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The influence of cupric ions on porphyrin-induced photodynamic membrane damage in human red blood cells

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Photooxidation of various susceptible substrates with hematoporphyrin derivative (photofrin) as sensitizer was strongly inhibited by simultaneous addition of cupric acetate to the reaction mixture. With sulfhydryl-containing compounds, however, an increased rate of photooxidation was observed under these experimental conditions. Preincubation of photofrin and cupric acetate at equimolar concentrations for 24 h at room temperature yielded a stable photofrin-Cu^{2+} complex. This complex did not act as photosensitizer with histidine, tryptophan, tyrosine, methionine or guanosine as substrates. With dithiothreitol, however, the photofrin-Cu^{2+} complex still acted as a photosensitizer, with an efficiency of about 30% as compared to free photofrin. Also in red blood cell membranes only sulfhydryl groups were photooxidized with the photofrin-Cu^{2+} complex as sensitizer. Illumination of intact erythrocytes in the presence of the photofrin-Cu^{2+} complex resulted in K^+ leakage and, ultimately, photohemolysis. Pretreatment of the cells with *N*-ethylmaleimide and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) inhibited this photodynamically induced K^+ loss. Considering recent studies on the reactivity of distinct membrane SH-groups with various sulfhydryl reagents this suggests that a sulfhydryl group, located in the 17 kDa membrane-bound fragment of band 3, is involved in photodynamic K^+ leakage with the photofrin-Cu^{2+} complex as sensitizer.

Introduction

In many studies the erythrocyte has been used as a model to investigate photodynamic membrane damage. As photodynamic cell killing may be caused primarily by membrane damage [1,2], such studies are relevant in the context of photoradiation therapy of cancer [3,4] and the pathogenesis of hypersensitivity of the skin to light in various porphyrias [5]. Photohemolysis is a colloid-osmotic process, based on increased passive cation permeability of the membrane [6]. This type of damage is caused by photooxidation of membrane proteins [7]. With various porphyrins as sensitizers, photodynamic treatment of red blood cell membranes resulted in oxidation of SH-groups, histidine, tryptophan, methionine and tyrosine residues [8]. This makes it very difficult to evaluate the role of each of these photooxidized residues in disturbed membrane function.

Recently, Sommer et al. demonstrated that during prolonged incubation of porphyrins with cupric ions a stable porphyrin-Cu^{2+} complex is formed [9]. According to these authors, this porphyrin-Cu^{2+} complex did not act as photosensitizer. In previous, unpublished studies we found, however, that photohemolysis still occurred upon illumination of erythrocytes in the presence of a porphyrin-Cu^{2+} complex. To elucidate this apparent contradiction, the influence of cupric ions on photodynamic reactions induced by photofrin

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PCMBS, *para*-chloromercuriphenylsulfonic acid.

(hematoporphyrin derivative) was further explored. As discussed in this paper, free Cu^{2+} inhibited photooxidation of most substrates, whereas photooxidation of SH-groups, on the contrary, was stimulated. The photofrin-Cu^{2+} complex appeared to be a sulfhydryl-specific photosensitizer with a rather low efficiency. Presumably, photofrin-Cu^{2+}-induced photohemolysis is caused by photooxidation of a distinct class of SH-groups, located in the membrane-bound fragment of band 3.

Materials and Methods

Photofrin (hematoporphyrin derivative) was obtained from Oncology Research and Development, Cheektowaga, NY. All other chemicals were of analytical grade and used without further purification.

Formation of the photofrin-Cu^{2+} complex was achieved by incubating photofrin (0.5 mM) and cupric acetate (0.5 mM) in 50 mM phosphate buffer (pH 7.4) at room temperature in the dark. Complex formation was followed by recording the optical absorbance spectrum of the solution as described by Sommer et al. [9] and was complete in 22 h.

Heparinized human blood was centrifuged shortly after collection. The red blood cells were washed three times in buffered isotonic NaCl solution. Ghosts were prepared by the gradual osmotic lysis method of Weed et al. [10].

