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Effect of Long-Term Ammonia Starvation on the Oxidation of Ammonia and Hydroxylamine by *Nitrosomonas europaea*[1]

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Axenic cultures of the ammonia-oxidizing bacterium *Nitrosomonas europaea* were starved of ammonia (energy source) for up to 342 d. During this time the bacteria retained the ability to respond instantly to ammonia (1 mM) or hydroxylamine (0.1 mM) amendment by oxidizing it to nitrite without initial protein synthesis. *In vivo*, the ability to oxidize amended ammonia stayed almost constant during the starvation period, but a drop in the hydroxylamine oxidation rate (to 33%) was observed after 4 wk of starvation when exogenous hydroxylamine was supplied as sole energy source. In contrast, it has been shown that the level and *in vitro* activity of hydroxylamine oxidoreductase were not significantly affected during the starvation period. Only minor changes were detected between the protein patterns on one-dimensional SDS-PAGE of growing and starved cells. Thus, it is concluded that the activities of the energy-generating enzymes in *N. europaea* were not affected during long-term ammonia starvation.

Key words: ammonia and hydroxylamine oxidation, ammonia starvation, hydroxylamine oxidoreductase, *Nitrosomonas europaea*.

Ammonia-oxidizing bacteria are chemolithotrophs which get chemical energy from oxidizing ammonia to nitrite through a two-step process when grown aerobically (*1, 2*). The first step is the oxidation of ammonia to hydroxylamine catalyzed by ammonia monooxygenase (AMO), and the second step is the oxidation of hydroxylamine to nitrite by hydroxylamine oxidoreductase (HAO).

The redox couple NH_3/NO_2^- lies at redox potentials "near the practical limit of an aerobic energy source" (*1*). Hence the biomass yield (*3*) of ammonia-oxidizers such as *Nitrosomonas* is low (0.6 g d.w./mol N) in comparison to heterotrophic bacteria such as *Escherichia coli* (15 g d.w./mol C). Thus, growth rates of ammonia-oxidizers are low even in the presence of excess ammonia. Despite these energy limitations, populations of these bacteria efficiently overcome periods of ammonia depletion (and other stress situations) in various habitats.

Whereas other non-spore forming heterotrophic bacteria may use their cellular structural components or storage materials for energy production and adjust their metabolism (*4-6*), there were few hints as to how nitrifiers such as *N. europaea* adjust to energy depletion. Jones and co-workers have investigated the adaptation of the marine bacterium *Nitrosomonas cryotolerans* to long-term energy starvation (2 to 10 wk). They have shown that the endogenous respiration of *N. cryotolerans* fell to undetectable levels after depletion of the sole energy source (ammonia), and the energy charge of the cells was reduced (*7*). When ammonia was added to a culture starved for 5 wk, they detected immediate incorporation of $^{14}CO_2$ and NO_2^- production. In addition, cell densities of cultures and the amount of cellular compounds hardly declined during 5 wk of energy deprivation (*7-9*). Sayavedra-Soto *et al.* (*10*) investigated the starvation and recovery response of *N. europaea* starved for 3 d and found that the RNA transcripts of the energy-producing enzymes AMO and HAO were totally degraded a few hours after depletion of ammonia, while *N. europaea* cells starved for ammonia up to 72 h, contained a considerable amount of active HAO (*11*). These findings imply that AMO and HAO are stable during prolonged ammonia starvation as *de novo* synthesis of these enzymes was not necessary to resume generation of energy.

In this communication, we report the kinetics of ammonia and hydroxylamine oxidation by *N. europaea* cells starved of ammonia for up to 342 d. Our findings indicate that starved cells still maintain high levels of active AMO and HAO, but some cells may already have lost viability.

MATERIAL AND METHODS

Bacteria and Culture Media—*N. europaea* ATCC 19718 was purchased from the American Type Culture Collection (Rockville, MD) and cultured in batches of sterile medium

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Abbreviations: SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; d.w., dry weight.

221 (ATCC) at 25°C (*12*). These bacteria were used for experiments unless indicated otherwise. *N. europaea* ATCC 25196T was continuously cultivated in a retentostat as described by Tappe *et al.* (*13*).

