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Inhibition of autoxidation of egg yolk phosphatidylcholine in homogeneous solution and in liposomes by oxidized ubiquinone

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The aim of this study was to obtain a quantification of the antioxidant activity of ubiquinone. To this purpose the oxidation of egg yolk phosphatidylcholine both in solvent and in liposomes initiated by an azocompound has been studied either in the absence or in the presence of ubiquinone-3, using α-tocopherol as a reference antioxidant. The two experimental systems gave similar results. In the presence of ubiquinone-3 the oxidation rate was reduced with respect to control experiments but was faster than that in the presence of α-tocopherol. The amount of ubiquinone required to decrease the autoxidation rate was so high as to prevent detection of the induction period. The stoichiometric factor was greater than 2 and the rate constant of inhibition was two orders of magnitude lower than that of α-tocopherol. It is concluded that high concentrations of ubiquinone are required to exhibit significant antioxidant activity. A possible mechanism compatible with the stoichiometric factor larger than 2 for the inhibiting effect of ubiquinone is also suggested.

Introduction

There is evidence, derived from experiments on mitochondrial [1], submitochondrial [2] and model [3] membranes that Q may act as an antioxidant in addition to its numerous other roles in energy metabolism. This protective function might be important since Q is increasingly employed as a therapeutic agent in a great number of disease states where free radical toxicity may be involved [4].

In our previous works [5,6] the effect of physiological concentrations of ubiquinones on liposome peroxidation induced by ultrasonic irradiation or iron salts has been shown. The experimental results have demonstrated that ubiquinones exert a protective effect against peroxidation. However, it was not possible to obtain a quantification of the antioxidant activity or to understand the mechanism of the antioxidant action because of the poorly controlled experimental parameters. To obtain quantitative data on the kinetics of the autoxidation, both in the presence and absence of an inhibitor, the autoxidation chain reaction must be initiated at a uniform and reproducible rate. Initiators fulfilling this requirements are azocompounds which decompose thermally even at relatively low temperatures, i.e., at nearly physiological conditions. An azoderivative particularly indicated for autoxidation studies of liposomes is DMVN, which, owing to its lipophilicity, gives rise to radical chain initiation within the lipid bilayer [7].

In the present work the antioxidant activity of a representative ubiquinone was investigated both in an organic solution of egg PC and in multilamellar liposomes. The ubiquinone chosen was Q_3, since its side-chain length is similar to that of tocopherol and its concentration in the hydrophobic phase of multilamellar liposomes shows a linear dependence with the amount of quinone added during the preparation. Ubiquinones with a long side-chain, such as ubiquinone-9, show a sigmoidal dependence instead [8].

Materials and Methods

Egg PC was purchased from Lipid Products (Redhill, U.K.) and was stored as a solution in chloroform at −20 °C under nitrogen. The composition of fatty acids in egg PC was determined by gas-liquid chromatog-

Abbreviations: Q, ubiquinone; Q_3, ubiquinone-3; egg PC, egg-yolk phosphatidylcholine; α-T, α-tocopherol; DMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); LOOH, lipid hydroperoxide; AH, phenolic antioxidant.

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raphy after hydrolysis and esterification with HCl-ethanol [9] and was found to contain (in %): 16 : 0, 28.1; 16 : 1, 2.2; 18 : 0, 11.4; 18 : 1, 28.0; 28 : 2, 13.8; 20 : 4, 3.2; 22 : 4, 0.36; 22 : 4, 0.36; 22 : 5, 1.33; and 22 : 6, 2.69.

Q_3 was kindly supplied by Eisai (Tokyo, Japan) and stored as a 10–20 mM solution in ethanol at $-20\,^{\circ}C$. DMVN was from Polysciences (Warrington, PA) and α-T from Sigma (St. Louis, MO). The lipid-soluble initiator, DMVN, was stored as a 0.8 M solution in benzene at $-20\,^{\circ}C$ in the dark. All other chemicals of the highest available purity were from Merck. Phosphate buffer (0.01 M, pH 7.4) containing 10^{-5} M Na_2EDTA was passed through a Chelex 100 column (BioRad), 200–400 mesh, sodium form, to remove any trace of metal ions. The rate of oxidation in CCl_4 solution was determined by monitoring the oxygen concentration with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). The reaction mixture (3 ml) contained 16 mM egg PC, 13 mM DMVN and, when present, either α-T (7 or 14 μM) or Q_3 (16 or 72 μM). The reaction vessel was carefully temperature controlled at $40\,^{\circ}C$ and maintained in the dark. The total oxygen concentration in air-saturated CCl_4 at $40\,^{\circ}C$ was taken as $2.4 \cdot 10^{-3}$ M [10].

