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Thermodynamics and Kinetics of Propidium Binding to Poly(A)·Poly(U) in Aqueous Solution

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The propidium binding to poly(A)·poly(U) has been investigated thermodynamically and kinetically in buffers containing different concentrations of NaCl at various temperatures. Free propidium had an absorption maximum at 493 nm, and this shifted to about 530 nm for propidium fully bound to poly(A)·poly(U) in all solutions. The association constant for the reaction was 1.42×10^{6} mol^{-1} dm^{3} at 2.02×10^{-2} mol dm^{-3} of Na^+ concentration, and about 26 times larger than that at 1.02 mol dm^{-3} Na^+. The dependence of the association constants indicated the enthalpies for the reaction at the low and high Na^+ concentrations were 39.8 and −8.3 kJ mol^{-1}, respectively. The entropies were 251 and 63 J K^{-1} mol^{-1}, respectively. These thermodynamical results suggested that the association reaction of propidium to poly(A)·poly(U) may play different behaviors at the low and high salt concentrations. Kinetic results by a micro stopped-flow method indicated that the propidium binding to poly(A)·poly(U) was a single step at the low Na^+ concentration, while at the high Na^+ concentration it consisted of two steps which contained the slow unimolecular process after the binding step.

Recently the reactions of drugs binding to DNA's have been extensively studied in a different concentration of a salt. Breslauer and co-workers investigated the calorimetrically-drived characterization of the thermodynamics of ethidium and propidium binding to a series of DNA's.[1] It was reported that the binding constants of the drugs with synthetic and natural DNA's decreased with increasing the salt concentration. Wilson et al. who studied the reaction of propidium with poly- and oligodeoxyribonucleotides containing A-T base pairs showed the same dependence of the binding constants on the salt concentration.[2,3] We also found the same tendency at the cases of actinomycin D,[4] tetrakis(4-*N*-methylpyridyl)porphine,[5] and azure B[6] to DNA's. However, despite the importance, little is examined about the salt dependence of the reaction of the drugs binding to RNA's.[7]

In the present work, we have investigated thermodynamics and kinetics of propidium (PD) binding to poly(A)·poly(U) in 10^{-2} mol dm^{-3} Na_2HPO_4, 10^{-4} mol dm^{-3} Na_2EDTA, pH 7.0 containing different concentrations of NaCl at various temperatures by using a spectrophotometric and a micro stopped-flow method. The results have been compared with those for ethidium (ED) binding to nucleic acids. It should be noted that PD is a dication containing a large, charged side chain, while ED is a monocation having a neutral side chain as shown in Fig. 1.

Experimental

Materials. Poly(A)·poly(U) and propidium diiodide were obtained from Yamasa Shoyu Co. and Sigma Chemical Co., respectively. All solutions were prepared using a buffer consisting of 10^{-2} mol dm^{-3} Na_2HPO_4 and 10^{-4} mol dm^{-3} Na_2EDTA. Each buffer solution was adjusted to the desired final Na^+ concentration by adding NaCl up to 1 mol dm^{-3} followed by readjustment of the pH to 7.0. The Na^+ concentration was calculated as the sum of the concentrations from NaCl, Na_2HPO_4, and Na_2EDTA. The solutions of the nucleic acid were prepared and dialyzed against each buffer containing the desired concentration of the salt at 4 °C for 1—2 days. The propidium (PD) and poly(A)·poly(U) concentrations were determined spectrophotometrically using the following extinction coefficients; $\varepsilon_{493}=5.90\times10^{3}$ mol^{-1} dm^{3} cm^{-1} for PD[1] and $\varepsilon_{260}=6.34\times10^{3}$ mol^{-1} dm^{3} cm^{-1} for poly(A)·poly(U) listed in Yamasa's catalog No. 7078.

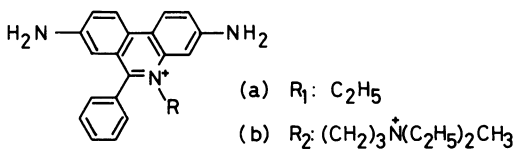

Fig. 1. The structures of (a) ethidium (ED) and (b) propidium (PD).

