Constitutive expression of a saturable transport system for non-esterified fatty acids in *Xenopus laevis* oocytes*

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In the presence of 150 μM BSA, uptake of [^{3}H]oleate by *Xenopus laevis* oocytes was a saturable function of the unbound oleate concentration ($V_{max.}$ 110 ± 4 pmol/h per oocyte; K_m 193 ± 11 nM unbound oleate). Oleate uptake was three orders of magnitude faster than that of another test substance, [^{35}S]bromosulphophthalein, and was competitively inhibited by 55 nM unbound palmitate ($V_{max.}$ 111 ± 14 pmol/h per oocyte; K_m 424 ± 63 nM unbound oleate) ($P < 0.01$). Oleate uptake was also inhibited by antibodies to a 43 kDa rat liver plasma-membrane fatty acid-binding protein, a putative transporter of long-chain fatty acids in mammalian cells; uptake of the medium-chain fatty acid [^{14}C]octanoate was unaffected. Immunofluorescence and immunoblotting demonstrated that the antiserum reacted with a single 43 kDa protein on the oocyte surface. Hence a protein related to the mammalian plasma-membrane fatty acid-binding protein may play a role in saturable uptake of long-chain fatty acids by *Xenopus* oocytes.

INTRODUCTION

The uptake of long-chain non-esterified fatty acids (NEFA), long considered a passive process [1,2], exhibits all of the kinetic properties of carrier-mediated transport in rat adipocytes [3–5], hepatocytes [6–8], jejunal enterocytes [9] and cardiac myocytes [10]. We have isolated and characterized a 43 kDa plasma-membrane fatty acid-binding protein ($FABP_{pm}$) from each of these cell types [11]. It is unrelated to either the smaller (about 14 kDa) cytoplasmic fatty acid-binding proteins ($FABP_c$s) present in these same tissues [12] or any product of the *fadL* gene complex that mediates NEFA uptake in *Escherichia coli* [13–15]. Antibodies to $FABP_{pm}$ selectively inhibit NEFA uptake, while having no effect on the transport of a number of compounds, including glucose, which are taken up by other carriers [16]. On the basis of this and other, kinetic, evidence, we have postulated that $FABP_{pm}$ is a component of a facilitated transport mechanism for NEFA uptake in mammals.

Xenopus laevis oocytes have been used successfully for the functional cloning of cDNAs for several plasma-membrane transport proteins, including the Na^+-dependent glucose [17] and bile acid transporters [18], amino acid transporters [19], and the Cl^--dependent bromosulphophthalein (BSP) transporter [20]. Before attempting the functional cloning of an $FABP_{pm}$ cDNA in this system, we studied uptake of [^{3}H]oleate into uninjected oocytes. The data suggest that *Xenopus* oocytes contain an endogenous NEFA-transport system mediated by a protein related to mammalian $FABP_{pm}$.

MATERIALS AND METHODS

Chemicals and isotopes

9,10-[^{3}H]Oleic acid (10.0 Ci/mmol), [^{14}C]octanoate (55 mCi/mmol), [^{14}C]BSA (26 mCi/g) and $H_2{}^{35}SO_4$ (carrier-free; 30–43 Ci/mg) were obtained from DuPont–New England Nuclear (Boston, MA, U.S.A.) and ^{125}I-Protein A (30 mCi/mg) was from Amersham (Arlington Heights, IL, U.S.A.). Non-radioactive oleate, BSA (NEFA-free), ouabain, phloretin, palmitic acid, trypsin, 4,4′-di-isothiocyanostilbene-2,2′-disulphonate (DIDS) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were from Sigma (St. Louis, MO, U.S.A.), collagenase A was from Boehringer-Mannheim (Indianapolis, IN, U.S.A.) and BCA protein assay reagents were from Pierce Chemical (Rockford, IL, U.S.A.). [^{35}S]BSP (2.3 Ci/mmol) was synthesized from phenoltetrabromophthalein and $H_2{}^{35}SO_4$ [21] and purified by h.p.l.c. [22].

