Electrophoretic Properties of Ovomucoid

By M. D. MELAMED*
Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2

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1. The nature of the electrophoretic heterogeneity of ovomucoid was investigated. Optimum resolution of the fractions on starch-gel electrophoresis occurred over a narrow range of pH and ionic strength. The pattern was not altered in the presence of 8M-urea but the bands were sharper. Ovomucoid–trypsin complex is stable at pH 4·6 but dissociated in 6M-urea. 2. The two major fractions of ovomucoid were eluted from the gels. One of these was virtually free of sialic acid and the other, which contained 0·4mole of sialic acid/mole of protein, split into two components on electrophoresis after neuraminidase treatment. It was concluded that these two components, and likewise the two major fractions of ovomucoid, differ by a unit charge/mol. Differences in sialic acid content account for only part of the electrophoretic heterogeneity of ovomucoid.

Ovomucoid, the trypsin-inhibiting glycoprotein from hen's-egg white, has long been known to be electrophoretically heterogeneous (for review see Melamed, 1966). The occurrence of proteins as a series of slightly different molecular forms is being observed with increasing frequency and is referred to variously as microheterogeneity, isoenzymes, polymorphism etc. The extreme stability of ovomucoid to denaturation makes it a convenient protein for the study of this phenomenon.

Most investigators have employed material obtained by acetone precipitation at pH 3·5, after removal of most other egg-white proteins with trichloroacetate (Lineweaver & Murray, 1947) or by various modifications of the method. Rhodes, Azari & Feeney (1958) introduced the use of chromatographic methods for the isolation of ovomucoid from egg white. These preparations have been further purified in a number of Laboratories (Fredericq & Deutsch, 1949; Bier, Terminiello, Duke, Gibbs & Nord, 1953; Noble, Legault-Demare & Jutisz, 1957; Feeney, Stevens & Osuga, 1963*b*; Chatterjee & Montgomery, 1962; Beeley & Jevons, 1965), but no preparations have been shown to consist of a single electrophoretic species.

In the present study, starch-gel electrophoresis was employed to investigate some of the properties of ovomucoid and its trypsin complex (Melamed, 1964). The technique has been used for separating egg-white proteins (Lush, 1961; Feeney, Abplanalp, Clary, Edwards & Clark, 1963*a*), but ovomucoid was not resolved from ovalbumin and could not be identified in the gels. In addition, the usual procedure for showing up the proteins with Naphthalene Black does not stain ovomucoid (Feeney *et al.* 1963*b*). The fact that starch-gel electrophoresis is possible in 6M- or 8M-urea (see e.g. Smithies, 1962) has been exploited in this study to investigate the effect of urea on the electrophoretic behaviour of ovomucoid and its trypsin complex. Ovomucoid has been subjected to electrophoresis in starch gels by Wise, Ketterer & Hansen (1964).

A more detailed understanding of the nature of the differences between electrophoretically distinct species of a protein requires their isolation. Attempts to achieve this by ion-exchange chromatography on substituted cellulose or Sephadex by published procedures (Jevons, 1960; Chatterjee & Montgomery, 1962; Feeney *et al.* 1963*b*) or by any variations of these (M. D. Melamed, unpublished work), were unsuccessful. A method was therefore devised for the simultaneous recovery of several bands from the starch gel by a second electrophoresis. A multi-membraned tray was employed, which is simpler to construct than apparatus previously described (e.g. Gordon, 1962), but which requires a longer period for elution owing to poor heat dissipation. Details of a similar apparatus have been published independently (Lloyd & Meares, 1964).

