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SIMULTANEOUS MEASUREMENTS OF GLUTATHIONE AND ACTIVATED SULPHATE (PAPS) SYNTHESIS RATES AND THE EFFECTS OF SELECTIVE INHIBITION OF GLUTATHIONE CONJUGATION OR SULPHATION OF ACETAMINOPHEN

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Abstract—The aim of the present study was to examine the effects of the hepatotoxic drug acetaminophen (AA) on the synthesis rates of glutathione (GSH), activated sulphate (PAPS; adenosine 3′-phosphate 5′-phosphosulphate) and the AA metabolites AA–GSH and AA–sulphate after selective inhibition of GSH biosynthesis or sulphation in isolated rat hepatocytes. Selective inhibition of the two interdependent metabolic pathways was accomplished by buthionine sulphoximine (BSO) and 2,6-dichloro-4-nitrophenol (DCNP). The synthesis rates of GSH and PAPS were determined simultaneously by a previously described method based on trapping of radioactivity (^{35}S) in the pre-labelled GSH and PAPS pools. Pre-incubation with 10 mM BSO for 30 min depleted GSH by 38% ($P < 0.05$) and PAPS by 27% ($P < 0.05$). The depletion resulted in increased PAPS synthesis at low, non-toxic [5–19 nmol/(10^6 cells·min)] ($P < 0.05$) and at high, toxic [7–30 nmol/(10^6 cells·min)] ($P < 0.05$) AA concentrations. In both cases sulphur is diverted from GSH biosynthesis to sulphoxidation and PAPS synthesis, thereby maintaining the PAPS pool and preserving the sulphation capacity. This corresponds to the finding that AA sulphation was unaffected by BSO irrespective of AA concentration [6 vs 5 and 20 vs 17 nmol/(10^6 cells·hr), respectively]. Even though the GSH synthesis was halved after BSO pre-incubation, the GSH conjugating capacity of AA was well preserved. Incubation with 200 μM DCNP and 5 mM AA diminished PAPS synthesis from 24 to 10 nmol/(10^6 cells·min) ($P < 0.02$) and reduced AA–sulphate synthesis by 67% compared to experiments without DCNP incubation [4.8 vs 14.7 nmol/(10^6 cells·hr)] ($P < 0.05$). GSH and AA–GSH synthesis rates did not change compared to control experiments in which sulphation was not inhibited [1165 vs 1487 nmol/(10^6 cells·min), respectively] and [1.7 vs 1.7 nmol/(10^6 cells·hr), respectively]. This indicates that increased sulphur availability due to decreased PAPS synthesis is unable to raise the cysteine pool and stimulate the γ-glutamyl cycle and GSH synthesis.

Sulphation and glutathione (GSH‡) conjugation represent major detoxification reactions involved in the biotransformation and deactivation of xenobiotics and carcinogens. Both reactions share cysteine as a common precursor. Inorganic sulphate deriving from cysteine is activated by formation of the coenzyme adenosine 3′-phosphate 5′-phosphosulphate (PAPS, activated sulphate) preceding the conjugation reaction catalysed by phenol-sulphotransferase [1]. The γ-glutamyl cycle utilizes cysteine in the formation of GSH, which is a substrate for glutathione-*S*-transferases, the enzymes catalysing the conjugation reaction [2]. The hepatotoxic analgesic acetaminophen (AA) is predominantly detoxified by sulphation, glucuronidation and cytochrome P450 oxidation/GSH conjugation [3]. A radioactive tracer method [4] measured simultaneously PAPS and GSH synthesis rates in isolated rat hepatocytes together with synthesis rates of two AA metabolites, AA–sulphate and AA–GSH. Data indicated that sulphation and GSH conjugation are regulated by cysteine dependent on the concentration of AA to be detoxified.

The aim of the present study was to evaluate further the regulation of these two important detoxification pathways by investigating the effects of selective inhibition of GSH biosynthesis by buthionine sulphoximine (BSO) and of sulphation by 2,6-dichloro-4-nitrophenol (DCNP). BSO [5] is a specific inhibitor of γ-glutamyl cysteine synthetase which is the rate-limiting enzyme in the γ-glutamyl cycle, normally generating GSH. DCNP is a highly effective selective inhibitor of sulphation [6].

