was treated with 30 mg of sodium dithionite in 1 mL of H_2O. Solid precipitated. This was removed by centrifugation and washed with three 10-mL portions of 0.5 M NaOH and then with three 10-mL portions of deionized water. The material was then hydrolyzed by 6.2 N HCl for 24 h in a sealed tube (evacuated to 50 M) and then subjected to automated amino acid analysis. The chromatogram showed two major peaks in addition to that due to lysine. One of these, C, was coincident with the peaks obtained from the hydrolysates of both bis-cross-linked RNase A and the linked tryptic fragments appearing at 176 min between phenylalanine (173 min) and lysine (182 min). The second peak at 157 min from the polylysine remains unidentified. Upon 36-h hydrolysis, this second peak was absent from the amino acid analysis and thus is presumed to be a partially hydrolyzed fragment of cross-linked polylysine.

Determination of Enzymatic Activity.[32] Cyclic cytidine 2′,3′-phosphate was purchased from Sigma Chemical Co. as the sodium salt. Tris buffer solution of constant ionic strength was prepared by dissolving 0.629 g of NaCl in 50 mL of 0.1 M Tris base solution and 44.2 mL of 0.1 M HCl and finally diluted to 100 mL with deionized water. The pH of the solution was determined by pH meter to be 7.32 at 25 °C. A 3.30-mg sample of the sodium salt of cyclic CMP was dissolved in 25 mL of the above buffer. Solutions of the enzymes to be assayed were also prepared in the same buffer solution. The concentrations of enzyme solution were 3.28 mg/mL for native RNase

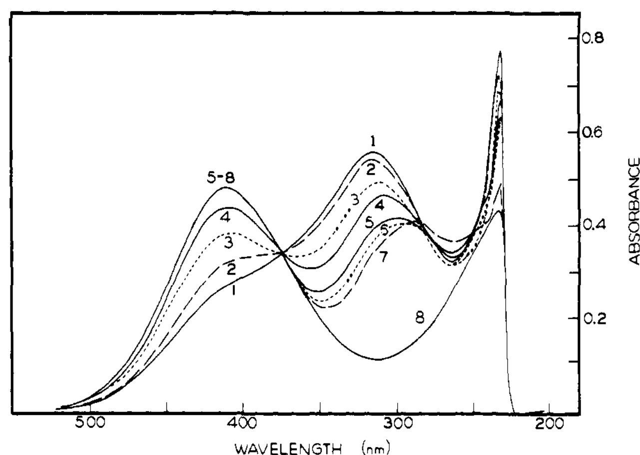


Figure 1. Consecutive UV spectra of the reaction of 2 mmol of cysteine with 0.1 mmol of ETAC-II in 20 mL of 0.1 M phosphate buffer of pH 8.0 at 25 °C. 40-μL samples were withdrawn at appropriate time intervals and diluted with 3.8 mL for spectral measurements: (1) 2.83 h after start of reaction; (2) after 4.08 h; (3) after 5.92 h; (4) after 9.42 h; (5) after 14.33 h; (6) after 16.67 h; (7) after 18.33 h; (8) after 58.33 h.

and 6.00 mg/mL for cross-linked RNase.

The hydrolysis reaction was carried out in stoppered quartz cells in a thermostatically controlled cell holder maintained at 25 ± 0.2 °C. To each of three quartz cells 3.0 mL of the substrate solution was added. The cells were placed in the cell compartment of a Beckman DU instrument and allowed to equilibrate for 10 min. A 10-μL portion of the RNase solution was added to cell no. 2 and an equal volume of the cross-linked RNase solution was added to cell no. 3. The contents of the cells were mixed rapidly by inversion and the increase in absorbance ΔA_t at 286 nm was measured with time t against the blank solution containing substrate alone. The slit width of the instrument was maintained at 0.51 throughout the experiment. The plots of log $(\Delta A_\infty - \Delta A_t)$ vs. time were straight lines: ribonuclease, slope = 0.33, and cross-linked ribonuclease, slope = ~0.001.

The ratio of activities for native RNase A and cross-linked RNase A is equal to the ratio of the slopes of the respective plots[32] from which the activity of cross-linked RNase was found to be 3.3% of that of native RNase A.

Dansyl End Group Determination. Dansyl end group determination was accomplished using the procedure of Weiner, Platt, and Weber.[33] Identification of dansyllysine and didansyllysine was accomplished using polyamide plates and the described solvent systems and, in addition, using electrophoresis on Eastman cellulose plastic-backed thin-layer plates, pH 1.9 acetic acid-formic acid buffer in a Desaga-Brinkman double chamber at 400 V.

Results

Results of model reaction studies of reagents **11** and **12** with various organic and bioorganic substrates confirmed the proposed scheme of equilibrium alkylation transfer. For example, reaction of **12** with excess cysteine in pH 8 phosphate buffer was followed by the release of mercaptonitrobenzoate (Ellman's reagent) and loss of the *p*-nitrostyrene chromophore (Figure 1, see Scheme III, N_1 = S-Cys). With either **11** or **12**, when the concentration of cysteine was limited, the monoadduct **14** (N_1 = S-Cys) could be isolated. Under these same conditions, with larger amounts of addend cysteine present (tenfold excess), both the vinyl protons (NMR) and the nitrostyrene chromophore (UV) completely disappear; the NMR of the solution implied the formation of a structure such as **15**. Still, only monoadduct **14** could be isolated on workup, suggesting the equilibrium relationship of **14** and **15**. However, treatment of such reaction mixtures with sodium dithionite trapped **15** by its reduction to the amine **16** (N_1 = S-Cys), which then allowed isolation. Additional examples of the exchange [**14**, N_1 = SBu, → **19**, $N_2 = SCH_2CH_2N(CH_3)_2$; **14**, N_1 = N-HBz → **19**, $N_2 = N(CH_3)_2$] and formation of adducts from *n*-butylmercaptan, dimethylaminoethylmercaptan,

