# A model for circadian oscillations in the *Drosophila* period protein (PER)

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**SUMMARY**

The mechanism of circadian oscillations in the period protein (PER) in *Drosophila* is investigated by means of a theoretical model. Taking into account recent experimental observations, the model for the circadian clock is based on multiple phosphorylation of PER and on the negative feedback exerted by PER on the transcription of the period (*per*) gene. This minimal biochemical model provides a molecular basis for circadian oscillations of the limit cycle type. During oscillations, the peak in *per* mRNA precedes by several hours the peak in total PER protein. The results support the view that multiple PER phosphorylation introduces times delays which strengthen the capability of negative feedback to produce oscillations. The analysis shows that the rhythm only occurs in a range bounded by two critical values of the maximum rate of PER degradation. A similar result is obtained with respect to the rate of PER transport into the nucleus. The results suggest a tentative explanation for the altered period of *per* mutants, in terms of variations in the rate of PER degradation.

## 1. INTRODUCTION

Study of the period (*per*) gene in *Drosophila* has led to remarkable advances in unravelling the molecular bases of circadian rhythms (Konopka & Benzer 1971; Bargiello *et al.* 1984; Zehring *et al.* 1984; Baylies *et al.* 1987; Yu *et al.* 1987; Hall & Rosbash 1988; Dunlap 1993; Baylies *et al.* 1993). The *per* gene product (PER) has homologies with transcription factors (Huang *et al.* 1993), which may explain how PER could direct the periodic expression of numerous other genes (Huang *et al.* 1993; Takahashi 1993; Sassone-Corsi 1994). An important clue to the mechanism by which *per* produces rhythmic behaviour is that *per* mRNA itself varies in a circadian manner (Hardin *et al.* 1990, 1992). The fact that PER also varies periodically, but follows the mRNA rhythm by several hours, suggested that the mechanism of circadian oscillations involves a negative feedback exerted by PER on the transcription of the *per* gene (Hardin *et al.* 1990, 1992). The view of the circadian clock as a cellular oscillator controlled by repression is consistent with the observation that only those cells in which PER is overproduced lose the rhythm in PER (Zeng *et al.* 1994). Post-translational regulation appears to be involved in the oscillatory mechanism, as PER is phosphorylated in a circadian manner (Edery *et al.* 1994). Here, based on multiple phosphorylation of PER and on repression of *per* transcription by a phosphorylated form of the protein, a theoretical model is proposed for circadian oscillations in the *Drosophila* PER protein and its mRNA.

An alternative, more detailed model for circadian PER oscillations in *Drosophila* has been developed independently by Abbott *et al.* (1995) (M. Rosbash, personal communication; results of that study were first presented at the 4th conference of the Society for Research on Biological Rhythms held in May 1994 in Amelia Island, Florida). That model, which is also based on the negative feedback exerted by PER on *per* transcription, takes into account a larger number of phosphorylated residues and focuses on the role of PER phosphorylation in delaying the entry of the protein into the nucleus (Curtin *et al.* 1995).

## 2. MINIMAL MODEL FOR CIRCADIAN OSCILLATIONS OF PER AND *per* mRNA

The model, schematized in figure 1, relies on the following assumptions: *per* mRNA, whose cytosolic concentration is denoted by $M$, is synthesized in the nucleus and transfers to the cytosol, where it is degraded; the rate of synthesis of PER is proportional to $M$. To take into account the fact that PER is multiply phosphorylated (Edery *et al.* 1994), and to keep the model as simple as possible (the precise number of phosphorylated residues is still unknown), only three states of the protein are considered: unphosphorylated ($P_0$), mono- ($P_1$) and bisphosphorylated ($P_2$). The model could readily be extended to include a larger number of phosphorylated residues. However, as shown by numerical simulations and stability analysis currently under progress, such an extension would unnecessarily complicate the model by increasing the number of variables, without significantly altering its dynamic behaviour (whether more

