Biochem. J. (1984) **220**, 221–226
Printed in Great Britain

Physicochemical properties and *N*-terminal sequence of eel lectin

Charles KELLY*
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(*Received 25 November 1983/Accepted 6 February 1984*)

Some physicochemical properties of the L-fucose-binding lectin from the serum of the European eel (*Anguilla anguilla*) were determined. The lectin is a dimer composed of identical subunits of M_r approx. 40000. In agreement with previous results [Hořejší & Kocourek (1978) *Biochim. Biophys. Acta* **538**, 299–315], the subunit was shown to comprise two non-glycosylated polypeptides of M_r approx. 20000 and linked by disulphide bonds. *N*-Terminal sequence analysis, carboxypeptidase digestion and peptide mapping indicated identity of the polypeptides. There were two L-fucose-binding sites per subunit with K_D 1.6×10^{-3}M for the lectin–fucose complex, as determined by equilibrium dialysis.

This study describes some of the physicochemical properties of eel lectin (the L-fucose-binding lectin from the serum of the European eel, *Anguilla anguilla*) isolated as described elsewhere (Kelly, 1980). That the serum of *A. anguilla* possessed haemagglutinating activity directed against human group-O erythrocytes was first shown by Jonsson (1944). Subsequently Watkins & Morgan (1952) reported that this agglutination could be inhibited by L-fucose, this being the first description of inhibition of a blood-group 'antibody' by a simple sugar.

Eel lectin has previously been characterized by Hořejší & Kocourek (1978) as having a subunit M_r of 44000. The subunit consisted of two polypeptides linked by disulphide bonds and each of M_r approx. 23000. Thin-layer gel filtration indicated that the M_r of the intact protein was 50000, a value that was anomalously low when compared with the $s_{20,w}$ value of 6.5S. In contrast, the lectin from the American eel (*Anguilla rostrata*) was shown to be a trimer (with $s_{20,w}$ of 7.2S) of non-covalently associated subunits each of M_r approx. 40000 (Bezkorovainy *et al.*, 1971). The subunits were composed of four polypeptide chains linked by disulphide bonds and of M_r 10000; *N*- and *C*-terminal analyses suggested that the polypeptides were of two different kinds (Springer & Desai, 1971). Matsumato & Osawa (1974) have also isolated eel lectin (the eel species was not stated) and determined the $s_{20,w}$ as 7.2S and the M_r, by gel filtration, as 140000.

In addition, on the basis of binding studies employing affinity electrophoresis, Hořejší *et al.* (1977) have reported that there are five eel isolectins.

Materials and methods

Materials

Eel lectin was prepared from pooled eel sera by affinity chromatography followed by gel filtration, and rabbit anti-(eel lectin) serum was prepared as described elsewhere (Kelly, 1980). Trypsin (diphenylcarbamoyl chloride-treated) (EC 3.4.21.4), carboxypeptidase A (EC 3.4.12.2) and carboxypeptidase B (di-isopropyl phosphorofluoridate-treated) (EC 3.4.12.3) were from Sigma Chemical Co. Clostripain (EC 3.4.22.8) was from Boehringer Corp. L-[6-^{3}H]Fucose (20–30 Ci/mmol) was from Amersham International. All other reagents were of analytical grade. Phosphate-buffered saline was 0.155M-NaCl/10mM-sodium phosphate buffer, pH7.0. Water was glass-distilled.

Reduction and alkylation of eel lectin

Lectin (10mg) was dissolved in 8M-urea/0.67M-Tris/HCl, pH8.6, and disulphide bonds were reduced with 2-mercaptoethanol and thiol groups alkylated with sodium iodoacetate (Crestfield *et al.*, 1963). After dialysis against 10mM-NH_4HCO_3, protein was recovered by freeze-drying.

* Present address: Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.

