METABOLISM OF THE MUCOPOLYSACCHARIDES OF CONNECTIVE TISSUE*†

ALBERT DORFMAN

Department of Pediatrics, University of Chicago and La Rabida Jackson Park Sanitarium, Chicago, Illinois

The term acid mucopolysaccharides has been widely used to denote a group of chemical compounds which appear to be high molecular weight polymers composed of the hexosamines, glucosamine or galactosamine and glucuronic acid, acetate, and in some cases esterified sulfate. To date, two of these substances, hyaluronic acid (H.A.) and chondroitinsulfuric acid (C.S.A.), have been well characterized in connective tissue. Although extensively investigated, the chemical nature of heparin is still the subject of some confusion, with evidence that there may be a family of such compounds. There also appears to be evidence for the existence of compounds whose composition is identical with that of C.S.A. of cartilage, but differing in other properties. Thus, Meyer and Chaffee (99) and Meyer and Rapport (101) have shown that chondroitin B, which exists in skin, differs from cartilage chondroitin (A) in its optical rotation and susceptibility to hydrolysis by testicular hyaluronidase. What appears to be a similar compound has been isolated by Marbet and Winterstein (94, 95) from lung and originally called β-heparin because of anticoagulant properties. A possibly identical substance has been isolated by Smith and Gallop (138) from hog gastric mucosa (polysaccharide B). In recent studies Schiller, Mathews, and Dorfman (131) have isolated in analytically pure form a sulfated polysaccharide which appears to be identical in composition with cartilage C.S.A., but gives a lesser color equivalent by the Dische carbazole reaction. Treatment of this substance with testicular hyaluronidase results in some apparent hydrolysis as measured by the turbidity method, suggesting that this may be a mixture of cartilage C.S.A. and so called chondroitin B or β-heparin. A fifth acid mucopolysaccharide, named chondroitin C, has also been postulated by Meyer and Rapport (101). This differs from chondroitin A in optical rotation, but unlike chondroitin B *is* hydrolyzed by testicular hyaluronidase. The existence of the previously postulated hyaluronosulfate in cornea has recently been denied by Meyer *et al.* (105), and instead the existence of a non-uronic acid containing sulfated polysaccharide named keratosulfate has been postulated. Orr (111) has recently separated from C.S.A., prepared from trachea, a fraction which differs from the remaining C.S.A. in respect to its infra-red spectrum and optical rota-

* Original investigations discussed were supported by grants from the National Heart Institute, United States Public Health Service, Chicago Heart Association, the Helen Hay Whitney Foundation and the Variety Club of Illinois.

† The following abbreviations are used in this discussion: H.A., hyaluronic acid; C.S.A., chondroitinsulfuric acid; A.T.P., adenosine triphosphate; CoA, coenzyme A.; UTP, uridine triphosphate; UDPAG, uridine diphosphate acetylglucosamine; UDPGA, uridine diphosphate glucuronic acid; UDPG, uridine diphosphate glucose.

tion. He has suggested that this compound differs from the remaining C.S.A. with respect to the position of the sulfate group.

In recent years an extensive biological and medical literature regarding the ground substance of connective tissue has appeared. Unfortunately, the term ground substance has been used loosely, some authors considering "ground substance" as a uniform chemical substance, while others consider the term ground substance as synonymous with extracellular fluid. With this confusion of definition, extensive theories regarding the behavior of connective tissues, mechanisms of action of hormones, etc., have been propounded on the basis of evidence obtained by the use of histochemical techniques which have not been validated. Thus, some workers (55, 56) now speak of polymerization and depolymerization of ground substance, although no direct evidence for such a transformation has yet appeared. The rise of serum mucoproteins has been attributed to an outpouring of material from depolymerized ground substance, although all available evidence would seem to indicate that serum mucoproteins are chemically quite distinct from connective tissue mucopolysaccharides (28).

In order to clarify our understanding of the nature and function of ground substance, some fundamental agreement regarding definitions seems desirable. Dorfman (34) has previously proposed that the term ground substance be reserved for the amorphous continuum separating the cells, fibers and vessels of connective tissue. This solution is made up of a variety of components, some of which may be regarded as peculiar to the connective tissue, while others may be regarded as in transit between the circulation and the parenchymal cells. The acid mucopolysaccharides appear to be peculiar to the connective tissue.

The fact that these substances are flexible chain polymers of high negative charge with a high affinity for cations and water molecules suggests that they may play a critical role in regulating the metabolism of inorganic ions and water. A change in concentration or molecular size of such a substance is obviously of great importance in modifying the capacity of connective tissue to bind water and salts. It is thus possible that the ground substance may act as a selective and controlled barrier between the circulation and parenchymal cells.

Recent evidence has indicated that a soluble precursor of collagen is present in the ground substance (136). The possible interaction of the acid mucopolysaccharides with soluble collagen requires further explanation. Jackson (67) has suggested that C.S.A. plays a significant role in the structure of collagen fibers in tendon.

Although a somewhat detailed understanding of the enzymatic mechanism of synthesis of homologous polysaccharides such as starch or glycogen has been achieved, little is known regarding heterologous polysaccharides to which group belong C.S.A. and H.A. The incorporation of alternating monosaccharide units poses certain fundamental problems not encountered in the homologous polysaccharides. In the last few years a number of contributions pertinent to these questions have appeared, which, together with older studies, begin at least to point out certain directions for pursuit of an understanding of the details of the metabolism of the mucopolysaccharides.

In view of the increasingly apparent importance of the mucopolysaccharides in certain fundamental physiological and pathological processes, it would seem worthwhile to attempt to bring together the various observations pertinent to these questions. To this end, this review is directed.

METABOLISM OF COMPONENTS

1. Glucuronic Acid. In view of its importance in the process of detoxification, studies of the metabolism of this compound have been numerous. The literature along these lines has been extensively reviewed by Artz and Osman (1). Certain fundamental metabolic questions regarding glucuronic acid remain of importance. These can be stated as follows:

1) What is the precursor of glucuronic acid and by what mechanism does conversion to glucuronic acid occur?
2) Does glucuronide synthesis involve the utilization of glucuronic acid,[1] or does some other glycoside intermediate form before oxidation of the C-6 of the carbon chain?
3) What is the relationship of β-glucuronidase to synthesis of glucuronides?
4) What is the relationship of glucuronic acid formed in the liver for detoxification to connective tissue polysaccharides containing glucuronic acid?
5) What is the relationship of glucuronic acid to ascorbic acid synthesis?

While space does not permit a detailed consideration of all of these problems, certain definitive information is available pertinent to these questions. Despite the extensive discussions of the older literature and the more recent claims (6, 32) of the role of three carbon particles as precursors of glucuronic acid, recent tracer studies conclusively demonstrate the derivation of glucuronic acid from glucose without previous scission of the glucose molecule. This conclusion was reached by Mosbach and King (106) who utilized uniformly labeled glucose in guinea-pigs fed borneol, Douglas and King (37) who utilized both C_1 and C_6 labeled glucose in the same test system, and Eisenberg and Gurin (50) who performed similar studies utilizing 1-C^{14}-glucose in rabbits receiving menthol. Roseman *et al.* (126) made similar observations regarding the origin of the glucuronic acid portion of H.A. formed by a strain of Group A hemolytic streptococcus. It thus seems quite clear that some pathway exists whereby glucose is converted to glucuronic acid without splitting of the carbon chain, although the enzymatic mechanism of this conversion is not clear (*vide infra*).

The question of whether glucuronic acid is first formed and then linked to phenols or whether oxidation of the C-6 carbon atom occurs after glycoside formation has been investigated for many years by various techniques with no convincing conclusion. Packham and Butler (112) have compared the incorporation of C^{14} into α-naphthol glucuronide in rabbits after the administration of labeled lactate, pyruvate, glucose and glucuronic acid. Since intraperitoneal administration of labeled glucuronic acid resulted in greater incorporation of isotope, it was concluded that administered glucuronic acid may be utilized for glucuronide synthesis. Similar results were not obtained when the

labeled glucuronic acid was administered orally. Following the administration of glucuronic acid to guinea-pigs receiving borneol, Douglas and King (36) observed that only a small amount of the radioactivity of the glucuronic acid was recovered in the urinary glucuronide. Some doubt arose whether or not this represented utilization of the glucuronic acid, since the isotope distribution of the excreted glucuronide differed from that of the administered glucuronide, suggesting asymmetric resynthesis from 3 carbon particles. Evidence of such asymmetric synthesis was found by Bidder (6) and Doerschuk (32), and has recently been demonstrated for the synthesis of glucose from glycerol by Schambye and Wood (129) and Swick and Nakao (142). In a recent study, Douglas and King (38) have reinvestigated this question in the guinea-pig and the albino rat, utilizing 6-C^{14}-glucuronic acid. Glucuronic acid was rapidly metabolized. A small amount of radioactivity was present in the excreted glucuronide which on degradation again showed concentration of radioactivity in the first 3 carbon atoms. It was of particular interest in this study to note that the C_6 of glucose isolated from the glycogen was quite active, suggesting the possibility that glucuronic acid may be converted to glucose. In a more recent study Packham and Butler (113) have attempted to reconcile their earlier results with those of Douglas and King (38). They observed a much greater incorporation of radioactivity in the excreted glucuronide following the administration of glucuronic acid rather than glucurone. They concluded that glucurone and glucuronic acid did not equilibrate in the body and that only glucuronic acid was utilized for conjugation. It was noted that glucurone was much more rapidly metabolized to CO_2. It is possible that the results of these authors might be explained by the more rapid renal excretion (a difference in renal threshold) of glucuronic acid, with an accompanying greater contamination of the isolated glucuronide. These authors have recently found that the labeled glucuronic acid used in these studies was contaminated with labeled naphthol glucuronide (114).

Further understanding of the mechanism of glucuronide synthesis has recently been suggested by the publications of Storey (141) and Dutton and Storey (40, 41), reporting that liver contains a thermostable factor necessary for the synthesis of o-aminophenol glucuronide by liver homogenates. This could not be replaced by a wide variety of co-factors, or metabolic intermediates, or by β-D-glucuronic acid-1-phosphate. These findings have now been confirmed and extended by Smith and Mills (139). They are of particular interest in view of the recent establishment of the role of uridyl compounds in a variety of metabolic reactions, as a result particularly of the studies of Park (116, 117), Leloir and his coworkers (26, 84, 115), and Kalckar and coworkers (71, 72, 107). Smith and Mills were able to show that the uridyl diphosphoglucuronic acid reacts stoichiometrically with o-aminophenol to produce the corresponding glucuronide. The enzyme which catalyzes this reaction can be extracted from liver equally well with water or with potassium chloride solution. It is not sedimented at 10,000 g. for 10 minutes and is active in glycylglycine, tris(hydroxymethyl-aminomethane) or phosphate buffer. Storage at 0–15°C. for 3 days causes marked loss of activity. The reaction is not inhibited by fluoride, iodoacetate, or cyanide

and is stimulated slightly by magnesium ions. The uronic acid containing cofactor was found to be quite unstable, being completely hydrolyzed at pH 2.0 in 10 minutes at 100°C. Under these conditions, there was a complete loss of activity for uronide synthesis, and an apparent liberation of uronic acid and uridine monophosphate.

The mechanism of formation of uridine diphosphoglucuronic acid (UDPGA) is not yet clear. Attempts to demonstrate the following reaction—

$$\text{UDPGA} + \text{pyrophosphate} \leftrightharpoons \text{UTP} + \text{PGA}$$

UTP = uridine triphosphate

PGA = phosphoglucuronic acid

show no evidence of the participation of either α-, or β-1-phosphoglucuronic acid. This is of interest in view of the previous demonstration of the analogous reactions involving glucose and N-acetylglucosamine (72).

$$\text{UDPG} + \text{pyrophosphate} \rightleftharpoons \text{UTP} + \text{glucose-1-phosphate}$$

and

$$\text{UDPAG} + \text{pyrophosphate} \rightleftharpoons \text{UTP} + \text{N-acetylglucosamine-1-phosphate}$$

In view of poor utilization of glucuronic acid in uronide (or polysaccharide) synthesis, and the good evidence that glucose serves as a precursor for the glucuronic acid portion of uronides, an intriguing mechanism which would serve both as a mechanism of uronic acid synthesis and formation of an intermediate for uronide synthesis would be as follows.—

$$\text{UDPG} + \overset{\text{oxidizing system}}{\longrightarrow} \text{UDPGA}$$

No attempts to study such a system have so far been reported.

