Chemical Modification of Avian Ovomucoids*

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Comparative physical and chemical studies were performed on three avian ovomucoids: chicken ovomucoid (a trypsin inhibitor), turkey ovomucoid (a trypsin and chymotryps ininhibitor), and pheasant ovomucoid (a chymotrypsin inhibitor). The amino acid composition and the carbohydrate contents have been determined. All three ovomucoids are stable to heating at 80° at pH < 9, but heating to 100° at pH 6 or to 80° at pH 9 results in a rapid loss of inhibitory activity for all three ovomucoids. They are also resistant to treatment with trichloroacetic acid–acetone and 9 M urea at 80°. Their complexes with the respective enzymes are not affected by 3 M urea. Extensive iodination has no effect on any of the inhibitory activities. Acetylation with acetic anhydride or carbamylation with KNCO destroy the trypsin inhibitory activity of turkey ovomucoid, while they leave its chymotrypsin inhibitory activity and the inhibitory activities of the other ovomucoids unaffected. It appeared that the structural requirements of turkey and chicken ovomucoids for inhibiting trypsin are different and that the inhibitory sites of turkey ovomucoid for trypsin and for chymotrypsin are independent.

Avian egg-white ovomucoids are members of a large and diverse group of proteins which have the capacity to inhibit proteolytic enzymes (Laskowski and Laskowski, 1954). Recently it has been reported that ovomucoids isolated from the egg whites of different avian species have different specificities (Rhodes *et al.*, 1960). Some inhibit trypsin (e.g., chicken ovomucoid); others are essentially specific for chymotrypsin (e.g., pheasant[1] ovomucoid); and some can inhibit both trypsin and chymotrypsin independently or simultaneously (e.g., turkey ovomucoid). These last are termed "double-headed" inhibitors and are believed to have two independent inhibitory sites (Rhodes *et al.*, 1960). Inhibitors with different specificities have also been obtained from other sources [*Ascaris lumbricoides* (Peanasky and Laskowski, 1960), soybean (Birk, 1961), sheep serum (Martin, 1962) and potato (Ryan ond Balls, 1962)], but in contrast to the ovomucoids the physical and chemical properties of these differ considerably from one another.

All the ovomucoids studied have been shown to be very similar to chicken ovomucoid in their physical and chemical properties (Rhodes *et al.*, 1960). Chicken ovomucoid has never been crystallized, but it has been highly purified and studied extensively. It has a molecular weight of approximately 28,000. (Lineweaver and Murray, 1947; Fredericq and Deutsch, 1949), and is a mucoprotein containing about 20% carbohydrate (Hartley and Jevons, 1962; Chatterjee and Montgomery, 1962). The specificity of chicken ovomucoid for trypsin has been questioned, but definite evidence has recently been presented to show that it inhibits bovine trypsin but has no demonstrable activity against bovine chymotrypsin (Feeney *et al.*, 1963). Chicken ovomucoid apparently also inhibits porcine and ovine trypsin (Vithayathil *et al.*, 1961) but not human trypsin (Buck *et al.*, 1962).

Inhibition of proteolytic enzymes by ovomucoids has been shown to be accompanied by the formation of a complex between enzyme and inhibitor. These complexes are demonstrable by physical means such as electrophoresis and ultracentrifugation (Rhodes *et al.*, 1960; Ram *et al.*, 1954; Feeney *et al.*, 1963). The ovomucoid-enzyme complex is therefore a good system for the study of protein-protein interaction. The variations in specificity of the ovomucoids with the different enzymes add to the attractiveness of this system. It is possible that these differences in specificity could eventually be related to small differences in structure of the various ovomucoids and in this way provide important information about the relation of structure to function in protein. The present paper gives comparative chemical analyses and also compares the effects of physical and chemical treatments on the inhibitory activities of three ovomucoids with different specificities.

EXPERIMENTAL PROCEDURES

Materials.—Salt-free crystalline preparations of bovine trypsin and chymotrypsin were purchased from Nutritional Biochemical Corporation and Worthington Biochemical Company, respectively. Both were used without further purification. The trypsin preparation was estimated to have approximately 75% the activity of pure trypsin (Rhodes *et al.*, 1957). The different ovomucoids were prepared as described earlier by trichloroacetic acid–acetone (1 vol 0.5 M trichloroacetic acid in water + 2 vol acetone) precipitation (Lineweaver and Murray, 1947), CM-cellulose[2] chromatography (Rhodes *et al.*, 1960), or a combination of both (Feeney *et al.*, 1963). The CM-cellulose and DEAE-cellulose were obtained from the Whatman Company. The trypsin substrate, TAME, was purchased from the Mann Research Laboratories. BTEE, the sub-

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[1] Pheasant ovomucoid. The ovomucoid used in this study was isolated from the egg whites of the offspring of a cross of golden pheasant with Lady Amherst pheasant. This ovomucoid is similar to that of golden pheasant (Rhodes *et al.*, 1960). It is an inhibitor of chymotrypsin but has very weak activity against trypsin. When measured with the protein substrate casein it shows complete specificity for chymotrypsin, but when measured with synthetic substrate it also shows approximately 20% activity against trypsin. The inhibition of trypsin by pheasant ovomucoid is being studied further and will be reported in detail elsewhere.

