Detection of Menaquinone-6 and a Novel Methyl-substituted Menaquinone-6 in *Campylobacter jejuni* and *Campylobacter fetus* subsp. *fetus*

By GEORGE M. CARLONE¹*† AND FRANK A. L. ANET²

¹Department of Microbiology and ²Department of Chemistry and Biochemistry, University of California at Los Angeles, Los Angeles, California 90024, U.S.A.

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Menaquinone-6 (2-methyl-3-farnesyl-farnesyl-1,4-naphthoquinone) and a methyl-substituted menaquinone-6 (2,[5 or 8]-dimethyl-3-farnesyl-farnesyl-1,4-naphthoquinone) were the major isoprenoid quinones found in membrane preparations of *Campylobacter jejuni* and *Campylobacter fetus* subsp. *fetus*. By reverse-phase high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) the faster-eluting menaquinone-6 co-chromatographed with a menaquinone-6 standard. The identity of menaquinone-6 was confirmed by UV spectrophotometry, mass spectrometry and nuclear magnetic resonance (NMR) analysis. The slower-eluting methyl-substituted menaquinone-6 co-chromatographed with a menaquinone-7 standard by reverse-phase TLC but eluted between menaquinone-6 and menaquinone-7 standards by HPLC. The UV spectrum of the methyl-substituted menaquinone-6 did not correlate with either authentic menaquinone or demethylmenaquinone. Mass spectra showed an increase of 14 mass units when compared to menaquinone-6, and indicated that a methyl substituent was on the naphthoquinone nucleus. NMR spectra confirmed the presence of a methyl substituent at a peri position (carbon-5 or -8) on the benzenoid ring.

INTRODUCTION

Respiratory quinones are a class of isoprenoid lipids separated on the basis of structural differences into naphthoquinones and benzoquinones (Collins & Jones, 1981). Naphthoquinones can be further separated into two types, phyloquinones (2-methyl-3-phytyl-1,4-naphthoquinone), which are found predominantly in green plants and in bacteria (Collins & Jones, 1981; Langemann & Isler, 1965), and menaquinones (2-methyl-3-polyprenyl-1,4-naphthoquinone), which are widely distributed in nature.

Menaquinones of bacteria are lipophilic components of the cytoplasmic membrane which undergo reversible oxidation and reduction to form a quinone or hydroquinone, respectively (Kroger, 1980; Kroger & Dad'ak, 1969; Taber, 1980). In bacteria they play important roles in the transfer of electrons in the respiratory chain (Bentley & Meganathan, 1982; Taber, 1980), and may function in oxidative phosphorylation and active transport (Collins & Jones, 1981; Taber *et al.*, 1981). Menaquinones are also associated with the reduction of fumarate in microorganisms capable of using fumarate as a terminal electron acceptor (Kroger, 1980; Taber, 1980; Thauer *et al.*, 1977). Demethylmenaquinones (DMK) appear to be interchangeable with menaquinones for fumarate reduction, whereas ubiquinones are ineffective (Hollander, 1981).

In *Campylobacter jejuni* and *C. fetus* subsp. *fetus*, microaerophilic pathogens of man and animals (Smibert, 1981), fumarate may serve as a terminal electron acceptor (Carlone & Lascelles, 1982). Both species have low- and high-potential forms of cytochrome *c* and cytochrome *b* and an assortment of membrane enzymes including formate dehydrogenase and

† Present address: 1054-1 Noble Vines Drive, Clarkston, GA 30021, U.S.A.

Abbreviations: Acetone *d*₆, deuterated acetone; DMK, demethylmenaquinone; MK-*n*, menaquinone with *n* isoprene units.

fumarate reductase (Carlone & Lascelles, 1982; Harvey & Lascelles, 1980; Hoffman & Goodman, 1982). Menaquinone isoprenologues associated with these organisms have not been clearly defined and the objective of this investigation was to characterize the respiratory quinones in *C. jejuni* and *C. fetus* subsp. *fetus*, grown under a microaerobic atmosphere containing hydrogen.

METHODS

Organisms and cultural conditions. Stock cultures of *C. jejuni* (ATCC 29428) and *C. fetus* subsp. *fetus* (ATCC 27374) were maintained in fluid thioglycolate medium (BBL) and were subcultured weekly. Working cultures were derived from 24- and 48-h thioglycolate cultures, respectively, with 1.0% inocula. *Campylobacter jejuni* working cultures were grown at 37 °C and *C. fetus* subsp. *fetus* at 30 °C.

