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Melatonin: Free Radicals and Metabolites Resulting by Emission and Consumption of Solvated Electrons (e_{aq}^-): Reaction Mechanisms

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Abstract. *Background/Aim. Melatonin not only regulates circadian rhythm, but also induces apoptosis in tumor cells. Hence, elucidation of the basic reaction mechanisms of melatonin and its metabolites is a matter of interest. Material and Methods. Melatonin dissolved in a mixture of water/ethanol=40/60 form associates (unstable complexes). For simulation of biological processes, melatonin was excited by UV light into the singlet state. Results. By using monochromatic UV light (λ=254 nm) melatonin ejects solvated electrons (e_{aq}^-), a part of which is scavenged by melatonin in ground state contained in the associates. Consequently, with increase of melatonin concentration a decrease of the determined quantum yield of emitted e_{aq}^-, Q(e_{aq}^-), is obtained. The complex molecular structure of melatonin contains functional groups which can emit e_{aq}^-, as well such consuming e_{aq}^-. As a succession of these processes various types of metabolites are generated, as well as degradation products, with lower molecular weight, are formed. Conclusion. Not melatonin per se, but the ejected e_{aq}^- and thereby resulting various metabolites are responsible for different biological properties of melatonin.*

Melatonin (*N-acetyl-5-methoxytryptamine*) is a hormone secreted by the pineal gland of vertebrates (1), as well as in the retina and the brain (2). Besides its effect in regulating the circadian rhythm, it also possesses various biological properties. Melatonin induces apoptosis of tumor cells (3), as well as inhibits the growth of human uveal melanoma cells (4-7). Melatonin and its metabolites also act as successful scavengers of free radicals (8, 9). Finally, it should be mentioned that the spectroscopic and kinetic characteristics of melatonin transients have been studied by pulse radiolysis (10, 11). The rate constant of the reaction of melatonin with e_{aq}^- has been determined to be k= 4.2×10^8 l/mol/s and of that with OH radicals of k=1.3×10^{10} l/mol/s (10).

The purpose of the present investigation concerns: (i) the emission of solvated electrons (e_{aq}^-) from melatonin dissolved in 40/60 water/ethanol when excited into the singlet sate; (ii) the effect of substrate concentration on the quantum yield of emitted e_{aq}^- [Q (e_{aq}^-)]; (iii) determination of melatonin metabolites arising as a consequence of e_{aq}^- emission; (iv) possible reaction mechanisms based on the obtained results.

Materials and Methods

Melatonin of the highest purity available (>98%) was purchased from Sigma Aldrich (Vienna, Austria) and used as obtained. Melatonin is essentially not soluble in water, hence a solvent mixture of triple-distilled water/ethanol (40/60) was used. In order to avoid oxidation of substrate during salvation, the solvent mixture was first saturated with highest-purity argon in an irradiation vessel for 20 min followed by adding the substrate under intense stirring. A 4π-geometry double-wall irradiation vessel in combination with a low-pressure mercury lamp (HNS 12, OSRAM, 12 W; Osram GmbH, Vienna, Austria) with incorporated VYCOR filter to remove the line at 185 nm was used for irradiation (12). The intensity of the monochromatic UV-light (λ=254 nm; 4.85 eV/hv; 1 eV=23 kcal/mol) was determined by monochloric acid actinometer (13).

Samples containing different melatonin concentrations and treated with different UV doses (hv/l) were analyzed immediately after irradiation. The e_{aq}^- ejected by melatonin at given UV dosage and melatonin concentrations were scavenged by chloroethanol (1×10^{-2} mol/l) and the resulting chloride ions were determined spectrophotometrically (14):

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$e_{aq}^- + ClC_2H_4OH \rightarrow Cl^- + {}^{\bullet}C_2H_4OH$ Eq.1
$k=6.9\times10^9$ l/mol/s

$Q(Cl^-) = Q(e_{aq}^-)$ Eq.2

The spectrophotometric measurements were performed with a double-beam spectrophotometer.
The metabolites resulting from the e_{aq}^- emission by melatonin at different concentrations were analyzed using liquid chromatography/mass spectroscopy (LC/MS). The handling of the samples and LC/MS analysis are described elsewhere (15).