Studies of rat hepatocytes [7], incubated with a toxic AA concentration, demonstrated increased PAPS concentration without a corresponding increase of AA sulphation. Increased clearance of AA–sulphate was demonstrated *in vivo* [8] after administration of AA to rats depleted of GSH by BSO. It was suggested that cysteine was more available for oxidation to inorganic sulphate, but a dissociation between increased PAPS synthesis or increased sulphotransferase activity was not established. By the present approach we are able to determine whether PAPS synthesis or PAPS concentration is the regulator of AA sulphation, and to determine the influence of GSH inhibition on the shunting of sulphur from GSH to PAPS synthesis.

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‡ Abbreviations: AA, acetaminophen; GSH, glutathione; PAPS, activated sulphate, adenosine 3′-phosphate 5′-phosphosulphate; BSO, buthionine sulphoximine; DCNP, 2,6-dichloro-4-nitrophenol.

Table 1. Median (interquartile distances) turnover rates, concentrations, synthesis rates of PAPS, GSH, AA–sulphate and AA–GSH in isolated rat hepatocytes pre-incubated with 10 mM BSO prior to incubation with 0.1 or 5.0 mM AA (N = 6) and compared with experiments without BSO incubation (N = 11)

		Turnover rate /min		Concentration (nmol/10^6 cells)		Synthesis [nmol/(10^6 cells · min)]		Adduct synthesis [nmol/(10^6 cells · hr)]	
		PAPS		PAPS		PAPS		AA–sulphate	
Controls	low AA	0.7	(0.2–5.3)	2.3	(1.4–2.5)	4.9	(0.2–10.9)	5.5	(5.4–12)
	high AA	2.0	(1.5–3.2)	2.2	(1.7–2.7)	7.1	(1.2–12)	20.4	(18–24)
BSO	low AA	7.6†	(5.4–8.3)	2.4	(2.2–2.8)	18.8†	(16.2–19.7)	5.4	(5.4–5.4)
	high AA	11.3*	(10–15)	2.4	(2.2–2.8)	30.2*†	(27.2–32.9)	17.4*	(16.2–17.4)
		GSH		GSH		GSH		AA–GSH	
Controls	low AA	24.1	(9.7–35.4)	18.8	(15.8–20.6)	357	(234–793)	0.6	(0.4–1.2)
	high AA	18.2	(0.6–107)	17.4	(14.7–19.7)	301	(11–2218)	5.3	(3.6–5.4)
BSO	low AA	14.0	(8.9–20.0)	10.6†	(8.3–12.1)	123‡	(64–229)	0.4	(0.2–0.6)
	high AA	14.5	(5.8–20.0)	10.3†	(8.0–11.7)	121‡	(66–274)	3.8*	(2.6–4.7)

* Significant difference ($P < 0.05$) from paired experiments with low AA concentrations (within the BSO data).
† Significant difference ($P < 0.05$) from control experiments without BSO pre-treatment (non-paired experiments).
‡ Significant difference ($P < 0.05$) from control experiments without BSO pre-treatment if the difference in AA concentrations is disregarded.

Galinsky [8] demonstrated increased sulphation of harmol in BSO treated rats, due to increased catabolism of thiol-containing amino acids to inorganic sulphate. By the use of DCNP we wanted to establish whether the opposite effect was seen, i.e. if GSH turnover and synthesis were increased by inhibition of sulphation.

MATERIALS AND METHODS

Female Wistar rats (180–205 g, Møllegårdens Avlslaboratorium, Ll. Skensved, Denmark) were fasted for 16 hr before the experiments. The animals were kept in an air-conditioned environment with a controlled 12 hr light–dark cycle. They had free access to water. One hour before hepatocyte isolation, the rats were given [^{35}S]L-cysteine (80–130 μCi, sp. act. >600 Ci/mmol, Amersham Denmark ApS) intraperitoneally.