Illuminations were carried out with white light, as described previously [11]. Solubilized substrates (2 mM) were illuminated in 50 mM phosphate buffer (pH 7.4). Ghosts were illuminated at a final protein concentration of 1 mg/ml in 10 mM phosphate buffer (pH 7.4). Intact red blood cells were illuminated at a final hematocrit of 5% in buffered isotonic NaCl solution. The sensitizer, either photofrin or the photofrin-Cu^{2+} complex, was present at final concentrations as indicated in Results. Oxygen consumption was measured on 3-ml samples with an YSI oxygen monitor, equipped with a Clark-type oxygen electrode and a time-base recorder.

Protein was measured according to the method of Lowry et al. [12] with bovine serum albumin as a standard.

Photooxidation of sensitive residues in ghosts was assayed by quantitative analysis of histidine, tryptophan, tyrosine, methionine and thiol groups, before and subsequent to illumination in the presence of sensitizer. Results were expressed as percentage of initial values in untreated ghosts. Histidine was determined according to Sokolovski and Vallee [13], tryptophan as described by Spies and Chambers [14], tyrosine according to Uehara et al. [15], methionine according to McCarthy and Paille [16] and sulfhydryl groups by the method of Sedlak and Lindsay [17]. Lipid peroxidation was assayed by measuring thiobarbituric acid-reactive products, as described by Stocks and Dormandy [18].

K^+ efflux from red blood cells was determined by flame-photometry. The initial K^+ content of the cells was measured by hemolysis of samples in distilled water. Pretreatment of 10% erythrocyte suspensions with diamide (5 mM, 60 min) was carried out as described by Deuticke et al. [19]. Pretreatment with *N*-ethylmaleimide plus DTNB was performed as follows. Incubation of 10% erythrocyte suspensions with *N*-ethylmaleimide (10 mM, 45 min) was done as described by Lukacovic et al. [20]. Subsequently, DTNB was added to the suspension at 5 mM and incubation was continued for another 30 min. After preincubation, the erythrocytes were washed twice with buffered isotonic NaCl and resuspended at 5% final hematocrit.

Results

The influence of increasing concentrations of Cu^{2+} on the velocity of photooxidation of histidine and dithiothreitol, with photofrin as sensitizer, is shown in Fig. 1. In these experiments, photofrin and cupric acetate were added to the reaction mixture simultaneously. Illumination and measurement of oxygen consumption were started immediately thereafter and continued for 4 min. Oxygen consumption was linear with time during this period and no measurable formation of the photofrin-Cu^{2+} complex occurred, as judged from optical absorbance spectra. Apparently, cupric ions strongly inhibited photooxidation of histidine. Virtually identical inhibitions were found with tryptophan, tyrosine, methionine and guanosine as substrates. Surprisingly, the results with dithio-

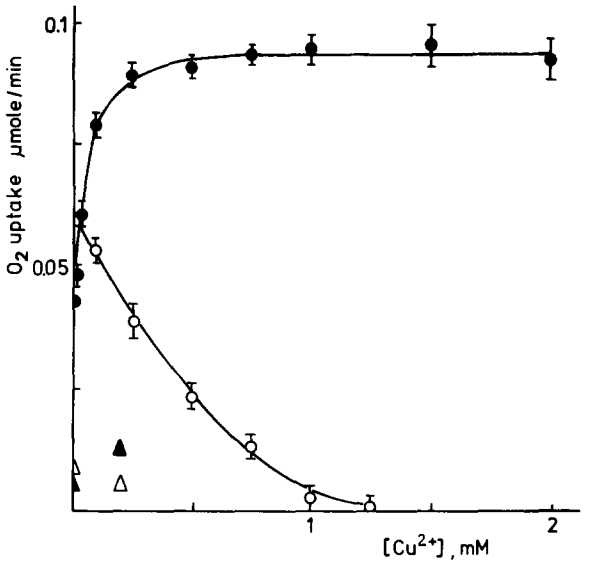

Fig. 1. Photooxidation of dithiothreitol (●) and histidine (○) in the presence of cupric ions. Photofrin (0.08 mM) and cupric acetate were added simultaneously. The influence of sodium azide (2.5 mM) on the photooxidation of dithiothreitol (▲) and histidine (△) is shown in the absence and in the presence of 0.2 mM cupric acetate.

