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GENERATION OF HYDROXYL RADICAL BY ANTICANCER QUINONE DRUGS, CARBAZILQUINONE, MITOMYCIN C, ACLACINOMYCIN A AND ADRIAMYCIN, IN THE PRESENCE OF NADPH-CYTOCHROME P-450 REDUCTASE

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Abstract—The generation of hydroxyl free radicals in the system consisting of purified NADPH-cytochrome P-450 reductase and anticancer quinone drugs, such as carbazilquinone, mitomycin C, aclacinomycin A and adriamycin, has been confirmed by two methods. In the spin trapping study, using *N-tert*-butyl-α-phenylnitrone as the spin trapping agent, four drugs generated hydroxyl radical-trapped signals, and the formation of the spin adduct was dependent on time and the enzyme concentration. Among the four drugs, the generation time of signal was in the order of carbazilquinone, aclacinomycin A, adriamycin and mitomycin C, but the magnitude of signal intensity was different. In both aclacinomycin A and adriamycin, the signal disappeared in a few minutes. Catalase completely inhibited the formation of the spin adduct, while superoxide dismutase did not significantly inhibit, but effected in some manner. The generation of hydroxyl radical was also confirmed by the ethylene production from methional. Among the four drugs, the order of the magnitude of ethylene production was different from that of signal intensity by ESR study. Catalase potently inhibited the ethylene production, while superoxide dismutase effected in some manner. From these results, the interactions of anticancer quinone drugs with NADPH-cytochrome P-450 reductase and oxygen, and the possible relations of the enzymes to the radical related actions of these drugs are discussed.

The anticancer quinone drugs (Fig. 1) show many biological effects [1–5], and recent studies are focusing on their molecular mechanism [3, 6–14]. In studies on the metabolism of anticancer quinone drugs, we have found that NADPH-cytochrome P-450 reductase (EC 1.6.2.4) and some enzymes which are active on quinone compounds catalyze the reductive glycosidic cleavage of anticancer anthracycline antibiotics, such as aclacinomycin A, adriamycin and daunomycin, and the reductive activation of anticancer aminoquinone antibiotic, mitomycin C, under anaerobic conditions. And under aerobic conditions, hydrogen peroxide was produced from the mixture of NADPH-cytochrome P-450 reductase and anticancer anthracyclines [13–16]. From these studies, it was suggested that the contribution of these enzymes to quinone drug metabolism is the reduction of quinone moiety of these drugs to semiquinone form, and the production of hydrogen peroxide is the result of the reduction of molecular oxygen by semiquinone form of the drug [13, 14].

On the other hand, there are reports that the anthracycline antibiotics and mitomycin C show the stimulation of lipid peroxidation and of strand scission of DNA where the reductions were carried out by NADPH-cytochrome P-450 reductase or chemical agents [1, 3, 4, 11]. In these systems, the action mechanism of these drugs had been considered as the generation of superoxide or hydroxyl radicals by reduced drugs. Recently, Lown *et al.* [7] reported ESR detection of the hydroxyl spin adduct in the system containing sodium borohydride and mitomycin C.

In this paper, we have measured both the ESR spectrum, using *N-tert*-butyl-α-phenylnitrone (BPN) as a spin trapping agent, and ethylene production from methional, in order to investigate the production of hydroxyl radicals in systems consisting of purified NADPH-cytochrome P-450 reductase and anticancer quinone drugs under aerobic conditions. A preliminary report of the present study has appeared previously [17].

MATERIALS AND METHODS

NADPH-cytochrome P-450 reductase which was solubilized with Emulgen 913 from rabbit liver microsomes was purified by the method described by Taniguchi *et al.* [18] and the purity of the enzyme was confirmed by acrylamide gel electrophoresis. Bovine blood superoxide dismutase (EC 1.15.1.1), type I, twice-crystallyzed bovine liver catalase (EC 1.11.1.6), and methional were obtained from Sigma Chemical Co. (St Louis, MO). Aclacinomycin A hydrochloride, adriamycin hydrochloride and carbazilquinone were the kind gift of Dr Oki,

