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Cellular mechanisms of dinoflagellate cyst development and ecdysis – many questions to answer

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Summary

Dinoflagellates are characterized by the ability to form different types of cysts (resting and temporary) ensuring their viability under unfavorable environmental conditions. Although cyst production was described in many species and is considered as one of the key adaptations of these organisms, relatively little information on the cellular mechanisms of dinoflagellate cyst development and the related process of ecdysis is available. This article reviews the data obtained so far and emphasizes the questions that need to be answered in future in order to improve our knowledge about dinoflagellate physiology and ecological success.

Key words: dinoflagellate, ecdysis, resting cysts, temporary cysts

Introduction

Among diverse phytoplankton groups, dinoflagellates stand out as the organisms possessing a remarkable number of unique cellular traits ranging from specific DNA compaction to the complex organization of a cell covering (Hackett et al., 2004). The discovery of such traits boosted interest to these organisms and thus has led to publication of many cytological studies well-known nowadays (e.g. Morrill and Loeblich, 1983; Rizzo, 1991, 2003; Bhaud et al., 2000). Nevertheless, most of the cellular processes that make dinoflagellates unique remain enigmatic and still need close attention of cell biologists.

Dinoflagellates are also exceptional in terms of their ecological relevance being one of the largest and diverse groups of planktonic microorganisms. They are essential components of aquatic food webs as both primary producers and consumers and one of the most significant drivers of biogeochemical cycling in the marine systems (Saldarriaga and Taylor, 2017). Furthermore, many dinoflagellate species can cause harmful algal blooms, a serious and expanding environmental problem of the last decades (Hallegraeff, 1993; Anderson et al., 2002; Heisler et al., 2008; Glibert and Burford, 2017). Therefore, it is natural that these organisms have received major attention from plankton ecologists resulting in numerous studies of dinoflagellate distribution, nutrient uptake, growth and bloom dynamics (e.g. Anderson, 1997; Kudela and Cochlan, 2000; Fan et al., 2003; Li et al., 2010; Kibler et al., 2015).

A combination of the two approaches paying attention to the cellular mechanisms behind the ecologically relevant processes has remarkable perspectives and will be likely exploited more often in the near future. For instance, a huge amount of data on the nitrogen uptake kinetics and uptake

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rates by dinoflagellates in natural and laboratory systems has been accumulated (e.g. Sciandra, 1991; Fan et al., 2003; Collos et al., 2004; Matantseva et al., 2016, 2017; Jauzein et al., 2017). However, the information about the transporters responsible for the nitrogen uptake and regulation of their expression is still limited (Dagenais-Bellefeuille and Morse, 2013, 2016; Matantseva et al., 2016, 2017). Another example is that inhibition of the nitrate uptake by ammonium in some dinoflagellate species is well documented (Lomas and Glibert, 1999; Jauzein et al., 2008; Glibert et al., 2016), but the cellular mechanisms behind such inhibition remain unknown.

This review considers the data on dinoflagellate cysts and ecdysis. These phenomena are widely spread among the great number of dinoflagellate species and undoubtedly ensure their high adaptive potential (Bravo and Figueroa, 2014). Although cellular mechanisms of encystment, cyst dormancy and quiescence, excystment, and ecdysis that is involved in cyst physiology are still vague, some highly valuable data were accumulated over the last decades. Further, we focus on these data, but first briefly describe the process of ecdysis, types of dinoflagellate cysts and their potential functions.

The phenomenon of dinoflagellate ecdysis

Dinoflagellates possess a peculiar cell covering structure (Dodge and Crawford, 1970; Dodge, 1971; Loeblich, 1970; Morrill and Loeblich, 1983). Their cell covering called amphiesma consists of a plasma membrane and amphiesmal vesicles beneath it. In armored species, amphiesmal vesicles contain cellulosic thecal plates. Moreover, in some species, an additional pellicular layer is observed in amphiesmal vesicles. On the transmission electron microscopy images (cross sections of cells) amphiesma appears as a set of three membranes with thecal plates lying between the outer amphiesmal vesicle and inner amphiesmal vesicle membranes. Such images sometimes lead to confusion regarding which membrane represents the plasma membrane. Obviously, the plasma membrane is the outermost membrane often termed ‘outer membrane’ in the literature, since it is the only continuous membrane encircling the entire cell including eukaryotic flagella. For an explicit review of the dinoflagellate amphiesma and the history of its ultrastructural studies please see the work by Pozdnyakov and Skarlato (2012) and references therein.