M_r determination

The M_r of purified eel lectin was determined by fast protein liquid chromatography gel filtration on a TSK-G3000SW column. Elution was with phosphate-buffered saline at a flow rate of 0.5ml/min. The column was calibrated with the following proteins: β-galactosidase, phosphorylase *b*, human serum transferrin, bovine serum albumin, hen ovalbumin, myoglobin and cytochrome *c*. Eel serum was fractionated on a column (1.3cm × 125cm) of Sephadex G-200, which was eluted with phosphate-buffered saline at a flow rate of 6ml/h. Fractions were assayed for lectin by fused rocket immunoelectrophoresis (Svendsen, 1973), with the rabbit anti-(eel lectin) serum. The column was calibrated with catalase, lactate dehydrogenase, human serum transferrin, bovine serum albumin, hen ovalbumin and ribonuclease A.

The subunit M_r of eel lectin was determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate by using the system of Fairbanks *et al.* (1971) with slab gels (16cm × 20cm × 0.1cm) of 7.5% (w/v) polyacrylamide. The M_r markers were: hen ovotransferrin, bovine serum albumin, hen ovalbumin, carbonic anhydrase and cytochrome *c*.

Amino acid analysis

Samples were hydrolysed in constant-boiling HCl at 110°C for 24, 48 and 72h and analysed on a Rank–Hilger Chromaspek J180 ion-exchange chromatograph. Cysteine (plus half-cystine) was determined as carboxymethyl-cysteine after hydrolysis of reduced and alkylated eel lectin. Tryptophan was determined after hydrolysis in 3M-toluene-*p*-sulphonic acid/0.2% (w/v) 3-(2-aminoethyl)indole (Liu, 1974).

Carbohydrate analysis

This was performed by Professor J. R. Clamp, Department of Medicine, University of Bristol, Bristol. After methanolysis and conversion of liberated sugars into the *O*-trimethylsilyl ethers, samples were analysed by g.l.c. (Clamp, 1977).

N-Terminal sequence analysis

Amino acid sequences were determined by Professor P. Osinski (Unit of Experimental Medicine, International Institute of Cellular and Molecular Pathology, Brussels, Belgium) by using the Beckman 890C sequencer with programmes modified from that of Brauer *et al.* (1975). A 100–150nmol portion of polypeptide was used for each determination. Thiazolinone fractions were converted manually into the phenylthiohydantoin derivatives, which were identified by reverse-phase high-pressure liquid chromatography on a Zorbax cyanopropylsilane column (Johnson *et al.*, 1979).

A 3nmol portion of reduced and alkylated eel lectin was also analysed on the gas-phase sequencer (Hewick et al., 1981) by Dr. M. D. Waterfield and Mr. N. F. Totty (Imperial Cancer Research Fund, Lincoln's Inn Fields, London). Amino acid phenylthiohydantoin derivatives were identified by reverse-phase high-pressure liquid chromatography on a Zorbax Dupont C_8 column.

Carboxypeptidase digestion

Reduced and alkylated lectin (4mg) was dissolved in 2ml of 0.1M-sodium phosphate buffer, pH8.0, containing 0.1mM-norleucine as internal standard. The solution was placed on ice, and carboxypeptidases A and B were added in equal amounts such that the enzyme/substrate ratio was 1:20. Samples (300μl) were taken from the solution before and immediately after addition of enzyme. The sample was then incubated at 37°C, with further samples being taken after 2, 4, 6 and 18h. These were each added to 200μl of 0.25M-HCl, heated at 80°C for 3min and centrifuged, after which the supernatants were analysed on the amino acid analyser. A control solution of enzyme alone was treated in parallel. Two separate determinations of the *C*-terminal amino acids were made.

Peptide mapping

(*i*) *Trypsin digestion.* A 2mg portion of eel lectin, oxidized with performic acid (Means & Feeney, 1971), was digested with trypsin in 100μl of 50mM-NH_4HCO_3 at an enzyme/substrate ratio of 1:25 for 4h at 37°C. The digest was then loaded on Whatman 3MM paper for two-dimensional peptide mapping. Separation in the first dimension was by electrophoresis at pH6.5 for 1h at 3kV (Ambler, 1963) and in the second by descending chromatography with butan-1-ol/acetic acid/water (3:1:1, by vol.) as solvent. The chromatogram was developed for 12h and then stained with fluorescamine (Mendez & Lai, 1975).