Results

Emission of e_{aq}^- from melatonin. The emission of solvated electrons, e_{aq}^-, from melatonin dissolved in air-free 40/60=water/ethanol mixture and photo-excited in the singlet state (UV: λ=254 nm) was studied for three melatonin concentrations (1×10^{-5}, 5×10^{-5} and 1×10^{-4} mol/l). Firstly, the formation of unstable melatonin complexes similar to other hormones (15, 16) was examined. Hence, the molar extinction coefficient, ε254 nm (l/mol/cm) depends on the melatonin concentration (Figure 1). This indicates that some of the emitted e_{aq}^- are scavenged by melatonin molecules in the ground state, contained in the complexes, where k (melatonin + e_{aq}^-)=4.2×10^8 l/mol/s (10). Therefore, the proportion of e_{aq}^- scavenged by melatonin in complexes rises with increasing melatonin concentration, leading to a decrease of the corresponding e_{aq}^- quantum yield, Q (e_{aq}^-), and the formation of free radicals. Photodegradation of substrate progresses simultaneously to the formation of free radicals (Figure 1).

Studying the emission of e_{aq}^- from 1×10^{-5} mol/L melatonin as a function of absorbed UV dose, two peaks were observed (Figure 2), similar to the findings for other hormones (15, 16). This effect can be attributed to the fact that the primary metabolites are excited in the solution with higher concentration and are able to eject e_{aq}^-, but with much lower Q (e_{aq}^-) (Figure 2). Simultaneously, the pH of the irradiated media decreased significantly, which indicates that the e_{aq}^- emission is related to H^+ ion formation. Thereby some of the ejected e_{aq}^- are scavenged by the H^+ ions leading to Q (e_{aq}^-) reduction:

$e_{aq}^- + H^+ \rightarrow H$; $k=2.3\times20^{10}$ l/mol/s (17) Eq.3

A similar course of the e_{aq}^- emission from 5×10^{-5} mol/l melatonin was observed depending on the absorbed UV dose, depicted in Figure 3. Here, only a slight pH-decrease with the increase of the absorbed UV dose was registered, indicating the role of the process expressed by Eq.3. Moreover the determined Q (e_{aq}^-) values for peaks A and B are one order of magnitude lower compared to those of 1×10^{-5} mol/l melatonin (Figure 2). This observation

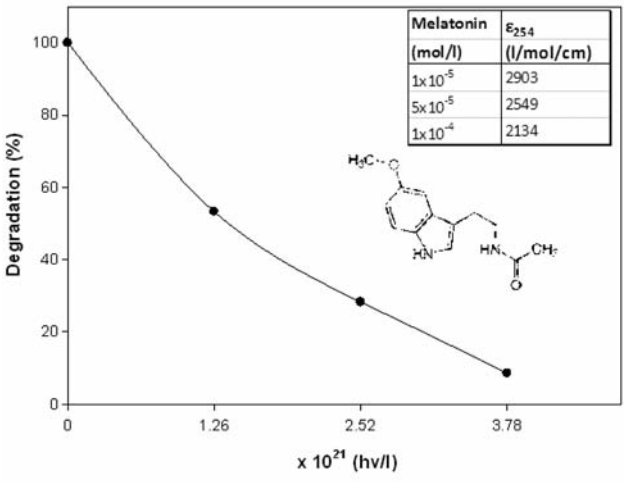
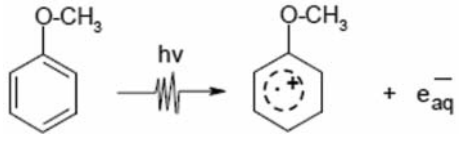


Figure 1. *Photo-induced (UV: λ=254 nm) degradation of 5×10^{-5} mol/l melatonin as a function of absorbed UV-dose (hv/l). Inset: Molar extinction coefficient, ε_{254nm} (l/mol/cm) for three melatonin concentrations. Structure formula of melatonin.*

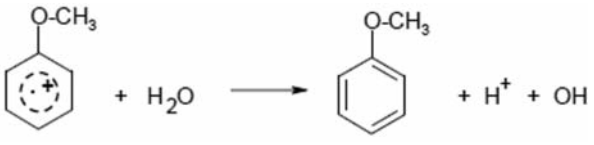
underlines once more the scavenging effect of complexes and H^+ ions on the e_{aq}^-.

