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Dextran Sulfate Complexes with Potassium Phosphate to Interfere in Determinations of High-Density Lipoprotein Cholesterol

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The interference of dextran sulfate with the colorimetric determination of free cholesterol causes an increase in the absorbance that is proportional to the dextran sulfate concentration in the sample. This makes estimation of the free cholesterol concentration unreliable in high-density lipoprotein-containing supernates obtained after plasma precipitation with dextran sulfate/$MgCl_2$. Analysis of the absorption spectrum demonstrated that the absorbance increase was due to turbidity. We observed this effect with colorimetric reagents based on potassium phosphate buffer but not with those based on sodium phosphate or Tris buffers. This effect is ascribed to dextran sulfate because neither the omission of Mg^{2+} ions nor their chelation with EDTA prevented turbidity, whereas precipitation of dextran sulfate with Ba^{2+} counteracted this effect. Therefore, potassium phosphate-buffered reagents appear unsuitable for colorimetric lipid determinations in samples containing dextran sulfate.

Additional Keyphrases: *analytical error* · *colorimetry*

The well-documented inverse correlation between the concentration of plasma high-density lipoprotein (HDL) cholesterol and the incidence of atherosclerosis and coronary artery disease (*1*) has led to the development of several techniques for ready measurement of HDL.[2] Preparative ultracentrifugation is still considered the most accurate method for separation of HDL and its two major subclasses HDL_2 and HDL_3; however, lipoprotein(a) is also partially separated within the HDL density range (*2*), and the procedure is too laborious and expensive to be practical for routine analysis. Several alternatives are available, but those preferred in the clinical laboratory are the precipitation methods based on the interaction of polyanions and sulfated polysaccharides with the different lipoproteins in the presence of divalent cations (*3–11*). Some methods allow quantification of HDL subclasses and have gained popularity because of their simplicity, but they are not free of drawbacks, e.g., the deleterious effects of Mn^{2+} on the colorimetric determinations of HDL lipids (*12, 13*) or the specificity for lipoprotein precipitation (*14–17*). Dextran sulfate/$MgCl_2$ is probably one of the most widely used precipitating agents, but it presents some interferences in the enzymatic determination of cholesterol (*18*).

To investigate the nature of potential interferences in this methodology, we studied the effect of potassium phosphate buffer on the enzymatic determination of free cholesterol in supernates from plasma precipitated with dextran sulfate/$MgCl_2$. Our results indicate that complexes of dextran sulfate–potassium form, producing turbidity and interference in absorbance measurements.

Materials and Methods

Plasma Lipoprotein Precipitation

We followed the method of Warnick et al. (*7*) to compare two different precipitating solutions: DS-I, 10 g of dextran sulfate (M_r 50 000 ± 5000; Sochibo, Vélizy Villacoublay, France) and 0.5 mol of $MgCl_2 \cdot 6H_2O$ (Merck, Darmstadt, F.R.G.) per liter; and DS-II, 10 g of dextran sulfate and 1.5 mol of $MgCl_2$ per liter. The solutions were brought to pH 7.0 with diluted NaOH, and contained 25 mg of NaN_3, 250 mg of gentamicin sulfate, and 500 mg of chloramphenicol per liter as preservatives. We prepared these solutions weekly and stored them at 4 °C.

To precipitate the lipoproteins, we added 100 μL of DS-I solution to 1.0 mL of human plasma (final concentrations: dextran sulfate, 0.9 g/L; $MgCl_2$, 45 mmol/L). The samples were then vortex-mixed, incubated for 15 min at room temperature, and centrifuged at 1500 × *g* for 30 min. The clear supernate containing the HDL was aspirated carefully and used for free cholesterol determination and for HDL_2 precipitation. For precipitation we added 50 μL of DS-II solution to 0.5 mL of the supernate (final concentrations: dextran sulfate, 1.73 g/L; $MgCl_2$, 178 mmol/L). We mixed the samples and centrifuged as above and used the clear supernates containing HDL_3 for enzymatic determination of free cholesterol.

In some instances, to prepare blanks of the precipitating solutions, we omitted plasma and substituted an equal volume of 0.15 mol/L NaCl. We termed these samples DS-I and DS-II blank supernates, respectively, and processed them the same way as the supernates from precipitated plasmas.

