Secretion of β -N-Acetylglucosaminidase Isoenzymes by Normal Human Fibroblasts

By PAUL WILLCOX

Department of Biochemistry, University of Auckland, Auckland, New Zealand

(Received 14 November 1977)

1. Secretion of the lysosomal enzyme β -N-acetylglucosaminidase (EC 3.2.1.30) by normal human fibroblast cultures was linear with respect to time up to 96 h. 2. Two forms of the A isoenzyme of β -N-acetylglucosaminidase were found in the culture medium. One form was similar to the isoenzyme found in other extracellular fluids, such as plasma and tears, the other resembled the intracellular (lysosomal) enzyme. The presence of the two isoenzymes in the culture medium appears to reflect two distinct secretory processes. 3. It is suggested that plasma acid hydrolases may be destined for incorporation into lysosomes in a manner analogous to that described for the packaging of lysosomal enzymes by fibroblasts.

Lysosomal acid hydrolases are mainly involved in intracellular digestion and are not usually released from normal cells. However, exceptions have been documented, e.g. Vaes (1969) described the secretion of lysosomal enzymes by osteoclasts during parathyroid-hormone-induced bone resorption. More recently several workers have shown that polymorphonuclear leucocytes and macrophages can release a large proportion of their acid hydrolase content into the surrounding medium under a variety of conditions (Davies & Allison, 1976; Henson, 1976; Werb & Dingle, 1976). Similarly, acid hydrolases are released by cultured fibroblasts into the surrounding medium, and Hickman & Neufeld (1972) have proposed that packaging of lysosomal enzymes by fibroblasts involves their secretion into the medium followed by specific recognition and uptake by neighbouring cells. The molecular form of the secreted enzyme has received little attention in many studies and it is not known if the extracellular form is derived from extruded lysosomal contents or whether a specific secretory pathway is involved.

A condition analogous to the cell-culture situation applies *in vivo*, where acid hydrolases are found in most extracellular fluids in addition to their intracellular lysosomal location. The extracellular and tissue forms of many acid hydrolases appear to be closely related, since both activities are usually absent from patients with lysosomal storage diseases. It has recently been shown, however, that in many cases the enzymes are not identical, plasma acid hydrolases differing from the liver enzymes in several respects, notably in position of elution from DEAE-cellulose and in their susceptibility to neuraminidase treatment (Willcox & Renwick, 1977). The presence of acid hydrolases in plasma therefore appears to result from an active secretion rather than from 'leakage' due to cellular damage.

To investigate further the nature and function of extracellular acid hydrolases, a thorough study of the secretion of the acid hydrolase β -N-acetylglucosaminidase (EC 3.2.1.30) from normal fibroblasts has been undertaken, with particular reference to the molecular form of the enzyme found in fibroblasts and in the culture medium.

Materials and Methods

Materials

4-Methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside was supplied by Koch-Light Laboratories (Colnbrook, Bucks., U.K.). Whatman DEAE-cellulose (type DE-52) was obtained from W. and R. Balston (Maidstone, Kent, U.K.). Human albumin and neuraminidase (*Clostridium perfringens*, type V; EC 3.2.1.18) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Medium 199, trypsin and glutamine were obtained from Grand Island Biological Co. (Grand Island, NY, U.S.A.), foetal bovine serum was from Laboratory Services (Auckland, N.Z.). Penicillin and streptomycin were supplied by Glaxo (N.Z.) Ltd. (Palmerston North, N.Z.), and Hanks balanced salt solution was supplied by Commonwealth Serum Laboratories (Parkville, Vic., Australia).

Cell culture

Normal skin-fibroblast cultures were obtained by standard procedures. Fibroblasts from a patient with G_{M2} gangliosidosis type 2 (Sandhoff disease) were kindly supplied by Dr. A. D. Patrick (Institute of Child Health, London, W.C.1, U.K.). A second fibroblast culture from a patient with Sandhoff disease (GM-470) was obtained from the Human

Genetic Mutant Cell Repository (Campden, NJ, U.S.A.).

All cultures were maintained in medium 199 containing antibiotics (penicillin, 60 µg/ml, and streptomycin, 100 µg/ml), added glutamine (1.0 mM) and 15% (v/v) foetal bovine serum in an atmosphere of CO₂/air (1:19), at 37°C.