The role of the enzyme β-glucuronidase has attracted a great deal of attention. This work has been extensively reviewed by Fishman (52, 53), and questions regarding its possible relationship to biosynthesis of glucuronides and significance of variation in its level in tissues are discussed in some detail by that author. From the point of view of metabolism of mucopolysaccharides, Meyer *et al.* (104) have presented evidence that β-glucuronidase serves to degrade to monosaccharides the disaccharide which is formed as a result of the action of testicular hyaluronidase on hyaluronic acid. What role that action has under physiological conditions is not as yet clear. There is no conclusive evidence that β-glucuronidase plays a significant role in the synthesis of uronides. Indeed, Dutton and Storey (42) have recently pointed out that the properties of the uronide synthesizing system of liver are quite different from β-glucuronidase and that the activity of the latter enzyme is unaffected by the presence of UDPGA.

Although it is apparent that glucuronic acid plays a major role in the process of detoxification and in the structure of the acid mucopolysaccharides, the interrelationship between these two functions has not been explored. Smith and Mills (140) have suggested that compounds such as UDPGA may play a role in the

biosynthesis of H.A., while Kalckar (71) points out the possibility that uronic acid of H.A. may serve as a source for formation of UDPGA. A number of studies have demonstrated that glucuronide synthesis probably occurs in the liver, while it seems most likely that mucopolysaccharide formation occurs as a result of the metabolic activity of connective tissue cells (fibroblasts, mast cells). In the studies of Douglas and King (38) little radioactivity of exogenous glucuronic acid was fixed in tissues.

The relationship of ascorbic acid to connective tissue is of considerable interest and importance. It is beyond the scope of this review to detail all pertinent observations along these lines, but it is worthwhile to point out some biochemical relationships between glucuronic acid and ascorbic acid. On the basis of studies utilizing C^{14}-labeled glucuronic acid, Horowitz and King (65) concluded that glucuronic acid is probably an intermediate in the synthesis of ascorbic acid from glucose in the rat.

Such a mechanism has received striking support from the recent studies of Isherwood, Chen and Mapson (66) who found that of many related carbohydrate derivatives only L-gulono-, L-galactono- and D-glucurono-γ-lactones, and D-galacturonic acid methyl ester were converted to ascorbic acid by cress seedlings and by rats. In a subsequent study Mapson, Isherwood and Chen (93) demonstrated the *in vitro* conversion of L-galactono-γ-lactone to ascorbic acid by mitochondria obtained from pea seedlings.

Older studies on the metabolism of glucuronic acid in humans need not be reviewed in detail in view of the relatively crude methods employed. Brox (24) studied the normal excretion of glucuronides, as well as the stimulation produced by the administration of menthol. Increased uronide excretion was found in pregnancy, associated with toxicity, and in diabetes mellitus. In diseases associated with severe damage to the liver parenchyma decreased excretion was found. Fretwurst and Ahlhelm (54) have studied the fate of glucuronic acid administered orally and intravenously to humans. The presence of liver disease resulted in an increase in the portion of administered glucuronic acid which is excreted. This evidence suggests that glucuronic acid is both synthesized and degraded in the liver.

2. Hexosamines. Continually accumulating evidence points to the widespread natural occurrence of the two amino hexoses, glucosamine (chitosamine), and galactosamine (chondrosamine). In addition to their well established presence in the mucopolysaccharides, the hexosamines are found in varying amounts in a large number of proteins, being at particularly high levels in the mucoproteins such as blood group substances and serum mucoproteins.

Recent studies utilizing isotopic tracer techniques have made it quite clear that glucosamine derives from glucose without previous scission of the carbon chain of glucose in at least several biological systems. Thus, studies in the author's laboratory by Roseman *et al.* (124) have demonstrated that the incorporation of 1-C^{14}-glucose or 6-C^{14}-glucose into a semi-synthetic medium, upon which a strain of Group A streptococcus is grown, leads to the formation of H.A., the glucosamine of which contains C^{14} of the same activity (in the appropriate car-

bon atom) as the starting glucose. Identical results were obtained by Topper and Lipton (144), utilizing the same organism. Likewise, the studies of Becker and Day (4), with regard to the formation of glucosamine in serum proteins of the rat, led to similar conclusions. Studies by Rieder (119) of the synthesis of the glucosamine of the egg proteins (principally in the ovomucoid and ovomucin) also indicated the utilization of the intact glucose molecule for the synthesis of glucosamine. These authors found that glycine (labeled with N^{15} and C^{14}) did not serve as a glucosamine precursor.

The mechanism of amination has not been entirely clarified. Lowther and Rogers (89), in a preliminary report, found that glutamine or glutamic acid plus ammonia were necessary for the synthesis of H.A. by resting cell suspensions of hemolytic streptococci, indicating that the amide group of glutamine serves as the precursor of the amino group of glucosamine. Leloir and Cardini (85) have studied the mechanism of amination in extracts of *Neurospora crassa* as part of a study of the mechanism of synthesis of chitin. Evidence was found that glutamine is necessary for amination; the reaction presumably being between hexose-6-phosphate and glutamine to yield glucosamine-6-phosphate.

Becker and Day (4) have proposed that glucosone serves as an intermediate in the conversion of glucose to glucosamine. This conclusion was reached on the basis of the finding that the administration of 1-C^{14}-glucosone to rats resulted in the incorporation of a greater amount of C^{14} into glucosamine isolated from the serum proteins than was obtained as a result of the administration of an equivalent amount of 1-C^{14}-glucose. A similar conclusion was reached by Topper and Lipton (144) who found that the addition of glucosone to a medium containing 1-C^{14}-glucose resulted in a decrease in the radioactivity of the glucosamine portion of the H.A. formed by hemolytic streptococci. Dorfman *et al.* (35) have reinvestigated this question and found that 1-C^{14}-glucosone is a relatively inefficient precursor of glucosamine. It was postulated that the apparent conversion of glucosone to glucosamine may result from the prior conversion of glucosone to glucose. Support for this idea was found in the data of Becker and Day (4), which show that the glucosone carbon acts as a precursor of liver glycogen. In order to test this hypothesis, Dorfman *et al.* (35) utilized uniformly labeled glucosone in experiments with streptococci. It had previously been demonstrated that glucose serves not only as a precursor of glucosamine, but also as a precursor of the glucuronic acid portion of the H.A. molecule. If glucosone is incorporated *via* glucose, the glucuronic acid should be labeled to the same extent as the glucosamine. This was found to be the case. It was suggested that the explanation of the more efficient utilization of glucosone than of glucose observed by Becker and Day (4) may lie in the more rapid metabolism of glucose. It may be concluded that present evidence does not substantiate the role of glucosone as an intermediate in the synthesis of glucosamine from glucose.

Detailed information regarding the enzymes responsible for the degradation of glucosamine and N-acetylglucosamine in mammalian tissue is as yet lacking. A number of older studies suggest that glucosamine may be deaminated in the liver to yield glucose (39, 58, 128, 157, 158). Other older studies on the degrada-

tion of glucosamine both *in vitro* and *in vivo* are reviewed by Kawabe (75) who also conducted studies of both the *in vivo* and *in vitro* degradation of glucosamine (74, 75). Although evidence of degradation of glucosamine was obtained, the methods used and the results obtained permit no more quantitative or detailed conclusion. In a series of similar studies Watanabe (146–149) investigated the metabolism of N-acetylglucosamine both *in vivo* and *in vitro*. By *in vivo* techniques they found little evidence of N-acetylglucosamine utilization, although evidence of the existence of a deacetylase in liver was found *in vitro*.

Bergfeld and Kapfhammer (5) have reviewed the literature concerned with the question as to whether or not glucosamine is converted to glucose, and restudied this question in human subjects. Using relatively crude methods, no positive evidence for such a conversion was found.

In a more recent study, carried out with considerably better enzyme techniques, Lutwak-Mann (91) observed that the addition of amino sugars resulted in an increase in oxygen uptake and ammonia production by renal cortex, testis, brain cortex, ovaries, retina and, to a lesser extent, lung tissue. Liver, intestine, mucus membrane, cartilage, spleen, erythrocytes, plasma and synovial fluid had no such activity. Glucosamine, galactosamine and N-acetyl-glucosamine were all utilized. Somewhat lower production of ammonia was obtained, as a result of action on the acetylated sugar, by all tissues except brain cortex which did not metabolize this compound. In all of the tissues studied, the utilization of galactosamine was less than that of the glucosamine. The oxygen uptake in animal tissues (rat and rabbit) is accompanied by ammonia liberation and acid formation, but not by liberation of CO_2. In yeast and in certain bacteria (*B. coli*, *Streptococcus faecalis*, and *Proteus vulgaris*) evidence of similar metabolism was found, but, unlike what was seen in the mammalian tissues, this proceeded anaerobically as well as aerobically. The liberation of acid corresponded to the oxygen uptake, but not to ammonia production, suggesting that oxidation may occur before deamination. Glucosaminic acid, however, was not utilized. The metabolic products were not identified.

Deacetylation of N-acetylglucosamine in bacteria was suggested by the finding of a volatile acid in the medium, following the action of a variety of strains of streptococci on N-acetylglucosamine. Direct confirmation of this is furnished by the demonstration of radioactivity in the acetic acid of the medium, after a strain of Group A streptococcus has been grown in carboxyl labeled N-acetylglucosamine (35). A cell free preparation of such a deacetylase has been obtained from *B. coli* (125). As part of a study of the metabolism of H.A. by bacteria, Sallman *et al.* (127) demonstrated oxygen consumption when either N-acetylglucosamine or glucosamine were utilized as substrate for a strain of *B. coli*.

Several studies have recently concerned themselves with the phosphorylation of hexosamines. The first report of such phosphorylation was that of Harpur and Quastel (64) who found evidence of phosphorylation of glucose, fructose, and glucosamine by an acetone powder of white matter of brain. Since the phosphorylation of the three hexoses was not additive, it was concluded that a common enzyme was responsible. The rate of reaction was considerably slower for

glucosamine than for glucose and for fructose, while the approximate Michaelis constants were comparable for glucosamine and fructose; both constants were somewhat higher than that for glucose. N-acetylglucosamine was not phosphorylated and appeared to act as an inhibitor (apparently competitive in nature) of the phosphorylation of glucosamine, glucose and fructose. Further evidence of the phosphorylation of glucosamine was obtained by Grant and Long (59) who showed that yeast hexokinase, in the presence of adenosine triphosphate, magnesium chloride, potassium fluoride, and potassium phosphate, catalyzed the formation from glucosamine of a substance, the analyses of which suggested a monophosphoglucosamine. The ester was found to be stable to normal acid and contained a reducing group and a free amino-group. Brown (22) studied this phosphorylation in somewhat greater detail, utilizing crystalline yeast hexokinase and succeeded in identifying the product of the phosphorylation as glucosamine-6-phosphate. Subsequent studies by Brown (23) showed that phosphoglucosamine, obtained from rabbit muscle (both crystalline and amorphous fractions were used, the latter being more active), catalyzed the conversion of glucosamine-6-phosphate to a compound with properties consistent with a structure of glucosamine-1-phosphate. This reaction was found to attain equilibrium at pH 7.53 and 30°C. with the conversion of 19.2 per cent of the glucosamine-6-phosphate. It could not be ascertained whether or not 1–6 glucosediphosphate acted as a coenzyme for this reaction (as is the case for equilibrium between glucose-6-phosphate and glucose-1-phosphate), but the author proposes that the reaction probably does proceed through a diester. The rate of mutation of the glucosamine ester was found to be very much slower than that resulting from the action of phosphoglucomutase on the corresponding glucose ester.

A somewhat different pathway of formation of glucosamine phosphate esters has been proposed by Leloir and Cardini (85). These authors demonstrated amination of glucose-6-phosphate to form a compound which was thought to be glucosamine-6-phosphate. It was proposed that mutation to the 1-phosphate ester occurs following acetylation, although details of this mutation have not yet been published, but it is said to be accelerated by glucose-1,6-diphosphate.

Relatively little information is as yet available regarding the metabolism of galactosamine, although Cardini and Leloir (27) have recently demonstrated its phosphorylation in the presence of adenosine triphosphate and liver and brain extracts, as well as a yeast enzyme. The enzyme activity appeared to be parallel to the galactokinase activity, suggesting the possibility that the same enzyme was responsible for both activities. The product formed, although not positively identified, appeared to be galactosamine-1-phosphate. Pontis Videla (118) has recently demonstrated the presence of a uridine diphosphate acetylgalactosamine compound, and has suggested that this is involved in the transformation of glucosamine to galactosamine, in a manner similar to the transformation of glucose to galactose.

The amino sugars are usually found to be acetylated. An exception appears to exist in the case of heparin, where the amino group is thought to be sulfonated (155, 156). Chou and Soodak (29) showed that glucosamine and chondrosamine

(galactosamine) can be acetylated by an enzyme prepared from pigeon liver. In crude extracts acetylation was demonstrated in the presence of adenosine triphosphate, coenzyme A and acetate, while in purified preparations acetyl phosphate and transacetylase were used as the acetyl donor system. It was suggested that the glucosamine acetylating enzyme differs from that involved in the acetylation of arylamines. Acetylation of glucosamine by bacterial enzyme preparations has also been reported (73, 143).