In dinoflagellates, ecdysis is a process of cell covering rearrangement that includes shedding of the old plasma membrane, outer amphiesmal vesicle membranes and thecal plates (in armored species), accompanied by the concurrent formation of the new plasma membrane from fused inner amphiesmal vesicle membranes and maturing of the new amphiesmal vesicles (Pozdnyakov and Skarlato, 2012). Ecdysis can be induced in response to different environmental stressors, e.g. mechanical disturbance. This truly astonishing process was morphologically described in many dinoflagellate species (Morrill, 1984; Morrill and Loeblich, 1984; Bricheux et al., 1992; Höhfeld and Melkonian, 1992; Sekida et al., 2001, 2004; Berdieva et al., 2016), but it is significantly underexplored from the standpoint of cell biology. Nevertheless, it is already clear that ecdysis is an essential part of the dinoflagellate life cycle that have a high adaptive value, for example, in many cases it is involved in the formation and germination of their cysts.

Types of dinoflagellate cysts and their ecological relevance

Many dinoflagellate species are able to form cysts either in response to unfavorable environmental conditions or as a stage of their progression through the life cycle. There are two basic types of cysts distinguished based on their morphology, way of formation and dormancy period (Bravo and Figueroa, 2014). Further, we provide only a brief comparative description of their properties important in the context of this review but do not focus on details of their structure and general biology.

Very thick or moderately thick walls often consisting of several layers that are highly resistant to external impact, e.g. chemical treatment, characterize resting, or thick-walled, cysts. In turn, temporary, or thin-walled, cysts possess relatively thin walls. Resting cysts ensure cell survival over long periods of time lasting from months to years, whereas temporary cysts usually function over shorter time scales. The formation of resting cysts is often linked to the sexual process in dinoflagellates whereby the fusion of motile haploid gametes results in diploid planozygotes which can develop into cysts (Steidinger, 1975; Anderson and Wall, 1978; Figueroa et al., 2006). At the same time, resting cysts were also shown to be formed independently of the sexual process (Kremp and Parrow, 2006). Temporary cysts develop in response to perturbations

in environmental conditions, such as mechanical impact, changes in temperature, nutrients, light regime, and this ability was repeatedly demonstrated both in nature and laboratory studies (e.g. Balzer and Hardeland, 1992; Garcés et al., 2002, 2004; Figueroa et al., 2007). Ecdysis seems to be a necessary stage of the formation and/or germination of dinoflagellate temporary cysts that are even sometimes referred to as ecdysal cysts (Bravo et al., 2010). However, some studies demonstrated that ecdysis also accompanied the formation of resting cysts (Kokinos and Anderson, 1995; Bravo et al., 2010). Thus, this process is very important to understand different aspects of dinoflagellate cyst development.

Resting cysts enable long-term survival of dinoflagellates, for example, overwintering, and are involved in their bloom dynamics and dispersion. The ecological role of temporary cysts is less clear and usually interpreted as a stress response allowing to survive short-term unfavorable conditions (Bravo and Figueroa, 2014). Remarkably, some field data indicate that temporary cyst formation also can be implicated in population and bloom dynamics of dinoflagellates (Garcés et al., 1999, 2002; Basterretxea et al., 2005). Overall, it is quite clear that both types of dinoflagellate cysts represent one of the powerful adaptive strategies employed by these widely distributed and diverse microorganisms. Therefore, knowledge about the cellular mechanisms of dinoflagellate cyst maintenance and ecdysis is a key to understand their ecological success.

Current data on cellular mechanisms of cyst development and ecdysis in dinoflagellates

Although the studies focused on the cellular and molecular details and mechanisms of ecdysis and cyst development (i.e. formation, dormancy, quiescence, and germination) are still rare, a significant amount of information has been obtained. This knowledge can be used as a solid platform for future research and is summarized in Fig. 1. One part of this information is related to the resting cysts whereas the other – to the temporary cysts. Here we consider both types of dinoflagellate cysts, since it is likely that physiological aspects accompanying different stages of their development are similar or at least may overlap to some extent.

