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Organophosphate Insecticide Chlorfenvinphos Affects Enzymatic and Nonenzymatic Antioxidants in Erythrocytes and Serum of Rats

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Abstract

Chlorfenvinphos, 2-chloro-1-(2,4-dichlorofenyl)vinyl diethyl phosphate, is an organophosphate insecticide widely used in Poland singly or in mixture. The present study was undertaken to determine chlorfenvinphos-induced lipid peroxidation and to show whether acute intoxication with chlorfenvinphos alters the antioxidant system in the erythrocytes and serum. The study was conducted on male Wistar rats divided into two groups. The animals of control group were given olive oil intragastrically by a stomach tube, the animals of experimental group received oil solution of chlorfenvinphos (CVP) at a dose of 0.02, 0.1 or 0.5LD_{50}. The blood was collected via heart puncture at the 1st, 24th and 48th hour after treatment. We determined the erythrocytes concentration of glutathione, the activities of glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase as well as the serum concentration of ascorbic acid, α-tocopherol and malondialdehyde. We observed the stimulation of enzymatic and nonenzymatic antioxidant system and lipid peroxidation in the erythrocytes and serum of chlorfenvinphos intoxicated rats.

Keywords: chlorfenvinphos, erythrocytes, serum, GSH, GPx, GR, G6PDH, Vit C and E, MDA

Introduction

Organophosphate pesticides are extensively used to control agricultural, household and structural pests. Their primary toxicological action arises from inhibition of the enzyme acetylcholinesterase [1]. Since reactive oxygen species (ROS) are known mediators of many toxin-induced organ injuries, the influence of organophosphate on ROS generation is worth studying.

Mammalian cells are equipped with both enzymatic and nonenzymatic defence system to minimize cellular damage from action of reactive oxygen species. The overproduction of reactive oxygen species leads to cellular and tissue damage [2]. Free oxygen unstable radical species that have at least one unpaired electron in an outer orbital, are by-products of normal mitochondrial and endoplasmic reticulum metabolism. The primary product of oxygen reduction is superoxide anion, which is a precursor of hydrogen peroxide, and highly reactive hydroxyl radicals [3, 4]. Mitochondria are continually exposed to ROS, which cause peroxidation of membrane lipid [5]. An increase in the oxidative stress may be due to a decrease in the antioxidant defences or due to an increase in the proceses that produce oxidants [6].

Chlorfenvinphos, 2-chloro-1-(2,4-dichlorofenyl)vinyl diethyl phosphate, is an organophosphate insecticide widely used singly or in mixture in Poland. In our earlier works we observed that acute intoxication with chlorfenvinphos led to the inhibition of serum cholinesterase activity [7]. In the earlier papers we reported the increased ratio lactate/pyruvate in the livers reffered to as an enhancement of the anaerobic glycolysis [8, 9]. The

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increased level of the serum lactate was observed at the same time of intoxication. Therefore, our results indicate state of tissue hypoxia.

There is evidence indicating that hypoxia alters intracellular redox status inducing free radicals [10]. We have also previously reported that acute intoxication with chlorfenvinphos affected the activity of superoxide dismutase and catalase in the liver as well as in the erythrocytes [11].

The present study was undertaken to determine chlorfenvinphos-induced lipid peroxidation and to show whether acute intoxication with chlorfenvinphos alters the glutathione level and glutathione-dependent enzymes system in the erythrocytes as well as vitamins C and E, and MDA level in the serum.

Experimental Procedures

The present study was conducted on 95 male Wistar rats weighing on average 200±10 grams. Rats of all groups were given standard feed and water *ad libitum*. They were divided into two groups. Animals of the control group were given olive oil intragastrically by a stomach tube, the animals of experimental group received oil solution of chlorfenvinphos (CVP) at the dose of 0.02, 0.1 or 0.5 LD_{50}. Blood was collected via heart puncture at the 1st, 24th and 48th hour after treatment. Red blood cells and serum were obtained from heparinized rat blood. Our study was approved by the Local Ethical Commission.

