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Transport mechanisms of nucleosides and the derivative, 6-mercaptopurine riboside across rat intestinal brush-border membranes

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Abstract

Na^+-driven nucleoside transport processes across rat intestinal brush-border membrane vesicles were investigated. 6-Mercaptopurine riboside (6-MPR), an analogue of purine-nucleoside such as adenosine and inosine, was recognized by its purine- and pyrimidine-nucleosides transport system, but their nucleo-bases did not entirely inhibit the 6-MPR transport. The analysis according to the Hill equation of the curve for Na^+ activation of 6-MPR uptake was consistent with the notion of a Na^+/6-MPR coupling stoichiometry of 1:1. The expressed transport activities of adenosine, uridine, and 6-MPR were Na^+-dependent and saturable, and their affinity constants (K_m value) obtained by Eadie-Hofstee analysis were approx. 20, 15 and 100 μM. Moreover, the uptake of radiolabeled adenosine and uridine was trans-stimulated by 6-MPR inside vesicles in the absence of an inwardly directed Na^+-gradient. On the other hand, uridine did not exhibit any inhibitory effects on the uptake of adenosine despite the fact that adenosine was a potent inhibitor for uridine uptake by intestinal brush-border membrane vesicles. These differences in the inhibition may be explained by the multiplicity of the nucleoside transport systems.

Keywords: Nucleoside transport; 6-Mercaptopurine riboside; Sodium ion dependence; Brush-border membrane; Small intestine; (Rat)

1. Introduction

It is widely accepted that carrier-mediated transport systems participate in the uptake of nucleosides into many types of mammalian cells. Na^+-dependent nucleoside transport systems have been demonstrated in jejunum [1,2], isolated enterocytes [3,4], membrane vesicles from intestinal and renal epithelia [5–9], and in other tissue cells [10–12]. On the basis of substrate selectivity, two principal Na^+-dependent nucleoside transport systems have been recognized. One system, N1, is generally purine-specific, the other, N2, is pyrimidine-specific. Adenosine and uridine are reported to be substrates for both transporters. Furthermore, Huang et al. [13] reported that another broad specificity system (N3), which was distinguished from N1 and N2 Na^+-linked nucleoside transporters, has been detected in the oocytes of *Xenopus laevis* following injection with rat intestinal mRNA.

On the other hand, 6-mercaptopurine riboside (6-MPR) is an analogue of purine-nucleosides such as adenosine and inosine (Fig. 1), and its chief use is in continuation therapy for acute lymphoblastic leukaemia. This drug is almost exclusively given by the oral route because of its fine absorbability. It is likely that 6-MPR is absorbed via the nucleoside-transport carrier system from the gastrointes-

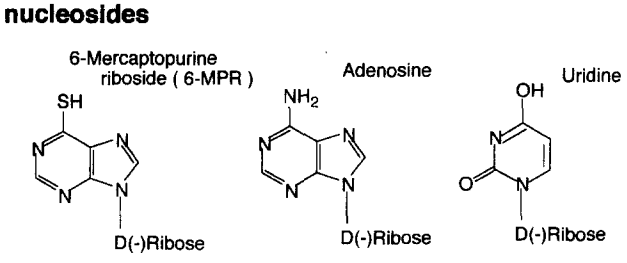

Fig. 1. Chemical structures of nucleosides and their related compounds.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; 6-MPR, 6-mercaptopurine riboside.

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tinal tract. However, due to the fact that transports of nucleosides across animal cell membranes have been reported to be mediated by a number of different pathways, few details are available on the carriers responsible for the transport of 6-MPR and pyrimidine nucleosides in the intestine. The present study was undertaken to characterize the Na^+-dependent nucleoside transport in brush-border membrane vesicles prepared from rat small intestinal epitheliums, and to estimate the physiological and pharmacological significance of this transport system using 6-MPR.

2. Materials and methods

2.1. Chemicals

[2,8-^{3}H]Adenosine (1.3 TBq/mmol) and [5,6-^{3}H]uridine (1.7 TBq/mmol) were purchased from Amersham (Buckinghamshire, UK). 6-MPR, 1H-purine riboside, and 1H-purine were obtained from Sigma Chemicals (St. Louis, MO). The following chemicals were from Wako Pure Chemicals: 6-mercaptopurine, adenine, inosine, hypoxanthine, guanosine, and D(−)ribose. All other chemicals were of the highest grade available.

