The Amino Acid Sequence of a Carbohydrate-Containing Fragment of Hen Ovotransferrin

By I. BARRY KINGSTON* and JOHN WILLIAMS

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

(Received 26 November 1974)

1. Hen ovotransferrin was treated with CNBr and fractionated by gel filtration. 2. After further treatment by reduction and carboxymethylation a carbohydrate-containing fragment of molecular weight 11990 was obtained (fragment BCd). 3. The amino acid sequence of this fragment was determined. It consists of a single chain of 94 residues. 4. The structure of a tryptic glycopeptide derived from whole ovotransferrin permitted a further eight residues to be assigned at the *N*-terminus of fragment BCd. 5. Heterogeneity was found at two positions. 6. Further evidence has been deposited as Supplementary Publication SUP 50045 (19 pages) at the British Library (Lending Division), Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, U.K., from whom copies may be obtained on the terms indicated in *Biochem. J.* (1975), 145, 5.

It is still not known whether the transferrins possess a duplicated structure. The possibility of duplication was first suggested because the transferrins bind two atoms of iron per molecule and because human serum transferrin possesses two identical carbohydrate groups. However, Jeppson's (1967) claim that there are two identical subunits was disproved by ultracentrifuge studies which showed that the transferrins consist of a single polypeptide chain of molecular weight about 77000 (Greene & Feeney, 1968; Mann *et al.*, 1970). Since peptide 'maps' have shown fewer spots than would be expected from a non-repeating sequence of this molecular weight, it has been suggested that the chain contains similar halves (Baker *et al.*, 1968; Greene & Feeney, 1968; Mann *et al.*, 1970). The possibility of a simple duplicate structure was disproved, however, by the finding of 34 unique cysteic acid-containing peptides in digests of performic acid-oxidized hen ovotransferrin (Elleman & Williams, 1970) and by CNBr cleavage experiments (Bezkorovainy & Grohlich, 1974; Tsao *et al.*, 1974; Sutton & Brew, 1974). Graham & Williams (1975) found that in several transferrins there are two carbohydrate groups with identical sugar compositions, but the amino acid sequences of the glycopeptides show no obvious similarity. It has recently been found that in hen ovotransferrin the binding sites for the first and second atoms of iron are formed by the *N*-terminal and *C*-terminal regions of the protein respectively (Williams, 1974; J. Williams, unpublished work). Metal-binding fragments representing the *N*-terminal and *C*-terminal regions,

prepared by limited proteolysis, were found to differ in their metal-binding properties, amino acid compositions, peptide 'maps' and immunological properties. The single major carbohydrate group of this protein is located in the *C*-terminal region. This evidence further supports the conclusions that no exact duplication of structure exists. There is, however, a clear need for more information on the primary structure of the transferrins. In this paper we report the amino acid sequence of a 94-residue fragment obtained from hen ovotransferrin by CNBr cleavage.

Materials and Methods

Enzymes and reagents

Trypsin (type XI, diphenylcarbamoyl chloride-treated), chymotrypsin (type II), subtilisin (proteinase type VIII) and carboxypeptidases A and B (di-isopropyl phosphorofluoridate-treated) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Thermolysin was obtained from Calbiochem, San Diego, Calif., U.S.A. *S*-Ethyl thioltrifluoroacetate was obtained from Pierce Chemical Co., Rockford, Ill., U.S.A.

Ovotransferrin

Hen ovotransferrin was prepared as described by Williams (1968).

CNBr cleavage

Ovotransferrin (500mg) was dissolved in 5ml of 70% (v/v) formic acid, and CNBr (500mg) was

* Present address: University of Indiana, Department of Zoology, Jordan Hall 208, Bloomington, Ind. 47401, U.S.A.

added. After 24h at room temperature the mixture was diluted with 1 vol. of water, deaerated with a water vacuum pump, and applied to a column (80cm×4cm) of Sephadex G-100 in 5% (v/v) formic acid. The eluate was monitored continuously at 276nm and fractions (10ml) were collected. Peak fractions were pooled, dialysed against water and freeze-dried.

