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ON THE METABOLISM OF MUCOPOLYSACCHARIDES

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Abstract—Aspects of the biosynthesis of mucopolysaccharides are discussed. Substances dealt with include the hexosamine-containing compounds which occur in mammalian tissues; these are hyaluronic acid, chondroitin and the chondroitin sulphates, heparin, heparitin sulphate and keratosulphate. Mechanisms of synthesis of the mucopolysaccharides have been derived from the use of radioactive precursors such as ^{14}C-labelled glucose or ^{35}S-labelled sulphate and by knowledge of the enzymatic reactions of the various constituents of the compounds. Experimental work concerning these two phases is discussed in some detail. Finally evidence is given of polysaccharide or macromolecule formation.

WHILE much of our knowledge about the chemistry of the acid mucopolysaccharides dates back to the nineteenth century, the current concepts about the metabolism of these substances have resulted mainly from the work of the past decade. However, in 1861, one of the earliest workers in the field of connective tissue chemistry, Boedeker, also contributed the first known metabolic experiment.[1] Boedeker, who studied the carbohydrate-containing material of cartilage, administered a cartilage jelly from autopsy material to volunteers and observed an increased excretion of sulphate in the urine.

The introduction of the isotope technique, in conjunction with the development of methods for small-scale isolation of mucopolysaccharides and their components, has increased enormously the possibilities of carrying out metabolic studies in the mucopolysaccharide field. There exists today a vast literature on the metabolism of mucopolysaccharides summarized in a number of recent reviews,[2–9] and this paper will be confined to a few aspects of the biosynthesis of these compounds. The group of substances, which will be dealt with, includes the following hexosamine-containing polysaccharides, which occur in mammalian tissues: hyaluronic acid, chondroitin, the three different chondroitin sulphates, heparin, heparitin sulphate and keratosulphate. Table 1 gives a survey of the compounds under discussion and of their components.

The first enzymic polysaccharide synthesis was the well-known phosphorylase reaction catalysing the formation of glycogen from glucose-1-phosphate.[10] Each successive step in the formation of glycogen involves the addition of a glucose residue from glucose-1-phosphate. In the case of the mucopolysaccharides, which belong to the group of heteropolysaccharides composed of dissimilar units, we are faced with the following problems: (1) Which are the precursors of the different constituents and how are they formed? (2) How is the macromolecule formed from its precursors with the concomitant alternation of the various units? With regard to the first of these

questions, a good deal of knowledge has now accumulated, whereas the answer to the second question is still completely unknown.

The study of mucopolysaccharide biosynthesis has derived great benefit from the application of results gained in other fields of carbohydrate metabolism and, especially, numerous examples of the participation of various uridine compounds in saccharide synthesis makes it likely that such substances are the specific precursors of the mucopolysaccharides as well. Some instances of polysaccharide synthesis have already

TABLE 1. CONSTITUENTS OF ACID MUCOPOLYSACCHARIDES

Name	Hexosamine	Uronic acid	Galactose	Sulphate
Hyaluronic acid	Acetylglucosamine	Glucuronic acid	—	—
Chondroitin	Acetylgalactosamine	Glucuronic acid	—	—
Chondroitin sulphate A	Acetylgalactosamine	Glucuronic acid	—	+
Chondroitin sulphate B	Acetylgalactosamine	Iduronic acid	—	+
Chondroitin sulphate C	Acetylgalactosamine	Glucuronic acid	—	+
Heparitin sulphate	Acetylglucosamine	Glucuronic acid	—	+
Heparin	Glucosamine	Glucuronic acid	—	+
Keratosulphate	Acetylglucosamine	—	+	+

been reported, where the immediate precursor is a uridine nucleotide, namely, glycogen,[11] cellulose,[12] chitin[13] and hyaluronic acid.[14, 15] In fact, it seems possible that the uridine diphosphate glucose–glycogen *trans*glucosylase reaction may represent the normal route of glycogen synthesis, while the phosphorylase reaction, though reversible, is presumably responsible mainly for the degradation to glucose-1-phosphate.

