fermenting, when used with the abbreviation of an amino acid and sugar, respectively: *S*, sensitivity to a lethal agent; *R*, resistance to a lethal agent.

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*THE SELECTIVITY OF BIOSYNTHESIS OF GLUCOSYL COMPOUNDS AS ILLUSTRATED BY AN E. COLI MUTANT DEFECTIVE IN UDPG SYNTHETASE**

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It has recently been reported that mutants of *Escherichia coli* K12 which are defective in the ability to synthetize UDPG synthetase (UDPG pyrophosphorylase) show also a great decrease in the amount of glucose incorporated into their cell-wall polysaccharides.[1, 2]

In their analysis of cell walls isolated by Salton's technique,[3] Fukasawa *et al.*[4] reported that the UDPG synthetase-defective mutants of *E. coli* K12, W4597, and W3142, contained only 15–20 per cent of the amount of glucose present in the wild-type strain W3110.

A study has been made in this laboratory of the cell-wall lipopolysaccharides (LPS) of various *E. coli* K12 mutants.[2] LPS was isolated by the hot phenol procedure of Westphal *et al.*,[5] and a preliminary investigation of its sugar composition was made by paper chromatography and by various colorimetric reactions. It was reported in this study that in the case of the UDPG synthetase-defective mutant Gal 23 (identical with W4597), glucose could not be detected in the LPS;[2] this was also the case of a galactokinaseless derivative of this strain, Gal 23 K^-.[2]

In the present study we wish to report some observations which seem to be of interest from the point of view of cellular physiology. By using the highly sensitive hexokinase-Zwischenferment assay for glucose, we have found that the LPS of Gal 23 contains only minute amounts of glucose (less than 0.1% of the lyophilized LPS), whereas in the corresponding parental strain (W3100), glucose is a major constituent, amounting to 8–12 per cent of the LPS.

We have found that in various strains of *E. coli* K12 there are, in addition to LPS, glucose-containing phosphorylated polysaccharides. We would like to refer to these acidic polysaccharides in the present article as "soluble polysaccharide fraction" or sPF. A subsequent publication will deal with the fractionation and the chemical makeup of this sPF.[6]

It seems noteworthy that sPF is present both in the original strain (W3100) as well as in the UDPG synthetase-defective mutant (Gal 23 K^-) and that in the latter the sPF contains 5–10 per cent of the amount of glucose found in wild-type sPF. (W3100 ferments both glucose and galactose and is considered here as "wild

type." Gal 23 K$^-$ derives via Gal 23 K$^+$ from W3100.) Since the absolute amount of sPF present in mutant and parental strains is approximately the same and usually reaches the same order of magnitude as the LPS fraction, the glucose present in this soluble fraction represents a sizable amount. This is especially remarkable in the case of the UDPG defective K12 mutant, which has only traces of glucose in the LPS.

Most recently, Segel and his co-workers[7, 8] have found that UDPG defectives starved for nitrogen are able to accumulate glycogen, and in amounts equaling those found in strains having normal biosynthesis and relatively high levels of UDPG. As will appear from the present study, sPF contains neither glycogen nor other compounds with α- or β-(1 $\rightarrow$ 4)-glucosidic linkages. Nevertheless, these observations raise the question of the occurrence in *E. coli* of glucosyl donors other than UDPG, and of the possible function of such donors in the biosynthesis of sPF and glycogen. In the case of LPS, however, the biosynthesis of glucosyl units seems to proceed exclusively through the mediation of UDPG.

Materials and Methods.—Bacterial strains: The strains used are derivatives of *Escherichia coli* K12 from the collection of Drs. E. and J. Lederberg, Stanford University, Cal.

Strain W3100 is a glucose and galactose fermenting strain. Gal 23 K$^-$ is a double defective mutant derived from W3100; it lacks both UDPG synthetase (UDPG pyrophosphorylase) and galactokinase.

Culture conditions: The strains were kept as slant cultures on bactonutrient agar at 0–2°. For large-scale cultures, bacteria were grown in ammonia mineral medium[9] with glycerol as sole carbon source, and were harvested in the late logarithmic phase. Details concerning culture conditions have been described previously.[10]

Enzymes: Hexokinase, type III from yeast (glucose-free) and α-amylase type II, crystalline, were purchased from Sigma Chemical Co., St. Louis, Mo. Glucose-6-phosphate dehydrogenase ("Zwischenferment") was obtained from C. F. Boehringer & Söhne, Mannheim, Germany.