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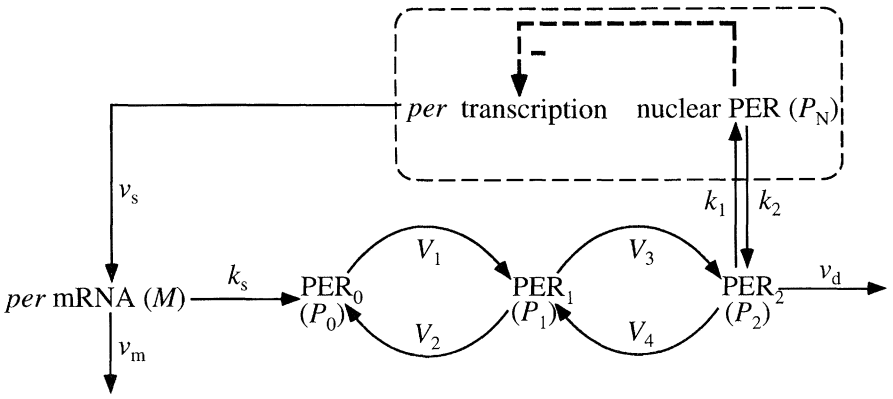


Figure 1. Scheme of the model for circadian oscillations in PER and *per* mRNA (see text). *per* mRNA ($M$) is synthesized in the nucleus and transfers to the cytosol, where it accumulates at a maximum rate $v_s$; there it is degraded by an enzyme of maximum rate $v_m$ and Michaelis constant $K_m$. The rate of synthesis of the PER protein, proportional to $M$, is characterized by an apparent first-order rate constant $k_s$. Parameters $V_i$ and $K_i$ ($i = 1, \ldots 4$) denote the maximum rate and Michaelis constant of the kinase(s) and phosphatase(s) involved in the reversible phosphorylation of $P_0$ into $P_1$ and $P_1$ into $P_2$, respectively. The fully phosphorylated form ($P_2$) is degraded by an enzyme of maximum rate $v_d$ and Michaelis constant $K_d$, and transported into the nucleus at a rate characterized by the apparent first-order rate constant $k_1$. Transport of the nuclear, bisphosphorylated form of PER ($P_N$) into the cytosol is characterized by the apparent first-order rate constant $k_2$. The negative feedback exerted by nuclear PER on *per* transcription is described by an equation of the Hill type (see first term in equation (1*a*)), in which $n$ denotes the degree of cooperativity, and $K_I$ the threshold constant for repression.

complicated oscillatory dynamics might nevertheless occur with a much higher number of variables remains to be seen).

The role of PER phosphorylation is still unclear. It has been suggested (Edery *et al.* 1994) that phosphorylation may control nuclear localization and/or degradation of PER. Here we assume that the fully phosphorylated form ($P_2$) is marked both for degradation and for reversible transport into the nucleus. In the absence of more detailed information, alternative assumptions could, at this stage, be retained. Thus, degradation of PER could also be directed at the nuclear form of PER ($P_N$), or at the unphosphorylated or monophosphorylated forms of the protein, both of which could also be transported into the nucleus. Such changes would probably produce only minor modifications in dynamic behaviour, but delaying PER entry into the nucleus and degradation until the protein is fully phosphorylated – as considered here – should favour the occurrence of sustained oscillations (see §5). The assumption that only the fully phosphorylated form of PER enters the nucleus introduces a delay, which is consistent with recent observations on delayed PER nuclear entry (Curtin *et al.* 1995); such a delay could be caused, at least in part, by PER phosphorylation.

Crucial to the mechanism of oscillations in the model is the negative feedback exerted by nuclear PER on the production of *per* mRNA. This negative feedback is described by an equation of the Hill type. To simplify the model, we consider that $P_N$ behaves directly as a repressor; activation of a repressor upon binding of $P_N$ would not significantly alter the results. The time evolution of the five-variable model is governed by the following kinetic equations, in which all parameters and concentrations are defined with respect to the total cell volume (see the figure 1 legend for a definition of the various parameters):

$$\frac{dM}{dt} = v_s \frac{K_I^n}{K_I^n + P_N^n} - v_m \frac{M}{K_m + M} \tag{1a}$$

$$\frac{dP_0}{dt} = k_s M - V_1 \frac{P_0}{K_1 + P_0} + V_2 \frac{P_1}{K_2 + P_1} \tag{1b}$$

$$\frac{dP_1}{dt} = V_1 \frac{P_0}{K_1 + P_0} - V_2 \frac{P_1}{K_2 + P_1} - V_3 \frac{P_1}{K_3 + P_1} + V_4 \frac{P_2}{K_4 + P_2} \tag{1c}$$

