

## Triton-Stimulated Nucleoside Diphosphatase Activity: Subcellular Localization in Corn Root Homogenates<sup>1</sup>

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### Summary

The distribution of latent UDPase activity (cold storage-activated) is similar to Triton-stimulated UDPase activity in membrane fractions separated by differential centrifugation as well as fractions purified by linear sucrose density centrifugation. The Triton-stimulated UDPase activity appears to be a specific marker for Golgi membranes in corn root homogenates. Detergent-activated UDPase activity provides a more reliable, less cumbersome way to monitor Golgi membranes compared to cold storage-activation and this marker can be used on fresh preparations.

**Keywords:** Golgi; Latency; Membranes; Nucleoside Diphosphatase; Triton X-100.

### 1. Introduction

Latent nucleoside diphosphatase (NDPase) activity is often used to monitor Golgi membranes in plant cell homogenates [24]. According to MORRÉ *et al.* [19], the latent activity is unique to the Golgi apparatus; however, there are several drawbacks when using this enzyme as a marker for Golgi membranes. The latent enzyme is typically activated by storing isolated membranes at 0–4 °C for approximately 4 days [13, 19, 25] and hence it is a cumbersome assay. Additionally, the kinetics of cold storage activation of the latent

enzyme are variable since enzyme activity may appear early [19] or only after longer periods of cold storage [13, 15]. In two reports, the latent enzyme could not be activated even after 8 days of storage [10, 21].

Current evidence indicates that membrane-bound latent enzymes of plants [10, 18] are often activated by detergent treatment. Several reports indicate that a latent NDPase is activated by Triton X-100 [1, 8, 20, 25]. In one report [8], the subcellular location of the detergent-activated NDPase was not known since assays were performed on a crude microsomal fraction (8,500 to 33,000 × g pellet). In other reports [1, 25], detergent activation was only performed on isolated Golgi fractions and it was not ascertained whether other subcellular membranes also contained a detergent activated NDPase. This paper shows Triton X-100-stimulated NDPase activity can be used in place of cold storage-activated NDPase activity to “mark” Golgi membranes in corn root homogenates.

### 2. Materials and Methods

#### 2.1. Plant Material

Corn seeds (*Zea mays* L. WF9Ht × Mol7Ht) were germinated in the dark for 4 days at 26 °C [14]. Primary roots (6–8 cm in length) were excised, washed twice in cold deionized distilled water and homogenized in a prechilled mortar and pestle. The homogenization medium contained 30 mM Tris-MES pH 7.7, 5 mM EDTA, 5 mM dithiothreitol (DTT) or β-mercaptoethanol in 0.5 M sucrose. Homogenization and all centrifugations were carried out between 0–4 °C. The homogenate was filtered through cheesecloth and centrifuged at 1,000 × g for 5 minutes.

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## 2.2. Centrifugation Procedures

Subcellular membranes and organelles of the 1,000 × g supernatant were initially isolated by differential centrifugation as indicated in the text. Further separation of subcellular components was achieved by equilibrium density centrifugation in linear sucrose gradients (20-50% sucrose, W/W; 3 mM Tris-MES pH 7.2; ± 1 mM DTT) at 80,000 × g for 15 hours (Beckman L5-B ultracentrifuge with SW28 rotor).

## 2.3. Enzyme Assays

K<sup>+</sup>-stimulated Mg<sup>2+</sup>-ATPase activity was measured as described by LEONARD and VAN DER WOUDE [15]. Latent NDPase activity was detected after cold storage (0-4 °C) of isolated membranes for 5 to 7 days and best activation occurred when DTT was excluded from the homogenization medium. Detergent activated NDPase was detected by the addition of Triton X-100 (0.03% final concentration) to the normal assay medium which contained 3 mM UDP, 3 mM MnSO<sub>4</sub> (except where indicated) and 30 mM Tris-MES pH 6.5 [22]. Since Triton X-100 interferes with the Fiske and Subba Row [4] procedure for determining inorganic phosphate [3, 23], it was unclear how previous workers resolved this problem [1, 8] when studying Triton-activated NDPase activity. Triton interference was eliminated by the addition of sodium dodecyl sulfate (SDS) to the Fiske and Subba Row reducing agent [3, 23]. With a 1 ml assay volume containing 0.03% Triton-X-100, we used 2.4 ml of the Fiske and Subba Row reagent which contained 1.5% SDS.

