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The Use of the Acetylenic Function for Direct Chemical Modification of Proteins[1]

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The possibility of using the acetylenic function for chemical modification of proteins was tested on chlorotetrolic acid and methyl chlorotetrolate. These compounds react under mild conditions with different functional groups analogous to those present in proteins. The ease with which the same nucleophile adds to the triple bond and substitutes the chlorine was compared, and the stability of the addition products was checked. The modification of amino groups by addition across the triple bond can be reversed in acidic medium. In reactions with small model molecules and with proteins, the tested compounds behave like bifunctional protein reagents.

INTRODUCTION

Acetylenic compounds have been so far seldom used for chemical modification of biological macromolecules. Several assays have been made to evaluate the utility of some derivatives of this type in protein chemistry. These investigations were based on two different approaches. The first consisted in exploiting the unique geometrical properties of the rigid linear —C≡C— system to obtain acetylenic substrate analogs (*1*), competitive inhibitors (*2*), or affinity labels (*3*) of restricted conformation, in which the triple bond played a structural but not a chemical role. The other guiding idea was to use acetylenic substrate analogs as irreversible enzyme-activated inhibitors or k_{cat} inhibitors (*4*, *5*) to perform the so-called dynamic affinity labeling of enzymes (*6*), in which the triple bond again does not react directly but is converted by the enzyme to an allenic group which eventually substitutes a nucleophile in the receptor site.

The present investigation was based upon a different, still unexplored concept: to evaluate the possibility of using the triple bond directly as a function with the ability to modify protein side chains.

The model acetylenic compounds used in this study were chlorotetrolic acid and its methyl ester, $ClCH_2$—C≡C—COOR (R = H or CH_3), ethynylogs of haloacetates commonly applied in protein chemistry as alkylating agents. A comparison could thus be made between the relative ease of the reactions of two func-

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tions of these products with a given nucleophile, i.e., addition across the triple bond and substitution of chlorine.

The synthesis of chlorotetrolic acid and some of its esters was described in 1958 (*7*). A few reactions of these compounds are known, such as displacement of chlorine by di- and trimethylamine, the latter leading to trimethyltetrolobetaine, the first example of an acetylenic betaine (*8*). The preparation of aminotetrolic acid proved more difficult (*9, 10*). Some other derivatives of chlorotetrolic acid were also described (*9–11*). In general, however, the chemical properties of this versatile compound remained largely unexplored.

This paper describes a more detailed chemical study of chlorotetrolic acid and ester performed on small model molecules containing nucleophiles analogous to those present in proteins and some preliminary examples of reactions of these compounds with proteins, suggestive of general utility of these products in protein chemistry. A short preliminary paper on some chemical properties of these compounds has appeared (*12*).

EXPERIMENTAL

Chlorotetrolic (chloro-2-butynoic) acid and methyl chlorotetrolate were obtained according to Olomucki (*7, 13*). 5-Mercaptomethylimidazole hydrochloride was prepared from 5-hydroxymethylimidazole (*14*) by conversion of the hydroxymethyl group successively to chloromethyl then benzylmercaptomethyl (*15*) followed by cleavage of the benzyl group according to Schneider (*16*). Cristalline yeast alcohol dehydrogenase was purchased from Boehringer, Mannheim, bovine pancreatic ribonuclease A from Nutritional Biochemical Company, A-grade NADH from Calbiochem and NAD from Fluka AG. Spectral determinations were performed on the following instruments: infrared (KBr discs), Perkin–Elmer 720; ultraviolet, Zeiss PMQ II or Cary 15; proton magnetic resonance, Varian FT 80A. Thiols were titrated by the method of Ellman (*17*).

Model Reactions: Comparison of the Reactivity of Chlorine and of the Triple Bond

(*a*) *Chlorotetrolic acid: Reaction with thiols.* The pH of a solution of 0.095 g (0.8 mmol) of chlorotetrolic acid in 2 ml water was adjusted to 8 with sodium hydroxide, 0.122 g (0.77 mmol) of *N*-acetylcysteine was added, the volume was made up to 10 ml with 0.2 *M* sodium bicarbonate, pH 9, and the solution was kept at room temperature until complete disappearance of thiol. The amount of mineralized halogen was then determined by Volhard titration.

(*b*) *Methyl chlorotetrolate: Reaction with thiols.* A 0.02 *M* solution of methyl chlorotetrolate and *N*-acetylcysteine in 0.2 *M* bicarbonate, pH 9, was prepared, a 0.1-ml aliquot was immediately added to 1.9 ml of a 0.002 *M* solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[4] in 0.1 *M* phosphate buffer, pH 7, and the

[4] Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NOE, nuclear Overhauser effect; CNTP, 3-carboxy-4-nitrothiophenate.

absorbance at 412 nm was determined. In an another experiment a similar 0.1 *M* solution was left for 10 min and ionic chlorine was determined.

Reaction with amines. 0.174 g (2 mmol) of *n*-amylamine was added to a solution of 0.405 g (3 mmol) of methyl chlorotetrolate in 10 ml of benzene. After 2 hr at room temperature thin-layer chromatography showed that the amine completely disappeared. The dark-brown solution was extracted 3 times with 10-ml portions of water and chloride ions were determined in the combined extracts.

