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Abstract

Experimental beta-alaninuria was induced in rats by injection of (aminooxy)acetate (AOA), a potent inhibitor of aminotransferases, in order to elucidate the pathogenesis of hyper-beta-alaninemia. A 27-fold increase of beta-alanine (BALA) excretion was induced by subcutaneous injection of 1 5 mg of AOA per kg of body weight. A 13-fold and a 9-fold increase of beta-aminoisobutyric acid (BAIBA) and gamma-aminobutyric acid (GABA), respectively, were also induced simultaneously by the AOA injection. Identification of BALA and BAIBA isolated from the rat urine was performed by chromatographic and mass spectrometric analyses. The effects of AOA injection on the tissue levels of these amino acids were also studied. Contents of BALA in the liver and kidney and GABA in the brain increased significantly in response to AOA injection. The present study indicates that BALA transaminase is involved in hyper-beta-alaninemia.

KEYWORDS: beta-alanine, beta-aminoisobutyric acid, ganma-amlnobutyric-acid, (aminooxy)acetate, aminotransferase

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Experimental β-Alaninuria Induced by (Aminooxy)acetate

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Experimental β-alaninuria was induced in rats by injection of (aminooxy)acetate (AOA), a potent inhibitor of aminotransferases, in order to elucidate the pathogenesis of hyper-β-alaninemia. A 27-fold increase of β-alanine (BALA) excretion was induced by subcutaneous injection of 15 mg of AOA per kg of body weight. A 13-fold and a 9-fold increase of β-aminoisobutyric acid (BAIBA) and γ-aminobutyric acid (GABA), respectively, were also induced simultaneously by the AOA injection. Identification of BALA and BAIBA isolated from the rat urine was performed by chromatographic and mass spectrometric analyses. The effects of AOA injection on the tissue levels of these amino acids were also studied. Contents of BALA in the liver and kidney and GABA in the brain increased significantly in response to AOA injection. The present study indicates that BALA transaminase is involved in hyper-β-alaninemia.

Key words: β-alanine, β-aminoisobutyric acid, γ-aminobutyric acid, (aminooxy)acetate, aminotransferase

Hyper-β-alaninemia (McKusick 237400) is a rare metabolic disorder characterized by elevated β-alanine (BALA) concentrations in blood plasma, urine and tissues, and only one case has been reported thus far (1, 2). Although the pathogenesis of this disorder has not yet been clarified, it has been suggested that its metabolic cause is related to defects in BALA transaminase (1). During our studies on the MP-pathway (3-mercaptopyruvate pathway) of cysteine metabolism (3), we found that injecting rats with (aminooxy)acetate (AOA), a potent inhibitor of aminotransferases (4), resulted in increases in excretion of taurine (β-aminosulfonic acid) and hypotaurine (β-aminosulfinic acid) (5). We noted that, besides taurine and hypotaurine, peaks corresponding to BALA, β-aminoisobutyric acid (BAIBA) and γ-aminobutyric acid (GABA) were also elevated in the chromatogram of reversed-phase high-performance liquid chromatography (RP-HPLC) of the same urine.

In the present paper, we report on the isolation and identification of BALA and BAIBA from the urine of rats injected with AOA in order to identify the urinary amino acids increased by AOA injection. A RP-HPLC method for the analysis of these amino acids has been reported elsewhere (6) and, using this method, the effect of AOA treatment on the tissue contents of these amino acids and GABA was studied further in order to elucidate the pathogenesis of hyper-β-alaninemia.

Materials and Methods

Materials. AOA was obtained from Sigma Chemical Co., St. Louis, MO, USA. 4-Dimethylaminoazobenzene-4'-sulfonyl chloride (dabsyl chloride) was obtained from Fluka Chemie AG, Buchs, Switzerland and crystallized once from acetone. BALA, BAIBA, GABA, D,L-ethionine (Eth) and acetonitrile (HPLC grade) were obtained from Wako Pure Chemical Ind., Osaka, Japan.

Male Wistar rats were maintained on a laboratory diet, MF, of the Oriental Yeast Co., Ltd., Tokyo, Japan and water *ad libitum*. These rats were used for experiments involving urine samples when they had grown to 225 ± 6 g of body weight and for experiments involving tissue samples when they had grown to 280 ± 12 g of body weight.