Multilamellar liposomes were prepared in a round-bottom tube by adding in the following order: DMVN and, when present, Q_3 or α-tocopherol followed by egg PC. After each addition the solvent was carefully removed with a stream of nitrogen in ice. The thin film obtained after evaporation was vortex stirred for 10 min with an aliquot of 0.01 M phosphate buffer (pH 7.4) in order to obtain 4 mM DMVN, 72 μM, 288 μM Q_3 or 16 μM α-tocopherol and 15 mM egg PC dispersions. Appropriate blanks lacking the thermal initiator were also prepared. The reaction vessels were immersed in a water bath at $37\,^{\circ}C$ and kept under air in the dark. Liposome autoxidation was measured by determining spectrophotometrically conjugated diene formation. Liposome aliquots were dissolved in ethanol and spectra in the wavelength range 200–320 nm were recorded. The increase in absorbance at 233 nm ($\varepsilon = 28\,000\ M^{-1} \cdot cm^{-1}$) [11] was taken as an indication of the appearance of conjugated dienes. Autoxidated samples were read against a blank containing the same amount of liposomes lacking the initiator and maintained in the same experimental conditions. The Q_3 amount remaining in the egg PC liposomes during the course of oxidation was extracted from liposomes [12] and analyzed by HPLC using an Altex ODS 5 μm column (15 × 0.46 cm). The column was eluted with water/methanol (1 : 9, v/v) containing 0.2% of 70% perchloric acid, at a flow rate of 1.5 ml/min. Detection was at 275 nm.

Results and Discussion

The autoxidation of egg PC has been studied both in homogeneous solution and in model membranes [13,14]. It has been shown that, when an azothermal initiator is present, the reaction proceeds via a radical chain mechanism, as exemplified in Eqns. 1–6:

Initiation

$$R\text{–}N{=}N\text{–}R \xrightarrow{k_i} N_2 + (1-e)R_2 + 2eR^{\cdot} \quad (1)$$

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \quad (2)$$

$$ROO^{\cdot} + LH \rightarrow ROOH + L^{\cdot} \quad (3)$$

Propagation

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (4)$$

$$LOO^{\cdot} + LH \xrightarrow{k_p} LOOH + L^{\cdot} \quad (5)$$

Termination

$$2LOO^{\cdot} \xrightarrow{k_t} \text{Non-radical products} \quad (6)$$

Where R–N=N–R is the azoinitiator, LH represents polyunsaturated fatty acid residues of egg lecithin, and $L^{\cdot}$ and $LOO^{\cdot}$ are alkyl and alkylperoxyl radicals generated therefrom.

In these equations e represents the efficiency of the initiation step and k_p and k_t are the rate constants for propagation and termination of the radical chain, respectively. When the rate of initiation is constant, i.e., when the new chains are started at a constant rate, the steady-state approximation holds and the rate of oxidation is given by Eqn. 7:

$$-\frac{d[O_2]}{dt} = \frac{d[LOOH]}{dt} = k_p(R_i/2k_t)^{1/2}[LH] \quad (7)$$

where R_i is the rate of chain initiation.