Isolation procedure for LPS and sPF: For the extraction of LPS and sPF the hot phenol-water procedure of Westphal and co-workers was used.[5] Washed cells were extracted at 67–68° with 45% phenol for 30 min, and the combined aqueous layers were dialyzed and concentrated about 10-fold. Upon centrifugation of the turbid solution in a Spinco model L ultracentrifuge for 3 hr at 40,000 rpm (105,000 *g*), the very high molecular LPS precipitates, whereas RNA and sPF stay in solution. The precipitate of LPS is contaminated with a small amount of RNA, which is removed by repeating the centrifugation procedure 5 or 6 times. The LPS is then redispersed and lyophilized. The analytical determinations reported here are based on this lyophilized material and are not corrected for the retained moisture (about 15%) in the lyophilizate. The supernatant obtained in the first cycle of the ultracentrifugation is yellow-colored and contains the bulk of the bacterial RNA and also of the soluble acidic polysaccharide fraction (sPF). No appreciable amounts of the latter could be found in the supernatants from subsequent cycles of the ultracentrifugation.

RNA (representing about 95% of the material in the first supernatant) was removed by acid precipitation: 3 *N* ice-cold HCl was slowly added to the ice-cold supernatant, to make the solution 0.5 *N* in HCl. RNA immediately formed a very dense precipitate which could easily be removed by centrifugation. sPF stayed in solution and this was filtered through sintered glass; the pH of this solution was 0.8. The HCl was removed by dialysis in the cold against distilled water, with frequent changes of water. The nondialyzable material was concentrated in a vacuum evaporator (40° water bath), and was then lyophilized. The faintly yellow powder had a slight UV-absorption, due to contamination by 2–5% by weight of RNA.

Analysis of neutral sugars: Samples of lyophilized materials (1–2 mg) were hydrolyzed with 1 *N* H_2SO_4 in sealed tubes at 100° for 4 hr. After neutralization with $Ba(OH)_2$ the samples were concentrated, and aliquots were taken for paper and thin-layer chromatography, and also for the enzymatic glucose determinations. Paper chromatography on Whatman no. 1 paper was carried out using the following solvents: (*i*) pyridine/n-butanol/water (4:6:3, v/v);[11] (*ii*) 95% aqueous

phenol;[12] (*iii*) pyridine/ethyl acetate/acetic acid/water (5:5:1:3, v/v) in a tank saturated with pyridine/ethyl acetate/water (11:40:6, v/v).[13] Also used for thin-layer chromatography (TLC) were: (*iv*) ethyl acetate/pyridine/water (12:5:4, v/v),[14] (*v*) pyridine/ethyl acetate/water (1:2:2, v/v, upper phase),[15] and (*vi*) n-butanol/acetic acid/water (4:1:5, v/v).[16] In solvents (*iv*) and (*v*) multiple development technique was usually chosen.

Thin-layer plates with cellulose layers[17] were prepared according to an unpublished method by Drs. E. and K. Randerath of this laboratory. Reference sugars were spotted in amounts of 1–2 μg, hydrolyzates in amounts containing at least 1 μg of each constituent.

Sugars were located on paper or thin-layer chromatograms by the alkaline silver nitrate reagent of Trevelyan,[18] using a dipping technique.[17] A *p*-anisidine spray was used for discriminating between heptoses and hexoses;[19] amino sugars were detected with a ninhydrin spray. For a tentative qualitative analysis of the neutral sugar constituents of the isolated polysaccharides, and for a quantitative determination of glucose in sPF, the method described by Dische[20] as primary and secondary reaction of hexoses with L-cysteine and sulfuric acid was used. Spectra were taken in a Cary-14 recording spectrophotometer, 1.5, 22, and 48 hr after addition of cysteine.