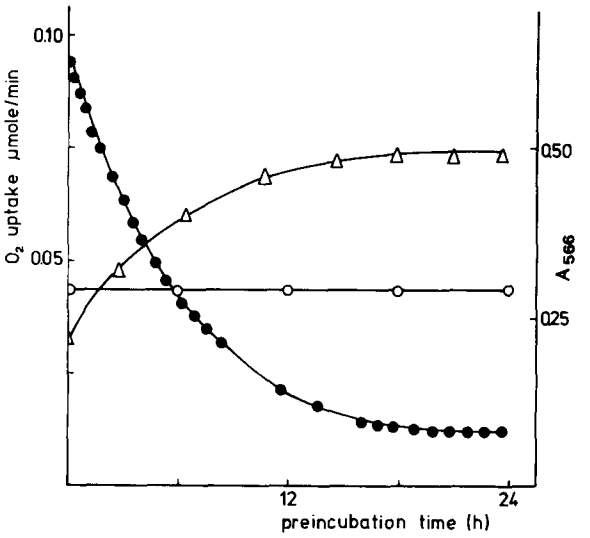

Fig. 2. Photooxidation of dithiothreitol by free photofrin (○) and by photofrin preincubated for the indicated time with an equimolar concentration of cupric acetate (●). Final photofrin concentration: 0.08 mM. △, Optical absorbance (566 nm) of the photofrin/cupric acetate mixture, used in these experiments.

threitol were quite different. Photooxidation of this substrate was significantly enhanced by cupric ions. In control experiments, no oxygen uptake was measured when photofrin was omitted from the reaction mixture. Both in the absence and in the presence of cupric acetate, photooxidation of histidine and dithiothreitol was about 85% inhibited by sodium azide (2.5 mM), indicating a type II, singlet oxygen-involving mechanism [21] in all these cases (Fig. 1).

In further experiments, photofrin and cupric acetate were preincubated for various times, as described in Materials and Methods, before addition of dithiothreitol. After short preincubations, photooxidation of dithiothreitol was increased, in accordance with the results shown in Fig. 1. After longer preincubation periods, however, the velocity of photooxidation decreased to a final level of about 30% of that in the absence of Cu^{2+} (Fig. 2). Concomitantly, an increase of the optical absorbance at 566 nm of the photofrin/cupric acetate mixture was observed, characteristic for the formation of a porphyrin-Cu^{2+} complex [9]. After 22 h, the absorbance at 566 nm reached its highest value, not responding to longer incubation or additional cupric acetate. As shown in Fig. 2, there was a close correlation between the decrease of the velocity of dithiothreitol photooxidation and the formation of the photofrin-Cu^{2+} complex.

With the pre-formed photofrin-Cu^{2+} complex as photosensitizer, no photooxidation could be measured with the substrates histidine, tyrosine, tryptophan, methionine and guanosine. With dithiothreitol, the complex was still active as photosensitizer, with an efficiency of about 30% as compared to free photofrin (Fig. 3). With glutathione and cysteine, similar results were obtained. In control experiments in the dark, no oxygen consumption occurred. Photooxidation of both histidine and dithiothreitol by free photofrin was strongly inhibited by sodium azide (Fig. 3). Photooxidation of dithiothreitol by the porphyrin-Cu^{2+} complex, however, was insensitive to azide (Fig. 3). Also mannitol (50 mM) had no influence on photooxidation of dithiothreitol by the photofrin-Cu^{2+} complex.

Prolonged illumination (2 h) of mixtures of dithiothreitol (2 mM) and either histidine (2 mM) or tryptophan (2 mM) in the presence of the photofrin-Cu^{2+} complex (0.08 mM) resulted in