[2] The abbreviations used in this paper are: CM-cellulose, carboxymethyl-cellulose; DEAE-cellulose, diethylaminoethyl-cellulose; TAME, p-tosyl arginine methyl ester; BTEE, benzoyl-tyrosine ethyl ester.

strate for chymotrypsin, was synthesized from benzoyl chloride and L-tyrosine ethyl ester according to the method described by Fox (1946) for the synthesis of benzoyldiiodotyrosine ethyl ester. Radioactive acetic anhydride–C^{14} and $KNC^{14}O$ were obtained from the New England Nuclear Co.

Determination of the Free Amino Groups.—Free amino groups were determined routinely by the ninhydrin method (Fraenkel-Conrat, 1957), standardized by the method of Peters and Van Slyke (1932), on control and acetylated samples of turkey ovomucoid.

Determination of Carbohydrate.—Total hexoses were determined by the anthrone method (Scott and Melvin, 1953), carried out directly on the protein. No corrections were made for interference by the protein moiety.[3] Hexosamines were determined as described by Boas (1953), after hydrolysis of the protein in 4 N HCl, 95–100°, for 8 hours in sealed tubes.[4] In order to check if hydrolysis under these conditions resulted in destruction of some of the liberated hexosamine, the hydrolysis was also performed in 2 N HCl, 95–100°, for 8 hours. The results obtained by the two methods of hydrolysis were essentially identical. Sialic acid was determined by the thiobarbituric acid assay method described by Warren (1959), after hydrolysis of the proteins with 0.05 M sulfuric acid for 2 hours at 80°. The details of this method, as applied to the proteins of egg white, have previously been described by Feeney *et al.* (1960).

Amino Acid Analysis.—Amino acid analyses were carried out by the Analytica Corporation (118 East 28th Street, New York 16, N. Y.) according to the method of Moore *et al.* (1958). All values refer to vacuum-dried material which was hydrolyzed with 1000 volumes of constant-boiling HCl for 22 hours at 110° ± 1° in an evacuated tube.

Determination of O-Acyl Groups. –The number of *O*-acyl groups introduced in the protein after acetylation was determined by the hydroxylamine method of Lipmann and Tuttle (1945) as modified by Uraki *et al.* (1957) for use in proteins. To 1 ml of a 2.5% solution of acetylated turkey ovomucoid were added 1 ml of 2 M hydroxylamine hydrochloride and 1 ml of 3.5 M NaOH. After incubation of the mixture at 25° for 2 minutes, 1 ml of a 2:1 dilution of concentrated HCl and 1 ml of ferric chloride reagent (0.37 M ferric chloride in 0.1 M HCl) were added and the color was read at 540 mμ. Ethyl acetate was used as a standard.

Removal of O-Acyl Bonds.—The *O*-acyl bonds were removed with hydroxylamine as described by Grossberg and Pressman (1963). A 2% solution (2.5 ml) of acetylated turkey ovomucoid was adjusted to *p*H 9.5 with 1 M NaOH. To this mixture was added 2.5 ml of 2 M hydroxylamine hydrochloride, previously neutralized. The *p*H of the mixture was then readjusted to *p*H 9.5 and the reaction mixture was kept at 5° for 18 hours. The product was isolated by dialysis and lyophilization.

Inhibitor Assays.—Unless otherwise indicated, assays were made by the spectrophotometric method described previously (Feeney *et al.*, 1963; Rhodes *et al.*, 1960) using synthetic substrates and an acid-base indicator (in a Beckman Model DB spectrophotometer with recording chart). For comparative purposes assays were also done according to the method of Sale *et al.* (1957) with casein as a substrate.

Heat Treatment of the Ovomucoids.—Ovomucoid samples at a concentration of 10 mg/ml were dissolved in 0.01 M sodium phosphate buffer, *p*H 6.0, or in 0.1 M glycine–sodium hydroxide buffer at *p*H 9.0; the samples were incubated at 100° and 80°, respectively, and at regular time intervals aliquots were taken and diluted with 0.006 M Tris buffer, *p*H 8.2, and assayed for remaining inhibitory activity.

