Biochem. J. (1971) 125, 1119–1129
Printed in Great Britain

Degradation of Heparin in Mouse Mastocytoma Tissue

By SÖREN ÖGREN and ULF LINDAHL
Institute of Medical Chemistry, University of Uppsala, Box 551, S75122 Uppsala, Sweden

(*Received 2 August* 1971)

1. Heparin was prepared from mouse mastocytoma tissue by mild procedures, including extraction of mast-cell granules with 2M-potassium chloride, precipitation of the extracted polysaccharide with cetylpyridinium chloride from 0.8M-potassium chloride and finally digestion of the isolated material with testicular hyaluronidase. The resulting product (fraction GE_H) represented approx. 40% of the total heparin content of the tissue. 2. Fraction GE_H was fractionated by gel chromatography on Sepharose 4B into three subfractions, with average molecular weights ($\bar{M}_w$) of approx. 60000–70000 (highly polydisperse material), 26000 and 9000 respectively. Treatment of each of the subfractions with alkali or with papain did not affect their behaviour on gel chromatography. Amino acid and neutral sugar analyses indicated that the two low-molecular-weight fractions consisted largely of single polysaccharide chains lacking the carbohydrate–protein linkage region. It was suggested that these heparin molecules had been degraded by an endopolysaccharidase. 3. Pulse labelling *in vivo* of mastocytoma heparin with [^{35}S]sulphate showed initial labelling of large molecules followed by a progressive shift of radioactivity toward fractions of lower molecular weight. Further, heparin-depolymerizing activity was demonstrated by incubating ^{35}S-labelled heparin *in vitro* with a mastocytoma 10000 *g*-supernatant fraction. Appreciable degradation of the polysaccharide occurred, as demonstrated by gel chromatography. In contrast, no depolymerization was observed on subjecting ^{14}C-labelled chondroitin sulphate to the same procedure.

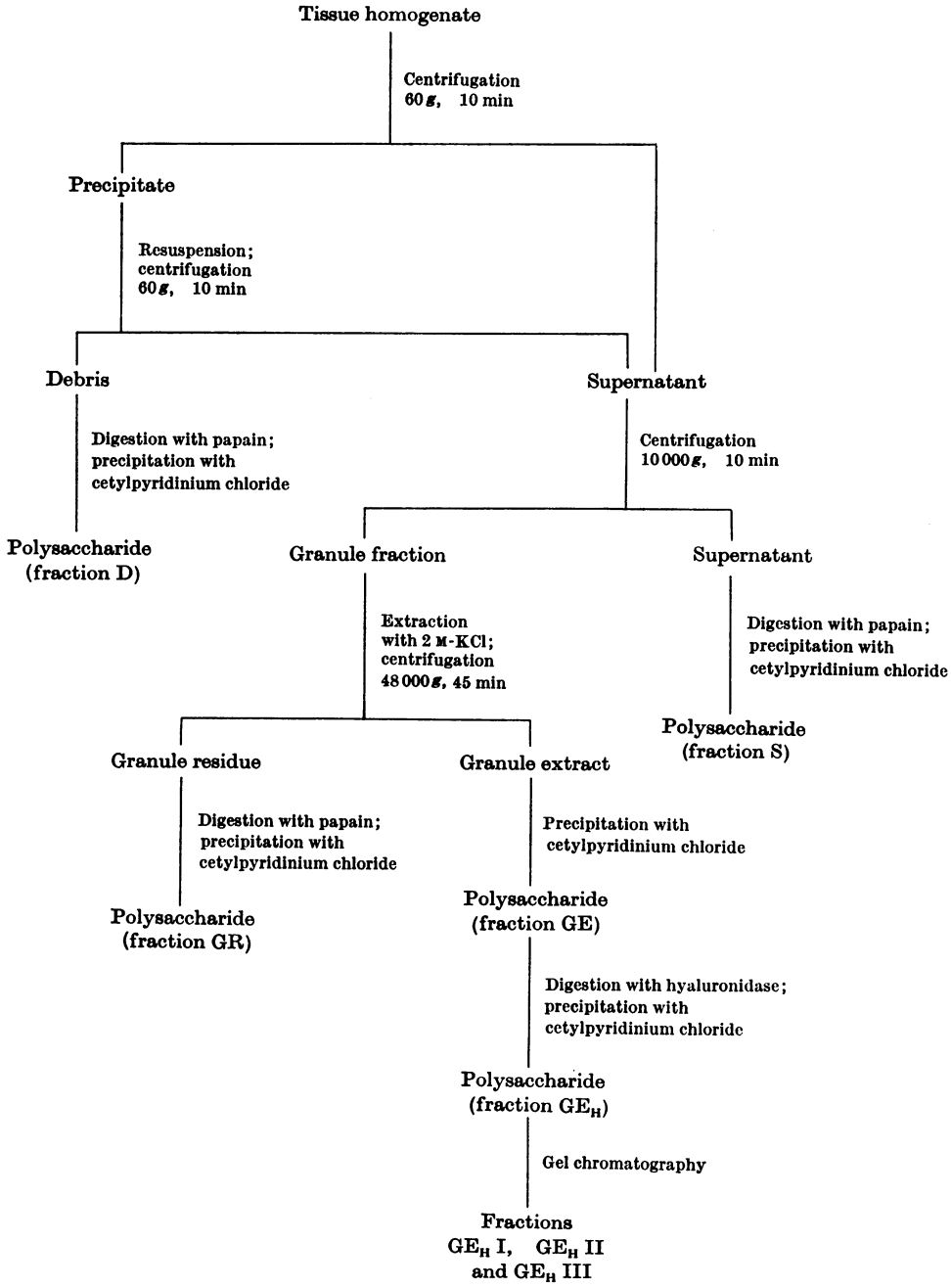
Most of the glycosaminoglycuronans of mammalian tissues, including the chondroitin 4- and 6-sulphates, dermatan sulphate (for references, see Lindahl, 1970*b*) and heparan sulphate (Jansson & Lindahl, 1970), occur as multichain proteoglycans consisting of several polysaccharide chains in covalent linkage to a single polypeptide. Little is known of the mechanisms responsible for the breakdown *in vivo* of these macromolecules, although various proteases and glycosidases have been implicated in the degradation of chondroitin sulphate proteoglycans (Aronson & Davidson, 1968; Platt & Stein, 1969; Morrison, 1970; Dingle, Barrett & Weston, 1971). The physiological breakdown mechanisms for dermatan sulphate, heparan sulphate and heparin are unknown.

