# Modification of Arginines in Trypsin Inhibitors by 1,2-Cyclohexanedione*

Woo-Hoe Liu, Gad Feinstein,† David T. Osuga, Royce Haynes,‡ and Robert E. Feeney

ABSTRACT: 1,2-Cyclohexanedione was employed to chemically modify the arginyl residues of several inhibitors of proteolytic enzymes. The inhibitors were ovomucoids from egg whites of various avian species, a lima bean trypsin inhibitor, and a soybean trypsin inhibitor. Nearly all of the trypsin-inhibitory activity of chicken ovomucoid and of soybean trypsin inhibitor was abolished by modification with 1,2-cyclohexanedione. The trypsin-inhibitory activities of these two inhibitors were not destroyed by modification of their amino groups. Only small losses in trypsin-inhibitory activity were detected with lima bean trypsin inhibitor and with turkey, duck, rhea, cassowary, and ostrich ovomucoids by modification with 1,2-cyclohexanedione. The trypsin-inhibitory activities of these six inhibitors were destroyed by modification of their amino groups. During modification with 1,2-cyclohexanedione a yellow color appeared, even with proteins containing no arginine. This appears to be due to the reaction of 1,2-cyclohexanedione with the ε-amino groups of lysine in proteins. This side reaction explains the small losses in trypsin inhibitory activity of those ovomucoids in which the ε-amino groups have been found to be essential. This explanation was confirmed by spectral studies of native chicken ovomucoid, 1,2-cyclohexanedione chicken ovomucoid, amidinated and guanidinated chicken ovomucoid, 1,2-cyclohexanedione-amidinated chicken ovomucoid and 1,2-cyclohexanedione lysine, and also by amino group determination using 2,4,6-trinitrobenzenesulfonic acid.

The reagent 1,2-cyclohexanedione (CHD)[1] has been reported as a reagent for modifying the guanidino groups of arginyl residues in proteins (Toi *et al.*, 1965, 1967). The peptide bonds involving the carboxyl groups of the modified arginyl residues were not susceptible to hydrolysis by trypsin. These authors suggested CHD as a reagent for modifying arginyl residues in proteins prior to tryptic hydrolysis in order to restrict the specificity of trypsin to the lysyl bonds. Although the reaction was done in 0.2 N sodium hydroxide, exposure to such strongly alkaline conditions apparently did not interfere with subsequent tryptic hydrolyses for peptide mapping.

Both lysyl and arginyl residues have been implicated as important for the interaction of the protein inhibitors with trypsin. A variety of trypsin inhibitors have been reported to be inactivated by chemical modification of their amino groups (Fraenkel-Conrat *et al.*, 1952; Haynes and Feeney, 1967, 1968; Haynes *et al.*, 1967). With several avian ovomucoids and with a lima bean inhibitor, one particular amino group was shown to be essential for the interaction with trypsin (Haynes *et al.*, 1967; Haynes and Feeney, 1967). Finkenstadt and Laskowski (1965) and Ozawa and Laskowski (1966) have shown the enzymatic release of arginine from chicken ovomucoid and from a soybean trypsin inhibitor (STI) by sequential treatment with trypsin and carboxypeptidase B. In the modification studies of Haynes *et al.* (1967) chicken ovomucoid and STI were not affected by modification of their amino groups.

We have used CHD to study the essentiality of arginyl residues in inhibitors of trypsin. The inhibitors used in this study include several which have previously been shown to require lysyl residues and therefore should not be sensitive to modification with CHD, and two that were found to be insensitive to the reagents for lysine and might therefore be inactivated by this reagent. In the course of this investigation, certain modifications of the methods of Toi *et al.* (1965, 1967) were developed; a partial characterization of a side reaction in the modification was made.

## Materials and Methods

*Materials.* Salt-free crystalline preparations of bovine trypsin and α-chymotrypsin were purchased from Worthington Biochemical Corp. Crystalline STI and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were from Nutritional Biochemicals Corp. The ovomucoids used in this study were isolated from the egg whites of chicken (*Gallus gallus*), cassowary (*Casuarius aruensis*), tinamou (*Eudromia elegans*), duck (*Anas platyrhynchos*), turkey (*Maleagris gallopavo*), emu (*Dromiceius* n. *hollandiae*), rhea

* From the Department of Food Science and Technology, University of California, Davis, California 95616. *Received January 8, 1968.* Supported in part by U. S. Public Health Service Grant HD-00122-04. Part of the material was taken from theses of G. F., R. H., and W. H. L., submitted to the Graduate Division of the University of California in partial fulfillment of M.S. and Ph.D. degrees.

† Permanent address: Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel.