The concentration of erythrocyte glutathione level, activities of glutathione peroxidase (GPx, EC 1.11.1.9) and reductase (GR, EC 1.6.4.2) were performed using BIOXYTECH Assay kits produced by OXIS International, Inc., Portland, USA. The GSH assay method is based on a chemical reaction which proceeds in two steps. The first step leads to formation of substitution products between a patented reagent (OXIS) and all mercaptans which are present in sample; the second is a β-elimination reaction which takes place under alkaline solution.

Determination of GPx was done on the basis of oxidation of NADPH to $NADP^+$, which is accompanied by a decrease in absorbance. A unit of enzyme is defined as that which will cause the consumption of one μmol NADPH per minute at pH 7.6, 25°C.

Determination of GR was done on the basis of the oxidation of NADPH to $NADP^+$ catalyzed by a limiting concentration of glutathione reductase. One GR activity unit is defined as the reduction of one μmol of GSSG per minute at pH 7.5, 25°C.

The activity of glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) was assayed using assay kit produced by Sigma Diagnostics, Inc., St. Louis, USA. Determination of G6PDH was done on the basis of the formation of NADPH, which is proportional to the activity of G6PDH and is measured spectrophotometrically as an increase in absorbance at 340 nm. One G6PDH activity unit is the amount of G6PDH activity that will convert one μmol of substrate per minute.

The serum concentration of ascorbate was assayed by HPLC, with UV detection at 262 nm [12]. The serum α-tocopherol concentration was measured by HPLC using γ-tocopherol (Sigma Chemical Co) as an internal standard. Peaks were detected and analysed using a Merck-Hitachi Fluorescence spectrophotometer with detection at 296 nm [13]. The serum concentration of MDA was determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buega and Aust [14].

Data for the ten groups of animals were compared using one way analysis of variance (ANOVA). The probability of $p < 0.05$ was considered significant.

Results

The rats' erythrocytes GSH level showed no difference, in comparison to the control group, after administration of chlorfenvinphos at a dose of 0.02 LD_{50} (Tab. 1). Two higher doses of chlorfenvinphos resulted in reduced glutathione concentration in the erythrocytes of treated rats. However, there were no differences in GSH concentration between the control group and the group of rats examined 48 hours after treatment with the chlorfenvinphos at a dose of 0.1 LD_{50}. After intoxication with chlorfenvinphos at a dose of 0.5 LD_{50}, the concentration of glutathione was higher at the 48th hour than in the earlier period after treatment.

There were no significant differences in the activity of erythrocyte glutathione peroxidase between the chlorfenvinphos at a dose of 0.5 LD_{50} treated rats and the control group (Tab. 1). In contrast, in the erythrocytes of rats treated with lower dose of chlorfenvinphos, the activity of GPx increased (100-200% over control) during the whole examined period for the dose of 0.1 LD_{50} and at the 48th hour for the dose 0.02 LD_{50}. At the 1st and 24th hour of intoxication with the lowest dose of insecticide GPx activity decreased. The highest activity was observed at the 24th hour after intoxication with chlorfenvinphos at a dose of 0.1 LD_{50}.

The treatment of rats with the lower doses of chlorfenvinphos resulted in an enhanced activity of glutathione reductase in the erythrocytes. The highest value was observed at the 24th hour after intoxication, at the same time point as it was demonstrated for a glutathione peroxidase. There was no significant change in an activity of this enzyme at the 1st and 24th hours after intoxication with chlorfenvinphos at a dose of 0.5 LD_{50}, but at the 48th hour the activity decreased (Tab. 1).

The activity of G6PDH in the erythrocytes of the lowest dose of chlorfenvinphos treated rats did not change statistically significantly, (Tab. 2). The activity G6PDH increased significantly compared with the control group at the 24th and 48th hours after treatment with chlorfenvinphos at a dose of 0.5 LD_{50} and at the 1st and 24th hour after intoxication at a dose of 0.1 LD_{50}.

The results showed decreased serum concentration of vitamin C after intoxication with chlorfenvinphos in comparison to the control group. The concentration of serum vitamin C in the two lower doses of chlorfenvinphos

Table 1. GSH (mmol/l) concentration , GPx (U/l) and GR (U/l) activity in erythrocytes of rats after acute intoxication with chlorfenvinphos.