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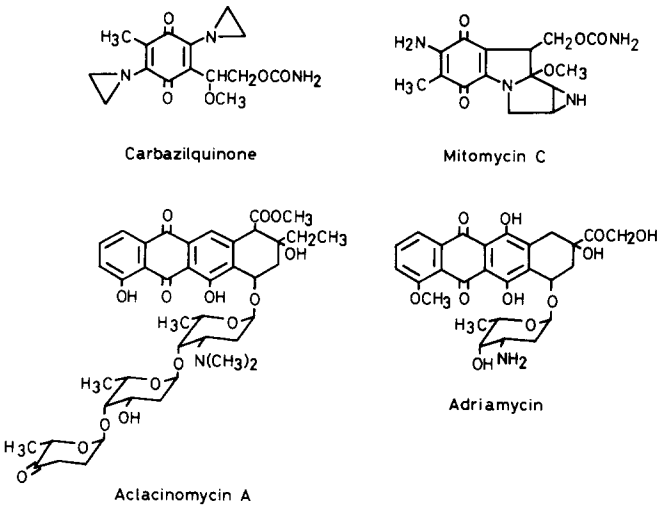

Fig. 1. Structures of the anticancer quinone drugs examined.

Sanraku-Ocean Co., Ltd. (Japan), Dr Arcamone, Falmitalia-carloerba S.p.A. (Italy) and Sankyo Co., Ltd (Japan), respectively. Mitomycin C and NADPH were obtained from Kyowa Hakko Kogyo Co., Ltd (Japan), and Oriental Yeast Co., Ltd. (Japan), respectively. BPN and Prapack type Q were obtained from Aldrich Chemical Co., Ltd. (Milwaukee, WI) and Waters Associated Inc. (U.S.A.), respectively. All other chemicals were analytical grade. The stock solutions of carbazilquinone, 10 mM for ESR study and 0.5 mM for ethylene production method were prepared by dissolution in dimethylsulfoxide and 0.1 M Tris–HCl, pH 7.5. Mitomycin C, aclacinomycin A hydrochloride and adriamycin hydrochloride were dissolved in distilled water. All experiments were carried out at least three times.

ESR study. Complete system in a final volume of 0.2 ml in a quartz flat cell, consisted of 0.1 M Tris–HCl, pH 7.5, 2 mM NADPH, 80 mM BPN, 0.1 mM anticancer quinone drug, and 2.2 μg of NADPH-cytochrome P-450 reductase. In some experiments, superoxide dismutase and catalase were added to the complete system at the conditions of 40 μg of superoxide dismutase and 25 μg of catalase in carbazilquinone study, and 70 μg of superoxide dismutase and 12.5 μg of catalase in mitomycin C, aclacinomycin A and adriamycin studies. X-band ESR spectra were recorded with a Joel JES-FE3X at 25° using modulation amplitude 0.8 G, microwave power 10 mW, and time constant 0.03 sec.

Ethylene production. Ethylene production was measured by gas chromatography using a modification of the method described by Beauchamp and Fridovich [19]. The assay mixture, in a final volume of 1.0 ml in a 11.5 ml test tube with silicone rubber stopper, consisted of 0.1 M Tris–HCl, pH 7.5, 2 mM NADPH, 1 μl of methional, 0.1 mM anticancer quinone drug, and 10 μg of NADPH-cytochrome P-450 reductase. In some experiments, 40 μg of superoxide dismutase and catalase were added. This concentration of methional showed no effect on cytochrome *c* reductase activity of NADPH-cytochrome P-450 reductase. Reaction was carried out at 37° for 60 min with reciprocal shaking at 120 rpm, and 0.5 ml of gaseous phase was analyzed with a Shimadzu GC-3BF flame ionization gaschromatograph equipped with 3 × 1000 mm column packed with Porapack type Q. The quantity of ethylene released during each incubation was calculated from the height of the peak using calibration chromatograms.

