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Compounds I of Catalase and Horse Radish Peroxidase: π-Cation Radicals

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ABSTRACT **Two-electron oxidation of cobaltous octaethylporphyrin [Co(II)(Et)$_8$P] yields a stable π-cation radical [Co(III)(Et)$_8$P]$^{2+\cdot}$, the optical spectrum of which exhibits spectral changes dependent upon the nature of the counterion. Comparison of these spectra with those of Compounds I of horseradish peroxidase and catalase leads us to propose that these Compounds I contain a π-cation radical of the heme prosthetic group. This proposal explains the oxidation level, optical spectra, and stability of the primary compounds without recourse to properties such as stoichiometric mixtures of special porphyrins, stable Fe(V) porphyrins, or unique conformers of heme porphyrins. Explanations are advanced to account for the missing electron spin resonance signal of Compound I of horseradish peroxidase.**

Recent work on the oxidation of metalloporphyrins has provided (1–4) examples in which one or two electrons are given up by the organic moiety to yield π-cations, as well as examples of oxidations in which one electron is abstracted from the metal and the other from the organic ring (4). The esr spectra of the π-cation radical of zinc *meso*-tetraphenylporphyrin [Zn(II)(Ph)$_4$P]$^{+\cdot}$ ClO_4^-, and that of magnesium octaethylporphyrin [Mg(II)(Et)$_8$P]$^{+\cdot}$ ClO_4^-, clearly establish electron abstraction from the porphyrin ring. Comparison of the optical spectra of a number of such π-cation radicals reveals that they fall in two categories typified by either Mg(Et)$_8^{+\cdot}$ or Zn(Ph)$_4^{+\cdot}$. The Mg(Et)$_8^{+\cdot}$ visible spectrum is characterized by a major absorption peak near 700 nm with a high-energy shoulder, while Zn(Ph)$_4$P$^{+\cdot}$ exhibits a broad, nearly featureless absorption in the region 500–700 nm. These differences have been ascribed previously (4) to two close-lying ground states of the radicals: $^2A_{1u}$ (class 1) for Mg(Et)$_8$P$^{+\cdot}$, and $^2A_{2u}$ (class 2) for Zn(Ph)$_4^{+\cdot}$.* We present here results on the chemical and electrochemical oxidation of Co(II)(Et)$_8$P, which undergoes reversible metal and ring oxidations to yield [Co(III)(Et)$_8$P]$^+$ and [Co(III)(Et)$_8$P]$^{2+\cdot}$. This radical possesses the additional feature that it exhibits class 1 and 2 spectra interchangeably as a function of the counter ion. Correlation of the stability and the optical properties of the metalloporphyrin cation radicals with the known properties of Compounds I of catalase and peroxidase leads us to propose that these forms of the enzymes contain a π-cation radical of the heme prosthetic group.

Abbreviations: HRP, horse radish peroxidase; (Ph)$_4$P, tetraphenylporphyrin; (Et)$_8$P, octaethylporphyrin; sce, aqueous saturated calomel electrode.

* Class 1 spectra are shown by [Mg(II)(Et)$_8$P]$^{+\cdot}$ ClO_4^- and Br^-; [Ni(II)(Ph)$_4$P]$^{+\cdot}$ ClO_4^- and Cl^-; [Zn(II)(Et)$_8$P]$^{+\cdot}$ ClO_4^- and Br^-; [Ag(II)(Et)$_8$P]$^{+\cdot}$ ClO_4^-: Class 2 spectra are shown by [Zn(II)(Ph)$_4$P]$^{+\cdot}$ ClO_4^- and Br^-; [Co(III)(Ph)$_4$P]$^{2+\cdot}$2ClO_4^-, 2Br^-, and 2Cl^-; [Cu(II)(Ph)$_4$P]$^{+\cdot}$ ClO_4^- [Mg(II)(Ph)$_4$P]$^{+\cdot}$ ClO_4^-; [Cu(II)(Et)$_8$P]$^{+\cdot}$ ClO_4^- and Br^-; [Pd(II)(Et)$_8$P]$^{+\cdot}$ ClO_4^-; [Ni(II)(Et)$_8$P]$^{+\cdot}$ ClO_4^- and Cl^- (the last three examples are discussed in ref. 2).

