Glycoconj J (2010) 27:201–209
DOI 10.1007/s10719-009-9254-9

REVIEW ARTICLE

Glycosaminoglycan metabolism before molecular biology: reminiscences of our early work

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Received: 27 May 2009 / Revised: 24 June 2009 / Accepted: 7 August 2009 / Published online: 2 September 2009

Abstract This article concerns personal reminiscences of research on proteoglycans accomplished by Jeremiah Silbert and his co-investigators over a 25–30 year period beginning in 1961. Radiolabeled substrates were prepared and incubated with subcellular particles from mast cells and cartilage to determine pathways and organization of heparin and chondroitin glycosaminoglycan formation together with sulfation. Microsomal/Golgi fractions were examined for localization and organization of synthesis. Cell surface heparan sulfate and chondroitin were examined for preliminary information regarding potential function, and techniques were developed to alter sulfation processes.

Keywords Heparin · Heparan sulfate · Chondroitin sulfate · Sulfation · Sugar nucleotides · Microsome/Golgi

Introduction and overview

Research concerning the biosynthesis of polysaccharides owes its foundation to the Nobel Prize-winning (Chemistry 1970) work of Argentine Luis Leloir who described the formation of the sugar nucleotides UDP-glucose (1950), UDP-*N*-acetylglucosamine (1953) and GDP-mannose (1954) as donor intermediates. By 1959 when I began my fellowship to study the biosynthesis of heparin and heparan sulfate, glycogen and starch synthesis from UDP-glucose had been demonstrated, and there were several publications concerning bacterial polysaccharide formation, such as hyaluronic acid, from nucleotide sugar intermediates. Nevertheless, formation of the polysaccharides of glycoproteins and glycosaminoglycans other than hyaluronic acid had not been demonstrated. It was obvious that sugar nucleotides were the probable intermediates, but this had not been shown nor was there any information concerning the cellular localization or organization of synthesis.

My earliest work was to prepare radioactive UDPGlcNAc (UDP-*N*-acetylglucosamine), UDPGalNAc (UDP-*N*-acetylgalactosamine), and PAPS (3'-phosphoadenosine 5'-phosphosulfate) and to utilize microsomal preparations from mast cell tumors and chick embryo cartilage to define the biosynthesis of heparin and chondroitin sulfate glycosaminoglycans. Working by myself, I showed that heparin polymer formation was through an acetylated glycosaminoglycan intermediate with replacement of the acetyl by sulfate during polymer formation in the presence of PAPS with an "all or none" sulfation pattern of heparin later confirmed to be a proteoglycan. Similarly my colleagues and I showed that polymer formation and sulfation of chondroitin sulfate proceeded together in an "all or none" sulfation pattern. These investigations accompanied and were followed by experiments that provided fundamental information concerning the localization, organization, and interaction of Golgi membrane-bound substrates and enzymes in the synthesis of chondroitin sulfate. This basic research on biosynthesis has provided a starting point for further studies on biosynthesis by others and the subsequent molecular biology and genetic modification research that now has provided so much more.

We also provided the first demonstration of heparan sulfate and chondroitin sulfate formation on normal cell

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surfaces and initiated early studies that indicated an interaction of fibronectin, laminin, and lipoprotein lipase with cell surface heparan sulfate. These studies were an introduction to the extraordinary research by many others on heparan sulfate metabolism and structure/function that has followed. In addition, our examination of the under-sulfation of heparan sulfate and chondroitin sulfate when cells were grown in low sulfate media or with chlorate has provided a useful tool for examination of potential roles for sulfate conjugation in glycobiology.