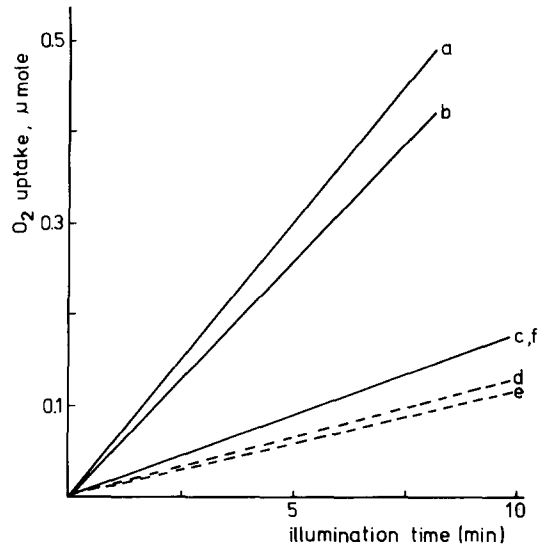

Fig. 3. Photooxidation of histidine and dithiothreitol under various experimental conditions. (a) Histidine plus photofrin; (b) dithiothreitol plus photofrin; (c) dithiothreitol plus photofrin-Cu^{2+} complex; (d) as a, plus sodium azide; (e) as b, plus sodium azide; (f) as c, plus sodium azide. Final concentrations of photofrin and the photofrin-Cu^{2+} complex: 0.08 mM. Final concentration of sodium azide, where present: 2.5 mM.

complete oxidation of sulfhydryl groups, without measurable decrease of histidine or tryptophan.

Illumination of red cell membranes in the presence of the porphyrin-Cu^{2+} complex resulted in limited photooxidation of SH-groups, without any decrease of histidine, tryptophan, tyrosine and methionine residues (Fig. 4). No generation of thiobarbituric acid-reactive products was observed, indicating that no lipid peroxidation took place. Maximal decrease of sulfhydryl groups amounted to 20% of the total number, which was achieved after an illumination period of about 20 min. With photofrin instead of the photofrin-Cu^{2+} complex as sensitizer, a decrease of all photooxidizable amino acid residues was observed. As shown in Fig. 4, the initial velocity of SH-oxidation with 5 µM photofrin more or less equaled the velocity with 40 µM photofrin-Cu^{2+}. With photofrin, photooxidation continued much longer, however, and about 75% of the membrane sulfhydryl groups had disappeared after 80 min of illumination.

Illumination of intact erythrocytes in the presence of the photofrin-Cu^{2+} complex resulted in K^+ leakage to the medium (Fig. 5). Incubation of the cells with the porphyrin-Cu^{2+} complex in the dark did not cause K^+ loss. Pretreatment of erythrocytes with diamide resulted in increased K^+ efflux as compared to control cells. When red blood cells, pretreated with diamide were subsequently illuminated in the presence of the photofrin-Cu^{2+} complex, the photodynamic effect was more or less additive to the diamide-induced K^+ loss (Fig. 5).

Pretreatment of intact erythrocytes with *N*-ethylmaleimide caused a slow but significant K^+ loss. Again, photodynamically and *N*-ethylmaleimide-

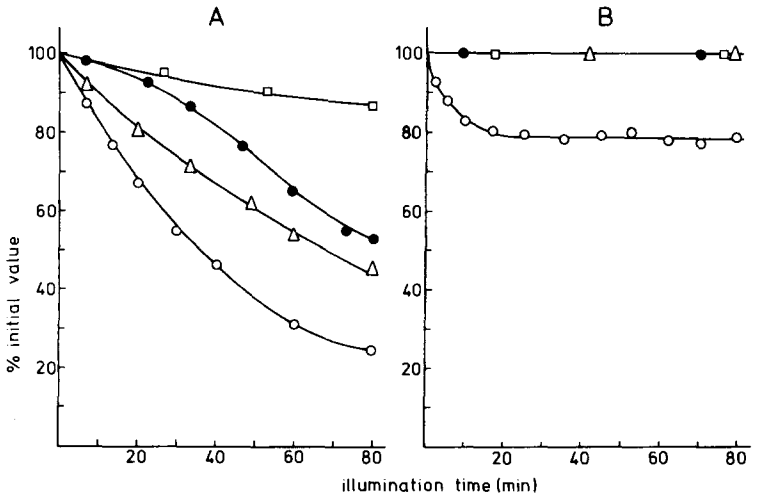

Fig. 4. Photooxidation of amino acid residues in red cell ghosts. ○, sulfhydryl groups; △, histidine; ●, tryptophan; □, tyrosine. (A) Illumination in the presence of photofrin (5 µM). (B) Illumination in the presence of the photofrin-Cu^{2+} complex (40 µM).

