NUCLEOTIDE INTERMEDIATES IN THE BIOSYNTHESIS OF HETEROPOLYMERIC POLYSACCHARIDES

JACK L. STROMINGER
From the Department of Pharmacology, Washington University School of Medicine, St. Louis

ABSTRACT The role of nucleotides as "carriers" of small molecules for biosynthetic reactions is discussed. Following this introduction, the particular problem of nucleotide intermediates in chondroitin sulfate synthesis is presented. The egg shell of the hen contains a form of chondroitin sulfate and particular emphasis is placed on the biosynthesis of sulfated polysaccharides in the hen oviduct, which has been studied in the author's laboratory.

Although a great deal is now known about the mechanism of synthesis of polysaccharides containing a single monomer, such as glycogen, comparatively less is known about the synthesis of polysaccharides containing two or more monomers. In opening my discussion of this subject, I would like to emphasize that the heteropolymeric polysaccharides being considered today, which are composed of two or three monomers, are relatively simple, compared to some other polysaccharides in nature, which can contain eight to ten different monomers in the polymer. Even the knowledge of the synthesis of polysaccharides containing two monomers is extremely limited, however.

Modern progress in this field was initiated in 1949 when Leloir isolated uridine diphosphoglucose, the cofactor for the transformation of galactose into glucose. Soon after this, it was suggested by Calvin from isotope experiments in plants that uridine diphosphoglucose might be a precursor for the synthesis of the disaccharide, sucrose, in plants. This hypothesis was soon experimentally verified by Leloir, who demonstrated directly that uridine diphosphoglucose could be a donor of glucose for the synthesis of sucrose, and, since that time, a large number of other oligosaccharides and polysaccharides containing glucose have been shown to be synthesized in reactions involving uridine diphosphoglucose.

A large number of small molecules can be activated for synthetic reactions as their nucleotide derivatives (Fig. 1). For example, some acids, such as sulfate and

ACTIVATION OF SMALL MOLECULES AS NUCLEOTIDES FOR SYNTHETIC REACTIONS

1. Adenine nucleotides—acids
   e.g. sulfate, amino acids
2. Uridine nucleotides—sugars
   e.g. glucose, acetylglucosamine
3. Guanosine nucleotides—sugars
   e.g. mannose, fucose
4. Cytidine nucleotides—alcohols, sugars
   e.g. choline, acetylneuraminic acid
5. Thymidine nucleotides—sugars
   e.g. rhamnose, unusual amino sugars

FIGURE 1

amino acids, are activated as adenine nucleotides for synthetic reaction. Sugars can be activated as uridine nucleotides, as guanosine nucleotides, as cytidine nucleotides, or as thymidine nucleotides. Alcohols such as choline or glycerol are activated as cytidine nucleotides for synthetic reactions.

These nucleotide intermediates are synthesized in reactions catalyzed by enzymes termed pyrophosphorylases, the first of which was originally described by Kornberg (Fig. 2). In this type of reaction, a nucleoside triphosphate reacts with an acid to yield the nucleoside monophosphate derivative of that acid and as a second product, inorganic pyrophosphate. In the first example (Fig. 2) inorganic sulfate is activated

SYNTHESIS OF NUCLEOTIDE INTERMEDIATES

Pyrophosphorylases

Nucleoside triphosphate + acid $\rightleftarrows$
Nucleoside monophospho-acid + pyrophosphate

e.g. ATP + sulfate $\rightleftarrows$ AMP-sulfate + PP
UTP + glucose-1-phosphate $\rightleftarrows$ UDP-glucose + PP

FIGURE 2

by reaction with adenine triphosphate to form adenyl sulfate and inorganic pyrophosphate. In the second example, the acid, glucose-1-phosphate, is activated by reaction with uridine triphosphate to form uridine diphosphoglucose and inorganic pyrophosphate.

Once activated at its nucleotide derivative, the sugar can be modified in a number of different ways. One modification of sugars which occurs at the level of the nucleotide derivatives and which is important in the biosynthesis of mucopolysaccharides is epimerization, for example the epimerization of UDP-acetylglucosamine

to yield UDP-acetylgalactosamine or epimerization of UDP-D-glucuronic acid to yield UDP-L-iduronic acid (Fig. 3). These two epimerizations occur at carbon atoms 4 and 5, respectively.

MODIFICATION OF A NUCLEOTIDE-LINKED SUGAR BY EPIMERIZATION

UDP-*N*-acetylglucosamine $\rightleftarrows$ UDP-*N*-acetylgalactosamine

UDP-D-glucuronic acid $\rightleftarrows$ UDP-L-iduronic acid

FIGURE 3

In addition to modification by epimerization, sugars linked to nucleotides can also be modified by oxidation. Two examples of such oxidation, in fact, the only two known, are the oxidation of uridine diphosphoglucose to yield UDP-glucuronic acid, another intermediate which is important in the biosynthesis of mucopolysaccharides, and the oxidation of guanosine diphosphomannose to yield GDP-mannuronic acid, a compound which is important in the biosynthesis of a bacterial polysaccharide (Fig. 4).

