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The Influence of Calcium Ions, Acetate and L-Glycerate Groups on the Gellan Double-Helix

R. Chandrasekaran & V. G. Thailambal

Whistler Center for Carbohydrate Research, Smith Hall, Purdue University, West Lafayette, Indiana 47907, USA

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ABSTRACT

A computer-assisted linked-atom least-squares program has been used to visualize the crystal structure of calcium gellan and to examine the ability of acetate and L-glycerate groups in native gellan to sustain the double-helix and to maintain the potassium gellan crystal packing. The results explain the strong and brittle gelation behavior at low ionic strength of calcium gellan and suggest that the weak and rubbery gelation behavior of native gellan is perhaps due to the L-glycerate groups.

INTRODUCTION

Gellan is a microbial polysaccharide, potentially important industrially because of its ability to form firm and brittle aqueous gels. The de-acylated polymer, which is the commercial form and which is generally the polymer termed gellan, has a linear anionic tetrasaccharide repeating unit

$$[\rightarrow 3)\beta\text{-D-Glc}(1\rightarrow 4)\beta\text{-D-GlcA}(1\rightarrow 4)\beta\text{-D-Glc}(1\rightarrow 4)\alpha\text{-L-Rha}(1\rightarrow]$$

and exhibits cation dependent gelation properties. Native gellan contains acetate at C6 on approximately 50% of the O-3 substituted glucose residues and L-glycerate groups at C2 on all of the same glucose residues (Kuo *et al.*, 1986). Rheological studies show that this material, in contrast with the de-acylated polymer, forms only weak gels (Moorhouse, 1987). Throughout this paper, native gellan will refer to the acylated polymer as secreted by the bacterium; gellan will refer to the de-acylated polymer which is commercially known as gellan gum.

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The way in which the bulky substituents interfere with gelation is unknown. In the case of gellan, solution studies (Sanderson & Clark, 1983; Crescenzi *et al.*, 1986; Grasdalen & Smidsrod, 1987; Sanderson, in press) indicate that gels can be formed with divalent (Ca^{2+}) ions at drastically reduced concentrations than are required for gelation with monovalent ions (K^+). An explanation for this behavior based on an X-ray structure analysis of the potassium salt of gellan has recently been proposed (Chandrasekaran *et al.*, 1988*a*). The salient features are that the gellan molecule exists as a half-staggered, parallel double-helix and the crystal structure is stabilized by double-helix—K^+—water—K^+—double-helix interactions. It is conjectured that, in the presence of divalent ions, (e.g. Ca^{2+}), there would be direct cross-links between the ions and the double-helices giving rise to much stronger double-helix—Ca^{2+}—double-helix interactions. Aggregates of double-helices, and hence gels would therefore be formed at very low divalent ion concentrations.

The structural roles of calcium ions and the two substituents on the relative stability of the gellan double-helix and of the experimentally determined crystal structure of the potassium form were investigated. Using a computer model building approach (Smith & Arnott, 1978), all the critical interactions in the double-helix—Ca^{2+}—double-helix cross-links were visualized. Further, the results reveal that the acetate groups can be more readily accommodated than can L-glycerate groups, in the double-helix and also in the potassium crystal structure. The implication of the latter findings is that, perhaps the glycerate groups, and not the acetyl groups, are impairing gelation.

MOLECULAR MODEL BUILDING

A schematic representation of the repeating motif of native gellan is shown in Fig. 1. The potassium crystal structure (Chandrasekaran *et al.*, 1988*a*) has confirmed that the gellan molecule forms a half-staggered, parallel, double-helix in which each polysaccharide chain has a left-handed, three-fold helix symmetry of pitch 5·63 nm. Potassium ions are coordinated to the carboxylate groups. Two molecules are packed in a trigonal unit cell ($a = b = 1{\cdot}575$ nm, $c = 2{\cdot}815$ nm), in an antiparallel fashion such that double-helix—K^+—water—K^+—double-helix interactions stabilize the aggregation of polymer chains.

