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Characterization of bovine serum albumin/chlorogenic acid solution mixtures by analytical ultracentrifugation

Abstract The aim of this work was to investigate a possible interaction between bovine serum albumin (BSA) and chlorogenic acid (CGA) in KH_2PO_4/K_2HPO_4 buffer (pH 7.0; ionic strength 0.1 mol/l) depending on the BSA/CGA molar ratio by analytical ultracentrifugation (sedimentation velocity) using an OPTIMA XL-A AUC equipped with a UV–VIS absorption optical system. The protein concentration of all the solution mixtures with BSA-to-CGA molar (mass) ratios of 1:19 (10:1) up to 1:95 (2:1) was 0.50 g/l. The investigations were carried out under experimental conditions where the possibility of derivatization of the BSA (covalent bonding) is minimized (pH, temperature, investigation of the solutions immediately after mixing). At 280 nm both sedimenting BSA and BSA with non-covalent-bound CGA can be detected. In the region of 320 nm BSA shows no absorption, and thus only BSA with bound CGA (absorption maximum at 325 nm) can be monitored. The results indicate weak interaction between BSA and CGA. At a scan wavelength of 280 nm sample solutions with a BSA-to-CGA molar ratio of 1:19 revealed a major component of 4.2 S similar to the major component of the BSA control sample. However a further component with a lower $s_{c,20^\circ}$ value (2.3–2.5 S) appears and this is not present in the BSA control sample. At 320 nm the sedimentation of a major component with an $s_{c,20^\circ}$ value of 1.8–2.0 S followed by a 3.1 S component could be demonstrated. This indicates that the binding of CGA to BSA results in considerably lower $s_{c,20^\circ}$ value components. The cause could be a more asymmetric particle, a "swollen" structure and/or ligand-induced partial unfolding of the BSA molecule. Similar results have been obtained with BSA/CGA solutions up to a molar ratio of (1:95). UV–VIS absorption measurements and studies of intrinsic tryptophan fluorescence of BSA/CGA solution mixtures at various CGA concentrations also indicate such interactions between BSA and CGA. Some possible physiological consequences of the BSA/CGA interaction are considered.

Keywords Analytical ultracentrifugation · Sedimentation velocity · Interactions · Proteins · Chlorogenic acid

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Introduction

Chlorogenic acid (CGA) is widely distributed in the plant kingdom. For example, green coffee beans contain 6–10% CGA, and a 200-ml cup of coffee may have 20–675 mg CGA, depending on the roasting and brewing conditions [1]. In potato tubers CGA constitutes up to 90% of the total phenolic content [2]. CGA plays an important role in

many biological and metabolic processes, for example, antiviral activity and inhibition of tumour promotion. Because of its chemical structure CGA (Fig. 1) may interact with proteins by "weak" interactions (e.g., hydrogen, hydrophobic, ionic bonding) [3, 4, 5, 6, 7, 8]. Under defined reaction conditions (pH 9) nucleophilic binding to proteins can be attained, resulting in protein derivatives (covalent bonding) [8, 9, 10].

The aim of the work was to investigate the possible interaction between bovine serum albumin (BSA) and CGA depending on the BSA-to-CGA molar ratio by analytical ultracentrifugation (sedimentation velocity) under experimental conditions where the possibility of derivatization of the BSA (covalent bonding) is minimized.

Materials and Methods

Materials

BSA [Assay ≥98%; crystallized; lyophilized; $H_2O \leq 5\%$; ash ≤ 5%; $M_r \approx 67{,}000$ (lit)] and CGA hemihydrate (pure; ~97%; $M_r = 363{,}32$) were employed as supplied by Fluka Chemie, Buchs, Switzerland. Potassium dihydrogen phosphate and dipotassium hydrogen phosphate (p.a. Merck, Darmstadt, Germany) were dried overnight at 120 °C before use.

Solutions

The KH_2PO_4/K_2HPO_4 buffer solution (pH 7.0; ionic strength 0.1 mol/l) was prepared according to the method in Ref. [11] and gave a measured pH of 6.98.

