Biochem. J. (1973) **135**, 631–637
Printed in Great Britain

The Molecular-Weight Distribution of Glycosaminoglycans

By JOHN J. HOPWOOD* and H. CLEM ROBINSON

Department of Biochemistry, Monash University, Clayton, Vic. 3168, *Australia*

(*Received* 1 *May* 1973)

1. A rapid and sensitive method for the accurate estimation of the molecular-weight distribution of keratan sulphate and chondroitin sulphate isolated from adult bovine nasal septum and intervertebral disc is described. The method utilizes gel chromatography of reductively labelled glycosaminoglycan and end-group estimation of number-average molecular weight for each fraction across the peak of eluted glycosaminoglycan. 2. Chain-length distribution data obtained by this procedure are used to evaluate mechanisms of chondroitin sulphate biosynthesis.

The chain length and size distribution of glycosaminoglycans are of considerable interest to the biochemist, since these may provide insight into mechanisms involved in polysaccharide biosynthesis and degradation. As most glycosaminoglycans occur *in vivo* covalently bound to protein (Rodén, 1970) it is first necessary to isolate glycosaminoglycans as single chains to determine their molecular weight and size distribution. This is commonly accomplished by proteolytic or alkaline digestion of tissue or extracted proteoglycan. However, during these isolation procedures, the glycosaminoglycan chains may be degraded, and the average molecular weight and size distribution of the final product may, therefore, be lower and have a wider distribution of molecular sizes than those occurring *in vivo* (Robert *et al.*, 1962; Whistler & Rowell, 1966; Blumberg & Ogston, 1957, 1958). On the other hand the preparative procedure may not be effective in degrading the protein core of the proteoglycan complex and the resulting glycosaminoglycan product will then have a higher average molecular weight and a broader size distribution than will free glycosaminoglycan chains (Luscombe & Phelps, 1967; Mathews, 1971). Robinson & Hopwood (1973) and Hopwood (1972) have shown that cleavage in 0.5M-KOH at 4°C yields single chondroitin sulphate and keratan sulphate chains that closely represent their average size and composition *in vivo*.

Gel filtration is of considerable value for the determination of the average molecular weight and size distribution of polysaccharides (Wasteson, 1969; Constantopoulos *et al.*, 1969). This procedure relies on an established relationship between (*a*) the size and conformation of a macromolecule, and (*b*) its elution volume during gel-filtration chromatography (Determann, 1968; Laurent & Granath, 1967). Minor variations in experimental conditions such as ionic strength and the flow rate of the eluting buffer, the operating column temperature and the amount of material applied can, however, influence the elution behaviour of macromolecules (Wasteson, 1971). In addition, the established relationship may only operate within a limited range of molecular size or for polysaccharide identical in composition with that used to establish the relationship between elution volume and solute molecular size. Thus, two major concerns when using the technique of gel filtration to determine the molecular size of macromolecules are (*a*) the possible inconsistencies of gel filtration, and (*b*) the need to prepare and accurately characterize suitable reference substances for the calibration of the gel columns.

The present paper describes a rapid and convenient method by which the molecular-weight distribution of glycosaminoglycan chains may be accurately determined without: (*a*) calibration of the gel column or (*b*) its operation under strictly controlled conditions. Chondroitin sulphate or keratan sulphate chains isolated from cartilage by treatment with dilute alkali and end-labelled with NaB^3H_4 were separated on Sephadex G-200 into a series of fractions with different average molecular weights. The molecular weight of the end-labelled glycosaminoglycan in each fraction was then readily determined by the technique of end-group estimation (Robinson & Hopwood, 1973; Hopwood, 1972). A preliminary communication of these results has been published (Hopwood & Robinson, 1971).

* Present address: Department of Pediatrics, University of Chicago, Chicago, Ill. 60637, U.S.A.

Experimental

Preparation of end-labelled keratan sulphate and chondroitin sulphate

Bovine nasal-septum cartilage and intervertebral disc tissue were pooled from ten animals (2–5 years) within 1 h of slaughter and powdered in a Wiley mill. Stock NaB^3H_4 solution was prepared and diluted with 9 vol. of 0.5M-KOH immediately before use to yield a solution approximately 0.02M in borohydride. The specific radioactivity of this 0.02M-borohydride solution was 6.65×10^5 d.p.m./μmol of cyclohexanol (Robinson & Hopwood, 1973).

