Oxidants and antioxidants of erythrocytes

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ABSTRACT

Erythrocytes contain reactive forms of oxygen (superoxide anion, hydrogen peroxide, hydroxyl radical) and reactive form of nitrogen (nitric oxide anion, S-nitrosothiols, peroxynitrite anion). Reactive oxygen species and reactive nitrogen species inactivate enzymatic (methemoglobin reductase, Cu, Zn-Superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (glutathione, alpha-tocopherol, beta-carotene, ascorbate) antioxidants. Their quantity in erythrocytes increases in case of exposure to xenobiotics, in erythrocytes containing pathological hemoglobin, in erythrocytes with the enzymatic defects of the glycolytic or pentose cycle, in erythrocytes found in arterial and venous thrombi, and in the blood extravasated to tissues and body cavity. In such cases are observed in erythrocytes: structure modification of hemoglobin and membrane proteins, and lipids peroxidation. These processes cause changes of shape, decrease of flexibility, decrease of resistance to hemolysis, Heinz's bodies production and shorten the life span of red cells.

Key words: Erythrocytes; reactive oxygen species; reactive nitrogen species; antioxidants.

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INTRODUCTION

There are three phases in the development of cells in the red-blood-cell/erythrocyte system: production and maturation of erythroblasts and reticulocytes in the bone marrow transport of mature erythrocytes in the circulating blood, and aging as well as elimination of erythrocytes from the blood by mononuclear phagocytes of the spleen. Erythroblasts possess nuclei, mitochondria and ribosomes and they are the place of hemoglobin biosynthesis.

Reticulocytes, on the other hand, do not possess nuclei, but they contain fragments of mitochondria and ribosomes, whose quantity decreases up to disappearance together with maturation [1]. They synthesize only small amounts of hemoglobin. Reticulocytes comprise only 0.03-0.15% of mature erythrocytes in the circulating blood. Erythrocytes are dominant among blood cells, and their ratio to blood platelets and leukocytes is 700:40:1. There are 4.5 million of erythrocytes in 1mm$^3$. Erythrocytes total count in the circulating blood equals approximately 25 billion. Due to the structure, chemical composition and metabolism, erythrocytes are adapted to oxygen and carbon dioxide transport. Energy, stored as ATP, is obtained by anaerobic glycolysis (90%) and pentose cycle (10%). Glycolysis provides reduced nicotinamide adenine dinucleotide (NADH) and pentose cycle – reduced nicotinamide adenine dinucleotide phosphate (NADPH), essential for, among others, reduction of methemoglobin and oxidized glutathione.

Hemoglobin and heme iron

The mass of one erythrocyte is 82.8pg. Hemoglobin is a dominant chemical component of erythrocytes and comprises 33% of total mass, 94% of dry mass, and 97% of protein mass of the erythrocyte (Tab. 1).

Table 1. Chemical composition of erythrocyte [5]*

<table>
<thead>
<tr>
<th>Component</th>
<th>Contents, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
</tr>
<tr>
<td>water</td>
<td>65.0</td>
</tr>
<tr>
<td>remaining compounds</td>
<td>35.0</td>
</tr>
<tr>
<td>hemoglobin</td>
<td>33</td>
</tr>
<tr>
<td>non-hemoglobin proteins</td>
<td>~ 1</td>
</tr>
<tr>
<td>lipids</td>
<td>0.5</td>
</tr>
<tr>
<td>heme iron</td>
<td>0.11</td>
</tr>
<tr>
<td>glutathione</td>
<td>~ 0.1</td>
</tr>
<tr>
<td>peroxide dismutase</td>
<td>0.007</td>
</tr>
<tr>
<td>other compounds</td>
<td>~ 0.4</td>
</tr>
</tbody>
</table>

* - mean approximate values

One erythrocyte contains 32x10$^{-12}$g and all erythrocytes of the human body contain approximately 800g of hemoglobin. Hemoglobin appears in two conformational forms: relaxed (R) of high oxygen affinity and tense (T) of low oxygen affinity [3].