Leloir and Cardini (85) found evidence of acetylation by a similar system with an enzyme obtained from *Neurospora crassa*, but in this case acetylation apparently also occurs with glucosamine-6-phosphate as substrate. It is not as yet clear, whether or not physiological synthesis of polysaccharides involves acetylation before or after phosphorylation. Leloir and Cardini favor the idea that acetylation occurs after phosphorylation of glucosamine. This view is in keeping with the finding by Dorfman *et al.* (35) that no evidence could be obtained for the direct utilization of N-acetylglucosamine in the synthesis of H.A. by hemolytic streptococci. The utilization of acetate for acetylation by crude pigeon liver preparations, and by the system derived from *Neurospora crassa*, is in accord with the finding in this laboratory that radioactive acetic acid is incorporated into the acetyl group of H.A. The data discussed in a later section of this review on mammalian skin suggest that a similar situation obtains with respect to synthesis of H.A. and to the sulfated mucopolysaccharide of rabbit skin.

Before leaving the subject of the enzymatic reactions of glucosamine, mention should be made of the demonstration by Park (116, 117) of a uridine nucleotide which contains an amino sugar, as well as of the more recent isolation by Cabib *et al.* (25) of a uridine diphosphate acetylglucosamine compound from yeast, and of an apparently identical compound from liver by Smith and Mills (139). It is suggested that this compound may play some role in the metabolism of hexosamines, although no definite role has so far been established (*vide infra*). Smith *et al.* (140) have demonstrated that, in the presence of an enzyme derived from liver nuclei, this compound undergoes pyrophosphorylysis, as previously indicated. The importance of the uridine nucleotides in the synthesis of polysaccharides is emphasized by the report of Leloir and Cabib (87) of the synthesis of trehalose phosphate as a result of the interaction of uridine diphosphate glucose and glucose-6-phosphate, and the report of Leloir and Cardini (86) of the synthesis of sucrose as a result of the interaction of uridine diphosphoglucose and fructose.

BIOSYNTHESIS OF MUCOPOLYSACCHARIDES IN STREPTOCOCCI

On the basis of the known structure of the acid mucopolysaccharides, it is obvious that many possible pathways of biosynthesis may be postulated. Variation in the order of occurrence of specific steps permits many permutations in pathways between remote precursors and final products. Final answers can only be obtained after isolation and study of the enzyme systems involved. To a limited extent, however, some data are now available pertinent to these ques-

tions. These results have been obtained, to a large extent, using carbon-14 tracer techniques in the study of the synthesis of H.A. by a strain of Group A streptococcus.

A first approach was attempted utilizing carboxyl labeled N-acetylglucosamine, since the demonstration of the incorporation of this compound would obviate the necessity of the study of the incorporation of glucosamine. When N-acetylglucosamine, labeled in the carboxyl group, was added to a semisynthetic medium containing glucose, the H.A. produced by Group A streptococci was found to be labeled in the acetyl group, thus suggesting that N-acetylglucosamine could indeed be directly incorporated. Such a conclusion had previously been reached by Topper and Lipton (144) on the basis of the decrease in the radioactivity of H.A., synthesized in the presence of 1-C^{14}-glucose when inactive N-acetylglucosamine was added to the medium.

This conclusion, however, seemed open to question when the following facts were ascertained. When labeled N-acetylglucosamine was added to the medium, acetate isolated from the growth medium was found to be highly radioactive. Furthermore, when carboxyl labeled acetic acid was incorporated into the medium, the acetyl group of the H.A. was found to be highly radioactive. These findings suggested that the apparent incorporation of N-acetylglucosamine may actually be due to the following set of reactions:

$$\text{N-acetylglucosamine} \leftrightarrows \text{glucosamine} + \text{acetate}$$

$$\text{Acetate} + X \rightarrow \text{acetyl } X \rightarrow \text{polysaccharide}$$

X may be glucosamine or any other intermediate (such as a disaccharide or phosphorylated glucosamine intermediate). To test this hypothesis, additional experiments were performed in which inactive acetic acid was added in addition to the carboxyl labeled N-acetylglucosamine. If N-acetylglucosamine is directly incorporated into the polysaccharide, the presence of inactive acetate should not affect the degree of labeling, while if the mechanism proposed above were responsible for labeling, the presence of inactive acetate should result in a decrease of the radioactivity of the H.A. formed. The latter was found to be the case, thus making impossible the conclusion from this type of experiment that N-acetylglucosamine is a direct precursor. This is consistent with the finding by Harpur and Quastel (64) and by Leloir and Cardini (85) that N-acetylglucosamine, in contrast to glucosamine, is not phosphorylated by hexokinase and adenosine triphosphate.

Further experiments were performed to determine whether glucosamine is utilized for polysaccharide synthesis. For this purpose, C^{14}, N^{15} doubly labeled glucosamine was prepared by incorporating 1-C^{14}-glucose and $N^{15}H_4^+$ in a medium upon which *Aspergillus niger* was grown. The doubly labeled glucosamine, which was isolated from the chitin of the mycelium, was utilized for the synthesis of H.A. by the streptococcus. The ratio of C^{14}/N^{15} was found to be almost identical in the glucosamine placed in the medium and in the glucosamine isolated from the H.A., thus unequivocally demonstrating that glucosamine is a precursor of H.A.

The origin of the glucuronic acid portion of the polysaccharide molecule is not clear. Incorporation of labeled glucuronolactone into the growth medium has so far not resulted in incorporation of radioactivity into the molecule. Variation of the pH of the medium seemed to play no role in this reaction. The lack of direct utilization of glucuronic acid is consistent with the apparent lack of utilization of this compound in uronide synthesis in the mammal (*vide infra*).

ACTION OF HYALURONIDASES

The widespread occurrence and ready availability of hyaluronidases has focused attention on the role of this enzyme in the metabolism of H.A. The literature on the action of hyaluronidase has been previously reviewed by Meyer (98), Dorfman (33), and Meyer and Rapport (102), and will not be considered in detail here. Although striking effects of hyaluronidase have been demonstrated both *in vivo* and *in vitro*, the physiological or pathological role of these enzymes is not as yet clear. In the mammalian organism the presence of hyaluronidase has been conclusively demonstrated only in testes, although claims of its presence in other tissues have been advanced (98).

There is at present no clearcut evidence to indicate that hyaluronidase plays a significant role in the metabolism of the mucopolysaccharides in the mammal; nevertheless, it is important to consider the nature of the action of hyaluronidases.

The appearance of reducing substances upon hydrolysis of H.A. by hyaluronidases has indicated that the primary linkages attacked are of a glycosidic nature. In view of the fact that H.A. is composed of equimolecular amounts of N-acetylglucosamine and glucuronic acid, it is necessary that at least two glycosidic linkages must exist within the molecule. The usual specificity of glycosidases suggests that at least two enzymes are required for the splitting of H.A. to monosaccharide units.

That more than one enzyme activity is involved in the breakdown of H.A. became apparent from the failure of several early studies to show good correspondence between the liberation of reducing substance and the reduction of viscosity by different hyaluronidase preparations. Thus, Meyer *et al.* (100) observed that pneumococcal hyaluronidase hydrolyzed H.A. to give almost one hundred per cent of theoretical reducing substances, whereas a testicular hyaluronidase preparation, which showed a much more rapid rate of lowering of viscosity, yielded only about fifty per cent of theoretical reducing substances. More striking discrepancies have been reported by Meyer (98) for leech enzyme and by Madinaveitia and Quibell (92) and McClean and Hale (97).

Other evidence for the difference in action of different enzymes has been reported by Rogers (120, 121). This investigator found that H.A., or H.A. digested by incubation with testicular enzyme, stimulated the production of hyaluronidase by streptococci or *Cl. welchii*, while H.A. digested by streptococcal hyaluronidase did not stimulate enzyme production. On the basis of these facts, and of other ancillary evidence, several investigators (100, 102) suggested that the breakdown of H.A. to constituent monosaccharides required the participa-

tion of at least two enzymes, an enzyme which brings about depolymerization of the high polymer to smaller units and another which hydrolyzes these smaller units to monosaccharides. Meyer made the more specific suggestion that one enzyme was required to bring about hydrolysis to aldobionic units, while a second is concerned with the hydrolysis of the aldobionic units to monosaccharides.

More direct evidence for this concept was furnished by Hahn (60–63) who carried out an extensive series of investigations of the action of hyaluronidase obtained from testicular extract, leech heads, and filtrates of *Cl. welchii*. Using $(NH_4)_2SO_4$ and lead acetate, this investigator claimed to have separated the enzymatic activity of testicular extract into two fractions which he designated as mucopolysaccharase (MP) and muco-oligosaccharase (MO). The MP activity was measured in terms of viscosity reduction, while the MO activity was measured by liberation of reducing groups. By various fractionation procedures it was possible to concentrate the mucopolysaccharase (*i.e.*, increase viscosity-reducing activity), while the muco-oligosaccharase activity (liberation of reducing substances) was decreased. The addition of MO to a product, obtained by the action of MP on vitreous humor H.A., resulted in an increase in reducing power. It should be pointed out that these increases were relatively small. Further evidence as to the nature of the reaction products was obtained by analysis of the products by the method of frontal absorption analysis of Tiselius, utilizing carbon as an adsorbent and ephedrine as the eluting agent. Under these conditions, the product obtained as a result of the action of MO behaved in a manner characteristic of monosaccharides, while that obtained by the action of MP was strongly absorbed and could not be eluted by ephedrine.

Since the product of the reaction produced by MP gives the Elson-Morgan color reaction, which presumably requires a free aldehyde group in the glucosamine portion of the molecule, Hahn concluded that the MP enzyme, which breaks H.A. to some smaller particle, acts on the glucosaminide linkage.

It was thus tentatively suggested that MP is a glucosaminidase which hydrolyzes H.A. to a disaccharide containing a free reducing group in the glucosamine portion of the molecule. The MO activity is then presumed to be a glucuronidase.

East, Madinaveitia and Todd (49) have shown that testicular extract, as well as other hyaluronidase preparations, contains an enzyme which splits β-phenyl-N-acetylglucosamine, but that this activity is distinct from the depolymerizing activity (as measured by viscosity reduction) of testicular enzyme. Other sources of β-phenyl glucosaminidase are devoid of hyaluronidase activity (East *et al.*, see above). More recently, Roseman and Dorfman (123) have demonstrated the existence in testicular extract of an enzyme which splits α-phenyl-N-acetylglucosaminide and which is distinct from both hyaluronidase (as measured by the turbidity reducing method), and β-phenyl glucosaminidase. This suggests that if hyaluronidase is a glucosaminidase, it is specific with respect to substrate (or molecular size).

Since H.A. contains equimolecular amounts of N-acetylglucosamine and glu-

curonic acid, the splitting, to yield a product with a free aldehyde group in N-acetylglucosamine portions, should yield a product which retains a glucuronide linkage. The further splitting of this product would then require the action of a glucuronidase. On this basis, Hahn (63) suggested that the MO activity might consist of a glucuronidase, but found that the β-glucuronides of menthol, borneol, and pregnanediol are not broken down by a water extract of testis.

Hahn (62) points out that there is as yet no evidence to indicate whether the glucuronide linkage in H.A. is α or β in nature. No evidence of the existence of an α-glucuronidase has yet appeared. More recently, however, Weissmann and Meyer (152) have suggested that the glucuronide linkage is β in nature.

Meyer, Linker and Rapport (104) have reiterated the suggestion that β-glucuronidase is responsible for the breakdown of H.A. After treatment of H.A. with relatively crude hyaluronidase, the reaction mixture was precipitated with alcohol and then subjected to further digestion by less pure testicular enzyme and by a crude liver enzyme prepared according to the method of Fishman and Talalay (52) to contain β-glucuronidase. The product of this reaction was subjected to chromatographic analysis by the method of Whistler and Durso (154) employing a carbon column and elution with water, alcohol, and pyridine mixtures. Monosaccharides are eluted with water and 5 per cent ethanol. Two peaks were obtained, the first appearing predominantly in the water eluate and having properties suggestive of glucuronic acid, while the second, which was eluted with 5 per cent ethanol, behaved as N-acetylglucosamine. Further evidence of the nature of these substances was obtained by paper chromatography. In other experiments the rates of release of reducing substances from the products prepared by the action of crude testicular enzyme, crude liver enzyme, and a purified liver enzyme, were found to be similar. Crude testis enzyme was found to hydrolyze 8-hydroxyquinoline glucuronide at the same rate as does a purified liver β-glucuronidase preparation. Crude testicular enzyme was found also to hydrolyze the glucuronides of borneol, menthol, and phenolphthalein. The evidence of these authors would seem to once again reiterate the fact that H.A. is enzymatically split to monosaccharides. The role of β-glucuronidase is highly suggestive; however, it cannot be considered conclusively demonstrated because no pure enzymes were used. The correspondence of the rate curves is not conclusive.