Oocyte isolation

Oocytes were isolated from mature *Xenopus laevis* females (Xenopus I, Ann Arbor, MI, U.S.A.) as previously described [23,24]. After overnight incubation at 20°C in buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Hepes, 50 units/ml penicillin, 50 μg/ml streptomycin and 125 ng/ml amphotericin, pH 7.5), healthy stage V and VI oocytes [23] of similar size were selected for uptake studies and membrane isolation.

Isolation of $FABP_{pm}$ and preparation of antisera

$FABP_{pm}$ was isolated from sinusoidally enriched rat liver plasma membranes and used to prepare monospecific antisera in outbred New Zealand White rabbits as previously described [6].

Uptake studies

[^{3}H]Oleate-uptake studies were performed in solutions containing 150 μM BSA and oleate concentrations of 13.5–450 μM, prepared in HH buffer, pH 7.4, as described [3,7,10,25]. The oleate/BSA molar ratio employed in the various studies ranged from 0.09:1 to 3:1. The unbound oleate concentration in each solution was estimated by the stepwise equilibrium method [26,27] using the oleate/BSA-binding constants of Spector et al.

Abbreviations used: NEFA, long-chain non-esterified fatty acid; $FABP_{pm}$, plasma-membrane fatty acid-binding protein; $FABP_c$, cytosolic fatty acid-binding protein; BSP, bromosulphophthalein; DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonate; FITC, fluorescein isothiocyanate; HH buffer, Hanks' solution supplemented with 10 mM Hepes and 4.2 mM $NaHCO_3$; V_0, initial uptake velocity.

* This paper is dedicated to Professor Gustav Paumgartner in honour of his 60th birthday.

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[28]. Oocytes, cultured overnight at 20 °C, were washed in HH buffer twice at room temperature. Five to eight oocytes were then incubated at 22 °C in individual vessels containing 1 ml of the [^{3}H]oleate-uptake medium. At 1 h intervals for 4 h, uptake by individual oocytes was stopped by adding 1 ml of ice-cold stop solution (5% BSA plus 200 μM phloretin in HH buffer), and oocytes were separated from the medium by rapid vacuum filtration [6]. Individual oocytes were washed on the filters with a further 10 ml of stop solution and placed into scintillation vials containing 0.25 ml of 10% SDS. Then 5 ml of scintillation fluid (Amersham) was added and the oocytes allowed to dissolve for 2 days before determination of oocyte-associated radioactivity by liquid-scintillation counting. Preliminary studies established that larger volumes of stop/wash solution did not change the oocyte-associated radioactivity remaining on the filter. 'Zero-time' values were measured for each study [6]. Filter blanks were also determined, and contained negligible radioactivity.

Inhibitory effects of ouabain (1 mM), phloretin (200 μM), DIDS (300 μM) and trypsin (0.6 mg/ml) on [^{3}H]oleate influx were tested using previously described protocols [7]. Controls were incubated with the same buffer as the potential inhibitor.

To establish that oocyte-associated radioactivity represented cellular uptake and not simply non-specific binding or entrapment, the following preliminary studies were performed using oocytes from a single harvest. (1) When oleate uptake was measured, as above, from a solution containing 220 μM [^{3}H]oleate and 150 μM [^{14}C]BSA, oocyte-associated ^{14}C never exceeded background over 4 h, whereas [^{3}H]oleate-uptake velocity was 21.0 pmol/h per oocyte. Hence, oocyte-associated [^{3}H]oleate represents material separated from the BSA to which it was bound during incubation. (2) Oleate uptake from a solution containing 220 μM [^{3}H]oleate and 150 μM BSA was determined at 0, 1, 5 and 60 min. The uptake versus time curve was linear (slope 29 $\pm$ 0.8 pmol/h per oocyte; $r = 0.999$) with a y intercept not significantly different from zero (0.07 $\pm$ 0.04 pmol; $0.1 > P > 0.05$). If 5% BSA was omitted from the stop solution, the uptake curve was parallel (slope 31 $\pm$ 0.8 pmol/h per oocyte; $r = 0.998$) but exhibited a positive intercept (0.40 $\pm$ 0.07 pmol). Thus, as demonstrated in mammalian cells [6], inclusion of 5% BSA in the stop solution removes any [^{3}H]oleate that is merely surface-bound before determination of oocyte-associated radioactivity. (3) Groups of 10–15 oocytes were incubated for 1 or 4 h in 220 μM [^{3}H]oleate plus 150 μM BSA and then mechanically disrupted. Lipids were extracted in chloroform/methanol (2:1, v/v), separated by t.l.c. and detected by exposure to iodine vapour [29]. [^{3}H]Oleate extracted from the incubation medium migrated as a single band. In the oocyte extracts, nine bands in addition to oleate were identified and scraped from the plates; radioactivity in each was measured by liquid-scintillation spectrometry. Unmetabolized [^{3}H]oleate accounted for only 1.6% of oocyte-associated radioactivity at 1 h, and 0.3% at 4 h, demonstrating that oocyte-associated [^{3}H]oleate had entered intracellular metabolic pathways. No detailed efforts were undertaken to identify the labelled bands. However, by comparison with R_F values of known standards, they included phospholipid, triolein and cholesteryl oleate [29].