Urea Treatment of the Ovomucoids.—Ovomucoid samples at a concentration of 10 mg/ml were dissolved in 9 M urea, buffered with 0.1 M phosphate buffer at *p*H 7.2. The samples were incubated in a water bath at 80° and at appropriate times samples were removed, diluted with 0.006 M Tris, *p*H 8.2, and assayed for residual inhibitory activity.

Chemical Modifications.—(a) Iodination.—Iodination was essentially as described by Azari and Feeney (1961). Chilled 0.053 M I_2 solution in 0.24 M KI was added to a cold solution of 1–10% protein in 0.1 M sodium carbonate buffer, *p*H 9.3. The vessels were then stoppered and kept at 0° for 15 minutes. The reaction was stopped by addition of a few drops of 1 M sodium sulfite solution and the reaction product was then dialyzed. The degree of iodination was estimated using I^{131}-labeled KI in the reaction mixture and then determining the amount of radioactivity introduced into the protein.

(b) Acetylation.—Acetylation essentially was according to the method described by Fraenkel-Conrat (1957; Fraenkel-Conrat *et al.*, 1949). Solutions of 1–10% ovomucoid were made in half-saturated sodium acetate and were cooled in an ice bath. An amount of acetic anhydride approximately equal to the weight of the protein was added in three to six increments over the course of 1 hour. The product was dialyzed. In some experiments, to obtain more extensive reaction, the amount of acetic anhydride used was increased to about two times the weight of the protein present.

(c) Carbamylation.—Carbamylation was done according to Stark *et al.*, (1960) with KNCO, but with modification of concentration of reagent, time, and temperature as indicated. Samples of protein at a concentration of 10 mg/ml were incubated with 0.1 M KNCO in 0.05 M Tris buffer, *p*H 8.1, for 0, 2, 4, and 8 hours at 50°. After extensive dialysis and subsequent lyophilization, the inhibitory activities and the number of residual free amino groups were determined. The modification of turkey ovomucoid was also carried out using C^{14}-labeled reagent to determine the extent of reaction.

Paper Electrophoresis.—Paper electrophoresis was in a Spinco Durrum cell, using Spinco B_2 Veronal buffer at *p*H 8.6. The radioactivity on the strips was counted with a Nuclear Chicago automatic strip counter with thin-window gas-flow detector.

Radioactivity Measurements.— I^{131} was counted with a Baird Atomic scaler with a Geiger tube; C^{14} was determined with a Nuclear Chicago gas-flow counter.

Results

Chemical Analysis of Chicken, Turkey, and Pheasant Ovomucoids.—Table I gives the amino acid composition of the three ovomucoids. The results are expressed in number of residues/10,000 g of vacuum-dried protein. The values have not been corrected for possible destruction of some amino acids residues during acid hydrolysis. The gross composition of the three ovomucoids is very similar, but slight differences can be found.

[3] The proteins remained completely soluble during the determination and also are devoid of tryptophan, which is known to interfere with the determination.

[4] The results of our hexosamine determination were compared with those of Chatterjee and Montgomery (1962). We gratefully acknowledge a sample of chicken ovomucoid from Dr. Rex Montgomery of the State University of Iowa. The hexosamine content of this sample was 15.4–15.7% as determined by him and 13.6–14.0% as determined by us.

TABLE I
AMINO ACID COMPOSITION OF OVOMUCOIDS[a]

Amino Acid	Ovomucoids			
	Chicken 1[b]	Chicken 2[c]	Turkey[c]	Pheasant[c]
Alanine	2.6	3.56	3.04	3.24
Arginine	2.1	1.92	2.03	1.62
Aspartic acid	9.8	9.52	7.18	10.45
Cysteine	5.5	5.84	4.66	6.32
Glutamic acid	4.4	4.71	5.91	7.18
Glycine	5.1	4.94	4.91	6.69
Histidine	1.38	1.56	2.15	1.72
Hydroxyproline	—	—	—	—
Isoleucine	1.1	1.07	1.50	1.67
Leucine	3.9	3.66	4.20	4.41
Lysine	4.1	4.10	3.91	4.15
Methionine	0.64	0.70	0.77	0.88
Phenylalanine	1.76	1.51	1.22	1.56
Proline	2.36	2.03	2.89	3.63
Serine	4.0	3.55	3.18	3.92
Threonine	4.6	4.53	4.62	5.18
Tryptophan	<0.15	<0.15	<0.15	<0.29
Tyrosine	1.76	1.90	2.05	3.17
Valine	5.1	5.00	4.48	5.57

[a] Results in residues/10,000 g protein. When calculated on a weight percentage basis, the amino acids make up 77% of the total weight of chicken ovomucoid, 75% of the total weight of turkey ovomucoid, and 92% of the total weight of pheasant ovomucoid. [b] Values calculated from the results of Lewis *et al.* (1950). The analysis was performed on chicken ovomucoid prepared by the method of Lineweaver and Murray (1947). [c] Values obtained in this study with the exception of the tryptophan values, which were taken from Rhodes *et al.* (1960).

