Characterization of Proteins Structurally Related to Human N-Acetyl-β-D-glucosaminidase

By MARK CARROLL*

*Department of Biochemistry, University of Nairobi, P.O. Box 30197,
Nairobi, Kenya*

Those proteins of human liver that cross-reacted with antibodies raised to apparently homogeneous hexosaminidases A and B were detected by immunodiffusion. Cross-reacting proteins with high molecular weights (greater than 200000) and intermediate molecular weights (70000–200000) were present both in the unadsorbed fraction and in the 0.05–0.2 M-NaCl eluate obtained by DEAE-cellulose chromatography at pH 7.0. The unadsorbed fraction also contained a cross-reacting protein of low molecular weight (10000–70000). The possible structural and functional relationships between hexosaminidase and the cross-reacting proteins are discussed. An apparently cross-reacting protein present in the 0.05 M-NaCl eluate from the DEAE-cellulose column was serologically unrelated to hexosaminidase, but it gave a reaction of immunological identity with one of the apparently cross-reacting proteins having the charge and size characteristics of hexosaminidase A. It is suggested that immunochemical methods may provide criteria for the homogeneity of enzyme preparations superior to those of conventional methods.

The immunochemical properties of several human lysosomal enzymes have been examined, including hexosaminidase (Carroll & Robinson, 1973), arylsulphatase (Neuwelt *et al.*, 1971), α-mannosidase (Phillips *et al.*, 1975), α-glucosidase (Van Hoof *et al.*, 1972) and cathepsin D (Dingle *et al.*, 1971). Such studies have in general had two aims. Firstly, serological reactions of identity or non-identity between different molecular forms of the enzyme provide information on the possible structural and genetic interrelationships of the isoenzymes. Secondly, the presence or absence of serologically cross-reacting protein in the enzyme-deficient states, the lysosomal storage diseases, should shed some light on the associated genetic lesions. Also, the absence of cross-reacting protein might cause immunological complications for affected individuals undergoing the proposed treatment for such diseases, enzyme-replacement therapy.

The N-acetyl-β-D-glucosaminidase (hexosaminidase, EC 3.2.1.30) activity of human tissues has been particularly well studied in this respect (Carroll & Robinson, 1973; Srivastava & Beutler, 1974). The two major isoenzymes, hexosaminidases A and B, show a reaction of complete immunological identity, although a specific anti-(hexosaminidase A) serum could be prepared by absorption with purified hexosaminidase B of those antibodies common to

both forms (Carroll & Robinson, 1972; Srivastava & Beutler, 1972). These immunochemical characteristics have been explained in structural terms (Robinson & Carroll, 1972; Srivastava & Beutler, 1973). The A and B isoenzymes are multimeric proteins which share a common subunit (β); the A-form is characterized by a unique subunit (α). Isolated α-subunits aggregate spontaneously to form a highly acidic isoenzyme, hexosaminidase S, which is also present in low activity in normal individuals (Beutler & Kuhl, 1975; Srivastava *et al.*, 1975).

Two lysosomal storage diseases are associated with a specific deficiency of hexosaminidase (O'Brien, 1973). In Tay–Sachs disease the A-form is missing but the B-form is present in increased amounts; in the clinically more severe Sandhoff's disease both isoenzymes are absent. The associated genetic lesions probably affect the α-subunit in Tay–Sachs disease and the β-subunit in Sandhoff's disease (Robinson & Carroll, 1972). There have been conflicting reports about the presence in the enzyme-deficient states of serologically cross-reacting protein corresponding to hexosaminidase A. Carroll & Robinson (1973) found no such protein in one case of Tay–Sachs disease and one of Sandhoff's disease. Srivastava & Beutler (1974) confirmed this finding in three cases of Tay–Sachs disease, but claimed that it was present in one case of Sandhoff's disease; in another patient with Sandhoff's disease cross-reacting protein with the electrophoretic characteristics of hexosaminidase B was also detected. A low-molecular-weight cross-reacting protein has been isolated from human liver

* Present address: Department of Biochemistry, The London Hospital Medical School, Turner Street, London E1 2AD, U.K.

and partially characterized (Carroll & Robinson, 1974); it may be the putative β-subunit.