Theories about the synthesis of the mucopolysaccharides today rest on a composite basis which is formed partly of knowledge about the enzymic reactions of the various constituents of the mucopolysaccharides, partly of studies using radioactive precursors like ^{14}C-labelled glucose or ^{35}S-labelled sulphate. Furthermore, the mere existence of certain compounds such as uridine diphosphate galactosamine sulphate[16] seems to indicate that they may be involved in mucopolysaccharide formation. Finally, there is a smaller amount of knowledge more directly concerned with the formation of the macromolecule.

ENZYMIC FORMATION OF MONOSACCHARIDE MOIETIES

The synthesis of mucopolysaccharides from glucose has for a long time been considered to follow a pathway, in which uridine nucleotides represent the final stage before polymerization to a macromolecule. This has so far been proven only in two instances, i.e. the formation of chitin[13] from uridine diphosphate acetylglucosamine and of hyaluronic acid[14, 15] from uridine diphosphate acetylglucosamine and uridine diphosphate glucuronic acid. Several uridine nucleotides exist, however, which are likely precursors in the biosynthesis of mucopolysaccharides, and the following discussion of the enzymic formation of the monosaccharide moieties will therefore be carried through the enzymic synthesis of these various compounds.

The characteristic constituent of the mucopolysaccharides is hexosamine, which may be either glucosamine or galactosamine. In addition, a uronic acid is present in

the substances under discussion except for keratosulphate, which contains galactose instead. In some of the mucopolysaccharides, which are sulphated, the introduction of the sulphate group into the molecule presents a special problem implying the activation of the sulphate group to adenosine 3′-phosphate-5′-phosphosulphate[17] and the subsequent transfer of the sulphate group to a carbohydrate acceptor. The question of the sulphate activation has been discussed at length in another symposium at this meeting[18] and will therefore be touched on only briefly.

Hexosamine synthesis

The first evidence concerning the enzymic formation of hexosamine was obtained by Leloir and Cardini.[19] From the chitin-producing mould *Neurospora crassa* they prepared an enzyme system, which catalysed the synthesis of glucosamine-6-phosphate from glutamine and glucose-6-phosphate—the key intermediate of glucose metabolism. Subsequent studies of this reaction by Blumenthal *et al.*[20] showed that fructose-6-phosphate was in fact the substrate for the amination. An enzyme catalysing the same reaction has also been isolated from liver by Pogell and Gryder[21]. Though Pogell maintained that glucose-6-phosphate is the substrate of the mammalian enzyme rather than fructose-6-phosphate, it has been claimed by Roseman *et al.*[22] that fructose-6-phosphate is also the substrate for the liver enzyme. In extracts from cartilage formation of glucosamine-6-phosphate from glucose-6-phosphate and glutamine could also be demonstrated.[23]

An alternate pathway for the hexosamine formation exists, in which ammonia substitutes for glutamine as the nitrogen donor, while fructose-6-phosphate, even in this reaction, is the hexose component.[24, 25] In contrast to the equilibrium conditions prevailing in the glutamine reaction,[19] which favour hexosamine synthesis, the equilibrium of the ammonia reaction is displaced towards fructose-6-phosphate and ammonia. This does not, however, necessitate the conclusion that under physiologic conditions the latter reaction does not operate in the direction of hexosamine synthesis. On the contrary, it has been suggested[25] that the acetylation of the reaction product, glucosamine-6-phosphate, *in vivo* pulls the reaction towards the formation of the amino sugar phosphates. The enzyme catalysing this reaction, glucosamine-6-phosphate acetylase, was first isolated from yeast,[26] but it has also been found in several animal tissues.[27]

Other routes leading to the formation of hexosamine-6-phosphates can be considered, i.e. the phosphorylation of glucosamine[28–31] or N-acetylhexosamine,[28, 32–34] yielding glucosamine-6-phosphate and N-acetylglucosamine-6-phosphate, respectively. The former reaction is catalysed by the non-specific glucokinase, while the latter requires a specific enzyme. Under *in vivo* conditions, however, these reactions are probably not operating to a large extent, an assumption which is supported by the fact that both free and acetylated hexosamines are absent from tissues and body fluids except possibly in one instance, namely, cerebrospinal fluid.[35]

In the next step toward a hexosamine-containing uridine nucleotide, N-acetylglucosamine-6-phosphate is transformed to N-acetylglucosamine-1-phosphate in a reaction, which is catalysed by an enzyme similar to phosphoglucomutase.[36] A partial separation of the phosphoglucomutase and phosphoacetylglucosamine mutase activities has been obtained, indicating that the two reactions are catalysed by different enzymes.

