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EFFECT OF MANGANESE ON TYROSYLPROTEIN SULFOTRANSFERASE ACTIVITY IN PC12 CELLS

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Abstract—Recent studies in our laboratory have revealed that Mn^{2+} is capable of promoting cell spreading and neurite outgrowth in PC12 cells, a process which is dependent on Mn^{2+} stimulation of the interaction between extracellular matrix (ECM) components and their corresponding integrin receptors. Since the major ECM proteins implicated in the Mn^{2+}-induced morphogenesis, fibronectin and vitronectin, are both tyrosine sulfated, it was of interest to determine whether Mn^{2+} can regulate the activity of the enzyme responsible for tyrosine sulfation, tyrosylprotein sulfotransferase (TPST). Results of the present studies demonstrated that Mn^{2+} can suppress TPST activity in PC12 cells in both a time and concentration-dependent manner at concentrations of Mn^{2+} (0.1 to 1 mM) that promote morphological changes in PC12 cells. Since uptake of Mn^{2+} may occur via the Ca^{2+} channel, $LaCl_3$, an inhibitor of Ca^{2+} transport, was examined and found not to prevent the suppression of TPST activity induced by Mn^{2+}, suggesting that Mn^{2+} may function at an extracellular site. Suppression of TPST activity occurred with cells plated in serum-free medium on a substrata consisting of either serum or polylysine, suggesting that attachment to extracellular matrix was not an absolute requirement for regulation of activity. Consistent with this is the fact that an RGD-containing pentapeptide did not prevent suppression of TPST activity. Results from the present study demonstrated that Mn^{2+} is capable of promoting the suppression of TPST activity in PC12 cells at concentrations that have been shown to induce cell spreading and neurite outgrowth. However, unlike the Mn^{2+}-induced morphological changes, the presence of ECM proteins is not an absolute requirement for suppression of TPST activity.

Key words: manganese; tyrosylprotein sulfotransferase; PC12 cells; pheochromocytoma cells; tyrosine sulfation; sulfation

Tyrosine sulfation is now known to be a widespread post-translational modification of proteins and peptides [1, 2]. The majority of the sulfated proteins and peptides are of a secretory nature and range from small peptide hormones such as CCK† and gastrin to much larger proteins like immunoglobulins, coagulation factors, and ECM proteins. Tyrosine sulfation is catalyzed by the Golgi enzyme TPST, which has been identified and characterized in several species and tissues [3–8]. TPST is a lipid-dependent [9], integral membrane-bound protein whose activity is distributed unevenly in the rat, with the highest activities expressed in liver, pituitary, and cerebellum, and almost no activity in the testes [5].

TPST activity is differentially increased several-fold in both liver and cerebellum of neonatal rats during the first few days after parturition [10]. The regulation of TPST in the nervous system is of particular interest, because tyrosine sulfation occurs in many proteins and peptides important to the development and functional state of the nervous system. The biological activities of several neuropeptides, such as CCK [11] and enkephalin [12], are known to be modified by tyrosine sulfation. In addition, tyrosine sulfation has been demonstrated in many ECM components such as vitronectin [13], fibronectin [14] and entactin [15], and the membrane-bound β-amyloid precursor protein [16]. These proteins have all been demonstrated to promote cell–substratum or cell–cell adhesion and provide guidance for neuronal morphogenesis and migration [17, 18]. In addition, tyrosine sulfation has also been proposed to regulate the proteolytic processing, intracellular sorting and rates of secretion of several secretory proteins [1].

PC12 cells, a clonal line developed from rat pheochromocytoma, have been used extensively as a model system for neuronal differentiation because of their ability to undergo neuronal differentiation upon stimulation by nerve growth factor [19, 20]. Lee and Huttner [7] have reported that PC12 cells express TPST activity capable of sulfating several proteins that are present in neurons, such as the secretogranins and the β-amyloid precursor protein [1]. Therefore, PC12 cells provide a good model for investigating the regulation of TPST activity during neuronal development. Prior studies [8] in our laboratory have demonstrated that treatment of PC12 cells with 2-chloroadenosine, an adenosine receptor agonist [21, 22], results in decreased TPST activity in a time- and dose-dependent fashion.