Only 12.5cm$^3$ of oxygen is dissolved in 5 L of blood in temperature of 37°C. Hemoglobin in this volume of blood binds as much as 1000cm$^3$ of oxygen, i.e. 80 times more than is physically dissolved. Thus, 1g of hemoglobin binds 1.34cm$^3$ and 1g of heme iron – binds 400cm$^3$ of oxygen. In arterial blood there is 95-98% of oxygenated hemoglobin and in venous blood 67-75% of oxygenated hemoglobin. The relaxing organism demand for oxygen at rest is 250cm$^3$/min. During physical exercise, it is up to 10 fold higher, i.e. it increases to 2500cm$^3$/min.

Deoxygenated/oxygenated hemoglobin, under the influence of specific chemical agents, is changed into derivatives: methemoglobin, S-nitrosohemoglobin, carbonous oxide hemoglobin, sulphhemoglobin, cyanomethemoglobin [3]. These derivatives are incapable of carrying oxygen. Some of them, specifically methemoglobin and S-nitrosohemoglobin, play a significant role in oxidative/antioxidative processes.

Hemoglobin in erythrocytes contains approximately 2.6g of iron, which comprises 60% of systemic organism iron. The organism requires 20-36mg of iron for hemoglobin synthesis per 24 hours. It comes mainly from used up erythrocytes and tissue reserves and only to a small extent from iron consumed with food. Thus, iron balance seems to be a closed system to a large extent.

The content of hemoglobin and its derivatives, heme iron and cation iron Fe$^{2+}$/Fe$^{3+}$, with the domination of trivalent iron, is increased locally, e.g. in inflammatory focuses, in blood extravasated to tissues and body cavities, at the site of hemoglobin infiltration to the walls of arterial and venous vessels and arterial and venous thrombi [4, 40, 43, 65].

Reactive oxygen species

Transport of significant amounts of oxygen by erythrocytes favors production of reactive oxygen species. They occur in forms of superoxide anion (O$_2^-$), chargeless radicals, such as hydroxyl radical (OH) and reactive oxygen species such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (O$_2$). The oxidation reaction of Fe$^{2+}$ of hemoglobin to Fe$^{3+}$ of methemoglobin is the main source of superoxide anion in erythrocytes. One electron is transferred from ferrous cation of an oxyhemoglobin molecule to molecular oxygen (Fig.1).
Bivalent iron is oxidized to trivalent iron and an unstable indirect compound is produced – superoxide anion of methemoglobin. It undergoes dissociation to produce methemoglobin and superoxide anion:  

\[
\text{Hb-Fe}^{2+} - \text{O}_2 \leftrightarrow \text{MetHb-Fe}^{3+} - \text{O}_2 \leftrightarrow \text{MetHb-Fe}^{3+} + \text{O}_2^-
\]

Approximately, 1.2% of hemoglobin undergoes this change on physiological conditions. In twenty four hours, about \(10^{10}\) molecules of superoxide anion are produced in one erythrocyte and \(250 \times 10^{15}\) in all erythrocytes of the human body [5]. Production of superoxide anion is increased in congenital methemoglobinemia and methemoglobin reductase deficiency as well as acquired methemoglobinemia, e.g. after sulfonamides and antimalarial drugs and in nitrite compounds and aniline intoxication, after hemodialysis, ultrasound effect, thermal shock, and physical exercises. The rate of forming superoxide anion increases together with the increase in acidity of the environment and in the presence of anions in the sequence of: \(\text{Cl}^- < \text{F}^- < \text{OCN}^- < \text{SCN}^- < \text{N}_3^- < \text{CN}^-\) [6].

Not only, the superoxide anion reduces ferric cations to ferrous cations [7]:  

\[
\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2
\]

but also ascorbate, NADH, NADPH, glutathione, cysteine, and protein-thiolic groups.