Equilibrium Mesurements. Absorbance measurements in the UV-visible region were made on a Hitachi U-3200 programable spectrophotometer. Wavelength scans were made in cells having a path length of 10 mm. Cell holders were thermostated by a Hitachi SPR-7 temperature controller. In all experiments temperature was controlled within ±0.1 °C.

The absorbance of PD obeyed the Beer-Lambert law at the concentration range used in the experiment (10^{-5}—10^{-4} mol dm^{-3}). The absorption change of the drug at 480 nm with increasing the concentration of poly(A)·poly(U) was measured in each buffer to make a spectrophotometric titration. Following each addition of the nucleic acid, a time scan was made on the absorbance of the drug at the wavelength to confirm that the equilibrium was reached, and then the absorbance was recorded. The value of r which was defined as the average fraction of lattice binding sites of the nucleic acid occupied by the drug was calculated from the titration curve, finally being obtained an association constant of the drug with the nucleic acid in each solution.

Kinetic Measurements. The kinetic experiments were performed on an Otuka Denshi RA-401 stopped-flow apparatus and a micro stopped-flow one (Wakenyaku Co.) interfaced to an NEC PC-9801-VX computer. Only 1.7×10^{-5} dm^{3} of each sample solution was needed to measure each kinetic run on the micro stopped-flow apparatus.

The rate of the association of PD with poly(A)·poly(U) in 2.02×10^{-2} and 1.02 mol dm^{-3} Na^+ was determined by

monitoring the change of absorbance with time at 480 nm. In the experiments the initial concentration of the nucleic acid was always regulated to large excess over that of the drug, and temperature was kept at 25.0±0.1 °C. The obtained kinetic runs were analyzed with nonlinear least-squares fitting to single or double exponential curves.

Results and Discussion

Spectra and Association Constants at 25 °C. Free PD had an absorption maximum at 493 nm at 25 °C, which shifted to about 530 nm for PD fully bound to poly(A)·poly(U) in solution. The spectral shapes at 2.02×10^{-2} mol dm^{-3} Na^+ were shown in Fig. 2, and the spectra were very similar at each temperature and in each solution containing a different Na^+ concentration. The absorbance of PD at 480 nm decreased with the increasing poly(A)·poly(U) concentration, and the minimal absorbance was reached by about 10^{-3} mol dm^{-3} poly(A)·poly(U).

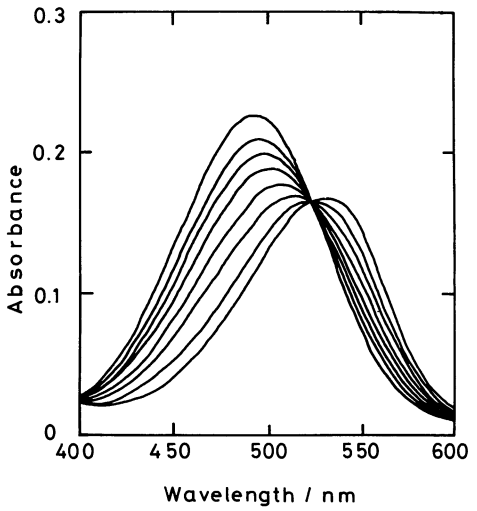

Fig. 2. Spectrophotometric titration of PD with poly(A)·poly(U) in 2.02×10^{-2} mol dm^{-3} Na^+, pH 7.0 at 25 °C. The constant concentration of PD is 3.62×10^{-5} mol dm^{-3}. The concentration range of poly(A)·poly(U) is from zero at the top curve to 2.48×10^{-4} mol dm^{-3} at the bottom curve at 480 nm.

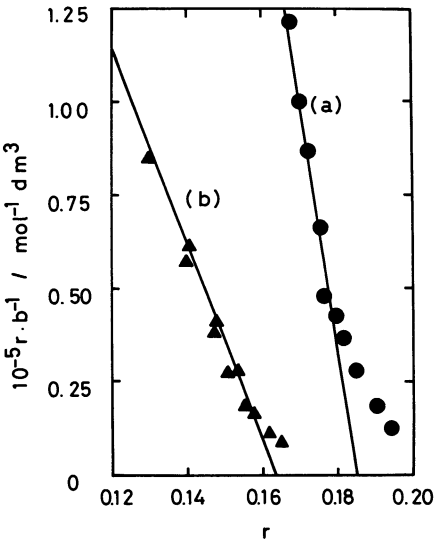

Fig. 3. Binding isotherms of PD to poly(A)·poly(U) in (a) 2.02×10^{-2} and (b) 1.20×10^{-1} mol dm^{-3} Na^+ at 25 °C.