Reduction and alkylation

Freeze-dried material (1g) was dissolved in a mixture of 100ml of 8M-urea, 5ml of 0.2% EDTA and 30ml of 3M-Tris-HCl, pH8.6. β -Mercaptoethanol (1 ml) was added and the solution was left under N₂ for 5h at room temperature. Iodoacetic acid (6.5g in 25ml of 1M-NaOH) was added slowly and the pH was maintained at 8.6 by additions of 1M-NaOH. After 15min, dialysis of the solution against water was begun and this was continued for 24h. The volume of the solution was decreased by partial freeze-drying and it was then dialysed against 5% (v/v) formic acid for a further period of 24h before being applied to a column (80cm×4cm) of Sephadex G-100 in 5% (v/v) formic acid. Fraction BCd (see Fig. 2) was further purified on a column (86cm×3 cm) of Sephadex G-50 (superfine-grade) in 5% (v/v) formic acid.

Determinations of molecular weight

Molecular weight was determined by polyacrylamide-gel electrophoresis and by gel filtration. Gel electrophoresis was carried out as described by Fairbanks *et al.* (1971) with the use of 0.1% (w/v) sodium dodecyl sulphate. Gel filtration was carried out on a column (90cm×1.8cm) of Sephadex G-50 (superfine-grade) as described by Andrews (1964). The solvent was 1% (w/v) sodium dodecyl sulphate-1mM-EDTA adjusted to pH8.0 with 1M-NaOH. Protein samples were dissolved in the same solvent containing 2% (v/v) β -mercaptoethanol and heated in a boiling-water bath for 2min before gel filtration.

Enzyme digestions

Digestions were carried out in 0.2M-N-ethylmorpholine acetate buffer, pH8.4. Enzymes were used at an enzyme/substrate ratio of 1:50 (w/w) and incubation was carried out for 5h at 37°C.

Purification of peptides

Peptides were fractionated on a column (100cm×0.6cm) of Dowex 50 (Bio-Rad AG50W-X4, 20–30 μ m) with volatile pyridine-acetate buffers and a concave buffer gradient from pH3.1 to 5.1 (Schroeder, 1967). Fractions (80 drops) were collected and in most

experiments the eluate was monitored continuously at 276nm. Samples from each fraction were tested by the ninhydrin method, after alkaline hydrolysis (Rudloff & Braunitzer, 1961). High-voltage paper electrophoresis was used for further purification of peptides. Peptides were detected on paper by the cadmium acetate-ninhydrin stain of Heilmann *et al.* (1957), and a specific stain for tryptophan was also used (Smith, 1953). Peptides were eluted from the paper with 1% (w/v) NH₃ solution.

Amino acid and carbohydrate analyses

Proteins and peptides were hydrolysed with 5.7M-HCl in sealed evacuated tubes at 105°C for 16–72h. Amino acid compositions were determined with a Technicon TSM-1 amino acid analyser. The compositions of peptides are given as molar ratios of the amino acids. Tryptophan was measured by the method of Spies & Chambers (1949). The hexosamine content of fragment BCd was determined by the method of Randle & Morgan (1955). The moisture content of the sample was determined by drying to constant weight in an oven at 105°C.

Amino acid sequence determination

Sequence determination was carried out by the dansyl-Edman method as described by Gray (1967a,b). During this process peptides containing tryptophan gave a characteristic purple colour with trifluoroacetic acid (Uphaus *et al.*, 1959). Dansyl-amino acids were identified by t.l.c. on polyamide layers cut into squares (6.5cm×6.5cm). Solvents I and II of Woods & Wang (1967) and the solvent of Crowshaw *et al.*, (1967) were used. In some cases peptides were subjected to further enzyme digestion. Thermolysin digestion was carried out for 2h at 60°C in 0.05M-ammonium acetate-5mM-CaCl₂ (pH8.5). Digestion with other enzymes was carried out at 37°C for 2h in 0.2M-N-ethylmorpholine acetate, pH8.4. Mobilities of peptides at pH6.5 were measured relative to that of aspartic acid. The sign given indicates the nature of the charge. Amide groups were assigned by Offord's (1966) electrophoretic-mobility method.