MODIFICATION OF A NUCLEOTIDE-LINKED SUGAR BY OXIDATION

$$\text{UDP-D-glucose} \xrightarrow{\text{DPN}^+} \text{UDP-D-glucuronic acid}$$

$$\text{GDP-D-mannose} \xrightarrow{\text{DPN}^+} \text{GDP-D-mannuronic acid}$$

FIGURE 4

Similarly, reduction of sugars linked to nucleotides can occur. One example is the reduction of guanosine diphosphomannose in a complex reaction which leads to the formation of guanosine diphospho-L-fucose, an intermediate in the biosynthesis of milk oligosaccharides, for example. Similarly, in bacteria, thymidine diphosphoglucose can be reduced to thymidine diphospho-L-rhamnose in a similar reaction (Fig. 5).

MODIFICATION OF A NUCLEOTIDE-LINKED SUGAR BY REDUCTION

$$\text{GDP-D-mannose} \longrightarrow \xrightarrow{\text{TPNH}} \text{GDP-L-fucose}$$

$$\text{TDP-D-glucose} \longrightarrow \xrightarrow{\text{TPNH}} \text{TDP-L-rhamnose}$$

FIGURE 5

Once activated as its nucleotide derivative and then, perhaps, modified, the sugar can be transferred in a reaction which leads to the formation of a new glycosidic linkage. These reactions are catalyzed by enzymes termed "transferases." One example of a transferase (Fig. 6) involves uridine diphosphoglucose. The glucose

SYNTHETIC REACTIONS OF NUCLEOTIDES

Transferases

UDP-glucose + acceptor ⇄
Acceptor-glucose + UDP
CDP-choline + acceptor ⇄
Acceptor-P-choline + CMP

FIGURE 6

moiety of the nucleotide is transferred to an acceptor, leading to the formation of a glycosidic linkage and uridine diphosphate. A second type of transfer reaction of such nucleotide derivatives, which is important in the biosynthesis of phospholipids, involves the transfer of phosphoryl choline from CDP-choline to an acceptor, leading to formation of a derivative of phosphoryl choline and cytidine monophosphate.

Glucuronide synthesis illustrates a typical reaction cycle, which leads to such a synthetic reaction (Fig. 7). The first reaction in the cycle is a reaction in which

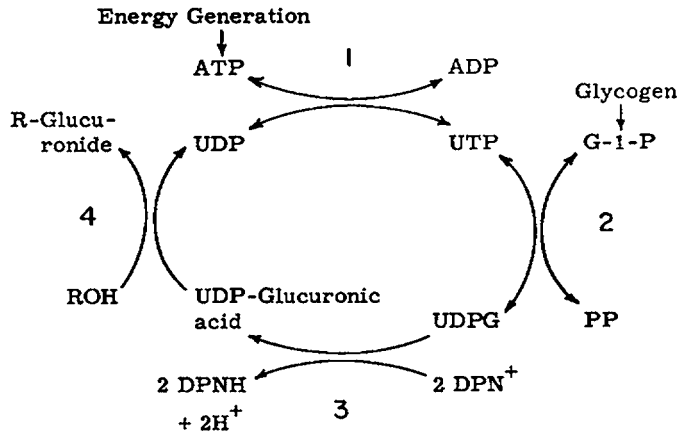

FIGURE 7 Cyclic mechanism for the synthesis of glucuronides. The enzymes which participate in this cycle are (1) nucleoside diphosphokinase, (2) UDP-D-glucose pyrophosphorylase, (3) UDP-D-glucose dehydrogenase, and (4) a variety of transferases utilizing UDP-D-glucuronic acid.

energy "enters" the cycle. It is the phosphorylation of uridine diphosphate by adenosine triphosphate, leading to the formation of uridine triphosphate. In the second reaction, the sugar is activated as its nucleotide derivative. The formation of uridine diphosphoglucose from UTP and glucose-1-phosphate takes place. Then the activated sugar is modified, in this case by oxidation, to UDP-glucuronic acid, and, finally, the UDP-glucuronic acid can be transferred to an acceptor, leading to the formation of a glucuronide or to the formation of a glycosidic linkage in polysaccharides, such as hyaluronic acid and chondroitin sulfate.

It may be useful, briefly, to compare two mechanisms for the synthesis of glycosidic linkages. These two mechanisms are the synthesis of glycosides from uridine nucleotides and the synthesis of glycosides from hexose phosphates. Sucrose synthesis is an example of synthesis of a disaccharide which can occur by each of these mechanisms (Fig. 8). In plants, sucrose is synthesized by the

SUCROSE SYNTHESIS

Plants

UDP-glucose + fructose ⇆
sucrose + UDP

Bacteria

Glucose-1-phosphate + fructose ⇆
sucrose + Pi

FIGURE 8

transfer of glucose from UDP-glucose to fructose, while, in bacteria, sucrose can be synthesized by the transfer of glucose from glucose-1-phosphate to fructose. There are several important differences between these two reactions. In the first place, the equilibrium of the transfer from UDP-glucose is displaced far to the right, while the equilibrium of the synthesis of sucrose from glucose-1-phosphate is only slightly to the right. Second, one of the products of the reaction which proceeds from the uridine nucleotide is uridine diphosphate, a comparatively unusual constituent of tissues. Moreover, the uridine diphosphate formed can be removed by phosphorylation, thus tending to pull the reaction even further toward the right; *i.e.*, toward the side of synthesis of the glycosidic linkage. On the other hand, the second product of the reaction proceeding from glucose-1-phosphate is inorganic phosphate, which occurs at high concentrations in many cells, and this reaction can therefore proceed only under conditions where the inorganic phosphate content is comparatively low.