The results of the spectrophotometric titrations at 480 nm were converted to binding isotherms. Typical plots at 2.02×10^{-2} and 1.20×10^{-1} mol dm^{-3} Na^+ were shown in Fig. 3. The data were analyzed with the theory for site-exclusion binding.[8] For this kind of binding, Eq. 1 is often used;

$$r/b = K(1 - nr)^n (1 - (n - 1)r)^{1-n}, \quad (1)$$

where r is the average fraction of lattice binding sites of poly(A)·poly(U) occupied by PD, b the molar concentration of free PD, K the binding constant for propidium to poly(A)·poly(U), and n the number of consecutive lattice residues made inaccessible by the binding of a single PD molecule. In Eq. 1 the intercept on the r/b axis is K and the intercept on the r axis is $1/n$. But the determination of n by this method is subject to large uncertainties, and these affect the values of K.[9] Therefore, in this work the derivative of Eq. 1 in the limit of low r was used to obtain the values of K:

$$\lim_{r\to0}[d(r/b)/dr] = -K(2n-1). \quad (2)$$

The obtained values of K and n at 2.02×10^{-2}, 1.20×10^{-1}, and 1.02 mol dm^{-3} Na^+ at 25 °C were listed in Table 1 with those at the other temperatures.

The value of K at 2.02×10^{-2} mol dm^{-3} Na^+ was 1.42×10^6 mol^{-1}dm^3 at 25 °C corresponding to $\Delta G°=-35.1$ kJ mol^{-1}. This value of the free energy was close to that for ethidium (ED) to poly(A)·poly(U) which had been determined calorimetrically to be −36.4 kJ mol^{-1} at 1.6×10^{-2} mol dm^{-3} Na^+ at 25 °C.[9] In addition, the binding constant of PD to poly(A)·poly(U) was 4.3×10^5 mol^{-1} dm^3 at 1.20×10^{-1} mol dm^{-3} Na^+ at 25 °C, and the value was about 20 times larger than the value of K of PD binding to poly(dA)·poly(dT).[2] This different affinity of PD to these RNA and DNA polymers was about the same in the case of ED.[10] The values of n at 2.02×10^{-2} and 1.20×10^{-1} mol dm^{-3} Na^+ were 5.4 and 6.1, respectively, which was consistent with 5.9 for the case of ED in 4×10^{-2} mol dm^{-1} Tris buffer.[11] Therefore, these results suggest that the behavior of PD in the binding step at a low Na^+ concentration may be very similar to the intercalation of ED to base pairs of the nucleic acids.

Salt and Temperature Effects on Association Constants. The values of K for PD binding to poly(A)·poly(U) decreased with increasing Na^+ concentrations at the given temperature as shown in Table 1. It suggests the binding is not only stacking but electrostatic in origin.[12]

Table 1. The Dependence of K and n for Propidium Binding to Poly(A)·Poly(U) on Na^+ Concentration at Several Temperatures[a]

T/°C	Low Na^+ concn[b]		High Na^+ concn[c]	
	$10^{-5}K$/mol^{-1} dm^3	n	$10^{-4}K$/mol^{-1} dm^3	n
25.0	14.2 (4.3)[d]	5.4 (6.1)[d]	5.42	10.9
20.0	11.4	5.2	5.29	9.0
15.0	7.81	5.0	6.69	7.8
10.0	6.29	4.8	6.25	8.0
$\Delta H°$/kJ mol^{-1}	39.8		−8.3	
$\Delta S°$/J K^{-1} mol^{-1}	251		63	
$\Delta G°_{25}$/kJ mol^{-1}	−35.1		−27.1	

a) Accurate to within ±15% for K and n. b) 2.02×10^{-2} mol dm^{-3} Na^+. c) 1.02 mol dm^{-3} Na^+. d) 1.20×10^{-1} mol dm^{-3} Na^+.