Hepatocyte isolation from rats was performed after collagenase perfusion [9, 10]. The yield of each preparation was $0.1–8.0 \times 10^8$ cells/liver, measured by counting the final cell suspension in a Buerker chamber. Immediately after isolation, 80–91% of the cells excluded Trypan blue. Incubation was performed at 37° under a 95% O_2/5% CO_2 atmosphere at a cell concentration of 10×10^6 cells/mL. The incubation medium was a Krebs–Ringer buffer ($MgSO_4$ 1.2 mM, pH 7.4) supplemented with glucose (10 mM) and albumin (1% w/v). In the BSO experiments cells were pre-incubated for 30 min with the drug (10 mM) (the Sigma Chemical Co., St Louis, MO, U.S.A). AA (0.1 and 5.0 mM) was added to shaken incubation flasks immediately before starting the experiments. In control experiments the BSO pre-incubation period was omitted.

A stock solution of DCNP was made by dissolving 13.7 mg DCNP (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) in 7 mL propylene glycol (Sigma). The final concentration in the cell suspension was 200 μM (23 μL DCNP stock solution/mL cell suspension), chosen from pilot experiments in order to obtain a non-toxic inhibition of sulphation. The final cell suspension was divided into two fractions which were incubated for 30 min with 200 μM DCNP and 5 mM AA or 5 mM AA and the dissolving agent (propylene glycol) alone (23 μL/mL cell suspension). In one experiment the cell suspension was divided into three fractions and in addition to the above, the third fraction contained hepatocytes incubated with 5 mM AA without DCNP or propylene glycol.

Analytical methods. All the analytical analyses were performed according to our previous study [4]. PAPS was measured using a modification of a method of Hazelton *et al.* [11]. Total GSH was measured by the GSH reductase 5,5′-dithiobis (2-nitrobenzoic acid) assay of Tietze [12]. AA metabolites were measured and fractionated on HPLC (Waters Associates, Milford, MA, U.S.A.) and radioactivity was determined by liquid scintillation spectrometry (Packard Instruments).

Calculations. Turnover rates of PAPS and GSH were determined by the decline of specific activities in AA–sulphate and AA–GSH by calculations of the turnover rate constants k_{PAPS} and k_{GSH}. These rates were multiplied with the mean concentrations of PAPS and GSH, respectively, during the incubation period to give the synthesis rates [4].

All values are expressed as medians with interquartile distances. The differences between turnover rates, concentrations and synthesis rates within the BSO and DCNP data are tested using a Wilcoxon test for paired observations. Differences between BSO data and control experiments are tested using a Mann–Whitney test for unpaired observations. The level of significance was 0.05.

RESULTS

Inhibition by BSO

The GSH concentration in freshly isolated hepatocytes was 20.3 (18.5–20.6) nmol/10^6 cells (data not shown). Pre-incubation with BSO (10 mM) for 30 min resulted in a 38% reduction of hepatocellular GSH to 12.6 (10.8–12.7) nmol/10^6 cells (data not shown) ($P < 0.05$). Although BSO

Table 2. Turnover rates, concentrations, synthesis rates of PAPS and AA–sulphate in isolated rat hepatocytes incubated with 200 μM DCNP and 5.0 mM AA or 5.0 mM AA without DCNP

Experiment No.	Turnover rate of PAPS /min Control	DCNP	Concentration of PAPS (nmol/10⁶ cells) Control	DCNP	Synthesis of PAPS [nmol/(10⁶ cells·min)] Control	DCNP	Synthesis of the AA–sulphate adduct [nmol/(10⁶ cells·hr)] Control	DCNP
1	11.7	9.4	2.2	2.2	26.1	21.1	19.3	17.4
2	9.2	3.6	2.6	2.8	24.0	10.0	9.3	1.6
3	2.2	1.1	3.9	4.0	8.3	4.5	16.7	5.8
4	15.5	7.2	4.6	4.8	72.0	34.4	15.1	5.5
5	2.1	1.1	6.1	6.0	13.0	6.5	12.6	4.1
6	2.5	1.1	5.9	5.6	14.9	6.0	14.7	4.3
7	18.2	7.2	5.2	5.1	93.9	36.9	13.8	4.8
Median	9.2	3.6*	4.6	4.8	24.0	10.0*	14.7	4.8*
Quartiles	(2.2–15.5)	(1.1–7.2)	(2.6–5.9)	(2.8–5.6)	(13.0–72.0)	(6.0–34.4)	(12.6–16.7)	(4.1–5.8)

* Significant difference ($P < 0.05$) from controls.

should be a specific inhibitor of GSH biosynthesis, pre-incubation reduced the PAPS concentration by one third from 3.32 (3.24–3.56) to 2.41 (2.24–2.45) nmol/10^6 cells (data not shown) ($P < 0.05$).

