ORNITHINE DECARBOXYLASE ACTIVITY IN RETINAL EXPLANTS OF GOLDFISH UNDERGOING OPTIC NERVE REGENERATION

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SUMMARY

Retinal explants cultured in the presence of fetal calf serum (FCS) exhibit an increase in the activity of ornithine decarboxylase which is maximal several hours after explantation. The measured activity of this enzyme is higher in explants of retinas whose optic nerve had been crushed several days previously (post-crush, PC) than in control (normal, N) retinas. The addition of rabbit antibodies against nerve growth factor (NGF) to the incubation medium does not block this stimulatory effect of FCS, a result suggesting that factors other than NGF present in FCS are responsible for the observed stimulation. An inhibitor of ODC synthesis, diaminopropane (DAP), and an irreversible inhibitor of ODC activity, α-DL-difluoromethylornithine (α-DFMO), each suppressed the FCS-stimulated ODC activity when added to the culture medium.

Since FCS addition also promotes neuritic outgrowth from PC goldfish retinal explants, we explored the possible relationship of the stimulated ODC activity and the ability of explants to extend neurites. Concentrations of DAP or α-DFMO that block ODC activity also suppress neuritic outgrowth. Possible non-specific actions of the drugs unrelated to the block of ODC are examined.

While the increases in ODC activity seen in PC and N goldfish retinas explanted into FCS-containing medium may be a requisite, they cannot be sufficient to support neuritic outgrowth. Intrinsic changes in the retinal explant secondary to crush of its optic nerve as well as factor(s) present in FCS that may be unrelated to the stimulation of ODC activity also appear necessary for neuritic outgrowth.

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INTRODUCTION

Recently, we observed that enhancement of ODC activity, specifically related to nerve injury, occurs following unilateral crush of the goldfish optic nerve[12]. In contrast to the unilateral nature of several retinal biochemical changes previously seen[1,3,8,11], ODC activity is increased in both PC and N retina, albeit that ODC activity in the post-crush (PC) retina is significantly higher than in the normal (N) retina[12]. In previous studies, we have demonstrated that explant cultures of PC retinas support extensive outgrowth of neurites onto the substratum, while explants from N retinas do not[14,15]. In the present study, we have employed the explants to further examine the possible role of ODC in regeneration.

MATERIALS AND METHODS

Materials

L-[1-^{14}C]ornithine hydrochloride (59 mCi/mmol) was purchased from Amersham (Arlington Heights, Ill.). Fetal calf serum (FCS) and Leibowitz L-15 medium were the products of Gibco (Grand Island, N.Y.). Anti-NGF antiserum was obtained from Collaborative Research, (Waltham, Mass.). β-NGF was the generous gift of Dr. Eric Shooter, Stanford University. α-DL-Difluoromethylornithine (α-DFMO, RMI71782) was the gift of Dr. Peter McCann, Merrell Research Center, Cincinnati, Ohio. Tricaine methanesulfonate was obtained from Ayerst Laboratories (New York, N.Y.). All other chemicals were obtained from Sigma Chemical (St. Louis, Mo.).

Animals

Goldfish (*Carassius auratus*), 6–7 cm in body length, obtained from Ozark Fisheries (Stoutland, Mo.) were anesthetized with tricaine methanesulfonate. Intraorbital crush of the right optic nerve was performed as previously described[8].

Explant cultures

Ten to fourteen days following optic nerve crush, PC and N retinas were removed and cut into 0.5 mm squares as previously described[15]. The retinal explants were placed in culture dishes coated with poly-L-lysine in Leibowitz nutrient (L-15) medium supplemented with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, pH 7.2), 0.1 mM FudR (5'-fluorodeoxyuridine), 0.2 mM uridine and 0.1 mg/ml gentamicin sulfate. Unless otherwise stated, the medium also contained 10% FCS. In experiments in which one or more of the following compounds were added to the incubation medium; 1,3-diaminopropane (DAP), putrescine, S-adenosyl methionine (SAM) or α-DFMO, the final pH was adjusted to 7.2 with 1 N HCl or 1 N NaOH. Cutures were maintained at 20–22 °C.

Sequential neuritic growth indices were based on the density and extent of outgrowth[15].

