

## Mitochondrial citrulline synthesis from ammonia and glutamine in the liver of ureogenic air-breathing catfish, *Clarias batrachus* (Linnaeus)

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The possible synthesis of citrulline, a rate limiting step for urea synthesis via the ornithine-urea cycle (OUC) in teleosts was tested both in the presence of ammonia and glutamine as nitrogen-donating substrates by the isolated liver mitochondria of ureogenic air-breathing walking catfish, *C. batrachus*. Both ammonia and glutamine could be used as nitrogen-donating substrates for the synthesis of citrulline by the isolated liver mitochondria, since the rate of citrulline synthesis was almost equal in presence of both the substrates. The citrulline synthesis by the isolated liver mitochondria requires succinate at a concentration of 0.1 mM as an energy source, and also requires the involvement of intramitochondrial carbonic anhydrase activity for supplying  $\text{HCO}_3^-$  as another substrate for citrulline synthesis. The rate of citrulline synthesis was further stimulated significantly by the isolated liver mitochondria of the fish after pre-exposure to 25 mM  $\text{NH}_4\text{Cl}$  for 7 days. Due to possessing this biochemical adaptational strategy leading to the amelioration of ammonia toxicity mainly by channeling ammonia directly and/or via the formation of glutamine to the OUC, this air-breathing catfish could succeed in surviving in high external ammonia, which it faces in its natural habitat in certain seasons of the year.

**Keywords:** Carbamyl phosphate synthetase, Carbonic anhydrase, Ornithine urea cycle, Succinate, Ureogenesis

The formation of urea in freshwater teleosts via the ornithine-urea cycle (OUC), which is the major source of urea formation for detoxification of ammonia in higher vertebrates, was thought impossible as the OUC was found to be either incomplete or non-functional in teleosts<sup>1-3</sup>. However, in recent years, there has been renewed interest on studies of the OUC in teleosts after the reports on high expression of OUC enzymes accompanied by active ureogenesis in several teleosts species such as the marine toadfishes (*Opsanus beta* and *Opsanus tau*)<sup>4,5</sup>, the alkaline lake-adapted tilapia (*Alcolapia grahami*)<sup>6</sup>, two Indian freshwater air-breathing catfishes (*Heteropneustes fossilis* and *Clarias batrachus*)<sup>7-9</sup> and the gobiid fish (*Mugilogobius abei*)<sup>10</sup>. Some of these species also excrete significant amount of urea in response to various adverse environmental circumstances such as confinement (stress), severely alkaline water, ammonia loading and exposure to air or while living inside mud peat under semidry conditions<sup>6,11-20</sup>.

One important finding in relation to OUC in teleosts is the presence of a different type of carbamyl

phosphate synthetase (CPS), the CPS III activity rather than the presence of a typical OUC-related CPS I activity, reported for the first time in largemouth bass (*Micropterus salmoides*)<sup>21</sup>, elasmobranch (*Squalus acanthias*)<sup>22</sup>, and subsequently followed by some other ureogenic teleosts<sup>23</sup>. CPS III differs from CPS I with respect to substrate preference and utilizes glutamine as nitrogen-donating substrate rather than ammonia, but like CPS I, it also requires N-acetyl-L-glutamate (AGA) as coenzyme and is located in the mitochondrial matrix<sup>24</sup>. Most interestingly, in two ureogenic air-breathing catfishes (*H. fossilis* and *C. batrachus*), in addition to the presence of a typical fish-type CPS III activity, CPS I-like activity (normally found in mammals, amphibians and also in certain lungfishes) has also been demonstrated in the mitochondria of both hepatic and certain non-hepatic tissues<sup>25,9</sup>. The presence of CPS III activity was not unusual in these catfishes with their known ureotelic capacity but the presence of a significant ammonia- and AGA-dependent CPS I-like activity is unique from that observed in other teleosts, and the presence of both types of OUC-related CPSes in a single vertebrate species was not reported earlier.