Table 1 shows turnover rates, concentrations and synthesis rates of PAPS, GSH, AA–sulphate and AA–GSH in paired experiments with BSO pre-incubated hepatocytes incubated with a toxic and a non-toxic AA concentration and control experiments in which hepatocytes were not pre-incubated with BSO. There was no difference in PAPS concentration in BSO treated hepatocytes compared to controls at toxic or non-toxic AA concentrations. However, PAPS synthesis increased about four times at low, non-toxic as well as at high, toxic AA concentrations corresponding to increased PAPS turnover rates. Although the PAPS synthesis increased significantly, the rate of AA sulphation was unchanged, i.e. there was no difference in AA–sulphate synthesis in BSO treated hepatocytes compared to control hepatocytes at low or high AA concentrations.

The GSH concentration was reduced in BSO pre-incubated hepatocytes compared to control hepatocytes at low AA concentrations (44%) and at high AA concentrations (41%). The GSH synthesis was reduced by 50% in BSO pre-incubated hepatocytes. This halving of the GSH synthesis reached statistical significance if the difference in AA concentration was disregarded, and was not followed by a corresponding reduction in the synthesis of the AA–GSH metabolite.

Figure 1 shows the relation between the GSH concentration and the increment in AA–GSH synthesis if hepatocytes are incubated with increased AA concentrations (from 0.1 to 5.0 mM AA) in control and BSO pre-incubated hepatocytes.

Inhibition by DCNP. Tables 2 and 3 show turnover rates, concentrations and synthesis rates of PAPS and GSH together with synthesis rates of AA–sulphate and AA–GSH in control and in DCNP pre-incubated hepatocytes.

AA–sulphate formation was linear during the entire experiment with or without DCNP pre-incubation. However, DCNP resulted in a 67% reduction of AA–sulphate synthesis. The PAPS concentration was unaltered by DCNP, but the PAPS synthesis was reduced by 58% corresponding with a 61% reduction of PAPS turnover rates.

DCNP did not affect GSH concentration of AA–GSH synthesis rates. Neither was GSH synthesis affected by DCNP. However, in experiments four and seven GSH synthesis rates were increased after DCNP pre-incubation. In these two experiments the decrement of PAPS synthesis was 38 and 57 nmol/(10^6 cells·min), respectively, in contrast to the rest of the experiments in which the decrement of PAPS synthesis never exceeded 14 nmol/(10^6 cells·min).

DCNP had no effect on AA glucuronidation. AA–glucuronide synthesis in DCNP experiments was 31 (27–33) compared to 31 (27–31) nmol/(10^6 cells·hr) in control experiments (data not shown). However, both AA–glucuronide and AA–GSH synthesis rates were lower than in our previous studies. In a control experiment (data not shown), in which the isolated hepatocytes were divided into three fractions (one incubated with AA, DCNP and propylene glycol; one with AA and propylene glycol and one with AA alone) AA–GSH synthesis was reduced by 51%, AA–glucuronide synthesis by 40%, and AA–sulphate synthesis was unchanged, except for the expected decrease due to DCNP treatment. However, differences observed in the present study are considered to be the result of DCNP treatment alone due to the experimental design, which only compares intra-experimental data.

DISCUSSION

In the first series of experiments in which GSH synthesis was inhibited we found a 4-fold increase in PAPS synthesis irrespective of AA concentration suggesting that more sulphur from cysteine was available compared to situations with intact GSH synthesis. This effect on PAPS synthesis was the result of increased PAPS turnover rates rather than increased PAPS concentration.