Glucose was also determined in hydrolyzates of LPS and sPF by an enzymatic reaction involving hexokinase and glucose-6-phosphate dehydrogenase. The reaction mixture contained in 1 ml: Tris-buffer pH 7.5 (50 μmole), $MgCl_2$ (5 μmole), TPN (0.5 μmole), glucose-6-phosphate dehydrogenase (0.14 unit), hexokinase (30 K.M. units), and 0.05–0.1 μmole glucose. The formation of TPNH was followed by reading the optical density at 340 mμ, using 400 mμ as an isobestic point. Glucose and glucose-6-phosphate were used as standards. Since neither fructose nor mannose could be detected chromatographically in any of the samples, this method could be used for the determination of glucose, in spite of the fact that some commercial hexokinase preparations contain significant amounts of glucose-6-phosphate isomerase.

For a quantitative determination of hexosamines the Rondle-Morgan variation[21] of the classical Elson-Morgan reaction was used. Samples were hydrolyzed for 6 or 12 hr in 4 *N* HCl. The values reported here are based on a glucosamine standard, but are not corrected for the partial destruction of hexosamine during acid hydrolysis. Burton[22] reports a decrease of 20% in glucosamine when standards are kept under the conditions of the hydrolysis of the samples (6 hr in 6 *N* HCl).

Periodate oxidation:[23] A standard of liver glycogen and a sample of sPF derived from strain W3100 (32% glucose) in 0.1% concentration in unbuffered aqueous solutions were treated with $NaIO_4$ (0.02 *M*) for 19 hr at 4° in the dark. The excess of periodate was then destroyed by adding ethylene glycol, and the resulting polyaldehydes were reduced by a fourfold excess of sodium borohydride (calculated for glycogen), added in two equal portions 1 hr apart.[24] After a total reduction period of 3 hr, the solutions were slightly acidified with glacial acetic acid, concentrated to dryness, redissolved in 0.1% methanolic HCl, and again brought to dryness; the evaporations were repeated several times in the presence of methanol, for complete removal of borate. The oxidized and reduced samples were hydrolyzed for 1.5 hr in 1 *N* H_2SO_4, and after neutralization with $Ba(OH)_2$ were deionized with a Dowex mixed bed resin. The concentrated solutions were examined in solvent (*vi*) on cellulose thin-layer plates, with glucose, erythritol, and glycerol as references.

Incubation experiment with bacterial α-amylase:[25] 3.2 mg of bacterial glycogen (from Gal 23 K^+, kindly supplied by Dr. I. H. Segel, Davis, Cal.) and 4.4 mg of sPF from W3100 were dissolved each in 0.3 ml of a 0.02 *M* sodium phosphate buffer (pH 6.9) containing 0.006 *M* NaCl. These solutions were each divided into three aliquots. One aliquot was incubated with 0.1 ml of an aqueous solution of crystalline bacterial α-amylase (0.1 mg enzyme/1 ml). The second aliquot was diluted with 0.1 ml H_2O. The third aliquot of the sPF solution was mixed with the third aliquot of the glycogen solution, and 0.1 ml of the α-amylase solution was added, thus providing an internal standard. All samples were treated with 10 μl of toluene and kept in a 37° incubator. After 15 hr the reaction was stopped by heating the samples for 3 min at 100°. The incubation mixtures were then examined directly by thin-layer chromatography in solvent (*iv*), to detect any release of glucose.

Thiobarbituric acid reaction: The method of Cynkin and Ashwell[26] was used to test for 2-keto-3-deoxyoctonate[27] on partial hydrolyzates of LPS and sPF (0.1 *N* H_2SO_4 for 10 min at 100°). Absorption spectra were recorded in the range of 410–850 mμ in a Cary-14 spectrophotometer.

Protein was measured by the method of Lowry.[28] *Phosphorus* was determined by a micro-adaptation[29] of the Fiske-Subba-Row method, using glucose-6-phosphate as reference. *Hexuronic acids* were measured by the carbazole-H_2SO_4 method of Dische.[30]

Results.—Table 1 compares the glucose contents of LPS and of sPF from strain W3100 and from the UDPG synthetase-defective mutant Gal 23 K^-. It should be noted that LPS and sPF listed in parallel were all obtained from the same large-scale cultures. In addition, Table 1 shows the total amount of glucose bound in LPS and in sPF, and expressed in mg per 10 gm of bacterial dry weight.