The capacity of synthesizing citrulline, the product formed by the involvement of the first two mitochondrial enzymes of the OUC, the CPS and

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ornithine transcarbamoylase (OTC) from bicarbonate, ornithine and ammonia using succinate as an energy source, by isolated liver mitochondria has been employed to study the regulatory and related properties of the first two steps of the OUC in mammalian species<sup>26-28</sup>. In the present study, to obtain further evidence for the involvement of both glutamine and ammonia as nitrogen-donating substrates for CPS, so as to find out whether both the substrates individually, or in combination can be used for urea synthesis via the OUC due to the presence of both types of OUC-related CPS activities, the mitochondrial citrulline synthesis has been studied in the liver of one of the ureogenic air-breathing catfishes, *Clarias batrachus* both in control fish and also in the fish pre-exposed to high external ammonia (25 mM NH<sub>4</sub>Cl) for 7 days. Further, the dependency of citrulline synthesis by the isolated liver mitochondria on succinate, an energy source for carbamyl phosphate formation<sup>29-31</sup>, and carbonic anhydrase (CA) enzyme, which supplies HCO<sub>3</sub><sup>-</sup> for CPS, has been tested by using acetazolamide (a known inhibitor of CA).

### Materials and Methods

**Animal**—The air-breathing walking catfish (*C. batrachus*), weighing 85 ± 15 g were purchased from commercial sources, and acclimatized in the laboratory for approximately one month at room temperature (28°±2 °C) in 12:12 hr light and dark photoperiod. Minced pork liver and rice bran (5% of the body wt.) was supplied as food and water was changed on alternate days. No sex differentiation of the fish was done while performing this experiment. Food was withdrawn 24 hr prior to experiment.

**Chemicals**—All the substrates and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, USA. All other chemicals used were of analytical grades and obtained locally. Deionized double glass distilled water was used in all preparations.

**Experimental protocol**—A set of acclimatized fish of similar size were placed in a plastic aquarium of known volume of 25 mM NH<sub>4</sub>Cl solution (pH 6.95±0.11), prepared in bacteria-free filtered stream water, and exposed for seven days. Another set of acclimatized fish were kept in a plastic aquarium containing only bacteria-free filtered stream water (pH 7.04±0.10), which served as controls. Both, NH<sub>4</sub>Cl solution and water from each aquarium were

replaced with fresh medium at one day interval. After 7 days, fishes from each treatment were removed, sacrificed immediately by decapitation, livers from each fish were excised and dipped in liquid nitrogen before storing at -60°C.

**Mitochondrial preparation**—Livers from 4 to 5 fishes of both control and NH<sub>4</sub>Cl-treated fish were pooled separately, minced and a 20% homogenate (w/v) of the liver was prepared in a homogenizing buffer containing 50 mM Na-phosphate buffer (pH 7.4), 300 mM mannitol, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 100 mM KCl using a motor-driven Potter-Elvehjem glass homogenizer with a loosely fitted Teflon pestle, and the mitochondria from each set of liver were isolated by differential centrifugation method following Dkhar *et al.*<sup>32</sup>. The mitochondrial pellets were resuspended in a known volume of original homogenizing buffer containing bovine serum albumin (BSA, 5 mg/ml), so that the final concentration of BSA in the solution was equivalent to 0.2 ml/g of liver. The suspended mitochondria were then immediately used for the analysis of citrulline synthesis under different experimental conditions. All the steps were carried out at 4°C.

**Citrulline synthesis by the isolated mitochondria**—The reaction mixture for the analysis of mitochondrial citrulline synthesis was prepared following the method of Anderson and Walsh<sup>33</sup> with certain modifications. The reaction mixture in a final volume of 750 µl contained 50 mM Na-phosphate buffer (pH 7.4), 100 mM mannitol, 90 mM KCl, 5 mM NaHCO<sub>3</sub>, 5 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 0.1 mM Na-succinate, 5 mM ATP, 5 mM L-ornithine, 20 mM glutamine/NH<sub>4</sub>Cl and 100 µl mitochondrial fraction.