METHODS

The techniques for bromine oxidations, controlled potential electrolysis, coulometry, and for optical and esr measurements (as well as for purification and preparation of materials) have been described (4). For anion-transfer reactions, a quartz esr tube was attached to an ampoule with two side arms, one of which contained a degassed solution of [Co(III)(Et)$_8$P]$^{2+\cdot}$ 2Br^- in chloroform and the other dry $AgClO_4$ isolated by a fritted disc. After the spectrum of the bromide salt was obtained, the solution was shaken in the $AgClO_4$ compartment to form the [Co(III)(Et)$_8$P]$^{2+\cdot}$ 2ClO_4^- salt. Relative concentrations of free radicals were determined by double integration by computer of the first-derivative spectra obtained under comparable experimental conditions. The mass spectrum was recorded on an AEI MS-9 mass spectrometer operating at 70 eV, and a source temperature of 250°C. The nuclear magnetic resonance (nmr) spectra were measured in chloroform and recorded on a Varian HA-100 spectrometer, with tetramethylsilane as internal reference. Attempts to oxidize ferric porphyrins to a formal Fe(V) oxidation state were unsuccessful due to solvent breakdown encountered at the high potentials required [>1.5 V vs. aqueous saturated calomel electrode (sce)].

RESULTS

Electrolysis of Co(II)(Et)$_8$P in CH_2Cl_2 containing 0.1 M *n*-$(C_3H_7)_4NClO_4$, at 0.77 V versus sce required 1.0 (±0.1) elec-

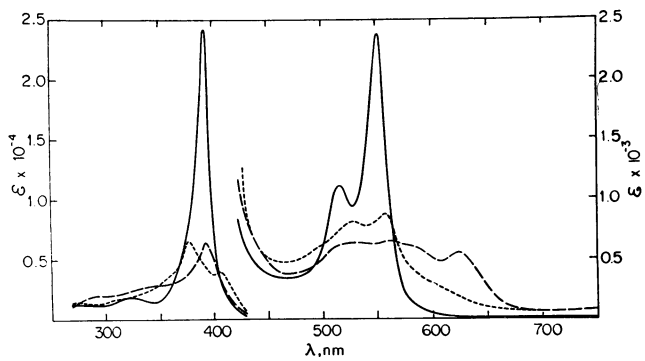


FIG. 1. Optical absorption spectra (in CH_2Cl_2/*n*-C_3H_7)$_4$-$NClO_4$) of Co(II) (Et)$_8$P (——); [Co(III)(Et)$_8$P]$^+$ ClO_4^-(---); and [Co(III)(Et)$_8$P]$^{2+\cdot}$ 2ClO_4 (— — —).

trons (determined by coulometry) to yield a red solution (Fig. 1). Continued electrolysis at 0.97 V versus sce resulted in the removal of a second electron (1 ± 0.1) to yield a green solution (Fig. 1). This new species required two electrons for reduction, via the cation, to the initial porphyrin (with complete recovery of Co (II)$(Et)_8P$):

$$[Co(III)(Et)_8P]^{2+\cdot} \xrightarrow{+e^-} [Co(III)(Et)_8P]^{+} \xrightarrow{+e^-} Co(II)(Et)_8P.$$

The changes in the optical spectra (Fig. 1), and the lack of an esr signal, are consistent with an initial oxidation of the cobaltous to a cobaltic state $[Co(III)(Et)_8P]^+$. Moreover, both the $[Co(III)(Et)_8P]^+ Br^-$ and $[Co(III)(Et)_8P]^+ ClO_4^-$ give nmr spectra (for the monobromide: 1.87 (triplet, 24 protons of eight methyl groups); 4.09 (quartet, 16 protons of eight methylene groups); 9.96 (singlet, four protons of 4 *meso*-hydrogens)) without paramagnetic broadening or contact shifts, consistent with a low-spin diamagnetic Co(III) (5).