For studies of enzyme secretion by fibroblasts, confluent cultures (35 cm²) were carefully washed four times with Hanks balanced salt solution. The cells were then maintained for the specified length of time (24 h unless otherwise stated) in medium 199 (10 ml) containing no foetal bovine serum but with added albumin (1.0 mg/ml). The medium was then removed, filtered through a Millipore filter (pore size 0.2 µm) and either frozen immediately or desalted and concentrated. After removal of the medium, the cell layer was treated with trypsin solution (0.1%, w/v) in Dulbecco's phosphate-buffered saline. The detached cells were suspended in Hanks balanced salt solution (5.0 ml), centrifuged at 500g for 10 min, and the cell pellet was washed with 2.0 ml of ice-cold 0.9% NaCl. The pellet was covered with cold water (100 µl) and quickly frozen in a methanol/solid CO₂ bath and stored at -20°C.

Enzyme preparations

Fresh plasma was obtained from healthy donors, with the use of sodium citrate as anti-coagulant. The plasma was dialysed against water overnight at 4°C and then centrifuged at 2.5×10^4 g-min. The supernatant was stored at -20°C.

Culture medium from secretion experiments was desalted and concentrated (10–20 times) by means of either an Amicon standard stirred cell, model 52, or an Amicon micro-ultrafiltration system, model 8MC (Amicon Corp., Lexington, MA, U.S.A.). Both systems were fitted with an Amicon Diaflo membrane filter, type PM10 (nominal mol.wt. cut-off 10000).

Fibroblasts were homogenized in water and then rapidly frozen and thawed three times. The homogenate was centrifuged at 2.5×10^4 g-min and the supernatant removed.

Neuraminidase treatment

Enzyme samples (1.0 ml) were incubated with 1.0 ml of a 0.5 mg/ml neuraminidase solution (0.25 unit/ml, with *N*-acetylneuraminlactose as substrate) for 3 h at 37°C in 0.1 M-citric acid/0.2 M-disodium hydrogen phosphate buffer, pH 5.0. Under these conditions the neuraminidase released over 90% of the total bound sialic acid from all the enzyme preparations, as measured by the thiobarbituric assay method of Warren (1959). Total sialic acid content

was determined after hydrolysis of the preparations by 0.1 M-H₂SO₄ at 80°C for 1 h (Warren, 1959). Control samples, containing buffer but no neuraminidase, were incubated in a similar manner. After incubation, all samples were dialysed against deionized water overnight at 4°C and then applied to ion-exchange columns.

At the concentration used, the neuraminidase preparation had no detectable β -*N*-acetylglucosaminidase activity. The stated proteinase activity of the preparation was 1 munit/mg of protein, where 1 unit hydrolyses casein to produce colour equivalent to 1.0 µmol of tyrosine/min.

Enzyme assay

Enzyme samples (100 µl) were incubated with 100 µl of 5 mM-4-methylumbelliferyl 2-acetamido-2-deoxy- β -glucopyranoside containing 0.1% human albumin in 0.2 M-disodium hydrogen phosphate/0.1 M-citric acid buffer, pH 4.5, for up to 3 h at 37°C. After incubation, 1.0 ml of 0.5 M-glycine/NaOH buffer, pH 10.4, was added to stop the reaction and to maximize the fluorescence of the liberated 4-methylumbelliferone. Fluorescence was measured in an Aminco-Bowman spectrophotofluorimeter with excitation set at 358 nm and emission at 448 nm.

All enzyme activities are reported in nmol of substrate transformed/min per ml or per mg of protein. Protein was measured by the method of Lowry *et al.* (1951), with human serum albumin as standard.

Ion-exchange chromatography

Whatman DEAE-cellulose (type DE-52) was equilibrated with 10 mM-sodium phosphate buffer, pH 6.0, and packed in 10 ml (65 mm × 14 mm) disposable syringes (Becton, Dickenson and Co., Rutherford, NJ, U.S.A.). Two columns (bed volumes 4.8 ml), supplied by a single gradient-former and flow-inducer, were run simultaneously. To ensure that identical salt gradients were applied to each column the buffer stream was divided immediately before entering the columns (Willcox & Renwick, 1977).