Milled tissue (3.0g dry weight) was stirred with 50ml of 0.02M-NaB^3H_4 at 4°C for 10 days. The acidified reaction mixtures were desalted on a Bio-Gel P-4 column and fractionated on ECTEOLA-cellulose. All of the hexuronate-positive material from each tissue was recovered from ECTEOLA-cellulose as a single peak corresponding to a single peak of radioactivity. This peak also contained all of the keratan sulphate of each tissue (Robinson & Hopwood, 1973).

Separation of chondroitin sulphate and keratan sulphate

Several methods were used to separate these glycosaminoglycans. Initially the chondroitin sulphate in the nasal-septum preparation after chromatography on ECTEOLA-cellulose was purified by precipitation with cetylpyridinium chloride in 0.05M-LiBr. The precipitate was then redissolved in 2.0M-LiBr and the chondroitin sulphate isolated as the lithium salt by precipitation with 3 vol. of ethanol. The white precipitate was washed thoroughly with ethanol and with ether and dried to constant weight over P_2O_5 at 70°C *in vacuo*. Secondly, the chondroitin sulphate in the intervertebral-disc preparation, after chromatography on ECTEOLA-cellulose, was separated from ketatan sulphate by ion-exchange chromatography on a column of Dowex 1 X4 (Cl^- form; 36cm×2cm diam.). The column was eluted with a linear gradient of NaCl generated from 500ml of 0.02M-imidazole–HCl buffer at pH5.7 and 500ml of 2.0M-NaCl in the same buffer. The column was then eluted further with 500ml of 5M-NaCl. Chondroitin sulphate was eluted at a NaCl concentration of 1.0M whereas keratan sulphate was eluted with 5.0M-NaCl. The chondroitin sulphate and keratan sulphate fractions were desalted on a column of Bio-Gel P-4. Column chromatography on Dowex 1 X4 of the nasal-septum preparation failed to separate the chondroitin and keratan sulphate. Thirdly, the hexuronate-positive ECTEOLA-cellulose fractions from both nasal septum and intervertebral-disc preparations were digested with *Proteus vulgaris* chondroitin sulphate lyase and then each digest was separated into keratan sulphate–chondroitin sulphate linkage-region oligosaccharide and unsaturated chondroitin sulphate disaccharide components on a column of Sephadex G-50 (Robinson & Hopwood, 1973). The keratan sulphate fraction was then reapplied to a column of ECTEOLA-cellulose (Cl^- form; 36cm×2cm diam.) and eluted with a linear gradient of NaCl generated from 500ml of 0.02M-imidazole–HCl, pH5.7, and 500ml of 2.0M-NaCl in the same buffer. The hexose-positive and radioactive material eluted as a single broad peak and was desalted on a column of Bio-Gel P-4 and freeze-dried. Typical chemical compositions of these chondroitin sulphate and keratan sulphate preparations have been described (Robinson & Hopwood, 1973; Hopwood, 1972).

Sephadex G-200 chromatography of chondroitin sulphate and keratan sulphate

Chondroitin sulphate (10–15mg dry weight), end-labelled with ^3H as described above, was dissolved in 1.0ml of 0.02M-NaCl buffered with 0.02M-imidazole–HCl at pH6.0 and applied to a column of Sephadex G-200 (fine grade; 90cm×2.5cm diam.) equilibrated and eluted with the same buffered salt solution. The column was eluted at a flow rate of 13ml/h and 5.1ml fractions were collected. Each fraction was assayed for radioactivity and chondroitin sulphate content; the applied chondroitin sulphate sample was used as standard in the Dische (1947) hexuronate assay procedure.

Keratan sulphate (10–20mg dry weight) end-labelled with ^3H was fractionated on Sephadex G-200 as described above. Fractions were assayed for radioactivity and keratan sulphate content and the applied keratan sulphate sample was used as standard in the hexose assay procedure (anthrone method) described by Trevelyan & Harrison (1952).

Aqueous samples (0.5ml) were mixed with 10ml of scintillation mixture (Bruno & Christian, 1961) and the radioactivity was assayed in a Phillips liquid-scintillation analyser PW 4510. Molecular weights of various chondroitin sulphate and keratan sulphate preparations were determined by sedimentation equilibrium as previously described (Robinson & Hopwood, 1973).

