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A Kinetic Study of the Self-degradation of *o*-Carboranylalanine to *nido*-Carboranylalanine in Solution

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Dedicated to Professor Göran Bergson on the occasion of his 65th birthday

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The spontaneous degradation of racemic *o*-carboranylalanine [3-(1,2-dicarba-*closo*-dodecaboran(12)-1-yl)-2-aminopropanoic acid], a carborane analogue of the amino acid phenylalanine, to the corresponding diastereomeric, racemic pairs of *nido*-carboranylalanine [containing the dodecahydro-7,8-dicarba-*nido*-undecaborate (−1) cage] has been studied in buffered water–methanol solution at three temperatures. The parent compound and the two diastereomeric reaction products were separated off-line by liquid chromatography.

The reaction was found to be first order with respect to *o*-carboranylalanine. The rate is pH dependent with a broad maximum around pH 5, where the zwitterion form predominates. Rate constants and activation parameters for the formation of the two *nido*-compounds were determined. The activation entropies close to zero (0 and 3 J K^{-1} mol^{-1}, respectively) show that the loss of one boron atom from *o*-carboranylalanine is a unimolecular process.

Amino acids substituted with polyhedral boron clusters are presently of considerable interest for use in boron neutron capture therapy (BNCT) of cancer, owing to their high boron content.[1–3] Carborane analogues of phenylalanine such as *o*-carboranylalanine and *p*-carboranylalanine are of particular interest.[1–5] It has previously been shown that *o*-carboranylalanine[4] and 2-methyl-*o*-carboranylalanine[5] spontaneously undergo degradation to the corresponding diastereomeric *nido*-analogues (**1** and **2**) in methanol or water solutions according to Scheme 1. The higher homologues [5-(1,2-dicarba-*closo*-dodecaboran(12)-1-yl)-2-aminopentanoic acid (**3**) and 5-(2-methyl-1,2-dicarba-*closo*-dodecaboran(12)-1-yl)-2-aminopentanoic acid (**4**)] were stable under these conditions. Crossover experiments with mixtures of **3** or **4** with *o*-carboranylalanine resulted only in the degradation of the latter.[4]

For *o*-carboranylalanine the reaction was studied in some detail.[4] The kinetics of the degradation was studied by nuclear magnetic resonance (NMR) spectroscopy and the rate was found to depend on the pH of the solution. The spectra clearly showed the formation of the two diastereoisomers of the *nido*-compounds. Experiments showed that the degradation is intramolecular but it was not possible to determine the individual rate constants with such accuracy that activation parameters could be determined. Chromatographic separation of the reaction mixture would be needed to achieve this goal.

The reaction can be quenched by strong acidification. Porous graphitic carbon (PGC) is one of the few packing materials for chromatographic columns compatible with strongly acidic samples and mobile phases. The PGC surface is composed of flat carbon sheets, has no functional groups and is highly hydrophobic.[6] Aqueous acetonitrile with addition of trifluoroacetic acid (TFA) has been shown to give fast separations of closely related ionic compounds on PGC[7,8] and this mobile phase also separated the present reaction mixture.

The *o*-carboranylalanine and its *nido*-analogues can be detected and quantified without derivatization by combining liquid chromatography (LC) with inductively coupled plasma atomic emission spectrometry (ICP-AES) and monitoring a boron atomic emission line. However, coupling of a chromatographic system to an ICP is not straightforward. The plasma has a low tolerance towards high contents of organic solvents in the

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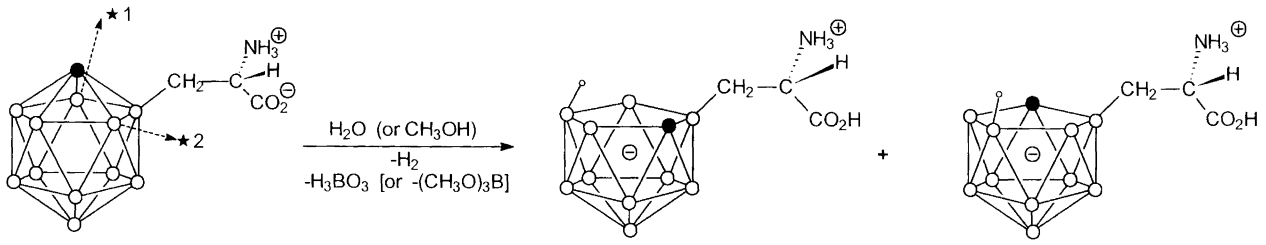


o-carboranylalanine *nido*-carboranylalanine 1 *nido*-carboranylalanine 2

● = CH ○ = C ○ = BH o = H

★1 and ★2 represent the diastereotopic boron atoms that are removed in the formation of the *nido*-carboranylalanines **1** and **2** respectively

