The Amino Acid Sequences of Cysteic Acid-Containing Peptides from Performic Acid-Oxidized Ovotransferrin

By T. C. ELLEMAN AND J. WILLIAMS

Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

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1. The half-cystine content of ovotransferrin, measured as cysteic acid, was 31 mol/80 000 g of protein. 2. The amino acid sequences of cysteic acid-containing peptides from performic acid-oxidized ovotransferrin were studied. 3. 34 unique cysteic acid residues were identified. 4. It is concluded that hen ovotransferrin does not consist of two identical halves or subunits.

The transferrins are a group of related proteins that bind two atoms of iron/molecule of protein with approximately equal binding constants (Aisen, Leibman & Reich, 1966; Wenn & Williams, 1968). Estimates of their molecular weights range from 68 000 (Charlwood, 1963) to 95 000 (Bezkorovainy & Rafelson, 1964). Some workers have suggested that the transferrin molecule consists of two similar or identical subunits. This idea is supported by two findings. First, both rabbit transferrin (Baker, Shaw & Morgan, 1968) and human transferrin (Jeppsson, 1967) appear to give fewer spots on peptide 'maps' after trypsin digestion than would be expected from their arginine and lysine contents. Secondly, the molecular weight of reduced and carboxymethylated human transferrin in 8M-urea was reported by Jeppsson (1967) to be about 40 000. On the other hand, Greene & Feeney (1968) found that the molecular weights of several reduced and carboxymethylated transferrins in 8M-urea were about 78 000, which shows that they contain a single polypeptide chain. Similarly, Bezkorovainy, Zschocke & Grohlich (1969) found that after reduction, carboxymethylation and succinylation the molecular weights of human serum transferrin and hen ovotransferrin were 84 000 and 72 000 respectively. W. C. Parker, A. M. Crestfield & A. G. Bearn (unpublished work quoted by Bearn & Parker, 1966) and A. C. Cox & C. Tanford (unpublished work, quoted by Leibman & Aisen, 1967) have obtained a similar result. Fraenkel-Conrat & Porter (1952) obtained 1 mol of dinitrophenyl-alanine/87 000 g of ovotransferrin, and Erikson & Sjöquist (1960) found 1 mol of valine phenylthiohydantoin/88 000 g of human serum transferrin.

The present study attempts to count the unique cysteic acid residues in hen ovotransferrin after performic acid oxidation. Since the protein con-

tains 31 mol of half-cystine/80 000 g and 34 unique cysteic acid residues have been characterized, ovotransferrin does not consist of two identical halves.

MATERIALS AND METHODS

Enzymes. Trypsin and chymotrypsin were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pepsin, subtilisin (type VII), carboxypeptidase A (di-isopropyl phosphorofluoridate-treated) and carboxypeptidase B (di-isopropyl phosphorofluoridate-treated) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Ovotransferrin. The iron complex of hen ovotransferrin was prepared as described by Williams (1968), from eggs purchased from a supermarket.

Performic acid oxidation. Before digestion with trypsin, ovotransferrin was oxidized with performic acid. The protein (1 g) was dissolved in 5 ml of formic acid (98%, v/v) and cooled in ice. Hydrogen peroxide [1 ml of 30% (w/v) solution] was added to 5 ml of formic acid and the mixture left at room temperature for 1 h. It was then cooled and added to the protein solution. Oxidation was allowed to occur for 4 h at 0°C. The reaction mixture was then freeze-dried to remove excess of reagents. In the case of chymotryptic digests performic acid oxidation was carried out after the enzyme digestion, so that the digestion would not be impeded by destruction of tryptophan residues.

Enzymic digestion. Enzymes were used at an enzyme/substrate ratio 1:50 (w/w) and incubation was carried out overnight at 37°C. Pepsin digestion was carried out in 5% (v/v) formic acid. For trypsin digestion the insoluble oxidized ovotransferrin was suspended in 0.2M-N-ethylmorpholine-acetate buffer, pH 8.4. Before digestion with chymotrypsin, ovotransferrin was denatured by being heated in 1% Na₂SO₄ solution at 100°C for 15 min. The precipitated protein was washed free of salt, then suspended in 0.2M-N-ethylmorpholine-acetate buffer, pH 8.4, and digestion carried out. Generally 2 g of protein was used for pepsin or chymotrypsin digestion.

Fractionation of peptides. A preliminary fractionation of the peptides was carried out by ion-exchange chromatography on a column (150 cm $\times$ 2 cm) of Dowex 50 (X2) (Bio-Rad AG 50W-X2, 200–400 mesh), with volatile pyridine-acetic acid buffers (Schroeder, Jones, Cormick & McCalla, 1962). The column was loaded with 2 g of protein hydrolysate and peptides were eluted with a linear buffer gradient from pH 3.1 (pyridine concn. 0.2 M) to pH 5.0 (pyridine concn. 2.0 M); 2.5 l of each buffer was used in forming the gradient. The column was water-jacketed at 37°C. A flow rate of approx. 30 ml/h was used and 7 ml fractions were collected. Peptides were detected manually by the ninhydrin reaction as described by Rudloff & Braunitzer (1961), and the peptide-containing eluates were concentrated by rotary evaporation.

Further purification of the peptides was performed by high-voltage paper electrophoresis as described by Ambler (1963). The cadmium acetate-ninhydrin reagent of Heilmann, Barrolier & Watzke (1957) was used to detect peptides on paper. Elution of peptide bands was performed with aq. 1% (w/v) NH_3 . Portions of each eluate were subjected to total acid hydrolysis and the hydrolysate was tested for the presence of cysteic acid by high-voltage electrophoresis at pH 2.1.

Amino acid analysis. Proteins and peptides were hydrolysed with 5.7 M-HCl in sealed evacuated tubes at 105°C for 24 h. Amino acid compositions were determined either qualitatively by high-voltage paper electrophoresis at pH 2.1 or quantitatively with the Technicon amino acid analyser. Amino acids were detected on paper electrophoretograms by staining with 0.25% ninhydrin–2% collidine in ethanol. Results are expressed as no. of residues/molecule of peptide.

The CM-cysteine* content of ovotransferrin was determined after reduction and carboxymethylation. Ovotransferrin (2 g) was dissolved in 80 ml of 67 mM-tris–67 mM- NaHCO_3 –7 M-urea, pH 8.5. β -Mercaptoethanol (1.2 ml, providing a 20-fold excess over half-cystine residues) was added and after 4 h at room temperature a solution containing 9.0 g of iodoacetic acid adjusted to pH 3.0 was added (providing a threefold excess over total thiol groups). The pH was maintained at 8.0–8.6 with NaOH. Dialysis against water was begun 15 min after completion of addition of iodoacetic acid. The preparation was finally freeze-dried. CM-cysteine was determined with the amino acid analyser after total acid hydrolysis.

The total half-cystine content of ovotransferrin was also measured with 5,5'-dithiobis-(2-nitrobenzoic acid) after reduction of the disulphide bonds as described by Cavallini, Graziani & Dupré (1966).

Amino acid sequence determination. The main method of amino acid sequence determination used here was the 'dansyl'-Edman method as described by Gray & Hartley (1963) and Gray (1967), and, unless otherwise stated, the sequences reported in the Results section were obtained by this method. In some cases peptides were subjected to further enzymic digestion in 0.2 M-*N*-ethylmorpholine-acetate buffer, pH 8.4, to which was added 0.05 ml of

enzyme solution (1 mg/ml). Partial acid hydrolysis was performed by treatment of the peptide with conc. HCl at 37°C for 3 days. The position of amide groups in peptides was determined by Offord's (1966) electrophoretic-mobility method. The mobilities of peptides at pH 6.5 are given relative to that of aspartic acid, and the sign indicates the nature of the charge.

RESULTS

Half-cystine content of ovotransferrin

The cysteic acid yield from ovotransferrin after performic acid oxidation was 31.0 mol/80 000 g of protein. After reduction and alkylation the yield of CM-cysteine was 28.1 mol/80 000 g of protein. Titration of total half-cystine residues with 5,5'-dithiobis-(2-nitrobenzoic acid) gave 27.8 mol/80 000 g. This method was also applied to the native protein and to a pepsin hydrolysate and showed that no free thiol groups were present.

Tryptic peptides from oxidized ovotransferrin

Chromatography of a trypsin digest of oxidized ovotransferrin gave the elution pattern shown in Fig. 1. Fractions 1–14 were examined for the presence of cysteic acid-containing peptides, and these were found only in fractions 1, 2, 4, 5, 6, 7 and 8. A total of 20 cysteic acid-containing peptides was recovered and they contained 23 cysteic acid residues.

Fraction 1. This fraction gave four cysteic acid-containing peptides.

(i) Peptide T_1 ($m = -0.4$). This peptide stained pink with cadmium-ninhydrin reagent. Its amino acid composition was: CySO_3H (2.0), Ser (0.7), Glu (3.0), Pro (2.0), Gly (3.0), Ile (1.0), Leu (2.0), Arg (0.7). The *N*-terminal residue was leucine.

Fig. 1. Elution profile on Dowex 50 (X2) of a trypsin digest of oxidized ovotransferrin. The numbers 1–14 show the fractions taken for further study.

* Abbreviations: CM-cysteine, carboxymethylcysteine; PITC, phenyl isothiocyanate; Asp(CHO), carbohydrate attached to aspartic acid; CySO_3H , cysteic acid; MetSO_2 , methionine sulphone; m , electrophoretic mobility of peptide relative to that of Asp ($= -1.0$) at pH 6.5.

Digestion with subtilisin produced four fragments.

Peptide T_{1s_1} had the amino acid composition: Glu (1.0), Pro (2.0), Gly (2.0), Ile (1.0), Arg (1.0). It was neutral at pH 6.5, showing it to contain glutamic acid, and the sequence was Gly-Gly-Ile-Pro-Pro-Glu-Arg. Partial acid hydrolysis produced glycine and arginine, and the three peptides Glu-Arg, (Ile,Pro₂,Glu) and (Ile,Pro₂,Glu,Arg).

Peptide T_{1s_2} ($m = -0.28$) had the composition: CySO₃H (0.9), Ser (1.0), Glu (2.2), Pro (2.3), Gly (3.0), Ile (1.0), Arg (0.7). The mobility indicated the presence of one amide group. The sequence was CySO₃H-Glx-Gly-Ser-Gly-Gly-Ile-Pro-Pro-Glx-Arg. Removal of the first three residues by PITC left a neutral yellow-staining peptide, indicating that glutamine was the second residue and glutamic acid the penultimate residue. A partial split in this peptide had occurred after serine to produce fragments T_{1s_1} and T_{1s_4} .

Peptide T_{1s_3} ($m = -0.46$) stained pink with cadmium-ninhydrin reagent, whereas the other fragments all stained yellow, indicating this peptide to be derived from the *N*-terminus of peptide T_1 . Its composition was: CySO₃H (0.9), Glu (1.1), Leu (2.0). The mobility indicated only one negative charge, showing a glutamine residue to be present. The sequence was Leu-CySO₃H-Gln-Leu.

Peptide T_{1s_4} ($m = -0.59$) had the composition: CySO₃H (0.9), Ser (0.8), Glu (1.0), Gly (1.0) and its sequence was CySO₃H-Gln-Gly-Ser.