After application of the sample, 10 mM-sodium phosphate buffer, pH 6.0, was applied and six 3.0 ml fractions were collected. Elution was continued with a linear KCl gradient (0–150 mM-KCl in 10 mM-sodium phosphate buffer, pH 6.0) and a further 38 fractions were collected. Chromatography was carried out at room temperature (20°C) and columns took approx. 2 h to run. Preliminary experiments showed that, when two identical samples were chromatographed simultaneously, the maximum peak difference was one fraction. Conductivity was measured with a Radiometer CDM 2e conductivity meter.

Results

Human fibroblasts are usually cultured in medium containing foetal bovine serum, which contains large amounts of acid hydrolases, including β -N-acetylglucosaminidase. Secretion experiments must therefore be carried out in either serum-free medium or in medium containing heat-inactivated serum.

Fig. 1. Secretion of β -N-acetylglucosaminidase by normal and Sandhoff-disease fibroblasts

Confluent cultures of two normal fibroblast cell lines (Δ , $\bullet$) and two cultures initiated from patients with Sandhoff disease ($\circ$, $\square$) were maintained in serum-free medium (10 ml). At timed intervals, 0.5 ml portions of medium were removed and assayed for β -N-acetylglucosaminidase activity as described in the Materials and Methods section. After 96 h the cultures were harvested with trypsin and the protein content of the cells was determined. The total enzyme activity of the medium is expressed as nmol of 4-methylumbelliferone liberated/min per mg of cell protein.

Although identical results were obtained with either medium, serum-free medium was routinely used to preserve a more closely defined experimental system. Human serum albumin (β -N-acetylglucosaminidase-free) was added to the medium at a concentration of 0.1% to stabilize the secreted enzyme. Cellular breakdown, as reflected by leakage of lactate dehydrogenase (EC 1.1.1.27), could only be demonstrated after prolonged exposure of the cultures to the medium (i.e. in excess of 72 h).

The appearance of β -N-acetylglucosaminidase activity in the medium of cultured fibroblasts could possibly result, not from secretion, but from the progressive elution of adsorbed enzyme derived from foetal bovine serum. To obviate this possibility, two experiments were performed. In the first experiment, samples of serum-free medium were removed from two normal cell lines at time intervals up to 96 h and assayed for β -N-acetylglucosaminidase activity. At the same time the secretion of the enzyme from two cell lines derived from patients with Sandhoff disease was followed. In Sandhoff disease the β -N-acetylglucosaminidase activity of the cells is diminished to less than 5% of the normal value and they would therefore be expected to secrete greatly decreased amounts of the enzyme into the medium. If the appearance of the enzyme in the medium resulted from the elution of adsorbed enzyme, values similar to those obtained from normal cells would be expected. Fig. 1 shows that secretion of β -N-acetylglucosaminidase by normal cells was linear up to 96 h and secretion from Sandhoff-disease fibroblasts was less than 5% of the value for normal cells.

A second experiment was carried out in which the DEAE-cellulose elution profile of the β -N-acetyl-

Fig. 2. Comparison of β -N-acetylglucosaminidase components of foetal bovine serum and normal fibroblast culture medium. Normal fibroblasts were maintained in serum-free medium for 24 h. The medium was then removed, filtered, desalted and concentrated. The elution profile of the β -N-acetylglucosaminidase activity of the secreted medium ($\circ$) was compared with that of dialysed foetal bovine serum (1.0 ml) ($\bullet$) by using twin columns of DEAE-cellulose. All experimental procedures are as described in the Materials and Methods section. Enzyme activity is expressed as nmol of 4-methylumbelliferone liberated/min per ml of each fraction. ---, KCl gradient.

glucosaminidase activity of foetal bovine serum was compared with that of the enzyme secreted into the medium by normal cells. Fig. 2 shows that the elution pattern of foetal bovine serum is markedly different from that of secreted medium.