EXPERIMENTAL

Materials

Two preparations of ovomucoid were employed. One was prepared chromatographically (Rhodes, Bennet & Feeney, 1960) and was similar to the material described by Neuberger & Papkoff (1963). The other was prepared by

* Present address: Department of Biological Chemistry, U.C.L.A. Centre for the Health Sciences, Los Angeles, Calif. 90024, U.S.A.

acetone precipitation at pH 3·5 (Lineweaver & Murray, 1947). Trypsin (Novo Industrials) was obtained from Globe Products (Accrington, Lancs.). The neuraminidase used was a solution (100 units/ml. in acetate buffer, pH 5·5) from L. Light and Co. (Colnbrook, Bucks.) and the standard *N*-acetylneuraminic acid was from the same source. The agarose (batch 21; Seravac Laboratories Ltd., Maidenhead, Berks.) had been prepared by the method of Russell, Mead & Polson (1964). When a solution of the agarose (0·8%, w/v, in 0·01 M-acetate buffer, pH 4·5) was prepared by heating in a boiling-water bath and kept at 45° for about 1 hr., a contaminating material came out of suspension as a flocculent precipitate and could be removed by centrifugation. The clear colourless supernatant was stored and reheated in a boiling-water bath when required. Partially hydrolysed starch (Connaught Medical Laboratories, Toronto, Canada) was used for gel electrophoresis. The dye Eriochrome Black T (equivalent to Solochrome Black WDFA; Colour Index no. 14645) was obtained from E. Gurr Ltd. (London, S.W. 14). The urea was a commercial grade (British Drug Houses Ltd., Poole, Dorset) and was used without further purification.

METHODS

Starch-gel electrophoresis. The vertical apparatus (Smithies, 1959*a*) was used with soft porous plastic (Spontex) for wicks, and a starch concentration of 12% (Smithies, 1959*b*). For applying the samples the agarose gel (0·8% in 0·01 M-acetate buffer, pH 4·5) was rendered fluid in a boiling-water bath and then placed in a bath at 45°. The protein solution (at 45°) was mixed with an equal volume of the agarose and pipetted into the slot, where it solidified almost immediately. The band-sharpening effect observed when the protein is applied in solution is not diminished by the use of the agarose gel. The exposed surface of the gel was sealed with a thin sheet of polythene and the gel subjected to electrophoresis for 12–20 hr. with a total power not exceeding 3·5 w in the gel. The Eriochrome Black T dye (Clark, 1962) was used in the same way as had been developed for Naphthalene Black (Smithies, 1955). The stained gel was washed with the methanol–water–acetic acid solution (5:5:1, by vol.) until the background was clear (2–4 hr. minimum, but preferably overnight). A total protein concentration of 2% produced optimum loading of the gels, but the minor components of ovomucoid were only visible at double this loading.

Electrophoresis in the presence of urea. Electrophoresis of ovomucoid in the presence of high concentrations of urea posed special problems, owing to the very narrow range of conditions of pH and ionic strength over which good resolution was attainable. A modified procedure was thus devised for preparation of the gels containing 6 M- or 8 M-urea, whereby Zeo-Karb 225 resin (H^+ form) is present in the mixture as long as any heating is required, but is removed before addition of the buffer (Melamed, 1967).

Preparative electrophoresis. The starch-gel (12%) was prepared in sodium acetate buffer (pH 4·6–4·7 and *I* 0·015) and poured in the usual way, but only one slot of 12 cm. was made, which occupied almost the full width of the gel. The gel was loaded with 30 mg. of crude ovomucoid in 0·8 ml. of the usual 0·4% agarose at 45° and subjected to electrophoresis (150 v, 25 mA) for 20–24 hr. The electrode chambers contained sodium acetate buffer (*I* 0·3) as did the chambers adjacent to the gel (*I* 0·05), all at the same pH as the gel. After electrophoresis, those regions in the gel that contained protein, as indicated by reference to locating strips (and taking into account the shrinkage that occurs during the