Using 1×10^{-4} mol/l melatonin, a further decrease of Q(e_{aq}^-) for peaks A and B was ascertained (Figure 4) but the pH of the irradiated solution hardly decreased. This indicates that associates were formed and that Eq.1 is valid.

Sites of the melatonin molecule ejecting and consuming e_{aq}^-. The structure of melatonin molecule is complex (Figure 1). There are spots, which emit e_{aq}^- and there are other sites which act as scavengers for e_{aq}^-. Considering the structural formula of melatonin, it is clear that the first ring corresponds to anisole. On the other hand, it has been previously reported (18) that UV excitation in of anisole in the S1 state in aqueous solution gave Q (e_{aq}^-)=0.025 under formation of a radical cation.



Eq.4



Eq.5

melatonin + OH → degradation of melatonin Eq.6

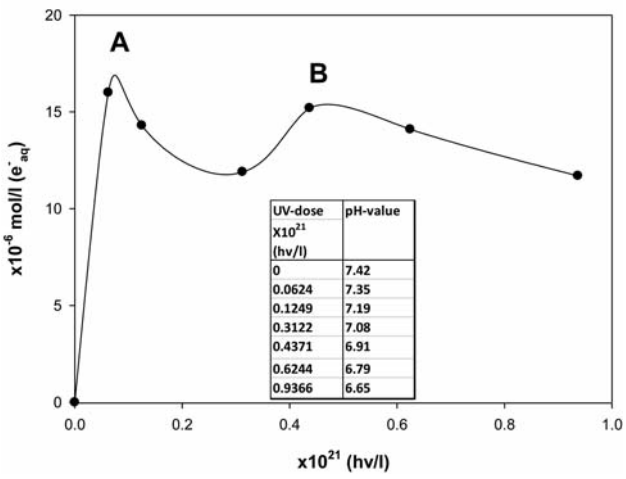


Figure 2. *Emission of e_{aq}^- (mol/l) from 1×10^{-5} mol/l melatonin in 40/60=water/ethanol at 37°C as a function of absorbed UV-dose (hv/l) of monochromatic UV-light (λ=254 nm). Q(e_{aq}^-)-yields at peak A and B: 0.1543 and 0.0210 respectively. Inset: pH-change in dependence of absorbed UV-dose.*

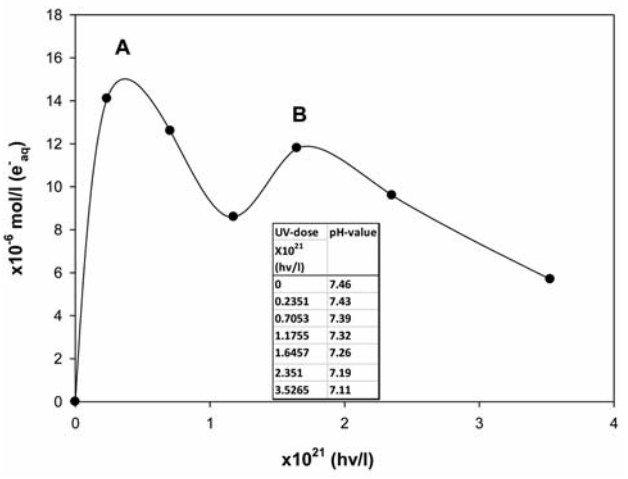


Figure 3. *Emission of e_{aq}^- (mol/l) from 5×10^{-5} mol/l melatonin in 40/60=water/ethanol at 37°C as a function of absorbed UV-dose (hv/l) of monochromatic UV-light (λ=254 nm). Q(e_{aq}^-)-yields at peak A and B: 0.0241 and 0.0047 respectively. Inset: pH-change in dependence of absorbed UV-dose.*

Further, it has been previously found (18) that the imino-groups of the pyrol ring as well as of the side chain can act as e_{aq}^- emission spots also, where on the other hand carbonyl group scavenges e_{aq}^- [$k=1.7\times10^9$ l/mol/s] (17)]. Based on these facts a simplified scheme of the competing processes is presented in Figure 5.

$$R1 + H_2O \rightarrow \text{melatonin} + H^+ + OH \qquad \text{Eq.7}$$

$$\text{melatonin} + e_{aq}^- \rightarrow \text{free radicals;}\quad k=4.2\times10^8 \text{ l/mol/sec (10)} \qquad \text{Eq.8}$$

$$CO + e_{aq}^- \rightarrow CO^{\bullet-} ;\ k=1.7\times10^9 \text{ l/mol/sec (17)} \qquad \text{Eq.9}$$

The resulting free radicals lead to the formation of various metabolites dependent on the given medium conditions.