The polyelectrolyte theory of Manning[13] and Record et al.[14] is very useful to interpret the effects of counterion concentration on ligand binding to polyelectrolytes. From this theory, Eq. 3 can be obtained:

$$(\partial \log K/\partial \log [Na^+]) = -m'\psi. \quad (3)$$

Here m' is the number of ion pairs that form with the polyelectrolyte on binding of one ligand, and ψ is the sum of fraction of counterions which are directly condensed onto the polyelectrolyte and the fraction of screening counterions per phosphate.

When the average axial charge spacing along the helical axis for poly(A)·poly(U) is assumed to be 0.16 nm,[6] the value of ψ is calculated to be 0.89 at 25 °C. Therefore, the value of m' between 2.02×10^{-2} and 1.02 mol dm^{-3} Na^+ is about 0.94. The result suggests that one ion-pair contributes mainly to a formation of the complex between PD and poly(A)·poly(U) when the same reaction mechanism at the low and high salt concentrations is assumed.

The changes of enthalpy ($\Delta H°$), entropy ($\Delta S°$), and free energy at 25 °C ($\Delta G°_{25}$) for the association of PD with poly(A)·poly(U) at 2.02×10^{-2} and 1.02 mol dm^{-3} Na^+ are shown in Table 1. The value of $\Delta G°_{25}$ for PD binding to poly(A)·poly(U) at low Na^+ concentration is slightly smaller than −34.7 and −31.2 kJ mol^{-1} for PD binding to $tRNA^{Phe}$[7] and poly(I)·poly(C),[15] respectively. As shown in Table 1, this negative value of $\Delta G°_{25}$ is due to a very favorable entropic driving force. The favorable entropic driving force PD binding may reflects the fact that the binding releases many counterions from the duplex of poly(A)·poly(U) and/or induces desolvation of the drug and the duplex because PD has two positive charges and a large side chain which can disrupt the layers of hydration and Na^+ for the duplex. This disruption of hydration and Na^+ layers can be envisioned as an endothermic process, thereby reducing the exothermicity of the bulk drug-binding, especially at the low salt concentration. This could result in the observed positive value of $\Delta H°$ at 2.02×10^{-2} mol dm^{-3} Na^+.

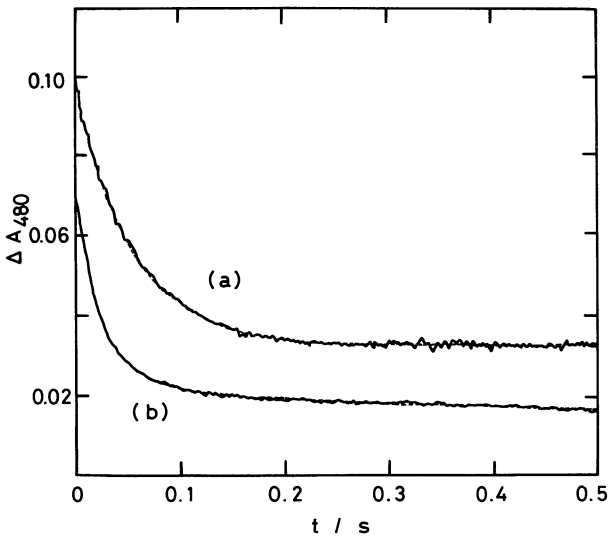

Fig. 4. Stopped-flow kinetic traces for the association reaction of PD with poly(A)·poly(U) at 25 °C in (a) 2.02×10^{-2} and (b) 1.02 mol dm^{-3} Na^+. The RNA concentration kept 1.9×10^{-3} mol dm^{-3} in both buffers was about 50 times larger than PD concentration. The smooth dotted lines represent nonlinear least-squares fitting to single and double exponential curves for curves a and b, respectively.

Kinetics at Low Na^+ Concentration. The positive and negative values of $\Delta H°$ at the low and high Na^+ concentrations, respectively, indicate that a mechanism of the reaction might be different in the low and high salt concentrations. Therefore, association kinetics of PD to poly(A)·poly(U) have been investigated to provide insights into salt effect on the mechanism of the binding behaviors. Figure 4 shows typical kinetic runs at 2.02×10^{-2} and 1.02 mol dm^{-3} Na^+ at 25 °C. The kinetic run at the low Na^+ concentration was fitted with a single exponential function, but the run at the high Na^+ concentration was not, as shown in Fig. 4. On the kinetic curve at the high Na^+ concentration a fast step finished within about 50 ms and was followed by the slower step.

