

# Safety of UVA–Riboflavin Cross-Linking of the Cornea

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**Purpose:** To study potential damage to ocular tissue during corneal collagen cross-linking (X-linking) by means of the riboflavin/UVA (370 nm) approach.

**Methods:** Comparison of the currently used technique with officially accepted guidelines regarding direct UV damage and the damage created by the induced free radicals (photochemical damage).

**Results:** The currently used UVA radiant exposure of 5.4 mJ/cm<sup>2</sup> and the corresponding irradiance of 3 mW/cm<sup>2</sup> is below the known damage thresholds of UVA for the corneal endothelium, lens, and retina. Regarding the photochemical damage caused by the free radicals, the damage thresholds for keratocytes and endothelial cells are 0.45 and 0.35 mW/cm<sup>2</sup>, respectively. In a 400- $\mu$ m-thick cornea saturated with riboflavin, the irradiance at the endothelial level was 0.18 mW/cm<sup>2</sup>, which is a factor of 2 smaller than the damage threshold.

**Conclusions:** After corneal X-linking, the stroma is depopulated of keratocytes  $\sim$ 300  $\mu$ m deep. Repopulation of this area takes up to 6 months. As long as the cornea treated has a minimum thickness of 400  $\mu$ m (as recommended), the corneal endothelium will not experience damage, nor will deeper structures such as lens and retina. The light source should provide a homogenous irradiance, avoiding hot spots.

**Key Words:** cross-linking, keratoconus, UV, cornea, keratectasia, damage, collagen

(*Cornea* 2007;26:385–389)

Cross-linking (X-linking) of the cornea is a new approach to increase the mechanical and biochemical stability of the stromal tissue.<sup>1–4</sup> Thus far, the clinical indication for X-linking is limited to melting processes of the cornea and corneal thinning disorders such as keratoconus, pellucid marginal degeneration, and iatrogenic keratectasia after laser in situ keratomileusis (LASIK).<sup>5–7</sup> The aim of this treatment is to

create additional chemical bonds inside the corneal stroma by means of a photopolymerization in the anterior stroma while minimizing exposure to the surrounding structures of the eye.<sup>8</sup> Because the UV light causes an effect only where it is absorbed, it is desirable that the treatment be designed so that as much as possible of the irradiation is absorbed in the corneal stroma tissue. This is achieved by the selection of the wavelength of the used UV light at 370 nm, a wavelength that corresponds to one of the absorption maxima of the riboflavin chromophore (Fig. 1). Riboflavin acts as a photomediator, creating free radicals to induce new chemical bonds. The photochemical process is schematically depicted in Figure 2.

In this study, the physical and clinical treatment parameters of the currently clinically applied X-linking procedure were compared with accepted safety guidelines. Special attention must be given to the 2 different potential damage mechanisms: the UV irradiation alone and the action of the photochemically induced free radicals (photochemical damage).

## UVA–RIBOFLAVIN X-LINKING PROCEDURE

After an abrasion of the cornea epithelium of 9-mm diameter, drops of a 0.1% riboflavin solution in 20% dextran are instilled onto the cornea every 3 minutes for 30 minutes. By means of a slit-lamp inspection using blue light, the surgeon has to assure that riboflavin has appeared in the anterior chamber before the UV irradiation is started. The cornea is exposed to UV light with a wavelength of  $370 \pm 5$  nm and an irradiance of 3 mW/cm<sup>2</sup> for a total time of 30 minutes; this corresponds to a total dose of 3.4 J or a total radiant exposure of 5.4 J/cm<sup>2</sup> to the cornea. The cropped light beam has a diameter of 9 mm. During the irradiation time, the cornea is rinsed with riboflavin/dextran solution and topical anesthetic every 5 minutes. After the treatment, antibiotic ointment (ofloxacin) and a bandage lens soaked with 0.3% ofloxacin are applied until the epithelium is healed.