Injection of AOA and preparation of samples for analyses. AOA was dissolved in water, neutralized with sodium hydroxide solution and adjusted at a concentration of 2.5 mg/ml. After sterilization by filtration through a membrane filter (pore size, 0.2 μm), the solution was injected subcutaneously into the rats' backs at a dose of 15 mg per kg of body weight. Rats

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were housed separately in metabolic cages and 24-h urine samples were collected in 100 ml Erlenmeyer flasks containing one ml of toluene and 5 ml of water.

When tissue samples were analyzed, rats were anesthetized with ether and killed by taking blood by heart puncture at 2 or 4 h after the injection of AOA. The following procedures were performed at 0–4 °C. Ten % EDTA solution was used as an anticoagulant and blood plasma was separated by centrifugation at 1,200 × *g* for 10 min. Liver, kidney and whole brain were removed and washed with 0.9 % sodium chloride solution. Blood plasma, liver, kidney and whole brain were homogenized with 10 volumes of 5.5 % trichloroacetic acid (TCA) and centrifuged at 10,000 × *g* for 10 min. TCA-extracts thus obtained were used for RP-HPLC analysis as described below.

Precolumn derivatization and RP-HPLC of BALA and related compounds. Precolumn derivatization and RP-HPLC analysis of BALA and related compounds were performed according to the method described elsewhere (6). Briefly, amino acids in the urine and TCA extracts were derivatized with dabsyl chloride. A known amount of Eth was added as an internal standard to sample solutions before derivatization. Dabsyl-amino acids formed were analyzed by the RP-HPLC system of the Tosoh Company (Tokyo, Japan) which consists of a CCPM pump, a UV-8010 detector and a Chromatocorder 12 integrator. The column used was a TSKgel ODS-80Ts (4.6 × 250 mm) with a guard column of TSKguardgel ODS-80Ts (3.2 × 15 mm). Chromatography was performed at a flow rate of 0.7 ml per min with a linear gradient prepared from 50 mM sodium acetate buffer (pH 4.15) (solvent A) and acetonitrile (solvent B) as previously reported (6). Routine determination of BALA, BAIBA and GABA were performed with RP-HPLC.

Purification of BALA from the urine of rats injected with AOA. In order to identify a peak on RP-HPLC corresponding to BALA, the amino acid was purified from the urine of 7 rats injected with AOA (15 mg/kg of body weight). The urine after AOA administration was collected for 2 days in an Erlenmeyer flask containing 1 ml of toluene as an antiseptic. The pooled urine (271 ml) was centrifuged at 1,200 × *g* for 20 min and filtered through a filter paper. The filtrate was adjusted to pH 2 with 2 M hydrochloric acid and applied to a column of Dowex 50 W (X8, 200–400 mesh, H^+ form, 3.3 × 47 cm). After washing with water, amino acids were eluted with 2 M ammonia and the eluate was evaporated to dryness with a flash evaporator at 45 °C. The dried residue was dissolved in 5 ml of water and applied to a column of Dowex 50 W (X8, 200–400 mesh, Na^+ form, 2.6 × 90 cm). Chromatography was performed with 0.2 M sodium acetate, pH 4.50, collecting 20 ml-fractions. Fractions 67–91 containing a peak corresponding to BALA were pooled and concentrated to about 10 ml with a flash evaporator. The concentrate was again chromatographed using the same column and buffer as above. The fractions containing a peak corresponding to BALA were pooled and desalinized with a Dowex 50 W column (X8, 200–400 mesh, H^+ form, 1.5 × 10 cm). The eluate with 2 M ammonia from the column was evaporated to dryness with a flash evaporator and the residue was dissolved in 8 ml of water.

A portion (2 ml) of the above solution was subjected to high-voltage paper electrophoresis using 1 Chr paper (Whatman) (7 × 40 cm) and a buffer (95 % pyridine-acetate-water, 1:20:179, pH 3.1) (7) at 3 kV for 45 min at 10 °C. The area containing a band corresponding to BALA was cut out and the band was eluted with water. This procedure was repeated 4 times and the eluates were combined and applied to a Dowex 50 W column (X8, 200–400 mesh, H^+ form, 1.5 × 10 cm). After washing with water, the column was eluted with 2 M ammonia. The ammonia fraction was evaporated to dryness with a flash evaporator and the dried residue (sample A) was used for further analyses.