$$\frac{dP_2}{dt} = V_3 \frac{P_1}{K_3 + P_1} - V_4 \frac{P_2}{K_4 + P_2} - k_1 P_2 + k_2 P_N - v_d \frac{P_2}{K_d + P_2} \tag{1d}$$

$$\frac{dP_N}{dt} = k_1 P_2 - k_2 P_N \tag{1e}$$

The total (nonconserved) quantity of PER protein, $P_t$, is given by:

$$P_t = P_0 + P_1 + P_2 + P_N \tag{2}$$

## 3. THE PER CIRCADIAN CLOCK AS LIMIT CYCLE OSCILLATOR

Numerical integration shows that in a large domain of parameter values, instead of evolving toward a stable steady state, the system governed by equations (1*a–e*) reaches a regime of sustained, periodic oscillations. The temporal variation in *per* mRNA and the variation in nuclear PER are shown in figure 2; also shown is the periodic variation in the total amount of PER protein and in the unphosphorylated and

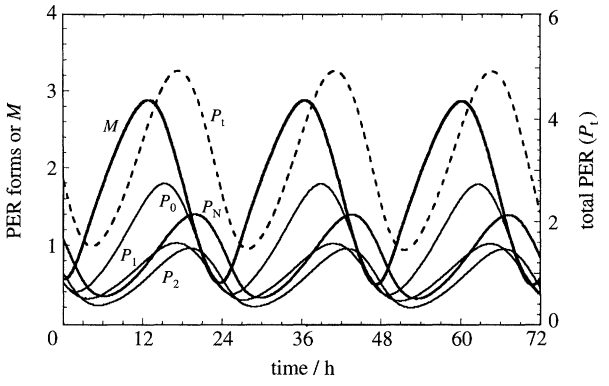


Figure 2. Sustained oscillations generated by the model based on negative regulation of *per* mRNA synthesis by the PER protein in *Drosophila*. The temporal variation in *per* mRNA ($M$) and in the total amount of PER protein ($P_t$) is shown, together with the variation in nuclear PER ($P_N$) and in the unphosphorylated ($P_0$) and phosphorylated, cytosolic ($P_1$ and $P_2$) forms of the protein. The curves are obtained by numerical integration of equations (1*a–e*); $P_t$ is given by equation (2). Parameter values are: $v_s = 0.76$ μM $h^{-1}$, $v_m = 0.65$ μM $h^{-1}$, $K_m = 0.5$ μM, $k_s = 0.38$ $h^{-1}$, $v_d = 0.95$ μM $h^{-1}$, $k_1 = 1.9$ $h^{-1}$, $k_2 = 1.3$ $h^{-1}$, $K_I = 1$ μM, $K_d = 0.2$ μM, $n = 4$, $K_1 = K_2 = K_3 = K_4 = 2$ μM, $V_1 = 3.2$ μM $h^{-1}$, $V_2 = 1.58$ μM $h^{-1}$, $V_3 = 5$ μM $h^{-1}$, $V_4 = 2.5$ μM $h^{-1}$. The model can also produce sustained oscillations for $n = 2$ or $n = 1$, in a domain in parameter space smaller than for $n = 4$. In the absence, at this stage, of detailed information on concentrations and kinetic constants, the above parameter values have been chosen so as to yield a period close to 24 h; the concentration scale is given tentatively in μM.

