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Thiol and Disulphide Contents of Hen Ovalbumin

C-TERMINAL SEQUENCE AND LOCATION OF DISULPHIDE BOND

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1. The thiol and disulphide contents of hen ovalbumin were investigated by *p*-chloromercuribenzoate titration, by determination of cysteic acid content after performic acid oxidation, by measurement of uptake of radioactive iodoacetic acid, and by assay of *S*-aminoethylcysteine after reaction with ethyleneimine. All results showed that ovalbumin had 6 half-cystine residues. Experiments with and without reducing agents demonstrated that there were 4 thiol groups and 1 disulphide bond. 2. A peptide containing equimolar amounts of *S*-carboxymethylcysteine, serine, valine and proline, but no lysine or arginine, was obtained by radioactive labelling of the cysteine residues with iodo[^{14}C]acetic acid followed by electrophoretic and chromatographic separation of tryptic digests. It was concluded that the *C*-terminal sequence of ovalbumin is -Cys-Val-Ser-Pro. 3. The location of the disulphide bond was studied by using a double-labelling technique. It was shown that one end of the disulphide was located in this *C*-terminal peptide.

Considerable uncertainty about the number of thiol and disulphide groups in hen ovalbumin has existed owing to lack of agreement in published results (Cecil & McPhee, 1959). The results have varied depending on the method used, on the presence or absence of denaturing agents, and on the authors reporting the results. It has become apparent that the thiol groups of hen ovalbumin are not all equally reactive. Some 'thiol reagents' such as nitroprusside, porphyrindin or ferricyanide do not react at all with native hen ovalbumin (Heffter, 1907; Greenstein, 1938; Mirsky & Anson, 1936), but will react with the denatured ovalbumin. The values reported have varied from 2.2 thiol groups/molecule of ovalbumin (by using porphyrindin with heat-denatured ovalbumin) to 4.8 thiol groups/molecule (by using the same reagent, but with guanidine-denatured ovalbumin) (Greenstein, 1938). Some other reagents, however, will react with some but not all of the thiol groups of native hen ovalbumin. Iodoacetic acid, iodoacetamide and *p*-CMB* have been reported to react with 3 or sometimes 4 thiol groups in native hen ovalbumin (Lontie & Beckers, 1956; MacDonnell, Silva & Feeney, 1951; Boyer, 1954; Cunningham, Nuenke & Strayhorn, 1957). Denaturation of the hen ovalbumin usually allowed 1 or 2 more thiol groups to react (MacDonnell *et al.* 1951; Lontie & Beckers, 1956; Cunningham *et al.* 1957).

* Abbreviation: *p*-CMB, *p*-chloromercuribenzoate.

The disulphide content of hen ovalbumin is also uncertain. Amperometric titrations of hen ovalbumin with silver nitrate both before and after reduction with sulphite indicated the presence of 5 thiol groups and 1.5 disulphide bonds (Belitser & Lobachevskaya, 1960, 1961). To explain these results it was postulated that ovalbumin consists of two proteins, one with 4 thiol groups and 2 disulphide bonds, the other with 6 thiols and 1 disulphide. Winzor & Creeth (1962) used methylmercuric chloride or mercuric chloride to determine the number of thiol groups before and after the addition of sulphite. They reported 4 thiol groups and 2 disulphide bonds. However, the results obtained by these two groups do not balance with the sulphur and methionine contents that have been established for ovalbumin (Neuberger & Marshall, 1966).

In view of the apparent difficulty in the determination of the thiol and disulphide contents of hen ovalbumin, four different methods were employed: (1) titration of native and denatured ovalbumin with *p*-CMB; (2) oxidation by performic acid followed by cysteic acid determination; (3) measurement of uptake of radioactive iodoacetic acid, (4) reaction with ethyleneimine followed by determination of *S*-aminoethylcysteine.

The position of the disulphide bond was also investigated by isolating tryptic peptides that contained radioactively labelled cysteine residues.

Preliminary results have already been published (Gilmore & Fothergill, 1967; Fothergill & Gilmore, 1968).