Purification of BAIBA from the urine of rats injected with AOA. Thirty-one ml of the urine obtained after AOA treatment was desalinized with a Dowex 50 W column (X8, 200–400 mesh, H^+ form, 2.3 × 8 cm). The eluate with 2 M ammonia from the above column was evaporated to dryness with a flash evaporator at 45 °C and dissolved in 3 ml of water. After adjusting to pH 3 with 2 M hydrochloric acid, the solution was applied to a Dowex 50 W column (X8, 200–400 mesh, 2.6 × 90 cm, Na^+ form) and chromatography was performed with 0.2 M sodium acetate (pH 4.50). Ten-ml fractions were collected and checked by ninhydrin reaction. Fractions 201–210 containing a peak corresponding to BAIBA were pooled and evaporated to dryness with a flash evaporator. The dried residue was dissolved in a small amount of water (approximately 2 ml) and subjected to high-voltage paper electrophoresis as described above. The band corresponding to BAIBA was eluted with water. The eluate was concentrated and subjected to

paper chromatography using 1 Chr paper and a solvent system (isopropanol-formate-water, 64:3:16, by volume). The band corresponding to BAIBA was eluted with water and evaporated to dryness as above. This final residue (sample B) was used for further analyses.

Amino acid analysis. A Hitachi KLA-5 amino acid analyzer equipped with a column (0.9 × 55 cm) of Hitachi custom ion-exchange resin 2613 was used. Amino acid analysis was performed using 0.2 N sodium citrate buffer (pH 3.22) until 105 min and then 0.2 N sodium citrate buffer (pH 4.50) containing 24 % ethanol at a flow rate of 60 ml/h at 55 °C.

High-voltage paper electrophoresis. Routine high-voltage paper electrophoresis was performed in a pyridine acetate buffer (95 % pyridine-acetic acid-water, 1:20:179; pH 3.1) at a potential gradient of 100 volts/cm for 30 min. Amino acids were visualized with ninhydrin.

Thin layer chromatography. Thin layer chromatography was performed using microcrystalline cellulose (Funacel SF, Funakoshi Co., Ltd., Tokyo, Japan) and a solvent system of isopropanol-formic acid-water (64:3:16, v/v). Amino acids were stained with ninhydrin.

Fast-atom-bombardment mass spectrometry (FAB-MS). FAB-MS was performed with a Shimadzu 9020-DF gas chromatograph-mass spectrometer with a SCAP data system (Shimadzu Seisakusyo, Kyoto, Japan). The target surface was bombarded by a beam of energetic argon atoms at 5 keV. Glycerol was used as a matrix.

Results and Discussion

Identification of BALA and BAIBA. Purification of BALA and BAIBA was followed by thin layer chromatography, high-voltage paper electrophoresis, amino acid analysis and RP-HPLC. The purified samples A and B yielded a single spot or band, respectively, on the chromatograms of thin layer chromatography and paper electrophoresis, and exhibited the same behavior as authentic BALA and BAIBA, respectively. On chromatograms of amino acid analysis and RP-HPLC, the isolated compounds (samples A and B) exhibited single peaks. Figs. 1a and 1b are typical examples of RP-HPLC. Retention times of sample A (co-eluted with authentic BALA) and sample B (co-eluted with authentic BAIBA) in amino acid analysis were 207 and 209 min. Corresponding times in RP-HPLC were 45 and 61 min.