Ammonia Starvation—A 10-liter batch culture of *N. europaea* ATCC 19718 at the end of the exponential phase of growth was starved of ammonia after harvesting cells by centrifugation and washing them with sterile NH_4^+-free medium 221 (ATCC) until no NH_4^+ or NO_2^- could be detected. Finally, cells were resuspended in 200 ml of NH_4^+-free medium. The suspension was divided into two aliquots of 100 ml which were transferred to 250-ml Erlenmeyer flasks and kept at 25°C in the dark. Samples (5 ml) were withdrawn after 0, 7, 16, 28, 44, and 61 d of ammonia starvation to be tested for activity as described below. In a different set of experiments, the effluent suspension of *N. europaea* ATCC 25196T from continuously fed cultures with partial biomass retention (*13*) was collected in sterile 1-liter flasks and kept air-tight at 25°C in darkness. As this part of this work was carried out in Germany, the suspension of cells that settled in the flasks was transferred to sterile, air-tight 5-ml test tubes and sent to the laboratory in Israel for further analysis. These cultures were NH_4^+-free (<0.01 mM detection limit) but contained 4.4 mM NO_2^-. Therefore, these cells were washed 3 times with NH_4^+-free medium 221 prior to further experiments.

Ammonia and Hydroxylamine Oxidation—Aliquots of 1 ml of exponential-phase cultures or starved cultures (described in the previous section) were washed 3 times in NH_4^+-free medium 221. Pelleted cells were resuspended in 5 ml of sterile medium 221 modified by addition of either 1 mM NH_4^+ (corresponding to an initial NH_3 concentration of about 0.1 mM at pH 8.3) or 0.1 mM hydroxylamine. In other experiments, various amounts of chloramphenicol were added to media containing NH_4^+. The cultures were incubated in sterile 20-ml flasks on a rotary shaker (150 rpm) at 25°C. At intervals, 0.5-ml samples were withdrawn and cells were pelleted. Nitrite concentration in the supernatant was determined, and the cells were analyzed for protein and HAO activity. The concentrations of NH_4^+ and NO_2^- were determined by standard methods as previously described (*14*).

Protein Analysis and Detection of HAO Activity—Analysis of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (*15*). For the detection of HAO activity, bacterial cells were combined with sample buffer without β-mercaptoethanol but with SDS. The samples were subjected to 10% SDS-PAGE, then used directly for the detection of HAO activity as previously described (*11*).

For immunoblot analysis, proteins were electrophoretically transferred to nitrocellulose filters, and reacted with rabbit antibodies against HAO, followed by alkaline phosphatase-conjugated goat anti-rabbit antibodies. The production of rabbit antibodies against active HAO was previously described (*11*). Total protein was determined by the Lowry assay (*16*).

RESULTS AND DISCUSSION

Protein Pattern—Protein patterns of *N. europaea* cells after ammonia starvation were analyzed by SDS-PAGE (Fig. 1), and only minor changes between growing and starving cells were detected. Since HAO aggregated during preparation due to boiling and application of β-mercaptoethanol (*11*), it settled in the first band at top of the gel (P1; Fig. 1). A new protein band (P2) appeared solely in starving cells. It is possible that this polypeptide is a degradation product of a larger protein or *de novo* synthesized in the early stages of the starvation period, when a very low concentration of intracellular ammonia was available. Some pronounced bands from growing cells became weaker after 107 d of starvation (between P3 and P4). Most of the prominent bands around 45 and 31 kDa did not show any significant intensity shift. Thus the kind of extended protein synthesis and re-organization of the cellular metabolism and regulation that is found in many heterotrophic bacteria (*4*) could not be proved, and this result is in accordance with expectations raised by others (*7-9, 17*). It is reasonable to assume that there is no extended turn over of proteins in starved *N. europaea* cells, and the proteins produced when energy sources were available were stable for prolonged periods of ammonia starvation. Consequently, the machinery for the immediate utilization of ammonia was always present. Nevertheless, the investigators cited above and Tomaschewski (*18*) also observed a decline in the ability of starved cells to oxidize ammonia, which could indicate some discrepancy with our finding of a stable protein pattern. Therefore, we investigated whether the key enzymes, AMO and HAO, had been affected in our experiments.