The present study is an attempt to characterize those proteins of human liver that cross-react with antibodies raised to apparently homogeneous preparations of human hexosaminidases A and B. The results cast some doubt on the interpretation of the structural and immunochemical studies reported by Beutler and co-workers (Srivastava & Beutler, 1974; Srivastava *et al.*, 1974*a,b*). It is suggested that currently accepted criteria of enzyme homogeneity may be inadequate.

Experimental

All materials and methods with the following exceptions were as described previously (Carroll & Robinson, 1973). Urease, lactate dehydrogenase, bovine serum albumin and cytochrome *c* were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A.

Tissue and antisera

Liver from normal individuals was obtained at post mortem and stored at −20°C until required. Placenta was obtained from a local maternity ward and stored likewise. Tissue homogenates (20%, w/v) were prepared in a Potter–Elvehjem homogenizer by using a Teflon pestle rotating at 250rev./min. The homogenates were prepared in 0.01M-potassium phosphate buffer, pH7.0, and centrifuged in a Sorvall SS-1 centrifuge for 30min at 10^4g ($r_{av.}$ 107mm) and 4°C, the supernatants being used as the enzyme preparations. The γ-globulin fractions from antisera raised to apparently homogeneous hexosaminidases A and B from human placenta (Srivastava & Beutler, 1974) were provided as freeze-dried powders by Professor S. K. Srivastava, University of Texas Medical Branch, Galveston, TX, U.S.A. Each freeze-dried preparation was dissolved in phosphate-buffered saline containing 0.2% (w/v) NaN₃ at a concentration of 50mg/ml and the solutions were stored at 4°C.

Enzyme assays

Samples (50μl) were incubated with 0.45ml of 5mM-4-nitrophenyl *N*-acetyl-β-D-glucosaminide (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) in 0.05M-sodium citrate buffer, pH4.4. After 30min at 37°C the reaction was stopped by the addition of 0.5M-glycine/NaOH buffer, pH10.4 (2.5ml). The A_{420} of the liberated aglycone was measured in a Zeiss PMQII spectrophotometer.

Ion-exchange chromatography

Columns (9.0cm×2.8cm) of Whatman DEAE 11 ion-exchange cellulose (W. and R. Balston, Maidstone, Kent, U.K.) were equilibrated in 0.01M-potassium phosphate buffer, pH7.0, before chromatography of tissue supernatants in the same buffer at 4°C. Unadsorbed proteins were eluted with buffer and adsorbed proteins were eluted with a stepwise salt gradient (50ml each of 0.05M-, 0.2M- and 1.0M-NaCl in the buffer).

Gel filtration

Samples (3–5ml) were applied to a column (90cm×2.6cm) of Sephadex G-200 equilibrated at 4°C in 0.01M-potassium phosphate buffer, pH7.0, containing 5mM-NaCl. The column was eluted with the same buffer at a flow rate of 17ml/h, and after a volume of 180ml had emerged fractions (4.3ml) were collected. The molecular weight of hexosaminidase isoenzymes was estimated by calibration of the column with urease (mol.wt. 490000), lactate dehydrogenase (mol.wt. 140000), bovine serum albumin (mol.wt. 67000) and cytochrome *c* (mol.wt. 12400). After gel filtration of samples containing hexosaminidase, fractions were pooled as follows: fractions 1–18 (corresponding to mol.wts. greater than 200000; pre-hexosaminidase); fractions 19–33 (70000–200000mol.wt.; hexosaminidase); fractions 34–60 (10000–70000mol.wt.; post-hexosaminidase). Pre- and post-hexosaminidase fractions were free of detectable enzyme activity. After either gel filtration or ion-exchange chromatography, fractions were adjusted to 95% saturation with (NH₄)₂SO₄ (Dixon & Webb, 1958). The precipitate recovered by centrifugation (30min at 10^4g) was dissolved in a small volume of the appropriate buffer and, if necessary, dialysed against the same buffer overnight at 4°C.