## RIBOFLAVIN DIFFUSION INTO THE EYE

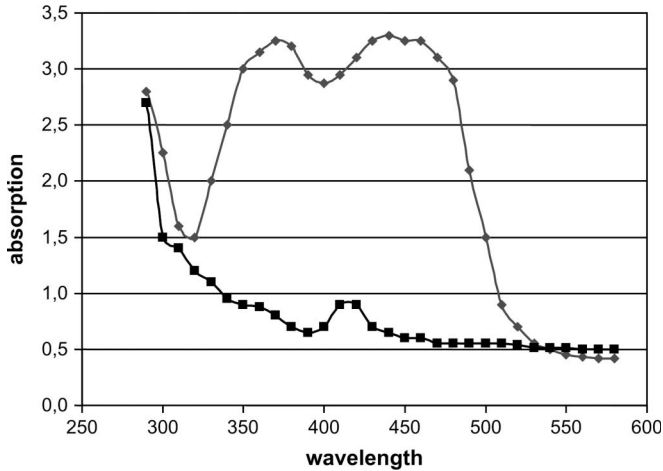
Both the time course of the diffusion process and the concentration of the superficially applied riboflavin solution are relevant for the absorption of the riboflavin into the cornea. Applied riboflavin must diffuse into the cornea stroma, and this process requires a certain time. The intact epithelium is a barrier that slows the absorption of riboflavin (molecular weight, 376.37 g/mol) into the cornea so it penetrates slowly and incompletely. For that reason, the epithelium should be debrided within the intended treatment area because this

Received for publication April 27, 2006; revision received October 22, 2006; accepted December 25, 2006.

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**FIGURE 1.** Absorption spectrum of the porcine cornea with and without riboflavin. Three absorption maxima could be selected, but the one <300 nm is not acceptable because of potential DNA damages, and the maximum at ~450 nm may be dangerous because of the blue light hazard to the retina.

simple procedure removes a diffusion barrier for the riboflavin molecule and speeds saturation of the corneal stromal tissue.<sup>9</sup>

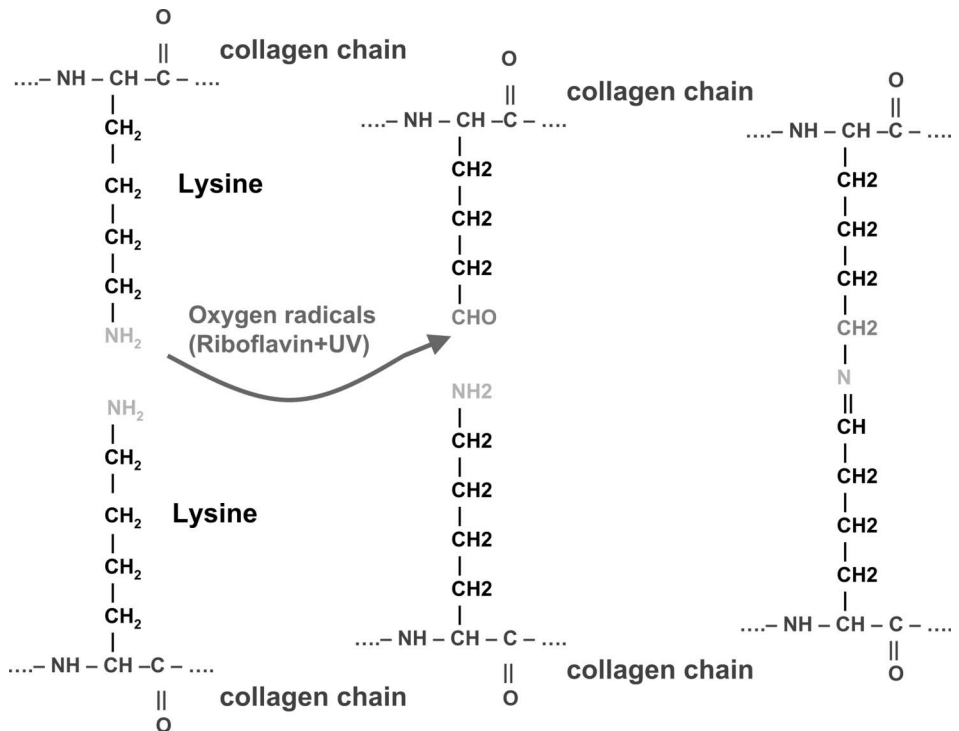
The diffusion process of 0.1% riboflavin in the stroma can theoretically be described by the time-dependent one-dimensional diffusion equation,<sup>10</sup> assuming a diffusion coefficient of  $D = 6.5 \times 10^7 \text{ cm}^2/\text{s}$ . Because unpolar substances with the same molecular weight have the same diffusion coefficient, the known diffusion coefficient for sodium fluorescein (molecular weight, 376 g/mol) was used. The concentration of the riboflavin can be calculated for each stromal depth because it depends on elapsed

time (Fig. 3). It is obvious that the riboflavin needs a certain time until it has reached the posterior cornea. The intrastromal riboflavin distribution is shown in Figure 4 as it varies with depth in the cornea and time. Even after 30 minutes, the concentration gradient through the cornea gets still flatter with time; however, the concentration at the endothelium level exceeds 0.04%.