GROUP	GSH	GPx	GR
Control	8.46±1.73 (n=8)	685.91±101.79 (n=9)	29.74±5.30 (n=10)
CVP-0.02LD$_{50}$			
1 h	7.21±1.51 (n=8)	404.41±137.35 ^{a} (n=8)	66.64±8.92 ^{a} (n=8)
24 h	9.01±0.64 ^{b} (n=8)	485.17±65.04 ^{a} (n=8)	34.01±4.70 ^{b} (n=8)
48 h	8.20±0.82 (n=8)	1092.18±165.84 ^{abc} (n=8)	64.41±7.91 ^{ac} (n=8)
CVP-0.1LD$_{50}$			
1 h	3.07±0.28 ^{abcd} (n=8)	1377.89±225.34 ^{abc} (n=8)	37.62±13.46 ^{bd} (n=7)
24 h	2.88±0.17 ^{abcd} (n=8)	2779.33±272.30 ^{abcde} (n=8)	58.41±19.45^{ace} (n=8)
48 h	9.03±0.13 ^{ef} (n=8)	1468.74±105.17^{abcdef} (n=8)	49.74±15.13^{a} (n=8)
CVP-0.5LD$_{50}$			
1 h	3.09±0.25 ^{abcdg} (n=8)	777.41±198.45 (n=6)	26.34±3.00 ^{bcdfg} (n=7)
24 h	3.56±0.75 ^{abcdg} (n=8)	586.32±197.80 (n=7)	30.33±6.32 ^{bdfg} (n=6)
48 h	4.76±0.21 ^{abcdefghi} (n=8)	576.22±106.11 (n=7)	17.34±6.21^{abcdefghi} (n=6)

values expressed as mean±SD

statistically significant in comparison with: a- control, b- 0.02 LD50-1h, c- 0.02 LD50-24h, d- 0.02 LD50-48h, e- 0.1 LD50-1h, f- 0 1LD50- 24h, g- 01 LD50-48h, h- 0.5LD50-1h, i- 0.5 LD50-24h

Table 2. G6PDH activity (U/l) in erythrocytes of rats after acute intoxication with chlorfenvinphos.

GROUP	G6PDH
Control	29.81±6.64 (n=8)
CVP-0.02LD$_{50}$	
1 h	24.02±4.88 (n=8)
24 h	26.31±4.29 (n=8)
48 h	27.62±1.89 (n=8)
CVP-0.1LD$_{50}$	
1 h	79.90±10.31 ^{abcd} (n=6)
24 h	83.21±13.50 ^{abcd} (n=6)
48 h	36.71±5.87 ^{bcdef} (n=6)
CVP-0.5LD$_{50}$	
1 h	44.94±10.70 ^{abcdef} (n=8)
24 h	64.20±14.68 ^{abcdg} (n=6)
48 h	54.28±10.32 ^{abcd} (n=6)

values expressed as mean±SD

statistically significant in comparison with: a- control, b- 0.02 LD50-1h, c- 0.02 LD50-24h d- 0.02 LD50-48h, e- 0.1LD50-1h, f- 01 LD50- 24h, g- 01 LD50-48h,

treated rats were about 50% lower than those of the highest dose of chlorfenvinphos treated rats (at the 1st and at the 24th hour) (Tab. 3).

There were no significant difference in the concentration of a vitamin E serum between the control group and the group of rats intoxicated with the lowest and highest doses of chlorfenvinphos, except the 1st hour after treatment (Tab. 3). At this time, the serum concentration of α-tocopherol was lower than in the control group. The serum concentration of vitamin E in the group at the dose of 0.1 LD$_{50}$ treated rats was decreased in comparison to the value observed in control group during the whole examined period.

As reported in Table 3, the concentration of serum malondialdehyde was increased during the whole examined period after intoxication with the lowest dose of chlorfenvinphos and at the 1st and 48th after treatment at the dose of 0.1 LD$_{50}$ and 0.5 LD$_{50}$, respectively. After 24 hours of intoxication with chlorfenvinphos at the dose of 0.1 LD$_{50}$, MDA concentration in the serum decreased (but not statistically significantly) with recovery to control within 48 hours. At the earlier period after intoxication with chlorfenvinphos at the highest dose the MDA concentration decreased.