A second example of this type involves the two enzymes known to catalyze the synthesis of glycogen, namely a uridine nucleotide transferase and glycogen phosphorylase, the enzyme discovered many years ago by the Coris, which synthesizes

glycogen in a reaction involving glucose-1-phosphate (Fig. 9). It is now believed,

GLYCOGEN SYNTHESIS

$$\text{UDP-glucose} + (\text{polyglucose})_n \rightleftarrows (\text{polyglucose})_{n+1} + \text{UDP}$$

$$\text{Glucose-1-phosphate} + (\text{polyglucose})_n \rightleftarrows (\text{polyglucose})_{n+1} + \text{Pi}$$

FIGURE 9

of course, that glycogen synthesis in animal cells occurs primarily by the first reaction, while glycogen phosphorylase serves primarily for the degradation of glycogen rather than for its synthesis.

Uridine diphosphoacetylglucosamine (Fig. 10) illustrates the structure of one

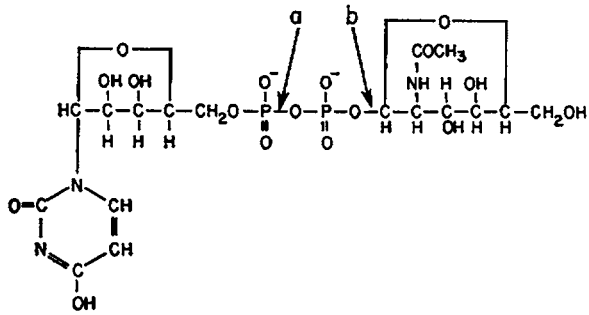

FIGURE 10 Uridine diphosphoacetylglucosamine.

of these nucleotides. It contains uridine linked through a pyrophosphate bridge to the sugar. The sugar may be considered as a fragment which is activated by the nucleotide for the synthetic reaction.

There are several groups of sugars which are activated as nucleotides. One group contains the hexose derivatives of uridine nucleotides, a principal member of which is uridine diphosphoglucose (Fig. 11). This key compound, as already mentioned, is formed by a reaction of UTP with glucose-1-phosphate. Once formed, it can be transformed in a number of ways, for example, by oxidation to UDP-glucuronic acid and then by epimerization of the glucuronic acid either to iduronic acid or to galacturonic acid. Furthermore, UDP-glucose itself can be epimerized to form UDP-galactose. Each of these sugars, linked to nucleotides, can act as a glycosyl donor for the synthesis of polysaccharides. For example, UDP-glucuronic acid participates in hyaluronic acid synthesis, UDP-glucose in glycogen synthesis, and UDP-galactose in the biosynthesis of complex bacterial polysaccharides. There are two congenital lesions which occur in man in reactions shown in Fig. 11. The first of these is the

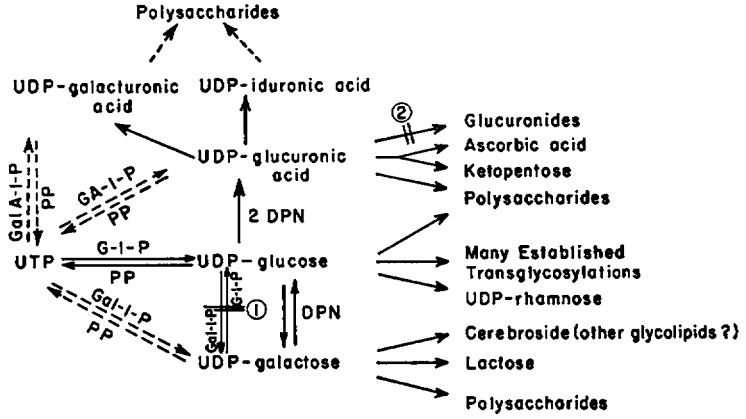

FIGURE 11 Uridine diphosphohexoses.

defect in the glucuronyl transferase, which leads to an inability to conjugate bilirubin as a glucuronide; this defect results in the disease known as congenital non-hemolytic jaundice. The second defect, an inability to transform UDP-galactose to UDP-glucose, produces the disease, congenital galactosemia.

There is another pathway for UDP-glucuronic acid metabolism (Fig. 12). In this

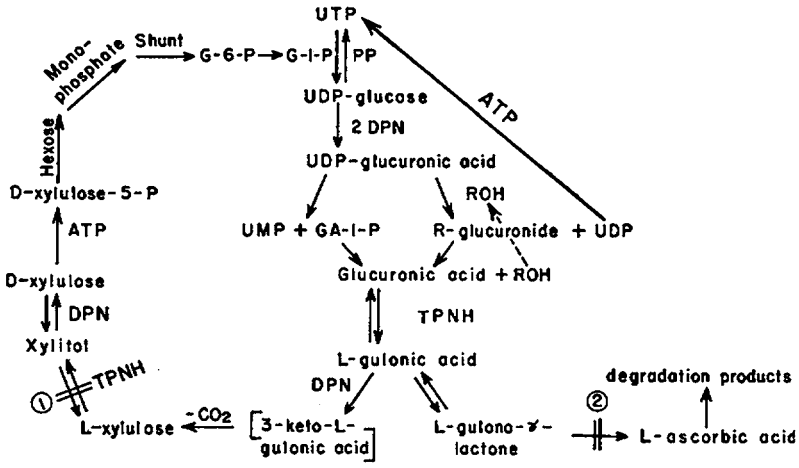

FIGURE 12 The glucuronic acid cycle.

pathway, the glucuronic acid is released from the nucleotide. After it is released, it can cycle through xylulose back to hexose phosphate, or it can lead to formation of ascorbic acid. Two more defects known in man, both benign, occur in the metabolism of these compounds. One of these is the inability to reduce L-xylulose to xylitol, which results in the benign condition, congenital pentosuria. The second

defect is shared by man with monkeys and guinea pigs, and is the inability to synthesize asorbic acid.

