

METABOLISM OF ACID MUCOPOLYSACCHARIDES

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ABSTRACT The biosynthesis of the acid mucopolysaccharides, hyaluronic acid and chondroitin sulfuric acid, occurs by way of uridine nucleotides which contain the monosaccharide units of the respective polysaccharides. The mechanism of alternation of groups is as yet unknown. Certain of the acid mucopolysaccharides are covalently bound to protein by way of serine. In the case of the protein-polysaccharide complex of cartilage, there is evidence to suggest that the polysaccharide may be linked to the serine by way of galactose. Chondroitin sulfuric acid B may be isolated almost free of amino acids from the tissues and urine of patients with the Hurler syndrome without the use of proteolytic enzymes, acid, or alkali. This contrasts markedly with the tight binding of this compound to protein in normal tissue. It is suggested that the metabolic defect in this disease may reside in a defect of the peptide or linkage of the peptide to polysaccharide resulting in failure of the acid mucopolysaccharide to be fixed normally in connective tissue. Such a defect may result in interference with normal regulation of polysaccharide synthesis with a resultant increased synthesis. It is proposed that such a mechanism may obtain in other heritable connective tissue diseases as well as other storage diseases.

One of the problems that confronts the biochemist interested in biosynthetic pathways of acid mucopolysaccharides is the choice of adequate materials for study, since connective tissue although everywhere, is not separated from other tissues. I refer to the difficulty of obtaining connective tissue with a high density of cells appropriate for conventional biochemical studies.

For this reason, when, some 12 years ago, Dr. Saul Roseman and I began to investigate the biosynthesis of hyaluronic acid (HA), we chose to use rather than connective tissue, the group A hemolytic streptococcus. The capsule of this organism contains a polysaccharide chemically indistinguishable from the HA of mammalian connective tissue.

The earlier studies which now seem to be obvious, were concerned with the establishment of the remote precursors of the fourteen unique carbon atoms of HA. It was demonstrated, somewhat laboriously, that glucose was the precursor of both the hexosamine and the uronic acid portions of the molecule, without breakdown of the hexose chain (1-3). In view of the knowledge which has accumulated regard-

ing glycosyl transfer *via* uridine nucleotides, the explanation of these findings is clear.

Following the discovery of the uridine nucleotides by Leloir and coworkers (4) and the demonstration of their function as glycosyl donors, compounds of this type became reasonable candidates for intermediates in HA synthesis. Studies with Dr. Cifonelli (5) demonstrated that a strain of group A streptococcus, which synthesized abundant HA, contained both uridine diphospho-*N*-acetylglucosamine (UDPGNAc) and uridine diphosphoglucuronic acid (UDPGA), the nucleotides that might be expected to participate in HA synthesis. During the course of these studies, a large amount of UDPGA became available as a result of the extraction of a large batch of commercially grown streptococci apparently derived from a mutant strain which accumulated UDPGA. Tritiation by the Wilzbach procedure (6) produced a radioactive product which was used for biosynthetic studies.

When a suspension of streptococci was disrupted by sonic oscillation by Dr. Markovitz, a cell-free enzyme preparation was obtained which requires for synthesis of HA, only UDPGNAc, UDPGA, and Mg^{++} . The enzyme was found to sediment at $100,000 \times g$. With this enzyme, not only was radioactivity incorporated in HA from labeled UDPGNAc and UDPGA, but also net synthesis was demonstrated (7).

The difficulty encountered in solubilizing this enzyme raised the question of its *in vivo* localization. This seemed of particular importance in view of growing evidence of macromolecular syntheses at membrane surfaces.

About that time, an enzyme originally discovered by Maxted and extensively studied by Krause and Friemer *et al.* (8, 9) became available. This enzyme, produced by Group C streptococci following phage infection, hydrolyzes the cell wall of Group A streptococci. It serves as a convenient tool for the preparation of protoplasts from Group A streptococci. When protoplasts prepared in this fashion were osmotically lysed and washed, the HA synthesizing activity was found to be associated with the membranes. Treatment of such preparations with RNase and DNase did not interfere with HA synthesis (10). These findings are of interest in view of the suspicion that the protoplast membrane of bacteria may be functionally homologous to the endoplasmic reticulum of mammalian cells.