MATERIALS AND METHODS

Chemicals. *S*-Aminoethylcysteine, *S*-carboxymethylcysteine, DL-cysteic acid, GSH, iodoacetic acid and $NaBH_4$ were all reagent grade and were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. The iodoacetic acid was purified by recrystallization from light petroleum (b.p. 60–80°C). *p*-CMB sodium salt (pure) and ethyleneimine monomer (pure) were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. The *p*-CMB was purified by dissolving it in 1.0M-NaOH, centrifuging it to remove any insoluble material, and precipitation with 1.0M-HCl. The procedure was repeated twice and the *p*-CMB was washed three times with water by centrifugation. The *p*-CMB was finally dried over P_2O_5. 2-Mercaptoethanol was from Kodak Ltd., London W.C.1, U.K. The following chemicals were scintillation grade and were obtained from Nu clear Enterprises (G.B.) Ltd., Edinburgh, U.K.: 2,5-diphenyloxazole (PPO), 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP), naphthalene and 1.0M-Hyamine hydroxide in methanol. All chemicals used were of analytical-reagent quality, and sodium salts were employed unless otherwise specified.

Radioactive chemicals. The following chemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.: iodo[2-^{14}C]acetic acid (6.5, 7.0 and 15.5mCi/mmol) and iodo[2-^{3}H]acetic acid (93mCi/mmol).

Enzyme. Trypsin (EC 3.4.4.4) (twice crystallized, salt-free) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Ion-exchangers. Analytical-grade cation-exchange resin AG 50W (X8; H^+ form; 200–400 mesh) came from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The resin was washed with 1.0M-NaOH, 1.0M-HCl and water before use. CM-cellulose was prepared from Solka-Floc SW-40-B (40–100 mesh) as described by Peterson & Sober (1961) except that the drying procedure was omitted.

Sephadex. Sephadex G-25 (fine grade) was obtained from Pharmacia, Uppsala, Sweden.

Polyamide layers. Polyamide layers were from the Cheng-Chin Trading Co., Taipei, Taiwan.

Preparation of ovalbumin. Hen ovalbumin was prepared from egg white by CM-cellulose chromatography (Fothergill & Perrie, 1966), followed by precipitation by 60% saturation with $(NH_4)_2SO_4$. The ovalbumin was isolated from eggs laid by only one White Leghorn hen, to minimize problems of genetic polymorphism. All ovalbumin preparations were demonstrated to be pure by Ouchterlony double diffusion, immunoelectrophoresis and starch-gel electrophoresis. A molecular weight of 45000 (Neuberger & Marshall, 1966) has been assumed in all the calculations in this paper.

p-CMB titrations. Modifications of the method described by Cunningham *et al.* (1957), which was based on Boyer's (1954) spectrophotometric method, were employed to determine the thiol contents of different ovalbumin samples. Native, heat-denatured and urea-denatured ovalbumin were examined. To determine the thiol content of native ovalbumin, 0.03ml of ovalbumin solution (2.06mM) was added to 3ml of 0.184mM-*p*-CMB in 50mM-sodium phosphate buffer, pH7.0. The solution was titrated by adding 0.01ml portions of a standard solution of freshly prepared GSH (5.83mM). The extinction was measured at 250nm after each addition of GSH. A graph was constructed by plotting extinction as a function of the volume of GSH added, and the end point was taken to be that point at which the addition of GSH produced no increase in extinction.

The heat-denatured ovalbumin was prepared for assay by heating the ovalbumin–*p*-CMB mixture in a water bath at 82°C for 15min, followed by centrifugation. The urea-denatured ovalbumin was prepared by mixing ovalbumin solution with a *p*-CMB solution that was also 8M with respect to urea and 0.5M with respect to Na_2SO_4.

Cysteic acid determination. For the determination of total thiol plus disulphide contents of the ovalbumin, the protein was oxidized with performic acid and hydrolysed with HCl, and the cysteic acid was separated and measured (Schram, Moore & Bigwood, 1954). Ovalbumin (25mg) was treated with 10ml of performic acid for 4h at 0°C. The performic acid was then rapidly removed by rotary evaporation, and 5ml of 5.5M-HCl was added. After refluxing for 20h, the HCl was removed by rotary evaporation, and 2ml of 0.1M-sodium citrate buffer, pH3.4, was added. The pH was adjusted to 3–4 if necessary. The solutions were filtered through Whatman no. 1 paper and made to 5ml with the citrate buffer. The cysteic acid was isolated by applying 1ml of the hydrolysate to a column (1cm×20cm) of cation-exchange resin 50W (X8) equilibrated with 0.1M-citrate buffer, pH3.4. Elution was carried out with the same buffer and fractions of volume 1.0ml were collected and subjected to ninhydrin assay by the method of Yemm & Cocking (1955) as modified by Matheson & Tattrie (1964). The first amino acid eluted was demonstrated to be cysteic acid by paper chromatography with butan-1-ol–acetic acid–water (3:1:1, by vol.).