RESULTS

The spin-trapped ESR spectrum generated by NADPH-cytochrome P-450 reductase and carbazilquinone in the presence of BPN is shown in Fig. 2(a). The signal which appeared immediately after

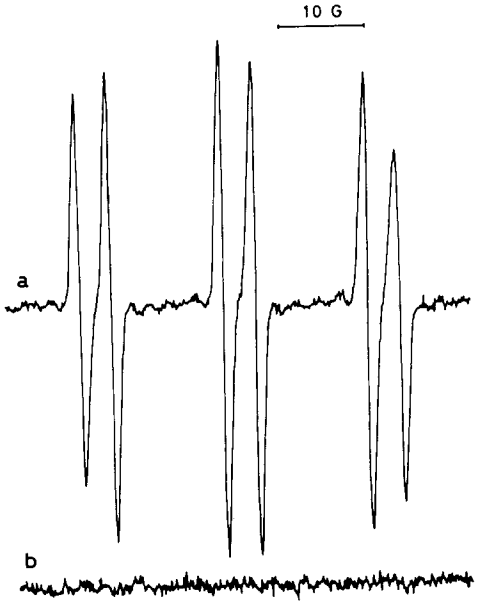

Fig. 2. The ESR spectrum of the BPN-hydroxyl radical spin-adduct obtained by carbazilquinone and NADPH-cytochrome P-450 reductase in the presence of BPN: (a) complete system, and (b) complete system + catalase. The signal was recorded after 15 min in (a) and 10 min in (b).

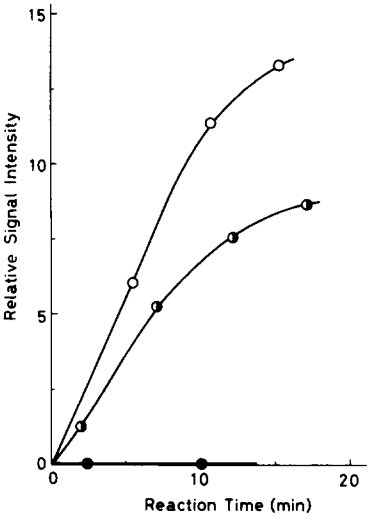

Fig. 3. Time course of the formation of carbazilquinone derived BPN-hydroxyl radical spin-adduct and the effects of superoxide dismutase and catalase. The vertical scale of relative signal intensity in Figs 3, 5, 7 and 9 is the same. ○, complete system; ◑, complete system + superoxide dismutase, and ●, complete system + catalase.

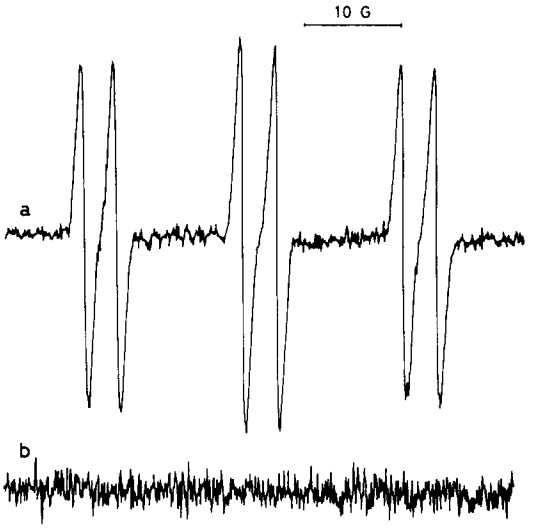

Fig. 4. The ESR spectrum of the BPN-hydroxyl radical spin-adduct obtained by mitomycin C and NADPH-cytochrome P-450 reductase in the presence of BPN: (a) complete system, and (b) complete system + catalase. The signal was recorded after 30 min in (a) and 25 min in (b).

the addition of the enzyme as indicated in Fig. 3, and consisted of a triplet of doublets with the *g*-value and hyperfone splitting constants as summarized in Table 1. As shown in Figs 2(b) and 3, the formation of the spin-adduct was completely inhibited by the addition of catalase. Superoxide dismutase decreased in the intensity of the spin-adduct (Fig. 3). The following properties were observed as to the four anticancer quinone drugs: (i) a dependence on enzyme concentration of the generation of the ESR signal and (ii) the absence of an ESR signal when either the enzyme or the drug was omitted from the system.

The ESR spectrum of BPN spin-adduct which was generated by NADPH-cytochrome P-450 reductase and mitomycin C in the presence of BPN is shown in Fig. 4(a). The signal consisted of a triplet of doublets, as in the case of carbazilquinone; the *g*-value and hyperfine splitting constants determined are shown in Table 1. The ESR absorption appeared slowly as shown in Fig. 5, and catalase completely inhibited the generation of the signal as indicated in Figs 4(b) and 5. Superoxide dismutase slightly accelerated the appearance of the signal (Fig. 5).