Unlike protein synthesis, RNA does not seem requisite to HA synthesis. Recently, Dr. Sara Schiller has demonstrated HA synthesis in an extract of embryonic rat skin. Although this enzyme has not been yet studied in detail, it appears to be associated with a particulate fraction (11).

Other properties of the enzyme which synthesizes HA are of interest. Polysaccharide synthesis usually cannot be demonstrated with monosaccharide units, but larger molecular weight acceptors are necessary. In our system, no primer requirement has been demonstrated. The possibility that minute amounts of primer are attached to the enzyme must be considered. In a series of experiments the enzyme was made highly radioactive by growth of the organisms in radioactive acetate. Hyaluronic acid synthesis was carried out with the radioactive enzyme and non-

labeled nucleotides. The radioactivity of the HA formed indicated that less than 1 per cent of the polysaccharide was derived from the enzyme. These results do not rule out a primer requirement, but if such exists, it is below the level of detection by available methods (11).

From the point of view of enzyme mechanism, the problem of alternation of groups is of interest. One possible mechanism is the formation of a disaccharide attached to a nucleotide. Polysaccharide formation may then occur by polymerization of such units. Another possible mechanism involves the alternate addition of monosaccharide units from nucleotides to form the polysaccharide.

Some years ago, Dr. Cifonelli (12) isolated small amounts of a nucleotide which appeared to contain a disaccharide, but the amounts were so small that this work could not be carried out short of some massive system for growth of organisms.

More recently, Jourdan *et al.* (13) and Kobata (14) have found nucleotides containing more than one sugar. This finding reopens the possibility that such an intermediate may be involved in HA synthesis.

We may now turn to a consideration of some of the problems of the biosynthesis of the chondroitin sulfates, some of which have been discussed by Dr. Strominger. I would like to speak of other aspects of the problem. Fig. 1 indicates the structure of the disaccharide repeating unit of chondroitin sulfuric acid A.

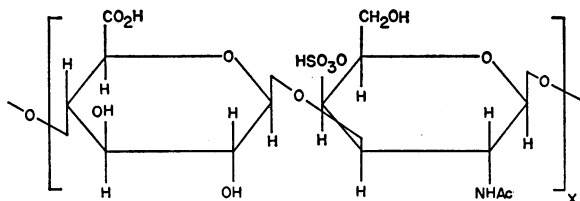


FIGURE 1 Repeating disaccharide unit of chondroitin sulfuric acid A.

The problems of synthesis of the chondroitin sulfuric acids are more complicated than the synthesis of HA in several respects. These may be enumerated as follows:—
 (a) The formation of galactosamine in all three chondroitin sulfuric acids. Starting with the glucose configuration, an epimerization of C-4 is required. In the case of chondroitin sulfuric acid B, another epimerization reaction for the formation of L-iduronic acid is required. (b) The addition of sulfate groups to the 4 or 6 positions in chondroitin sulfuric acids A and C respectively (c). The linkage to proteins and the relationship of polysaccharide to protein synthesis to form the chondromucoprotein.

Several years ago, the late Dr. John Gross studied the turnover rate of the protein and polysaccharide portions of the chondroitin sulfuric acid–protein complex in rat costal cartilage. This was accomplished by labeling the protein with lysine- C^{14} and

the polysaccharide with S^{35} -sulfate. Both portions of the molecule were found to turn over at about the same rate (15). Such experiments do not contribute to the understanding of the pathways of biosynthesis, but do indicate that the entire complex is probably renewed in the matrix as a unit.

Since the original work of D'Abramo and Lipmann (16), now amply confirmed in a number of laboratories, it has become apparent that cartilage contains a sulfation system which transfers sulfate from phospho-adenosine-phosphosulfate (PAPS) to an appropriate acceptor which is apparently polysaccharide. Enzyme preparations which demonstrate sulfation show no evidence of polysaccharide synthesis.

In order to learn more about several aspects of the synthesis of cartilage matrix, we have recently undertaken a series of experiments with epiphyses of 13 day old chick embryos. For the original studies carried out primarily by Dr. Frank K. Thorp, chondrocyte suspensions were prepared by treatment of tibial and femoral epiphyses with trypsin (17). This method, originally used by Moscona and Moscona (18), and now widely used in tissue culture work, serves to remove the matrix.