[^{35}S]BSP was dissolved in HH at a concentration of 2 μM, and BSP uptake was determined over 4 h as described for oleate.

Competitive inhibition

To study the effects of another NEFA on oleate uptake by *Xenopus* oocytes, 150 μM BSA was combined with predetermined quantities of palmitate and [^{3}H]oleate chosen to yield an unbound palmitate concentration of 55 nM and unbound oleate concentrations identical with those used in the uptake studies described above. Concentrations of unbound palmitate and oleate in these solutions were calculated by a modification (D. Sorrentino, K. Van Ness, A. J. Schwab, D. Stump, C. A. Goresky and P. D. Berk, unpublished work; details available on application to the authors) of the stepwise equilibrium method [26,27,30], using the binding constants of Spector et al. [28].

Antibody inhibition

Oocytes were washed in HH buffer and incubated at 22 °C for 1 h with a 1:10 dilution of rabbit anti-(rat $FABP_{pm}$) serum or the same dilution of preimmune rabbit serum as control. They were then washed three times in HH buffer and incubated with [^{3}H]oleate/[^{14}C]octanoate/BSA (each 150 μM) in HH buffer to determine fatty acid uptake, as above.

Kinetic analysis

The initial oleate or BSP-uptake velocity (V_0) was taken to be the slope of the uptake versus time curve over the first 3 h, determined by least squares. The correlation coefficient in all cases was 0.9 or greater. To examine the kinetic features of oleate uptake, the data were fitted directly to the Michaelis–Menten equation using the SAAM program of Berman and Weiss [31], and to the Woolf linear transformation of the Michaelis–Menten equation. No significant differences resulted from the alternative fitting procedures.

Immunofluorescence

Oocytes were washed three times in PBS buffer, pH 7.5, fixed in 10% formaldehyde, sectioned and mounted on glass slides. The slides were incubated with 10% normal goat serum for 1 h, washed three times in PBS, and incubated with anti-$FABP_{pm}$ serum diluted 1:200 in 5% normal goat serum. Controls employed preimmune serum instead of anti-$FABP_{pm}$. After extensive washing in PBS, detection was with FITC-conjugated goat anti-rabbit immunoglobulin diluted 1:100 in PBS, using standard methods.

Immunoblotting of $FABP_{pm}$

Crude membrane fractions were prepared from 10–20 healthy oocytes by loose Dounce homogenization and differential centrifugation [32]. Membrane proteins were extracted by gentle sonication (30 min on ice) in 1% Triton X-100 and separated by 5–25% gradient SDS/PAGE. Standard Western-blotting methods were used to assess immunological reactivity between the membrane proteins and anti-$FABP_{pm}$ serum [6,33].

RESULTS AND DISCUSSION

Fatty acid-uptake kinetics

The time course of cumulative [^{3}H]oleate or [^{35}S]BSP uptake was linear for at least 3 h (Figure 1). [^{3}H]Oleate-uptake velocity from a solution containing 75 μM oleate and 150 μM BSA (unbound oleate 43 nM) [8.2 $\pm$ (S.D.) 1.0 pmol/h per oocyte] was nearly three orders of magnitude faster than that of 2 μM BSP in the absence of BSA (12.5 $\pm$ 4.0 fmol/h per oocyte), and similarly exceeded uptake velocities for glucose [17] and taurocholate [18]. This led us to suspect the existence of a facilitated NEFA-uptake mechanism. When V_0 for oleate was plotted as a function of the unbound oleate concentration, saturation of uptake was observed (Figure 2). The computed $V_{max.}$ was 110 $\pm$ 4 pmol/h per