The rate of citrulline synthesis by the isolated liver mitochondria was initially tested at different concentrations of succinate, and 0.1 mM succinate was found to be the optimal concentration for citrulline synthesis (Fig. 1). Therefore, in all subsequent experiments, 0.1 mM succinate was taken in the reaction mixture. In another set of experiments, acetazolamide, an inhibitor for CA, was added to the final reaction mixture to study the role of intramitochondrial CA activity on citrulline synthesis. The reaction mixture in a final volume of 750 µl was identical to the one mentioned above except for the addition of 3.25 µmoles of acetazolamide. The reaction mixtures containing acetazolamide (without the substrate) were first pre-incubated in different

microcentrifuge tubes for 5 min at 30°C, followed by addition of different nitrogen-donating substrates such as glutamine (20 mM), NH<sub>4</sub>Cl (20 mM) or NH<sub>4</sub>Cl plus glutamine (20 mM each), incubated for 20 min at 30°C, and the reaction was stopped by adding 10 µl of 70% perchloric acid (PCA). The effects of various nitrogen-donating substrates on citrulline synthesis by the isolated liver mitochondria were also studied in a similar way in the same reaction mixture but by having different concentrations of each substrate. In each experiment, a reagent blank was always prepared by adding 10 µl of 70% PCA to the reaction mixture prior to the addition of the substrate. The precipitated protein was separated out by centrifugation for 10 min at 10,000 g and the supernatant was used for the estimation of citrulline spectrophotometrically at 490 nm by the method of Moore and Kauffman<sup>34</sup>.

**Protein estimation**—Protein was estimated following the dye-binding method of Bradford<sup>35</sup> using bovine serum albumin as the standard.

**Statistical analysis**—The data were calculated from at least five observations at each point and presented as mean ± SE (*n* = number of experiments). Comparisons of the unpaired mean values between the experimental one and respective controls were made using unpaired Student's *t*-test and differences with *P* < 0.05 were regarded as statistically significant.

## Results

**Effects of succinate concentrations on citrulline synthesis**—Initially, the rate of citrulline synthesis by the isolated liver mitochondria of the catfish was tested at different concentrations of succinate (0.05 to 10 mM) *in vitro* conditions (Fig. 1). Interestingly, both with ammonia and glutamine as nitrogen-donating substrates, the peak of citrulline synthesis was achieved at 0.1 mM succinate, followed by a sharp decrease of citrulline synthesis at higher concentrations.

**Role of carbonic anhydrase (CA) activity on citrulline synthesis**—The role of CA in citrulline synthesis, which supplies HCO<sub>3</sub><sup>-</sup> as one of the substrates for CPS, by the isolated liver mitochondria of *C. batrachus* was tested by using acetazolamide, a known inhibitor of CA. When glutamine and ammonia were taken separately as nitrogen-donors almost similar rates of citrulline synthesis were observed (Table 1), but when glutamine and ammonia were taken together in the reaction mixture some additive effect on citrulline synthesis was observed.

Acetazolamide inhibited the rate of citrulline synthesis significantly by 70% (*P* < 0.001) when ammonia and glutamine were taken separately as nitrogen-donating substrates, and by 65% when both glutamine plus ammonia were taken together.

**Effects of different concentrations of NH<sub>4</sub>Cl and glutamine on citrulline synthesis**—The citrulline synthetic rates by the liver mitochondria, isolated from control fish as well as from the fish pre-exposed to 25 mM NH<sub>4</sub>Cl for 7 days, were studied with different concentrations of ammonia and glutamine separately (Figs 2 and 3). With ammonia as a nitrogen-donating substrate, the rates of citrulline synthesis increased linearly with increasing ammonia concentrations until it reached a V<sub>max</sub> of 230 nmoles/mg protein/hr at 3 mM concentration, followed by no further increase at higher concentrations (Fig. 2). Almost similar pattern of citrulline synthetic rates were observed with ammonia by the fish pre-exposed to NH<sub>4</sub>Cl, but with higher