Beginning

Following a Medical Internship and a year of Residency at Barnes Hospital in St. Louis, I began a two year Fellowship in 1959 in Carl Cori's Department of Biochemistry at Washington University. I met with David Brown who had published some work on heparan sulfate (initially called heparitin sulfate), described as a heparin-like substance that accumulated in tissues of children with lethal Hurler mucopolysaccharidosis, and a good topic for biochemistry research with a direct relationship to clinical medicine. It had been shown that heparan sulfate contained less sulfate than heparin, and contained a mixture of some *N*-acetylated glucosamine as well as the *N*-sulfated glucosamine found in heparin. The disaccharide repeating units of glucosamine and uronic acid (later determined to be iduronic, formed by epimerization of glucuronic after its incorporation into the glycosaminoglycan polymer) sugars and their linkages appeared to be identical to those of heparin. Brown thought that heparan sulfate could be an intermediate in the formation of heparin, and that the genetic defect in Hurler children might be a deficiency in enzymes converting *N*-acetylglucosamine to the *N*-sulfated structure.

The Department was small and allowed for close contact with a diverse and extraordinarily gifted group of investigators. Gerty Cori had died the previous year, but Carl Cori was still working in the laboratory (They were 1947 Nobel Laureates for "discovery of the course of the catalytic conversion of glycogen" and Luis Leloir had been in their laboratory in 1944). Other faculty were Mildred Cohn who moved to University of Pennsylvania while I was a Fellow, Sidney Velick who later became Chairman of Biochemistry at Utah, Robert Crane who became Chairman at Rutgers, Luis Glaser who later became Chairman at Washington University and then Provost at University of Miami, Barbara Brown, Carl Frieden, and George Drysdale who all remained at Washington U. Frieden, Glaser and Drysdale were walking encyclopedias of biochemistry, seeming to know and be able to critique everything currently and previously published. There were only two other post-doctoral fellows and only one graduate student, Rosalind, who had married Stuart Kornfeld, a medical student who was working summers with Luis Glaser. The Kornfelds had already demonstrated exceptional abilities. A Swiss physician, Max Burger, came as a fellow while I was there, but immediately decided to obtain a PhD. A stellar group, to say the least.

I knew from my medical training that heparin was found in granules of mast cells, that mice had peritoneal mast cells, and that mastocytomas were a common tumor in dogs. Transplantable mast cell tumor lines had been produced in two strains of mice, and were clearly the best source for tissues that would be producing heparin. I wrote to Thelma Dunn at NIH regarding the Dunn-Potter tumors in the DBA line, and to Jacob Furth at Children's Hospital in Boston regarding the Furth LAF line, and then traveled to Boston and Bethesda after they graciously agreed to give me animals with the tumors. Subsequently, I found the DBA tumor to be easier to use since these grew more quickly, and produced about 3 mg of heparin in a tumor growing from a subcutaneous inoculum to as much as 500–1000 mg before the animals died 14 or 15 days later. I continued for almost 15 years to inoculate 4 to 6 mice each week and harvested tumors as needed at 12 to 14 days. Initially I used only fresh tumors, but later found that frozen stored tumors worked just as well for subcellular experimentation.

Luis Glaser together with David Brown [1] had utilized a 10,000×G supernatant from Rous sarcoma homogenates to demonstrate formation of [^{14}C]hyaluronic acid when incubated with UDP-*N*-[^{14}C]acetylglucosamine (UDPGlcN [^{14}C]Ac) and UDP-glucuronic acid (UDPGlcA). Furthermore UDP-glucosamine (UDPGlcN) had been shown to be formed when GlcN-1-*P* and UTP were incubated with UDP-glucose (UDPG) pyrophosphorylase [2]. It seemed reasonable that UDPGlcA and UDPGlcN might be the precursors for formation of heparin. I then utilized commercially available [^{14}C]GlcN as well as unlabeled GlcN to synthesize labeled and unlabeled GlcN-6-*P* and GlcN-1-*P* with commercially available hexokinase and phosphoglucomutase, and then incubated labeled and unlabeled GlcN-1-*P* together with UTP and a 105,000×G supernatant from mast cell tumor homogenates to form UDPGlcN and UDP [^{14}C]GlcN [3]. I also found small amounts of UDPGlcN in extracts of tumor, reinforcing the idea that it might be a precursor for heparin.

