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Effects of dietary polyunsaturated fatty acids on adenylate cyclase, 5'nucleotidasa and Na^+K^+-ATPase activities in rat brain-plasma membrane

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SUMMARY. The incidence of polyunsaturated fatty acids (PUFA) in human nutrition is now generally accepted. As essential membrane components, PUFA may act as enzyme activity modulators. In this study, four different diets, in which PUFA type was the only modifying factor, were evaluated on 5'nucleotidase, adenylate cyclase and Na^+/K^+ATPase activities in rat brain plasma membranes. Animals fed the total PUFA deficient diet exhibited significant lower body weight and lower brain weight than did the control group. The specific activities of 5'nucleotidase and Na^+/K^+ATPase in brain plasma membrane were slightly modified by dietary PUFA. The catalytic unit of adenylate cyclase in total PUFA deficient animals presented augmented enzyme activity and animals receiving diets deficient in n-6 PUFA showed reduced activity in relation to the control animals. Our results showed that the epinephrine receptors, in the case of adenylate cyclase are not modified by dietary PUFA, but rather the catalytic unit seems to be altered by dietary PUFA. These results can be partially explained by the fluidity that PUFA confers to membranes facilitating the proximity of enzyme-substrate. The physiological consequences of dietary PUFA incidence on enzyme activity needs further study.

Key words: PUFA deficiency, brain plasma membrane, adenylate cyclase, 5'nucleotidase, Na^+K^+ATPase.

RESUMEN. Efecto de los ácidos grasos poliinsaturados dietarios en la actividad de la adenilato ciclasa 5'nucleotidasa y Na^+K^+-ATPasa de la membrana plasmática del cerebro de ratas. Los efectos de los ácidos grasos poliinsaturados (AGPI) en la nutrición humana son hoy en día ampliamente aceptados. Como componentes esenciales de las membranas, los AGPI pueden actuar modificando actividades enzimáticas. Se estudiaron cuatro dietas distintas, en las cuales el tipo de AGPI fue el único componente distinto, en las actividades de la 5'nucleotidasa, Na^+K^+aTPasa y adenilato ciclasa de la membrana plasmática de cerebro de rata. Los animales alimentados con la dieta totalmente deficiente en AGPI presentaron peso corporal y peso del cerebro inferior al encontrado en los animales del grupo testigo. Las actividades específicas de la 5'nucleotidasa y la Na^+K^+ATPasa de la membrana plasmática del cerebro fueron ligeramente modificadas por los lipidos dietarios. La unidad catalítica de la adenylato ciclasa aumentó su actividad en los animales que recibieron la dieta totalmente deficiente en AGPI, mientras que esta actividad disminuyó en los animales con la dieta deficiente en AGPI n-6, en relación a la actividad del grupo testigo. Nuestros resultados muestran que los receptores de la epinefrina, en el caso de la adenilato ciclasa, no se modifican por los lípidos dietarios, pero éstos si afectan la unidad catalítica de la enzima. Estos resultados pueden ser parcialmente explicados por la fluidez que los AGPI confieren a la membrana, facilitando así la proximidad de enzima-sustrato. Las consecuencias fisiológicas de los efectos de los AGPI dietarios en las actividades enzimáticas requiere de mayor estudio.

Palabras clave: Deficiencia de AGPI, membrana plasmática cerebral, adenilato ciclasa, 5'nucleotidasa, Na^+K^+ATPasa.

INTRODUCTION

The role of n-6 and n-3 polyunsaturated fatty acids (PUFA) has been largely studied, however, the specific mechanisms of action are yet to be outlined. As essential membrane components, PUFA may act as enzyme activity modulators. Polyunsaturated fatty acids esterified to glycerolphosphoryl bases are, with cholesterol, the main components of the lipid matrix of biological membranes. Their relative proportions determine the biophysical and physiological properties of these membranes (1). However, the composition of brain lipids is generally less readily modified by dietary factors compared with other organs (2). Nevertheless, dietary modifications induced, albeit moderate, appear to alter membrane enzyme activity and other physiological conditions (3).