A second large group of sugars which can be activated as nucleotide derivatives is the amino sugars (Fig. 13). The central compound in this scheme is UDP-

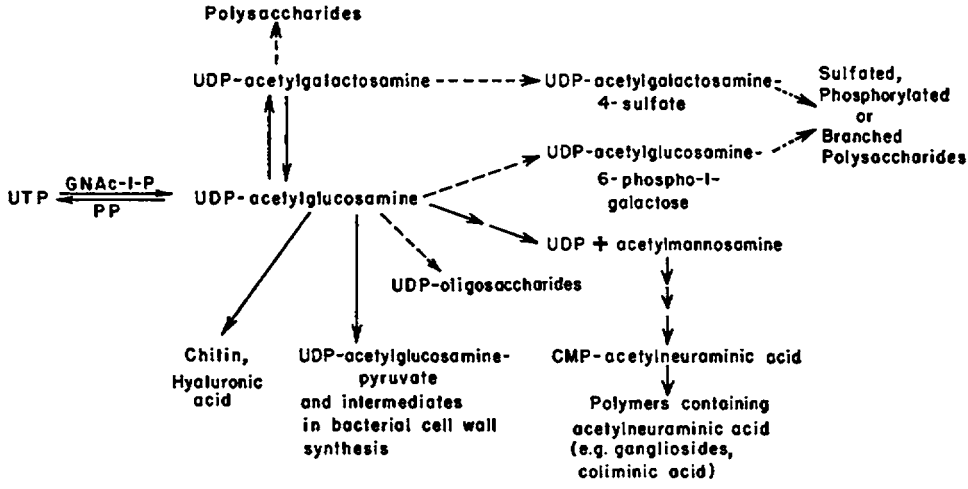

FIGURE 13 Uridine diphosphoacetylamino sugars.

acetylglucosamine. This compound, like UDP-glucose, is formed in a reaction between uridine triphosphate and acetylglucosamine-1-phosphate. The synthesis of chitin and the synthesis of hyaluronic acid are two reactions for which UDP-acetylglucosamine is a substrate. This compound is, however, also a precursor in a very complex reaction cycle which leads to the synthesis of bacterial cell walls. Furthermore, it participates in a reaction which leads to the synthesis of acetylmannosamine in animal tissues, the mechanism of which is still not understood. Acetylmannosamine can then be transformed into acetylneuraminic acid, and acetylneuraminic acid is then, in turn, activated as a cytidine nucleotide, CMP-acetylneuraminic acid.

Like UDP-glucose, UDP-acetylglucosamine can be epimerized, to UDP-acetylgalactosamine. Moreover, several substituted derivatives of UDP-acetylglucosamine and UDP-acetylgalactosamine are known. Two of these derivatives have been isolated only from the oviduct of the laying hen, which will be discussed in more detail in a moment. These two compounds are UDP-acetylglucosamine-phosphogalactose and UDP-acetylgalactosamine-4-sulfate. It is believed that nucleotides of these general types are intermediates in the biosynthesis of sulfated polysaccharides, although this fact has never been directly demonstrated experimentally. In the remainder of the time available, some attempts to investigate biosynthesis of chondroitin sulfate in the oviduct of the laying hen will be discussed.

The oviduct of the laying hen is a remarkable factory (Fig. 14). It is an assembly line, geared for the synthesis of the egg and of the egg shell. In the first 30 cm of this

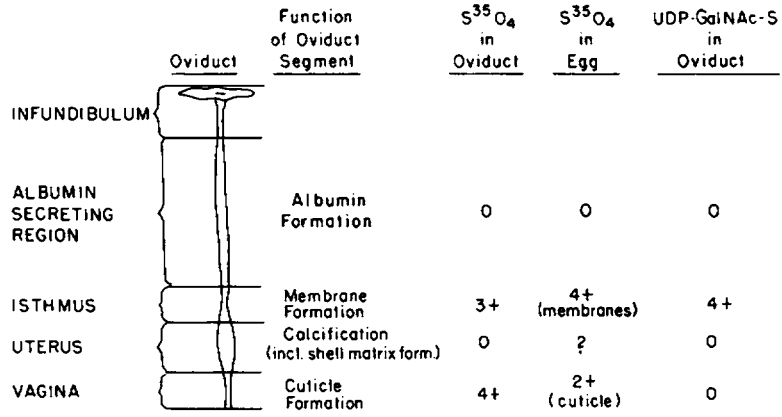

FIGURE 14 The oviduct of the laying hen.

assembly line, the albumin is secreted; in the next few centimeters the inner egg shell membranes are deposited. The inner egg shell membranes are sulfated mucopolysaccharides, related to chondroitin sulfates, but their chemistry has never been completely elucidated. On administration of S^{35}-inorganic sulfate to hens, isotope was rapidly incorporated into the isthmus of the oviduct, and, moreover, it also appeared in the egg shell membranes. The terminal region of the oviduct, the vagina, also incorporated inorganic sulfate into the glandular epithelium. This region also synthesizes a polysaccharide, the cuticle (which forms the smooth outer surface of the egg shell). The cuticle also contains some ester-bound sulfate.