Brown did not plan to continue work on heparin and had no problem with my taking the project with me when I moved to the Massachusetts General Hospital in 1961 for a three year rheumatology research fellowship including some clinical work in the arthritis unit where Stephen Krane had just become Director. Jerome Gross and his collagen group were down the hall, while Roger Jeanloz and his carbohydrate chemistry group were in the next laboratory, all part of the Robert W. Lovett Memorial for the Study of Diseases Causing Deformities. Although Krane's interests were not

in proteoglycans (then called mucopolysaccharides), he was very supportive and provided excellent advice and commentary on my work and publications from his unit. Jeanloz and several of his fellows were extremely helpful with advice on chemical syntheses and column separations.

Preparation of sugar nucleotide substrates for glycosaminoglycan biosynthesis

There had been some investigations of hyaluronic acid synthesis in other laboratories using labeled sugar nucleotides with homogenates of tissues, but results were limited since concentrations and specific activities were too low to provide adequate information. No biosynthesis of heparin or chondroitin sulfate had been reported. In order to approach this, I considered it to be imperative to prepare labeled substrates with high specific activities and to use tissue fractions rather than homogenates. At that time [^{14}C] glucose was available from New England Nuclear, but it was expensive and the specific activity was much lower than what I wanted. Therefore, with instruction from Donald Comb who was working in the same building (he later founded New England Biolabs), I prepared 5 millicuries of uniformly labeled high specific activity [^{14}C] glucose by use of a fresh tobacco leaf exposed in the dark to high specific activity $^{14}CO_2$ in a closed flask (this was done in a special radioisotope preparation room in the building). It turned out to be especially fortunate, since New England Nuclear was in short supply of [^{14}C]glucose, and its owner, Seymour Rothschild, had heard that I had prepared this large amount. He contacted me and I agreed to provide 4 mCi to New England Nuclear with the understanding that my laboratory would be credited at the New England Nuclear [^{14}C]glucose selling price for equivalent costs of any radioisotopes that I would order in the future. Subsequently, I received multiple orders during the next five or more years that would have cost upwards of 10,000 dollars (double my yearly pay in those days).

I utilized the [^{14}C]glucose to prepare significant amounts of high specific activity UDP[^{14}C]GlcA by sequential incubations with commercially available hexokinase, phosphoglucomutase, UDPG pyrophophorylase, and UDPG dehydrogenase, isolating products at each step in order to obtain maximal synthesis. At the same time I prepared larger amounts of GlcN-1-*P* chemically [2], and after acetylating it chemically with high specific activity [^{3}H]acetic anhydride, reacted it with UMP-morpholidate [4] to form UDPGlcN [^{3}H]Ac. Similarly I also prepared UDP[^{14}C]GlcNAc chemically starting with [^{14}C]GlcNAc-1-*P* that I prepared, and also prepared UDP[^{14}C]GlcNAc by use of commercial UDPGlcNAc pyrophosphorylase. Products were separated and identified by various paper chromatographic [5] and column chromatographic [6] techniques followed by isolation and identification of the sugar components.

At the same time that I prepared labeled UDPGlcNAc, I prepared [7] unlabeled and labeled GalN-1-*P* and UDPGalNAc (UDP-*N*-acetylgalactosamine)(not commercially available) in order to examine biosynthesis of chondroitin. A few years later, my laboratory colleague Silvana DeLuca and I examined mast cell tumor subcellular fractions for formation of UDP[^{14}C]GlcA from UDP[^{14}C]Glc, and to prepare UDP[^{14}C]Xyl from the UDP[^{14}C]GlcA [8] since Xyl had been shown to be the sugar attaching to the core protein of chondroitin proteoglycans. Formation of the UDP[^{14}C]GlcA, as expected, was essentially all in the 105,000×G supernatant of homogenates, but UDP[^{14}C]Xyl was formed in microsomal fractions with none in the 105,000×G supernatant. To my knowledge, this is the only proteoglycan precursor sugar nucleotide to be formed with a microsomal rather than a cytosomal enzyme.