Free Cholesterol Determination

We measured free cholesterol in total HDL- or HDL_3-containing supernates enzymatically, using two methods that differed basically in the buffer. Reagent T contained 0.1 mol/L Tris · HCl, pH 7.7, supplemented with, per liter, 23 mmol of sodium cholate as detergent

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[2] Nonstandard abbreviations: HDL, high-density lipoproteins, *d* 1.063 to 1.21; HDL_2, *d* 1.063 to 1.125; HDL_3, *d* 1.125 to 1.21; DS-I, dextran sulfate 10 g/L, $MgCl_2$ 0.5 mol/L solution; and DS-II, dextran sulfate 10 g/L, $MgCl_2$ 1.5 mol/L solution.

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and 50 mmol of phenol and 25 mmol of 4-aminoantipyrine for color development. Reagent P contained the same components as Reagent T except that Tris was replaced with 0.4 mol/L potassium phosphate buffer, pH 7.7. Immediately before use, the reagents were supplemented with cholesterol oxidase (from *Nocardia erythropolis*, EC 1.1.3.6; final activity >70 U/L) and horseradish peroxidase (EC 1.11.1.7; final activity >1000 U/L), both from Sigma Chemical Co., St. Louis, MO.

We used commercial kits in some experiments: (*a*) Reagent B: "free cholesterol enzymatic" (no. A-01371; Biotrol, Paris, France), containing 0.1 mol/L phosphate buffer (the cation is not indicated by the manufacturer); (*b*) Reagent BM: "free cholesterol, CHOD-PAP enzymatic" (no. 310328; Boehringer Mannheim, Mannheim, F.R.G.), containing 0.4 mol/L potassium phosphate buffer.

In the other experiments, we used two plain 0.4 mol/L phosphate buffer solutions at pH 7.7 instead of the colorimetric reagents. One was made with potassium salts and the other with sodium salts. In some cases, these solutions were supplemented with EDTA, 8.8 mmol/L, prepared with disodium and tetrasodium salts as described by Steele et al. (*12*).

For the assay, we mixed 20 μL of the HDL-containing supernates or blanks with 0.5 mL of the respective reagent. After incubation at 37 °C for 30 min, we measured the absorbance at 492 nm in a DU-6 spectrophotometer (Beckman Instruments, Palo Alto, CA). Precinorm L (Boehringer Mannheim) was used as the standard.

In some cases, to precipitate the excess of dextran sulfate in the tubes, once the color development was completed, we supplemented the tubes with different volumes of 10 g/L $Ba(OH)_2$, and then centrifuged at 1500 × *g* for 15 min. We used the supernates obtained for absorbance measurements.

Results

To study the effect of the dextran sulfate/$MgCl_2$ precipitant on the enzymatic determination of free cholesterol, we used two different dextran sulfate solutions and several reagent solutions. The absorbances at 492 nm of the HDL-containing supernates from plasma precipitated with the two dextran sulfate/$MgCl_2$ solutions, as well as their respective blanks (no plasma present), are shown in Table 1. The absorbance of blanks was much higher with reagent solutions containing 0.4 mol/L potassium phosphate buffer (Reagent P and commercial Reagent BM) than with solutions containing 0.1 mol/L phosphate buffer (commercial Reagent B) or 0.1 mol/L Tris · HCl (Reagent T). In addition, the DS-II blanks produced higher absorbances with phosphate-buffered reagents (but not with the Tris · HCl buffered reagent) than did the DS-I blanks. A similar pattern was observed with plasma samples (Table 1). Also, with Reagent P, the absorbances of the supernates obtained with DS-II (which contained HDL_3) were paradoxically lower than their respective blanks and higher than for the supernates obtained with DS-I (which contained total HDL). On the contrary, when we used either Reagent B or Reagent T, the absorbance values of plasma samples precipitated with DS-II were lower than those precipitated with DS-I (Table 1).

