The Biosynthesis of Glycosaminoglycans

C. F. PHELPS

Department of Biochemistry, The Medical School, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

The sugar components of acidic glycosaminoglycans have long been known to be derived from glucose without alteration of the sugar ring (Topper & Lipton, 1953). The discovery of the nucleotide sugars by Caputto et al. (1950) and Park (1952) radically transformed the outlook on the biosynthesis of polysaccharides. It became rapidly apparent, not only that nucleotide sugars were the 'activated' donors for polysaccharide synthesis, but that a great many interconversions between sugar species were performed at this level of conjugation (Ginsburg, 1964). Here the concern is with the nucleotide sugars associated with glycosaminoglycan synthesis, namely the UDP derivatives of glucuronate, iduronate, xylose, N-acetylglucosamine, N-acetylgalactosamine and galactose, and with their fate up to the polymerization and subsequent export of the finished polymer.

Formation of nucleotide sugar

The sites of nucleotide conjugation are few. Two pyrophosphorylase reactions are sufficient to account for the occurrence of the variety of sugar nucleotides previously cited. The pathways for each are different. Whereas UDP-glucose arises from a direct conjugation of glucose 1-phosphate with UTP by a pyrophosphorylase whose specificity has been studied by Albrecht et al. (1968), the hexosamine pathway is far more complex, involving the amination of fructose 6-phosphate by glutamine, the subsequent acetylation, mutation and, only then, conjugation by a specific pyrophosphorylase to form UDP-N-acetylglucosamine. Whereas considerable information is available on the UDP-glucose pyrophosphorylase, information on the enzymology of the hexosamine pathway is scant. Fructose 6-phosphate-glutamine aminotransferase has been purified extensively from rat liver and its steady-state kinetic parameters have been established (Winterburn & Phelps, 1970, 1971). The acetylation by acetyl-CoA has been thoroughly investigated by P. J. Winterburn & A. Corfield (unpublished work), but scant information is available on the specific mutase interconverting N-acetylglucosamine 6-phosphate and its 1-phosphate counterpart (Fernandez-Sorenson, 1968), and detailed information is similarly lacking on the synthesis of UDP-N-acetylglucosamine from this last intermediate and UTP (Strominger & Smith, 1959).

Interconversion of nucleotide sugars

One of the most potentially productive areas of research in this field involves the study of the vast variety of interconversion reactions of which nucleotide sugars are capable. These involve precise inversions, dehydrogenations, reductions, decarboxylations and simultaneous performances of these reactions.

Of immediate concern in the context of glycosaminoglycan biosynthesis is the formation of UDP-galactose from UDP-glucose by the 4'-epimerase. This enzyme has been studied in a variety of tissues. The mammalian enzyme is potently inhibited by NADH, though requiring catalytic amounts of NAD+ for its reaction (Maxwell, 1957).

UDP-xylose arises from the decarboxylation of UDP-glucuronic acid and has been demonstrated in a wide variety of tissues, e.g. hen oviduct (Bdolah & Feingold, 1965), mouse cell mastocytoma (Silbert & Deluca, 1967) and cartilage (Castellani et al., 1967). The enzyme from Cryptococcus laurentii and yeast has been intensively and elegantly studied by Schutzbach & Feingold (1970), who proposed an interesting mechanism of reaction. The enzyme is potently inhibited by NADH and UDP, though containing tightly bound NAD+.

UDP-glucuronic acid arises by dehydrogenation of UDP-glucose (Strominger et al., 1957). The reaction is noteworthy for being a four-electron oxidation. The enzyme from bovine liver contains six identical subunits, and some details of its protein chemistry have
been documented (Gainey et al., 1972). Nelsestuen & Kirkwood (1971) reported that synthetic UDP-glucose 6-aldehyde was used as an intermediate in this reaction, and further studies on the mechanism of action of this enzyme have been carried out (P. A. Gainey & C. F. Phelps, unpublished work). Much interest has been centred on the finding by Neufeld & Hall (1965) that this enzyme is potently and allosterically inhibited by UDP-xylose. It should be remembered, however, that this inhibitor is in many ways a perfect substrate analogue, differing only in the absence of that very side-chain primary alcohol that is modified by enzyme action. Gainey & Phelps (1972) have documented the activity of this enzyme, and the cellular concentration of UDP-xylose, in a number of tissues engaged in glycosaminoglycan biosynthesis, and there is a striking negative correlation between the concentrations of inhibitor and the incorporation of the glucuronide moiety in the polymers produced by these tissues.