Stock solutions of BSA (about 1.0 g/l) and CGA (exactly 1.00 g/l related to the weighed amount) were prepared by dissolving them in the buffer at room temperature. After determination of the protein content of the BSA stock solutions, the BSA control solutions and BSA/CGA solution mixtures of different BSA-to-CGA molar (mass) ratios of 1:19 (10:1), 1:38 (5:1) and 1:95 (2:1) were prepared. The protein concentration in all the solutions was 0.50 g/l. To minimize any possibility of derivatization of the BSA (covalent bonding) a moderate pH condition was applied and the solutions were investigated with the analytical ultracentrifuge (AUC) immediately after mixing (start of the runs within 2 h).

Methods

Analytical ultracentrifugation

The AUC employed was the OPTIMA XL-A (Beckman/Coulter) with a UV–VIS absorption optical system (190–800 nm). The solutions were investigated at 45,000 rpm (approximately 160,000*g*) in double-sector cells which contained the solution and the reference solution (buffer solution or CGA solutions). CGA solutions in the reference sector were used because of the large basic CGA absorbance of more than 1.5 with increasing CGA content in the BSA/CGA solution mixtures. This experimental procedure gave absorbance values below 1.5 and also made it possible to scan the solutions in the region conventionally used for recording protein absorption (280 nm). During the runs "radial scans" were carried out in periods of 6–9 min, which provide figures of the absorbance as a function of radial displacement (distance from the centre of rotation) in the solution. The main aim of such high-speed sedimentation velocity investigations was to determine sedimentation coefficients and their distributions at the given concentration at 20.0 °C ($s_{c,20°}$; unit 10^{-13} s≡1 S [Svedberg]). The $s_{c,20°}$ values characterize the hydrodynamic behaviour of the BSA molecules in solution in the presence of CGA and in comparison to the reference molecule (BSA) dissolved in the same buffer. The sedimentation coefficient is a function of the mass, size and shape of the dissolved particles, of the properties of the solvent and of the interaction properties within the system. Additional runs were performed at 3,000 rpm prior to the high speed sedimentation velocity investigations to check if any possible higher molecular weight "impurities" could be formed in the preparations. Further "wavelength scans" (190–800 nm) were carried out at 3,000 rpm, giving figures of the absorbance depending on the wavelength at a fixed radius in the solution.

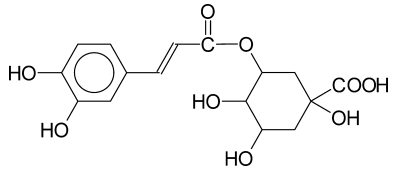

Fig. 1 Structural formula of chlorogenic acid (*CGA*)

Two distinguishing features of the BSA and CGA molecules were exploited in the investigations. The first one is that the CGA molecules with a molar mass of about 350 are too small to sediment at the centrifugal fields generally realized with an AUC, whereas CGA bound to BSA by "weak" interactions can sediment. Secondly, CGA shows an absorption maximum at about 320 nm, but BSA does not absorb at this wavelength. By recording the absorbance in the region of 320 nm, it is thus possible to provide evidence for BSA with bound CGA. At 280 nm, both BSA and CGA absorb. A comparative evaluation of the sedimentation figures at wavelengths of 280 and 320 nm thus provides the basis of our assay procedure.

The evaluation of the results on the basis of the absorbance/radius figures was carried out with the program SEDFIT5 [12, 13] according to the least-squares $g^*(s)$ method, which facilitates the evaluation of a $g^*(s)$ distribution, based on direct boundary fitting and algebraic systematic noise deconvolution. The result of this analysis is an apparent sedimentation coefficient equivalent to the $g(s^*)$ analysis of dc/dt [14]. However, there is no need to differentiate the data and calculate dc/dt here, thus eliminating a source of broadening from finite time differences between the scans. Therefore, this method is, as a consequence, far more tolerant of large time steps, such as commonly occur in absorbance optical data. The model requires the following user input:

1. Resolution: This specifies the increments of the s values for the calculation. The recommended value of 100 was applied, with a value of 200 chosen as a check. Generally, with higher values, more noise is introduced into the distribution, but this can be efficiently reduced by Tikhonov–Phillips regularization [15].
2. s_{min} and s_{max}: These values should confine the range of s values expected for the sample. In all evaluations s_{min} and s_{max} were 1 and 20 S, respectively.
3. Confidence level (F ratio): This input is required for the statistical adjustment of the regularization constraint. The recommended values of 0.68 (one standard deviation) or 0.95 (two) were applied.