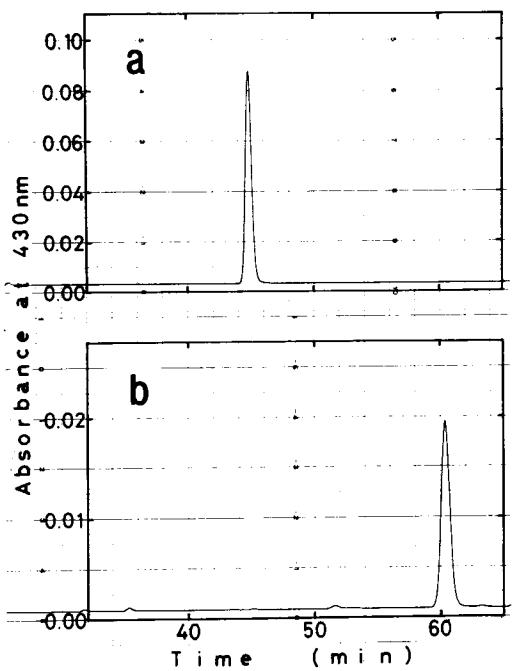

Fig. 1 Elution profiles of reversed-phase high-performance liquid chromatography of isolated compounds corresponding to β-alanine (**a**) and β-aminoisobutyric acid (**b**). See text for details.

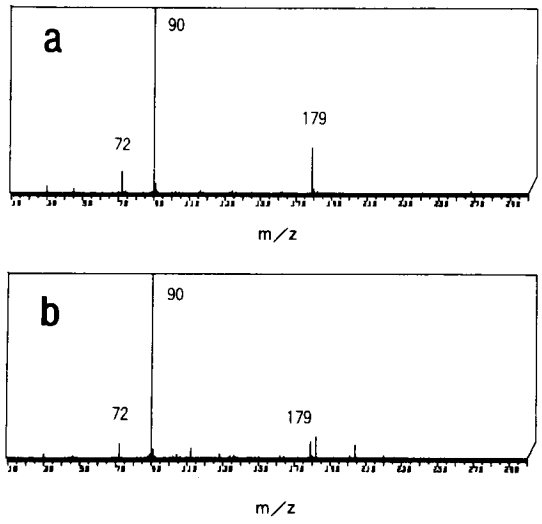

Fig. 2 Fast-atom-bombardment mass spectrometry of authentic β-alanine (**a**) and isolated compound (**b**). See text for details.

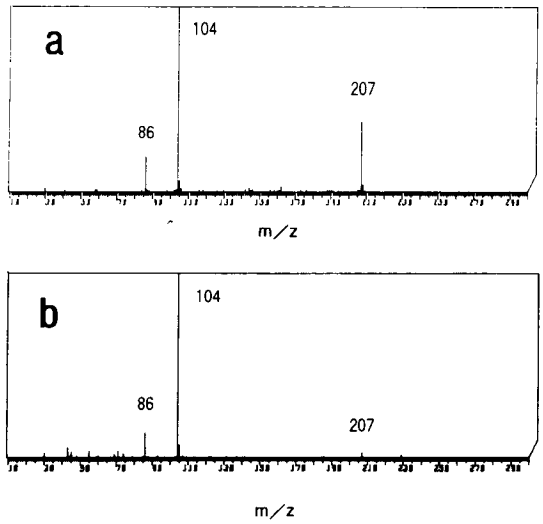

Fig. 3 Fast-atom-bombardment mass spectrometry of authentic β-aminoisobutyric acid (**a**) and isolated compound (**b**). See text for details.

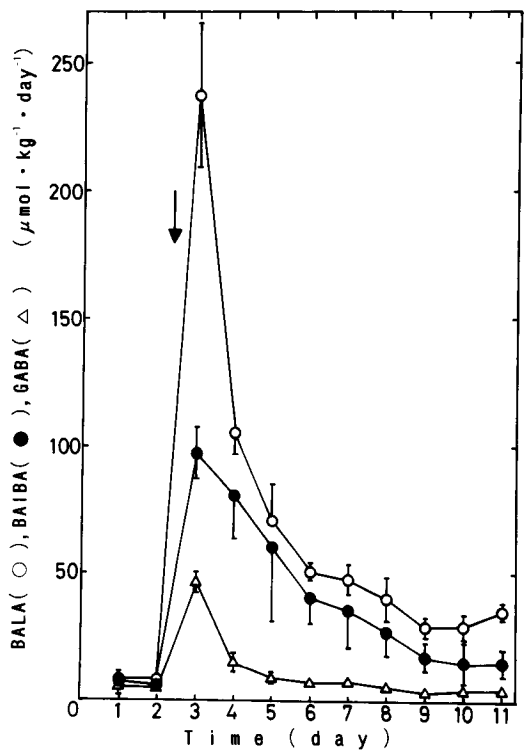

Fig. 4 Urinary excretion of β-alanine (BALA), β-aminoisobutyric acid (BAIBA) and γ-aminobutyric acid (GABA) after (aminooxy) acetate (AOA) administration to rats. Fifteen mg of AOA per kg of body weight was injected subcutaneously at day 2 (arrow) and urine was collected at 24 h intervals for amino acid content determination. Data are shown as mean $\pm$ SD (n = 7).