Biosynthesis of glycosaminoglycans, the polymerization steps

My first experiment was to incubate mast cell granule fractions (600–10,000×G precipitate) or microsomal fractions (20,000–105,000×G precipitate) with UDP[^{14}C]GlcA together with UDPGlcN or UDPGlcNAc as a control. In order to have as high a concentration of high specific activity substrates with as much of the mast cell fractions as feasible, the total reaction volumes were kept to 0.05 ml. [9]. Unexpectedly, radioactive product from UDP[^{14}C] GlcA was found when UDPGlcNAc was added as a control, but none was found with UDPGlcN, and granules had no activity, while incorporation was excellent with the microsomal material. More detailed experiments [10] demonstrated that there was equimolar incorporation of labeled GlcA and GlcNAc and that the product differed from hyaluronic acid since it was not degradable by hyaluronidase, but was degradable by a heparinase preparation from *Flavobacter heparinum.* Products were easily separated by spotting a fraction or the entire reaction mixtures (usually 0.025 ml) on Whatman paper for overnight descending paper chromatography with an ethanol ammonium acetate eluent [5]. With this system the only labeled material that remains at the origin is the proteoglycan or glycosaminoglycan, while all the intact or degraded substrates move various distances down the paper allowing for determination of any degradation of substrates during the incubations. The radioactive glycosaminoglycan products at the origin could then be removed by use of 0.1 M alkali or a crude protease that degrades the proteoglycan products to provide the soluble glycosaminoglycan. As many as

eight to ten samples could be spotted on one paper sheet, and six or more sheets could be chromatographed at the same time in one chromatography tank. Thus fifty or more samples could be spotted and run at the same time, and labeled glycosaminoglycan products then recovered for analysis by easy and accurate techniques. This technique of separation and assay provided for the optimization of multiple substrate concentrations, and the determination of amounts of product as little as 25 cpm synthesized at multiple time points in a single controlled experiment that could have as much as 10^6 cpm of substrate.

The labeled glycosaminoglycan products were further characterized by use of DEAE-cellulose column chromatography (suggested by Anund Hallen from the University of Uppsala, a post-doctoral fellow in the Jeanloz laboratory) using logarithmic gradients of 1 or 2 M LiCl to separate by charge, and use of a Sephadex G-200 column to characterize by size. Results indicated true biosynthesis of labeled glycosaminoglycan polymers that were found in fractions similar to hyaluronic acid with both columns and were well separated from standards of chondroitin sulfate and heparin.

I also tried incubations of labeled substrates with microsomal preparations from rat cartilage and from Englebreth-Holm-Swarm mouse sarcoma, which had been described as producing chondroitin sulfate. Neither was successful. Unfortunately, when I incubated the sarcoma microsomal preparations with labeled UDPGlcA and UDPGalNAc, I did not use UDPGlcNAc as a control as I had with the previous heparin investigation. Had I done so, I would have synthesized heparan sulfate, which later was shown to be produced by the sarcoma rather than chondroitin sulfate [11], and which I later did use to demonstrate biosynthesis of heparan sulfate [12]. I then found that epiphyseal cartilage from 12–14 day chick embryos was an excellent source for synthesis of chondroitin, which I produced with microsomal preparatrions and characterized [13] in the same fashion as the heparin precursor.

Some years later I incubated penta- and hexasaccharides derived from chondroitin sulfate together with microsomal preparations containing UDP[^{14}C]GlcA or UDPGalN[^{3}H]Ac, respectively, for addition of a single sugar [14], and also with both substrates together to determine the capacity for polymerization on these soluble acceptors. There was no true polymerization, but only a delayed addition of a second sugar to about 50% of the product after addition of the first sugar. Furthermore, these oligosaccharides inhibited formation of microsomal-bound chondroitin in incubations containing both sugar nucleotides. These experiments demonstrated that even though oligosaccharides had full access to the microsomal enzymes for polymerization, membrane-bound chondroitin primer was necessary for forming polymers of chondroitin.