The two-electron oxidation product, on the other hand, shows changes in its visible absorption spectrum, which now resembles the $Zn(Ph)_4P$ radical (class 2), and exhibits the esr signal shown in Fig. 2. We conclude, therefore, that the dication is a cobaltic species complexed by a porphyrin π-cation radical: $[Co(III)(Et)_8P]^{2+\cdot}$. As in the $[Co(III)(Ph)_4P]^{2+\cdot}$ case previously reported (1, 3), spin density is transferred from the oxidized ring onto the metal, and the esr spectrum can be simulated using a hyperfine-splitting constant of 1.2 ± 0.2 G for cobalt (I = 7/2). (This represents a small spin density on the cobalt ion.)

Co(II)$(Et)_8P$ in chloroform, upon treatment with bromine, also undergoes two distinct, reversible oxidations (1):

$$Co(II)(Et)_8P \xrightarrow{1/2\ Br_2} [Co(III)(Et)_8P]^+ Br^- \xrightarrow{1/2\ Br_2} [Co(III)(Et)_8P]^{2+\cdot}\ 2Br^-$$

The absorption spectrum (Fig. 3) of the latter dication resembles that of the $Mg(Et)_8P^{+\cdot}$ radical (class 1). No esr is observed at room temperature, but as the solution is cooled, a broad signal appears that narrows with further cooling; at −50°C the spectrum shown in Fig. 2 is observed. The bromide ligands thus cause significant changes in both the optical and esr spectra. The spin-lattice broadening of the esr spectrum indicates a strong coupling of the Br^- to the cation radical. In fact, the bromide ions are so strongly complexed to the dication that the parent peak in the mass spectrum (m/e 749) is 1/10 as intense as the base peak ($Co(Et)_8P$), whereas the parent peaks of either $[Fe(III)(Et)_8P]^+ Cl^-$ or $[Mn(III)(Et)_8P]^+ Cl^-$ are only 1/200 as intense as the base peak ($Fe(Et)_8P$ and $Mn(Et)_8P$, respectively) (6).

The class 1 spectrum of $[Co(III)(Et)_8P]^{2+\cdot}\ 2Br^-$ is transformed to the class 2 spectrum of $[Co(III)(Et)_8P]^{2+\cdot}\ 2ClO_4^-$ when the dibromide is treated with excess $AgClO_4$ (Fig. 3) to give the perchlorate salt and insoluble AgBr. At the same time, a narrow esr signal is again observed at room temperature. No change in radical concentration (determined at −50°C) is caused by the ligand exchange.

In conclusion, Co(II)$(Et)_8P$ can be reversibly oxidized in two distinct steps in which the first electron is removed from the metal and the second from the porphyrin ring: in addition, the second oxidation stage (which contains the porphyrin radical) shows two different optical spectra, depending upon the counter ion. *Both of these spectra are typical of π-cation radicals.*

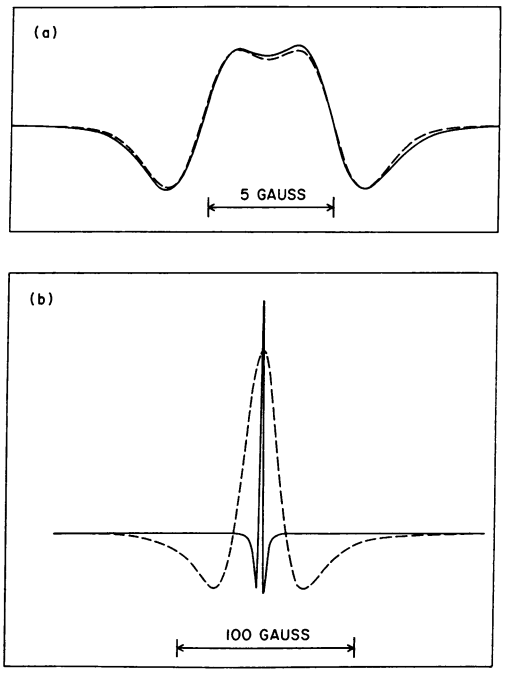


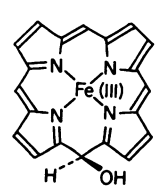
FIG. 2. Second-derivative esr spectra of (*a*) $[Co(III)(Et)_8P]^{2+\cdot}\ 2ClO_4$ in CH_2Cl_2 at 25°C (—), and a computer simulation (—) using a_{Co} = 1.2 G and a linewidth of 4.2 G (*b*) $[Co(III)(Et)_8P]^{2+\cdot}\ 2Br^-$ (—) and $[Co(III)(Et)_8P]^{2+\cdot}\ 2ClO_4^-$ (—) in $CHCl_3$ at −50°C (at different concentrations).