Model Compounds

n-Amylaminotetrolic Acid (**1**). *n*-Amylamine (8.7 g, 0.1 mol) was added to a stirred, ice-cold solution of 2.4 g (0.02 mol) of chlorotetrolic acid in 25 ml of benzene and kept for 1 hr in ice, then for 24 hr at room temperature. During the first few hours of reaction an absorption at 280 nm appeared in the mixture and was then gradually replaced by a peak at 310 nm. The dark-brown solution was extracted twice with 50 ml of water and the yellow aqueous extract was evaporated. The residual oil was suspended in 1 vol of ether and acetone was slowly added with slight heating; the oil gradually dissolved and a precipitate appeared. The suspension was chilled, filtered, and the solid washed with acetone. Yield 1.12 g (30%), mp 212–215°C. After recrystallization in methanol–acetone, mp 215°C. IR: 2250, 1620, 1560, 1305, 1080, 802 cm^{-1}. *Anal.* Calcd for $C_9H_{15}NO_2$: C, 63.87; H, 8.93; N, 8.27. Found: C, 63.8; H, 8.9; N, 8.1.

The benzene solution contains a product absorbing at 310 nm which could not be purified.

Imidazol-5-yl-methylthiotetrolic acid (**2**). Ten milliliters 1 *N* sodium hydroxide were added to a solution of 1.2 g (0.01 mol) of chlorotetrolic acid in 5 ml water, followed by 1.6 g (0.0106 mol) of 5-thiomethylimidazole hydrochloride and 18 ml 1 *N* sodium hydroxide. The solution was kept under nitrogen at room temperature and the pH was maintained at 9.5 by periodical additions of sodium hydroxide. After 6 hr titration showed that no more thiol was present. The pH was brought to 4 by 1 *N* hydrochloric acid, the solution evaporated, and the residual oil treated with acetic acid. The insoluble salt was filtered off, the filtrate evaporated, and the residue treated with ethanol and filtered. Acetone was then added until turbid, the solution chilled, and the solid filtered off, washed with acetone, and recrystallized in aqueous ethanol. Yield 1.2 g (66%), mp 194–195°C. IR: 2240, 1600, 1550, 1415, 1338, 1310, 1090, 820 cm^{-1}. NMR (DMSO-d_6, HMDS): δ 3.47 (s, CH_2—C≡), 3.76 (s, CH_2—S), 7.01 (s, imidazole C_4H), 7.79 ppm (s, imidazole C_2H). The spectrum gradually changes; after 19 hr the same solution showed: δ 3.88 (s), 4.24 (s), 6.28 (s), 6.76 (s), 8.3 ppm (s) (see below, compound **3**). *Anal.* Calcd for $C_8H_8N_2O_2S$: C, 48.96; H, 4.11; N, 14.28; O, 16.30; S, 16.34. Found: C, 48.8; H, 4.1; N, 14.3; O, 16.4; S, 16.0.

Cyclization of compound **2** *in DMSO: 3-Carboxymethyleneimidazo[1, 5-d]perhydrothiazine* (**3**). A solution of 0.25 g of compound **2** in 5 ml dimethyl sulfoxide was kept at 30°C for 48 hr. The solvent was then evaporated and the residual solid recrystallized in a methanol–ethanol mixture, yielding 0.12 g of product, mp 180°C. In thin-layer chromatography (silica gel, *n*-butanol–acetic

acid–water 5 : 3 : 2) this product has a higher R_f than compound **2** and, unlike the latter, can be visualized on the chromatogram by uv irradiation. UV: λ_{max} (CH_3CN) 235 nm (ϵ 7000). IR: 1485, 1320, 825 cm^{-1}, no acetylenic band. NMR (DMSO-d_6, HMDS): δ 3.91 (s, C_8H), 4.25 (s, C_2H), 6.26 (s, C_9H), 6.81 (m, C_7H), 8.30 ppm (m, C_5H); NOE on C_9H by irradiation of C_5H: 15%. *Anal.* Calcd for $C_8H_8N_2O_2S$: C, 48.96; H, 4.11; N, 14.28; O, 16, 30; S, 16, 34. Found: C, 48.8; H, 4.3; N, 14.4; O, 16.3; S, 16.0.

2-Carboxymethylene-1,4-dithiane (**4**). A solution of 1.2 g (0.01 mol) of chlorotetrolic acid in 10 ml methanol was added to 1 g (0.01 mol) of 1,2-dithioethane, followed by 20 ml 1 *N* sodium hydroxide. After 6 hr at room temperature the mixture was acidified to pH 3–4 with hydrochloric acid and concentrated. The precipitated solid was filtered off, dissolved in hot methanol, filtered, and the filtrate chilled, yielding 0.7 g (40%) of a solid, mp 230°C. UV: λ_{max} (CH_3CN) 285 nm (ϵ 10750). IR: 1675, 1550, 1420, 1220, 840, 820 cm^{-1}. NMR (DMSO-d_6, HMDS): δ 3.01 (br s, C_5H, C_6H), 3,52 (d, $J_{3,7}$ = 0.7 Hz, C_3H), 5.78 ppm (t, $J_{3,7}$ = 0.7 Hz, C_7H); NOE on C_7H by irradiation of C_3H: 38%. *Anal.* Calcd for $C_8H_8O_2S_2$: C, 40.88; H, 4.57; O, 18.15; S, 36.40. Found: C, 41.0; H, 4.6; O, 18.2; S, 36.1.