Identification of the isolated compounds was further performed with FAB-MS as shown in Figs. 2b and 3b. Sample A exhibited signals of m/z 72, 90 and 179, and sample B m/z 86, 104 and 207. These signals of samples A and B were assigned to be m/z 72 ($MH^+ - H_2O$), 90 (MH^+) and 179 ($2MH^+$) of BALA and m/z 86 ($MH^+ - H_2O$), 104 (MH^+) and 207 ($2MH^+$) of BAIBA, respectively, as the same signals were exhibited in FAB-MS of authentic BALA and BAIBA as shown in Figs. 2a and 3a, respectively.

Thus, the isolated compounds A and B, namely, the two urinary compounds increased by AOA injection were identified as BALA and BAIBA, respectively. As GABA has been well characterized (8), its isolation and identification were not performed in this study.

Urinary excretion of BALA, BAIBA and GABA before and after AOA administration. As reported previously (6), urinary excretion of BALA, BAIBA and GABA in normal rats were low at levels of 9.13 ± 1.55, 5.81 ± 0.69 and 5.02 ± 1.24 μmol/kg of body weight per day, respectively. In the present study, similar urinary levels of these amino acids were reconfirmed: 8.63 ± 2.95, 7.54 ± 1.95 and 5.33 ± 3.26 μmol/kg of body weight per day (n = 7), respectively.

Table 1 Contents[a] of β-alanine (BALA), β-aminoisobutyric acid (BAIBA) and γ-aminobutyric acid (GABA) in rat tissues and their increase at 2 and 4 h after (aminooxy) acetate (AOA) injection[b]

		BALA	BAIBA	GABA
Liver	Control	0.177 ± 0.046	0.000 ± 0.000	0.000 ± 0.000
	2	1.892 ± 0.195*[c]	0.415 ± 0.025*	0.252 ± 0.063**
	4	2.879 ± 0.337*	0.571 ± 0.087*	0.514 ± 0.100*
Kidney	Control	0.123 ± 0.025	0.000 ± 0.000	0.048 ± 0.015
	2	0.594 ± 0.085*	0.066 ± 0.023**	0.028 ± 0.009
	4	1.238 ± 0.203*	0.123 ± 0.013*	0.072 ± 0.009***
Brain	Control	0.082 ± 0.021	0.000 ± 0.000	3.844 ± 0.342
	2	0.115 ± 0.036	0.000 ± 0.000	5.261 ± 1.611
	4	0.170 ± 0.021**	0.000 ± 0.000	6.892 ± 1.856***
Blood plasma	Control	6.400 ± 3.991	0.000 ± 0.000	0.700 ± 0.096
	2	13.545 ± 5.396	1.451 ± 0.196*	0.959 ± 0.225
	4	18.765 ± 8.515	3.088 ± 1.436***	1.966 ± 0.971

a: Contents in tissues (μmol/g) and blood plasma (nmol/ml) are expressed as mean ± SD of data obtained from 3 (control) or 4 (AOA-treated) animals.

b: Fifteen mg of AOA per kg of body weight was injected subcutaneously.

c: Statistical difference between controls and the AOA-treated animals was calculated by the Student's *t* test: *$P < 0.001$; **$P < 0.01$; ***$P < 0.05$.

Fig. 4 shows increases in the urinary excretion of BALA, BAIBA and GABA after subcutaneous injection of 15 mg of AOA per kg of body weight. BALA excretion increased to 237.55 ± 28.03 μmol/kg of body weight per day during the 24 h after the AOA injection. This remarkable increase in BALA excretion after AOA injection in the present and previous (6) studies indicates that BALA is actively metabolized through transamination reaction and that BALA is rapidly excreted in the urine. After this, the BALA excretion decreased gradually, but it was still at a higher level even at 10 days later than it had been before AOA injection. This suggests that the inhibitory effect of AOA on BALA-metabolizing transaminase(s) continued for at least 10 days after the AOA injection.