Cells were grown in campylobacteria formate/fumarate broth composed of the following, per litre: 10 g peptone (Difco), 10 g tryptone (Difco), 10 g yeast extract (Difco) and 0.1 g sodium bisulphite. Carbon sources were added to the medium before sterilization to a final concentration of 10 mM for sodium formate and 50 mM for potassium fumarate; the pH was adjusted to 7.2 with 10 M-KOH. The cultures were in 200 ml volumes, in 500 ml flasks, in an atmosphere containing (by vol.) approximately 5% O₂, 10% CO₂, 30% H₂ and the balance N₂. The inoculated flasks were placed into GasPak jars with the GasPak generating system without a catalyst (BBL) to provide this atmosphere, and the jars were shaken for 24 h on a gyratory shaker at 200 r.p.m.

Preparation of membrane fractions. Cells were harvested during late-exponential phase (22 to 26 h) by centrifugation at 4000 g for 20 min at 4 °C and then washed twice in 0.1 vol. 40 mM-Tris/HCl buffer, pH 7.5, and centrifuged at 12000 g for 10 min. Pellets were pooled and resuspended in 1 ml buffer per 100 ml culture. Cell-free extracts were prepared by two passages of cells through a French pressure cell (845 kg cm⁻²) with the addition of DNAase and RNAase (10 µg ml⁻¹).

Cell debris and unbroken cells were removed by centrifugation at 12000 g for 10 min, and the supernatant fluid was centrifuged at 126000 g for 90 min at 4 °C. The pellets were washed once with 40 mM-Tris/HCl buffer, pH 7.5, at 0 °C and suspended in this buffer containing 20% (v/v) glycerol with a polytetrafluoroethylene tissue homogenizer. The suspension was adjusted to contain 5 to 10 mg membrane protein ml⁻¹. This crude membrane fraction was kept at 0 °C until used or stored at -20 °C. The membrane fractions were rich in menaquinones, whereas cytoplasmic fractions contained only trace amounts which were attributable to unsedimented membrane fragments. All subsequent work was with crude membrane fractions.

Menaquinone extraction. Menaquinones were extracted from 1 ml of membrane preparation by a modified method of Kroger & Dad'ak (1969), using 3.6 ml methanol and 2.4 ml petroleum ether in light-protected 15 ml glass centrifuge tubes. The mixtures were shaken intermittently for 15 min at room temperature. Acetone (1 ml) was added and shaking was continued for 15 min. The upper layer was recovered after centrifugation (1400 g for 5 min) and the residue reextracted with 1 ml petroleum ether. The combined extracts were evaporated to dryness prior to menaquinone purification.

Thin-layer chromatography (TLC). Authentic standards and membrane-extracted menaquinones in acetone (5 µl) were separated on C-18 reverse-phase thin-layer plates (Analtech, Newark, Del., U.S.A.) with a solvent system of methanol/acetone (50:50, v/v). Development was complete in approximately 20 min; separated menaquinones were detected with 254 nm UV light.

High performance liquid chromatography (HPLC). Authentic standards and membrane-extracted menaquinones were dissolved in acetone and separated by HPLC using a Waters System 272 equipped with a U6K injector (Waters Associates, Inc., Milford, Mass., U.S.A.) and a model 1305 variable-wavelength detector (Bio-Rad). An RCM-100 radial compression module fitted with a Radial Pak A C-18 reverse-phase 10 µm column (Waters) was used in the separation. Menaquinones were chromatographed in a methanol/isopropanol (75:25, v/v; 2 ml min⁻¹) isocratic solvent system at room temperature. The eluent was monitored at 269 nm and collected. Purity of isolated menaquinones was determined by re-chromatography with HPLC and reverse-phase TLC.