TABLE II
CHEMICAL ANALYSIS OF OVOMUCOIDS

	Ovomucoid		
	Chicken	Turkey	Pheasant
Nitrogen (%)	13.3	12.7	13.5
Hexoses (%)[a]	5.1	5.9	4.6
Hexosamines (%)[b]	13.6	10.4	8.0
Sialic Acid (%)	0.4	2.4	0.6

[a] Hexoses were calculated as % glucose. [b] Hexosamines were calculated as % glucosamine.

TABLE III
HEAT INACTIVATION OF OVOMUCOIDS AT 100°, *p*H 6.0[a]

Heating Time (min)	Inhibitory Activity of Ovomucoids			
	Chicken against Trypsin (%)	Turkey against Trypsin (%)	Turkey against Chymotrypsin (%)	Pheasant against Chymotrypsin (%)
0	100	100	100	100
15	30	55	55	77
30	10	20	25	64
45	<5	10	15	35
60	<1	5	10	20
120	<1	<1	5	5

[a] Conditions are as described in the text.

None of the three ovomucoids apparently contains hydroxyproline or tryptophan. The largest differences in composition can be observed in the contents of aspartic acid, glutamic acid, and tyrosine.

The results of analyses for N, hexose, hexosamine, and sialic acid content are given in Table II. The values for total hexoses and also for hexosamines of chicken ovomucoid are approximately 10% less than those reported by Chatterjee and Montgomery (1962). The significance of these differences remains unexplained.

TABLE IV
HEAT INACTIVATION OF OVOMUCOIDS AT 80°, *p*H 9.0[a]

Heating Time (min)	Inhibitory Activities of Ovomucoids			
	Chicken against Trypsin (%)	Turkey against Trypsin (%)	Turkey against Chymotrypsin (%)	Pheasant against Chymotrypsin (%)
0	100	100	100	100
30	6	45	35	20
60	<1	24	16	10
90	<1	12	12	5

[a] Conditions are as described in the text.

TABLE V
EFFECT OF 9 M UREA ON THE OVOMUCOIDS AT *p*H 7.4, 80°[a]

Incubation Time (min)	Inhibitory Activities of Ovomucoids			
	Chicken against Trypsin (%)	Turkey against Trypsin (%)	Turkey against Chymotrypsin (%)	Pheasant against Chymotrypsin (%)
0	100	100	100	100
30	>90	55	100	>90
60	>90	10	>90	>90
90	80	<5	85	>90

[a] Conditions are as described in the text.

Heat Stability of the Three Ovomucoids.—At a *p*H below *p*H 9 and a temperature of 80° none of the three ovomucoids loses its inhibitory activity for periods up to 60 minutes. Below *p*H 9 at 100° the activity is slowly lost, while at *p*H 9 and 80° the loss of activity for all three ovomucoids is very rapid. The results are summarized in Tables III and IV.

Effect of 9 M Urea on the Inhibitory Activity of the Ovomucoids.—As can be seen from the results given in Table V, incubation of the ovomucoids with 9 M urea, *p*H 7.4, at 80° for periods up to 90 minutes does not have any considerable effect on their inhibitory activities, with the possible exception of the trypsin inhibitory activity of turkey ovomucoid. This inactivation can, however, be explained by reaction with cyanate present in the urea. Determination of amino groups of turkey ovomucoid after incubation with 9 M urea showed that essentially all the free amino groups had been lost.

Stability to Trichloroacetic Acid–Acetone Treatment.—The three ovomucoids were completely stable to trichloroacetic acid–acetone (1 volume of 0.5 M trichloroacetic acid + 2 volumes of acetone) and also to 50–75% aqueous acetone as used in the purification of ovomucoid by the Lineweaver-Murray method (1947).