3-Carboxymethylene-5-carboxy-1,4-thiazine (**5**). Cysteine hydrochloride monohydrate (1.75 g, 0.01 mol) was added to a solution of 1.2 g (0.01 mol) of chlorotetrolic acid in 5 ml water and 10 ml 1 *N* sodium hydroxide. The pH of the mixture was brought to 8.2 by adding 15 ml of 1 *N* sodium hydroxide and kept constant during 10 hr at room temperature. The pH was then lowered to 3–4 with 1 *N* hydrochloric acid, the precipitate was filtered and washed with 0.1 *N* hydrochloric acid and with acetone. The product was purified by dissolving in sodium hydroxide and precipitating with hydrochloric acid. Yield 0.55 g (27%). The product decomposes above 320°C. UV: λ_{max} (0.2 *M* bicarbonate) 270 nm (ϵ 9000). IR: 1635, 1580, 1400, 1350, 1310, 1250, 1225, 845, 815 cm^{-1}. NMR (NaOD, external HMDS): δ 3.24, 3.48 (AB part of ABX, $J_{A,X}$ = 5.4 Hz, $J_{B,X}$ = 6.6 Hz, C_6H), 3.94 (br s, C_2H), 4.05 (X part of ABX, C_5H), 6.19 ppm (br s, C_7H); NOE on C_7H by irradiation of C_2H: 25%. *Anal.* Calcd for $C_7H_9NO_4S$: C, 41,38; H 4.47; N, 6.90; S, 15.75. Found: C, 41.3; H, 4.5; N, 6.8; S, 15.6.

3,4-bis-(2-Amino-2-carboxyethylthio)crotonic acid (**6a**). The filtrate obtained after precipitation of crude product **5** was evaporated, the residual oil was treated with acetic acid and the insoluble salts filtered. The filtrate was evaporated, the residue was suspended in warm chloroform, and ethanol was added slowly. A solid gradually crystallized and became abundant after chilling. The product was filtered and recrystallized in a water–ethanol mixture. Yield 0.5 g (31%), mp 208–210°C. With ninhydrine this compound gives the color reaction characteristic of primary amines. UV: λ_{max} (H_2O) 280 nm (ϵ 3000). IR: 1660, 1620, 1580, 1530, 1415, 1330, 1315, 1225, 905, 833, 798 cm^{-1}. NMR (D_2O, external HMDS): δ 3.47, 3.93 (AB parts of two ABX, CH_2—S—CH_2—C= and CH_2—S—C=), 4.09 (s, CH_2—C=), 4.53 (m, CH—N), 6.58 ppm (s, =CH). *Anal.* Calcd for $C_{10}H_{16}N_2O_6S_2$: C, 36.89; H, 5.14; N, 8.59; O, 29.78; S, 19.60. Found: C, 37.07; H, 4.97; N, 8.63; O, 29.59; S, 19.77.

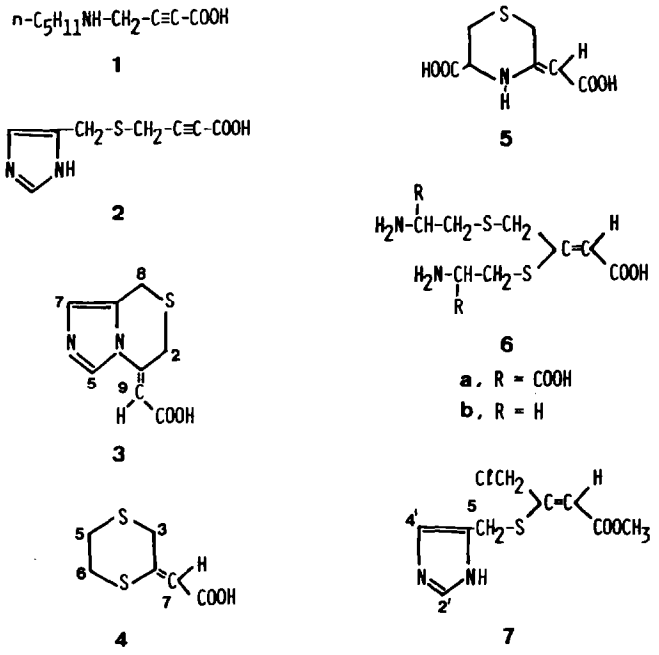
3,4-bis(2-Aminoethylthio)crotonic acid dihydrochloride (**6b**). Cysteamine hy-

drochloride (1.14 g, 0.01 mol) in 15 ml ethanol was mixed with 1.2 g (0.01 mol) of chlorotetrolic acid in 10 ml ethanol, 15 ml 1 *N* sodium hydroxide were added, and the mixture was incubated for 12 hr at room temperature, the pH being kept at 8.5 by periodical additions of 1 *N* sodium hydroxide. The solution was acidified to pH 3–4 with 1 *N* hydrochloric acid and evaporated. The residue was extracted with acetic acid, filtered, and the filtrate evaporated. The residual oil was suspended in chloroform and methanol was added dropwise with warming until a clear solution was obtained. After chilling 0.99 g (64%) of product, mp 175–180°C was collected and recrystallized in a methanol–chloroform mixture yielding a ninhydrine-positive compound, mp 184–185°C. UV: λ_{max} (CH_3OH) 270 nm (ϵ 12,000). IR: 2030, 1680, 1580, 1480, 1390, 1310, 1200, 939, 855, 795 cm^{-1}. NMR (D_2O, external HMDS): δ 3.21, 3.56 (A and B parts of AA'BB', splitting 6 Hz), 3.64 (s) (CH_2—CH_2), 4.02 (s, S—CH_2—C=), 6.50 ppm (s, =CH); NOE on =CH by irradiation of S—CH_2—C=: 23%. *Anal.* Calcd for $C_8H_{18}N_2O_2S_2Cl_2$: C, 31.06; H, 5.87; N, 9.06. Found: C, 31.01; H, 5.91; N, 8.97.