Plasma membrane proteins (enzymes, receptors, and ionic channels) are directly related to the membrane lipid bilayer. Examples of these are the 5'nucleotidase, adenylate cyclase and Na^+/K^+ATPase 5'nucleotidase catalyzes the dephosphorylation of adenosine 5'monophosphate (5'AMP). Adenylate cyclase is directly related to the cellular communication and as such plays a fundamental role in regulating cellular metabolism via the cyclic adenosine

monophosphate (cAMP). Na^+/K^+ATPase is necessary for the ionic shuttle across the membrane cell, therefore, it plays a crucial role in controlling the ionic environment essential for neuronal activity. To our knowledge, very little research has been published on the effect of dietary PUFA on the activity of adenylate cyclase in brain plasma membrane (4). These authors reported no change in adenylate cyclase activity in neuroblastoma cells rich in 18:2 n-6. However, later on, the research group of Murphy (5) reported a high adenylate cyclase activity of neuroblastoma under the same conditions. The effects of dietary PUFA on the activity of 5'nucleotidase although largely studied, the results are contradictory. In fact, while Bernshon and Spitz (6) observed a reduced activity in 5'nucleotidase activity in brain plasma membrane depleted of PUFA, others (7) reported no change in this activity under the same diet. Na^+/K^+ATPase activity has been reported to be elevated in animals receiving a diet deficient in total PUFA (8). The n-3PUFA deficient diet produced a 40% reduction in Na^+/K^+ATPase activity from rat synaptosomes (9). However, very frequently comparison between reported results are not as evident due to the discrepancies in control groups. Nevertheless, the dependence of these three enzymes on PUFA membrane environment appears important. Therefore In this study, the effects of four different diets on the 5'nucleotidase adenylate cyclase and Na^+/K^+ATPase activities in rat brain plasma membranes are compared.

MATERIALS AND METHODS

Experimental animals

Wistar rats were given a semisynthetic diet in which lipids were supplied by 5% of the respective oil (w/w). The protocols used were as described previously (10). In general, lipids were supplied by either peanut and rapeseed oil mixture (control), peanut oil (n-3 PUFA deficient), cod liver oil (n-6 PUFA deficient) or partially hydrogenated palm oil (total PUFA deficient). The diet and the fatty acid composition of dietary lipids are presented in Tables 1 and 2. Ten animals were put on each diet right after weaning and until adult age (90 d), when killed by decapitation. Brain were immediately removed, weighed and homogenized for membrane preparation as described in the following section.

Membrane purification

Brain plasma membranes were separated by a modified method (11). Briefly, 2g of brain were homogenized with 10 ml of cold buffer (Hepes:KOH, 5 mM; pH=7.4) with a teflon potter homogenizer (Thomas C) using 15 up and down strokes at 1 000 rpm. The homogenate was filtered through a tissue and centrifuged at 10 000 rpm (12 000 xg) for 10 minutes in a Beckman JA20 centrifuge. The supernatant, diluted to 75% with Percoll, was then placed at the bottom of the tube followed by a step Percoll gradient (30, 25, 18, 10 and 0% v/v) (Pharmacia) and centrifuged at 20 000 rpm (48 000 xg) for 4 minutes at 4°C. The plasma membrane fraction was collected at the 10%-0% interface. Then it was diluted with a Percoll solution (75%, v/v) to a ratio of 2 ml of Percoll per 5 ml of membrane fraction. Seven ml of this membrane fraction were placed again at the bottom of the tube followed by a step gradient (18%, 10%, 0% v/v) and centrifuged using the conditions mentioned above. The plasma membranes obtained were immediately used for enzyme activity measurements. Protein analysis was done after the Bradford method (12).

TABLE 1
Diet composition (g/kg)

Composition %	Group 1 Control	Group 2 n-3 PUFA Deficient	Group 3 n-6 PUFA Deficient	Group 4 Total PUFA Deficient
Casein	22.0	22.0	22.0	22.0
DL methionine	0.16	0.16	0.16	0.16
Cellulose	2.0	2.0	2.0	2.0
Starch	43.90	43.90	43.90	43.90
Sucrose	21.94	21.94	21.94	21.94
Vitamin mixture[a]	1.0	1.0	1.0	1.0
Mineral mixture[b]	4.0	4.0	4.0	4.0
Hydrogenated palm oil	-	-	-	5.0
Peanut oil	2.5	5.0	-	-
Cod liver oil	-	-	5.0	-
Rapeseed oil	2.5	-	-	-