Structural studies on commercially available heparin preparations suggested that heparin occurs in the native state as a proteoglycan. The heparin–protein linkage region was found to be identical with that of the chondroitin 4-sulphate proteoglycan (Rodén & Smith, 1966), involving the trisaccharide galactosylgalactosylxylose, in glycosidic linkage with the hydroxyl group of serine (Lindahl, 1966, 1967). In view of the close analogy between heparin and chondroitin 4-sulphate, with respect to structure of polysaccharide–protein linkage region as well as mode of biosynthesis (Rodén, 1971), it seemed reasonable to expect the heparin proteoglycan to exhibit multichain properties. However, an attempt to isolate a native heparin proteoglycan from bovine liver capsule yielded single polysaccharide chains and polysaccharide–peptides only; no multichain proteoglycan was detected (Lindahl, 1970*b*). It was suggested that part of the isolated material had been degraded by an endopolysaccharidase.

Transplantable mast-cell tumours have been extensively employed in studies on the biosynthesis of heparin (see review by Rodén, 1971, for references). In the present work the macromolecular properties and degradation of mouse mastocytoma heparin have been investigated. Evidence will be presented suggesting that the newly synthesized polysaccharide is rapidly degraded *in vivo* by a heparinase behaving as an endo-enzyme. A preliminary report of the work has been published (Lindahl, 1970*a*).

MATERIALS AND METHODS

The mast-cell tumour (Furth, Hagen & Hirsch, 1957) used in this study was generously supplied by Dr N. Ringertz, Institute of Cellular Physiology, Karolinska Institutet, Stockholm, Sweden. It has been maintained in this laboratory as a solid tumour in (A/Sn × Leaden)F_1 mice by routine subcutaneous and intramuscular trans-



Scheme 1. Flow diagram for the preparation of glycosaminoglycans from mastocytoma tissue. The symbols used in denoting the various polysaccharide preparations refer to the corresponding subcellular tissue fractions.

plantations every 11–13 days. Tumour tissue was frozen immediately after dissection and stored at −20°C.

Pronase (lot no. 45550) was purchased from Calbiochem, Los Angeles, Calif., U.S.A., and chondroitinase ABC (EC 4.2.–.–) from Miles Laboratories Inc., Elkhart, Ind., U.S.A. Additional enzymes used have been described earlier (Lindahl, 1970*b*). D-[^{14}C]Glucose (uniformly labelled, 260μCi/μmol) and carrier-free sodium [^{35}S]-sulphate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

The glycosaminoglycan preparations employed have been described by Lindahl (1970*b*). A sample of chondroitin 4-sulphate isolated from bovine nasal cartilage was kindly provided by Dr Å. Wasteson of this Institute.

^{14}C-labelled chondroitin sulphate was prepared as follows. Epiphyses from the femurs and tibias of 96 14-day-old chick embryos were incubated for 6h at 37°C with 500μCi of [^{14}C]glucose in 20ml of Krebs–Ringer phosphate solution (Umbreit, Burris & Stauffer, 1964). The incubation mixture was heated at 100°C for 10min and was then digested with papain as described by Herbai & Lindahl (1970). The digest was clarified by passage through a pad of Celite and polysaccharides were precipitated from the filtrate by the addition of 80ml of water and 34ml of a 1% (w/v) solution of cetylpyridinium chloride in water. After two additional precipitations with cetylpyridinium chloride from 0.2M-KCl the polysaccharide was isolated as its sodium salt by precipitation from 2M-NaCl with 3vol. of ethanol. The resulting product (corresponding to 4.0mg of uronic acid) showed a specific radioactivity of 5400c.p.m./μg of uronic acid and migrated like authentic chondroitin sulphate on electrophoresis in either barium acetate or HCl (see below).

Isolation of heparin from mastocytoma tissue. A flow diagram of the fractionation procedure is shown in Scheme 1. All procedures preceding the first cetylpyridinium chloride-precipitation step were carried out at 0–4°C.

Frozen tumour tissue was homogenized by passage through a bacteria press (Edebo, 1960); this procedure was repeated three times. Homogenized tumour (85g) was suspended in 400ml of ice-cold water and gently agitated until the suspension appeared homogeneous. After centrifugation of the homogenate at 60*g* for 10min, the resulting precipitate was resuspended in 200ml of ice-cold water and centrifugation was repeated. Granules were collected from the pooled supernatants by centrifugation at 10000*g* for 10min (Korn, 1959).

The granule fraction was suspended in 350ml of ice-cold 2M-KCl and extracted at 0°C for 75min. After centrifugation at 48000*g* for 45min the precipitate was again extracted with 2M-KCl (200ml), and the two extracts were passed separately through pads of Celite, equilibrated with 2M-KCl. Each filter pad was rinsed with 40ml of 2M-KCl, and the filtrates were analysed for carbazole-reacting material. The second extract contained practically no uronic acid and was therefore discarded.

Glycosaminoglycans were isolated from the granule extract by repeated precipitation with cetylpyridinium chloride from 0.8M-KCl. The procedure has been outlined in detail in a previous paper (Lindahl, 1970*b*). The resulting preparation (fraction GE; see Scheme 1) represented 43% (based on uronic acid) of the total glycosaminoglycan content of the tumour tissue (Table 1). Additional polysaccharide was recovered from the final 60*g* precipitate (fraction D), the 10000*g*-supernatant liquid (fraction S) and the KCl-extracted granules (fraction GR), accounting for another 39% of the total mastocytomal glycosaminoglycan (see Table 1 and Scheme 1). For fraction GE the glucosamine/total hexosamine molar ratio was 0.67.

Table 1. *Yields of glycosaminoglycan fractions*

The fractions refer to Scheme 1. Fractions S, GR and D were isolated after digestion of the corresponding tissue fractions (see Scheme 1) with papain (Lindahl, 1970*b*). The total glycosaminoglycan content of the mastocytoma tissue, as determined by a similar procedure, corresponded to 2.8mg of uronic acid/10g wet weight.

Fraction	Yield (mg of uronic acid/10g of tissue wet wt.)
S	0.2
GE	1.2
GE_H	0.9
GR	0.5
D	0.4

Portions of fraction GE were digested with highly purified testicular hyaluronidase (Lindahl, 1970*b*), the amount of enzyme used being 1mg/mg of uronic acid. The resistant glycosaminoglycans were recovered by precipitation with cetylpyridinium chloride from 0.8M-KCl and converted into the sodium salt. The hyaluronidase-digestion step was repeated once, yielding fraction GE_H, which accounted for 75% of fraction GE (Table 1). Electrophoresis in barium acetate of fraction GE_H showed heparin as the major component, along with a trace of chondroitin sulphate. Further analysis indicated (expressed as percentage of dry weight): hexosamine (not corrected for losses during hydrolysis), 24; uronic acid, 40; glucosamine/total hexosamine molar ratio, 0.91; sulphate/hexosamine molar ratio, 2.0.