We have now employed the linked-atom least-squares procedure (Smith & Arnott, 1978) to create a calcium gellan crystal structure by replacing each pair of monovalent potassium ions by a single divalent calcium ion. Likewise, we have appended the acetyl groups (Fig. 1) on

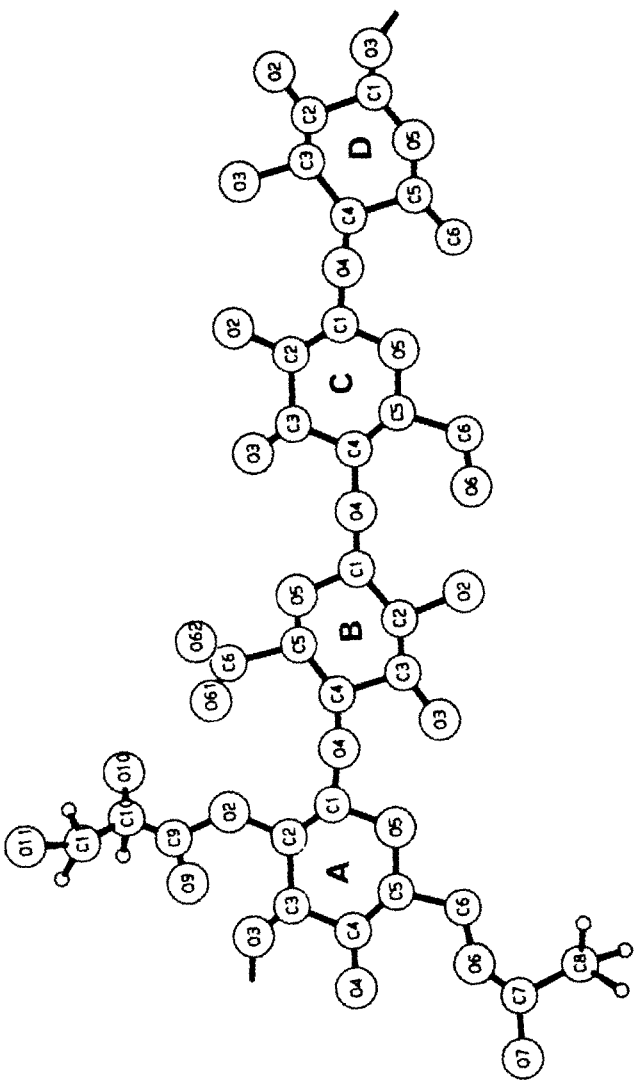

Fig. 1. Schematic representation, and atom labeling, of the tetrasaccharide repeating unit of native gellan. The 3-linked glucose residue (A) contains L-glycerate at C2 and acetate at C6.

the gellan double-helix and visualized an acetyl gellan crystal structure. Furthermore, the influence of L-glycerate groups on the 'canonical' potassium crystal structure has been modeled.

In the least-squares program, the positions of calcium ions and water molecules were varied; the conformation angles of the acetate and glycerate groups were refined, but their bond lengths and bond angles were fixed at standard values. The function minimized in producing a compression-free molecular, and/or crystal model is given by

$$\Omega = \sum_i e_i({}_0\theta_i - \theta_i)^2 + \sum_j c_j({}_0d_j - d_j)^2$$

The first term allows the program to drive a conformation angle θ_i towards one of its preferred values ${}_0\theta_i$ with weight e_i. The second term is responsible not only to trap hydrogen bonds and ligands to cations, but also to pull apart atoms with short non-bonded contacts d_j towards ${}_0d_j$ which are slightly larger than the usually accepted contact limits.

Unless stated otherwise, all calculations were done by treating the gellan double-helix as a rigid body which has the morphology previously determined (Chandrasekaran *et al.*, 1988*a*).

Attempts were made to retain the same potassium gellan crystal packing arrangement as far as possible in order to make meaningful comparisons.