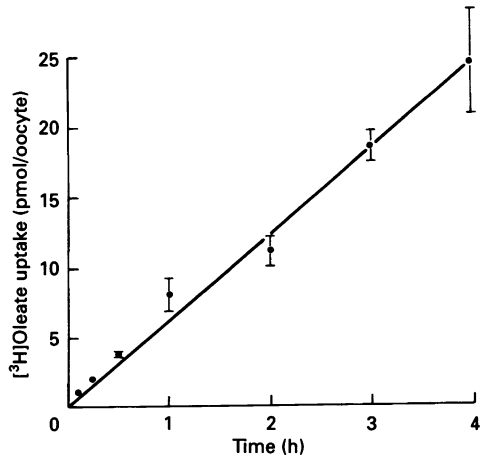

Figure 1 [^{3}H]Oleate uptake versus time

In the studies illustrated, BSA concentration was 150 μM and total oleate concentration 75 μM. Data represent means $\pm$ S.D. of three to five separate determinations in two separate oocyte harvests.

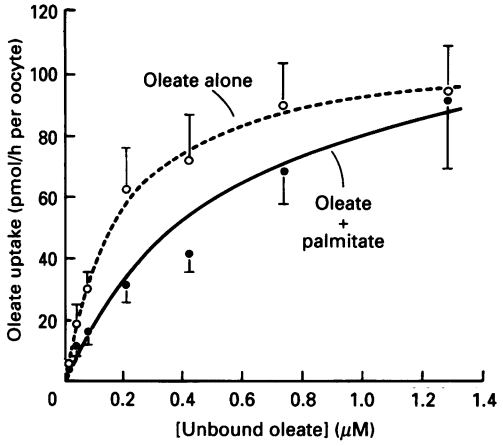

Figure 2 Oleate uptake as a function of the unbound oleate concentration in the presence (●) and absence (○) of 55 nM unbound palmitate

Data points represent means of 10–20 separate measurements in oocytes from at least two separate harvests. Curves illustrate best computer fits of the data to the Michaelis–Menten equation. Results indicate saturation of uptake with increasing concentrations of unbound oleate, and inhibition of oleate uptake by palmitate.

oocyte and the K_m 193 $\pm$ 11 nM unbound oleate. Palmitate competitively inhibited oleate uptake (Figure 2). In the presence of 55 nM unbound palmitate, the $V_{max.}$ was 111 $\pm$ 14 pmol/h per oocyte ($P > 0.5$, not significant) and the K_m was 424 $\pm$ 63 nM ($P < 0.01$). These data offer further support for the proposed transport system. The K_m for oleate uptake in *Xenopus* oocytes is similar to that in rat hepatocytes (153 $\pm$ 55 nM) [34], suggesting that saturable transport in both systems is mediated by proteins with similar affinity for the substrate.

Ouabain, phloretin, trypsin and DIDS did not inhibit oleate uptake in *Xenopus* oocytes. Although the first three of these have been reported to inhibit NEFA uptake in rat hepatocytes [6,7], the observations in oocytes are consistent with those in other mammalian cell types, such as adipocytes [3–5].

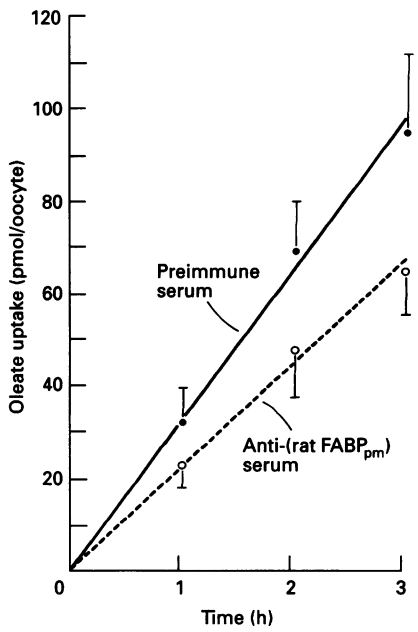

Figure 3 Effect of rabbit anti-(rat liver FABP$_{pm}$) serum (○) on [^{3}H]oleate uptake by *Xenopus* oocytes

Experimental conditions are described in the text. Data points represent the means of 20–30 separate determinations using oocytes from at least two separate harvests.