Analytical methods. The methods used for the determination of amino acids and neutral sugars have been reported by Lindahl (1970*b*). Analyses of total hexosamine were performed by a modification (Gardell, 1953) of the Elson–Morgan reaction, after hydrolysis of saccharides in 4M-HCl at 100°C for 14h. Glucosamine/galactosamine molar ratios were obtained in the course of amino acid analyses. Uronic acid was determined as described by Bitter & Muir (1962) and sulphate by the method of Antonopoulos (1962). Anticoagulant activity was determined by the *British Pharmacopeia* (1968) assay method.

Radioactivity was determined with a Beckman model LS-250 liquid-scintillation counter; the scintillation liquid contained 5g of 2,5-diphenyloxazole and 100g of naphthalene/l of dioxan. Electrophoretograms were analysed for radioactivity with a Packard model 7201 strip scanner.

The identification of neutral sugars in polysaccharides by paper chromatography has been described by Lindahl (1970*b*).

Electrophoresis of glycosaminoglycans was carried out on strips of cellulose acetate in 0.1M-barium acetate (2.7V/cm for 6h) (Wessler, 1968) or in 0.1M-HCl (1.9V/cm for 2h) (Wessler, 1971). The separation obtained by electrophoresis in barium acetate depends primarily on the structure of the polysaccharide backbone, whereas the

sulphate content is of minor importance. In dilute HCl the migration rate of a polysaccharide essentially reflects the degree of sulphation. Preliminary treatment of polysaccharide fractions with alkali (Lindahl, 1970*b*) did not affect the electrophoresis patterns.

High-voltage electrophoresis was performed with Whatman 3MM paper, in 0.08M-pyridine–0.046M-acetic acid, pH 5.3, at 70V/cm for 20min. Papers were stained by a silver-dip procedure (Smith, 1960).

Gel chromatography of glycosaminoglycans was carried out on columns (see legends to figures for dimensions) of Sepharose 4B or Sephadex G-200. Samples were eluted with the eluents indicated, at a rate of about 4ml/h. V_0 and V_t of the columns were determined by chromatography of Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) and tritiated water respectively.

Molecular weights of glycosaminoglycan preparations were determined by equilibrium ultracentrifugation (Yphantis, 1964), as described in detail in Table 2.

Incorporation in vivo of [^{35}S]sulphate into mastocytoma glycosaminoglycans. Tumour-bearing mice were injected intraperitoneally with 100μCi of $Na_2{}^{35}SO_4$ and were then killed after various periods of time. The times of injection were chosen so that all tumours were of similar size (approx. 1g of tissue) when harvested. Tumours from four or five mice were combined, minced, suspended in 15ml of 0.05M-tris–HCl–0.01M-$CaCl_2$ buffer, pH 7.2, and digested with 100mg of Pronase at 55°C. After 12h another 50mg of enzyme was added, and the incubation was continued, with stirring, for a total of 36h. The digestion mixture was then heated at 100°C for 2min and filtered through a pad of Celite, which was subsequently rinsed with 4ml of 2M-KCl. To the combined rinse and filtrate were added 15ml of water followed by 4ml of 1% (w/v) cetylpyridinium chloride in water. The resulting precipitate, collected by centrifugation, was dissolved in 5ml of 2M-KCl, reprecipitated from 0.8M-KCl and finally converted into the sodium salt of the polysaccharide.

Incubation of glycosaminoglycans with mastocytoma 10000g supernatant. Frozen mastocytoma tissue (2g) was thawed and homogenized at 0°C with 2ml of 0.05M-tris–HCl–0.01M-$CaCl_2$ buffer, pH 7.2, in a Potter–Elvehjem glass homogenizer. The homogenate was centrifuged at 10000*g* for 1h in the cold and the supernatant was retained.

The supernatant liquid was incubated at 37°C with ^{35}S-labelled heparin or with ^{14}C-labelled chondroitin sulphate from embryonic chick cartilage. Samples of the respective polysaccharide solutions, in each case corresponding to 20μg of uronic acid, were evaporated to dryness before addition of the supernatant fraction (2ml). After 12h incubation the mixtures were heated at 100°C for 5min and finally digested with 30mg of Pronase, as described above. The heat-inactivated digests were centrifuged and the supernatants were subjected to gel chromatography, as described in the legends to Figs. 6 and 10.

RESULTS

Macromolecular properties of fraction GE_H

Gel chromatography. Gel chromatography on Sepharose 4B showed fraction GE_H to be polydisperse, having an average molecular size considerably exceeding that of commercial heparin (Fig. 1). This observation was consistent with the average molecular weights of the subfractions, GE_H I, GE_H II and GE_H III, as determined by ultracentrifugation (Table 2). Fractions GE_H I and GE_H II apparently contained heparin exclusively, since their galactosamine contents were negligible; fraction GE_H III contained larger amounts of this amino sugar. All three fractions showed sulphate/hexosamine ratios characteristic for heparin (Table 2) and migrated like a sample of commercial heparin on electrophoresis in 0.1M-HCl. In addition, fraction GE_H III contained minor amounts of a slower-migrating component, possibly chondroitin sulphate partially degraded by hyaluronidase. The anticoagulant activity of the mastocytomal heparin varied with the molecular weight of the polysaccharide, fraction GE_H I having the highest and fraction GE_H III the lowest specific activity (Table 2; see also Laurent, 1961).

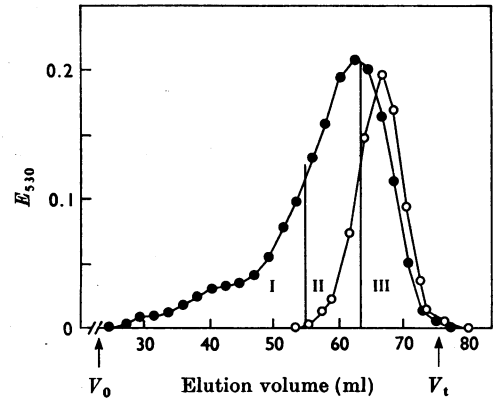


Fig. 1. Gel chromatography on Sepharose 4B of fraction GE_H (50mg; ●) and commercial heparin (○). The column (1.2cm×75cm) was eluted with 2M-KCl. Effluent fractions were analysed for uronic acid and combined as indicated by the vertical lines, yielding fractions GE_H I, GE_H II and GE_H III.