RESULTS

Ca^{2+} Gellan

In the potassium gellan crystal structure, the pair of ions K1 and K2 belonging to the up- and down-pointing double-helices, which have the fractional coordinates, 0·4503, 0·2910, −0·0624, and 0·3594, 0·4987, −0·0884, respectively, are separated by only 0·43 nm. The carboxylate oxygen atoms O61B of the two molecules around these cations are only 0·51 nm apart. As speculated in the previous study (Chandrasekaran *et al.*, 1988*a*), these results suggest that a single calcium positioned at, 0·4163, 0·4028, −0·0493, can replace the two potassium ions, in such a way that it readily cross-links two double-helices using one or both carboxylate oxygen atoms from each double-helix. In total there are four ligands, two from each double-helix. Whereas O61B and O2A are the ligands from the up-pointing molecule, both carboxylate oxygen atoms of the down-pointing molecule are coordinated to the calcium ion. In addition, two water molecules are also involved. Water molecule W1

already exists in the potassium gellan crystal structure and W9 is a newly introduced water molecule at, 0·3078, 0·2342, −0·1247. As Table 1(a) shows, W9 is stabilized by hydrogen bonds with the carboxylate oxygen atoms of the up-pointing double-helix and with another water molecule, W7 which is already involved in the formation of intrachain water bridges.

The cross-linking of the double-helices by calcium ions can be easily visualized from Fig. 2. This also shows a distorted octahedral coordination for calcium consisting of six ligands — four from gellan and two from water molecules. The sharing of calcium ions by neighboring

TABLE 1

Attractive Interactions[a] in the Proposed Double-Helical Models[b] of (a) Calcium Gellan and (b) Potassium Native Gellan

Type	*Atom*[c] *X*	*Atom Y*	*X...Y (nm)*	*Precursor (P)*	*Angle (°) P—X...Y*
(a) *Ca^{2+} gellan*					
Calcium	O61B(I,1)	Ca	0·310	C6B	149
Coordination	O2A(I,1)	Ca	0·297	C2A	138
	O61B(II,1)	Ca	0·260	C6B	110
	O62B(II,1)	Ca	0·327	C6B	77
	W1	Ca	0·265	—	—
	W9	Ca	0·312	—	—
Water bridges	O62B(I,1)	W9	0·285	C6B	90
	O61B(I,1)	W9	0·282	C6B	91
	W7	W9	0·275		
(b) *K^+ native gellan*					
Intrachain	O3D	O10A	0·282	C3D	78
Interchain	O6C	O62B	0·278	C6C	113
	O10A	O2B	0·272	C10A	105
Potassium	O61B(2)	K	0·282	C6B	100
Coordination	O62B(2)	K	0·309	C6B	87
	O6C(1)	K	0·331	C6C	169
	W1	K	0·300	—	—
	O11A(2)	K	0·293	C11A	111
Interchain	O10A(1)	W1	0·318	C10A	71
Water bridges	O11A(1)	W1	0·298	C11A	82
	O2B(2)	W1	0·274	C2B	124
	O3B(2)[d]	W1	0·308	C3B	105

[a]These are supplementary to data given in Table V of Chandrasekaran *et al.* (1988*a*).
[b]The 'up' and 'down' molecules are distinguished by I and II in (a); the two chains in the duplex are labelled as 1 and 2 in (a) and (b). These labels are given in parentheses.
[c]X is the donor atom, Y the acceptor, and P the precursor to X.
[d]The corresponding values of the hydrogen bond missed out in Table V Chandrasekaran *et al.* (1988*a*) are 0·293 nm and 74°.

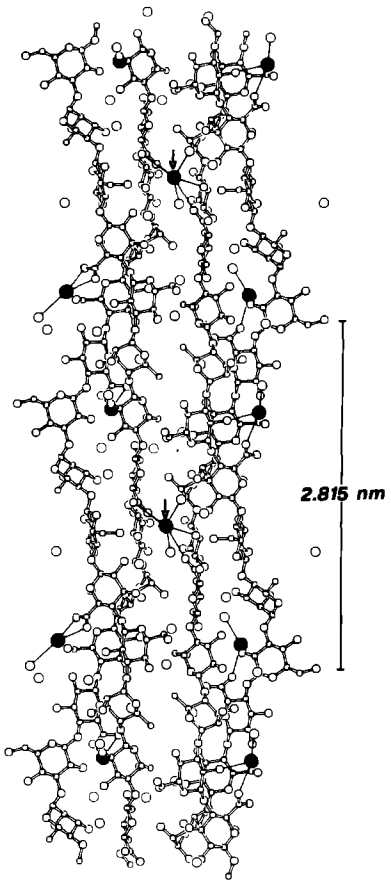

Fig. 2. Two adjacent, up- and down-pointing gellan double-helices, cross-linked at the arrows by calcium ions and the associated water molecules in the unit cell viewed normal to the c-axis. The calcium ions and water molecules are shown by filled and open circles, respectively, and the ligands are drawn in thin lines.

double-helices in the unit cell can further be seen in Fig. 3. It should be emphasized that the packing arrangement of the experimentally determined potassium gellan crystal structure (Chandrasekaran *et al.*, 1988*a*) is preserved (Figs. 2 and 3).