UDP-iduronate arises, allegedly, from an inversion at the 5' position of UDP-glucuronic acid (Jacobson & Davidson, 1962). This enzyme also requires the presence of catalytic quantities of NAD+. NADH and UDP-N-acetylglucosamine both inhibit the enzyme (Jacobson & Davidson, 1963). The mechanism of inversion of this enzyme is at present unclear, and Lindahl et al. (1972) have reported that such inversion may occur at the polymer level in heparin. This most important implication, strengthened by the report of a similar event in alginic acid biosynthesis (Haug & Larsen, 1971), may explain why UDP-iduronic acid has never been isolated (Hardingham & Phelps, 1968).

Finally, inversion at the 4' position of UDP-N-acetylglucosamine has yet to be studied.

Metabolic control of the nucleotide sugar pathway

Before I pass on to the details of polymerization, it is relevant to discuss certain aspects of the control of this pathway. Mainly, the reactions occur in the cytosol, and the curb on extravagant overproduction of these nucleotide sugars offers one of the best examples of biochemical homeostasis. All ranges of control are evidenced, and may be briefly summarized as follows.

(a) By the supply of hexose monophosphate precursor. This is seen both in the effect of [glucose 6-phosphate]/[fructose 6-phosphate] ratios on the activity of the glucosamine synthetase (Winterburn & Phelps, 1971) and on the UDP-glucose pyrophosphorylase response to glucose 1-phosphate concentrations.

(b) By the nucleotide phosphate 'potential'. Nucleotide triphosphate is a stoicheiometric reactant in the formation of nucleotide sugars, and the ratio of nucleotide triphosphate to monophosphate is well indicated in the studies on glucosamine synthetase (Winterburn & Phelps, 1971) and in the UDP-glucose pyrophosphorylase reaction (M. Luscombe & C. F. Phelps, unpublished work).

(c) By the nicotinamide nucleotide redox potential. In discussion of many of the above-mentioned enzymes it is to be noted that, whereas NADH potently inhibits (sometimes with K_i values of 1 μM), NAD^+ is an essential cofactor. It is to be expected that changes in the cytosol [NADH]/[NAD^+] ratio will profoundly influence the activity of many of the nucleotide sugar interconversions.

(d) As if this were not enough, some of the most elegant examples of feedback inhibition by end products of specific pathways on the first enzyme of that pathway are to be found in nucleotide sugar metabolism. Kornfeld et al. (1964) first showed that UDP-N-acetylglucosamine inhibits the fructose 6-phosphate-glutamine aminotransferase, and Neufeld & Hall (1965) have raised the question of UDP-xylose inhibition on UDP-glucose dehydrogenase. What is still unclear is the mechanism of 'cross-talk' between the uronic and hexosamine pathways that would be expected to exist in systems synthesizing polymers containing equal proportions of each.

Polymerization reactions

Physiologically, the glycosaminoglycans exist as protein-polysaccharide entities, with the possible exception of hyaluronate. The province of the present communication is to
discuss the biosynthesis of the carbohydrate portions of these molecules, and it is thus incumbent on any discussion to present evidence on chain initiation, propagation and termination.

**Chain initiation.** This involves a linkage in the large number of glycosaminoglycans involving a short tetrasaccharide shown below:

\[
\begin{pmatrix}
\text{Glucuronate} \\
\text{or}
\end{pmatrix}
\begin{pmatrix}
\text{N-acetylglucosamine} \\
\text{or}
\end{pmatrix}
\begin{pmatrix}
\text{N-acetylgalactosamine} \\
\text{Iduronate}
\end{pmatrix}
\]

\[\text{glucuronate}-\text{galactose}-\text{galactose}-\text{xylose-O-serine}\]

**linkage tetrasaccharide**

Robinson *et al.* (1966) have shown that the 10000g supernatant of cartilage homogenate catalyses the incorporation of xylose from UDP-[\(^{14}\text{C}\)]xylose in trichloroacetic acid-precipitable material. Fractionation showed that the activity was equally distributed between 105000g supernatant and pellet. After proteolytic digestion of the trichloroacetic acid-precipitable material, material with chromatographic and electrophoretic identity with O-\(\beta\)-D-xylosyl-L-serine was isolated. Stoolmiller (1971) finds the xylosyltransferase from chick-embryo cartilage in the cytosol, and argues that, as its activity is independent of the presence of membranes or other glycosyltransferases, it may well play a key regulatory role in chain initiation. Baker *et al.* (1971) have shown that native proteoglycan protein prepared by the Smith degradation of proteoglycan can act as an acceptor for xylose in the xylosyltransferase preparation described by Robinson *et al.* (1966).