Cold storage-activated UDPase was determined by subtracting the activity at day 0 from that measured after cold storage. Triton-stimulated (or activated) UDPase was determined by subtracting the activity in the absence of detergent from that detected in the presence of detergent. All assays were performed at 37 °C for 15 to 20 minutes with 20-30 μg of membrane protein [22]. All nucleoside diphosphates and triphosphates were treated with Dowex 50X proton exchange

resin to remove sodium. Tris salts of phosphorylated substrates were prepared as described [7].

NADH cyt *c* reductase (± antimycin A) and cyt *c* oxidase activities were assayed as described previously [7, 16]. Protein was estimated according to LOWRY *et al.* [17].

## 3. Results and Discussion

### 3.1. Differential Centrifugation

In an effort to determine if the cold storage-activated NDPase activity and Triton-stimulated NDPase were distributed the same during subcellular fractionation, a series of differential centrifugations were performed. The data in Table 1 indicated the distribution of cold storage-activated UDPase and the Triton-stimulated UDPase were similar. However, we did not obtain the same results as those reported for crude homogenates of onion root tip [9] where detergent-activation and cold storage-activation produced that same level of enzyme activity. In experiments with onion roots, isolated membrane fractions were preincubated in detergent before enzyme assay [9] compared to our procedure of adding detergent to the enzyme assay. Both the highest specific activity and highest percent total activity for latent UDPase were observed in the 13,000 to 80,000 × g fraction (Table 1) although fractions collected between 1,000 to 13,000 × g contained between 30-40% of the total activity. This is not in agreement with a previous report which did not detect

Table 1. The distribution of cold storage-activated and Triton X-100-stimulated UDPase activity in fractions separated from corn root homogenates by differential centrifugation. Triton-stimulated activity was determined by subtracting the activity in the absence of detergent from that measured in its presence at Day 0. Cold storage-activated UDPase activity was determined by subtracting the activity at Day 0 (- T) from that measured at Day 7 (- T). See Materials and Methods for assay conditions

Fraction	Day 0		Day 7		Triton-stimulated			Cold Storage-Activated		
	SA <sup>1</sup>		SA		UDPase Activity			UDPase Activity		
	- T <sup>2</sup>	+ T	- T	SA	TA <sup>3</sup>	%TA <sup>4</sup>	SA	TA	%TA	
0-1,000 × g × 5 min	3.49	5.18	3.37	1.69	2.52	1.5	0	0	0	
1,000-6,000 × g × 20 min	3.10	7.69	6.28	4.59	27.75	16.5	3.18	19.23	15.7	
6,000-13,000 × g × 15 min	4.58	15.49	10.53	10.91	32.90	19.6	5.95	17.94	14.6	
13,000-80,000 × g × 35 min	7.54	23.98	17.41	16.44	94.20	56.1	9.87	79.47	64.8	
80,000-120,000 × g × 35 min	5.27	13.55	10.03	8.28	10.60	6.3	4.76	6.09	4.9	
120,000 × g Supernatant	88.76	86.90	83.00	0	0	0	0	0	0	

<sup>1</sup> SA = Specific Activity (μmoles/mg protein · hr).

<sup>2</sup> T = ± 0.03% Triton X-100.

<sup>3</sup> TA = Total Activity (μmoles/fraction · hr).

<sup>4</sup> %TA = Percent Total Activity.

