Biochem. J. (1972) **127**, 775–780
Printed in Great Britain

Replacement of Asparagine by Aspartic Acid in Hen Ovalbumin and a Difference in Immunochemical Reactivity

By R. L. WISEMAN,* J. E. FOTHERGILL and L. A. FOTHERGILL
Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, U.K.

(*Received* 10 *November* 1971)

Two forms of hen ovalbumin that exist as genetic variants were compared by physical and immunochemical techniques. The two ovalbumins could be distinguished by electrophoretic mobility and by antisera that had been pretreated with heterologous antigen. Peptide 'maps' of chymotrypsin digests of the two ovalbumins revealed that three of the peptides were different. These were isolated and analysed. One form of ovalbumin (type B) contains the sequence:

-Ser-Ser-Ala-Asp-Leu-Ser-Gly-Ile-Ala-Glu-Ser(Ser,Leu)-

whereas the other form (type A) contains an asparagine residue in place of the aspartic acid residue. The -Asn-Leu-Ser- sequence of type A is not glycosylated. Chymotrypsin readily cleaved the leucine–serine bond in the asparagine-containing peptide, but not in the aspartic acid-containing variant.

Hen ovalbumin has been used in many immunochemical studies of the physical properties and chemical structure of protein antigens. Investigation of those structural features of the protein that contribute to immunochemical activity has been retarded by failure of the degradation fragments of ovalbumin to react with anti-ovalbumin antibodies. Enzymic digestion of the native protein and separation of the liberated peptides through dialysis membranes (Porter, 1957) was unsuccessful. A study of enzymic and chemical degradation of the native or chemically modified protein and Sephadex fractionation of the cleavage products (Wiseman, 1967) was unrewarding, and showed that a report of splitting of ovalbumin by trypsin into immunologically active products (Kaminski, 1960) could not be confirmed quantitatively. Shmakova & Kaverzneva (1969) also failed to obtain immunologically active proteolytic fragments of ovalbumin, and confirmed our observation (Wiseman, 1967) that the carbohydrate moiety of ovalbumin does not contribute to the antigenic specificity of the whole molecule.

The discovery of a genetic variant (type B) of hen ovalbumin that can be distinguished from the more common ovalbumin (type A) by its greater anionic mobility in starch-gel electrophoresis at pH 5.0 (Lush, 1964) has provided an opportunity to examine the effect of a small structural difference on the immunological activity of this protein. We have detected a difference in the reaction of the two variants with rabbit antibodies, and defined the difference in structure between these two ovalbumins.

* Present address: Department of Molecular Biophysics and Biochemistry, Box 1937, Yale University, New Haven, Conn. 06520, U.S.A.

Some aspects of this work have been published in a preliminary form (Wiseman & Fothergill, 1966, 1967).

Materials and Methods

Eggs

Eggs from hens (either heterozygous or homozygous for the ovalbumin variants) were obtained from Mr. J. Manson, Edinburgh School of Agriculture, Edinburgh, U.K., and Mr. W. Johnston, Newton of Fintray, Aberdeenshire, U.K.

To confirm the genetic nature of this ovalbumin variant, breeding experiments were done with hens homozygous for type B ovalbumin and a cock that was thought to have the B-type gene. The female offspring of these matings were typed by electrophoresis of egg white collected during the following season.

Isolation of ovalbumin

Egg white was separated from the yolk and was gently homogenized to break the membranes and yield a freely running liquid. Ovalbumin was separated from whole egg white by fractionation on CM-cellulose (Fothergill & Perrie, 1966).

Characterization of ovalbumin

(*a*) *Starch-gel electrophoresis.* Electrophoresis in starch gel at pH 5.0, as described by Lush (1964), was done on all egg white and ovalbumin samples. This allowed classification into the slower-moving or common form of ovalbumin (type A), the faster-moving form of ovalbumin (type B), or a mixture

of approximately equal amounts of types A and B characteristic of the hen heterozygous for this gene. Type A or type B ovalbumin was always prepared from egg whites of birds homozygous for this gene.

(*b*) *Ultracentrifugation.* The method of Fothergill & Fothergill (1970*a*) was used.

(*c*) *Immunoelectrophoresis.* The method of Scheidegger (1955) was used.

(*d*) *Ouchterlony double-diffusion.* The method of Ouchterlony (1953) was used.