Attention was directed to the synthetic processes which occur in the isthmus, however, because it was found that all of the UDP–acetylgalactosamine-sulfate found in the oviduct is localized in this region, where the inner egg shell membranes are synthesized. At about this time, Robbins, Gregory, and Lipmann had worked out the mechanism of sulfate activation and transfer (Fig. 15). This reaction sequence involves, first, the activation of sulfate to form adenyl sulfate, second, the phosphorylation of the active sulfate to yield phosphoadenosine phosphosulfate (PAPS).

ACTIVATION AND TRANSFER OF SULFATE

1. ATP + sulfate $\leftrightarrows$ AMP-sulfate + PP
   (APS)
2. AMP-sulfate + ATP $\rightarrow$ phospho-AMP-sulfate + ADP
   (PAPS)
3. Phospho-AMP-sulfate + acceptor $\rightleftarrows$ phospho-AMP + acceptor-sulfate
   (PAP)

FIGURE 15

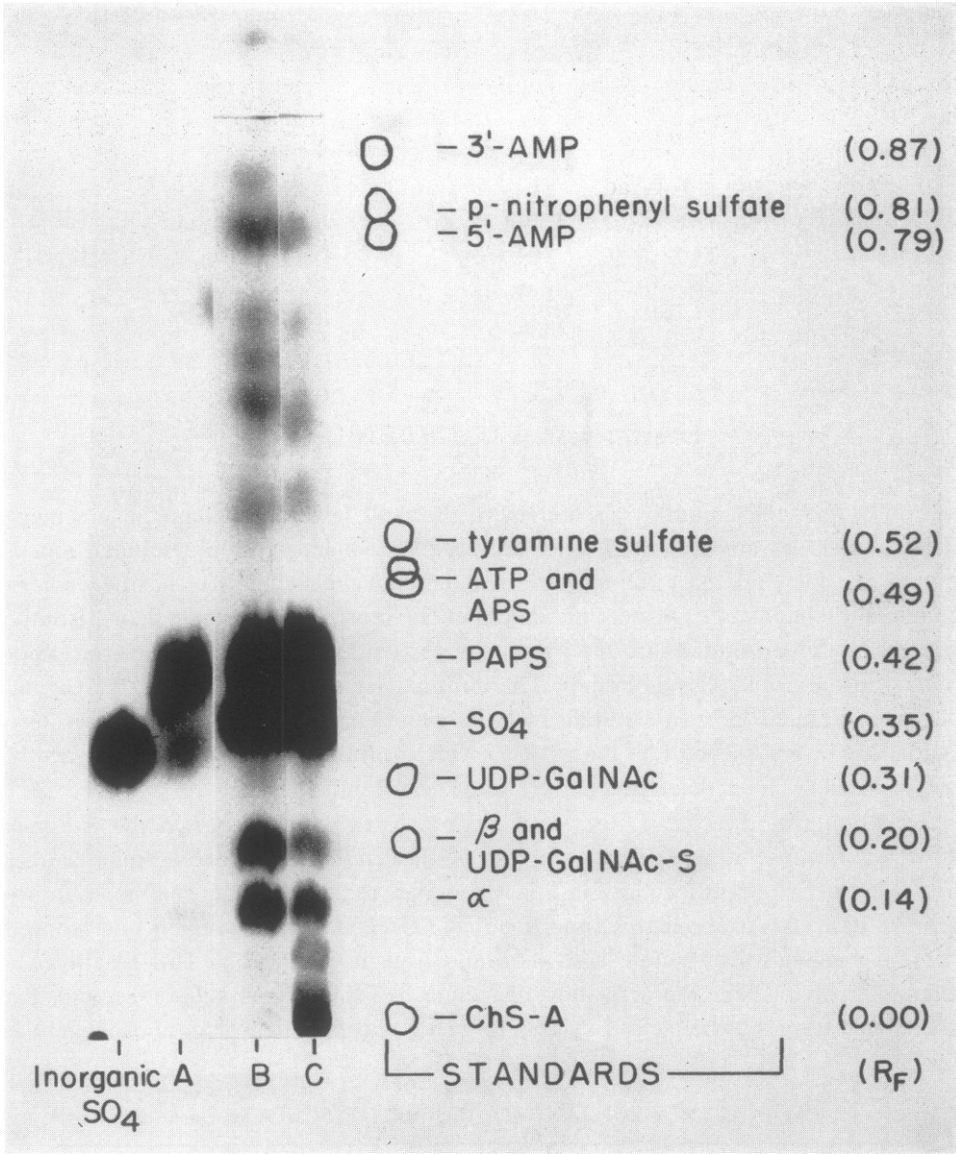

FIGURE 16 Radioautogram of a chromatogram of incubation mixtures containing $PAP^{35}S$ and an enzyme preparation from the isthmus of hen oviduct. (A) $PAP^{35}S$ and an enzyme preparation heated before incubation. (B) $PAP^{35}S$ and enzyme preparation. (C) As B but with 50 μg chondroitin sulfate A that had been partially hydrolyzed with hyaluronidase added. The substance with $R_f = 0$ is the polymer, the spots between $R_f = 0$ and 0.14 correspond to sulfated oligosaccharides. If the polymer is not treated with hyaluronidase before incubation, only the spot at $R_f = 0$ is found. α and β are unidentified radioactive products. β is not UDP-GalNAc-S although it has the same mobility in this solvent.