Carboxymethylation. The uptake of radioactive iodoacetic acid by thiol groups was used to measure the thiol and disulphide contents of ovalbumin. By adding radioactive iodoacetic acid to reduced ovalbumin a measure of the total number of thiol plus disulphide groups was obtained. However, if no reducing agent was present, the uptake of radioactivity was due only to thiol groups from cysteinyl residues. In the double-labelling experiments the object was to label the cysteinyl thiol groups with iodoacetic acid containing one isotopic label and then, after removal of excess of reagent, to reduce the ovalbumin and label the thiol groups derived from the disulphide residues with iodoacetic acid containing a different isotopic label.

The procedure for carboxymethylation with reducing agent was the method described by Crestfield, Moore & Stein (1963) except that iodo[2-^{14}C]acetic acid (5.21μCi/mmol) was used. Carboxymethylation without reducing agent used ethanol instead of 2-mercaptoethanol. Doubly-labelled reduced carboxymethylated ovalbumin was prepared by initial carboxymethylation with iodo[2-^{14}C]acetic acid (26.9μCi/mmol) in the absence of reducing agent. After removal of excess of reactants by gel filtration, the carboxymethylated ovalbumin was reduced and carboxymethylated with iodo[2-^{3}H]acetic acid (130μCi/mmol).

Reaction with ethyleneimine. The procedure used was based on that described by Raftery & Cole (1963). Ovalbumin (45mg) was dissolved in 0.5ml of water and then treated for 2h at room temperature with 2ml of 8M-urea, pH8.6, which was 25mM with respect to mercaptoethanol. The reaction was carried out under N_2. After addition of 0.25ml. of 5.0M-tris–HCl buffer, pH8.6, 25μl of ethyleneimine was added, and reaction was allowed to continue for 30min at room temperature. The aminoethylated ovalbumin was dialysed against several changes of distilled water. The *S*-aminoethylcysteine content of the modified ovalbumin was determined by amino acid analysis with a Technicon amino acid analyser.

Isolation of cysteine-containing peptides. Samples of reduced carboxymethylated ovalbumin that had been labelled with radioactive iodoacetic acid were digested with trypsin and subjected to high-voltage electrophoresis, and the radioactive peptides were isolated.

A freeze-dried sample of ovalbumin was suspended in 1–2ml of water and the pH was adjusted to 8–9 before digestion. The digestions were carried out in an automatic titrator (model TTT1) adjusted to function as a pH-stat (Radiometer, Copenhagen, Denmark). The ovalbumin was placed in a water-jacketed vessel at 37°C, and the pH was adjusted to 9.1 and maintained at that value by the automatic addition of 0.2M-NaOH. The solution was stirred continuously. After an initial equilibration period of 15min, a portion of aqueous trypsin solution was added and digestion was allowed to proceed for 1–2h. A second portion of enzyme was then added and a further digestion for 1–2h was carried out. The final enzyme/ovalbumin ratio was 1:50 by weight. The digestion was stopped by adjusting the pH to 6.5, which usually caused the formation of a small amount of precipitate. The precipitate was removed by centrifugation after freezing and thawing.

The trypsin-digested ovalbumin was applied along the centre of a 46cm×57cm sheet of Whatman 3MM paper (Brown & Hartley, 1966). Usually 50mg of digested protein in 7ml was separated in a single run. The electrophoresis equipment used was a liquid-cooled system based on that originally described by Michl (1951), with toluene as the cooling liquid. Pyridine–acetic acid–water (25:1:225, by vol.), pH6.5, was used to wet the electrophoresis paper and in the electrode tanks. Electrophoresis was for 50min at a voltage gradient of 60V/cm. Peptides were detected by staining with cadmium–ninhydrin reagent (Heilman, Barollier & Watzke, 1957) and by scanning for radioactivity.