The sequence of peptide T_1 was concluded to be Leu-CySO₃H-Gln-Leu-CySO₃H-Gln-Gly-Ser-Gly-Gly-Ile-Pro-Pro-Glu-Arg.

In other batches of ovotransferrin the *C*-terminal residue of peptide T_1 appears to be variable. From one batch, peptide T_1 was prepared containing lysine (0.6 residue) and arginine (0.5 residue). The peptide T_{1s_1} was isolated, with the composition: Glu (1.2), Pro (2.0), Gly (2.0), Ile (1.0), Lys (0.6), Arg (0.5). Carboxypeptidase B released both lysine and arginine, leaving a residual yellow-staining peptide ($m = -0.35$).

(ii) Peptide T_2 ($m = -0.55$). This peptide stained yellow with cadmium-ninhydrin reagent. Its amino acid composition was: CySO₃H (2.0), Asp (3.0), Thr (2.0), Ser (2.7), Glu (2.2), Pro (3.0), Gly (3.0), Ala (1.0), Tyr (0.8), Phe (2.0), Arg (1.0). The *N*-terminal residue was threonine. Further digestion with chymotrypsin gave rise to two products T_{2c_1} and T_{2c_2} , which were separated by electrophoresis at pH 6.5.

Peptide T_{2c_1} ($m = -0.25$) stained pink with cadmium-ninhydrin reagent. Its amino acid composition was: CySO₃H (1.0), Asp (1.0), Ser (2.6), Glu (1.2), Pro (2.7), Gly (2.0), Ala (0.9), Phe (0.9), Arg (1.0). The mobility showed one charge to be present and the sequence was Phe-Ser-Glx-Gly-CySO₃H-Ala-Gly-Ser-Pro-Pro-Asx-Ser(Arg). Re-

moval of the first four residues from the *N*-terminus produced a neutral peptide that stained yellow with cadmium-ninhydrin reagent. The neutrality of this remaining peptide showed that an asparagine residue was present and that glutamic acid had been removed. Removal of the cysteic acid residue produced a peptide with $m = +0.3$, which confirmed the presence of an asparagine residue. Digestion of peptide T_{2c_1} with subtilisin produced two fragments. Peptide $T_{2c_1s_2}$ ($m = +0.5$) was found to be Ser-Arg; peptide $T_{2c_1s_1}$ ($m = -0.53$) represented the remainder of the original peptide T_{2c_1} . The amino acid composition was: CySO₃H (0.6), Asp (1.1), Ser (1.6), Glu (1.3), Pro (2.2), Gly (2.0), Ala (1.0), Phe (0.7). Partial acid hydrolysis of peptide T_{2c_1} gave several products, one of which was a peptide that stained yellow with cadmium-ninhydrin reagent and had the amino acid composition: Asp (1.0), Ser (0.8), Pro (2.0). Its sequence was Ser-Pro-Pro-Asp. The mobility (-0.5) indicated that the amide group had been lost.

Peptide T_{2c_2} ($m = -0.75$) stained yellow with cadmium-ninhydrin reagent. Its amino acid composition was: CySO₃H (0.8), Asp (1.8), Thr (1.6), Glu (1.0), Gly (1.0), Tyr (0.7), Phe (0.9). The *N*-terminal residue was threonine, indicating this fragment to be from the *N*-terminus of peptide T_2 . The mobility indicated the presence of three negative charges and the sequence was Thr-Gly-Thr-CySO₃H-Asx-Phe-Asx-Glx-Tyr. Removal of the first five residues from the *N*-terminus produced a peptide with $m = -0.7$ at pH 6.5, indicating that two charges were present and that an asparagine residue had been removed.

From these data the structure of peptide T_2 was concluded to be Thr-Gly-Thr-CySO₃H-Asn-Phe-Asp-Glu-Tyr-Phe-Ser-Glu-Gly-CySO₃H-Ala-Gly-Ser-Pro-Pro-Asn-Ser-Arg.

(iii) Peptide T_3 ($m = -0.55$). With this peptide the cadmium-ninhydrin reagent gave a yellow colour that rapidly changed to orange. The *N*-terminal residue was threonine. Its amino acid composition was: CySO₃H (2.2), Asp (2.0), MetSO₂ (1.0), Thr (1.0), Ser (1.8), Glu (2.2), Pro (1.0), Gly (1.1), Ile (1.0), Leu (2.0), Phe (1.0), Lys (1.0). Chymotryptic digestion of peptide T_3 produced four fragments.

Peptide T_{3c_1} ($m = 0$) had the composition: Glu (1.0), Gly (1.0), Leu (1.0), Lys (1.0). Its sequence was Leu-Glu-Gly-Lys.

Peptide T_{3c_2} ($m = -0.28$) had the composition: CySO₃H (0.6), MetSO₂ (1.0), Ser (1.0), Glu (2.0), Gly (1.0), Leu (0.8), Phe (0.8), Lys (1.0). Its mobility indicated one negative charge and its amino acid sequence was Glx-MetSO₂-CySO₃H-Ser-Phe-Leu-Glx-Gly-Lys.

Peptide T_{3c_3} ($m = -0.43$) had the composition: CySO₃H (1.0), MetSO₂ (1.0), Ser (1.0), Glu (1.2),

Phe (0.9). Its mobility indicated one negative charge, which showed this fragment to contain a glutamine residue. Its sequence was Gln-MetSO₂-CySO₃H-Ser-Phe. Peptides T_{3c3} and T_{3c1} appear to have arisen by partial cleavage of peptide T_{3c2}.

Peptide T_{3c4} ($m = -0.67$) stained yellow with cadmium-ninhydrin reagent. Its amino acid composition was: CySO₃H (1.0), Asp (1.7), Thr (0.6), Ser (1.0), Pro (1.2), Ile (0.8), Leu (1.0), and the sequence was Thr-CySO₃H-Asx-Ser-Asx-Pro(Ile-Leu). That the *C*-terminal residue was leucine, as would be expected from the specificity of chymotrypsin, was shown by its release with carboxypeptidase A. The mobility indicated the presence of one asparagine residue. Removal of the first three residues by the Edman degradation produced a peptide with $m = -0.44$, which indicated that the remaining peptide of mol.wt. 543 possessed one negative charge, the asparagine having been removed. The structure of peptide T_{3c4} was concluded to be Thr-CySO₃H-Asn-Ser-Asp-Pro-Ile-Leu.

From these data the structure of peptide T₃ was concluded to be Thr-CySO₃H-Asn-Ser-Asp-Pro-Ile-Leu-Gln-MetSO₂-CySO₃H-Ser-Phe-Leu-Glu-Gly-Lys.

(iv) Peptide T₄ ($m = -0.69$). This peptide stained only very faintly with cadmium-ninhydrin reagent and failed to react with 1-dimethylaminonaphthalene-5-sulphonyl chloride at the *N*-terminus. The amino acid composition was: CySO₃H (1.0), Thr (1.0), Ser (1.8), Glu (2.0), Pro (1.0), Ile (0.9), Lys (1.0). The mobility of this peptide indicates a net negative charge of three, but is inconsistent with the amino acid composition, which would allow a maximum net negative charge of two.

A chymotryptic peptide (C₁₈) of identical amino acid composition was later found during this work, with the sequence CySO₃H-Thr-Ile-Ser-Ser-Pro-Glu-Glu-Lys. Peptide C₁₈ had $m = -0.5$ at pH 6.5, indicating a net negative charge of two, and it reacted with 1-dimethylaminonaphthalene-5-sulphonyl chloride. It seems probable that peptides T₄ and C₁₈ were derived from the same part of the protein and that the *N*-terminus of the tryptic peptide had been blocked, possibly by a damaged tryptophan residue, destroyed by performic acid oxidation; pyrrolidonecarboxylic acid is unlikely as the *N*-terminus since this would have shown as a third glutamic acid residue in the analysis.

Fraction 2. This fraction gave only one cysteic acid-containing peptide, with $m = -0.55$. It stained pink with cadmium-ninhydrin and had the composition: Glu (1.0), CySO₃H (1.0), Lys (1.0). No *N*-terminal residue could be detected by the 'dansyl' method, and the structure of this peptide was not investigated further.

Fraction 4. This fraction contained four cysteic acid-containing peptides.

(i) Peptide T₅ ($m = -0.19$). This peptide had the composition: CySO₃H (1.0), Thr (0.6), Ser (1.4), Glu (2.0), Pro (1.0), Gly (1.0), Ala (1.8), Val (1.0), Ile (1.0), Phe (2.0), Lys (1.0). The *N*-terminal residue was phenylalanine. Digestion with chymotrypsin removed both phenylalanine residues, showing that the *N*-terminal sequence Phe-Phe was present. The 'dansyl'-Edman method showed that peptide T₅ had the partial structure Phe-Phe-Ser-Ala-Ser-CySO₃H-Val(Pro,Gly,Ala,Thr,Ile,Gln,Glu)-Lys.

Digestion of peptide T₅ with subtilisin produced fragments that were neutral, basic and acidic on electrophoresis at pH 6.5. (a) Three peptides were purified from the neutral zone by electrophoresis at pH 3.5 and pH 2.1. Peptide T_{5s1} was found to be Ile-Glx-Glx-Lys. Peptide T_{5s2} was Ser-Ala-Ser. Peptide T_{5s3} was Phe-Phe-Ser-Ala-Ser. (b) Peptide T_{5s4} ($m = +0.56$) was the dipeptide Gln-Lys. (c) Peptides T_{5s5} and T_{5s6} were acidic. Peptide T_{5s5} ($m = -0.65$) was the dipeptide Ile-Glu. Peptide T_{5s6} ($m = -0.53$) stained yellow with cadmium-ninhydrin reagent and the sequence was CySO₃H-Val-Pro-Gly-Ala-Thr. Partial acid hydrolysis produced threonine and a trace of alanine together with two peptides whose amino acid compositions were (CySO₃H,Val,Pro,Gly) and (CySO₃H,Val,Pro,Gly,Ala).

This evidence shows that peptide T₅ had the structure Phe-Phe-Ser-Ala-Ser-CySO₃H-Val-Pro-Gly-Ala-Thr-Ile-Glu-Gln-Lys.

(ii) Peptide T₆ ($m = -0.4$). This peptide stained pink with cadmium-ninhydrin reagent. Total acid hydrolysis for 48 h gave the following amino acid composition: CySO₃H (1.0), Asp (1.0), Ile (1.8), Lys (1.1). The mobility indicated one net negative charge. The amino acid sequence was Asp-CySO₃H-Ile-Ile-Lys.

(iii) Peptide T₇ ($m = -0.65$). This peptide stained yellow with cadmium-ninhydrin reagent. Its amino acid composition was: CySO₃H (1.0), unresolved Asp + MetSO₂ (5), Thr (1.0), Glu (2.4), Gly (1.6), Leu (3.2), Phe (1.0), Arg (1.0). The *N*-terminal sequence was Asx-Leu. Since the peptide stained yellow, it was probable that the *N*-terminal residue was asparagine. Subtilisin digestion produced fragments that were separated into four bands by electrophoresis at pH 6.5.