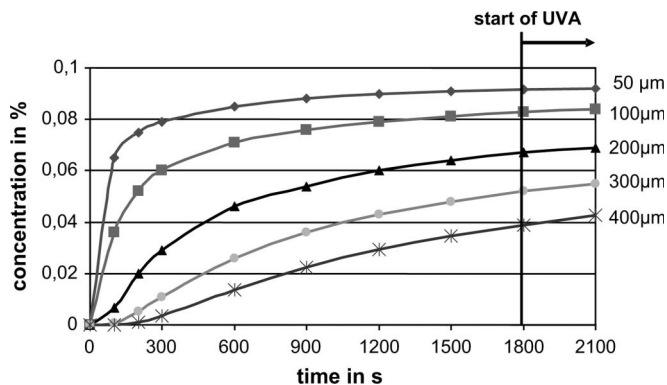
After the riboflavin has traversed the cornea, it enters the anterior chamber. Whether the endothelium represents a diffusion barrier is unknown; however, most probably it does not because the endothelium contains gap junctions that permit good penetration of most drugs.<sup>11</sup> The aqueous humor without riboflavin does not have any relevant absorption at 370 nm, but clinically it starts to stain after ~5 minutes of surface exposure to riboflavin. In a rabbit study, this was further examined where aqueous humor was sampled, and the absorption coefficient was measured. Thirty minutes after riboflavin application onto the deepithelialized cornea, an absorption coefficient of  $0.7 \text{ cm}^{-1}$  could be measured, corresponding to a concentration of 0.002% riboflavin in the aqueous humor (unpublished results). This leads to a further reduction of the UV light by 20%, considering an anterior chamber depth of 3 mm and the measured absorption coefficient of  $0.7 \text{ cm}^{-1}$ . Because of the low riboflavin concentration, the shielding effect caused by riboflavin in the anterior chamber is not significant. The yellow staining of the anterior chamber serves more as a safety feature, indicating that the riboflavin has penetrated the cornea and the cornea is saturated thoroughly.

**DIRECT UV DAMAGE**

The damage mechanism from the UV light depends on its wavelength, its irradiance, and the irradiation time.

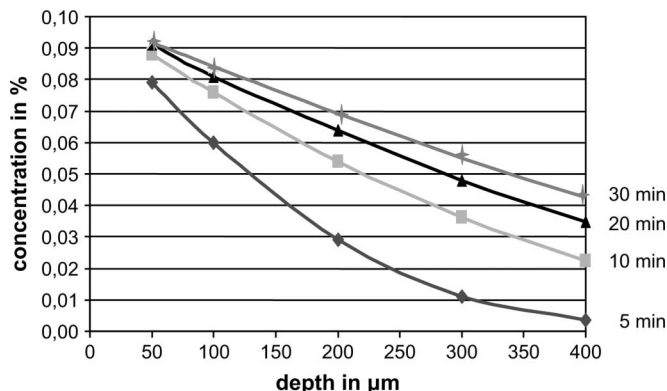


**FIGURE 2.** Schematic of the photochemical reaction of cross-linking of collagen caused by production of oxygen radicals by riboflavin and UVA light inducing a change at the end of an amino group. Afterward, these reactive groups can form new covalent bonds.



**FIGURE 3.** Theoretical modeling of the diffusion dynamics of riboflavin in the corneal stroma. From the diffusion constant of fluorescein in corneal stroma, the local concentration is determined as a function of time. After approximately 30 minutes, a concentration of 0.04% is achieved 400 μm deep in the stroma.