Serological methods

Immunodiffusion was performed in 1.5% (w/v) agarose gel in phosphate-buffered saline. The precipitin lines of washed gels were stained for protein with 0.25% (w/v) Coomassie Blue (Sigma Chemical Co.) in methanol/acetic acid/water (5:1:5, by vol.) and destained in the same solvent. In immuno-absorption experiments anti-hexosaminidase serum (50μl) was incubated with the appropriate fraction (150μl) or with bovine serum albumin (1.2mg/ml) as a control. After 1h at 37°C and 4h at 4°C absorbed antiserum was tested by immunodiffusion.

Results and Discussion

Although the antisera used in the present study were raised to purified hexosaminidases A and B from human placenta, the enzymes from placenta and liver were immunologically identical in immunodiffusion gels (see also Srivastava *et al.*, 1974*a*). Liver was used

as the source of hexosaminidase and serologically related proteins in all subsequent experiments.

The total hexosaminidase activity of a liver supernatant was fractionated by DEAE-cellulose chromatography at pH 7.0 into the B-form (unadsorbed fraction; 28–30% of the applied activity), the I-form (0.05M-NaCl eluate; 0.7–1.2%), the A-form (0.2M-NaCl eluate; 45–52%) and the S-form (1.0M-NaCl eluate; 6–8%) (cf. Carroll & Robinson, 1973; Price & Dance, 1972; Beutler et al., 1975). The association of each isoenzyme with the respective fraction was confirmed by Cellogel electrophoresis as described by Braidman et al. (1974). The recovery of protein was 75–77% of that applied (range from three experiments). Under these conditions the serologically unrelated hexosaminidase C is not recovered from the DEAE-cellulose column (Braidman et al., 1974). When the concentrated fractions were examined by immunodiffusion with anti-hexosaminidase serum, several precipitin lines were observed (Fig. 1a). The B-fraction contained at least one major cross-reacting protein in addition to hexosaminidase B, which gave rise to an enzymically active precipitin line. The I-fraction contained one cross-reacting protein devoid of hexosaminidase activity; the serological reaction of this protein was more pronounced with anti-(hexosaminidase A) serum than with anti-(hexosaminidase B) serum, but it also gave a second blurred line with the latter not clearly apparent in Fig. 1a owing to loss of definition on reproduction). The A-fraction contained two prominent cross-reacting proteins, one of which was associated with enzymic activity. The other protein formed a precipitin line which fused with that formed by the cross-reacting protein in the I-fraction. No cross-reacting protein was detected in the S-fraction under the conditions used here. There was no evidence of any cross-reacting protein specifically related serologically to either hexosaminidase A or hexosaminidase B in any of the fractions. The two antisera gave essentially identical results, except for the reaction with the I-fraction noted above; any difference in the relative positions of precipitin lines can be ascribed to the higher potency of the antiserum raised to the B-isoenzyme.

The concentrated A-, B- and I-fractions were further fractionated by gel filtration in Sephadex G-200. The molecular weight of each isoenzyme was in the range 120000–140000, in agreement with earlier reports of gel-filtration experiments (Wässle & Sandhoff, 1971; Srivastava et al., 1974b). The concentrated pooled fractions were examined by immunodiffusion. The cross-reacting material of the B-fraction was separated into four proteins, one with a mol.wt. greater than 200000, two of mol.wt. 70000–200000 (one corresponding to hexosaminidase B) and one of mol.wt. 10000–70000 (Fig. 1b). This low-molecular-weight cross-reacting protein