Resting (thick-walled) cysts

Almost 30 years ago, Binder and Anderson (1990) published their findings on physiological changes accompanying the formation, dormancy, quiescence, and germination of the resting cysts of *Scrippsiella trochoidea*. In particular, they investigated chemical composition, respiration and photosynthetic activity of vegetative cells and cysts at the different stages of their development. It was shown that *S. trochoidea* cysts contained the reduced amount of proteins and chlorophyll a, whereas the amount of carbohydrates in the newly formed cysts was an order of magnitude higher than in vegetative cells indicating an intensive carbohydrate synthesis prior to encystment. The accumulated carbohydrates along with lipids served as an energy source, since their content gradually declined during different stages of cyst development. An estimated respiration rate was rather low in the resting cysts and constituted 10% of that in vegetative cells at the initial phase of cyst development and only 1.5% - in quiescent cysts. The reduction in the respiration rate measured as oxygen consumption was also shown for the *S. trochoidea* resting cysts in a recent independent study (Deng et al., 2017). Remarkably, germination of cysts was preceded by the increase in respiration followed by the activation of photosynthesis (Binder and Anderson, 1990).

Deng et al. (2017) performed a transcriptomic analysis of *S. trochoidea* to reveal processes that regulate resting cyst formation and dormancy. They discovered that more than 3800 genes (2.32% of all genes) were differentially expressed between cysts and vegetative cells, with 134 genes expressed in cysts only. According to the obtained data and similar to the observations of Binder and Anderson (1990), photosynthesis was repressed, whereas general energy metabolism, i.e. glycolysis, tricarboxylic acid cycle, glyoxylate cycle and β-oxidation of fatty acids, was still active in the resting cysts. Genes responsible for the resistance to reactive oxygen species (ROS) and usually considered as markers of stress were upregulated in cysts. At the same time, the expression of three genes encoding heat shock proteins (HSPs) was reduced, possibly reflecting the reduced requirements for the functioning of chaperones. Expression of genes encoding two putative cyclin-dependent kinases (CDKs) was downregulated, whereas expression of two putative cyclins – upregulated. Moreover, 21 putative phytohormone-encoding genes were differentially expressed. The most remarkable finding of Deng et al. (2017) shedding light on the resting cyst physiology was related to the importance of abscisic acid (ABA) for the cyst formation and dormancy. ABA is a well-known plant hormone responsible