Methyl 3-(imidazol-5-yl-methylthio)-4-chlorocrotonate hydrochloride (**7**). To a solution of 1.6 g (0.106 mol) of 5-thiomethylimidazole in 20 ml acetone and 20 ml 0.1 *M* phosphate buffer, pH 7.2, there was added 1.32 g (0.01 mol) of methyl chlorotetrolate and the mixture was stored for 18 hr at room temperature. Hydrochloric acid was added until the pH decreased to 3–4 and the solution was evaporated. The residue was digested with acetic acid, the insoluble material was filtered, and the filtrate was evaporated. The residual sirup was dissolved in hot acetone and a small volume of ethanol was added, followed by petroleum ether until turbid. The solid precipitated in the cold (2.1 g, 74.5%, mp 190–191°C) was recrystallized in ethanol. MP 192°C. UV: λ_{max} (CH_3CN) 290 nm (ϵ 7700). IR: 1685, 1615, 1575, 1435, 1340, 1210, 1155, 835, 790 cm^{-1}. NMR (DMSO-d_6, TMS): δ 3.64 (s, CH_3), 4.42 (d, $J_{4',5}$ = 0.5 Hz, C_5H), 4.74 (d, $J_{2,4}$ = 0.5 Hz, C_4H), 6.33 (t, $J_{2,4}$ = 0.5 Hz, C_2H), 7.66 (dt, $J_{2',4'}$ = 1 Hz, $J_{4',5}$ = 0.5 Hz, $C_{4'}H$), 9.09 ppm (d, $J_{2',4'}$ = 1 Hz, $C_{2'}H$); NOE on C_2H by irradiation of C_4H: 33%. *Anal.* Calcd for $C_9H_{11}N_2O_2SCl_2$: Cl^- 12.54. Found: Cl^- 12.43.

RESULTS

Both the triple bond and the chloromethyl group of chlorotetrolic acid and ester can easily react with various nucleophiles. In several tests the reactions of different thiols with chlorotetrolic acid were followed by periodical titrations by DTNB of remaining SH groups. Under the mild conditions of the experiments shown in Fig. 1, the half-life of cysteine, cysteamine, or thiomethylimidazole is about 15 min. For the less nucleophilic thiol *N*-acetylcysteine the half-reaction time is 3 hr. In the latter case titration of ionic chlorine at the end of the reaction showed that 65% of the thiol reacted by substitution of chlorine. The higher reactivity of the chloromethyl group with respect to the triple bond in chlorotetrolic acid was also shown by the model reaction of this compound with thiomethylimidazole, in which the chlorine-substitution product **2** was isolated in a 66% yield.



Imidazol-5-yl-methylthiotetrolic acid (**2**) was also used as a model compound to test the reactivity of the triple bond in a protein-crosslinking reaction of chlorotetrolic acid starting with substitution of chlorine. The half-reaction time of a thiol (cysteamine) with product **2** is 2 hr, i.e., longer than in the case of chlorotetrolic acid (Fig. 1). On the other hand, reaction of chlorotetrolic acid with thiomethylimidazole, followed by cyclization of compound **2** to give product **3**, can be considered as a model of the intramolecular reaction of SH-imidazole crosslinking in proteins. The intramolecular addition of the imidazole moiety across the triple bond in compound **2** seems to proceed rather easily, since the nmr spectrum of **2** could only be recorded on fresh solutions of this product. Aging of the solutions

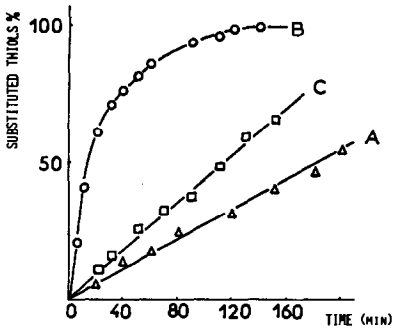


FIG. 1. Reactions of thiols with chlorotetrolic and imidazol-5-yl-methylthiotetrolic acids. SH groups were periodically titrated in 0.02 *M* solutions of acetylenic acid and thiol in 0.2 *M* bicarbonate, pH 9, kept in room temperature. A, chlorotetrolic acid + *N*-acetylcysteine; B, chlorotetrolic acid + cysteine or cysteamine or thiomethylimidazole; C, imidazol-5-yl-methylthiotetrolic acid (**2**) + cysteamine.

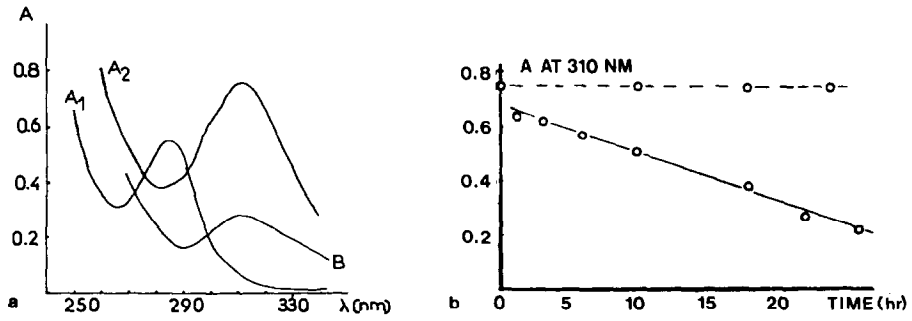


FIG. 2. (a) Reactions of n-amylamine with chlorotetrolic and n-amylaminotetrolic acid. A solution of 87 mg (1 mmol) of n-amylamine and 54 mg (0.45 mmol) of chlorotetrolic acid in 10 ml 0.2 *M* bicarbonate, pH 9, was incubated for 5 hr at room temperature. Curve A_1, uv spectrum of an aliquot diluted 20-fold in 0.1 *M* bicarbonate, pH 8.4; curve A_2, the same after an additional incubation at 50°C for 1 hr; curve B, a similar solution containing 67 mg (0.4 mmol) of amylaminotetrolic acid and 72 mg (0.83 mmol) of amylamine was incubated at 50°C for 1 hr and the uv spectrum was recorded on an aliquot diluted 50-fold as above. (b) 0.1-ml aliquots of the latter solution were diluted 20-fold either in 0.1 *M* bicarbonate, pH 8.4 (---), or in 1 *N* hydrochloric acid (—) and the time evolution of the absorbance at 310 nm was followed.

accompanied by a complication of the nmr spectrum occurs rapidly but after several hours a clear spectrum of compound **3** appears.