PAPS can transfer sulfate to a variety of acceptors, leading to the formation of sulfate esters. Radioactive PAPS was prepared and utilized in an attempt to trace the pathway of chondroitin sulfate synthesis in the isthmus of the hen oviduct, hoping that this reaction sequence would lead through the sulfated uridine nucleotide.

Using paper chromatography for the assay, it could readily be demonstrated that the radioactivity from radioactive phosphoadenosine phosphosulfate could be incorporated in the presence of an added acceptor into a high molecular weight compound, which remained at the origin of the chromatogram and could be shown to be a radioactive chondroitin sulfate (Fig. 16). If no acceptor was added, no transfer reaction was demonstrable. The system was activated by a boiled supernatant factor; it seemed possible that this might contain a uridine nucleotide which was essential for the transfer reaction. There was an absolute dependence on addition of polysaccharides as acceptors, and adenosine phosphosulfate would not substitute for PAPS as the sulfate donor (Fig. 17).

EXPERIMENT 1
SYSTEM WITH CHONDROITIN SULFATE A AS ACCEPTOR

	Radioactivity of the polysaccharide
	CPM
1. Complete	3320
2. Minus chondroitin sulfate A (added after incubation)	30
3. Minus $PAP^{35}S$ (added after incubation)	25
4. Boiled enzyme control	30
5. $AP^{35}S$ substituted for $PAP^{35}S$	100

EXPERIMENT 2
VARIOUS ACCEPTORS

	Radioactivity of the polysaccharide
	CPM
Chondroitin sulfate A	1750
Chondroitin sulfate B	1330
Chondroitin sulfate C	1060
Heparitin sulfate	1000
Heparin, hyaluronic acid, glycogen, or charonin sulfate	40 to 50

FIGURE 17

A variety of polysaccharides served as acceptors in this reaction; most of these compounds were very kindly given to us by our Chairman, Dr. Meyer. Chondroitin

from bovine cornea, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C (from human chondrosarcoma), heparitin sulfate, chondroitin sulfate D (from shark cartilage), and several other compounds would all act as sulfate acceptors in this reaction.

In considering the mechanism of this transfer reaction, there were two prominent possibilities (Fig. 18). One possibility was that sulfate was first transferred to a

TRANSFER OF SULFATE TO POLYSACCHARIDE VIA URIDINE NUCLEOTIDE

$$\text{UDP-GalNAc} \xrightarrow{\text{PAPS}} \text{UDP-GalNAc-S}$$

$$\text{Polysaccharide} \xrightarrow{\text{UDP-GalNAc-S}} \text{Polysaccharide-GalNAc-S}$$

DIRECT TRANSFER OF SULFATE TO POLYSACCHARIDE

$$\text{Polysaccharide} \xrightarrow{\text{PAPS}} \text{Polysaccharide-S}$$

FIGURE 18

uridine nucleotide present in the enzyme preparation (or in the boiled supernatant which was employed) leading to the formation of UDP-acetylgalactosamine-sulfate, and that, then, the acetylgalactosamine-sulfate moiety of the nucleotide was transferred to the acceptor, leading to the addition of a new monosaccharide unit on the polysaccharide acceptor. Then, by a sequential reaction involving also the participation of UDP-glucuronic acid, the chain length of the polysaccharide could be lengthened. There was, however, a second possibility, namely, that PAPS was transferring sulfate directly to the acceptor, leading to the formation of a higher sulfated polysaccharide.

It was impossible to study the mechanism of this reaction, using the high molecular weight compounds as acceptors, because the addition of monosaccharide sulfate to a high molecular weight compound could not be distinguished from the addition of sulfate alone. In order to study the mechanism of this reaction the sulfation of small oligosaccharides was, therefore, examined. Radioactive oligosaccharides were prepared by incubation of PAPS with several oligosaccharides. A new radioactive product was formed which, in each case, had a slower mobility than the acceptor which was employed (Fig. 19). These radioactive products were then isolated, and their nature determined by paper chromatography and electrophoresis (Fig. 20). In every case, it was found that the reaction was a simple addition of sulfate to the oligosaccharide acceptor, without the participation of a uridine nucleotide intermediate. For example, sulfate was transferred directly to the tetrasaccharide