(*ii*) *Clostripain digestion.* A 1.5mg portion of reduced and alkylated lectin was digested with clostripain as described by Mitchell & Harrington (1970) at an enzyme/substrate ratio of 1:100 for 3h at 37°C. A sample of the digest was taken for analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the remainder was analysed by paper electrophoresis and chromatography as above.

(*iii*) *CNBr cleavage.* CNBr cleavage (Gross & Witkop, 1962) was performed on 1mg of reduced and alkylated lectin. Cleavage products were analysed by sodium dodecyl sulphate/polyacryla-

mide-gel electrophoresis and paper electrophoresis.

(*iv*) *Dimethyl sulphoxide/HBr cleavage.* Dimethyl sulphoxide/HBr cleavage (Savige & Fontana, 1977) was performed on 4mg of reduced and alkylated lectin. The products were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and partially separated on a column (100cm × 1.5cm) of Sephadex G-50, with elution with 5% (w/v) formic acid.

Equilibrium dialysis

Micro dialysis cells were made (in the Medical School Workshop, University of Bristol, Bristol) and assembled as described by Englund *et al.* (1969). A 25μl volume of lectin solution (4mg/ml) in phosphate-buffered saline was introduced into each chamber on one side of a dialysis membrane, and 25μl of solutions of L-[6-^{3}H]fucose (10μCi/μmol) in phosphate-buffered saline (at concentrations in the range 0.1–5mM) was introduced into the chambers on the other side of the membrane. The cells were incubated at 4°C for 6h with slow rotation, after which 15μl samples were taken from each chamber, 10μl of each then being transferred to scintillant for determination of radioactivity (20min for each sample) in an Isocap 300 counter.

Results

Two peaks (of comparable magnitude), of M_r 51000 and 78000, were observed on gel filtration of purified eel lectin. On gel filtration of eel serum, the lectin (identified by fused rocket immunoelectrophoresis) showed an apparent M_r of 80000, being eluted at the position of the human serum transferrin marker (Fig. 1). Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that the subunit M_r was 38000 (±2000, from 12 determinations). After reduction with 2-mercaptoethanol, a single band of M_r 18000 (±2000) was observed; similarly, reduced and alkylated eel lectin ran as a single band of M_r 18000. These results confirm those obtained by Horejší & Kocourek (1978), showing that the eel subunit is composed of two polypeptides linked by disulphide bonds. They also indicate that the native lectin is a dimer of non-covalently associated subunits. Some dissociation of this dimer is apparent, after purification of the lectin, this possibly being due to partial denaturation.

The amino acid composition of eel lectin is shown in Table 1, together with the results of carbohydrate analysis. The values are expressed as mol of amino acid residue per mol of lectin, assuming the M_r of the latter to be 40000. No carbohydrate was detected (other than trace amounts of glucose). Again, the results are in good agreement with those reported by Horejší & Kocourek (1978) and with those reported by Springer & Desai (1971) determined for the lectin from *A. rostrata*.

The sequence of the first 18 amino acid residues is shown in Fig. 2(*a*), together with the yield of amino acid phenylthiohydantoin derivative at each step (the yields are those obtained with the gas-phase sequencer). Residue 8 was identified as threonine, although the instability of the phenylthiohydantoin derivative meant the yield could not