When the chromatographic behaviour of plasma and liver β -*N*-acetylglucosaminidase is compared directly by DEAE-cellulose chromatography, the A isoenzyme of plasma is eluted at a lower salt concentration than is the A isoenzyme of liver (Ikonne & Ellis, 1973). A similar comparison was made of the same enzyme obtained from normal fibroblasts and the enzyme recovered from the medium of the cells. Similar elution patterns were obtained (Fig. 3a), although the A isoenzyme of

secreted medium was eluted at a marginally lower salt concentration, and in a much wider peak, than the isoenzyme from fibroblasts.

After preincubation with neuraminidase, the β -*N*-acetylglucosaminidase A isoenzyme of plasma is eluted from DEAE-cellulose at an appreciably lower salt concentration than an untreated sample, whereas the liver isoenzyme is unaffected by the treatment (Ikonne & Ellis, 1973). The effect of neuraminidase on the β -*N*-acetylglucosaminidase A activity of both secreted medium and normal fibroblasts was similarly investigated. Neuraminidase treatment had no effect on the elution profile of fibroblast β -*N*-acetylglucosaminidase activity (Fig. 3b), whereas the broad peak of β -*N*-acetylglucos-

Fig. 3. Resolution of fibroblast and secreted medium β -*N*-acetylglucosaminidase components. Elution profiles from twin columns of DEAE-cellulose of: (a) $\circ$, fibroblast extract; $\bullet$, medium from normal fibroblasts; (b) $\circ$, fibroblast extract incubated with neuraminidase for 3 h at 37°C and pH 5.0; $\bullet$, fibroblast extract incubated in the absence of neuraminidase; (c) $\bullet$, medium from normal fibroblasts incubated with neuraminidase; $\circ$, medium from normal fibroblasts incubated in the absence of neuraminidase; ----, KCl gradient. Experimental details are as described in the Materials and Methods section.

Fig. 4. β -N-Acetylglucosaminidase components of normal fibroblast culture medium

Elution profiles from twin columns of DEAE-cellulose of: (a) ●, medium from normal fibroblast culture treated with neuraminidase; ○, normal fibroblast extract; (b) ●, medium from normal fibroblast culture treated with neuraminidase; ○, dialysed human plasma (1.0 ml); ----, KCl gradient. Experimental details are as described in the Materials and Methods section.

aminidase A activity of secreted medium was resolved into two components (Fig. 3c).

The presence of two components after neuraminidase treatment suggested that both a 'plasma-like' component and a 'tissue' component were present in secreted medium. This was supported by the observation that, when an enzyme preparation derived from normal fibroblasts was compared with the enzyme found in the culture medium and treated with neuraminidase, the most anionic of the two secreted medium isoenzymes was eluted at a salt concentration identical with that at which the A isoenzyme from fibroblasts was eluted (Fig. 4a). Similarly, the position of the A isoenzyme of a plasma sample that had been treated with neuraminidase was very similar to the least acidic of the two secreted medium components (Fig. 4b).

Control experiments in which fibroblast β -N-acetylglucosaminidase was incubated in the culture medium eliminated the possibility that any new properties were a result of the culture conditions.

Discussion

Acid hydrolases are present in most extracellular fluids of the body, including plasma, synovial fluid,

urine, tears and saliva. Elevated activities of the enzymes are often associated with tissue damage, either experimentally induced or resulting from a variety of diseased states. A few examples have also been documented where lysosomal enzymes are released from cells during the resorption or remodelling of tissues. In many instances, however, both the origin and the function of extracellular acid hydrolases are unknown.

Under a variety of experimental conditions, phagocytic cells in short-term culture can release a large proportion of their acid hydrolase content into the surrounding medium. This release usually results from an extrusion of the lysosomal contents by fusion of the lysosomal membrane with the cell membrane. A non-destructive secretion of acid hydrolases over longer periods of time has been documented with fibroblast cultures (Hickman & Neufeld, 1972; von Figura & Kresse, 1974). The origin of the extracellular enzymes has, however, received little attention and it is not known if the enzymes are lysosomal in origin or whether an alternative secretory pathway is involved.

The present study has been concerned with documenting more precisely the secretion of lysosomal

enzymes by fibroblasts. The enzyme β -N-acetylglucosaminidase was chosen for these investigations, because it is the most comprehensively studied lysosomal acid hydrolase and Ellis *et al.* (1975) reported differences between fibroblast and secreted forms of the enzyme.