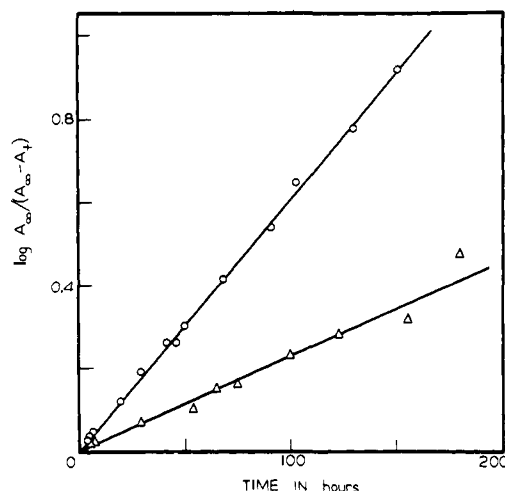


Figure 2. Kinetic study of (Δ) the reaction of lysine (1.0 mmol) with ETAC-II (0.1 mmol) (molar ratio 10:1) in 0.1 M phosphate buffer (5 mL) of pH 10.5 at 39 °C, and of (O) the reaction of *N*-acetylcysteine (1 mmol) with ETAC-II (0.1 mmol) (molar ratio 10:1) in 0.1 M phosphate buffer (20 mL) of pH 10.5 at 25 °C, measured by following the change of absorbance at 412 nm indicating the release of mercaptonitrobenzoate anion reduced Ellman's thiol.[17] In each case, 50 μL of reaction was diluted to 3 mL.

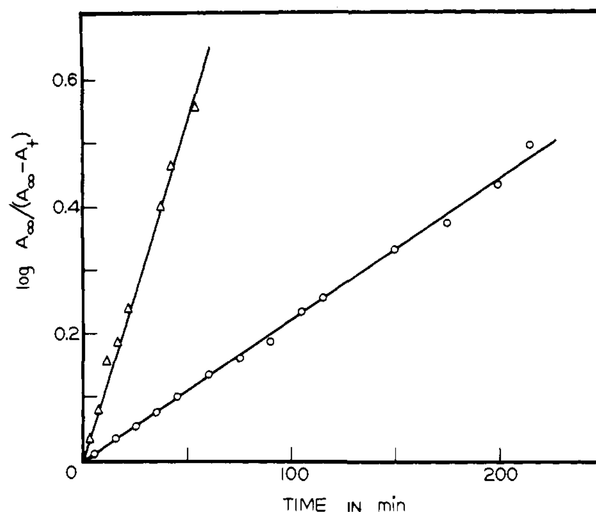


Figure 3. Kinetic study of the reaction of cysteine (1.0 mmol) with ETAC-II (0.1 mmol) (molar ratio 10:1) (O) in 20 mL of 0.1 M Tris buffer of pH 8.0 at 25 °C, and (Δ) in 20 mL of 50:50 v/v Me_2SO and 0.1 M Tris buffer of pH 8.0 at 25 °C measured by following the change in absorbance at 412 nm indicating the release of reduced Ellman's thiol. In each case, 40 μL of reaction was diluted to 3.8 mL.

dimethylamine, and benzylamine, as well as cysteine and lysine, are described in the Experimental Section. Representative rate plots of the release of mercaptonitrobenzoate anion from ETAC-II with model compounds are given in Figures 2 and 3. Since a large excess of addend is present, the plots show pseudo-first-order behavior (**13** → **14** + mercaptonitrobenzoate). In the absence of excess addend, the kinetic plots show a reflection of the superimposed second-order first step (**12** + N_1 → **13**). These same complex rate effects are observed in protein reactions. We observe significant differences in rate between addition of thiols and amines (Figures 2 and 3). Ammonia and primary amines enhance the rate of addition of thiols to ETAC II. A rather nice demonstration of the equilibrium nature of the reaction was observed using α-(diethylaminomethyl)-4-nitrostyrene **14** ($N_1 = NEt_2$). Saturating a methanol solution of this material with dimethylamine gave the α-(dimethylaminomethyl)-4-nitrostyrene **19** [N_2 =

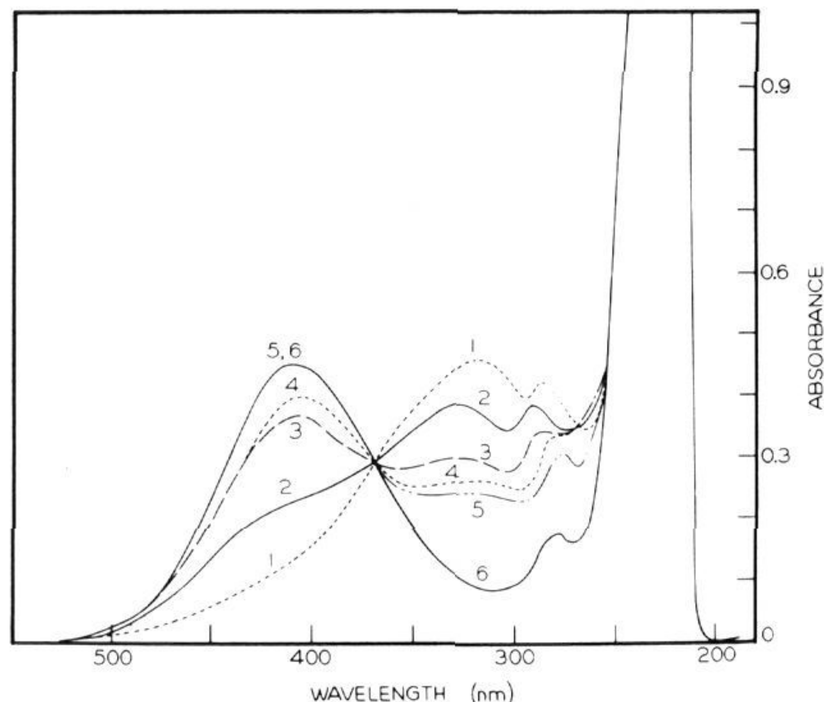


Figure 4. Consecutive UV spectra of the reaction of 8.76 μmol of ETAC-II with 4.83 μmol of RNase in 0.1 M phosphate buffer of pH 10.5 at 42 °C. 50-μL samples were withdrawn at appropriate time intervals and diluted to 3.0 mL for UV measurements: (1) 1.0 h after start of reaction; (2) after 1.5 h; (3) after 2.0 h; (4) after 2.25 h; (5) after 2.5 h; (6) after 36.0 h.

$N(CH_3)_2$] and diethylamine. Warming the solution so that dimethylamine evaporated reversed the equilibrium to re-form the original compounds (**14** ⇄ **17** ⇄ **19**). The sequence could be monitored by NMR over numerous repeated exchanges.