CATALASE AND PEROXIDASE

During the catalytic cycles of both catalase and horseradish peroxidase, the resting ferrihemoproteins undergo reversible two-electron oxidations to their green primary compounds (Catalase I and HRP I) (7, 8). Within the limitations imposed by the magnetic-susceptibility measurements (9), which show S = 3/2 and a formal oxidation state Fe(V), a number of suggestions have been made concerning the constitution and electronic configuration of these primary compounds.

It was initially suggested (10) that the primary compounds were enzyme–substrate complexes of the type Fe(III)OOH. George (11) has challenged this concept because *non*peroxidatic oxidants can give rise to green compounds optically identical to the so-called peroxide complexes.

Winfield (12) proposed that the primary compounds should be considered as a complex of pentavalent Fe(V) stabilized by an O^- ion. Brill and Williams (13) suggested that the primary compounds might be mixtures of iron compounds with an average spin of 3/2: a hemoprotein having a hydroperoxide as a sixth ligand coordinated to the iron, and a complex (**A**) having an sp^3 *meso*-carbon atom substituted by a hydroxyl group.



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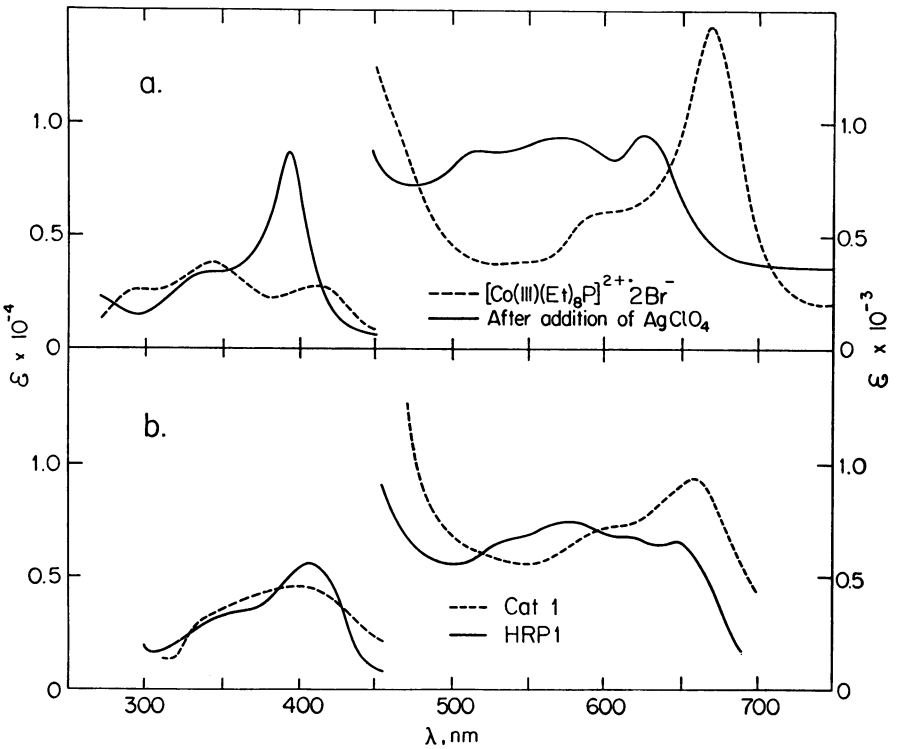

FIG. 3. A comparison of the optical absorption spectra of (*a*) $[Co(III)(Et)_8P]^{2+\cdot}\ 2Br^-$, and $[Co(III)(Et)_8P]^{2+\cdot}\ 2ClO_4$ in $CHCl_3$, (*b*) Catalase I and HRP I. The HRP I spectrum is taken from ref. 18, and that of Cat I from ref. 13. Catalase is tetrameric and the ϵ shown is per hematin.

However, complex **A** is in effect an isoporphyrin, whose optical absorption spectra (14) possess a characteristic infrared absorption (~900 nm) not seen with the primary compounds (G. R. Schonbaum, personal communication).