A model reaction of crosslinking two vicinal SH groups was performed using dithioethane, leading to the cyclic derivative **4.** With cysteine, compound **5** was obtained as a model of SH—NH_2 crosslinking. However, an equal amount of product **6a** was also formed in this reaction as a result of an interaction of the SH group of cysteine with both the chloromethyl group and the triple bond of chlorotetrolic acid. With cysteamine only a product of the latter type, **6b**, was formed in a yield equal to the sum of the yields of **5** and **6a.**

The thiol adducts **4, 6a, 6b, 7,** and the primary amine adduct **5** have *Z* configuration, whereas the imidazole adduct **3** has the *E* form. These assignments are based on nmr spectra and the nuclear Overhauser effect observed for these products. They are also corroborated by the uv spectra, the λ_{max} of product **3** being lower and in most cases of lower intensity than those of the other adducts, in accordance with the uv spectral properties of cis–trans compounds (*18*). This stereochemistry is consistent with the rules of addition to triple bonds, i.e., the "trans-addition rule" of thiols and primary amines and the cis addition of imidazole (*19*).

In a model reaction of an amine with chlorotetrolic acid, n-amylamine gave the chlorine-substitution derivative **1,** which could be isolated in a 30% yield. In the reaction mixture, however, another product appears simultaneously having an absorption peak at 280 nm characteristic of compounds resulting from reactions of addition across the triple bond. This band gradually shifts to 310 nm. This sequential formation of derivatives absorbing at 280 and 310 nm is also clearly shown in Fig. 2a, curves A_1 and A_2. When amylaminotetrolic acid **1,** which has an absorption band at 210 nm but does not absorb in the 280 nm region, is treated with amylamine, an absorption peak at 310 nm again appears (Fig. 2a, curve B). It can be assumed that the derivative absorbing at 280 nm results from addition of amine

across the triple bond of chlorotetrolic acid, and the product absorbing at 310 nm is formed by reaction of two molecules of amylamine with both the triple bond and the chlorine. The primary attack of amine can be directed to either of these two functions of chlorotetrolic acid.

The band at 310 nm was also observed in the reaction of chlorotetrolic acid with ethylene diamine. In this case as well as in the preceding one, however, we could not isolate the reaction products. In mixtures of chlorotetrolic acid with amines dark tars appear which often complicate the isolation of pure products (*9, 13*).

The bands characteristic of triple bond-addition derivatives were also used to test the stability of these compounds. Enamines hydrolyse to ketones in acidic medium. Figure 2b shows that the product formed by reaction of amylaminotetrolic acid with amylamine is stable in alkaline solution, but in the presence of acid the 310-nm absorption peak gradually decreases and after 17 hr reaches half of its initial value. Under the same conditions, compounds resulting from addition of thiols across the triple bond are stable both in alkaline and acidic medium (not shown).

In contrast to chlorotetrolic acid, in methyl chlorotetrolate the activated triple bond is much more reactive, and the difference in reactivity between this function and the chloromethyl group is distinctly larger. Thus, when equivalent amounts of *N*-acetylcysteine and methyl chlorotetrolate are mixed together, the thiol entirely disappears in one minute, the reaction consisting solely in addition across the triple bond (no ionic chloride formed). In the reaction of 5-thiomethylimidazole with methyl chlorotetrolate only product **7** resulting from addition of thiol across the triple bond was obtained in a high yield.

A comparison of the reactivity of methyl chlorotetrolate, chlorotetrolic acid, and methyl tetrolate with thiols is shown in Fig. 3. Since 3-carboxy-4-nitrothiophenate, formed in the reaction of DTNB with thiols, reacts itself with both

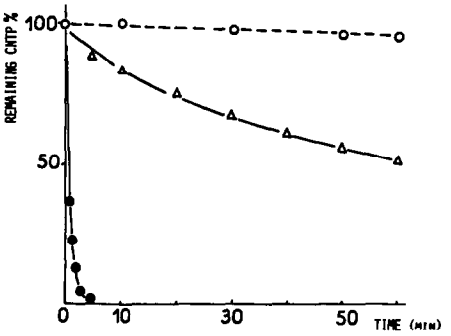


FIG. 3. Reactions of methyl chlorotetrolate, chlorotetrolic acid, and methyltetrolate with 3-carboxy-4-nitrothiophenate (CNTP). A mixture of 0.2 ml of a 0.01 *M* ethanolic solution of DTNB and 1.8 ml 0.05 *N* sodium hydroxide was incubated at room temperature until complete hydrolysis as indicated by the final absorbance at 412 nm of a 10-fold diluted aliquot (found: 1.32; calculated absorbance: 1.36). To 0.2 ml of this solution were added 1.6 ml of 0.1 *M* bicarbonate, pH 8.4, then 0.2 ml of a 0.02 *M* acetone solution of methyl chlorotetrolate and the decrease of 3-carboxy-4-nitrothiophenate was followed by measuring the absorbance at 412 nm (●). The same experiments were performed with chlorotetrolic acid (○) and with methyl tetrolate (△).

acetylenic esters, the disappearance of thiols could not be followed by the usual DTNB titration; and the phenate, prepared by hydrolysis of DTNB, was used in these reactions as a model thiol. Besides the expected difference in reactivity between chlorotetrolic acid and ester, the activating influence of chlorine can also be clearly seen (half-lives of 1.5 and 60 min, respectively, for methyl chlorotetrolate and tetrolate).