2.2. Preparation of brush-border membrane vesicles

Brush-border membrane vesicles were prepared from rat small intestine (Wistar, male; 190–230 g) by the $CaCl_2$ precipitation method [14], as described previously [15,16]. The purified vesicles were suspended in an ice-cold medium containing 100 mM KCl, 100 mM D-mannitol, and either 20 mM Mes-Tris (pH 5.5) or Hepes-Tris (pH 7.5). They were used on the same day of preparation. Protein was assayed according to Lowry et al. [17] with bovine serum albumin as a standard.

2.3. Transport studies

Uptake experiments were performed at 25°C by a rapid filtration technique with the use of membrane filters (type HAWP, 0.45 μm pore size, Millipore Corp.), as described previously [15,16]. Uptake was initiated by mixing a 20 μl aliquots of membrane vesicles (160–240 μg protein) with 100 μl of uptake buffer containing the substrates with/without inhibitors. The uptake buffer in most experiments was 100 mM NaCl, 100 mM D-mannitol, and either 20 mM Mes-Tris (pH 5.5) or 20 mM Hepes-Tris (pH 7.5). The uptake was terminated by adding 4 ml of the ice-cold stop buffer, which consisted of 150 mM NaCl buffered with either 20 mM Mes-Tris (pH 5.5) or 20 mM Hepes-Tris (pH 7.5). The mixture was then filtered rapidly. The filter which captured the membrane vesicles was washed quickly with 8 ml of ice-cold stop buffer and was transferred to a counting vial.

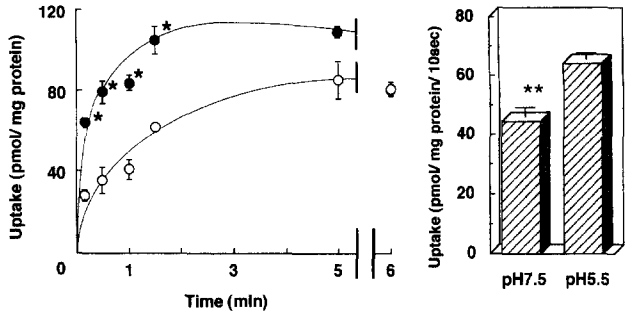

Fig. 2. Effect of an inward Na^+-gradient on the uptake of 6-MPR (100 μM). (Left panel) Time-course of 6-MPR uptake by rat intestinal brush-border membrane vesicles at pH 5.5. 6-MPR uptake was initiated by adding vesicles to media containing (final concentrations) 100 μM 6-MPR, 20 mM Mes-Tris (pH 5.5), 100 mM D-mannitol, and either 100 mM NaCl (●) or 100 mM KCl (○). Each point represents the mean with S.E. of 5–6 determinations: * $P < 0.01$. (Right panel) Uptake of 6-MPR at pH 7.5 (20 mM Hepes-Tris) compared to pH 5.5 (20 mM Mes-Tris) in the presence of Na^+-gradient: ** $P < 0.001$.

2.4. Analytical procedure

For assays of [^{3}H]adenosine and [^{3}H]uridine uptake, the radioactivity retained on the filter was determined using a liquid scintillation counter. Uptake was expressed relative to membrane protein. As a blank, a membrane vesicle-free incubation medium was handled in an identical manner. For the 6-MPR measurement, the drug trapped on the filter was extracted with 300 μl of 50 mM $Na_2B_4O_7$(pH 10) to avoid nonspecific adsorption of 6-MPR to the filter. The concentration of the extracted 6-MPR was determined by HPLC equipped with a UV detector at 320 nm. Separation was achieved on a reverse phase column (Nucleosil 100-C6H5,5 μm, 4.6 i.d. ×250 mm) using a mobile phase consisting of methanol/0.02 M KH_2PO_4 (9:1, v/v) at a flow rate of 0.7 ml/min.