Discussion

Many in vitro and in vivo studies have shown that several parameters of red blood cells are negatively af-

Table 3. Ascorbic acid (mg/l), α-tocopherol (mg/l) and MDA (μmol/l) concentration in serum of rats after acute intoxication with chlorfenvinphos.

GROUP	ascorbic acid	α-tocopherol	MDA
Control	23.82±2.99 (n=8)	5.03±0.36 (n=7)	3.33±0.58 (n=15)
CVP-0.02LD_{50}			
1 h	7.94±2.01[a] (n=8)	4.28±0.29 [a] (n=8)	6.14±1.10 [a] (n=8)
24 h	8.05±1.68 [a] (n=8)	4.73±0.89 (n=8)	5.62±0.89 [ab] (n=8)
48 h	12.30±2.05 [a] (n=8)	4.98±0.87 (n=8)	5.20±1.77 [a] (n=8)
CVP-0.1LD_{50}			
1 h	8.50±1.18[a] (n=6)	4.37±0.34[a] (n=8)	4.08±0.55[abcd] (n=9)
24 h	8.97±1.10[a] (n=8)	3.81±0.69[acd] (n=6)	2.66±0.69[bcd] (n=8)
48 h	9.83±0.69[a] (n=7)	3.84±0.48[acd] (n=7)	3.51±1.08[bcd] (n=8)
CVP-0.5LD_{50}			
1 h	15.03±1.76[abcefg] (n=6)	3.45±0.62[abce] (n=6)	1.95±0.56[abcdeg] (n=11)
24 h	14.96±0.58[abcefg] (n=5)	4.96±0.97 (n=6)	2.27±0.45[abcde] (n=9)
48 h	8.99±3.35[adhi] (n=5)	4.90±0.27 (n=6)	4.21±1.08[abcdfh] (n=9)

values expressed as mean±SD

statistically significant in comparison with: a- control, b- 0.02 LD50-1h, c-0.02 LD50-24h ,d- 0.02 LD50-48h, e- 0.1 LD50-1h, f- 01 LD50-24h, g- 01LD50-48h, h- 0.5LD50-1h, i- 0.5 LD50-24h

fected by increased oxidative stress. Imbalance between ROS production and antioxidant cell defences has been reported to occur in several pathological conditions [15]. However, little is known about the influence of organophosphorus compounds on antioxidant systems in erythrocytes.

In the present study the stimulation of nonenzymatic antioxidant systems as well as enzymes involved in glutathione metabolism were demonstrated in the erythrocytes and serum of chlorfenvinphos-intoxicated rats.

The reduced glutathione is a key cellular antioxidant that detoxifies oxygen-reactive species. Reduced glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is a tripeptide that is present in nearly all animal cells and is predominant as an intracellular low-molecular-weight thiol compound [16, 17]. GSH is very important in the protection of cells against toxic insults, because it participates in the detoxification of electrophilic metabolites of xenobiotic and is a very efficient free radicals scavenger [17, 18, 19].

Numerous enzymes participate in glutathione metabolism. The glutathione-dependent antioxidant system consists of glutathione and two enzymes: glutathione peroxidase and glutathione reductase [20, 21, 22].

In this study we reported a decreased glutathione level in the red blood cells after treatment of rats with the higher doses of chlorfenvinphos. We also observed the changed activity of GPx, but only in the erythrocytes of rats intoxicated with lower doses of chlorfenvinphos. This GSH-dependent enzyme detoxicated hydrogen peroxides and a wide variety of organic peroxides, including lipid peroxides. Thus, the increase of an erythrocytes GPx activity might be a protective response against oxidative stress generated by lower-doses of chlorfenvinphos. From the data for GPx activity, it can be concluded that this protective effect has not been observed after intoxication with the highest dose of insecticide. When the activity of GPx increased the concentration of GSH decreased as it was observed in the red blood cells of rats intoxicated with chlorfenvinphos at a dose of 0.1 LD_{50}, at the 1st and 24th hours. These data are in agreement with other authors, who reported that reduced glutathione is consumed by peroxidases, and its also protects protein sulfhydryl groups against oxidation [23, 24, 25]. Glutathione peroxidase catalyzes the formation of oxidized glutathione (GSSG) during the reduction of hydroperoxides [16, 17, 18].