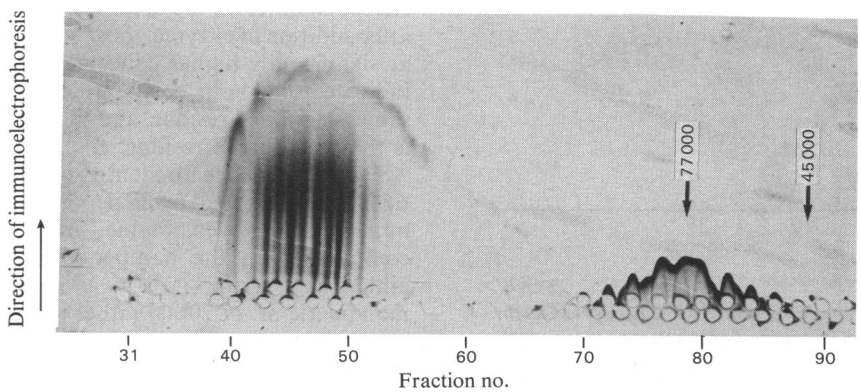

Fig. 1. *Distribution of eel lectin, determined by fused rocket immunoelectrophoresis, after gel filtration of eel serum*
Samples (5μl) from each fraction were applied to wells in the region of the agarose (1%, w/v) gel that contained no antiserum. Electrophoresis into gel containing 1% (v/v) rabbit anti-(eel lectin) serum was at 3V/cm for 16h with Tris/barbitone buffer, pH8.6. The gel was dried and stained with Coomassie Blue. The positions of the M_r markers (human serum transferrin and ovalbumin) are shown. The fainter precipitin peak in the larger-M_r fractions arises from reaction between the antiserum and a non-lectin eel serum component (present in trace amounts in the lectin preparation used for immunization).

Table 1. *Amino acid and carbohydrate composition of eel lectin*

Amino acid values were determined after hydrolysis for 24, 48 and 72h, and only the corrected (to the nearest integer) results are shown. The values given assume the lectin M_r to be 40000.

Component	Composition (mol/mol of eel lectin)
Asx	39
Thr	24
Ser	26
Glx	46
Pro	29
Gly	29
Ala	29
Cys	22*
Val	26
Met	4
Ile	20
Leu	23
Tyr	14
Phe	8
His	15
Trp	2†
Lys	16
Arg	15
D-Glucose	0.2‡

* Determined as carboxymethyl-cysteine after hydrolysis of reduced and alkylated eel lectin.

† Determined after hydrolysis in 3M-toluene-*p*-sulphonic acid.

‡ From g.l.c. analysis.

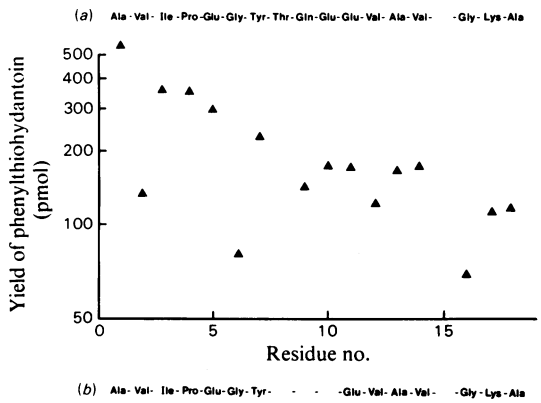

Fig. 2. *N-Terminal amino acid sequence of eel lectin*
(*a*) Automated Edman degradations were performed on 3nmol of eel lectin with the gas-phase sequencer, and amino acid phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography. No quantification of residue 8, threonine, could be made. (*b*) *N*-Terminal sequence of a 7000-M_r peptide produced by cleavage of eel lectin with dimethyl sulphoxide/HBr. The sequence (determined on 100nmol on the Beckman 890C sequencer) identifies it as the *N*-terminal peptide.

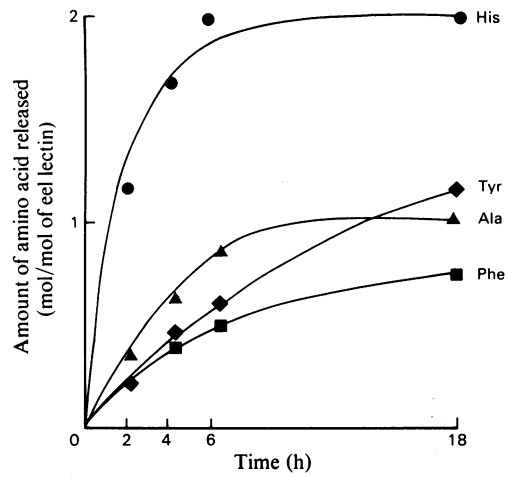

Fig. 3. *C-Terminal analysis of eel lectin*
The time course of release of amino acids, on digestion with carboxypeptidases A and B, is shown.