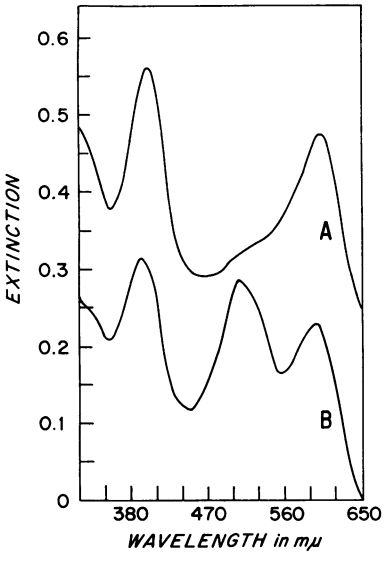

FIG. 1.—Dische reaction for heptoses. (Secondary L-cysteine-H_2SO_4 reaction, readings taken 48 hr after adding cysteine.) (*A*) sPF from W3100 (450 μg). (*B*) LPS from W3100 (470 μg).

The figures listed in Table 1 show that the incorporation of glucose into LPS is much more affected by the mutation than is the incorporation into sPF. It should also be mentioned that, of the values obtained in the two different determinations of glucose, the higher ones, given by the Dische cysteine-H_2SO_4 reaction, are probably more correct. It is doubtful that the 1 *N* H_2SO_4 hydrolysis used to liberate glucose for the enzymatic assay would also liberate all the glucose which is either phosphorylated or linked to hexosamine.

As appears from the data, the incorporation of glucose into LPS in the UDPG synthetase-defective mutant is only 0.5–1 per cent of that in strain W3100, whereas the level of glucose in the sPF of the mutant strain still amounts to 5–10 per cent of that found in sPF from W3100.

Comparison of LPS and sPF in the Dische reaction: The primary and secondary cysteine sulfuric acid reaction was run in parallel on samples of sPF and LPS obtained from the same batch of bacteria. Figure 1 shows clearly that heptose—a main constituent of the LPS in W3100—is not demonstrable in sPF (*A* in Fig. 1); the peak at 505 mμ, which is characteristic for heptoses in the secondary cysteine

TABLE 1

	Glucose content of LPS (enzymatically), %	Glucose content (in absolute amounts per 10 gm of bacterial dry weight), mg
W3100		
(*a*)	11.4	14.8
(*b*)	7.8	11.7
Gal 23 K^-		
(*a*)	<0.1	<0.1
(*b*)	0.08	∽0.1

	Glucose Content of sPF, %		Glucose content (in absolute amounts per 10 gm of bacterial dry weight), mg
	Enzymatically	Dische reaction	
W3100			
(*a*)		32.0	5.7
(*b*)	20.8		5.8
Gal 23 K^-			
(*a*)		2.7	0.6–0.7
(*b*)	1.5		0.3

sulfuric acid reaction, is strongly marked for LPS (*B*, Fig. 1), but totally absent in the case of sPF prepared from the same bacterial culture (*A*, Fig. 1).

It has recently been described[2, 26, 31] that KDO (2-keto-3-deoxy-octonate) is a ubiquitous constituent of the LPS of *Enterobacteriaceae*, which can be detected by the thiobarbituric acid reaction. Figure 2 shows that samples of sPF from both Gal 23 K$^-$ (*B* in Fig. 2) and W3100 (*C* in Fig. 2) failed to form the 549-mμ maximum, characteristic of KDO, whereas this maximum was obtained when a sample of LPS from Gal 23 K$^-$ was handled in the same way[2, 26] (*A* in Fig. 2).

The absence of any detectable amounts of heptose and KDO in sPF preparations indicates clearly that sPF is different from LPS. Table 2, which summarizes some analytical data concerning sPF, lists glucose as the only neutral sugar present in sPF of both bacterial strains studied. It may be recalled[2] that galactose, in addition to glucose and heptose, is one of the main constituents of the polysaccharide part of LPS from W3100. There was no evidence for even trace amounts of galactose in the sPF samples, which once more underlines the fact that sPF is different from LPS.