To demonstrate the relationship between the concentration of dextran sulfate/$MgCl_2$ in the tube and the magnitude of the absorbance, we performed the colorimetric assay with 20 μL of DS-I or DS-II blank supernates, which could be conveniently diluted to different concentrations, and 500 μL of Reagent P or Reagent T separately, in the absence of free cholesterol. The absorbance values obtained were plotted against the concentration of dextran sulfate in the sample (Figure 1). Dextran sulfate at concentrations up to 1.73 g/L (which corresponds to that in DS-II supernates) did not modify the absorbance of Tris · HCl-buffered reagent, whereas there was a linear increase in the absorbance with the phosphate-buffered reagent (Figure 1).

To evaluate whether this interference was due to the formation of a colored compound absorbing at 492 nm or just to light scattering due to turbidity, we scanned the absorbances of Reagent P from 350 to 700 nm. Compared with the reagent alone (Figure 2, curve *a*), the addition of 20 μL of DS-I blank supernate to the reagent produced an increase of the absorbance along the full spectrum (Figure 2, curve *b*), which was even more accentuated with the DS-II blank (Figure 2, curve *c*). As also shown in Figure 2 (curves *d* and *e*), the precipitation of dextran sulfate with Ba^{2+} produced a decrease in the absorbance pattern, the values obtained being very similar to those observed in the samples not containing dextran sulfate originally (Figure 2, curve *a*). The effects of dextran sulfate and Ba^{2+} (Figure 2*A*) were also observed in samples supplemented with free cholesterol (Figure 2*B*).

To confirm that precipitation of dextran sulfate with Ba^{2+} counteracted the interference, we mixed 20 μL of DS-I or DS-II blank supernates with 0.5 mL of Reagent

Table 1. Absorbances at 492 nm of HDL-Containing Supernates after Precipitation with Dextran Sulfate/$MgCl_2$

	Plasmas, mean ± SD (n = 5)		Blanks[b]	
Reagents[a]	DS-I	DS-II	DS-I	DS-II
Reagent P	0.124 ± 0.010	0.262 ± 0.015	0.155	0.435
Reagent T	0.076 ± 0.005	0.052 ± 0.004	0.005	0.007
Reagent BM	0.221 ± 0.030	0.517 ± 0.035	0.164	0.387
Reagent B	0.118 ± 0.016	0.089 ± 0.006	0.008	0.015

[a] Composition as described in the text. [b] Isotonic saline (NaCl 9 g/L) instead of plasma.

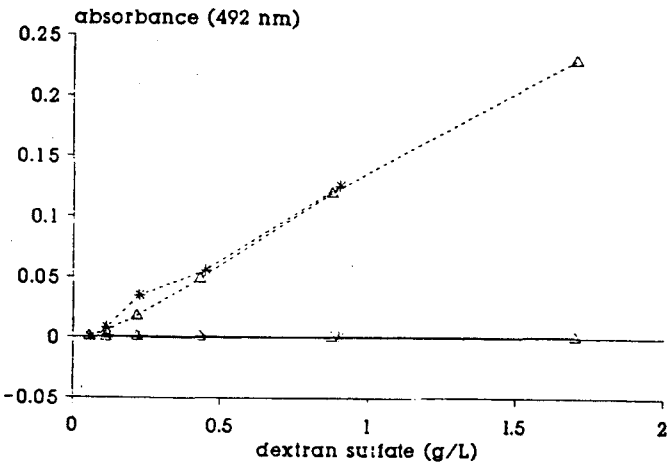

Fig. 1. Effect of dextran sulfate/$MgCl_2$ on the absorbance at 492 nm of two reagents for determination of free cholesterol

DS-I (—*—, —*—) and DS-II (△) blank supernates (20 μL) were mixed with 500 μL of cholesterol assay reagents that contained 0.1 mol/L Tris · HCl (——) or 0.4 mol/L potassium phosphate (-----) as buffer. The absorbance of each reagent was adjusted to 0. Results are plotted against the dextran sulfate concentration in the supernate solutions and correspond to the mean of duplicates of a representative experiment

