Turkish Journal of Biology

Volume 23 | Number 2 Article 2

1-1-1999

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Tr. J. of Biology
23 (1999) 143-151

On The Kinetics of Human Erythrocyte Glutathione Disulfide Reductase: Does The Enzyme Really Play 'Ping-Pong'?

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Received: 20.11.1997

Abstract:: Human erythrocyte glutathione disulfide reductase studied at 37°C in 100 mM potassium phosphate buffer, pH 7.4, is subject to partial inhibition by GSSG at low [NADPH]. Saturation by NADPH is hyperbolic at low [GSSG] and becomes increasingly sigmoidal at high [GSSG]. NADP+ is a competitive inhibitor with respect to NADPH at 20-1000 µM GSSG and an uncompetitive inhibitor with respect to GSSG at 33-120 µM NADPH. Studied under special conditions (10 µM NADPH and 1000 µM GSSG), NADP+ exhibits a two-fold effect, inhibition being preceded by activation in the 0-50 µM effector concentration range. The data are consistent with the ping-pong / sequential ordered hybrid model diverging at the level of E.NADPH. A composite sequential ordered mechanism is suggested, involving two conformational states of free enzyme at equilibrium. While one state is selective for NADPH as the first substrate, the second state adds GSSG to initiate the catalytic cycle. The pathway proceeding through E.GSSG has an apparently lower turnover number than that proceeding through E.NADPH.

Key Words: Glutathione disulfide reductase, kinetics, mechanism, NADP+ inhibition, human erythrocytes.

İnsan Eritrosit Glutatyon Disülfit Reduktazının Kinetiği Üzerine Enzim Gerçekten 'Ping-Pong' Mekanizma mı Gösterir?

Özet: İnsan eritrositi glutatyon disülfit redüktazı, 100 mM potasyum fosfat (pH 7.4, 37°C) tamponunda çalışıldığında, düşük [NADPH] derişiminde GSSG'nin kısmi inhibisyon yaptığı bulundu. Düşük GSSG derişimlerinde, NADPH doygunluk grafiğinin hiperbolik olduğu ancak GSSG derişimi arttıkça sigmoidisitenin de arttığı gözlendi. NADP+, 20-1000 µM GSSG derişimlerinde, NADPH'la kompetitif; 33-120 µM NADPH derişimlerinde ise GSSG ile unkompetitiftir. Özel koşullarda çalışıldığında (10 µM NADPH ve 1000 µM GSSG), NADP+'nin iki tür etkisinin olduğu; inhibisyondan önce, 0-50 µM effektör derişiminde, bir aktivasyonun olduğu görüldü. Elde edilen bulgular, E.NADPH düzeyinde ayrılan 'ping-pong/ardışık sıralı hibrid' modeli ile uygunluk göstermektedir. Bu bulgular, serbest enzimin, birbirleri ile dengede, iki konformasyonu içeren ardışık sıralı mekanizma imgelemektedir. Bu konformasyonlardan birisi ilk substrat olarak NADPH'ı seçerken, diğer konformasyon GSSG'yi bağlayarak katalitik siklusu başlatmaktadır. E.GSSG üzerinden giden yolun, E.NADPH üzerinden gidene göre, daha düşük 'turnover' sayısının olduğu açıktır.

Anahtar Sözcükler: Glutatyon disülfit redüktaz, kinetik, mekanizma, NADP+ inhibisyonu, insan eritrositi.

