

# Contribution of zinc and other metals to the biological activity of the serum thymic factor

(thymus/hormone/peptide/thymulin/chelation)

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**ABSTRACT** The serum thymic factor (FTS) utilized in its synthetic or natural form loses its biological activity in a rosette assay after treatment with a metal ion-chelating agent, Chelex 100. This activity is restored by the addition of Zn salts and, to a lesser extent, certain other metal salts. FTS activation is secondary to the binding of the metal to the peptide. The metal-to-peptide molar ratio of 1:1 provides the best activation. These data indicate the existence of two forms of FTS. The first one lacks Zn and is biologically inactive; the second one contains Zn and is biologically active, for which we propose the name of "thymulin" (FTS-Zn). The presence of Zn in synthetic FTS was confirmed by atomic absorption spectrometry. The interaction between Zn and FTS was further suggested by microanalysis demonstrating the presence of this metal in thymic reticuloepithelial cells.

The thymus gland produces a number of polypeptidic hormones that can induce T-cell markers and functions in immature cells and, consequently, are considered to be physiologically involved in T-cell differentiation (1). One of these hormones, *facteur thymique sérique* (FTS; serum thymic factor), was initially isolated from serum (2). Its amino acid sequence was determined (<Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn), and the synthetic peptide was shown to be fully biologically active (3). More recently, the presence of high-affinity FTS receptors was demonstrated on lymphoblastoid T-cell lines (4), and direct evidence was produced for the thymic origin of FTS by using an indirect immunofluorescence technique (5, 6) (with polyclonal and monoclonal antibodies produced against synthetic FTS) and by chemical analysis (down to the amino acid composition) of thymic extracts (7). Several laboratories have synthesized FTS and confirmed its high biological activity (8). However, the fortuitous preparation of inactive or instable lots has suggested that FTS might exist in two forms—one biologically active and the other one inactive. Data reported in this article indicate that the active form contains a metal, probably Zn, whereas the inactive form is deprived of metal. We propose to give the name "thymulin" for the Zn-associated, biologically active form of FTS.

## MATERIALS AND METHODS

**Chemical.** FTS was synthesized by an original method using homogeneous-phase synthesis (unpublished results). Labeled FTS was obtained by replacing lysine with its derivative 2,6-diamino-4-hexynoic acid (9). This derivative was labeled after catalytic tritiation (10). Specific activity was 120 Ci/mmol (1 Ci =  $3.7 \times 10^{10}$  becquerels). Metal salts (chloride, acetate, or sulfate) of the highest reagent grade were purchased from the Pas-

teur Institute (Paris, France). Azathioprine, used in its sodium salt form, was obtained from Burroughs Wellcome (Research Triangle Park, NC).

**Mice.** C57BL/6 mice (5–10 weeks old) were obtained from the Centre National de la Recherche Scientifique, Orléans la Source, France. Adult thymectomy was performed by suction under nembutal anesthesia.

**Chelation Experiments.** Serum (100  $\mu$ l) containing natural or synthetic FTS (1  $\mu$ M) were incubated for 30 min at room temperature with an equal volume of Chelex 100 at 50 mg/ml in distilled water. At the end of the incubation, the mixture was centrifuged at  $12,000 \times g$  for 2 min in order to eliminate the chelating resin, and the biological activity in the supernatant of Chelex 100-treated FTS was measured.

**Metal Treatment.** The supernatant obtained after chelation was incubated with different metals in the form of chloride, sulfate, or nitrate salts. The incubation of FTS with metal salts was performed with variations in temperature, pH, and duration. The metal/peptide molar ratio varied from 0.01 to 100.

**Chromatography on Bio-Gel P-2 Column.** Tritiated FTS (50 ng) was incubated with Chelex 100 as described above. After removal of Chelex 100 by centrifugation, [ $^3$ H]FTS was added to 0.01 mCi of radioactive  $^{65}$ Zn in 0.1 M Tris buffer (pH 7.6) and incubated for 30 min at room temperature. This mixture was applied to a Bio-Gel P-2 column (50  $\times$  0.9 cm) and eluted with distilled water. Fractions (1 ml) were collected, and the biological activity of each fraction was tested by the rosette assay. The radioactivity incorporated was evaluated for  $^{65}$ Zn in a gamma counter and for [ $^3$ H]FTS in a liquid scintillation counter. [ $^3$ H]FTS alone and  $^{65}$ Zn alone were applied to a Bio-Gel P-2 column under the same conditions as described for the FTS/Zn mixture.