Oxidation of Ammonia and Hydroxylamine by Starved N. europaea—Starved and growing cells of *N. europaea* were washed in ammonia-free medium, then incubated in media containing ammonia. Nitrite accumulation represents the consecutive *in vivo* activities of AMO and HAO. In addition, recent publications have demonstrated that *N. europaea* can grow on hydroxylamine (*19, 20*). Therefore, the ability of the starved cells to oxidize exogenously added hydroxylamine was studied. To the best of our knowledge, this is the first study to report upon the effect of long-term energy starvation on hydroxylamine oxidation.

The results shown in Fig. 2 represent the time courses of nitrite accumulation by two preparations of *N. europaea* cells starved of ammonia for 61 d and 342 d upon provision of ammonia and hydroxylamine. The oxidation of ammonia (1 mM) and hydroxylamine (0.1 mM) by both preparations was detectable within minutes after their addition. Saturation of NH_4^+/NH_3 oxidation with about 20% residues after approximately 8 h (Fig. 2A) can be explained by the acidification of the medium, which limited the availability of NH_3 as the regular substrate of AMO (*21*). The lower level of nitrite accumulated by cells starved for 342 d (Fig. 2B) is due to the smaller biomass in these samples. This also explains why the linear increase of nitrite accumulation was extended up to 20 h (lower acidification rate). However, the possibility that cells starved for 342 d may exhibit different recovery kinetics cannot be excluded. Batchelor *et al.* (*22*) have reported that *N. europaea* cells starved for 42 d exhibited a lag phase of 153 h prior to exponential nitrite production. These findings are contradictory to the results presented in Fig. 2, where nitrite accumulation was measured immediately upon addition of ammonia or hydroxylamine. This indicates that the recovery of *N. europaea* cells from energy starvation is a