be quantified. The identification was based on the characteristic elution pattern of two double peaks, one of which is eluted before and the other after the position of the phenylthiohydantoin derivative of tyrosine (Waterfield *et al.*, 1983). No assignment of residue 15 could be made. No sequence heterogeneity was observed. Fig. 2(*b*) shows the *N*-terminal sequence of a peptide obtained from cleavage with dimethyl sulphoxide/HBr (see below); the sequence identified it as the *N*-terminal peptide.

Digestion with carboxypeptidase resulted in the rapid release of histidine, which reached a maximum value of 2mol/mol of eel lectin (assuming the lectin M_r to be 40000), as shown in Fig. 3. Although other amino acids were present from 4h after addition of enzyme, those shown were present at significantly higher concentrations. The result indicates that both polypeptide chains have the same *C*-terminal residue, and suggests that alanine is the penultimate residue.

Further evidence for identity of the two polypeptides was obtained from peptide mapping. The number of peptides obtained in each case, when compared with the number expected from the amino acid composition, was consistent with a monomeric M_r of 20000 rather than 40000. Thus, after cleavage with trypsin, 17 peptides were resolved on the two-dimensional map (Fig. 4). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis analysis of the products of clostripain cleavage showed complete destruction of the original polypeptide. Paper electrophoresis resolved six bands, one migrating towards the anode and the remainder towards the cathode. All were single peptides except band 3 (numbering from the

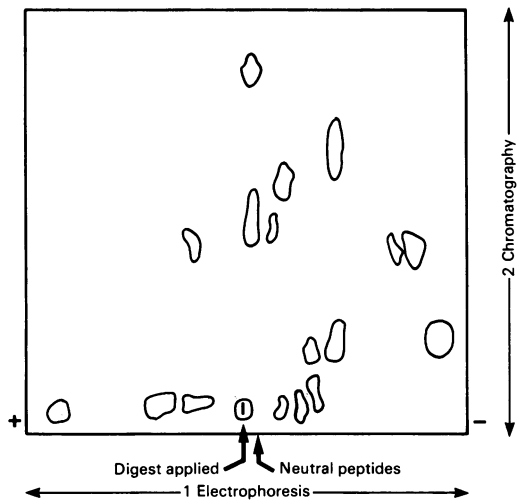

Fig. 4. *Peptide map of trypsin digest of performic acid-oxidized eel lectin*

After digestion of eel lectin (2mg) with trypsin, peptides were separated by electrophoresis (1) and chromatography (2). Some (presumably) undigested material remained at the origin. 'Neutral' peptides, i.e. those in the region arrowed, were not completely resolved.

cathode), which was resolved as two peptides by chromatography. No apparent change in M_r was observed on sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis after cleavage with CNBr. However, paper electrophoresis showed two lightly staining peptides that migrated towards the anode plus a strongly staining band at the origin. Cleavage with dimethyl sulphoxide/HBr (resulting in cleavage of the peptide bond *C*-terminal to tryptophan), produced a peptide of M_r 13000 plus one of M_r approx. 7000, together with some uncleaved lectin. The smaller-M_r peptide was isolated, as described, and identified as *N*-terminal by sequence analysis.

Fig. 5 shows the results obtained from equilibrium dialysis, where they have been analysed by a plot of the Scatchard equation (Scatchard *et al.*, 1950, 1957):

$$\frac{R}{[\text{Fucose}]_{\text{free}}} = n \cdot K_a - R \cdot K_a$$

where R is the ratio of bound fucose concentration to the total lectin concentration, K_a is the association constant for the binding of fucose by lectin and n is the number of binding sites per lectin molecule. A value of 40000 has been assumed for the M_r of eel lectin. The calculated line of best fit gives n as 2.14 and $K_a = 6.3 \times 10^2\,\text{M}^{-1}$, i.e. there are two identical L-fucose-binding sites per subunit with K_D of 1.6×10^{-3} M.