Isolation of N-terminal peptide

The N-terminal peptide of fragment BCd was isolated by the maleylation method of Bruton & Hartley (1970).

Isolation of a glycopeptide from ovotransferrin

Ovotransferrin (2g) was dissolved in 100ml of 0.1M-Tris-HCl (pH8.6)–8M-urea and heated at 100°C for 5min. After cooling, the solution was

added slowly, with stirring, to 300ml of 0.1M-Tris-HCl (pH8.6) containing 20mg of trypsin. Incubation was carried out at 37°C for 4h. A further 20mg of trypsin was added and the incubation continued for 3h. The digest was dried by rotary evaporation, dissolved in 20ml of water and applied to a column (80cm×4cm) of Sephadex G-50 in 1% (w/v) NH_4HCO_3 . Fractions containing hexose, detected by the orcinol- H_2SO_4 reaction (Winzler, 1955), were pooled and the glycopeptide fraction was further purified by ion-exchange chromatography. Since precipitation occurred in acid solutions chromatography was first carried out on a column (100cm×0.6cm) of Dowex 1 (Bio-Rad AG1-X2) as described by Kasper (1970). A single hexose-containing fraction was eluted quickly but paper electrophoresis showed it to be heterogeneous. Since this material did not precipitate at low pH, chromatography on Dowex 50-X4 was carried out as described above.

Trifluoroacetylation and trypsin digestion

Trifluoroacetylation of fragment BCd (140 mg) was carried out as described by Goldberger & Anfinsen (1962) and by Taniuchi *et al.* (1967). After freeze-drying the trifluoroacetylated material was digested with trypsin. The digest was then freeze-dried and redissolved in 7ml of ice-cold 1M-piperidine to remove the blocking groups. Acetic acid (4M) was added to the solution to give pH 5.0 and the solution was applied to a column (90cm×1.8cm) of Sephadex G-25 (superfine grade) in 0.1M-acetic acid. Peptides were detected by the ninhydrin method, after alkaline hydrolysis (Rudloff & Braunitzer, 1961).

Results

CNBr cleavage

Fig. 1 shows the elution pattern given by CNBr-treated ovotransferrin on gel filtration on Sephadex G-100. In some experiments the resolution of peaks B and C was less complete than in Fig. 1 and in these cases they were pooled. After reduction and alkylation of the pooled material (BC), gel filtration gave the elution pattern shown in Fig. 2.

In other experiments isolated peak-B material was subjected to reduction and alkylation. The product was eluted from Sephadex G-100 in the same position as untreated peak-B material. Peak BCb (Fig. 2) was also eluted in this position. The molecular weight of BCb was 35000 and it gave *N*-terminal alanine. The maleylation method, when applied to peak BCb, gave an *N*-terminal peptide with the amino acid sequence Ala-Pro-Pro-Lys-Ser. This has already been found to be the amino acid sequence at the *N*-terminus of hen ovotransferrin (Graham & Williams, 1975). It is likely therefore that fragment BCb represents the *N*-terminal half of the protein.

Fig. 1. Gel-filtration pattern of CNBr-treated ovotransferrin on Sephadex G-100 in 5% (v/v) formic acid

For details see the text.

Fig. 2. Gel-filtration pattern of reduced carboxymethylated pooled material BC (see Fig. 1) on Sephadex G-100 in 5% (v/v) formic acid

For details see the text.

Molecular weight of fragment BCd

Three estimates of the molecular weight of fragment BCd by gel electrophoresis gave values of 11 700, 14 800 and 17 800. A single estimation by gel filtration gave 11 350.

Composition of fragment BCd

Table 1 shows the amino acid and carbohydrate composition of fragment BCd. Although the mannose and hexosamine values are lower than those obtained earlier for hen ovotransferrin (Williams, 1968) they indicate that fragment BCd contains the main carbohydrate group of the protein.