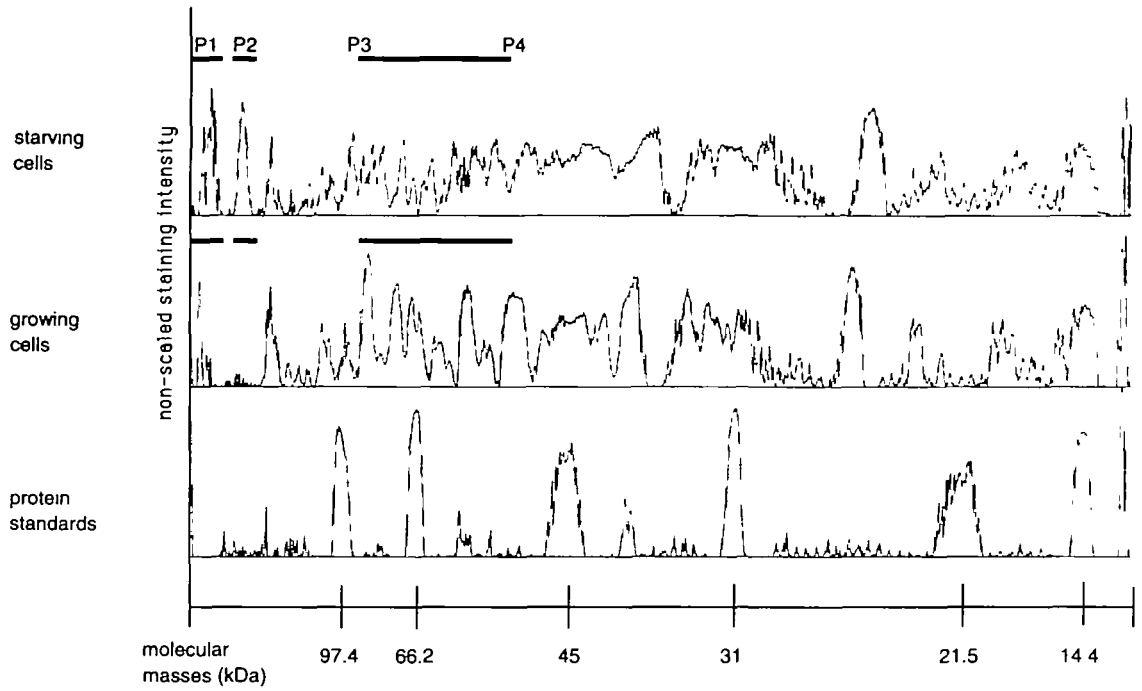

Fig. 1. **Pattern of proteins isolated from proliferating and starved cells (107 d) of *N. europaea* after separation by SDS-PAGE.** Proteins were stained with Coomassie Brilliant Blue R-250. A video image of the gel was recorded and the densitometric profiles were measured. Preparation was carried out according to "MATERIALS AND METHODS." Proteins were extracted with sample buffer (*15*) containing β-mercaptoethanol. Under these conditions HAO polymerized and did not enter the gel (*11*). Each well was loaded with 140 μg of protein for SDS-PAGE.

complex process which can be affected by growth conditions prior to starvation. Oxidation of hydroxylamine reached a calculated level of 88 to 100% depletion in any trial. Low concentrations of hydroxylamine were used to avoid toxic effects.

The experiment was repeated with cultures starved for different periods of time, and the initial rates of ammonia and hydroxylamine oxidation were calculated from the slope of the accumulating nitrite concentration within the first 5 h (compare Fig. 2A). The initial oxidation rate of NH_4^+ seemed to be stable for over 60 d of ammonia starvation (Fig. 3). In contrast, the rate of hydroxylamine oxidation decreased significantly to below 33% of the initial rate after 28 d. Johnstone and Jones (*7*) reported similar results for ammonia-oxidation by *N. cryotolerans*, whereas Jones and Morita (*9*) measured a slight decrease (to 78%) of the ammonia-oxidation rate within a starvation period of about 6 wk. The latter authors reported a stronger decline when lower ammonia concentrations (less than 0.07 mM) were applied and suggested that their observation was due to the physiological state of the bacteria rather than a direct loss of AMO activity. However, comparable data about HAO activity were not presented. Johnstone and Jones (*8*) measured instant $^{14}CO_2$ incorporation by *Nitrosomonas* cells starved for 5 wk as soon as ammonia was provided. We examined the possibility that the initial activity of the AMO and HAO is partly due to an instant *de novo* synthesis of these proteins by adding chloramphenicol prior to ammonia to the medium. We did not detect any inhibition of nitrite production up to a concentration of 400 mg/liter chloramphenicol (data not shown). On the other hand, when *N. europaea* was inoculated in medium supplemented with

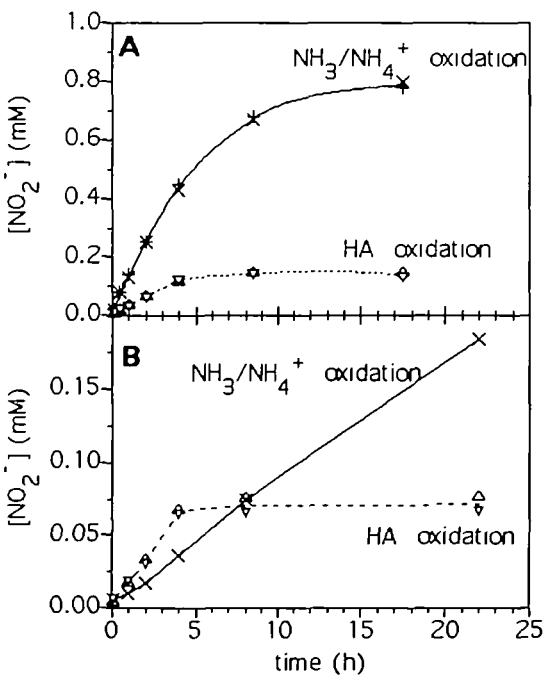

Fig. 2. **Oxidation of NH_4^+/NH_3 and hydroxylamine by *N. europaea* after starvation.** *N. europaea* ATCC 19718 (A) and *N. europaea* ATCC 25196T (B) were starved for ammonia of 61 d and 342 d, respectively. The starved cells were transferred to media amended with NH_4^+ (1 mM) or hydroxylamine (0.1 mM). The production of NO_2^- was recorded to follow the oxidation of the nitrogen compounds (HA = hydroxylamine). Duplicate experiments are shown by symbols, while lines depict mean values.