**Biological Assays.** The rosette assay for thymic hormone evaluation used in these studies has been described (11). It is based upon the changes induced by thymic hormones on the minority of spleen cells from adult thymectomized mice that form spontaneous rosettes with sheep erythrocytes. The assay consists of detecting the lowest concentration of serum that renders spleen rosette-forming cells sensitive to anti-theta serum or to azathioprine (2). In brief, the samples were incubated for 90 min at 37°C with spleen cells from C57BL/6 mice, thymectomized 10–15 days before the test. Azathioprine (10  $\mu$ g/ml) was added simultaneously with the sample. At the end of the incubation time,  $12 \times 10^6$  sheep erythrocytes were added to the spleen cells, which were centrifuged and resuspended by rotation on a roller (diameter, 10 cm) at low speed (10 rpm). Rosette-forming cells were counted in a hemacytometer at low magnification ( $\times 250$ ). In the presence of FTS activity, azathioprine at

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Abbreviation: FTS, serum thymic factor (*facteur thymique sérique*).

10  $\mu\text{g}/\text{ml}$  inhibits rosette formation. The lowest concentration of the test sample that induces rosette inhibition by azathioprine is considered as the active concentration.

In *in vivo* studies, the serum level of FTS activity was evaluated by using the method just described with serum samples from adult thymectomized mice collected at different times after an intraperitoneal injection of 0.1, 1, or 10 ng of the chemical to be tested. Test sera were filtered through a CF50 Amicon membrane in order to eliminate high molecular weight molecules previously shown to behave like FTS inhibitors (11). The FTS activity of the ultrafiltrate was then tested by the rosette assay, and the activity of the serum was expressed as the highest dilution of the serum inducing the inhibition of 50% of the rosette-forming cells.

**Zn Determination.** Zn was determined by atomic absorption spectrometry (IL Metter instrument). The standard ranged from 0 to 500 ppm of  $\text{ZnCl}_2$  in distilled water.

**Microprobe Technique.** Electron-probe microanalysis of ultrathin sections of mouse thymus and cultured thymic epithelial cells was carried out with a Castaing microprobe, type Camebax (electron microscope coupled to two wavelength-dispersion spectrographs). After selection of the areas of the sections with the electron microscope, the path of the electron beam was modified for the analysis of elements in the X spectrograph. This technique has been described in detail (12).

## RESULTS

**Loss of Biological Activity of FTS in the Presence of a Chelating Agent, Chelex 100.** Incubation of synthetic FTS with Chelex 100 resulted in the loss of its biological activity as measured by the rosette assay. The minimal active concentration of synthetic FTS, which initially was 0.1 pM, increased to >1  $\mu\text{M}$  after incubation with Chelex 100. The same results were observed with natural FTS present in ultrafiltrates of normal mouse serum. The maximal active dilution of serum, which was

Table 1. Effect of Chelex-100 treatment and  $\text{ZnCl}_2$  addition on the biological activity of FTS as assessed by the rosette assay

Treatment	<i>In vitro</i> activity*	<i>In vivo</i> activity†
Synthetic FTS, 1 $\mu\text{M}$	2–5 $\times 10^{-6}$	1:80,000–1:160,000
Chelex 100-treated synthetic FTS	1–4	1:128–1:256
Chelex 100-treated synthetic FTS + 1 $\mu\text{M}$ $\text{ZnCl}_2$	1–2 $\times 10^{-7}$	1:10 <sup>6</sup> –2:10 <sup>6</sup>
$\text{ZnCl}_2$ , 1 $\mu\text{M}$	No activity	1:2–1:4
Normal mouse serum (ultrafiltrate)	1:64–1:128	ND
Chelex 100-treated normal serum	1:2–1:4	—
Chelex 100-treated normal serum + $\text{ZnCl}_2$	1:64–1:128	—
ATx-mouse serum	1:2–1:4	—
ATx-mouse serum + $\text{ZnCl}_2$	1:2–1:4	—

ATx, adult thymectomized; ND, not determined. Treatment with Chelex 100 was performed by incubating 0.2 ml of FTS solution (100 ng) with 0.2 ml of Chelex-100 suspension (50 mg/ml) and eliminating Chelex 100 by centrifugation (12,000  $\times g$ ). Treatment with  $\text{ZnCl}_2$  was performed by mixing 10 ng of  $\text{ZnCl}_2$  with 100 ng of synthetic FTS (or 0.1 ml of serum ultrafiltrate for 10 min at 20°C). Results are the maximal and minimal values obtained in triplicate experiments.