Fig. 3. Amino acid sequence of CNBr fragment BCd

Peptides derived by tryptic digestion (T) are shown above the sequence and by chymotryptic digestion (C) and tryptic digestion of trifluoroacetylated fragment (TFA) below the sequence. The *N*-terminal tryptic peptide (TG) was obtained as a glycopeptide after tryptic digestion of whole ovotransferrin. Vertical arrows show peptides produced by secondary digestion: H, thermolysin, X, CNBr. Full lines indicate quantitative amino acid analysis. Sequences obtained by the dansyl-Edman method are indicated by —; amino acids released by carboxypeptidase A or B are indicated by - - -; — indicates final residues of peptides identified as the free amino acid after the removal of the remainder by successive dansyl-Edman cycles. * See the Discussion section.

Table 1. *Amino acid and carbohydrate analysis of fragment BCd*

Results are shown as residues per molecule. Tryptophan was determined colorimetrically. *N*-Acetylglucosamine and mannose values are the results of a single determination by g.l.c. Results were calculated on the basis of a molecular weight of 10471 derived from the sequence.

Amino acid or carbohydrate	Residues per molecule			Sequence
	24h hydrolysis	48h hydrolysis	72h hydrolysis	
CmCys	6.4	5.1	5.3	5
Asp	9.8	8.8	9.2	9
Thr	4.8	4.3	4.6	5
Ser	6.0	5.3	5.0	7
Glu	11.3	10.8	11.6	11
Pro	5.3	5.2	5.4	4*
Gly	11.7	12.1	12.1	12
Ala	6.2	6.3	6.3	6
Val	5.1	5.5	5.3	5
Ile	1.9	2.1	2.1	2
Leu	6.0	6.5	6.2	6
Tyr	2.9	2.8	2.6	3
Phe	3.9	3.9	3.9	4
Lys	5.1	5.9	5.4	5-6
His	2.8	2.8	2.8	3
Arg	4.3	5.0	4.7	3-4
Trp	0.7			1
Hse	+			1
<i>N</i> -Acetyl glucosamine	4.4			
Mannose	2.9			

* See the Discussion section.

N-Terminal peptide of fragment BCd

The maleylation method gave only the dipeptide Gly-Leu.

Amino acid sequence of fragment BCd

Reduced and alkylated samples of fragment BCd were digested with trypsin and with chymotrypsin. In a further experiment lysine residues were modified by trifluoroacetylation before trypsin digestion was carried out. Fig. 3 summarizes the evidence for the proposed amino acid sequence of fragment BCd. Detailed evidence for this structure has been deposited as Supplementary Publication SUP 50045. This evidence consists of: (1) figures of the elution patterns given by enzyme digests of fragment BCd on chromatography on Dowex 50; (2) tables showing the amino acid compositions, electrophoretic mobility at pH 6.5, yields and cadmium-ninhydrin colours of all the peptides used in establishing the amino acid sequence of fragment BCd (this includes some peptides that are not shown in Fig. 3); (3) a description of the results of carboxypeptidase digestion of peptide

Fig. 4. *Elution profile of tryptic glycopeptides of ovotransferrin on Dowex 50*

For details see the text. Shaded areas represent carbohydrate-containing fractions.

Table 2. *Amino acid composition of the tryptic glycopeptides from whole ovotransferrin*

Results are expressed as molar ratios of amino acids. The presence of tryptophan was detected on paper after staining with Ehrlich reagent. 'm' indicates the mobility relative to aspartic acid ($m = -1.00$).

	Peptide 1	Peptide 3	Peptide 4
Asp	0.9	0.9	0.9
Thr	0.3	0.8	0.8
Pro	0.9	1.0	1.1
Gly	1.7	1.9	1.8
Ala	0.8	0.9	0.8
Val	0.6	0.6	0.7
Met	0.7	0.8	0.8
Ile	1.2	1.4	1.5
Leu	0.9	1.0	1.0
His	0.7	0.8	0.8
Arg	1.0	1.1	1.2
Trp	+	++	++
Glucosamine	++	++	++
m	0	+0.2	+0.18
Colour with cadmium-ninhydrin	Pink	Yellow	Yellow

TFA2 (Fig. 3) which establish the C-terminal sequence of that peptide as Ala-Asp-Trp-Ala-Lys.