Amino acid analysis. Ovalbumin samples were prepared for analysis by being precipitated and washed with 5% (w/v) trichloroacetic acid. The washed ovalbumin was suspended in 1ml of 5.5M-HCl and transferred to a thick-walled Pyrex tube. The suspensions were degassed under vacuum and the tubes sealed and heated at 110°C for 20h. Peptide samples were dissolved in a small volume of water, placed in a hydrolysis tube and freeze-dried before the addition of 0.5ml of 5.5M-HCl. Automatic amino acid analysis was performed in a Technicon amino acid analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey, U.K.).

Identification of N-terminal amino acids. The 'dansyl' method described by Gray (1967) was used. The DNS-amino acid was identified after 5.5M-HCl hydrolysis by chromatography on polyamide thin layers (Woods & Wang, 1967).

Detection of radioactivity. A Packard model 3003 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., La Grange, Ill., U.S.A.) was used. All aqueous samples and most dry ones were counted with the following scintillation fluid: 1.3g of 2,5-diphenyloxazole, 26mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 20.8g of naphthalene, 100ml of toluene, 100ml of dioxan and 60ml of methanol (Schall & Turba, 1963–64). Some dry samples used a scintillation fluid containing 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 4g of 2,5-diphenyloxazole and 1litre of toluene (Skehel & Burke, 1968). Hyamine hydroxide was added to all samples containing protein. Determinations of specific radioactivity were carried out in duplicate, and usually the results of two or three separate 10min counting periods were collected for each sample. Standard solutions of radioactive iodoacetic acid were prepared from the samples of iodoacetic acid that were used to carboxymethylate the ovalbumin. All vials in a given series contained identical amounts of water, Hyamine hydroxide and other quenching materials.

Electrophoresis strips containing ^{14}C-labelled peptides were sometimes scanned for radioactivity in a model 1002 paper radiochromatography system, i.e. an Actigraph III (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.). The strips scanned were 2.8cm wide, and markers of ^{147}Pm were placed in suitable positions on the strips. The settings listed below were found to give best detection with least background noise: full-scale deflexion 300 c.p.m., scan speed 30cm/h, collimator slit width 1.5mm, response time 10s.

Electrophoresis strips containing both ^{14}C- and ^{3}H-labelled peptides were cut into 1cm segments, which were placed in separate vials and counted by using the liquid-scintillation technique.

The procedure described by Hendler (1964) was used for the simultaneous assay of ^{14}C and ^{3}H.

Determination of protein. A micro method for the determination of nitrogen by nesslerization, based on that described by Lanni, Dillon & Beard (1950), was used to estimate amounts of protein or peptide. It was assumed that all protein samples contained 16% of nitrogen. The Nessler reagent was prepared as described by Koch & McMeekin (1924).

RESULTS

Thiol and disulphide contents. The following thiol contents were determined by titration with *p*-CMB under various reaction conditions: native ovalbumin, 3.1 thiol groups/molecule; heat-denatured ovalbumin, 4.1 thiol groups/molecule; urea-denatured ovalbumin, 3.0 thiol groups/molecule. These results indicated that ovalbumin has 4 thiol groups, 1 of which reacts less readily with *p*-CMB than the other 3.

The cysteic acid content of performic acid-oxidized ovalbumin was shown to be 6.0 residues/molecule of ovalbumin (0.622μmol of cysteic acid was recovered from 0.104μmol of ovalbumin).

The carboxymethylation experiments (Table 1)

Table 1. *Carboxymethylation of unreduced and reduced ovalbumin with iodo*[^{14}C]*acetic acid*

The procedure is described in the text. The molecular weight of ovalbumin was assumed to be 45000. Results are expressed as means ±S.E.M. of the c.p.m. above background/μmol of ovalbumin. Counting efficiency was 76.2±2.7% (S.E.M. of nine measurements). The specific radioactivity of the iodo[^{14}C]acetic acid was 5.21 μCi/mmol.