100 mg/liter chloramphenicol, growth was not observed within 30 d of incubation. Hyman and Arp (*23*) showed that protein synthesis is inhibited by chloramphenicol in growing cells of *N. europaea* or cells starved for a short period, and that a loss in AMO activity in stationary phase was not due to protein degradation. We further focused our work on the HAO by testing the activity of this enzyme *in vitro*, since, in contrast to AMO, comparable data are not available for HAO.

In Vitro HAO Activity and Enzyme Levels—The activity and levels of HAO were studied in starved *N. europaea* cells after prolonged starvation of ammonia. The procedure is based upon fractionating of *N. europaea* proteins by SDS-PAGE and detection of HAO activity on the gel and protein levels by Western blotting. HAO and its activity were detected in *N. europaea* cells starved of ammonia for up to 223 d (Fig. 4A). This method did not indicate significant losses of HAO-activity during starvation, in contrast to our previous findings (compare Fig. 3). Similarly, the HAO level was not effected beyond the error limits of the method, as demonstrated by SDS-PAGE and subsequential Western blotting (Fig. 4B). Therefore, the decrease of the *in vivo* oxidation rate of exogenously supplied hydroxylamine (Fig. 3) could not be attributed to a direct loss of enzyme activity or degradation of HAO.

The activity of HAO, on SDS-polyacrylamide gels, and the HAO-protein level, on Western blot analysis, did not increase within 1 d when cells that had been starved for 7 d up to 342 d were transferred to ammonia-amended media (Fig. 5, data for 28 d of starvation is shown). Recently published findings for short-term starved cultures (up to 72 h starvation period) indicated an almost immediate synthesis of HAO after amendment of hydroxylamine (*11*). Therefore, it is likely that prolonged periods of starvation may result in prolonged lag phases for the synthesis of HAO. Batchelor *et al.* (*22*) made a consistent observation following the NO_2^- production in reference to the growth of *N. europaea* cells recovering from starvation in ammonia-amended media. The lag period of growth-associated nitrite production increased from 8 h after short starvation periods to about 150 h when cells were starved for 42 d.

The results reported in this paper and those published before (*7-11, 21, 23*) prove that the protein pattern of *N. europaea* cells is extremely stable over prolonged periods of starvation. In addition, it has been shown for *N. cryotolerans* that the amount of other cellular components (RNA, DNA) as well as cell size did not change much during starvation (*7*). We further demonstrated *in vitro* that the pool of the energy-utilizing enzyme HAO neither lost activity nor decreased significantly. Nejidat *et al.* (*11*) suggested that the degradation of HAO and AMO in *Nitrosomonas* cells may proceed through an ATP-dependent mechanism and is coupled to energy production. Thus the energy-generating enzymes are excluded from turnover if the energy source is depleted. Data on the ATP content, energy charge, and the electron transport system activity reveal a dynamic pattern during the first 4 wk of energy deprivation (*7*). In addition to the minor changes in protein

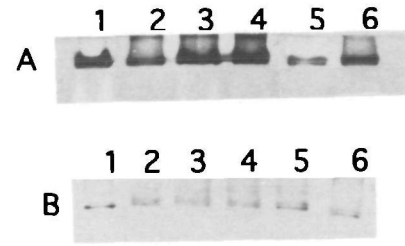

Fig. 4. **HAO activity (A) and protein level (B) in *N. europaea* cells starved of ammonia for up to 223 d.** Samples were withdrawn from starved cultures of *N. europaea* and subjected to SDS-PAGE and Western blotting as described in "MATERIALS AND METHODS." A: Lanes 1 to 5 were loaded with equal amounts of protein (70 μg): Cells were sampled during growth, after 7, 16, 28, and 61 d of starvation, respectively, and lane 6 (loaded protein not determined) cells were starved for 223 d after sampling from continuous culture as described in "MATERIALS AND METHODS." B: Western blot. Lanes 1 to 6 were loaded with equal amounts of protein (70 μg): Cells were sampled during growth after 7, 16, 28, 61, and 81 d of starvation.