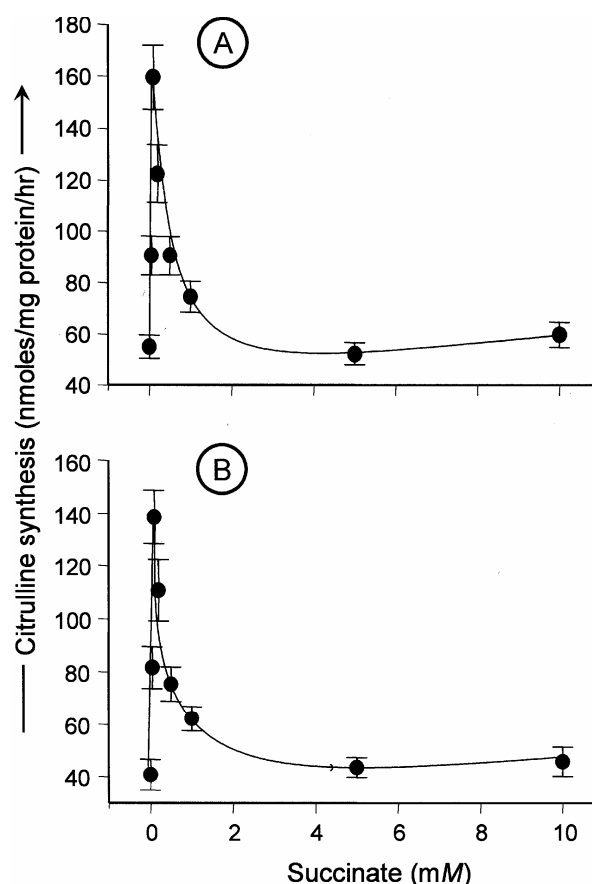


Fig. 1—Effects of different concentrations of succinate on citrulline synthesis by the isolated liver mitochondria of *C. batrachus* in presence of (A) NH<sub>4</sub>Cl (20 mM) and (B) glutamine (20 mM). Values are plotted as mean ± SE (*n* = 5).

Table 1—The rate of citrulline synthesis (nmoles/mg protein/hr) by the isolated liver mitochondria of *C. batrachus* in presence/absence of different substrates (20 mM) and acetazolamide (5 mM).

[Values are mean  $\pm$  SE from 5 experiments]

Components in the reaction mixture	Citrulline synthesis (nmoles/mg protein/hr)
Glutamine	138.24 $\pm$ 21.11
Glutamine + Acetazolamide	41.56 $\pm$ 4.35* (- 70%)
NH <sub>4</sub> Cl	155.27 $\pm$ 16.35
NH <sub>4</sub> Cl + Acetazolamide	47.39 $\pm$ 5.71* (- 70%)
NH <sub>4</sub> Cl + Glutamine	197.32 $\pm$ 24.65
NH <sub>4</sub> Cl + Glutamine + Acetazolamide	69.51 $\pm$ 8.12* (- 65%)

Percentage decrease (-) of citrulline synthesis in presence of acetazolamide is given in parenthesis.

\*Significantly different at  $P < 0.001$  level due to the presence of acetazolamide.

The incubation mixture contained everything as mentioned under materials and methods, plus 0.1 mM succinate, and different combinations of nitrogen donating substrates and acetazolamide.

$V_{max}$  of 265.3 nmoles/mg protein/hr (15%) at 3 mM concentration compared to the control fish.

With glutamine as a nitrogen donating substrate, the rates of citrulline synthesis by the control fish liver mitochondria also increased linearly with increasing concentrations of glutamine until it reached a  $V_{max}$  of 162 nmoles/mg protein/hr at 4 mM concentration, followed by no further increase at higher concentrations (Fig. 3). Almost similar pattern of increase of citrulline synthetic rates were observed with glutamine by the fish pre-exposed to NH<sub>4</sub>Cl, but with a higher  $V_{max}$  of 226 nmoles/mg protein/hr (40%) at 4 mM concentration compared to the control fish.

## Discussion

The peak of citrulline synthesis by the isolated liver mitochondria of walking catfish was obtained at 0.1 mM concentration of succinate, which is known as an energy source for carbamyl phosphate formation<sup>29-31</sup>, either in the presence of ammonia or glutamine as nitrogen-donating substrates. But, the citrulline production was inhibited at higher succinate concentrations, thus showing an inverse relationship with the concentration of succinate beyond the limit of 0.1 mM at least up to 3 mM concentration. Similar observations were also made in the toadfish (*O. beta*)<sup>31</sup> at least up to 5 mM concentration of succinate. The possible mechanism(s) of inhibition of mitochondrial citrulline production beyond 0.1 mM

