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Mitochondrial Respiratory Enzyme Function and Superoxide Dismutase Activity following Brain Glutathione Depletion in the Rat

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ABSTRACT. In substantia nigra from patients with Parkinson's disease, there are decreased levels of reduced glutathione (GSH) and diminished activities of mitochondrial complex I and α-ketoglutarate dehydrogenase (α-KGDH), along with increased activity of superoxide dismutase (SOD). However, the interrelationship among these events is uncertain. We now report the effect of decreased brain GSH levels on SOD and mitochondrial respiratory enzyme activity in rat brain. In addition, we have investigated the ability of thioctic acid, an endogenous antioxidant, to alter these parameters. Unilateral or bilateral intracerebroventricular (ICV) administration of buthionine sulphoximine (BSO; 1 × 3.2 mg or 2 × 1.6 mg) over a 48-hr period reduced cortical GSH by 55–70%. There was no change in the activity of complex I, II/III, or IV or of citrate synthase in cortex. Similarly, there was no alteration of mitochondrial or cytosolic SOD activity. Thioctic acid (50 or 100 mg/kg IP) alone had no effect on cortical GSH levels in control animals and did not reverse the decrease in GSH levels produced by unilateral or bilateral ICV BSO administration. Thioctic acid (50 or 100 mg/kg IP) had no overall effect on complex I, II/III, or IV or on citrate synthase activity in control animals. Thioctic acid also did not alter cortical mitochondrial respiratory enzyme activity in BSO-treated rats. At the lower dose, thioctic acid tended to increase mitochondrial and cytosolic SOD activity in control animals and in BSO-treated rats. However, at the higher dose, thioctic acid tended to decrease mitochondrial SOD activity. Overall, there was no consistent effect of thioctic acid (50 or 100 mg/kg IP) on SOD activity in control or BSO-treated animals. This study shows that BSO-induced glutathione deficiency does not lead to alterations in mitochondrial respiratory enzyme activity or to changes in SOD activity. GSH depletion in Parkinson's disease therefore may not account for the alterations occurring in complex I and mitochondrial SOD in substantia nigra. Thioctic acid did not alter brain GSH levels or mitochondrial function. Interestingly, however, it did produce some alterations in SOD activity, which may reflect either its antioxidant activity or its ability to act as a thiol–disulphide redox couple. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1657–1663, 1996.

KEY WORDS. glutathione; SOD; mitochondria; complex I; Parkinson's disease

The cause of nigral neuronal degeneration in Parkinson's disease remains unknown, but there is current interest in the involvement of free radicals and oxidative stress. In substantia nigra, there is increased lipid peroxidation [1, 2] and an increase in 8-hydroxy-2-deoxyguanosine levels, suggesting DNA damage [3]. Increased levels of iron in substantia nigra in Parkinson's disease [4–7] may result in increased hydroxyl radical formation. However, alterations in iron are not specific to Parkinson's disease [8, 9], and other biochemical changes may be more important. These include a specific decrease in complex I activity of the mitochondrial respiratory chain [10], a decrease in α-KGDH§ [11], an increase in SOD activity [12, 13], and a decrease in GSH levels in the substantia nigra [14–17].

The decrease in GSH levels may be of particular relevance, because this also occurs in substantia nigra in incidental Lewy body disease (presymptomatic Parkinson's disease) [18]. Decreased GSH levels are the earliest biochemical change associated with cell death in Parkinson's disease and occur at a time when complex I activity and iron levels are normal. Decreased GSH levels might, therefore, lead to impairment of complex I and increased SOD activity. Indeed, GSH depletion in neonatal rats induced by the inhibition of γ-glutamylcysteine synthetase by BSO causes swelling and distortion of mitochondria in the brain [19]. Furthermore, recent evidence has shown that BSO-induced depletion of whole brain GSH content inhibits complex IV activity in preweanling rats [20]. However, at present it is not known whether brain GSH depletion re-

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§ *Abbreviations:* GSH, reduced glutathione; α-KGDH, α-ketoglutarate dehydrogenase; SOD, superoxide dismutase; ICV, intracerebroventricular; BSO, buthionine sulphoximine; 6-OHDA, 6-hydroxydopamine.