Structure of a glycopeptide from ovotransferrin

Ion-exchange chromatography of the glycopeptide fraction from a tryptic digest of hen ovotransferrin gave the elution pattern shown in Fig. 4. The amino acid compositions of the three peaks containing hexose are given in Table 2. Attempted dansyl-Edman degradation of peptide 1 gave no results. Peptide 3 gave the partial sequence shown in Fig. 5 and it contained tryptophan. Peptide 4 had *N*-terminal threonine and was cleaved by CNBr into peptides 4.1 and 4.2 (Fig. 5 and Table 3).

The C-terminal part of the glycopeptide corresponds to the *N*-terminal part of fragment BCd. The

Fig. 5. Amino acid sequence of glycopeptides 3 and 4

For details see the text. The symbols are as in Fig. 3.

Table 3. Peptides obtained by treatment of glycopeptide 4 with CNBr

The peptides were separated by electrophoresis at pH 1.9.

Peptide	Amino acid composition (molar ratios)
4.1	Thr(0.9), Pro(1.1), Gly(1.2), Ala(1.1), Val(0.8), Ile(0.8), Trp(+), Hse(++)
4.2	Asp(1.0), Gly(1.0), Ile(0.9), Leu(1.0), His(0.9), Arg(1.1), Glucosamine(++)

probable location of the *N*-terminal part of the glycopeptide was identified by using the sequence Thr-Ala-Gly-Trp as a marker. Ovotransferrin was digested successively with trypsin and chymotrypsin and chromatographed on Dowex 50. Several fractions containing tryptophan were obtained as judged by u.v. absorption at 276nm and by the use of a specific stain for tryptophan on paper. One such fraction contained a single tryptophan-containing peptide which gave the sequence Thr-Ala-Gly-Trp. This peptide was not found in similar digests of fragments BCa, BCb, BCc or BCD but was obtained from fragment BCE. It is therefore likely that the tryptic glycopeptide joins the *C*-terminus of fragment BCE to the *N*-terminus of fragment BCD.

Discussion

Residues 9 to 23 of the sequence shown in Fig. 3 were first determined by Williams (1968). They were confirmed by Elleman & Williams (1970) who also extended the sequence to residue 60. In the present work most of these 52 residues were redetermined and no disagreement with the earlier work was found. Nevertheless, as the following paragraph shows, the earlier work contained a possible error which was

also overlooked in the course of the present work. The structure of the tryptic glycopeptide shows that the tryptic glycopeptide described by Williams (1968) was produced by cleavage of a Met-Gly bond. It is likely that this was due to contamination of the trypsin by chymotrypsin.

Table 1 shows that 5.3 (average) mol of proline/mol of protein were found by amino acid analysis of fragment BCD but only 4 were found by sequence determination. This discrepancy can be traced to the region of residues 33–36 (Fig. 3). All the peptides in which this region is represented (T2, T2Cl, C4, C5 and TFA7) contain an excess of proline residues (average value 2.86) over the two assigned to positions 32 and 33. As Fig. 3 shows, sequence determination in the present work proceeded only as far as the proline residue at position 32. In the work of Elleman & Williams (1970) the tryptic peptide T2 contained 3.0 residues of proline/mol of peptide by amino acid analysis but sequence determination gave the structure Ser-Pro-Pro-Asn-Ser-Arg. In view of the consistent results of amino acid analysis it seems likely that a third residue of proline exists in this region and it would be desirable to study this part of the sequence again.

Fig. 3 shows that at position 51 both lysine and arginine were found, confirming earlier observations (Elleman & Williams, 1970). In a trypsin digest of fragment BCD peptides T1 and T3 were obtained in amounts of 558 and 343nmol respectively. Peptide T1 contained lysine (0.9 residue/molecule) but no arginine. Peptide T3 contained arginine (1.1 residues/molecule) but no lysine. In a second trypsin digest the yields of these peptides were 588nmol and 1715nmol respectively. Except for the basic amino acids the compositions of peptides T1 and T3 showed no significant differences. In a chymotryptic digest of fragment BCD, peptide C7 (833nmol) contained lysine