Finally, in complete analogy with the reactions incorporating glucose into uridine diphosphate glucose, N-acetylglucosamine-1-phosphate reacts with uridine triphosphate yielding uridine diphosphate acetylglucosamine.[37–39] In addition to its participation in the synthesis of chitin and hyaluronic acid, uridine diphosphate acetylglucosamine can also be expected to be the precursor of the N-acetylglucosamine moieties of keratosulphate and heparitin sulphate. Besides N-acetylated glucosamine residues, the latter substance also contains N-sulphated glucosamine.[40] Though the precursor of this non-acetylated glucosamine moiety is not known, it seems likely that its synthesis occurs via uridine diphosphate glucosamine, the formation of which has been demonstrated in yeast.[38] This substance could likewise be expected to be a heparin precursor, as most of the amino groups of heparin are also sulphated.

Concerning the biosynthesis of the galactosamine moiety of chondroitin and the three chondroitin sulphates nothing is known, but quite recently the normal pathway for its formation seems to have been found by the discovery of an enzyme which catalyses the reaction:

uridine diphosphate acetylglucosamine
↓
uridine diphosphate acetylgalactosamine[41, 42]

This enzyme effects a change similar to the conversion of uridine diphosphate glucose to uridine diphosphate galactose, and thus, a close analogy has again been established between the transformations of the neutral hexoses and those of the hexosamines.

Uronic acid

Several uronic acids are now known to occur in nature. In the acid mucopolysaccharides the uronic acid has always been found to be D-glucuronic acid except in one instance, chondroitin sulphate B, which contains L-iduronic acid—the 5-epimer of D-glucuronic acid. So far, no enzymic reactions concerned with the formation of L-iduronic acid have been reported.

The biosynthesis of glucuronides has been the subject of investigation since the days of Fischer, when the formation of conjugated glucuronic acid was considered to proceed through a glucoside, which was subsequently oxidized to a uronic acid[43, 44]. Later work with tissue slices[45] seemed to indicate a synthesis via three-carbon metabolites, and *in vivo* experiments with ^{14}C-labelled precursors (lactate, pyruvate, glycerol and glucose),[46, 47] which were administered together with glucuronidogenic substances, pointed in the same direction.

Results at variance with this conclusion were obtained, however, in other experiments utilizing glucose-1-^{14}C and glucose-6-^{14}C,[48–50] in which incorporation of the glucose without rearrangement of the chain occurred. The problem was finally solved by the isolation of a thermostable factor involved in glucuronide formation in cell-free liver suspensions, which was subsequently identified as uridine diphosphate glucuronic acid.[51–54] The original hypothesis of Fischer and Piloty was subsequently shown to contain a nucleus of truth by the demonstration of Strominger *et al.*[55] that uridine diphosphate glucuronic acid is formed by way of oxidation of uridine diphosphate glucose by a diphosphopyridine nucleotide-dependent enzyme, uridine diphosphate glucose dehydrogenase. It is obvious that uridine diphosphate glucuronic acid

is a likely precursor of the glucuronic acid moiety of mucopolysaccharides, and its participation in streptococcal hyaluronic acid synthesis has been demonstrated.[15]

ISOTOPIC STUDIES

It is natural that the introduction of isotope methods, which have revolutionized the whole field of metabolic studies, has also proved to be very fruitful in the elucidation of mucopolysaccharide metabolism. The isotope methods have been used mainly for two purposes. First, the origin of the different moieties of the mucopolysaccharides has been investigated in bacteria and in mammals by using precursors such as ^{35}S-sulphate, ^{14}C-acetate and variously labelled ^{14}C-glucose. Secondly, the turnover of the mucopolysaccharides has been studied in normal animals as well as under the influence of various endocrine agents. The first studies of this kind were made by Dziewiatkowski *et al.*[56], who studied the ^{35}S-fixation in cartilage *in vivo*, and by Layton and Frankel[57] who observed on ^{35}S-fixation *in vitro*. This work was later extended by Dziewiatkowski[58] and Boström[59], who demonstrated that the ^{35}S-incorporation into cartilage occurred in the sulphate group of chondroitin sulphuric acid. Later on, studies on the carbon skeleton[60–62] established that the glucose molecule is incorporated without scission of the chain into hyaluronic acid of streptococci in such a way that C(1) of the glucose becomes C(1) in glucosamine as well as in glucuronic acid. Similar results pertain to the formation of hyaluronic acid of animal tissues.[63]