In kinetic measurements at 2.02×10^{-2} mol dm^{-3} Na^+

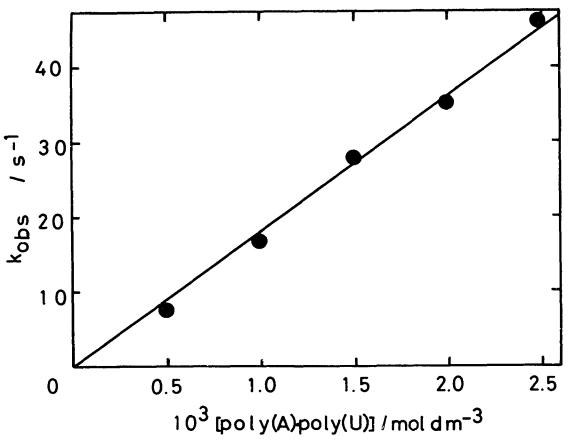

Fig. 5. Dependence of k_{obs} on poly(A)·poly(U) concentration in 2.02×10^{-2} mol dm^{-3} Na^+ at 25 °C.

the stopped-flow trace obeyed a first-order kinetic equation when the poly(A)·poly(U) concentration was in large excess over the PD concentration. The plots of the observed rate constant, k_{obs}, against the initial concentration of poly(A)·poly(U), $[B]_0$, at 25 °C were linear in agreement with Eq. 4 as shown in Fig. 5:

$$k_{obs} = k_1[B]_0 + k_{-1} \quad (4)$$

In Eq. 4, k_1 and k_{-1} were the association and dissociation rate constants, respectively. The value of k_1 determined from the dependence of k_{obs} on $[B]_0$ by a least-squares method was 1.81×10^4 mol^{-1} dm^3 s^{-1}. On the other hand, the value of k_{-1} which was the intercept on the x axis in this treatment was near zero and was too small to be reliable. The value of k_{-1} can be calculated to be 1.3×10^{-2} s^{-1} by the relation that $k_{-1}=k_1/K_1$ and $K_1=1.42\times10^6$ mol^{-1} dm^3 in Table 1.

The association constants of PD at 2×10^{-1} mol dm^{-3} NaCl at 15 °C were reported as 1.25×10^6 and 1.06×10^6 mol^{-1} dm^3 s^{-1} for poly(dAT)·poly(dAT) and calf thymus DNA, respectively.[2] However, the association constant with poly(dA)·poly(dT) was quite small (4.8×10^4 mol^{-1} dm^3 s^{-1}).[2] This different behavior between the altenating and nonalternating A/T polymers was also found in the results with oligomers: That is, the association constants at 5 °C were 2.1×10^5 and 4.3×10^4 mol^{-1} dm^3 s^{-1} for d(A–T)$_6$ and d(A$_6$–T$_6$), respectively.[3] In the present work, the value of 1.81×10^4 mol^{-1} dm^3 s^{-1} for poly(A)·poly(U) is very small even at the high temperature (25 °C). This might be due to the nonalternating A/U sequence, and/or the difference between RNA and DNA.

Kinetics at High Na^+ Concentration. The association reaction at 1.02 mol dm^{-3} Na^+ gives the two observed rate constants of $k_{obs,1}$ and $k_{obs,2}$. Figure 6 shows the dependences of $k_{obs,1}$ and $k_{obs,2}$ on $[B]_0$ at 25 °C. These linear and hyperbolic concentration dependences of $k_{obs,1}$ and $k_{obs,2}$, respectively, at the high Na^+ concentration cannot be explained by the simple one-step mechanism at 2.02×10^{-2} mol dm^{-3} Na^+. It is known that poly(A)·poly(U) suffers disproportionation into a poly(A)·poly(U) triple helix and a single poly(A) strand at a very high ionic strength.[16] However, the disproportionation hardly occurs in the present salt concentration at 25 °C.[17] In addition, in about 3 mol dm^{-3} Na^+ in which a poly(A)·poly(U) triple helix mainly exists, a binding isotherm shows a very different behavior from Fig. 3b: The value of r/b increases with increasing r at low r.[18] These suggest that the disproportion does not contribute the present reaction at 1.02 mol dm^{-3} Na^+. It could be also considered that there is an allosteric conversion, discussed in DNA,[19] from some nonstandard RNA state to a usual A-form double helix, before the binding of the drug. Again, the allosteric conversion cannot give the probable explanation for the linear and hyperbolic dependences of $k_{obs,1}$ and $k_{obs,2}$, respectively, on the poly(A)·poly(U) concentration.[20]