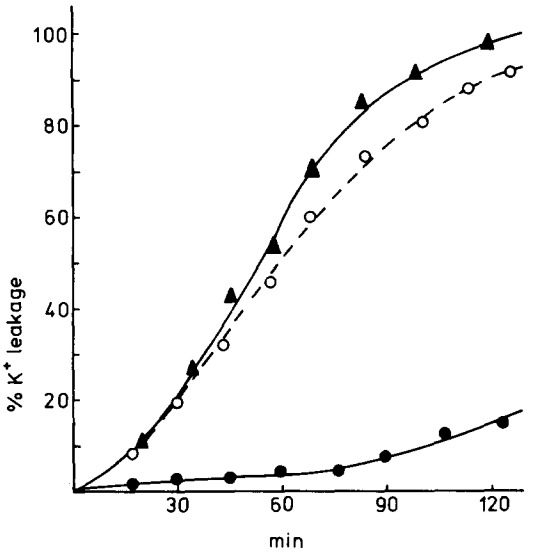

Fig. 5. K^+ leakage from red blood cells. ●———●, Pretreated with diamide; O------O, illuminated in the presence of the photofrin-Cu^{2+} complex (30 μM); ▲———▲, pretreated with diamide and subsequently illuminated in the presence of the photofrin-Cu^{2+} complex (30 μM).

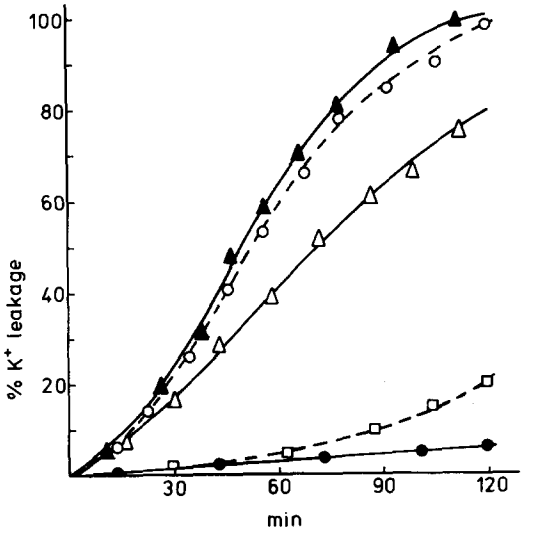

Fig. 6. K^+ leakage from red blood cells. ●———●, Pretreated with *N*-ethylmaleimide; □-----□, pretreated with *N*-ethylmaleimide plus DTNB; O------O, illuminated in the presence of the photofrin-Cu^{2+} complex (30 μM); ▲———▲, pretreated with *N*-ethylmaleimide and subsequently illuminated in the presence of the photofrin-Cu^{2+} complex (30 μM); △———△, pretreated with *N*-ethylmaleimide plus DTNB and subsequently illuminated in the presence of the photofrin-Cu^{2+} complex (30 μM).

induced K^+ loss were additive (Fig. 6). Pretreatment of red blood cells with *N*-ethylmaleimide and subsequently with DTNB was slightly more effective in causing K^+ leakage than incubation with *N*-ethylmaleimide alone. Illumination of *N*-ethylmaleimide plus DTNB pretreated cells in the presence of the porphyrin-Cu^{2+} complex resulted in a K^+ leakage that was considerably lower than with erythrocytes, not pretreated with these SH-reagents (Fig. 6). These results were reproducible with six blood samples, obtained from different donors.

Discussion

The influence of cupric ions on photofrin-induced photooxidation comprises at least two aspects: the effects of free cupric ions and the consequences of the formation of a porphyrin-Cu^{2+} complex. The effects of cupric ions, not bound to the sensitizer, are depicted in Fig. 1. It is not clear why photooxidation of dithiothreitol is enhanced, whereas photooxidation of all other substrates, used in these studies, is inhibited by Cu^{2+}. Although chelation of cupric ions by the substrate molecules may be involved in these effects, it should be emphasized that near-maximal effects are observed at Cu^{2+} concentrations considerably lower than the substrate concentrations. In all these experiments, sodium azide caused a strong inhibition of photooxidation, indicating a singlet oxygen-involving mechanism both in the presence and in the absence of cupric ions. Further studies will be needed to elucidate the mechanism of these effects of cupric ions.

