Loss of Type Antigen in a Type III Streptococcus and Identification of the Determinant Disaccharide of the Remaining Antigen

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A Note on the Nomenclature of Certain Polysaccharides Resembling the Group Antigens of Streptococci

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SUMMARY

By subcultivating a streptococcal z3 III strain in medium containing anti-III serum a strain lacking the type antigen was isolated. Evidence is given that this strain possesses only z3 antigen. From partial acid hydrolysates of formamide extracts of both z3 and z3 III bacteria the same disaccharide was isolated. The most probable structure of the disaccharide is 3-*O*- α -*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine. It was 250 times more active than α -methyl-*N*-acetylglucosamine in the inhibition of the z3/anti-z3 system. This denotes that it is an important part of the determinant group of the z3 antigen. By ethanol fractionation of a formamide extract of z3 III bacteria two distinct fractions were isolated. The first fraction reacted only with type III antiserum and consisted of glucose, galactose and rhamnose in the ratio 5:3:1. The second polymer was composed of rhamnose, glucosamine and galactosamine in relative amounts of about 2:1:1. Chemical and serological evidence suggests very strongly that this is the z3 antigen. The similarities between z and true group antigens are discussed.

INTRODUCTION

Ottens & Winkler (1962) described several strains of haemolytic and non-haemolytic streptococci carrying, apart from the group antigen F, one of five type antigens of polysaccharide nature. These type antigens have repeatedly been found in strains belonging to other serological groups, for instance in groups C, G and T (Ottens & Winkler, 1962), A (Jablon, Brust & Saslaw, 1965), and L (Willers, Ottens & Michel, 1964*b*). The availability of type-specific antisera led to the discovery of other strains in which no known group antigen could be detected. These strains were designated by Ottens & Winkler (1962) as OI, OII, OIII and OIV, where O stands for zero. Whether zero denoted the absence of a group antigen or the presence of a hitherto unknown antigen was not known. Since it has been shown in several cases that zero

stands for an unknown carbohydrate antigen it seems practical to discontinue the use of O for zero so as to avoid confusion between zero-antigens and group O antigens. In this paper we will therefore allude to strains containing type and no group as zI, zII etc. The lower case z was deliberately chosen to avoid any suggestion of group or type status for these antigens. A numerical indicator is used to differentiate between different z antigens. For example, the symbol OIII is replaced by z₃ III (see Addendum).

In group B streptococci four polysaccharide type antigens have also been described. Lancefield (1934) separated the type antigens in group B from the group antigen by fractional precipitation with ethanol. Lancefield (Curtis & Krause, 1964) isolated a group B strain lacking type antigen by subcultivating a BI strain in the presence of homologous type-specific antiserum.

We have used both methods for obtaining type antigen and a z antigen in a pure form from a z₃III strain; the results are reported here.

METHODS

Streptococcal strains. A non-haemolytic streptococcal strain z₃III was isolated by Dr C. E. de Moor (National Institute of Public Health, Utrecht, the Netherlands) and tentatively designated as *Streptococcus* MG 216.

Culture conditions. Organisms were grown for 36 hr at 37° in Todd-Hewitt broth containing g./l.: 17 Todd-Hewitt (Difco) medium, 6.4 glucose, 3 sodium bicarbonate. The bacteria were collected by centrifugation in a continuous flow centrifuge MSE-17 at 17,000 rev./min. and washed once with distilled water.

Formamide extraction. The antigenic polysaccharides were extracted from the bacteria with formamide according to Fuller (1938). After centrifugation the extraction was repeated and the extracts combined. The extract was further treated as described by Willers, Michel, Sijsma & Winkler (1964a). In one experiment purification and fractionation as described below was done.

Purification and fractionation. The crude formamide extract was dialysed against several changes of distilled water; insoluble material in the dialysis residue was discarded after centrifugation. Nucleic acids were removed with streptomycin sulphate according to Hu, Wolfe & Reithel (1959). After treatment with trypsin, the extract was dialysed against distilled water and finally purified on DEAE-cellulose (Willers *et al.* 1964a).