The gel-chromatography patterns of fraction GE_H and of its subfractions, GE_H I–III, were not affected by previous treatment of the polysaccharide with 0.5M-NaOH at 4°C for 20h (Anderson, Hoffman & Meyer, 1965). Similarly, digestion of the fractions with papain did not result in any significant degradation, as the slight retardation of the digested as compared with the untreated materials could be reproduced by incubating the fractions at 65°C, in the medium employed for papain digestions (Lindahl, 1970*b*), but with omission of enzyme.

Amino acid and neutral sugar analyses. Amino acid analyses of fraction GE_H are shown in Table 3.

Table 2. *Analytical results of subfractions obtained by gel chromatography of fraction GE_H*

Average molecular weights ($\bar{M}_w$) were determined by equilibrium ultracentrifugation, as described previously (Lindahl, 1970*b*). Fractions GE_H I, GE_H II and GE_H III were centrifuged for 20, 19 and 12h at 12590, 15220 and 42040rev./min, respectively. For the two low-molecular-weight fractions, GE_H II and GE_H III, straight plots were obtained of the logarithm of fringe displacement against r^2, indicating fair homogeneity of the samples. Fraction GE_H I, on the other hand, showed the curved plot typical of a polydisperse material, as expected from the gel chromatogram shown in Fig. 2. The $\bar{M}_w$ value given for this material, 69000, should be considered as a rough approximation; it was calculated from the slope of that portion of the plot corresponding to the material located closest to the meniscus.

Fraction	Uronic acid/hexosamine molar ratio	Sulphate/hexosamine molar ratio	Glucosamine/total hexosamine (%)	$\bar{M}_w$	Anticoagulant activity (B.P. units/mg)
GE_H I	1.47	2.0	>99	69000	139
GE_H II	1.66	2.0	>99	26000	109
GE_H III	1.53	2.4	80	9000	48

Table 3. *Amino acid analysis of fraction GE_H: (A) untreated; (B) digested with papain (Lindahl, 1970b)*

Before analysis, sample B was precipitated with cetylpyridinium chloride from 0.5M-KCl and recovered as sodium salt. The analytical values are expressed as residues/100 residues of hexosamine, determined by the Elson–Morgan reaction. Owing to irregularities of the base-line, lysine and histidine could not be accurately determined by the single-column system used; however, in both samples the basic amino acids generally amounted to less than 0.4 residue/100 residues of hexosamine.

	Residues/100 residues of hexosamine	
Amino acid	A	B
Aspartic acid	0.50	0.27
Threonine	0.32	0.13
Serine	2.34	1.68
Glutamic acid	0.47	0.21
Proline	Trace	Trace
Glycine	1.42	0.86
Alanine	0.39	0.23
Half-cystine	—	—
Valine	0.58	0.36
Methionine	Trace	Trace
Isoleucine	0.16	0.11
Leucine	0.29	Trace
Tyrosine	0.09	Trace
Phenylalanine	0.09	Trace

Serine was the most abundant amino acid (column A), followed by glycine, whereas other amino acids were present in smaller amounts. Similar amino acid patterns were observed previously with heparin preparations isolated from various tissues by mild procedures (Lindahl, Cifonelli, Lindahl & Rodén, 1965; Lindahl, 1970*b*). Digestion of fraction GE_H with papain, followed by re-isolation of polysaccharide by precipitation with cetylpyridinium chloride, resulted in a moderate decrease of the amounts of polysaccharide-bound amino acids (column B). The serine content was decreased by 27% only, whereas the other amino acids were more extensively separated from the polysaccharide. The relatively high yield of cetylpyridinium chloride-precipitable amino acids after the papain treatment clearly suggested that the peptide components of fraction GE_H were covalently bound to the polysaccharide (see also Lindahl, 1969, 1970*b*).

Paper chromatography of a hydrolysate of fraction GE_H indicated the presence of galactose and xylose.

Amino acid and neutral sugar analyses of fractions GE_H I, GE_H II and GE_H III, obtained from fraction GE_H by gel chromatography, are shown in Fig. 2. As the molecular weights had been estimated (Table 2) the analytical results could be expressed as number of residues per polysaccharide chain. Calculations were based on the assumption that the polysaccharide species of fractions GE_H I, GE_H II and GE_H III contained 120, 46 and 16, respectively, repeating disaccharide units (molecular weight, 563, estimated for the sodium salt of a disaccharide with 2.0 residues of sulphate) per chain. [As stated in Table 2 the molecular weight value assigned to fraction GE_H I does not provide an accurate basis for the calculation of the average number of repeating disaccharide units per chain. An estimation of the number of serine or xylose residues per polysaccharide molecule should thus be evaluated with caution (see also the Discussion section).] Serine and glycine were the predominant amino acids in each of the subfractions GE_H I–III, as in the parent fraction, GE_H. However, the three subfractions differed drastically with respect to amino acid content, fraction GE_H I apparently containing more serine residues than polysaccharide molecules, whereas fractions GE_H II and GE_H III contained one serine residue for every 2 and 4 polysaccharide chains respectively (column A). Treatment of fraction GE_H I with alkali destroyed 38% of the serine residues (column B), whereas the glycine content was unchanged. On re-isolation of

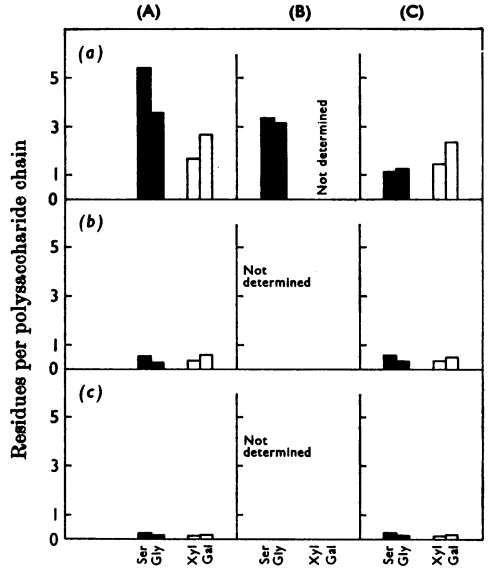


Fig. 2. Amino acid and neutral sugar analyses of fractions GE_H 1(*a*), GE_H II (*b*) and GE_H III (*c*). Amino acid (for the sake of clarity only serine and glycine are included) and neutral sugar contents are expressed as residues per polysaccharide chain; for details, see the text. (A) Untreated material; (B) alkali-treated (0.5 M-NaOH, 4°C for 20h) material (entire reaction mixture subjected to amino acid analysis); (C) alkali-treated material (polysaccharide re-isolated before analysis by precipitation with cetylpyridinium chloride from 0.5 M-KCl). The amounts, in each sample, of repeating disaccharide units were estimated from the hexosamine contents.