(1.9 residues/molecule) but no arginine, whereas peptide C9 (55nmol) contained lysine (0.9 residue/molecule) and arginine (1.0 residue/molecule). Peptide C12 also contained residue number 51. Its composition showed lysine (1.7 residues/molecule) but no arginine. No corresponding peptide could be found in which both lysine and arginine were present. In a trypsin digest of trifluoroacetylated fragment BCd two peptides containing residue number 51 were recovered. Peptide TFA6 contained arginine (1.2 residues/molecule) but no lysine; from the composition and from the *N*-terminal sequence Leu-CmCys it appears to correspond to the sequence from position 37 to position 51. Another peptide from this digest (peptide TFA6a, which is not described in the Supplementary Publication) corresponded to the sequence from position 37 to position 68, residue number 51 being lysine. After deblocking and further trypsin digestion peptide TFA6a was obtained (Supplementary Publication, Table 4). This peptide contained lysine (1.1 residues/molecule) but no arginine and represented the sequence from position 37 to position 51. The electrophoretic mobilities at pH6.5 of the variant peptides containing residue number 51 were very similar but a clear separation was obtained by ion-exchange chromatography. The lysine-containing form of each pair of peptides was eluted before the arginine-containing form.

Fig. 3 also indicates the possibility of polymorphism at position 79. The evidence for this is suggestive but not as clear as for position 51. In trypsin hydrolysates of fragment BCd, peptides T11 and TFA2 had valine at this position and no other tryptic peptides containing this position were found. In a chymotryptic hydrolysate, however, there were three peptides, well separated by ion-exchange chromatography, in which the residue at position 79 appeared to be the *N*-terminus. (1) Peptide C26 contained valine (1.8 residues/molecule) and valine was the sole *N*-terminal residue. This peptide represented the sequence from position 79 to position 95. A yield of 245nmol was obtained. (2) Peptide C27 (225nmol) contained valine (1.0 residue/molecule) and isoleucine (0.8 residue/molecule) and the sole *N*-terminal residue was isoleucine. Since, except for the Val-Ile values, the compositions of peptide C27 and its products on digestion with trypsin were the same as for peptide C26 it was considered to represent a variant form of the sequence from position 79 to position 95. (3) Peptide C14 (78nmol) contained valine (1.1 residues/molecule) and isoleucine (0.9 residue/molecule). The *N*-terminal sequence was Ile-Glx-His-Ser and from the composition it appears to represent the sequence from position 79 to position 92. In view of the failure of the trypsin hydrolysates to reveal heterogeneity at position 79, however, the question is still unresolved.

The chymotryptic digest yielded peptides C10 (1860nmol) and C13 (110nmol) which were well separated by ion-exchange chromatography and which also possessed different electrophoretic mobilities at pH6.5 (C10, $m = -0.28$; C13, $m = -0.20$). The compositions of these peptides differed only in that C10 had phenylalanine (1.0 residue/molecule) whereas C13 had phenylalanine (2.0 residues/molecule). The amino acid sequences were found to be identical except that peptide C13 had an extra residue of phenylalanine at the *C*-terminal end. There is clearly insufficient evidence to decide whether the extra residue represents an insertion between positions 78 and 79 or is a third variant at position 79. In Fig. 3 the latter alternative is tentatively indicated.

We conclude that fragment BCd prepared from different batches of ovotransferrin has consistently shown polymorphism at position 51 and that there is possible polymorphism at position 79. These variants would not be expected to cause electrophoretic differences in the protein but electrophoretically distinct varieties of ovotransferrin have been described in populations of hen (Ogden *et al.*, 1962; Stratil, 1966), pigeons (Mueller *et al.*, 1962), pheasants (Baker *et al.*, 1966) and other avian species (Baker, 1967).

Tsao *et al.* (1974) found that CNBr cleavage of hen ovotransferrin gave eight polypeptides and free homoserine. Their fragment RCMCF1-I, which had *N*-terminal alanine, appears to correspond to fragment BCd in the present work. Fragment RCMCF2-I corresponds to fragment BCd in its amino acid composition and in possessing *N*-terminal glycine. The molecular weight of fragment RCMCF2-I, estimated from the amino acid composition, was 13453 as compared with 10471 given by the amino acid sequence of fragment BCd. The addition of a carbohydrate group consisting of three residues of mannose and five residues of *N*-acetylglucosamine (Wenn, 1975) increases the molecular weight of fragment BCd to 11990.