Hydroxide radical is formed in the Fenton reaction, in which hydrogen peroxide is reduced under the influence of ferrous cation [8]:  

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{Fe}^{2+}} \text{OH}^- + \text{OH}^-
\]

The formation of hydroxyl radical in the Fenton reaction is locally specific [9]. The heme ring containing cations \(\text{Fe}^{3+}\) is situated at the bottom of the hydrophobic split of alpha- and beta-globins and hydrogen peroxide, a compound well-transferable in the environment, gets there, unlike antioxidants that are no capable of getting to the alpha- and beta-globin chain. Then, a high concentration of \(\text{OH}\) and destructive action to amino-acid residues at the site of appearance take place.

Hydroxyl radical is also formed in the Haber-Weiss reaction with the participation of hydrogen peroxide, superoxide anion and ferrous cation [10]:  

\[
\text{Fe}^{2+}/\text{Fe}^{3+}
\]

\[
\text{H}_2\text{O}_2 + \text{O}_2^- \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2
\]

In the reaction of \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\), singlet oxygen \(\text{O}_2\), hydroxide radical \(\text{OH}^-\) and hydroxide anion \(\text{OH}^-\) are formed:  

\[
\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2\text{H}^+ + \text{OH}^- + \text{OH}^-
\]

**Reactive nitrogen species**

Reactive nitrogen species, such as nitrogen oxide radical \(\text{(NO)}\), nitrogendioxide radical \(\text{(NO}_2\text{)}\), nitrosic cation \(\text{(NO)}^+\), nitroxylic anion \(\text{(NO)}^-\) and peroxinitrite \(\text{(ONOO)}^-\) are present in erythrocytes [11]. Nitrogen oxide is synthesized in the cells of the vascular endothelium, from where it is transported to the cells of vascular smooth muscles and to the plasma [12]. From the plasma it passes to erythrocytes, where it is bonded to hemoglobin. The reaction of binding nitrogen oxide to hemoglobin comprises S-nitrosilation of Cys93 residue in the beta-globin chain and formation of S-nitrosohemoglobin (HbSNO) [13]. The reaction takes place with oxygenated hemoglobin in R conformation [14]. Deoxygenation of hemoglobin and its passage to T conformation releases NO* back to the plasma. Due to these processes, venous blood contains approximately 10fold less HbSNO than arterial blood with S-nitrosilohemoglobin being an NO* transporter. Nitrogen oxide is transported from HbSNO, in the S-transnitrosilation, to fine particulate thioles such as cysteine and glutathione with the formation of nitrosocysteine (CysNO) and nitrosogluthathione (GSONO) [15]. S-nitrosilation is easier while denitrosilation is harder in case of glycated hemoglobin [16]. S-nitrosilation of pathological hemoglobin S (HbS) hinders its polymerization. Increased content of S-nitrosohemoglobin can be observed in endotoxia.

In the condition of oxidative stress, the reaction of nitrogen oxide and oxyhemoglobin ferrous cation with superoxide anion, takes place. The reaction of nitrogen oxide and oxyhemoglobin iron leads to the formation of methemoglobin and nitrates [17]:  

\[
\text{HbO}_2 + \text{NO}^- \rightarrow \text{MetHb} + \text{NO}_3^-
\]

Peroxinitrite anion is the product of the reaction of nitrogen oxide and superoxide anion [18]:  

\[
\text{O}_2^- + \text{NO}^+ \rightarrow \text{ONOO}^-
\]

Spontaneous protonation of the peroxynitrite leads to the formation of nitrogen dioxide \(\text{(NO}_2\text{)}\) and hydroxyl radical \(\text{(OH)}\):  

\[
\text{ONOO}^- + \text{H}^+ \rightarrow \text{NO}_2^+ + \text{OH}^-
\]
In the reaction of peroxynitrite with sulphydryc compounds, their nitro-derivatives are formed [19]:
$$\text{ONOO}^- + \text{RSH} \rightarrow \text{RSNO} + \text{O}_2 + \text{H}^+$$
Reactions mentioned above make the circulating blood poorer in nitrogen oxide.