a. Total vitamin supplement, United States Biochemical corp. Cleveland, OH.
b. Composition g/100 g: $CaHPO_4.2H_2O$, 38.0; K_2HPO_4, 24.0; $CaCO_3$,18.0; NaCl, 6.9; MgO, 2.0; $MgSO_4.7H_2O$, 9.0; $FeSO_4.7H_2O$, 0.086; $ZnSO_4.H_2O$, 0.5; $MnSO_4.H_2O$, 0.5; $CuSO_4$, $5H_2O$,0.1; NaF, 0.08; $CrK(SO_4)_2.H2O$, 0.05; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.002; KI, 0.004; $CoCO_3$, 0.002; $NaSeO_3.5H_2O$, 0.002.

TABLE 2
Fatty acid composition of dietary lipids

Fatty Acids (%)	Group 1 Control	Group 2 n-3 PUFA Deficient	Group 3 n-6 PUFA Deficient	Group 4 Total PUFA Deficient
Fatty acids/ 100 g of diet:				
n-6PUFA (mg)	930.6	935.3	136.3	84.6
n-3PUFA (mg)	188.0	4.7	1113.9	-

Control diet = mixture of peanut and rapeseed oil, n-3 PUFA deficient diet = peanut oil, n-6 PUFA deficient diet = cod liver oil, total deficient diet = hydrogenated palm oil.

Plasma membrane lipid analysis

Total lipids from plasma membrane were extracted by the Folch et al. procedure (13) in the presence of butyl hydroxytoluene (BHT) at a 0.02% (w/v).

Total phospholipid fatty acid methyl esters (FAME) were

analyzed by gas chromatography under the conditions described previously (10).

Enzyme activity

5'Nucleotidase (E.C.3.1.3.5)

5'nucleotidase activity of brain plasma membrane was determined by measuring the degradation of (3H)AMP (adenosine monophosphate) in a liquid scintillation detector (Packard model TRI-CARB 1500). The assay was carried at 37°C during 60 min in an incubation mixture containing Tris/HCl 50 mM, MgCl2 180 M, AMP(Na) 254 M and (3H) cAMP 15 Ci/nmole pH=7.4. The enzyme activity was calculated taking into account the isotope specific activity (33x110-6 dpm corresponding to 1 nmole of (3H)AMP hydrolyzed) (14).

Adenylate Cyclase (E.C. 4.6.1.1.)

Adenylate cyclase activity of brain plasma membrane was determined after Salesse technique (15) by measurement of labeled cyclic (3H) AMP generated from the substrate ATP in the presence of 20 mg protein. The reaction was carried out at 37°C during 20 min. The incubation mixture contained creatine phosphate (100 mM), creatine phosphokinase (375 U/ml), myokinase (850 µg / ml), ATP 0.1 M, adenosine deaminase (1000 U/ml), isobutylmethylxanthine (200mg / 200µl DMSO). Basal adenylate cyclase activity was measured in the presence of GTP (10 mM) and stimulated adenylate cyclase activity was determined replacing GTP by epinephrine, GppNHp (10mM), forskoline (300 mM) and forskoline+ GDPßs (0.003 M). The cyclic AMP produced was separated from the incubation mixture by fixing it to the protein kinase (4 mg/ml) in the presence of saturated 3H cAMP (2.2 pmoles/assay). The complex was stabilized by addition of protein kinase inhibitor (3 mg/ml). The cyclic AMP produced was measured by liquid scintillation after separation from the incubation mixture.

Na^+/K^+ATPase (E.C. 3.6.1.37)

The activity of Na^+/K^+ATPase was measured after a modified Fujita et al. technique (16). The analysis was carried out in 20 µg of membrane protein and 1 ml of an incubation mixture containing Tris /HCl 54 mM, NaCl 125 mM, KCl 21.5 mM, MgCl2 5mM, and ATP 10mM in the presence and abscence of ouabaine 0.5mM. The reaction was done at 37°C during 30 min using a mixture of TCA (20%) and ascorbic acid (4%) to stop the reaction. The phosphorus produced was measured by the Baginski et al. (17) technique: an aliquot of 0.3 ml was mixed with 0.7 ml of TCA-Ascorbic acid solution at 30%. The addition of 0.5 ml of ammonium molybdate (1%) and arsenate-citrate solution favors the color reaction which was measured after 15 min at 840 nm.