In 1964 I ended my fellowship at the MGH, to continue my career-long work on proteoglycans at VA hospitals in Boston and the Boston area. A benefit of working next to the Jeanloz laboratory, had been my good fortune to meet and become friends with Nathan Sharon, who was spending a sabbatical year there. Sharon was lavish in his invitations to come to Israel to spend time in his laboratory at the Weizmann Institute in Rehovot. After getting established at the Boston VA Medical Center and extending my experiments to sulfation of heparin and chondroitin, I proceeded with my wife and two small children (2 and 4 years old) to accept the invitation. We spent six superb months from late 1965 to early 1966 at the Weizmann, where I worked with one of Sharon's graduate students to synthesize TDPGlcNAc and GDPGlcNAc [15]. These substances were used in the course of investigations related to bacillosamine [16], but did not turn out to be of importance. However, these 6 months led to a close friendship with the Sharon family that has continued and expanded through the years.

Sulfation/deacetylation of heparin

Back in Boston, I turned to the question of the mechanism of heparin sulfation. For this purpose I examined the capacity for the mastocytoma microsomal fraction to sulfate endogenous glycosaminoglycans [17], using incubations with non-labeled and ^{35}S-labeled PAPS (3'-phosphoadenosine 5'-phosphosulfate) that I synthesized enzymatically as had been previously described [18], since PAPS and PAP^{35}S were not commercially available. When the PAP^{35}S was added to incubations containing non-labeled UDPGlcA and UDPGlcNAc, almost three times more ^{35}S was incorporated than in the absence of sugar nucleotides [19], indicating sulfation of newly formed glycosaminoglycan as well as endogenous glycosaminoglycan. DEAE-cellulose chromatography of this ^{35}S-glycosaminoglycan indicated that sulfation appeared to be essentially complete since almost all of it was found in fractions indicating a degree of sulfation equal to or greater than that of a heparin standard. Subsequently, we incubated the microsomal preparations with UDPGlcA and UDPGlcN[^{3}H]Ac with or without PAPS to determine what happened to the labeled acetyl [20]. When PAPS was added to incubations after formation of [^{3}H]acetyl-labeled glycosaminoglycan about 50% of the [^{3}H]acetyl was lost, demonstrating an exchange of sulfate for acetyl in formation of the *N*-sulfate of heparin. This suggested the possibility of a single enzyme protein that catalyzed the linked reactions of de-acetylation and *N*-sulfation. This

indeed was later shown to be the case [21], and was subsequently extended [22] by disruption of the *N*-deacetylase/*N*-sulfotransferase gene in transgenic mice. In any event it was apparent that the polymerization, deacetylation, *N*-sulfation, and *O*-sulfation occurred in juxtaposition in the same microsomal location. Moreover, none of the nascent heparin products could be removed from the microsomal preparation without use of alkali to separate the glycosaminoglycan from its core protein. Thus there appeared to be an attachment of enzymes and nascent proteoglycan to the same microsomal membrane location during the process of glycosaminoglycan synthesis. Subsequently, work in Lindahl's and Kjellen's laboratories have expanded greatly on our early findings, including a demonstration that GlcNAc and GlcA transglycosylase resided on a single protein [23], and with detailed examination of multiple aspects of heparin and heparan structure, sulfation, and biological activity.