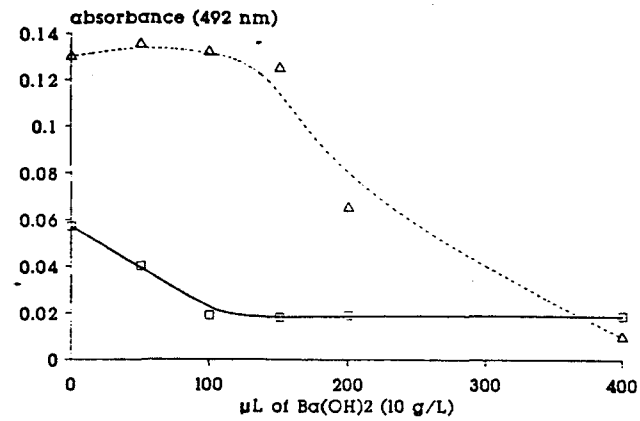

Fig. 3. Effect of Ba^{2+} ion on the interference of dextran sulfate/$MgCl_2$ with a potassium phosphate-buffered reagent

DS-I (—□—) and DS-II (---△---) blank supernates (20 μL) were mixed with 500 μL of a reagent for the colorimetric determination of free cholesterol containing 0.4 mol/L potassium phosphate, and then various volumes of 10 g/L $Ba(OH)_2$ were added for dextran sulfate precipitation, the precipitates being removed by centrifugation. The supernates were processed for the measurement of absorbance at 492 nm. Results correspond to the mean of duplicates of a representative experiment

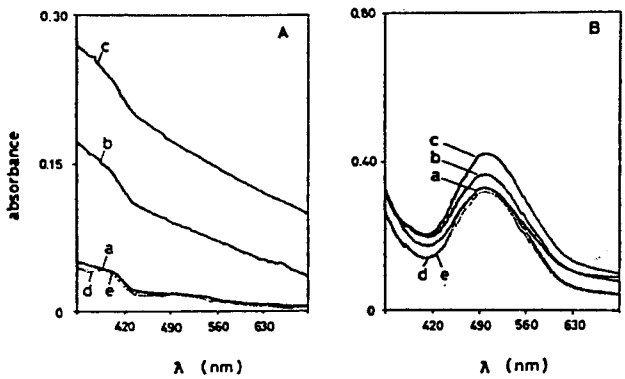

Fig. 2. Effect of dextran sulfate/$MgCl_2$ on the absorption spectrum of a potassium phosphate-buffered reagent

Samples in *A* correspond to mixtures of 500 μL of a reagent containing 0.4 mol/L potassium phosphate and 20 μL of the following solutions: (*a*) NaCl (9 g/L), (*b*) DS-I blank supernate, (*c*) DS-II blank supernate, (*d*) DS-I blank supernate and 0.4 mL of 10 g/L $Ba(OH)_2$, and (*e*) DS-II blank supernate and 0.4 mL of 10 g/L $Ba(OH)_2$. Samples in *B* are equivalent to those in *A*, but also contain 20 μL of a cholesterol solution (2 g/L in isopropanol)

P and, after color development was completed, added increasing volumes of 10 g/L $Ba(OH)_2$ solution for dextran sulfate precipitation, which was removed by centrifugation. As shown in Figure 3, the addition of 100 μL or more of Ba^{2+} solution to the tubes corresponding to DS-I blanks restored the absorbance values to that of Reagent P, whereas 400 μL of Ba^{2+} was needed for the DS-II blanks.

To finally evaluate the origin of this interference, we used as reagents two 0.4 mol/L phosphate solutions at pH 7.7, one made with potassium salts and the other with sodium salts (containing no chromogen or other additives). We performed these experiments in the absence and in the presence of EDTA, 3.8 mmol/L, for chelating divalent ions. After mixing 500 μL of any of these solutions with the samples and leaving them to stand for 30 min, we monitored the absorbance at 492 nm. With the 0.4 mol/L potassium phosphate solution, the addition of either DS-I or DS-II blank supernates gave higher absorbances than distilled water, as in previous experiments (Table 2). Dextran sulfate plain solutions, at the concentrations used for the precipitating purposes, showed absorbances very similar to the respective blank supernates, the one observed with 0.9 g/L dextran sulfate reagent being twofold that with 1.73 g/L. However, solutions of $MgCl_2$, at 45 or 178 mmol/L, did not increase the absorbance of the reagent. The mixture of dextran sulfate and $MgCl_2$ again produced high absorbance measurements, as did dextran sulfate alone. For comparison, we included in this series of experiments the addition of $MnCl_2$ to reproduce the well-known interference of this cation in the presence of phosphates. Actually, the addition of $MnCl_2$ (92 mmol/L) produced the expected turbidity, which was reflected in a marked increase in the absorbance. When formation of insoluble $MnPO_4$ was prevented by previous addition of EDTA, the $MnCl_2$ no longer produced light scattering. However, the inclusion of EDTA did not arrest the effect of dextran sulfate (Table 2).