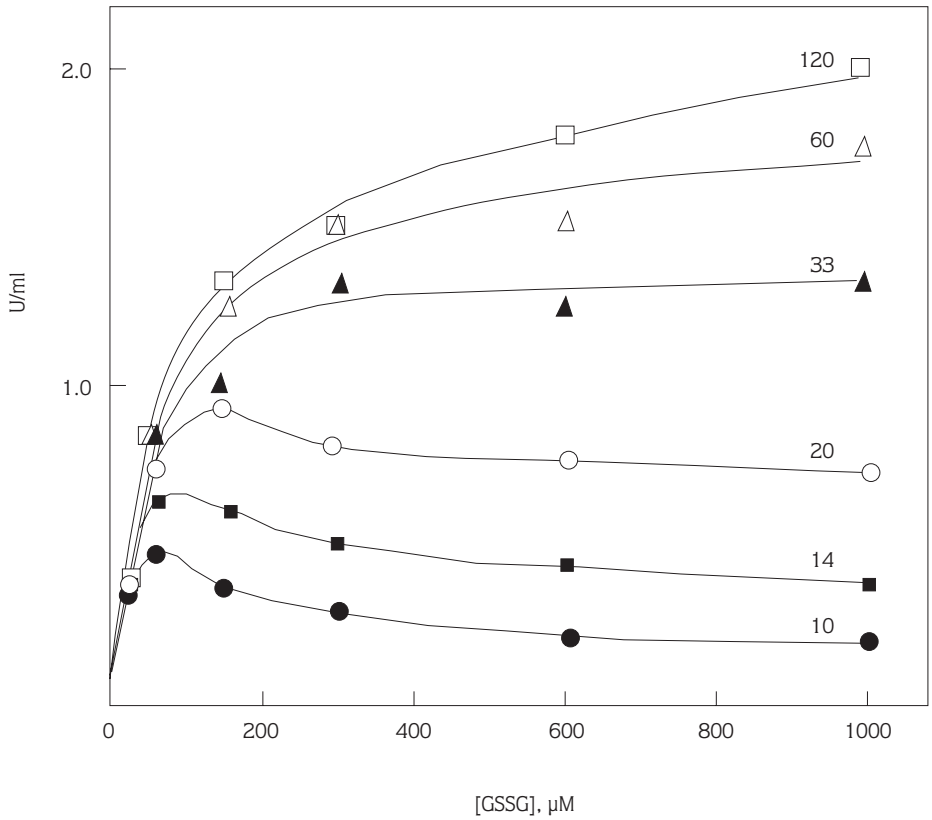


Figure 1. Dependence of initial rate on GSSG concentration. The fixed concentrations of NADPH (in μM) are marked on the curves.

Introduction

Complex kinetic patterns obtained with glutathione disulfide reductase from a variety of sources [1-7] have generally been interpreted in terms of a branched mechanism [3] involving the partitioning of E.NADPH between ping-pong and sequential ordered pathways. However there is some indication than E.GSSG may also be a catalytically competent species [6]. In the following study we have investigated the same problem, using human erythrocyte glutathione disulfide reductase (GSSGR, NAD(P)H; oxidized glutathione oxidoreductase, E.C. 1. 6. 4. 2.). The results suggest that kinetic complexity arises from the existence of alternative sequential ordered pathways comprising a system analogous to the steady-state random system with nonequivalent halves [8].

Table 1. Predictions relating to the inhibition of glutathione disulfide reductase by NADP+

Mechanism	Type of Inhibition: [NADPH] varied [NADPH]: low / saturating	Type of Inhibition: [GSSG] varied [NADPH]: low/saturating
1. Ping-pong NADPH NADP$^+$ GSSG 2GSH _↓_____↑_____ ↓____↑_	M[a]/-[b]	C[c]/C
2. Sequential order a) NADPH adds first; GSH leaves last NADPH GSSG NADP$^+$ 2GSH _↓_____↑_____ ↓____↑_	M/U[d]	M/M
b) NADPH adds first; NADP+ leaves last NADPH GSSG 2GSH NADP$^+$ _↓_____↑_____ ↓____↑_	C/C	M/-[b]
c) GSSG adds first; GSH leaves last GSSG NADPH NADP$^+$ 2GSH _↓_____↑_____ ↓____↑_	M/-[e]	M/U
d) GSSG adds first; NADP+ leaves last GSSG NADPH 2GSH NADP+ _↓_____↑_____ ↓____↑_	M/-[b]	C/C
3. Random order	M/M	M/M

[a] Mixed type inhibition
[b] No inhibition
[c] Competitive inhibition
[a] Uncompetitive inhibition
[e] May look competitive, depending on the relative magnitudes of the kinetic constants.