The reaction of methyl chlorotetrolate with amine was tested by following the absorbance at 295 nm of 100-fold diluted aliquots of a 0.02 *M* solution of *n*-amylamine and the ester in 0.2 *M* bicarbonate, pH 9, incubated at room temperature. The half-life was about 1 hr; and the reaction was again oriented mainly toward the triple bond, since only 8% of chlorine was mineralized at the end of the reaction. When the aliquot was diluted in 0.05 *M* hydrochloric acid, the λ_{max} shifted immediately to 275 nm, a value characteristic of β-dicarbonyl compounds (e.g., ethyl acetoacetate, 275 nm; acetylacetone, 274 nm). The same shift was observed when the solution was heated to 50°C. After these treatments, the reaction media show the presence of a new product giving positive tests of ketones, e.g., formation of a dinitrophenylhydrazone. Thus, the enamine formed by reaction of an amine with methyl chlorotetrolate is much less stable than the one obtained from chlorotetrolic acid.

Although more slowly than thiols and amines, phenols also react with methyl chlorotetrolate as shown by the test with *N*-acetyltyrosine (Fig. 4). During the reaction the absorption at 280 nm of the phenol gradually decreases and is replaced by a 250-nm peak of the reaction product. The latter is also unstable in acidic medium in which the 250-nm absorption band disappears. This conversion, however, is less rapid than in the case of the amine-addition product.

In view of the properties of methyl chlorotetrolate, reaction of the triple bond should be the first step of a protein crosslinking performed with this product. It seemed therefore interesting to test the reactivity of chlorine in a triple bond-

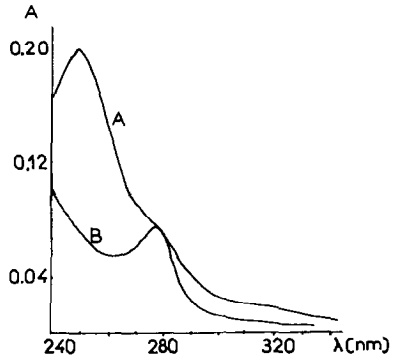


FIG. 4. Reaction of methyl chlorotetrolate with *N*-acetyltyrosine. A solution of methyl chlorotetrolate (2 m*M*) and *N*-acetyltyrosine (0.65 m*M*) in 0.1 *M* bicarbonate, pH 8.4, was incubated at room temperature until the disappearance of the absorbance of phenol at 280 nm (48 hr). The spectrum of an aliquot diluted 200-fold in 0.1 *M* bicarbonate, pH 8.4, was then recorded against a blank of the ester treated under the same conditions (curve A). Another aliquot was similarly diluted in 0.05 *N* hydrochloric acid, incubated at room temperature until the peak at 250 nm disappeared (2 hr) and the spectrum was recorded (curve B).

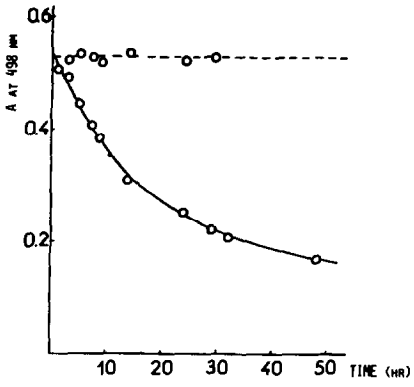


FIG. 5. Cyclization of compound **7**. A 0.01 *M* solution of compound **7** in 0.1 *M* bicarbonate, pH 8.4, was incubated at room temperature and the remaining monosubstituted imidazole was periodically titrated by diazotation (the diazo derivative absorbs at 498 nm) with diazosulfanilic acid (—). For comparison, methyl chlorotetrolate was incubated under the same conditions with *N*-acetylhistidine (---).

addition product of methyl chlorotetrolate. Periodical DTNB titrations of thiol in 200-fold diluted aliquots of a 0.01 *M* solution of cysteamine and compound **7** in 0.2 *M* bicarbonate, pH 9, containing 25% ethanol showed that at room temperature the thiol entirely disappears in 20 min (half-life 2.5 min). The mobility of chlorine in product **7** thus appears quite high compared, e.g., to chlorotetrolic acid (half-life 15 min for the reaction involving both the chlorine and the triple bond). Another illustration of the reactivity of chlorine in compound **7** is the instability of this product due to intramolecular substitution of chlorine by the imidazole moiety (Fig. 5).

The properties of chlorotetrolic acid and ester were further tested on proteins. Methyl chlorotetrolate inhibits yeast alcohol dehydrogenase rapidly and efficiently. When the enzyme is treated with this product (final concentrations 0.75×10^{-6} *M* and 2×10^{-4} *M*, respectively, in 0.2 *M* bicarbonate, pH 9, room tempera-

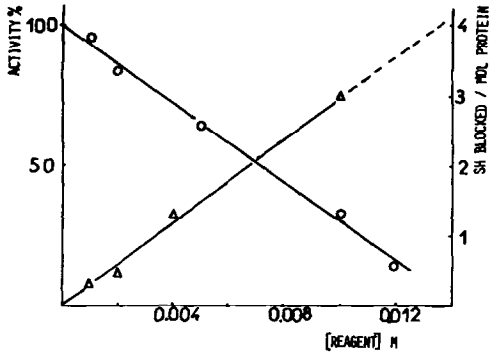


FIG. 6. Reactions of chlorotetrolic acid with yeast alcohol dehydrogenase. Mixtures of protein (final concentration 1.2×10^{-6} *M*) with chlorotetrolic acid at different concentrations in 0.2 *M* bicarbonate, pH 9, were incubated for 1 hr at 25°C and the enzymic activity (O) and remaining SH groups (Δ) were determined in each solution.