Sulfation of chondroitin, membrane organization, chain termination

Together with Sylvana DeLuca, we utilized PAP^{35}S to examine incorporation of [^{35}S]sulfate into endogenous proteochondroitin sulfate in microsomal preparations of chick cartilage [24], and the formation of some highly sulfated [^{14}C]chondroitin sulfate when UDP[^{14}C]GlcA, UDPGalNAc, and PAPS were incubated together with the microsomal preparation [25]. The size distribution of the endogenous chondroitin sulfate was determined by separate incubations with labeled UDPGlcA, UDP GalNAc, or PAPS in order to tag the receptor by adding a single radioactive residue [26]. We estimated that only a few percent of the endogenous material was less than 8,000 Mol Wt, while most ranged in size from 16–28,000 Mol Wt. with a small amount of sulfate acceptor that was as large as 38,000 Mol Wt. When incubations contained both sugar nucleotides, biosynthesis of two products averaging over 30,000 Mol Wt was found [27]. About 40% of the radioactivity consisted of the addition of chondroitin of about 20,000–35,000 Mol Wt on primer less than 8000 Mol Wt, while about 60% consisted of the addition of about 10,000 Mol Wt product onto endogenous chondroitin sulfate of about 16–28,000 Mol Wt. When we examined the product of an incubation with PAPS present together with the two sugar nucleotides, we found that about 80% of the new chondroitin product formed was 95% sulfated (all 6-sulfate), with only small amounts of non-sulfated product formed on endogenous acceptors. In other experiments we demonstrated that sulfation at timed intervals during polymerization produced increasing amounts of fully sulfated polymer rather than partial sulfation [28], demonstrating that full sulfation took place in an "all or none" manner. As with heparin, this was consistent with a membrane-bound complex of enzymes localized and working together in the cell with membrane-bound primers for rapid formation of each chondroitin sulfate polysaccharide chain. At the same time another colleague, Richard Lewis, and I examined microsomal preparations from cultured mast cells that synthesized chondroitin sulfate rather than heparin [29], and found similar results except for 4-sulfation instead of 6-sulfation [30].

Together with Geetha Sugumaran in my laboratory we found that incorporation of [^{35}S]sulfate from PAP^{35}S when oligosaccharides or chondroitin were added to microsomal preparations was somewhat different from the limited incorporation of GlcA and GalNAc to oligosaccharides. Sulfation was found to proceed to a considerable extent on oligosaccharides [31] and on added chondroitin, with some sulfation of added proteochondroitin if microsomes were incubated in the presence of detergent [32]. However, the degree of sulfation was much lower than the 95% found with the chondroitin formed by microsomal preparations. Sulfation of exogenous chondroitin proceeded somewhat with soluble enzyme in a partial "all or none" pattern consisting of added chondroitin being sulfated 50% or not at all [33]. We also cultured chondroitin sulfate-producing mast cells in the presence of methylumbelliferyl xyloside, which provided a lipophylic primer [34] for mast cell chondroitin synthesis [35], in order to examine the sulfation pattern of this chondroitin in comparison to the pattern formed on endogenous primer [36]. Sulfation of chondroitin formed on the xyloside was incomplete and required higher sulfate concentrations than the concentrations needed for complete sulfation on endogenous primer. This indicated that endogenous acceptor-membrane organization was important for sulfation as well as for polymerization. In other experiments we demonstrated that the position of sulfation was determined only by the specific sulfotransferase and not by differences in microsomal proteoglycan acceptors [32].

With Lawrence Freilich, who was trained in cell biology, we used cell fractionation and electron microscopy to show that the location for chondroitin polymerization, as well as for the addition of single sugars to penta and hexasaccharides, was in the Golgi [37,38]. Subsequently, Geetha Sugumaran and I pursued further fractionation, and she was able to characterize the portion of Golgi involved for separate addition of each GlcA-Gal-Gal-Xyl protein linkage sugar as well as polymerization [39,40]. She also incubated ^{14}C-labeled substrates and PAPS with microsomal preparations for short time intervals using low temperature to slow

polymerization [41]. This demonstrated that sulfation occurred while the chondroitin polymer was growing, thus confirming the same Golgi location for polymerization and sulfation.