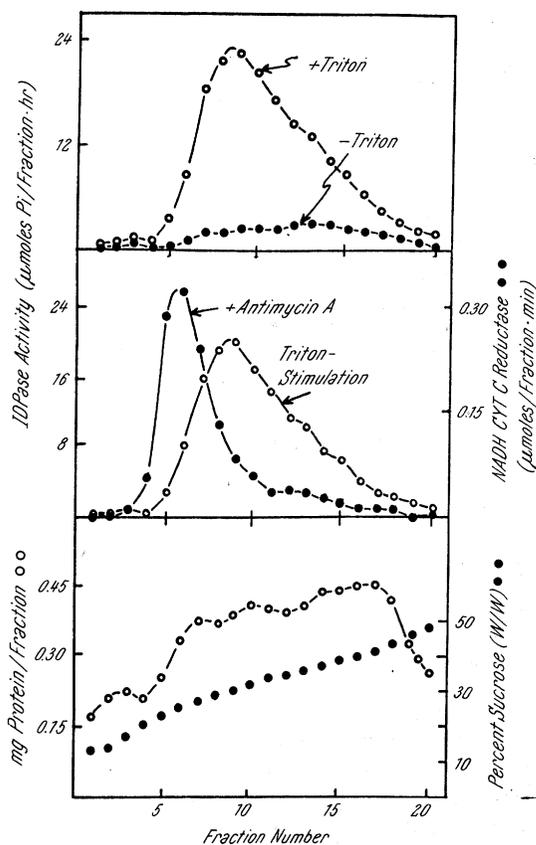


Fig. 1. Distribution of Triton-stimulated IDPase activity in a linear sucrose gradient overlaid with a 13,000 to 80,000  $\times$  g pellet isolated from a corn root homogenate. IDPase activity was determined at pH 7.5 in the presence of 50 mM KCl, 3 mM MgSO<sub>4</sub>  $\pm$  0.03% Triton X-100. Antimycin A insensitive NADH Cyt *c* reductase activity is a marker for endoplasmic reticulum

latent NDPase activity in the 1,000  $\times$  g to 13,000  $\times$  g fraction isolated from oat roots [13].

Since the 120,000  $\times$  g supernatant contained no latent UDPase activity and the 1,000  $\times$  g and 120,000  $\times$  g pellets only contained a small percentage of the total activity (Table 1), only fractions collected between 1,000  $\times$  g to 80,000  $\times$  g were used for further analyses. To determine the subcellular location of the Triton-stimulated UDPase in comparison to the cold storage-activated UDPase, isopycnic sucrose density centrifugation was performed.

### 3.2. Linear Sucrose Density Gradient Centrifugation of the 13,000 to 80,000 $\times$ g Fraction

All initial experiments including assays of fractions obtained by differential centrifugation were performed with 3 mM IDP (inosine 5'-diphosphate) and 3 mM

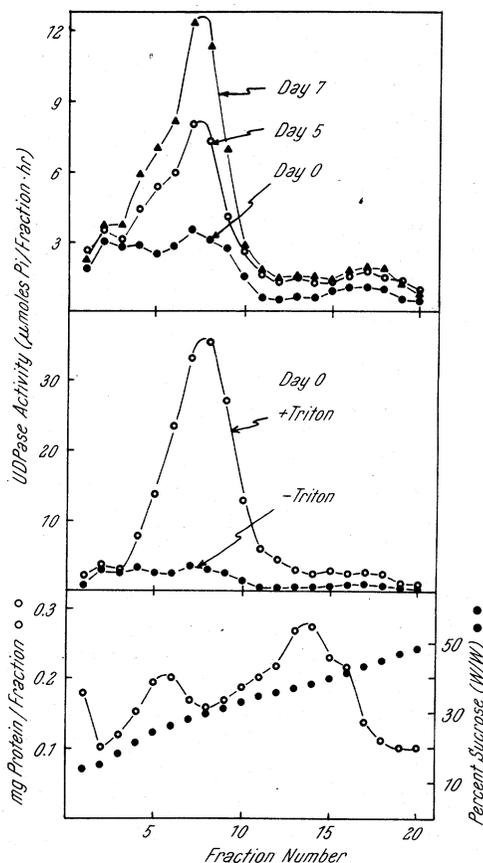


Fig. 2. Distribution of UDPase activity in a linear sucrose density gradient overlaid with a 13,000 to 80,000  $\times$  g pellet isolated from a corn root homogenate. UDPase activity was determined in the presence and absence of Triton X-100 at day 0 and was also determined in the absence of detergent at day 5 and day 7