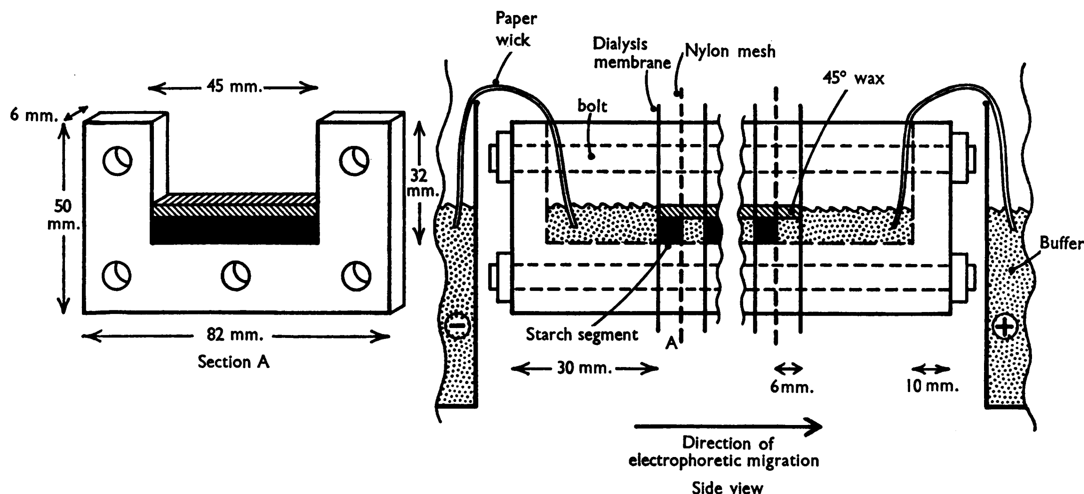

Fig. 1. Apparatus for electrophoretic elution of proteins from starch gel. Starch segments containing the two major electrophoretically separated fractions of ovomucoid were placed in each alternative segment as indicated, acetate buffer (pH 5) was added to the same level throughout, and molten wax at 45° was pipetted on to the segments and adjoining buffer. Electrophoretic elution was performed for 24 hr. at 20 mA. The full apparatus is suitable for simultaneous elution of six segments. This can be doubled by placing a second segment on top of each one and adjusting the buffer levels and the total power accordingly.

staining), were cut out, yielding two segments of gel for each protein band (one from each side of the central locating strip). Each of these was then placed in a separate compartment of a tray designed for electrophoretic elution.

The elution tray (Fig. 1) was made from Perspex; it consisted of two end pieces and 12 segments that could be assembled together to form the tray and held in place by brass rods furnished with washers and nuts at each end. During assembly, a section of dialysis membrane (previously soaked and punched) and a section of nylon mesh (50–100 openings/in., similarly punched) were inserted alternately between the segments. The use of silicone grease between each face and membrane or nylon mesh ensured a watertight fit; and once set up the apparatus could be used repeatedly as long as the membranes were prevented from drying out. A piece of starch gel was placed in every alternate segment such that the protein migrated through the nylon mesh and was trapped by the dialysis membrane. The segments not containing the starch as well as the end pieces were filled with acetate buffer (pH 5·0 and I 0·01) to the level of the gel slices. Each gel segment as well as each pool of buffer (except that in the end pieces) was covered with molten paraffin wax at 45° to prevent evaporation; the end compartments were connected with filter-paper wicks to the electrode vessels (capacity 5 l.) filled with sodium acetate buffer, pH 5·0 and I 0·05. It was important that the buffer level in both electrode vessels and the elution tray was the same to prevent siphoning. Electrophoretic elution was performed for 24 hr. at a current of 20 mA.

At the end of the run each compartment was washed out and the appropriate washings were pooled, dialysed and freeze-dried. The resulting materials were considerably contaminated with soluble starch and the preparations were therefore further purified by column chromatography on DEAE-cellulose columns. Material eluted from the two major bands from two gels (total starting material 60 mg.) were pooled into one 'fast' fraction and one 'slow' fraction (the terms 'fast' and 'slow' referring to their relative rates of migration towards the anode). The solutions were dialysed against phosphate buffer (pH 7·0 and I 0·005) and adsorbed on to a DEAE-cellulose column (1 cm. × 40 cm.) equilibrated with the same buffer. Elution was effected by a linear increase in Na_2SO_4 concentration from 0 to 0·25 M over 200 ml. of the phosphate buffer. The fractions containing protein were pooled, dialysed and freeze-dried. The final yield was 14 mg. of slow fraction and 8·5 mg. of fast fraction. After correcting for the material removed in the guide strips, this represents a recovery of about 50% of the remainder. The proportions of fast material and slow material isolated are roughly in accord with their relative staining intensity as judged by eye.