Effect of Urea on the Complex.—The effect of urea on the complexes of chicken ovomucoid with trypsin and of turkey ovomucoid with trypsin and chymotrypsin was tested. The complexes, consisting of 30 μg (a slight excess) of ovomucoid and 24 μg of enzyme, first were formed in buffer as in the routine assay procedure. The mixtures were then made 1, 2, or 3 M in urea by addition of an equal amount of buffer containing urea at twice the final desired concentration. The substrate-indicator solution, which also was made 1, 2, or 3 M in urea, was added to the mixtures as in

TABLE VI
IODINATION OF CHICKEN, TURKEY, AND PHEASANT OVOMUCOIDS

Ovomucoid	Level[a] of Iodination	Iodine Introduced[b] (atoms/mole)	Inhibition against Trypsin (%)	Inhibition against Chymotrypsin (%)
Chicken	Low	3.3	100	—
	Medium	6.3	100	—
	High	6.3	100[c]	—
Turkey	Low	4.2	100	100
	Medium	7.8	100	100
	High	9.0	100[c]	100[c]
Pheasant	Low	4.2	—	100
	Medium	7.5	—	100
	High	9.2	—	100[c]

[a] Levels of iodination are described in the text and were obtained by varying the concentrations of I_2. [b] Amount of iodine introduced was obtained by using radioactive I^{131} and determining the radioactivity of products. [c] With extensive iodination the inhibitory activities were essentially 100% at low levels of inhibitor to enzyme, but when the ratio of inhibitor to enzyme approached unity the activity decreased to 75%.

TABLE VII
ACETYLATION OF CHICKEN, TURKEY, AND PHEASANT OVOMUCOIDS

Ovomucoid	Level of Acetylation[a]	Inhibition against Trypsin (%)	Inhibition against Chymotrypsin (%)
Chicken	None	100	—
	Intermediate	100	—
	High	100	—
Turkey	None	100	100
	Intermediate	0	100
	High	0	100
Pheasant	None	—	100
	Intermediate	—	100
	High	—	100

[a] Intermediate level of acetylation was obtained by adding 0.025 ml of acetic anhydride to 0.2 ml of a 10% protein solution. High level was obtained by addition of 0.05 ml of acetic anhydride to 0.2 ml of a 10% protein solution.

the routine assay. Under these conditions the enzymes were still inhibited, since no increase in enzymatic activity over the slight activities (5–15%) which these enzyme-inhibitor complexes normally show (Gorini and Audrain, 1953; Rhodes *et al.*, 1960) was detected. In control experiments both trypsin and chymotrypsin were fully active in the presence of 3 M urea, indicating that urea, in the concentrations used, does not dissociate the enzyme-inhibitor complexes.

Iodination.—Iodination of all three ovomucoids was carried out at three different levels of iodine. The I_2 concentrations in the final reaction mixtures were 0.0018 M, 0.0036 M, and 0.0072 M, respectively. The ovomucoid concentration was 10 mg/ml. The results of a quantitative experiment, using I^{131}-labeled reagent, are given in Table VI. Even with extensive reaction, iodine caused no substantial inactivation of any of the three ovomucoids.

Acetylation.—Acetylation with acetic anhydride completely destroyed the trypsin-inhibitory activity of turkey ovomucoid, but had no effect on its chymotrypsin-inhibitory activity. Also, acetylation did not destroy the inhibitory activities of chicken or pheasant ovomucoid (Table VII). Twice the concentration of reagent which gave complete inactivation of the trypsin-inhibitory activity of turkey ovomucoid had no effect on the other inhibitory activities. The experiments with chicken and turkey ovomucoid were repeated except that the samples were assayed with casein as substrate. The results were essentially the same as those obtained with synthetic substrates.

The reaction of acetic anhydride with turkey ovomucoid was investigated in more detail using C^{14}-labeled reagent. Table VIII compares the extent of modification at different levels of reagent as determined by the number of free amino groups lost and by the amount of reagent introduced (measured by radioactivity incorporation). A loss of about 50% of the total amino groups resulted in a loss of about 50% of the trypsin inhibitory activity. At this level of reaction the number of amino groups lost agreed quite well with the number of reagent molecules introduced. At higher levels of reaction, however, the amount of acetyl introduced was always in excess of the amount of amino groups lost. Extensively acetylated turkey ovomucoid was shown to contain approximately 4 *O*-acyl bonds per mole. After removal of these by treatment with hydroxylamine, there was no recovery of trypsin-inhibitory activity and the chymotrypsin-inhibitory activity was still 100%. In a control experiment, unmodified turkey ovomucoid was also treated with hydroxylamine and this treatment did not result in any changes in the inhibitory activities. Attempts were made to separate partially (40%) active acetylated turkey ovomucoid into active and inactive material by chromatography on DEAE-cellulose. The result of this chromatographic experiment is shown in Figure 1, and the properties of the fractions are described in Table IX. Only one peak, almost symmetrical, was obtained, and only very small differences in free amino groups and radioactivity were