Results and Discussion

Chain-length distribution data

Fig. 1 and Fig. 2 show the elution profiles from Sephadex G-200 of end-labelled keratan sulphate isolated from adult bovine intervertebral disc and chondroitin sulphate from adult bovine nasal septum respectively. Fig. 2 has two peaks of radioactivity; the material in the most retarded and minor peak (fractions 54–70), which contained no hexuronate-positive material and all of the applied glucosamine,

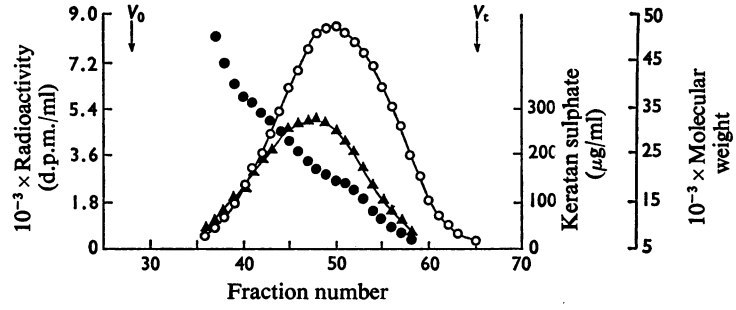

Fig. 1. *Elution from Sephadex G-200 of keratan sulphate fractionated from adult bovine intervertebral disc after digestion with chondroitin sulphate lyase as described in the Experimental section*

Fractions were 5.0ml; keratan sulphate (▲), radioactivity (○) and calculated molecular-weight values (●) are shown.

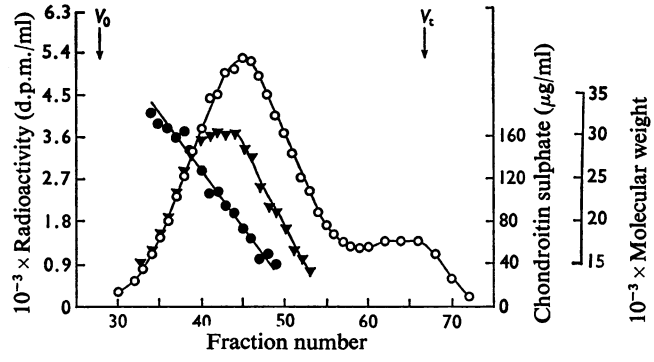

Fig. 2. *Elution from Sephadex G-200 of chondroitin sulphate fractionated from adult bovine nasal septum*

The chondroitin sulphate used was the hexuronate-positive peak from ECTEOLA-cellulose, isolated as outlined in the Experimental section. Radioactivity (○), chondroitin sulphate (▼) and calculated molecular-weight values (●) are shown.

was identified as keratan sulphate on the basis of its hexosamine and galactose content. Labelled chondroitin sulphate isolated from bovine nasal septum and purified by precipitation with excess of cetylpyridinium chloride (as outlined in the Experimental section) was free of keratan sulphate, because all of the radioactivity was eluted from Sephadex G-200 as a single peak together with the hexuronate-positive material. This result is consistent with the reported solubility of the cetylpyridinium chloride complex of keratan sulphate in cetylpyridinium chloride (Antonopoulos *et al.*, 1961; Anseth *et al.*, 1970).

In Fig. 1 and Fig. 2 it is apparent that the peaks of ^3H and hexose- or hexuronate-positive material no longer coincide exactly and that fractionation of each polysaccharide on a molecular-size basis has occurred. The number-average molecular weight (M_n) of the polysaccharide in each Sephadex G-200 fraction was determined by end-group analysis as described in the Experimental section. This method of molecular-weight estimation assumes one specific radioactivity label per polysaccharide chain (Robinson & Hopwood, 1973) and derives M_n from the ratio of polysaccharide weight to ^3H d.p.m. in each fraction. The weight of chondroitin sulphate or keratan sulphate was estimated from the hexuronate or hexose assayed in each fraction. From these molecular-weight data for chondroitin sulphate or keratan sulphate preparations we constructed the chain-size distribution profiles of the type illustrated in Fig. 3. From these data number-average (M_n), weight-average (M_w) and z-average (M_z) molecular-