‡ Present address: Department of Biochemistry, University f Washington, Seattle, Wash. 98105.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CHD, 1,2-cyclohexanedione; STI, soybean trypsin inhibitor; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

(*Rhea americana*), and ostrich (*Struthio camelus*). All of these ovomucoids were prepared by trichloroacetic acid–acetone precipitation (one volume of 0.5 M trichloroacetic acid in water plus two volumes of acetone) according to the method of Lineweaver and Murray (1947) followed by CM- and DEAE-cellulose chromatography (Rhodes *et al.*, 1960; Feeney *et al.*, 1963; Osuga and Feeney, 1968). Lima bean trypsin inhibitor was the fraction corresponding to peak 6 from the DEAE-Sephadex chromatography of Haynes and Feeney (1967). The following materials were purchased: CM- and DEAE-cellulose, from Whatman Co.; CHD, from J. T. Baker Chemical Co.; and *O*-methylisourea sulfate, from K & K Laboratories. Ethyl acetimidate was synthesized according to the method of McElvain and Nelson (1942) and stored in an evacuated desiccator. The trypsin substrate, *p*-tosyl-L-arginine methyl ester, was purchased from Sigma Chemical Co. The chymotrypsin substrate, benzoyl-L-tyrosine ethyl ester, was prepared according to the method of Fox (1946) for the synthesis of benzoyldiiodotyrosine ethyl ester.

*Assays for Inhibitory Activities against Trypsin and α-Chymotrypsin.* Trypsin and α-chymotrypsin assays were done spectrophotometrically according to the methods of Rhodes *et al.* (1957, 1960) and Feeney *et al.* (1963). The indicator was *m*-nitrophenol. The enzymic activities were measured by the rate of change in per cent transmission; a Bausch & Lomb Spectronic 600 with a recorder was used. The per cent inhibition of trypsin or α-chymotrypsin by the ovomucoids or STI was determined from the residual trypsin or α-chymotrypsin activity. Routinely, 0.3 ml of a solution containing 24–28 μg of trypsin or α-chymotrypsin in 0.004 M acetic acid and 0.02 M $CaCl_2$ was added to 0.7 ml of a solution containing 2–28 μg of inhibitor in 0.006 M Tris buffer (pH 8.2). The mixture was incubated for at least 3 min, then 2 ml of a solution containing substrate (0.0092 M) and indicator (0.012%) in 0.006 M Tris buffer (pH 8.2) was added. Change in per cent transmission at 395 mμ was followed by the recorder.

*Determination of Arginine.* The arginyl residues in the native and modified inhibitors were determined by a modified Sakaguchi reaction (Izumi, 1965a,b). No major change has been made except for doubling the amounts of reagents and samples.

*Amino Acid Analyses.* Protein samples were hydrolyzed in 6 N HCl in sealed tubes for 22 hr at 110° (Moore and Stein, 1962). The amino acid compositions of the hydrolysates were determined by ion-exchange chromatography using a Technicon Autoanalyzer.

*Chemical Modification of Inhibitors with CHD.* The arginyl residues of the inhibitors were chemically modified using the reagent CHD (Toi *et al.*, 1965, 1967). The inhibitors (10–25 mg) were dissolved in 4.5 ml of 0.1 M buffer (triethanolamine buffer was used for modification at pH 7–9; triethylamine buffer, at pH 10–12). CHD (10 mg) in 0.5 ml of water was added. In the control, 0.5 ml of water without CHD was added. The reactions were allowed to proceed at room temperature in the dark for 12 hr. The reaction mixtures were then dialyzed in the cold against deionized water for 24–48 hr and lyophilized. The lyophilized proteins were assayed for their inhibitory activities against trypsin or α-chymotrypsin, or both.

*Determination of Free Amino Groups.* The determination of free amino groups was done essentially according to the methods of Habeeb (1966) and Haynes *et al.* (1967).

*Preparation of the Product of CHD and Arginine.* Arginine-HCl (105 mg) and CHD (80 mg) were dissolved in 10 ml of 2.0 N NaOH and the reaction was allowed to proceed overnight. The reaction was stopped by neutralizing with 1 N HCl. The mixture was then transferred to a Dowex 50-X2 column previously equilibrated with 0.1 N HCl. The column was eluted with $H_2O$ to wash off the unreacted CHD and was then eluted with 1 N $NH_4OH$. The eluate was heated gently to evaporate the $NH_3$ and lyophilized. The residue obtained after lyophilization, which contained more than 90% of the product of CHD-arginine, was dissolved in 50% ethanol; acetone was added until the solution became turbid. The product crystallized in the refrigerator after about 10 min. The CHD-arginine was collected by filtration and dried in a desiccator for 48 hr. The structure of CHD-arginine has been previously identified by independent chemical synthesis to be $N^5$-(4-oxo-1,3-diazaspiro[4,4]non-2-ylidene)-L-ornithine (Toi *et al.*, 1967). No attempt was made to identify our product.