The activity of erythrocyte GR increased or decreased after treatment with different doses of chlorfenvinphos. The increased activity of GR can lead to the enhancement of GSH level, but in this study such phenomenon was observed only at the 48th hour after intoxication at a dose of 0.1 LD_{50}. In the erythrocytes of rats treated at the same dose of chlorfenvinphos, the 24th hour after intoxication, GR activity increased while GSH level remained low. This appears to be due to the increased activity of glutathione peroxidase, which has been observed at the same time. Increase in the activity of GSH peroxidase induces a

decrease in GSH concentration. The other authors showed that increased activity of enzymes involved in GSSG reduction can lead to an increased GSH concentration [16, 17, 18]. The results seen in this paper are consistent with this suggestion. Increased activity of GR seems to prevent the reduced glutathione level after treatment of rats with the lowest dose of chlorfenvinphos.

Variations of glutathione levels during oxidative stress may result from modification in its synthesis and/or loss. The mechanism responsible for maintenance of GSH homeostasis in different tissues is poorly documented, especialy in pathological conditions, but the significance of glutathione redox cycle in the protection against toxic oxygen compounds is well known [23, 24, 25].

The exact mechanism by which GSH protects against oxidative stress is difficult to explain. However, it has been shown that GSH and GSH-dependent enzymes are affected by acute intoxication with chlorfenvinphos. Taken together, these results show also that changes of GSH-dependent enzymes are greater after intoxication with chlorfenvinphos at the lower doses.

In this study, the effect of acute intoxication with chlorfenvinphos of different doses on the activity of red blood cells G6PDH was also examined.

Glucose-6-phosphate dehydrogenase is the key enzyme of pentose phosphate pathway that is responsible for the generation of NADPH. This enzyme plays an essential role in the regulation of oxidative stress by regulating NADPH level, the main intracellular reductant. For this reason, G6PDH is required for the antioxidant defence system [6, 26].

Glucose-6-phosphate dehydrogenase catalyzes the first step in the pentose phosphate shunt, oxidizing glucose-6-phosphate to phosphogluconate and reducing NADP to NADPH [6, 27]. In the case of genetic G6PDH deficiency, or other circumstances where NADPH is in short supply, the reduction of GSSG is impaired, there is a deficiency of GSH, and hemolytic anaemia [17].

The results obtained in this study show increases in the activity of G6PDH in red blood cells after treatment of rats with the higher (0.1 and 0.5 LD_{50}) doses of chlorfenvinphos. It has been recently suggested that the primary physiological role of G6PDH in mammalian cells is the defence against oxidative stress injury [26]. The formation of GSH from its oxidized form, GSSG, is dependent on NADPH produced by the pentose phosphate pathway. This pathway can be activated in response to GSH depletion [26]. This study shows that decreased levels of GSH are accompanied by increased activity of G6PDH. Thus, the G6PDH seems to be activated in response to a decreased level of glutathione, as discussed above.

As is reported by other authors, the activities of G6PDH and GPx are linked in their capacity to prevent peroxidative tissue damage from oxidants. GPx converts toxic lipid hydroperoxides and H_2O_2 using reducing equivalents generated by G6PDH [28, 29]. However, in this study the increased activity of these two enzymes was observed at the same time after intoxication with chlorfenvinphos at the dose of 0.1 LD_{50} only. After treatment with chlorfenvinphos at the dose of 0.5 LD_{50}, the activity of G6PDH enhanced at the further period but the activity of GPx did not change statistically significantly whithin the whole examined period. The lowest dose of chlorfenvinphos did not change G6PDH activity at all, probably because of the unaffected level of GSH. These results suggest that rapid increase in G6PDH activity is a response to an increased erythrocytes demand for NADPH, however the method of using of this reductant is due to chlorfenvinphos' dose.

Besides the glutathione level and activity of enzymes involved in its metabolism, the concentrations of ascorbic acid and α-tocopherol were determined in the serum of chlorfenvinphos-treated rats.

These two vitamins are most important among many low molecular weight compounds which can act as biological antioxidants [28, 29, 30].