Sialic acid content and electrophoretic behaviour of isolated fractions. Stock solutions were prepared of both fractions and of the ovomucoid starting material by adding water (0·1 ml.) to 3 mg. of each preparation. N-Acetylneuraminic acid was liberated by mild acid hydrolysis or by neuraminidase, and the isolated fractions were subjected to re-electrophoresis on starch gel before and after enzymic liberation of sialic acid.

For acid hydrolysis 10 μl. of stock solution was mixed with an equal volume of 0·2 N-H_2SO_4, sealed into a short length of melting-point capillary tubing and maintained at 80° for 60 min. (Warren, 1959). Recovery of standard samples after this treatment was 90%.

The rate of enzymic liberation of sialic acid from ovomucoid was determined by incubating a sample (7 mg.) of the starting material with 0·5 ml. of enzyme solution at 37° and pH 5·5 and analysing samples (0·05 ml.) at various times. The enzyme/substrate ratio was 7 units/mg. of ovomucoid or 0·7 unit/μg. of N-acetylneuraminic acid. (One unit liberates 1 μg. of N-acetylneuraminic acid from a human serum glycoprotein substrate in 15 min. at 37°.) The results obtained, as a percentage of sialic acid in the sample and as a percentage of the final value for the experiment were: 1 hr., 0·43% (76%); 3 hr., 0·53% (93%); 6 hr., 0·57% (100%); 16 hr., 0·57% (100%).

For determination of sialic acid in the two isolated ovomucoid fractions by enzymic liberation, samples were incubated with enzyme/substrate ratios 2–3 units/μg. of N-acetylneuraminic acid. Stock solution (10 μl.) was incubated with 40 μl. of neuraminidase solution and 2 μl. of toluene at 37° and pH 5·5 overnight. As a check on the activity of the enzyme and on the absence of aldolases in the preparation, a sample of sialyl-lactose, containing 19% of bound sialic acid, was treated similarly. Recovery of control N-acetylneuraminic acid after enzymic treatment as above was 100% and of sialic acid from the sialyl-lactose 90%.

Liberated sialic acid was determined by the method of Aminoff (1961) on a reduced scale (sample volume, 50 μl.) except that the chromophore was extracted with cyclohexanone as in the Warren (1959) procedure. This modification (suggested by Dr E. R. B. Graham) results in more complete extraction of the chromophore. The final solution was heated at 100° for 15 min. and then cooled in an ice bath. Conc. HCl (10 μl.) and cyclohexanone (500 μl.) were finally added, and after vigorous shaking the mixture was cooled in ice and centrifuged at 0°. The organic layer was pipetted off and its absorption spectrum recorded in a 1 cm. micro-cell. Determination of standard N-acetylneuraminic acid over the range 0·2–1·5 μg. in 0·05 ml. resulted in a linear calibration with a molar extinction coefficient at 549 mμ of $9{\cdot}1 \times 10^4$ and blank readings of about 0·02.

Both the isolated fractions of ovomucoid were subjected to starch-gel electrophoresis. A sample of each fraction was also subjected to electrophoresis after treatment with neuraminidase. Stock solutions (20 μl. of the fast fraction and 30 μl. of the slow fraction) were each incubated with 10 μl. of neuraminidase (not less than 0·5 unit/μg. of N-acetylneuraminic acid) and 2 μl. of toluene at 37° and pH 5·5 for 24 hr., and the whole mixture was applied.

RESULTS

Starch-gel electrophoresis. The use of Eriochrome Black T resulted in very satisfactory and permanent staining of all proteins used and gave low background staining. Good resolution of the different components of ovomucoid by starch-gel electrophoresis required approximately the same conditions as did free-boundary electrophoresis (Bier *et al.* 1953). Best results were obtained at pH 4·65 and I 0·01. Both ovomucoid preparations gave two major and one or two minor bands moving towards the anode and fairly evenly spaced. The intensity of staining was highest for the slowest-moving band and consistently less for each band

further from the origin. In addition two contaminants (conalbumin, lysozyme) were identified in the ovomucoid prepared by acetone precipitation at pH 3·5.