Production of antisera

Antisera against whole hen's-egg white were prepared in rabbits by intramuscular injection of 0.25ml of egg white emulsified with an equal volume of Freund's complete adjuvant, followed 3 weeks later by injection of 0.75ml of the same egg white, also with adjuvant. Antisera against type A or type B ovalbumin were obtained by a similar method by using 0.2 and 0.6ml of ovalbumin solution (50mg/ml) in place of the egg white for the two injections.

Quantitative precipitation

The method of Fothergill & Fothergill (1971) was used.

Pre-treatment of anti-ovalbumin antisera

Preliminary treatment of anti-ovalbumin antisera was done by adding sufficient type B ovalbumin to an anti-(type A ovalbumin) antiserum to precipitate approx. 75% of the total anti-type B ovalbumin antibodies. The precipitate was incubated at 37°C for 40min and left at 2°C overnight, and then removed by centrifugation. The supernatant was used as antiserum in further quantitative precipitation experiments. Pre-treatment of anti-(type A ovalbumin) with type A ovalbumin was done as a control. An analogous series of experiments was performed on anti-(type B ovalbumin) antiserum.

Digestion of ovalbumins with trypsin and chymotrypsin

Ovalbumins were heat-denatured at pH7.5 and 82°C for 15min (Cunningham *et al.*, 1957) and were then digested (100mg of ovalbumin/2mg of enzyme) at 37°C with trypsin or chymotrypsin in a Radiometer pH-stat assembly maintained at pH9.1 with 0.2M-NaOH.

Digestion of peptides with thermolysin

The method of Ambler & Meadway (1968) was used.

Separation of peptides

(*a*) *Peptide 'mapping'.* Peptides were separated in two dimensions by electrophoresis at pH6.4 and chromatography by the method of Ingram (1963). Peptides were detected by staining with cadmium–ninhydrin reagent (Heilman *et al.*, 1957) or by illumination with u.v. radiation (Phillips, 1948).

Neutral peptides that were unresolved at pH6.4 were eluted and separated by electrophoresis at pH2.0 followed by chromatography.

(*b*) *One-dimensional electrophoresis.* Larger quantities of two of the chymotryptic peptides were obtained by electrophoresis (see Fothergill & Fothergill, 1970*b*).

Amino acid analysis of peptides

The method of Fothergill & Fothergill (1970*b*) was used. Spectrophotometry of the intact peptides was used to confirm the absence of aromatic amino acids.

Identification of N-terminal amino acids

The 'dansyl' method described by Gray (1967*a*) was used. The Dns-amino acid was identified after hydrolysis by chromatography on 5cm×5cm polyamide thin layers (Woods & Wang, 1967).

Edman degradation

Successive *N*-terminal amino acid residues were removed from peptides by the Edman method as described by Gray (1967*b*).

Sephadex chromatography of peptides

The peptides, isolated from the unstained peptide 'maps', were passed through a column (1.1cm× 105cm) of Sephadex G-25 (medium grade) in 0.1M-acetic acid to estimate the molecular weights (Andrews, 1965).

Results

Isolation of ovalbumins

Elution of hen's-egg white at pH4.5 from CM-cellulose gives an initial peak at 0.05M-sodium acetate and a further small peak. Neither of these peaks contains significant amounts of ovalbumin. Increasing the sodium acetate concentration to 0.15M produces a large protein peak that contains most of the doubly phosphorylated ovalbumin. Egg whites from homozygous A or B type hens give similar results.

Characterization of ovalbumins

(*a*) *Starch-gel electrophoresis.* At pH5.0, starch-gel electrophoresis clearly distinguishes the two forms of ovalbumin (Fig. 1). It shows, in each sample from

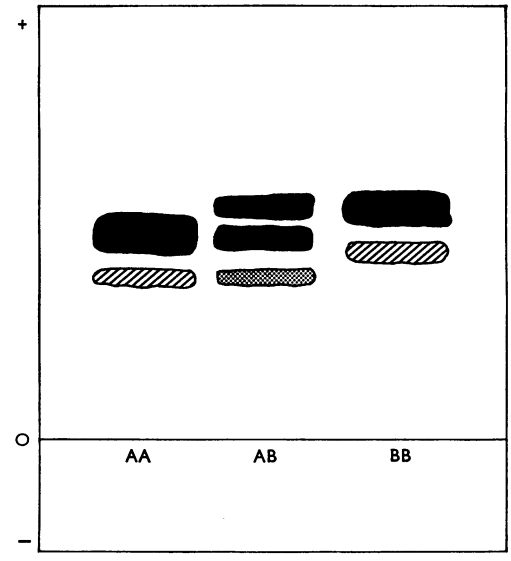

Fig. 1. *Starch-gel electrophoresis at pH5.0 of egg whites from hens with three different genotypes*

Only the anionic components are shown. AA, Hen homozygous for the A allele; AB, hen heterozygous; BB, hen homozygous for the B allele. O is the origin.