In the case of thiol addition, we were able to capture and isolate the intermediate mixed adduct of dimethylaminoethanethiol and butylmercaptan [**18**, N_1 = $SCH_2CH_2N(CH_3)_2$, N_2 = S-n-C_4H_9] using dithionite to quench the reaction at just the point when all the vinyl protons had disappeared. Longer reaction times gave mostly the symmetrical adducts (**21**, N_2 = S-n-C_4H_9). However, all attempts to prepare bisamine adducts by similarly trapping the intermediate species were unsuccessful with intermolecular reactions, presumably because the high pH necessary to have unprotonated amino groups allows facile reverse Michael reactions. The bislysine adduct **16** (N_1 = ϵ-Lys) was obtained by cross-linking of polylysine with ETAC-II, followed by dithionite reduction and acid hydrolysis. Because of the observation that the equilibrium constant for the double addition to the reagent was relatively unfavorable in the model cases, we anticipated the best assessment of the cross-linking and transfer character of our reagents would be with an actual protein[3] where the second addition would be intramolecular. Ribonuclease was chosen because of the extensive cross-linking experience with this enzyme,[1,5] its primary structure,[23] and its well-established X-ray structure,[22] and also because the molecule had a significant tendency to aggregate[33,34] allowing the evaluation of intermolecular reactions. Also, the presence of ten lysines, the absence of cysteine, and yet the presence of four reducible cystine disulfide bonds promised to provide a number of alternative sites for attachment of the reagent.

Incubation of ETAC reagent **12** (8.76 μmol) with ribonuclease (4.38 μmol, 43.8 μmol of lysine residue) in 0.1 M phosphate buffer at pH 10.5 (37 °C) yielded the release of 2 equiv of reduced Ellman's reagent (increase at 412 nm) and the corresponding decrease of absorption at 320 nm (see Figure 4), indicating the establishment of an average of two cross-links per monomeric ribonuclease unit. No reaction was observed when the same experiment was carried out at pH 8. Reductive fixing of the cross-link (**3**) was accomplished by addition of sodium dithionite (26 μmol, 5 min). Desalting and removal of low molecular weight organic materials were accomplished on Sephadex G-25. Lyophilization was followed by analytical $NaDodSO_4$ gel electrophoresis carried out at pH 6.1 (Figure 5) using essentially the procedure of Weber and Osborn.[30]

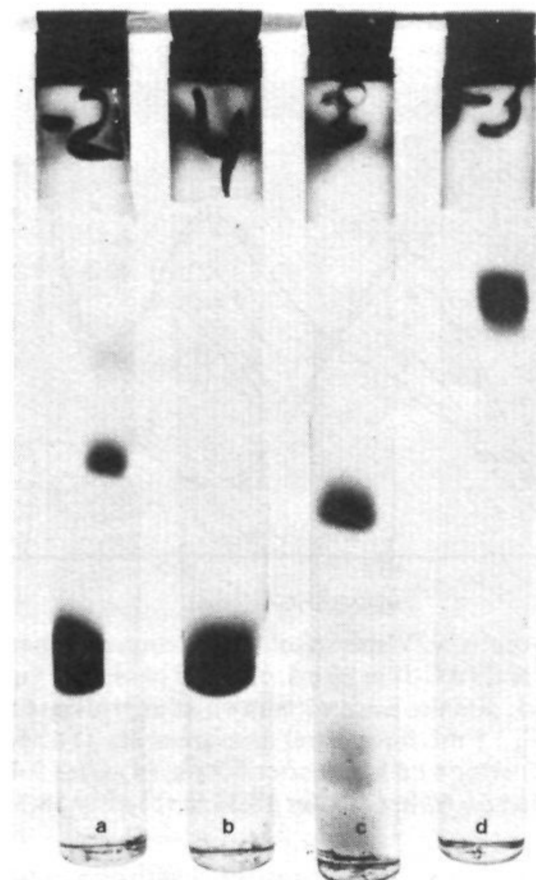


Figure 5. $NaDodSO_4$-polyacrylamide gel electrophorograms on Biophore 12% gels; the protein bands are stained with Coomassie brilliant blue. From left to right: (a) RNase A treated with ETAC-II (molar ratio 1:2) in 0.1 M phosphate buffer of pH 10.5 at 37 °C until complete disappearance of UV absorption at 320 nm was observed (48 h) and then treated with 6 molar equiv of sodium dithionite followed by desalting on Sephadex G-25 (see Table III for mobilities). (b) Native RNase A treated with sodium dithionite (molar ratio 1:6) and then dialyzed prior to application on gel. (c) Trypsin, untreated standard. (d) Ovalbumin, untreated standard. Proteins were applied on the gels as described by Weber and Osborn.[30]

Coomassie blue stained gels showed (Figure 5) bands corresponding to monomer (69%), dimer (21%), trimer (10%), and tetramer (trace). Since the ratio of the reagent to protein was 2:1, 2 equiv of Ellman's thiol mercaptonitrobenzoate anion had been released, and there was also the eventual loss of both equivalents of the attached nitrostyrene chromophore, we concluded that the monomer fraction must contain a significant number of intramolecular cross-links. For similar reasons, dimer and trimer fractions would likely have intra- as well as the necessary intermolecular links, though multiple intermolecular cross-links were also possible. $NaDodSO_4$ gel electrophoresis of ETAC treated ribonuclease (as determined by 412- and 320-nm absorptions), which had not been subjected to the reductive fixing step using dithionite, gave diffuse and broad bands suggestive of a continued equilibrium between cross-linked monomer and aggregate forms during the $NaDodSO_4$ gel separation process. Incubation over 24 h in the Tris (pH 6.4)-dithioerythritol buffer prior to electrophoresis of this material gave a single band moving at the ribonuclease position.