Experimental conditions	Radioactivity (c.p.m.)	^{14}C incorporated (g-atoms/mol)
Mercaptoethanol omitted	37382±1581 (3)	4.2
Mercaptoethanol included	58143±2724 (3)	6.6

Table 2. *Carboxymethylation of ovalbumin with iodo*[^{14}C]*acetic acid under non-reducing conditions followed by carboxymethylation with iodo*[^{3}H]*acetic acid under reducing conditions*

The procedure is described in the text. The molecular weight of ovalbumin was assumed to be 45000. The counting efficiency was 57% for ^{14}C and 65% for ^{3}H. The specific radioactivity of the iodo[^{14}C]acetic acid was 26.9 μCi/mmol; the specific radioactivity of the iodo[^{3}H]-acetic acid was 130 μCi/mmol. Results are expressed as c.p.m. above background.

	With iodo[^{14}C]acetic acid	With iodo[^{3}H]acetic acid
Radioactivity incorporated (c.p.m./μmol of ovalbumin)	135900	396000
^{14}C or ^{3}H incorporated (g-atoms/mol of ovalbumin)	4.1	2.1

indicate that mercaptoethanol-reduced ovalbumin is labelled with approx. 2 more g-atoms of ^{14}C/mol than the unreduced ovalbumin. Table 2 shows the results of a double-labelling experiment on a single ovalbumin sample. In non-reducing conditions when the ovalbumin was allowed to react with iodo[^{14}C]acetic acid approx. 4 thiol groups were labelled. Subsequent reduction of the same sample and treatment with iodo[^{3}H]acetic acid enabled 2 more thiol groups to be labelled.

Amino acid analysis of reduced carboxymethylated ovalbumin (Table 3) showed that there were 6.1 residues of *S*-carboxymethylcysteine/molecule of ovalbumin. No unchanged cystine was detected.

Amino acid analysis of ovalbumin treated with ethyleneimine (Table 3) showed that there were 6.3 residues of *S*-aminoethylcysteine/molecule of ovalbumin, and that no unchanged cystine was present.

Cysteine-containing peptides. Tryptic peptides from ovalbumin that had been reduced and carboxymethylated with iodo[^{14}C]acetic acid were separated electrophoretically at pH 6.5. Electrophoretic strips were stained with cadmium–ninhydrin reagent and then scanned for radioactivity as shown in Fig. 1. Peptides corresponding to regions I, II and III were eluted and purified by gel filtration, electrophoresis, or chromatography, or a combination of these. The amino acid compositions and *N*-terminal amino acids of peptides I and III are shown in Table 4. Region II was found to consist of at least two peptides, which were not further purified.

Double-labelled ovalbumin was prepared to determine which peptides were involved in the disulphide bond. The ovalbumin was treated initially with iodo[^{14}C]acetic acid under non-reducing conditions, and then with iodo[^{3}H]acetic acid in the presence of mercaptoethanol. Tryptic peptides derived from the double-labelled ovalbumin were separated electrophoretically at pH 6.5. An electrophoresis strip (3 cm × 57 cm) was cut into 1 cm segments, which were assayed for ^{14}C and ^{3}H. The results are presented in Fig. 2, and are compared with the adjacent electrophoresis strip, which was stained with cadmium–ninhydrin reagent. Band I contained approx. 32% of the ^{3}H radioactivity, but only about 9% of the ^{14}C radioactivity. Since the ^{3}H labelled peptides were those that were derived from the disulphide bond, it was apparent that peptide I was involved in the disulphide bond. Approx. 37% of the ^{3}H radioactivity occurred in the neutral region, and it seemed probable that the other half of the disulphide bond was one of the neutral peptides. Band III contained only about 14% of the ^{3}H radioactivity, or less than half of that present in either peptide I or the neutral region.

DISCUSSION

The results obtained from the *p*-CMB titrations were in good agreement with those reported by other workers. MacDonnell *et al.* (1951) found 2.9 thiol residues/molecule in native ovalbumin and 4.1 residues/molecule in guanidine-denatured ovalbumin. Cunningham *et al.* (1957) reported values of 3.0 and 4.0 for native and heat-denatured ovalbumin respectively. Other authors, however, have published rather higher values for the *p*-CMB titration of denatured ovalbumin: 5.5 residues/molecule for urea-denatured ovalbumin (Lontie & Beckers, 1956) and 4.7 residues/molecule for ovalbumin treated with Duponol PC (Anson, 1941). There are inherent disadvantages in the *p*-CMB technique for the determination of thiol residues

Table 3. *Amino acid analysis of reduced carboxymethylated ovalbumin and of aminoethylated ovalbumin*

The number of residues of each amino acid was calculated on the basis of a total of 387 residues/molecule, including 3 residues of tryptophan/molecule (Neuberger & Marshall, 1966). Serine and threonine were corrected for 10 and 5% destruction respectively. CM-Cys, *S*-carboxymethylcysteine; AE-Cys, *S*-aminoethylcysteine.