Peptide T_{7s1} stained orange with cadmium-ninhydrin reagent. Its amino acid composition was: Asp (1.0), Glu (1.0), Leu (1.0). The neutrality of this peptide at pH 6.5 indicated the presence of asparagine and glutamine, and the sequence was Asn-Leu-Gln.

Peptide T_{7s2} ($m = -0.52$) stained yellow with cadmium-ninhydrin reagent. Its amino acid

composition was: CySO_3H (0.8), Asp (1.1), Thr (0.8), Gly (1.1), Arg (0.8). The mobility indicated that the aspartic acid carried a negative charge and the sequence was $\text{CySO}_3\text{H-Thr-Asp-Gly-Arg}$. Two other bands with $m = -0.72$ and -0.85 were found, but both appeared to be heterogeneous and there was insufficient material to effect further purification.

The partial structure of peptide T_7 was concluded to be $\text{Asn-Leu-Gln(Asp}_2\text{,MetSO}_2\text{,Glu,Gly,Leu}_2\text{,-Phe)CySO}_3\text{H-Thr-Asp-Gly-Arg}$.

(iv) Peptide T_8 ($m = -0.76$). This peptide had the amino acid composition: CySO_3H (0.8), Asp (2.0), Ser (0.7), Glu (2.1), Gly (1.1), Leu (3.0), Tyr (0.8), Arg (1.0). The *N*-terminal residue was aspartic acid. Subtilisin digestion produced two fragments that were separated by electrophoresis at pH 6.5.

Peptide T_{8s_1} ($m = -0.47$) had the composition: CySO_3H (1.0), Asp (1.0), Ser (1.0), Gly (1.0), Leu (1.0), Arg (1.0), and the sequence was $\text{CySO}_3\text{H-Leu-Asp-Gly-Ser-Arg}$.

Peptide T_{8s_2} ($m = -0.87$) had the composition: Asp (1.0), Glu (2.0), Leu (2.0), Tyr (1.0). The mobility indicated three negative charges to be present, and the sequence was $\text{Asp-Glu-Tyr-Glu-Leu-Leu}$.

Hence the structure of peptide T_8 was concluded to be: $\text{Asp-Glu-Tyr-Glu-Leu-Leu-CySO}_3\text{H-Leu-Asp-Gly-Ser-Arg}$.

Fraction 5. This fraction gave four cysteic acid-containing peptides.

(i) Peptide T_9 ($m = 0$). The amino acid composition of this peptide after acid hydrolysis for 48 h was: CySO_3H (0.8), Thr (1.0), Ser (0.6), Glu (1.1), Ala (0.4), Val (1.0), Ile (1.0), Leu (1.0), Lys (1.0). The neutrality suggested that one residue of glutamine was present. The 'dansyl'-Edman method gave the sequence $\text{Ile-Ala-Leu-Thr-CySO}_3\text{H-Val-Gln-Lys}$.

Treatment of peptide T_9 with subtilisin gave six main products. Peptide T_{9s_1} ($m = +0.52$) was found to be the dipeptide Gln-Lys . Peptide T_{9s_2} ($m = +0.46$) had the amino acid composition: Glu (1.0), Val (1.0), Lys (1.0). The sequence was Val-Gln-Lys . Peptide T_{9s_3} ($m = 0$) contained isoleucine and alanine. Peptide T_{9s_4} ($m = 0$) was the dipeptide Ile-Ser . Peptide T_{9s_5} ($m = 0$) was the dipeptide Leu-Thr . Peptide T_{9s_6} ($m = -0.10$) stained yellow with the cadmium-ninhydrin reagent and had the following composition: CySO_3H (0.9), Glu (1.0), Val (1.0), Lys (1.0). Its sequence was $\text{CySO}_3\text{H-Val-Gln-Lys}$.

The recovery of the dipeptide sequence Ile-Ser (peptide T_{9s_4}) agrees with the presence of serine in the peptide T_9 and suggests the possibility that peptide T_9 may be heterogeneous, the second residue being either alanine or serine.

(ii) Peptide T_{10} ($m = -0.46$). This peptide gave

a yellow colour with the cadmium-ninhydrin reagent. Its amino acid composition was: CySO_3H (1.0), Glu (1.0), Leu (1.0), Val (1.0), Lys (1.0). Its sequence was $\text{CySO}_3\text{H-Leu-Val-Glu-Lys}$.

(iii) Peptide T_{11} ($m = -0.52$). This peptide stained pink with the cadmium-ninhydrin reagent and had the composition: Glu (1.1), CySO_3H (0.9), Lys (0.9). No reaction with PITC or dansyl chloride at the *N*-terminal residue could be obtained, which suggests that the *N*-terminal residue is pyrrolidonecarboxylic acid (Pyr). The reaction with ninhydrin may be attributed to the lysine residue that presumably occurs at the *C*-terminus. The structure $\text{Pyr-CySO}_3\text{H-Lys}$ that was tentatively assigned to peptide T_{11} agrees with the observed mobility and it appears to be very similar to the cysteic acid-containing peptide found in fraction 2. These peptides behaved differently on ion-exchange chromatography, but it may be suggested that both were derived from the same glutamine-containing peptide, in which partial cyclization of the glutamine residue occurred during the chromatography on Dowex 50 (X2). The remaining unchanged peptide may have undergone cyclization of the glutamine residue after elution from the resin and during the rotary evaporation. Smyth, Stein & Moore (1962) have observed cyclization of glutamine residues in tryptic peptides of ribonuclease during chromatography on Dowex 50 (X2).

(iv) Peptide T_{12} ($m = -0.77$). The amino acid composition of this peptide was: CySO_3H (1.1), Asp (2.0), Ser (1.4), Glu (2.0), Tyr (0.9), Lys (1.0). From the electrophoretic mobility this peptide appeared to have a net negative charge of three, suggesting the presence of one amide group. The sequence was $\text{Tyr-Asx-Asx-Glx-Ser-Glx-CySO}_3\text{H-Ser-Lys}$. The position of the amide group was subsequently found in two overlapping chymotryptic peptides (peptides C_{25} and C_{36}), which showed the second glutamic acid residue to be present as its amide. Peptide T_{12} therefore has the structure $\text{Tyr-Asp-Asp-Glu-Ser-Gln-CySO}_3\text{H-Ser-Lys}$.

Fraction 6. This fraction yielded five cysteic acid-containing peptides.

(i), (ii) and (iii) Peptides T_{13} , T_{14} and T_{15} ($m = -0.1$). These three peptides, which were resolved by electrophoresis at pH 3.5, stained yellow with cadmium-ninhydrin. Their sequences were: T_{13} , $\text{CySO}_3\text{H-Gly-Lys}$; T_{14} , $\text{CySO}_3\text{H-Val-Lys}$; T_{15} , $\text{CySO}_3\text{H-Ala-Val-Gly-Lys}$.

(iv) Peptide T_{16} ($m = -0.09$). This peptide stained yellow with cadmium-ninhydrin reagent and had the amino acid composition: CySO_3H (0.9), Asp (2.3), Leu (1.0), Arg (1.0). The peptide was neutral at pH 6.5, indicating that both aspartic acid residues had been derived from asparagine. The sequence was $\text{CySO}_3\text{H-Asn-Asn-Leu-Arg}$.

Table 1. *Amino acid sequences of cysteic acid-containing peptides obtained by trypsin digestion of performic acid-oxidized ovotransferrin*

Peptides	Sequence
T ₁	Leu-CySO ₃ H-Gln-Leu-CySO ₃ H-Gln-Gly-Ser-Gly-Gly-Ile-Pro-Pro-Glu-Lys-Arg
T ₂	Thr-Gly-Thr-CySO ₃ H-Asn-Phe-Asp-Glu-Tyr-Phe-Ser-Glu-Gly-CySO ₃ H-Ala-Gly-Ser-Pro-Pro-Asn-Ser-Arg
T ₃	Thr-CySO ₃ H-Asn-Ser-Asp-Pro-Ile-Leu-Gln-MetSO ₂ -CySO ₃ H-Ser-Phe-Leu-Glu-Gly-Lys
T ₄	(CySO ₃ H, Thr, Ile, Ser ₂ , Pro, Glu ₂ , Lys)
T ₅	Phe-Phe-Ser-Ala-Ser-CySO ₃ H-Val-Pro-Gly-Ala-Thr-Ile-Glu-Gln-Lys
T ₆	Asp-CySO ₃ H-Ile-Ile-Lys
T ₇	Asn-Leu-Gln(Asp ₂ , MetSO ₂ , Glu, Gly, Leu ₂ , Phe)CySO ₃ H-Thr-Asp-Gly-Arg
T ₈	Asp-Glu-Tyr-Glu-Leu-Leu-CySO ₃ H-Leu-Asp-Gly-Ser-Arg
T ₉	Ile-Ser-Ala-Leu-Thr-CySO ₃ H-Val-Gln-Lys
T ₁₀	CySO ₃ H-Leu-Val-Glu-Lys
T ₁₁	Pyr-CySO ₃ H-Lys
T ₁₂	Tyr-Asp-Asp-Glu-Ser-Gln-CySO ₃ H-Ser-Lys
T ₁₃	CySO ₃ H-Gly-Lys
T ₁₄	CySO ₃ H-Val-Lys
T ₁₅	CySO ₃ H-Ala-Val-Gly-Lys
T ₁₆	CySO ₃ H-Asn-Asn-Leu-Arg
T ₁₇	Ala-Thr-Tyr-Leu-Asp-CySO ₃ H-Ile-Lys
T ₁₈	CySO ₃ H-Ala-Arg
T ₁₉	Leu-CySO ₃ H-Arg
T ₂₀	CySO ₃ H-Leu-Phe-Lys

(v) Peptide T₁₇ ($m = -0.26$). This peptide had the amino acid composition: CySO₃H (0.7), Asp (1.3), Thr (0.8), Ala (0.9), Ile (0.9), Leu (0.9), Tyr (0.9), Lys (1.1). Hydrolysis for 72h produced no increase in isoleucine. The *N*-terminal residue was alanine. Digestion of peptide T₁₇ with chymotrypsin produced two fragments, peptides T_{17c1} and T_{17c2}. Peptide T_{17c1} ($m = 0$) was Ala-Thr-Tyr. Peptide T_{17c2} ($m = -0.43$) was Leu-Asp-CySO₃H-Ile-Lys.

The sequence of peptide T₁₇ was concluded to be Ala-Thr-Tyr-Leu-Asp-CySO₃H-Ile-Lys.

Fraction 7. This fraction gave only one cysteic acid-containing peptide.

Peptide T₁₈ ($m = -0.1$). This stained yellow with cadmium-ninhydrin. Its composition was: CySO₃H (0.9), Ala (1.0), Arg (1.0). The sequence was: CySO₃H-Ala-Arg.

Fraction 8. This fraction gave two cysteic acid-containing peptides.

(i) Peptide T₁₉ ($m = 0$). This peptide stained pink with cadmium-ninhydrin reagent. The amino acid composition showed cysteic acid, leucine and arginine present in approximately equimolar amounts, and the sequence was Leu-CySO₃H-Arg.