probably corresponds to the putative β-subunit described by Carroll & Robinson (1974). A cross-reacting protein with a mol.wt. greater than 200000 has been described previously (Robinson et al., 1973), but it was not further characterized. It may represent membrane-bound or aggregated hexosaminidase in an inactive form, or a macromolecular protein serologically related to the β-subunit. The single cross-reacting protein in the I-fraction had a mol.wt. in the range 70000–200000. The cross-reacting material of the A-fraction was separated into four proteins, one eluted in the pre-hexosaminidase fraction and three in the hexosaminidase fraction (Fig. 1c). There was no evidence of a low-molecular-weight cross-reacting protein which might correspond to the α-subunit of hexosaminidase A. However, the α-subunit is known to undergo spontaneous aggregation forming hexosaminidase S (Beutler & Kuhl, 1975).

The possibility could not be excluded that some of the cross-reacting proteins detected by immunodiffusion were artifacts serologically unrelated to hexosaminidase. Such a situation might arise if the preparations used to raise the antisera were not in fact homogeneous, although they were judged to be so on the basis of a single protein band after electrophoresis in polyacrylamide gel and a single schlieren boundary in the ultracentrifuge (Srivastava et al., 1974a,b). A small amount of a highly immunogenic contaminant in the antigen preparation might give rise to specific antibodies which would form a precipitin line when tested against a crude preparation in immunodiffusion gels. This was indeed found to be so. When anti-(hexosaminidase A) serum was absorbed with the I-fraction (prepared by DEAE-cellulose chromatography and containing a single apparently cross-reacting protein), it lost its ability to form a precipitin line with the I-fraction in immunodiffusion gels, but its ability to precipitate hexosaminidase A was unaffected (Fig. 1d). The A-fraction removed antibodies to hexosaminidase A, as expected, and also abolished the formation of a precipitin line between the antiserum and the I-fraction. Thus it appears that that protein in the I-fraction detected by immunodiffusion is serologically unrelated to hexosaminidase A. It does, however, appear to be immunologically identical with a protein having the charge and size characteristics of the A-isoenzyme.

If the above conclusion is correct, it follows that the purified hexosaminidase A preparation used to raise the antiserum was not in fact homogeneous, as has been claimed by Srivastava et al. (1974a). This inevitably casts some doubt on their interpretation of the amino acid analysis of the 'homogeneous' A- and B-forms. The small differences in amino acid composition, which were considered to be significant (Beutler & Srivastava, 1973), might be caused by the
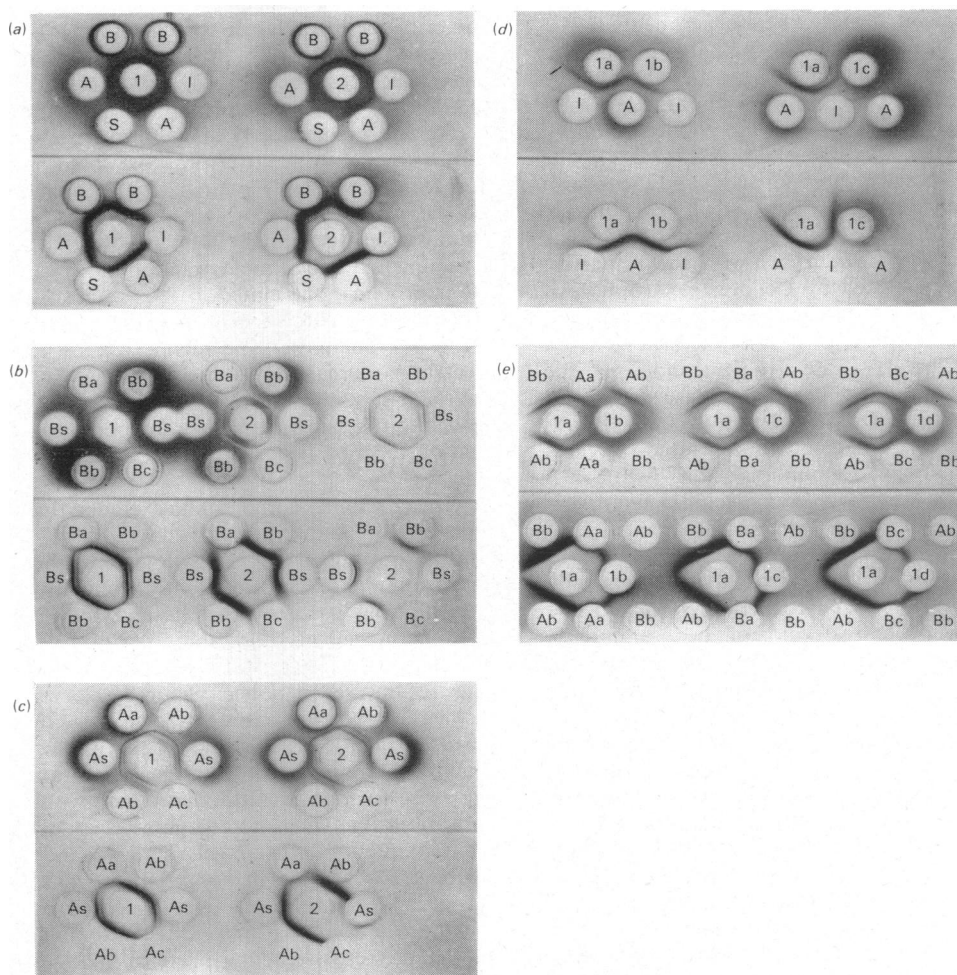
Fig. 1. *Immunodiffusion gels*