ODC assay

Following in vitro incubation for various times, culture dishes were gently rinsed 3 times with phosphate-buffered salts[4] and then drained and stored at —20 °C prior to assay. Thawed explanted tissue was mechanically dislodged from the culture dish, homogenized in 50 mM Tris·HCl buffer (pH 7.6) containing 1 mM dithiothreitol and 100 μM pyridoxal phosphate. The homogenates were centrifuged at 30,000 × g for 20 min and the resulting supernatant fractions were used for ODC assay, measured by release of $^{14}CO_2$ from labeled ornithine (final concentration, 100 μM, 0.5 μCi)[12]. Specific activities are based on protein determinations according to Lowry et al.[16]. In a typical assay of ODC activity the blank value was about 50 cpm and experimental values varied from 300 to 1000 cpm.

RESULTS

ODC activities

When PC retinal explants were cultured in medium containing FCS, ODC activity reached a maximum level approximately 6 h after explantation, then gradually returned to the basal level (Fig. 1). Cultures obtained from normal (N) retinas also
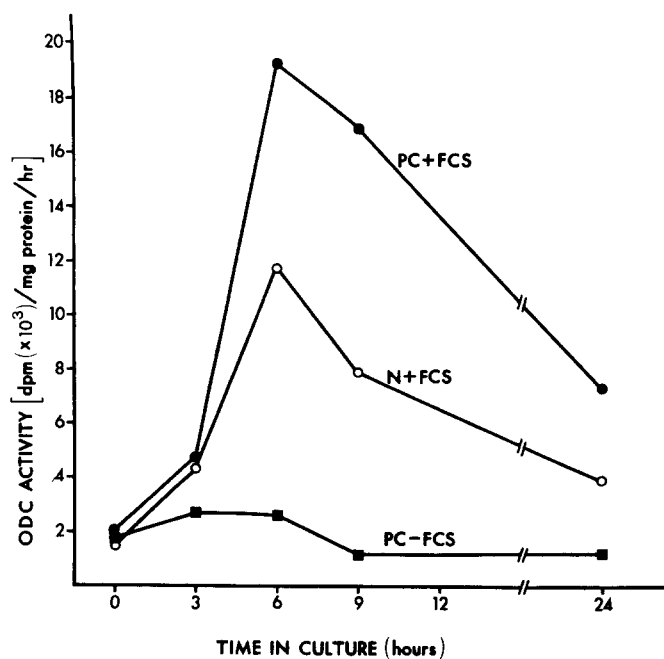

Fig. 1. Stimulation of ODC activities in retinal explants incubated with FCS. Explants obtained from PC retinas were incubated in medium with (●) and without (■) 10% FCS, and ODC activity was measured at different times following explantation. The ODC activities of explants obtained from N retina (○) incubated with FCS were also measured. Results are from a single experiment. In 4 experiments in which the 6 h time point was examined, the ratio PC + FCS:PC—FCS = 8.51 ± 0.82; PC + FCS:N + FCS = 1.56 ± 0.07.
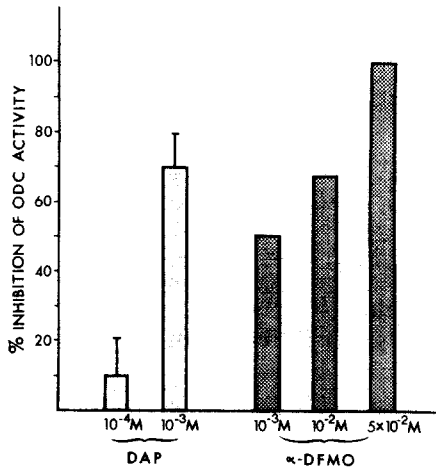
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Fig. 2. Effect of ODC inhibitors on FCS-stimulated ODC activity. Explants were cultured with FCS and the inhibitors indicated. Six h later, cultures were rinsed and ODC activity measured. Per cent inhibition of ODC activity was expressed according to the following equation:

$$\frac{(\text{s.a.})_{\text{FCS}} - (\text{s.a.})_{\text{FCS} + \text{inhibitor}}}{(\text{s.a.})_{\text{FCS}}} \times 100$$

where s.a. (specific activity) was expressed as dpm·mg protein^{-1}·h^{-1} for the various preparations. Values are means from 3 DAP and 2 α-DFMO experiments.