3. Results

3.1. Na^+-dependence of 6-MPR uptake by the brush-border membrane vesicles and the saturation kinetics

The effect of an inwardly directed Na^+-gradient on the uptake of 6-MPR was studied in rat intestinal brush-border membrane vesicles (Fig. 2). The uptake of 6-MPR (100 μM) in the presence of an inward Na^+-gradient was significantly greater than that under the K^+ medium condition ($[K^+]in = [K^+]out$). Furthermore, as shown in Fig. 2 (right panel), the acidic condition of medium (pH 5.5) gave more stimulative uptake by Na^+-gradient compared as the neutral medium pH (7.5). These results demonstrated the high sodium specificity of the uptake of the nucleoside analogue by brush-border membrane vesicles at pH 5.5. Therefore, the further experiments were investigated under the acidic condition (pH 5.5).

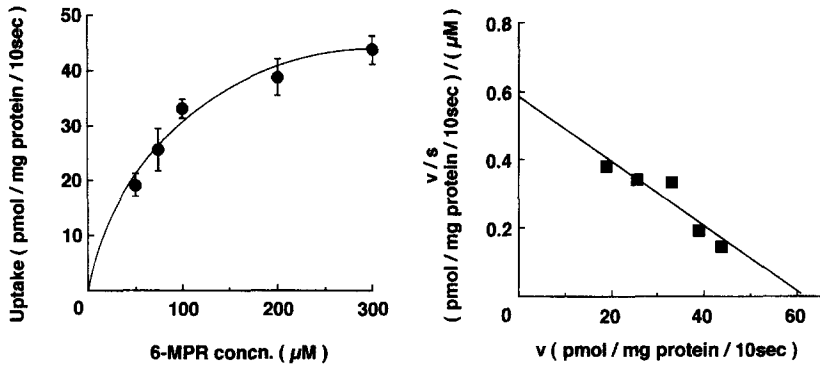

Fig. 3. Saturability (●) and Eadie-Hofstee plot (■) of the Na^+-dependent uptake of 6-MPR by rat intestinal brush-border membrane vesicles. In order to determine the Na^+-dependent uptake, the uptake of 6-MPR measured in the absence of an Na^+-gradient was subtracted from the respective total uptakes which were measured in the presence of an inwardly directed Na^+-gradient. Each point represents the mean with S.E. of three determinations. In the right figure, a linear regression program has been used to fit the transformed data. The intercepts of the line with x- and y-axes are V_{max} ($V_{max} = 61.04 \pm 15.41$ pmol/mg protein/10 s) and V_{max}/K_m ($K_m = 103.70 \pm 16.94$ μM).

In order to compare the transport kinetics of 6-MPR and other nucleosides, furthermore, the concentration dependence of adenosine and uridine uptakes in the presence of a Na^+-gradient was studied at pH 5.5. Both Na^+-dependent nucleoside uptakes exhibited saturability on increasing the concentration of the substrates. The resulting linearity of the Eadie-Hofstee plots (initial Na^+-dependent uptake/substrate concentration vs. substrate concentration) indicates that the data conform to a transport system with a single component over the concentration range studied. For three or four separate experiments, least-squares analysis of the Eadie-Hofstee plot yielded apparent K_m values (mean ± S.E.) of 21.52 ± 1.90 μM for adenosine and 15.72 ± 1.41 μM for uridine with V_{max} values (mean ± S.E.) of 30.17 ± 4.12 pmol/mg protein per 10 s for adenosine and 69.65 ± 9.15 pmol/mg protein per 10 s for uridine.

3.2. Saturability of 6-MPR uptake by the brush-border membrane vesicles

The concentration dependence of the Na^+-dependent uptake of 6-MPR is shown in Fig. 3. The Na^+-dependent component, which was obtained by subtracting the 6-MPR uptake rate in the absence of Na^+-gradient from that in the presence of Na^+-gradient, exhibited distinct saturation kinetics (Fig. 3, left). In addition, an Eadie-Hofstee transformation of the corrected data resulted in values (mean ± S.E.) for K_m and V_{max} of 103.70 ± 16.94 μM and 61.04 ± 15.41 pmol/mg protein per 10 s, respectively (Fig. 3, right).