Photokeratitis<sup>12</sup> has been shown to occur in the cornea at wavelengths of 270 to 315 nm (UVB), with dose densities ranging from 0.12 to 0.56 J/cm<sup>2</sup>. For the development of cataract, various dose values were presented for the wavelengths between 290 and 365 nm in the literature.<sup>13–15</sup> The retina is damaged by thermal or blue light–induced photochemical damage in the wavelength range of 400–1400 nm.<sup>16</sup> In short UV treatment times up to 120 minutes, the photochemical damage is reduced by antioxidative protective mechanisms in the lens.<sup>17</sup> Under these circumstances, a balance between the cell damage, the biologic protective mechanisms, and the biologic repair mechanisms is achieved.<sup>18,19</sup> Without the presence of a photosensitizer, light at wavelengths 350 nm and higher and an irradiance of 3 mW/cm<sup>2</sup> will not cause damage to the endothelium.<sup>20</sup> In rabbits, the threshold radiant exposure for damage has been shown to be 70 J/cm<sup>2</sup> for the lens and 42 J/cm<sup>2</sup> for the cornea, and the



**FIGURE 4.** Riboflavin concentration gradients in the corneal stroma at different times after riboflavin application at the surface. The diffusion process of 0.1% riboflavin in the cornea can theoretically be described by the time-dependent 1-dimensional diffusion equation,<sup>10</sup> assuming a diffusion coefficient of  $D = 6.5 \times 10^7$  cm<sup>2</sup>/s.

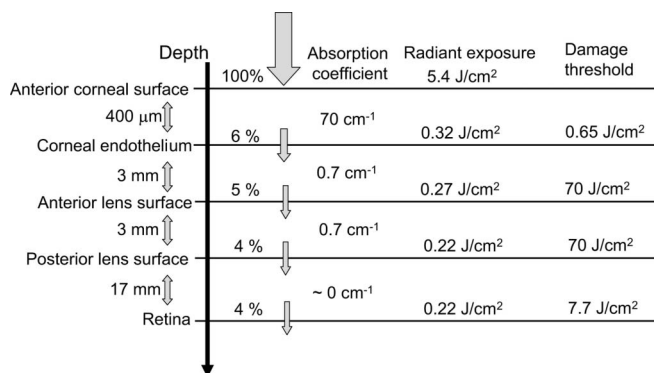
threshold irradiance for retinal damage was 4.3 mW/cm<sup>2</sup>.<sup>21,22</sup> Comparing these thresholds with the UV irradiance and dose used during the X-linking procedure (3 mW/cm<sup>2</sup>, 5.4 J/cm<sup>2</sup>), we would not expect any damage to the corneal endothelium, the lens, or the retina (even in the absence of riboflavin) except photokeratitis, which is not relevant because the corneal epithelium is removed anyway.

Recently, the most restrictive threshold was recommended by the “Guidelines on Limits of Exposure to Ultraviolet Radiation of Wavelengths Between 180 nm and 400 nm”: For longer UV irradiation times, the limiting radiant exposure of 1 J/cm<sup>2</sup> should not be exceeded.<sup>23</sup> This level is recommended for occupational and chronic exposures and is considerably lower than the damage thresholds that we have discussed. The radiant exposure of 5.4 J/cm<sup>2</sup> applied during the X-linking clearly exceeds this threshold; however, when taking the riboflavin into account (Fig. 5), this guideline is met regarding the corneal endothelium and deeper structures.