To 1 vol. of purified formamide extract 2 vol. of ethanol 96% (v/v) in water were added. After standing at 4° for at least 2 hr the precipitate was removed by centrifugation. Two additional volumes of ethanol were added to the supernatant fluid and after removal of the precipitate formed the volume of the last supernatant fluid was concentrated in a flash evaporator to the original volume of the formamide extract. Addition of 5 vol. of acetone gave a precipitate. All precipitates were dissolved separately in 30–50 ml. distilled water and refractionated with ethanol and acetone. Finally the fractions were freeze-dried.

Hydrolysis conditions. For the qualitative and quantitative sugar analysis, 1 ml. of a solution containing 50 mg. polysaccharide was hydrolysed in 2 N-HCl at 100° for 5 hr. After cooling the hydrolysed material was neutralized with Dowex 1 in the carbonate form.

Paper chromatography was done as described by Willers *et al.* (1964*a*). The following solvents were used: solvent A, *n*-butanol + acetic acid + water (60 + 10 + 20, by vol.); solvent B, 2·4–2·5 lutidine + water (65 + 35, by vol.); solvent C, *n*-butanol + pyridine + water (60 + 40 + 30, by vol.).

Determination of sugars and aminosugars. Glucose was determined with glucose oxidase (Hugett & Nixon, 1957), galactose with the galactose oxidase reaction (Avigad, Amaral, Asensio & Horecker, 1962) and rhamnose with the thioglycollic acid + sulphuric acid reaction according to Gibbons (1955). Total hexosamines were estimated with the modified Elson & Morgan reaction (Rondle & Morgan, 1955). Glucosamine and galactosamine determinations were kindly done by Dr J. A. F. Op den Kamp (Laboratory of Biochemistry, State University of Utrecht) by gas chromatography by a method described by Perry (1964) and modified by Op den Kamp & van Deenen (1966). *N*-acetyl-hexosamine was determined by the modified Morgan & Elson reaction (Reissig, Strominger & Leloir, 1956).

Isolation of a streptococcal strain which lacked the type antigen. The z3III strain was subcultivated 30 times in a medium containing 0·25 ml. Todd–Hewitt broth and 0·25 ml. type III antiserum. After the last passage the streptococci were inoculated into Todd–Hewitt broth without antiserum. After incubation for 16 hr a slide was made and tested with fluorescent type III antiserum. None of the bacteria showed fluorescence, proving that streptococci carrying the type III antigen had disappeared from the culture. This culture of bacteria lacking in type III antigen was stable for this property after subcultivation several times in Todd–Hewitt broth.

Biochemical tests. Biochemical reactions of the z3III and z3 strain were kindly made by Dr C. E. de Moor (National Institute of Public Health, Utrecht, the Netherlands). The streptococci were grown in trypticase + yeast extract + cystine broth containing 1 % of one of the following compounds: glucose, lactose, saccharose, maltose, salicin, trehalose, raffinose, starch, inulin, mannitol, glycerol, sorbitol, aesculin, arginine, sodium hippurate. Reactions were read after 1 and 5 days.

Preparation of antisera and quantitative precipitin inhibition technique. These were as described by Willers *et al.* (1964*a*).

Capillary precipitin reactions with all streptococcal grouping sera were kindly done by Dr C. E. de Moor.

Partial hydrolysis and isolation of two oligosaccharides. Controlled hydrolysis of formamide extracts of z3III and z3 streptococci prepared as described by Willers *et al.* (1964*a*) was done by hydrolysis at pH 3, 2·5, 2, 1·5 and 1, and at temperatures of 60°, 70°, 80° and 90° for each pH value, every hydrolysis step taking 30 min. After each step the mixture was dialysed against distilled water with stirring for at least 4 hr. All diffusates were combined and neutralized with *N*-sodium hydroxide. The volume of the diffusate was reduced to about 50 ml. by evaporation *in vacuo*.