Scheme 1. The degradation reaction is illustrated for one enantiomer of *o*-carboranylalanine.

mobile phase. Such solvents affect the temperature of the plasma and may even extinguish it when a conventional Meinhard nebulizer is mounted. Further, the flow rate of the mobile phase from the LC device is often smaller than required by the ICP instrument and, in addition, the band broadening in the spray chamber may be large, which results in broad chromatographic peaks and poor resolution.

One of the recently introduced nebulizers which operate at low flow rates is the microconcentrical nebulizer (MCN-100) from Cetac Technologies.[9,10] A lower sample uptake rate may disturb the plasma to a lesser extent and thus make it more compatible with organic solvents. Few studies have been published on the performance of the MCN-100 in the presence of other than pure aqueous solvents. Augagneur *et al.*[11] reported the determination of rare earth elements in wine with addition of ethanol to a concentration of up to 60% at a sample flow rate of 30 μl min^{-1}. In unpublished studies in this laboratory, the MCN-100 was run with up to 100% octanol at a flow rate of 150 μl min^{-1}.

Experimental

Instruments. An ICP-AES instrument (Spectroflame P, Spectro Analytical Instruments, Kleve, Germany) equipped with an MCN-100 nebulizer (Model M-2, Cetac Technologies, Omaha, NE, USA) was used. The boron signal was optimised with respect to the observation height, the nebulizer gas flow rate (0.70 l min^{-1}) and the effect (1290 W, 27 MHz). Boron was monitored at the 249.773 nm atomic emission line. Collection and integration of the emission signal was performed over 0.4 s intervals with the Spectro Software 2.10 provided by the manufacturer.

The LC system consisted of a pump (Jasco PU180i, Tokyo, Japan) and a six-port injector (Cheminert Valco C2–2346, Houston, TX, USA) with a 20 μl sample loop. The column (100 × 2 mm i.d., particle size 5 μm) of porous graphitic carbon (Hybercarb) was obtained from Shandon Scientific, Runcorn, UK. All tubing within the LC system was made of PEEK with inner diameters of 170–250 μm. The LC-system was connected to the spectrometer by a 45 mm long and 175 μm i.d. PEEK tube encapsulated in FEP Teflon provided by the manufacturer of the nebulizer. The mobile phase consisted of 0.5% TFA in 35% aqueous acetonitrile and it was pumped at a rate of 150 μl min^{-1} through the column at room temperature. The selected flow rate was a compromise between sensitivity on one side and analysis time and argon consumption on the other.

Measurements of pH were made with a Radiometer PHM 84 (Radiometer, Copenhagen, Denmark) equipped with a combined glass electrode. The electrode was calibrated with aqueous NIST buffers.

Chemicals. Argon gas was of quality 4.5 (purity 99.995%). All chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). Racemic *o*-carboranylalanine hydrochloride was synthesised as previously described.[12] ^{1}H NMR (400 MHz, CD_3OD): 2.74 (dd, 1 H, β-CH), 3.14 (dd 1 H, β-CH), 4.15 (dd, 1 H, α-CH), 4.72 (br s, 1 H, BCH).

Kinetic experiments. The main series of experiments was carried out in a medium prepared by mixing equal volumes of 0.2 M disodium hydrogen citrate, pH 5.00, and methanol. About 3.9 mg of *o*-carboranylalanine hydrochloride was weighed into a 4.5 ml vial and dissolved in 3 ml of the thermostatted (< ±0.1 °C) reaction medium. This amount of the substance is fairly close to its solubility in 50% methanol. The vial was sealed by a screw cap with a septum for gas chromatography and placed in a thermostat. Sampling was at regular intervals and on each occasion 40 μl portions of the reaction mixture were transferred with a Hamilton gas-tight syringe to vials containing 40 μl of 1 M hydrochloric acid. The samples were diluted with 420 μl of 0.2% TFA in 35% aqueous acetonitrile, shaken and stored in a freezer until analysis was carried out, at least in duplicate. The

addition of strong acid and the freezing quenches the reaction.