Ascorbate, a well-known antioxidant in biological systems, is capable of reducing a variety of oxidative compounds, especially free radicals [31]. Vitamin C is an electron donor. When ascorbate donates its two high-energy electrons to scavenge free radicals, much of the resulting dehydroascorbate is reduced to ascorbate and therefore can be used repeatedly [28, 32]. α-Tocopherol is the primary radical scavenger in biological membranes, and is continously regenerated at the expense of ascorbate and GSH oxidation, with ascorbate and GSH being regenerated by NADH and NADPH respectively, in the presence of relevant reductases [17].

In the present study were demonstrated decrease in the serum concentration of ascorbic acid and α-tocopherol after treatment of rats with chlorfenvinphos. The serum concentration of vitamin C was lower in the intoxication with chlorfenvinphos at the two lower doses in comparison to the values observed at the 1st and 24th hour after treatment of rats at the highest dose of chlorfenvinphos. Simultaneously, the lipid peroxidation index (MDA) was higher in these experimental groups. The concentration of vitamin E lowered to the very similar level during the whole examined period after intoxication with chlorfenvinphos of 0.1 LD_{50} dose. After intoxication with chlorfenvinphos at the higher and lower doses, the concentration of vitamin E decreased significantly in comparison to the control, at the 1st hour only. Accordingly to the other authors, the consumption of ascorbate can be high, under oxidative stress [32]. In the present study enhanced lipid peroxidation was related to depletion of ascorbic acid. Both decreased levels of vitamin C and increased levels of MDA suggest the induction of oxidative stress in the blood of rats acute intoxicated with the lower doses of chlorfenvinphos. Thus, the change of vitamin C concentration show that the pro- and antioxidant balance moved toward the prooxidant side, especially after intoxication with chlorfenvinphos at the two lower doses and at the further period after intoxication at the highest dose.

Peroxidation of lipids occurs when prooxidant compound reacts with unsaturated fatty acids of biological

membranes. Their oxidative modification causes changes in the physical and chemical properties of the membranes [15, 33]. In this study the index of lipid peroxidation was measured as thiobarbituric acid reactive substances. The acute intoxication with chlorfenvinphos affected the concentration of MDA serum. Generation of MDA serum was not related to the dose of insecticide but rather to the time after intoxication. The increase in the concentration of malondialdehyde was greater after treatment of rats with chlorfenvinphos at the lowest dose in comparison to that observed after treatment of rats at the higher dose. Inactivation of antioxidative enzyme, such as GPx, probably result in the enhancement of serum lipid peroxidation in this group of rats. After treatment of rats with chlorfenvinphos at the dose of 0.5 LD_{50}, the MDA concentration decreased at the earlier period of intoxication and increased further. In the serum of rats treated at the two lower doses of chlorfenvinphos, MDA increased as early as at the 1st hour.

This indicates that the prooxidant effect of the highest dose of chlorfenvinphos becomes significant only after 48 hours of intoxication, while prooxidant effect of the lower doses of this compound is evident much earlier.

The decrease in MDA level that occurs, at the 48th hour, in response to treatment with chlorfenvinphos at the dose of 0.1 LD_{50} may be related to the normalization of GSH concentration and increased activity of GPx at the same time point. On the basis of these observations, we suggest that normalization of GSH level and high activity of GPx prevent lipid peroxidation.

The results reported here clearly show that acute intoxication with chlorfenvinphos caused disturbance in oxidative-antioxidative balance in the erythrocytes and serum of rats. Enhanced lipid peroxidation and decreased levels of vitamin C and E, which have been observed after intoxication with chlorfenvinphos at the two lower doses, are probably associated with acute oxidative stress. Interestingly, the treatment of rats with chlorfenvinphos at the highest dose induced the decrease in the concentration of MDA. These data suggest that the highest dose of chlorfenvinphos probably caused hypoxia at an early period after treatment. The induction of oxidative stress occurs at the 48th hour.

In conclusion, the results obtained suggest that reoxygenation following hypoxia occur in the acute intoxication with chlorfenvinphos at the highest dose (0.5 LD_{50}) whereas oxidative stress with tendency to normalization occur in the red blood cells of rats treated with the lower doses (0.02 and 0.1 LD_{50}) of this insecticide.

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