Table 1. The *g*-values and hyperfine splitting constants determined for radicals generated by anticancer quinone drugs and NADPH-cytochrome P-450 reductase in the presence of the spin-trap BPN

Compound	*g*-value	a^N/Gauss	a_β^H/Gauss
Carbazilquinone	2.006	16.4	3.65
Mitomycin C	2.006	16.2	3.36
Aclacinomycin A	2.006	16.2	3.39
Adriamycin	2.006	16.2	3.38

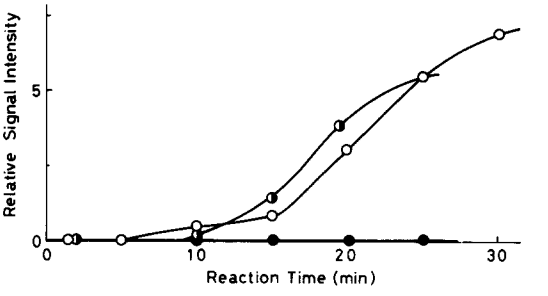

Fig. 5. Time course of the formation of mitomycin C derived BPN-hydroxyl radical spin-adduct and the effects of superoxide dismutase and catalase. The symbols are same as in Fig. 3.

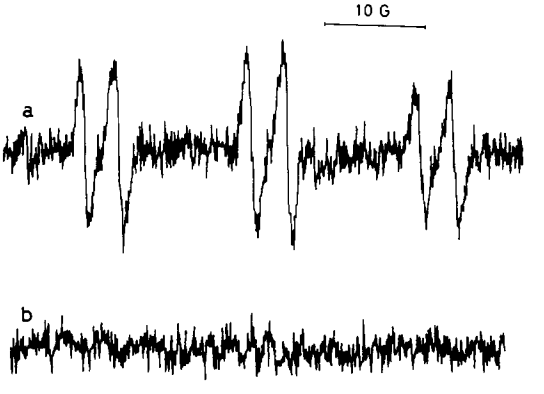

Fig. 6. The ESR spectrum of the BPN-hydroxyl radical spin-adduct obtained by aclacinomycin A and NADPH-cytochrome P-450 reductase in the presence of BPN: (a) complete system, and (b) complete system + catalase. The signal was recorded after 6 min in (a) and 5 min in (b).

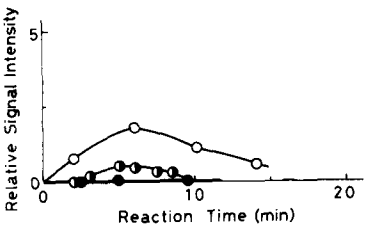

Fig. 7. Time course of the formation of aclacinomycin A derived BPN-hydroxyl radical spin-adduct and the effects of superoxide dismutase and catalase. The symbols are same as in Fig. 3.

Figure 6(a) shows the ESR result for the aclacinomycin A system; the *g*-value and hyperfine splitting constants are summarized in Table 1. In the case of aclacinomycin A, the signal intensity was the smallest among the present four anticancer quinone drugs examined, and this signal disappeared rapidly (Fig. 7). The behaviour was not changed even at a high concentration of the drug (ten times) and a high concentration of the enzyme (eight times). Catalase also inhibited the formation of the BPN spin-adduct and superoxide dismutase reduced the signal intensity (Figs 6(b) and 7).

The ESR spectrum recorded for the system containing adriamycin is shown in Fig. 8(a), and Table 1 summarizes the obtained ESR parameters. In the case of adriamycin, the signal intensity observed was stronger than that witnessed for aclacinomycin A