In the presence of a phenolic chain breaking antioxidant the chain length is reduced and termination occurs by reaction of the peroxyl radicals with the antioxidant itself, Eqns. 8,9:

$$LOO^{\cdot} + AH \xrightarrow{k_{inh}} LOOH + A^{\cdot} \quad (8)$$

$$(n-1)LOO^{\cdot} + A^{\cdot} \rightarrow \text{Non-radical products} \quad (9)$$

where n represents the stoichiometric factor for the antioxidant, i.e., the number of radicals trapped by each molecule of inhibitor. Under these conditions the rate of oxidation is expressed by Eqn. 10:

$$-\frac{d[O_2]}{dt} = \frac{d[LOOH]}{dt} = \frac{k_p R_i[LH]}{nk_{inh}[AH]} \quad (10)$$

and the rate of disappearance of the inhibitor by Eqn. 11:

$$-\frac{d[AH]}{dt} = k_{inh}[LOO^{\cdot}][AH] = \frac{R_i}{n} \quad (11)$$

Integration of the last equation gives:

$$[AH]_0 - [AH]_t = R_i t/n \qquad (12)$$

R_i can therefore be easily determined by measuring the duration of induction period, τ, i.e., the length of time in which the peroxidation of the lipid is strongly reduced by a known amount of the inhibitor.

Autoxidation in homogeneous solution

When the lipid substrate has two double bonds (linoleic chains) peroxidation occurs at the bisallylic hydrogens and the resulting lipid hydroperoxides, LOOH, are conjugated dienes absorbing in the UV spectrum. Therefore, the rate of oxidation can be determined by measuring either the rate of oxygen uptake or the rate of conjugated diene formation [14]. In the case of egg PC, which contains fatty acid residues with three or more double bonds, other products resulting from intramolecular cycloaddition of the peroxyl radical can be formed [15]. Therefore, the rate of oxygen uptake and that of conjugated dienes formation does not provide equivalent results. Nevertheless, it has been shown [14] that the two rates vary linearly, although the latter is always smaller than the rate of oxygen consumption. This has been confirmed by studying the initiated autoxidation of egg PC in CCl_4 at 40 ° C. By measuring both the rate of oxygen consumption electrochemically and the rate of conjugated diene formation spectrophotometrically we obtained straight lines (see Fig. 1). However, in the latter case the slope was smaller by a factor of 2.9, this meaning that only approx. 1/3 of the oxidation products are peroxides containing two conjugated double bonds. Hence, the two methods give equivalent results, provided a suitable correction factor is taken into account. All other experiments performed in CCl_4 were carried out by measuring the oxygen uptake.

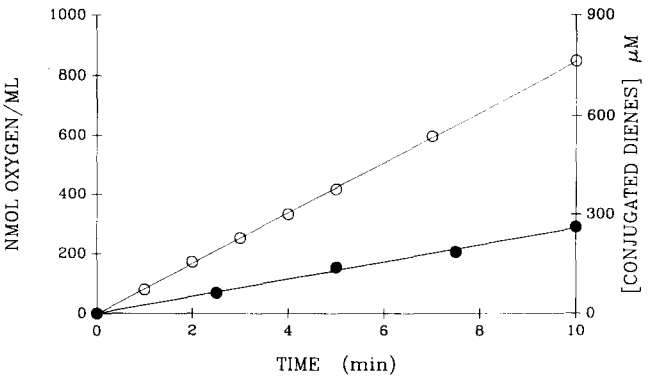


Fig. 1. Rates of oxygen uptake (○) and conjugated diene formation (●) in CCl_4 during autoxidation of 16 mM egg PC initiated by 13 mM DMVN.

At first, the rate of initiation, R_i, was determined by measuring the induction period τ of solutions containing known amounts of α-T (see Fig. 2a) for which a stoichiometric factor $n = 2$ was assumed [16]. The efficiency of the initiation step, $e = R_i/2k_i[DMVN]$, was also determined as 0.639 from the known rate, k_i, of DMVN decomposition [17].

From the trace of Fig. 2a the oxidizability, $k_p/(2k_t)^{1/2}$ and the chain length, v, were also determined (see Table I). These values, i.e., 0.29 $M^{-1/2} \cdot s^{-1/2}$ and 21 respectively, are in good agreement with those measured by Barclay and Ingold for initiated autoxidation of egg PC in chlorobenzene [13]. From the slope of the oxygen uptake traces in the presence of tocopherol the ratio k_{inh}/k_p could be derived as $6.7 \cdot 10^2$ from Eqn. 10. As far as we know there are no reports on the determination of the propagation rate constant for the oxidation of egg PC; therefore, no absolute value of the inhibition rate constant can be given. Since, however, we are interested in obtaining the relative efficiency of Q as inhibitor with respect to α-T, the ratio