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sults in the onset of complex I inhibition or an increase in SOD activity in the adult rat.

At present, there is no effective means for preventing the cascade of events leading to oxidative damage in the substantia nigra in Parkinson's disease. If depletion of GSH is an important and early component of the pathological process, strategies aimed at raising low GSH levels may be important. For this reason, thioctic acid (α-lipoic acid) could be of interest. Thioctic acid is a naturally occurring antioxidant, which, with its reduced form, dihydrolipoic acid, forms a thiol–disulphide redox couple. Thioctic acid is also a cofactor for α-KGDH, maintaining coenzyme Q in its reduced form [21], and it may replenish GSH in brain under some circumstances [22–24].

In this study we have utilised the ability of BSO to inhibit γ-glutamylcysteine synthetase so as to deplete GSH levels in rat brain following its ICV administration [25]. We have investigated whether such a reduction in GSH levels alters mitochondrial complex activity or SOD activity. In addition, we have studied the ability of thioctic acid to alter GSH levels and mitochondrial respiratory chain and SOD activity following GSH depletion.

MATERIALS AND METHODS

Surgical Procedure

Male Wistar rats (Bantin & Kingman, Hull, U.K.; 180–200 g) were anaesthetised (Sagatal; sodium pentobarbitone, 60 mg/kg IP) and placed in a Kopf stereotaxic frame with the nosebar raised to 2.5 mm above horizontal. Unilateral or bilateral administration of BSO into the lateral ventricles was carried out at the following coordinates: A −0.11, V −0.34, and L ±0.12 [26]. L-Buthionine-[*S*,*R*]-sulphoximine (Sigma, Poole, U.K.) was dissolved in distilled water (80 mg/mL) and administered ICV either unilaterally (3.2 mg/20 μL) or bilaterally (1.6 mg/20 μL). Animals that received BSO bilaterally received a bilateral treatment with BSO (1.6 mg/20 μL) 48 hr later. Thioctic acid (50 mg/mL; Asta Medica, Germany) was dissolved in 0.1 M sodium hydroxide and neutralised with 1 M hydrochloric acid to approximately pH 7. Thioctic acid was administered 12, 24, and 45 hr after BSO treatment in doses of either 50 or 100 mg/kg IP. Animals were killed 48 hr after the final administration of BSO.

Tissue Preparation

Rats were killed by decapitation and the brain was removed and cut transversely at the level of the optic chiasm. The brain tissue anterior to this point minus the striatum was rapidly frozen in precooled isopentane and stored at −70°C. This brain material is referred to as the cortical shell. These tissues were homogenised in 9 volumes of ice-cold medium (320 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4) in a glass mortar/PTFE pestle for 40 up-and-down strokes. Aliquots (approximately 1 mL) were quickly frozen on dry ice and stored at −70°C.

Measurement of GSH

Samples of homogenate were diluted (1:2) with ice-cold 0.44 M perchloric acid containing 40 mg/L diethylenetriamine penta-acetic acid, and the internal standard, cysteic acid (1 mM), was added. After centrifugation for 10 min at 12,500 rpm and 4°C, the supernatant was removed for analysis of GSH.

GSH was determined by HPLC with ultraviolet detection [27]. Iodoacetic acid (0.44 μM) and an excess of sodium bicarbonate were added to the supernatant (0.5 mL), mixed, and left for 60 min in the dark at room temperature. After this time, 1.5% alcoholic fluorodinitrobenzene (0.5 mL) was added, mixed, and left for a further 4 hr in the dark at room temperature. Diethyl ether (1.0 mL) was added to the sample after 4 hr, mixed, and centrifuged for 20 min at 4000 rpm. The top ether layer was discarded and the lower aqueous layer was analysed by HPLC (model 501, Waters, Watford, U.K.) at a wavelength of 365 nm with a UV detector (model 411, Waters).