UV absorption spectrophotometry. All spectroscopic measurements were made with a Cary 14 spectrophotometer (Applied Physics Corp., Monrovia, Ca., U.S.A.). Menaquinones purified by HPLC were evaporated to dryness and dissolved in absolute ethanol. Concentrations of menaquinone with six isoprene units (MK-6) were determined by difference spectroscopy. A molar difference absorbance coefficient ($\Delta\epsilon$) of 22.8 mM⁻¹ cm⁻¹ was applied to MK-6 using wavelength pairs of 248–254 nm (Kroger *et al.*, 1971); a coefficient was not determined for methyl-substituted MK-6. Reduction of menaquinones was achieved by the addition of 5 µl of an aqueous solution of KBH₄ (5 mg ml⁻¹, kept at 0 °C) to 1 ml of the ethanolic solution containing 5 µl 0.5 M-sodium acetate buffer, pH 5.2 (Kroger & Dad'ak, 1969).

Mass spectra. High- and low-resolution mass spectra were obtained using an AEI MS9 mass spectrometer with an acceleration potential of 8 kV and electron ionization voltages of 70 eV and 16 eV. The analyses were run at 220 °C under computer control with an automatic data acquisition system.

Fig. 1. Separation of authentic menaquinone standards and menaquinones from membrane extracts of *Campylobacter jejuni* and *C. fetus* subsp. *fetus*. Reverse-phase thin-layer chromatograms were developed in methanol/acetone (50:50, v/v). The chromatographic identities of menaquinones in B and C were based on R_F values observed on chromatograph A (authentic menaquinone standards, MK-3 to MK-10). B, menaquinones from membrane extracts of (a) *C. jejuni* and (b) *C. fetus* subsp. *fetus*. C, *C. jejuni* and *C. fetus* subsp. *fetus* menaquinones purified by HPLC: (a) MK-6 from both species were mixed and chromatographed, (b) authentic menaquinone standards MK-5, MK-6 and MK-7, and (c) methyl-substituted MK-6 from both species were mixed and chromatographed. Menaquinones were detected with 254 nm UV light.

Nuclear magnetic resonance (NMR) spectra. Proton magnetic resonance spectra were recorded on a Bruker high-resolution multinuclear spectrometer at 500 MHz. Samples were dissolved in acetone- d_6 with tetramethylsilane as an internal standard. Chemical shifts are reported in parts per million (p.p.m.) downfield from tetramethylsilane.

Protein determination. Protein was determined by a modification of the Lowry method with bovine serum albumin as the standard (Dulley & Greene, 1975).

RESULTS

Separation of menaquinones with reverse-phase TLC. The system used to establish chromatographic identity clearly and reproducibly resolved unsaturated menaquinones MK-3 to MK-10 (Fig. 1, A); no other authentic menaquinone standards were examined. Standard R_F values decreased linearly as a function of increasing isoprene unit number, and the R_F values ($\times 100$) were as follows: MK-3, 82.7; MK-4, 75.9; MK-5, 68.5; MK-6, 59.9; MK-7, 50.0; MK-8, 40.0; MK-9, 30.2; and MK-10, 22.2. Membrane extracts from *C. jejuni* and *C. fetus* subsp. *fetus* contained menaquinones that co-chromatographed with authentic MK-6 and MK-7 standards in the methanol/acetone solvent system (Fig. 1, B) and in the less polar methanol/isopropanol and the more polar methanol/acetonitrile solvent system. The identity of MK-6 extracted from membranes was verified for both organisms (details given below). In contrast, material identified chromatographically as MK-7 was found to be a methyl-substituted MK-6 (2, [5 or 8]-dimethyl-3-farnesyl-farnesyl-1,4-naphthoquinone) with six unsaturated isoprene units. The methyl-substituted MK-6 appeared to be less polar than MK-6, as indicated by the lower R_F value. Mixtures of purified MK-6 from *C. jejuni* and *C. fetus* subsp. *fetus* were separated and found to develop as a discrete spot (Fig. 1, C); this was also observed with mixtures of methyl-substituted MK-6.

Separation of menaquinones by HPLC. Mixtures of authentic menaquinone standards (MK-4 to MK-9) were separated under isocratic conditions, and chromatographed as a function of isoprene unit number. All six menaquinone standards were separated within 25 min (Fig. 2). No significant difference in selectivity or sensitivity was found when menaquinones were monitored at 269 nm (quinonoid absorbance) or 248 nm (benzenoid absorbance).