Deficiency of nitrogen oxide and its highly reactive metabolic products specifically peroxinitrite, cause the membrane proteins damage and decrease erythrocytes flexibility [20, 21]. The metabolism of reactive nitrogen species in erythrocyte is presented in Fig. 2 [19].

![Figure 2. Nitric oxide metabolism in erythrocyte according [37]. Explanatory notes in the text.](image)

**Antioxidants**

Both reactive oxygen species and reactive nitrogen species that are formed in erythrocytes are inactivated by antioxidative enzymes and non-enzymatic antioxidants.

Due to the antioxidants activity, the amount of methemoglobin in erythrocytes does not exceed 0.6-1.8% of hemoglobin in erythrocytes. There are three kinds of reductases that reduce trivalent iron of methemoglobin, NADH is the co-enzyme of the first one, NADPH – the second one, and the third one acts with participation of ferrocytochrome b5 [22].

Reductase of methemoglobin: NADH catalyses the reaction of:
$$\text{MetHb} + \text{Fe}^{3+} + \text{NADH} \rightarrow \text{Hb} + \text{Fe}^{2+} + \text{NAD}$$

The course of reaction catalyzed by reductase of methemoglobin:NADPH goes as follows:
$$\text{MetHb} + \text{Fe}^{3+} + \text{NADPH} \rightarrow \text{Hb} + \text{Fe}^{2+} + \text{NADP}$$

Hemoglobin, comprising about 10% of catalase activity of erythrocyte, decomposes hydrogen peroxide in the non-enzymatic reaction at the lower rate than catalase herself does [25]. Due to high content of hemoglobin, its antioxidative activity has a great impact on the regulation of erythrocyte oxidative-antioxidative processes.

Glutathione thiolic groups [26] and protein thiolic groups [27] have an important antioxidative role in the erythrocyte and comprise approximately 0.1% of total mass of these cells with reduced glutathione (GSH) of 99.8% and oxidized glutathione (GSSG) – 0.2% of its content. Oxidized glutathione also occurs in combination with thiolic groups of hemoglobin and other proteins of the erythrocyte, in the form of mixed disulphides (PSSG). GSSG and PSSG contents in erythrocytes increase in the oxidative stress [28].

Reduced glutathione decomposes hydrogen peroxide directly and in the reaction catalyzed by selenium-dependent glutathione peroxidase [26]:
$$\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}$$

GSH also participates in the reaction of decomposition of alkylohydroxides (ROOH) to alcohol (ROH) [29]:
$$\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{GSSG}$$

Moreover, GSH is coupled with hydroxyl derivatives of xenobiotics (X-OH) in the reaction catalyzed by S-transferase of glutathione [29]:
$$\text{X-OH} + \text{GSH} \rightarrow \text{X-SG} + \text{H}_2\text{O}$$

Reducetase oxidized glutathione: NADPH reduces oxidized glutathione:
$$\text{GSSG} + \text{NADPH} \rightarrow \text{GSH} + \text{NADP}$$

Ascorbate, cysteine and ergothioneine are fine particulate hydrophilic antioxidants of the erythrocyte, localized in cytosol [26]. Alphatocopherol [30] and beta-carotene [31] are fine particulate hydrophobic antioxidants, localized in the erythrocyte membrane. Ascorbate, alphatocopherol, beta-carotene and ergothionein are exogenic antioxidants.

The reaction of reactive nitrogen species and thiolic groups, with formation of S-nitrosothiol (RSNO), leads to their detoxication [32].

**Oxidative-antioxidative balance**

Reactive oxygen species and reactive nitrogen species are natural products of erythrocyte metabolism and are in a dynamic balance with enzymatic and non-enzymatic antioxidants. Inhibition of dismutase activity by hydrogen superoxide and inhibition of catalase activity by superoxide anion prove co-dependence of these systems [33].

Singlet oxygen and hydroxyl radical are the most reactive forms of oxygen while peroxinitrite – the most reactive form of nitrogen. They are characterized by low specificity and react with almost every organic molecule: they attach or...
separate an electron to or from the molecule and form an appropriate free radical.