HPLC was undertaken at room temperature with a mobile phase flow rate of 1.25 mL/min at a pressure of about 2700 psi. Aliquots (10 μL) were injected onto a Spherisorb S-5 amino ODS column (25 cm × 4.6 mm, 5 μm particle size; Phase Separations, Deeside, U.K.) and eluted along a continuous ammonium acetate gradient of methanol (80%) and glacial acetic acid, methanol, and water (pH 5.05). The internal standard, cysteic acid (1 mM), was used to quantify GSH concentrations in the samples.

Mitochondrial Enzyme Activity

Enzyme assays were performed by using a Shimadzu 2101PC dual beam spectrophotometer thermostatically controlled at a temperature of 22°C. Citrate synthase activity was determined as previously described [28]. The cortical homogenate was diluted 1:40 in ice-cold medium (320 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4). Samples (1.0 mL) consisted of Tris-HCl (100 mM), Triton X-100 (0.1%), 5,5′-dithio-bis-nitrobenzoic acid (200 μM), acetyl CoA (200 μM), and cortical homogenate (10 μL). The reaction was initiated by the addition of oxaloacetate (100 μM) and measured at a wavelength of 412 nm. The activity of complex I (NADH ubiquinone-1 reductase) was assayed according to the method of Ragen and colleagues [29]. The cortical homogenate was diluted 1:2 in ice-cold medium (320 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4). Samples (1.0 mL) consisted of phosphate buffer (20 mM, pH 7.2), bovine serum albumin (2.5 mg/mL), NADH (150 μM), and cortical homogenate (10 μL). The reaction was initiated by the addition of ubiquinone-1 (50 μM; Eisai Co., Tokyo, Japan) and measured at a wavelength of 340 nm. Rotenone (10 μM) was added to the assay mixture to define the change in absorbance due to complex I activity. The combined activity of complexes II and III (succinate cytochrome *c* reductase) was measured according to King [30]. The cortical homogenate was diluted 1:2 in ice-cold

TABLE 1. Effects of BSO and thioctic acid on the GSH content, mitochondrial enzyme complex activity, and SOD activity in rat brain cortex 48 hr after unilateral ICV (3.2 mg in 20 μL) administration

	Control	Thioctic acid treatment alone	BSO treatment alone	BSO + thioctic acid treatment
A. 50 mg/kg thioctic acid				
GSH	1.36 ± 0.05	1.44 ± 0.07	0.66 ± 0.04**	0.73 ± 0.06**
Citrate synthase	430 ± 32	564 ± 41*	534 ± 41	539 ± 27
Complex I	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.004
Complex II/III	0.08 ± 0.01	0.06 ± 0.004*	0.07 ± 0.01	0.07 ± 0.01
Complex IV	0.02 ± 0.003	0.02 ± 0.001	0.02 ± 0.001	0.01 ± 0.001
Particulate SOD	1.21 ± 0.15	1.69 ± 0.10**	1.48 ± 0.10	1.60 ± 0.10**
Cytosolic SOD	10.53 ± 0.51	16.31 ± 1.60	11.25 ± .059	19.68 ± 2.4*
B. 100 mg/kg thioctic acid				
GSH	1.70 ± 0.14	1.55 ± 0.07	0.71 ± 0.03**	0.57 ± 0.02**
Citrate synthase	548 ± 19	561 ± 20	549 ± 23	559 ± 11
Complex I	0.13 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.15 ± 0.005*
Complex II/III	0.07 ± 0.003	0.07 ± 0.003	0.07 ± 0.003	0.08 ± 0.001
Complex IV	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001
Particulate SOD	1.54 ± 0.08	1.51 ± 0.11	1.46 ± 0.10	1.24 ± 0.04*
Cytosolic SOD	16.36 ± 2.1	12.72 ± 1.03	14.57 ± 1.5	14.10 ± 2.4*

Animals received thioctic acid, 50 (A) or 100 mg/kg IP (B), 12, 24, and 45 hr after ICV administration. Units are as follows: GSH, μmol/g tissue; mitochondrial enzymes, nmol/min/mg protein; SOD, units/mg protein. The data are mean ± SEM (N = 7). *$P < 0.05$; **$P < 0.01$, compared with controls using the Mann-Whitney *U*-test.