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† Abbreviations: CCK, cholecystokinin; DMEM, Dulbecco's Modified Eagle's Medium; DTT, dithiothreitol; ECM, extracellular matrix; MES, 2-[*N*-morpholino]-ethanesulfonic acid; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; and TPST, tyrosylprotein sulfotransferase.

However, its ability to specifically suppress TPST activity in PC12 cells did not appear to be associated with either the adenosine A_1 or A_2 receptor but was, instead, mediated through a novel and uncharacterized cell-surface receptor.

Recent studies [23] have revealed that Mn^{2+} is capable of promoting cell spreading and neurite outgrowth in PC12 cells and is also capable of modifying their triacylglyceride metabolism [24]. The morphological transformation of PC12 cells results from Mn^{2+} stimulation of the interaction between ECM components and integrin receptors; however, the mechanism responsible for Mn^{2+}-induced changes in lipid metabolism has not been established. Since TPST is a lipid-dependent enzyme [9], it is possible that Mn^{2+}-induced changes in lipid composition of PC12 cells may consequently affect TPST activity. In the present study, we report that Mn^{2+} decreased TPST activity in PC12 cells but, in contrast to PC12 cell differentiation, TPST-suppression induced by Mn^{2+} may be independent of both cell attachment and ECM–integrin interaction.

MATERIALS AND METHODS

Materials. DMEM, penicillin and streptomycin were obtained from GIBCO, Grand Island, NY. Fetal bovine serum was purchased from Hyclone Laboratories, Logan, UT, and heat-inactivated horse serum from Hazelton Laboratories, Lenexa, KS. Sucrose, Triton X-100, NaF, and β-mercaptoethanol were purchased from J. T. Baker, Phillipsburg, NJ. [^{35}S]PAPS (1.5 to 2.5 Ci/mmol), [^{14}C]UDP-galactose ([galactose-^{14}C(U)], 270 μCi/μmol) and [α-^{32}P]ATP were purchased from New England Nuclear, Boston, MA, and Sep-Pak C_{18} cartridges were from Waters, Milford, MA. 2-Chloroadenosine, 5′-AMP, ATP, unlabeled PAPS, fluorescamine, phosphonoacetic acid, HEPES, MES, EDTA, *N*-acetylglucosamine, and Dowex-2 (200–400 mesh) were obtained from the Sigma Chemical Co., St. Louis, MO. P359A,* a synthetic peptide based on the sequence of the C-terminus of the C4 component of complement, was provided by Dr. Glen Hortin, Department of Pediatrics, Washington, University, St. Louis, MO.

Maintenance and treatment of cell culture. Both the wild-type PC12 cells and the mutant strain A126–1B2–1, which is deficient in protein kinase A activity [25], were gifts from Dr. Wagner (Cornell University School of Medicine). Cell cultures were maintained in DMEM supplemented with 10% fetal bovine serum, 5% heat-inactivated horse serum and 100 U/mL of penicillin and 100 μg/mL of streptomycin at 36.5° with 5% CO_2 and 85% humidity and subcultured every 3–4 days at 80–90% confluency. $MnCl_2$ treatments were initiated by adding an appropriate volume of stock solution to the culture medium to achieve the final concentration indicated. For experiments using defined growth medium, DMEM was supplemented with 1 μM insulin, 100 U/mL of penicillin and 100 μg/mL of streptomycin. For these experiments, cells maintained in normal growth medium were harvested, washed twice with DMEM, and replated in defined medium onto culture dishes coated with either 50 μg/mL poly-D-lysine or normal cell culture medium containing 10% fetal bovine serum and 5% horse serum. Appropriate volumes of $MnCl_2$ stock solution were added to the cultures to initiate treatments.