showed increased ODC activity in the presence of FCS in the culture medium. The maximal level of ODC activity attained, however, was significantly lower than that seen in PC retinal explants. At 6 h, the ratio of ODC specific activities for PC to N retinas was 1.6:1.0. The ratio of ODC activities in PC retinas explanted in FCS-
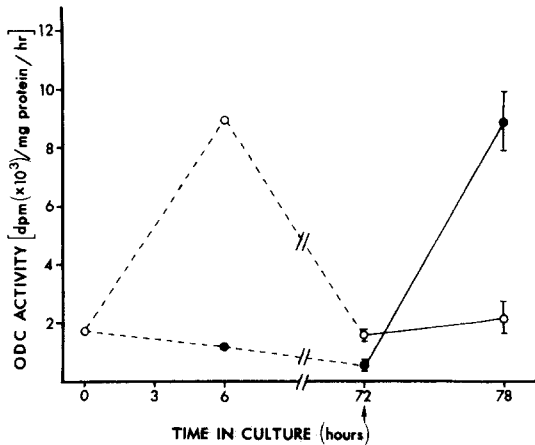

Fig. 3. Effect of DAP on FCS-stimulated ODC activity in explants. Explants were incubated with FCS in the presence (●) or absence (○) of 10^{-3} M DAP. For zero and 6 h time points, n = 2. In separate experiments, n = 4, the medium of the 2 experimental groups was replaced with FCS only, and ODC was measured immediately (72 h) or 6 h later (78 h).
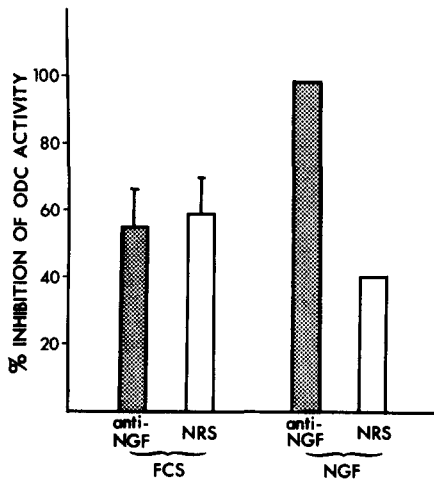
Fig. 4. Effect of anti-NGF sera on FCS-stimulated ODC activity. PC retinas were explanted in medium containing either FCS and anti-NGF antisera (1:20) or FCS and normal rabbit serum (NRS, 1:20). 6 h following explantation, ODC activity was assayed. Results are expressed as average of 4 separate experiments. As a control, the effect of anti-NGF antisera on NGF-stimulated ODC activity was measured (duplicate samples from a single experiment). Per cent antibody inhibition was calculated as described previously[12].

containing medium to that in FCS-deficient medium at 6 h was 7.3:1.0. Further experiments employed only PC retinas.

The ODC activity observed in the FCS-fortified PC retinal cultures was completely blocked when a specific inhibitor of the enzyme, a-DL-difluoromethylornithine (a-DFMO, 5×10^{-2} M), was present in the culture medium. Lower concentrations had a lesser effect on ODC activity (Fig. 2). The ODC synthesis inhibitor,
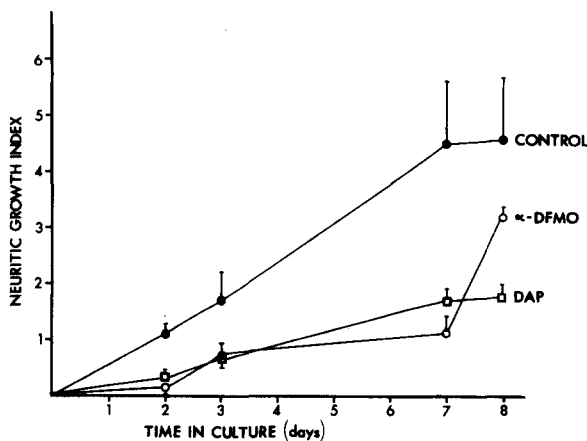