(ii) Peptide T₂₀ ($m = -0.1$). This peptide contained cysteic acid, leucine, phenylalanine and lysine in approximately equimolar amounts. Cysteic acid was *N*-terminal and removal of this residue by Edman degradation produced a new *N*-terminal residue, leucine. Lysine was presumed to be *C*-terminal and the sequence was concluded to be CySO₃H-Leu-Phe-Lys.

The amino acid sequences of the above 20 tryptic peptides are shown in Table 1.

Oxidized chymotryptic peptides from ovotransferrin

Chromatography of the oxidized chymotryptic digest of ovotransferrin on Dowex 50 (X2) gave the elution pattern shown in Fig. 2. Fractions 1-28 were tested for the presence of cysteic acid-containing peptides. Fractions 1-7 inclusive, 10-17 inclusive and 21 yielded 33 cysteic acid-containing peptides in good yield and small amounts of six cysteic acid-containing peptides.

Fraction 1. This fraction, on electrophoresis at pH 6.5, gave a very streaky pattern from which five peptides were purified.

(i) Peptide C₁ ($m = -0.17$). This peptide stained yellow with cadmium-ninhydrin reagent. The amino acid composition was: CySO₃H (1.3), Asp (2.0), MetSO₂ (1.0), Phe (0.9), Ser (0.9), GlcN (+). The 'dansyl'-Edman method gave the sequence CySO₃H-MetSO₂-Asx-Asx-Ser-Phe. Removal of the first three residues left a yellow-staining neutral peptide containing asparagine, serine and phenylalanine, but lacking glucosamine. The sequence was concluded to be CySO₃H-MetSO₂-Asp(CHO)-Asn-Ser-Phe. The mobility of this peptide indicates a carbohydrate unit of approx. mol.wt. 1600, comparable in size with the oligosaccharide moieties found in other parts of the protein (basic site 1519 and neutral site 1680; R. V. Wenn, unpublished work). Peptide C₁ thus contains a third point on the ovotransferrin molecule at which carbohydrate may be attached.

Fig. 2. Elution profile on Dowex 50 (X2) of an oxidized chymotryptic digest of ovotransferrin. The numbers 1–28 show the fractions taken for study.

(ii) Peptide C_2 ($m = -0.48$). This peptide stained yellow with cadmium–ninhydrin reagent. The amino acid composition was: $CySO_3H$ (1.8), Ser (1.0), Glu (2.0), Pro (2.0), Gly (2.8), Ala (1.0), Val (0.9), Ile (0.9), Arg (1.0). The *N*-terminal residue was cysteic acid, and carboxypeptidase A released alanine and a small amount of valine. The ‘dansyl’–Edman procedure gave the *N*-terminal sequence $CySO_3H$ -Glx-Gly. Removal of the *N*-terminal cysteic acid decreased the mobility to -0.19 and removal of the second residue left a yellow-staining peptide of unchanged mobility, which indicated that the second residue was glutamine. Peptide C_2 therefore appeared to have the partial structure $CySO_3H$ -Gln-Gly(Ser, Gly, 2-Ile, Pro, 2-Glu, Arg, $CySO_3H$)Val-Ala.

(iii) Peptide C_3 ($m = -0.63$). This peptide stained pink with cadmium–ninhydrin reagent. The amino acid composition after hydrolysis for 48 h was: $CySO_3H$ (1.6), Asp (3.7), $MetSO_2$ (1.0), Thr (1.7), Ser (1.7), Glu (2.3), Gly (1.3), Val (4.3), Ile (2.4), Lys (1.7). The ‘dansyl’–Edman method showed the *N*-terminal sequence to be Ser-Val-Val-Ser-Asp-Asp. Digestion with trypsin produced three fragments.

Peptide C_3t_1 ($m = 0$) was Ile- $MetSO_2$, and was derived from the *C*-terminus of peptide C_3 .

Peptide C_3t_2 ($m = -0.4$) was identified as Asp- $CySO_3H$ -Ile-Ile-Lys.

Peptide C_3t_3 ($m = -0.72$) had the composition: $CySO_3H$ (1.0), Asp (3.0), Thr (1.4), Ser (1.4), Glu (1.9), Gly (1.0), Val (3.8), Lys (1.2). Digestion of peptide C_3t_3 with subtilisin produced four fragments. (a) Peptide $C_3t_3s_1$ ($m = -0.34$) was Val-Val-Asp-Glu-Thr-Lys. (b) Peptide $C_3t_3s_2$ ($m = -0.75$) had the composition: Asp (2.0), Ser (1.3), Glu (1.0), Gly (1.1), Val (2.4). (c) Peptide $C_3t_3s_3$ ($m = -0.83$)

had the composition: $CySO_3H$ (1.0), Asp (2.0), Glu (0.9), Thr (1.0), Ser (1.4), Gly (0.9), Val (2.0). (d) Peptide $C_3t_3s_4$ ($m = -0.83$) had the composition: $CySO_3H$ (1.0), Thr (1.0).

Peptide C_3 was assigned the partial sequence: Ser-Val-Val-Ser-Asp-Asp(Glu, Gly)($CySO_3H$, Thr)-Val-Val-Asp-Glu-Thr-Lys-Asp- $CySO_3H$ -Ile-Ile-Lys-Ile- $MetSO_2$.

(iv) Peptide C_4 ($m = -0.67$). This peptide, which was present in low yield, stained yellow with cadmium–ninhydrin reagent. The amino acid composition was: $CySO_3H$ (1.0), Asp (2.0), Ser (1.0), Ile (0.8), Leu (0.8), Pro (+). Removal of the first residue left a yellow-staining peptide with $m = -0.37$. The *N*-terminal sequence was $CySO_3H$ -Asn-Pro-Ser-Asp-Ile(Leu); no continuity was established between isoleucine and the terminal leucine residue.

(v) Peptide C_5 ($m = -0.7$). This peptide was also present in low yield. It stained yellow with cadmium–ninhydrin reagent and had the composition: $CySO_3H$ (1.52), Asp (0.9), $MetSO_2$ (+), Thr (0.6), Ser (1.0), Glu (1.0), Pro (+), Gly (0.9), Ala (1.2), Val (1.0), Ile (0.9), Leu (0.6). The *N*-terminal sequence was $CySO_3H$ -Asx-Ile.

Fraction 2. This fraction yielded six cysteic acid-containing peptides.

(i) Peptide C_6 ($m = -0.48$). This peptide stained yellow with cadmium–ninhydrin and its amino acid composition was: $CySO_3H$ (0.8), Thr (0.8), Gly (2.0), Ala (1.0), Val (1.0), Leu (1.1). The sequence was Thr-Ala-Gly-Val- $CySO_3H$ -Gly-Leu.

(ii) Peptide C_7 ($m = -0.50$). This peptide stained pink with cadmium–ninhydrin. Its composition was: $CySO_3H$ (0.9), Gly (2.0), Ala (1.0), Val (1.1), Leu (1.0). The sequence was Ala-Gly-Val- $CySO_3H$ -(Gly, Leu).

Peptide C_7 appears to differ from peptide C_6 only in the absence of the *N*-terminal threonine residue.

(iii) Peptide C_8 ($m = -0.58$). This yellow-staining peptide was found to be Thr- $CySO_3H$ -Val-Gln.

(iv) and (v) Peptides C_9 and C_{10} ($m = -0.60$). These peptides also stained yellow with cadmium–ninhydrin and had the structures: C_9 , $CySO_3H$ -Val-Gln; C_{10} , $CySO_3H$ -Gln-Leu.

(vi) Peptide C_{11} ($m = -0.80$). This was the dipeptide $CySO_3H$ -Leu and stained yellow.

Fraction 3. There were five cysteic acid-containing peptides in this fraction.

(i) Peptide C_{12} ($m = -0.50$). This peptide stained yellow and its amino acid composition was: $CySO_3H$ (1.5), Asp (1.1), Thr (0.9), Ser (1.7), Glu (2.2), Pro (+), Ile (1.0), Lys (2.0). The *N*-terminal residue was cysteic acid. Digestion with trypsin produced two fragments.

Peptide $C_{12}t_1$ ($m = 0$) stained pink and had the sequence Lys- $CySO_3H$ -Asn. The neutrality of this peptide showed the aspartic acid to have been derived from asparagine.

Peptide $C_{12}t_2$ ($m = -0.55$) stained yellow and contained the remaining *N*-terminal sequence of peptide C_{12} . The mobility indicated a net negative charge of two. The sequence was $CySO_3H$ -Thr-Ile-Ser-Ser-Pro-Glu-Glu-Lys.

The sequence of peptide C_{12} was concluded to be $CySO_3H$ -Thr-Ile-Ser-Ser-Pro-Glu-Glu-Lys-Lys- $CySO_3H$ -Asn.

(ii) Peptide C_{13} ($m = -0.55$). This yellow-staining peptide was recovered in low yield and had the sequence $CySO_3H$ -Ser-Phe-Leu.

(iii) Peptide C_{14} ($m = -0.60$). This was the tripeptide $CySO_3H$ -Leu-Phe.

(iv) Peptide C_{15} ($m = -0.66$). This was the tripeptide $CySO_3H$ -Ser-Phe.

(v) Peptide C_{16} ($m = -0.80$). This peptide stained yellow and the amino acid composition was: $CySO_3H$ (1.0), Asp (1.7), Glu (1.4), Phe (1.0), Tyr (0.4). The mobility indicated the presence of one amide group. The 'dansyl'-Edman method gave the sequence $CySO_3H$ -Asx-Phe-Asx(Glx,Tyr).

Fraction 4. Two cysteic acid-containing peptides were recovered from this fraction.

(i) Peptide C_{17} ($m = -0.23$). This peptide stained yellow and the *N*-terminal residue was serine. The amino acid composition was: $CySO_3H$ (1.0), Thr (0.9), Ser (1.4), Glu (2.0), Pro (+), Gly (1.1), Ala (1.7), Val (0.9), Ile (0.9), Leu (0.9), Lys (1.0). The mobility indicated that one glutamine residue was present. Trypsin digestion gave two products, free leucine, which was presumed to be derived from the *C*-terminus of peptide C_{17} , and peptide $C_{17}t_1$ ($m = -0.27$), which stained yellow and had the composition: $CySO_3H$ (1.1), Thr (1.0), Ser (2.0), Glu (2.4), Pro (+), Gly (1.4), Ala (2.0), Val (1.0), Ile (1.0), Lys (1.0). Its sequence was Ser-Ala-Ser- $CySO_3H$ -Val-Pro-Gly-Ala-Thr-Ile(Glu,Gly)Lys.

The sequence of peptide C_{17} was concluded to be Ser-Ala-Ser- $CySO_3H$ -Val-Pro-Gly-Ala-Thr-Ile(Glu,Gln)Lys-Leu.

(ii) Peptide C_{18} ($m = -0.51$). This peptide stained yellow. The amino acid composition was: $CySO_3H$ (0.8), Thr (1.0), Ser (1.6), Glu (2.2), Ile (0.8), Pro (+), Lys (0.8). This and the mobility suggested that peptide C_{18} was identical with peptide $C_{12}t_2$, an incomplete cleavage having occurred after the lysine residue of peptide C_{12} . This assumption was verified by the 'dansyl'-Edman method, which gave the sequence $CySO_3H$ -Thr-Ile-Ser-Ser-Pro-Glu-Glu-Lys.