Weissmann *et al.* (153) have suggested the formation of a variety of different products from H.A. by the action of partially purified testicular hyaluronidases, with a free reducing group on the N-acetylglucosamine moieties. On the basis of chromatographic studies, evidence was obtained of the formation of a series of oligosaccharides, the smallest of which was proven to be a disaccharide, since it was converted to a methyl ester of heptaacetylhyalobiuronic acid which had been previously satisfactorily characterized by Weissmann and Meyer (152). However, even upon extensive digestion by the enzyme, only 10 per cent of the H.A. was converted to disaccharide, while as much as 74 per cent was found in the fraction which was thought to contain tetrasaccharides. The presence of a variety of molecular sizes is attributed to transglycosidation reactions analogous

to those recently demonstrated for other glycosidases. No definitive evidence indicating the occurrence of such reaction with hyaluronidase has so far appeared.

The possible difference in action of hyaluronidases from different sources has been suggested by a number of different observations (102). Hahn (60, 61) compared the behavior of partially purified enzymes obtained from *Cl. perfringens*, leech heads, and testicular extract. Leech enzyme was found to release only 26 per cent of reducing substances (calculated as glucose), and a product which gave the Morgan-Elson reaction only after treatment with alkali at high temperatures. Enzyme obtained from *Cl. perfringens* cultures gave a higher reducing value and yielded a substance which on absorption analysis behaved as N-acetylglucosamine, but no evidence of a hexuronic acid could be obtained.

The difference in action of certain hyaluronidases has recently been studied in some detail by Meyer and his coworkers. They have pointed out that the pneumococcal (and streptococcal) enzymes differ from testicular enzyme in substrate affinity, in extent of hydrolysis, and in temperature sensitivity. Linker, Weissmann and Meyer (88) have reported that hyaluronidases derived from pneumococci, streptococci, staphylococci, or *Cl. perfringens* produce a disaccharide which is similar in composition to N-acetyl hyalobiuronic acid, but differs from the latter in R_f value, optical rotation, and hypoiodite reduction. When the tetrasaccharide obtained by hydrolysis of H.A. by testicular enzyme is hydrolyzed by pneumococcal enzyme, equal amounts of the bacterial disaccharide and N-acetyl hyalobiuronic acid are obtained, but when the bacterial enzymes act directly on H.A. only the bacterial disaccharide is obtained.

Further study on the nature of the "bacterial disaccharide" indicates that it contains glucosamine, takes up 1 mole of bromine, 2 moles of hydrogen, and absorbs ultraviolet light at 230 mμ. The hydrogenated product reduces ferricyanide, but no longer gives the carbazole reaction for uronic acid, and has lost the ultraviolet absorption.

The authors suggest that these properties are due to the formation of an α-β unsaturation of the uronic acid which occurs concomitantly with the hydrolysis of the glucosaminidic bond. It is of interest to point out that this α-β unsaturation would represent the same position in which a double bond is produced in the conversion of glucuronic acid to ascorbic acid. It is difficult to conclude that this action is actually due to the hydrolytic enzyme, in view of the fact that crude enzymes were used in this study.

Problems regarding the specificity of hyaluronidase are still somewhat obscured by the lack of pure enzyme preparations. Earlier studies along these lines are detailed in previous reviews. It remains of importance to note that several studies (103) indicate that testicular hyaluronidase brings about hydrolysis of C.S.A. of cartilage, although enzymes obtained from bacterial sources do not possess such actions. It is to be noted that in the latter study highly purified enzyme preparations were used.

It is apparent from the above discussion that recent work, particularly by Meyer and coworkers, has served to increase considerably the understanding of

the mechanism of action of hyaluronidases, although many problems remain unsolved. It is not yet apparent what role hyaluronidase plays in the metabolism of the mucopolysaccharides. Recent studies on glycosidases suggest that transglycosidation may occur quite generally as a result of the action of hydrolytic enzymes. That this may occur with N-acetylglucosamine glycosides, is indicated by the report of Wallenfels (145) concerning the formation of galactosido-N-acetylglucosamine as a result of the action of lactase on a mixture of lactose and N-acetylglucosamine. A similar type of synthesis involving the production of a growth factor for *Lactobacillus bifidus* (*Pennsylvania strain*) has recently been reported by Zilliken *et al.* (159).

METABOLISM OF MUCOPOLYSACCHARIDES IN MAMMALS

It is obvious from the preceding discussion that of the meager information available regarding the metabolism of mucopolysaccharides, little has been derived from study of mammalian connective tissue. This deficiency derives to a great extent from the difficulty of securing adequate material for such studies. Although connective tissue is widely distributed, its localization is such that it is impossible to secure adequate material for the study of its metabolism by the customary biochemical techniques. A possible exception to this is cartilage, which has been studied to a somewhat greater extent than other connective tissues.

There is as yet no certain definition of the cells responsible for the synthesis or degradation of the mucopolysaccharides in connective tissue. It seems reasonably certain that the cartilage C.S.A. (chondroitin A of Meyer and Rapport (101)) is produced by chondroblasts. It has been widely assumed that H.A. is produced by fibroblasts, although no definitive proof of this has been presented. Asboe-Hansen (2) has suggested instead, that mast cells are responsible for the formation of H.A. That these cells play a role in the formation of heparin, was suggested some time ago by Jorpes (68, 69, 70) and has been accepted by many, because of the presence of metachromatic granules in them. It is beyond the scope of this paper to consider the problems of cellular origins in detail.

The availability of S^{35} presents a highly advantageous tool for the study of metabolism of mucopolysaccharides. This isotope has many advantages for such studies, among which can be listed the following: 1) it is relatively cheap and easily available; 2) its low-energy β-emission is relatively easy to count and presents a minimum of health hazard; 3) it can be readily obtained as sulfate, and can be used as such without requiring further synthesis; 4) incorporation as ester sulfate can be readily determined by hydrolysis and precipitation of $BaSO_4$; and 5) the localization of the ester sulfate can be achieved by radioautography.

These advantages are counterbalanced by certain disadvantages, *e.g.* 1) the method gives no information regarding the metabolism of H.A.; 2) conclusions must be confined to changes of the sulfate ester group, since it is possible that these are not correlated with changes in other parts of the molecule; 3) there is as yet no clear definition of all of the ester sulfate compounds present. Thus, the existence of more than one type of C.S.A. may result in confusion regarding

conclusions with respect to metabolism. However, despite these difficulties, considerable useful information has recently accumulated, as a result of studies utilizing radioactive sulfate.

Singher and Marinelli (137) originally showed that the injection of radioactive sulfate resulted in the fixation of radioactivity in bone marrow, as well as in cartilage. Numerous other studies have now confirmed the incorporation of radioactive sulfate in cartilage. Dziewiatkowski *et al.* (43) showed that 20–25 per cent of labeled sulfate, administered to 7 day old suckling rats, was retained after 48 hours. Of the S^{35} retained at 24 hours, a concentration many times that present in the blood and 14 times that present in the whole carcass, was found in the cartilage of the knee joints. An increase in the concentration of S^{35} in the cartilage was observed up to 24 hours after administration of the labeled sodium sulfate, and little decrease was observed up to the 164th hour. In a subsequent study, Dziewiatkowski (48) showed that the C.S.A. isolated from the knee cartilage by the method of Bray *et al.* (20, 21) showed a high radioactivity, thus indicating that the S^{35} in cartilage is present in the C.S.A. fraction. In this isolation it was necessary to use carrier C.S.A., but the material was reprecipitated to constant activity. However, it was not pure C.S.A. as evidenced by the published analyses.

In order to determine the chemical specificity of the localization of administered sulfate, Boström and Åqvist (11) administered $Na_2S^{35}O_4$ intraperitoneally to a series of rats, groups of which were sacrificed at 2, 8, 24, and 48 hours after a single injection. From pools of each group of animals the C.S.A. of the ribs, and the taurine, cystine, and methionine of the liver, were isolated for determination of the radioactivity. High activity was found in the C.S.A., low (about 3 per cent), but significant, activity in the taurine, and none in the methionine and cystine. It is thus apparent that administered sulfate is preferentially incorporated into the C.S.A. (as compared with the other compounds studied). However, the presence of radioactive sulfate in other sulfate esters was not excluded (see studies on nervous system and liver discussed below).

Using a somewhat different method of preparation, Boström (8, 9) isolated the C.S.A. from rib cartilage of the rat without use of carrier. On the basis of analytical data the purity of the C.S.A. appeared somewhat greater than that reported by Dziewiatkowski, but varied considerably in different preparations (note variation of sulfur analyses of 4.09 to 5.68 per cent). The amount of 1.5 mgm. of S^{35}-labeled Na_2SO_4 was injected intraperitoneally into nine groups of 20 rats each. The animals were sacrificed at varying intervals after the initial injection, C.S.A. was isolated, and total blood sulfur and free blood sulfate of intercostal muscles were determined. The radioactivity of both the total blood sulfur and the sulfate of the intercostal muscles rose rapidly after injection of the labeled sulfate, but fell to low levels in 24 hours, while the activity of the C.S.A. rose to a maximum at about 24 hours and fell slowly from this point. On extrapolation, the C.S.A. curve appears to drop to half its maximum value by the seventeenth day. Boström *et al.* (10) have attempted to study the rate of metabolism of C.S.A. in tracheal cartilage utilizing quantitative radioautogra-

phy. Although the rate of decrease of radioactivity in general simulates that obtained by chemical methods, the data are not sufficiently exact for the determination of half-life times.

Dziewiatkowski (44, 45, 47) has utilized radioautographic techniques to study the distribution of S^{35} in different portions of bone and its variation with time. Of considerable importance in these studies is the finding that the distribution of radio-sulfate, as revealed by radioautography, varies with methods of fixation used. This finding indicates the difficulties of drawing conclusions, regarding the metabolism of individual compounds, on the basis of radioautographic studies.

The finding of the fixation of radioactive sulfate in cartilage led Layton and Frankel (76), and Gottschalk and Allen (57) to study the concentration of S^{35} in chondrosarcomata of human subjects. In two patients, marked uptake of radioactive sulfate in such neoplasms was demonstrated, the extent of uptake being apparently greater in one of the tumors, which had a larger amount of ground substance.

In addition to the studies on the metabolism of C.S.A. by cartilage, a variety of reports have dealt with the distribution of radioactive sulfate in various other tissues. Thus, Odeblad and Boström (109), in a study of a variety of tissues of adult rats and rabbits, found the sulfate concentrated in those tissues which are known to contain sulfated mucopolysaccharides. In a separate study on the brain of rats, Boström and Odeblad (13) found greater fixation of sulfate in the gray matter than in the white matter of the cortex, as well as greater fixation in the cerebellum than in other portions of the brain. The largest concentration of radioactivity was found in the choroid plexus. No attempt was made in this study (autoradiographic) to delineate the types of compounds responsible for this fixation; it was, however, suggested that sulfolipids may be partly responsible.

Boström and Odeblad (12) studied the distribution of radioactive sulfate in the genital organs of female rats and rabbits by radioautographic techniques, and found localization in the granulosa layer of the follicle, as well as in the follicular fluid. Odeblad (108) found only small amounts of radioactive sulfate in the lutein cells, but, as might be expected, larger amounts in the connective tissue surrounding the lutein cells. In other parts of the genital system S^{35} was found in areas known to be of high mucus content. In the corpus uteri the endometrial stroma contained considerable amounts of S^{35} after estrogen stimulation.

Boström and Gardell (18) have attempted to study the turnover rate of mucopolysaccharides of adult rat skin utilizing chemical methods. The sulfated mucopolysaccharides were separated by an electrophoretic method. The maximum radioactivity of the ester sulfate of the polysaccharide, which was reached about 20 hours after a single injection, gradually declined at a rate suggesting a half-life time of about 10 days. It should be pointed out, however, that the analyses of the polysaccharides studied did not indicate that pure compounds were obtained. For reasons which will be discussed below, there is considerable likelihood that mixtures of polysaccharides were studied. In a separate study (19), localization of radioactivity in skin was investigated by radioautographic meth-

ods. The localization in the corium was consistent with previous chemical studies, but, in addition, considerable uptake of S^{35} was noted in the hair follicles, the walls of small vessels, and mast cells. The S^{35} of the hair follicles appeared to disappear at a more rapid rate than that of the corium. It is of interest that metachromatic material has been noted in the hair follicles; its chemical nature has not been satisfactorily elucidated.