When ovomucoid was mixed with a slight excess of trypsin and then subjected to electrophoresis, all the negatively charged material disappeared and a diffuse band appeared at a position intermediate between that of the trypsin and that of the original bands. Thus the group of evenly spaced anodic bands were all ovomucoid and the complex with trypsin is stable at pH 4·6 (cf. Sri-Ram, Terminiello, Bier & Nord, 1954). A second band smaller and more cathodic than the main new band also appeared. This material possibly corresponds with the $(trypsin)_2$–ovomucoid complex (Sri-Ram *et al.* 1954).

Electrophoresis in the presence of urea. As with the aqueous gels, in the presence of urea, ovomucoid only showed clear banding over a narrow range of conditions. The pattern of bands and their relative staining intensity was not affected by the presence of urea, but the bands were much sharper. The ionic strength again had to be about 0·01 and the best results were obtained between pH 4·7 and 5·0 in 6M- or 8M-urea. A pH value 0·2 unit higher was required in the presence of urea to obtain bands with the same apparent electrophoretic mobilities as in its absence.

When the ovomucoid–trypsin complex was subjected to electrophoresis in starch gels containing 6M-urea, the trypsin was recovered and the ovomucoid appeared in the gels in a slightly altered position. The complex was apparently completely absent. This is consistent with the general conclusion that only enzymically active trypsin can form complexes with naturally occurring trypsin inhibitors. Further, 6M-urea will bring about dissociation of the enzyme–inhibitor complex. The unexpected position of the ovomucoid may be due to the complex first migrating into the gel and then dissociating, leaving the ovomucoid significantly displaced, or, possibly, to the ovomucoid's being modified by the trypsin during the process of association and dissociation (Finkenstadt & Laskowski, 1965).

Sialic acid content and re-electrophoresis of isolated fractions. The sialic acid results are summarized in Table 1. A considerable proportion of the total sialic acid of the starting material is not accounted for in the two fractions isolated from the starch gel. This is probably due to the removal of fractions containing much higher proportions of sialic acid, such as the sialic acid-rich ovomucoid (Rhodes *et al.* 1960) and possibly ovoglycoprotein (Ketterer, 1965).

The results of re-electrophoresis on the two fractions before and after neuraminidase treatment are shown in Fig. 2. The fractions are electrophoretically homogeneous with negligible cross-contamination. The slow fraction is virtually sialic acid-free and, as expected, is not altered by neuraminidase treatment. The original fast fraction contained about 0·4 mole of sialic acid/mole of protein and ran as two fractions after neuraminidase incubation: the F_f fraction, in the same position as the original fast fraction, and the F_s fraction, in the same position as the original slow fraction. This is consistent with the explanation that the original fast fraction is a mixture of two species of ovomucoids, which both have the same net charge at the pH of the experiment. One of these is free of sialic acid (F_f fraction) and the other probably contains 1 mole of sialic acid/mole of protein (F_s fraction). If this is so, the difference between the F_f and F_s fractions, after neuraminidase treatment, is

Table 1. *Sialic acid content of ovomucoid fractions isolated from starch-gel electrophoresis*

Sialic acid was determined after its liberation by acid (0·1 N-H_2SO_4 at 80° for 60 min.) or by neuraminidase (pH 5·5 at 37° for 16 hr.).

	Residues of sialic acid/mol. of ovomucoid	
	Released by acid	Released by enzyme
Starting material	0·81	0·66
Slow fraction	0·05	0·06
Fast fraction	0·35	0·37

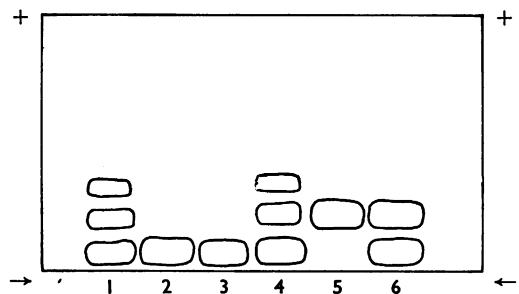

Fig. 2. Effect of neuraminidase on re-electrophoresis of ovomucoid fractions isolated from starch gel. Starch-gel electrophoresis was performed in acetate buffer, pH 4·65 and *I* 0·015, at 5 v/cm. for 20 hr. Slots 1 and 4 contained ovomucoid prepared by the method of Lineweaver & Murray (1947). Slots 2 and 3 contained the isolated slow fraction, the material in slot 2 being neuraminidase-treated (pH 5·5 at 37° overnight). Slots 5 and 6 contained the fast fraction, slot 6 being the neuraminidase-treated material. Arrows mark application line for samples.