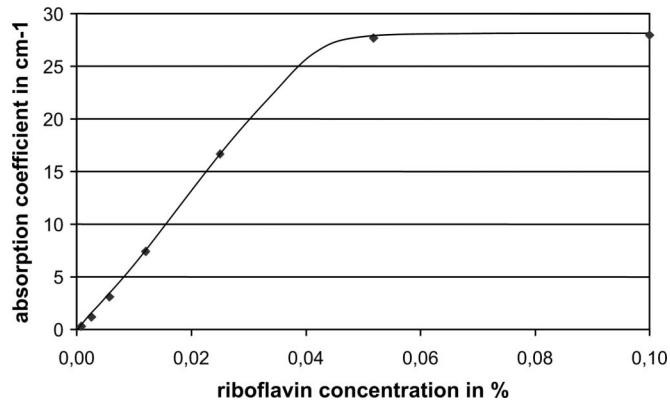
**RIBOFLAVIN SHIELDING**

The absorption coefficient of riboflavin solutions increases linearly up to a concentration of 0.04% and remains constant at 28 cm<sup>-1</sup> for concentrations between 0.05% and 0.1% (Fig. 6). Thirty minutes after application of riboflavin, the concentration of riboflavin exceeds 0.04% at any level up to 400 μm deep (Fig. 4). Lambert–Beer law yields a reduction of the irradiance caused by absorption in a 400-μm-thick layer, with a riboflavin distribution (Fig. 4; 30-minute curve) by a factor of 5.5. Therefore, because of the additional riboflavin shielding, all structures behind the corneal stroma, including corneal endothelium, anterior chamber, iris, lens and retina are exposed to a residual UV radiant exposure that is less than 1 J/cm<sup>2</sup>. This value is recommended as a safety threshold by the guidelines mentioned above.

To confirm these calculations, we measured the absorption coefficient of rabbit, porcine, and human corneas saturated with a 0.1% riboflavin concentration (Table 1). The riboflavin imbibed in the corneal stroma enhances the absorption coefficient by a factor of ~5, which limits the UV irradiance through a 400-μm-thick stroma to 0.18 mW/cm<sup>2</sup> at the endothelial level.



**FIGURE 5.** Radiant exposures, transmission, and damage thresholds for different ocular media for a human eye after 30 minutes of riboflavin application.



**FIGURE 6.** Measured nonlinear absorption of riboflavin solution in water. In solutions with concentrations of  $>0.04\%$ , the absorption does not increase more.

### PHOTOCHEMICAL DAMAGE TO CORNEAL CELLS

The photopolymerization process inducing additional X-links in the corneal stroma is believed to be carried out by free radicals mediated by the riboflavin irradiated with UV light (Fig. 2). Such radicals can create cell damage that may be accepted at the keratocytes but not at the corneal endothelium. The cytotoxicity of the riboflavin–UVA treatment on keratocytes and endothelium cells was studied by Wollensak et al.<sup>24–26</sup>

In rabbit corneas 24 hours after standard riboflavin/UVA treatment, keratocyte apoptosis was found 300  $\mu\text{m}$  deep. Smaller irradiances led to shallower cell depth, following Lambert–Beer law.<sup>24</sup> In cell cultures established from porcine keratocytes, the damage threshold of the irradiance of UVA in combination with 0.025% riboflavin solution was determined to be 0.45  $\text{mW}/\text{cm}^2$ , which is 10 times lower than UVA alone.<sup>25</sup> According to Lambert–Beer law, this irradiance is achieved after passing through  $\sim 300$   $\mu\text{m}$  of stroma saturated with riboflavin. In agreement with these experimental data, confocal microscopy of 10 clinically X-linked corneas revealed cell death of keratocytes 270–350  $\mu\text{m}$  deep in the stroma, but 6 months after X-linking, a repopulation of the whole stroma with a normal keratocyte density had taken place.<sup>27,28</sup> No damage was detected at the corneal endothelium at any time after treatment.

An experimental setup similar to that with the keratocytes was used to measure the damage threshold for porcine endothelial cells.<sup>26</sup> At an irradiance of 0.3  $\text{mW}/\text{cm}^2$ , no signs of cell damage were detected, whereas at 0.35  $\text{mW}/\text{cm}^2$ , 98% of the cells stained positively for both trypan blue and

Yopro in their nuclei. This threshold for photochemical damage is approximately a factor of 2 higher than the 0.18  $\text{mW}/\text{cm}^2$  that occurs at the cornea endothelium level during standard X-linking of a cornea with a thickness of 400  $\mu\text{m}$ .

### DISCUSSION

UV light can damage the cornea, the lens, and the retina. The UV exposure can induce a photokeratitis at the cornea or a cataract in the lens, and thermal or photochemical damage can occur in the retina.<sup>29</sup> Furthermore, the free radicals and oxidizing agents liberated during the exposure of riboflavin-soaked corneal tissues also pose a photochemical risk to anterior-segment structures. An overview of the applied radiant exposures and the damage thresholds for different ocular media is shown in Figure 5.