Arginine and CHD-arginine were compared by paper chromatography on Whatman No. 4 filter paper. The chromatograms were developed with 80% phenol–water for 16 hr at room temperature and were then dried. The spots were located using ninhydrin spray. The $R_F$ of the CHD-arginine was 0.770 and the $R_F$ of arginine was 0.385.

*Spectral Studies.* Absorption spectra of proteins and their modified derivatives were measured using a Cary Model 15 spectrophotometer. All protein samples, unless otherwise indicated, were dissolved in 0.1 M sodium phosphate buffer (pH 7.2) and the spectra of the samples were measured against this same buffer at a spectral range from 550 to 250 mμ.

*Starch gel electrophoresis* was done using the standard procedure used in this laboratory (Osuga and Feeney, 1967). The starch gel was made with Tris–citrate buffer (pH 5.1) and the bridge buffer was succinic acid–NaOH (pH 5.0). Electrophoresis was run in the cold at 450 V and 100–125 mA. The stain used was 0.5% aniline blue or nigrosine.

*Amidination of Chicken Ovomucoid.* Chicken ovomucoid (50 mg) was dissolved in 10 ml of 0.1 M sodium borate buffer (pH 9.5) and 0.5 M *O*-ethyl acetimidate. The solution was incubated at 4° for 6 hr, then dialyzed, and lyophilized.

*Guanidination of Chicken Ovomucoid.* Chicken ovomucoid (50 mg) was dissolved in 10 ml of 0.5 M *O*-methylisourea (pH 9.5). The solution was incubated at 25° for 60 hr, then dialyzed, and lyophilized.

## Results

*Selection of Conditions for Reaction with CHD.* In initial studies with chicken ovomucoid and STI, extensive losses of activity were found in controls without

TABLE I: Effect of Modification[a] with 1,2-Cyclohexanedione on the Inhibitory Activities of Proteolytic Enzyme Inhibitors.

| Types of Inhibitors | Inhibitory Activities Remaining[b] | |
|---|---|---|
| | % of Trypsin | % of Chymotrypsin |
| Trypsin | | |
| Chicken ovomucoid | 19 | *d* |
| Amidinated chicken ovomucoid[c] | 10 | *d* |
| Guanidinated chicken ovomucoid[c] | 10 | *d* |
| STI | 20 | *d* |
| Cassowary ovomucoid | 76 | *d* |
| Lima bean inhibitor[e] | 72 | *f* |
| Chymotrypsin | | |
| Tinamou ovomucoid | *d* | 83 |
| Trypsin and Chymotrypsin | | |
| Duck ovomucoid | 75 | 100 |
| Ostrich ovomucoid | 67 | 100 |
| Turkey ovomucoid | 69 | >95 |
| Rhea ovomucoid | 75 | 100 |
| Emu ovomucoid | 100 | 100 |

[a] All these modification reactions were carried out at pH 11 using 0.1 M triethylamine buffer. [b] The values for STI and chicken, duck, and ostrich ovomucoids are averages of triplicate determinations. The values for cassowary, turkey, and rhea ovomucoids are averages of duplicates. Others are single values. [c] Amidinated chicken ovomucoid contains 0.7% of amino groups, while guanidinated chicken ovomucoid contains 4.4% of amino groups, as determined by TNBS. [d] These inhibitors either do not inhibit the indicated enzyme or inhibit them very weakly. [e] This was fraction 6 of Haynes and Feeney (1967). [f] Not tested.

CHD at pH values of 12 and above. Reaction conditions, therefore, had to be found which would be less drastic than 0.2 N sodium hydroxide used by Toi *et al.* (1965, 1967). In early attempts with reactions at pH 10 or 11 extensive inactivation of the control samples was still observed, possibly due, in part, to inactivation by traces of metal ions (Feeney *et al.*, 1956). A buffer of 0.1 M carbonate–bicarbonate (pH 10) containing 0.01 M EDTA was found to give little or no loss of inhibitory activities of either chicken ovomucoid or of STI when incubated for several hours at room temperature. Under the latter conditions approximately two-thirds of the inhibitory activity was destroyed in the presence of the reagent while only about 20% loss of activity occurred in the control. Attempts to use this particular buffer at pH 11 were not successful.

The triethylamine buffer at pH 11 was found to be superior to the carbonate–EDTA buffer. Samples were incubated at room temperature in the dark for 12 hr. Chicken ovomucoid and STI both lost more than 80% of their activity with reagent, while controls retained full activity. At pH 12 complete inactivation occurred in the absence of reagent. Lower pH values (9 and 10) were unsatisfactory, giving much lower degrees of modification. In the case of chicken ovomucoid, the inhibitory activities were 67% at pH 9 and 45% at pH 10, as compared with 15% activity at pH 11.