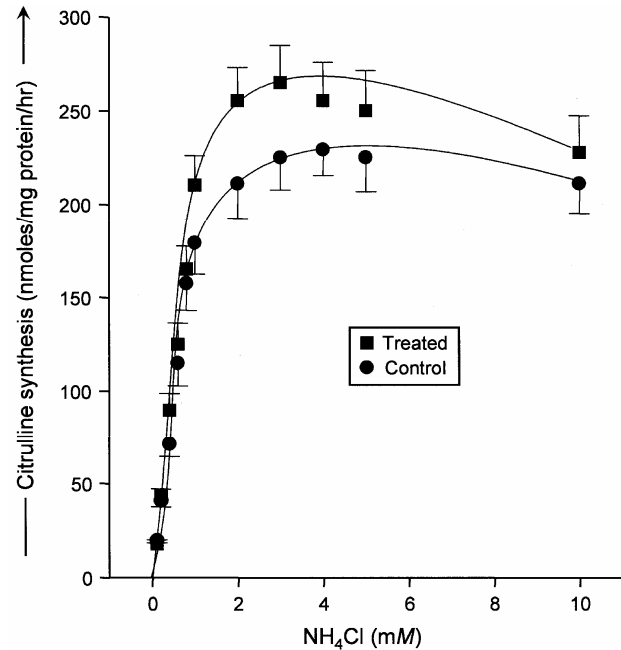


Fig. 2—Effects of different concentrations of NH<sub>4</sub>Cl on citrulline synthesis by the mitochondria isolated from both the liver of control and 25 mM NH<sub>4</sub>Cl pre-exposed *C. batrachus*. Values are plotted as mean  $\pm$  SE (n = 5).

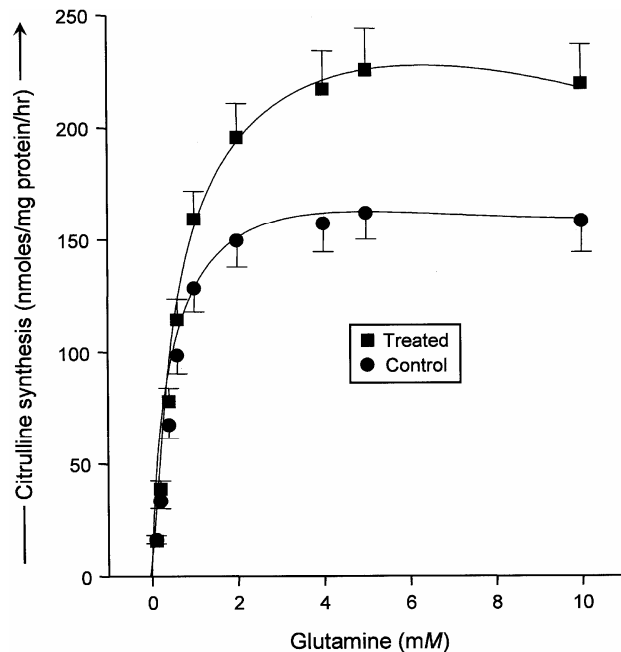


Fig. 3—Effects of different concentrations of glutamine on citrulline synthesis by liver mitochondria isolated from the liver of both control and 25 mM NH<sub>4</sub>Cl pre-exposed *C. batrachus*. Values are plotted as mean  $\pm$  SE (n = 5).

concentration of succinate could be explained as either due to competitive inhibition of glutamine and ammonia transport into the mitochondria and/or due to inhibition of one of the reactive pathways of

citrulline synthesis such as the carbamyl phosphate synthetase (CPS) or ornithine transcarbamoylase (OTC), or due to inhibition of N-acetyl-L-glutamate (AGA) synthetic pathway, the allosteric modulator of both CPS I and III. Furthermore, the optimal concentration of succinate to achieve the *in vivo* production of citrulline to a maximum rate could be much lower than 0.1 mM, because it is not clear in this experiment about how much succinate could get entry into the mitochondria from the assay medium. Thus, it needs a detailed investigation in knowing the possible mechanism(s) of regulation of citrulline synthesis by succinate.