Tracer methods thus may give valuable hints about possible reaction sequences in the biosynthetic pathways, and this type of work should be regarded as preliminary to a closer investigation of the enzymic reactions involved. So far the pilot investigations on the origin of the carbon skeleton carried out with the isotope methods have generally been confirmed by later enzymic studies. In the case of L-iduronic acid such a pilot study is the only investigation so far of the origin of this substance, for which no enzymic reactions are known.[63] Even in this case, the glucose molecule is incorporated without cleavage into the L-iduronic acid of chondroitin sulphuric acid B.

In addition to the usefulness in the studies of mucopolysaccharide precursors, the isotopic methods are indispensable in providing information concerning the turnover of the different mucopolysaccharides *in vivo*. The first studies of this kind were made by Boström in 1952 on costal cartilage of rats.[59] A large number of rats were given ^{35}S-sulphate, and at different times after the injection a number of animals were killed and the radioactivity of various sulphur fractions measured. The labelling of inorganic sulphate decreased very rapidly. The chondroitin sulphuric acid curve had, however, another shape and from the descending part of this curve the biological half-life was found to be be around 16 days. In a similar study on chondroitin sulphuric acid prepared from skin, Boström and Gardell found a half-life of chondroitin sulphuric acid in this tissue of 9–10 days.[64]

The knowledge gained from the ^{35}S studies was subsequently confirmed by Schiller *et al.* using ^{14}C-labelled glucose and acetate to study the turnover of skin mucopolysaccharides.[65, 66] The outcome of these studies was the demonstration of the similar turnover rates of the different moieties of the chondroitin sulphuric acid molecule, the sulphate, hexosamine, glucuronic acid and acetyl groups, which indicates that the whole molecule turns over as an entity. The use of ^{14}C-labelled precursors also made it

possible to study the turnover of the hyaluronic acid, which contains no sulphate. This mucopolysaccharide showed a particularly rapid turnover rate with a half-life time of approximately 2–3 days.

While *in vivo* studies utilizing labelled precursors may give valuable clues to the existence of specific enzymic steps, it is also sometimes possible to corroborate by the isotopic methods the existence *in vivo* of an enzymic reaction demonstrated *in vitro* and to estimate its quantitative significance. In the era of enzymology it seems justified to keep in mind that what we really want to know is what is going on within the living, unbroken tissue.

As our group has made extensive studies of the metabolism of slices of cartilage especially utilizing ^{35}S-labelled sulphate, we would like to exemplify the possibilities of the isotopic methods from our own experience. There are several advantages of the ^{35}S technique over ^{14}C methods among which can be mentioned the following:

(1) The low isotope costs, amounting to only 1 per cent or less of those of suitable ^{14}C precursors.

(2) The specificity of ^{35}S methods due to the very limited incorporation of the isotope into compounds other than sulphate esters.

(3) The high specific activity of the isotope, which gives the ^{35}S methods a very high sensitivity.

The *in vitro* system in question was developed by Boström in collaboration with Månsson in 1952.[67] Working with this system Boström and Månsson found in 1953 that the addition of a liver homogenate greatly increased the incorporation of ^{35}S.[68] After some laborious purification work we were able to crystallize the factor responsible for this effect and prove its identity with glutamine.[69, 70] It so happened that one of our best purification steps was a fractional precipitation with mercuric salt, which was essentially the same method as that used 75 years ago by Schulze and Bosshard, in the first isolation of glutamine.[71]