Further, a floating baseline was allowed.

UV–VIS absorption spectra

The UV–VIS absorption spectra of BSA, CGA and the solution mixtures were determined using a UV–VIS spectrophotometer

(Spekol, Carl Zeiss Jena, Germany) and additionally using the AUC as described earlier.

Protein content

The protein content of the BSA solutions was determined according to the method in Ref. [16] with the modification described in Ref. [17]. BSA was used to calibrate the regression curve after determining its protein content by semimicro Kjeldahl analysis (Kjeldatherm System KT40, Gerhardt Laboratory Instruments, Bonn, Germany).

Tryptophan fluorescence

Intrinsic fluorescence emission spectra between 300 and 800 nm were recorded with a JASCO FP 920 fluorescence detector using an excitation wavelength of 290 nm and quartz optical cells of 10 mm path length. Excitation and emission bandwidths were set at 18 and 40 nm, respectively. The protein concentrations were adjusted to 0.50 g/l. Corrections were made for the buffer blanks. The BSA/CGA solution mixtures (BSA-to-CGA mass ratio 0.50/0.01–0.10 g/l) were prepared freshly and the fluorescence was measured as described earlier.

Results and discussion

At a scan wavelength of 280 nm, both free BSA and BSA with bound CGA can be detected – if present. A comparison of the apparent sedimentation coefficients of the BSA/CGA(1:19) solution with the BSA solution investigated during the same run indicates for both scans a major component at 4.2 S (Table 1). In the case of the BSA/CGA(1:19) solution further components appear. The component with an essentially lower $s_{c,20^\circ}$ value of 2.3–2.5 S (''shoulder''), which is not present in the BSA control sample, demonstrates a stronger change of the hydrodynamic behaviour (slower sedimentation).

That may be due to a more asymmetric particle, a ''swollen'' structure and/or partial unfolding of the BSA molecule. This observation is also confirmed by a larger proportion of this component with increasing concentration of CGA in the BSA/CGA(1:38) sample solution. The increase of the CGA concentration in the solution mixtures also results in more distinct components (maxima) owing to the binding of CGA to BSA. Proof of weak interactions between BSA and CGA is given by the fact that at 320 nm where only BSA with bound CGA can be registered (BSA does not absorb at 320 nm, CGA has an absorption maximum at 325 nm) sedimentation of a number of components from between 2 S to 11 S occurred [Table 1; BSA/CGA(1:19) solution sample]. The low $s_{c,20^\circ}$ values of the major sedimenting component of 1.8–2.0 S (shoulder) and a second 3.1 S (maximum) component support the preceding discussion. Because of the large basic CGA absorbance of more than 1.5, BSA/CGA solution samples with molar ratios of 1:38 and 1:95 had to be scanned at a higher wavelength, but gave a consistent picture (Fig. 2). All the BSA/CGA solution samples exhibited maxima of the sedimentation coefficient distribution at about 2.3 S and a strong increase of the concentration of components below 2.3 S in the least-squares $g^*(s)$–$s_{c,20^\circ}$ plot. Only 19 molecules of CGA per molecule of BSA produce this change in the hydrodynamic behaviour. Considering that BSA may interact with smaller molecules and ions owing to at least six binding regions on the protein molecule [18], there is an excess of CGA with about the 3 times number of CGA molecules per BSA molecule being present in the BSA/CGA(1:19) solution.

The BSA/CGA(1:19) and BSA/CGA(1:95) solutions show components in the region of the BSA absorption (4.2–4.3 S). This may be due to BSA molecules with a relatively small amount of bound CGA, so the sedimentation behaviour is not significantly different. Surprisingly the sample with a medium BSA-to-CGA

Table 1 Sedimentation coefficients of bovine serum albumin (*BSA*)/chlorogenic acid (*CGA*) solution mixtures of different BSA-to-CGA molar ratios. Analytical Ultracentrifugation conditions 45,000 rpm (≈160,000*g*), 20.0 °C