Enzyme preparation and TPST assay. Control and treated cells were harvested by trituration in PBS and pelleted by centrifugation at 1000 *g* for 4 min. The cell pellets were washed once with PBS and then homogenized in 0.5 M sucrose containing 10 mM HEPES, pH 7.0, and 1 mM DTT, using a Dounce homogenizer (20 strokes). The homogenate was then centrifuged at 200,000 g_{max} for 50 min. The resulting pellet was resuspended in homogenization buffer and assayed immediately for TPST activity using the methods of Rens-Domiano *et al.* [26] with minor modifications as described [8]. Briefly, the reaction mixture contained, in a final volume of 100 μL, 40 mM MES, pH 6.5, 20 mM $MnCl_2$, 50 mM NaF, 1 mM 5′-AMP, 1 mM phosphonoacetic acid, 0.5% Triton X-100, 20 μM P359A and 4 μM [^{35}S]-PAPS (0.2 to 0.3 μCi/assay). The reactions were initiated by the addition of 50–150 μg protein of the enzyme preparation and terminated after incubation for 20 min at 30° by addition of 400 μL of ice-cold 75 mM EDTA followed by placing the tubes on ice. The sulfated products were separated from PAPS and its degradative metabolites by chromatography using Sep-Pak C_{18} cartridges as described [8]. To correct for sulfation of endogenous substrates, blank values were determined from incubations performed in the absence of the peptide P359A. Under these conditions, the reactions were linear with both time of reaction and the amounts of enzyme preparation added. Galactosyltransferase activity was assayed as described previously [27].

RESULTS AND DISCUSSION

In this study, we examined the regulation of TPST activity during Mn^{2+}-induced morphogenesis. PC12 cells were treated with Mn^{2+} at concentrations that have been demonstrated previously [23] to induce cell spreading and neurite outgrowth (i.e. 0.1 to 1.0 mM). As shown in Fig. 1, there was a concentration-dependent decrease of TPST activity after 24 hr of treatment with Mn^{2+}. Approximately 50% inhibition was achieved after 24 hr of treatment with 0.3 mM Mn^{2+} and, as illustrated in the inset in Fig. 1, the decrease in TPST activity was first order with respect to Mn^{2+} concentration. This suppression of TPST activity observed could not have resulted from direct inhibition by Mn^{2+}, since $MnCl_2$ has been shown to stimulate TPST activity and is routinely included in the *in vitro* assay to measure TPST activity. Therefore, the reduction of TPST activity is likely to be the result of specific cellular events triggered by Mn^{2+}.

The time course for the suppression of TPST activity is illustrated in Fig. 2. As indicated, the decrease of TPST activity occurred as early as 3 hr after initiation of Mn^{2+} treatment, and the level of activity continued to decrease with sustained treatment, reaching about 50–70% inhibition by 24 hr. Although not shown, suppression of TPST activity was first order with respect to time. In

* The structure of P359A is: EANEDFEDYEFDE.

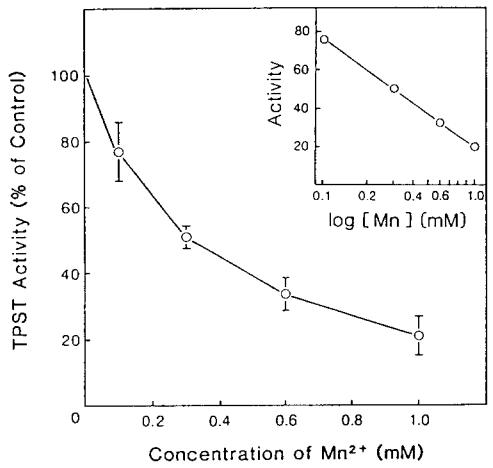

Fig. 1. Effect of Mn^{2+} concentration on the activity of TPST. PC12 cells were treated for 24 hr with Mn^{2+}, as indicated. Cells were harvested and assayed for TPST activity as described in Materials and Methods. Data are the means ± SEM of five separate experiments in which the control specific activity ranged from 0.39 to 0.95 pmol/min/mg protein.

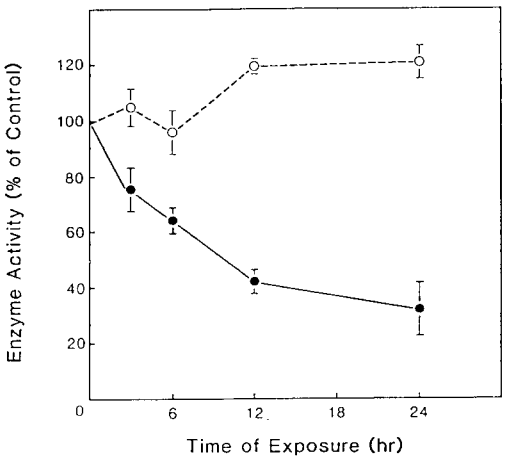

Fig. 2. Effect of time on TPST (-●-) and galactosyltransferase (-○-) activity in PC12 cells treated with 1 mM Mn^{2+}. Results are the means ± SEM of four separate experiments in which the control activities for TPST and galactosyltransferase ranged from 0.66 to 0.89 pmol/min/mg protein and 0.41 to 0.56 nmol/min/mg protein, respectively.