Finally, the results may be consistent with a two-step mechanism in which the fast bimolecular association process is followed by a slow unimolecular process:

$$\text{PD} + \text{poly(A)}\cdot\text{poly(U)} \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} \text{Complex 1} \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} \text{Complex 2} \quad (5)$$

where Complexes 1 and 2 are the different types of the PD–poly(A)·poly(U) complex. When the poly(A)·poly(U) concentration was in excess over the PD concentration, $k_{obs,1}$ and $k_{obs,2}$ for this mechanism are given by Eqs. 6 and 7, respectively:[21]

$$k_{obs,1} = k_1[B]_0 + k_{-1} \quad (6)$$

$$k_{obs,2} = k_2[B]_0/([B]_0+1/K_1) + k_{-2} \quad (7)$$

Here, k_1, k_{-1}, k_2, and k_{-2} are the rate constants as indicated in Eq. 5, and $K_1=k_1/k_{-1}$. The linear plot in Fig. 6a by a least-squares method gives 1.91×10^4 mol^{-1} dm^3 s^{-1}, 33.8 s^{-1}, and 5.65×10^2 mol^{-1} dm^3 for k_1, k_{-1}, and K_1, respectively. The curve in Fig. 6b by a nonlinear least-squares method gives 17.5 s^{-1} and 8.78 s^{-1} for k_2 and k_{-2}, respectively.

It is known that conversions of Z to B-form[22] and of A to B-form[23] of nucleic acids are very slow. However, in the present reaction, the unimolecular process in Eq. 5 is quite faster than these conversions. The result suggests that poly(A)·poly(U) in about 1 mol dm^{-3} Na^+ and 25 °C may have small structural variations induced by PD such a A′ conformation,[16] and the structual variations may be minor in comparison with the major conformational variations. The study for poly(I)·poly(C) will be able to show whether this initial results on poly(A)·poly(U) has the generality in the other ribo-homopolymers. Although there are

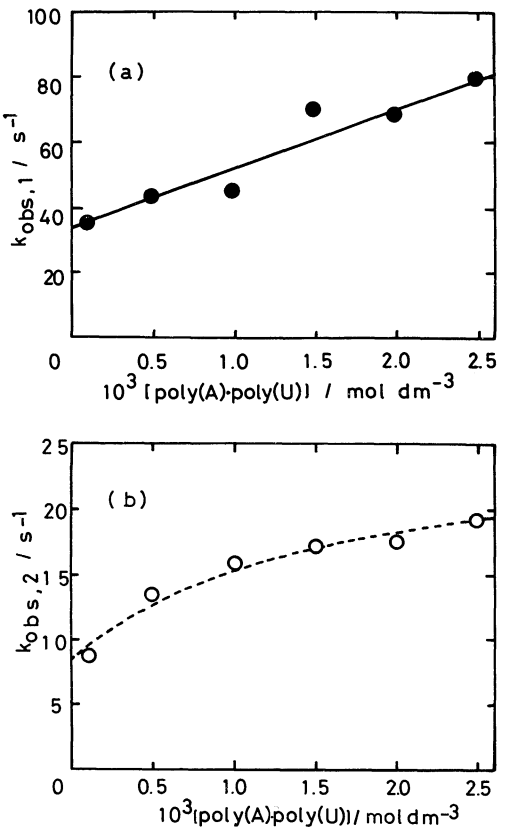

Fig. 6. Dependence of (a) $k_{obs,1}$ and (b) $k_{obs,2}$ on poly(A)·poly(U) concentration in 1.02 mol dm^{-3} Na^+ at 25 °C.

other possible explanations for the complex kinetics of the association, there can be no question that the association displays different behaviors at the low and high salt concentrations.

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