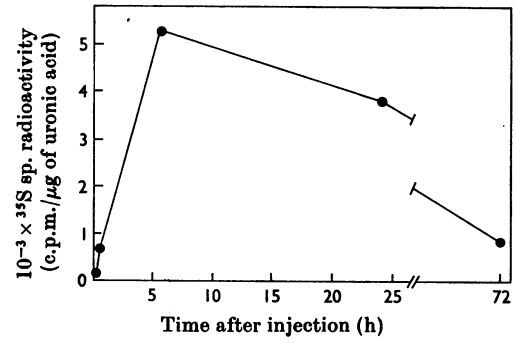


Fig. 3. Incorporation *in vivo* of [^{35}S]sulphate into mastocytomal glycosaminoglycans.

the alkali-treated fraction GE_H I, only 24% and 36% of the original amounts of serine and glycine, respectively, remained covalently bound to the polysaccharide (column C). In contrast, the peptide moieties of fractions GE_H II and GE_H III were not affected by alkali (column C), suggesting that these peptides might differ with respect to structure from those of fraction GE_H I.

The neutral sugar contents of the subfractions GE_H I–III were found essentially to parallel those of the amino acids. Thus, fraction GE_H I appeared to contain 1.7 xylose residues per polysaccharide molecule, whereas the corresponding values for fractions GE_H II and GE_H III were only 0.4 and 0.1, respectively (Fig. 2, column A). Neither the xylose nor the galactose contents of fractions GE_H I–III were significantly affected by subjecting the materials to alkaline conditions (column C).

Gel chromatography of mastocytoma glycosaminoglycans labelled in vivo *with* [^{35}S]*sulphate*

Fig. 3 shows the specific radioactivities of glycosaminoglycan preparations isolated from mastocytoma tissue at various times after injecting mice with [^{35}S]sulphate. The maximal specific radioactivity was attained approx. 5h after administration of the label.

Gel chromatograms of the labelled polysaccharide preparations are shown in Fig. 4. Each of the isolated samples yielded a uronic acid distribution similar to that of fraction GE_H (Fig. 1). In contrast, the various preparations differed considerably with respect to the distribution of [^{35}S]sulphate. In preparations from animals killed within the first few hours after receiving the radioactive sulphate the largest components showed the highest specific radioactivity (Figs. 4*a* and 4*b*). With prolonged periods of labelling *in vivo* a shift of the radioactivity toward the more low-molecular-weight portion of the chromatograms was observed (Figs. 4*c* and 4*d*). Gel chromatography of material isolated 72h after administration of the label showed the bulk of radioactive material to be retarded in relation to that of the total polysaccharide (Fig. 4*d*).

The labelled glycosaminoglycans were identified by electrophoresis in barium acetate. In all preparations the major portion of radioactive material behaved like heparin, and a minor portion migrated like chondroitin sulphate. The presence of chondroitin sulphate was further demonstrated by digestion with chondroitinase ABC. By this procedure chondroitin 4- and 6-sulphates as well as dermatan sulphate are degraded to unsaturated disaccharides, whereas heparin and heparan sulphate remain intact (Yamagata, Saito, Habuchi & Suzuki, 1968). On gel chromatography, the digested preparations showed a distinct peak of low-molecular-weight material, which did not appear with untreated controls (Fig. 5). The amounts of chondroitin [^{35}S]sulphate, as determined from the size of this peak, corresponded to less than 20% of the total radioactivity in each of two preparations,

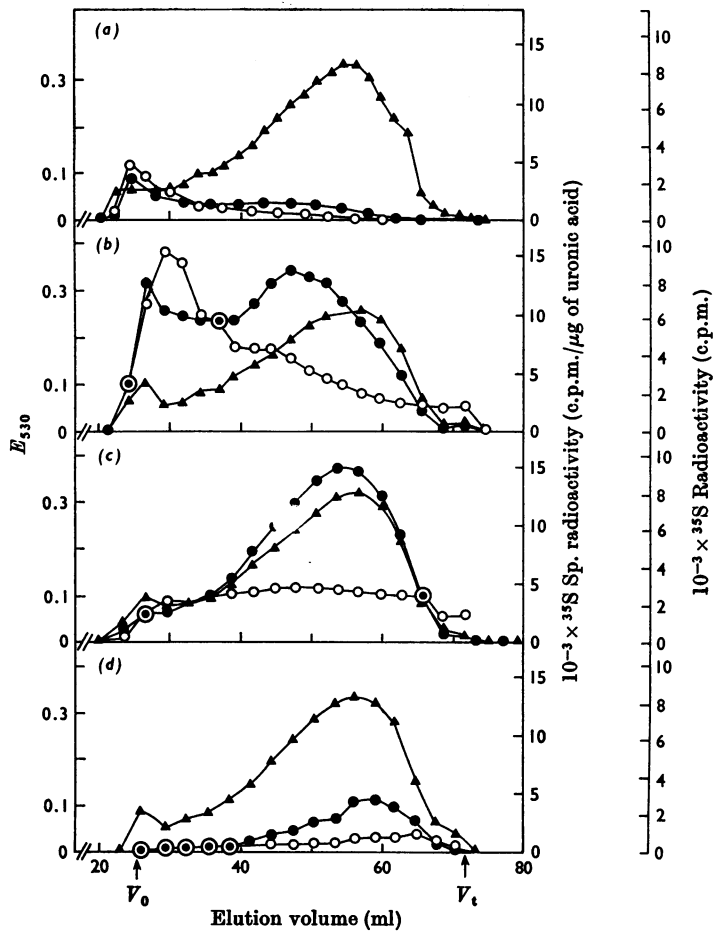


Fig. 4. Gel chromatography on Sepharose 4B of glycosaminoglycans labelled *in vivo*, isolated from mastocytoma tissue (*a*) 30min, (*b*) 5.5h, (*c*) 24h and (*d*) 72h after the injection of [^{35}S]sulphate. ▲, Uronic acid; ●, radioactivity; ○, specific radioactivity. The column (1.2cm × 70cm) was eluted with 0.2M-KCl.

obtained 5.5 and 24h respectively after administration of the label.