comparison to the morphological changes induced by Mn^{2+}, the decrease in TPST activity appeared prior to neurite outgrowth which was observed between 6 and 12 hr after initiating treatment with 1 mM Mn^{2+} [23].

To examine whether inhibition of TPST activity by Mn^{2+} was part of a general suppression of cellular enzyme activities, the effect of Mn^{2+} on the activity of another trans-Golgi enzyme, galactosyltransferase,

Table 1. Effect of $LaCl_3$ on TPST activity in PC12 cells

Divalent cation	TPST activity (pmol/min/mg protein)	% of Control
None	0.61	100
Mn^{2+} (1 mM)	0.16	26
$LaCl_3$ (10 μM)	0.53	87
Mn^{2+} + $LaCl_3$	0.19	31

PC12 cells were treated with $MnCl_2$ and/or $LaCl_3$ for 18 hr at which time the cells were harvested and assayed for TPST activity as described in Materials and Methods. Percent activity is calculated relative to the untreated cells. Results are from a typical experiment performed three times in duplicate.

was also investigated (Fig. 2). In contrast to TPST, the activity of galactosyltransferase was not altered significantly throughout the entire course of Mn^{2+} treatment, although a slight increase was observed at the later time points. The time course for suppression of TPST activity by Mn^{2+} was similar to that reported previously [8] in PC12 cells treated with 2-chloroadenosine, whereas the increase in galactosyltransferase activity was considerably smaller than that observed upon exposure to this adenosine derivative. Prior studies have demonstrated that induction of galactosyltransferase activity in PC12 cells is dependent on cAMP [27], and, therefore, the small increase in activity seen upon Mn^{2+} treatment may have resulted from stimulation of adenylate cyclase [28]. These results suggest that the regulation of enzyme activities by Mn^{2+} in PC12 cells is highly specific.

Since Mn^{2+} can be taken up by the cells through Ca^{2+} channels [29, 30], it is possible that Mn^{2+} may interfere with transmembrane Ca^{2+} movement by competing with Ca^{2+} for the ion channels. To examine whether this was, in fact, the mechanism through which Mn^{2+} decreased TPST activity, we examined the effects of $LaCl_3$, a Ca^{2+}-channel blocker [31], on the activity of TPST. As shown by the data in Table 1, $LaCl_3$ at a concentration that is known to cause complete blockade of the Ca^{2+} channels had only a minor effect on TPST activity and did not prevent Mn^{2+} from suppressing TPST activity. These results suggest that Mn^{2+} may decrease TPST activity via its interaction with cellular structures on the membrane rather than with intracellular cytosolic elements, similar to the mechanism proposed for spreading and neurite outgrowth in PC12 cells [25].

To determine whether other divalent cations also mimic the effects of Mn^{2+}, TPST activity was measured in cells treated with a number of divalent cations. The results of this experiment, shown in Table 2, indicate that only Mn^{2+} and Co^{2+} were capable of suppressing TPST activity, while Zn^{2+} and Cd^{2+} were ineffective, suggesting that the suppression of TPST was not a general effect of divalent cations. It is also worth noting that, in our previous studies [23] on the morphological effects of these divalent cations, Co^{2+} was the only other

Table 2. Effects of various divalent cations on TPST activity in PC12 cells

Divalent cation	TPST activity (pmol/min/mg protein)	% of Control
None	1.19	100
Mn^{2+} (1 mM)	0.61	51
Co^{2+} (0.3 mM)	0.44	37
Mn^{2+} + Co^{2+}	0.26	22
Zn^{2+} (10 μM)	1.08	90
Cd^{2+} (1 μM)	1.03	86

PC12 cells were treated with various cations for 18 hr at which time the cells were harvested and assayed for TPST activity as described in Materials and Methods. Results are from a typical experiment performed three times in duplicate.