Peisach *et al.* (15) have recently suggested an intermediate spin (S = 3/2) d^5 ferric complex for HRP I in which the two oxidizing equivalents are stored temporarily at a methine-bridge carbon atom or at a pyrrole carbon atom of the porphyrin. Brill (16) has noted that the formal oxidation state of of Fe(V) could be satisfied by a low-spin (S = 1) Fe(IV) complex with a free radical on the porphyrin ring or protein, or by a low-spin ferric complex with a biradical on the porphyrin or protein. However, structures such as a low-spin d^5 and a biradical, or low-spin d^4 and a single radical, have been considered unlikely by both Winfield (12, 17) and Brill (16) and were rejected by Peisach *et al.* (15) who considered that the absence of a low temperature esr signal (18) and the stability of the primary compounds was inconsistent with the expected properties of radical-type structures.

The demonstrated stability of metalloporphyrin π-cation radicals (4) invalidates the previous criticism that such structures are too unstable to be the chromophores of the primary compounds of catalase and peroxidase. *To the contrary, it is our contention that the optical spectra of these primary compounds characterize them as porphyrin π-cation radicals: Thus, the spectrum of HRP I is similar to $[Co(III)(Et)_8P]^{2+\cdot}\ 2ClO_4^-$ (class 2), while that of Catalase I is similar to $[Co(III)(Et)_8P]^{2+\cdot}\ 2Br^-$ (class 1), (Fig. 3.).*

One of the two oxidizing equivalents of the primary compounds is accounted for by the loss of an electron from the porphyrin π-orbitals. The second oxidation may then occur from the porphyrin ring, the protein, or the metal.

(*1*) Further oxidation of the ring would result in π-dications. Optical spectra of the dications (4) are so different from those of HRP I and Catalase I that they can be excluded.

(*2*) Oxidation of the protein would result in an esr spectrum characteristic of an organic free radical; such spectra are not observed in these systems.†

(*3*) We conclude, therefore, that the second oxidizing equivalent can be accounted for by the oxidation of the ferric iron to quadrivalent Fe(IV). The absence of nuclear-hyperfine structure in the low-temperature Mössbauer spectra of HRP I (21) and of Japanese-radish peroxidase (22) also is compatible with an integral-spin form of iron, but not with half-integral configurations (23). The overall electronic configurations of the primary compounds are then represented as:

$$[Fe(IV)HRP]^{3+\cdot} \text{ and } [Fe(IV)Catalase]^{3+\cdot}$$

where the charge on the metal can be balanced either with anionic ligands supplied by the protein or with hydroxide ions.

† It should be noted (however) that Compound I of cytochrome-c peroxidase has a formal oxidation state of Fe(V), but possesses an optical spectrum similar to those of HRP II and Catalse II, and exhibits an esr signal (19) compatible with an organic free radical. In *this* case [and in agreement with Yonetani *et al.* (20)] we account for the two oxidizing equivalents of *this* primary compound as (*i*) a one-electron oxidation of the Fe(III) to Fe(IV), and (*ii*) a one-electron oxidation of the protein, but not of the porphyrin.

One-electron reduction of the green primary compounds forms the pale-red secondary compounds HRP II and Catalase II. The considerable changes in the optical spectrum (reappearance of a typical metalloporphyrin spectrum) associated with this reduction are consistent with the addition of an electron to the porphyrin π system. Thus, if the primary compounds are π-cation radicals of a quadrivalent iron porphyrin, then the *secondary compounds are complexes of quadrivalent iron.*

These hypotheses concerning Compounds I and II require that the oxidation state of the iron (Fe(IV)) to be the same in both systems. The same conclusion has been reached by Ehrenberg *et al.* (21) for HRP I and HRP II and by Maeda and Morita (22), working with Japanese-radish peroxidase, who have shown by Mössbauer spectroscopy that the electronic configuration of the iron is the same in Compounds I and II.

Formulation of HRP I as quadrivalent iron complexed with a porphyrin radical must account for the failure to observe an esr signal arising from the radical. We now present possible explanations to account for the failure to elicit esr signals.