The addition of the next two galactose residues occurs each by a specific galactosyltransferase, studied by Helting & Rodén (1968, 1969a,b). They have shown the transfer of galactose from UDP-[\(^{14}\text{C}\)]galactose to xylosylserine as acceptor, and have confirmed that, with exogenous acceptors, the rate of incorporation of galactose is dependent on UDP-xylose and on xylosyltransferase activities. The second galactose residue is transferred to galactosylxylose to give the trisaccharide 3-\(O\)-\(\beta\)-D-galactosyl-4-\(O\)-\(\beta\)-D-galactosyl-D-xylose.

The glucuronate residue in the linkage region is added by a distinct, particulate, glucuronyltransferase studied in embryonic cartilage, where galactosylgalactosyl-xylosyl-L-serine was the most efficient acceptor molecule of those synthetic substrates tested.

Much interest is presently expressed in the possible involvement of lipids in polysaccharide biosynthesis, either as potential donors or as environments in which the glycosidic bond can be synthesized away from the aqueous milieu. Schwartz & Rodén (1972) treated a crude glycosyltransferase complex from chick-embryo cartilage with phospholipase C, with the result that 50% of the galactosyltransferase activity studied disappeared. Other glycosyltransferases were unaltered by this treatment. Total reactivation was observed on addition of phosphatidylcholine (lecithin) or detergent immediately after digestion. This result suggests an involvement in environment of the phospholipid.

**Chain elongation.** Three experimental areas are yielding the first clues to the mechanism whereby the alternating sugar residues are polymerized into chains, which may be as long as 30000 disaccharide units in hyaluronate or 40 in the chondroitin sulphate proteoglycans. These areas are: hyaluronate synthesis in A and C streptococci, chondroitin sulphate synthesis in chick-embryo cartilage and heparin production in mast-cell tumours. Early work was disappointing because of the minute amounts of radioactivity incorporated: amounts so low as to leave the matter of net synthesis, as opposed to incorporation, still questionable.

Valuable work on the hyaluronate synthetase has been done by Stoolmiller & Dorfman (1969) with streptococcal preparations that are activated by Mg\(^{2+}\) and appear specific for the two nucleotide sugar donors. In such difficult experimental systems the rate of acquisition of new information is small. Nevertheless it seems clear that, in bacterial
hyaluronate synthesis, polymerization proceeds by sequential addition of each respective nucleotide sugar to the non-reducing terminus of the chain, and that primer is not needed, in contradistinction to glycogen synthesis. There does not appear to be any evidence supporting the involvement of lipid in the biosynthesis of these polymers, in contrast with the synthesis of Salmonella O-antigen or bacterial murein peptidoglycan, although work by Behren & Leloir (1970) supports the involvement of polyprenol sugar carriers in the glycosylation of mammalian glycoproteins, and it would be wise at present to preserve an openness of mind on this point.

Similar work on the other tissues has not yet produced any new formulation of ideas about the mechanism of chain elongation.

Chain termination. This aspect of polymer synthesis is still shrouded in mystery. Is it necessary? For hyaluronate the answer could well be 'no', since a statistical distribution of molecular weights is frequently found. However, for the proteoglycans there is evidence that the polysaccharide chain length is remarkably constant, hinting at some process capable of achieving such uniformity. This process may either take place synthetically or be a tailoring of the molecule by degradative processes after synthesis. The attractive suggestion was made by Marler & Davidson (1965) of kinetic competition between, e.g., sulphation processes (which are believed to occur at the polymer level) and polymerase activity, such that steady increase in the sulphation of the still-growing polymer with time progressively inhibited more strongly the polymerizing enzyme.

Tailoring of the molecule

The previous discussion has already introduced the involvement of sulphate ester groups in all of the glycosaminoglycans except hyaluronate. Since the discovery by Robbins & Lipmann (1957) of the enzymic path involving 3'-phosphoadenosine 5'-phosphatide in biological sulphation reactions and in the sulphation of polysaccharides (D'Abramo & Lipmann, 1957), it has become a matter of deep interest to uncover the mechanism of these specific and complicated sulphations. The evidence obtained in vitro favours sulphation occurring at the polymer level, since UDP-N-acetylgalactosamine 4-sulphate is not used in chondroitin sulphate biosynthesis (Hardingham & Phelps, 1970), and Perlman et al. (1964) showed that polysaccharide formation could take place in the absence of sulphation. The specificity of the sulphotransferases is also in some doubt. Whereas Suzuki et al. (1961) could separate the sulphotransferase activity of the hen oviduct isthmus into three fractions, each with a specific transfer to heparin, chondroitin 4-sulphate and chondroitin 6-sulphate, Meezan & Davidson (1967) found that cartilage sulphotransferase used not only the chondroitin and dermatan molecules as acceptors but also keratan and heparin. In this delicate area of membrane-bound transferases it seems that the manipulative history of the enzyme preparation may be critically important in deciding between true and spurious acceptors.