The presence of mucopolysaccharides in the aorta has previously been demonstrated, and changes in their nature have been suggested as of importance from the point of view of arteriosclerosis. Odeblad and Boström (110) have demonstrated the localization of S^{35} in the aorta of adult rabbits and have estimated its half-life as 14 days, on the basis of quantitative radioautography.

Mast cells have been considered of importance in the metabolism of mucopolysaccharides on the basis of the presence of metachromatic granules and of an abundance of material stainable with Fuchsin sulfurous acid after periodate oxidation. Various authors, particularly Jorpes *et al.* (68–70), have suggested that mast cells are responsible for the synthesis of heparin, while Asboe-Hansen (2) has suggested that these cells are responsible for the synthesis of H.A. Using radioautographic techniques, Jorpes (70) demonstrated that S^{35} accumulates in the mast cells of the subcutaneous tissue of the rat. It was not possible to estimate a half-life time for the granules, since little evidence of decrease in radioactivity was found after 18 days. This problem has recently been further investigated by Asboe-Jansen (3), studying papillomas produced in mice by painting with 9,10-dimethyl-12-benzanthracene. In such tumors the dermal connective tissue shows a large concentration of mast cells. Marked accumulation of radioactive sulfate was demonstrated in these cells. The author suggests that the sulfated polysaccharide shown to be present may serve as a precursor of both heparin and H.A., although the experimental evidence merely demonstrates the presence of radioactive sulfate in the cell.

A variety of experiments have been concerned with the fixation of radioactive sulfate by fetal tissues. Layton *et al.* (77) demonstrated that the administration of radioactive sulfate to pregnant rats results in deposition of radioactivity in the tissues of the fetus, thus establishing transfer of radioactive sulfate across the placental barrier. Secretion of radioactive sulfate in milk was also demonstrated. Similar studies have been carried out by Dziewiatkowski (45), and Boström and Odeblad (14), who studied the distribution of S^{35} in the various tissues of the embryo. Dziewiatkowski (45) showed that the concentration of S^{35} in the cartilaginous portions of humeri of the fetal skeleton was about thirty times that of the maternal sternum.

It has now been demonstrated that fixation of radioactive sulfate occurs *in vitro* as well as *in vivo*. Layton (78, 79) showed that tissue of explants of chick embryos, incubated in Tyrode's solution containing $S^{35}O_4^{=}$, exhibited easily demonstrable fixation of radioactive sulfate. That this was due to actual incorporation into tissue components was demonstrated by the following facts: a) exhaustive washing of the tissues does not remove the radioactive sulfate, b) fixation does not occur when incubation is carried out at 0°C., c) previous freezing of

tissues prevents fixation. In addition to embryonic tissues, it was found that granulation tissue also effectively fixes radioactive sulfates. In another publication, Layton and Frankel (76) have demonstrated that explants of chondrosarcomata fix large amounts of sulfate, as compared with surrounding tissues. It was suggested that this technique may be useful for the diagnosis of such tumors, relying on culture of biopsy specimens. Layton (83) has demonstrated that the fixation of sulfate *in vitro* by chick tissues is similar to that observed *in vivo*, the greatest fixation occurring in known locations of acid mucopolysaccharides, such as cartilage and aorta. An exception to this is the active esterifying system which is present in embryonic liver (81). This system, which appeared to be more active at 20°C. than at 37°C., was completely inactivated after 40 hours at 37°C. The esterification was accelerated by the addition of phenol, suggesting that this represents a system for the esterification of phenols.

Another approach to the *in vitro* metabolism of the sulfated mucopolysaccharides has been the use of cartilage slices by Boström and Mansson (17). When slices of costal cartilage of suckling calves were incubated in Krebs-Ringer's phosphate or bicarbonate containing S^{35}, incorporation in relatively small amounts of radioactive sulfate in the isolated C.S.A. could be demonstrated. The reaction appeared to be enzymatic in nature, since, a) incubation in 93.5 per cent N_2·6.5 per cent CO_2 markedly reduced the extent of incorporation; b) the system was inactivated by heating between 43°C. and 47°C., and the rate of reaction was increased with increasing temperature (below the inactivation range); c) freezing and thawing results in inactivation; and d) the amount of incorporation of S^{35} is progressive with time. The authors studied the effect of a large number of commonly used enzyme inhibitors, and found that those reagents which combine with sulfhydryl groups were more effective in inhibiting this system than metal and carbonyl inhibitors. Moderate inhibition was also observed with salicylates, but the concentrations required were in the range in which salicylates act as protein denaturants. Boström and Mansson (17) have reported that this fixation of S^{35} is stimulated by a heat stable factor found in liver homogenates.

The incorporation of C^{14} from acetate in cartilage slices, at a rate somewhat slower than that of sulfate, has been reported by Boström and Mansson (16). The incorporation was not linear with time. Although methyl-labeled acetate was used, the acetate isolated from the C.S.A. showed distribution of the C^{14} between the methyl and carboxyl group. The latter finding suggests that under these conditions the rate limiting reaction was not necessarily polysaccharide synthesis.

In view of the limitations of the information that can be obtained by the use of S^{35}, a series of studies has been undertaken in the author's laboratory utilizing C^{14} either alone or in combination with S^{35}. Before studies of metabolic rates could be undertaken, it was necessary to devise methods for the isolation of the acid mucopolysaccharides free of other contaminating carbon compounds, as well as their separation from each other. Schiller *et al.* (132) have devised methods for the preparation from rabbit skin of both H.A. and a sulfated mucopoly-

saccharide of high analytical purity. By all available methods, the H.A. behaves like that isolated from other sources, but the sulfated fraction shows certain discrepancies in behavior when compared with cartilage C.S.A. These are, a lower color equivalent by the carbazole reaction, different behavior toward testicular hyaluronidase, and difference in optical rotation. The exact nature of the sulfated mucopolysaccharides has not as yet been elucidated.

Using these methods, it has been possible to study rates of incorporation of C^{14}, following the administration of carboxyl labeled acetate and labeled glucose. In the first set of experiments, C^{14} carboxyl labeled acetate was administered in three divided doses daily for various intervals. The rabbits were sacrificed at the end of the periods and the H.A. and sulfated polysaccharides were isolated from the skin of the individual animals for radioactivity analyses. The rate of incorporation of C^{14} into H.A. was found to be about 3 times as rapid as that observed in the sulfated mucopolysaccharides.

In another set of experiments, approximately 0.75 mc. of carboxyl-labeled acetate was administered in divided doses on one day and pairs of animals were sacrificed on the first, fourth, eighth and twelfth days after the beginning of injections (133). The initial radioactivity (18 hours after the last administered dose) of the H.A. was found to be considerably greater than that of the sulfated polysaccharides. The rate of decrease of this activity was much greater for the H.A. than for the sulfated polysaccharides. Actual calculation of half-life time was not entirely satisfactory because of the complexity of the kinetics involved over the period of the experiment. Assuming first order kinetics, however, a half-life time of about two days for H.A., and a considerably longer period for the sulfated polysaccharides, was indicated. When polysaccharides from duplicate animals for each time period were pooled and degraded, it was possible to demonstrate that almost all of the radioactivity appears in the acetyl group. This is in keeping with the previous demonstration of the utilization of acetate for the N-acetyl group in the synthesis of H.A. by Group A streptococcus, and the relative inefficiency of acetate as a precursor for glucose (which presumably acts as the precursor of the hexosamine and glucuronic acid portions of the polysaccharide molecule).

The finding of a rapid rate of turnover of H.A. in the skin of adult rats is somewhat surprising in view of the usually assumed stability of connective tissue. However, it makes better understandable the known rapidity of changes that apparently occur under certain physiological and pathological stimuli.

The marked discrepancy of turnover time in the H.A. and the sulfated polysaccharides deserves special comment. The data so far available suggest the possibility that the sulfated polysaccharide isolated from skin may represent a mixture of substances, thus, the apparent turnover rates would be a function of more than one rate. It is apparent, that at least one sulfated substance is present which turns over much more slowly than H.A. The actual rates observed appear to be of the same order of magnitude as that observed by Boström and Gardell (18) utilizing S^{35}.

That these half-life times are not due solely to exchange reactions of the ace-

tate and sulfate groups is demonstrated by more recent experiments in which uniformly labeled glucose has been used, and the turnover rates of the hexosamine moieties have been studied. Almost identical half-life times have been obtained (134).

The marked difference in the rates of synthesis of H.A. and C.S.A. is of considerable physiological interest, in view of the differences in physical chemical behavior that might be expected of these compounds as a result of the presence of the sulfate ester group in the sulfated polysaccharides.

EFFECTS OF HORMONES

There exists an extensive biological literature regarding the effects of various biological agents on connective tissues. Most of this is based on biological behavior or histochemical studies. No attempt will be made here to review these various studies. This discussion will be confined to a brief consideration of those studies which have utilized more direct chemical methods for the study of the effects of hormones on the metabolism of mucopolysaccharides.

The striking clinical effects of adrenocorticotropic hormone and cortisone on a variety of diseases which are characterized by changes in connective tissue has led many investigators to study the effects of these hormones on connective tissue. The author (34) has previously reviewed this literature. Layton (82) found that the administration of cortisone to rats (20 mg. per kilo) resulted in a drop to 25 per cent of control levels in the incorporation of S^{35} in the skin. Concomitantly, there was an increase in excretion of ester bound sulfate in the urine. In another study, this author (80) found that cortisone alcohol inhibited the esterification of $S^{35}O_4^=$ by explants of chick embryo tissues. It was claimed that inhibition of synthesis occurred in the absence of inhibition of fibroblast growth.

Boström and Odeblad (15) have studied the effect of cortisone on incorporation of $S^{35}O_4$ both *in vivo* and *in vitro*. After the administration of cortisone to rats there was a 60 per cent decrease in the incorporation of $S^{35}O_4^=$ in rib cartilage. A decrease in incorporation into skin was also demonstrated by radioautography. Addition of cortisone alcohol to cartilage slices resulted in inhibition of *in vitro* incorporation of sulfate of up to 60 per cent. The inhibition was proportional to the concentration of cortisone up to a level of 10 mg. per cent. It should be pointed out, however, that this represents a relatively huge concentration of cortisone. No data are given with respect to the effect of other steroids under similar conditions.

Clark and Umbreit (30) have recently found that although cortisone alcohol inhibits the synthesis of C.S.A. by cartilage slices, no such inhibition is produced by hydrocortisone or its acetate, although desoxycorticosterone acetate did produce marked inhibition. These data cast considerable doubt on whether or not the observed inhibition by cortisone has any significance in explaining the *in vitro* effects of cortisone upon connective tissue.

Schiller *et al.* (130) showed that although testosterone stimulated the synthesis of mucopolysaccharides in the cock's comb (as measured by hexosamine

analyses), as previously reported by Boas (7), no inhibition by cortisone was observed. It was pointed out that the effect of hormones may vary, depending upon local factors. A striking confirmation of this local effect was the finding by Schiller and Dorfman (135) that, although testosterone caused a marked increase in hexosamine in chick comb, no such change was observed in the skin of the same animals.

Dziewiatkowski (46) studied the effect of thyroxine and thiouracil on the S^{35} deposition in articular cartilage of suckling rats. Thiouracil resulted in a decrease in the uptake of S^{35}, but no stimulation of uptake by thyroxine could be demonstrated, although the hormone did counteract the effects of thiouracil. Interpretation of these results in terms of direct effects on the metabolism of the mucopolysaccharides is difficult in view of the effects of the hormones on differentiation of the cartilage cells.

Ellis *et al.* (51) have recently demonstrated that hypophysectomy in rats results in a decrease in the rate of incorporation of S^{35} in C.S.A. in rats, and this can be partially restored by administration of growth hormone.

Several studies by Watson and Pearce (150, 151) have demonstrated the large increase of mucopolysaccharides in pretibial myxedema. Along similar lines Ludwig *et al.* (90) showed an increase in hexosamine and metachromatic material in orbital contents in experimental exophthalmos in the guinea pig.

SUMMARY

An attempt has been made to bring together a variety of observations pertinent to the general subject of the metabolism of the mucopolysaccharides of connective tissue. This has necessitated the consideration of biochemical and biological phenomena which superficially, at least, appear unrelated. It is obvious that at this stage of knowledge no definitive picture can be drawn of either the mechanisms of synthesis or the influence of various biological factors thereon. There are, however, an increasing number of indications of directions for promising investigations aimed at the elucidation of the metabolism of these compounds, although many unsuspected surprises will undoubtedly appear.

In order to correlate some of the information which has been indicated in the text and to bring this information in relation to well established reactions of carbohydrate metabolism, the accompanying figures have been constructed.