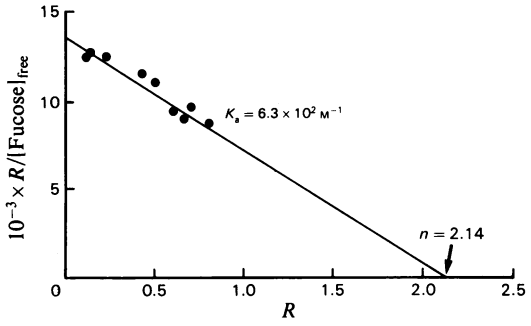

Fig. 5. *Scatchard analysis of results from equilibrium dialysis*

Experimental details are in the text. The data were analysed by the Scatchard equation (see the text) and the calculated line of best fit gives n, the number of binding sites per lectin subunit, as 2.14, with K_a, the association constant of fucose-binding, as $6.3 \times 10^2\,\text{M}^{-1}$.

Discussion

In summary, the results indicate that eel lectin is a dimer of non-covalently associated identical subunits of M_r approx. 40000. The subunit is non-glycosylated and comprises two identical polypeptide chains (of M_r 20000), which are linked by disulphide bonds. There are two identical L-fucose-binding sites per subunit, which show rather low affinity for the monosaccharide.

The results of *N*-terminal sequence analysis, *C*-terminal analysis and peptide mapping argue strongly that the polypeptide chains are identical. There are problems in interpreting peptide maps arising from factors such as incomplete hydrolysis, non-specific hydrolysis, incomplete separation of peptides etc. However, given these reservations, the peptide maps revealed no major structural heterogeneity that could have resulted in an increased number of peptides, and therefore do not provide evidence for more than one gene product, i.e. isolectins.

Although isolectins have been demonstrated in some cases, e.g. phytohaemagglutinin, the lectin from the red kidney bean (Allen *et al.*, 1969; Yachnin & Svenson, 1972), in others initial reports have not been substantiated by subsequent structural analyses. Thus Richardson *et al.* (1978) sequenced the α-subunit of pea lectin and found no evidence for the earlier suggestion based on isoelectric-focusing data, that there were two isolectins (Trowbridge, 1974). Similarly Kaplan *et al.* (1977) found no sequence heterogeneity in the *N*-terminal 86 residues of limulin, although on isoelectric focusing three bands were resolved. In the present example, the presence of multiple bands on affinity electrophoresis provided the

main evidence for the report of five eel isolectins (Hořejší *et al.*, 1977). However, affinity preparations of eel lectin are likely to include components capable of binding to the lectin that are co-purified (Kelly, 1980) and that could give rise to apparent binding heterogeneity reflecting lectin species with no, one, two etc. binding sites occupied. In keeping with this suggestion, the K_D value determined in the present study, although of the same order, is slightly lower than those determined by Hořejší *et al.* (1977).

The amino acid composition of the lectins from *A. anguilla* and *A. rostrata* are strikingly similar, as might be expected for two species of the same genus. However, there are differences in the subunit structure, that of *A. rostrata* comprising polypeptides of M_r 10000 rather than 20000. Eel lectin shows no obvious physicochemical similarity to other vertebrate lectins. The fish lectins isolated by Krajhanzl *et al.* (1978*a*,*b*), for example, differ in M_r and amino acid composition, as do the D-galactosyl-binding lectins isolated from various sources (Teichberg *et al.*, 1975; de Waard *et al.*, 1976; Den & Malinzak, 1977; Powell, 1980).

With a few exceptions, such as those involved in catabolism of serum glycoproteins (Ashwell & Morell, 1974; Stahl *et al.*, 1978), the physiological functions of lectins are not known, although many have been proposed. Structural studies, although giving few clues as to function, have shown that lectins are an extremely diverse grouping and thus are likely to have equally diverse functions.

This work forms part of a Ph.D. Thesis submitted to the University of Bristol. I am grateful to my supervisors, Dr. J. Williams and Dr. E. R. Gold, for advice and to the University of Bristol for financial support.