(*a*) Reaction of anti-(hexosaminidase A) serum (1) and anti-(hexosaminidase B) serum (2) with fractions of human liver obtained by DEAE-cellulose chromatography at pH 7.0 and containing hexosaminidase B, I, A or S. (*b*) Reaction of anti-(hexosaminidase A) serum (1) and anti-(hexosaminidase B) serum (2) with fractions obtained by gel filtration in Sephadex G-200 of hexosaminidase B (Bs) prepared by DEAE-cellulose chromatography at pH 7.0. Fractions were pooled corresponding to mol.wt. of greater than 200000 (Ba), 70000–200000 (Bb) and 10000–70000 (Bc). The fractions were either undiluted (left and centre patterns) or diluted sixfold with phosphate-buffered saline (right pattern). (*c*) As (*b*), but with fractions (Aa, Ab, Ac) obtained by gel filtration of hexosaminidase A (As). (*d*) Reaction of hexosaminidase I fraction (I) and hexosaminidase A fraction (A) with anti-(hexosaminidase A) serum absorbed with bovine serum albumin (control; 1a), with hexosaminidase I (1b) or with hexosaminidase A (1c). (*e*) Reaction of fractions from gel filtration in Sephadex G-200 of pre-hexosaminidase (Aa, Ba), hexosaminidase (Ab, Bb) and post-hexosaminidase (Bc) [all designations as in (*b*) and (*c*) above] with anti-(hexosaminidase A) serum absorbed with bovine serum albumin (control; 1a), with fraction Aa (1b), with fraction Ba (1c) or with fraction Bc (1d). The upper gel of each pair was stained for protein with Coomassie Blue; the lower gel of each pair was stained for enzymic activity with Naphthol ASBI glucosaminide.

presence of the contaminating protein in the hexos-aminidase A preparation. In addition, it is clear that, in this particular case, conventional physical methods such as electrophoresis in polyacrylamide gel and sedimentation in the ultracentrifuge do not provide adequate criteria of homogeneity of an enzyme

preparation. If the enzyme is purified only on the basis of its physicochemical properties, then the final preparation is likely to be contaminated with proteins having similar charge, size and solubility characteristics. Conventional methods may not be sensitive enough to detect small amounts of such contaminating proteins. However, the formation of a discrete precipitin line in immunodiffusion gels, which is based on the serological specificity and concentration of the protein concerned, appears to offer a greater degree of sensitivity. Purification schedules which incorporate an affinity-chromatography step, such as that described for placental hexosaminidase by Geiger & Arnon (1976), are advisable when the enzyme of interest does not possess exceptional physicochemical properties and when the source of the enzyme is a tissue such as liver.