Antibody-inhibition studies

Rabbit antisera to rat liver FABP$_{pm}$ inhibited [^{3}H]oleate uptake by *Xenopus* oocytes by up to 31% ($P < 0.02$) (Figure 3). The antibody had no effect ($P > 0.2$) on uptake of [^{14}C]octanoate (results not shown), a medium-chain fatty acid which enters mammalian cells by mechanisms different from these employed by fatty acids of chain length 12 or more [4]. Although highly significant, antibody inhibition of oleate uptake by oocytes was less than that seen in rat tissue adipocytes (63%) [5] and hepatocytes (65%) [7], and in mouse 3T3-L1 adipocytes (55%) [16]. This may simply reflect a relatively larger proportion of non-specific uptake in oocytes than in mammalian cells [34].

Immunofluorescence studies

When stained with rabbit anti-(rat liver FABP$_{pm}$) serum, both intact (Figure 4a) and sectioned (Figure 4b) *Xenopus* oocytes demonstrated an intense pattern of specific plasma-membrane immunofluorescence, similar to that observed with rat hepatocytes [6–8], tissue adipocytes [3–5] and 3T3-L1 adipocytes [16]. Oocytes also exhibited positive cytoplasmic staining. In view of immunological cross-reactivity between FABP$_{pm}$ and the mitochondrial isoenzyme of aspartate aminotransferase [33], the cytoplasmic signal may be from mitochondria. Oocytes incubated with preimmune serum exhibited only minimal background fluorescence.

Identification of FABP$_{pm}$ in oocyte membranes

In immunoblots of *Xenopus* membrane protein extracts, FABP$_{pm}$ antisera detected only a single 43 kDa protein band, which migrated identically with authentic purified rat liver FABP$_{pm}$ (Figure 5). An identical band was seen with corresponding extracts of mouse and rat liver and adipose tissue, and purified FABP$_{pm}$ protein from both species (not shown), demonstrating

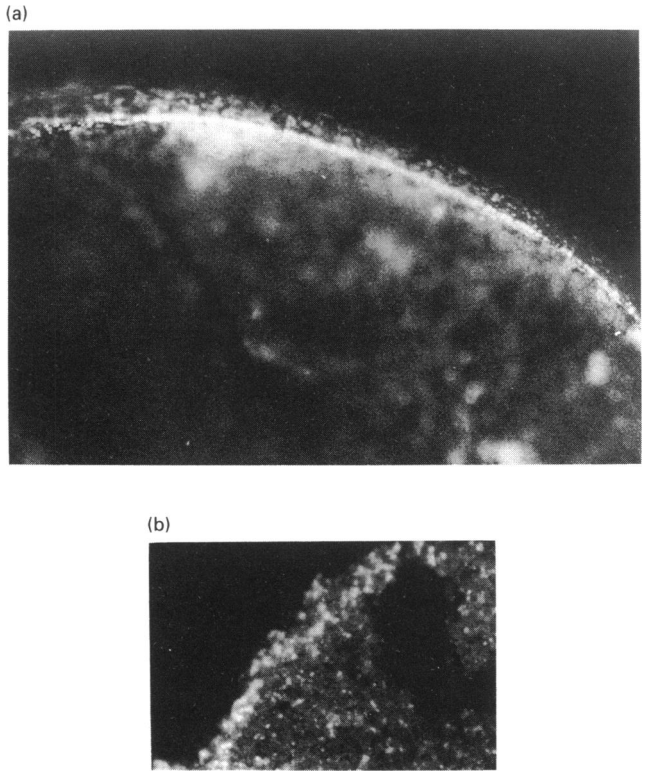

Figure 4 Immunofluorescent staining of *Xenopus* oocytes with rabbit anti-(rat liver $FABP_{pm}$) serum using FITC-conjugated goat anti-rabbit immunoglobulin for detection

Experimental details are provided in the text. Substitution of preimmune rabbit serum for the specific antibody resulted in only minimal background immunofluorescence. (**a**) Intact oocyte (magnification × 58), emphasizing staining of the peripheral membrane. (**b**) Portion of a transverse section (magnification × 58) demonstrating both intense membrane staining and some interior staining, presumably in mitochondria.