The use of sodium phosphate instead of potassium phosphate rendered quite different results (Table 2). Still, the turbidity created by manganese salts and the minimizing effect of EDTA on this were observed. However, neither blank supernates or dextran sulfate alone nor the presence of Mg^{2+} ions produced any significant increase in the absorbance of this sodium-based reagent (Table 2).

To study the concentration effect, we varied the potassium phosphate concentration from 0.01 to 0.4 mol/L. The increase in the absorbance produced by adding either DS-I and DS-II blank supernates was detected at concentrations >0.05 mol/L, the maximum effect being reached at 0.2 mol/L potassium phosphate (Figure 4).

Once the implication of dextran sulfate and potassium

Table 2. Effect of Dextran Sulfate, $MgCl_2$, and $MnCl_2$ on Absorbance of Phosphate Buffers

Additive[a]	$A_{492\ nm}$, 0.4 mol/L phosphate buffer, pH 7.7[b]			
	K	K, EDTA	Na	Na, EDTA
Distilled water	0.008	0.009	0.000	0.000
DS-I blank supernate[c]	0.097	0.104	0.000	0.001
DS-II blank supernate[c]	0.201	0.217	0.000	0.000
Dextran sulfate, 0.9 g/L	0.128	0.127	0.001	0.000
Dextran sulfate, 1.73 g/L	0.210	0.221	0.000	0.001
$MgCl_2$, 45 mmol/L	0.013	0.003	0.011	0.000
$MgCl_2$, 178 mmol/L	0.008	0.001	0.000	0.000
Dextran sulfate, 0.9 g/L, plus $MgCl_2$, 45 mmol/L	0.104	0.108	0.000	0.000
Dextran sulfate, 1.73 g/L, plus $MgCl_2$, 178 mmol/L	0.201	0.220	0.000	0.000
$MnCl_2$, 46 mmol/L	0.020	0.018	0.025	0.000
$MnCl_2$, 92 mmol/L	0.696	0.018	0.516	0.008

[a] 20 μL added to 500 μL of buffer plus 50 μL of distilled water or EDTA, 100 mmol/L.
[b] Mean of duplicates of a representative experiment.
[c] Isotonic saline instead of plasma.

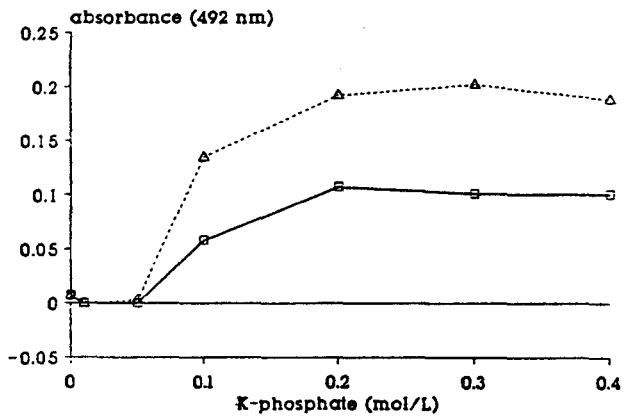

Fig. 4. Effect of potassium phosphate on the absorbance of dextran sulfate blank supernates

DS-I (—□—) and DS-II (---△---) blank supernates (20 μL) were mixed with 500 μL of potassium phosphate buffer or distilled water (0 concentration) and the absorbance at 492 nm was monitored 30 min afterwards. Results correspond to the mean of duplicates of a representative experiment

ions on this effect was demonstrated, we checked whether some free sulfate might contribute to turbidity formation. We exhaustively dialyzed dextran sulfate solutions against distilled water and then immediately measured their absorbance after mixing them with the potassium phosphate buffer, as above. The results showed no differences between dialyzed and nondialyzed dextran sulfate solutions (data not shown), indicating that free sulfate is not the cause of the observed effect.