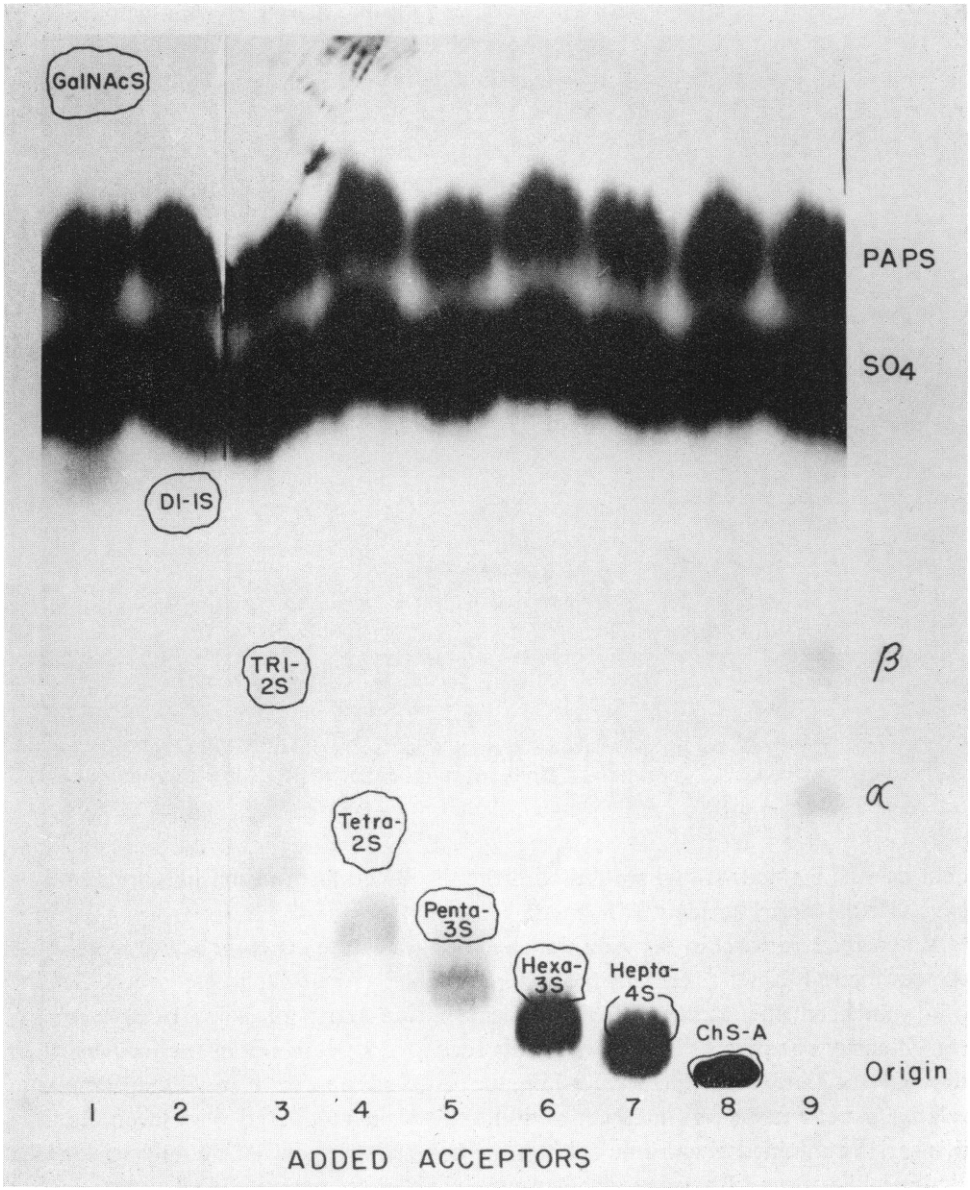

FIGURE 19 Preparation of radioactive oligosaccharides. Acceptors employed are encircled; *e.g.*, Tetra-2S is the tetrasaccharide disulfate derived from chondroitin sulfate A. The products formed on incubation of these acceptors with radioactive PAPS and enzyme are seen in this radioautogram of a chromatogram of these incubation mixtures.

containing no sulfate residues to form a tetrasaccharide containing one sulfate residue. If one started with the monosulfated tetrasaccharide, a disulfated tetrasac-