Reactions which have been demonstrated are indicated by a solid arrow, postulated reactions by a dotted arrow, and reactions which, on the basis of available evidence, probably do not occur are indicated by cancelled arrows. Figure 1 is concerned with reactions of hexosamines. The schemes illustrated have been indicated on the basis of reactions which have been demonstrated in a variety of tissues. It is obviously impossible at this stage of development to speak of a pathway for any given tissue. For purposes of simplicity most of the chart is concerned only with glucosamine, although it is likely that similar pathways apply to galactosamine, but these have hardly been studied at all. Phosphorylation of galactosamine by galactokinase has been demonstrated. It is to be noted that N-acetylglucosamine is shown to be converted to uridine diphos-

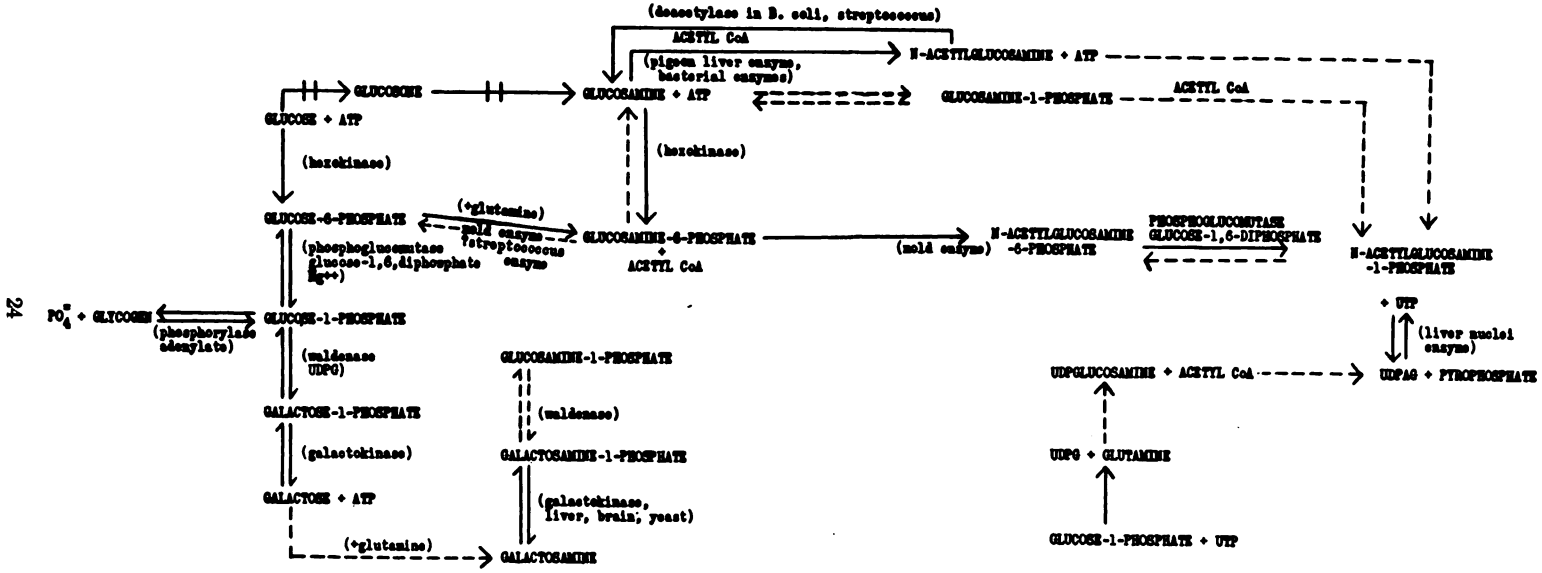

FIG. 1. Pathways of metabolism of hexosamines

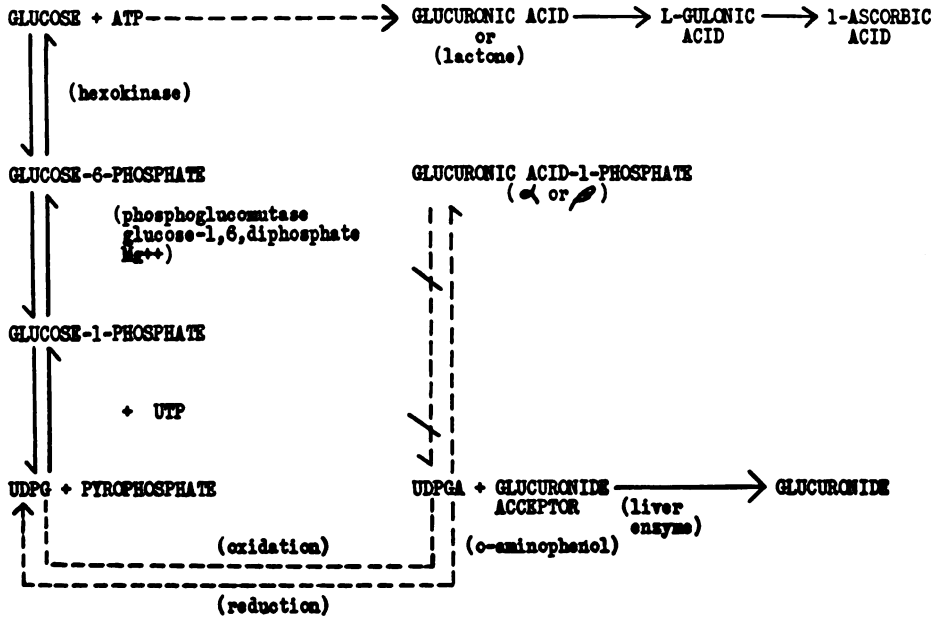

FIG. 2. Possible pathways of formation of uronides

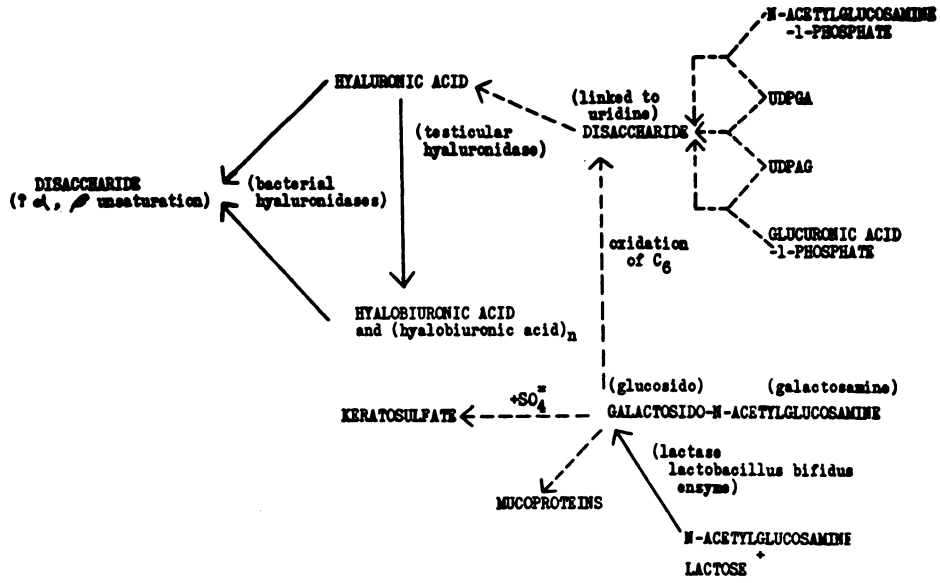

FIG. 3. Pathways of metabolism of mucopolysaccharides

pho N-acetylglucosamine. The demonstration of the pyrophosphorolysis of UDPAG by an enzyme from liver nuclei, and the demonstration of the role of uridine compounds in the synthesis of other heterologous glycosides (sucrose, o-amino-phenol glucuronide) suggest the possible importance of this compound,

although no direct evidence of the participation in mucopolysaccharide metabolism has yet been obtained. It should also be noted that UDPAG may arise by the amination and acetylation of UDPG.

Figure 2 illustrates some of the reactions involving the uronide portion of the molecule. Of importance to note is that, although α- and β-1-phosphoglucuronic acid have been made synthetically, there is, so far, no evidence of their participation in enzymatic reactions. It should again be emphasized that the predominance of evidence indicates that glucuronic acid *per se* does not enter into uronide synthesis. For these reasons it is suggested that oxidation of C_6 may occur after glycoside formation.

In figure 3 an attempt is made to depict possible mechanisms involved in the metabolism of the entire polysaccharide molecule. The reactions given involve only H.A., it being assumed that similar reactions may be involved in the biosynthesis of C.S.A. which, of course, would, in addition, involve esterification of sulfate, the mechanism of which is entirely unknown.

The possible role of the uridine compounds in the biosynthesis of these heterologous polysaccharides is illustrated in only a very general way, since there are no data to point to probable mechanisms. It is obvious that this problem is complicated by the necessity of formation of different alternate bonds. In addition to the possible role of the uridine compounds, another mechanism is illustrated. This involves the formation of a glycoside of N-acetylglucosamine. Two reports have indicated that such glycosides may be formed by the action of hydrolytic enzymes (145, 159). A similar compound isolated by Meyer *et al.* (105) from cornea has been named keratosulfate. If mechanisms for the oxidatior of the C_6 carbon atom exist, such compounds might serve as intermediates in the synthesis of mucopolysaccharides, while without oxidation of the C_6 carbon such compounds may play a role in the synthesis of other hexosamine containing compounds such as serum mucoproteins or blood group substances.

The schemes proposed in figures 1, 2, and 3 are not meant to lay definite claims to actual pathways, but to be used as a framework for the correlation of many scattered observations, and to point to certain suggestive pathways.

Kalckar and Strominger (personal communication) have recently isolated an enzyme from liver which brings about the oxidation of UDPG to UDPGA as postulated in this review.

REFERENCES

1. Artz, N. E. and Osman, E. M.: Biochemistry of glucuronic acid. Acad. Press, New York 1950.
2. Asboe-Hansen, G.: The origin of synovial mucin. Ehrlich's mast cell—a secretory element of the connective tissue. Ann. Rheumat. Dis., 9: 149–158, 1950.
3. Asboe-Hansen, G.: Autoradiography of mast cells in experimental skin tumors of mice injected with radioactive sulfur (S^{35}). Cancer Res., 13: 587–589, 1953.
4. Becker, C. E. and Day, H. G.: Utilization of glucosone and the synthesis of glucosamine in the rat. J. Biol. Chem., 201: 795–801, 1953.
5. Bergfeld, W. and Kapfhammer, J.: Belastungsversuche mit Glucosamin bei Mensch und Tieren. Deutsche Ztschr. Verdauungskr., 8: 113–117, 1944.
6. Bidder, T. G.: The origins of glucuronic acid. J. Am. Chem. Soc., 74: 1616–1617, 1952.
7. Boas, N. F.: Isolation of hyaluronic acid from the cock's comb. J. Biol. Chem., 181: 573–575, 1949.
8. Boström, H.: On the metabolism of the sulfate group of chondroitinsulfuric acid. J. Biol. Chem., 196: 477–481, 1952.