Materials and Methods

Glutathione disulfide reductase (with a specific activity of 140 units/mg protein) was purified from expired human blood by modification of a previously documented procedure [9]. Activity was determined at 37°C, in 100 mM potassium phosphate buffer (pH 7.4), containing 4 mM

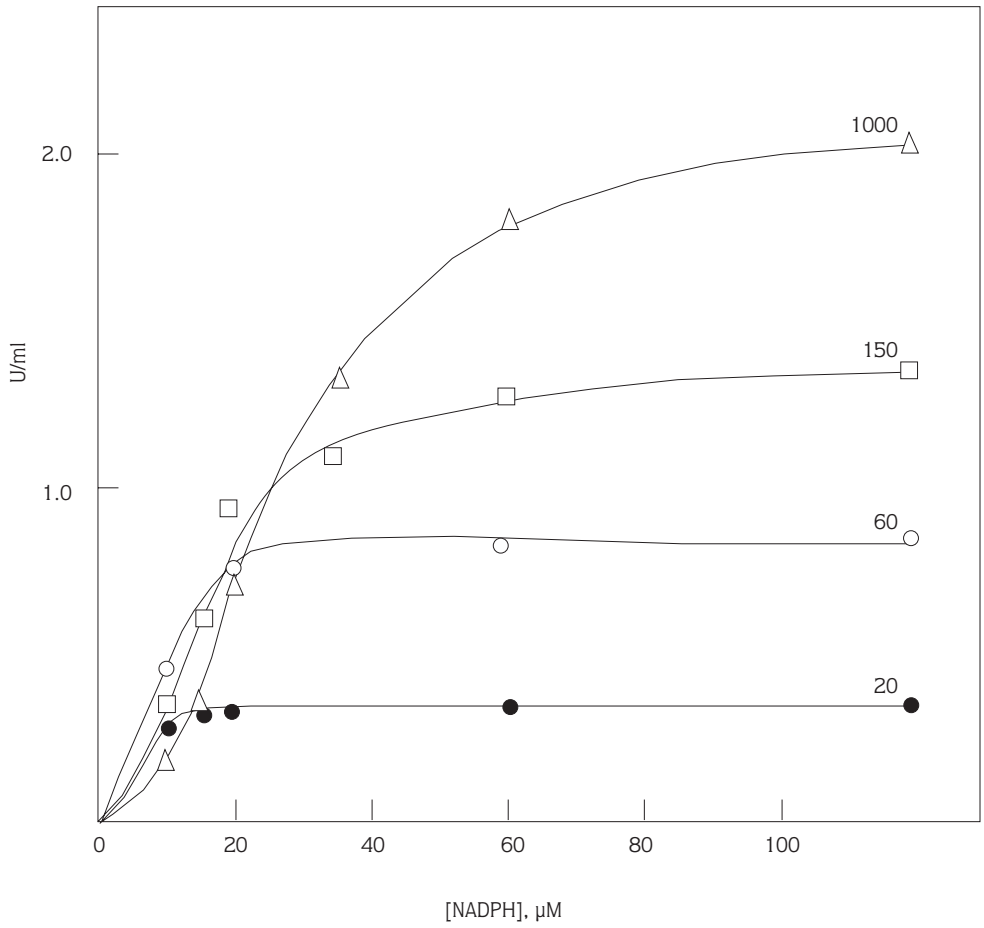


Figure 2. Dependence of initial rate on NADPH concentration. The fixed concentrations of GSSG (in µM) are marked on the curves.

EDTA. The reaction was initiated by adding enzyme (to give a final concentration of about 3.3 nM) and followed by monitoring the change in absorbance at 340 nm.

All biochemicals were from Boehringer Mannheim (FRG) or Sigma (U.S.A).

Results and Discussion

The variation of the initial rate of [GSSG] at fixed [NADPH] is given in Figure 1. The system is marked by partial substrate inhibition by GSSG at low [NADPH]. Correspondingly, saturation