The activity of oxidative and antioxidative factors of the blood plasma has an effect on oxidative-antioxidative balance of the erythrocytes. The activity of erythrocyte antioxidative enzymes is significantly higher than that of blood plasma (Tab. 2) [34].

**Table 2. Activity of antioxidative enzymes of erythrocytes and blood plasma [34].**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythrocytes U/10(^{10}) *</td>
<td>Plasma U/ml</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>550-800</td>
<td>5-20</td>
</tr>
<tr>
<td>Catalase</td>
<td>3800-5400</td>
<td>small quantity</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>7.8-10.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>2.7-3.7</td>
<td>0.03</td>
</tr>
<tr>
<td>Glutathione transferase</td>
<td>1.5-2.5</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* - number of erythrocytes contained in 1 ml.

The content of fine particulate antioxidants is also significantly higher in erythrocytes than in blood plasma [35]. Thus, internal surface of erythrocyte membranes is better protected by reactive oxygen species than the external surface. A significant antioxidative role is fulfilled by the binding of hemoglobin through haptoglobin [36] and ferric cations through transferrin [37].

**Erythrocyte aging, apoptosis, hemoglobin degradation**

Elimination of cells of the red-blood-cell system, at each stage of their development, occurs in the course of apoptosis [38]. Causative and executive factors as well as the range and course of apoptosis are different in different developmental phases of the erythrocyte. As far as erythroblasts are concerned, total apoptosis concerns only damaged, mutated, or infected cells. The process of apoptosis is initiated by typical apoptotic factors and performed by typical apoptosis executors leading to mitochondria, cellular nucleus and the whole cell damage. Erythropoietin protects non-damaged erythroblasts against total apoptosis [39]. There are only limited apoptotic processes in non-damaged erythroblasts which concern cellular organelles and lead to their fragmentation and decomposition. The processes of limited apoptosis are continued in reticuocytes. Total decomposition of ribosomes and mitochondria occurs and their fragments are eliminated in form of coated vesicles.

Mature erythrocytes without cellular organelles are metabolically stable. Only old and used up erythrocytes, which life span in blood reached about 120 days, undergo apoptosis and elimination from the bloodstream. In 24 hours, 0,21x10\(^{12}\) erythrocytes (0.83% of total) are eliminated from blood and about 8g of hemoglobin (1% of total) is degraded [40]. Deferoxamine protects against elimination processes [41]. In pathological conditions, e.g. in hemolytic anemia and in intoxication with certain xenobiotics, life span of erythrocytes in circulating blood is shortened and they undergo apoptosis earlier.

In aging erythrocytes, apoptosis is induced by appearance of increased amount of reactive oxygen species [42] and increased content of calcium cations [43]. The calcium cations activate procaspase-3 and procaspase-8 [2] while the procaspase-8 activate calpain I (μ-calpain) [44]. The activity of above enzymes is inhibited by specific inhibitors [45]. In aging occur changes in erythrocytes characteristic for apoptosis, such as decrease in affinity to hemoglobin, decrease in activity of enzymes of glycolytic cycle and pentose cycle, oxidation and fragmentation of proteins. Apoptotic changes in erythrocytes concern also: the chemical composition (decrease in content of glutathione and calcium cations and increase in content of methemoglobin, oxidized lipids and sodium cation), the structure of erythrocyte membrane (translocation of phosphatidylserine from the internal to the external monolayer, loss of peripheral membrane proteins, disturbance of lipid asymmetry) and morphological features (change of shape from biconcave to spherical, decrease in size through shrinking, changes of surface from smooth to that with bulge. Translocation of phosphatidylserine to the external layer of the membrane and forming bulges on its surface is the most important change [46]. Above described changes in external layer of the red cell membrane begin the process of apoptosis and enables recognition as well as binding of erythrocyte by receptors of mononuclear phagocytes of sinusoid vessels of the spleen where decomposition of apoptotic erythrocytes total occur.