The identity of the liver factor with glutamine clearly indicated that the effect on ^{35}S incorporation did not primarily involve the process of sulphate esterification as the amide group of glutamine had been shown to be the precursor for hexosamine nitrogen in two microbial systems. The first demonstration of a role for glutamine in hexosamine synthesis was the finding of Leloir and Cardini that an extract of *Neurospora crassa* could synthesize glucosamine-6-phosphate from glucose-6-phosphate and glutamine.[19] Subsequently Lowther and Rogers showed that glutamine was a precursor in the formation of hyaluronic acid in a resting cells suspension of streptococci.[72, 73] These findings actually led us on the right track in the isolation of the liver factor stimulating the incorporation of ^{35}S in chondroitin sulphate. Evidence that glutamine was not specifically involved in the sulphate esterification was obtained by studying its influence on the formation of other parts of the chondroitin sulphate molecule as well, and it was also found that the incorporation of glucose-^{14}C and acetate-^{14}C was enhanced by glutamine.[74] In addition, the incorporation of glucose-^{14}C into the galactosamine moiety of chondroitin sulphate was increased much more than the increase in labelling of the uronic acid moiety. Furthermore, glucosamine stimulated the ^{35}S uptake to approximately the same extent as glutamine.[75] The simultaneous presence of the two substances did not result in a further increase in ^{35}S uptake. Therefore, it seems justified to conclude that the observed effects of

glutamine on the cartilage system can be fully explained by assuming that glutamine participates in the synthesis of the galactosamine moiety of the chondroitin sulphate. As was pointed out previously two reactions have been described, which use glutamine and ammonia, respectively, as nitrogen donors for the hexosamine formation.[19, 24, 25] On the basis of our results we believe that the glutamine reaction is the one used by the cartilage. This assumption is further strengthened by the fact that the decline in the

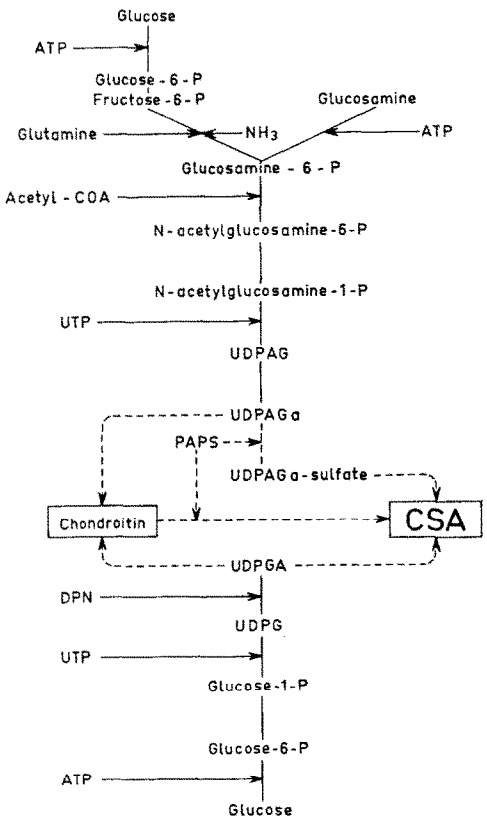

FIG. 1 Biosynthesis of CSA. Possible pathways

Abbreviations used: P for phosphate; ATP for adenosine triphosphate; Acetyl-CoA for acetyl-coenzyme A; UTP for uridine triphosphate; UDPAG for uridine diphosphate acetylglucosamine; PAPS for adenosine 3′-phosphate-5′-phosphosulphate; CSA for chondroitin sulphate; UDPGA for uridine disphosphate glucuronic acid; DPN for diphosphopyridine nucleotide and UDPG for uridine diphosphate glucose.

rate of ^{35}S incorporation with time can be abolished by the addition of glutamine to give a straight line for several hours.[74]

POSSIBLE INTERMEDIATES IN POLYSACCHARIDE FORMATION

Some indirect evidence has also been brought forward in the discussion of different possible pathways of biosynthesis of the mucopolysaccharides (Fig. 1). In particular, the mere existence of certain compounds seems to indicate the occurrence of various

enzymic reactions, which could possibly participate in the formation of a mucopolysaccharide molecule. One such compound is the galactosamine and sulphate-containing uridine nucleotide of Strominger[16], uridine diphosphate acetylgalactosamine sulphate. It is very tempting to assume that this substance is a metabolite on the main pathway in the synthesis of chondroitin sulphate. On the other hand, Meyer *et al.*[76] have isolated polysaccharide fractions from the cornea with a sulphur content that varies between zero and 1 mole of sulphate per disaccharide unit. This fact may indicate that the sulphation occurs after the polymerization of the molecule and not on the monomer stage. Another compound also isolated by Strominger[16] is uridine diphosphate acetylglucosamine-6-phosphate, a compound for which no obvious function yet can be conceived.