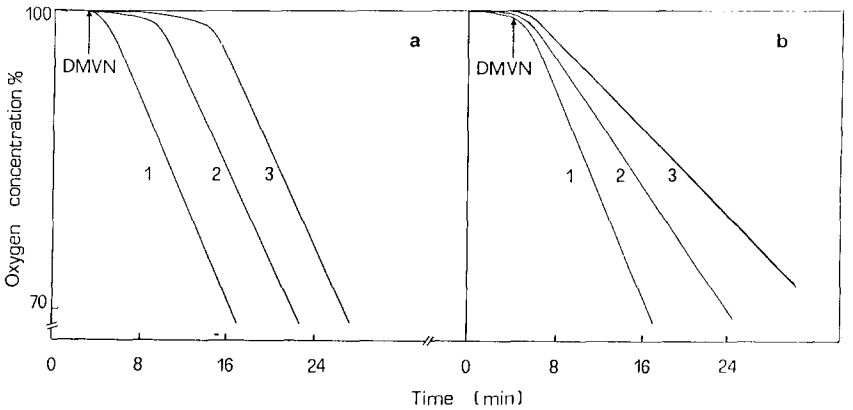


Fig. 2. Inhibition of oxidation of egg PC in CCl_4 by α-T (a) and Q_3 (b) at 40 ° C. (a) 1, uninhibited reaction; 2, inhibited by 7 μM α-T; 3, inhibited by 14 μM α-T. (b) 1, uninhibited reaction; 2, inhibited by 72 μM Q_3; 3, inhibited by 288 μM Q_3. The DMVN concentration was 13 mM.

TABLE I

Autoxidation of egg PC in CCl_4 and in liposomes initiated by DMVN [a] in the presence and in the absence of α-T and Q_3

System	Antioxidant	$R_i(M \cdot s^{-1})$	e	$k_p(2k_t)^{-1/2}(M^{-1/2} \cdot s^{-1/2})$	v	n	k_{inh}/k_p
CCl_4	none	–	–	0.29	21	–	–
	7 μM α-T	$5.1 \cdot 10^{-8}$	0.63	–	–	2 [b]	$6.7 \cdot 10^2$
	72 μM Q_3	–	–	–	12	approx. 7	8.7
Liposomes [c]	none	–	–	$5.2 \cdot 10^{-3}$ [d]	24 [d]	–	–
	16 μM α-T	$4.9 \cdot 10^{-8}$	0.043	–	–	–	$3.1 \cdot 10^2$ [d]
	72 μM Q_3	–	–	–	17 [d]	3.7 [e]	2.5
	288 μM Q_3	–	–	–	11 [d]	3.7 [e]	1.6

[a] The DMVN concentration was 13 mM in CCl_4 and 4 mM in liposomes.
[b] Assumed.
[c] Rates are expressed for the hydrophobic bilayer rather than for the total solution.
[d] Determined from the rate of conjugated diene formation in the assumption that the ratio $(-d[O_2]/dt)/(d[LOOH]/dt)$ is 2.9 as in CCl_4.
[e] Average value for the two concentrations of Q_3.

$k_{inh}(\alpha\text{-T})/k_{inh}(Q)$ is equivalent to the ratio between k_{inh}/k_p for the two antioxidants.

The autoxidation of egg PC was then studied in the presence of Q. The trace obtained by using 72 μM Q_3 (Fig. 2b) shows that oxygen consumption, although reduced with respect to the control experiments, is faster than that in the presence of α-T. Moreover, the amount of quinone required to observe a significant decrease of $-d[O_2]/dt$ is so high as to prevent detection of the induction period. From the plot of Fig. 2b the average length of the radical chain, $(-d[O_2]/dt)/R_i$, in the presence of Q_3 was calculated as 12. This value, compared to that of 21 in control experiments, suggests that quinone shows some antioxidant properties. In order to clarify the mechanism by which Q_3 can behave as an antioxidant it is important to know its stoichiometric factor, n. Since the induction period could not be detected, n was calculated from Eqn. 12 by measuring the amount of unreacted quinone at different times, and was found to be about 7. Repeated experiments confirmed this very high value. The ratio k_{inh}/k_p was then calculated as 8.7, i.e., nearly 80-times less than that of tocopherol.