Experiments performed at other pH and methanol concentrations were carried out in a similar way. The total buffer concentration (citrate, phosphate, borate) was around 0.15 M in order to keep the pH of the medium constant as protons are formed in the degradation.

Potentiometric titration. The acidity constants were determined by potentiometric titration of an 1.5 mM solution of racemic *o*-carboranylalanine hydrochloride in 25 ml of 25% aqueous methanol with 0.021 M carbonate-free sodium hydroxide in an atmosphere of dinitrogen. Carbonate was removed from a 0.1 M stock solution of sodium hydroxide by addition of 0.5 g calcium oxide to 250 ml of solution.[13] The titrator was standardized against 0.100 M nitric acid.

Evaluation of chromatograms. The areas of the chromatographic peaks were calculated by the peak fitting module of the program Origin™ 5.0 (Microcal Software Inc., Northampton, MA, USA). The peaks of the *nido*-diastereomers were not completely baseline-separated and the asymmetrical double sigmoidal function was fitted to the data before integration.

Separate measurement of a sample containing known amounts of *o*- (A) and *nido*-carboranylalanine (B and C) showed that the signal area per boron atom is the same for A, B, and C, to within a few per cent. This agrees with measurements in the laboratory on other organic boron compounds, including boron cages. An almost constant area per boron atom was found. Let A_A, A_B and A_C denote the areas measured at time t after the start of the reaction. The concentration variables needed for the determination of the rate constants can then be found from eqn. (1),

$$\frac{[A]_t}{[A]_0} = \frac{0.9A_A}{0.9A_A + A_B + A_C} \quad \text{and} \quad \frac{[B]_t}{[C]_t} = \frac{A_B}{A_C} \qquad (1)$$

where $[\,]_t$ is the concentration at time t and $[\,]_0$ the initial concentration.

Results and discussion

An LC system with a PGC separation column connected to the spectrometer via an MCN-100 nebulizer provided sufficient separation efficiency and analytical sensitivity for the determination of the boron-containing amino acids. A chromatogram of a sample collected 18 h after the start of the reaction at 45.5 °C is shown in Fig. 1. The separation was complete within 20 min, with an average resolution, R_s, of 1.5 between the two *nido*-diastereomers. Most of the band-broadening seemed to occur in the detector system, since injection of decreasing sample volumes containing the same amount of boron did not have a large effect on the peak widths.

An emphasis was to determine the rate constants in the reaction scheme below. It is not known which of the diastereomers, *nido*-carboranylalanine (B) or *nido*-carboranylalanine (C), corresponds to the *nido*-compounds **1** and **2** in Scheme 1.

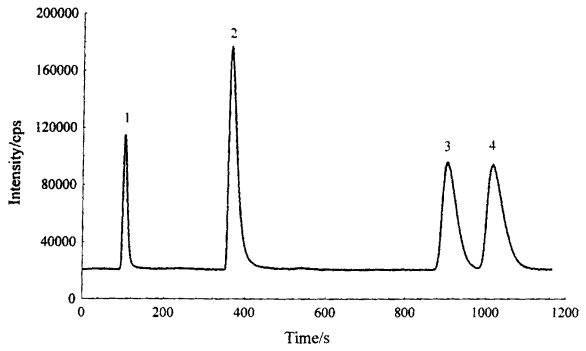
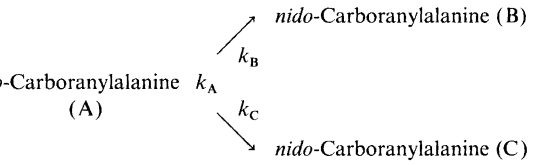


Fig. 1. HPLC-ICP-AES chromatogram of a sample collected 18 h after the start of the degradation of *o*-carboranylalanine at 45.5 °C. Column: porous graphitic carbon 2 × 100 mm. Sample volume: 20 µl. Mobile phase: 0.5% trifluoroacetic acid in 35% aqueous acetonitrile. Flow-rate: 150 µl min^{-1}. Measured wavelength: 249.773 nm (boron). Peak identification: (1) boric acid trimethyl ester; (2) *o*-carboranylalanine (A); (3) *nido*-carboranylalanine (B); (4) *nido*-carboranylalanine (C).