Fig. 2. High-performance liquid chromatograms of authentic menaquinone standards MK-4 to MK-9 and membrane-extracted menaquinones MK-6 and methyl-substituted *MK-6. (a, b) Membrane-extracted menaquinones from *Campylobacter jejuni* (a) alone and (b) added to a mixture of authentic menaquinone standards, MK-4 to MK-9. (c, d) Membrane-extracted menaquinones from *C. fetus* subsp. *fetus* (c) alone and (d) added to a mixture of authentic menaquinone standards, MK-4 to MK-9. Menaquinones from both *C. jejuni* and *C. fetus* subsp. *fetus* had elution times of 8.3 (MK-6) and 10.0 min (methyl-substituted *MK-6). Menaquinones were separated with a Waters C-18 reverse-phase radial compression cartridge using methanol/isopropanol (75:25, v/v; 2 ml min⁻¹) isocratically at room temperature. The eluent was monitored at 269 nm. Menaquinones MK-4 to MK-9 were resolved at the elution times of 4.5, 6.3, 8.3, 11.8, 16.8 and 24.1 min, respectively. Bar, absorbance unit; I, injection point.

Two major menaquinones, one which co-chromatographed with MK-6 and the other between MK-6 and MK-7 standards, were detected in both species of *Campylobacter* (Fig. 2). The peak eluting at 8.3 min was verified as a MK-6, and the peak eluting at 10.0 min was verified as that of methyl-substituted MK-6 (2, [5 or 8]-dimethyl-3-farnesyl-farnesyl-1,4-naphthoquinone) with six unsaturated isoprene units (details given below). From the relative peak heights in Fig. 2(a) it appeared that the two menaquinones extracted from *C. jejuni* were produced in approximately equal amounts (assuming that both menaquinones were extracted and separated with similar efficiencies), whereas *C. fetus* subsp. *fetus* had approximately five times more MK-6 than methyl-substituted MK-6 (Fig. 2c).

Fig. 3. UV absorption spectra of menaquinones in absolute ethanol. (a) MK-6 and (b) methyl-substituted MK-6. —, Oxidized menaquinones; ---, reduced menaquinones obtained after reduction of solution a, difference spectra of menaquinones (reduced vs oxidized) obtained with oxidized sample in reference cuvette and reduced sample in sample cuvette. Menaquinones were reduced by adding 5 μ l of an aqueous solution of KBH_4 (5 mg ml^{-1}) to 1 ml of the ethanolic solution, which contained 5 μ l 0.5 M-sodium acetate buffer (pH 5.2).

UV absorption spectra of menaquinones. UV spectra of ethanolic solutions of menaquinone purified from *Campylobacter* were recorded at room temperature from 220 to 340 nm (Fig. 3). Menaquinone-6 in *C. jejuni* was present at approximately 3.5 μmol (g membrane protein) $^{-1}$, whereas in *C. fetus* subsp. *fetus* MK-6 was approximately 2.3 μmol (g membrane protein) $^{-1}$.

The maxima for oxidized MK-6 and methyl-substituted MK-6 were at 242 and 248 nm (benzenoid absorbance) and 261 and 269 nm (quinonoid absorbance) (Fig. 3, solid lines). Upon reduction (dashed lines), the MK-6 spectrum changed to one characteristic of a hydroquinone (substantial loss of absorption bands at 261 and 269 nm and increased intensity at 245 nm), whereas the methyl-substituted MK-6 spectrum appeared as an intermediate to that of the reduced MK-6 spectrum. The absorption loss at 261 and 269 nm was not complete, and there was little increase at 245 nm. Isosbestic points of the difference spectra for MK-6 were at 254 and 288 nm and for methyl-substituted MK-6 at 250 and 275 nm (Fig. 3, dotted lines). The maximum absorbance decrease for both MK-6 and methyl substituted MK-6 occurred 2 min after reduction and remained constant for up to 1 h. Addition of more KBH_4 and/or acetate buffer, to ensure complete reduction of substituted MK-6, resulted in little change of the absorption.