Metabolite analysis. The degradation of 5×10^{-5} mol/L melatonin as a function of absorbed UV-dose is illustrated in Figure 1. As expected the number of metabolites and degradation products increases with increasing absorbed UV dose. This also indicates that, by the emission of e_{aq}^-, the melatonin free radicals expected to be formed (Figure 5) undergo reactions with each other, resulting in various metabolites. Simultaneously with these reactions, some of the primary-formed melatonin metabolites are decomposed by emitting e_{aq}^- (Figures 2-4), leading to the formation of secondary metabolites and to numerous low molecular weight degradation products. All these processes are superimposed

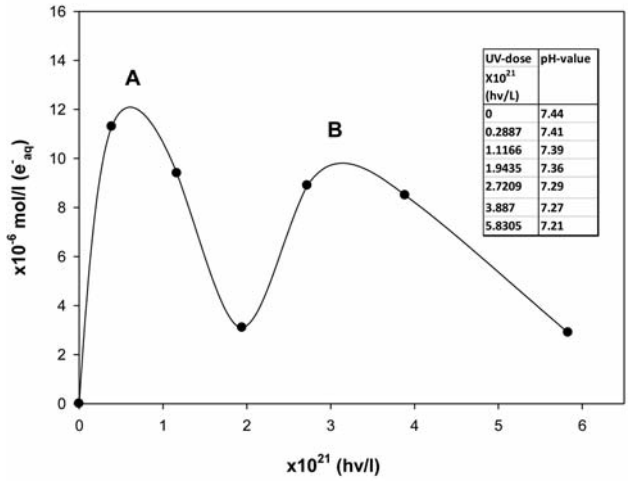


Figure 4. *Emission of e_{aq}^- (mol/l) from 5×10^{-4} mol/l melatonin in 40/60=water/ethanol at 37°C as a function of absorbed UV-dose (hv/l) of monochromatic UV-light (λ=254 nm). Q(e_{aq}^-)-yields at peak A and B: 0.0113 and 0.0021 respectively. Inset: pH-change in dependence of absorbed UV-dose.*

and depend on the specific concentrations of the reaction partners and the corresponding reaction rate constants. In other words, the increase of the absorbed UV dose results in propagation of the number of final products with different molecular weight. The structure formulas of main metabolites formed by using 5×10^{-5} mol/l melatonin with various UV