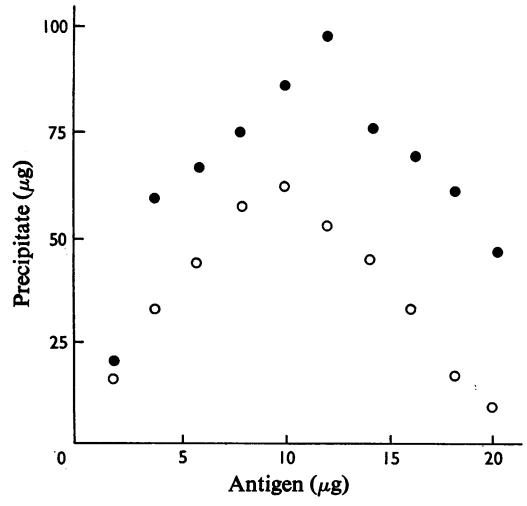

Fig. 2. *Quantitative precipitin assay of ovalbumins with anti-(type A ovalbumin) antiserum pre-absorbed with type B ovalbumin*

The methods are described in the text. o, Type B ovalbumin; ●, type A ovalbumin.

hens homozygous for either gene, the presence of a major component and of a small amount of a slower component that are presumably the doubly and singly phosphorylated forms (Perlmann, 1952). The difference in mobility between type A and type B ovalbumins is slightly less than that between the differently phosphorylated forms in the same preparation.

Egg whites collected from the female offspring of the breeding experiment were characterized as described above and were distributed as follows: type AB, 25; type BB, 21. These results suggest that the cock was of AB genotype, and confirms the evidence of Lush (1964) that this variant ovalbumin is inherited according to simple Mendelian genetics.

(*b*) *Ultracentrifugation.* Both A and B type ovalbumins showed single symmetrical peaks of identical sedimentation coefficient ($s_{20,w} = 3.24$S).

(*c*) *Immunoelectrophoresis and* (*d*) *double diffusion.* Both these techniques demonstrated the purity and identity of the ovalbumins. No differences could be found between A and B type ovalbumins with antisera prepared against egg whites containing either type of ovalbumin.

Immunochemical distinction between ovalbumins

When both A and B type ovalbumins were tested by quantitative precipitation against anti-(ovalbumin A) or anti-(ovalbumin B) there was very little difference between the homologous and heterologous systems. However, when the supernatants from antisera pre-treated with heterologous ovalbumin were used, a substantial difference between the homologous and heterologous systems was obtained (Fig. 2). Supernatants from antisera pre-treated with homologous ovalbumin showed no differences.

Peptide 'maps'

Peptide 'maps' of the tryptic peptides sometimes showed a single difference between the two ovalbumins, but the different peptides were not consistently obtained, presumably because of marginal solubility. However, the chymotryptic peptides that were soluble at pH6.4 showed a consistent single difference between the two ovalbumins among the anionic peptides (Fig. 3). These peptides are designated A acidic (A_A) and B acidic (B_A) peptides. An additional peptide, neutral at pH6.4 (A_N), was found in the ovalbumin A digest after electrophoresis at pH1.9 and the usual chromatography in the second dimension (Fig. 4). All three peptides stained yellow–orange with the cadmium–ninhydrin reagent. Peptide 'maps' of the tryptic digests of the peptides insoluble at pH6.4 from the chymotrypsin digests gave identical peptide patterns for the two ovalbumins.

One-dimensional electrophoresis

Electrophoresis at pH6.4 of chymotryptic digests of A and B ovalbumins showed yellow–orange-staining acidic peptides of different mobilities corresponding to the differences found in the peptide 'maps'. These peptides were purified by electrophoresis at pH1.9 followed by electrophoresis at pH6.5 for a longer time for the A_A peptide.

Analysis of 'variant' peptides

(*a*) *Amino acid content.* Table 1 shows the results of amino acid analysis of peptides A_A, B_A and A_N.

(*b*) *Sephadex chromatography.* The elution volumes of the three peptides correspond to that expected of peptides of the size indicated by the results of amino acid analysis shown in Table 1.