Cell suspensions prepared in this manner may be washed free of uronic acid-containing material and used for a variety of metabolic experiments. Radioactive sulfate is readily incorporated by such preparations. When chondrocyte suspensions are incubated in a tissue culture medium, polysaccharide rapidly accumulates in the medium. In earlier experiments, despite evidence of sulfate incorporation and polysaccharide accumulation in the medium, no incorporation of radioactive acetate indicative of polysaccharide synthesis was observed. These data suggested that the polysaccharide which appears in the medium was not synthesized *de novo* during the incubation, but rather originated from a preformed pool within the cells. When estimations were made of the amount of chondroitin sulfate present within the cells before and after incubation, it became clear that this supposition was true. Whether this phenomenon resulted from cell breakdown or excretion is difficult to determine.

These findings indicated the need for a better characterization of intracellular chondroitin sulfate. Using appropriate methods, it was possible to isolate intracellular chondroitin sulfate and compare this with material isolated from the matrix. The distinction between chondroitin sulfuric acids A and C was carried out by Dr. Mathews by a colorimetric method (19). The results were confirmed by infrared spectroscopy. It is to be noted that both intracellular and extracellular chondroitin sulfuric acid consist of a mixture of the 4 and 6 sulfate isomers. A portion of this material is not sulfated.

The fact that conditions have been found under which sulfation occurs without polysaccharide formation, suggests that polysaccharide formation precedes sulfation. This concept originally proposed by Davidson and Meyer (20) is in accord with recent studies by Mathews and Hinds on tadpole metamorphosis (21).

The problem of the biosynthesis of chondroitin sulfuric acid by chondrocyte suspensions has been pursued further in our laboratory by Dr. Robert L. Perlman.

For reasons which are not yet clear to us, under what were apparently identical conditions previously used by Dr. Thorp, Dr. Perlman found excellent incorporation into chondroitin sulfuric acid of acetate-1-C¹⁴. The data of one such experiment are illustrated in Table I. These data indicate polysaccharide synthesis.

TABLE I
INCORPORATION OF S³⁵O₄⁻ AND C¹⁴-ACETATE

Sample		1st purification	2nd purification	3rd purification
		specific activity*	specific activity*	specific activity*
S ³⁵ O ₄ ⁻	4°	0.2	0.1	0.2
	37°	51.7	55.8	52.6
C ¹⁴ -acetate	4°	0.2	0.1	0.1
	37°	89.5	86.5	80.1

* Counts/minute/μg uronic acid.

The next series of experiments was performed in order to determine the pathway of biosynthesis of chondroitin sulfuric acid utilizing chondrocyte suspensions. In the course of other work in progress in our laboratory, Dr. Eugene N. Fox had found that Group A streptococci became permeable to ATP following treatment with trypsin. Other studies by Dr. Howard Holtzer and his colleagues had shown that trypsinization leads to changes in permeability of mammalian cells (22). It seemed possible that trypsinized chondrocyte suspensions are permeable to phosphorylated intermediates.

For this reason, a series of experiments was conducted to determine whether certain appropriate phosphorylated intermediates were incorporated into chondroitin sulfuric acid when incubated with trypsinized chondrocyte suspensions. The data obtained in such experiments are presented in Table II. In all cases, the compounds

TABLE II
INCORPORATION OF GNAC-1-P, GalNAc-1-P, AND UDPGNAC

Sample		1st purification	2nd purification	3rd purification	Incorporation
		specific activity*	specific activity*	specific activity*	<i>per cent</i>
GNAC-1-P	4°	248	58		
	37°	11,000	13,000		0.51
GalNAc-1-P	4°	9	19		
	37°	522	539		0.06
UDPGNAC	4°	37	98	24	
	37°	1,988	2,014	2,035	0.14

* Counts/minute/mg uronic acid.

were labeled in the acetyl groups with tritium. To be certain that incorporation was not by way of free acetate as a result of hydrolysis, acetate was isolated from the reaction mixture. There was little radioactivity in this fraction. The data in Table II indicate that the *N*-acetylglucosamine-1-PO₄, UDPGNac, and to a much lesser extent *N*-acetylgalactosamine-1-PO₄ serve as precursors of chondroitin sulfate.¹

These data suggest the following pathway for the synthesis of chondroitin sulfates in cartilage.