Separation of monosaccharides and salts from oligosaccharides was done on a charcoal column prepared as described by Schiffman, Howe & Kabat (1958). The diffusate obtained after partial hydrolysis was slowly adsorbed on a column (50 × 2·5 cm) containing 40 g. Darco G60 + 40 g. Celite 535. Elution was started with water (3 l.) to remove the salts and monosaccharides, followed by 3 l. of 5 % (v/v) ethanol in water. The 5 % (v/v) ethanol fraction was evaporated to dryness and redissolved in 5 ml. distilled water. This fraction was tested for inhibitions in the quantitative precipitin reactions of the z3III and z3 systems. The 5 % (v/v) ethanol fraction was

further fractionated on washed Whatman paper no. 3 MM, with solvent C as eluent.

Borohydride reduction and periodate oxidation was done as described by Michel & Willers (1964).

RESULTS

Properties of the strain lacking type III antigen. The strain which was isolated after subcultivation in Todd-Hewitt broth containing type III antiserum, and which did not react any longer with type III antiserum, was tentatively called z3.

Table 1. *Qualitative sugar analysis of formamide extracts of z3 III and z3 streptococci*

Sugars	Formamide extract of	
	z3 III	z3
Glucose	+	+
Galactose	+	—
Rhamnose	+	+
Glucosamine	+	+
Galactosamine	+	+

Table 2. *Inhibition by sugars of the quantitative precipitin reactions of z3 III/anti-type III and z3/anti-z3.*

Sugars	z3 III/ anti-type III	z3/anti-z3
Glucose	90*	.†
Galactose	90	.
<i>N</i> -acetyl-glucosamine	.	3

* The number of μ moles of sugar necessary to obtain 50 % inhibition is given.

† Inhibition with 90 μ moles less than 10 %.

A formamide extract of z3 bacteria did not react with any known group antiserum. An antiserum against this strain could be prepared. Both strains gave identical reactions in the biochemical tests; they did not ferment trehalose, a reaction which is nearly always positive with group F streptococci. A comparison of the sugar composition of the formamide extracts of z3 III and z3 bacteria showed that both had the same composition except that galactose was absent in the z3 formamide extract (Table 1). As previously described, the determinant group of the type III antigen contains probably glucose and galactose as the most important sugars (Willers *et al.* 1964*a*). In the z3/anti-z3 system however glucose and galactose did not give any inhibition of the quantitative precipitin reaction, while *N*-acetylglucosamine was a good inhibitor (Table 2).

Partial acid hydrolysis of formamide extracts of z3 III and z3 formamide extracts resulted in both cases in the production of inhibitory material. From the 5% (v/v) ethanol effluent an oligosaccharide, called compound A2, was isolated in both cases. This material had a R_f value in solvent A of 0.55 and in solvent B of 1.15. Further purification was obtained through chromatography on Whatman paper no. 3 MM with solvent C as eluent, and on a small charcoal column.

Complete hydrolysis of compound A2 showed it to be composed of equal amounts of glucosamine and galactosamine. When the substance was first reduced with sodium borohydride and then hydrolysed, the glucosamine content remained unchanged and the galactosamine content became negligible. From this it was inferred that compound A2 is a disaccharide containing *N*-acetylgalactosamine at its reducing end.

Evidence of the presence of the hexosamines in the acetylated form in the disaccharide was obtained as follows. When 0.5 mg. of the disaccharide was put on a Dowex 50 H⁺ column and eluted with water, all the material was recovered from the water eluate. De-acetylated hexosamines are not eluted from a Dowex 50 H⁺ column with water (Gardell, 1953). The amount of colour produced by this disaccharide in

Fig. 1

Fig. 2

Fig. 1. The Morgan & Elson reaction of: $\times-\times$, *N*-acetyl-glucosamine; $\bigcirc-\bigcirc$, *N*-acetyl-galactosamine; and $\bullet-\bullet$, compound A2. Compound A2 was isolated from partial acid hydrolysates of formamide extracts of z3III and z3 bacteria.