*Effect of Modification with CHD on Inhibitory Activities.* Chicken ovomucoid and STI showed extensive loss of activity after modification with CHD (Table I, Figures 1 and 2). The plots of amount of inhibitor added *vs.* the residual trypsin activity were more similar to those expected from a mixture of completely inactive inhibitor and a small amount of active inhibitor than they were to those expected from an inhibitor with weak activity.[2] Activities of these particular preparations of modified inhibitors were calculated to be 16% for the CHD chicken ovomucoid and 8% for the CHD-STI.[3]

In addition to chicken ovomucoid and STI, several other inhibitors with different specificities were modified with CHD. These were another trypsin inhibitor, cassowary ovomucoid; a chymotrypsin inhibitor, tinamou ovomucoid; and six inhibitors which inhibit both trypsin and chymotrypsin, a lima bean inhibitor fraction and duck, ostrich, turkey, rhea, and emu ovomucoids. Only chicken ovomucoid and STI showed extensive loss of trypsin-inhibitory activity. With the exception of tinamou ovomucoid, which showed a 17% loss of activity against α-chymotrypsin, the other inhibitors showed no losses of activity against α-chymotrypsin. Some losses against trypsin were noted, however, varying from no detectable loss to as much as 33%. All of these inhibitors have been classified as lysine inhibitors since they are inactivated by reagents which modify amino groups. As will be described below, the losses of activity in these lysine inhibitors was most likely due to reaction of the reagent with amino groups.

*Effect of Modification of Inhibitors with CHD on the Amino Acid Contents.* Table II lists the amino acid contents of the control and the CHD-modified chicken ovomucoid, STI, lima bean inhibitor, and turkey ovomucoid. For chicken ovomucoid and STI, comparisons are given for modifications in the bicarbonate buffer and the triethylamine buffer. The only amino acid showing appreciable change was arginine, which showed approx-

[2] In studies currently underway in this laboratory comparisons are being made of the properties of inhibitors with weak and strong inhibitory activities. It is admittedly difficult to distinguish between a preparation with inherently low inhibitory activity and one with low activity due to contamination of mostly inert protein with a strong inhibitor (Feeney *et al.*, 1963).

It is also possible that an inhibitor might have two active inhibitory sites, a strong site, which is normally operative, and a weak site, which is normally inactive. If chemical modification were to destroy the normally functional strong site without affecting the normally nonfunctional weak site, then the inhibitor might be changed from a strong inhibitor to a weak inhibitor.

[3] In a detailed study on one arginine trypsin inhibitor, comparable losses of activity after modification with CHD have been found by both esterolytic and casein assays.

TABLE II: Amino Acid Composition of 1,2-Cyclohexanedione-Modified Proteins.[a]

| | Residues of Amino Acid/10,000 g | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Chicken Ovomucoid[b] | | | STI | | | Turkey Ovomucoid[b] | | Lima Bean Inhibitor | |
| Amino Acid | Control | Modified | | Control | Modified | | Control | Modified | Control | Modified |
| Aspartic acid | 15.6[c] | 15.4[c] | 15.7 | 14.1[c] | 14.7[c] | 13.4 | 13.4[c] | 13.6[c] | 15.1[c] | 15.8[c] |
| Threonine | 7.1 | 6.3 | 7.2 | 3.6 | 3.8 | 3.5 | 7.0 | 7.0 | 5.0 | 5.3 |
| Serine | 5.2 | 5.3 | 5.1 | 5.2 | 5.5 | 5.9 | 4.5 | 4.7 | 13.8 | 14.4 |
| Glutamic acid | 7.5 | 7.6 | 7.9 | 11.3 | 12.1 | 11.3 | 10.0 | 10.4 | 8.5 | 8.8 |
| Glycine | 7.7 | 7.6 | 6.9 | 8.7 | 9.1 | 8.7 | 8.3 | 8.3 | 1.6 | 1.7 |
| Alanine | 5.5 | 5.6 | 5.5 | 4.2 | 4.5 | 4.2 | 4.1 | 4.3 | 3.6 | 3.6 |
| Valine | 7.3 | 7.6 | 7.3 | 6.4 | 6.9 | 6.4 | 7.2 | 7.5 | 1.3 | 1.4 |
| Cystine (half) | 8.3 | 8.3 | 8.3 | 2.2 | 1.6 | 2.6 | 8.0 | 7.9 | 15.9 | 15.4 |
| Methionine | 0.9 | 0.9 | 0.9 | 1.4 | 1.3 | 1.6 | 1.0 | 1.0 | 0 | 0 |
| Isoleucine | 1.4 | 1.5 | 1.6 | 6.8 | 7.3 | 6.6 | 2.1 | 2.1 | 4.7 | 4.9 |
| Leucine | 5.5 | 5.7 | 5.1 | 7.7 | 8.1 | 7.7 | 6.7 | 6.8 | 3.9 | 4.0 |
| Tyrosine | 2.7 | 2.6 | 2.6 | 2.1 | 2.0 | 2.1 | 3.3 | 3.1 | 1.1 | 0.9 |
| Phenylalanine | 2.4 | 2.5 | 2.5 | 4.6 | 4.9 | 4.3 | 1.5 | 1.5 | 1.7 | 1.8 |
| Lysine | 6.4 | 6.0 | 5.6 | 5.6 | 5.6 | 4.9 | 5.7 | 5.3 | 5.0 | 4.9 |
| Histidine | 2.0 | 2.1 | 2.1 | 1.1 | 1.9 | 1.2 | 2.5 | 2.5 | 6.7 | 7.1 |
| Arginine[d] | 3.0 | 1.2 | 1.2 | 4.8 | 2.4 | 2.4 | 2.9 | 1.2 | 2.5 | 1.2 |