\* Expressed as minimal active concentration (ng/ml or dilution) in the rosette assay.

† Serum dilution active in the rosette assay 30 min after intraperitoneal administration of 1 ng of FTS.

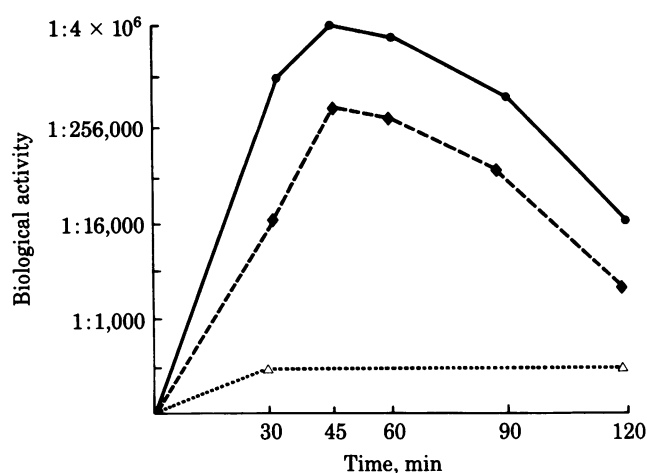


FIG. 1. Serum FTS activity as measured by the rosette assay after injection into thymectomized mice of 1 ng of Chelex 100-treated FTS with (—) or without (---) preincubation with  $\text{ZnCl}_2$ . Synthetic FTS was injected as the control (···). Biological activity is expressed as the minimal active dilution of serum.

initially 1:128, decreased to 1:4 after Chelex treatment (Table 1). In all of these experiments, Chelex 100 was removed before testing FTS activity in the rosette assay. The absence of an effect by residual Chelex was demonstrated by the full activity in the rosette assay of the mixture of FTS and of Chelex supernatant that had been prepared as described in chelating experiments but without FTS—i.e., under conditions where putative residual Chelex would be present.

**Effect of Zn on the Biological Activity of FTS.** The addition of  $\text{ZnCl}_2$  (1  $\mu\text{M}$ ) to synthetic or natural FTS (normal mouse serum ultrafiltrate) previously inactivated by Chelex-100 treatment totally restored *in vitro* and *in vivo* the biological activity of FTS (Fig. 1 and Table 1). In the case of synthetic FTS, the activity became even superior to that of the initial preparation tested before Chelex 100 treatment (Table 1 and Fig. 1).  $\text{ZnSO}_4$  also reactivated FTS (down to 10 pM) but less than did  $\text{ZnCl}_2$ , which was effective down to 0.1 pM.  $\text{Zn}(\text{OAc})_2$  did not provide any restoration of the biological activity of Chelex 100-treated FTS. Importantly, Zn reactivation of FTS was optimal at a neutral or slightly alkaline pH (7–8.5) and was obtained both at room temperature and at 37°C. Full FTS reactivation with both  $\text{ZnCl}_2$  and  $\text{ZnSO}_4$  was obtained after 5 min (it was not better after 30 min of incubation). However, the need for a direct contact between Zn and FTS was demonstrated by the absence of FTS reactivation observed in *in vivo* experiments, when Chelex 100-treated FTS and Zn were injected with a 1-min interval.