We are indebted to Professor J. R. Clamp for the carbohydrate analyses by g.l.c. We thank the Science Research Council for financial support and for a Research Studentship (I.B.K.).

References

- Andrews, P. (1964) *Biochem. J.* **91**, 222-233
- Baker, C. M. A. (1967) *Comp. Biochem. Physiol.* **20**, 949-973
- Baker, C. M. A., Manwell, C., Labinsky, R. F. & Harper, J. A. (1966) *Comp. Biochem. Physiol.* **17**, 467-499
- Baker, E., Shaw, D. C. & Morgan, E. H. (1968) *Biochemistry* **7**, 1371-1378
- Bezborovainy, A. & Grohlich, D. (1974) *Comp. Biochem. Physiol.* **47B**, 787-797

- Bruton, C. J. & Hartley, B. S. (1970) *J. Mol. Biol.* **52**, 165–178
- Crowshaw, K., Jessup, S. J. & Ramwell, P. W. (1967) *Biochem. J.* **103**, 79–85
- Elleman, T. C. & Williams, J. (1970) *Biochem. J.* **116**, 515–535
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Goldberger, R. F. & Anfinsen, C. B. (1962) *Biochemistry* **1**, 401–405
- Graham, I. & Williams, J. (1975) *Biochem. J.* **145**, 263–279
- Gray, W. R. (1967a) *Methods Enzymol.* **11**, 139–151
- Gray, W. R. (1967b) *Methods Enzymol.* **11**, 469–475
- Greene, F. C. & Feeney, R. E. (1968) *Biochemistry* **7**, 1366–1371
- Heilmann, J., Barrolier, J. & Watzke, E. (1957) *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 219–220
- Jeppsson, J.-O. (1967) *Acta Chem. Scand.* **21**, 1686–1694
- Kasper, C. B. (1970) in *Protein Sequence Determination* (Needleman, S. B., ed.), pp. 172–175, Chapman and Hall, London
- Mann, K. G., Fish, W. W., Cox, A. C. & Tanford, C. (1970) *Biochemistry* **9**, 1348–1354
- Mueller, J. O., Smithies, O. & Irwin, M. R. (1962) *Genetics* **47**, 1385–1392
- Offord, R. E. (1966) *Nature (London)* **211**, 591–593
- Ogden, A. L., Morton, J. R., Gilmour, D. G. & McDermid, E. M. (1962) *Nature (London)* **195**, 1026–1028
- Rondle, C. J. M. & Morgan, W. T. J. (1955) *Biochem. J.* **61**, 586–589
- Rudloff, V. & Braunitzer, G. (1961) *Hoppe-Seyler's Z. Physiol. Chem.* **323**, 129–144
- Schroeder, W. A. (1967) *Methods Enzymol.* **11**, 351–361
- Smith, I. (1953) *Nature (London)* **171**, 43–44
- Spies, J. R. & Chambers, D. C. (1949) *Anal. Chem.* **21**, 1249–1266
- Stratil, A. (1966) *Proc. Eur. Conf. Anim. Blood Groups Biochem. Polymorphisms 10th*, pp. 241–243
- Sutton, M. R. & Brew, K. (1974) *Biochem. J.* **139**, 163–168
- Taniuchi, H., Anfinsen, C. B. & Sodja, A. (1967) *J. Biol. Chem.* **242**, 4752–4758
- Tsao, D., Azari, P. & Phillips, J. L. (1974) *Biochemistry* **13**, 397–403
- Uphaus, R. A., Grossweiner, L. I., Katz, J. J. & Kopple, K. D. (1959) *Science* **129**, 641–643
- Wenn, R. V. (1975) *Biochem. J.* **145**, 281–285
- Williams, J. (1968) *Biochem. J.* **108**, 57–67
- Williams, J. (1974) *Biochem. J.* **141**, 745–752
- Winzler, R. J. (1955) *Methods Biochem. Anal.* **2**, 279–311
- Woods, K. R. & Wang, K. T. (1967) *Biochim. Biophys. Acta* **133**, 369–370