TABLE VIII
ACETYLATION OF TURKEY OVOMUCOID. COMPARISONS OF AMINO GROUPS LOST AND ACETYL GROUPS INTRODUCED

Level of Acetylation[a]	Amino Groups[b] Found (moles/mole)	Amino Groups[b] Lost (moles/mole)	Acetyls[b] Introduced (mole/mole)	Inhibition against Trypsin (%)	Inhibition against Chymotrypsin (%)
None	10	—	—	100	100
Low	4–5	5–6	6–7	45	100
Intermediate	3	7	12	15	100

[a] Low level of acetylation was obtained by adding 0.025 ml of acetic anhydride to 2 ml of a 1% protein solution in 50% saturated sodium acetate. High level of acetylation was obtained by adding the same amount of acetic anhydride to 0.2 ml of a 10% protein solution. [b] Amino groups determined by ninhydrin. Acetyls introduced obtained by using radioactive C^{14}-labeled reagent and determining radioactivity introduced.

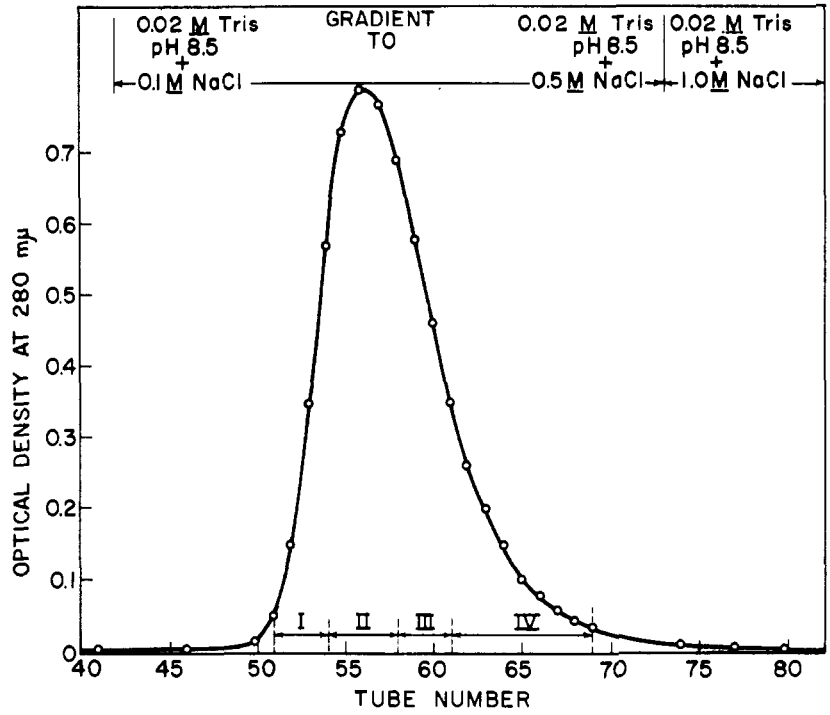

FIG. 1.—Chromatography of acetylated ovomucoid. One hundred mg partially acetylated turkey ovomucoid was chromatographed on 3 g DEAE-cellulose in a column 1-cm in diameter and 15-cm high. The starting buffer was 0.02 M Tris, *p*H 8.5. First a linear gradient of 0.02 M Tris, *p*H 8.5, to 0.02 M Tris, *p*H 8.5, + 0.1 M NaCl (100 ml total volume) was run through the column; then a linear gradient from 0.02 M Tris, *p*H 8.5, +0.1 M NaCl to 0.02 M Tris *p*H 8.5, +0.5 M NaCl; and finally the column was washed through with 0.2 M Tris, *p*H 8.5, +1 M NaCl. The flow rate was approximately 0.8 ml/min and 5 ml was collected per tube. Fractions of the peak were collected as indicated in the figure and then dialyzed, lyophilized, and weighed.

found between fractions from the ascending and descending limbs of the peak.

Carbamylation.—The results of carbamylation (Table X) agreed qualitatively with those of acetylation. With the exception of the trypsin-inhibitory activity of turkey ovomucoid, all inhibitory activities were unaffected. The reaction with turkey ovomucoid was studied again in greater detail using C^{14}-labeled KNCO. The results are presented graphically in Figure 2. In this experiment there was an even greater discrepancy between amino groups lost and reagent introduced than there was in the acetylation experiment. A sample of radioactive carbamylated turkey ovomucoid was dialyzed against 0.1 M urea in acetate buffer at *p*H 4.0 for 2 hours and then dialyzed extensively against water. This did not result in any decrease in radioactivity. Absorption spectra of turkey ovomucoid and carbamylated turkey ovomucoid were compared in the region of 240–340 mμ in 0.1 M HCl, 0.1 M NaOH, and 0.02 M phosphate buffer at *p*H 7.4. The spectra were found to be identical, indicating that no phenolic groups were involved in the reaction. The discrepancy between amino groups lost and reagent introduced remains unexplained.