It is important to note that a single electron localized on the porphyrin ring will couple, via an exchange interaction, with spin localized on the metal (24); for example $(Cu(II)(Ph)_4P)^{+\cdot}ClO_4$ is a ground state triplet (3), rather than two doublets. Thus, a description of the spin coupling for the formulation $(Fe(IV)HRP)^{3+\cdot}$ with S = 3/2 is envisaged as either a high spin (S = 2) or low spin (S = 1) Fe(IV) coupled to a porphyrin radical (S = 1/2). Peisach *et al.* have suggested (15), for a system with S = 3/2, that the $Sz = \pm 1/2$ states that are involved in magnetic transitions are thermally inaccessible at the temperature (1.5°K) of their esr experiment (18). If this suggestion is extended to data at 77°K, the relevant spin Hamiltonian would require a large, negative zero-field splitting parameter in order to explain the lack of esr (25). The existing data on HRP I do not rule out such a possibility.‡

An alternative phenomenon also may cause disappearance of an esr signal. Should aggregation of the protein occur at the reduced temperatures of the esr experiments, exchange coupling between the S = 3/2 spin systems of HRP I could affect the esr signal. Since the resting enzyme in frozen solutions displays the prominent esr features of high-spin ferric ion, exchange coupling between spins localized on metal ions may be neglected. However, a similar conclusion is not warranted should spin be *delocalized on the porphyrin ring* as is the case with π-cation radicals (4). In this latter instance, weak exchange coupling between oxidized porphyrin rings of HRP I ($\lesssim 30$ cm^{-1}), caused by either direct interaction at distances of about 5–10 Å or indirect exchange via sigma-bonding electrons (26), would still yield¶ an effective magnetic moment at room temperature corresponding to that of uncoupled S = 3/2 spin systems. However, on the scale of energy appropriate to esr measurements, the aggregates (presumed to consist primarily of dimers) would behave as even-electron systems. The resulting thermally-populated paramagnetic states with S = 1 2, and 3 would be subject to zero-field splitting. The consequent signal anisotropy could make detection of a magnetic transition extremely difficult (or—in the case of a zero-field splitting in excess of the microwave energy—improbable). The postulated weak coupling would have no dramatic effect upon the electronic spectrum, and room-temperature optical spectra would appear (15) similar to those obtained at lower temperatures. The present proposal *does* require that a portion of porphyrin ring be exposed to the exterior of the protein in order to allow two cation radicals to interact with one another. Evidence of exchange-coupled porphyrin cation radicals is found (4) in the dimerization of $(Mg(II)(Et)_8P)^{+\cdot}$, with a concomitant loss of the esr signal. In this example, the radicals are *not* bound to protein, approach of the two porphyrins can be close, and dramatic spectral changes occur.

The existence of stable porphyrin π-cation radicals in both catalase and horseradish peroxidase suggests that the electron transfers associated with their catalytic cycles may occur via the porphyrin ring. Such a concept is supported by the diffusion-controlled rate of decomposition of hydrogen peroxide by catalase (27). It is difficult to envisage such a fast rate if the substrate (H_2O_2) must approach the iron atom, which is sterically hindered by both the tetradentate porphyrin and axial ligands (7). However, approach of the substrate to the large periphery of the porphyrin ring will encounter less steric constraint. Similarly, it is possible that electron transfer in the hemoprotein enzymes cytochrome *c* and b_5, which results in an overall oxidation or reduction of the iron, also occurs via the porphyrin ring, because x-ray structures (28, 29) show that although in both cases the iron is "buried" within the porphyrin and protein, an edge of the porphyrin remains exposed to the exterior of the enzyme.

In summary, we have presented spectral evidence that Compounds I of HRP and Catalase contain π-cation radicals of the heme prosthetic groups. As a corollary to this proposal, and in agreement with Mössbauer spectra, the heme iron is presumed to be Fe(IV) in Compounds I and II.

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‡ We have found no published esr studies on Catalase I. High-field Mössbauer spectra (23) and low-temperature magnetic susceptibilities of Compounds I and II would help to understand the zero-field energy distribution of the oxidized hemes.

¶ The magnetic moment, μ, in units of a Bohr magneton is found to be $\mu^2 = 2(2x + 30x^2 + 84x^3)/(1 + 3x + 5x^2 + 7x^3)$, where $x = \exp(-J/kT)$ and J is the coupling constant. The formula neglects zero-field splittings within the spin multiplets.

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