The spectral data, shown in Fig. 2, indicate the slow formation of a porphyrin-Cu^{2+} complex during incubation of photofrin with cupric acetate, in accordance with previous results of Sommer et al. [9]. As shown in Results, the porphyrin-Cu^{2+} complex is ineffective as photosensitizer with all substrates tested, except sulfhydryl-containing compounds. Photooxidation of dithiothreitol by the porphyrin-Cu^{2+} complex was insensitive to sodium azide and mannitol, indicating that neither singlet oxygen nor hydroxyl radicals were involved.

It has been shown that under certain experimental conditions free radical metabolites are generated during thiol oxidation [22–25]. For in-

stance, photooxidation of SH-groups to cysteic acid residues by a type I reaction proceeds with $S^{\cdot}$, $SO_2^{\cdot}$ and $SO_4^{\cdot}$ radicals as intermediates [25]. Such free radical metabolites might induce oxidation of other amino acid residues in a secondary chain reaction. However, the data presented in Results do not reveal such a mechanism in photooxidation of thiols, induced by the porphyrin-Cu^{2+} complex. Both in red cell membranes and in solutions containing dithiothreitol plus histidine or tryptophan, only a decrease of reactive SH-groups was found, without a concomitant decrease of the other oxidizable amino acid residues. Therefore, the porphyrin-Cu^{2+} complex seems to be a sulfhydryl-specific sensitizer.

It is not clear why only about 20% of ghost membrane SH-groups are reactive with the porphyrin-Cu^{2+} complex as sensitizer, whereas with free photofrin a much higher percentage is photooxidized (Fig. 4). It is possible that the accessibility of certain membrane region is lower for the photofrin-Cu^{2+} complex than for free photofrin.

The results with isolated membranes make it highly probable that the photodynamic K^+ leakage, induced by the porphyrin-Cu^{2+} complex (Figs. 5 and 6), is caused by photooxidation of a limited number of membrane sulfhydryl groups. Therefore, further experiments were performed as an attempt to localize the SH-groups, involved in this process. As shown by Deuticke et al., oxidation of certain erythrocyte membrane SH-groups by diamide causes enhancement of membrane permeability for hydrophilic non-electrolytes and ions [19,26]. The thiol groups involved in these diamide effects are presumably localized in spectrin [19,26]. Under the present experimental conditions, almost all diamide-sensitive thiol groups were converted to disulfides during preincubation [19]. Disulfides are insensitive to photooxidation. Therefore, it should be expected that diamide-pretreated cells would be much less sensitive to photodynamic attack, assuming that the same SH-groups are involved in diamide and in porphyrin-Cu^{2+} plus light-induced K^+ leakage. The results shown in Fig. 5 contradict this assumption. The photodynamically induced K^+ leakage is additive to the diamide-induced K^+ leakage, suggesting that different thiol groups are involved in these two processes.

N-Ethylmaleimide is very reactive with many classes of cellular SH-groups [27], but causes only a minor enhancement of passive permeability of the erythrocyte membrane [19]. In *N*-ethylmaleimide-pretreated cells, both PCMBS [20,28] and DTNB [27] react exclusively with a particular SH-group, localized in the membrane-bound 17 kDa fragment of band 3. This SH-group does not react with *N*-ethylmaleimide [27]. Under these conditions, PCMBS causes a very pronounced K^+ leakage [20]. These results are in accordance with observations of Grinstein and Rothstein, also indicating that the 17 kDa fragment of band 3 is involved in PCMBS-induced K^+ leak [29]. DTNB, reacting with the same SH-group [27], induces only a very moderate K^+ leakage (Fig. 6). As DTNB reacts with SH-groups by sulfhydryl-disulfide exchange, it should be expected that the modified membrane SH-group becomes insensitive to photooxidation. As shown in Fig. 6, pretreatment of erythrocytes with *N*-ethylmaleimide plus DTNB actually protected against K^+ loss by subsequent illumination in the presence of the porphyrin-Cu^{2+} complex. It should be stressed that pretreatment with *N*-ethylmaleimide alone was not protective. Therefore, these results strongly suggest that the SH-group, located in the 17 kDa fragment of band 3, is involved in photodynamically induced K^+ leakage with the porphyrin-Cu^{2+} complex as sensitizer. These results do not rule out the possibility that photooxidation of other thiol groups contributes to the ultimate effect.

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