Fraction 5. Only one cysteic acid-containing peptide was obtained from this fraction.

Peptide C_{19} ($m = -0.18$). This peptide stained yellow with cadmium-ninhydrin reagent, and its amino acid composition was: $CySO_3H$ (0.9), Asp (1.0), Ser (2.6), Glu (1.1), Pro (>1), Gly (2.1), Ala (1.0), Leu (1.0), Arg (1.0). The *N*-terminal residue was serine. The 'dansyl'-Edman method gave the

N-terminal sequence Ser-Glx-Gly- $CySO_3H$ -Ala-Gly. After removal of five residues by the Edman method the residual peptide, which stained yellow, had $m = +0.36$, indicating the presence of asparagine. Digestion with trypsin produced two fragments, free leucine, which was presumed to have been cleaved from the *C*-terminus of peptide C_{19} , and peptide $C_{19}t_1$ ($m = -0.27$) which stained yellow. Its amino acid composition was: $CySO_3H$ (0.9), Asp (0.9), Ser (2.2), Glu (0.9), Pro (>1), Gly (1.8), Ala (1.0), Arg (1.0). The mobility indicated the presence of one amide group.

The partial structure of peptide C_{19} is therefore Ser-Glu-Gly- $CySO_3H$ -Ala-Gly(Ser₂,Pro_{>1},Asn)Arg-Leu.

Fraction 6. Two cysteic acid-containing peptides were obtained from this fraction.

(i) Peptide C_{20} ($m = 0$). This peptide stained yellow and was found to be the tripeptide Ser- $CySO_3H$ -His.

(ii) Peptide C_{21} ($m = -0.22$). This peptide stained pink and the amino acid composition was: $CySO_3H$ (0.7), Asp (1.6), Thr (0.9), Ser (1.0), Pro (+), Ile (1.0), Leu (1.2), Lys (0.8). The mobility indicated the presence of one amide group. Lysine was *N*-terminal, and removal of this residue with PITC produced a yellow-staining peptide. The sequence of peptide C_{21} was found to be Lys-Thr- $CySO_3H$ -Asn(Ser,Asp,Pro,Ile,Leu).

The position of the amide group was shown by the fact that after removal of four residues by the Edman method the residual peptide had mobility $m = -0.44$.

Fraction 7. Four cysteic acid-containing peptides were obtained from this fraction.

(i) Peptide C_{22} ($m = -0.17$). This peptide had the amino acid composition: $CySO_3H$ (1.0), Asp (1.1), Ser (2.0), Glu (1.1), Gly (1.9), Pro (>1), Ala (0.9), Leu (1.0), Phe (0.9), Arg (1.0). The mobility indicated the presence of one amide group. The *N*-terminal residue was phenylalanine.

From the amino acid composition and mobility peptide C_{22} appeared to represent peptide C_{19} with the addition of an *N*-terminal residue of phenylalanine.

(ii) Peptide C_{23} ($m = -0.40$). This peptide stained yellow and the *N*-terminal residue was cysteic acid. The amino acid composition was $CySO_3H$ (0.9), Asp (2.7), Ser (0.9), Glu (1.1), Pro (1.0), Gly (1.0), Val (0.9), Leu (1.0), Tyr (0.7), Arg (1.0). Digestion with trypsin produced two acidic fragments.

Peptide $C_{23}t_1$ ($m = -0.31$) stained pink and had the composition: Asp (2.0), Glu (1.0), Pro (1.0), Val (1.0), Tyr (1.0). The mobility indicated the presence of two amide groups. Glutamic acid was *N*-terminal. Removal of this with PITC produced a peptide with $m = -0.41$, which indicated that the glutamic acid had been derived from glutamine.

The remaining sequence was determined by the 'dansyl'-Edman method as Pro-Val-Asx-Asx-Tyr.

Peptide $C_{23}t_2$ ($m = -0.42$) stained yellow. The amino acid composition was: $CySO_3H$ (1.0), Asp (1.0), Ser (1.0), Gly (1.0), Leu (1.0), Arg (1.0). The sequence was $CySO_3H$ -Leu-Asp-Gly-Ser-Arg.

The sequence of peptide C_{23} was concluded to be $CySO_3H$ -Leu-Asp-Gly-Ser-Arg-Gln-Pro-Val(Asp, Asn)Tyr.

(iii) Peptide C_{24} ($m = -0.40$). This peptide stained pink. The amino acid composition was: $CySO_3H$ (1.4), Asp (1.0), Thr (0.8), Glu (1.8), Pro (1.0), Ala (0.8), Val (0.7), Leu (1.0), Arg (1.0). The sequence was Arg-Glx- $CySO_3H$ -Asx-Leu-Ala-Glx-Val(Pro,Thr). The mobility indicated the presence of one amide group. Removal of three residues by the Edman method left a yellow-staining peptide of $m = -0.34$, which indicated that the second residue removed was glutamic acid. Removal of the fourth residue had very little effect on the mobility, which showed it to be asparagine. The sequence was concluded to be Arg-Glu- $CySO_3H$ -Asn-Leu-Ala-Glu-Val(Pro,Thr).

(iv) Peptide C_{25} ($m = -0.59$). This peptide had the amino acid composition: $CySO_3H$ (1.0), Asp (2.9), Thr (1.0), Ser (2.4), Glu (3.0), Pro (>1), Ala (0.9), Tyr (0.8), Lys (1.0), Arg (1.0). Aspartic acid was *N*-terminal, and digestion with carboxypeptidase A produced serine and tyrosine in approximately equimolar quantities. Digestion with trypsin produced two fragments.

Peptide $C_{25}t_1$ ($m = -0.25$) had the composition: Asp (1.0), Thr (1.0), Ser (1.0), Glu (1.0), Pro (1.0), Ala (1.0), Tyr (1.0), Arg (1.0). The sequence was Thr-Asp-Glu-Arg-Pro-Ala-Ser-Tyr, and this peptide therefore appeared to represent the *C*-terminal portion of peptide C_{25} .

Peptide $C_{25}t_2$ ($m = -0.83$) had the composition: $CySO_3H$ (1.0), Asp (2.0), Ser (2.0), Glu (2.0), Lys (1.0). It was further digested with subtilisin and produced two fragments. (a) Peptide $C_{25}t_2s_1$ ($m = -0.1$) was $CySO_3H$ -Ser-Lys; (b) peptide $C_{25}t_2s_2$ ($m = -1.02$) had the composition: Asp (2.0), Ser (1.0), Glu (2.0). It presumably represents the *N*-terminal section of peptide C_{25} . The mobility showed that one amide group was present. After successive removal of three residues from the *N*-terminal end by Edman degradation, the mobility of the residual peptide changed as follows: $-1.02 \rightarrow -0.90 \rightarrow -0.57 \rightarrow 0$. This shows that the residues removed were acidic. The amino acid sequence of peptide $C_{25}t_2s_2$ was not determined, but it appears to correspond to part of the peptide T_{12} and to part of peptide C_{36} .

The sequence of peptide C_{25} was concluded to be Asp(Asp,Glu)(Ser,Gln) $CySO_3H$ -Ser-Lys-Thr-Asp-Glu-Arg-Pro-Ala-Ser-Tyr.

Fraction 10. This fraction provided one cysteic acid-containing peptide.

Peptide C_{26} ($m = -0.64$). This peptide had the amino acid composition: $CySO_3H$ (0.6), Asp (2.0), Glu (1.0), Tyr (0.8), Arg (0.8). The mobility indicated one amide group. The sequence was Asx-Tyr-Arg-Glx- $CySO_3H$ -Asx. Removal of the first four residues left a peptide that stained yellow and had $m = -0.8$, which indicated the presence of an asparagine residue and the sequence Asp-Tyr-Arg-Glu- $CySO_3H$ -Asn.

This peptide showed a four-residue overlap with peptide C_{24} , showing the incomplete cleavage by chymotrypsin of the bonds Tyr-Arg and Asn-Leu.

Fraction 11. Three cysteic acid-containing peptides were found in this fraction.

(i) Peptide C_{27} ($m = 0$). This peptide stained yellow and the amino acid composition was: $CySO_3H$ (0.8), Asp (1.1), Pro (1.6), Ala (1.8), Tyr (0.6), Arg (1.0). Cysteic acid was *N*-terminal and trypsin produced two neutral fragments, identified as $CySO_3H$ -Ala-Arg and Asn-Ala-Pro-Tyr. The sequence of peptide C_{27} was concluded to be $CySO_3H$ -Ala-Arg-Asn-Ala-Pro-Tyr.

(ii) Peptide C_{28} ($m = -0.24$). This peptide had the amino acid composition: $CySO_3H$ (0.8), Asp (1.3), Thr (0.9), Glu (2.1), Pro (+), Ala (1.0), Val (1.0), Leu (1.0), His (0.8), Arg (0.8). The mobility indicated one amide group. The 'dansyl'-Edman method showed the *N*-terminal sequence to be Arg-Glu, and carboxypeptidase A released one residue of histidine from the *C*-terminal end. This identified the peptide as a one-residue extension of peptide C_{24} . The quantitative release of histidine by carboxypeptidase A suggests the *C*-terminal sequence -Pro-Thr-His.

The sequence was concluded to be Arg-Glu- $CySO_3H$ -Asn-Leu-Ala-Glu-Val-Pro-Thr-His.

(iii) Peptide C_{29} ($m = -0.42$). This peptide contained $CySO_3H$ (+), Asp (+), Glu (+) and Arg (+). The mobility indicated one amide group. The sequence was Arg-Glu- $CySO_3H$ -Asn, previously found within peptides C_{24} and C_{26} . The *C*-terminal asparagine was identified by electrophoresis at pH 2.1 after three applications of the Edman procedure.

Fraction 12. This fraction gave low yields of two cysteic acid-containing peptides.

(i) Peptide C_{30} ($m = -0.10$). This was the tripeptide $CySO_3H$ -Arg-Gln.

(ii) Peptide C_{31} ($m = -0.10$). This peptide stained yellow and had the composition: $CySO_3H$ (1.4), Glu (1.0), Lys (0.9), Arg (1.0). The sequence was $CySO_3H$ -Arg-Gln- $CySO_3H$ -Lys.

Fraction 14. Only one cysteic acid-containing peptide was obtained from this fraction.

Peptide C_{32} ($m = 0$). This was the tripeptide Arg-CySO₃H-Leu.

Fraction 15. This fraction gave only one cysteic acid-containing peptide.

Peptide C_{33} ($m = 0$). This peptide had the composition: CySO₃H (0.8), Asp (1.2), Thr (0.8), Leu (1.6), Lys (1.6). Its sequence was Lys-Asp-Leu-Thr-Lys-CySO₃H-Leu.

Fraction 16. Three cysteic acid-containing peptides were obtained from this fraction.

(i) Peptide C_{34} ($m = +0.40$). The amino acid composition of this peptide was: CySO₃H (0.7), Ser (0.9), Lys (1.3), His (1.0). The sequence was Lys-Ser-CySO₃H-His.