References

Allen, L. W., Svenson, R. H. & Yachnin, S. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 334–341
Ambler, R. P. (1963) *Biochem. J.* **89**, 349–378
Ashwell, G. & Morell, A. G. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol.* **41**, 99–128
Bezkorovainy, A., Springer, G. F. & Desai, P. R. (1971) *Biochemistry* **10**, 3761–3764
Brauer, A. W., Margolies, M. N. & Haber, E. (1975) *Biochemistry* **14**, 3029–3035
Clamp, J. R. (1977) *Biochem. Soc. Trans.* **5**, 1693–1695
Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627
Den, H. & Malinzak, D. A. (1977) *J. Biol. Chem.* **252**, 5444–5448
de Waard, A., Hickman, S. & Kornfeld, S. (1976) *J. Biol. Chem.* **251**, 7581–7587
Englund, P. T., Huberman, J. A., Jovin, T. M. & Kornberg, A. (1969) *J. Biol. Chem.* **244**, 3038–3044
Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2602–2617
Gross, E. & Witkop, B. (1962) *J. Biol. Chem.* **237**, 1856–1860
Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990–7997
Hořejší, V. & Kocourek, J. (1978) *Biochim. Biophys. Acta* **538**, 299–315
Hořejší, V., Ticha, M. & Kocourek, J. (1977) *Biochim. Biophys. Acta* **499**, 290–300
Johnson, N. D., Hunkapiller, M. W. & Hood, L. E. (1979) *Anal. Biochem.* **100**, 335–338
Jonsson, B. (1944) *Acta Pathol. Microbiol. Scand. Suppl.* **54**, 456–464
Kaplan, R., Li, S. S-L. & Kehoe, J. M. (1977) *Biochemistry* **16**, 4297–4303
Kelly, C. (1980) Ph.D. Thesis, University of Bristol
Krajhanzl, W., Hořejší, V. & Kocourek, J. (1978*a*) *Biochim. Biophys. Acta* **532**, 209–214
Krajhanzl, A., Hořejší, V. & Kocourek, J. (1978*b*) *Biochim. Biophys. Acta* **532**, 215–224
Liu, T-Y. (1974) *Methods Enzymol.* **25**, 44–55
Matsumato, I. & Osawa, T. (1974) *Biochemistry* **13**, 582–588
Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp. 160–161, Holden-Day, San Francisco
Mendez, E. & Lai, C. Y. (1975) *Anal. Biochem.* **65**, 281–292
Mitchell, W. M. & Harrington, W. F. (1970) *Methods Enzymol.* **19**, 635–642
Powell, J. T. (1980) *Biochem. J.* **187**, 123–129
Richardson, C., Behnke, W. D., Freisheim, J. H. & Blumenthal, K. M. (1978) *Biochim. Biophys. Acta* **537**, 310–319
Savige, W. E. & Fontana, A. (1977) *Methods Enzymol.* **47**, 459–469
Scatchard, G., Scheinberg, I. H. & Armstrong, S. H. (1950) *J. Am. Chem. Soc.* **72**, 535–540
Scatchard, G., Coleman, J. S. & Shen, A. L. (1957) *J. Am. Chem. Soc.* **79**, 12–20
Springer, G. R. & Desai, P. R. (1971) *Biochemistry* **10**, 3749–3761
Stahl, P., Rodman, J. S., Miller, M. J. & Schlesinger, P. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1399–1403
Svendsen, P. J. (1973) *Scand. J. Immunol.* **2**, *Suppl.* **1**, 69–70
Teichberg, V. I., Silman, I., Beitsch, D. D. & Resheff, G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1383–1387
Trowbridge, I. S. (1974) *J. Biol. Chem.* **249**, 6004–6012
Waterfield, M. D., Scrace, G. T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J. S. & Deuel, T. F. (1983) *Nature (London)* **304**, 35–39
Watkins, W. M., & Morgan, W. T. J. (1952) *Nature (London)* **169**, 322–328
Yachnin, S. & Svenson, R. H. (1972) *Immunology* **22**, 871–873