- (1) $\text{GNAc}-1-\text{P} + \text{UTP} \rightarrow \text{UDPGNAc} + \text{UDP}$
- (2) $\text{UDPGNAc} \rightarrow \text{UDPGalNAc}$
- (3) $\text{GalNAc}-1-\text{P} + \text{UTP} \rightarrow \text{UDPGalNAc}$
- (4) $\text{UDPGalNAc} + \text{UDPGA} \rightarrow \text{Chondroitin}$
- (5) $\text{Chondroitin} + \text{PAPS} \rightarrow \text{CSA}-4-\text{SO}_4$
- (6) $\text{Chondroitin} + \text{PAPS} \rightarrow \text{CSA}-6-\text{SO}_4$

Reactions 1 and 2 and reaction 3 represent alternate pathways for the formation of UDPGalNAc. Reaction 3 is included in view of low but definite incorporation of radioactivity from GalNAc-1-P. It is also possible, however, that UDPGalNAc formation occurs by way of a uridyl transferase enzyme.

It is apparent that we are approaching an elucidation of the pathway of biosynthesis of the chondroitin sulfates. However, the mechanism responsible for the formation of the protein-polysaccharide linkage remains unknown.

We may now turn to a consideration of the metabolism of acid mucopolysaccharides from another point of view. Since 1956 we have been interested in the Hurler syndrome or gargoylism. This heritable disease of connective tissue is characterized by a massive deposition of acid mucopolysaccharides with a consequent striking structural and functional distortion of tissues. The afflicted individuals show marked bony abnormalities, enlarged liver and spleen, limitations of motion in various joints, hernias, corneal clouding, cardiac disease, mental retardation, and numerous other abnormalities. The disease appears to occur in two different genetic forms, an autosomal recessive and a sex-linked recessive, each of which has somewhat different clinical characteristics.

In 1952, Brante (23) in Sweden, isolated, from the livers of two patients with Hurler's syndrome, a large amount of polysaccharide which analytically corresponded to chondroitin sulfuric acid. In 1957, we isolated from the urine of a patient with the Hurler syndrome, a large amount of a mixture of chondroitin sul-

¹ Since this paper was presented it has been possible to isolate a cell-free enzyme preparation from cartilage which results in the incorporation of radioactivity from both UDPGNac and UDPGalNAc into chondroitin sulfate in the presence of UDPGA and Mg⁺⁺. Once again the enzyme appears to be particulate. The radioactivity is highest in a fraction eluted from a Dowex 1 column at 0.5 M NaCl suggesting that polymerization precedes sulfation (Perlman, R. L., and Dorfman, A., *Fed. Proc.*, 1963, **22**, 413). More recently the polymerase and sulfate transferase have been completely separated.

furic acid B and heparitin monosulfuric acid (24). Since that time, numerous reports have indicated that these two polysaccharides occur in both the urine and tissues of patients with this disease (25). Unexplained as yet, is the variation in proportion of the two polysaccharides in different patients.

The genetically determined metabolic defect which results in this massive excretion and deposition of normally occurring connective tissue mucopolysaccharides is not yet clear. A variety of hypotheses involving absence of degradative enzymes, abnormal cells, and control mechanisms has been offered. In view of the existent knowledge of the binding of sulfated mucopolysaccharides to protein, it seemed of interest to investigate more fully the state of binding of the polysaccharides excreted and deposited in tissues in the Hurler syndrome.

Dr. Klaus von Berlepsch has isolated chondroitin sulfuric acid B from the urine of two patients by the use of chromatographic and electrophoretic methods which do not rupture peptide bonds. Similarly, Miss Pei-Lei Ho has isolated polysaccharide from the spleen of one patient using water extraction and equally mild methods of purification. Analyses of two fractions obtained from the spleen and of two urine preparations are given in Table III. Striking is the fact that in all cases there is little

TABLE III

	Hurler's				Human skin CS-B, papain- digested
	Spleen 1.0	Spleen 1.3	Urine L.H.	Urine v.W.	
Aspartic acid	1.85	0.29	0.20	0.22	2.84
Threonine	2.39	0.22	Tr	0.23	0.92
Serine	2.56	0.65	1.03	0.82	3.50
Glutamic acid	2.06	0.45	0.21	0.25	3.09
Glycine	2.14	0.57	0.22	0.34	5.88
Alanine	1.37	0.19	0.14	Tr	2.77

All results expressed as moles of amino acid per 100 moles of galactosamine.