Fig. 5. Effect of ODC inhibitors on neuritic outgrowth. Explants obtained from PC retinas were cultured in medium containing FCS (●), FCS with DAP (10^{-3} M, □) or FCS with a-DFMO (10^{-2} M, ○). The density and extent of growth was determined at different times. Results are expressed by their neuritic growth index ± S.E.M.[14,15]. Each point represents values of 3 or more culture dishes, each containing 16–20 explants.
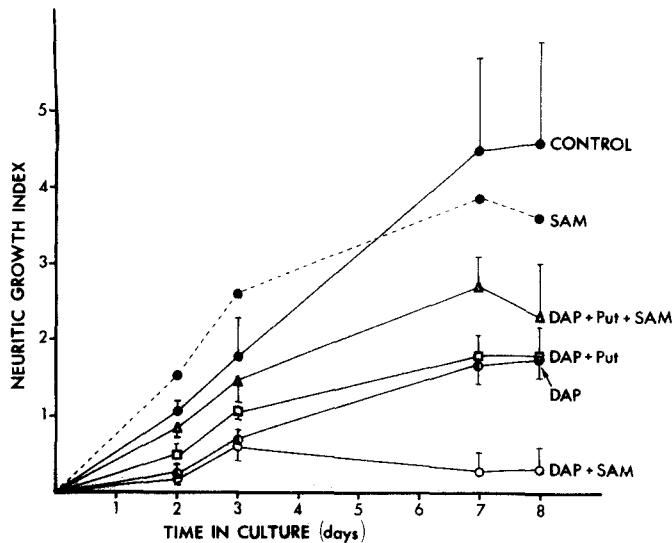
Fig. 6. Polyamine levels and neuritic outgrowth. PC retinas were explanted in FCS-containing medium to which was added: 10^{-3} M DAP (●); 10^{-3} M DAP + 10^{-5} M SAM (○); 10^{-3} M DAP + 10^{-5} M SAM + 10^{-6} M putrescine (Put) (△); or 10^{-5} M SAM (●---●). At times shown following explantation, neuritic growth indices were determined. Results represent mean ± S.E.M. of 2–4 culture dishes, each containing at least 16–20 explants.

diaminopropane (DAP, 10^{-3} M), produced a 70% decrease of ODC activity in the PC retinal explants (Fig. 2).

We next examined whether the DAP inhibition was reversible. Cultures were incubated for 3 days in medium containing both FCS and DAP, and were then washed with medium containing FCS, but not DAP, and further incubated in this medium for 6 h (Fig. 3). An increase in ODC activity was seen similar to that found on initial explantation. On the other hand, if the 3 day incubation mixture contained only FCS, the replenishment produced only a marginal increase in ODC.

NGF addition (1 μg/ml) in the absence of FCS produced a similar magnitude of increase in ODC activity of PC retina at 6 h as produced by FCS. However, addition of anti-NGF antisera did not block the FCS stimulation of ODC activity beyond a non-specific effect attributable to the presence of normal rabbit sera (Fig. 4).

Neuritic outgrowth

In the absence of added FCS, PC retinas show poor attachment and little or no neuritic outgrowth[13,15]. When cultures of PC retinas were explanted in the presence of FCS and 10^{-4} M DAP, there was no detectable effect on neuritic outgrowth (not shown), while 10^{-3} M DAP significantly suppressed outgrowth (Fig. 5). α-DFMO (10^{-3} M) did not affect growth, while (10^{-2} M) α-DFMO produced significant suppression which, however, diminished after 1 week in culture (Fig. 5).