The reactivation of FTS by  $\text{ZnCl}_2$  was studied at different Zn/

Table 2. Reactivation of Chelex 100-treated FTS (1  $\mu\text{M}$ ) by Zn at different concentrations

Synthetic FTS treatment	<i>In vitro</i> activity*
Control, 1 $\mu\text{M}$ FTS	2–5 $\times 10^{-6}$
Chelex 100-treated	1–4
Chelex 100-treated	
+ Zn, 100 $\mu\text{M}$	1–2 $\times 10^{-4}$
+ Zn, 10 $\mu\text{M}$	2–4 $\times 10^{-5}$
+ Zn, 1 $\mu\text{M}$	1–2 $\times 10^{-7}$
+ Zn, 0.1 $\mu\text{M}$	2–4 $\times 10^{-7}$
+ Zn, 0.01 $\mu\text{M}$	2–4 $\times 10^{-5}$

These experiments were done in triplicate for each zinc concentration. \* Expressed as minimal active concentration (ng/ml) in the rosette assay.

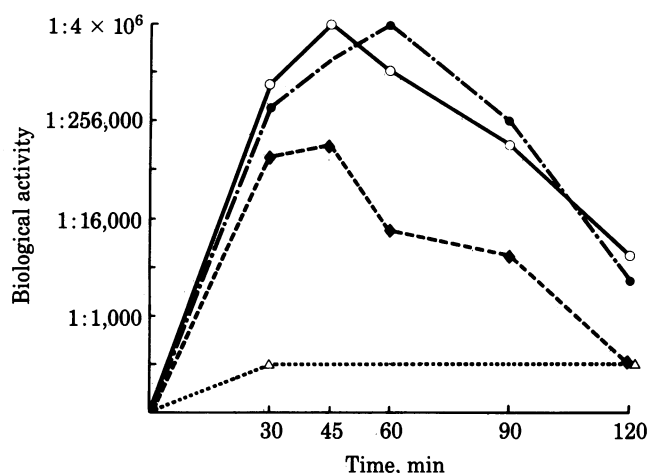


FIG. 2. Serum FTS activity as measured by the rosette assay after injection into thymectomized mice of Chelex 100-treated FTS preincubated with  $\text{ZnCl}_2$  at different Zn/FTS molar ratios: 10 (—), 1 (---), and 0.1 (· · ·). Chelex 100-treated FTS without Zn was injected as the control (— · —). Biological activity is expressed as the minimal active dilution of serum.

FTS molar ratios in order to define the optimal conditions for the reaction. FTS ( $1 \mu\text{M}$ ) was mixed with graded concentrations of  $\text{ZnCl}_2$  ( $0.01$ – $10 \mu\text{M}$ ) for 30 min. The results (Table 2 and Fig. 2) indicate that the best *in vitro* reactivation was obtained when FTS was mixed with Zn in a molar ratio of 1:1.

**Reactivation by Other Metals (Table 3).** The experiments were repeated with metal salts other than Zn at a FTS-to-metal molar ratio of 1:1. The following metals showed no effect on Chelex 100-treated FTS: Mg, Ca, Co, Cd, In, Sn, Tl, and Pb. Some metals proved capable of reactivating FTS significantly, but to a lesser extent than Zn: Cr, Mn, Fe, Ni, and Cu. Only two metals, Al and Ga, proved to be as active as Zn.

**Direct Demonstration of Zn Binding to the FTS Molecule.** Evidence of Zn binding to Chelex 100-treated FTS was demonstrated by using  $^3\text{H}$ FTS and  $^{65}\text{Zn}$ . As has been reported above for unlabeled FTS,  $^3\text{H}$ FTS lost its biological activity after incubation with Chelex 100. This activity was fully restored by  $\text{ZnCl}_2$ . The binding of  $^{65}\text{Zn}$  to  $^3\text{H}$ FTS was directly demonstrated by chromatography of the  $^{65}\text{ZnCl}_2$ / $^3\text{H}$ FTS mixture on a Bio-Gel P-2 column.

Whereas  $^{65}\text{Zn}$  eluted as a single peak ( $V_e/V_o = 2.2$ ) when tested alone, it eluted in two peaks (with respective  $V_e/V_o$  values of 1.3 and 2.2) when the  $^3\text{H}$ FTS/ $^{65}\text{ZnCl}_2$  mixture was used. The first of these peaks was coeluted precisely with the FTS biological activity (as assessed in the rosette assay) and the first two-thirds of the  $^3\text{H}$ FTS radioactivity peak. One may thus

Table 3. Reactivation of Chelex 100-treated FTS ( $1 \mu\text{M}$ ) by various metals in a 1:1 metal-to-FTS mole ratio