9. Boström, H.: Chemical and autoradiographic studies on the sulfate exchange in sulfomucopolysaccharides. Arkiv Kemi, 6: 43–57, 1953.
10. Boström, H., Odeblad, E. and Friberg, U.: A quantitative autoradiographic study of the incorporation of S^{35} in tracheal cartilage. Arch. Biochem., 38: 283–286, 1952.
11. Boström, H. and Åqvist, S. E. G.: Utilisation of S^{35}-labeled sodium sulfate in the synthesis of chondroitinsulfuric acid, taurine, methionine and cystine. Acta chem. scandinav., 6: 1557–1559, 1952.
12. Boström, H. and Odeblad, E.: Autoradiographic observations on the uptake of S^{35} in the genital organs of the female rat and rabbit after injection of labeled sodium sulfate. Acta endocrinol., 10: 89–96, 1952.
13. Boström, H. and Odeblad, E.: Autoradiographic observations on the uptake of S^{35}-labeled sodium sulfate in the nervous system of the adult rat. Acta psychiat. et neurol. scandinav., 28: 5–8, 1953.
14. Boström, H. and Odeblad, E.: Autoradiographic observations on the incorporation of sulfur^{35}-labeled sodium sulfate in the rabbit fetus. Anat. Rec., 115: 105–113, 1953.
15. Boström, H. and Odeblad, E.: The influence of cortisone upon the sulphate exchange of chondroitinsulfuric acid. Arkiv Kemi, 6: 39–42, 1953.
16. Boström, H. and Mansson, B.: Exchange of the acetyl group of CSA in slices of cartilage. Acta chem. scandinav., 6: 1559–1561, 1952.
17. Boström, H. and Mansson, B.: The demonstration of a liver factor stimulating the sulphate exchange of chondroitinsulfuric acid. Acta chem. scandinav., 7: 1014, 1953.
18. Boström, H. and Gardell, S.: Uptake of sulfates in mucopolysaccharides esterified with sulfuric acid in the skin of adult rats after intraperitoneal injection of sulfur^{35}-labeled sodium sulfate. Acta chem. scandinav., 7: 216–222, 1953.
19. Boström, H., Odeblad, E. and Friberg, U.: A qualitative and quantitative autoradiographic study on the uptake of S^{35}-labeled sodium sulphate in the skin of the adult rat. Acta path. et microbiol. scandinav., 32: 516–521, 1953.
20. Bray, H. G., Gregory, J. E. and Stacey, M.: Chemistry of tissues. I. Chondroitin from cartilage. Biochem. J., 38: 142–146, 1944.
21. Bray, H. G.: β-Glucuronic acid in metabolism. Advanc. Carbohyd. Chem., 8: 251–275, 1953.
22. Brown, D. H.: The phosphorylation of D (+)-glucosamine by crystalline yeast hexokinase. Biochim. et biophys. acta, 7: 487–493, 1951.
23. Brown, D. H.: Action of phosphoglucomutase on D-glucosamine-6-phosphate. J. Biol. Chem., 204: 877–889, 1953.
24. Brox, G.: Beiträge zum Glukuronsäurestoffwechsel. I. Mitt. Zur Physiologie. Deutsche Ztschr. Verdauungskr., 13: 99–111, 1953.
25. Cabib, E., Leloir, L. F. and Cardini, C. E.: Uridine diphosphate acetyl-glucosamine. J. Biol. Chem., 203: 1055–1070, 1953.
26. Caputto, R., Leloir, L. F., Cardini, C. E. and Paladini, A. C.: Isolation of the coenzyme of the galactose phosphate-glucose phosphate transformation. J. Biol. Chem., 184: 333–350, 1950.
27. Cardini, C. E. and Leloir, L. F.: Enzymic phosphorylation of galactosamine and galactose. Arch. Biochem., 45: 55–64, 1953.
28 Catchpole, H. R.: Serum and tissue glycoproteins in mice bearing transplantable tumors. Proc. Soc. Exper. Biol. & Med., 75: 221–223, 1950.
29. Chou, P. C. and Soodak, M.: The acetylation of D-glucosamine by pigeon liver extracts. J. Biol. Chem., 196: 105–109, 1952.
30. Clark, I. and Umbreit, W. W.: Effect of cortisone and other steroids upon *in vitro* synthesis of chondroitin sulfate. Proc. Soc. Exper. Biol. & Med., 86: 558–561, 1954.
31. Dische, Z.: Method for determination of hexuronic acid. J. Biol. Chem., 167: 189–198, 1947.
32. Dobrschuk, A. P.: Radiotracer studies of the biosynthesis of conjugated D-glucuronic acid. J. Biol. Chem., 195: 855–859, 1952.
33. Dorfman, A.: The action of serum on hyaluronidase. Ann. New York Acad. Sc., 52: 1098–1104, 1950.
34. Dorfman, A.: The effects of adrenal hormones on connective tissues. Ann. New York Acad. Sc., 56: 698–703, 1953.
35. Dorfman, A., Roseman, S., Ludowieg, J. L., Moses, F. E., Ciponelli, J. A. and Mayeda, M.: Unpublished results.
36. Douglas, J. F. and King, C. G.: The metabolism of uniformly labeled D-glucuronic acid-C^{14} in the guinea pig. J. Biol. Chem., 198: 187–194, 1952.
37. Douglas, J. F. and King, C. G.: The conversion of C^{14}-labeled glucose to glucuronic acid in the guinea pig. J. Biol. Chem., 202: 865–871, 1953.
38. Douglas, J. F. and King, C. G.: The metabolism of C^{14}-labeled D-glucuronic acid in the guinea pig and the albino rat. J. Biol. Chem., 203: 889–894, 1953.
39. Drury, D. R. and Salter, W. T.: The effect of glucose derivatives upon animals (rabbits) following hepatectomy. Am. J. Physiol., 107: 406–413, 1934.
40. Dutton, G. J. and Storey, I. D. E.: Glucuronide synthesis in liver homogenates. Biochem. J., 48: xxix, 1951.
41. Dutton, G. J. and Storey, I. D. E.: The isolation of a compound of uridine diphosphate and glucuronic acid from liver. Biochem. J., 53: xxxvii, 1953.
42. Dutton, G. J. and Storey,. I D. E.: Uridine compounds in glucuronic acid metabolism. 1. The formation of glucuronides in liver suspensions. Biochem. J., 57: 275–283, 1954.
43. Dziewiatkowski, D. D., Benesch, R. E. and Benesch, R.: On the possible utilization of sulfate sulfur by

the suckling rat for the synthesis of chondroitin sulfate as indicated by the use of radioactive sulfur. J. Biol. Chem., 178: 931–938, 1949.

44. Dziewiatkowski, D. D.: Radioautographic visualization of sulfur-35 disposition in articular cartilage and bone of suckling rats following administration of labeled sodium sulfate. Fed. Proc., 10: 178, 1951.
45. Dziewiatkowski, D. D.: Radioautographic visualization of sulfur-35 disposition in the articular cartilage and bone of suckling rats following injection of labeled sodium sulfate. J. Exper. Med., 93: 451–458, 1951.
46. Dziewiatkowski, D. D.: Effect of thyroxine and thiouracil on S^{35} deposition in articular cartilage. J. Biol. Chem., 189: 717–727, 1951.
47. Dziewiatkowski, D. D.: Radioautographic studies of sulfate-sulfur (S^{35}) metabolism in the articular cartilage and bone of suckling rats. J. Exper. Med., 95: 489–496, 1952.
48. Dziewiatkowski, D. D.: Sulfate-sulfur metabolism in the rat fetus as indicated by sulfur-35. J. Exper. Med., 98: 119–128, 1953.
49. East, M. E., Madinaveitia, J. and Todd, A. R.: The β-glucosaminase activity of testicular extracts and its bearing on the problem of diffusing factors. Biochem. J., 35: 872–876, 1941.
50. Eisenberg, F. and Gurin, S.: The biosynthesis of glucuronic acid from 1-C^{14}-glucose. J. Biol. Chem., 195: 317–325, 1952.
51. Ellis, S., Huble, J. and Simpson, M. E.: Influence of hypophysectomy and growth hormone on cartilage sulfate metabolism. Proc. Soc. Exper. Biol. & Med., 84: 603–605, 1953.
52. Fishman, W. H. and Talalay, P.: A simplified method of preparing active extracts of β-glucuronidase. Science, 105: 131–132, 1947.
53. Fishman, W. H.: β-Glucuronidase. In "The Enzymes", vol. I, part 1, pp. 635–652, ed. by Sumner, J. B. and Myrbäck, K. Acad. Press, New York 1950.
54. Fretwurst, F. and Ahlhelm, H. A.: Untersuchungen über den Glucuronsäure-Stoffwechsel beim Menschen nach peroraler und intravenöser Zufuhr. Arch. exper. Path. u. Pharmakol., 217: 382–398, 1953.
55. Gersh, I.: Ground substance and the plasticity of connective tissue. Harvey Lect., 45: 211–241, 1949.
56. Gersh, I. and Catchpole, H. R.: The organization of ground substance and basement membrane and its significance in tissue injury, disease and growth. Am. J. Anat., 85: 457–521, 1949.
57. Gottschalk, R. G. and Allen, H. C.: Uptake of radioactive sulfur by chondrosarcomas in man. Proc. Soc. Exper. Biol. & Med., 80: 334–339, 1952.
58. Grafe, E. and Meythaler, F.: Beiträge zur Kenntnis der Regulation der Insulinproduktion. III. Mitteilung: Die Wirkung von Anhydrokohlenhydraten, Zuckerderivaten und Zuckerspaltungsprodukten. Arch. exper. Path. u. Pharmakol., 136: 360–369, 1928.
59. Grant, T. P. and Long, C.: The enzymic phosphorylation of D-glucosamine. Biochem. J., 50: xx, 1952.
60. Hahn, L.: Ueber das mucolytische Enzym des Säugerhodens. Biochem. Ztschr., 315: 83–96, 1943.
61. Hahn, L.: On the splitting of hyaluronic acid by mucopolysaccharases of leeches and *Cl. perfringens*. Arkiv Kemi Min. Geol., 19A: No. 33, 1945.
62. Hahn, L.: On the mucopolysaccharide-splitting enzyme system of the mammalian testis. Arkiv Kemi Min. Geol., 21A: No. 1, 1945.
63. Hahn, L.: Ueber die enzymatische Spaltung der Hyaluronsäure. II. Mitteilung: Abbau zu Monosacchariden. Biochem. Ztschr., 318: 138–148, 1947.
64. Harpur, R. P. and Quastel, J. H.: Phosphorylation of d-glucosamine by brain extracts. Nature, London, 164: 693–694, 1949.
65. Horowitz, H. H. and King, C. G.: Glucuronic acid as a precursor of ascorbic acid in the albino rat. J. Biol. Chem., 205: 815–821, 1953.
66. Isherwood, F. A., Chen, Y. T. and Mapson, L. W.: Synthesis of L-ascorbic acid in plants and animals. Biochem. J., 56: 1–15, 1954.
67. Jackson, D. S.: The nature of collagen-chondroitin sulfate linkages in tendon. Biochem. J., 56: 699–703, 1954.
68. Jorpes, J. E.: The origin and the physiology of heparin: the specific therapy in thrombosis. Ann. Int. Med., 27: 361–370, 1947.
69. Jorpes, J. E., Werner, B. and Åberg, B.: The fuchsin-sulfurous acid test after periodate oxidation of heparin and allied polysaccharides. J. Biol. Chem., 176: 277–282, 1948.
70. Jorpes, J. E., Odeblad, E. and Boström, H.: An autoradiographic study on the uptake of S^{35}-labelled sodium sulphate in the mast cells. Acta haemat., 9: 273–276, 1953.
71. Kalckar, H. M.: Role of phosphoglycosyl compounds in the biosynthesis of nucleosides and nucleotides. Biochim. et biophys. acta, 12: 250–264, 1953.
72. Kalckar, H. M.: The enzymes of uridine polyphosphates. Science, 119: 479–480, 1954.
73. Katz, J., Lieberman, I. and Barker, H. H.: Acetylation of amino acids by enzymes of *Clostridium kluyveri*. J. Biol. Chem., 200: 417–429, 1953.
74. Kawabe, K.: Biochemical studies on carbohydrates. VII. On an enzyme which disintegrates glucosamine. J. Biochem., 20: 233–241, 1934.
75. Kawabe, K.: Biochemical studies on carbohydrates. VIII. The distribution of glucosamine among tissues when it is administered into the animal body subcutaneously or parenterally, and its renal threshold. J. Biochem., 20: 243–251, 1934.
76. Layton, L. L. and Frankel, D. R.: Labeled inorganic sulfate in the diagnosis of cartilaginous tumors and their metastases. Cancer, 2: 1089–1092, 1949.
77. Layton, L. L., Frankel, D. R. and Scapa, S.: Maternal sulfate utilized by mammalian embryos and suckling young. Arch. Biochem., 28: 142–144, 1950.