Preparative Sephadex chromatography (G-75, 0.05 M NH_4HCO_3) of the "fixed" mixture of monomer and aggregates allowed the separation and isolation of two monomeric species in the ratio of 6:1, as well as the dimeric and trimer conjugates (Figure 6). The molecular weights of each component from the chromatography were verified by $NaDodSO_4$ gel electrophoresis and corresponded to individual bands of the mixture (Table I). The faster moving monomeric component (peak 3) from Sephadex chromatography had two cross-links as determined by a loss of four lysine residues on amino acid analysis as compared to non-cross-linked ribonuclease (Table I). The slower moving monomeric component (peak 4) from Sephadex chromatography had three cross-links as determined by loss of six lysines by amino acid difference analysis (see

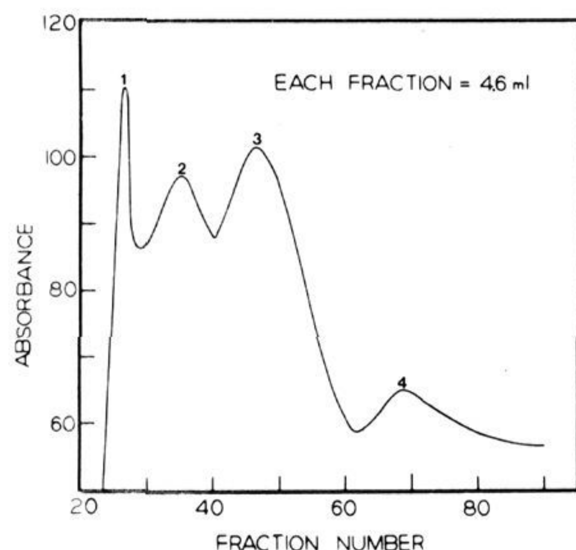


Figure 6. Elution curve of gel permeation chromatography of the reaction mixture of RNase A treated with 2 molar equiv of ETAC-II followed by reduction with 6 molar equiv of sodium dithionite. The eluate was monitored continuously by UV at 280 nm. See text for experimental details.

Table I. Amino Acid Analyses of GPC Fractions of Figure 6[a]

amino acid	peak 2, fraction 30–42, dimer[b]	peak 3, fraction 45–60, monomer[c]	peak 4, fraction 60–80, monomer[d]	RNase standard[c]	theor value
Lys	3.60	6.17	3.91	10.15	10
His	2.46	2.69	2.56	3.24	4
Arg	3.72	3.55	3.73	3.50	4
Asp	14.60	14.27	14.63	14.36	15
Thr	9.24	9.21	9.20	8.80	10
Ser	12.14	12.04	12.26	12.26	15
Glu	11.87	12.00	11.80	12.28	12
Pro	4.00	3.36	3.70	3.68	4
Gly	3.27	3.53	3.31	3.69	3
Ala	11.88	12.02	12.07	11.85	12
½-Cys	7.38	7.35	7.20	7.34	8
Val	8.10	8.40	8.38	8.51	9
Met	3.36	2.72	3.01	2.11	4
Ile	1.86	2.06	2.03	2.40	3
Leu	2.20	2.02	2.20	2.20	2
Tyr	1.28	1.89	1.14	4.25	6
Phe	7.64[e]	4.68[e]	6.79[e]	3.23	3

[a] The analysis of the trimer ($NaDodSO_4$ mobility 0.31 cm) could not be repeated owing to the small quantities of this material isolated. [b] Per RNase unit; $NaDodSO_4$ mobility 0.52 cm. [c] $NaDodSO_4$ mobility 0.71 cm. [d] $NaDodSO_4$ mobility 0.73 cm. [e] These values for phenylalanine contain the contribution of an overlapping cross-linked lysine peak and are, therefore, higher than the theoretical value.

Table I). The trimeric (peak 1) and dimeric (peak 2) cross-linked ribonuclease derivatives each appeared to have both intra- as well as intermolecular cross-links from their preliminary amino acid analyses, though the amount of trimer was too small to obtain multiple analyses. The protein reagent stoichiometry inferred from spectroscopy and amino acid analyses compared well. In each of the automated amino acid analyses of the cross-linked derivatives, a peak corresponding to the standard cross-linked lysine residues appeared. The position was at 175–178 min, between (but sometimes overlapping) the phenylalanine peak (173 min) and the lysine peak (182 min). The standard bis-cross-linked lysine (**16**, N_1 = ε-Lys) was that synthesized by reaction of ETAC-II (**12**) with polylysine, monitoring of the reaction by UV, reduction by sodium dithionite after addition and cross-linking were complete, and finally acid hydrolysis. The linked cysteines (**16**, N_1 = Cys) elute at 246 min on these same columns.

Partially reduced ribonuclease (mercaptoethanol reduction, four SH groups by Ellman's procedure[17]) incubated with ETAC-I (3 equiv) at pH 8 afforded ribonuclease derivatives having an average of two cysteine–cysteine cross-links and one

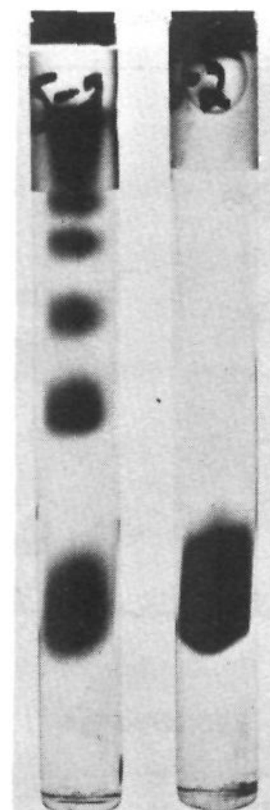


Figure 7. $NaDodSO_4$–polyacrylamide gel electrophorograms on "Biophore" 12% gels; the protein bands are stained with Coomassie brilliant blue. From left to right: (a) RNase A reduced with DTE to give 8 thiol groups and then treated with ETAC-II (molar ratio 1:4) in 0.1 M phosphate buffer of pH 8.0 followed by treatment with 12 molar equiv of sodium dithionite and desalting on Sephadex G-25. (b) Native RNase A treated with sodium dithionite (molar ratio 1:12) and then dialyzed prior to application on gel.

Table II. Amino Acid Analysis for Reaction of Partially Reduced (4SH) RNase with 3 Equiv of ETAC-I; Hydrolysis with Me_2SO

amino acid	no. of residues: cross-linked RNase	control RNase	theor for RNase
Lys	8.11	10.02	10
His[a]			4
Arg	3.66	3.50	4
Cys	4.16	7.89	8
Asp	14.69	14.39	15
Thr	9.00	9.00	10
Ser	12.72	12.89	15
Glu	12.20	11.57	12
Pro[b]			4
Gly	3.04	3.65	3
Ala	11.80	11.89	12
Val	8.92	8.76	9
Met[a]			3
Ile	2.84	3.02	3
Leu	1.22	2.44	2
Tyr[a]			6
Phe	2.38	2.33	3

[a] Residues destroyed by oxidative hydrolysis. [b] Equipment failure prevented quantitation.

lysine–lysine cross-link as determined by amino acid difference analysis compared to native reduced ribonuclease (Table II). Similarly, fully reduced ribonuclease[29] (dithioerythritol reduction, 8 SH groups by Ellman's procedure) reacted rapidly (total appearance of 412 nm, total loss 320-nm peaks within 30 min) with ETAC-II at pH 8. $NaDodSO_4$ gel electrophoresis of the dithionite reduced product of these conditions involving unfolded protein showed (Figure 7) significantly more intermolecular cross-linking.