3.3. Effect of sodium concentration on 6-MPR transport

The sodium dependence of 6-MPR transport was explored further by measuring the 10-s uptake rate of 6-MPR

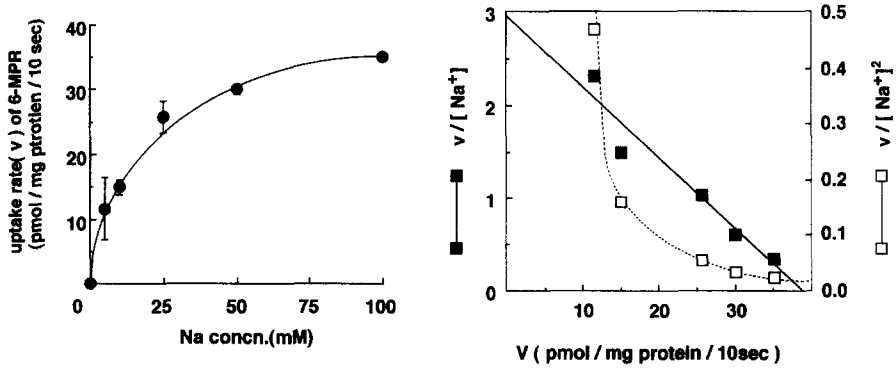

Fig. 4. Effect of extravesicular Na^+ concentration of 6-MPR (100 μM) uptake by rat intestinal brush-border membrane vesicles (●, left panel) and replot of data for Na^+ dependent 6-MPR uptake (■, □, right panel) according to Hill equation. (Left panel) The membrane vesicles were suspended in 0–150 mM NaCl, 150–0 mM KCl, and 20 mM Mes-Tris, pH 5.5. The osmolarity was maintained constant by a variant of the KCl concentration. The Na^+-dependent uptake components were obtained by subtracting the uptake values in the absence of an Na^+-gradient from the respective total uptakes which were measured in the presence of an inwardly directed Na^+-gradient. Each point represents the mean with S.E. of 3–6 determinations. (Right panel) Plots of uptake rate $(v)/[Na^+]^n$ vs. uptake rate (v) for $n = 1$ (■), and 2 (□). Linearity of plot for $n = 1$ is indicative of involvement of ~ 1 Na^+ per 6-MPR transport event.

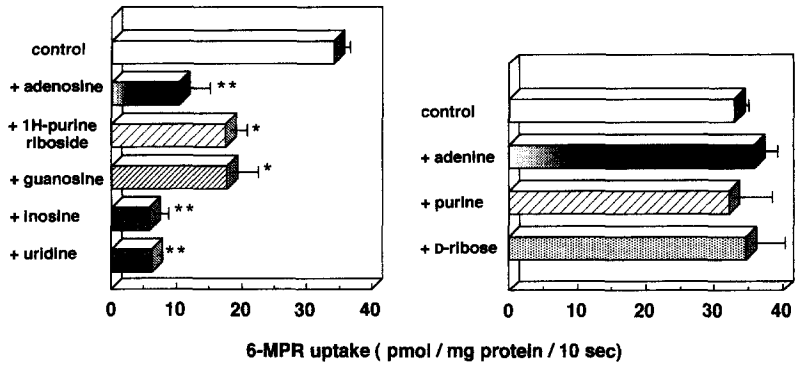

Fig. 5. Inhibitory effects of nucleosides (left), nucleo-bases and D(−)ribose (right) on the Na^+-driven uptake (10 s) of 6-MPR by intestinal brush-border membrane vesicles. The uptake study was performed in the buffer containing 100 μM 6-MPR with/without 1.0 mM inhibitors. To clarify the effects of inhibitors on the Na^+-dependent 6-MPR uptake, the uptake values of 6-MPR measured in the absence of an Na^+-gradient was subtracted from the respective total uptakes which were measured in the presence of an inwardly directed Na^+-gradient. Each column represents the mean with S.E. of 5–6 determinations: * $P < 0.01$, ** $P < 0.001$.