The excretion rates of BAIBA and GABA in 24 h following AOA injection were 97.37 ± 10.22 and 46.32 ± 4.01 μmol/kg of body weight per day, respectively. Excretion patterns of these amino acids were similar to that of BALA as shown in Fig. 4.

Tissue contents of BALA, BAIBA and GABA before and after AOA administration. Table 1 shows the increase in BALA, BAIBA and GABA concentrations in liver, kidney, brain and blood plasma at 2 and 4 h after AOA injection. BALA in the liver increased 10-times at 2 h after the AOA injection and increased further at 4 h. BALA contents in the kidney, brain and blood plasma exhibited similar patterns. BAIBA was not detected in these tissues, but it was detected at 2 h after AOA injection and increased further at 4 h. In the brain, however, BAIBA was not detected even at 4 h after AOA administration. GABA in the liver exhibited the same pattern as that of BAIBA. In the brain, GABA content was much higher than the content of these β-amino acids and it increased further by AOA injection.

BALA is a catabolite of uracil (9) and is contained in coenzyme A and in β-alanyl dipeptides, carnosine and anserine (2). Coenzyme A is an indispensable factor in fatty acid metabolism, and thus, BALA is a physiologically important cell constituent. Carnosine is contained in the human skeletal muscle and anserine in the skeletal muscle of birds and mammals such as rabbits, rats and whales. Although the functions of these β-alanyl dipeptides are not fully understood, they seem to play important roles in muscle function (2).

BAIBA occurs in mammals in two forms: *R*- and *S*-BAIBA. The former is a catabolite of thymine and the latter of valine (9). Although the configuration of BAIBA was not studied in the present study, the increased BAIBA after AOA injection seems to be mainly *R*-form because *S*-BAIBA is formed from L-valine by transamination reactions (9), which are also supposed to be inhibited by AOA. Absence of BAIBA in the brain even after

AOA injection seems to indicate that metabolism of thymine to BAIBA in the brain is negligibly low if any.

It has been reported that BALA-oxoglutarate aminotransferase (4-aminobutyrate transaminase, EC 2.6.1.19) from the rat liver is identical with L-β-aminoisobutyrate (*S*-BAIBA) aminotransferase, and that the enzyme is inactive against *R*-BAIBA (10). *R*-BAIBA is thought to be metabolized by D-3-aminoisobutyrate-pyruvate aminotransferase (EC 2.6.1.40) in the rat liver (11). It seems that this enzyme was inhibited by AOA in the present study.

GABA is an important neurotransmitter in the brain, and it is also formed in small amounts in the liver and other tissues. GABA is mainly formed by α-decarboxylation of L-glutamic acid by glutamate decarboxylase (EC 4.1.1.15) and metabolized through a transamination reaction (12). It has been reported that GABA transaminase (EC 2.6.1.19) in the brain of rats is inhibited by AOA (4, 13). In mice, the activity of GABA transaminase was reduced to near zero within 30 min of AOA administration, whereas the activity of glutamate decarboxylase was not influenced *in vivo*, although AOA had a pronounced inhibitory effect on both enzymes *in vitro* (14). These facts explain the increase in GABA concentration by AOA administration in the mouse brain, and this may also be possible in the rat brain. GABA transaminase preparations from the mouse brain and liver catalyzed transamination of BALA (15) and GABA transaminase in human tissues transaminated GABA and BALA equally well (16). BALA transaminase isolated from the rabbit liver catalyzed transamination of BALA, GABA and BAIBA, and it was concluded that BALA transaminase from the rabbit liver closely resembled GABA transaminase from the liver and brain (17). These observations are in accordance with the similar excretion patterns of GABA, BAIBA and BALA in the present study. These excretion patterns are similar to those in the urine of the patient with hyper-β-alaninemia (1). Thus, the present study indicates that BALA transaminase is involved in the pathogenesis of this metabolic disorder. The mechanisms producing symptoms such as seizures, hypotonia, hyporeflexia and somnolence observed in the patient with hyper-β-alaninemia are not clear at present, but the animals treated with AOA seem to be useful for the study of their pathogenesis.

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