excess nitrogen representing amino acid contamination. These preparations were subjected to amino acid analysis on a technicon autoanalyzer by Dr. Lennart Rodén. The results obtained are compared to a preparation of chondroitin sulfuric acid B isolated from human skin after extensive digestion with papain. It is to be noted that all the Hurler preparations contain significantly fewer amino acids than does the preparation obtained from normal skin. The spleen 1.0 preparation was least pure as indicated by the lower optical rotation and a slightly higher contamination with glucosamine. In the best of these preparations, there were fewer than three amino acid residues per 100 moles of galactosamine. Other studies in this laboratory by Rodén *et al.* (26) have shown that a number of chondroitin sulfuric acid B preparations isolated from various sources prepared after extensive proteolytic digestion

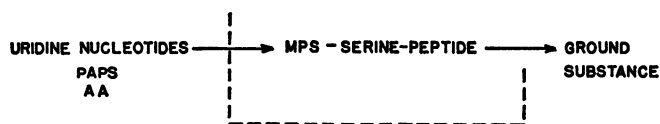
still contained considerably larger amounts of amino acids than were found in the preparations from Hurler's syndrome.

Before continuing this discussion of the Hurler syndrome, it would be useful to digress briefly to consider some recent findings regarding the mucopolysaccharide-protein link. Muir (27) originally discovered that following proteolytic digestion of a preparation of chondromucoprotein from cartilage, hydrolysis of the residual polysaccharide showed the presence of serine. Dr. Lennart Rodén and Dr. John Gregory (28, 29), in work originating in Uppsala, and more recently carried out in our laboratories and at The Rockefeller Institute, have pursued this question further. By means of digestion of chondromucoprotein with testicular hyaluronidase and proteolytic enzymes, they have isolated material with a molecular weight of about 1080 as determined in the ultracentrifuge by Dr. Torvard Laurent. This material contains in addition to galactosamine and uronic acid, galactose and serine. Paper chromatograms of the sugar components also show a spot with the staining properties of a pentose. The origin of this material is not clear. Depending on the extent of treatment with proteolytic enzymes, small amounts of other amino acids are present. Reduction of this material with borohydride does not decrease the galactose, hexosamine, or uronic acid content indicating the absence of a terminal reducing group.

These experiments suggest that sulfated mucopolysaccharides are firmly bound to protein probably through a serine residue. Many problems concerning this structure remain unresolved. Dr. Schubert has already discussed some of the problems of the structure of chondromucoprotein.

If we may reason by analogy, it seems highly likely that chondroitin sulfuric acid B in skin is also bound to protein. The excretion and deposition in the Hurler syndrome of chondroitin sulfuric acid B, relatively free of protein, raise the possibility that the metabolic defect in Hurler's syndrome may be due to a defect in protein binding. This may follow the conventional pattern so well illustrated by the hemoglobins, of a defect in the peptide or may be due to an enzymatic defect in bringing about linkage to protein. Defective linkage to protein may also be consequent to overproduction of polysaccharide due to a mutant regulator gene. In either case, the overproduction of mucopolysaccharides may be regarded in terms of the scheme illustrated in Fig. 2. The suggestion is made that following or simultaneous with polymerization of the appropriate nucleotides and sulfation, the carbohydrate is linked to a peptide which in turn polymerizes to form the normal matrix material of ground substance. Whether final formation of the macromolecular complex occurs intracellularly or extracellularly and whether some other protein moiety is involved are at present unknown. The occurrence of chondromucoprotein with variable protein content has led us to suspect that some protein, other than that directly attached to the mucopolysaccharide, may be involved in forming the macromolecule. It is suggested that normally deposition of appropriate amounts of ground substance results in some type of feedback inhibition which controls mucopolysaccharide synthesis.

NORMAL



HURLER'S SYNDROME

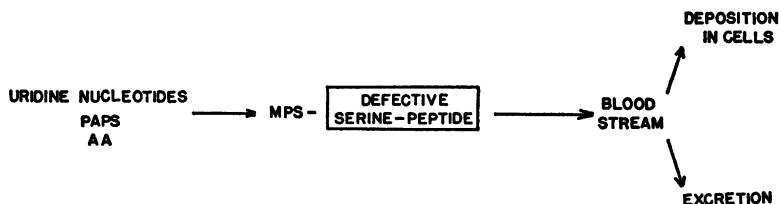


FIGURE 2 Proposed metabolic defect in the Hurler syndrome.

If the formation of normal complex is defective in Hurler's syndrome, the unbound mucopolysaccharide may readily diffuse to the blood stream and consequently may be deposited in many types of cells as well as excreted in the urine. Under these conditions, feedback inhibition does not occur at a rapid rate.