medium (320 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4). Samples (1.0 mL) consisted of phosphate buffer (0.1 M, pH 7.4), EDTA (0.3 mM), cytochrome *c* (0.1 mM), sodium azide (1 mM), and cortical homogenate (10 μL). The reaction was initiated by addition of succinate (20 mM) and measured at a wavelength of 550 nm. Antimycin (10 μg/mL) was added to the reaction mixture to define the change in absorbance due to complex II/III activity. Complex IV (cytochrome *c* oxidase) activity was determined according to Wharton and Tzagoloff [31]. Samples (1.0 mL) consisted of phosphate buffer (0.01 M, pH 7.0) and cortical homogenate (20 μL). The reaction was initiated with reduced cytochrome *c* (50 μM) and measured at a wavelength of 550 nm. The mitochondrial enzyme data were expressed corrected for citrate synthase activity.

Superoxide Dismutase Activity

Cortical homogenates were diluted 1:11 with ice-cold sucrose medium (0.32 M). The homogenate was centrifuged at 100,000 × *g* for 1 hr on a fixed-angle rotor in a Kontron Centrikon T-1055 ultracentrifuge. The supernatant was retained for determination of cytosolic SOD, and the pellet was resuspended in 10% (w/v) ice-cold sucrose medium (0.32 M) for the determination of mitochondrial SOD.

Superoxide dismutase activity was determined as previously described [32]. Assay samples (3.0 mL) consisted of sodium carbonate buffer (0.05 M, pH 10.2) and EDTA (0.1 mM), and the reaction was initiated by addition of (−)-adrenaline (+)-bitartrate (0.3 mM; Sigma) at a wavelength of 480 nm at 25°C in an LKB single beam spectrophotometer. This rate represented the blank, defined as 100% oxidation of adrenaline to adrenochrome. The addition of bovine erythrocyte SOD (50 ng/mL, Sigma) inhibited the rate of auto-oxidation by 56%. Aliquots of mitochondrial (50 μL) and cytosolic (15 μL) preparations were added to the reaction mixture to produce an inhibition of between 40 and 60%.

Protein Determination

The protein concentration of the tissue homogenate and the mitochondrial and cytosolic fractions was determined according to Lowry and colleagues [33].

Statistical Analysis

The effect of BSO and/or thioctic acid on GSH levels, mitochondrial enzyme activity, and superoxide dismutase activity was analysed by using the Mann–Whitney *U*-test.

RESULTS

Effect of BSO Treatment

Unilateral or bilateral ICV administration of BSO reduced cerebral cortical GSH levels to approximately 45% and 30% of control values, respectively (Tables 1, 2). Similarly, administration of BSO either unilaterally or bilaterally did not alter citrate synthase activity and had no effect on complex I, II/III, and IV activity. The levels of mitochon-

TABLE 2. Effects of BSO and thioctic acid on the GSH content, mitochondrial enzyme complex activity, and SOD activity in rat brain cortex 48 hr after bilateral ICV (1.6 mg in 20 μL × 2) administration

	Control	Thioctic acid treatment alone	BSO treatment alone	BSO + thioctic acid treatment
A. 50 mg/kg thioctic acid				
GSH	1.70 ± 0.05	1.60 ± 0.1	0.66 ± 0.04**	0.57 ± 0.05**
Citrate synthase	650 ± 34	676 ± 21	646 ± 53	661 ± 57
Complex I	0.12 ± 0.008	0.11 ± 0.01	0.12 ± 0.009	0.10 ± 0.01
Complex II/III	0.07 ± 0.005	0.06 ± 0.003	0.07 ± 0.001	0.07 ± 0.008
Complex IV	0.02 ± 0.001	0.02 ± 0.001	0.02 ± 0.001	0.02 ± 0.001
Particulate SOD	1.65 ± 0.10	1.40 ± 0.05*	1.50 ± 0.09	1.69 ± 0.12
Cytosolic SOD	12.94 ± 0.98	40.13 ± 8.7**	11.77 ± 0.96	27.99 ± 4.0**
B. 100 mg/kg thioctic acid				
GSH	1.98 ± 0.30	1.72 ± 0.2	0.45 ± 0.04**	0.46 ± 0.03**
Citrate synthase	406 ± 60	621 ± 56*	527 ± 35	540 ± 25*
Complex I	0.19 ± 0.04	0.14 ± 0.02	0.11 ± 0.02	0.14 ± 0.02
Complex II/III	0.10 ± 0.02	0.07 ± 0.004*	0.07 ± 0.004	0.07 ± 0.004*
Complex IV	0.02 ± 0.03	0.02 ± 0.001	0.02 ± 0.001	0.02 ± 0.001
Particulate SOD	1.73 ± 0.09	1.36 ± 0.08*	1.69 ± 0.05	1.18 ± 0.09**
Cytosolic SOD	25.41 ± 2.1	18.76 ± 0.67	24.72 ± 4.8	12.81 ± 0.64**