A further corollary is that the antisera used in the present study are not monospecific for hexosaminidase. This finding complicates the conclusion drawn by Srivastava & Beutler (1974) that there is cross-reacting protein corresponding to inactive hexosaminidase in the liver of patients with Sandhoff's disease. The precipitin lines that they observed after immunoelectrophoresis might have arisen from serologically unrelated proteins. One such protein has the charge and size characteristics of hexosaminidase A. The lack of specificity of the antisera also severely limits their application in further studies, such as enzyme localization by using immunofluorescence techniques. A more specific antiserum could be prepared by removing the enzymically active precipitin line from immunodiffusion gels and using that as an antigen preparation. Such antigen–antibody precipitates are highly immunogenic, as has been shown for the cathepsin D system of chicken liver (Weston, 1969).

The two high-molecular-weight cross-reacting proteins were indeed serologically related to hexosaminidase, as was the one of low molecular weight (Fig. 1e). Each protein absorbed the hexosaminidase-specific antibodies of either antiserum. It was difficult to reach any definite conclusion about the serological status of those apparently cross-reacting proteins of intermediate molecular weight (except for the one with the charge and size characteristics of hexosaminidase A, as noted above). They formed indistinct precipitin lines in immunodiffusion gels after absorption of the antiserum with either bovine serum albumin as a control or one of the other cross-reacting proteins. This may be a consequence of the fourfold dilution of the antiserum that the absorption step entailed. The antibody/antigen ratio might then have been far from equivalence.

The results imply that there are at least three, and possibly as many as six, proteins of human liver that are structurally related to hexosaminidase. Since each of the cross-reacting proteins reacts equally well

with antiserum raised to either hexosaminidase A or B, the structural resemblance is presumably with the β-subunit rather than the α-subunit. As the β-subunit bears an active site, the cross-reacting proteins themselves may have hexosaminidase activity that is not detected by the artificial substrate used in the present study. The common identity of hexosaminidase A and the GM2-ganglioside N-acetyl-β-D-galactosaminidase has not been conclusively established, and there are probably as yet undiscovered hexosaminidases involved in the catabolism of glycosaminoglycans and glycoproteins (see, e.g., Neufeld et al., 1975; Dorfman & Matalon, 1976). Such a situation would have obvious benefits in terms of cellular economy. The substrate specificity of a common 'hexosaminidase subunit' would be directed by association with a 'specifier subunit' (cf. Robinson & Carroll, 1972). Just such an enzyme–modifier association has been described for the lactose synthetase of mammary gland (Brew et al., 1968).

The low-molecular-weight cross-reacting protein reported here and partially characterized by Carroll & Robinson (1974) may be identical with the β-subunit; in addition, it has charge, size and stability characteristics very similar to those of lysozyme. Lysozyme itself is an acid hydrolase which acts primarily as a muramidase, but it also has associated endohexosaminidase activity (Rupley & Gates, 1967). Although no serological cross-reaction was observed between human lysozyme and hexosaminidase (Carroll & Robinson, 1974), the immunodiffusion method may not be sensitive enough to detect a low degree of structural homology between related proteins. A more sensitive serological method may be required, such as the immobilized-antibody technique with which Truffa-Bachi et al. (1975) analysed the structurally related enzymes of the aspartate kinase system of Escherichia coli. The low-molecular-weight cross-reacting protein is also structurally related to chitobiase (NN'-diacetylchitobiase), for the two proteins show a reaction of immunological identity (Carroll, 1973). Chitobiase itself is functionally related to the hexosaminidases (Stirling, 1974). Thus the picture that emerges is that of a family of proteins having structural and/or functional relationships with the hexosaminidases. It will be interesting to see whether the cross-reacting structurally related proteins described here have any affinity for N-acetyl-β-D-glucosamine. Such a prediction could be readily tested by using a suitably substituted affinity-chromatography adsorbent, such as that described by Geiger & Arnon (1976).