In paper electrophoresis the modified turkey ovomucoid moved as a single spot which became more acidic as the extent of reaction increased. A radioactivity strip counter revealed only one radioactive peak. Patterns of radioactivity and protein staining were identical.

DISCUSSION

Previous studies have shown that ovomucoids from different avian species have different biochemical specificities but that they have many physical and chemical properties in common (Rhodes *et al.*, 1960). This study shows that they also differ in their susceptibilities to chemical inactivation. Acetylation or carbamylation inactivated the trypsin-inhibitory activity

TABLE IX

PROPERTIES OF CHROMATOGRAPHIC FRACTIONS OF PARTIALLY ACETYLATED TURKEY OVOMUCOID

Fraction[a]	Amount (mg)	Amino Groups[b] Found (moles/mole)	Amino Groups[b] Lost (moles/mole)	Acetyls Introduced[c] (moles/mole)	Inhibition against Trypsin (%)	Inhibition against Chymotrypsin (%)
Starting	85	4.2	5.8	7.8	40	100
I	13	6.3	3.7	5.6	45	100
II	27	4.9	5.1	7.3	45	100
III	13	4.8	5.2	7.7	45	100
IV	10	5.1	4.9	7.8	40	100

[a] Chromatography is described in Fig. 1. Fractions are denoted in Fig. 1. The starting material was turkey ovomucoid partially acetylated with C^{14}-labeled acetic anhydride. [b] Amino groups determined by ninhydrin method. [c] Acetyls introduced calculated from radioactivity of products.

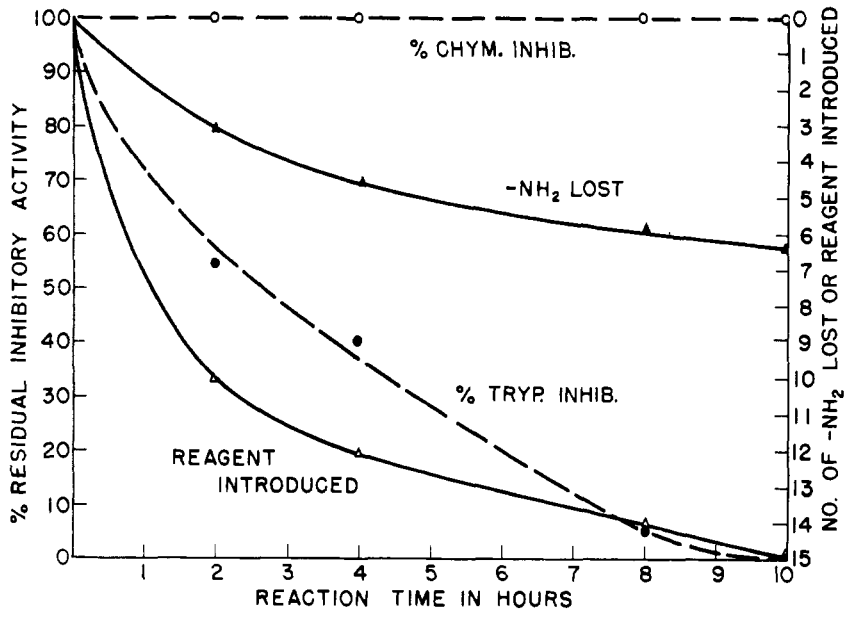

FIG. 2.—Carbamylation of turkey ovomucoid. Comparison of amino groups lost and reagent molecules introduced. O = chymotrypsin inhibitory activity; ● = trypsin inhibitory activity; ▲ = number of free amino groups lost; △ = number of reagent molecules introduced.