POLYSACCHARIDE FORMATION

Turning to the formation of the macromolecules this is a field which has only recently been opened up. It was early suggested by Leloir[19] that uridine diphosphate acetylglucosamine could possibly participate in the formation of chitin. Storey and Dutton[53] likewise suggested that uridine diphosphate glucuronic acid was a precursor of uronic acid-containing mucopolysaccharides. These hypotheses are now being substantiated by the demonstration of the participation of uridine nucleotides in the formation of several polysaccharides. The first well-documented example of the participation of a uridine nucleotide in polysaccharide synthesis is the demonstration of chitin synthesis from ^{14}C-labelled uridine diphosphate acetylglucosamine in a cell-free extract of *Neurospora crassa*.[13] The need of a primer in this reaction in the form of short-chain chitodextrins was also demonstrated. A similar study was also made on the formation of cellulose in an extract of *Acetobacter xylinum* using uridine diphosphate glucose as a substrate and cellodextrin as primer.[12]

Three years ago Leloir and Cardini[11] showed that a liver enzyme can catalyse a net synthesis of glycogen from uridine diphosphate glucose in the presence of primer glycogen or soluble starch. It was suggested that the essentially irreversible uridine diphosphate glucose reaction is used for the synthesis of glycogen, while the reversible phosphorylase reaction should act on the degrading side of glycogen metabolism.

Concerning the mucopolysaccharides under discussion information is more scarce. In 1955, Glaser and Brown observed incorporation of label into hyaluronic acid in an extract of Rous chicken sarcoma using acetyl-labelled uridine diphosphate acetylglucosamine and unlabelled uridine diphosphate glucuronic acid as precursors, though considerable loss of label occurred during purification of the hyaluronic acid.[14]

A more potent preparation has been obtained by Markovitz *et al.*[15] from streptococci. The precursors used were labelled with tritium using the elegant Wilzbach technique which consists simply of incubating a non-radioactive sample of the substance to be labelled with tritium gas followed by repurification.[77] In this way incorporation of tritium-labelled uridine diphosphate glucuronic acid, uridine diphosphate acetylglucosamine and acetylglucosamine-1-phosphate into hyaluronic acid could be achieved.

Concerning the formation of the other non-sulphated polysaccharide, chondroitin, nothing is known.

The formation of the sulphated mucopolysaccharides poses the special problem of the sulphate acceptor, and some progress has been made in this respect. The first

demonstration of the synthesis of a sulphated polysaccharide in a cell-free system was D'Abramo's and Lipmann's demonstration of an incorporation of ^{35}S-sulphate and ^{35}S-labelled "active sulphate", adenosine 3′-phosphate-5′-phosphosulphate, into chondroitin sulphate in an extract of embryonic cartilage.[78] This work gave no information, however, about the possible site of the sulphation. In subsequent work by Suzuki and Strominger[79] it could be shown that a soluble extract of oviduct was able to catalyse the incorporation of label from ^{35}S-labelled "active sulphate" into four sulphated polysaccharides including the three different chondroitin sulphates. The presence of primer was necessary for the reaction. Continued work along these lines by Suzuki[80] has shown that a transfer of sulphate can be obtained to certain oligosaccharides derived from chondroitin sulphate by enzymatic hydrolysis.

Recent work by Korn[81] and by Spolter and Marx[82] on the formation of heparin in cell-free systems from mouse mastocytomas seems to indicate that sulphation can occur on the polymer stage. The same view is also favoured by the work of Greiling and Bauditz[83], who showed that chondroitin sulphates and desulphated chondroitin sulphates could serve as sulphate acceptors in particle-free extracts prepared from liver. Still more conclusive in this respect are the experiments preliminarily reported by Delbrück and Lipmann[84] who showed that a purified enzyme preparation from embryonic cartilage, obviously free from carbohydrate precursors, could transfer sulphate from "active sulphate" to the three chondroitin sulphates or to chondroitin but not to hyaluronic acid, heparin or keratosulphate.

Concerning the biosynthesis of the carbon skeleton of the sulphated polysaccharides in cell-free systems nothing is known as yet, but the existence of several such systems makes it probable that these secrets are also beginning to be unravelled.

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