In used up erythrocytes, oxidative modification of the residues of certain amino-acids takes place as well as disturbs of the spatial structure of hemoglobin and membrane proteins, which increases their susceptibility to proteases activities [47]. Oxidatively modified hemoglobin is degraded by multi-catalytic proteasomal system [48,49] and modified membrane proteins – by membrane proteases [50]. Fragmentation of hemoglobin and membrane proteins is observed, with end products of fine particulate peptides and amino-acids, resulting in erythrocyte decomposition. Heme, with its oxidative activity, is released in erythrocyte decomposition processes. Heme...
Oxidants antioxidants erythrocytes

In erythrocytes with metabolic defects, containing pathological hemoglobin, increased methemoglobin content, with deficiency of heme oxidase and enzymes reducing oxidized glutathione, deep changes in protein structure and morphological changes take place [53, 54], which is presented in Figure 3. In Figure 3 arrow number 1 indicates factors modifying HB – congenital molecular defects and those caused by reactive oxygen species; arrow number 2 – indicates factors increasing the formation of reactive oxygen species: increased oxygen supply, ionizing radiation, ultraviolet, and heat radiation, ultrasounds, xenobiotics, certain medicines; arrow number 3 indicates factors decreasing reactive oxygen species decomposition: enzymes deficiency (glucose-6-phosphate dehydrogenase, glutathione peroxidase, glutathione reductase and synthetase, catalase, superoxide dismutase), fine particulate compound deficiency (alpha-tocopherol, ascorbate, beta-carotene, glutathione). The oxidation of thiolic groups of hemoglobin leads to the formation of disulphuric bindings inside subunits and between subunits of hemoglobin, which leads to the formation of intramolecular cross-binding and intermolecular bindings as well as the formation of insoluble aggregates [55]. Proteins modified in above described way lose their ability to solubility and are resistant to protease activity [56]. Protein losses and bodies composed of denaturated hemoglobin and proteins of erythrocyte stroma, called Heinz bodies, occur [57, 58]. On the other hand, uncontrolled oxidative and proteolytic fragmentation of erythrocyte membrane proteins leads to their hemolysis [9].

Erythrocytes in blood extravasated to tissues also undergo apoptosis. The inflammatory process and necrosis appear only when apoptotic changes are accompanied by bacterial infection and there occurs leukocyte infiltration.

Figure 3. Oxidative-antioxidative system of erythrocyte, connections with glycolytic cycle and pentose cycle, regulation and its disturbances leading to heine bodies formation and hemolysis according to [26]. Factors: 1- modifying HB, 2- increasing formation of reactive oxygen species, 3- decreasing reactive oxygen species decomposition were mentioned in the text.
Methodological points

Methods of identification and quantitative determination of reactive forms of oxygen and nitrogen, activity of enzymes participating in their formation, contents of products of activities of reactive oxygen species and reactive nitrogen species to lipids, proteins and nucleic acids as well as activity of antioxidative enzymes and concentration of non-enzymatic antioxidants are described by many authors [59, 60].

Determination of activity of most components in erythrocytes does not require modification although hemoglobin can interfere with determination of certain components. Superoxidative dismutase can be determined only after prior removal of hemoglobin using the mixture of chloroform and ethanol [61]. Thiolic compounds are determined after the reaction with 4,4′-ditiopiprydynyde and absorbance can be measured at 324 nm, which makes the result hemoglobin-independent [62]. Cysteine is determined using ninhydrinic agent in strongly acidic environment [63]. Ergothioneine is determined using spectrophotometric method, after the reaction with disulfide 2,2′-dipyrydole [64].

The evaluation of components of the erythrocyte oxidative-antioxidative system should be performed in the most recently collected blood samples and isolated erythrocytes.

The results are counted per 1ml of packed erythrocytes, containing 10¹⁰ of these cells. Erythrocyte life span, osmotic resistance, ability to change size, shape, stainiability and occurrence of intraerythrocyte aposomes are used to evaluate the effects of activity of reactive oxygen species to erythrocytes [65,66].

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Conflicts of interest

Actual or potential conflicts of interest do not exist.

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