Mass spectrometry. The molecular ions (M^+) for MK-6 and methyl-substituted MK-6 were at mass-to-charge ratios (m/e) of 580 and 594, respectively (Fig. 4). Fragmentation peaks for MK-6 (Fig. 4a) were at m/e 580 (M^+), 511, 443, 375, 307, 239, 225 and 187. For methyl-substituted MK-6 (Fig. 4b), peaks were at m/e 594 (M^+), 525, 457, 389, 321, 253, 239 and 201. Fragmentation of the polyprenyl side chains showed the loss of the terminal isoprenyl unit (69 mass units) followed by successive losses of 68 mass units. Saturation of olefinic bonds within the side chain was not observed. In Fig. 4(b) R and R_1 represent a methyl group and a proton, not necessarily respectively (details given below).

NMR spectroscopy. Table 1 and Fig. 5 summarize the data obtained from the 500 MHz proton NMR spectra of MK-6 and methyl-substituted MK-6.

DISCUSSION

Campylobacter jejuni and *C. fetus* subsp. *fetus* contained two respiratory quinones, a menaquinone-6 (2-methyl-3-farnesyl-farnesyl-1,4-naphthoquinone) and a methyl-substituted menaquinone-6 (2,[5 or 8]-dimethyl-3-farnesyl-farnesyl-1,4-naphthoquinone). Mass spectro-

Fig. 4. Mass spectra and chemical structures of membrane-extracted menaquinones. Spectra and structures of (a) MK-6 and (b) methyl-substituted MK-6. In the chemical structures shown in (b), R and R₁ are a methyl group and a proton in the peri position (not necessarily respectively). The molecular ion (M⁺) for MK-6 was at *m/e* 580 and for methyl-substituted MK-6 at *m/e* 594. The fragmentation pattern of the proprenyl side chains showed the loss of the terminal isoprenyl unit (69 mass units) followed by successive losses of 68 mass units. Olefinic bonds in the polyprenyl side chain were unsaturated. Analysis was made with an acceleration potential of 8 kV and ionization potential of 16 eV at 220 °C.

metry and NMR spectra of substituted MK-6 indicated that a methyl substituent was located on the naphthoquinone nucleus (C-5 or C-8) and that the polyprenyl side chain was unsaturated. To assign the methyl group it will be necessary to synthesize appropriate model compounds and to carry out NMR spectral comparisons.

The NMR spectrum and chemical shift assignments of MK-6 (Table 1) corresponded to other menaquinone isoprenologues obtained by previous workers (Langemann & Isler, 1965; Thomson, 1971). The menaquinones had almost symmetrically substituted naphthoquinone moieties so that the two peri aromatic protons (H-5 and H-8) had nearly the same chemical shifts as one another. The same situation also applied to the other two aromatic protons (H-6 and H-7). The spectrum of the methyl-substituted MK-6 (Table 1) was very similar to that of MK-6, except for the aromatic proton region, where there was only three protons instead of four. The splitting pattern of the aromatic protons in methyl-substituted MK-6 (Fig. 5) revealed that the substituent was a methyl group in a peri position on the aromatic ring. This methyl group caused a shielding of the three aromatic protons (H₁, H₂, and H₃), with the ortho proton (H₃) the most shielded.

The assignments of the aromatic protons were made on the basis of a large (7.5 Hz) ortho coupling constant in the signal of H₁ and H₃, and two such coupling constants in the signal of H₂. In addition, the deshielded proton signal at δ 7.97 clearly corresponded to a peri proton.

Fig. 5. Partial proton NMR spectra of menaquinones. (a) Experimental spectrum of methyl-substituted MK-6 ($R = \text{methyl}$) in the aromatic proton region. (b) Theoretical first-order spectrum corresponding to (a), but without long-range coupling to the benzenoid (R) methyl group ($J_{1,2} = J_{2,3} = 7.5 \text{ Hz}$; $J_{1,3} = 2 \text{ Hz}$). (c) Chemical shifts (mean of H_1 and H_4 and mean of H_2 and H_3) for the aromatic protons in Mk-6 ($R = H_4$). In the structure shown, R' and R'' are methyl and polyprenyl groups (not necessarily respectively).

Table 1. Chemical shifts and splitting patterns for the NMR spectra of menaquinone-6 and methyl-substituted menaquinone-6

Chemical shifts (δ) are given as p.p.m. downfield from tetramethylsilane in deuterated acetone- d_6 . Splitting patterns are denoted as follows: s, singlet; bs, broad singlet (line at half peak height about 3 to 4 Hz); d, doublet; t, triplet; and m, multiplet arising from two or more chemical shifts as well as from spin-spin coupling.