Synthetic FTS treatment	<i>In vitro</i> activity*
Control, $1 \mu\text{M}$ FTS	$2\text{--}5 \times 10^{-6}$
Chelex 100-treated	1–4
Chelex 100-treated	
+ Mg, Ca, Co, Cd, Tn, Sn, Pd	$1\text{--}2 \times 10^{-6}$
+ Cr, Mn, Fe, Ni, Cu	$0.5\text{--}2 \times 10^{-4}$
+ Al	$1\text{--}2 \times 10^{-6}$
+ Ga	$2\text{--}4 \times 10^{-7}$
+ Zn	$1\text{--}2 \times 10^{-7}$

These experiments were done in triplicate.

\* Expressed as minimal active concentration (ng/ml) in the rosette assay.

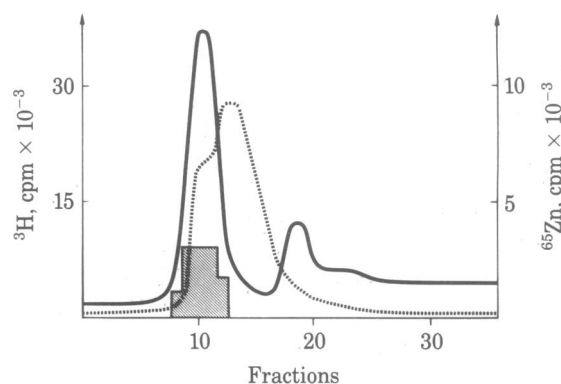


FIG. 3. Gel filtration of Chelex 100-treated  $^3\text{H}$ FTS ( $0.2 \mu\text{M}$ ) incubated with  $^{65}\text{ZnCl}_2$  (molar ratio, 1:1), applied to a Bio-Gel P-2 column, and eluted with  $\text{H}_2\text{O}$ . Fractions (1 ml) were collected. ■, Biological activity as measured by the rosette assay; —,  $^{65}\text{Zn}$  elution; · · ·,  $^3\text{H}$ FTS elution.

assume that it corresponded to FTS-bound Zn. The second peak corresponded to that of Zn tested alone (2.2) and represented free Zn (Fig. 3).

**Presence of Zn in Thymic Epithelium as Detected by Microprobe Analysis.** The presence of Zn in small quantities recognizable by its specific wavelength ( $K\alpha_1$ , 8.63 KeV emission) was detected in some thymic epithelial cells, in a few thymic macrophages, in normal thymic sections, and in cultured epithelial cells. In epithelial cells, Zn was concentrated in dense granules and dense rings inside the clear intracytoplasmic vacuoles (Fig. 4).

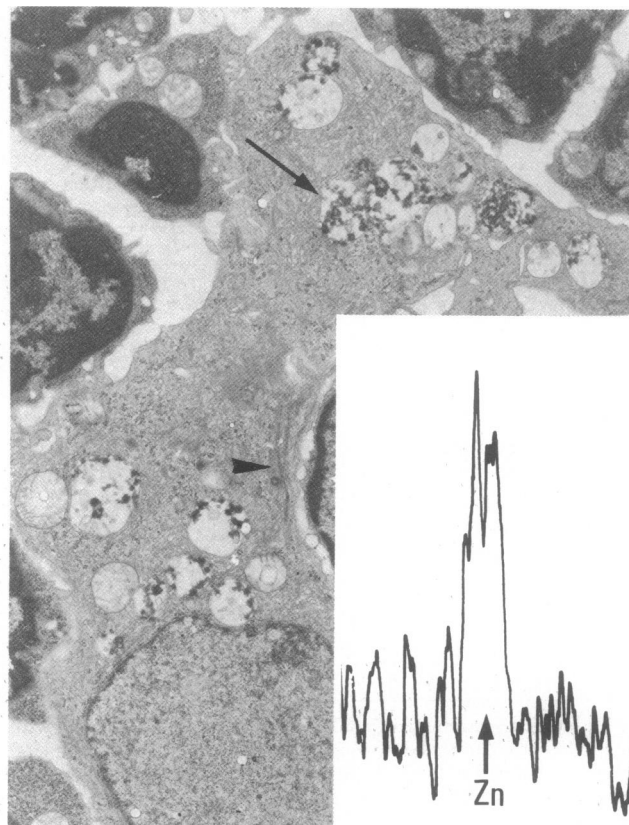


FIG. 4. Micrograph of a thymic epithelial reticular cell, identified by the presence of tonofilaments (arrowhead). The granular material in the clear cytoplasmic vacuoles (arrow) was identified as containing Zn by electroprobe analysis. (Uranyl acetate/lead citrate stain;  $\times 7,500$ .) (Inset) The corresponding Zn wavelength.