ture) the activity is entirely lost after 10 min. As expected, the reaction of chlorotetrolic acid with yeast alcohol dehydrogenase is much slower. Figure 6 shows that the inhibition is a linear function of the concentration of the reagent and is

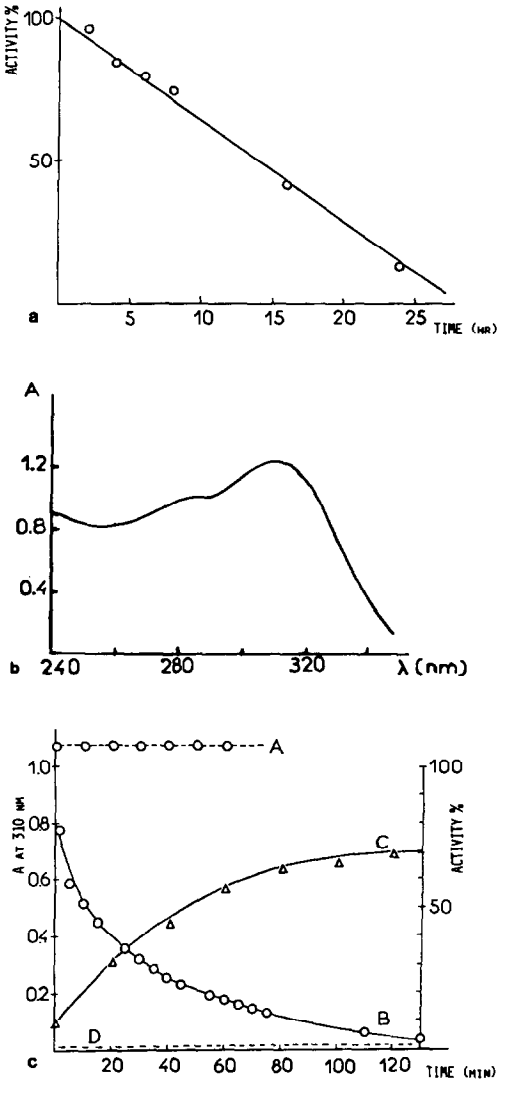


FIG. 7. Reaction of methyl chlorotetrolate with ribonuclease. (a) A solution of the enzyme and methyl chlorotetrolate in 0.1 *M* bicarbonate, pH 8.4, containing 2% ethanol (final concentrations: ribonuclease 0.13 m*M*, reagent 10 m*M*) was incubated at room temperature and the enzymic activity was periodically determined. (b) After 24 hr the uv spectrum of the solution was recorded. (c) After 24 hr the evolution of the absorbance at 310 nm was followed in aliquots of the solution diluted 10-fold in 0.1 *M* bicarbonate, pH 8.4 (curve A), or in 0.05 *N* hydrochloric acid (curve B), and the enzymic activity was simultaneously determined in the acidic solution (curve C). Another aliquot was diluted in acid after 48 hr of incubation and the enzymic activity was followed (curve D); the evolution of the peak at 310 nm was the same as in curve B.

proportional to the number of substituted SH groups. Extrapolation of the two straight lines shows that the enzyme is completely inactivated when four SH groups are substituted, in agreement with the existence of four essential thiol groups in yeast alcohol dehydrogenase.

The reactions of both compounds with protein nucleophiles other than SH groups were tested on bovine pancreatic ribonuclease. The inhibition of this enzyme by methyl chlorotetrolate is shown in Fig. 7a. By comparison, with a 20 times higher concentration of chlorotetrolic acid, the other conditions being the same, the loss of activity after 26 hr is only 26%. In both cases the modified proteins show an absorption peak at 310 nm (see Fig. 7b) characteristic of reactions of the triple bond and the chlorine of the reagents with nitrogen groups. This band is stable in alkaline medium but in the case of methyl chlorotetrolate-treated protein it disappears rather easily in slightly acidic solution (Fig. 7c) or even by simple dialysis of the treated protein against acetate buffer, pH 5.6. If the mixture is acidified after 24 hr of incubation, the disappearance of the 310-nm band is accompanied by a reactivation of the enzyme up to 70%. It can be assumed that the inhibition was due to the addition of the NH_2 group of the essential lysine-41 residue of ribonuclease across the triple bond of the ester. To check this supposition we used the known (*20*) possibility of a specific substitution of this single amino group of the enzyme by 2,4-dinitrophenylation.

Figure 8 shows that the reaction of fluoro-2,4-dinitrobenzene with methyl chlorotetrolate-treated ribonuclease is sluggish, but after incubation of the modified protein in acidic medium the reaction again becomes possible and proceeds to about the same extent as with the native enzyme. The fact that the activity of

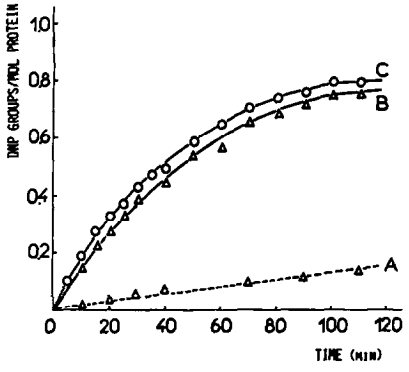


FIG. 8. Reaction of fluoro-2,4-dinitrobenzene with methyl chlorotetrolate-treated ribonuclease before and after reactivation in acidic medium. Curve A: To 0.2 ml of a solution of ribonuclease, treated for 24 hr with methyl chlorotetrolate as described in the legend to Fig. 7a, there were added 1.6 ml of 0.1 *M* bicarbonate, pH 8.4, and 0.2 ml of 0.02 *M* solution of fluoro-2,4-dinitrobenzene in ethanol. The mixture was incubated at room temperature and the binding of dinitrophenyl (DNP) groups was monitored by following the absorption at 360 nm. Curve B: A 0.2-ml sample of methyl chlorotetrolate-treated ribonuclease was diluted 10-fold with 0.05 *M* hydrochloric acid and incubated for 2 hr at room temperature. The pH was then adjusted to 8.4 with 1 *N* sodium hydroxide, 0.2 ml of the fluoro-2,4-dinitrobenzene solution were added and the experiment was continued as above. Curve C: Dinitrophenylation of native ribonuclease under the same conditions.