1 charged group/mol. The sialic acid-free F_f component must therefore also differ from the original slow fraction by 1 charge/mol.

DISCUSSION

In general, the behaviour of ovomucoid on electrophoresis in starch gel is not very different from that under free-boundary conditions (Bier *et al.* 1953). One discrepancy is that in the present study (as also in the results of Wise *et al.* 1964) the most basic component is present in highest concentration, whereas in free-boundary electrophoresis it is the second most basic one (O_2) that is the predominant component.

In the electrophoretic behaviour of proteins in concentrated urea solutions, their isoelectric points may be significantly different from those in water. Bull, Breese, Ferguson & Swenson (1964) have postulated that urea drastically decreases the activity of H^+ ions, leaving the activities of other ions more or less unchanged. It was surprising therefore that the isoelectric point of ovomucoid did not appear to be much altered in the presence of 8M-urea, but this conclusion is subject to the determination of endosmotic effects in the urea gels.

A better resolution of the different components of ovomucoid was obtained on starch-gel electrophoresis than on ion-exchange chromatography, as was similarly found for the α_1-acid glycoprotein of serum (Schmid, Binette, Kamiyama, Pfister & Takahashi, 1962). This is possibly due to the fact that, in chromatography, only surface charges (and not those in sterically inaccessible regions of the protein, such as those covered by carbohydrate) are operative. In spite of its low isoelectric point ovomucoid is only very weakly bound to DEAE-cellulose and is eluted at relatively low ionic strength. It is likely that the very hydrophilic carbohydrate groups of the molecule are situated on the outside of a highly cross-linked globular protein core, and that only very few charges are sterically sufficiently accessible to form salt bridges with the resin. It is also possible that the degree of accessibility of the charged groups to the resin is conformation-dependent, and this may complicate the chromatographic behaviour of ovomucoid.

From the re-electrophoresis of the isolated ovomucoid fractions after treatment with neuraminidase, it is clear that there is 1 charge difference/mol. between F_s and F_f. Thus the original fast fraction and slow fraction also differ by 1 charge/mol. Assuming these correspond to the two main fractions O_1 and O_2 (Bier *et al.* 1953), their isoelectric points are 4·41 and 4·28 respectively. At pH 4·41 there would be no charge on the slow fraction and one full negative charge on the fast fraction. The 29 side-chain carboxyl groups on the protein must therefore, on average, each undergo a 3·5% change in their degree of ionization for this shift in pH. As this would only happen at 0·7 unit from the p*K*, the side-chain carboxyl groups of ovomucoid appear to have an average p*K* about 3·7. Further, from the total of 22 positive and 29 negative charges on the molecule (Marshall & Neuberger, 1960), at the isoelectric point (pH 4·2, on average for all the different species) the negative groups must be 76% ionized, i.e. 0·5 pH unit above their p*K*. This again gives an average p*K* for the carboxyl groups of about 3·7. Titration (Marshall & Neuberger, 1960) indicated 18 groups with p*K* 3·45 and 11 groups with p*K* 4·9. Protein side-chain carboxyl groups have an expected p*K* 4·6 and groups of such low p*K* appear to be unusual (Tanford, 1962).

Ovomucoid free from sialic acid has also been reported by Montreuil, Castiglioni, Adam-Chosson, Caner & Queval (1965), but, as they do not describe electrophoretically homogenous fractions, it is difficult to relate their results exactly to those described here. The conclusion that sialic acid alone does not account for all the electrophoretic variability in ovomucoid is in accord with the results of Beeley & Jevons (1965), who found two major components of ovomucoid corresponding to fractions O_1 and O_2 (Bier *et al.* 1953) were still present on free-boundary electrophoresis of ovomucoid treated with neuraminidase. If this is so then the nature of the other groups responsible is of interest. As the difference between the species is 1 full charge/mol. at pH 4·6, it cannot be accounted for in terms of a single normal side-chain carboxyl group. There are three other possible explanations for the differences in electrophoretic mobility: (i) a carboxyl side chain of abnormally low p*K*; (ii) a basic amino acid; (iii) two carboxyl side chains of p*K* 4·6; the second is the simplest and involves the least degree of surmise.