	Amino acid composition of reduced carboxymethylated ovalbumin		Amino acid composition of aminoethylated ovalbumin	
Amino acid	(μmol)	(no. of residues)	(μmol)	(no. of residues)
Asp	0.319	31.7	0.552	32.4
Thr	0.143	15.0	0.258	15.1
Ser	0.337	36.9	0.735	43.1
Glu	0.512	50.9	0.806	47.2
Pro	0.156	15.5	0.237	13.9
Gly	0.187	18.5	0.296	17.4
Ala	0.345	34.3	0.580	34.0
Val	0.298	29.6	0.476	27.9
CyS	0	0	0	0
Met	0.134	13.3	0.249	14.6
Ile	0.247	24.6	0.382	22.4
Leu	0.323	32.1	0.540	31.7
Tyr	0.099	9.8	0.160	9.4
Phe	0.201	20.0	0.321	18.8
Lys	0.192	19.1	0.355	20.8
His	0.080	8.0	0.159	9.3
Arg	0.188	18.7	0.337	19.8
CM-Cys	0.061	6.1	0	0
AE-Cys	0	0	0.108	6.3
Trp		3		3
Total		387		387

in proteins. At high concentrations proteins absorb strongly at 250nm, and the measurements of small increments in extinction are thus made over a relatively high background extinction. Another disadvantage is that thiol groups vary in their rates of reaction, especially at pH values above 7.0 (Boyer, 1954).

The cysteic acid assay provided a relatively reliable procedure, although the method does not distinguish between thiol and disulphide groups. Possible low yields of cysteic acid might be due to incomplete oxidation of the cysteine residues or to destruction of the cysteic acid by excess of performic acid. High results might be obtained if there were any other strongly acidic amino acids present in the hydrolysate applied to the ion-exchange column that were eluted with the cysteic acid. However, we have found that serine phosphate, which is the most likely contaminant, is largely destroyed by hydrolysis.

The carboxymethylation of ovalbumin with radioactive iodoacetic acid with and without reducing agent allowed the distinction to be made between disulphide and thiol groups. The single-isotope-labelling experiments (Table 1) indicated that 4.2g-atoms of ^{14}C were incorporated with 1mol of ovalbumin under non-reducing conditions. An additional 2.4g-atoms of ^{14}C/mol were incorporated when mercaptoethanol was present in the reaction mixture. Amino acid analysis of the reduced carboxymethylated sample (Table 3) showed that there were 6.1 *S*-carboxymethylcysteine residues and no unchanged cystine residues/molecule of ovalbumin. It is known that iodoacetic acid can undergo side reactions with methionine, lysine, histidine and tyrosine (Crestfield *et al.* 1963), which may account for the rather high, non-integral, labelling values obtained.

The results of the double-labelling experiments also indicated that ovalbumin contains 4 thiol groups under non-reducing conditions plus an additional 2 thiol groups in the presence of mercaptoethanol.

The yields of *S*-aminoethylcysteine (6.3 residues/molecule of ovalbumin) agreed well with the results obtained by the other methods. Under similar

Table 4. *Amino acid composition and N-terminal amino acids of peptides I and III*

Results are expressed as μmol of each amino acid detected. CM-Cys, *S*-carboxymethylcysteine.

Peptide	Composition	*N*-Terminus
I	Ser (0.105), Pro (0.138), Val (0.129), CM-Cys (0.108), Lys (0), Arg (0)	Cysteic acid*
III	Asp (0.091), Pro (0.097), Ala (0.092), Ile (0.102), Leu (0.096), Phe (0.164), Lys (0.115), His (0.104), CM-Cys (0.073)	Alanine

* *N*-Terminal analysis was performed on performic acid-oxidized peptide I.