Animals received thioctic acid, 50 (A) or 100 mg/kg IP (B), 12, 24, and 45 hr after ICV administration. Units are as follows: GSH, μmol/g tissue; mitochondrial enzymes, nmol/min/mg protein; SOD, units/mg protein. The data are mean ± SEM (N = 7). *$P < 0.05$; **$P < 0.01$, compared with controls using the Mann-Whitney *U*-test.

drial and cytosolic SOD activity were also unaltered following either unilateral or bilateral BSO administration (Tables 1, 2).

Effects of Thioctic Acid Treatment Alone

Administration of thioctic acid (50 or 100 mg/kg IP) did not alter brain GSH levels (Tables 1, 2). Similarly, thioctic acid had no consistent effect on mitochondrial complex activity or citrate synthase. There was a trend for thioctic acid to increase citrate synthase and to inhibit complex II/III, but this was not consistent among experiments. Thioctic acid (50 or 100 mg/kg IP) produced some alterations in mitochondrial SOD activity (both increases and decreases), but there was no consistent effect. In contrast, while thioctic acid at 50 mg/kg IP caused marked increases in cytosolic SOD, the higher dose tended to decrease enzyme activity (Tables 1, 2).

Effects of Thioctic Acid in BSO-Treated Rats

Following unilateral BSO treatment, administration of thioctic acid (50 or 100 mg/kg IP) had no effect on GSH levels compared with animals receiving BSO alone (Table 1). Similarly, after bilateral BSO administration, treatment with thioctic acid (50 mg/kg or 100 mg/kg IP) had no effect on GSH levels (Table 2).

Following unilateral BSO treatment, thioctic acid (50 or 100 mg/kg IP) had no effect on mitochondrial enzyme activity (Table 1). After bilateral administration of BSO, thioctic acid treatment (50 mg/kg IP) did not alter mitochondrial enzyme activity. The higher dose of thioctic acid (100 mg/kg IP) tended to increase citrate synthase and to decrease complex II/III activity compared with control animals, but there was no significant change compared with the effect of BSO treatment alone (Table 2).

Following unilateral or bilateral administration of BSO, there was a trend for thioctic acid treatment (50 mg/kg IP) to raise both cytosolic and mitochondrial SOD activity. However, this effect was no different from that produced by thioctic acid alone (Tables 1A, 2A). Similarly, following bilateral administration of BSO, treatment with thioctic acid (100 mg/kg IP) tended to decrease both cytosolic and mitochondrial SOD activity, but again this was not significantly different from the effects produced by thioctic acid alone (Table 2A).

DISCUSSION

The interrelationship among the indices of oxidative stress and mitochondrial enzyme activity in Parkinson's disease remain unknown. The early changes in GSH levels suggest that it has an important role in initiating a cascade of events leading to nigral cell death [18]. We therefore investigated whether depletion of GSH in brain could be responsible for the inhibition of complex I and the increased SOD activity found in the substantia nigra in Parkinson's disease. In this study, the cortical shell rather than substantia nigra was employed to provide sufficient tissue for the assays undertaken. While GSH reductions are limited to the substantia nigra in Parkinson's disease, there is

a general depletion of GSH throughout the brain following ICV administration of BSO, so the effect on mitochondrial function will be similar in any brain region investigated. Similarly, the actions of thioctic acid are not limited to basal ganglia. Indeed, following systemic administration, thioctic acid alters neuronal function in many brain regions, including cerebral cortex, as judged by alterations in glucose utilisation [34].