(*c*) *Electrophoretic mobility.* At pH6.4, peptide A_N has zero net charge, indicating that it contains an asparagine rather than an aspartic acid residue. The mobilities of peptides A_A and B_A relative to aspartic acid (see Offord, 1966) are 0.30 and 0.42. This corresponds to molecular weights of 800 and 1300, if peptide A_A contains a glutamic acid residue and peptide B_A contains a glutamic acid residue and an aspartic acid residue. These molecular weights agree with the gel-filtration and amino acid-analysis results.

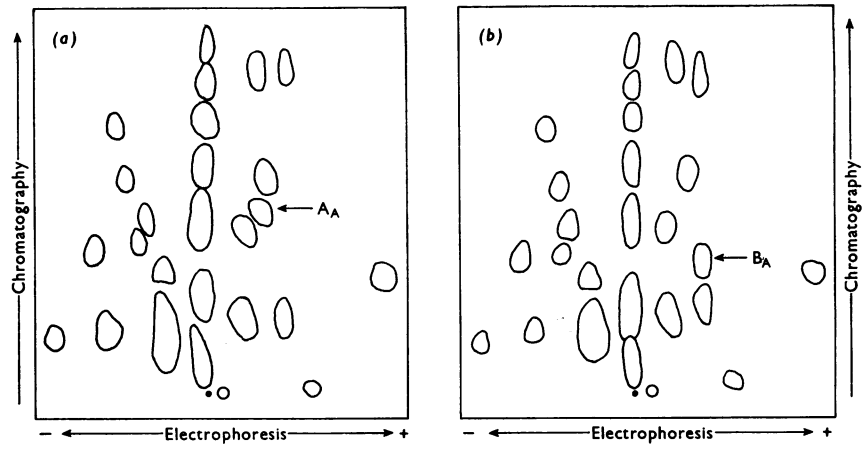

Fig. 3. *Peptide 'maps' at pH6.4 of the soluble chymotryptic peptides of type A and B ovalbumins*

The methods are described in the text. (*a*) Type A ovalbumin; (*b*) type B ovalbumin. A_A, Acidic peptide from type A ovalbumin; B_A, acidic peptide from type B ovalbumin; O, origin.

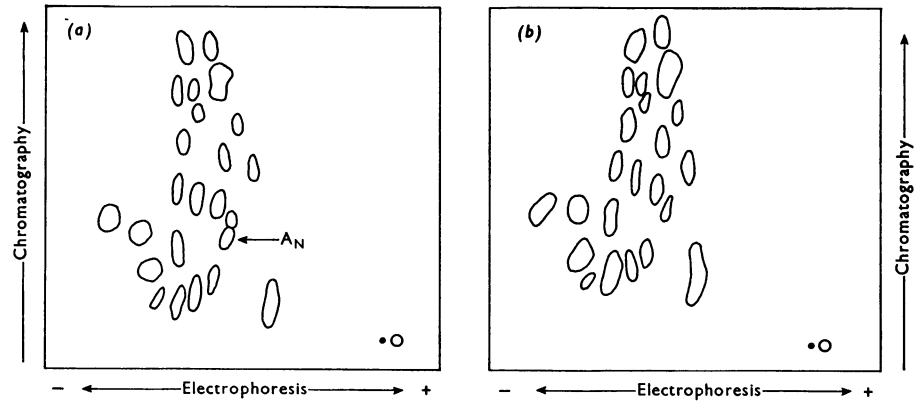

Fig. 4. *Peptide 'maps' at pH1.9 of the chymotryptic peptides neutral at pH6.4 from type A and B ovalbumins*

The methods are described in the text. (*a*) Type A ovalbumin; (*b*) type B ovalbumin, A_N, Neutral peptide from type A ovalbumin; O, origin.

Sequence of 'variant' peptides

Both acidic peptides were obtained in sufficient quantity for sequence determination by the dansyl–Edman procedure, and partial sequences of both were determined, to give Ser-Ser-Ala- for peptide B_A and Ser-Gly-Ile- for peptide A_A. Additionally, peptide B_A was digested with thermolysin and the subpeptides were separated by electrophoresis, and were then analysed and their sequences determined. Two thermolysin digests were done for 30min and for 3h at 37°C, resulting in two sets of peptides (a, c, d and 1, 2, 3, 4; see Table 2).

Discussion

The results of the amino acid and sequence analyses can be combined to give a structure for the 'variant' peptides. The results of the studies of peptides B_A, A_A and A_N can be written as shown in Fig. 5.