MgSO<sub>4</sub> at pH 7.5 in the presence of 50 mM KCl (data not shown). Because an earlier report with corn root homogenates [15] used these assay conditions for detecting Golgi associated latent IDPase activity (cold storage-activated) in sucrose gradients overlaid with a 13,000 to 80,000  $\times$  g pellet, we assayed under the identical conditions in the presence and absence of Triton X-100. The distribution of IDPase activity in the presence of detergent (Fig. 1) was virtually identical to the distribution of the cold storage-activated IDPase reported by LEONARD and VAN DER WOUDE [15]. A major peak of activity was observed at 1.12 g/ccm although a broad shoulder of activity was apparent between 32-37% sucrose (compare Fig. 1 with Fig. 5 of reference 15). When the NDPase was assayed with UDP and MnSO<sub>4</sub> at pH 6.5 in the presence of Triton (optimal conditions as determined by reference 22), only a single sharp peak of activity was observed at a

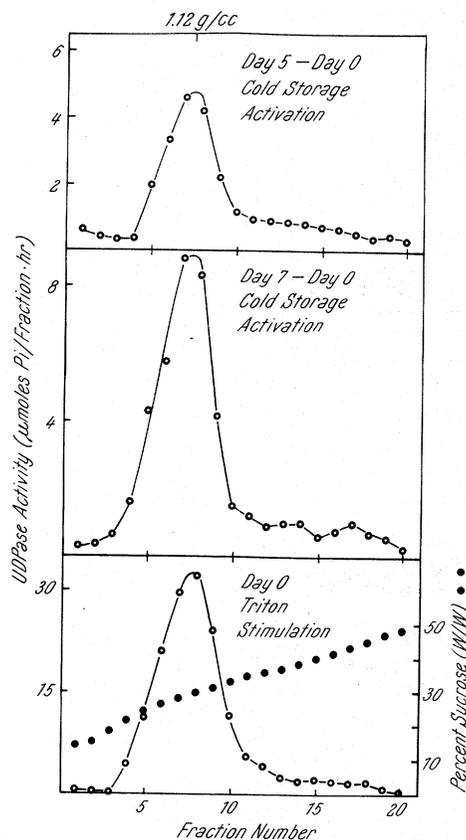


Fig. 3. Distribution of cold storage activated UDPase activity and Triton-stimulated UDPase activity in a linear sucrose gradient overlaid with a 13,000 to 80,000  $\times$  g pellet isolated from a corn root homogenate. Data from Fig. 2 is presented by subtracting the activity measured at day 0 from the activity measured at day 5 and day 7. Triton-stimulated UDPase activity (day 0) is determined by subtracting the activity detected in the absence of Triton X-100 (0.03%) from that measured in its presence

density of 1.12 g/ccm (Fig. 2). It was unclear why the distribution of UDPase (optimal conditions) and IDPase were not completely similar although the results in Fig. 1 and 2 were not obtained from the same gradient (protein distribution and levels of protein were not identical in the gradients).

The distribution of UDPase activity at day 0, day 5, and day 7 was compared to the UDPase activity measured in the presence and absence of detergent at day 0 (Fig. 2). When the actual latent increment of activity [19, 24] was compared to the Triton-stimulated activity (Fig. 3) only single sharp coincident peaks were observed (1.12 g/ccm). The higher levels of Triton-stimulated UDPase activity compared to the cold storage-activated UDPase activity (Fig. 3) was consistent with a recent report on the detergent activation of membrane associated NDPase [20]. These