The rate of degradation, k_A, was found from the decline of the *o*-carboranylalanine (A) concentration. The degradation reaction was followed at three temperatures, *viz.* 14.8, 29.7 and 45.5 °C. The plots of $\ln([A]_t/[A]_0)$ against time were linear which signifies that the degradation at pH 5 is first order with respect to *o*-carboranylalanine. Straight lines were fitted to the data by least-squares regression. The results are presented in Table 1.

Table 2 shows the (pseudo)-first-order rate constants in h^{-1}. Since the quotient between the rates of formation, k_B and k_C, of the diastereomers (B, C) of *nido*-carboranylalanine equals the ratio between the concentrations of B and C, the individual values could be calculated from $k_A = k_B + k_C$. The ratio between the rate constants of the

Table 1. Statistics at the 95% confidence level of the linear regression of $\ln([A]_t/[A]_0)$ with time (h) at three different temperatures (A = *o*-carboranylalanine). The degradation was measured in aqueous disodium hydrogen citrate (pH 5.00)–methanol (1 : 1).

T/°C	Slope/h^{-1}	Intercept	Correlation coefficient	Number of experimental points
14.8	−0.00146(3)	−0.01(2)	−0.9995	12
29.7	−0.0094(2)	−0.01(2)	−0.9996	11
45.5	−0.056(1)	−0.03(5)	−0.9995	14

Table 2. First-order rate constants, k, for the degradation of *o*-carboranylalanine and the formation of its corresponding *nido*-analogues at three different temperatures. The average values of k_B/k_C are given with 95% confidence intervals.

	k/h^{-1}			Average $n \geqslant 13$
T/°C	k_A	k_B	k_C	k_B/k_C
14.8	0.00146	0.00070	0.00076	0.912(3)
29.7	0.00944	0.00446	0.00498	0.895(5)
45.5	0.056	0.026	0.030	0.892(5)

nido-analogues was found to be independent of time. This quotient was also about the same whether a UV detector or an ICP-AES was used for detection. The means of k_B/k_C in Table 2 were calculated from experimental points where more than 35% of the *o*-carboranylalanine had reacted in order to reduce the influence of experimental error on the value of the quotient when A_B and A_C were small. The ratio between the rates of formation of the diastereomers tends to change with temperature in a non-linear fashion.

Theoretical, relative concentration vs. time curves were calculated from eqn. (2)

$$\frac{[A]_t}{[A]_0} = e^{-k_A t} \quad \text{and} \quad \frac{[B, C]_t}{[A]_0} = \frac{k_{B,C}}{k_A}(1 - e^{-k_A t}) \qquad (2)$$

with rate constants from Table 2 and compared with the experimental points. The agreement between the calculated curves and the experimental data was found to be good as exemplified by Fig. 2.

o-Carboranylalanine is present in solution as HA^+, HA and A^-, where HA represents the zwitterion form of the amino acid. The speciation is governed by the acidity constants. Mixed acidity constants defined by eqn. (3)

$$K_1^H = \frac{^{aq}\{H^+\}[HA]}{[H_2A^+]} \quad \text{and} \quad K_2^H = \frac{^{aq}\{H^+\}[A^-]}{[HA]} \qquad (3)$$

were determined from potentiometric titration in 25% methanol at low ionic strength (>0.005 M) with a glass indicator electrode. Fig. 3. The notation $^{aq}\{H^+\}$ serves to indicate that measured proton activities refer to the water scale, as the electrode was calibrated with aqueous pH standards. In the evaluation of the acidity constants from the titration data concentrations were used instead of activities as the ionic strength was low. Since the methanol concentration was only 25%, the free proton concentration was calculated as $[H^+] = 10^{-pH}$.[14] The result was $pK_1^H = 1.9 \pm 0.2$ and $pK_2^H = 7.76 \pm 0.05$ with the uncertainties estimated from replicate determinations. The corresponding distribution diagram is drawn in Fig. 4.