Incubation of [^{35}S]*heparin and* [^{14}C]*chondroitin sulphate with mastocytoma* 10000*g supernatant*

The [^{35}S]heparin employed as substrate in the degradation *in vitro* experiments was obtained by gel chromatography, after digestion with chondroitinase ABC, of a preparation of mastocytoma polysaccharide labelled *in vivo* (for further details see the legend to Fig. 5). Treatment of this material with a mastocytoma 10000*g* supernatant followed by gel chromatography resulted in the pattern shown in Fig. 6. A broad peak, substantially retarded in relation to that of the untreated polysaccharide was observed. The retarded fractions were recovered (see Fig. 6) and subjected to electrophoresis in barium acetate and in 0.1M-hydrochloric acid respectively. The resulting electrophoretograms (Figs. 7 and 8) clearly indicated that the depolymerized polysaccharide retained the migration properties of heparin. Further identification was obtained by deamination with nitrous acid (Lagunoff & Warren, 1962). Paper electrophoresis of the products formed in this procedure showed components migrating somewhat faster than free glucuronic acid monosaccharide (Fig. 9), similar to the sulphated disaccharides obtained on treatment of heparin with nitrous acid (Lindahl & Axelsson, 1971). In addition, the deamination mixture contained inorganic [^{35}S]sulphate (Fig. 9), presumably derived from [^{35}S]sulphamino groups of the intact polysaccharide.

As described in the Materials and Methods section, samples of [^{35}S]heparin were digested with Pronase subsequent to incubation with the mastocytoma 10000*g* supernatant. The proteolytic treatment alone resulted in a slight degradation of the polysaccharide, as demonstrated by gel chromatography (cf. Horner, 1971); however, the extent of depolymerization was insignificant in relation to that obtained with the mastocytoma supernatant fraction. A mastocytoma 10000*g* supernatant, which had been heated at 100°C for 10min before incubation with [^{35}S]heparin, failed to depolymerize the polysaccharide, as evident from subsequent gel chromatography.

Unlike the tumour heparin (Fig. 6), a sample of chondroitin sulphate from embryonic chick cartilage apparently retained its average chain length during incubation with the mastocytoma 10000*g* supernatant. The gel chromatograms obtained before and after treatment of the polysaccharide

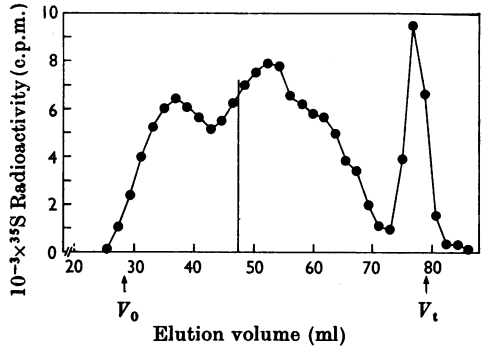


Fig. 5. Gel chromatography on Sepharose 4B of a ^{35}S-labelled preparation of mastocytomal polysaccharide (sample *b* in Fig. 4) after digestion with chondroitinase ABC. Material corresponding to approx. 0.4mg of uronic acid was incubated with 1.0 unit of enzyme, under the conditions described by Yamagata *et al.* (1968). After 15h of incubation the digest (1.7ml) was heated at 100°C for 2min and centrifuged. The supernatant liquid was subjected to gel chromatography, essentially as described in the legend to Fig. 4. The high-molecular-weight fractions (to the left of the vertical line) were combined, dialysed and used as substrate in the experiment on degradation *in vitro* shown in Fig. 6.

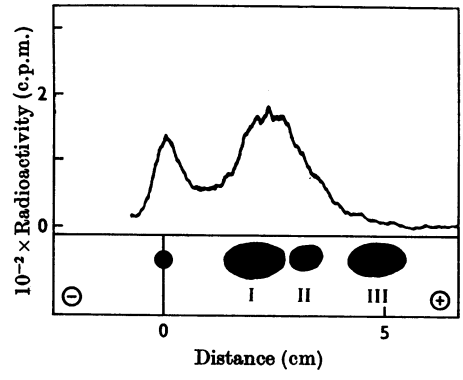


Fig. 7. Electrophoresis in barium acetate of depolymerized [^{35}S]heparin (see Fig. 6 for details). The radioscan pattern may be correlated with that of the authentic standards below the tracing: I, heparin; II, hyaluronic acid; III, chondroitin 4-sulphate. The radioactivity remaining at the origin probably represents heparin precipitated at the site of application (cf. Wessler, 1968).

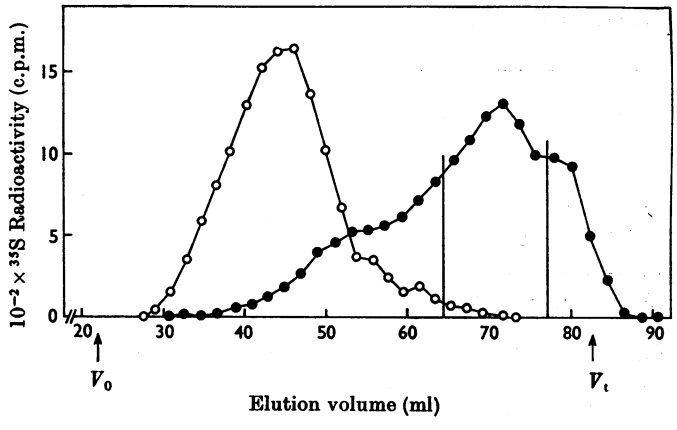


Fig. 6. Gel chromatography on Sepharose 4B of ^{35}S-labelled heparin (see the legend to Fig. 5 for details) after incubation *in vitro* with mastocytoma 10000*g* supernatant (●); undigested control (○). Samples were eluted from a gel column (1.2cm×80cm) with 0.2M-NaCl in 0.05M-tris–HCl buffer, pH 8.0. The effluent fractions falling between the vertical lines were pooled, dialysed and retained for further characterization (see Figs. 7–9).

with the tumour fraction were almost identical (Fig. 10).