Statistical methods

Results were analyzed statistically by standard analysis of variance (ANOVA) at a probability level of $p < 0.05$

RESULTS AND DISCUSSION

Body and brain weight

Animals fed diets containing partially hydrogenated palm oil exhibited significantly lower body weight (294 ± 18 g) and lower brain weight (1.73 ± 0.08 g) than did animals from the control group (372 ± 14 g, 1.97 ± 0.04 g). A similar body weight reduction was observed in animals receiving 12% corn oil diet, due mainly to differences in energy intakes (18). Protein per g of brain was around 21 mg independently of diet (Table 3).

TABLE 3
Dietary fatty acid effects on body and brain weight

	Diets			
	Control	n-3 PUFA Deficient	n-6 PUFA Deficient	Total PUFA Deficient
Body weight (g)	372±14[a]	355±33[a]	378±27[a]	294±18[b]
Brain weight (g)	1.97±0.04[a]	1.98±0.09[a]	1.97±0.05[a]	1.73±0.08[b]
Protein/brain (mg/g)	21.8±1.6[a]	20.5±1.6[a]	21.3±1.7[a]	21.2±1.9[a]

Results are the mean ±standard deviation
Control diet = mixture of peanut and rapeseed oil, n-3 PUFA deficient diet = peanut oil, n-6 PUFA deficient diet = cod liver oil, total deficient diet = hydrogenated palm oil.
Means with different letter are statistically different ($p < 0.05$).

Total phospholipid fatty acid composition

In general, total saturated fatty acids were not modified by dietary lipids as was expected since de novo synthesis also contributes to saturated fatty acid pool. Monoenes were slightly modified by the diet, indeed total MUFA were maintained around 35.2%. The n-3 PUFA deficient diet produced, on the contrary, a significant ($p<0.01$) increase in the n-6 PUFA family. This was due primarily to the increase in 22:5 n-6 which is the longest fatty acid in this family. This effect was compensated by a reduction in 22:6 n-3 (10.1vs4.1%) the most important of the n-3 PUFA. The diet rich in fish oil produced higher levels of the n-3 PUFA members than those reported in the control group. The reduction of the n-6 PUFA was also a characteristic of the fish oil rich diet. These fatty acid modifications corresponded to the expected effects of each diet (Table 4).

5'Nucleotidase activity

The specific activity of 5'nucleotidase in brain plasma membrane was in a range from 29.4 to 32.7 µmol PO4/mg protein. h (Table 5). Statistical analysis showed a slightly difference in 5'Nucleotidase activity between that of the control group (30.2) and the n-6PUFA (32.7) and total PUFA deficient animals (32.5).

TABLE 4
Effect of dietary lipids on brain membrane total phospholipid fatty acid composition