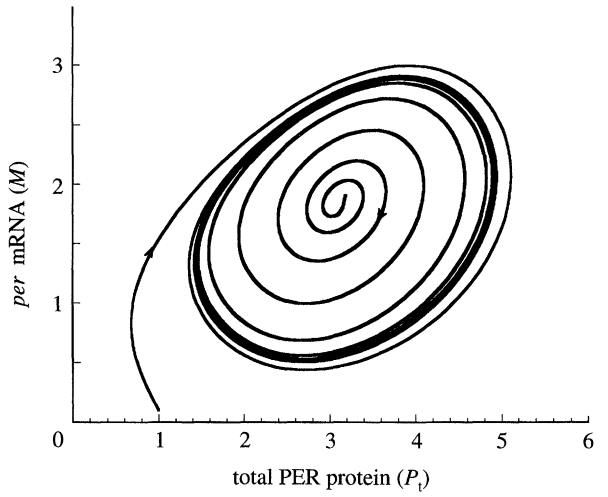


Figure 3. Sustained oscillations in PER protein and *per* mRNA correspond to the evolution toward a limit cycle in the ($M$, $P_t$) plane. Starting from two different sets of initial conditions, the system eventually reaches a unique, closed curve characterized by a period and amplitude that are fixed for the given set of parameter values. The initial conditions, located outside or inside the limit cycle, are, respectively, in μM (tentative scale): $M = 0.1$, $P_0 = P_1 = P_2 = P_N = 0.25$ ($P_t = 1$), and $M = 1.9$, $P_0 = P_1 = P_2 = P_N = 0.8$ ($P_t = 3.2$). The trajectories are obtained as in figure 2, for the same set of parameter values.

phosphorylated, cytosolic forms of PER. For a period close to 24 h, under the conditions of figure 2, the phase shift between the peaks in *per* mRNA and nuclear PER is of the order of 7 h, whereas the phase difference between total PER and *per* mRNA is shorter: about 4.5 h. Such a result is consistent with the observation that the maximum total PER protein follows the peak in *per* mRNA by about 4 h (Zeng *et al.* 1994). The model should prove useful in investigating the way various parameters control the duration of the delay between the mRNA and the various forms of PER.

The sensitivity of the model to parameter variation has been investigated by determining how the period alters when each of the parameters in turn is varied by ±5%. The results show that in the conditions of figure 2, in response to such a variation in any of the parameters, the largest change in period is less than ±2.7%.

Sustained oscillations in PER and *per* mRNA correspond to the evolution toward a limit cycle, away from the unstable steady state. This is demonstrated in figure 3 where the level of *per* mRNA is plotted as a function of the total amount of PER protein for two different initial conditions, one located inside and the other outside the limit cycle. In each case, the projection of the trajectory followed by the five-variable system shows that the system evolves towards the same, unique closed curve in the phase plane ($M$, $P_t$). Limit cycle oscillations are particularly stable because they are characterized by a unique amplitude and frequency for a given set of parameter values, regardless of initial conditions (Minorsky 1962; Nicolis & Prigogine 1977). The limit cycle nature of the oscillations, long considered for circadian rhythms, allows for their suppression by a critical perturbation that brings the oscillatory system back to its singularity, i.e. the steady state (Winfree 1980). Limit cycle models of an abstract mathematical nature or borrowed from physical sciences, as in the case of the Van der Pol oscillator, have often been used to analyse properties of circadian clocks (Wever 1972; Pavlidis 1973; Kronauer *et al.* 1982; Lakin-Thomas *et al.* 1991). The present model provides a molecular basis for circadian oscillations of the limit cycle type.

## 4. DEPENDENCE OF THE PERIOD ON THE RATE OF PER DEGRADATION

Repression by PER is at the core of the oscillatory mechanism and therefore the maximum rate $v_d$ at which PER is degraded is a key control parameter of the model. The model predicts that sustained oscillations occur in a window bounded by two critical values of this parameter. In that window, under the conditions of figure 2, the period of the oscillations increases from 19.3 h up to 64 h. Such long periods have not been reported for *Drosophila*, but periods approaching 50 h have been found at low light intensity in the $per^l$ mutant (Konopka *et al.* 1989). The period range in the window of $v_d$ values depends on other parameters. Thus, for a larger value of the rate of protein synthesis measured by parameter $k_s$, the period varies as a function of $v_d$ from 15.9 to 62.1 h (see figure 4). The period of PER oscillations rises with the rate of PER degradation as a result of the longer time required