Autoxidation in liposomes

The inhibiting efficiency of Q_3 was then studied in model membranes. To this purpose the measurements were repeated in egg PC vesicles. In these experiments all rates were calculated by using concentrations expressed for the hydrophobic bilayer rather than for the total solution, by assuming an average molecular weight of 800 and a density of 0.8 for egg PC.

R_i was determined by measuring the induction period of liposomes containing 16 μM α-T (see Fig. 3), and found to be $4.9 \cdot 10^{-8}$ $M \cdot s^{-1}$. The efficiency of the initiation step, i.e., 0.043, was much lower than in CCl_4. Nevertheless, this low efficiency is consistent with that found in model membranes for other azoinitiators [13,14,18]. The plot of Fig. 3 referring to the oxidation of control liposomes may allow the determination of the average length of the radical chains, v, and of the oxidizability, $k_p/(2k_t)^{1/2}$, in the assumption that the ratio between $-d[O_2]/dt$ and d[LOOH]/dt (rate of conjugated diene formation) is the same as in CCl_4 solution, i.e., 2.9. By doing that, we found 24 for v and $5.2 \cdot 10^{-3}$ $M^{-1/2} \cdot s^{-1/2}$ for the oxidizability. The latter value is about 3-times smaller than that found by monitoring the rate of oxygen uptake by Barclay and Ingold [13] and about 5-times smaller than that found by Niki et al. [14]. This means that the ratio between the rate of oxygen uptake and conjugated diene formation is larger in liposomes than in CCl_4.

The autoxidation was then repeated in liposomes containing 72 and 288 μM Q_3, concentrations close to physiological values. In fact, 72 μM is about one half of the physiological content of the quinone in mito-

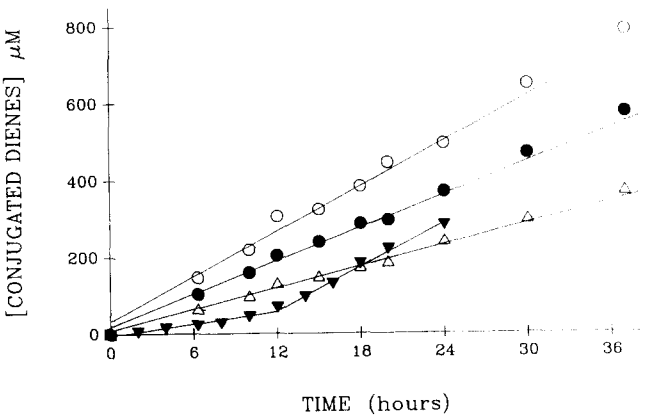


Fig. 3. Inhibition of oxidation of egg PC liposomes by α-T and Q_3 at 37°C. Oxidation was induced by 8 mM DMVN. ○, control liposomes; ●, 72 μM Q_3 containing liposomes; △, 288 μM Q_3 containing liposomes; ▼, 16 μM α-T containing liposomes.

TABLE II

Time dependence of Q_3 consumption during liposome peroxidation initiated by 4 mM DMVN

Q_3 was exctracted from liposomes according to Takada et al. [12] and analyzed by HPLC (see the section Materials and Methods).

Time (h)	72 μM Q_3	288 μM Q_3
0	72	288
6	71	290
12	68	276
20	62	273
24	58	271
31	55	261
37	53	249

chondria, where the quinone/lipid ratio is about 1 : 100. Under these experimental conditions the rate of conjugated diene formation showed a substantial decrease with respect to control experiments. However, autoxidation proceeded with the same rate even after 36 h, this meaning that complete consumption of the inhibitor did not occur, as previously observed in CCl_4. We therefore measured the amount of unreacted quinone along the amount of dienes formed at each time, as reported in Table II. From these data the stoichiometric factor, n, for the inhibitory effect of Q_3 could be calculated by means of Eqn. 12, as 3.7 ± 0.7. This value is smaller than in CCl_4, but still remarkably high. In the presence of quinone the chain length is reduced from 24 to 17 and 11 for the two concentrations of Q_3. These values, although subject to large errors, show that Q exerts some inhibitory effect on peroxidation also in model membranes.