Fatty acids (%)	Diets			
	Control	n-3 PUFA Deficient	n-6 PUFA Deficient	Total PUFA Deficient
16:0	20.7±1.5^{a}	21.5±0.8^{a}	20.9±1.2^{a}	20.9±1.0^{a}
18:0	18.4±0.7^{a}	16.8±0.6^{b}	17.1±0.3^{b}	17.8±0.2^{a}
ΣSFA	**40.9±1.4^{a}**	**40.5±0.7^{a}**	**41.0±0.9^{a}**	**40.5±0.6^{a}**
18:1 n-9	23.1±0.7^{a}	22.0±0.5^{b}	25.9±0.6^{c}	21.7±0.5^{b}
18:1 n-7	5.2±0.2^{a}	5.5±0.2^{b}	5.2±0.1^{a}	5.1±0.2^{a}
20:1 n-9	3.4±0.3^{a}	3.1±0.2^{ab}	3.0±0.2^{b}	2.6±0.2^{c}
20:1 n-7	1.1±0.1^{a}	1.1±0.1^{a}	1.0±0.1^{a}	0.9±0.1^{a}
ΣMUFA	**35.1±1.2^{a}**	**34.1±0.9^{ac}**	**37.8±0.8^{b}**	**33.5±1.0^{c}**
18:2 n-6	0.5±0.1^{a}	0.6±0.1^{a}	0.6±0.2^{a}	0.6±0.1^{a}
20:3 n-6	0.3±0.1^{a}	0.3±0.1^{a}	0.3±0.1^{a}	0.2±0.1^{a}
20:4 n-6	8.7±0.5^{a}	9.6±0.3^{b}	6.0±0.4^{c}	8.5±0.4^{a}
22:4 n-6	3.2±0.4^{a}	3.9±0.2^{b}	1.5±0.2^{c}	2.8±0.2^{a}
22:5 n-6	0.9±0.2^{a}	6.8±0.5^{b}	0.9±0.5^{a}	6.2±0.5^{b}
ΣPUFA n-6	**13.7±1.0^{a}**	**21.3±0.8^{b}**	**9.2±1.1^{c}**	**18.3±0.9^{d}**
20:5 n-3	- -	- -	0.4±0.1	- -
22:5 n-3	0.1±0.0	- -	0.9±0.1	- -
22:6 n-3	10.1±1.1^{a}	4.1±0.7^{b}	11.6±1.1^{a}	5.8±0.5^{c}
ΣPUFA n-3	**10.3±1.1^{a}**	**4.1±0.7^{b}**	**13.0±1.1^{c}**	**6.0±0.6^{d}**
Σn-6+n-3	24.1±1.9^{ab}	25.4±0.9^{a}	22.1±1.1^{b}	24.3±0.6^{a}
n-6/n-3	1.3±0.1^{a}	5.2±0.9^{b}	0.7±0.1^{c}	3.1±0.4^{d}
22:5 n-6/22:6 n-3	0.09±0.0^{a}	1.69±0.30^{b}	0.07±0.02^{a}	1.07±0.17^{c}

Results are the mean ±standard deviation of ten animals in each group
control diet = mixture of peanut and rapeseed oil, n-3 PUFA deficient diet = peanut oil, n-6 PUFA deficient diet = cod liver oil, total deficient diet = hydrogenated palm oil.

TABLE 5
Dietary fatty acid effects on brain plasma membrane 5'nucleotidase and Na^+K^+ATPase activities

Activity μmoles PO4 / mg prot. h	Diets			
	Control	n-3 PUFA Deficient	n-6 PUFA Deficient	Total PUFA Deficient
5´Nucleotidase	30.2±1.4^{a}	29.4±2.5^{a}	32.7±2.4b	32.5±3.2b
Na^+K^+ATPase	131.0±30.0^{ab}	114.0±20.0^{a}	138.0±13.6^{b}	1160±24.0^{a}

Results are the mean ±standard deviation
control diet = mixture of peanut and rapeseed oil, n-3 PUFA deficient diet = peanut oil, n-6 PUFA deficient diet = cod liver oil, total deficient diet = hydrogenated palm oil.
Means with different letter are statiscally different ($p < 0.05$)

It has been reported no diet-related difference in the activity of the enzyme in myelin membrane (6, 19). The low activity of the enzyme reported by others (4, 6) was not confirmed in this study.

5´nucleotidase activity has been reported to be dependent on PI microenvironment . In this study, PI levels were not modified by the individual n-6 and n-3 PUFA deficiency (data not shown), probably helping to maintain enzyme activity unchanged. It is also important to mention that the n-6/n-3 ratio depicted in this particular phospholipid (PI) was the highest of all other PL. Does this mean that the PL microenvironment was sufficient to maintain enzyme activity unchanged? Phospholipid proportions in other studies reporting 5´nucleotidase modification by dietary lipids are not reported.

Na^+/K^+ATPase

The specific activity of Na^+K^+ATPase in brain plasma membranes varied between 114 and 138 μmol PO4/ mg protein h. The activity of this enzyme appeared not to be modified by any of the dietary deficiencies in relation to the control group. However, a slight difference was observed between the activity of animals getting the individual (n-6 and n-3) PUFA deficiencies and the total PUFA deficient group (Table 5). The elevated Na^+K^+ATPase activity reported (20) during n-6 PUFA deficiency, however, seems to be related to the animals general altered physiology (hair loss, skin problems, etc.) rather than dietary manipulations only.