Fig. 2. The Elson & Morgan reaction of: $\times-\times$, *N*-acetyl-glucosamine; $\bigcirc-\bigcirc$, glucosamine; and $\bullet-\bullet$ compound A2. Compound A2 was isolated from partial acid hydrolysates of formamide extracts of z3III and z3 bacteria.

the Morgan & Elson reaction was about the sum of that obtained by equimolar concentrations of *N*-acetylglucosamine and *N*-acetylgalactosamine; glucosamine and galactosamine are negative in this reaction (Fig. 1). Equimolar concentrations of glucosamine, *N*-acetylglucosamine and the disaccharide were tested by the Elson & Morgan reaction. Figure 2 shows that the acetylated hexosamine and the disaccharide behaved similarly in this reaction, while glucosamine produced a higher amount of colour.

To obtain information about the linkage between the two *N*-acetylhexosamines periodate oxidation was done. The disaccharide consumed 2.7 mole periodate/mole after 7.5 hr and a total of 3.7 mole in 24 hr. After 24 hr the formaldehyde liberated from the sample was 1 mole/mole. These results indicate a 1-3 linkage as the most probable structure. The α_D^{20} in water was $+80^\circ$. In accordance with this was the good inhibition given by α -methyl-*N*-acetylglucosamine. Assuming that both hexosamines are in the D-configuration, the most probable structure of compound A2 is 3-*O*- α -*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine (Fig. 3).

From the partial acid hydrolysate of the formamide extract of z3 bacteria a second oligosaccharide, consisting of rhamnose and glucosamine in the relative amounts of

2:1 was isolated. The elution of the oligosaccharide from the charcoal column with 5% (v/v) ethanol in water and a R_f value in solvent C of 1.25 point to a trisaccharide.

Fig. 3. 3-*O*- α -*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine isolated from partial acid hydrolysates of formamide extracts of z3 III and z3 bacteria. The dotted lines indicate the diol linkages which are broken on periodate oxidation (theoretical 4 mole/mole, found 3.7 mole). On breakage of these linkages formic acid is formed and formaldehyde (theoretical 1 mole/mole, found 1 mole) is released.

Fig. 4

Fig. 5

Fig. 4. Inhibitions of the antigen-antibody reaction of the z3/anti-z3 system by: x—x, β -methyl-*N*-acetyl-glucosamine; o—o, α -methyl-*N*-acetylglucosamine; and ●—●, 3-*O*- α -*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine.

Fig. 5. Quantitative precipitin reactions of: x—x, acetone fraction of z3/anti-z3 serum; o—o, acetone fraction of z3 III/anti-z3 serum; and ●—●, alcohol fraction of z3 III/anti-type III serum.

Serological activity of the disaccharide. The inhibitory activity of 3-*O*- α -*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine is shown in Fig. 4 and compared with the inhibitions given by α -methyl-*N*-acetyl-glucosamine and β -methyl-*N*-acetyl-glucosamine. From Fig. 4 it can be seen that with 0.007 μ mole the disaccharide gave 50% inhibition of the antigen/antibody reaction, whereas 1.7 μ mole of α -methyl-*N*-acetyl-

glucosamine was necessary to obtain 50% inhibition. β -methyl-*N*-acetyl-glucosamine did not reach the 50% inhibition.

Fractionation of a formamide extract of z3 III bacteria. A purified formamide extract was fractionated with ethanol and acetone. On qualitative analysis by paper chromatography no galactose was found in the acetone fraction. The ethanol fractions contained rhamnose, glucose, galactose and small amounts of hexosamines. Refractionation with ethanol gave two main fractions. The first fraction was precipitable with 2 vol. of ethanol and contained glucose, galactose and rhamnose. The second fraction was precipitated with acetone and contained rhamnose and both hexosamines. Both final fractions were freeze-dried. By applying the same fractionation technique to a formamide extract of z3 bacteria, the main fraction precipitated with acetone. The quantitative sugar analysis of these fractions is presented in Table 3. In the first (2 vol. ethanol) fraction of z3 III glucose, galactose and rhamnose were present in the ratio of about 10:7:2. The composition of the acetone fractions of z3 III and z3 bacteria were much alike; the relative amounts of rhamnose, glucosamine and galactosamine were 2:1:1, with slightly more glucosamine than galactosamine (Table 3).