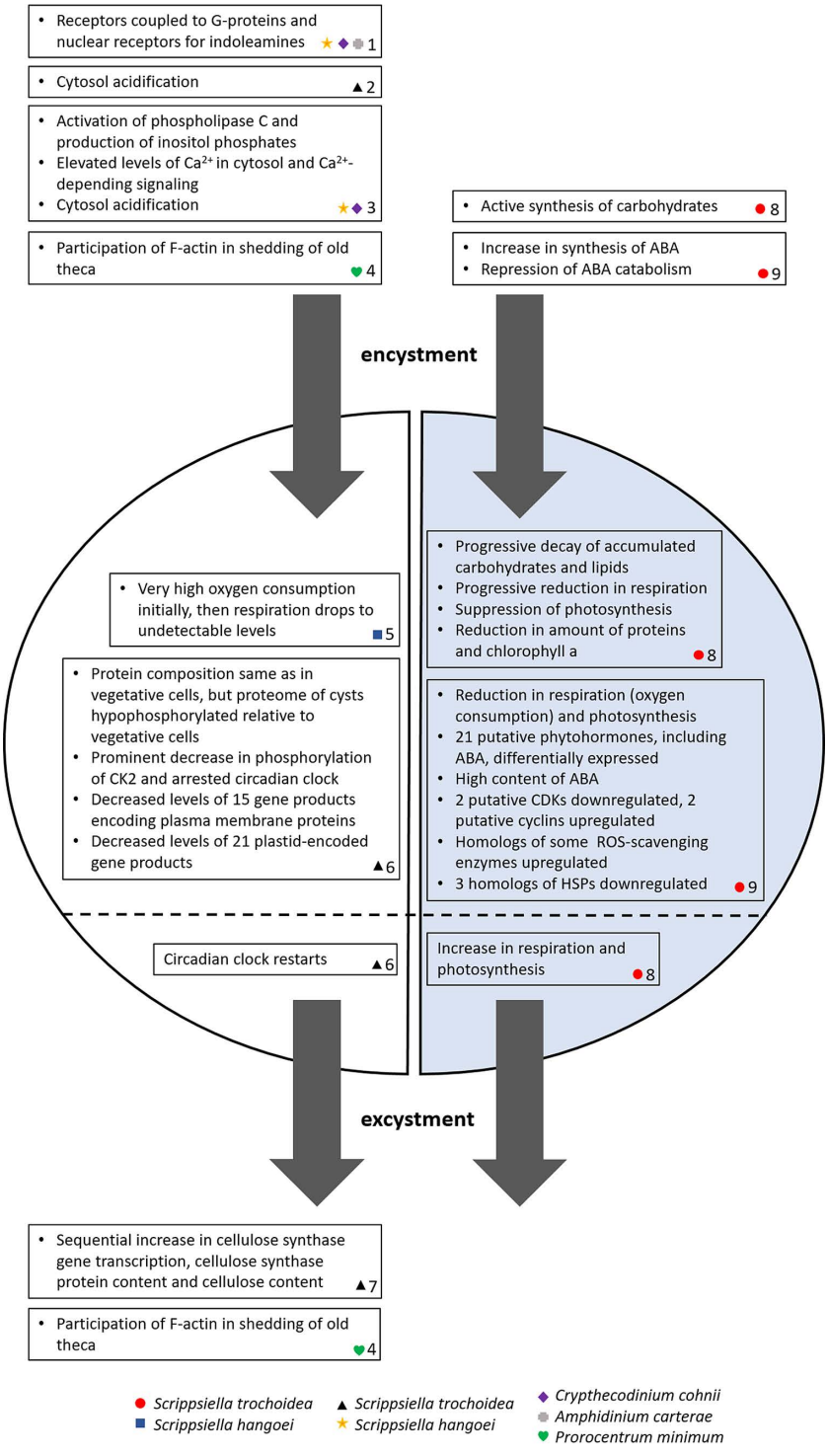

Fig. 1. A scheme summarizing current knowledge on the cellular aspects of cyst development in dinoflagellates. Left side (white) – temporary, or thin-walled, cysts, right side (blue) – resting, or thick-walled, cysts. Dashed line demarcates the events taking place shortly prior to excystment. Symbols in the lower right corner designate the investigated dinoflagellate species which are listed in the legend. Numbers in the lower right corner correspond to the following references: 1 – Tsim et al., 1996b; 2 – Hardeland et al., 1995; 3 – Tsim et al., 1997; 4 – Berdieva et al., 2018; 5 – Rintala et al., 2007; 6 – Roy et al., 2014; 7 – Chan et al., 2019; 8 – Binder and Anderson, 1990; 9 – Deng et al., 2017. *Abbreviations*: CK2 – Casein Kinase 2, ABA – abscisic acid, CDKs – cyclin-dependent kinases, ROS – reactive oxygen species, HSPs – heat shock proteins.

for the seed and bud dormancy in higher plants (Kermode, 2005; Nambara et al., 2010). The authors showed that ABA synthesis was increased during the transition state from dinoflagellate vegetative cells to cysts. Furthermore, ABA content remained high in mature resting cysts, which was achieved by both intensified biosynthesis and suppressed catabolism of ABA during cyst dormancy.

Taken together, these results suggest that resting cysts remain metabolically active during their dormancy and quiescence, although some processes, e.g. photosynthesis, are expectedly repressed. The demonstration of ABA functioning in dinoflagellate encystment is one of the rare studies of the role of this phytohormone in microalgae, on the one hand, and one of the few discoveries of the particular mechanisms involved in the resting cyst formation, on the other hand.