Since DAP blocks ODC activity, the amounts of the metabolic products, putrescine and spermidine, are presumably reduced in its presence. If the inhibitory
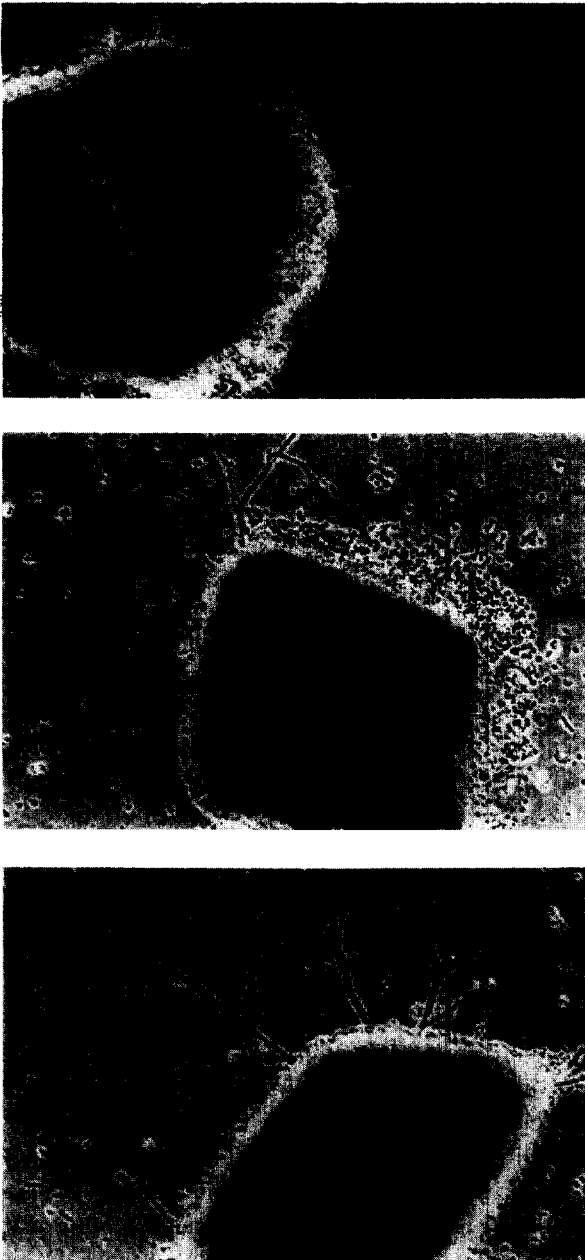
Fig. 7. Representative photomicrographs of PC retinal explants grown in the presence of FCS-containing medium with no additions (A), with 10^{-3} M DAP (B), and with 10^{-3} M DAP, 10^{-5} M SAM and 10^{-6} M putrescine (C). Space bar = 119 μm. Photographs were taken 7 days post-explantation.

effects of ODC blockers on growth are due to reduced polyamine production, the exogenous addition of putrescine should overcome the inhibitory effects on growth[18]. The addition of S-adenosyl methionine (SAM), precursor of the propylamine function

of spermidine and spermine, might be expected to further restore function. Addition of putrescine to the retinal explant medium did not of itself restore the inhibition of growth produced by DAP, although it largely restored the level seen in the presence of DAP + SAM (Figs. 6 and 7). The addition of SAM alone had no significant effect on outgrowth, and markedly increased the inhibitory effect of added DAP.

DISCUSSION

While polyamines have long been implicated in growth processes in eukaryotic cells, molecular bases for their roles in cellular metabolism remain unclear. A vast literature testifies to enhancement of polyamine synthesis in a number of growth processes, including mitosis, differentiation and hypertrophy, and further, that the enzyme ornithine decarboxylase is rate-limiting in their synthesis (for reviews, see refs. 10,21,22,24). In the developing nervous system, one finds an additional type of growth, neuronal process extension. The study of nerve regeneration in the adult provides the opportunity to investigate such outgrowth under conditions in which conventional growth processes in the remainder of the nervous system are relatively static.

Biochemical studies have thus far revealed that following unilateral optic nerve crush, N retinas appear unchanged, while within several days retinas whose optic nerves have been crushed show an increase in tubulin labeling[8], tubulin mRNA[2], and in enzymes involved in the sequential phosphorylation of uridine to UTP[11]. Retinal ODC levels increase 2- to 3-fold in both the PC and N retinas, with a peak of activity 5 days following crush[12] and return to basal levels by 7 days. The effect is not limited to the retinas; increases are also seen in brain and kidney. There is evidence that in addition to the generalized elevation, some of the ODC increase is specific to regenerating tissue, since enzyme levels are significantly higher in PC retinas than in N retinas[12]. This conclusion is supported by the finding in the present study that FCS produces a greater ODC response in PC than in N retinas.