This hypothesis must be regarded as conjectural at this time until more evidence is available. It is attractive in that it makes the Hurler syndrome understandable in terms of extant knowledge regarding other human genetic defects. It also opens the possibility for the understanding of other heritable diseases of connective tissue and forms a link between the known mechanisms of metabolic heritable defects and heritable defects characterized by structural abnormalities. It is of interest to point out that lipids found in the lipid storage diseases are normally bound to protein. Failure of such binding may play a role in the lipoidoses.

Many aspects of the Hurler syndrome need further clarification in these terms. Why are two mucopolysaccharides, heparitin monosulfate and chondroitin sulfuric acid B, affected? More data are needed regarding the structure of heparitin sulfate and its binding to protein. How may the two genetic forms of the disease be explained? If present genetic concepts of these two forms are correct, do they represent mutations on two different chromosomes? Perhaps the genetic data must be re-examined in view of the Lyon hypothesis (30). If the defective gene is on the X chromosome, it is possible that the apparent two forms of the disease may be reconciled in these terms.

SUMMARY

An attempt has been made to review the current status of knowledge regarding

the biosynthesis of acid mucopolysaccharides. The pathway of formation of HA from glucose *via* uridine nucleotides has now been largely elucidated although important questions remain regarding the mechanism of alternation of monosaccharide units. The pathway of biosynthesis of chondroitin sulfates is gradually becoming clear. It seems highly likely that polymerization precedes sulfation.

There is considerable evidence that the sulfated acid mucopolysaccharides are bound covalently to protein by way of a serine linkage. The relationship of polysaccharide synthesis to peptide synthesis is unknown. From both tissues and urine of patients with the Hurler syndrome, chondroitin sulfuric acid B may be isolated almost free of protein without the use of proteolytic enzymes. It is suggested that the metabolic defect in this disease may reside in a defect of the peptide, or linkage of the peptide to polysaccharide resulting in failure of the acid mucopolysaccharides to be fixed normally in connective tissue. Such a defect may result in increased polysaccharide synthesis. It is proposed that such a mechanism may obtain in other heritable connective tissue diseases as well as in other storage diseases.

The original investigations referred to in this manuscript were aided by grants from the United States Public Health Service (No. AH-5996), The National Foundation, and The Chicago Heart Association.

BIBLIOGRAPHY

1. ROSEMAN, S., MOSES, F. E., LUDOWIEG, J., and DORFMAN, A., The biosynthesis of hyaluronic acid by group A streptococcus. I. Utilization of 1-C¹⁴ glucose, *J. Biol. Chem.*, 1952, **203**, 213.
2. ROSEMAN, S., LUDOWIEG, J., MOSES, F. E., and DORFMAN, A., The biosynthesis of hyaluronic acid by group A streptococcus. II. Origin of the glucuronic acid, *J. Biol. Chem.*, 1954, **206**, 665.
3. DORFMAN, A., ROSEMAN, S., MOSES, F. E., LUDOWIEG, J., and MAYEDA, M., The biosynthesis of hyaluronic acid by group A streptococcus. III. Origin of the *N*-acetylglucosamine moiety, *J. Biol. Chem.*, 1955, **212**, 583.
4. LELOIR, L. F., and CARDINI, C. E., Uridine nucleotides, in *The Enzymes*, (P. D. Boyer, H. Lardy, and K. Myrback, editors), New York, Academic Press Inc., 1960, **2**, 39.
5. CIFONELLI, J. A., and DORFMAN, A., The biosynthesis of hyaluronic acid by group A streptococcus. V. The uridine nucleotides of group A streptococcus, *J. Biol. Chem.*, 1957, **228**, 547.
6. WILZBACH, K. E., Tritium-labeling by exposure of organic compounds to tritium gas, *J. Am. Chem. Soc.*, 1957, **79**, 1013.
7. MARKOVITZ, A., CIFONELLI, J. A., and DORFMAN, A., The biosynthesis of hyaluronic acid by group A streptococcus. VI. Biosynthesis from uridine nucleotides in cell-free extracts, *J. Biol. Chem.*, 1959, **234**, 2343.
8. KRAUSE, R. M., Studies on the bacteriophages of hemolytic streptococci. II. Antigens released from the streptococcal cell wall by a phage-associated lysin, *J. Exp. Med.*, 1958, **108**, 803.
9. FRIEMER, E. H., KRAUSE, R. M., and McCARTY, M., Study of L forms and protoplasts of group A streptococci, *J. Exp. Med.*, 1959, **110**, 853.
10. MARKOVITZ, A., and DORFMAN, A., Synthesis of capsular polysaccharide (hyaluronic acid) by protoplast membrane preparations of group A streptococcus, *J. Biol. Chem.*, 1962, **237**, 273.