**Zn Determination in Synthetic FTS.** This determination was made for four batches of synthetic FTS. The amount of Zn was <200 ppm in two of these batches, which proved to be biologically inactive. In the two other batches, which were fully active, the amount of Zn was respectively 2,181 and 3,623 ppm.

## DISCUSSION

These data demonstrate the importance of metals in the biological activity of FTS; this peptide loses its biological activity after incubation with a chelating agent and recovers it after addition of various metal salts, notably Zn, Al, and Ga salts. The specificity of the effect was assessed by the absence of biological effect in the assay when Zn was tested alone.

The interaction between Zn and FTS was directly shown by gel chromatography of a mixture of Chelex 100-treated [<sup>3</sup>H]FTS and <sup>65</sup>Zn<sup>2+</sup> on Bio-Gel P-2: the [<sup>3</sup>H]FTS and bound <sup>65</sup>Zn were coeluted precisely with the FTS biological activity. The binding affinity was sufficient to allow a separation of free and FTS-bound Zn on Bio-Gel P-2. The best activation was obtained when the peptide and the metal were mixed at a molar ratio of 1:1, which suggests a stoichiometric interaction between zinc and FTS. The coelution of the FTS biological activity and of bound <sup>65</sup>Zn in the first two-thirds of the radioactivity peak of [<sup>3</sup>H]FTS indicates that FTS probably exists in two forms. The first one is biologically active and contains Zn (corresponding to the fastest migrating radioactive FTS in the preceding experiment) and the second is metal-deprived and corresponds to the slower migrating radioactive fraction. We propose "thymulin" as the name for this active Zn-FTS complex.

These studies indicate that metal-deprived FTS is essentially biologically inactive. Synthetic FTS probably acquires its activity by binding the traces of metallic contaminants in some of the reagents used for its synthesis. The presence of Zn at >2,000 ppm has indeed been demonstrated in two active lots of synthetic FTS, whereas two inactive batches showed <200 ppm of Zn. Zn also could be the metal present in natural FTS because it is present in large amounts in mammals (concentrations in human serum range between 0.082 and 0.158 mg/100 ml) and because Zn deprivation induces a thymic involution (13) associated with a decreased level of biologically active FTS (14, 15). The direct proof of the presence of Zn in native FTS will be difficult to obtain because of the quantities of material necessary for the isolation of a sufficient amount of FTS to enable Zn detection. However, an indirect argument in favor of the possible role of Zn activation within the thymus has been provided by electron-microprobe studies that show the presence of Zn in cultured thymic epithelial cells or on thymic sections. Interestingly, an increase of Zn-dense granules in the intracytoplasmic cell vacuoles of Zn-treated animals was noted (unpublished observations). Because the presence of FTS in thymic epithelial cells has been demonstrated by immunofluorescence using heterologous antisera or monoclonal antibodies produced

against synthetic FTS (5, 6), it may be hypothesized that the interaction of Zn and FTS takes place inside the thymic epithelial cells.

Our experiments do not fully document the type of binding between FTS and the metal ions. However, one may note that only three metals (Zn, Al, and Ga) are able to induce full reactivation of the molecule. These metals are all known to induce tetracoordinated complexes with organic molecules. These metals could stabilize the precise conformation of the peptide that is fully biologically active. A better understanding of the coordination geometry of metal ions and FTS will probably be derived from physicochemical methods such as magnetic circular dichroism and nuclear magnetic resonance.

Finally, thymulin, similar to insulin (16) and numerous enzymes (17, 18), appears to be a naturally occurring metalloprotein. The importance of the presence of Zn for the biological activity of the circulating hormone strongly indicates the future use of thymulin rather than Zn-free FTS, for biological evaluation, clinical testing, and radioimmunological studies performed on biological samples.

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