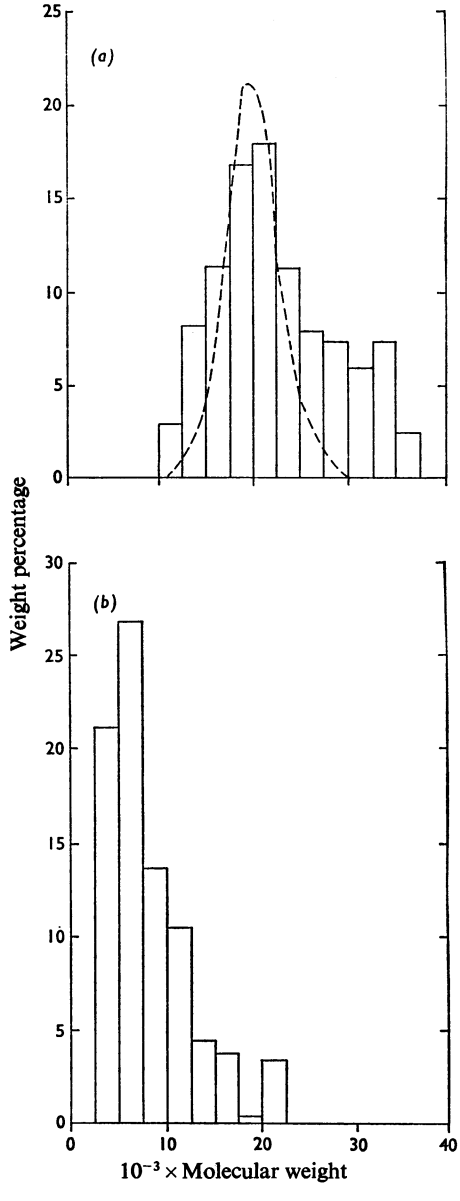

Fig. 3. *Molecular size distribution of (a) chondroitin sulphate and (b) keratan sulphate components of adult bovine nasal septum constructed from average molecular weight values of each of 20–25 fractions obtained from gel filtration of these two glycosaminoglycans on Sephadex G-200*

A value of $\gamma = 86$, the number of monomer units per polymer chain of average molecular weight 21500, was used to calculate a theoretical Poisson distribution as outlined in the Results and Discussion section; the distribution for $\gamma = 86$ (---) is shown in (*a*) to the same scale.

weight values for the whole chondroitin sulphate or keratan sulphate preparation were calculated. By this procedure values for the average molecular weights of chondroitin sulphate or keratan sulphate preparations are readily and accurately obtained, since one Sephadex G-200 fractionation conveniently yields a large number of essentially monodisperse fractions each with a different molecular size. Values of M_n, M_w and M_z for chondroitin sulphate and keratan sulphate from bovine intervertebral disc and nasal septum agree closely with those obtained by equilibrium sedimentation (Table 1). The M_n and M_w of a nasal-septum chondroitin sulphate preparation, determined four separate times, were 21000 and 23800 and the corresponding standard deviations were 0.15 and 0.31% respectively.

A convenient measure of the molecular-weight distribution of a polymer may be obtained from M_w/M_n ratios (Flory, 1953). A monodisperse preparation has a M_w/M_n ratio of 1.00. The M_w/M_n ratio obtained for chondroitin sulphate prepared from bovine nasal septum by extractions with dilute alkali is 1.09 (Table 1) whereas the ratio for chondroitin sulphate prepared from the same tissue by papain digestion is 1.22–1.28 (Wasteson, 1969, 1971). This difference may simply reflect the different techniques used to measure M_n and M_w; however, it is also possible that the higher ratio observed for chondroitin sulphate isolated by papain proteolysis may result from either the incomplete degradation of proteoglycan to yield single polysaccharide chains or random cleavage of glycosidic bonds within polysaccharide chains (Blumberg & Ogston, 1957, 1958; Gregory & Rodén, 1961; Hoffman *et al.*, 1967; Luscombe & Phelps, 1967; Meyer, 1970; Rodén, 1970; Hopwood, 1972).