References

Beutler, E. & Kuhl, W. (1975) *Nature (London)* **258**, 262–264

Beutler, E. & Srivastava, S. K. (1973) *Isr. J. Med. Sci.* **9**, 1335–1337

Beutler, E., Kuhl, W. & Comings, D. (1975) *Am. J. Hum. Genet.* **27**, 628–638

Braidman, I., Carroll, M., Dance, N. & Robinson, D. (1974) *Biochem. J.* **143**, 295–301

Brew, K., Vanaman, T. C. & Hill, R. L. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **59**, 491–497

Carroll, M. (1973) Ph.D. Thesis, University of London

Carroll, M. & Robinson, D. (1972) *Biochem. J.* **126**, 17P

Carroll, M. & Robinson, D. (1973) *Biochem. J.* **131**, 91–96

Carroll, M. & Robinson, D. (1974) *Biochem. J.* **137**, 217–221

Dingle, J. T., Barrett, A. J. & Weston, P. D. (1971) *Biochem. J.* **123**, 1–14

Dixon, M. & Webb, E. C. (1958) *Enzymes*, p. 40, Academic Press, New York

Dorfman, A. & Matalon, R. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 630–637

Geiger, B. & Arnon, R. (1976) *Biochemistry* **15**, 3484–3493

Neufeld, E. F., Lim, T. W. & Shapiro, L. J. (1975) *Annu. Rev. Biochem.* **44**, 357–376

Neuwelt, E., Stumpf, D., Austin, J. & Kohler, P. (1971) *Biochim. Biophys. Acta* **236**, 333–346

O'Brien, J. S. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 191–199

Phillips, N., Robinson, D. & Winchester, B. G. (1975) *Biochem. J.* **151**, 469–475

Price, R. G. & Dance, N. (1972) *Biochim. Biophys. Acta* **271**, 145–153

Robinson, D. & Carroll, M. (1972) *Lancet* i, 322–323

Robinson, D., Carroll, M. & Stirling, J. L. (1973) *Nature (London)* **243**, 415–416

Rupley, J. A. & Gates, V. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **57**, 496–510

Srivastava, S. K. & Beutler, E. (1972) *Biochem. Biophys. Res. Commun.* **47**, 753–759

Srivastava, S. K. & Beutler, E. (1973) *Nature (London)* **241**, 463

Srivastava, S. K. & Beutler, E. (1974) *J. Biol. Chem.* **249**, 2054–2057

Srivastava, S. K., Awasthi, Y. C., Yoshida, A. & Beutler, E. (1974a) *J. Biol. Chem.* **249**, 2043–2048

Srivastava, S. K., Yoshida, A., Awasthi, Y. C. & Beutler, E. (1974b) *J. Biol. Chem.* **249**, 2049–2053

Srivastava, S. K., Wiktorowicz, J., Klebe, R. & Awasthi, Y. C. (1975) *Biochim. Biophys. Acta* **397**, 428–436

Stirling, J. L. (1974) *FEBS Lett.* **39**, 171–175

Truffa-Bachi, P., Guiso, N., Cohen, G. N., Theze, J. & Burr, B. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1268–1271

Van Hoof, F., Hue, L., de Barsy, Th., Jacquemin, P., Devos, P. & Hers, H. G. (1972) *Biochimie* **54**, 745–751

Wässle, W. & Sandhoff, K. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1119–1133

Weston, P. D. (1969) *Immunology* **17**, 421–428