	Wavelength of scans (nm)	$s_{c,20^\circ}$ values of the components (S)[a,b,c,d]						
		Components at decreasing concentration						
		1 (major component)	2	3	4	5	6	7
BSA/CGA(1:19)	280	4.2	5.2–5.4[b]	2.3–2.5[b]	7.3	8.9		
	320	1.8–2.0[b]	3.1	6.2	4.5	5.4–5.6[b]	8.3	10.8
BSA/CGA(1:38)	280	4.2	2.2	5.2–5.4	6.2–6.4	8.5	10.0	
BSA control	280	4.2	7–9.5[c]					

[a]Maxima from the least-squares $g^*(s)$–$s_{c,20^\circ}$ diagrams
[b]Hidden maxima with ''shoulder(s)''
[c]Hidden maxima without ''shoulder(s)''
[d]All the results are from the same run; further runs show similar results
Evaluation program SEDFIT5 [12, 13]; method: least-squares $g^*(s)$; resolution 100; *F* ratio 0.68; $s_{c,20^\circ}$ values of least-squares $g^*(s)$ values below 0.005 are not given. Molar masses: BSA 67,000, CGA 354.31; solvent KH_2PO_4/K_2HPO_4 buffer (pH 7.0; ionic strength 0.1 mol/l); BSA concentration in all solutions 0.50 g/l

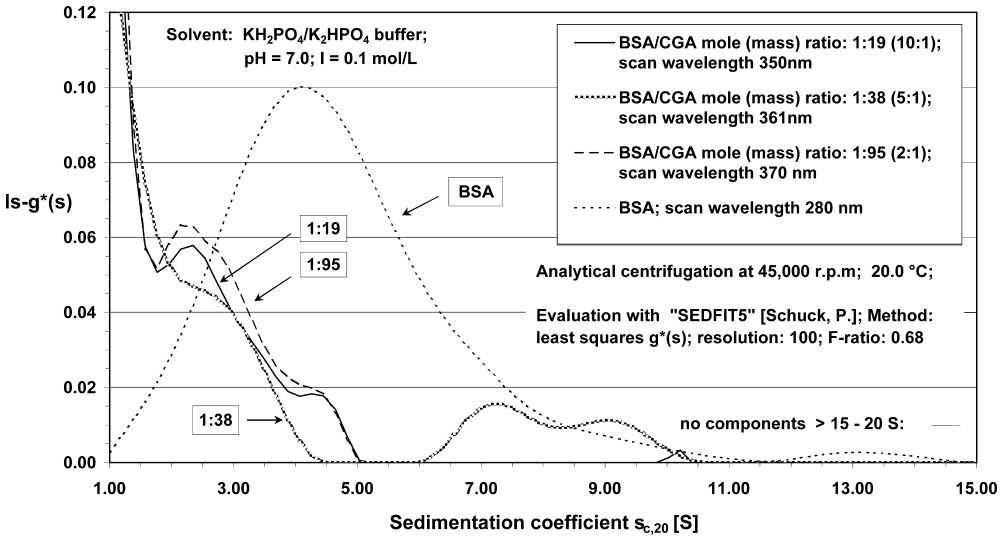

Fig. 2 Sedimentation coefficient distributions of bovine serum albumin (*BSA*)/CGA solution mixtures as a function of CGA concentration

molar ratio of 1:38 shows no component in the BSA region but two components at 7.1–7.3 and 9.1 S. This reveals that there is no simple relation between the formation of such components and the BSA-to-CGA molar ratio.

The results described could also be confirmed by further measurements of BSA/CGA solution mixtures of different molar ratios by including experiments at scan wavelengths in the BSA absorption region; the latter being made possible by means of CGA added to the reference sector.

Investigations on haze-active proteins and polyphenols [19, 20] propose a model for protein–polyphenol interactions, which includes the formation of different structures strongly depending on the concentrations of protein and polyphenol and their ratios. The authors proceed from a fixed number of binding ends of the polyphenol and binding sites of each protein. With excess polyphenol relative to protein, they assume all of the protein binding sites would be occupied, but the likelihood that bridging would occur would be low because each free polyphenol end would have a small chance of finding a free binding site on a protein molecule. They conclude, too, that this would result in small aggregates. Our results also indicate the formation of different structures depending on the CGA concentration. The BSA/CGA(1:19) and BSA/CGA(1:95) solutions show no components above 5 S (Fig. 2). The formation of greater aggregates consisting of two or more BSA molecules with bound CGA should be analogous to the aggregate formation of the native BSA (Fig. 2, small amount in the region of 13 S) and results in the formation of aggregates with $s_{c,20^\circ}$ values greater than the $s_{c,20^\circ}$ value of BSA (4.2 S). In the case of the BSA/CGA(1:38) solution the two maxima at 7.3 and 9.1 S indicate the formation of similar aggregates.