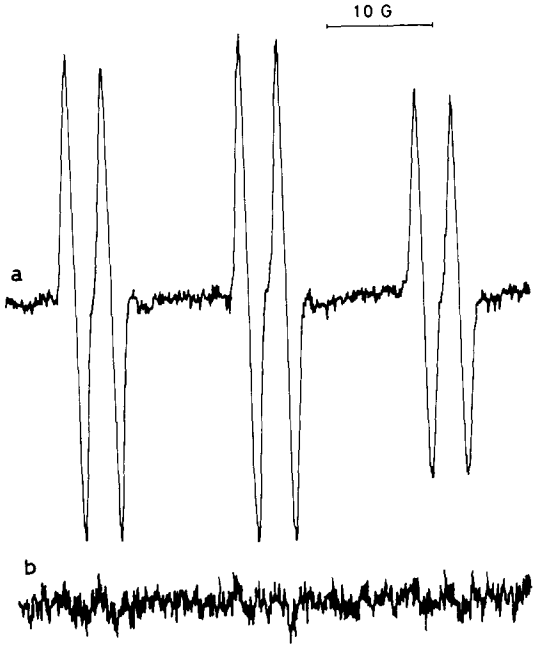

Fig. 8. The ESR spectrum of the BPN-hydroxyl radical spin-adduct obtained by adriamycin and NADPH-cytochrome P-450 reductase in the presence of BPN: (a) complete system, and (b) complete system + catalase. The signal was recorded after 13 min in (a) and 15 min in (b).

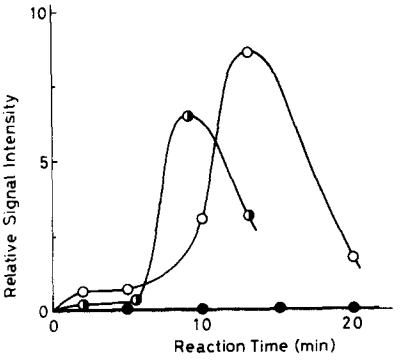

Fig. 9. Time course of the formation of adriamycin derived BPN-hydroxyl radical spin-adduct and the effect of superoxide dismutase and catalase. The symbols are same as in Fig. 3.

and its disappearance was rapid as in the case of aclacinomycin A (Fig. 9).

The results of the ethylene production from methional in the system consisting of NADPH-cytochrome P-450 reductase and the anticancer quinone drugs examined are shown in Table 2. Four anticancer quinone drugs effectively produced ethylene and the magnitude of ethylene production was in the order: adriamycin, aclacinomycin A, carbazilquinone and mitomycin C. Superoxide dismutase slightly inhibited the production of ethylene with mitomycin C and adriamycin, and slightly enhanced that with carbazilquinone and aclacinomycin A, whilst about 90% of the ethylene production was inhibited by catalase (80% with mitomycin C). Addition of both superoxide dismutase and catalase almost completly inhibited the ethylene production, and this result indicates a dependence upon free radicals, especially hydroxyl radical, for the production of ethylene in these systems. Assay system in which the drugs was absent produced 94 pmoles of ethylene.

Table 2. Production of ethylene from methional by anticancer quinone drugs and NADPH-cytochrome P-450 reductase

Assay system	Ethylene (pmoles/system)
Carbazilquinone	1320
+ superoxide dismutase	1680
+ catalase	100
+ superoxide dismutase + catalase	27
Mitomycin C	585
+ superoxide dismutase	309
+ catalase	125
+ superoxide dismutase + catalase	20
Aclacinomycin A	1380
+ superoxide dismutase	1640
+ catalase	194
+ superoxide dismutase + catalase	0
Adriamycin	2650
+ superoxide dismutase	2130
+ catalase	211
+ superoxide dismutase + catalase	22

DISCUSSION

Oxygen toxicity is an important problem for chemotherapy. In previous studies, we have suggested that the reduction of quinone drugs by NADPH-cytochrome P-450 reductase produces the respective semiquinone radicals by a one electron-transfer mechanism [13, 14]. Indeed, our ESR studies have shown the formation of semiquinone free radical of anticancer quinone drug by purified NADPH-cytochrome P-450 reductase [12], and other workers, using microsomes or a trypsin-digested enzyme, also observed ESR signals for the semiquinone radicals of anticancer quinone drugs [6, 8, 9]. However, evidence for the reduction of oxygen molecules by one electron-transfer from semiquinone and following reactions remained obscure. In studies of anticancer quinone drugs, Lown *et al.* [7] reported the generation of hydroxyl radicals from chemically reduced mitomycin C using BPN as a spin trap, and Kalyanaraman *et al.* [9] detected the generation of superoxide using both BPN and 5,5-dimethyl-1-pyrroline-1-oxide as trapping agents in the microsome-mitomycin C and daunomycin system.