The present results also indicated that the activities of both CPS I and III are dependent on a second enzyme for substrate supply, namely the intramitochondrial CA, required to supply  $\text{HCO}_3^-$  for carbamyl phosphate synthesis, since the presence of acetazolamide (a known inhibitor for intramitochondrial CA) inhibited the citrulline production both from ammonia and glutamine individually or in combination by 65-70%. Similar observations were also made while studying the citrulline production from glutamine by the isolated liver mitochondria of toadfish<sup>31</sup> and from ammonia by the isolated liver mitochondria of guinea pig<sup>36</sup>. CA is normally localized both in the mitochondria and cytosol of fish hepatocytes. Therefore, the question arises whether both cytosolic and mitochondrial CAs are responsible for mitochondrial citrulline production. To clarify this point Henry and Walsh<sup>31</sup> added extra bovine CA in the reaction mixture while studying the citrulline production by the isolated liver mitochondria of ureogenic toadfish. However, they could not detect any increase of citrulline production, thus suggesting that citrulline synthesis is mainly dependent on intramitochondrial  $\text{CO}_2$  production and on the activity of matrix CA to maintain chemical equilibrium between  $\text{CO}_2$  and  $\text{HCO}_3^-$  for continuous supply of substrate to CPS. This probably is also true in the ureogenic *C. batrachus* since the mitochondrial citrulline synthesis was inhibited significantly by inhibiting the mitochondrial CA activity even though extra  $\text{NaHCO}_3$  was added in the reaction medium.

The presence of both the OUC-related CPSes, the typical fish-type glutamine-dependent CPS III, and the most uniquely the ammonia-dependent CPS I activity has been reported in *C. batrachus*<sup>9</sup>. This was further evidenced from the present results of utilizing both ammonia and glutamine as nitrogen-donating

substrates in citrulline synthesis by the isolated liver mitochondria of the catfish (Figs 2 and 3). Additionally, presence of both ammonia and glutamine in the reaction mixture showed some additive effect on citrulline synthesis (Table 1). Further, the  $V_{\max}$  of citrulline synthesis was obtained at 3 and 4 mM concentrations of ammonia and glutamine, respectively, when used separately as nitrogen-donating substrates, with  $K_m$  values (determined by Lineweaver-Burk plot) of 0.58 and 0.43 mM, respectively, thus suggesting that both the substrates can equally contribute for mitochondrial citrulline synthesis in this catfish as a unique adaptation. In the ureogenic toadfish, the  $V_{\max}$  of citrulline synthesis by the isolated liver mitochondria was obtained at 5 mM glutamine, but when ammonia and glutamate were taken together in the assay mixture, the citrulline synthesis was significantly inhibited<sup>31,33</sup>. In toadfish, the glutamine synthetase (GS), which catalyzes the synthesis of glutamine from ammonia and glutamate, is partly localized in the mitochondria and partly in the cytosol of hepatic cells<sup>33</sup>. Further, citrulline synthesis was studied only in the isolated mitochondria, and the level of mitochondrial GS was recorded to be lower than the mitochondrial CPS III activity in toadfish liver<sup>31</sup>, hence there was a limited supply of glutamine for citrulline synthesis by CPS III and OTC enzymes. However, the walking catfish liver possesses high levels of GS activity in the mitochondrial matrix<sup>9</sup> in addition to the presence of both OUC-related CPS I and III activities<sup>9,37</sup>. Thus, it is more evident from this experiment that both ammonia and glutamine can act as nitrogen-donating substrates for the synthesis of urea in *C. batrachus*.

Interestingly, the rate of mitochondrial citrulline synthesis increased significantly in the liver of the catfish after pre-exposure to 25 mM  $\text{NH}_4\text{Cl}$  for 7 days both in the presence of ammonia and glutamine, but with slight increase of  $K_m$  values to 0.65 and 0.57 mM for ammonia and glutamine, respectively (Figs 2 and 3). This was mainly because of upregulation of activities of CPS I and III and also the GS along with the increase of enzyme protein concentrations under hyper-ammonia stress reported recently in this catfish<sup>37</sup>.

Thus, the results obtained in present experiments further confirm that the *C. batrachus* possesses the unique capacity of utilizing both ammonia and glutamine as nitrogen donating substrates for

production of urea via the OUC with having the capacity of enhancing the process under hyper-ammonia stress. Due to possessing this unique biochemical adaptational strategy related to the amelioration of ammonia toxicity, this air-breathing catfish is able to survive successfully under high external ammonia, which it faces in its natural habitat during certain seasons of the year.

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