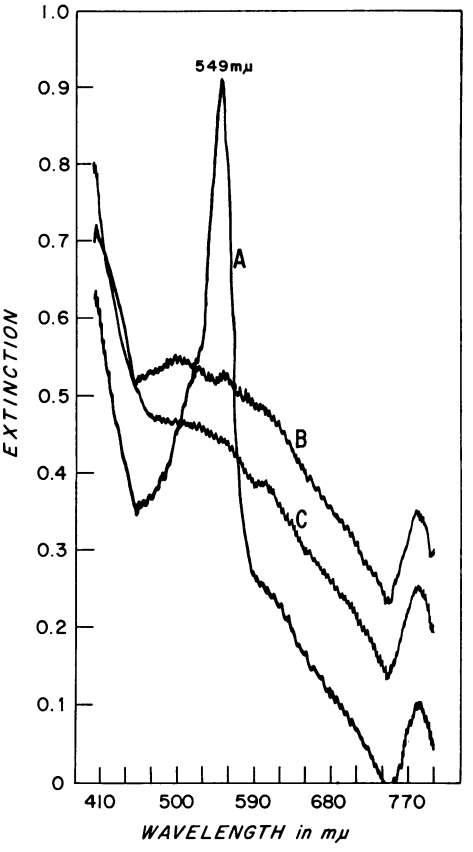

FIG. 2.—Thiobarbituric acid reaction for 2-keto-3-deoxyoctonate. (*A*) 55 μg of LPS from Gal 23 K$^-$. (*B*) 68 μg of sPF from Gal 23 K$^-$. (*C*) 59 μg of sPF from W3100.

Glucose was identified by paper and thin-layer chromatography. In all the solvents listed, hydrolyzates from sPF showed a reducing component having the same R_f value and the same color reactions as glucose. The reactivity of sPF hydrolyzates in the hexokinase-Zwischenferment assay in the absence of any de-

TABLE 2

CHEMICAL ANALYSIS OF sPF SAMPLES

	W3100	Gal 23 K$^-$
Neutral sugars	Glucose	Glucose
Glucose (Dische)	32%	2–5%
Hexosamine (as free glucosamine)	10.6%	42.1%
Hexosamine (as N-acetyl-glucosamine)	12.1%	50.6%
Protein (Lowry)	3.9%	1.8%
RNA (calculated from the readings at 260 mμ)	5.5%	2.6%
Phosphorus	2.1%	0.6%
Lipid	Not determined	
Uronic acids (carbazole-H_2SO_4)	Not demonstrable	
Heptoses	Not demonstrable	
Thiobarbituric acid-positive constituents (KDO, neuraminic acid, 3,6-dideoxyhexoses, 2-deoxyribose)	Not demonstrable	

tectable mannose or fructose provides a further proof, and also indicates that glucose is present in the D-configuration. Other neutral sugars were not detectable, with the exception of minute traces of ribose, apparently released from the contaminating RNA. Amino sugars reactive in the Rondle-Morgan test are by far the main constituents of Gal 23 K^- sPF, whereas both glucose and hexosamine are the major constituents of the sPF from strain W3100.

Experiments for the elucidation of the glucose linkage in sPF: Incubation of sPF with α-amylase: It has been reported that glycogen occurs in different strains of *Escherichia coli*,[32, 7] and even occasionally associated with the cell wall.[33] Recently, Segel and co-workers[8] found that, when starved for nitrogen, UDPG synthetase-defective mutants, including *E. coli* K12 Gal 23 K^+ which is derived from W3100, are able to accumulate glycogen to an extent which equals that in wild-type forms. The presence of high molecular glycogen was essentially ruled out by the fact that sPF was isolated from the supernatant of a 3-hr centrifugation at 105,000 *g*, under which conditions glycogen would have been precipitated.[33]

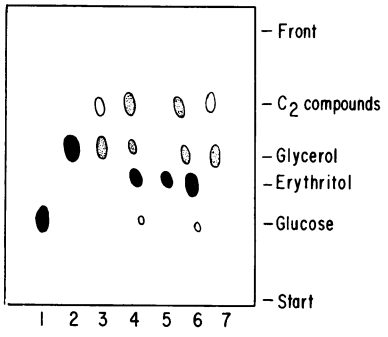

FIG. 3.—Thin-layer chromatography of hydrolyzates from sPF W3100 and liver glycogen after periodate oxidation and $NaBH_4$ reduction. (1) Glucose standard; (2) glycerol standard; (3) and (7) sPF W3100; (4) and (6) liver glycogen; (5) erythritol standard. The C_2-compounds might have been formed by the oxidation procedure, or may result from the ethylene glycol used to destroy the excess of periodate. Solvent (*vi*), 2 hr.