[a] Samples were modified under the conditions described in the text, except column *c*. [b] Values uncorrected for carbohydrate which amounts to about 25% of the weight of chicken ovomucoid. [c] The proteins were modified with CHD in 0.1 M carbonate–bicarbonate buffer (pH 10) plus 0.01 M EDTA. [d] Arginine contents in proteins corresponded to the values obtained by the Sakaguchi method.

imately a 60% decrease for chicken ovomucoid and a 50% decrease for STI.

Losses in arginine content were found to be of a similar order of magnitude using the Sakaguchi reaction with protein standards containing different amounts of arginine. The arginine content in the CHD chicken ovomucoid was 41% by amino acid analysis and 45% by the Sakaguchi reaction. Similar comparisons for the STI and CHD-treated STI gave a value of 50% by amino acid analysis and 52% by the Sakaguchi reaction.

Native chicken ovomucoid, STI, and their CHD-modified derivatives were compared on starch gel electrophoresis. The CHD-modified proteins showed greater anodic mobilities than the native ones.

*Characterization of Side Reaction Accompanying CHD Modification.* Modifications of the inhibitors with CHD was accompanied by a side reaction which gave the modified proteins a yellow color with an absorption maximum at 440 mμ. This yellow color was absent when the proteins were modified under the condition of Toi *et al.* (1967) or when chicken ovomucoid was amidinated

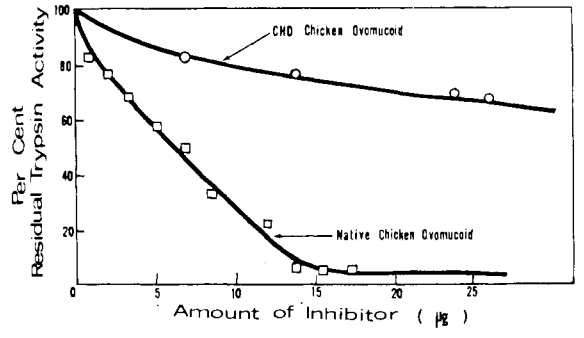


FIGURE 1: The trypsin-inhibitory activity of chicken ovomucoid modified with 1,2-cyclohexanedione. 1,2-Cyclohexanedione modification of chicken ovomucoid and assay for trypsin-inhibitory activity are described in text.

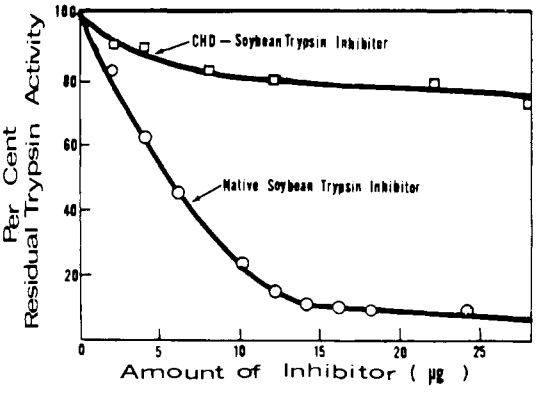


FIGURE 2: The trypsin-inhibitory activity of soybean trypsin inhibitor modified with 1,2-cyclohexanedione. 1,2-Cyclohexanedione modification of soybean trypsin inhibitor and the assay for trypsin inhibitory activity are described in text.