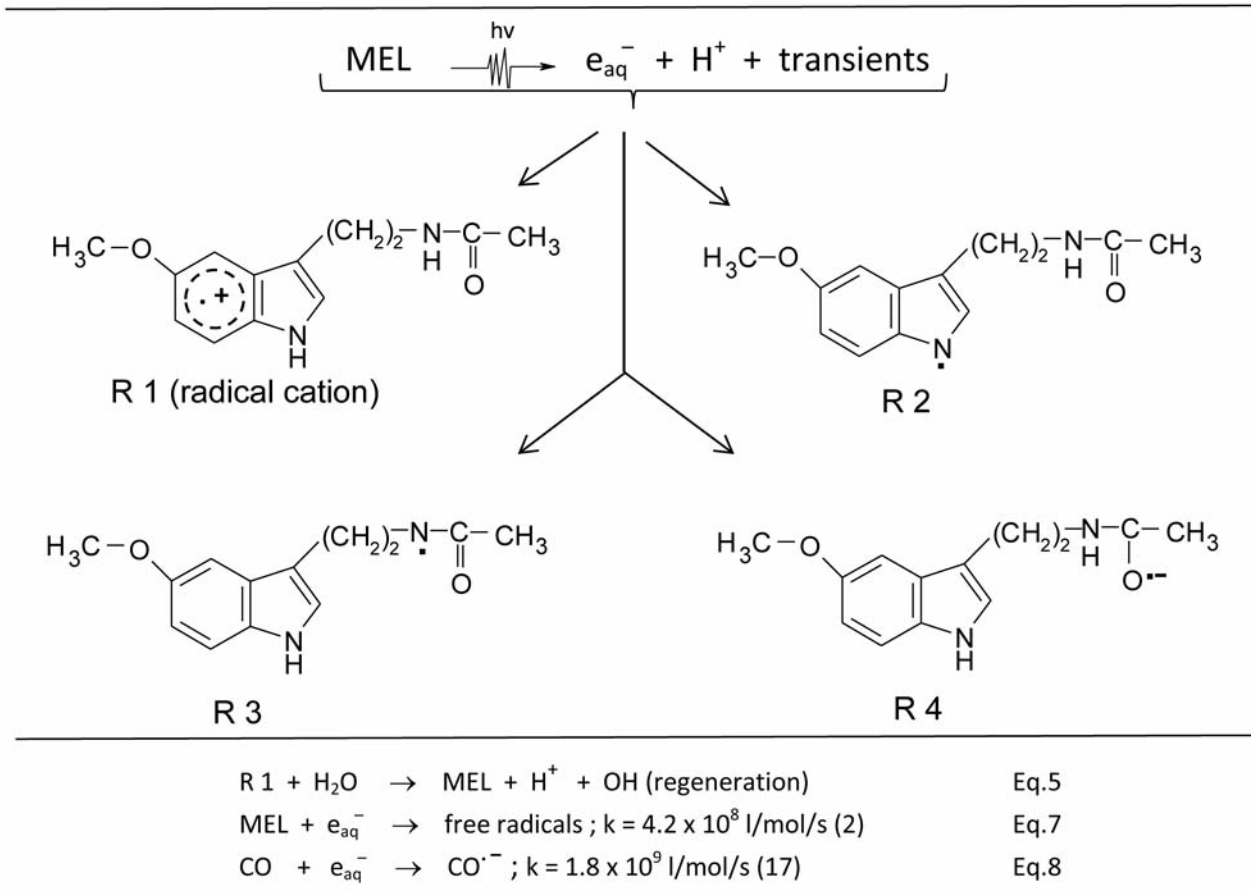


Figure 5. *Simplified scheme of the e_{aq}^- -emitting positions of melatonin and resulting transients R1 – R3 as well as R4 formed as e-aq-scavenging position.*

doses was determined (Figure 6). In addition to these, dimers with molecular weight of 437 and 473, as well as degradation products with lower molecular weights 32.02, 58.03, 175.0 *etc*. were also observed. This again demonstrates the complexity of the involved reaction mechanisms.

Conclusion

In summary, melatonin emits solvated electrons (e_{aq}^-), when excited into singlet state in polar media. As a sequence of this process free radicals are formed, which subsequently result into different metabolites and low-molecular degradation products. The findings also suggest that not melatonin *per se*, but the ejected e_{aq}^- and thereby resulting distingt metabolites, are determining factors for the different biological properties of melatonin.

References

1 Arendt J: Melatonin and the Mammalian Pineal Gland. Chapman & Hall, London, GB, 1995.

2 Roberts JE: Visible light induced changes in the immune response through an eye-brain mechanism (Photoneuroimmunology). J Photochem Photobiol B Biol *29*: 3-15, 1975.

3 Gonzalez R, Sanchez A, Ferguson JA, Balmer C, Daniel C, Cohn A and Robinson WA: Melatonin therapy of advanced human malignant melanoma. Melanoma Res *1*: 237-243, 1991.

4 Hu DN, Roberts JE: Melatonin inhibits growth of cultured human uveal melanoma cells. Melanoma Res *7*: 27-31, 1997.

5 Dzjelendziak A, Butler J, Hoey BM, Lea JS and Ward TH: Comparison of the structural and cytostatic activity of novel 2.5-bis(carbo-ethoxy-amino) 3.6-diaziridinyl-1.4-benzoquinone analogues. Cancer Res *50*: 2003-2008, 1990.