Acetyl groups in native gellan

Since the hydroxymethyl groups of glucose A residues occur on the periphery, computations show that the acetyl groups can be readily accommodated with no steric hindrance in an isolated gellan double-helix. The conformation angles of the acetyl group given in Table 2 indicate a folded geometry for this fragment. A stereo view of acetyl gellan double-helix is shown in Fig. 4.

As Fig. 5 illustrates, the acetyl groups exhibit substantial protrusion into neighboring unit cells and are stabilized merely by van der Waals interactions. Yet, surprisingly, they can be fitted into the potassium crys-

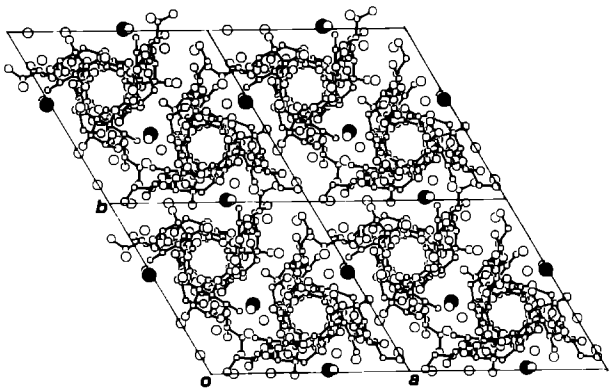

Fig. 3. A c-axis projection of the proposed model of the calcium gellan crystal structure. The calcium ions and water molecules are shown by filled and open circles, respectively.

TABLE 2
Conformation Angles[a] in the Acetate and L-Glycerate Groups of Native Gellan Double-Helix Model

Conformation angle	*Value (°)*
θ_2(C5A–C6A–O6A–C7A)	158
θ_3(C6A–O6A–C7A–C8A)	66
θ_4(O6A–C7A–C8A–H81A)	53
θ_5(C1A–C2A–O2A–C9A)	−73
θ_6(C2A–O2A–C9A–C10A)	−150
θ_7(O2A–C9A–C10A–C11A)	162
θ_8(C9A–C10A–C11A–O11A)	99

[a]These are supplementary to the data given in Table IV of Chandrasekaran *et al.* (1988*a*).

tal structure with minimum perturbation. In this process, only a crystalline water molecule W6, involved in intermolecular cross-linking, is expelled. This packing arrangement suggests that the acetate groups might have only a small weakening effect on the aggregation of gellan molecules.

L-Glyceryl groups in native gellan

Interestingly, the glycerate group attached to C2A, has both destabilizing and stabilizing influences on the double-helix. The intrachain O2A... O61B hydrogen bond is no longer possible because of the substitution. In addition, the atoms in the glycerate group are too close to the carboxylate group, potassium ion and the first shell water molecule W1.