With heparin, sulphation involves a de-N-acetylation of some glucosamine residues followed by a re-N-sulphation (Silbert, 1967).

Other tailoring reactions are in the even more speculative territory. Lindahl et al. (1972) have reported that the epimerization of glucuronate to iduronate may not occur at the nucleotide sugar level but in the polymer of heparin. A further question that should be raised concerns the possibility of migration of substituent groups, e.g. from (axial) sulphate on C-4 to (equatorial) sulphate on C-6 in the two chondroitin sulphates.

Cellular location of glycosaminoglycan synthesis

In the formation of proteoglycans, carbohydrate units are added to a specific protein core. Much of the available information suggests that the initial linkage sugars may be added to the nascent protein very soon after it leaves the ribosome and enters the cisternal space of the endoplasmic reticulum. It is at this stage that the material becomes effectively 'extracellular' (Winterburn & Phelps, 1972). Presumably a (membrane-bound) glycosyltransferase complex adds sequentially the specific linkage sugars to the initially
glycosylated protein, and at a later stage, in its movement through the smooth membranes to the Golgi complex, the polymerization of the glycosaminoglycan is achieved. Radioautographic studies have indicated that the Golgi body plays a major role in this process of export to the extracellular domain (Revel & Hay, 1963), although whether further synthesis and remodelling of the molecule proceeds here or passive accumulation occurs is at present unknown. In a study of the distribution of enzymes in subcellular membraneous fractions, Horwitz & Dorfman (1968) showed that xylosyltransferase and galactosyltransferase activities were effectively concentrated in the rough endoplasmic reticulum, whereas the polymerase for chondroitin sulphate was found approximately equally in the rough- and the smooth-membrane fractions, and sulphatetransferase activity was highest in the smooth-membrane fractions. It was therefore suggested that, after synthesis at ribosomal sites, the membrane-bound enzymes move slowly in their membranes through the smooth endoplasmic reticulum to the Golgi zone, with a halftime of days (Omura et al., 1967). Studies by Hardingham & Phelps (1968) and by Handley & Phelps (1972a,b) indicate that the rates of synthesis of the glycosaminoglycans in neonatal rat skin (predominantly hyaluronate), neonatal rat epiphyses (predominantly chondroitin 4-sulphate) and in bovine corneal stroma (predominantly keratan sulphate) are about 0.1-0.5 nmol of disaccharide addition/min per g wet wt. of tissue. These authors have also attempted to give a time-table of cellular events in the movement of isotopic label from glucose, through the nucleotide sugars and into the polymers.

Conclusion

Many aspects of glycosaminoglycan synthesis are not mentioned here, including hormonal effects, the involvement of other extracellular components and the excitement of explaining the mutual communication systems that exist between the inside and the outside those cells that synthesize connective-tissue components. The fundamental problem remains a simple one to state: how do molecules manufactured inside a cell get to the outside environment? This review indicates that considerable progress is being made.


1973
Sequestering Agents and Biochemical Systems: the Possibilities for Cross-Fertilization of Ideas between Industrial and Biological Chemistry

R. L. SMITH

28 Phoenix Lodge Mansions, Brook Green, London W6 7EG, U.K.

The subject of this communication is the chemistry of organic sequestering agents, although the occasion is a symposium on the biochemistry of calcium. This combination poses some problems, particularly to one whose whole experience has been in the industrial field. The chemistry of the organic sequestering agents is a very large topic, which cannot be fully discussed here. I must therefore select appropriate elements and limit the general treatment to matter appropriate to this selection. At first sight the combination of the uses of organic sequestering agents and the biochemistry of calcium might appear to be an unpromising combination. In industry the chelation of calcium has almost pride of place. However, the very extensive literature on organic sequestering agents in agriculture and animal biology is predominantly in relation to what are commonly called the trace metals, which are vital to the living systems, but generally present in very limited proportions. Calcium, on the other hand, presents at first sight the very opposite picture. Plants do not on the whole contain a great deal of calcium, although they are frequently grown in soils where calcium carbonate is a plentiful or even excessive constituent. When this is not the case then, except in very inaccessible regions, lime is the cheapest additive to the soil that the farmer could wish. In vertebrate animals calcium is present in such large overall proportion in bones and teeth that, if this essential structural need is fully served, then the bone marrow presents an extremely large reservoir of this element for other uses. Whereas infant mammals require their calcium in special form, such as milk, an adult diet deficient in calcium can be enriched with calcium carbonate, as was done in war-time bread. Thus the first impression is that plants are surrounded by calcium, do not need much of it and do not need the assistance of sequestering agents to acquire it, whereas animals need a lot, but again do not need sequestering agents to help them get it. If this were the whole picture then the chemistry of sequestering agents with special reference to the biochemistry of calcium would be rather uninteresting.