78. LAYTON, L. L.: In vitro sulfate fixation by granulation tissue and injured muscle tissue from healing wounds. Proc. Soc. Exper. Biol. & Med., 73: 570–572, 1950.
79. LAYTON, L. L.: Quantitative differential fixation of sulfate by tissues maintained *in vitro*. I. Sulfate fixation as a function of age for embryonic tissues. Cancer, 3: 725–734, 1950.
80. LAYTON, L. L.: Effect of cortisone upon chondroitin sulfate synthesis by animal tissues. Proc. Soc. Exper. Biol. & Med., 76: 596–598, 1951.
81. LAYTON, L. L. AND FRANKEL, D. R.: Sulfate esterifying activity of embryonic chick liver *in vitro*. Arch. Biochem., 31: 161–167, 1951.
82. LAYTON, L. L.: Cortisone inhibition of mucopolysaccharide synthesis in the intact rat. Arch. Biochem., 32: 224–226, 1951.
83. LAYTON, L. L.: The anabolic metabolism of radioactive sulfate by animal tissues *in vitro* and *in vivo*. Cancer, 4: 198–201, 1951.
84. LELOIR, L. F.: The enzymatic transformation of uridine diphosphate glucose into a galactose derivative. Arch. Biochem., 33: 186–190, 1951.
85. LELOIR, L. F. AND CARDINI, C. E.: Biosynthesis of glucosamine. Biochim. et biophys. acta, 12: 15–22, 1953.
86. LELOIR, L. F. AND CARDINI, C. E.: The biosynthesis of sucrose. J. Am. Chem. Soc., 75: 6084, 1953.
87. LELOIR, L. F. AND CABIB, E.: The enzyme synthesis of trehalose phosphate. J. Am. Chem. Soc., 75: 5445–5446, 1953.
88. LINKER, A., WEISSMANN, B. AND MEYER, K.: Hydrolysis of hyaluronate by bacterial hyaluronidases. Fed. Proc., 13: 840, 1954.
89. LOWTHER, D. A. AND ROGERS, H. J.: The relation of glutamine to the synthesis of hyaluronate or hyaluronate-like substances by streptococci. Biochem. J., 53: xxxix, 1953.
90. LUDWIG, A. W., BOAS, N. F. AND SOFFER, L. J.: Role of mucopolysaccharides in experimental exophthalmos. Proc. Soc. Exper. Biol. & Med., 73: 137–140, 1950.
91. LUTWAK-MANN, C.: Enzymic decomposition of amino-sugars. Biochem. J., 35: 610–636, 1941.
92. MADINAVEITIA, J. AND QUIBELL, T. H. H.: Studies on diffusing factors. 6. The action of testicular extracts on the viscosity of vitreous humour preparations. Biochem. J., 34: 625–631, 1940.
93. MAPSON, L. W., ISHERWOOD, F. A. AND CHEN, Y. T.: Biological synthesis of L-ascorbic acid: The conversion of L-galactono-γ-lactone into L-ascorbic acid by plant mitochondria. Biochem. J., 56: 21–28, 1954.
94. MARBET, R. AND WINTERSTEIN, A.: Probleme der Blutgerinnung. 4. Mitteilung: β-Heparin, ein neuer, blutgerinnungshemmender Mucoitinschwefelsäureester. Helvet. chim. acta, 34: 2311–2320, 1951.
95. MARBET, R. AND WINTERSTEIN, A.: Mucopolysaccharides with special reference to heparin. Experientia, 8: 41–44, 1952.
96. MATHEWS, M. B., ROSEMAN, S. AND DORFMAN, A.: Determination of the chondroitinase activity of bovine testicular preparations. J. Biol. Chem., 188: 327–334, 1951.
97. MCCLEAN, D. AND HALE, C. W.: Studies on diffusing factors. The hyaluronidase activity of testicular extracts, bacterial culture filtrates and other agents that increase tissue permeability. Biochem. J., 35: 159–183, 1941.
98. MEYER, K.: The biological significance of hyaluronic acid and hyaluronidase. Physiol. Rev., 27: 335–359, 1947.
99. MEYER, K. AND CHAFFEE, E.: The mucopolysaccharides of skin. J. Biol. Chem., 138: 491–499, 1941.
100. MEYER, K., CHAFFEE, E., HOBBY, G. L. AND DAWSON, M. H.: Hyaluronidases of bacterial and animal origin. J. Exper. Med., 73: 309–326, 1941.
101. MEYER, K. AND RAPPORT, M. M.: The mucopolysaccharides of the ground substance of connective tissue. Science, 113: 596–598, 1951.
102. MEYER, K. AND RAPPORT, M. M.: Hyaluronidases. Advances Enzymol., 13: 199–236, 1952.
103. MEYER, K. AND RAPPORT, M. M.: The hydrolysis of chondroitin sulfate by testicular hyaluronidase. Arch. Biochem., 27: 287–293, 1950.
104. MEYER, K., LINKER, A. AND RAPPORT, M. M.: The production of monosaccharides from hyaluronic acid by β-glucuronidase. J. Biol. Chem., 192: 275–291, 1951.
105. MEYER, K., LINKER, A., DAVIDSON, E. A. AND WEISSMAN, B.: The mucopolysaccharides of bovine cornea. J. Biol. Chem., 205: 611–616, 1953.
106. MOSBACH, E. H. AND KING, C. G.: Tracer studies of glucuronic acid biosynthesis. J. Biol. Chem., 185: 491–498, 1950.
107. MUNCH-PETERSON, A., KALCKAR, H. M., CUTOLO, E. AND SMITH, E. E. B.: Uridyl transferases and the formation of uridine triphosphate. Nature, London, 172: 1036–1037, 1953.
108. ODEBLAD, E.: Autoradiographic study with sulfur^{35} on the corpus luteum of the rabbit. Acta endocrinol., 11: 306–310, 1952.
109. ODEBLAD, E. AND BOSTRÖM, H.: An autoradiographic study of the incorporation of sulfur^{35}-labeled sodium sulfate in different organs of adult rats and rabbits. Acta path. et microbiol. scandinav., 31: 339–344, 1952.
110. ODEBLAD, E. AND BOSTRÖM, H.: Quantitative autoradiographic study on the uptake of labeled sulfate in the aorta of the rabbit. Acta chem. scandinav., 7: 233–234, 1953.
111. ORR, S. F. D.: Infra-red spectroscopic studies of some polysaccharides. Biochim. et biophys. acta, 14: 173–181, 1954.
112. PACKHAM, M. A. AND BUTLER, G. C.: Studies of the biological synthesis of glucuronides. J. Biol. Chem., 194: 349–357, 1952.
113. PACKHAM, M. A. AND BUTLER, G. C.: The fate of injected sodium glucuronate and glucurone in the rat. J. Biol. Chem., 207: 639–646, 1954.

114. Packham, M. A. and Butler, G. C.: Personal communication.
115. Paladini, A. C. and Leloir, L. F.: Studies on uridine-diphosphate-glucose. Biochem. J., 51: 426–430, 1952.
116. Park, J. T.: Uridine-5'-pyrophosphate derivatives. II. A structure common to three derivatives. J. Biol. Chem., 194: 885–895, 1952.
117. Park, J. T.: Uridine-5'-pyrophosphate derivatives. III. Amino-acid containing derivatives. J. Biol. Chem., 194: 897–904, 1952.
118. Pontis Videla, H. G.: Un nueva nucleotida de uridina. Ciencia e Investigacion, 10: 226–237, 1954.
119. Rieder, S. V.: Biosynthesis of D-glucosamine. Fed. Proc., 12: 258, 1953.
120. Rogers, H. J.: The conditions controlling the production of hyaluronidase by microorganisms grown in simplified media. Biochem. J., 39: 435–443, 1945.
121. Rogers, H. J.: The influence of hydrolysates of hyaluronate upon hyaluronidase production of microorganisms. Biochem. J., 40: 583–588, 1946.
122. Rogers, H. J.: An analysis of the products formed during the hydrolysis of hyaluronate by enzymes and acids, with observations on the nature of the amino-sugar released from the polysaccharide. Biochem. J., 40: 782–788, 1946.
123. Roseman, S. and Dorfman, A.: α-Glucosaminidase. J. Biol. Chem., 191: 607–620, 1951.
124. Roseman, S., Moses, F. E., Ludowieg, J. and Dorfman, A.: The biosynthesis of hyaluronic acid by group A streptococcus. I. Utilization of 1-C^{14}-glucose. J. Biol. Chem., 203: 213–225, 1953.
125. Roseman, S.: Hydrolysis of N-acetyl-D-glucosamine by extracts of *Escherichia coli*. Fed. Proc., 13: 283, 1954.
126. 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., 206: 665–669, 1954.
127. Sallman, B., Birkeland, J. M. and Gray, C. T.: Hyaluronic acid utilization by hemolytic streptococci in relation to possible hyaluronidase function in pathogenesis. Proc. Soc. Exper. Biol. & Med., 76: 467–471, 1951.
128. Salter, W. T., Robb, P. D. and Scharles, F. H.: Liver glycogen from derivatives of glucose. J. Nutrition, 9: 11–23, 1935.
129. Schambye, P., Wood, H. G. and Popjak, G.: Biological asymmetry of glycerol and formation of asymmetrically labeled glucose. J. Biol. Chem., 206: 875–882, 1954.
130. Schiller, S., Benditt, E. P. and Dorfman, A.: Effect of testosterone and cortisone on the hexosamine content and metachromasia of chick combs. Endocrinology, 50: 504–510, 1952.
131. Schiller, S., Mathews, M. B. and Dorfman, A.: Metabolism of the mucopolysaccharides of the skin. Fed. Proc., 13: 290, 1954.
132. Schiller, S., Mathews, M. B., Jefferson, H., Ludowieg, J. and Dorfman, A.: The metabolism of mucopolysaccharides in animals. I. Isolation from skin. J. Biol. Chem., 211: 717–724, 1954.
133. Schiller, S., Mathews, M. B., Goldfaber, L., Ludowieg, J. and Dorfman, A.: The metabolism of mucopolysaccharides in animals. II. Studies in skin utilising labeled acetate. J. Biol. Chem., in press.
134. Schiller, S., Goldfaber, L. and Dorfman, A.: Unpublished results.
135. Schiller, S. and Dorfman, A.: Unpublished results.
136. Schmitt, F. O., Gross, J. and Highberger, J. H.: A new particle type in certain connective tissue extracts. Proc. Nat. Acad. Sc., 39: 459–470, 1953.
137. Singher, H. O. and Marinelli, L.: Distribution of radioactive sulfur in the rat. Science, 101: 414–415, 1945.
138. Smith, H. and Gallop, R. C.: The 'acid polysaccharides' of hog gastric mucosa. Biochem. J., 53: 666–671, 1953.
139. Smith, E. E. B. and Mills, G. T.: Uridine nucleotide compounds of liver. Biochim. et biophys. acta, 13: 386–400, 1954.
140. Smith, E. E. B., Munch-Peterson, A. and Mills, G. T.: Pyrophosphorolysis of uridine diphosphoglucose and 'UDPX' by a rat liver nuclear fraction. Nature, London, 172: 1038–1039, 1953.
141. Storey, I. D. E.: The synthesis of glucuronides by liver slices. Biochem. J., 47: 212–222, 1950.
142. Swick, R. W. and Nakao, A.: The biological asymmetry of glycerol. J. Biol. Chem., 206: 883–886, 1954.
143. Tabor, H., Mehler, A. H. and Stadtman, E. R.: The enzymatic acetylation of amines. J. Biol. Chem., 204: 127–138, 1953.
144. Topper, Y. J. and Lipton, M. M.: The biosynthesis of a streptococcal capsular polysaccharide. J. Biol. Chem., 203: 135–142, 1953.
145. Wallenfels, K.: Enzymatische Synthese neuer Oligosaccharide mittels Hydrolasen. Angew. Chem., 65: 137, 1953.
146. Watanabe, K.: Biochemical studies on carbohydrates. XXI. Chemistry of assimilation and disintegration of N-monoacetylglucosamine. J. Biochem., 24: 287–295, 1936.
147. Watanabe, K.: Biochemical studies on carbohydrates. XXII. On Animal β-N-monoacetylglucosaminidase. Preliminary report. J. Biochem., 24: 297–303, 1936.
148. Watanabe, K.: Biochemical studies on carbohydrates. XXIII. Animal β-N-monoacetylglucosaminidase. 2. Purification of the enzyme. J. Biochem., 24: 305–313, 1936.
149. Watanabe, K.: Biochemical studies on carbohydrates. XXIV. Animal β-N-monoacetylglucosaminidase. 3. Kinetics of the enzyme reaction. J. Biochem., 24: 315–326, 1936.
150. Watson, E. M. and Pearce, R. H.: The mucopolysaccharide content of the skin in localised (pretibial) myxedema. Am. J. Clin. Path., 17: 507–512, 1927.
151. Watson, E. M. and Pearce, R. H.: The mucopolysaccharide content of the skin in localised (pretibial) myxedema. II. Am. J. Clin. Path., 19: 442–447, 1949.
152. Weissmann, B. and Meyer, K.: The structure of hyalobiuronic acid and of hyaluronic acid from umbilical cord J. Am. Chem. Soc., 76: 1753–1757, 1954.

153. Weissmann, B., Meyer, K., Sampson, P. and Linker, A.: Isolation of oligosaccharides enzymatically produced from hyaluronic acid. J. Biol. Chem., **206**: 417–429, 1954.
154. Whistler, R. L. and Durso, D. F.: Chromatographic separation of sugars on charcoal. J. Am. Chem. Soc., **72**: 677–679, 1950.
155. Wolfrom, M. L. and Montgomery, R.: Acetylative desulfation of carbohydrate acid sulfates. J. Am. Chem. Soc., **72**: 2859–2861, 1950.
156. Wolfrom, M. L., Montgomery, R., Karabinos, J. V. and Rathgeb, P.: The structure of heparin. J. Am. Chem. Soc., **72**: 5796–5797, 1950.
157. Yositake, M. J.: Biochemical studies on carbohydrates. LIII. Glucosamine as a glycogenetic substance in organisms. J. Biochem., **30**: 423–437, 1939.
158. Yositake, M. J.: Biochemical studies on carbohydrates. LIV. Hyperglycemia after glucosamine injection and the mechanism of its occurrence. J. Biochem., **30**: 439–449, 1939.
159. Zilliken, F., Smith, P. N., Rose, C. S. and György, P.: Enzymatic synthesis of a growth factor for *Lactobacillus bifidus* var. Penn. J. Biol. Chem., **206**: 299–305, 1954.