(ii) Peptide C_{35} ($m = -0.20$). This peptide stained orange and had the amino acid composition: CySO₃H (1.0), Asp (1.7), Ser (0.7), Glu (1.0), Gly (0.9), Ala (0.7), Val (0.8), Lys (2.0), Arg (0.9). The mobility indicated the absence of amide groups. This peptide was obtained only in very low yield, and the 'dansyl'-Edman method gave the sequence CySO₃H-Ala-Val-Gly-Lys-Asp-Glu(Asp, Ser, Lys, Arg).

(iii) Peptide C_{36} ($m = -0.48$). This peptide had the amino acid composition: CySO₃H (0.7), Asp (2.8), Thr (1.0), Ser (2.5), Glu (4.0), Pro (+), Ala (1.7), Tyr (1.2), Lys (1.1), Arg (2.0). The *N*-terminal residue was alanine. Carboxypeptidase A released serine and tyrosine in equal amounts. Digestion with trypsin produced three fragments.

Peptide C_{36t_1} ($m = 0$) had the sequence Ala-Glu-Arg, having been derived from the *N*-terminal end peptide C_{36} .

Peptide C_{36t_2} ($m = -0.28$) stained yellow with cadmium-ninhydrin reagent and the composition was: Asp (1.0), Thr (1.0), Ser (1.0), Glu (1.0), Pro (1.0), Ala (1.0), Tyr (1.0), Arg (1.0). The sequence was Thr-Asp-Glu-Arg-Pro-Ala-Ser-Tyr.

Peptide C_{36t_3} ($m = -0.73$) had the composition: CySO₃H (1.0), Asp (2.0), Ser (1.6), Glu (2.0), Tyr (1.0), Lys (1.0). The sequence was Tyr-Asp-Asp-Glu-Ser-Gln-CySO₃H-Ser-Lys.

The sequence of peptide C_{36} was concluded to be Ala-Glu-Arg-Tyr-Asp-Asp-Glu-Ser-Gln-CySO₃H-Ser-Lys-Thr-Asp-Glu-Arg-Pro-Ala-Ser-Tyr.

Fraction 17. Two cysteic acid-containing peptides were obtained from this fraction.

(i) Peptide C_{37} ($m = +0.20$). This peptide was obtained in low yield only. Its amino acid composition was identical with that of peptide C_{38} . The 'dansyl'-Edman procedure showed the *N*-terminal sequence to be CySO₃H-Thr-Asx. Peptide C_{37} appeared to differ from peptide C_{38} only in its mobility, which suggested the possibility of a labile amide group on the third residue.

(ii) Peptide C_{38} ($m = 0$). This peptide stained yellow and had the amino acid composition:

CySO₃H (1.0), Asp (2.0), Thr (1.0), Gly (1.1), Ala (0.9), Arg (2.0). The neutrality of this peptide indicated one asparagine residue. The *N*-terminal sequence was CySO₃H-Thr-Asx; removal of these residues by treatment with PITC left a yellow-staining peptide with $m = +0.72$, which contained the amide group. Digestion of peptide C_{38} with trypsin produced four fragments: free arginine, peptide C_{38t_1} ($m = +0.52$), which had the sequence Arg-Ala-Asn, peptide C_{38t_2} ($m = 0$), which was Ala-Asn, and peptide C_{38t_3} ($m = -0.47$), which stained yellow and had the sequence CySO₃H-Thr-Asp-Gly-Arg.

The sequence of peptide C_{38} was therefore CySO₃H-Thr-Asp-Gly-Arg-Arg-Ala-Asn.

Comparison of peptides C_{37} and C_{38} suggests that in peptide C_{38} an amide group has been lost from this peptide on the third residue. A similar instance of amide loss is noted in peptide P_6 . Both of these peptides contain the sequence Asn-Gly and several instances of loss of amide group from Asn-Gly sequences have been noted in sequence determinations on elastase (D. M. Shotton, personal communication). A further example of deamidation of this combination of amino acid residues has been reported by Ambler (1963).

Fraction 21. Only one cysteic acid-containing peptide was recovered in this fraction.

Peptide C_{39} ($m = +0.18$). This peptide stained yellow. Its amino acid composition was: CySO₃H (1.8), Asp (1.0), Thr (0.9), Glu (1.2), Gly (1.0), Pro (+), Lys (2.6), Arg (0.9). The *N*-terminal residue was cysteic acid, and the 'dansyl'-Edman method gave the sequence CySO₃H-Arg-Glx-CySO₃H-Lys-Gly-Asx(Pro,Thr,Lys₂). Digestion with trypsin produced four fragments.

Peptide C_{39t_1} ($m = +0.57$) had the sequence Thr-Lys.

Peptide C_{39t_2} ($m = 0$) stained yellow. The sequence was Gly-Asp-Pro-Lys.

Peptide C_{39t_3} ($m = -0.10$) stained yellow and contained: CySO₃H (1.9), Glu (1.0), Lys (1.0), Arg (1.2). The mobility indicated the presence of one amide group. The sequence was CySO₃H-Arg-Gln-CySO₃H-Lys.

Peptide C_{39t_4} ($m = 0$). This peptide arose by partial cleavage of peptide C_{39t_3} . Its amino acid composition was: Glu (1.0), CySO₃H (1.0), Lys (1.0). From its mobility it had one amide group. The sequence was Gln-CySO₃H-Lys.

The sequence of peptide C_{39} was therefore CySO₃H-Arg-Gln-CySO₃H-Lys-Gly-Asp-Pro-Lys-Thr-Lys. This peptide contains the sequences CySO₃H-Arg-Gln and CySO₃H-Arg-Gln-CySO₃H-Lys, previously found in low yield as the chymotryptic peptides C_{30} and C_{31} .

The amino acid sequences of these chymotryptic peptides are shown in Table 2.

Table 2. Amino acid sequences of cysteic acid-containing peptides obtained by chymotryptic digestion of performic acid-oxidized ovotransferrin

Pep- tide	Sequence
C ₁	CySO ₃ H-MetSO ₂ -Asp(CHO)-Asn-Ser-Phe
C ₂	CySO ₃ H-Gln-Gly(Ser, Gly ₂ , Ile, Pro ₂ , Glu, Arg, CySO ₃ H)Val-Ala
C ₃	Ser-Val-Val-Ser-Asp-Asp(Glu, Gly)(CySO ₃ H, Thr)Val-Val-Asp-Glu-Tyr-Lys-Asp-CySO ₃ H-Ile-Ile-Lys-Ile-MetSO ₂
C ₄	CySO ₃ H-Asn-Pro-Ser-Asp-Ile(Leu)
C ₅	CySO ₃ H-Asx-Ile(CySO ₃ H, MetSO ₂ , Thr, Ser, Glx, Pro, Gly, Ala, Val, Leu)
C ₆	Thr-Ala-Gly-Val-CySO ₃ H-Gly-Leu
C ₇	Ala-Gly-Val-CySO ₃ H(Gly, Leu)
C ₈	Thr-CySO ₃ H-Val-Gln
C ₉	CySO ₃ H-Val-Gln
C ₁₀	CySO ₃ H-Gln-Leu
C ₁₁	CySO ₃ H-Leu
C ₁₂	CySO ₃ H-Thr-Ile-Ser-Ser-Pro-Glu-Glu-Lys-Lys-CySO ₃ H-Asn
C ₁₃	CySO ₃ H-Ser-Phe-Leu
C ₁₄	CySO ₃ H-Leu-Phe
C ₁₅	CySO ₃ H-Ser-Phe
C ₁₆	CySO ₃ H-Asx-Phe-Asx(Glx, Tyr)
C ₁₇	Ser-Ala-Ser-CySO ₃ H-Val-Pro-Gly-Ala-Thr-Ile(Glu, Gln)Lys-Leu
C ₁₈	CySO ₃ H-Thr-Ile-Ser-Ser-Pro-Glu-Glu-Lys
C ₁₉	Ser-Glu-Gly-CySO ₃ H-Ala-Gly(Ser ₂ , Pro ₊₊ , Asn)Arg-Leu
C ₂₀	Ser-CySO ₃ H-His
C ₂₁	Lys-Thr-CySO ₃ H-Asn(Ser, Asp, Pro, Ile, Leu)
C ₂₂	Phe(Ser, Glu, Gly, CySO ₃ H, Ala, Gly, Ser ₂ , Pro ₊₊ , Asn, Arg, Leu)
C ₂₃	CySO ₃ H-Leu-Asp-Gly-Ser-Arg-Gln-Pro-Val(Asp, Asn)Tyr
C ₂₄	Arg-Glu-CySO ₃ H-Asn-Leu-Ala-Glu-Val(Pro, Thr)
C ₂₅	Asp(Asp, Glu, Ser, Gln)CySO ₃ H-Ser-Lys-Thr-Asp-Glu-Arg-Pro-Ala-Ser-Tyr
C ₂₆	Asp-Tyr-Arg-Glu-CySO ₃ H-Asn
C ₂₇	CySO ₃ H-Ala-Arg-Asn-Ala-Pro-Tyr
C ₂₈	Arg-Glu(CySO ₃ H, Asn, Leu, Ala, Glu, Val, Pro, Thr)His
C ₂₉	Arg-Glu-CySO ₃ H-Asn
C ₃₀	CySO ₃ H-Arg-Gln
C ₃₁	CySO ₃ H-Arg-Gln-CySO ₃ H-Lys
C ₃₂	Arg-CySO ₃ H-Leu
C ₃₃	Lys-Asp-Leu-Thr-Lys-CySO ₃ H-Leu
C ₃₄	Lys-Ser-CySO ₃ H-His
C ₃₅	CySO ₃ H-Ala-Val-Gly-Lys-Asp-Glu(Asp, Ser, Lys, Arg)
C ₃₆	Ala-Glu-Arg-Tyr-Asp-Asp-Glu-Ser-Gln-CySO ₃ H-Ser-Lys-Thr-Asp-Glu-Arg-Pro-Ala-Ser-Tyr
C ₃₇	CySO ₃ H-Thr-Asn(Gly, Arg ₂ , Ala, Asn)
C ₃₈	CySO ₃ H-Thr-Asp-Gly-Arg-Arg-Ala-Asn
C ₃₉	CySO ₃ H-Arg-Gln-CySO ₃ H-Lys-Gly-Asp-Pro-Lys-Thr-Lys

Fig. 3. Elution profile on Dowex 50 (X2) of a pepsin digest of ovotransferrin. Numbers 1-23 show the fractions taken for study.

Pepsin peptides from ovotransferrin

Fig. 3 shows the elution pattern given by a pepsin digest of ovotransferrin on ion-exchange chroma-

tography on Dowex 50 (X2). Twentythree ninhydrin-reacting peaks were observed, but examination of these fractions showed that many of the cystine peptides are not eluted from the resin by the buffer gradient used here, possibly because they are very basic. By using the diagonal electrophoretic technique of Brown & Hartley (1966) cystine-containing peptides were detected in fractions 9, 11, 13, 14, 15, 18, 19 and 20.

Fraction 9.

Peptide P₁ (*m* = 0). This peptide stained yellow and on oxidation with performic acid produced two yellow-staining bands.