Table 3. *Quantitative sugar analysis of the ethanol and acetone fractions of a formamide extract of z3 III streptococci and the acetone fraction of a formamide extract of z3 III streptococci*

Fraction	z3 III ethanol fraction	z3 III acetone fraction	z3 acetone fraction
Glucose	44	1	3.3
Galactose	31	.	.
Rhamnose	9	35.5	34
Glucosamine	} 0.5	{ 19	17
Galactosamine			15

The figures give the percentages of the sugars calculated on the dry weight of the polysaccharides before hydrolysis.

The serological activity of the different fractions was measured in quantitative precipitin reactions. In the reaction between the ethanol fraction of z3 III and type III antiserum a peak was obtained with 40 μ g. antigen (Fig. 5). The acetone fractions of z3 III and z3 did not react with type III antiserum but gave almost identical precipitin curves with z3 antiserum; in both curves a peak was obtained with 16 μ g. antigen. The quantitative precipitin reaction of the acetone fraction of z3 III with z3 antiserum was for 50% inhibited by 0.009 μ mole of 3-*O*- α -*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine.

DISCUSSION

To study a possible z antigen (Ottens & Winkler, 1962) without hindrance of the type antigens two techniques also used for the isolation of the group B antigen were used. Subcultivation of z3 III bacteria in medium, containing type III antiserum, resulted in a strain which was no longer reactive with type III antiserum. The formamide extract of these z3 streptococci did not contain galactose, in contrast to the extract of z3 III streptococci. The serological differences are more striking. The z3 III/anti-III reaction was inhibited by glucose and galactose, whereas *N*-acetyl-glucosamine was completely inactive (Table 2). In the z3/anti-z3 system *N*-acetyl-glucosamine was a very good inhibitor, whereas other sugars including glucose and galactose were inactive.

Partial acid hydrolysis of formamide extracts of both z3III and z3 streptococci yielded after purification and isolation a disaccharide composed of two *N*-acetylated hexosamines. The most probable formula of the disaccharide is 3-*O*- α -*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine (Fig. 3). In the inhibition reaction of the quantitative precipitation of the z3/anti-z3 system, the disaccharide was a very potent inhibitor. An amount as small as 0.007 μ mole gave 50% inhibition in a volume of 0.6 ml. containing 16 μ g. of antigen and 0.1 ml. of antiserum, giving strong evidence for the disaccharide being the determinant group of the z3 antigen.

Lancefield (1934) used ethanol fractionation as a means of separating group antigen from type antigens in group B streptococci. Michel & Krause (1967) were able to separate the group and the type antigens from an FII strain by ethanol fractionation on a cellulose column; the group antigen appeared in the acetone fraction. When the same technique was applied to an extract of a z2II strain pure type II and z2 antigen were obtained. The solubility in ethanol of the z2 antigen was similar to those found for regular group antigens.

In our case application of the ethanol fractionation to a formamide extract of z3III bacteria resulted in a separation in two chemically and serologically distinct fractions. The 2 vol. ethanol fraction was reactive only with type III antiserum and must therefore be considered to be the type antigen. It contains besides rhamnose, both the sugars inhibitory in the type III system, namely glucose and galactose. The relative amounts of glucose, galactose and rhamnose are about 5:3:1 which was unexpected. Since rhamnose is serologically not active it was expected to be the backbone of the molecule and to be present in higher amounts. A possible destruction of rhamnose during formamide extraction has to be considered.

The acetone fraction was not reactive with type III antiserum but only with z3 antiserum, and gave a peak in the quantitative precipitin reaction with only 16 μ g., indicating a high degree of purification. Fractionation of a formamide extract of z3 bacteria yielded mainly acetone-precipitable material; 16 μ g. of this material gave with z3 antiserum an amount of precipitate equal to that obtained with the acetone fraction of z3III. Further evidence for the identity of the acetone fractions of formamide extracts of z3III and z3 bacteria is given by the inhibition reactions of the quantitative precipitation with z3 antiserum. The disaccharide 3-*O*- α -*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine gave 50% inhibition with the acetone fraction of z3III with 0.009 μ mole and with the acetone fraction of z3 with 0.007 μ mole. The sugar analysis shows the same sugars for the acetone fractions of formamide extracts of z3III and z3 bacteria. The relative amounts of rhamnose, glucosamine and galactosamine of the acetone fraction of extracts of z3III and z3 bacteria are about 2:1:1, with slightly more glucosamine than galactosamine. This suggests that the z3 antigen consists of a rhamnose backbone, with on every second rhamnose a determinant group, while possibly on some rhamnoses only a *N*-acetyl-glucosamine is attached. This is also suggested by the isolation of a trisaccharide consisting of rhamnose and glucosamine in the ratio of 2:1.