Table 3. Effect of extracellular matrix and RGD on Mn^{2+}-induced suppression of TPST activity

Treatment	Specific activity (pmol/min/mg protein)	% Inhibition
Experiment 1		
Serum-coated	1.27	
PL-coated	1.42	0
Serum + Mn^{2+}	0.75	41
PL + Mn^{2+}	0.85	33
Experiment 2		
Control	0.84	
Mn^{2+}	0.46	45
RGD^{2+}	0.72	15
Mn^{2+} + RGD	0.50	41

Experiment 1: PC12 cells were grown in serum-free medium on serum-coated or poly-lysine-coated plates for 36 hr in the presence and absence of 40 μM Mn^{2+}. Experiment 2: PC12 cells were grown in 1% serum on polylysine-coated plates for 24 hr in the presence and absence of 0.2 mM Mn^{2+} and/or RGD-containing pentapeptide (0.1 mg/mL). TPST activity was assayed as described in Materials and Methods. Results are from a typical experiment performed three times in duplicate.

cation besides Mn^{2+} that was capable of stimulating cell spreading and neurite outgrowth, although its response was attenuated by its greater toxicity to the PC12 cells. It is therefore possible that the effects of these two cations on TPST activity are related to those on the morphology of PC12 cells. Since the morphological changes induced by Mn^{2+} are believed to involve the interaction between integrin receptors and ECM, it is interesting to note that Co^{2+} was shown to be capable of competing with Mn^{2+} for binding to the vitronectin receptor [32].

Accordingly, studies were performed to determine whether the presence of ECM proteins is required for Mn^{2+}-induced TPST suppression. Since ECM proteins are present in the sera to grow PC12 cells, additional studies were performed in defined serum-free medium consisting of DMEM supplemented with 1 μM insulin and penicillin/streptomycin. Cells were washed with DMEM and plated with this defined medium onto 100 mm culture dishes that were coated previously with either normal growth medium or 50 μg/mL poly-D-lysine. Polylysine-coated plates were used as the ECM-protein-free control for these assays because cells did not attach well to 100 mm dishes in serum-free medium at the plating density (approximately 1:4 from near confluent plates) used for TPST measurement. Cells in the serum-coated plates were attached primarily to either vitronectin or fibronectin, the two major ECM proteins in serum. Results of a typical experiment, shown in Table 3, demonstrate that Mn^{2+} was able to suppress TPST activity regardless of whether the PC12 cells were grown on serum-coated or polylysine-coated plates, whereas only the cells maintained on the serum-coated plates exhibited cell flattening and neurite outgrowth, as previously described [22]. The actual percent decrease in TPST activity in the Mn^{2+}-treated plates coated with polylysine was 40%, if compared with the cells grown on polylysine alone. These results suggest that cell differentiation is not a prerequisite for suppression of TPST activity and that the presence of ECM molecules is not an absolute requirement for this to occur.

In another related experiment, cells were exposed to Mn^{2+} in the presence of a synthetic pentapeptide Gly-Arg-Gly-Asp-Ser (GRGDS), which prevented cell attachment to the substratum presumably through occupying the RGD-dependent ECM binding sites on the cell membrane [33, 34]. To maintain cell attachment even in the presence of the RGD-containing peptide, cells were grown on poly-lysine-coated culture dishes. The results shown in Table 3 demonstrate that TPST activity was suppressed by Mn^{2+} in both control and RGD-containing media. There was a slightly smaller decrease in suppression of activity of approximately 30% when comparing Mn^{2+} plus RGD with control cells grown in the presence of RGD alone. This difference was caused by the fact that RGD by itself suppressed TPST activity approximately 15%. At the concentration of RGD employed in these studies, PC12 cell differentiation was essentially prevented, suggesting that TPST suppression caused by Mn^{2+} may be, at least, partially independent of its effects on RGD-dependent ECM–integrin interaction.