In order to determine how chains terminated in their formation, I incubated UDP[^{14}C]GlcA and UDP[^{14}C] GalNAc separately with commercial chondroitin sulfate, endogenous acceptors, and newly synthesized chondroitin polymer [42]. Products were degraded with chondroitin ABC lyase, yielding labeled [^{14}C]GalNAc that had been added to a terminal GlcA and labeled disaccharide when labeled [^{14}C]GlcA was added to a terminal GalNAc or GalNAc-6-*S*. Results with both endogenous and newly synthesized acceptors indicated that the chains terminated in a mixture of GlcA, GalNAc, and GalNAc-6-*S*. No GlcA could be added to terminal GalNAc-4-S. Later experiments demonstrated that no GalNAc could be added to GlcA if the penultimate GalNAc residues were 4-sulfated [43]. Thus terminal 4-sulfation could be considered as an inhibiter of polymerization. We also showed by isolation and identification of labeled tetrasaccharides that 4-sulfate and 6-sulfate could be found on the same chondroitin polymer [44].

Glycosaminoglycans of cells

Although our major subject had become the biosynthesis of chondroitin sulfate, we were also interested in the presence of heparan sulfate and chondroitin sulfate on cell surfaces as first described for some established tumor cell lines [45]. In collaboration with my wife, Cynthia Silbert, and Hynda Kleinman, who spent a year with us immediately after receiving her doctorate, before she moved to the NIH, we found that primary monolayer cultures of normal human skin fibroblasts synthesized and maintained heparan sulfate and chondroitin sulfate on their cell surfaces [46]. With my wife and Peggy Jo Gill in my laboratory, we showed that removal of these glycosaminoglycans from cell monolayers by use of crude heparanase from *F. heparinum* [47] did not disturb cell attachment or viability. (We had a large batch of the bacteria grown for us by the Tufts Enzyme Center, and we prepared a crude lysate that contained enzymes that degraded chondroitin sulfate and hyaluronic acid as well as heparin and heparan sulfate. Alfred Linker purified some heparinase for us). Subsequently, we showed that neither trypsin-treated skin fibroblasts nor trypsin-treated endothelial cells in suspension would reattach in the presence of the heparinase, while they would reattach in the presence of chondroitin lyase [48]. Furthermore, the presence of a fibronectin or laminin coating on the plate allowed reattachment even in the presence of heparinase, suggesting that these substances might be positioned by cell surface heparan sulfate to facilitate attachment [49]. We collaborated with Lloyd Culp in demonstrating that cell surface heparan sulfate did mediate some adhesion to fibronectin and other matrices [50]. We also collaborated with Barry Fanburg and Kazuyuki Shimada, a post-doctoral fellow in Fanburg's laboratory, to show that lipoprotein lipase bound to heparan sulfate on the surface of cultured endothelial cells [51].

In collaboration with Frank Austen and colleagues in his laboratory, Roger Yurt (he later became Chairman of Surgery at Cornell), and Dean Metcalf, we demonstrated that mast cell heparin, previously thought by some to be unattached to protein, was a proteoglycan [52] composed of heparin linked to a protease-resistant poly ser-gly portion that was 82% of the total protein [53], and was found as an intact proteoglycan in lung [54]. We also demonstrated that varying sizes of heparin proteoglycan in a tissue were mainly related to numbers of heparin chains rather than to sizes of the chains [53]. This demonstrated a relatively slow addition of chains in biosynthesis, but with rapid polymerization of each chain.