The effect of dietary alfa-linolenic acid and fish oil on Na^+/K^+ATPase isoenzymes were analyzed in brain (21,22) concluding that out of the three isoenzymes conforming the Na^+K^+ATPase, the sensitiveness of 2 and 3 isoenzymes to sodium were dependent of omega 3 fatty acids but no correlation was observed for the sensitiveness of 1 isoenzyme. Our results might be explained by the differences in ouabain sensitiveness that presented this enzyme, according to the results depicted above.

Recently, the incidence of dietary PUFA on neurotransmission has been related to an increase in dopamine and serotonin levels under diets deficient in n-6 and total PUFA respectively, suggesting a different mechanism of PUFA incidence in the neurotransmission system (23). The neurotransmission system disorder, during EFA deficiency, appears not to be related to enzyme activity, perhaps neurotransmitter molecule levels are impaired

When considering changes in enzyme activities very often reports present different control and therefore it is not surprising to find discrepancies in results. Na^+K^+ATPase is a key enzyme in the neurotransmitter system and for a long time it has been postulated (8) to be modulated by PUFA. Nevertheless, in our study only a slight change was observed in this enzyme when comparing the individual PUFA deficiency and the total PUFA deficiency group.

Adenylate Cyclase

Adenylate cyclase complex presents three different sites where PUFA may interfere: the receptor site (epinephrine), the regulating site (Protein G) and the catalytic site per se.

Basal adenylate cyclase activity in brain plasma membrane was not modified by dietary PUFA deficiencies. Forskoline and epinephrine stimulated adenylate cyclase (receptor site) was not influenced by dietary PUFA deficiencies.

On the other hand, the activity of the enzyme stimulated by the forskoline + GDP ßs which gives information on the

catalytic unit was reduced by the n-3 PUFA deficient diet in comparison to the activity from the total PUFA and n-6 PUFA deficient animals (Table 6). Murphy has been working extensively on the effect of PUFA on neuroblastoma cells and he concluded that the mechanisms involved in the stimulation of cAMP through PUFA was multifactorial (24). To our knowledge, no other studies have reported the incidence of dietary PUFA on adenylate cyclase activity in brain. Since, on one hand, this enzyme plays a fundamental roll in the metabolic cascade of carbohydrate and lipid through cAMP and, in the other hand carbohydrate and lipid metabolism have been reported to be altered during insulin-resistance syndrome, therefore, the study of dietary PUFA on this syndrome through cAMP might give us some insight as to mechanism involved in this type of disorder.

TABLE 6
Effect of dietary lipids on brain Adenylate Cyclase activity

Activity	Control	Diet[1] n-3 PUFA Deficient	n-6 PUFA Deficient	Total PUFA Deficient
Basale	68.0±19.7[a]	61 ± 21[a]	66 ± 18[a]	73 ± 18[a]
Epinephrine	95.0±33[a]	89 ± 28[a]	92 ± 26[a]	96 ± 30[a]
GppNHp[2]	252 ± 94[a]	231 ± 58[a]	259 ± 101[a]	244 ± 107[a]
Forskoline	438 ± 152[a]	434 ± 70[a]	406 ± 135[a]	496 ± 117[a]
Forskoline + GDPßs3	289 ± 58[ab]	312 ± 35[a]	253 ± 52[b]	346 ± 81[a]

Results are the mean ± standard deviation
control diet = mixture of peanut and rapeseed oil, n-3 PUFA deficient diet = peanut oil, n-6 PUFA deficient diet = cod liver oil, total deficient diet = hydrogenated palm oil.
Means with different letter are statiscally different ($p < 0.05$)

In conclusion, this study demonstrated the incidence of dietary lipids on the fatty acid profile of brain cells. These modifications on brain fatty acid composition were not sufficient to alter the activity of 5'nucleotidase. The activity of Na^+/K^+ATPase was not correlated to changes in total phospholipids fatty acid composition, recent studies, however, have reported a correlation between dietary PUFA and neurotransmitters (dopamine and serotonin) levels, which could account for the disturbances of the neurotransmission system reported under dietary PUFA deficiencies. The catalytic site of adenylate cyclase enzyme was altered during the n-3 PUFA deficiency, suggesting the need of these particular fatty acids for the functioning of the enzyme. Further research is needed to comprehend the physiological consequences of adenylate cyclase reduced activity under this dietary deficiency.

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