When the reaction of ETAC-II (2 equiv) was carried out in Tris buffer at pH 10 in concentrations identical with those in phosphate buffer, the additions and cross-linking reactions were now complete within 2 h. After reduction with dithionite, desalting on Sephadex G-25, and lyophilization, the protein

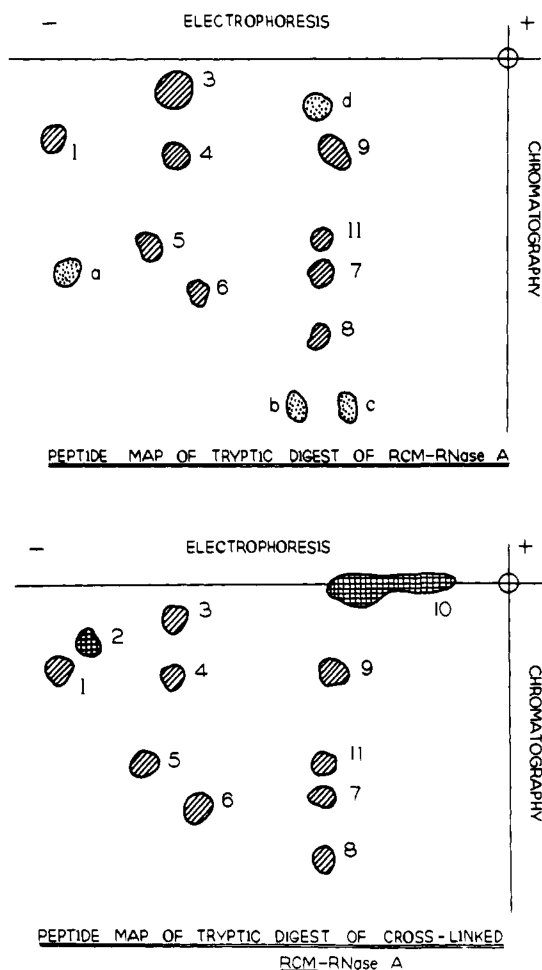


Figure 8. Peptide maps of RCM-RNase A and bis-cross-linked RCM-RNase A cross-linked at the lysine residues. The spots were detected with fluorescamine: diagonal lines, tryptic peptides common to both native RNase and bis-cross-linked RNase; dots, tryptic peptides obtained for RNase only; crossed lines, tryptic peptides obtained for bis-cross-linked RNase only. See details of mapping procedure in text.

was subjected to $NaDodSO_4$ gel electrophoresis. In contrast to the phosphate buffer conditions, only trace amounts of monomer and dimer bands were apparent. Almost all the protein was found in two closely associated bands corresponding approximately in molecular weight to trimer (mobilities 0.30 and 0.36). When this same sequence was attempted in borate buffer again at pH 10.5, there was no observable reaction as determined by production of the 412-nm absorption or loss of the 320-nm absorption.

Identification of the positions of the links by tryptic digest mapping has at this point only been carried out on the monomeric bis-cross-linked ribonuclease (peak 3 of the Sephadex G-75 gel chromatography). Trypsin digestion (Tos-$PheCH_2Cl$ treated)[35,36] of non-cross-linked, reduced, carboxymethylated ribonuclease (RCM RNase) followed by chromatography and orthogonal electrophoresis yielded 13 tryptic fragments.[37,38] Nine fragments coincident with the map of RCM RNase plus two new spots were observed by treating the bis-cross-linked derivative under the same conditions (see Figure 8). The map patterns indicated that the intramolecular cross-linking was not random and that a discrete bis-cross-linked derivative had been obtained. Interestingly, the number of fragments produced not only indicates that the cross-links are discrete, but that the introduced lysine–lysine cross-links do not perturb the tryptic digestion. A total of five maps of each material were run. One of each group was visualized with ninhydrin after fluorescamine, four maps using fluorescamine. Each peptide fragment was eluted from those maps visualized using fluorescamine alone. Acid hydrolysis of each of the eluted spots followed by automated amino acid analysis allowed the identification of every tryptic fragment but one (see Table III and Figure 9). These fragment spots of the tryptic digest of both ribonuclease and cross-linked ribonuclease account for all the 124 amino acids of each protein. Fragment spot 7 was not present in sufficient amounts to give any amino acid analysis data, but since this material appeared in both maps from treated and untreated ribonuclease, we presume this corresponds to a small amount of a partially digested, larger fragment. It cannot be involved in the segments involved in cross-linking since it appears in both maps. Residues 38–39 and 92–98 appear as the single spot 6. These two fragments are not separated from one another on ion exchange columns either.[36]

The fragments from each of the maps could easily be related to segments of the known structure and to segments observed in previous tryptic digestions,[37] even though there was some contamination of all the peptide map fragments by glycine. Spots a, b, c, and d are missing. These correspond, respectively, to residues 11–31, 1–7, 34–37, and 40–61 (Figures 8 and 9). The map of cross-linked ribonuclease contains a new spot, 2, corresponding in amino acid analysis (Table III) to the composition of spot b plus c with the substitution of an ETAC $(\epsilon\text{-Lys})_2$ group for two of the lysine residues. This means that fragments 1–7 and 34–37 must be interlinked by the reagent. Since fragments 11–31 and 40–61 are the only fragments also missing from the map, amino acid analysis indicates a second cross-link, and all other fragments are accounted for, we infer that 11–31 plus 40–61 represent the remaining link. This large cross-linked peptide presumably occurs along with other large, incompletely digested peptides and proteins in spot 10.

Dansyl chloride treatment of a sample of bis-cross-linked ribonuclease followed by acid hydrolysis allowed the identification of bis(dansyl)lysine (α,ϵ) as well as ϵ-dansyllysine. These were identified by thin-layer electrophoresis and thin-layer chromatography. The bis(dansyl)lysine can arise only from the N-terminal position of ribonuclease. This result indicates residues 1–7 and 34–37 are linked through Lys-7 and -37 rather than the alternative 1–37 link.