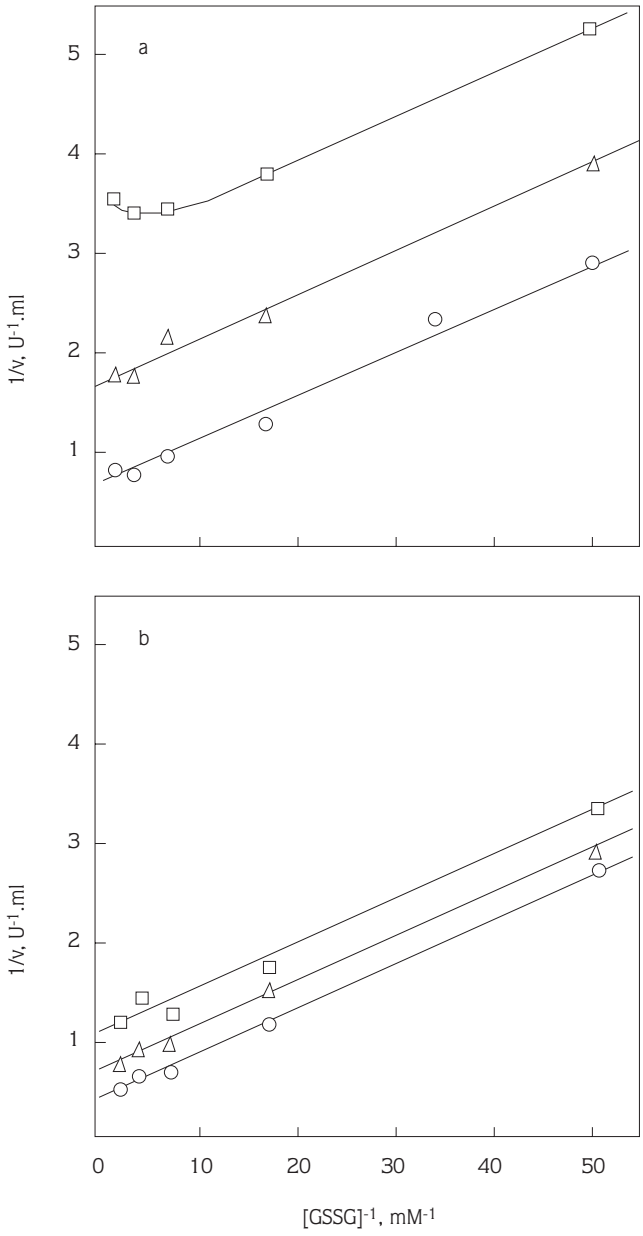


Figure 3. Lineweaver-Burk plots for GSSG. [NADPH] = (a) 33 µM and (b) 120 µM. $[NADP^+]$ = 0 (o, o), 300 (Δ, Δ) and 1000 (□, □) µM.

by NADPH exhibits a pronounced sigmoidal character at high [GSSG] (Figure 2). Taken at face value, these data fit the hybrid mechanism proposed previously [3], with the condition that the components branch at the level of E rather than E.NADPH (Scheme I), i.e. the E.NADPH complex