of turkey ovomucoid, but did not affect its chymotrypsin-inhibitory activity, nor did it affect the trypsin-inhibitory activity of chicken or the chymotrypsin-inhibitory activity of pheasant ovomucoid. The relative effects of these chemical treatments indicate that the structural requirements for inhibitory activity against trypsin are different in the chicken and the turkey ovomucoids. In addition, destroying the trypsin-inhibitory activity of turkey ovomucoid, while leaving its activity for chymotrypsin unaffected, substantiates the earlier hypothesis (Rhodes *et al.*, 1960) that the two inhibitory sites of turkey ovomucoid are independent and probably nonoverlapping. A sample of acetylated turkey ovomucoid, which still had 40% of its original activity against trypsin, could not be separated into active and inactive fractions. Extensively acetylated turkey ovomucoid contained 4 *O*-acyl bonds. Removal of these did not result in a recovery of trypsin-inhibitory activity, indicating that the loss of this activity was not due to acetylation of serine, threonine, or tyrosine residues. Therefore, the effects of acetylation indicated that free amino groups are important for the trypsin inhibitory activity of turkey ovomucoid. An attempt was made to substantiate these results by using cyanate, which has previously been used for modification of amino groups (Stark *et al.*, 1960, Chen *et al.*, 1962). Our results, however, have shown a large discrepancy between the loss of free amino groups and the amount of reagent introduced as measured by radioactivity. The discrepancies with KNCO were such that over twice as much reagent was introduced as calculated by losses of amino groups (Table VI), and over 30% more reagent was introduced as would be required to react with the total number of ε-amino groups of lysine and the N-terminal group. Sulfydryl groups are absent and phenolic hydroxyls do not appear to be modified. This indicates that other groups in the protein are also reacting, although Stark *et al.* (1960) did not find evidence for other reactions by examination of acid hydrolysates for amino acids by ion-exchange chromatography. Many possible reaction

TABLE X
CARBAMYLATIONS OF CHICKEN, TURKEY, AND PHEASANT OVOMUCOIDS

Ovomucoid	Reaction Time[a] (hours)	Amino Groups		Inhibition against	
		Found[b] (moles/mole)	Lost (moles/mole)	Trypsin (%)	Chymotrypsin (%)
Chicken	0	9.8	0	100	—
	2	8.1	1.7	100	—
	4	6.3	3.5	100	—
	8	5.0	4.8	100	—
Turkey	0	10	0	100	100
	2	6.6	3.4	60	100
	4	5.6	4.4	35	100
	8	4.3	5.7	12	100
Pheasant	0	9.3	0	—	100
	2	8.7	0.6	—	100
	4	5.9	3.4	—	110
	8	4.3	5.0	—	110

[a] Carbamylation was carried out as described in the text. To the 10% protein solutions in 0.05 M Tris buffer, *p*H 8.1, were added equal volumes of 0.2 M KNCO in the same buffer. The reaction mixture was incubated at 50° and the product was isolated by dialysis. [b] Determined by the ninhydrin method.

products might be hydrolyzed during the acid hydrolysis and might not be detected by such a procedure.

That the tyrosine residues probably are not critical for the inhibitory activities of the three ovomucoids studied was also substantiated by the iodination results. Under the conditions used iodination is preferentially directed toward modification of the tyrosines. However the possibility that other oxidative reactions could also take place has not been excluded.[5] The iodination results therefore indicate that even at levels of iodination where diiodo derivatives of all tyrosines could have been formed none of the ovomucoids loses its inhibitory activity.

In confirmation of previous studies (Rhodes *et al.*, 1960), no significant differences were noted in the physical properties of the three ovomucoids studied. All three are very heat-stable proteins. Both activities of turkey ovomucoid (against trypsin and chymotrypsin) are equally affected by the temperature treatment. This can be considered as further evidence for a single molecular species exhibiting both activities. That all three ovomucoids are very stable proteins is also evident from the results of the trichloroacetic acid–acetone and the 9 M urea treatments. Neither of these results in loss of activity. The loss of trypsin-inhibitory activity in turkey ovomucoid upon treatment with 9 M urea at 80° can be explained by reaction with cyanate. Stark *et al.* (1960) have shown that urea solutions are in equilibrium with cyanate and can therefore carbamylate the free amino groups in proteins. Since the trypsin-inhibitory activity of turkey ovomucoid was destroyed by treatment with cyanate and the free amino groups were lost after incubation with urea, 9 M urea itself probably does not affect the inhibitory activities of the three ovomucoids.

The chemical analyses on chicken, turkey, and pheasant ovomucoids reported in this paper show gross similarities in composition. The nitrogen content is nearly identical for all three ovomucoids. Pheasant ovomucoid is relatively low in carbohydrate content. The greatest differences are found in the hexosamine fraction, while the hexose content is virtually identical for all three proteins. Turkey ovomucoid contains much more sialic acid than the other two proteins. A detailed comparative chemical study on the carbohydrate moiety of the different avian ovomucoids is obviously desirable. Small differences in amino acid composition are observed, but do not explain the differences in biochemical specificity.

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[5] Studies now in progress show that the inhibitory activities of the ovomucoids are lost after reduction with mercaptoethanol and subsequent alkylation. Since, on the other hand, iodination does not result in loss of activity, it is reasonable to assume that it also does not affect the disulfide bonds, under the conditions used.

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