The unseparated cross-linked and fixed ribonuclease preparation had only small amounts of enzymatic activity (about 3%) remaining when assayed against the substrate cyclic cytidine 2′,3′-phosphate.[32] It is unlikely that any of the individual cross-linked proteins have significant activity.

Discussion

The concept that interaction of a protein molecule with any reagent probe is likely to be accompanied by a change in conformation, "however slight", is well established.[3] With respect to chemical modification, the precise conformational folding of a protein into its native structure has been beautifully summarized and discussed by Cohen.[3] All of the factors involved are determined primarily by the structures of the pendent amino acid functionalities, and any modification in their structure will lead to an alteration of the native conformation. Any interpretation of the proximity of residues by the use of cross-linking reagents therefore must allow for these considerations.[1] Thus, the usefulness of bifunctional reagents for the identification of spatial neighbors and estimating interresidue distances may be justifiably criticized by the argument that two nucleophilic residues on a protein with sufficient flexibility could become cross-linked (kinetically), even though the conformation having these groups in proximity was only a very small contributor to the conformational equilibrium.[3] Further, a particular protein conformation with two residues proximate might not make any contribution to the conformational equilibrium, yet after the attachment of the reagent and prior to

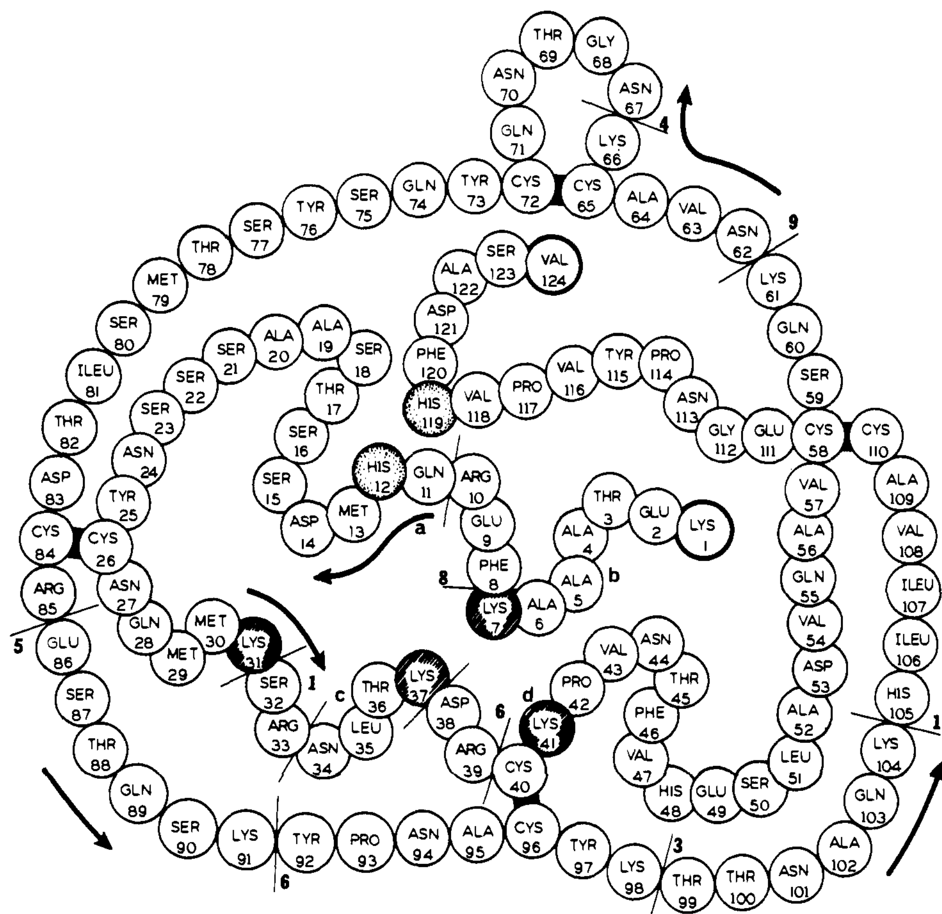


Figure 9. Schematic view of ribonuclease structure showing tryptic cleavage sites as slash lines. Histidine-12 and -119 of the active site are demarked. Lysine-7 and -37 and -31 and -41 are indicated showing their neighboring orientation for the cross-linking process. Numbers and letters refer to fragments found in maps (Figure 8).

cross-linking, a change in conformation caused by the reagent could bring these residues into spatial association. X-ray studies do not resolve this question irrevocably—the correspondence between interresidue distances obtained thereby and those estimated using bifunctional reagents is excellent in some cases[39-44] but unsatisfactory in others.[5,45-47]

Many traditional cross-linking reagents have been constructed so as to contain structural elements which provide water solubility.[1-6,44] These attached ionic groups would also be expected to provoke a major perturbation of the protein conformation during cross-linking. Still other reagents though not containing ionic functions would be expected to perturb the structure because of changes in the acidity or basicity of the side chains as the reagent becomes attached.[48,49] The structural characteristics of the present ETAC reagents allow the water solubilizing substituent to be contained in the leaving group of the first alkylation so that during the second alkylation (cross-linking) step, the protein conformation might not be so drastically affected by the introduction of a new ionic moiety. The second alkylation and cross-linking step cannot occur until the water solubilizing function has been eliminated to expose the latent double bond. In addition, these reagents, when attached to lysine ϵ-amino functions, only slightly change the basic character or hydrogen-bonding capability.

The equilibrium nature of ETAC reagents demonstrated with the model thiol and amino compounds should affect the cross-linking sites on proteins in a rather unique way, and comparison of the protein cross-links produced by an ETAC reagent with those of a traditional kinetic cross-linking agent is instructive. Consider a protein with just three nucleophilic sites, two close by one another in space (b and c) and a third, more exposed residue (a), which in the conformational meandering of the protein chain, side chain, or domains of native format[50] approaches one of these other two functions. The equilibrium concentration of this temporary orientation of the protein may be very small. Interaction of such a protein with a reagent such as, for example, difluorodinitrobenzene, would yield first a substituted protein followed by the cross-linking step.[51] Presuming the cross-linking agent did not perturb the conformational dynamics of the native protein, the intramolecular cross-linking reaction would eventually take place between the more exposed residue a and the residue which came into its proximity in the minor equilibrium distribution (b). The identification of this cross-link would imply that residues a and b were close by in space, a conclusion that would apply only to a conformation having a small contribution to the protein structure.[3]

ETAC reagent interaction with this same protein has the potential for attachment of the reagent to the more exposed residue (a), and, just as in the previous example, cross-linking in the minor conformational distribution to residue b. However, as noted in the Introduction, once this cross-link is established, a new equilibrium could allow the release of residue a leaving the reagent attached to only b. Still assuming the attached reagent did not affect the conformational distribution, reisomerization to the original structure would now allow cross-linking of b to c with the formation of the link between residues most closely associated in the native protein.