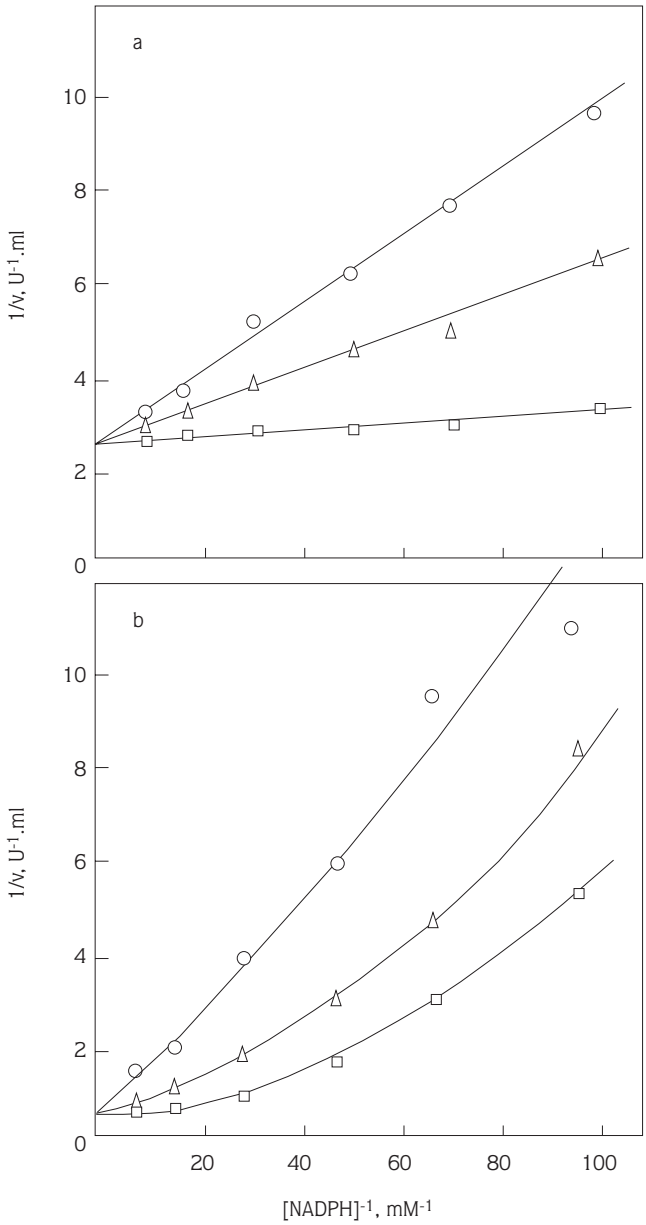


Figure 4. Lineweaver-Burk plots for NADPH. [GSSG] = (a) 20 µM and (b) 100 µM. [$NADP^+$] = 0 (□, □), 300 (Δ, Δ) and 1000 (○, ○) µM.

is committed to a ping-pong pathway, whereas the E:GSSG complex proceeds via a sequential ordered mechanism. Slow turnover of the sequential, relative to the ping-pong pathway, could account for the nonhyperbolic nature of the substrate saturation curves.

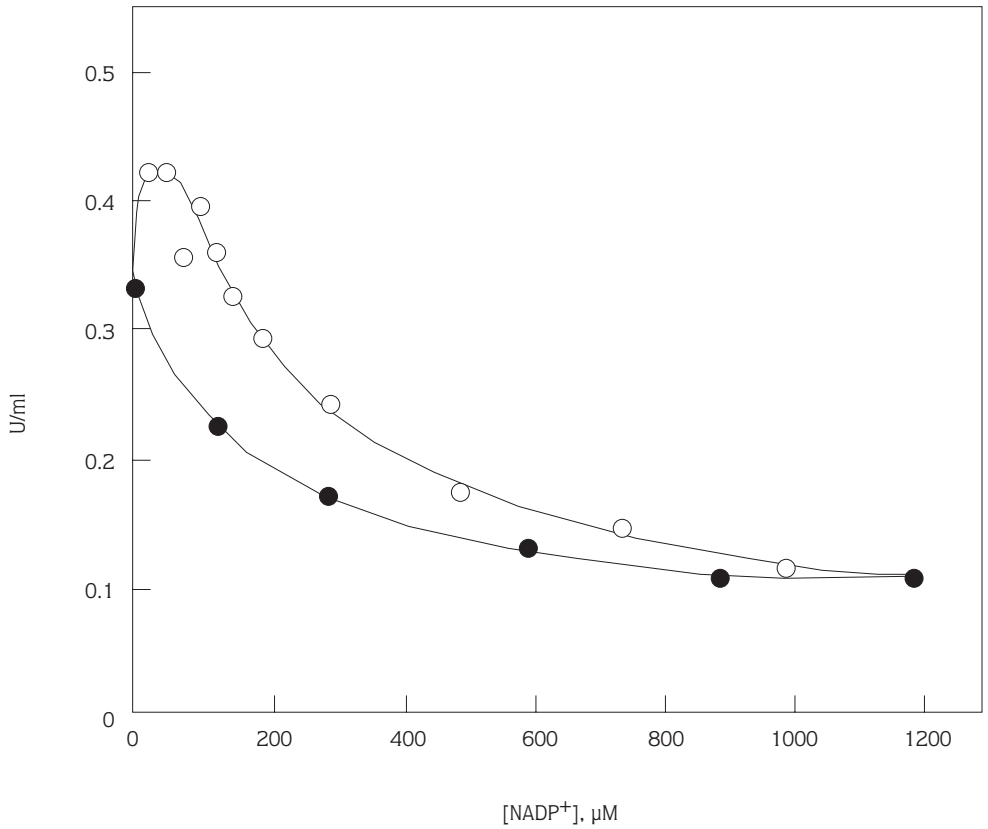
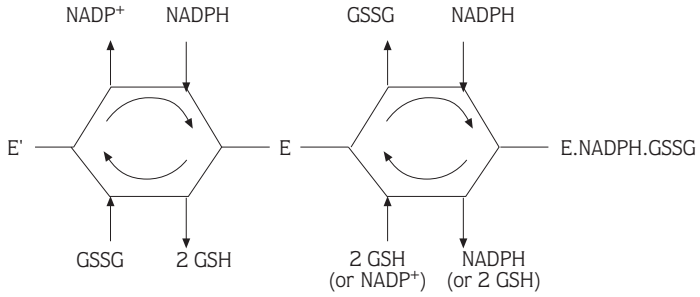