As evidenced by the two methods in this paper, purified NADPH-cytochrome P-450 reductase and the anticancer quinone drugs, carbazilquinone, mitomycin C, aclacinomycin A and adriamycin effectively generated the hydroxyl radical. In the ESR study, the time for the signal appearance among four anticancer drugs corresponds well to the effectiveness of the substrate towards NADPH oxidation catalyzed by NADPH-cytochrome P-450 reductase [14]. Although the a^N and a_β^H values determined (Table 1) were somwhat higher than previously reported values [20], observed parameters fall within the range expected for the nitroxide radical formed by spin-trapping the hydroxyl radical. Indeed, ESR parameters of nitroxide radicals are known to be solvent dependent [20, 21], and it is possible that the observed signal is an ethanol-derived radical adduct, since such an adduct is also feasible in this system.

In both our ESR and ethylene-production studies, the generation of hydroxyl radical was potently inhibited by catalase, and mildly affected by superoxide dismutase. From these results, the following reaction mechanism is proposed for the present system.

$$E_{ox} + NADPH + H^+ \rightarrow E_{red} + NADPH^+ \quad (1)$$
$$E_{red} + Q \longrightarrow E_{ox} + Q^{\cdot-} \quad (2)$$
$$Q^{\cdot-} + O_2 \longrightarrow Q + O_2^- \quad (3)$$
$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (4)$$
$$Q^{\cdot-} + H_2O_2 \longrightarrow Q + OH^- + OH\cdot \quad (5)$$

Here, E_{ox}, E_{red}, Q and $Q^{\cdot-}$ indicate the oxidized and reduced forms of the enzyme, and the quinone and semiquinone forms of the quinone drug, respectively. Reactions (1)–(4) are clearly consistent with the results of this study and previous works [7, 10, 12–14, 22]. Reaction (5) is not as common as either the Fenton type or Harber–Weiss reactions, but such a preferential possibility has been indicated by others [23].

For the four anticancer drugs examined the order of ESR signal intensity observed in our studies was different from that of the ethylene production, and this order did not simply reflect the substrate reactivity towards the enzyme [14]. In each method, the detection of hydroxyl radical depends upon a different mechanism, so the results in this study may reflect differences in these mechanisms. Superoxide dismutase affected in two ways: (i) in the ESR study, the acceleration of the spin-adduct formation and reducing the signal intensity, and (ii) in the ethylene production, enhanced production of ethylene and weak inhibition. In both methods, the first effects are explained in terms of enhanced production of hydrogen peroxide by the enzyme. The second effects may be the result of the balance of reactions (3)–(5), i.e. acceleration of reaction (4) by the enzyme thereby reducing the concentration of superoxide anion and accelerating reaction (3), hence less formation semiquinone form of the drug will proportionally prevent formation of hydroxyl radical by reaction (5). Further studies are clearly necessary. Of special interest are the rapid disappearance of the spin-adduct in systems contained the anthracycline antibiotics aclacinomycin A and adriamycin, and the low ESR signal intensity witnessed for aclacinomycin A. Although the sugar or aglycone moiety of the drug is likely to have an effect, such as radical scavengers, on the present rapid disappearance of the signal in anthracyclines and on weak signal in aclacinomycin A, further investigations would be required for the definitive explanation.

There are reports concerning the semiquinone radical-dependent effect such as anthracycline binding to nucleic acid [24], and the oxygen-dependent effects such as the cytotoxicity of anticancer drugs [25, 26], the cardiac toxicity of anthracyclines [1, 5, 27–29], and the strand scission of DNA by anticancer quinone drugs [3, 4, 11]. In addition to NADPH-cytochrome P-450 reductase, the anticancer quinone drugs interacted with some of enzymes which are active on quinone compounds, such as DT-diaphorase (EC 1.6.99.2) in soluble fraction, mitochondrial NADH dehydrogenase (EC 1.6.99.3), and xanthine oxidase (EC 1.2.3.2) [12–14, 30]. The semiquinone radical of the drugs and the oxygen free radicals generated by these enzymes through the reactions (1) to (5) may thus contribute to the radical-related actions of such anticancer quinone drugs *in vivo*.

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