These same types of events might be expected in the establishment of intermolecular cross-links in a monomer–aggregate equilibrium. The relative distribution between intramolecular cross-linked aggregates and intermolecular cross-linked de-

Table III. Amino Acid Analyses of Tryptic Peptides

peptide fragment[a]	1		3		4		5		6		8		9		11	
residues (Fig 9)[b]	residue 32–33 O-Trp-12		residue 99–104 O-Trp-8		residue 67–87 O·Trp·2		residue 86–91 O-Trp-6		residues 92–98 + 38, 39 O-Trp-14 + 7		residue 8–10 O·Trp-15		residue 62–66 O·Trp-5		residue 105–124 O-Trp-16	
	no. residues		no. residues		no. residues		no. residues		no. residues		no. residues		no. residues		no. residues	
	found	theor	found	theor	found	theor	found	theor	found	theor	found	theor	found	theor	found	theor
Lys			1.21	1	0.31		1.13	1	1.00	1	0.46		1.04	1	0.07	
His					0.10						0.13				1.78	2
Arg	1.0	1			1.19	1			0.98	1	1.0	1				
SCM-Cys			0.17		1.89	2			0.91	1			0.83	1	0.82	1
Asp	0.16		1.01	1	3.11	3			1.65	2	0.70		0.98	1	2.25	2
Thr	0.08		1.42	2	2.82	3	1.07	1	.51		0.37		0.30		0.32	
Ser	0.44	1			2.78	3	1.60	2			0.79		0.37		0.82	1
Glu	0.17		1.21	1	2.26	2	1.49	1			0.90	1	0.45		1.13	1
Pro									0.99	1	0.11				1.62	2
Gly	0.59		3.0		2.65	1	3.38	1			1.8		0.31		2.02	1
Ala	0.23		0.84	1	0.50		0.32		0.93	1	0.64		1.26	1	1.90	2
Val							0.22				0.50		1.24	1	3.89	4
Met					0.96	1	0.19				0.52		0.29		0.28	
Ile					0.91	1	0.27		0.50		0.35		0.25		1.89	2
Leu					0.38		0.35		0.41		0.36		0.30		0.37	
Tyr					1.80	2	0.16		1.68	2	0.34				1.03	1
Phe					0.03		0.23				0.90	1			0.66	1
$(Lys)_2$ETAC reduced																

peptide fragment[a]	a		b		c		d		2		undigested fragments	
residues (Fig 9)[b]	residue 11-31 O-Trp-4		residue 1–7 O-Trp-10		residue 34-37 O-Trp-11		residue 40–61 O-Trp-9		fragments b + c O-Trp·10 + 11			
	no. residues		no. residues		no. residues		no. residues		no. residues		no. residues	
	found	theor	found	theor	found	theor	found	theor	found	theor	found	theor
Lys	1.14	1	1.99	2	0.98	1	2.0	2	0.80	1	5.27	
His	1.02	1	0.46				0.65	1			2.4	
Arg							0.32				4.02	
SCM-Cys	0.89	1					1.89	2			7.39	
Asp	2.86	3	0.55		0.98	1	1.94	2	1.08	1	15.0	
Thr	1.13	1	1.11	1	1.39	1	1.0	1	1.83	2	9.02	
Ser	5.05	6			0.54		2.05	2			13.88	
Glu	1.84	2	1.19	1			3.50	3	1.01	1	12.60	
Pro							0.85	1			3.49	
Gly	4.49		1.46		1.72				2.21		6.06	
Ala	2.02	2	3.08	3			2.22	2	2.95	3	11.38	
Val	0.17				0.42		3.57	4			7.9	
Met	2.96	3									3.92	
Ile			0.58				0.34				2.42	
Leu			0.44		1.21	1	0.84	1	1.20	1	2.50	
Tyr	0.81	1					0.10				3.40	
Phe							0.83	1			2.45	
$(Lys)_2$ETAC reduced									1.0	1	1.3	

[a] Eluted from map (Figure 8). [b] Nomenclature according to Hirs and Moore.[37]

rivatives would depend not only on the relative propensity of the native protein to aggregate but also on the equilibrium between the individual intermolecular and intramolecular cross-linking processes and the protein concentration. This assumes the aggregates do not precipitate from the reaction solution.

Clearly, the introduction of two or more cross-links into a protein may accentuate the differences between the native structure and the cross-linked structure. The first cross-link may allow or cause a new conformational folding, so that the second link takes up an orientation not possible in the native structure. Thus, with many proteins ETAC reagents should give a pattern of multiple links distinct from those formed by kinetic reagents. We have observed distinctly different patterns of inter- and intramolecular cross-linked products in the ribonuclease ETAC reactions as conditions are varied. This is only observable after the reduction of the nitro function to prevent repositioning of the mobile links.

In phosphate buffer, cross-linking with 2 equiv of ETAC reagent afforded a major product in 69% yield having two discrete cross-links. A minimum in the equilibrium distribution appears to have been reached under these conditions. The bisintramolecular cross-link ribonuclease must have new connections between fragments 34-39 (c) and 1-7 (b) and fragments 11-31 (a) and 40-61 (d) at lysine residues (see Figure 9). These results imply that residues 7 and 37 form one link and residues 41 and 31 the other. The only alternative is involvement of residue 61 rather than 41. The X-ray structure of ribonuclease and previous cross-linking studies suggest this is unlikely. Comparison of the X-ray structure[22] with a model which contains these two cross-links suggests that the links may be accommodated without serious structural changes (see Figure 10). The 7-37 cross-link is a bridge of long span across the crevice to one side of the opening into the active site and undoubtedly requires some distortion from the crystal-structure position of this part of the chain. The 31-41 cross-link requires a folding or twisting of residue 41 back into the cleft away from the active site so as to interact with residue 31 across the loop. The two distortions may require cooperative processes with the formation of one link allowing the other to take place. The 7-37 link across the active-site crevice would seem to prevent the binding of substrate, and thus the negligible activity of cross-linked enzyme is not surprising. The simultaneous repositioning of both Lys-7 and -41 into a ridged, perturbed format may also have significant effect on enzyme activity. Nevertheless, the similarity of ORD curves of bis-cross-linked and native ribonuclease (Figure 11) implies that the major features of the ribonuclease structure are retained in this bis-cross-linked framework.