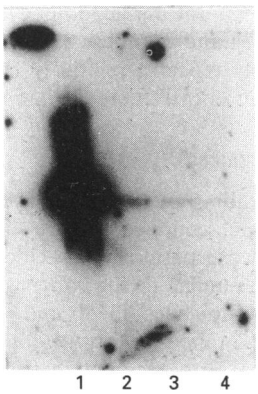

Figure 5 Immunoblots of Triton X-100 extracts of *Xenopus* oocyte membrane proteins

See the text for details. Lane 1, 1.0 μg of purified rat liver $FABP_{pm}$. Lanes 2–4, oocyte membrane extracts containing 29, 15 and 7.5 μg respectively of total protein. Detection was with a 1:1000 dilution of rabbit anti-(rat liver $FABP_{pm}$) serum followed by ^{125}I-Protein A.

immunological cross-reactivity among those species. The relative inhibition of oleate uptake, but not that of octanoate, by monospecific anti-(rat liver $FABP_{pm}$) serum, coupled with the detection by immunofluorescence and immunoblotting of a membrane protein related immunologically to $FABP_{pm}$, suggests that *Xenopus* oocytes not only contain an endogenous NEFA-transport system, but that transport is mediated by a protein related to the mammalian transporter described previously [25,33].

The mammalian hepatocyte has at least three distinct membrane transport systems for organic anion uptake. One of these mediates Na^+-dependent uptake of polar bile acids such as taurocholate, the second, uptake of non-bile acid cholephils such as BSP and bilirubin, and the third, NEFA uptake. The system mediating non-Na^+-dependent uptake of less polar bile acids has been uncertain, although some data suggested that this process uses the same system as BSP and bilirubin (reviewed in ref. [35]).

cDNAs for both the Na^+-dependent bile acid transporter [18] and the BSP/bilirubin transporter [20] have now been cloned successfully using an expression cloning strategy in *Xenopus laevis* oocytes. Expression in *Xenopus* of the cloned BSP/bilirubin transporter was associated not only with facilitated BSP uptake, but also with non-Na^+-dependent bile acid transport [20]. Using the rat liver Na^+-dependent bile acid-transporter cDNA as a probe, a corresponding mRNA was detected by Northern-blot analysis in livers of several mammalian species [36]. This message was not detected in the livers of non-mammalian species, including chicken, turtle, frog and small skate (*Raja erinacea*). By contrast, when RNA from the small skate was injected into *Xenopus* oocytes, only non-Na^+-dependent bile acid uptake was expressed, indicating the absence of an mRNA for the Na^+-coupled transport mechanism in the skate [36]. These data suggest that mammalian liver has evolved both Na^+-dependent and Na^+-independent transport systems for bile acids and other organic anions, whereas more primitive vertebrates have only the latter. This conclusion is consistent with comparative studies of hepatocyte bile acid-uptake kinetics [37,38], and also with comparative plasma-membrane bile acid-binding and photoaffinity-labelling studies in mammalian and non-mammalian species [37,39–41]. Although the Na^+-dependent bile acid transporter is new in evolutionary terms, the precise phylogenetic age of the older Na^+-independent bile acid/BSP/bilirubin-transport system has not yet been established.

Before the present studies, facilitated NEFA-uptake systems had been demonstrated principally in mammalian cells [3–11] and bacteria [13–15]. Based on the known properties of their putative transport proteins [15,33], the mammalian and bacterial systems appear to be unrelated. The demonstration in *Xenopus* oocytes of an endogenous NEFA-transport system mediated by a protein related to mammalian $FABP_{pm}$ suggests that this is, phylogenetically, a very old system. Its phylogenetic distribution will be of considerable interest, particularly in relation to the evolution of other plasma-membrane transport systems. The role of the facilitated NEFA-uptake system in the overall biology of *Xenopus*, and the possible existence in *Xenopus* of other putative mammalian NEFA transporters [42,43], remain to be determined.

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