DISCUSSION

The present investigation has demonstrated that extraction of glycosaminoglycans from mouse mastocytoma tissue, under conditions not promoting autolytic processes, yields a highly polydisperse heparin (fraction GE_H) with an average molecular weight considerably exceeding that of the commercially available material (Fig. 1). The high molecular weight of the tumour heparin does not seem to be related to the neoplastic character of the tissue, since heparin isolated from the skins of normal mice was shown to exhibit similar macromolecular properties (S. Ögren, U. Lindahl & Å. Wasteson, unpublished work). A heparin of unusually high molecular weight has also been isolated from rat skin (Horner, 1971).

Under alkaline conditions multi-chain proteoglycans containing *O*-seryl glycosidic linkages are degraded, by a β-elimination mechanism, to single polysaccharide chains (Anderson *et al.* 1965; Neuberger, Gottschalk & Marshall, 1966). A similar decrease in size occurs on splitting the polypeptide backbone of the proteoglycan with papain, thus yielding single polysaccharide–peptides. The resistance of fraction GE_H, and of its subfractions, toward both alkaline and proteolytic degradation thus indicated that this material did not contain multi-chain proteoglycans. Hence, the macromolecular properties of the highly polydisperse fraction GE_H I remain unclear. Recently, the larger components of this fraction were shown to contain seven or more xylose residues per molecule (S. Ögren, U. Lindahl & Å. Wasteson, unpublished work), suggesting the presence of material similar to the macromolecular heparin described by Horner (1971).

The lower-molecular-weight fractions, GE_H II and GE_H III, apparently consisted largely of single polysaccharide chains, free of peptide, as only 40% and 10%, respectively, of the molecules contained xylose; the amino acid contents were similarly low (Fig. 2). If it is assumed that all heparin molecules were initially synthesized as proteoglycans or peptidoglycans, fractions GE_H II and GE_H III must have been formed by cleavage at internal positions

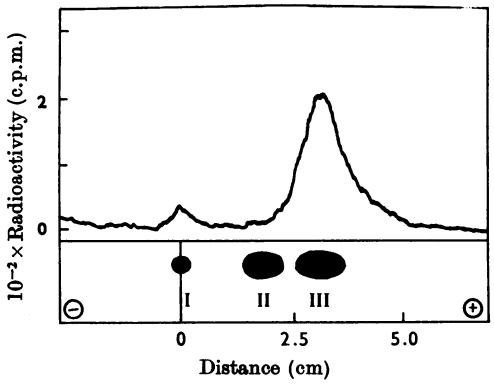


Fig. 8. Electrophoresis in HCl of depolymerized [^{35}S]-heparin (see Fig. 6 for details). The standards shown below the tracing are: I, hyaluronic acid; II, chondroitin 4-sulphate; III, heparin.

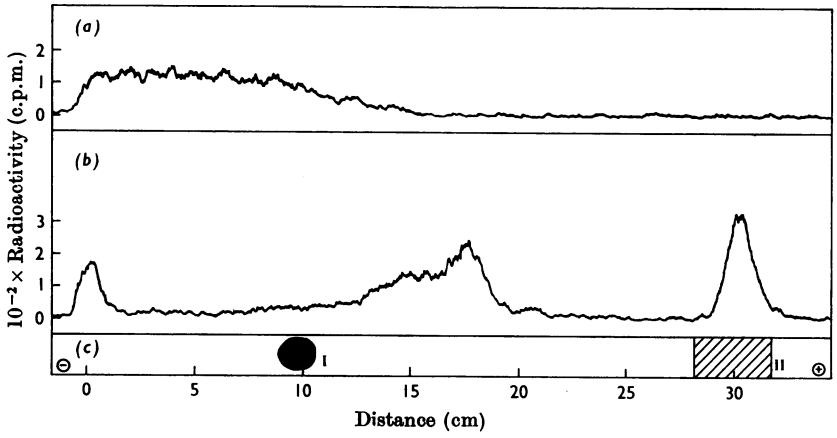


Fig. 9. High-voltage electrophoresis of depolymerized [^{35}S]heparin (see Fig. 6), (*a*) before; (*b*) after treatment with nitrous acid. Deamination was carried out as described previously (Lindahl & Axelsson, 1971). Standards: I, glucuronic acid; II, [^{35}S]sulphate.

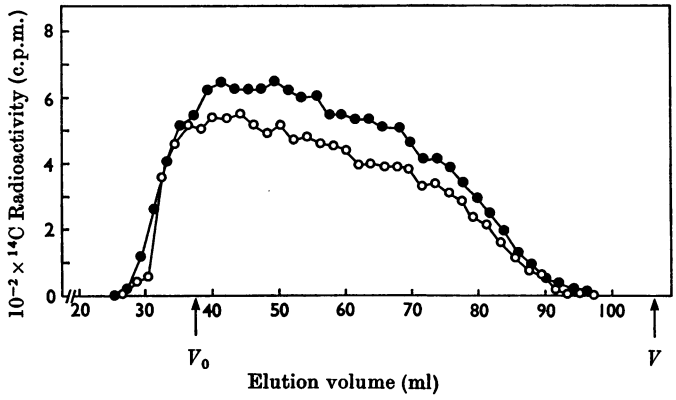


Fig. 10. Gel chromatography on Sephadex G-200 of ^{14}C-labelled chondroitin sulphate, after incubation *in vitro* with mastocytoma 10000 *g*-supernatant (●); undigested control (○). The gel column (1.4 cm × 80 cm) was eluted with 0.2 M-NaCl in 0.05 M-tris–HCl buffer, pH 8.0.

of the polysaccharide moieties of such compounds. Accordingly, fraction GE_H I would contain newly synthesized heparin molecules whereas fractions GE_H II and GE_H III would constitute degradation products. Evidence in support of this contention was obtained by pulse labelling *in vivo* of mastocytoma glycosaminoglycans with [^{35}S]sulphate. After an initial period of preferential incorporation into large molecules (cf. Olsson, Berg, Fransson & Nordén, 1970), a gradual shift of the radioactivity toward components of lower molecular weight was observed (Fig. 4). These findings are clearly compatible with the formation and subsequent degradation of a high-molecular-weight sulphated glycosaminoglycan. Moreover, characterization of the labelled polysaccharide preparations indicated heparin as the predominant component.