Higher molecular ''impurities'' could not be detected by additional runs performed at 3,000 rpm prior to the high-speed sedimentation velocity investigations.

The UV–VIS absorption spectra also support the occurrence of interactions between BSA and CGA. In order to check this with UV–vis absorption measurements on a spectrophotometer the product of the CGA concentration in the BSA/CGA solutions, c, and the cuvette layer thickness, d, was kept constant. In this case the number of absorbing molecules in the measured samples would not change and the absorbance values would remain constant. A change of the absorption properties of the BSA/CGA solution mixtures depending on the CGA concentration owing to interaction between BSA and CGA results in optical density (OD) values that are no longer constant.

The results in Fig. 3 of BSA/CGA solution mixtures with BSA-to-CGA molar ratios of 1:9.5, 1:19 and 1:95 illustrate a clear dependence on the CGA concentration. The highest OD values are shown by the BSA/CGA solution mixture with the lowest CGA concentration of 0.025 g/l. With increasing CGA concentration up to tenfold, the OD values decrease. This is due to the 5–8 times higher absorption level of CGA in comparison to BSA and the loss of ''free'' CGA by the binding to BSA through which the absorption properties of the solutions have changed. Pure CGA solutions show the opposite behaviour: increasing OD values with increasing CGA concentration. Similar UV–VIS absorption spectra were also obtained by wavelength scans recorded on the AUC at 3,000 rpm.

An intrinsic fluorescence study was performed to evaluate changes in tertiary structure caused by interactions of BSA with CGA. The maximum emission wavelength for unmodified BSA was 346 nm (Fig. 4, curve 1). The tryptophan residues are rather exposed on the protein surface, since its initial maximum of fluores-

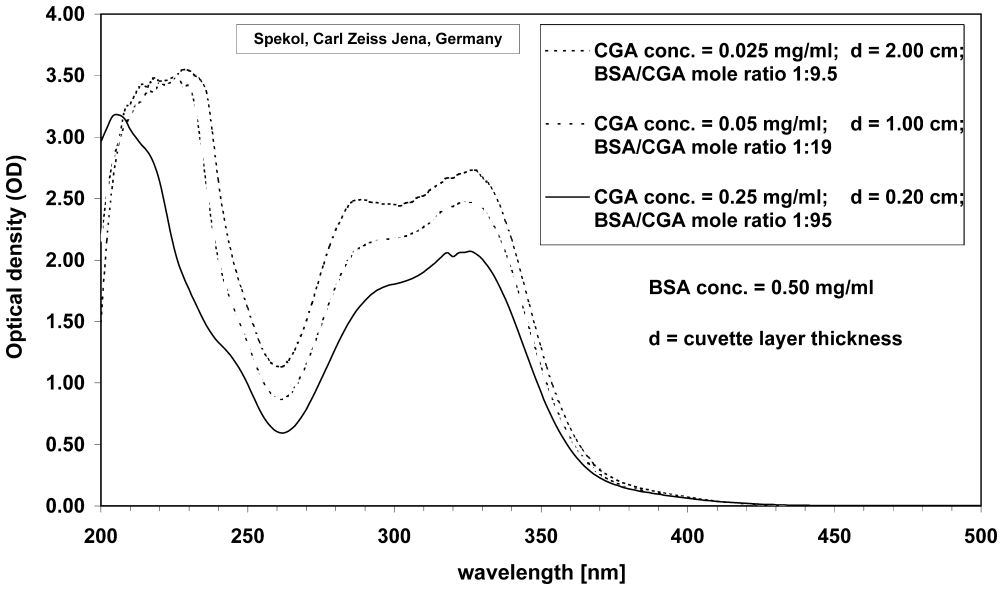

Fig. 3 UV–vis absorption spectra of BSA/CGA solution mixtures measured at the same value of CGA concentration times cuvette layer thickness