The experimental results show that two forms of the A isoenzyme of β -N-acetylglucosaminidase are found in the medium of cultured fibroblasts. One of the forms is very similar to the intracellular (fibroblast) enzyme, which is similar to the enzyme found in other tissues, including liver and leucocytes (Swallow *et al.*, 1974). The other isoenzyme differs from the tissue form, both in its pattern of elution from DEAE-cellulose and in its susceptibility to neuraminidase treatment; in both of these properties it resembles the isoenzyme found in plasma (Ikonne & Ellis, 1973; Willcox & Renwick, 1977).

Since secretion experiments are carried out in the absence of foetal bovine serum, it was considered possible that the tissue enzyme found in the culture medium could result from cell damage. We have, however, been unable to eliminate the appearance of this isoenzyme by shortening the time of the secretion experiment from 24 to 6 h, by increasing the albumin concentration of the medium, or by adding inactivated foetal calf serum to the medium. It is therefore probable that tissue β -N-acetylglucosaminidase A is a normal constituent of spent fibroblast culture medium. Its presence in the medium may be a consequence of 'regurgitation during feeding' (Weissmann *et al.*, 1975), where lysosomes are considered to fuse with endocytotic vacuoles that have not been completely interiorized, thus leaving a channel to the outside of the cell. Alternatively, the enzyme may be a constituent of 'residual bodies', which possibly expel their contents into the medium by exocytosis (Daems *et al.*, 1969). Mild cellular damage, induced by the addition of 0.5% ethanol to the culture medium, resulted in an increase in the proportion of tissue β -N-acetylglucosaminidase found in the medium (P. Willcox, unpublished work). This further suggests that the tissue isoenzyme is of lysosomal origin.

Spent culture medium contains a form of the A isoenzyme of β -N-acetylglucosaminidase that is different from the tissue isoenzyme, but that is similar in many respects to that found in plasma and also in tears (Ikonne & Ellis, 1973). This suggests that all extracellular hydrolases may have a common mode of origin. Most extracellular proteins are glycoproteins, and Eylar (1965) has proposed that glycosylation is associated with the export of proteins from cells. Extracellular acid hydrolases could therefore arise from the lysosomal enzymes by further glycosylation, the addition of a specific new carbohydrate determinant group acting as a 'pass-

port' to the outside of the cell. The plasma and tissue forms of β -N-acetylglucosaminidase A are certainly closely related, since both activities are absent from patients with Tay-Sachs disease (G_{M2} gangliosidosis type 1) (Okada & O'Brien, 1969). Also, although of similar size (Ikonne & Ellis, 1973), the extracellular isoenzyme differs from the intracellular form in that it contains sialic acid residues. Removal of the sialic acid, however, does not convert the extracellular isoenzyme into the tissue form; therefore additional carbohydrate residues would need to be involved.

There is now good evidence that, in fibroblast cell cultures, acid hydrolases are secreted into the medium and then endocytosed by neighbouring cells for packaging into lysosomes (Neufeld *et al.*, 1977). This process differs from the mechanism proposed for the synthesis, packaging and secretion of proteins by the exocrine pancreas (Palade, 1975). The sequence of events described by Palade (1975) has subsequently been extended to other cell types, including mouse peritoneal macrophages, where lysosomal enzymes appear to be synthesized and packaged in a similar manner (Cohn & Fedorko, 1969).

It is not known if secretion and subsequent uptake and packaging of lysosomal enzymes are unique to fibroblasts. In view of the similarities between the β -N-acetylglucosaminidase A isoenzyme secreted by fibroblast cultures and the isoenzyme found in plasma, it is tempting to draw an analogy and suggest that plasma acid hydrolases are also destined for incorporation into lysosomes. This would be in agreement with the hypothesis of Winterburn & Phelps (1972) that the function of the oligosaccharide units of plasma proteins is to determine the extracellular fate of the molecules. Supporting evidence comes from recent studies on the phenomenon of glycoprotein recognition. Morell *et al.* (1971) demonstrated that the presence of terminal sialic acid residues is essential for the maintenance of many glycoproteins in the circulation and that removal of sialic acid results in their rapid uptake by hepatocytes. More recently it has been shown that desialylation of serum β -N-acetylglucosaminidase facilitates its rapid uptake from the circulation (Bearpark & Stirling, 1977). Three additional terminal sugar residues, N-acetylglucosamine (Stahl *et al.*, 1976; Stockert *et al.*, 1976), mannose (Hieber *et al.*, 1976; Baynes & Wold, 1976) and mannose 6-phosphate (Kaplan *et al.*, 1977), have also been implicated in the recognition of various glycoproteins, including acid hydrolases, and there is some evidence that different tissues may recognize different terminal sugar residues of glycoproteins (Stockert *et al.*, 1976). At present, both the origin and fate of extracellular acid hydrolases *in vivo* is unknown; their function is also unknown.