Increased PAPS synthesis in BSO pre-incubated cells did not cause any change in AA–sulphate synthesis irrespective of AA concentration. In contrast, other studies [8] showed enhanced AA–sulphate synthesis in BSO treated rats suggesting that BSO results in accumulation of cysteine, which markedly stimulates cysteine dioxygenase activity, increasing oxidation of cysteine and production of PAPS for AA sulphation, thereby increasing both PAPS and AA–sulphate synthesis rates. Increasing

Table 3. Turnover rates, concentrations, synthesis rates of GSH and AA–GSH in isolated rat hepatocytes incubated with 200 μM DCNP and 5.0 mM AA or 5.0 mM AA without DCNP

Experiment No.	Turnover rate of GSH /min Control	DCNP	Concentration of GSH (nmol/10^6 cells) Control	DCNP	Synthesis of GSH [nmol/(10^6 cells · min)] Control	DCNP	Synthesis of the AA–GSH adduct [nmol/(10^6 cells · hr)] Control	DCNP
1	165	70	21	17	3521	1165	1.5	1.6
2	89	38	17	15	1487	579	1.0	0.4
3	51	23	27	26	1371	610	2.1	2.0
4	54	79	22	21	1181	1676	1.8	1.7
5	50	41	17	18	870	724	1.7	1.5
6	197	45	27	28	5410	1235	6.5	5.5
7	72	105	24	23	1717	2421	1.7	1.7
Median	72	45	22	21	1487	1165	1.7	1.7
Quartiles	(51–165)	(38–79)	(17–27)	(17–26)	(1181–3521)	(610–1676)	(1.5–2.1)	(1.5–2.0)

organic sulphate (Na_2SO_4) concentration in rat hepatocytes [6] resulted in higher PAPS concentration without increased sulphation of AA (1 mM). Our experiments in which increased sulphur was mobilized from increased oxidation of cysteine due to inhibition of GSH synthesis, demonstrated increased PAPS synthesis without a corresponding increase in PAPS concentration or AA sulphation, suggesting that two regulatory mechanisms may act synergistically, i.e. the PAPS depletion seen in our hepatocytes before exposure to AA together with increased sulphur availability resulted in increased *de novo* PAPS synthesis which tended to raise the PAPS concentration to a certain level thereby avoiding impaired sulphation.

PAPS is not only used for sulphation of xenobiotics. Maintenance of an extracellular matrix is related to macromolecules containing covalently linked sulphate groups [13, 14]. Increasing PAPS synthesis may therefore alternatively be due to an increased demand for sulphated macromolecules necessary for maintenance of the inter-cell stability disrupted by the isolation procedure.

In the present study the PAPS concentration decreased significantly during the BSO pre-incubation. Similar effects of other GSH depletory agents (phorone, vinylidene and diethyl maleate) on PAPS concentrations have been studied [15]. It has been suggested that GSH depletory agents, in particular BSO (the S-alkyl moiety), have higher binding affinities to the active site of γ-glutamyl cysteine synthetase than cysteine as the basis for inhibiting GSH formation [5]. A similar action of BSO on one of the PAPS generating enzymes, ATP-sulphyrulase could explain the decreased hepatocellular PAPS concentration.

Gregus *et al.* [15] found both decreased PAPS concentration (54%) and diminished sulphation if low (100 μmol/kg) and high (300 μmol/kg) concentrations of harmol [16] were given to rats depleted of GSH by phorone. However, both GSH and PAPS were more profoundly depleted than in our study, indicating the existence of a concentration threshold, by which both PAPS generation and sulphation of xenobiotics are severely compromised due to inability to generate sulphate from cysteine.

Even though the concentration and the synthesis of GSH were reduced in the BSO pre-incubated hepatocytes, they were still able to increase the GSH conjugation, when a toxic AA concentration was introduced to the cell suspension (Fig. 1). This is in accordance with our previous observations in isolated perfused rat livers [17]. Although the change in conjugation was not statistically different (P = 0.27), the data indicates that even if the hepatocellular GSH concentration approaches the AA–GSH concentration, the cells are still able to synthesize GSH substantially above the minimal rate necessary for AA conjugation, and that the GSH concentration itself does not reflect the ability of the hepatocytes to conjugate AA.

In the series of experiments in which sulphation was inhibited, we found unchanged GSH and AA–GSH synthesis rates irrespective of a potential increase in cysteine availability for GSH synthesis.