Hir's[39] early studies with ribonuclease showed lysine residue

Figure 10. Pictorial illustration of ribonuclease from the X-ray structure showing three-dimensional relationship of the four lysines which have been linked by the two ETAC moieties.

41 to be the most susceptible to arylation by fluorodinitrobenzene and lysine residue 7 becoming the next most susceptible to attack by this same reagent. Of the three monointramolecular cross-linked ribonuclease derivatives produced by reaction with the closely related difluorodinitrobenzene,[40] only the product having a 41–7 lysine cross-link has so far been identified.[41] Presumably Lys-41 is arylated followed by a sequential cross-linking to residue 7. It is significant that these studies were accomplished only in the absence of phosphate ions and that very dilute concentrations of protein were necessary in order to avoid polymer formation.

Frequently phosphate ion has been observed to protect Lys-41 from electrophilic attack in modification and cross-linking of ribonuclease.[52-56] These same phosphate buffer reaction conditions dramatically affect the rate of ETAC-II with ribonuclease, especially in the last cross-link step. Addition of 2 equiv of reagent and formation of one of the cross-links are complete in 3-4 h. The second, final cross-link is completed only after 48 h. By comparison, the ETAC-II reaction run under the same conditions in Tris buffer was completely over within 4 h. Again, the absence of phosphate changes the sequential rates of the ETAC reaction. However, this point alone is insufficient to allow comparison since the cross-linked products using Tris are almost completely trimeric inter-cross-linked proteins. Our analysis is that the enzyme has a very different distribution of monomer and aggregate forms in Tris from that in phosphate. Thus, the particular residues involved in the Tris buffer intra-cross-linking might be quite different from those in the inter-cross-linking in phosphate buffer. The equilibrium distribution of aggregate forms in phosphate buffer in the presence of cross-linking agent is undoubtedly different than that without cross-linking agent, but this latter equilibrium is so easily established that the distribution cannot easily be evaluated.[57]

The reaction conditions under which the identified ribonuclease bis-cross-links are produced parallel closely those used by Hartman and Wold (pH 10.5, phosphate buffer)[42] for the cross-linking of ribonuclease with dimethyl adipimidate. In their study, cross-linking occurred giving two different but unseparated mono-cross-linked derivatives having links be-

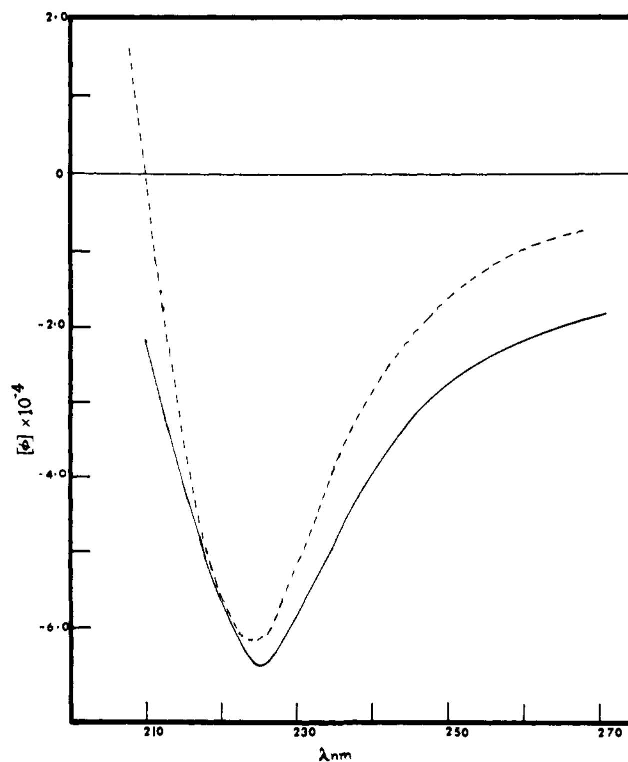


Figure 11. ORD curves in water of (- - -) native RNase A (0.60 mg/mL) and (—) bis-cross-linked RNase (0.66 mg/mL). Cells of 0.998-mm path lengths were used.

tween lysine residues 7 and 37 and residues 31 and 37. Dual links did not appear to be formed. It is of interest that these same three residues are involved in the dual cross-linking by ETAC reagents. The additional linking of normally protected Lys-41 may arise from perturbing or cooperative effects of the first link or from the transfer character of ETAC reagents. At this point the numerous equilibrium cross-link pathways are too numerous to speculate upon.[58] It is apparent that the long

chain lengths of the bisimidates are not an absolute necessity for the interlinking of the lysine 7-37 residues across the cleft of ribonuclease,[42] since the short span of an ETAC group bridges these residues.

Of great interest is the fact that tryptic cleavage of lysines linked by the 2-(*p*-aminophenyl)propyl moiety is still easily accomplished. Perhaps this fortunate circumstance is related to the situation of having no perturbing ionic substituent nearby the lysine ϵ-amino function and also of having the aromatic ring (aniline) insulated from the ϵ-amino group by two carbons. Benoiton and Denault[59] have demonstrated that the monomethyl-substituted derivatives of lysine do undergo slow but significant hydrolysis by trypsin. We rationalize that the tryptic cleavage at the cross-linked sites is observed because the modification of each lysine is in effect a monoalkyl substitution and because the aniline group is removed some distance from the recognition site with little change in basic character.[59] This observation, if sustained in other proteins and peptides, should make the identification of these types of cross-linked positions on proteins significantly easier than with other reagents.

The concept illustrated by these reagents should have wide application to the studies of protein subunits, multienzyme complexes, and protein structure.[60] Our study of these types of molecules and the attempt to specifically identify the transfer process are being continued in a variety of biochemical systems. We believe ETAC reagents such as **12** will be important chemical tools for the biochemist and molecular biologist.

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