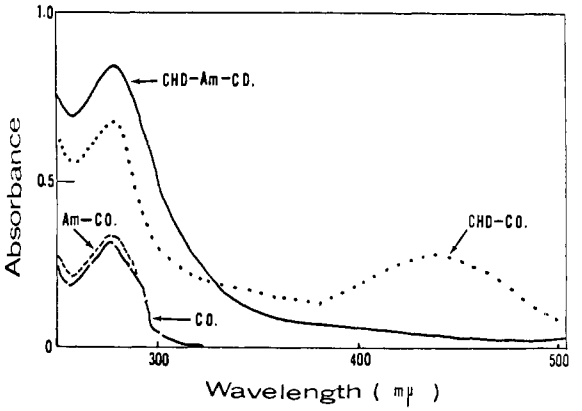


FIGURE 3: Absorption spectra of chicken ovomucoid and derivatives modified with 1,2-cyclohexanedione. Amidination of native chicken ovomucoid is described in text. Native and amidinated chicken ovomucoids were modified with 1,2-cyclohexanedione at pH 11 in 0.1 M triethylamine buffer. The concentration of protein was 1.0 mg/ml. (— —) Native chicken ovomucoid (CO); (- - - - - -) amidinated chicken ovomucoid (Am-CO); (· · · · · · · ·) CHD chicken ovomucoid (CHD-CO); (———) CHD-amidinated chicken ovomucoid (CHD-Am-CO).

(Figure 3) or guanidinated before CHD treatment. The intensity of the yellow color was dependent upon the pH during CHD modification. The absorption at 440 mμ formed by modification at different pH values increased from pH 7 to 11 and then decreased to nearly zero in 0.2 N NaOH (Table III). This yellow color also occurred on modification of rhea and emu ovomucoids, which contain no arginine (Figure 4).

A reaction of amino groups with CHD was evident from the spectrum of CHD-treated lysine (Figure 5). A peak at 440 mμ was found for CHD-treated lysine, but not for CHD-treated tyrosine, histidine, or arginine. A loss of amino groups was also found, when chicken ovomucoid was modified with CHD (Table IV). Similar losses of amino groups were found with the other inhibitors. These losses of amino groups ranging from 18 to 33% were not revealed by amino acid analysis. The lysine contents of the native and CHD-treated chicken ovomucoid and STI remained essentially the same. This might be due to the instability to acid hydrolysis of the product formed between CHD and amino groups. The losses in amino groups in the lysine inhibitors were almost directly proportional to the losses in inhibitory activities against trypsin.

TABLE III: Change in Absorption at 440 mμ Accompanying the Side Reaction in the Modification of Chicken Ovomucoid with 1,2-Cyclohexanedione.

| | Absorbance at 440 mμ[b] |
|---|---|
| CHD-arginine[a] | 0 |
| Chicken ovomucoid modified with CHD at | |
| pH 7 | <0.02 |
| pH 8 | 0.105 |
| pH 10 | 0.300 |
| pH 11 | 0.280 |
| pH 12 | 0.150 |
| 0.2 N NaOH | <0.02 |
| 1.0 N NaOH | <0.02 |

[a] CHD-arginine was obtained by treating arginine with CHD in 2.0 N NaOH; for details, see text. [b] Concentrations were 1 mg/ml in 0.1 M sodium phosphate buffer (pH 7.2).

TABLE IV: Losses of Amino Groups[a] in Chicken Ovomucoid after Modification with 1,2-Cyclohexanedione.

| Inhibitor | No. of Amino Groups Found | No. of Amino Groups Modified | % Amino Groups Modified[b] |
|---|---|---|---|
| Chicken ovomucoid, native | 14 | 0 | 0 |
| Chicken ovomucoid modified with CHD at | | | |
| pH 9 | 11.5 | 2.5 | 18 |
| pH 10 | 9.8 | 4.2 | 30 |
| pH 11 | 8.4 | 5.6 | 40 |

[a] Amino group determination was done using the reagent TNBS. [b] The values for the losses of amino groups in other inhibitors after modification at pH 11 were: 27% for cassowary ovomucoid, 23% for soybean trypsin inhibitor, 30% for tinamou ovomucoid, 18% for turkey ovomucoid, 33% for rhea ovomucoid, and 28% for ostrich ovomucoid.

The spectrum of CHD-arginine in 0.1 M sodium phosphate (pH 7.2) showed extensive absorption in the ultraviolet with a maximum at 213 mμ, which was very different from the spectrum for arginine. CHD chicken ovomucoid showed two absorption peaks (Figure 3). The absorption peak at 440 mμ is responsible for the observed yellow color. The second absorption maximum is at 278 mμ, the normal wavelength for native chicken ovomucoid, but it is much higher than that of either native or amidinated chicken ovomucoid. The increased absorbance at 278 mμ was less for CHD rhea and CHD emu ovomucoids than for CHD chicken ovomucoid (Figure 4). The nature of the reaction products between CHD and the arginyl groups in proteins at pH 11 have not been identified. New products corresponding to the losses of arginine were not found in amino acid analysis of acid hydrolysates.