(100 μM) as a function of the extravesicular Na^+ concentration (0–100 mM; Fig. 4, left). There was a hyperbolic relationship a minimum sodium/6-MPR stoichiometry of 1:1. The data were fit to Hill equation was used [18]:

$$\text{uptake rate}(v) = V_{max} \cdot [Na^+]^n / (K^n_{Na^+} + [Na^+]^n)$$

where K_{Na^+} is the Na^+ concentration ($[Na^+]$) which gives 50% of the maximal velocity (V_{max}), and n is the Hill coefficient. According to this equation, a plot of uptake rate $(v)/[Na^+]^n$ against uptake rate (v) for the correct value of n will yield a straight line. Fig. 4 (right) shows such a plot of the data in Fig. 4 (left) assuming $n = 1$. A straight line was observed with $n = 1$ and K_{Na^+} (mean ± S.E.) = 13.3 ± 0.77 mM; a result consistent with a single Na^+ binding site on the carrier. This stimulative uptake of the nucleoside by an extravesicular Na^+ was specific due to the fact that no enhanced uptakes were observed when the Na^+-gradient was replaced by Li^+ or choline (data not shown).

3.4. Inhibition behavior and substrate specificity

The substrate-specificity of the transport system of 6-MPR in intestinal brush-border membranes was investigated by an inhibition study using nucleosides and their related purine- or pyrimidine-bases. Fig. 5 indicates that the sodium-dependent component of 6-MPR (100 μM) uptake by intestinal brush-border membrane vesicles was significantly inhibited by nucleosides (1.0 mM) including pyrimidine nucleoside, and uridine, but not by their nucleo-bases (1.0 mM) and D(−)ribose (1.0 mM). Furthermore, as shown in Fig. 6, the Na^+-dependent uptake of ^{3}H-labeled adenosine was significantly reduced by the presence of extravesicular cold adenosine and 6-MPR, suggesting that a common transport system was shared between these purine-nucleosides.

On the other hand, 500 μM of uridine was not a potent inhibitor of the purine nucleoside transporter despite the fact that adenosine and 6-MPR inhibited the uptake of

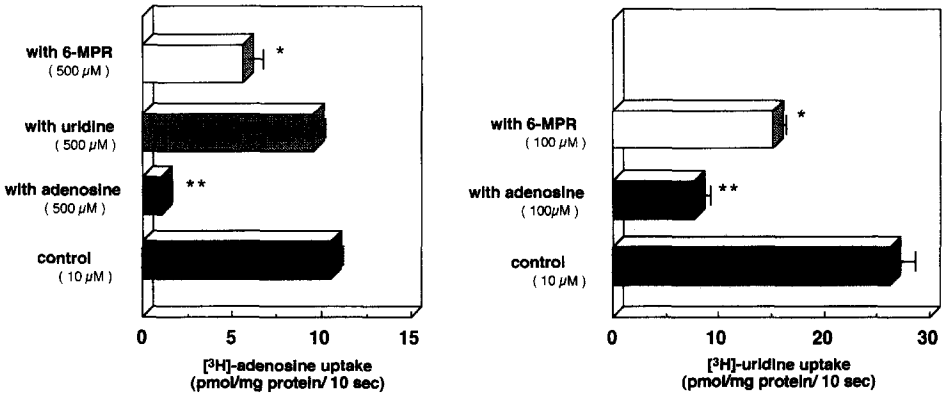

Fig. 6. Mutual inhibition behavior by nucleosides for their Na^+-dependent uptake (10 s) by intestinal brush-border membrane vesicles. Experimental conditions were similar to those in Fig. 5. Each column represents the mean with S.E. of 5–6 determinations: * $P < 0.01$, ** $P < 0.001$.

Table 1
Trans-stimulation effects of unlabelled nucleosides (100 μM) inside the membrane vesicles on the uptakes of [^{3}H]adenosine and [^{3}H]uridine (20 μM, respectively) by the intestinal brush-border membrane vesicles

	Uptake (pmol/mg protein/10 s)	% of control
[^{3}H]Adenosine uptake		
control	8.762 ± 0.391	100
adenosine	19.065 ± 0.185 *	217.59 ± 2.11
6-MPR	15.050 ± 0.729 *	171.77 ± 8.32
[^{3}H]Uridine uptake		
control	7.636 ± 0.183	100
adenosine	11.483 ± 0.341 *	150.38 ± 4.47
uridine	11.375 ± 0.861 *	148.96 ± 11.28
6-MPR	12.095 ± 0.728 *	158.40 ± 9.54

Values are means ± S.E. of at least three separate experiments in triplicate. Membrane vesicles (20 μl) were preloaded for 1 h in 100 mM KCl, 100 mM D-mannitol, and 20 mM Mes-Tris, pH 5.5, containing 100 μM of unlabelled nucleoside. The uptake media outside the vesicles contained (final concentration) 100 mM KCl, 100 mM D-mannitol, 5 μM unlabelled nucleoside and either 20 μM [^{3}H]adenosine or [^{3}H]uridine buffered with 20 mM Mes-Tris (pH 5.5): * $P < 0.001$.