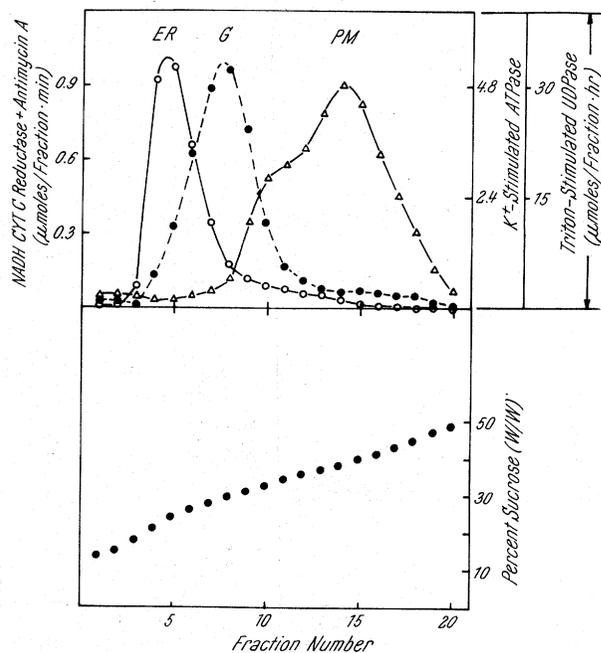


Fig. 4. Distribution of Triton-stimulated UDPase activity in a linear sucrose gradient overlaid with a 13,000 to 80,000  $\times$  g pellet isolated from a corn root homogenate. Distribution of the Golgi associated enzyme (Triton-stimulated UDPase  $\bullet-\bullet-\bullet$ ) to the distribution of the plasma membrane ( $K^+$ -stimulated ATPase  $\Delta-\Delta-\Delta$ ) and endoplasmic reticulum (NADH cytochrome *c* reductase  $\circ-\circ-\circ$ ) markers

workers found much higher detergent-stimulated activity in relation to the cold storage activation [20]. The density of ER (1.10 g/ccm) and PM (1.165 g/ccm) in a linear sucrose gradient (Fig. 4) confirmed an earlier report for corn root membrane fractions [15]. Antimycin A insensitive NADH cytochrome *c* reductase activity can be used as a reliable marker for ER [15, 24 and references therein]. The  $K^+$ -stimulated  $Mg^{2+}$ -ATPase activity assayed at pH 6.5 appears to be a reliable marker for PM in corn root subcellular fractions [15]. This was demonstrated by the excellent correlation between enzyme activity and percent plasma membrane as judged by the PM stain [15]. The detergent-activated UDPase was coincident with the cold storage-activated UDPase activity (Fig. 3) and was not coincident with the ER or PM markers (Fig. 4). The detergent-activated enzyme was specific for membranes with a density of 1.12 g/ccm and these membranes are believed to be Golgi membranes [15, 24 and references therein]. The distribution of tonoplast in sucrose gradients was not determined due to the lack of a reliable marker however the reported density of the vacuole membrane was less than the density of Golgi membranes [24, 26].

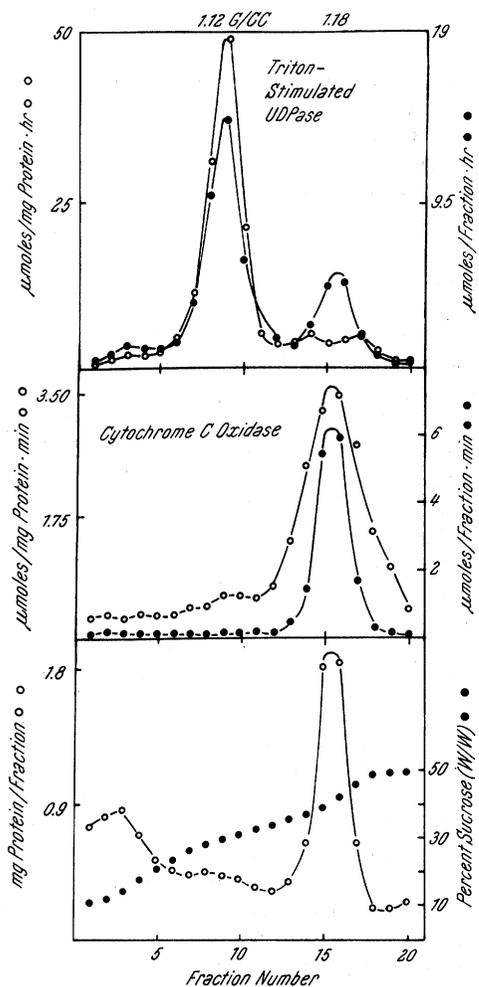


Fig. 5. Distribution of Triton-stimulated UDPase activity in a linear sucrose gradient overlaid with a crude mitochondrial fraction (1,000 to 13,000  $\times$  g pellet) isolated from a corn root homogenate

The specific localization of latent UDPase activity with Golgi membranes (Fig. 4) was consistent with an earlier report cytochemically localizing NDPase activity with the Golgi apparatus of corn roots [2]. This report [2]

and data in Figs. 3 and 4 are contrary to a recent investigation [20 and references therein] where a detergent activated NDPase was reported in association with ER and PM isolated from corn coleoptiles. These investigators could not rule out the possibility of Golgi contamination in these purified fractions [5, 6] and have acknowledged the fact that they could not specifically locate Golgi membranes in sucrose gradients [6].