11. SCHILLER, S., SLOVER, G., and DORFMAN, A., The synthesis of hyaluronic acid by cell-free extracts of embryonic skin, *Biochem. and Biophysic. Research Commun.*, 1961, **5**, 344.
12. CIFONELLI, J. A., and DORFMAN, A., Isolation of uridine nucleotides from group A streptococci, *Fed. Proc.*, 1955, **14**, 193.
13. JOURDIAN, G. W., SHIMZU, F., and ROSEMAN, S., Isolation of nucleotide oligosaccharides containing sialic acid, *Fed. Proc.*, 1961, **20**, 161.
14. KOBATA, A., Isolation and identification of two novel uridine nucleotide oligosaccharides from human milk, *Biochem. and Biophysic. Research Commun.*, 1962, **7**, 346.
15. GROSS, J. I., MATHEWS, M. B., and DORFMAN, A., Sodium chondroitin sulfate-protein complexes of cartilage. II. Metabolism, *J. Biol. Chem.*, 1960, **235**, 2889.
16. D'ABRAMO, F., and LIPMANN, F., The formation of adenosine-3'-phosphate-5'-phosphosulfate in extracts of chick embryo cartilage and its conversion into chondroitin sulfate, *Biochim. et Biophysica Acta*, 1957, **25**, 211.
17. THORP, F. K., and DORFMAN, A., The occurrence of intracellular chondroitin sulfate, *J. Cell Biol.*, 1963, **18**, 13.
18. MOSCONA, A., and MOSCONA, H., The dissociation and aggregation of cells from rudiments of early chick embryos, *J. Anat.*, 1952, **86**, 287.
19. MATHEWS, M. B., and INOUE, M., The determination of chondroitin sulfate C-type polysaccharides in mixtures with other acid mucopolysaccharides, *Biochim. et Biophysica Acta*, 1961, **53**, 509.
20. DAVIDSON, E. A., and MEYER, K., Chondroitin, a new mucopolysaccharide, *J. Biol. Chem.*, 1954, **211**, 605.
21. MATHEWS, M. B., and HINDS, L., Acid mucopolysaccharides and frog metamorphosis, *Fed. Proc.*, 1962, **21**, 167.
22. HOLTZER, H., Some further uses of antibodies for analyzing the structure and development of muscle, *Exp. Cell Research*, 1959, suppl. 7, 234.
23. BRANTE, G., Gargoylism—a mucopolysaccharidosis, *J. Clin. and Lab. Inv.*, 1952, **4**, 443.
24. DORFMAN, A., and LORINCZ, A. E., Occurrence of urinary acid mucopolysaccharides in the Hurler syndrome, *Proc. Nat. Acad. Sc.*, 1957, **43**, 443.
25. MEYER, K., GRUMBACH, M. M., LINKER, A., and HOFFMAN, P., Excretion of sulfated mucopolysaccharides in gargoylism (Hurler's syndrome), *Proc. Soc. Exp. Biol. and Med.*, 1958, **97**, 275.
26. RODÉN, L., LINDAHL, B., SCHILLER, S., and DORFMAN, A., unpublished results.
27. MUIR, H., The nature of the link between protein and carbohydrate of a chondroitin sulfate complex from hyaline cartilage, *Biochem. J.*, 1958, **69**, 195.
28. GREGORY, J. D., and RODÉN, L., Isolation of keratosulfate from chondromucoprotein of bovine nasal septum, *Biochem. and Biophysic. Research Commun.*, 1961, **5**, 430.
29. RODÉN, L., GREGORY, J. D., and LAURENT, T. C., Isolation of glycopeptides from the chondroitin 4-sulfate-protein complex of cartilage, *Fed. Proc.*, 1963, **22**, 413.
30. LYON, M. F., Gene action in the x-chromosome of the mouse (*Mus musculus* L.), *Nature*, 1961, **190**, 372.