The peptides resulting from thermolysin digestion have hydrophobic *N*-terminal residues, which is consistent with the specificity of this enzyme (Ambler & Meadway, 1968). However, the cleavage obtained after digestion for 3h shows unusual specificity in the production of peptide 2, which requires serine contributing the amino group of the cleaved bond. This may be a result of the long hydrolysis time or slight impurity in the thermolysin sample.

The proposed sequence is also consistent with the specificity of chymotrypsin, which is known to cleave preferentially at leucyl bonds (Neil *et al.*, 1966). By this criterion the last two residues are probably in the order -Ser-Leu. The presence of the free carboxyl group on the aspartic acid side chain in the B molecule must inhibit the action of chymotrypsin at the adjacent leucyl bond, whereas the asparagine side chain in the A molecule allows chymotryptic cleavage.

We conclude that the difference in structure between A and B type ovalbumins is the replacement of asparagine by aspartic acid. This is consistent with the observed difference in electrophoretic mobility in starch gel of rather less than 1 unit of charge at pH5.0, where the aspartic acid carboxyl group would be mainly in the ionized form. This amino acid replacement could result from a single base change in the triplet codon and has been found in many other proteins (see, e.g., Dayhoff, 1972). Separation of tryptic and chymotryptic peptides under several different conditions showed that only the B acidic (B_A) and the A acidic (A_A) and neutral (A_N) peptides were different between the two ovalbumins, suggesting that this was probably the only sequence difference between them.

The clear distinction between A and B type ovalbumins by pre-treated antisera suggests that this amino acid replacement is located on the surface of the molecule, as might be expected from the hydrophilic nature of the residues involved. However, it is not known if any conformational difference exists because of this amino acid replacement.

Table 1. *Amino acid composition of 'variant' peptides*

Experimental details are described in the text.

Amino acid	Peptide ...	Composition (mol/mol of peptide) B_A	A_A	A_N
Asp		1.0	—	1.0
Ser*		4.7	3.3	2.5
Glu		1.1	1.1	—
Gly		1.1	0.7	—
Ala		2.2	0.8	0.8
Ile		1.0	1.0	—
Leu		1.9	1.0	0.8
Number of residues		13	8	5

* Uncorrected for destruction during hydrolysis.

Table 2. *Thermolysin subpeptides of peptide B_A*

Experimental details are described in the text.

Peptide	Mobility at pH6.4	Amino acid composition (mol/mol of peptide) Asp	Ser*	Glu	Gly	Ala	Ile	Leu	Sequence
a	0	—	1.1	—	1.1	—	—	0.8	Leu-Ser-Gly
c	0.33	—	2.9	1.2	—	1.0	0.8	1.1	Ile-Ala-
d	0.45	0.9	2.2	—	—	0.9	—	—	—
1	0.72	1.1	—	—	—	0.9	—	—	Ala-
2	0.65	—	—	1.1	—	0.9	—	—	Ala-
3	0.58	—	1.6	1.3	—	1.0	—	—	Ala-
4	0	—	1.1	—	1.0	—	—	0.9	Leu-

* Uncorrected for destruction during hydrolysis.

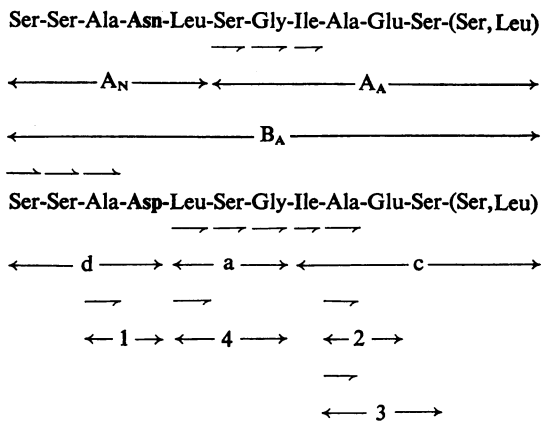

Fig. 5. *Suggested sequences of peptides A_N, A_A and B_A*

The amino acid replacement is shown in **bold** type. → indicates sequence determination by the dansyl–Edman procedure. Experimental details are given in the text.

It is not yet possible to locate this 13-residue peptide within the molecule of hen ovalbumin. It does not correspond to any of the published sequences, which contain only 85 of the total 387 residues (see Dayhoff, 1972). It is noteworthy, however, that the sequence -Ser-Gly-Ile-Ala- also occurs in the *N*-terminal sequence, where it is preceded by *N*-acetylated glycine (Narita & Ishii, 1962). The repetition of a tetrapeptide sequence is unlikely to occur by chance and is the first suggestion of gene duplication in hen ovalbumin.