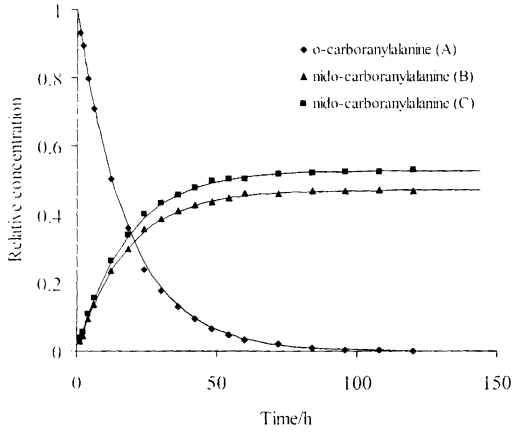


Fig. 2. Experimental (points) and calculated (lines) relative concentrations of *o*-carboranylalanine and its *nido*-analogues as a function of time during degradation at 45.5 °C.

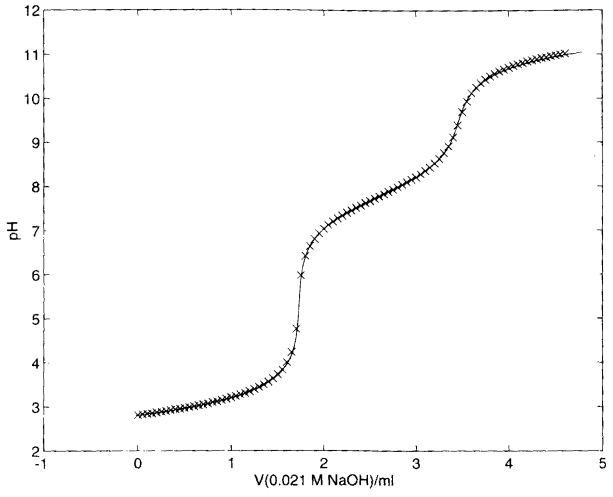


Fig. 3. Titration of 25 ml of a 1.5 mM solution of *o*-carboranylalanine in water–methanol (3:1) against 0.021 M sodium hydroxide. The graph was calculated with the acidity constants given in the text and the experimental points are represented by crosses.

The degradation rate will be pH-dependent if the stability of the various *o*-carboranylalanine forms varies. The results from experiments carried out in different buffers are shown in Fig. 5. The fastest degradation

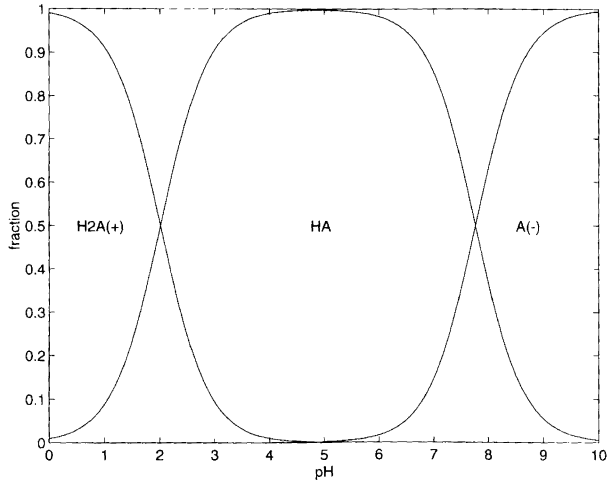


Fig. 4. Distribution diagram for *o*-carboranylalanine at different pH values.