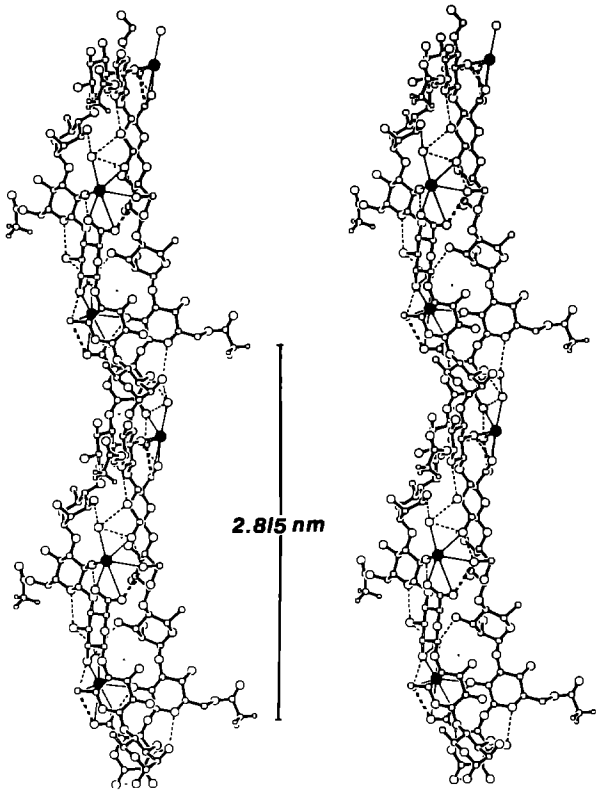

Fig. 4. A stereo view of acetylated potassium gellan double-helix, normal to the helix-axis, displaying the acetate groups in the periphery. Hydrogen atoms are shown only for the acetate groups. Potassium ions (filled circles) are connected to its ligands by thin lines. Interchain hydrogen bonds are shown in thick dashed lines and other hydrogen bonds in thin dashed lines.

This situation can, however, be relieved by making two major changes: (a) rotating the carboxylate groups about the C5B–C6B bond by roughly 30°, so that χ_2(C4B—C5B—C6B—O61B) becomes 38°, and (b) moving the potassium and water molecule W1 by about 0·2 nm from the original positions. These alterations provide enough room for the glycerate moiety to adopt a folded conformation (Table 2) such that all three of its oxygen atoms (O9A, O10A and O11A) achieve specific roles in stabilizing the slightly modified double-helix.

Table 1(b) summarizes the new features in the native gellan double-helix model. The loss of O2A...O61B is compensated by the formation of O3D...O10A intrachain hydrogen bond. The change in the carboxylate orientation has shortened the original interchain hydrogen bond O6C...O62B from 0·304 nm to 0·278 nm; there is a new, second, interchain hydrogen bond O10A...O2B (0·272 nm). Together, the two

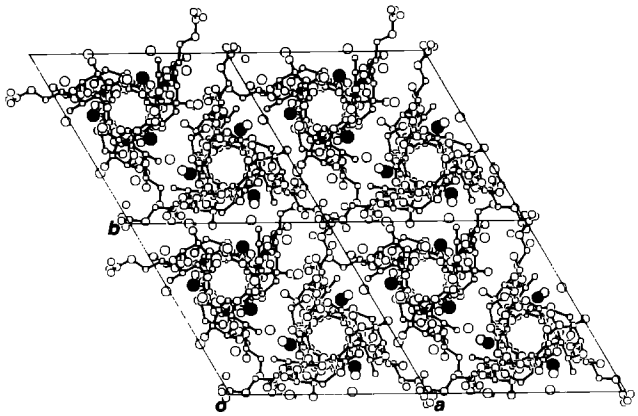

Fig. 5. Antiparallel packing of two acetyl gellan molecules in the K^+ gellan crystal structure, viewed down the c-axis. The ions are shown by filled circles and water molecules by open circles.

interchain hydrogen bonds offer strong stability to the native gellan molecule. The potassium ion is coordinated to O6C of one chain and to both carboxylate oxygens and, in addition, to O11A, of the other. The water molecule W1, which is held in position by four hydrogen bonds, two from each chain, serves as the fifth ligand.

A stereo view of the proposed model of native gellan double-helix with potassium ions and first shell water molecules is shown in Fig. 6.

Although the isolated double-helix of native gellan displays excellent stereochemical features, our extensive calculations do not provide any satisfactory proof that two such molecules could be packed in the trigonal unit cell of the potassium gellan crystal structure.

DISCUSSION

X-ray diffraction studies (Chandrasekaran *et al.*, 1988*a*,*b*) confirmed that the gellan molecule exists as a double-helix in the solid state and provided the molecular and crystal structures in the presence of monovalent ions, lithium and potassium. Further, X-ray diffraction patterns from other monovalent salt forms (e.g., sodium, rubidium and tetramethyl ammonium) strongly support the same double-helix and the same, or closely similar, packing arrangement as in the potassium gellan crystal structure (Chandrasekaran, in press).