Considerable clinical benefit can accrue from the X-linking treatment. X-linking may stabilize or even improve corneal disorders that significantly impair visual function.<sup>30</sup> In many cases, corneal transplantation is anticipated as the only alternative to riboflavin–UV X-linking. Early clinical results of X-linking in keratoconus eyes showed a halt in progression up to 3–5 years after the treatment.<sup>4,31</sup> Another indication is corneal melting, where the X-linked corneal collagen seems to be more resistant to proteinases.<sup>6</sup> This effect has also been shown experimentally.<sup>4</sup>

The cell population for which suffering damage either from UV light directly or from the free radicals is most critical is the corneal endothelium because it is immediately adjacent to the corneal stroma, and these cells in the normal human cornea have low regenerative capacity.<sup>11</sup> Any damage to these cells would endanger the procedure of corneal X-linking with the parameters used today. Wollensak et al<sup>26</sup> determined the damage threshold of the combination of UV and riboflavin to be 10 times lower than UV alone (0.35 vs. 4  $\text{mW}/\text{cm}^2$ ). However, because of the riboflavin shielding effect, the clinically applied UV irradiance at the endothelium level is at least a factor of 2 smaller than the damage threshold. These considerations thus far are from experimental and animal models, and there might be differences in the living human eye. On the other hand, Caporossi et al<sup>27</sup> and Mazzotta et al<sup>28</sup> were not able to detect any pathologic signs of endothelial damage by means of confocal microscopy in few keratoconus eyes treated with X-linking.

Keratocytes are exposed to free radicals and to UV light during the treatment. In animal experiments<sup>24</sup> and clinically,<sup>27,28</sup> it has been shown that keratocytes show cell death up to 350  $\mu\text{m}$  deep. After 6 months, this area was repopulated; in contrast to corneal endothelium cells, the keratocytes can reproduce. Such a transient cell loss in the corneal stroma is not considered critical and is reported after photorefractive keratectomy (PRK),<sup>32</sup> LASIK,<sup>33</sup> and corneal abrasion.<sup>34</sup> However, Erie et al<sup>35</sup> showed recently that even uneventful PRK and LASIK results in a significant loss of keratocytes. Long-term confocal microscopy studies are recommended to verify the repopulation of keratocytes, especially in cases of cross-linking, because of iatrogenic keratectasia after LASIK. In an experimental setup similar to that of endothelium cells, the damage threshold cell of porcine keratocytes was

**TABLE 1.** Absorption Coefficients of Different Corneas (unpublished data)

Cornea Type	Absorption Coefficient/ $\text{cm}^{-1}$	
	Without Riboflavin	With Riboflavin 0.1%
Porcine	13.6	59
Rabbit	13.8	63
Human	14	70

determined<sup>25</sup> to be  $\sim 0.5$  mW/cm<sup>2</sup> for the combination of UV and riboflavin compared with 5 mW/cm<sup>2</sup> for UV light alone. With the clinical irradiation parameters, this threshold is reached at a depth of  $\sim 300$   $\mu$ m, which is in good agreement with the damage shown by confocal microscopy.

All the above safety considerations are from an irradiance that is homogenous within the field of UVA application. However, if hot spots are present, the damage thresholds may be exceeded locally, leading to localized endothelial damage, although the average irradiance may be  $< 3$  mW/cm<sup>2</sup>. Therefore, clinically used light sources must guarantee a perfect homogeneity of the irradiance across the beam area.

Although only  $< 10\%$  of the UV light can penetrate the cornea and may interact with the aqueous that contains a small concentration of riboflavin, there is a minimal possibility to create free radicals in the anterior chamber. On the other hand, the aqueous contains a high concentration of ascorbate<sup>36</sup> that is believed to act as radical scavenger.

In summary, safe clinical application of X-linking must respect the following criteria: (1) to facilitate diffusion of riboflavin throughout the corneal stroma, the epithelium should be removed; (2) a 0.1% riboflavin solution should be applied for at least 30 minutes before the UV exposure (during the UV exposure, the riboflavin serves as both a photosensitizer and a UV blocker); (3) the UV irradiance of 3 mW/cm<sup>2</sup> and a wavelength of 370 nm must be homogenous; and (4) the cornea to be X-linked must have a minimal thickness of 400  $\mu$ m to protect the endothelium. Damage to the endothelium, the lens or the retina is not expected when these criteria are fulfilled.

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