Discussion

We report the effect of phosphate buffers on the colorimetric determination of free cholesterol in HDL-containing supernates from plasmas precipitated with dextran sulfate. The existence of this interference, caused by dextran sulfate/$MgCl_2$ in a CHOD-PAP method for cholesterol determination, has been pointed out by Demacker et al. (*18*), who observed a very high (0.31 mmol/L) "pseudocholesterol" concentration in their precipitation blanks (no cholesterol present). We have extended these observations and have found that dextran sulfate interferes only with reagents containing potassium phosphate. The interference, an increase in absorbance, is dependent on the concentration of potassium phosphate in the colorimetric reagent and on the concentration of dextran sulfate in the sample. This effect corresponds to the turbidity caused by the formation of an insoluble compound, probably between dextran sulfate and potassium ions.

While determining free cholesterol in the colorimetric assay, we observed that plasma supernates containing total HDL (which are obtained by treating plasma with the dextran sulfate/$MgCl_2$ precipitant reagent) (*7*) produced much lower absorbances than supernates containing HDL_3 (which are obtained in a second precipitation step at a higher concentration of dextran sulfate/$MgCl_2$). Obviously, these results made no sense and indicated the presence of an artifact in the colorimetric determination of free cholesterol. We observed similar inconsistent results with the respective assay blanks (Table 1). This phenomenon was clearly observed with a prepared colorimetric reagent and also with a commercial reagent, both of which contained 0.4 mol/L potassium phosphate buffer, but it did not appear with a Tris-buffered reagent.

The possibility of correcting this interference by subtracting the absorbance values of blanks from those of samples was not feasible, because with our reagent containing phosphate at 0.4 mol/L, the absorbance values of the blanks were even higher than those of plasma supernates. These results agree with those of Demacker et al. (*18*) and can be interpreted by assuming there is a lower concentration of dextran sulfate in the plasma samples than in the blanks, because some dextran sulfate is precipitated with the plasma lipoproteins.

The analysis of the absorbance spectrum showed that samples containing dextran sulfate/$MgCl_2$ had high absorbance values all along the visible spectrum, independent of the peak at 492 nm, which corresponded to the chromophore (Figure 2). This indicates that the origin of the interference was turbidity attributable to

the formation of an insoluble complex. In agreement with this interpretation, we observed that the interference could be partially eliminated by centrifuging the tubes once the color development was accomplished (results not shown).

Among the precipitating agents, dextran sulfate and not $MgCl_2$ appears to be responsible for this effect. First, dextran sulfate alone, when added to a 0.4 mol/L potassium phosphate solution, reproduced the effects of the DS-I or DS-II blank supernates, whereas $MgCl_2$ had no appreciable effect on the absorbance of the reagent (Table 2). Second, the increase in the absorbance of the reagent containing phosphate was linearly correlated to dextran sulfate concentration in the solution, and we observed no difference between those supernates despite their differences in $MgCl_2$ concentration (45 vs 178 mmol/L, respectively). Finally, when dextran sulfate was precipitated with Ba^{2+}, the DS-I and DS-II blank supernates lost their capacity to create turbidity (Figures 2 and 3).

On the other hand, we have demonstrated that the cation involved in this effect is potassium. First, turbidity was produced only with potassium phosphate (at concentrations >0.05 mol/L), not with sodium phosphate buffer solutions. Second, the possibility of the participation of a divalent cation is ruled out because, contrary to the effect on manganese salts, EDTA did not prevent the formation of the insoluble compound. Similar effects with EDTA were previously observed by Demacker et al. (*18*). Besides, the possibility that some free sulfate (coming from dextran sulfate decomposition) could contribute to turbidity by forming insoluble potassium sulfate is also ruled out on the basis of the results observed with freshly dialyzed dextran sulfate solution. Therefore, all the results together indicate that the insoluble compound is made up of dextran sulfate complexed to potassium.

The present work had its origin in our interest in measuring the free cholesterol content in HDL subfractions, given the value of free cholesterol in HDL as a cardiovascular risk factor (*19*). Surprisingly, and fortunately in this case, most of the available kits for total cholesterol determination do not use potassium salts. However, the methodological artifact described here is not limited to free-cholesterol determination. In general, for any colorimetric determination in samples containing dextran sulfate, the use of alternative buffers not containing potassium is strongly recommended.

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