Peptide P_{1a} (*m* = -0.28). This had the amino acid composition: CySO₃H (0.6), MetSO₂ (0.8), Thr (0.8), Gly (1.9), Ala (1.0), Val (2.4), Leu (1.0), Pro (1.0). The 'dansyl'-Edman procedure identified

this peptide as Thr-Ala-Gly-Val-CySO₃H-Gly-Leu(Val,Pro,MetSO₂).

Peptide P_{1b} ($m = -0.65$). This had the amino acid composition: CySO₃H (0.7), Ser (0.9), Phe (1.0). Its sequence was CySO₃H-Ser-Phe.

The structure of peptide P₁ therefore appeared to be:

Fraction 11.

Peptide P₂ ($m = 0$). This peptide stained yellow and after performic acid oxidation it gave two yellow-staining bands. One of these (P_{2a}) was identified as peptide P_{1b}. The other product, peptide P_{2b} ($m = -0.29$), was present in very small amount, so that its relationship to peptide P_{1a} could not be determined.

Fraction 13.

Peptide P₃ ($m = 0$). This peptide stained yellow and on performic acid oxidation it gave two products. (a) CySO₃H-Ser-Phe. (b) Peptide P_{3a} ($m = -0.30$), which stained pink and had the composition: CySO₃H (0.8), Thr (1.0), Gly (2.1), Ala (1.0), Val (1.9), Leu (0.8), Tyr (0.4). The 'dansyl'-Edman procedure gave the sequence Val-Thr(Ala,Gly,Val,CySO₃H,Gly,Leu,Tyr).

Peptide P_{3a} thus appears to overlap peptide P_{1a} and the absence of tyrosine from the latter peptide may be due to its destruction during acid hydrolysis. On the other hand, the tyrosine found in Peptide P_{3a} could be due to contamination. Both peptides, however, probably arise from the same cystine bridge:

Fraction 14.

Peptide P₄ ($m = 0$). Oxidation by performic acid vapour produced three bands.

Peptide P_{4a} ($m = -0.15$) stained very weakly with cadmium-ninhydrin and had the composition: CySO₃H (0.8), Asp (2.8), Thr (1.8), Glu (1.0), Gly (1.0), Ile (0.8), Tyr (0.3), Phe (0.9), His (0.9), Arg (1.0), GlcN (+++).

Peptide P_{4b} ($m = -0.3$) was present in very low yield and had the composition: CySO₃H (0.8), Asp (2.0), Thr (1.0), Ser (1.0), Pro (1.0), Leu (0.9), Lys (0.9). The *N*-terminal sequence was Leu-Lys-Thr-CySO₃H.

Peptide P_{4c} ($m = -0.3$) had a slightly greater electrophoretic mobility than peptide P_{4b} and

had the composition: CySO₃H (1.0), Asp (2.0), Thr (1.0), Ser (1.0), Pro (1.0), Lys (1.0). The *N*-terminal sequence was Lys-Thr-CySO₃H-Asx.

Peptide P₄ was assumed to be an extension of the glycopeptide described by Williams (1968).

Fraction 15. This fraction contained three peptides, which after oxidation produced the three peptides shown in Table 3.

Peptide P₅. This peptide was neutral. Oxidation yielded a peptide with $m = -0.32$. The *N*-terminal sequence was Leu-CySO₃H-Leu-Asx-Gly-Ser-Arg-Glx-Pro. Digestion by trypsin produced three fragments.

Peptide P_{5t₁} ($m = 0$) had the composition: Asp (2.0), Glu (1.0), Val (0.9), Tyr (0.8), Lys (1.0), Pro (1.0). The sequence was Glx-Pro-Val-Asx-Asx-Tyr-Lys. The mobility indicated two amide groups to be present.

Peptide P_{5t₂} ($m = -0.3$) had the composition: CySO₃H (1.0), Asp (1.0), Ser (1.0), Gly (1.0), Leu (2.0), Arg (1.0). The sequence was Leu-CySO₃H-Leu-Asp-Gly-Ser-Arg.

Peptide P_{5t₃} ($m = -0.56$) was Thr-CySO₃H-Asn. The overall sequence of peptide P₅ was therefore Leu-CySO₃H-Leu-Asp-Gly-Ser-Arg-Glx-Pro-Val-Asx-Asx-Tyr-Lys-Thr-Cys-Asn.

Peptides P₆ and P₇. These were present in very small quantities and had the *N*-terminal sequences Leu-CySO₃H-Leu-Asp-Gly-Ser-Arg and Leu-CySO₃H-Leu respectively. They appeared to differ from peptide P₅ in mobility only: $m = +0.2$ before oxidation and $m = -0.2$ after oxidation, peptide P₇ being slightly heavier than peptide P₆.

The absence of any apparent partners, the presence of two cysteic acid residues and the mobility changes

Table 3. Amino acid compositions of peptides P₅, P₆ and P₇

Amino acid	No. of residues/molecule of peptide		
	P ₅	P ₆	P ₇
CySO ₃ H	1.4	1.8	1.1
Asp	3.6	3.7	3.4
Thr	0.8	1.0	0.9
Ser	1.0	0.9	0.9
Glu	1.1	1.0	1.1
Pro	+	+	+
Gly	1.0	1.0	1.1
Val	0.9	1.0	0.9
Leu	1.8	1.8	1.5
Lys	0.9	1.0	1.0
Arg	1.0	1.0	0.9

indicate the presence of an internal cystine bridge in these peptides; thus:

ptides C₁₉ and C₂. The presence of a single band after oxidation and the mobility change show that the

An overlapping peptide, namely Thr-CMCys-Asn-Trp-Phe-Glx(Asx,Ser), has recently been found (J. Williams & J. M. Lowe, unpublished work) and a partial cleavage between Asn-Trp and Trp-Phe might explain the small difference between peptides P₆ and P₇. The charge difference between these peptides and peptide P₅ is probably due to the existence of an extra amide group in peptides P₆ and P₇. The extensive loss of amide groups from peptides containing the sequence Asn-Gly has already been mentioned in connexion with peptides C₃₇ and C₃₈.

Fraction 18.

Peptide P₈ (*m* = +0.12). Oxidation by performic acid vapour produced a single band (*m* = -0.3). The amino acid composition of the oxidized product was: CySO₃H (2.2), Asp (1.1), Ser (4.1), Glu (5.1), Pro (>3), Gly (4.6), Ala (2.1), Val (1.0), Ile (1.0), Leu (2.0), Phe (0.9), Lys (1.0), His (1.0), Arg (1.9). The *N*-terminal residue was phenylalanine, and carboxypeptidase B released lysine from the *C*-terminal end leaving a peptide with *m* = -0.42. Digestion of the oxidized peptide with trypsin produced three fragments.

Peptide P₈t₁ (*m* = -0.07) stained yellow and had the composition: CySO₃H (0.9), Ser (1.7), Glu (1.1), Ala (1.0), Val (1.0), Lys (0.9), His (1.0). Its sequence was CySO₃H-Val-Ala-Ser-Ser-His-Glu-Lys. Carboxypeptidase B released lysine, leaving a yellow-staining peptide with *m* = -0.35.

Peptide P₈t₂ (*m* = -0.22) had the composition: CySO₃H (0.7), Asp (1.0), Ser (2.3), Glu (1.2), Pro (2.0), Gly (1.8), Ala (1.0), Phe (0.8), Arg (1.0). Its partial structure was Phe-Ser-Glu-Gly-CySO₃H-Ala-Gly(Ser₂,Pro₂,Asn)Arg. This suggests that peptide P₈t₂ is identical with peptide T₂c₁.

Peptide P₈t₃ (*m* = -0.39) had the composition: CySO₃H (1.4), Ser (1.4), Glu (3.0), Pro (2.2), Gly (3.1), Ile (1.0), Leu (1.8), Arg (1.3). Its partial structure was Leu-CySO₃H-Glx-Leu-CySO₃H-Glx-(Gly₃,Ser,Ile,Pro₂,Glx)Arg. It thus appears to be identical with peptide T₁.

The end groups identified in peptide P₈ show that the order of the tryptic fragments is P₈t₂-P₈t₃-P₈t₁ and the required overlaps are provided by pep-

four half-cystine residues were joined together in internal disulphide bridges. Tryptic digestion of the unoxidized peptide produced no change in mobility, but subsequent oxidation produced the three fragments P₈t₁, P₈t₂ and P₈t₃. This shows that the half-cystine residues of peptide P₈t₃ were not linked by a disulphide bridge.

Fraction 19.

Peptide P₉ (*m* = 0). Oxidation of this peptide produced two new peptides, P_{9a} and P_{9b}.

Peptide P_{9b} (*m* = -0.38) had the composition: CySO₃H (0.6), Asp (1.0), Ala (1.0), Ile (1.0), Leu (1.0), Lys (1.0). Its sequence was Leu-Asp-CySO₃H-Ile-Lys-Ala.

Peptide P_{9a} (*m* = -0.14) had the composition: CySO₃H (0.6), Asp (0.7), Thr (1.0), Ser (2.5), Glu (2.5), Pro (2.0), Gly (0.5), Ala (1.1), Val (1.0), Ile (2.0), Lys (1.1), Arg (1.0). The *N*-terminal residue was alanine. Tryptic digestion of peptide P_{9a} produced two ninhydrin-reactive bands.

(a) Peptide P_{9a}t₁ (*m* = +0.53) had the composition Ala (1.0), Pro (1.7), Lys (1.0). The mobility suggested that only one proline residue was present and this fragment appears to be the *N*-terminal part of peptide P_{9a}. Its structure is thus Ala(Pro₁₋₂)Lys.

(b) Peptide P_{9a}t₂ (*m* = +0.44) had the composition: Ser (1.0), Val (1.0), Ile (1.0), Arg (1.0). Since this peptide stained yellow with cadmium-ninhydrin reagent, the sequence was probably Ser(Ile,-Val)Arg.

The remaining part of peptide P_{9a} was not recovered but it presumably contained CySO₃H₁-Thr₁,Ser₂,Glu₃,Pro(?),Ile₁,Asp(?),Gly(?). The failure to recover this fragment is not yet understood and peptide P₉ was tentatively assumed to have the structure shown below:

Fraction 20.

Peptide P₁₀ (*m* = -0.22). Performic acid oxidation of this peptide produced peptides P_{10a} and P_{10b}.

Peptide P_{10a} (*m* = 0) had the composition: CySO₃H (0.9), Asp (1.6), Thr (1.0), Leu (2.4), Phe (1.0), Lys (2.0). The *N*-terminal sequence of peptide P_{10a} was Phe-Lys. Tryptic digestion of peptide P_{10a} gave three fragments. (a) Peptide P_{10a}t₁

($m = +0.54$) was the dipeptide Phe-Lys. (b) Peptide $P_{10a}t_2$ ($m = 0$) was Asp-Leu-Thr-Lys. (c) Peptide $P_{10a}t_3$ ($m = -0.73$) was the dipeptide $CySO_3H$ -Leu. The sequence of peptide P_{10a} was therefore Phe-Lys-Asp-Leu-Thr-Lys- $CySO_3H$ -Leu.