Further proof for the presence in the tumour tissue of a heparin-degrading factor was obtained by incubation *in vitro* of ^{35}S-labelled mastocytoma heparin with a 10000*g*-supernatant fraction. This treatment resulted in appreciable degradation of the polysaccharide (Fig. 6), yielding products similar in size to the major portion of the unfractionated mastocytoma heparin (fraction GE_H; see Fig. 1). The depolymerizing activity was abolished by heating the supernatant fraction at 100°C for 10 min. It thus seems reasonable to conclude that mastocytoma tissue contains a heparinase with endo-enzyme properties. The linkage(s) cleaved by this enzyme has so far not been identified. It is notable, however, that the enzyme appeared unable to degrade chondroitin sulphate (Fig. 10) in view of the finding that both chondroitin sulphate and heparin are manufactured by the neoplastic mast cell (T. Helting, S. Ögren, U. Lindahl, H. Pertoft & T. Laurent, unpublished work).

Whereas a heparin-depolymerizing enzyme of bacterial origin has been purified and characterized in some detail (Linker & Hovingh, 1965; Dietrich, 1969; Hovingh & Linker, 1970) such an enzyme has not been demonstrated previously in mammalian tissues. However, the existence of a mammalian heparinase was implied by the observation of Green (1963), that the heparin released from neoplastic mast cells in tissue culture was largely dialysable. It is unclear whether the heparin-depolymerizing activity, demonstrated in the present study, occurs also in the normal mast cell. However, this possibility would seem to be favoured by the observation that heparin preparations isolated from various tissues by mild procedures do not contain amino acids in amounts sufficient to account for a peptide substituent on every polysaccharide chain (Lindahl *et al.* 1965; Cifonelli & Rodén, 1968; Lindahl, 1970*b*).

Finally, a point of physiological note may be considered. The results of the present investigation suggest that the molecular weight of the newly synthesized heparin considerably exceeds that of the polysaccharide stored in the mast-cell granules. The heparin released from the mast cell *in vivo* is believed to originate from these granules, thus representing partially degraded rather than intact polysaccharide chains. Although no specific function can at present be ascribed to a heparin-depolymerizing enzyme, the degradation process may be essential to the release of heparin from the mast cell (see also Horner, 1971).

This work was supported by grants from the Swedish Medical Research Council (B71-13X-2309-04C), the Swedish Cancer Society (53-B70-04XB), Gustaf V:s 80-årsfond and the Medical Faculty, University of Uppsala. The technical assistance of Miss G. Bäckström,

Miss I. Pettersson, Mrs L. Nirk and Mr E. Lindberg is gratefully acknowledged. We are indebted to Mr B. Ajaxon, AB Vitrum, Stockholm, for carrying out the anticoagulant activity assays. The ultracentrifugation analyses were kindly performed by Dr H. Pertoft.

REFERENCES

Anderson, B., Hoffman, P. & Meyer, K. (1965). *J. biol. Chem.* **240**, 156.
Antonopoulos, C. A. (1962). *Acta chem. scand.* **16**, 1521.
Aronson, N. N. & Davidson, E. A. (1968). *J. biol. Chem.* **243**, 4494.
Bitter, T. & Muir, H. M. (1962). *Analyt. Biochem.* **4**, 330.
British Pharmacopeia (1968) p. 1345. London: Pharmaceutical Press.
Cifonelli, J. A. & Rodén, L. (1968). *Biochim. biophys. Acta*, **165**, 553.
Dietrich, C. P. (1969). *Biochemistry, Easton*, **8**, 2089.
Dingle, J. T., Barrett, A. J. & Weston, P. D. (1971). *Biochem. J.* **123**, 1.
Edebo, L. (1960). *J. biochem. microbiol. Technol. Engng*, **2**, 453.
Furth, J., Hagen, P. & Hirsch, E. I. (1957). *Proc. Soc. exp. Biol. Med.* **95**, 824.
Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
Green, J. P. (1963). *Biochem. Pharmac.* **12**, 588.
Herbai, G. & Lindahl, U. (1970). *Acta physiol. scand.* **80**, 502.
Horner, A. (1971). *J. biol. Chem.* **246**, 231.
Hovingh, P. & Linker, A. (1970). *J. biol. Chem.* **245**, 6170.
Jansson, L. & Lindahl, U. (1970). *Biochem. J.* **117**, 699.
Korn, E. D. (1959). *J. biol. Chem.* **234**, 1325.
Lagunoff, D. & Warren, G. (1962). *Archs Biochem. Biophys.* **99**, 396.
Laurent, T. C. (1961). *Archs Biochem. Biophys.* **92**, 224.
Lindahl, U. (1966). *Biochim. biophys. Acta*, **130**, 368.
Lindahl, U. (1967). *Ark. Kemi*, **26**, 101.
Lindahl, U. (1969). *Biochem. J.* **113**, 569.
Lindahl, U. (1970*a*). In *Chemistry and Molecular Biology of the Intercellular Matrix*, vol. 2, p. 943. Ed. by Balazs, E. A. London: Academic Press (Inc.) Ltd.
Lindahl, U. (1970*b*). *Biochem. J.* **116**, 27.
Lindahl, U. & Axelsson, O. (1971). *J. biol. Chem.* **246**, 74.
Lindahl, U., Cifonelli, J. A., Lindahl, B. & Rodén, L. (1965). *J. biol. Chem.* **240**, 2817.
Linker, A. & Hovingh, P. (1965). *J. biol. Chem.* **240**, 3724.
Morrison, R. I. G. (1970). In *Chemistry and Molecular Biology of the Intercellular Matrix*, vol. 3, p. 1683. Ed. by Balazs, E. A. London: Academic Press (Inc.) Ltd.
Neuberger, A., Gottschalk, A. & Marshall, R. D. (1966). In *Glycoproteins*, p. 273. Ed. by Gottschalk, A. Amsterdam: Elsevier Publishing Co.
Olsson, I., Berg, B., Fransson, L. Å. & Nordén, Å. (1970). *Scand. J. Haematol.* **7**, 440.
Platt, D. & Stein, U. (1969). *Z. klin. Chem. u. klin. Biochem.* **7**, 374.
Rodén, L. (1971). In *Metabolic Conjugation and Metabolic Hydrolysis*, vol. 2, p. 345. Ed. by Fishman, W. H. New York: Academic Press Inc.
Rodén, L. & Smith, R. (1966). *J. biol. Chem.* **241**, 5949.
Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 252. New York: Interscience Publishers Inc.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964). *Manometric Techniques*, 4th ed., p. 131. Minneapolis: Burgess Publishing Co.
Wessler, E. (1968). *Analyt. Biochem.* **26**, 439.
Wessler, E. (1971). *Analyt. Biochem.* **41**, 67.
Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968). *J. biol. Chem.* **243**, 1523.
Yphantis, D. A. (1964). *Biochemistry, Easton*, **3**, 297.