Temporary (thin-walled) cysts

In the early 1990s it was shown that not only shortening of the day length (light period), but also treatment by melatonin and other indoleamines, such as 5-methoxytryptamine, can induce the formation of temporary cysts in the dinoflagellate species *Lingulodinium polyedra* (formerly *Gonyaulax polyedra* and *L. polyedrum*) (Balzer and Hardeland, 1991, 1992), *S. trochoidea, Crypthecodinium cohnii, Gymnodinium simplex, Aureodinium pigmentosum* and *Alexandrium tamarense* (formerly *Gonyaulax tamarensis*) (Wong and Wong, 1994). Indoleamines are well-studied regulators of photoperiodism in vertebrates and other groups of living organisms (e.g. Reiter, 1980; Tamarkin et al., 1985; Wetterberg et al., 1987; Vivien-Roels and Pévet, 1993), and the discovery of their presence and effects in dinoflagellates reinforced the hypothesis about evolutionary conservative functions of these substances (Vivien-Roels and Pévet, 1986). Moreover, it provided new details regarding the physiology of dinoflagellates.

Further research proposed that indoleamine-induced encystment of dinoflagellates does not rely on the cAMP-dependent signaling pathways (Tsim et al., 1996a), involves proton release from acidic vacuoles resulting in cytoplasm acidification (Hardeland et al., 1995; Tsim et al., 1997) and can be mediated via membrane receptors coupled to G-proteins, as well as via nuclear receptors (Tsim et al., 1996b). Moreover, the accurate study demonstrated an important role of calcium ions and calcium-dependent signaling including activation of phospholipase C for the process of indoleamine-induced encystment (Tsim et al., 1997).

It is not yet clear whether the findings described above are also applicable to the formation of temporary cysts induced by factors other than photoperiodism and indoleamines. Nevertheless, a significant amount of information concerning encystment and dormancy of other temporary cyst types is already available. Rintala et al. (2007) demonstrated that darkness-induced temporary cysts of the cold-water dinoflagellate *Scrippsiella hangoei* exhibited very high oxygen consumption rate shortly after encystment. Subsequently, respiration rate measured in cysts dropped to an almost undetectable level in agreement with the data on respiration in the resting cysts of other dinoflagellate species (Binder and Anderson, 1990; Deng et al., 2017). Remarkably, in the study of Rintala et al. (2007) temporary cysts remained viable and enabled the population survival over the long periods of unfavorable conditions similar to the resting cysts.

Cold-induced temporary cysts of *L. polyedra* were thoroughly investigated by Roy et al. (2014). A comparison of cysts and vegetative cells showed that their proteomes did not differ significantly in terms of protein composition, but cyst proteomes were hypophosphorylated relative to the vegetative cell ones (it must be noted that about 90 proteins were hyperphosphorylated in cysts). This tendency was especially prominent in the case of Casein Kinase 2 (CK2), a protein kinase that conservatively participates in the regulation of eukaryotic circadian clocks (Sugano et al., 1999; Lin et al., 2002; Tsuchiya et al., 2009). In the investigated cysts, a circadian clock was arrested as was indicated by the absence of rhythmical bioluminescence and changes in the content of Luciferin Binding Protein. Interestingly, the experiments showed that circadian clock restored its activity at excystment at the time corresponding to the start of the dark phase. In addition, Roy et al. (2014) demonstrated that the levels of 132 RNAs were significantly lower in cysts than in vegetative cells of *L. polyedra*, 21 of which represented plastid-encoded gene products and 11 – sequences of nuclear-encoded membrane proteins.

Ecdysis and regeneration of amhiesma

A recent investigation showed a key role of filamentous actin (F-actin) for the process of ecdysis, in particular, for the shedding of thecal plates (Berdieva et al., 2018). Cells of the dinoflagellate *Prorocentrum minimum* (syn. *Prorocentrum cordatum*) treated with

the actin-depolymerizing agent latrunculin B lacked their normal cortical layer of F-actin, and the level of ecdysis in response to mechanical disturbance (centrifugation) was very low in the latrunculin B-treated culture as compared to the untreated control.

It must be noted that theca shedding is thought to precede the cyst formation in many dinoflagellates, which seems to be typical for species developing a pellicle (Bricheux et al., 1992; Kwok and Wong, 2003; Sekida et al., 2001, 2004). However, in *P. minimum* it is not yet clear at which stage theca shedding occurs. According to Pozdnyakov et al. (2014) and Berdieva et al. (2016), ecdysis starts with the shedding of the old plasma membrane and outer amphiesmal vesicle membranes immediately after the mechanical treatment, and then cells remain immotile for at least 2 h in their old thecal plates, while no pellicle development occurs. Theca shedding takes place later, and cells released from the old thecal plates acquire motility shortly after it. Thus, in *P. minimum*, theca shedding can be considered as an indicator of excystment rather than encystment. For these reasons, on Fig. 1 participation of F-actin in the shedding of the old theca is placed both at the 'encystment' and 'excystment' parts of the scheme. Possibly, the position of theca shedding on the time scale of cyst development is species specific, and this issue has to be clarified.