Undersulfation of glycosaminoglycans

Further work in our laboratory using cell culture to investigate sulfate metabolism and sulfation was pursued together with my wife and Donald Humphries. Initially, we used [^{35}S]sulfate and [^{3}H]glucosamine to examine the degree of sulfation of chondroitin and heparan when endothelial cells were cultured in sulfate-depleted medium [55]. We found that chondroitin sulfation decreased 25% when sulfate concentrations were reduced to 0.11 mM and decreased 80% with 0.02 mM sulfate. Heparan sulfation was less affected, with an 11% decrease at 0.11 mM and a 52% decrease at 0.02 mM sulfate. The pattern of undersulfation with both chondroitin and heparan was a mixture of lesser sulfated glycosaminoglycans rather than the "all or none" distribution found in biosynthesis. We considered these results to be of potential clinical importance since humans have the lowest serum sulfate levels of any animals investigated to date, ranging from 0.2 to 0.5 mM, with occasional drops nearer to 0.11 mM. We also examined the effects of lower sulfate in cultures of human skin fibroblasts, and found that sulfation of chondroitin/dermatan sulfate decreased by 6% at 0.2 mM sulfate and 17% at 0.1 mM [56]. We found that the highly sulfated glycosaminoglycan formed with high concentrations of sulfate consisted mainly of sulfated dermatan residues with only a small amount of sulfated chondroitin residues, while the partially sulfated glycosaminoglycan formed when sulfate concentrations were low, consisted of lesser amount of dermatan that was all sulfated and larger amount of chondroitin that was all non-sulfated. This indicated that

epimerization from GlcA to IdA in formation of dermatan was linked to the sulfation of adjacent GalNAc, demonstrating that the epimerization to IdA and sulfation were performed at the same membrane location.

Donald Humphries in my laboratory developed an alternate method for lowering sulfation of glycosaminoglycans by the addition of chlorate to culture media [57]. This functions by blocking the formation of PAPS, which provides the advantage of culturing cells in routine culture media that contains serum with sulfate. Different degrees of under-sulfation were achieved by adding different amounts of chlorate resulting in controlled under-sulfation from a few % to 100%. Cells grew normally at low to medium levels of chlorate, and the ability to provide sulfation was quickly reversed by replacement with growth media containing no chlorate.

We questioned whether a decrease of sulfate levels *in vivo* might result in under-sulfation of chondroitin and loss of dermatan formation with possible deleterious effects upon cartilage, skin, and connective tissues. Sulfate is ordinarily available in some foods, but its main source for humans is methionine and cysteine, and its production had been thought to take place exclusively in liver. Although our results with cultures of bovine endothelial cells and human embryonic skin fibroblasts showed that these cells were not capable of producing sulfate from methionine and cysteine, we found that bovine aortic smooth muscle cells and human embryonic lung fibroblasts were capable of this production while bovine aortic endothelial cells were not [58]. We then examined the formation of dermatan sulfate when skin fibroblasts from six human adults were grown in sulfate-depleted medium, and found that the cells from two individuals were capable of forming sulfate, two had a partial capacity, and two appeared to be incapable of forming sulfate [59]. Thus there is a possibility for under-sulfation to have effects limited to some rather than all individuals. This remains to be examined.

Summary comments

Our laboratory provided the basic details of the biosynthetic pathways in polymerization and sulfation of heparin and chondroitin sulfate and the cellular localization and organization of this synthesis. In addition we made early contributions concerning the structure of heparin proteoglycan and the presence of and function of cell surface heparan sulfate. Subsequently, great strides assisted through molecular biology and genetic modifications have been made by others to define aspects of glycoprotein and glycosaminoglycan metabolism as well as mechanisms of structure/function. Proteoglycan investigators such as M Bernfield, J. Esko, L-A Fransson, J Gallagher, V Hascall, R. Iozzo, U. Lindahl, R. Rosenberg., K. Sugahara., and many others with their colleagues have expanded upon the early information and progressed much further in elegantly cloning enzymes of heparin, heparan sulfate, and chondroitin sulfate, and characterizing their metabolism as well as the pharmaceutical actions and functions of heparin and heparan sulfate. A special issue of Glycoconjugate Journal (vol 19, p 225–372) was published in 2003 to provide reports by 17 different laboratories on "Proteoglycans-Genetic Manipulation in Intact Organisms," and IUBMB Life contains current reviews of the biosynthesis of heparin/heparan sulfate [60] and chondroitin/dermatan sulfate [61].

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