The depletion of GSH following ICV administration of BSO confirmed previous studies utilising this technique [25]. The 55–70% decrease in GSH levels observed is in good agreement with earlier studies, but it is somewhat greater than that observed in Parkinson's disease (approximately 40%). The degree of GSH depletion produced by BSO enhances the toxicity of 6-OHDA to the nigrostriatal tract [35]. The toxicity of 6-OHDA is presumably increased because of decreased scavenging of free radical species and increased susceptibility of nigral cells to toxin action [36]. Consequently, BSO-induced GSH depletion may be sufficient to affect mitochondrial function and SOD activity.

However, the depletion of brain GSH by BSO in adult rats had no effect on mitochondrial function, including complex I activity. This suggests that the alterations in GSH content and complex I activity observed in Parkinson's disease are not related. Indeed, the results are compatible with the finding of decreased GSH in the presence of unaltered complex I activity in presymptomatic Parkinson's disease [18]. However, the results do not agree with previous reports on the effects of experimental depletion of brain GSH. Indeed, preweanling rats treated systemically with BSO for 10 days showed a decrease in mitochondrial complex IV activity [20]. Furthermore, administration of cyclohexen-1-one depleted forebrain GSH content, leading to changes in the composition of the mitochondrial membranes and the generation of free radical species [37]. In addition, treatment of neonatal rats with BSO caused mitochondrial damage and swelling in the brain [19.] In that study, the levels of mitochondrial enzymes were not measured, but the findings suggested that GSH depletion affects mitochondrial function. Indeed, in neonatal rats, the depletion of GSH produced by BSO exceeded 90% and should have reduced the levels of both cytosolic and mitochondrial GSH. In contrast, in the present study, brain GSH levels were only reduced by 70% and so may not have affected mitochondrial GSH. Indeed, mitochondrial GSH levels are presumably preserved at the expense of the cytosolic pool [38].

In the present study, BSO treatment did not alter mitochondrial or cytosolic SOD levels. This is compatible with the lack of effect of BSO on complex I and other mitochondrial enzymes, since their inhibition should lead to increased oxygen radical formation and so induce SOD activity [39–41]. The finding also supports the argument that BSO treatment did not deplete mitochondrial GSH content, since γ-glutamylcysteine synthetase is not present in mitochondria [42]. Thioctic acid had an inconsistent effect on SOD activity, although this appeared to be dependent on both the dose and the route of administration of BSO. Generally, 50 mg/kg of thioctic acid induced SOD activity, whereas 100 mg/kg decreased it. Maybe low doses of thioctic acid can neutralise excess superoxide radical generation, although this may [43] or may not [44, 45] occur *in vitro*. Alternatively, low doses of thioctic acid may stimulate superoxide radical formation, thus inducing SOD activity, whereas higher doses of thioctic acid neutralise, so SOD levels are unaffected.

The conclusion from this study is that a decrease in cerebral cortical GSH does not lead to an obvious impairment of complex I activity or a consistent change in SOD activity of the sort found in the substantia nigra in Parkinson's disease. However, the differences between the present study and the findings in Parkinson's disease require further elucidation. In Parkinson's disease, the decrease in GSH occurs in the presence of normal γ-glutamylcysteine synthetase activity [46] and thus may reflect ongoing oxidative stress rather than an impairment of GSH function. The changes in GSH induced in this study were relatively acute and may not mirror the effects of prolonged GSH depletion in Parkinson's disease. Additionally, it is not known whether the depletion of GSH in Parkinson's disease is due to a fall in mitochondrial or cytosolic GSH. If there is a decrease in mitochondrial GSH, this may still cause alterations in complex I and SOD levels. Decreased brain GSH levels may not be the cause of the cascade of events associated with oxidative stress precipitating nigral cell death. However, decreased GSH may render the nigro-striatal pathway sensitive to toxin action, as shown previously for 6-OHDA. Such vulnerability might subsequently lead to inhibition of complex I and increased SOD activity.

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