6 Hardeland R and Poeggeler B: Melatonin beyond its classical functions. The open Physiology J *1*: 1-22, 2008.

7 Escames G, Diaz-Casado ME, Doerrier C, Luni-Sanchez M, Lopez LC and Acuna-Castroviejo D: Early gender differences in the redox status of the brain mitochondria with age: effect of melatonin therapy. Horm Mol Biol Clin Invest *16*: 91-100, 2013.

8 Galano A: On the direct scavenging activity of melatonin towards hydroxyl and series of peroxyl radicals. Phys Chem *13*: 7178-7188, 2011.

9 Galano A, Tan DX and Reiter RJ: On the free radical scavenging activities of melatonin`s metabolites, AFMK and AMK. J Pineal Res *54*: 245-257, 2103.

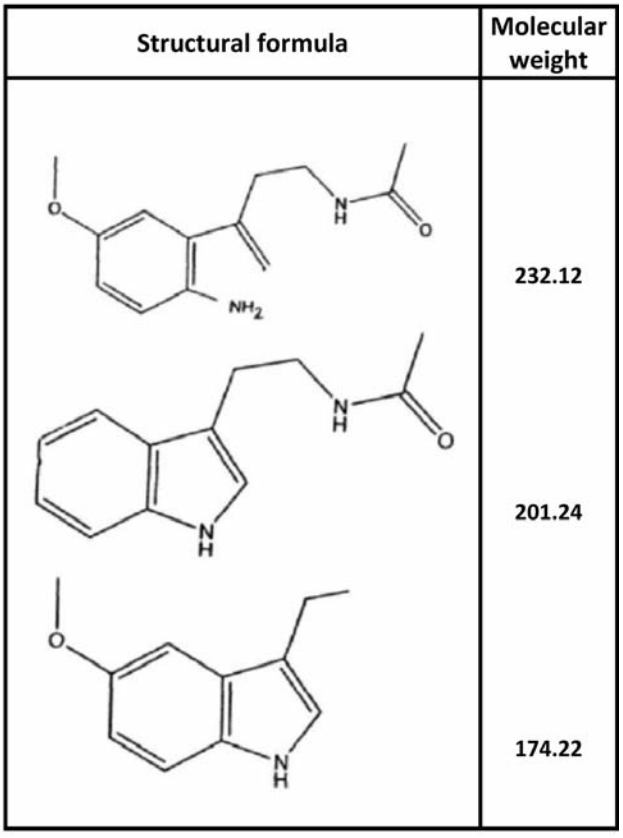


Figure 6. *Structure formulas of the main metabolites generated by e_{aq}^- emission from melatonin in water/ethanol=40/60 mixture.*

10 Roberts JE, Hu D-N and Wshart JF: Pulsradiolysis of melatonin and chloromelatonin. J Photochem Photobiol B Biology *42*: 125-132, 1998.
11 Stasica P, Ulanski P and Rosiak JM: Reactions of melatonin with radicals in deoxygenated aqueous solution. J Radioanalyt Nucl Chem *232*: 107-113, 1998.
12 Getoff N and Schenk GO: Primary products of liquid water photolysis at 1236, 1470 and 1849 Å. Photochem Photobiol *8*: 167-178, 1968.
13 Neumann-Spallert M and Getoff N: Photolysis of monochloric acetic acid at 253,7 nm in aqueous solution. Monatsh Chem *106*: 1359-1467, 1975 (in German).
14 Florence TM and Fara YT: Spectrophotometric determination of chloride at the part-per-billion level by mercury (II) thio cyanate method. Anal Chem Acta *54*: 373-377, 1971.
15 Brenn E, Mitulovic G, Quint RM and Getoff N: Corticosterone: Metabolites originating as consequence of solvated electrons (e-aq) emission. In Vivo *28*: 879-884, 2014.
16 Getoff N: Hormones: Electron emission, communication, mutual interaction, regeneration, melabolites, carcinogenesis and receptor action. Horm Mol Biol Clin Invest *12*: 363-375, 2012.
17 Buxton GV, Greenstock CL, Helman WR and Ross AB: Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals in aqueous solution. Phys Chem Ref Data *17*: 513-886, 1988.
18 Getoff N: A review of the relationship between $Q(e^-_{aq})$ and Q_F of excited compounds in aqueous solution. Radiat Phys Chem *34*: 711-719, 1989.

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