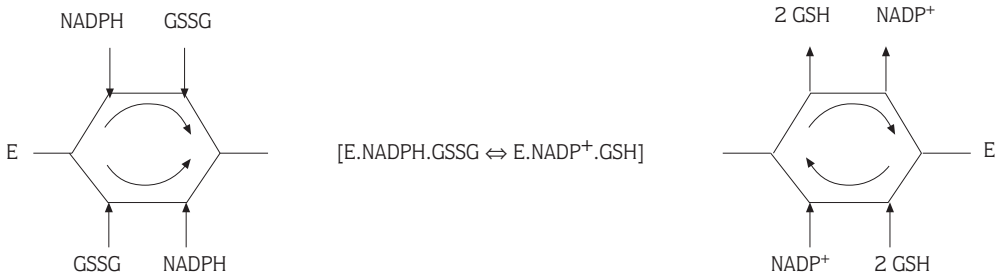
Figure 5. Inhibition by $NADP^+$. (•), at 14 µM NADPH and 20 µM GSSG. (O), at 10 µM NADPH and 1000 µM GSSG.

However, the inhibitory effect of NADP+ on the forward reaction indicates the lack of a ping-pong component: i. NADP+ is an uncompetitive inhibitor with respect to GSSG (Figure 3) even at 120 µM, an apparently saturating concentration (*cf.* Figure 2). ii. With NADPH as the varied substrate, competitive inhibition is observed (Figure 4) up to 1 mM GSSG. When checked against predictions based on different possible mechanisms (Table 1) [10], this inhibition pattern suggests catalysis by human erythrocyte glutathione disulfide reductase via a composite sequential ordered pathway (Scheme II, options 2b and 2c in Table I).

The mechanism in Scheme II is distinct from the random BI-BI system (where both substrate and products may be added and leave in any order), in that the order of departure of products is determined by which pathway is taken to reach the central complex. Such a 'restricted random' mechanism brings to mind the possibility that there may be two different conformational states of the enzyme [11], one favoring the pathway through E.NADPH and the other proceeding through E.GSSG. This argument is supported by the 'fine structure' or NADP+



Scheme I



Scheme II

inhibition (Figure 5). At low [GSSG], NADP+ acts as a partial inhibitor, in keeping with the mixed inhibition patterns relating to mechanism 2b or 2c in Table 1.

At high [GSSG], on the other hand, NADP+ is an activator at low concentration and shows its expected inhibitory effect only at higher concentrations. Such activation by a potential inhibitor is characteristic of cooperative systems where the inhibitor mimics the substrate in bringing about a conformational change [12]. In the particular case of glutathione disulfide reductase, one may therefore propose the following: (a) The dimeric enzyme exists in an R ⇔ T type conformational equilibrium. (b) The R state conformation is committed to an 'NADPH first' pathway (option 2b, Table I) while the T conformation is committed to the 'GSSG first' pathway (option 2c, Table I). (c) High [GSSG] shifts the equilibrium towards the catalytically inferior T state and (d) NADP+ shifts the system back to the R state in such a way that the potential inhibition by this product is initially compensated for by the higher activity of the R relative to the T state.

The significance of this restricted random / cooperative model is not immediately obvious.

The steady-state concentration of NADPH in the red blood cell has been reported to be 65 µM [13]. An upper limit for [GSSG] (based on [GSH]: 3.2 mM [14] and a minimal [GSH] / [GSSG] value of 18 [15]) is about 200 µM. The actual concentration is probably much lower [16]. Under these steady-state conditions the enzyme should function predominantly via the sequential ordered pathway involving E.NADPH (the upper route in scheme II). It seems likely that the kinetic complexity of the glutathione disulfide reductase reaction is a product of the unphysiological conditions employed in studying the enzyme *in vitro*.

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