Extraction of cartilage with dilute alkali, however, has been shown to yield single chondroitin sulphate and keratan sulphate chains by a reaction that specifically cleaves the glycosidic link between polysaccharide and protein without scission of other glycosidic bonds within the keratan sulphate or chondroitin sulphate structure (Robinson & Hopwood, 1973; Hopwood, 1972). In the present paper the recovery of keratan sulphate or chondroitin sulphate from nasal-septum cartilage or intervertebral disc was greater than 90% of the total polysaccharide released from the tissue by Pronase. Thus inaccuracies resulting from inefficient extraction, incomplete cleavage of glycosaminoglycan from protein or random degradation of glycosidic bonds are avoided, and, therefore, the molecular-weight distribution data reported in Table 1 should represent the chain-size distribution of chondroitin sulphate and keratan sulphate in the intact tissue.

It is interesting to note that the peak of nasal-septum chondroitin sulphate (maximum at fraction 45, Fig. 2) was eluted from the gel column well before the

Table 1. *Molecular-weight values for adult bovine nasal septum and intervertebral disc glycosaminoglycans*

Keratan sulphate preparations KS (1) and KS (2) were isolated from the alkaline digest of tissue by Dowex I chromatography and chondroitin sulphate lyase digestion respectively (see the Experimental section for details). Chondroitin sulphate preparation CS (1) was prepared by precipitation with cetylpyridinium chloride, preparation CS (2) represents desalted (Bio-Gel P-4) ECTEOLA-cellulose hexuronate-positive fraction and preparation CS (3) was separated from keratan sulphate by Dowex 1 chromatography. Values in parentheses give the values obtained by equilibrium sedimentation (Robinson & Hopwood, 1973). Molecular weight values were determined (A) by end-group estimation or calculated (B) from Sephadex G-200 fractions as outlined in the Results and Discussion section.

		$10^{-3} \times$ Molecular-weight values							
Tissue	Sample	M_n(A)	M_n(B)		M_w(B)		M_z(B)	$M_w(B)/M_n$(B)	
Bovine nasal septum	KS (2)	7.5	7.0		7.6		10.6	1.09	
	CS (1)	21.5	20.8	(21.7)	22.7	(22.3)	23.6	1.09	(1.03)
	CS (2)	—	21.0		22.8		23.8	1.09	
Intervertebral disc	KS (1)	20.5	21.9		24.8	(22.6)	28.5	1.13	
	KS (2)	19.1	20.3		23.1		26.7	1.14	
	CS (3)	13.5	15.0		17.0		19.0	1.13	

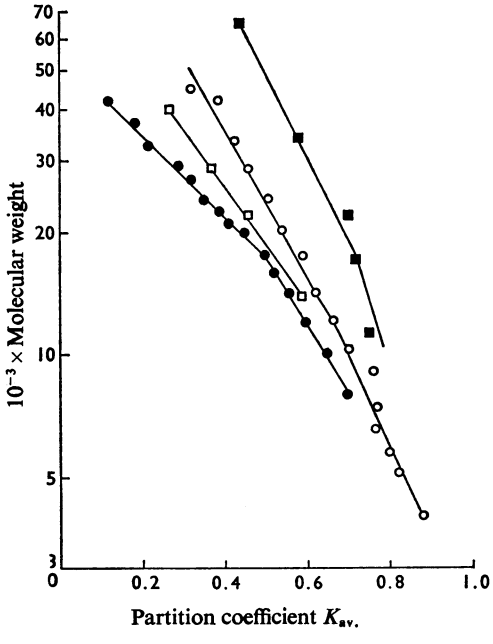

Fig. 4. *Fractionation coefficient ($K_{av.}$; Laurent & Killander, 1964) between Sephadex G-200 and buffer for a series of chondroitin sulphate and keratan sulphate fractions derived from bovine nasal septum and intervertebral disc as a function of molecular weight*

Chondroitin sulphate from both tissues (●), keratan sulphate also from both tissues (○), dextran (□) and globular protein (■) are shown. Dextran and protein values were taken from Laurent & Granath (1967) and Laurent & Killander (1964) respectively.

intervertebral-disc keratan sulphate (maximum at fraction 51, Fig. 1) although the average molecular weights of these two polysaccharides were equal (Table 1).

In Fig. 4 the partition coefficient $K_{av.}$ (Laurent & Killander, 1964) between Sephadex G-200 and buffer calculated for a number of chondroitin sulphate and keratan sulphate fractions is plotted against the calculated molecular weight of each polysaccharide sample. The plots illustrate a marked difference between keratan sulphate and chondroitin sulphate. The keratan sulphate plot, which lies between that of the linear chondroitin sulphate chains and that of globular protein structures, suggests that the keratan sulphate from nasal septum and intervertebral disc might have branched structure.