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REFERENCES

Aminoff, D. (1961). *Biochem. J.* **81**, 384.

Beeley, J. G. & Jevons, F. R. (1965). *Biochim. biophys. Acta*, **101**, 133.

Bier, M., Terminiello, L., Duke, J. A., Gibbs, R. J. & Nord, F. F. (1953). *Arch. Biochem. Biophys.* **47**, 465.

Bull, M. B., Breese, K., Ferguson, G. L. & Swenson, C. A. (1964). *Arch. Biochem. Biophys.* **104**, 297.

Chatterjee, A. K. & Montgomery, R. (1962). *Arch. Biochem. Biophys.* **99**, 426.

Clark, A. (1962). *Nature, Lond.*, **196**, 1097.

Feeney, R. E., Abplanalp, H., Clary, J. J., Edwards, D. L. & Clark, J. R. (1963*a*). *J. biol. Chem.* **238**, 1732.

Feeney, R. E., Stevens, F. C. & Osuga, D. T. (1963*b*). *J. biol. Chem.* **238**, 1415.

Finkenstadt, W. R. & Laskowski, M., jun. (1965). *J. biol. Chem.* **240**, PC 962.

Fredericq, E. & Deutsch, H. F. (1949). *J. biol. Chem.* **181**, 499.
Gordon, A. H. (1962). *Biochem. J.* **82**, 531.
Jevons, F. R. (1960). *Biochim. biophys. Acta*, **45**, 384.
Ketterer, B. (1965). *Biochem. J.* **96**, 372.
Lineweaver, H. & Murray, C. W. (1947). *J. biol. Chem.* **171**, 565.
Lloyd, H. M. & Meares, J. D. (1964). *Clin. chim. Acta*, **9**, 192.
Lush, I. E. (1961). *Nature, Lond.*, **189**, 981.
Marshall, R. D. & Neuberger, A. (1960). *Nature, Lond.*, **186**, 311.
Melamed, M. D. (1964). *Biochem. J.* **92**, 12 P.
Melamed, M. D. (1966). In *Glycoproteins: Their Composition, Structure and Function*, p. 317. Ed. by Gottschalk, A. Amsterdam: Elsevier Publishing Co.
Melamed, M. D. (1967). *Analyt. Biochem.* (in the Press).
Montreuil, J., Castiglioni, B., Adam-Chosson, A., Caner, F. & Queval, J. (1965). *J. Biochem., Tokyo*, **57**, 514.
Neuberger, A. & Papkoff, H. (1963). *Biochem. J.* **87**, 581.
Noble, E., Legault-Demare, J. & Jutisz, M. (1957). *Bull. Soc. Chim. biol., Paris*, **39**, 1077.
Rhodes, M. B., Azari, P. R. & Feeney, R. E. (1958). *J. biol. Chem.* **230**, 399.
Rhodes, M. B., Bennett, N. & Feeney, R. E. (1960). *J. biol. Chem.* **235**, 1686.
Russell, B., Mead, T. H. & Polson, A. (1964). *Biochim. biophys. Acta*, **86**, 169.
Schmid, K., Binette, J. P., Kamiyama, S., Pfister, V. & Takahashi, S. (1962). *Biochemistry*, **1**, 959.
Smithies, O. (1955). *Biochem. J.* **61**, 629.
Smithies, O. (1959*a*). *Biochem. J.* **71**, 585.
Smithies, O. (1959*b*). *Advanc. Protein Chem.* **14**, 65.
Smithies, O. (1962). *Arch. Biochem. Biophys.* Suppl. 1, 125.
Sri-Ram, J., Terminiello, L., Bier, M. & Nord, F. F. (1954). *Arch. Biochem. Biophys.* **52**, 451.
Tanford, C. (1962). *Advanc. Protein Chem.* **17**, 70.
Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
Wise, R. W., Ketterer, B. & Hansen, I. A. (1964). *Comp. Biochem. Physiol.* **12**, 439.