Since results shown in this study have demonstrated that the regulation of TPST activity by Mn^{2+} was independent of intracellular uptake of this cation by PC12 cells, these data imply that there may be other membrane sites with which Mn^{2+} can interact to cause the suppression of TPST activity. It is still possible that Mn^{2+} interacts directly with non-RGD-dependent integrins in the absence of ECM molecules to cause certain intracellular responses such as suppression of TPST activity, while observation of the full spectrum of responses, including the morphological changes, depends on the presence of ECM proteins or other factors in serum. The observation that Mn^{2+} and Co^{2+} are both capable of promoting morphological changes as well as suppressing TPST activity is consistent with this possibility. Conversely, we cannot rule out the possibility that Mn^{2+} could be interacting with other

Table 4. Effects of Mn^{2+} and 2-chloroadenosine on TPST activity in PC12 cells

Treatment	TPST activity (pmol/min/mg protein)	% of Control
Experiment 1		
None	0.747	100
Mn^{2+} (0.1 mM)	0.685	83
2-CADO (50 μM)	0.389	53
Mn^{2+} + 2-CADO	0.294	38
Experiment 2		
None	1.02	100
Mn^{2+} (0.3 mM)	0.527	52
2-CADO (50 μM)	0.429	42
Mn^{2+} + 2-CADO	0.242	24

PC12 cells were treated with Mn^{2+} and/or 2-chloroadenosine (2-CADO), as indicated, for 24 hr. TPST activity was assayed as described in Materials and Methods. Results are from a typical experiment performed three times in duplicate.

responsive elements on the cell membrane totally independent of its effects on the integrins.

In a previous study [8] suppression of TPST activity by the adenosine derivative, 2-chloroadenosine, was shown to be independent of cAMP but was instead apparently mediated through a novel membrane receptor other than the known adenosine receptor. To examine whether the effect of Mn^{2+} on TPST activity was also independent of cAMP, the effect of Mn^{2+} was examined in a mutant PC12 cell strain deficient in protein kinase A activity. Thus, to determine whether Mn^{2+} regulation of TPST activity was mediated through a mechanism similar to that of 2-chloroadenosine, we examined the interaction between the effects of 2-chloroadenosine and Mn^{2+}. The results reported in Table 4 indicate that the two responses are additive. In a typical experiment, 50 μM 2-chloroadenosine and 0.1 mM Mn^{2+} caused a decrease of TPST activity of 47 and 17%, respectively, whereas the combination of the two agents diminished TPST activity by 62%, approximating the sum of inhibition by each individual agent. At higher concentrations of Mn^{2+}, such as 0.3 mM, TPST activity was suppressed 48% by Mn^{2+} alone and 76% by the combination of Mn^{2+} and 2-chloroadenosine. Although this is less than the sum of the inhibition by each agent, this result is to be expected if the two agents modify TPST activity through independent mechanisms. For instance, our previous study [8] suggested that 2-chloroadenosine decreased TPST activity by inhibition of its synthesis. If Mn^{2+} decreased TPST activity through a different mechanism, e.g. through induction of an inhibitor, the inhibitory effects of Mn^{2+} would only be exerted on the existing enzyme activity after 2-chloroadenosine inhibition, or 50% of the original TPST activity. In this case, the combined effects of Mn^{2+} and 2-chloroadenosine would lead to only 75%, instead of 100%, suppression of TPST activity. Therefore, our results appear to indicate that Mn^{2+} suppression of TPST activity occurs through a mechanism different from that of 2-chloroadenosine.

In summary, results from the present study demonstrated that Mn^{2+} is capable of promoting the suppression of TPST activity in PC12 cells at concentrations that have been shown to induce cell spreading and neurite outgrowth. The precise mechanism for this cannot be ascertained from the studies presented herein but may possibly involve changes in the lipid composition within the Golgi membranes. TPST is a lipid-dependent enzyme, and extraction of the Golgi membranes with Triton or other detergents can result in complete loss of the enzyme activity. In addition, recent studies have confirmed that TPST activity is influenced by several lipids, including sphingosine and acidic phospholipids [9]. Since Mn^{2+} has been shown to modify triacylglycerol metabolism in PC12 cells [24], it is possible that alteration in the lipid composition of the Golgi apparatus may account for the decrease in TPST activity observed. Unlike the Mn^{2+}-induced morphological changes, the presence of ECM proteins does not appear to be a prerequisite for suppression of TPST activity, implying that a different mechanism may be responsible for the regulation of TPST activity and possibly triacylglycerol metabolism in PC12 cells.

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