	Menaquinone-6		Substituted menaquinone-6	
	δ (p.p.m.)	Pattern	δ (p.p.m.)	Pattern
Methyl groups (<i>trans</i> to olefinic-H)	1.544	bs	1.534	bs
	1.560	bs	1.547	bs
	1.584	bs	1.575	bs
	1.592*	bs	1.581*	bs
End-of-chain methyl (<i>cis</i> to olefinic-H)	1.650	bs	1.640	bs
Methyl (1st isoprene unit)	1.816	bs	1.794	bs
Allylic CH_2 groups	1.944–2.030	m	1.889–2.025	m
Benzenoid methyl	—	—	—†	—
Quinonoid methyl	2.184	s	2.165	s
Bisallylic CH_2	3.405	d‡	3.356	d‡
Olefinic CH groups	5.028–5.152	m	5.016–5.120	m
Aromatic-H (C-6, C-7)	7.82§	m	7.593	d
			7.645	t
Aromatic-H (C-5 and/or C-8)	8.06§	m	7.970	d

* Intensity corresponds to two methyl groups.

† The benzenoid methyl group was obscured by solvent lines ($\text{CD}_3\text{COCHD}_2$).

‡ $J(\text{CH}_2\text{-olefinic-H}) = 7.0 \text{ Hz}$.

§ Mean chemical shift.

|| Pattern caused by J_{ortho} (7.5 Hz).

This was consistent with aromatic chemical shifts in MK-6 (Fig. 5) and the known shielding effects of a methyl group on aromatic protons (Jackman & Sternhell, 1969). Also consistent with this assignment was the presence of a small (2 Hz) meta coupling of H_1 with H_3 . In the case of H_3 , the splitting was obscured by the presence of an expected long-range coupling to the methyl group (Jackman & Sternhell, 1969). Because of the solvent (acetone- d_6) used in the NMR

measurement, the methyl group on the aromatic ring was not directly visible in the NMR spectrum of substituted MK-6. However, the evidence presented unambiguously defined the peri placement of this group, although a differentiation between the two peri positions was not possible.

The intermediate reduction observed with UV absorption spectrometry may have resulted from incomplete quinone reduction. Electron-donating groups such as methyl and hydroxy tend to stabilize the oxidized (quinone) form of the naphthoquinone nucleus relative to the reduced (hydroquinone) form (Streitwieser & Heathcock, 1976). The electron-rich menaquinone, therefore, is not easily reduced and results in a lowering of the redox potential; the opposite effect is observed with electron-attracting groups. The methyl of substituted MK-6 (C-5 or C-8) probably stabilized the quinone and made it less easily reduced. This probably made the redox potential of substituted MK-6 lower than that of MK-6. A difference in redox potential may allow the menaquinones to participate in diverse cellular processes.

Separation of menaquinones by reverse-phase TLC is based primarily on the length and degree of unsaturation (Collins *et al.*, 1980; Collins & Jones, 1981; Dunphy & Brodie, 1971). However, when comparing R_F values of MK and DMK having the same polyprenyl side chain length, the addition of a methyl group to C-2 of DMK results in a decreased R_F value (Dunphy & Brodie, 1971). In this study, the substitution of a second methyl group on C-5 or C-8 of MK-6 also decreased the R_F value. With the solvent systems used for reverse-phase TLC, methyl-substituted MK-6 was not adequately separated from MK-7, whereas with reverse-phase HPLC, both were separated.

Menaquinone identity may be ambiguous when only reverse-phase TLC and UV spectrophotometry are used for identification. Additional techniques were required to characterize the novel quinone found in this study. Substituted MK-6 as well as other substituted menaquinone isoprenologues may, therefore, be more prevalent than reported to date, since many earlier studies have relied solely upon reverse-phase TLC and UV spectrophotometry. For accurate menaquinone identification, reverse-phase HPLC, UV spectrophotometry, mass spectroscopy and NMR analyses are needed.

Preliminary extraction and reincorporation experiments with *C. jejuni* and *C. fetus* subsp. *fetus* indicated that MK-6 and methyl-substituted MK-6 are obligatory components of the NADH:fumarate oxidoreductase system. An investigation of the hydrogen-fumarate and formate-fumarate oxidoreductase systems is in progress.

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