ribonuclease is not fully restored after acidic treatment could be due to a partial reaction of an essential histidine residue (His-119 or His-12) with the chloromethyl group of the reagent bound to the vicinal Lys-41. Actually, if ribonuclease is incubated with methyl chlorotetrolate for longer periods, the inhibition becomes irreversible; and even after complete hydrolysis of the enamine the enzyme remains inactive. Histidine residues were therefore titrated after 48 hr of incubation. The solution described in Fig. 7 was dialyzed for 24 hr against 0.1 *M* acetate buffer, pH 5.6 (the 310-nm peak disappeared), and free histidine residues were titrated by the diazonium coupling reaction (*21*). An apparent loss of 1.7 histidine residues was found in the treated protein with respect to native ribonuclease. Since the titration method used also detects tyrosine residues, it could be inferred that in addition to one histidine residue, tyrosine was also substituted; and this was confirmed by the similar shapes of the uv spectra of modified ribonuclease both in acidic and alkaline media (not shown) (*22*).

DISCUSSION

Two functions, the triple bond and the chloromethyl group, proved reactive in chlorotetrolic acid and methyl chlorotetrolate. In particular, in the latter product, reaction of the ester group was never observed. For both compounds a comparison was made of the relative reactivities of the two functional groups with different nucleophiles. In methyl chlorotetrolate the reactivity of the triple bond, activated by both the ester group and the chlorine, is high and clearly prevails over that of the chloromethyl group. In the case of chlorotetrolic acid, because of the lower reactivity of the triple bond the overall reaction is slower, and substitution of chlorine is relatively more favored. Possibly formation of a carboxylate anion in the alkaline reaction media retards addition of nucleophiles to the triple bond to a greater extent than affecting displacement of the chlorine. Thus, for example, the reaction of amine seems to be directed toward the two functions in a comparable degree.

Since the two compounds were considered as potential bifunctional protein reagents, most of their reactions were tested on model molecules containing two polar groups analogous to those present in proteins: thiol, amine, imidazole, etc. Compounds **3–5** are models of crosslinking of proximal nucleophiles by chlorotetrolic acid after a primary attack of thiol on chlorine. In the case of cysteine the second step of the reaction can be either intramolecular addition of the NH_2 group on the triple bond or intermolecular reaction of the latter with a SH group of a second cysteine molecule. These two reactions seem to be equally easy, since products **5** and **6a** were isolated in about the same yield. With cysteamine, in which the SH group is more nucleophilic (pK_{SH}: cysteamine, 8.35; cysteine, 8.7 (*23*)), only product **6b** was formed. In the reactions of 5-thiomethylimidazole with chlorotetrolic acid and methyl chlorotetrolate, the models of the first step of SH-imidazole crosslinking, i.e., respectively, the chlorine-substitution product **2** and the triple bond-addition product **7** could be isolated because of the lower reactivity of the imidazole ring. In both cases, however, cyclization by reaction of imidazole with the remaining function follows the primary attack.

The instability of the products of addition of amine across the triple bond, especially pronounced in the case of methyl chlorotetrolate, is an interesting property of this compound. Actually, a reverse situation exists in the case of the known protein reagent fluoro-2,4-dinitrobenzene: all the substitutions of various protein nucleophiles performed with this compound are reversible, except that of NH_2 groups. In this respect the properties of methyl chlorotetrolate are complementary to those of fluoro-2,4-dinitrobenzene.

The low reactivity of chlorotetrolic acid can be a useful feature in investigation of the highly nucleophilic SH groups in proteins, since it allows, for example, a precise determination of the number of essential thiols in enzymes or selective substitutions when many SH groups of different reactivity are present. Other cases in which thiol reagents of low activity are desirable have been described (*24, 25*).

The tests with ribonuclease were in accord with model reactions. The primary attack of methyl chlorotetrolate consists in addition of the reactive NH_2 group of Lys-41 across the triple bond, and this binding is followed by a slower irreversible substitution of chlorine by a vicinal histidine residue in the active site.[5] Blocking of the essential lysine can be reversed by lowering the pH; and, as long as histidine has not been entirely alkylated, partial reactivation of the enzyme is possible. These experiments illustrate the new possibility of exploring the vicinity of protein NH_2 groups after temporary substitution of the latter. Such a procedure utilizing acetylenic bifunctional reagents could probably be generalized.

In conclusion, our aim was to add a new dimension to the use of acetylenic reagents in protein chemistry by considering not only the geometry of such reagents but also the reactivity of the triple bond. Acetylenic compounds appear to be potential agents for chemical modification of proteins able to react with different nucleophiles with formation of chromophoric derivatives and to give reversible substitutions of NH_2 groups which can hardly be achieved with other protein reagents.

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[5] In previous papers we have described an irreversible crosslinking of lysine and histidine in the active site of ribonuclease by reagents having reactive centers at the same distance as methyl chlorotetrolate (*26, 27*).

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