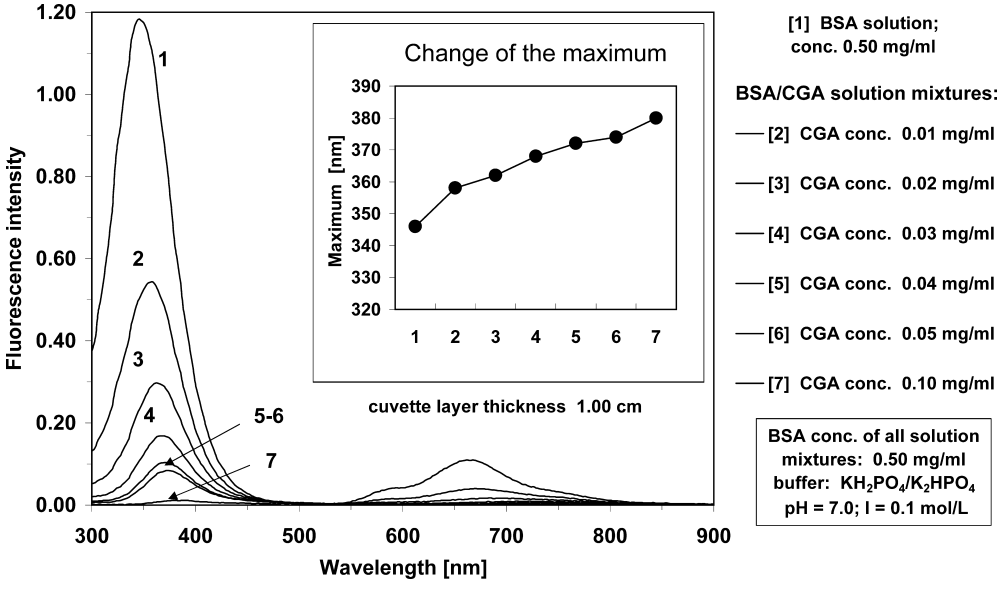

Fig. 4 Tryptophan fluorescence scans of BSA/CGA solution mixtures depending on the CGA concentration

cence emission appears at a wavelength close to that of exposed tryptophan model systems. However, buried tryptophan groups in close proximity to polar residues may be capable of forming an excited-state complex (exciplex), resulting in emission spectra characteristic of exposed residues with peaks close to 350 nm. Both tryptophan residues (Trp3, Trp158) present in the sequence of BSA are in close proximity to lysine residues, and first one (Trp3) is located very near the N-terminal. This resulting high exposure can be confirmed by the tryptophan quenching, where a low BSA-to-CGA ratio (Fig. 4, curve 2) caused a corresponding strong decrease of tryptophan fluorescence. The maximum emission also decreased in intensity as a function of the CGA concentration and a redshift of the maximum emission was observed (Fig. 4). From the progressive quenching and the redshift observed in the maximum fluorescence emission of BSA, it can be inferred that conformational changes are induced by interactions of CGA and BSA.

Conclusions

Sedimentation velocity in the AUC has been shown to be a useful tool for the detection and assay of the ''weak'' interactions in BSA/CGA solutions, particularly when it is shown that under the chosen experimental conditions the formation of BSA/CGA derivatives (covalent bond-

ing) is minimized. Weak interactions between CGA and BSA in BSA/CGA solution mixtures result in a clear change of the hydrodynamic behaviour of the BSA molecules and more distinct components depending on the concentration.

These observations are reinforced by fluorescence spectroscopy measurements, which show progressive quenching and a redshift in the maximum fluorescence emission, measurements which can be interpreted in terms of conformational changes induced by interactions of CGA and BSA.

Such observations may also be of physiological importance. The CGA concentrations in our investigations may approximate physiological concentrations resulting from coffee intake: if the interaction with BSA is nonspecific, it is reasonable to suppose that CGA at comparable concentrations may bind to endogenous proteins or enzymes and influence their functions in a deleterious manner. A quantity of 500 mg CGA per 200 ml (a cup of coffee) is equivalent to 2.5 g/l, a concentration that is, for example, 25 times higher than the concentration in the BSA/CGA(1:38) solution.

Further investigations should consider

Higher and lower BSA and CGA concentrations.
The application of other proteins (e.g., human serum albumin) and native phenolic compounds.
Other physiologically important pH values, including the type of buffer.

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