The following results show the similarity in properties between the z antigen and the other group antigens. A strain, z3, lacking type III antigen could be isolated. The sole antigen from strain z3 seems to be identical with the acetone-precipitable non-type antigen from strain z3III. This antigen has the solubility also found in other group antigens of streptococci. By injecting z3III streptococci in rabbits no anti-z antibodies

are formed. This is in accordance with the results obtained with strains carrying a group F and a type antigen (Ottens & Winkler, 1962), which give by injection in rabbits only anti-type antibodies. It is to be expected that more z antigens will be isolated and identified.

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A Note on the Nomenclature of Certain Polysaccharides Resembling the Group Antigens of Streptococci

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On the basis of serological reactions, Ottens & Winkler (1962) showed that the majority of streptococcal strains carrying the group F antigen possess one out of five additional type antigens. Accordingly these strains have been designated either FO or FI to FV. The antigens I and II presumably are identical with the type antigens described by Bliss (1937). All these type antigens are polysaccharides and are probably located on the surface of the cell wall. Antisera prepared in rabbits with vaccines of strains carrying both group and type antigen usually contain type-specific antibodies but no group-specific antibodies. Using type-specific antisera several type antigens have repeatedly been found in strains of other serological groups, FI in groups C, G and T (Ottens & Winkler, 1962), L (Willers *et al.* 1964*b*) and A (Jablon *et al.* 1965). The availability of type-specific antisera further led to the discovery of a substantial number of streptococcal strains carrying a type antigen but no known group antigen. Such strains have been designated OI, OII, OIII or OIV where O stands for zero. When these strains were described it was not clear whether zero denoted the absence of a group antigen or the presence of a hitherto unknown antigen.

Michel & Krause (1967) separated the group and type antigen present in an FII strain on the basis of a difference in alcohol solubility of the F and the II antigen. When the same technique was applied to extracts of an OII strain (Michel & Krause, 1967) and an OIII strain (Willers & Alderkamp, this paper) two different polymers were isolated in each instance. The polymers with the lowest solubility in alcohol were found to be serologically and chemically identical in both cases with known type antigens. The second set of polymers had the same physico-chemical properties as the group antigen F (Michel & Krause, 1967). It is therefore believed that the so-called 'O' (zero) polysaccharides have the same location in the cell wall as regular group antigens. They have not been recognized before because the 'O' polymers are exclusively found in strains carrying a type antigen. As indicated before the presence of type antigens generally suppresses the formation of group-specific antibodies. The masking effect of the presence of the type antigen on the antigenicity of an 'O' polymer was however clearly demonstrated by Willers *et al.* (this paper), with a mutant derived from strain OIII and lacking type antigen III and containing only the original 'O' antigen.

From this it appears that the symbol 'O' did not stand for the absence of a group

antigen but indicated the presence of an unknown group-like material. The zero polymers which have been isolated from three single strains (OI, OII and OIII) can clearly be differentiated from each other by their chemical composition. Moreover, the designation of several group-like materials denoted by capital O might give rise to confusion with the existing group O streptococcal polysaccharide. It is therefore suggested that a new provisional nomenclature for the 'O' antigens of streptococcal strains which can be found in association with a type antigen be introduced. It is proposed to replace the 'O' by a lower case 'z' followed by a numerical indicator. The lower case has been chosen to avoid any suggestion that these antigens are true group antigens, before they have been studied more elaborately. The first three indicators will be assigned to well investigated strains so that strain 18 formerly defined as O I will now be designated zI, strain HS 189 (OII) z2II and strain MG 216 (OIII) z3III. Unpublished observations indicate that different combinations of z and type polymers can be found and that the number of z polymers is larger than the number used here.

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