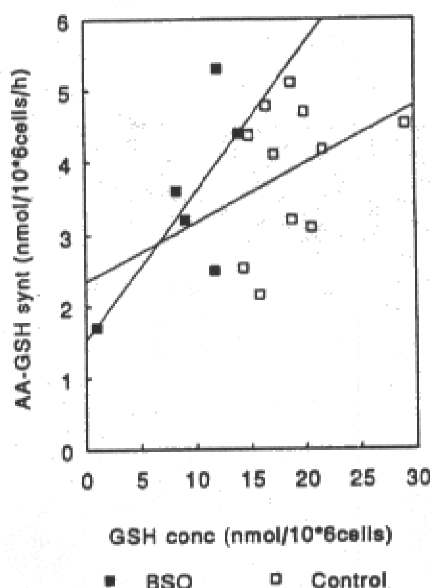

Fig. 1. Correlation between increment of AA–GSH synthesis (from low to high AA concentrations) and GSH concentration fitted by a linear regression line in control rat hepatocytes (open squares) ($Y = 0.34X + 1.52$), and in hepatocytes pre-incubated with 10 mM BSO (closed squares) ($Y = 0.08X + 2.35$).

Using a similar approach Lauterburg *et al.* [18] examined rats receiving a toxic AA concentration (1 g/kg) and found increased GSH synthesis if *N*-acetylcysteine was given 30 min after AA. However, the GSH synthesis was measured when GSH was substantially depleted, indicating that the depletion stimulated the GSH synthesis due to a feedback regulation of GSH on γ-glutamyl cysteine synthetase. Thus, the inability to stimulate GSH synthesis in the present study may be due to incomplete GSH depletion second to the relatively short time of investigation.

In contrast to the majority of experiments, inhibition of sulphation resulted in increased GSH synthesis in two experiments (four and seven). These two experiments showed the largest drop in PAPS synthesis, indicating that a substantial amount of sulphur deriving from cysteine under conditions in which sulphation was not inhibited is shunted from sulphation back into the cysteine pool with subsequently increased availability for incorporation into the γ-glutamyl cycle and increased GSH synthesis. Thus there seems to be a relation between the degree of PAPS inhibition and GSH synthesis which suggests that if PAPS synthesis is sufficiently inhibited, sulphur becomes available for the cysteine pool and GSH synthesis. The threshold level under the present experimental conditions seems to be between 14 and 38 nmol/(10^6 cells · min). Below this value inorganic sulphate availability can no longer maintain the cysteine pool which becomes depleted due to a counteracting effect that directs cysteine into the γ-glutamyl cycle and supports GSH synthesis for AA detoxification. Decreased substrate availability results in decreased GSH synthesis, eventually leading to decreased GSH concentration in the hepatocytes. Only when GSH is depleted beyond the threshold, the synthesis will be stimulated due to the feedback regulation of GSH on γ-glutamyl cysteine synthetase.

The large inter-experimental variation in GSH synthesis rates might alternatively be caused by a genetic polymorphism, well-known in humans [19, 20] in the expression of the GSH-*S*-transferase gene resulting in different ability to conjugate AA.

DCNP provides an instantaneous inhibition of sulphation of phenolic compounds [21] without evidence of metabolism in the liver. The mechanism of action has been suggested to be a competitive inhibition of phenol-sulphotransferases [22, 23]. However, the direct effect of DCNP has not been investigated in studies in which PAPS pool size and PAPS synthesis were measured simultaneously. We demonstrated, in accordance with Koster *et al.* [21], a 67% reduction of AA–sulphate synthesis in rat hepatocytes incubated with a toxic AA concentration. However, we also found significantly decreased PAPS synthesis which correlated to the decreased metabolite production. Although the correlation was weak, it indicated that DCNP may have an inhibitory effect on the PAPS generating enzymes as well. An alternative explanation for the reduced PAPS synthesis might be a substantial decrease in hepatocellular ATP necessary for generation of PAPS. It has previously been demonstrated that DCNP given to isolated rat hepatocytes causes ATP reduction [24–26].

Propylene glycol, the DCNP dissolving agent, decreased AA–glucuronide and AA–GSH synthesis rates, whereas AA–sulphate synthesis was unaltered as compared to our previous study. The dissociation of the propylene glycol effect on glucuronidation, GSH conjugation and sulphation makes a direct hepatotoxic effect unlikely. On the contrary and in accordance with a recent study [27] propylene glycol seems to protect the hepatocytes from the toxic effects of the oxidated AA metabolite.

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