Biological significance

The bimodal shape of the chain-size distribution profile for single chondroitin sulphate chains from bovine nasal septum (Fig. 3) suggests that two separate species of chondroitin sulphate may be present in this preparation. One species represents the major weight of approximately 20000 whereas the other minor species has an average molecular weight of approximately 30000. We have recently obtained evidence indicating the presence of two distinct pools of chondroitin sulphate (one with M_n 18000 and M_w/M_n ratio of 1.03 and the other with an M_n of 28000 and M_w/M_n ratio of 1.04) in adult bovine nasal septum (Hopwood, 1972). This is consistent with the evidence reported by Szirmai (1969), which suggested that nasal septum contains two major histologically distinct areas with chondroitin sulphate pools with different chain lengths.

Compared with the glycosaminoglycan isolated from nasal septum, broader molecular size distribution profiles and higher M_w/M_n ratios (Table 1) were obtained for chondroitin sulphate and keratan sulphate isolated from intervertebral disc. This result may reflect (*a*) the former tissue's more complex morphology, or (*b*) inherent differences between the several animals contributing to the pooled tissue. The latter proposition is strengthened by Szirmai (1970), who proposed on the basis of solubility profiles for the polysaccharides of human intervertebral disc that the average molecular sizes of both keratan sulphate and chondroitin sulphate components do not change significantly with morphological location within a single disc. The work of several authors (Szirmai, 1969; Lindahl & Wasteson, 1970; Hjertquist & Wasteson, 1972; Hjertquist & Lemperg, 1972) suggests that the average chain length of chondroitin sulphate decreases with animal age. Mason & Wusteman (1970) reported that keratan sulphate isolated from tracheobronchial cartilage contained a high-molecular-weight fraction that increased in relative amount with animal age. Some of the polydispersity present in keratan sulphate and chondroitin sulphate preparations may, therefore, reflect age variation in the ten animals that have contributed cartilage to the pooled tissue.

The chain-length distribution recorded in Fig. 3 for chondroitin sulphate of nasal septum, besides indicating that this preparation may contain at least two major species of chondroitin sulphate with different average molecular weights, also suggests that the distribution of chain sizes within each chondroitin sulphate pool is very narrow and approximates to a theoretical distribution of the Poisson type. The theoretical Poisson distribution shown in Fig. 3 was calculated as described by Flory (1940), assuming a value of 86 for γ, the average number of monomer units per polymer molecule. A value of $\gamma = 86$ assumes that chondroitin sulphate chain extension proceeds by the addition of one monosaccharide at a time (Telser *et al.*, 1965) to yield chondroitin sulphate chains with an average molecular weight of 21500. Marler & Davidson (1965) reported an M_w/M_n ratio determined by equilibrium sedimentation of 1.04 for a chondroitin sulphate preparation isolated by extraction of rib costal cartilage with dilute alkali. On the basis of this low ratio, they also concluded that the chain-length distribution of costal-cartilage chondroitin sulphate may be a Poisson distribution.

That chondroitin sulphate chains may have a Poisson distribution of sizes is an interesting observation because it makes it possible to evaluate proposed mechanisms of chain growth and termination of polymers. Flory (1940, 1953) calculated that polymers with a Poisson distribution of chain lengths are produced if conditions allow (*a*) a finite number of chains to extend at a constant rate throughout the polymerization process, (*b*) only one monomer to be added to the polymer at a time, (*c*) all sizes of polymer to have an equal rate and likelihood of reaction with monomer, (*d*) no chain transfer, interchange or termination reactions to occur.

The Poisson distribution observed for nasal-septum chondroitin sulphate suggests, therefore, that this polysaccharide is synthesized within the conditions described by Flory (1940, 1953), that is by the addition of glucuronic acid and *N*-acetylgalactosamine residues to the non-reducing end of the growing chain at a constant rate throughout the whole chain-extension process. Chain termination need not necessarily involve a specific termination reaction; a conceivable termination mechanism would result if the growing polysaccharide chain simply moved away from a fixed (membrane-bound) complex of glycosyltransferases (Dorfman, 1970) involved in chain extension. That is to say, chain size may be regulated by the rate at which the initiator molecule [an oligosaccharide covalently linked to peptide (Rodén, 1970)] is transported along the endoplasmic reticulum past a complex array of membrane-bound glycosyltransferases.