## Discussion

The inactivation of protein inhibitors of trypsin by modification of either arginyl residues or lysyl residues

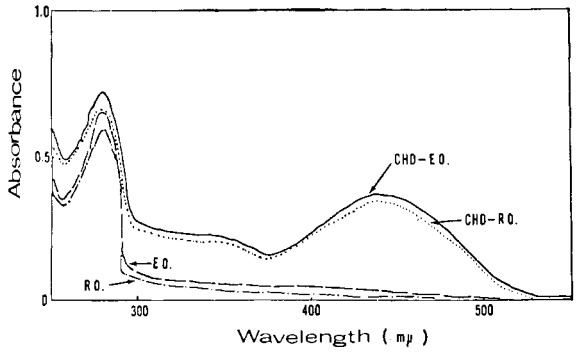


FIGURE 4: Absorption spectra of rhea and emu ovomucoids modified with 1,2-cyclohexanedione. Rhea and emu ovomucoids were modified with 1,2-cyclohexanedione at pH 11 in 0.1 M triethylamine buffer. The spectra were measured on Cary Model 15 spectrophotometer against 0.1 M phosphate buffer (pH 7.2) in which they were dissolved. (———) CHD-emu ovomucoid (CHD-EO); (· · · · · ·) CHD-rhea ovomucoid (CHD-RO); (— —) native emu ovomucoid (EO); (– · – · –) native rhea ovomucoid (RO).

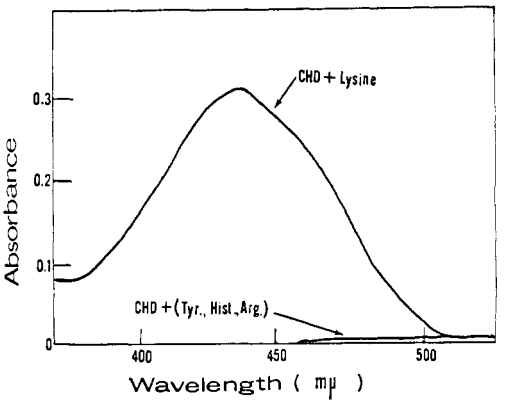


FIGURE 5: Absorption spectra of amino acids modified with 1,2-cyclohexanedione. Lysine, tyrosine, histidine, and arginine (5 mg) were individually incubated with CHD (5 mg) in 0.1 M triethylamine buffer pH 11 in the dark at room temperature for 16 hr. Spectra were measured against controls in which the amino acid was omitted.

has now been established. It would appear that these trypsin inhibitors may thus be classified as either arginine inhibitors or lysine inhibitors. The two most studied trypsin inhibitors, chicken ovomucoid and STI, have been shown in this study to lose their activities when the arginyl residues are modified with CHD. A variety of other trypsin inhibitors have been shown to require amino groups, and hence lysyl residues, rather than arginyl residues, are the functional groups (Haynes *et al.*, 1967). It has also been shown in this study that inactivations of lysine inhibitors by the arginyl reagent, CHD, occurs to only a partial extent and that this inactivation can be explained by a side reaction with essential lysines. In turn, the arginine inhibitors have been found to be relatively insensitive to modification of their amino groups. With several lysine inhibitors it has been possible to demonstrate that only one particular amino group is essential for inhibitory activity (Haynes *et al.*, 1967; Haynes and Feeney, 1967). A similar determination of a particular arginine or particular arginines was not done in the present study for the arginine-type inhibitors. Such an attempt is under way, but it would appear that the method employed for arginine modification may not be applicable for such time-course studies.

In the present study it was possible to modify the method of Toi *et al.* (1965, 1967) to retain nearly 100% activity in controls from which the reagent had been omitted. This required a particular buffer, triethylamine, and a pH of 11. As recognized by Toi *et al.* (1967), other reactions occurred under these conditions. They reported as many as four products between arginine and CHD in 0.05 N NaOH. In our studies there appeared to be at least two reactions. One resulted in a yellow color with a maximum extinction at 440 mμ. The yellow color of the products was associated with the reaction with amino groups. This was shown by the relationship between the numbers of amino groups modified and the corresponding (partial) losses in inhibitory activities of lysine inhibitors. In addition, the yellow color was definitely not associated with modification of the arginyl groups because two homologous ovomucoids, rhea and emu ovomucoids, are devoid of arginine and yet both showed nearly identical development of the yellow color on modification with CHD. The use of these ovomucoids is a further example of a successful application of the use of homologous proteins to determine the relationship between the structures and properties of proteins. Masking of amino groups by amidination or guanidination prior to treatment with the reagent prevented development of the color. Finally, in a mixture of several amino acids, only lysine gave the yellow color upon treatment with the reagent. No losses of lysine were observed in acid hydrolysates of treated proteins. This observation can easily be explained on the basis of the instability of the reaction product between the ε-amino group and the CHD. One of the most likely products between the reagent and amino groups could be the aldolamine. These compounds are readily hydrolyzed by acid. The second reaction noted was a small but definite increase in the extinction at 278 mμ.