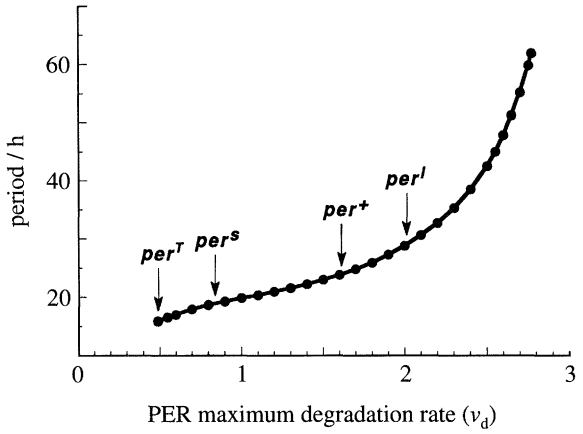


Figure 4. Dependence of the period of PER oscillations on the maximum rate of PER degradation, $v_d$. The data points are obtained by numerical integration of equations (1*a–e*) for different values of $v_d$ (in μM $h^{-1}$); other parameter values are as in figure 2, with $k_s = 0.78$ $h^{-1}$. Outside the indicated range of $v_d$ values, sustained oscillations do not occur and the system evolves toward a stable steady state. Arrows indicate the putative, approximate location of the mutants $per^T$, $per^s$ and $per^l$, and of the wild type $per^+$ as a function of $v_d$. In addition to (or instead of) changes in $v_d$, variations in other biochemical parameters of the model might underlie the changes in period seen in various *per* mutants.

to reach the threshold beyond which the protein significantly represses the transcription of its gene.

The results in figure 4 show that mutations affecting the structure of the PER protein (either hindering or enhancing its enzymic degradation), would result in a shortening or lengthening of the period, as in the $per^s$ and $per^l$ mutants, which have a period close to 19 h and 29 h, respectively (Konopka & Benzer 1971) (see arrows in figure 4). The ultrashort $per^T$mutant recently described (Konopka *et al.* 1994), which has a period close to 16 h, would also fall in the range shown in figure 4. The explanation of *per* mutants in terms of the PER degradation rate is only tentative and illustrates how the model may serve to pinpoint the parameters whose changes might cause the period alterations in the mutants. In the framework of the explanation in terms of $v_d$, it is tempting to predict, at first view, that the level of PER in $per^T$ and $per^s$ should be larger than in the wild type, because of the reduced value of $v_d$ in the mutants. Such differences in PER levels are not observed in the experiments (see, for example, Konopka *et al.* 1994). However, the link between the level of PER and $v_d$ is far from straightforward: indeed, a counterintuitive prediction of the model is that the maximum level of total PER protein reached in the course of oscillations rises as the maximum rate of PER degradation increases, because the time evolutions of PER and *per* mRNA are closely intertwined.

Another key parameter that controls the oscillations is the rate constant $k_1$ related to the transport of PER into the nucleus. Again, there is a range of $k_1$ values that produce sustained oscillations, but the period decreases as $k_1$ increases. If the rate of PER transport into the nucleus goes below a critical value, sustained oscillations disappear and the system evolves toward a stable steady state.

## 5. DISCUSSION

The model for circadian oscillations in PER protein and *per* mRNA presented here is closely related to the work of Goodwin (1965) who discussed the conditions in which a protein repressing the transcription of its gene can produce sustained oscillations in the levels of that protein and its mRNA. The equations originally proposed were not sufficiently nonlinear to give rise to limit cycle oscillations. Equations of that type were later investigated for limit cycle behaviour (Griffith 1968), and used explicitly for circadian rhythms to determine phase response curves with respect to transient perturbations (Drescher *et al.* 1982). Similar equations were also studied to predict how sustained oscillations occur in a metabolic pathway regulated by end-product inhibition (Morales & McKay 1967; Walter 1970; Hunding 1974; Rapp 1975; Tyson & Othmer 1978). These models showed that periodic behaviour is favoured, both by enlarging the length of the enzymatic chain that leads from the regulated step to the end-product, and by increasing the degree of cooperativity of negative feedback. These results bear on the model for PER oscillations. Here, as in a cascade model for the mitotic oscillator (Goldbeter 1991, 1995), the sequence of successive phosphorylations of the PER protein can be viewed as introducing a series of time delays, an effect similar to that of increasing the number of intermediate steps in the enzymatic chain regulated by end-product inhibition. Incorporating more than two phosphorylation steps into the model should therefore enlarge the domain of sustained oscillations. The model schematized in figure 1 can be seen as minimal compared with the model considered by Abbott *et al.* (1995), which treats multiple PER phosphorylation in a more comprehensive manner.