This work was supported by the Australian Research Grants Committee (Project no. D65/15893). J. J. H. thanks Monash University for a Monash Postgraduate Research Scholarship.

References

Anseth, A., Antonopoulos, C. A., Bjelle, A. & Fransson, L.-Å. (1970) *Biochim. Biophys. Acta* **215**, 522–526

Antonopoulos, C. A., Borelius, E., Gardell, S., Hamnström, B. & Scott, J. E. (1961) *Biochim. Biophys. Acta* **54**, 213–226

Blumberg, B. S. & Ogston, A. G. (1957) *Biochem. J.* **66**, 342–346

Blumberg, B. S. & Ogston, A. G. (1958) *Biochem. J.* **68**, 183–188

Bruno, G. A. & Christian, J. E. (1961) *Anal. Chem.* **33**, 1216–1218

Constantopoulos, G., Dekaban, A. S. & Carroll, W. R. (1969) *Anal. Biochem.* **31**, 59–70

Determann, H. (1968) *Gel Chromatography*, Springer-Verlag, Berlin

Dische, Z. (1947) *J. Biol. Chem.* **167**, 189–198

Dorfman, A. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 3, pp. 1421–1448, Academic Press, London

Flory, P. J. (1940) *J. Amer. Chem. Soc.* **62**, 1561–1564

Flory, P. J. (1953) *Principles of Polymer Chemistry*, p. 317, Cornell University Press, New York

Gregory, J. D. & Rodén, L. (1961) *Biochem. Biophys. Res. Commun.* **5**, 430–434

Hjertquist, S.-O. & Lemperg, R. (1972) *Calcif. Tissue Res.* **10**, 223–237

Hjertquist, S.-O. & Wasteson, Å. (1972) *Calcif. Tissue Res.* **10**, 31–37
Hoffman, P., Mashburn, T. A. & Meyer, K. (1967) *J. Biol. Chem.* **242**, 3805–3809
Hopwood, J. J. (1972) Ph.D. Thesis, Monash University
Hopwood, J. J. & Robinson, H. C. (1971) *Proc. Aust. Biochem. Soc.* **4**, 91
Laurent, T. C. & Granath, K. A. (1967) *Biochim. Biophys. Acta* **136**, 191–198
Laurent, T. C. & Killander, J. (1964) *J. Chromatogr.* **14**, 317–330
Lindahl, U. & Wasteson, Å. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 2, pp. 867–872, Academic Press, London
Luscombe, M. & Phelps, C. F. (1967) *Biochem. J.* **103**, 103–109
Marler, E. & Davidson, E. A. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 648–656
Mason, R. M. & Wusteman, F. S. (1970) *Biochem. J.* **120**, 777–785
Mathews, M. B. (1971) *Biochem. J.* **125**, 37–46
Meyer, K. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 1, pp. 5–24, Academic Press, London
Robert, L., Mednieks, M. & Winzler, R. J. (1962) *Biochim. Biophys. Acta* **63**, 327–333
Robinson, H. C. & Dorfman, A. (1969) *J. Biol. Chem.* **244**, 348–352
Robinson, H. C. & Hopwood, J. J. (1973) *Biochem. J.* **133**, 457–470
Rodén, L. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 2, pp. 851–858, Academic Press, London
Szirmai, J. A. (1969) *Aging of Connective and Skeletal Tissue; Thule Int. Symp. 1968* pp. 163–184
Szirmai, J. A. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 3, pp. 1279–1308, Academic Press, London
Telser, A., Robinson, H. C. & Dorfman, A. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 912–919
Trevelyan, W. E. & Harrison, J. R. (1952) *Biochem. J.* **50**, 298–303
Wasteson, Å. (1969) *Biochim. Biophys. Acta* **177**, 152–154
Wasteson, Å. (1971) *J. Chromatogr.* **59**, 87–97
Whistler, R. L. & Rowell, R. M. (1966) in *Glucuronic Acid Free and Combined* (Dutton, G. J., ed.), p. 139, Academic Press, New York