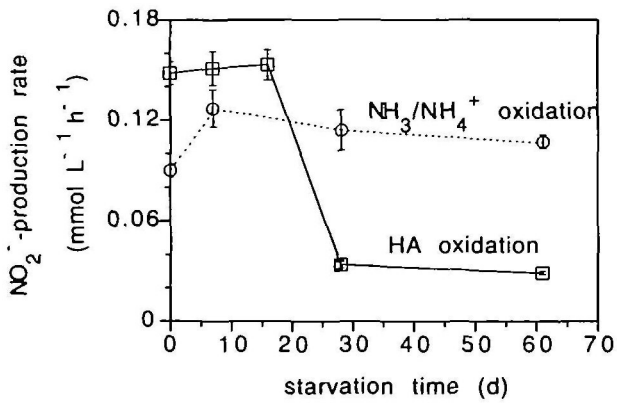

Fig. 3. **Effect of prolonged starvation on oxidation rates for ammonia and hydroxylamine by *N. europaea*.** Rates were calculated according to the production of NO_2^- as shown in Fig. 2 from the initial straight slope of the NO_2^- concentration in duplicate runs. In each run, 1-ml aliquots of the starved culture were incubated in fresh medium containing 1 mM ammonia or 0.1 mM hydroxylamine. Error bars refer to standard errors.

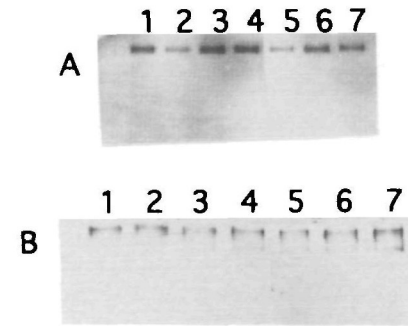

Fig. 5. **Time course of HAO activity in SDS-polyacrylamide gels (A) and protein levels (B) in *N. europaea* cells transferred to ammonia-amended medium after 28 d of starvation.** Samples were withdrawn from a culture of *N. europaea* (compare Fig. 2) and subjected to SDS-PAGE and Western blotting as described in "MATERIALS AND METHODS." A: Lanes 1 to 7 were loaded with equal aliquots from a culture recovering from starvation: Cells were sampled at 0.1, 1.8, 2.5, 3.5, 5.5, 8.6, and 21.5 h. B: Western blot. Lanes 1 to 7 were loaded with equal amounts of protein (70 μg) and correspond to lanes shown in Fig. 4A.

pattern that we observed (Fig. 1), a limited adjustment of the cell's physiology to starvation is indicated. Up to now, there are no data available to indicate whether specific genes are induced by starvation, as has been demonstrated for heterotrophic bacteria (*4–6*).

The decrease in hydroxylamine oxidation rate (Fig. 3), did not correspond to a loss in HAO activity *in vitro*. Therefore, other cellular structures may be damaged or modified during starvation. Since the hydroxylamine oxidation is affected only if hydroxylamine is supplied exogenously as sole energy source, damage may occur at the membrane or in the periplasm. Thus transport processes as well as the electron transfer chain or linked structures could be affected. Damage of membrane integrity could also cause toxic intracellular reactions when hydroxylamine entered the cytoplasm. Nevertheless, without energy supply, repairing or protecting mechanisms cannot work. Therefore, the stability of the key enzymes for energy generation, AMO and HAO, is necessary to ensure an energy supply and repair as soon as ammonia becomes available.

The survival strategy of *N. europaea* is a necessary and successful adaptation to the oligotrophic environment that provides only minimal amounts of energy for growth. No extended *de novo* synthesis is necessary to rearrange cellular metabolism in the case of fast-growing heterotrophic bacteria. On the other hand, the energy source itself forces *Nitrosomonas* and other chemolithotrophic ammonia oxidizers to cope with an extremely fast exploitation of the energy source, while the energy yield is so low that it hardly covers a fast rearrangement of the cellular metabolism. Thus a survival strategy based on a stable set of cellular components to overcome unfavorable environmental conditions, and with a high ability to generate energy as soon as the energy source is available, could explain why autotrophic nitrifiers are found in many habitats. In this paper we have demonstrated some physiological and enzymatic realizations for such a strategy in *N. europaea*. Nevertheless, it is still not known whether there are special components or structures that stabilize the proteins and how the cells adapt to other environmental stresses.

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