With regard to the role of nonlinear feedback control, the periodic behaviour shown in figure 3 occurs for a repression function characterized by a cooperativity coefficient $n$ equal to 4. A value of 2, or even 1, for $n$ can also give rise to sustained oscillations; however, the domain of oscillations in parameter space is then smaller than for $n = 4$. Thus, if the cooperativity of repression favours periodic behaviour, multiple phosphorylation of PER, by introducing a series of delays, reinforces, and could even substitute for the effect of such cooperativity in allowing for sustained oscillations. This conclusion supports the view (Curtin *et al.* 1995; Abbott *et al.* 1995) that, by gating PER entry into the nucleus, PER phosphorylation delays the negative feedback exerted by PER on *per* transcription, and thereby, at the same time, strengthens the capability of such feedback to produce robust oscillations and contributes to raise their period up to circadian values.

The behaviour of *per* mutants was interpreted in figure 4 in terms of variations in the maximum rate of PER degradation. However, the variation in PER degradation rate may not be the only factor – or even the main factor – responsible for the alteration in period seen in *per* mutants. Thus, recent observations show that the delay in nuclear entry is normal for the $per^s$ mutant, but increases in the mutant $per^l$ (Curtin

*et al.* 1995). Changes in the structure of the protein, possibly associated with modifications in the intra- or intermolecular interactions of PER (Huang *et al.* 1995) and with changes in the rate of PER degradation or phosphorylation, may underlie these differences.

In contrast to circadian rhythms in the wild type, which exhibit the property of temperature compensation, the period in the mutants $per^l$ and $per^s$ increases and decreases respectively with temperature (Konopka *et al.* 1989). A molecular explanation for the temperature compensation of circadian oscillations in *Drosophila* has recently been proposed (Huang *et al.* 1995). To address the mechanism of this phenomenon, the present theoretical model could be extended to include intra- and intermolecular interactions of PER, along the lines suggested by Huang *et al.* (1995). Also of interest is the effect of *per* gene dosage. The model indicates that in a certain range of parameter values, an increase in the rate of mRNA synthesis, measured by parameter $v_s$ (see figure 1), may produce a slight decrease in the period of PER oscillations. Such a result holds qualitatively with experimental observations (Smith & Konopka 1982) on the effect of increased gene dosage on the period of circadian rhythms.

Besides the circadian periodicity in eclosion and locomotor activity, *Drosophila* displays a rhythm of about 1 min period associated with the courtship song of the male. Surprisingly, mutations of the *per* gene also affect the latter rhythm (Kyriacou & Hall 1980). It is still unclear how PER may at the same time control the period of circadian rhythms and of these very rapid oscillations. PER behaves as a regulator of transcription (Huang *et al.* 1993) and therefore it might influence the synthesis of a number of different proteins. Perhaps the level of some key protein involved in the production of the song rhythm is altered in *per* mutants, with the consequence that a change in the period of this high frequency rhythm might occur.

The model can be used to study the mechanism of circadian rhythms in organisms other than *Drosophila*. The prominent role of protein synthesis is indeed attested by the observation, repeatedly made in different systems, that inhibitors of protein synthesis suppress circadian oscillations or at least produce phase shifts or modify their period (Taylor *et al.* 1982; Khalsa *et al.* 1992; Takahashi *et al.* 1993). Similar results have been obtained by means of an inhibitor of transcription (Raju *et al.* 1991). Together with the negative control exerted by the PER protein on the synthesis of its mRNA (Hardin *et al.* 1990, 1992; Zeng *et al.* 1994) and the delay introduced in that negative feedback loop (Curtin *et al.* 1995), possibly as a result of PER phosphorylation, protein synthesis is an integral part of the mechanism proposed for circadian oscillations in *Drosophila*. A role for protein covalent modification in the circadian clock mechanism is supported by observations in other organisms, such as *Gonyaulax*, where an inhibitor of protein phosphorylation was shown to block circadian oscillations (Comolli *et al.* 1994).

The present model, which involves both protein synthesis and post-translational modification, corroborates the view that the negative control exerted by PER on the synthesis of its mRNA plays a key role in the generation of circadian rhythms in *Drosophila*. Such a negative feedback on transcription has also been observed for the *frq* gene in *Neurospora* (Aronson *et al.* 1994), and could well prove to be a general feature (Takahashi 1993) of the circadian oscillatory mechanism in unicellular and multicellular organisms.

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