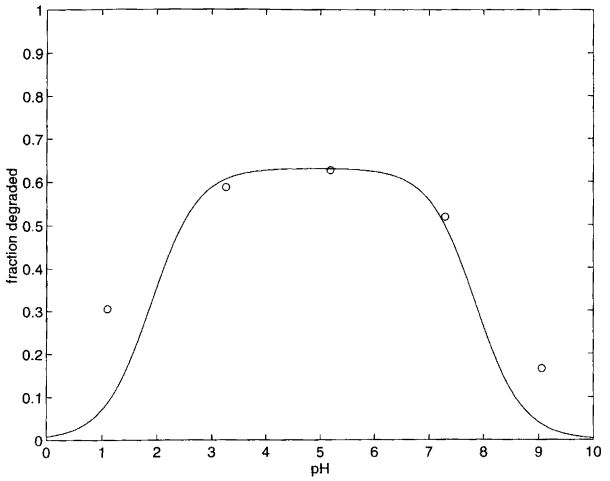


Fig. 5. The fraction of *o*-carboranylalanine degraded after storage in buffers of different pH for 120 h at 30 °C. The line was calculated on the assumption that the degradation reaction involves only the zwitterion.

occurs at pH 5. At this pH the zwitterionic form (HA) predominates according to the distribution diagram, and the resemblance of the variation of the instability of the amino acid and the fraction present as the zwitterion is intriguing. The line in Fig. 5 was calculated on the assumption that only HA decomposes. This hypothesis accounts for the results in the pH range 3–7. Outside this range, reactions involving H_2A^+ and A^-, respectively, appear to take place, as the fraction of *o*-carboranylalanine degraded is much larger than expected from the simple hypothesis.

The ratio between the two *nido*-diastereomers formed is also affected by pH. At pH 1 the ratio was 1.5, compared with 0.9 at pH 5. The ratio also increased somewhat under more basic conditions (pH 7 and 9), but did not exceed 1.

Arrhenius plots of the rate constants for the *nido*-diastereomers are shown in Fig. 6. The values of the

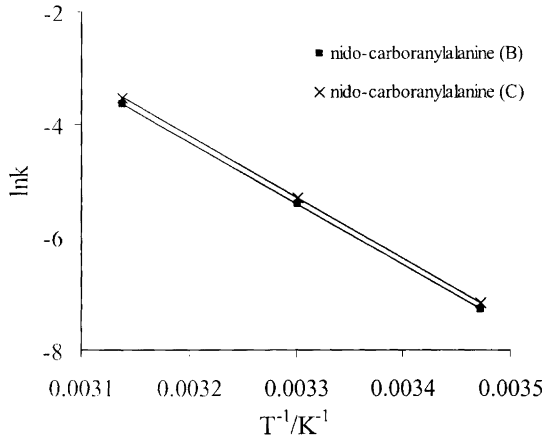


Fig. 6. Arrhenius plots of the temperature dependence of the rate constants for the formation of the *nido*-diastereomers in aqueous disodium hydrogen citrate buffer (pH 5.00)–methanol (1 : 1).

Table 3. Activation parameters for the formation of the *nido*-diastereomers B and C at pH 5 calculated from the Arrhenius plots in Fig. 3.

	E_A/kJ mol^{-1}	$\Delta H^{\circ\ddagger}$/kJ mol^{-1}	$\Delta S^{\circ\ddagger}$/J K^{-1} mol^{-1}
B	90±1	88±1	0±3
C	91±1	88±1	3±3

activation energy (E_A), calculated from a linear regression, the activation enthalpy ($\Delta H^{\circ\ddagger}$) and entropy ($\Delta S^{\circ\ddagger}$) are listed in Table 3.

In his pioneering work Hawthorne showed that the *o*-carborane cage degrades to the corresponding *nido*-cage when it is heated with strong bases such as hydroxide or alcoholate in water or alcohol solutions.[15,16] It was shown that the rate of the reaction was first order with respect to both carborane and base. In this case the low, but positive activation entropies (0 and 3 J K^{-1} mol^{-1}) support the hypothesis[4] that the rate-determining step in the degradation of *o*-carboranylalanine to its corresponding *nido*-analogues involves an intramolecular reaction and that both the carboxylate ion and the ammonium ion in carboranylalanine are needed for an optimum reaction rate.

Conclusions

It has been shown that *o*-carboranylalanine is degraded through an intramolecular, first-order reaction in a mixture of water and methanol under slightly acidic conditions where either of two diastereomeric *nido*-analogues are formed, one of them in excess. Which of the *nido*-diastereomers is found in excess depends on the acidity of the solution.

The boron containing amino acids could be separated on a porous graphitic carbon column at a pH around 1 with an aqueous TFA–acetonitrile mobile phase and detected by ICP-AES with a microconcentric nebulizer (MCN-100).

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