### 3.3. Linear sucrose density centrifugation of the 1,000 to 13,000 $\times$ g fraction

Results of differential centrifugation indicated that 30-40% of the total pelleted UDPase activity sedimented between 1,000 to 13,000  $\times$  g (Table 1). This suggested that either 40% of the Golgi membranes sedimented or the Triton-stimulated UDPase was also associated with other organelles such as mitochondria. When the membranes in the 1,000 to 13,000  $\times$  g pellet were further separated in a sucrose gradient (Fig. 5), a major peak of detergent activated UDPase ( $\mu$ moles/fraction  $\cdot$  hr) was observed at a density (1.12 g/ccm) identical to that of Golgi membranes (compare Figs. 3 and 5).

A second smaller peak was observed at a density of 1.18 g/ccm (Fig. 5) coincident with the mitochondrial marker (cyt *c* oxidase). This second peak was noticeable if total activity was plotted ( $\mu$ moles/fraction  $\cdot$  hr); however, when specific activity ( $\mu$ moles/mg protein  $\cdot$  hr) was plotted, only the Golgi membranes exhibited high activity (Fig. 5). This suggested that the Triton-stimulated activity associated with mitochondria may not be due to a UDPase but perhaps another phosphatase. Fractions 15 and 16, consisting of purified mitochondria (Fig. 5), were pooled and the substrate specificity of the mitochondrial associated phosphatase was determined at pH 6.5 in the presence of  $MnSO_4$ . Table 2 clearly shows that under these

Table 2. Substrate specificity of a Triton-stimulated phosphatase associated with mitochondria isolated from corn root homogenates. Mitochondria were purified by centrifuging a 1,000 to 13,000  $\times$  g pellet in a linear sucrose density gradient. See text for details

Substrate	pH 6.5 + $MnSO_4$ ( $\mu$ moles Pi/Mg protein $\cdot$ hr)		
	- Triton	+ Triton	Triton-stimulation
ATP	9.63	16.87	7.24
UDP	0.86	3.91	3.05
GDP	0.62	3.66	3.04
IDP	0.25	2.55	2.30
CDP	0.25	1.03	0.78
ADP	0.62	1.03	0.41

assay conditions, the preferred substrate is ATP (ATP >>> UDP = GDP > IDP > CDP > ADP). The Triton-stimulated phosphatase activity associated with mitochondria is probably due to the mitochondrial ATPase. In contrast, the Golgi associated Triton-stimulated NDPase prefers UDP over other nucleoside diphosphates and does not hydrolyze ATP to any substantial degree [22].

#### 4. Conclusions

The results of this study indicated that detergent treatment of isolated membranes activated a UDPase. The triton-stimulated enzyme was associated with Golgi membranes and provides a reliable marker for this organelle, at least in corn root homogenates. Triton-stimulated UDPase activity provides a less cumbersome and more reliable way to monitor Golgi membranes, compared to cold storage activation, and can be used on fresh preparations. To use this enzyme as a marker for Golgi membranes all appropriate controls must be performed since the possibility of more than one subcellular site for other plant tissue has been suggested [20].

KUHN *et al.* [12] suggested that the UDPase latency was a result of the enzyme's association with the luminal side of isolated Golgi vesicles. Detergents would in effect make the membranes leaky and UDPase activity in the absence of detergent reflects the presence of damaged or leaky vesicles in membrane preparations [20]. Studies with microsomal vesicles isolated from rat liver have shown that concentrations of Triton X-100 up to 0.08% (1.3 mM) can produce leaky vesicles without producing membrane disassembly [11]. The higher Triton-stimulated activity we observed compared to cold-storage activity (Fig. 3) can be explained by a more efficient creation of leaky vesicles by 0.03% Triton treatment compared to cold storage treatment. This assumes that both types of treatment activated the same enzyme.

The cold storage-activated NDPase and Triton-stimulated NDPase behave similarly during differential centrifugation and have an identical density in sucrose gradients. These results are consistent with but do not concretely show that the detergent treatment and cold storage treatment activate the same latent enzyme. We cannot rule out the possibility that the cold storage activated enzyme activity and detergent activated enzyme activity represent two different enzymes. The presence of several isozymes of NDPase have been reported [9] and it is conceivable that more than one is associated with Golgi membranes.

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