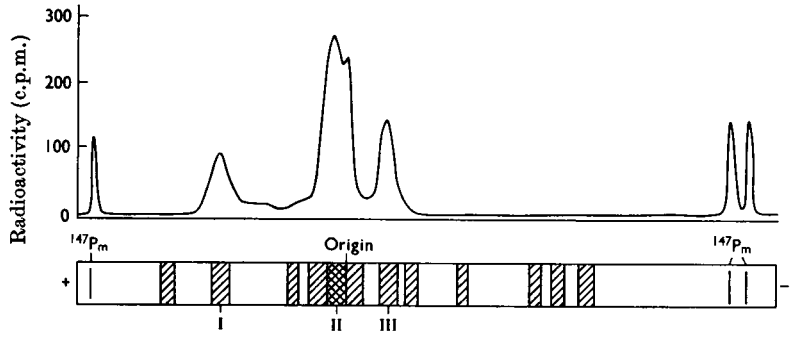

Fig. 1. Distribution of radioactivity along electrophoretic separation of tryptic peptides from ovalbumin reduced and carboxymethylated with iodo[^{14}C]acetic acid. The electrophoretic band I stained yellow with cadmium–ninhydrin reagent.

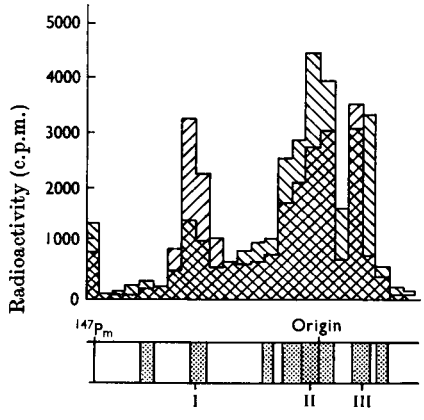

Fig. 2. Distribution of radioactivity along electrophoretic separation of double-labelled reduced carboxymethylated ovalbumin. ■, ^{14}C; ■, ^{3}H. Band I stained yellow with cadmium–ninhydrin reagent.

reaction conditions Raftery & Cole (1963) obtained quantitative yields of *S*-aminoethylcysteine from insulin B chain and from trypsinogen.

In summary, the results of all the methods used showed that hen ovalbumin has 4 thiol groups and 1 disulphide bond.

A possible reason for the variations in thiol and disulphide contents of hen ovalbumin that have been reported in the literature is the use of ovalbumin from pooled egg whites. Lush (1961, 1964*a*,*b*) observed intraspecific genetic polymorphism in ovalbumin from the domestic fowl, and polymorphisms of other egg-white proteins of the domestic fowl have been observed (Croizier, 1966; Feeney, Abplanalp, Clary, Edwards & Clark, 1963; Baker & Manwell, 1962; Ogden, Morton, Gilmour & McDermid, 1962). We have found that ovalbumin samples from different closely related species of birds vary considerably in their thiol and disulphide contents (L. A. Fothergill & J. E. Fothergill, unpublished work). It appears that there is little selective pressure to maintain a given thiol and disulphide composition, and it is possible that reported variations are at least partially due to genetic polymorphisms.

The amino acid analysis of peptide I indicated that it was *C*-terminal, since it was a tryptic peptide and contained no lysine or arginine, and had equimolar quantities of serine, proline, valine and *S*-carboxymethylcysteine. Niu & Fraenkel-Conrat (1955), using hydrazinolysis, reported that the *C*-terminal sequence of ovalbumin was -Val-Ser-Pro. Since it is known that the hydrazinolysis method has the disadvantage of complete loss of *C*-terminal

cysteine or cystine (Locker, 1954) it was possible for the cysteine to occur anywhere in the sequence of the *C*-terminal tetrapeptide. The fact that the peptide stained yellow with cadmium–ninhydrin reagent was consistent with the *N*-terminal amino acid being *S*-carboxymethylcysteine or cysteic acid, or being glycine or having its α-amino group suppressed in some way (Offord, 1966). The amino acid composition of the peptide indicated that the first possibility was the correct one. DNS-cysteic acid was identified as the *N*-terminal amino acid of peptide I derived from performic acid-oxidized ovalbumin. It is therefore possible to state unequivocally that the *C*-terminal sequence of ovalbumin is -Cys-Val-Ser-Pro.

The results from the double-labelling experiments indicated that one end of the disulphide bond was in the *C*-terminal tetrapeptide. The other end was one of the pH 6.5 neutral peptides, which cannot be located within the ovalbumin molecule at this time.

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