^{3}H-labeled uridine by the brush-border membrane vesicles in the presence of an inwardly directed Na^+-gradient.

3.5. Trans-stimulation effects of nucleosides on the transport systems

To further characterize the nucleoside transport systems, we determined whether the presence of unlabeled nucleosides inside the vesicles stimulated the uptake of radiolabeled adenosine or uridine (trans-stimulations). As shown in Table 1, in the absence of an inward Na^+-gradient, the uptake of 20 μM [^{3}H]adenosine and [^{3}H]uridine at 10 s was significantly greater in the vesicle preloaded with 6-MPR or unlabeled nucleosides (each 100 μM), than in the control vesicles. We also confirmed that equilibrated uptake values (30 min) were similar in the presence or absence of intravesicular 6-MPR and unlabeled nucleosides.

4. Discussion

Brett et al. [5] reported that cytosine arabinoside (Ara-C) and dideoxycitidine (ddT), which are nucleoside analogues with modifications on the ribose ring, were not recognized by the Na^+-driven nucleoside transport system in the human renal brush-border membrane. Our present data show that the uridine-nonsensitive transporter coupled with Na^+, in which the stoichiometry was 1:1, contributed to the uptake of adenosine and its analogue, 6-MPR, in rat intestinal brush-border membranes. The Na^+-dependent uptake of 6-MPR by the rat intestinal brush-border membrane vesicles was found to be inhibited by purine- and pyrimidine-nucleosides, but not by their nucleo-bases. Additionally, the uptakes of adenosine and uridine were trans-stimulated by pretreatment with 6-MPR, although these trans-stimulation may be also relating to an Na^+-independent transporter for this drug. These results suggest that 6-MPR interacts with the Na^+/nucleoside co-transporter(s) in rat intestinal brush-border membranes, and that the nucleoside structure is an important requirement for substrate specificity. These results agree with the reports of Jarvis [9] for rabbit intestinal brush-border membrane vesicles and Lee at al. [8] for renal brush-border membrane vesicles. Furthermore, uridine was also a potent inhibitor for 6-MPR uptake, suggesting that 6-MPR is able to be transported not only via the purine-nucleoside transport system, but also via the pyrimidine system.

On the other hand, uridine, which was potently inhibited by adenosine, did not affect the uptake of adenosine in the presence of an inward Na^+-gradient. The affinity constants (K_m) for those nucleosides (calculated from Eadie-Hofstee analysis) were 21.5 μM for adenosine and 15.7 μM for uridine. Therefore, it is unlikely that any effects of 500 μM uridine on the uptake of [^{3}H]adenosine (10 μM) were due to the lower concentration of uridine present in the incubation medium. Recently, the expression of the other Na^+-dependent nucleoside transporter (N3), in *Xenopus Laevis* oocytes injected with poly(A)$^+$ RNA isolated from rabbit choroid plexus, was determined by Wu et al. [19,20]. The N3 system was also found to be present in a human colon adenocarcinoma cell line (Caco-2) [21]. It has also been reported that apparent N1 and N3 activities are expressed in *Xenopus Laevis* oocytes following injection with mRNA from rat jejunum [13,22]. It seems that 6-MPR and adenosine may be taken up by Na^+-dependent pathways from the intestinal lumen via both N1 and N3 transport systems although it remains unclear whether the N3 system plays a functional role in the jejunum.

In conclusion, there seems to be at least two nucleoside transporters driven by Na^+-gradients in rat intestinal brush-border membranes. It is considered that adenosine and its analogue, 6-MPR, are transported via both systems, but that uridine is transported via either one or the other of these systems. However, it will be necessary to further investigate the classification of the transporters for these two nucleosides.

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