Another study was focused on the cyst-to-swarmer transition occurring shortly after theca shedding in the dinoflagellate *L. polyedra* (Chan et al., 2019). As in the study of Berdieva et al. (2018), temporary cysts were produced by means of centrifugation. Different stages of the new cellulose thecal plates synthesis that accompanied cyst-to-swarmer transition were investigated including transcription of the gene encoding cellulose synthase (an enzyme conducting cellulose synthesis at the plasma membrane), synthesis of cellulose synthase protein, synthesis of cellulose, as well as the formation of new cellulosic thecal plates. The authors found out that the increase in cellulose synthase transcript peaked in 4 h after release from a cyst (shedding of old theca) and remained high during the entire period of cellulose regeneration. The relative level of cellulose synthase protein increased 40% by 4 h after theca shedding, which preceded the increase in cellulose content. In 12 h most cells regained their cellulose. Thus, sequential activation of cellulose synthase transcription, synthesis and formation of thecal plates coordinated in time was observed. Knockdown of cellulose synthase (CesA1p) led to defects in newly formed thecal plates and postponement in successful transition into swarmer cells. Addition of 2,6-dichlorobenzonitrile (DCB), a chemical inhibitor of cellulose synthesis, suppressed regeneration of thecal plates in agreement with the observations made in the previous studies (Kwok and Wong, 2003; Pozdnyakov et al., 2014). Remarkably, Pozdnyakov et al. (2014) demonstrated that DCB-treatment itself induced ecdysis in the dinoflagellate *P. minimum*.

Still many questions to answer

There is a need to not only expand the so far obtained data described above but also find a common ground for the processes involved in the development of dinoflagellate cysts of both types. Relation between the appearance of resting and temporary cysts in the evolutionary history of dinoflagellates intuitively looks reasonable. Therefore, the mechanisms involved in cyst development and maintenance should have many similarities along with the discrepancies resulting in the two different cyst types. Already now it is known that resting and temporary cysts share some traits, i.e. pronounced reorganization of a cell covering, reduction of photosynthesis and respiration at the cyst stage, initiation of encystment triggered by external stressors.

Moreover, it is necessary to figure out to what extent cysts induced by various factors share the same developmental mechanisms. For example, the cellular mechanisms behind the indoleamine-induced encystment have been relatively well studied (Hardeland et al., 1995; Tsim et al., 1996a, 1996b, 1997), but are they also fair for the cold-induced or mechanically induced encystment? Although starting at different receptors, signaling pathways have to converge at some point to execute the program of the cyst formation.

Changes in a cell covering are one of the main events during encystment and excystment of dinoflagellates and hence should receive particular attention. Such changes are often accomplished through ecdysis, a dramatic process comprising, inter alia, the rapid replacement of the plasma membrane. We still know very little about the cellular features of ecdysis. What are the molecular mechanisms behind the shedding of several layers of a cell covering including the old plasma

membrane? How the reconstruction of the entire cell covering occurs? What signaling pathways are involved in ecdysis initiation and progression? Unraveling the mechanisms of ecdysis is not only pivotal to understand cyst development, but also has a fundamental relevance in terms of cell biology of eukaryotes due to the uniqueness of this process. Moreover, physiology of dinoflagellates is constrained by their complex cell covering in many respects, e.g. nutrition (Kalinina et al., 2018) and probably routine plasma membrane renovation. Therefore, ecdysis may represent an effective mechanism to modulate the molecular composition of the dinoflagellate plasma membrane in order to adequately respond to the environmental challenges, a hypothesis that requires thorough experimental testing.

Hopefully, the raised questions will be answered in the coming years. Meanwhile, the already obtained information pieces of the future mosaic should be very helpful on the way of broadening this realm of knowledge.

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