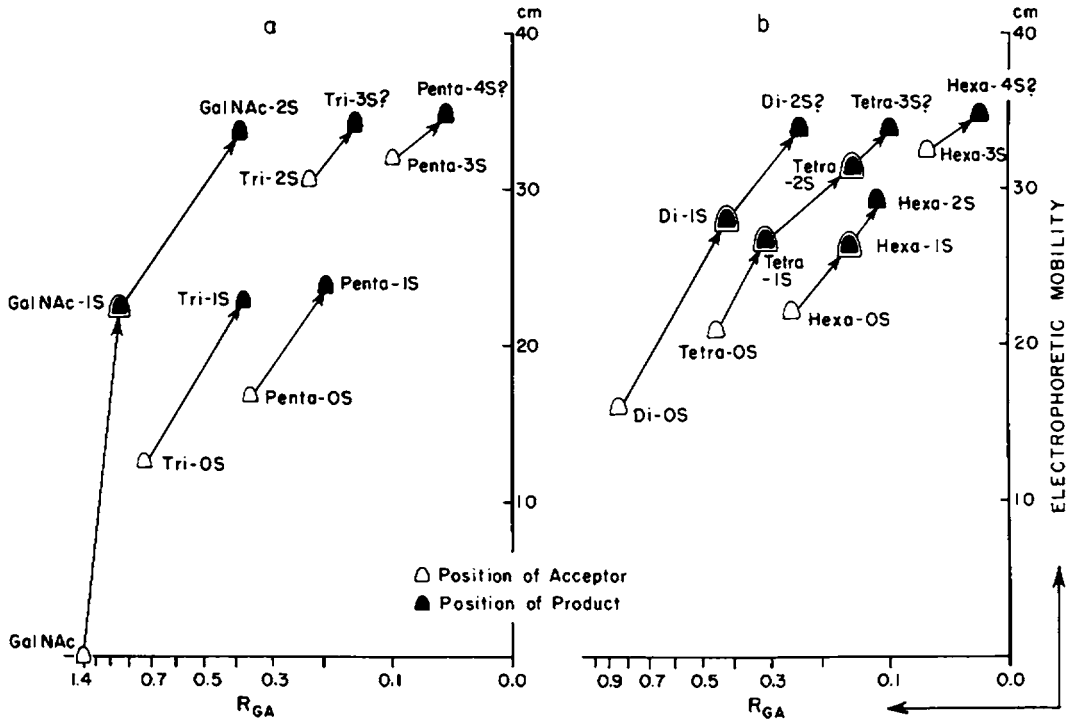

FIGURE 20 Products of sulfation of various oligosaccharides. Positions of the acceptors and products were derived from paper chromatography (abscissa) and electrophoresis (ordinate).

charide was formed. If one started with the disulfated tetrasaccharide, apparently a trisulfated tetrasaccharide was formed.

When the relationship between the chain length of the acceptor and the ability of the compound to act as an acceptor was examined (Fig. 21), it was found that the odd numbered oligosaccharides (*e.g.* trisaccharide and pentasaccharide) were extraordinarily effective as acceptors in this reaction, roughly equal in effectiveness to the polysaccharides themselves, while the even numbered oligosaccharides were relatively poor acceptors in the reaction. The even numbered oligosaccharides in this series contained glucuronic acid on the non-reducing end of the oligosaccharide chain, while the odd numbered oligosaccharides contained acetylgalactosamine on the non-reducing end of the polysaccharide chain. It may be, therefore, that one mechanism for the biosynthesis of the sulfate groups in the mucopolysaccharides involves the addition of sulfate at the non-reducing end of the growing polysaccharide chain when the acetylgalactosamine residue occurs at that non-reducing end.

The participation of uridine nucleotides in this process has not so far been directly demonstrated. However, a synthetic reaction of the sulfated uridine nucleotide

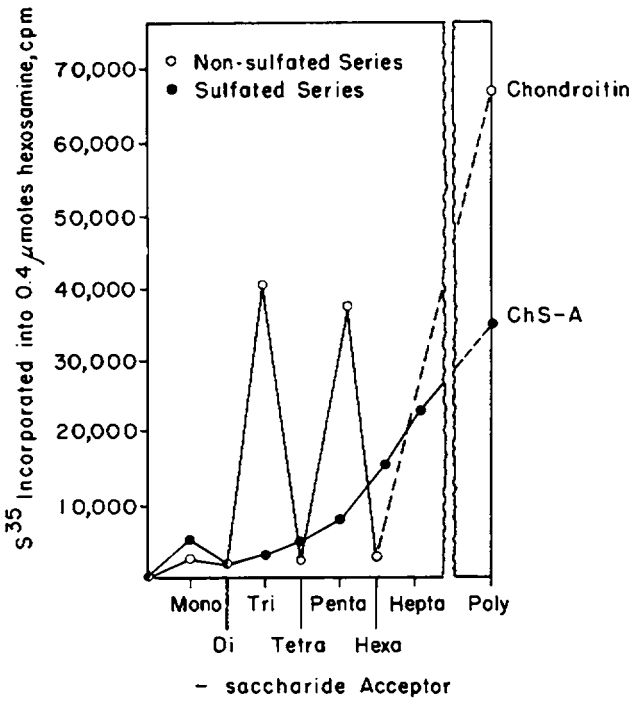

FIGURE 21 Relationship between chain length of the acceptor and the velocity of sulfate transfer.

certainly must exist, and there may, therefore, be several mechanisms for the introduction both of sugars and of sulfate into such polysaccharide chains. If the Heart Association has another meeting on this topic in 5 years, perhaps a great deal more information will be available about the mechanism of these reactions.

This paper is an edited transcript of the material presented. Work from the author's laboratory has been generously supported by research grants from the United States Public Health Service (NIAMD A-1158 and NIAID E-1902) and the National Science Foundation (G-18742).

REFERENCES

No references have been provided but a full discussion of these topics can be found in several excellent articles (1-3). The author has previously provided a complete list of references (4).

1. KORNBERG, A., Pyrophosphorylases and phosphorylases in biosynthetic reactions, *Adv. Enzymol.*, 1957, **18**, 191.
2. LELOIR, L. F., CARDINI, C. E., and CABIB, E., Utilization of free energy for the biosynthesis of saccharides, *in* Comparative Biochemistry, (M. Florkin and H. S. Mason, editors), New York, Academic Press Inc., 1960, **2**, 97.
3. LIPMANN, F., Biological sulfate activation and transfer, *Science*, 1958, **128**, 575.
4. STROMINGER, J. L., *Physiol. Rev.*, 1960, **40**, 55; *Angew. Chem., Internat. Ed.*, 1962, **1**, 134.