I thank Professor A. G. C. Renwick for helpful suggestions during the preparation of this manuscript. The excellent technical assistance of Ms. Cynthia Hay and Ms. Stephanie Rattray is also gratefully acknowledged. The work was financed by the Medical Research Council of New Zealand.

References

- Baynes, J. W. & Wold, F. (1976) *J. Biol. Chem.* **251**, 6016–6024
- Bearpark, T. & Stirling, J. (1977) *Biochem. J.* **168**, 435–439
- Cohn, Z. A. & Fedorko, M. E. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), vol. 1, pp. 43–63, North-Holland, Amsterdam and London
- Daems, W. Th., Wisse, E. & Brederoo, P. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), vol. 1, pp. 64–112, North-Holland, Amsterdam and London
- Davies, P. & Allison, A. C. (1976) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Dean, R. T., eds.), vol. 5, pp. 61–98, North-Holland, Amsterdam and London
- Ellis, R. B., Willcox, P. & Patrick, A. D. (1975) *Clin. Sci. Mol. Med.* **49**, 543–550
- Eylar, E. H. (1965) *J. Theor. Biol.* **10**, 89–113
- Henson, P. M. (1976) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Dean, R. T., eds.), vol. 5, pp. 99–126, North-Holland, Amsterdam and London
- Hickman, S. & Neufeld, E. F. (1972) *Biochem. Biophys. Res. Commun.* **49**, 992–999
- Hieber, U., Distler, J., Myerowitz, R., Schmickel, R. D. & Jourdien, G. W. (1976) *Biochem. Biophys. Res. Commun.* **73**, 710–717
- Ikonne, J. U. & Ellis, R. B. (1973) *Biochem. J.* **135**, 457–462
- Kaplan, A., Fischer, D., Achord, D. & Sly, W. (1977) *J. Clin. Invest.* **60**, 1088–1093
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J. & Ashwell, G. (1971) *J. Biol. Chem.* **246**, 1461–1467
- Neufeld, E. F., Sando, G. N., Garvin, A. J. & Rome, L. H. (1977) *J. Supramol. Struct.* **6**, 95–101
- Okada, S. & O'Brien, J. (1969) *Science* **165**, 698–700
- Palade, G. (1975) *Science* **189**, 347–358
- Stahl, P., Schlesinger, P. H., Rodman, J. S. & Doebber, T. (1976) *Nature (London)* **264**, 86–88
- Stockert, R. J., Morell, A. G. & Scheinberg, I. H. (1976) *Biochem. Biophys. Res. Commun.* **68**, 988–993
- Swallow, D. M., Stokes, D. C., Corney, G. & Harris, H. (1974) *Ann. Hum. Genet.* **37**, 287–302
- Vaes, G. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), vol. 1, pp. 217–253, North-Holland, Amsterdam and London
- von Figura, K. & Kresse, H. (1974) *Eur. J. Biochem.* **48**, 357–363
- Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975
- Weissmann, G., Goldstein, I., Hoffstein, S. & Tsung, P. K. (1975) *Ann. N.Y. Acad. Sci.* **253**, 750–762
- Werb, Z. & Dingle, J. T. (1976) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Dean, R. T., eds.), vol. 5, pp. 127–156, North-Holland, Amsterdam and London
- Willcox, P. & Renwick, A. G. C. (1977) *Eur. J. Biochem.* **73**, 579–590
- Winterburn, P. J. & Phelps, C. F. (1972) *Nature (London)* **236**, 147–151