The reaction responsible for the modification of arginine at pH 11 was not characterized. Toi *et al.* (1967) detected a new peak on chromatographic amino acid analysis of proteins treated with CHD in 0.2 N NaOH. This peak corresponded to the peak obtained on chromatography of the CHD-arginine product synthesized in 2 N NaOH. No evidence for such a product was obtained under our conditions of amino acid analysis.

Our studies have shown that the reagent, CHD, can be used quite successfully for the modification of arginyl residues in proteins which are stable at high pH. With proteins in which modifications of amino groups would be a serious side reaction, these groups might be reversibly blocked by some suitable reagent. However, the apparently successful use of TNBS for the rapid determination of numbers of amino groups remaining after treatment with the CHD, makes it possible to monitor easily the side reaction of amino groups. Of course, under conditions where retention of native structure is not essential, *e.g.*, peptide mapping, the 0.2 M NaOH

used by Toi *et al.* (1965, 1967) apparently avoids the reaction with amino groups. In an associated study it has been shown that CHD can also be used to modify homoarginine residues introduced into proteins by guanidination of ϵ-amino groups of lysine (Haynes and Feeney, 1968). This modification of chemically introduced guanidyl groups offers a novel approach to chemical modification procedures for proteins. It suggests that the introduction of guanidyl groups on other amino acid side chains followed by modification of these with the CHD may prove useful. It is obvious, however, that many proteins cannot be exposed to the alkaline conditions employed in this study. Further studies are planned on the possible use of the CHD under different conditions as well as the continued search for other methods of modifying arginines in proteins.

Acknowledgments

Appreciation is due to Gary Means and Dr. John R. Whitaker for helpful advice, and Mrs. Judy F. Miller and Mrs. Caroline O. Ouchida for technical assistance.

References

Feeney, R. E., MacDonnell, L. R., and Ducay, E. D. (1956), *Arch. Biochem. Biophys. 61*, 72.

Feeney, R. E., Stevens, F. C., and Osuga, D. T. (1963), *J. Biol. Chem. 238*, 1415.

Finkenstadt, W. R., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, PC962.

Fox, S. W. (1946), *J. Am. Chem. Soc. 68*, 194.

Fraenkel-Conrat, H., Bean, R. C., Ducay, E. D., and Olcott, H. S. (1952), *Arch. Biochem. Biophys. 37*, 393.

Habeeb, A. F. S. A. (1966), *Anal. Biochem. 14*, 328.

Haynes, R., and Feeney, R. E. (1967), *J. Biol. Chem. 242*, 5378.

Haynes, R., and Feeney, R. E. (1968), *Biochemistry 7*, 2879 (this issue; preceding paper).

Haynes, R., Osuga, D. T., and Feeney, R. E. (1967), *Biochemistry 6*, 541.

Izumi, Y. (1965a), *Anal. Biochem. 10*, 218.

Izumi, Y. (1965b), *Anal. Biochem. 12*, 1.

Lineweaver, H., and Murray, C. W. (1947), *J. Biol. Chem. 171*, 565.

McElvain, S. M., and Nelson, J. W. (1942), *J. Am. Chem. Soc. 64*, 1825.

Moore, S., and Stein, W. H. (1962), *Methods Enzymol. 6*, 819.

Osuga, D. T., and Feeney, R. E. (1967), *Arch. Biochem. Biophys. 118*, 340.

Osuga, D. T., and Feeney, R. E. (1968), *Arch. Biochem. Biophys. 124*, 560.

Ozawa, K., and Laskowski, M., Jr. (1966), *J. Biol. Chem. 241*, 3955.

Rhodes, M. B., Bennett, N., and Feeney, R. E. (1960), *J. Biol. Chem. 235*, 1686.

Rhodes, M. B., Hill, R. M., and Feeney, R. E. (1957), *Anal. Chem. 29*, 376.

Toi, K., Bynum, E., Norris, E., and Itano, H. A. (1965), *J. Biol. Chem. 240*, PC3455.

Toi, K., Bynum, E., Norris, E., and Itano, H. A. (1967), *J. Biol. Chem. 242*, 1036.