The computational results reported in this article extend the X-ray diffraction analyzes. The previous speculation on the ability of calcium ions to cross-link neighboring gellan double-helices in the potassium

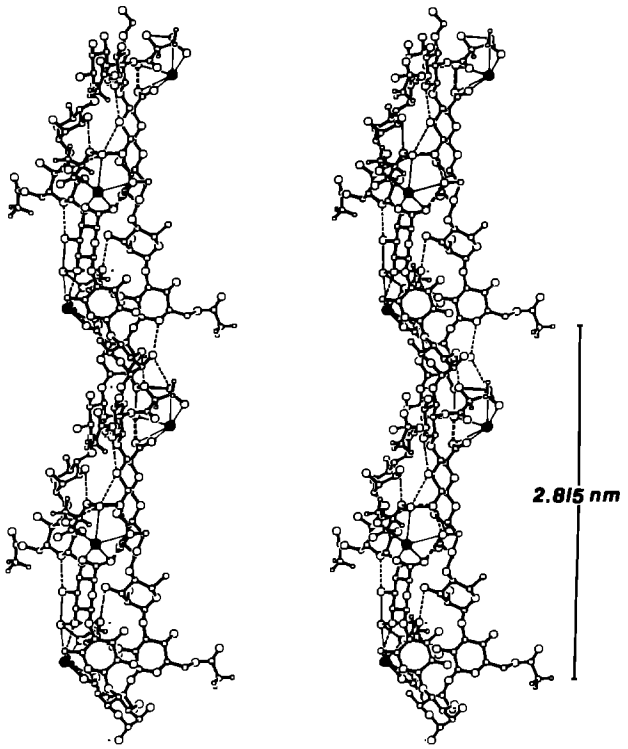

Fig. 6. A stereo view of the native gellan double-helix and the coordinating potassium ions (filled circles) and water molecules (open circles). The intrachain (thin dashed lines), interchain (thick dashed lines) hydrogen bonds and ligands to potassium (thin lines) stabilize the double-helix. Hydrogen atoms are shown for the L-glycerate and acetate groups.

crystal structure (Chandrasekaran *et al.*, 1988*a*) has now been visualized (Figs 2 and 3). The coordination of calcium between double-helices (Table 1(a)) is so strong that, in solution, aggregation of gellan molecules could happen readily even at very low ionic concentration.

Rheological studies on native gellan (Moorhouse, 1987) suggest considerable weakening of its gel properties, but provide no explanation. It is not known whether the acetate or L-glycerate groups, or both, are responsible for this effect. Our demonstration that acetylated gellan could be accommodated in the potassium gellan crystal structure would indicate only a passive role for the acetate groups in this process.

On the other hand, L-glycerate substitution does perturb the gellan double-helix due to its occurrence in the interior and its proximity to the carboxylate group, potassium ion and water molecule, which are all vital to the stability of the polymer duplex. Once again, the present study has revealed that the original double-helix could be suitably modified (Table

1(b); Fig. 6) by subtle movements of the above groups such that the native gellan double-helix has two, rather than one, interchain hydrogen bonds, lending additional stability.

The results suggest an important role for O9A, at very low pH, when the carboxylate group is protonated. Native gellanic acid, free from any cations, could still retain the same double-helix (of the unprotonated form), which is further stabilized by another intrachain O61B...O9A hydrogen bond (0·284 nm and 136°), O9A of the L-glycerate group serving as the acceptor.

Obviously the potassium gellan crystal structure offers, for the first time, all the intermolecular interactions that are typically involved in inducing the aggregation of the double-helices. It is not possible to mimic this packing arrangement with the modified native gellan double-helices without a significant lateral expansion of the unit cell so as to relieve the short intermolecular contacts involving the L-glycerate groups and the main chain atoms. This would effectively reduce and weaken the intermolecular aggregation compared to that in potassium gellan. Eventually this would result in modified gelation behavior.

Detailed X-ray investigations on calcium gellan and native gellan are currently in progress in order to gain a better insight into their structures and to correlate them with the observed physical properties of these important polymers.

CONCLUSIONS

Computer model building studies made it possible to visualize the most probable crystal structure for calcium gellan on the basis of the previously reported potassium gellan crystal structure. It is further shown that native gellan, which has an acetate group on C6 and an L-glycerate group on C2 of its 3-substituted glucose residues can also exist as a double-helix stabilized by monovalent ions (e.g. K^+). The glycerate, and not the acetate, groups of the double-helix are detrimental to crystal packing.

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