The suitability of explants for the study of regeneration is based on the previous observation that cultured PC retinas support neuritic outgrowth, while relatively little outgrowth occurs from N retinal explants[15]. It had also been observed that in the absence of FCS, initiation and maintenance of neuritic outgrowth is greatly diminished[13]. The present studies indicate that FCS also stimulates ODC activity, both in PC and N retinas. While ODC levels in PC explants stimulated by FCS are 64% higher than that seen in N retinas (Fig. 1), the difference in neuritic outgrowth between PC and N explants is several-fold. In a related study we found that ODC in goldfish retinal explants were enhanced by the addition of β-NGF to the growth medium and that PC retinas were more responsive than N retinas[12], a result similar to that seen with FCS in the present study. It is unclear at present whether NGF also supports neuritic outgrowth. Since antibodies to NGF did not block the FCS-mediated stimulation of ODC activity, the results thus far suggest for goldfish retina at least, that factors in FCS other than NGF support both the ODC increase and neuritic outgrowth. While a role for NGF in the goldfish visual system is not established,

Turner has reported that injection of NGF following crush accelerates morphometric indices of nerve regeneration[25]. Relevant to these results are previous studies with NGF in cultures known to be NGF-responsive: PC-12 cells[7] and explanted sympathetic ganglia[17]. Addition of NGF to these preparations results in an increase in ODC, and in PC-12 cells, neurite outgrowth as well. In order to study the possible interrelationship of ODC stimulation and neurite outgrowth in the retinal explants, we employed two ODC inhibitors whose mechanisms of action were different. DAP is believed to block ODC synthesis, as well as to activate an antizyme[5,19]. a-DFMO, on the other hand, blocks the enzyme active site[6]. Both agents are relatively non-toxic and have been used in vivo at relatively high concentrations (1–2%) in drinking water of rats[6]. These agents also significantly reduce ODC levels in culture systems[21,23]. DAP at 10^{-3} M produced 70% inhibition of ODC activity in the retinal explants and at the same time blocked neuritic outgrowth by approximately 50%. It was possible, despite this apparent correlation, that the block in outgrowth was not directly related to the inhibition of ODC activity but rather to other metabolic or physical effects. For example, the substratum for neurite outgrowth is a film of poly-L-lysine on the culture dish surface[9], and DAP, itself a bifunctional amine, might block outgrowth by preventing the negatively-charged neuritic membrane from adhering to the cationic substratum. This possibility seems unlikely, however, since a-DFMO, which has a net +1 charge at physiological pH, and therefore does not have this potentially detrimental effect on adhesion, also shows comparable inhibitions of neurite outgrowth and ODC activity. The possibility that the concentrations of DAP employed produced irreversible toxicity was examined in a recovery experiment. After 3 days of incubation with medium containing FCS + DAP, replacement with FCS medium elicited an increase in ODC activity, while cultures incubated with FCS medium alone were no longer responsive to renewal of the FCS medium (Fig. 3). DAP thus preserved the ability of explants to respond to FCS addition.

Putrescine, the product of ornithine decarboxylation, combines with the decarboxylated product of SAM to form spermidine and spermine. If the various inhibitors indeed prevent neuritic outgrowth by blocking putrescine and spermidine synthesis, one might hope to reverse the inhibitors' effects on neurite outgrowth by the addition of putrescine. Successful reversal would require that putrescine itself be neither toxic nor block neurite attachment on the basis of its bifunctional cationic nature. In these experiments, DAP was used as the inhibitor and SAM was added to further stimulate polyamine synthesis. As shown in Figs. 6 and 7, SAM had little effect on outgrowth, but unexpectedly appeared to potentiate the inhibitory effects of DAP. Addition of putrescine did not reverse the DAP-mediated block of neuritic outgrowth, although it did overcome the additional inhibition occasioned by the presence of SAM. The basis of the effects of SAM addition remain unexplained.

In summary, evidence is presented to indicate that a causal relationship exists between factors present in FCS that lead to increased ODC levels and neurite outgrowth in that two agents that block ODC activity by different mechanisms also block neurite outgrowth. This interpretation is complicated by the failure of added putrescine, the product of ODC action to reverse the drug-mediated inhibition of

growth. The incremental ODC response of post-crush retinal explants relative to normal retina may reflect some aspect of inferred intrinsic differences between the two kinds of explants that permit the former, but not latter, to extend neurites onto the surrounding substratum.

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