Preliminary investigations using gel filtration on Sephadex G-200 and high-voltage paper electrophoresis have shown that sPF consists of at least three different polysaccharides,[6] and the possibility therefore arose that some of the glucose in the sPF preparations might originate from contaminating lower-molecular-weight glycogen formed by partial hydrolysis during the preparation of sPF. To elucidate this question, sPF from both strains was incubated with bacterial α-amylase, and the incubation mixtures were analyzed for liberated glucose and oligosaccharides. Whereas a sample of bacterial glycogen from Gal 23 K^+ was extensively split to glucose, maltose, and higher homologues (even in the presence of an equal amount of sPF from W3100), the sPF of both strains failed to form any reducing compound when treated under the same conditions.

Investigation of the products formed from sPF after periodate oxidation and sodium borohydride reduction: Figure 3 shows the separation of the hydrolysis products from W3100 sPF and from liver glycogen, both treated in the same way. Whereas glycogen gave a large quantity of erythritol due to its predominantly (1→4)-linked glucose units and a rather small amount of glycerol from the nonreducing end groups, sPF formed no erythritol, and liberated glycerol only. Since the glucose in the sPF of W3100 was oxidized, but no erythritol was formed, one can conclude that the glucose units of sPF cannot have been linked (1 → 4), or (1 → 3), but must be linked (1 → 2) and/or (1 → 6), and hence that sPF is different from glycogen.

Experiments which will be described in detail in a subsequent article[6] have shown that in spite of its acidic character, sPF contains no hexuronic acid and does not precipitate with Cetavlon. This clearly indicates that sPF is also different

from the mucoid material and from capsular antigens recently found in *E. coli* strains.[38-40]

Discussion.—The finding that the defect in the UDPG synthetase system affects the glucose incorporation into LPS of Gal 23 K$^-$ to a much larger extent than its incorporation into sPF of the same organism is somewhat unexpected.

There seem to be at least three main possible explanations for this phenomenon: (1) A glucosyl donor other than UDPG is responsible for the synthesis of glycogen and sPF in these *E. coli* strains. This would be similar to the use of ADPG in starch biosynthesis.[34, 35] (2) Glycogen, or at least sPF, is synthesized through UDPG in *E. coli* mutants which have normal levels of this nucleotide, but another less efficient glucosyl donor is also able to serve. This could account for the decreased levels of glucose in the sPF of UDPG-defective mutants. Such a case is known for glycogen synthesis in liver, where ADPG can substitute for UDPG, although with a somewhat lower efficiency.[36] (3) The biosynthesis of sPF depends just as well as that of LPS on UDPG. However, feedback inhibition or other types of regulation stop the transfer of glucose to LPS when the UDPG concentration in the cell goes below a certain level. In the biosynthesis of sPF, the glucosyl transfer may not be subject to such a type of regulation, or to a less severe extent, permitting appreciable incorporation of glucose from even a small UDPG pool.

It is, of course, of interest that the LPS constitutes an important part of the cell wall and that defects in the sugar composition can, as shown by Fukasawa and Nikaido, affect the ability of the bacterial cells to adsorb phages.[37] It was first found by Adler (unpublished) that a group of mutants, later identified as defective in UDPG synthetase,[1, 2] are unable to become infected by the phage P1. If the inability of the phages to infect the UDPG defective hosts should be due to the lack of receptor sites, the almost complete loss of glucose (and of the more distal sugars)[2] from the LPS of these strains warrants some interest.

The localization of this glucoside fraction in the cell has not yet been determined; it might constitute the remaining glucose reported to be present in the cell wall of the UDPG defective mutants.[4] These questions should be subjects for further investigations, and all that can be stated at this time is that the sPF is different, not only from LPS and glycogenlike material, but also from mucoid material or capsular antigens.

Abbreviations: ADPG, adenosine diphosphoglucose; ATP, adenosine-5′-triphosphate; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; sPF, soluble polysaccharide fraction; TLC, thin-layer chromatography; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; and UDPG, uridine diphosphoglucose.

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