Peptide P_{10b} ($m = -0.5$) had the composition: $CySO_3H$ (0.8), Asp (2.7), Thr (0.8), Ser (2.4), Glu (4.0), Pro (+), Ala (1.9), Tyr (0.7), Lys (1.5), Arg (2.0). The *N*-terminal sequence of peptide P_{10b} was Ala-Glu-Arg. Trypsin digestion of peptide P_{10b} gave three fragments. (a) Peptide $P_{10b}t_1$ ($m = 0$) was the tripeptide Ala-Glu-Arg. (b) Peptide $P_{10b}t_2$ ($m = -0.29$) was Thr-Asp-Glu-Arg-Pro-Ala-Ser-Tyr. (c) Peptide $P_{10b}t_3$ ($m = -0.73$) contained one amide group and had the sequence Tyr-Asx-Asx-Glx-Ser-Glx- $CySO_3H$ -Ser-Lys. Peptide P_{10} therefore had the structure:

Further studies on cystine peptides

As noted in the previous section, most of the cystine peptides in a pepsin hydrolysate of ovotransferrin were not recovered from the ion-exchange column. The use of Dowex 1 was precluded because of the danger of disulphide interchange. A further difficulty was the low yield of peptides owing to the relatively low specificity of pepsin. Therefore ovotransferrin was digested successively with pepsin at pH 2.0 and trypsin at pH 6.5. The latter pH was chosen to avoid disulphide interchange. The diagonal technique of Brown & Hartley (1966) was used for purification of the peptides.

As the diagonal electrophoretic pattern was extremely complex (Fig. 4), individual cystine-containing peptides could not be purified, but in several cases it was possible to deduce the nature of the disulphide bridges from the pattern. Besides the neutral region, which was too complex to investigate by paper techniques, seven main bands of peptides moved off the diagonal (Fig. 4).

Band I. This contained two cysteic acid peptides present in good yield, and a further three present in low yield.

Peptide PT_1 ($m = -0.1$) stained yellow and was $CySO_3H$ -Ala-Arg.

Peptide PT_2 ($m = 0$) was Leu- $CySO_3H$ -Arg.

Peptide PT_3 ($m = +0.25$) stained yellow and had the sequence Thr-Ser- $CySO_3H$ -His-Thr-Gly-Leu-Gly-Arg. Digestion with chymotrypsin produced a yellow fragment ($m = +0.7$), which was Gly-Arg, and the neutral residual fragment.

Peptide PT_4 ($m = +0.3$) was Phe-His- $CySO_3H$ -Leu-Lys.

Peptide PT_5 ($m = +0.35$) was Lys- $CySO_3H$ -Asn-Asn-Leu-Arg.

The positions of these spots in the electrophoretogram and their relative intensities suggest that before oxidation peptides PT_1 and PT_2 were joined by a disulphide bridge and that peptides PT_3 and PT_4 were also joined by a disulphide bridge. No partner was found for peptide PT_5 .

Band II. This was resolved by electrophoresis at pH 3.5 into three bands.

(a) The least basic band, band IIa, on oxidation produced two fragments.

Peptide PT_6 ($m = -0.38$) was an acidic peptide of sequence Asp- $CySO_3H$ -Ile-Ile-Lys.

Peptide PT_7 ($m = 0$) had the composition: $CySO_3H$ (0.9), Glu (1.0), Gly (1.1), Ala (1.0), Val (1.0), Ile (0.8), Lys (1.1). The partial sequence was

determined as Ile-Gln-X- $CySO_3H$ -Ala(Val,Gly)Lys. The third residue in this sequence could not be identified, since no result was obtained by the 'dansyl' method. This residue may be tryptophan, since the tripeptide Ile-Gln-Trp has been found by J. Williams & J. M. Lowe (unpublished work).

Fig. 4. Diagonal electrophoretic pattern, carried out at pH 6.5, of a pepsin-trypsin digest of ovotransferrin.

(b) The middle band, band IIb, on oxidation produced two fragments.

Peptide PT_8 ($m = 0$) was $Gln-CySO_3H-Lys$.

Peptide PT_9 ($m = -0.2$) had the composition: $CySO_3H$ (0.8), Thr (0.7), Ser (1.8), Glu (2.0), Pro (+), Gly (1.2), Ala (1.8), Val (0.8), Phe (0.6), Lys (0.9). The *N*-terminal residue was phenylalanine and the composition suggests that peptide PT_9 differs from peptide T_5 only in the loss of one *N*-terminal residue of phenylalanine.

(c) The most basic band, band IIc, on oxidation produced two fragments.

Peptide PT_{10} ($m = -0.1$) stained yellow and was $CySO_3H-Ala-Val-Gly-Lys$.

The acidic fragments were found to have the following structures:

The neutral fragment contained the disulphide bridge of band V.

The presence of the acidic peptide Ile-Ser-Ser-Pro-Glu-Glu-Lys shows that band V possesses an extensive overlap with the chymotryptic peptide C_{18} . By subtracting the composition of the known sequences from the composition of peptide band V, the following partial structure can be deduced:

Peptide PT_{11} ($m = -0.38$) was identical with peptide PT_6 . Therefore it is likely that before oxidation disulphide bridges existed between peptides PT_6 and PT_7 , between peptides PT_8 and PT_9 , and between peptides PT_{10} and PT_{11} .

Band III. This produced two fragments on oxidation.

Peptide PT_{12} ($m = -0.5$) stained yellow and was $CySO_3H-Leu-Val-Glu$.

Peptide PT_{13} ($m = +0.25$) stained yellow and had the composition: $CySO_3H$ (0.6), Thr (0.9), Ser (1.0), Gly (1.2), Ala (1.1), Val (0.8), His (0.9), Arg (1.0). The sequence was $Ser-CySO_3H-His-Thr-Ala-Val-Gly-Arg$.

Band IV. This band gave rise to three oxidation products, peptides PT_{14} , PT_{15} and PT_{16} . Peptide PT_{14} was further fractionated by electrophoresis at pH 2.1 into peptides PT_{14a} and PT_{14b} , which differed only in the presence of a *C*-terminal tyrosine residue in peptide PT_{14b} . These four products were found to correspond to the tryptic-cleavage products of peptide P_8 , i.e. peptides P_{8t_1} , P_{8t_2} and P_{8t_3} respectively, and this observation confirms that peptide P_8 contains two internal disulphide bridges.

Band V. After performic acid oxidation only one ninhydrin-positive product was obtained, peptide PT_{17} , and this was found to have the structure $Leu-Asp-CySO_3H-Ile-Lys$. No partner could be detected.

Therefore the unoxidized band V was purified by electrophoresis at pH 3.5 and pH 2.1. The amino acid composition (after performic acid oxidation) was: $CySO_3H$ (1.5), Asp (3.1), Thr (2.0), Ser (1.4), Glu (3.1), Pro (++), Gly (0.9), Ala (0.9), Val (2.0), Ile (2.1), Leu (0.8), Lys (2.0). Digestion of the unoxidized band V with subtilisin gave rise to three acidic fragments and one that was neutral at pH 6.5.

Bands VI and VII. These bands appeared to contain the same disulphide bridge. Performic acid oxidation in both cases produced the acidic peptide PT_{18} ($m = -0.73$) $Tyr-Asp(Asp, Glu, Ser, Gln, CySO_3H, Ser)Lys$, previously encountered as peptide T_{12} , whereas the other side of the bridge was $CySO_3H-Leu$ (peptide PT_{19}) in band VII and $CySO_3H-Leu(Phe)Lys$ (peptide PT_{20}) in band VI.

The amino acid sequences of the cysteic acid-containing peptides found in this work may be placed to form 25 peptides in which there are 34 cysteic acid residues, as shown in Fig. 5. These residues are arbitrarily numbered and the probable constitution of 12 of the 17 disulphide bridges is as follows: 1-7, 2-3 or 4, 5-4 or 3, 6-8, 9-10, 11-30, 12-14, 15-24, 16-17, 18-19, 25-31 and 27-28.

DISCUSSION

It is well known that hen's-egg ovotransferrin consists of a major and a minor protein species that can be separated by starch-gel electrophoresis (Lush, 1961). It has been noted that the two species are similar in their amino acid compositions, immunological and iron-binding properties (Wenn & Williams, 1968). In the addendum to the present paper it is shown that they give identical patterns when studied by a radioactive peptide-'mapping' technique. It therefore seems probable that the two species possess closely similar or identical amino acid sequences and for the present study it did not appear necessary to isolate these forms.

The binding of iron by transferrins has been studied by equilibrium dialysis (Aasa, Malmström, Saltman & Vänngård, 1963), by free electrophoresis (Aisen *et al.* 1966) and by isoelectric focusing (Wenn & Williams, 1968). These studies suggest that the

Fig. 5. Amino acid sequences of cysteic acid-containing peptides from ovotransferrin. Peptides from tryptic digestion, —; peptides from chymotryptic digestion, ----; peptides from peptic digestion followed by tryptic digestion, -.-.-. The probable linkages of 12 of the 17 disulphide bridges are: 1-7, 2-3 or 4, 5-4 or 3, 6-8, 9-10, 11-30, 12-14, 15-24, 16-17, 18-19, 25-31, 27-28.

two metal-binding sites on the protein molecule are independent and equivalent; this could plausibly be associated with the presence of two closely similar or identical halves or subunits. The main experimental evidence for this idea is the finding that tryptic-peptide 'maps' of transferrins show fewer spots than would have been expected from the amino acid composition of the protein (Jeppsson, 1967; Baker *et al.* 1968). This approach is, however, probably less reliable than actual sequence determinations, and since 34 unique cysteic acid residues have been identified in the present work we conclude that hen ovotransferrin does not consist of identical halves or subunits. It is possible, however, that the present-day polypeptide chain has evolved from a shorter chain by a process of partial gene duplication with the subsequent accumulation of amino acid differences. Thus a certain similarity may be noted between peptides PT₃ and PT₁₃:

but more results are required before this can be evaluated.

It is difficult to know whether all the cysteic acid residues in the protein have been recovered, although this seems very likely since no evidence for the existence of additional cysteic acid-containing peptides was encountered. Since ovotransferrin contains 31.0 mol of cysteic acid/80 000 g of protein the recovery of 34 cysteic acid residues suggests that the minimum molecular weight is about 88 000.

In a previous study of the glycopeptides of hen ovotransferrin (Williams, 1968) it was found that 85% of the total hexose was attached to a basic site His-Asp(CHO)-Arg and the remaining 15% was present at neutral sites that were identified as Ala-Asp(CHO)-Leu-Thr, Asp(CHO)-Leu-Thr-Gly and Asp(CHO)-Leu-Thr-Tyr. An extra neutral glycopeptide C₁ has been found in the present work with the structure CySO₃H-MetSO₂-Asp(CHO)-Asn-Ser-Phe. It would be expected that a carbohydrate-free form of this peptide should also exist, but it has not been encountered so far.

At two sites a variable residue has been encountered. Thus at the C-terminus of peptide T₁ lysine and arginine appear in approximately equal amounts. Serine and alanine were found in the second position of peptide T₉. There appear to be three possible explanations of this heterogeneity: (a) the hens from

which the eggs were obtained may have been heterozygous; (b) genetically non-identical populations of hens may have contributed to the egg pool; (c) errors may have occurred in the synthesis of the protein.

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