To better quantify this inhibitory effect the ratio k_{inh}/k_p for Q_3 and α-T was calculated by measuring the amount of conjugated dienes formed after a time t by using the integrated form of Eqn. 10, i.e., Eqn. 13:

$$[LOOH]_t = -\frac{k_p[LH]}{k_{inh}} \ln\left(1 - \frac{t}{\tau}\right) \quad (13)$$

The inhibition time, τ, which is obtained straightforwardly as 12 h in the case of α-T, could not be directly determined for the Q, as mentioned above. Therefore, the value of τ was extrapolated at zero inhibitor concentration by measuring the amount of residual quinone at various times. For the 72 μM and 288 μM Q_3-containing vesicles τ resulted to be 152 and 324 h, respectively. The ratio k_{inh}/k_p was then determined a 2.5 and 1.6, respectively. This means that k_{inh} for Q_3 in liposomes is approx. 150-times smaller than that of α-T.

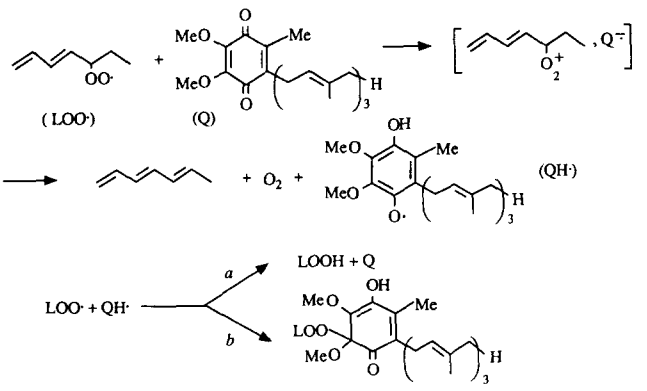
Mechanism of inhibition

It is apparent from the experimental data discussed in the previous section that ubiquinones may act as moderately effective antioxidants when used in concentrations close to the physiological values. However, a major question about the antioxidant role of ubiquinones concerns the mechanism by which these molecules inhibit the lipid peroxidation, since they do not bear easily abstractable hydrogens as the great majority of biological as well as chemical antioxidants. A possible explanation of this effect might be found on the low reduction potential of quinones. In fact, it is known that peroxyl radicals having next to their OO$^{\cdot}$ group a heteroatom with a lone electron pair or a double bond may give, with a variety of reactants characterized by low reduction potentials including quinones, an electron transfer reaction followed by a rapid elimination of a proton and an oxygen molecule [19].

We therefore suggest for the inhibiting action of Q_3 the mechanism exemplified in Scheme I.

Initially the peroxyl radical reacts with the quinone by electron transfer and subsequent deprotonation to give a semiquinone radical. For the sake of clarity, this reaction is depicted in the scheme as two distinct steps, but it may occur in a single step if the process is a dissociative electron transfer. The semiquinone radical may then react with a second peroxyl radical following two different routes: (a) disproportionation to regenerate the starting quinone and a hydroperoxide or (b) radical combination to give a cyclic diene.

The stoichiometric factor for the inhibitory effect of Q_3 equals infinity in the former case since no quinone molecules are consumed. If radical combination takes place the stoichiometric factor is 2. Therefore, depending on the relative importance of paths a and b, n may assume any value ranging from 2 to infinity. This mechanism can rationalize both the inhibitory effect of oxidized Q and the high stoichiometric factor. At present, however, we have no other experimental proof which could substantiate the above mechanism.



Scheme I.

Conclusions

The present results show that Q in its oxidized form, acts as an inhibitor of the autoxidation of egg PC, both in homogeneous solution and in model membranes. We hypothesize that the action of ubiquinone is that of a chain-breaking antioxidant, which intercepts the chain-carrying lipid peroxyl radical, thus reducing the chain length and preventing further peroxidative damage. Its inhibition rate constant is, however, about two orders of magnitude lower than that of α-T. Thus, high concentrations of Q_3 are required to exhibit significant antioxidant activity. Although the results of in vitro experiments cannot be applied directly to in vivo systems, these basic studies may provide important and quantitative information and serve as a basis for further investigations for understanding of the antioxidant role of ubiquinone.

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