The sequence -Asn-Leu-Ser- found in the common form of ovalbumin might be expected to carry carbohydrate on the asparagine residue. The electrophoretic mobility, the behaviour on Sephadex and the lack of glucosamine of A_N peptide indicate that this sequence is not glycosylated as is the -Asn-Leu-Thr- sequence found elsewhere in the molecule (Cunningham *et al.*, 1963). Moreover, evidence from glycopeptide studies suggests that all the carbohydrate is attached at this single asparagine residue (Neuberger & Marshall, 1966). Ovalbumin is thus an example of an unusual type of glycoprotein containing two sites of the -Asn-X-Ser/Thr type, where only one of the sites is glycosylated (see Hunt & Dayhoff, 1970), and is in marked contrast to the situation in pig ribonuclease where all three of such sites carry carbohydrate (Jackson & Hirs, 1970). This may be due to the difference between serine and threonine. The latter is usually found in glycopeptides containing the type of carbohydrate moiety that is present in ovalbumin, which perhaps reflects the specificity of the relevant glycosyltransferase. Alternatively, glycosylation near the serine-containing sequence may be sterically inhibited by the tertiary structure of the polypeptide chain.

We thank Mr. W. Johnston for carrying out the breeding experiments, Dr. R. P. Ambler for the gift of thermolysin and helpful discussions, and the Medical Research Council for financial assistance.

References

Ambler, R P. & Meadway, R. J. (1968) *Biochem. J.* **108**, 893
Andrews, P. (1965) *Biochem. J.* **96**, 595
Cunningham, L. W., Nuenke, B. J. & Strayhorn, W. D. (1957) *J. Biol. Chem.* **228**, 835
Cunningham, L. W., Clouse, R. W. & Ford, J. B. (1963) *Biochim. Biophys. Acta* **78**, 379
Dayhoff, M. O. (1972) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, Md.
Fothergill, J. E. & Perrie, W. T. (1966) *Biochem. J.* **99**, 58P
Fothergill, L. A. & Fothergill, J. E. (1970*a*) *Eur. J. Biochem.* **17**, 529
Fothergill, L. A. & Fothergill, J. E. (1970*b*) *Biochem. J.* **116**, 555
Fothergill, L. A. & Fothergill, J. E. (1971) *Comp. Biochem. Physiol.* **40A**, 445
Gray, W. R. (1967*a*) *Methods Enzymol.* **11**, 139
Gray, W. R. (1967*b*) *Methods Enzymol.* **11**, 469
Heilman, J., Barollier, J. & Watzke, E. Z. (1957) *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 219
Hunt, L. T. & Dayhoff, M. O. (1970) *Biochem. Biophys. Res. Commun.* **39**, 757
Ingram, V. M. (1963) *Methods Enzymol.* **6**, 831
Jackson, R. L. & Hirs, C. H. W. (1970) *J. Biol. Chem.* **245**, 624
Kaminski, M. (1960) *Ann. Inst. Pasteur Paris* **98**, 51
Lush, I. E. (1964) *Genet. Res.* **5**, 257
Narita, K. & Ishii, J. (1962) *J. Biochem. (Tokyo)* **52**, 367
Neil, G. L., Niemann, C. & Hein, G. E. (1966) *Nature (London)* **210**, 903
Neuberger, A. & Marshall, R. D. (1966) in *Glycoproteins* (Gottschalk, A., ed.), p. 307, Elsevier, Amsterdam
Offord, R. E. (1966) *Nature (London)* **211**, 591
Ouchterlony, O. (1953) *Acta Pathol. Microbiol. Scand.* **32**, 231
Perlmann, G. E. (1952) *J. Gen. Physiol.* **35**, 711
Phillips, D. M. P. (1948) *Nature (London)* **161**, 53
Porter, R. R. (1957) *Biochem. J.* **66**, 677
Scheidegger, J. J. (1955) *Int. Arch. Allergy Appl. Immunol.* **7**, 103
Shmakova, F. V. & Kaverzneva, E. D. (1969) *Biokhimiya* **34**, 445
Wiseman, R. L. (1967) Ph.D. Thesis, University of Aberdeen
Wiseman, R. L. & Fothergill, J. E. (1966) *Biochem. J.* **99**, 58P
Wiseman, R. L. & Fothergill, J. E. (1967) *Biochem. J.* **102**, 25P
Woods, K. R. & Wang, K. T. (1967) *Biochim. Biophys. Acta* **133**, 369