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USE OF PECTIC POLYSACCHARIDES FOR CRYOPRESERVATION OF BIOLOGICAL OBJECTS

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Abstract – The protectant activity of pectic polysaccharides derived from various plants was studied on *Saccharomyces cerevisiae* yeast-like fungi, human blood platelets and leukocytes, and the antihemolytic action of the same compounds was studied on red blood cells. The feasibility of cryopreservation of biological objects in the environment of pectic polysaccharide-containing cryoprotectant solutions was demonstrated.

Kew words: pectic polysaccharides; cryopreservation; leukocytes; platelet concentrate; red blood cells; *Saccharomyces cerevisiae*.

INTRODUCTION

The search for new approaches to the cryopreservation of biological objects is motivated by a practical need to introduce affordable, safe and economically viable methods in clinical medicine, science and various sectors of national economy. Traditional methods are based on single-purpose chemically synthesized protector substances that have toxic properties. It is well known (Belous and Grishchenko, 1994; Schrago et al., 1981) that monosaccharides (glucose, fructose, xylose, mannose, ribose), disaccharides (sucrose, maltose, lactose) and polysaccharides (raffinose, dextran) were used as substances having cryoprotective properties. However, Khotimchenko et al. (1999, 2001) found that sugars are more suited as blood substitutes than cryoprotectants, as they are not toxic and do not cause the agglomeration and sedimentation of the erythrocytes.

Individual messages about the efficiency of sugars in pure form as protective substances for the cryopreservation of blood cells and bone marrow have not been confirmed subsequently. Their presence in cryoprotectants is highly desirable, since sugar affects solution crystallization, as well as regulating osmotic and metabolic processes in cells. This contributes to more effective reparations of damaged structures of the blood cells in the cycle of freezing and warming.

According to the theory proposed by Shrago (1981), a substance with specific functional groups (-OH, -COOH, $-CH_3$, $=S=O-CH_3$, $-(CH_2-CH_2O)-OH$, etc) can have a cryoprotectant effect. The considerable branching of carbohydrate chains and high content of active -OH and -COOH radicals (Ovodov et al., 2009) makes it possible to assume that pectic polysaccharides have a significant cryoprotectant effect. Furthermore, they conform to the

basic requirements of cryoprotectants, i.e. they are nontoxic (no need to wash them off the bio object), water soluble and capable of stabilizing water molecules, cause no cell membrane and organelle breakage and lack an unpleasant odor (Svedentsov, 2010). The proven anti-inflammatory and immune-modulating effect of pectic polysaccharides (Markov et al., 2010) stimulated the development of a new class of cryopreservatives with therapeutic effect. Therefore, the purpose of this study was to research the cryoprotectant effect of several pectic polysaccharides incorporated into preservative solutions on diverse biological objects.

MATERIALS AND METHODS

The research material comprised the following pectic polysaccharides (Table 1, Fig. 1) isolated and described by the Immunology and Biotechnology Department, Institute of Physiology, Komi Science Centre, Urals Branch, Russian Academy of Sciences: comaruman from marsh cinquefoil (*Comarum palustre* L.), tanacetan from tansy (*Tanacetum vulgare* L.), rauwolfia from *Rauwolfia serpentina Benth*., bergenan from Siberian tea (*Bergenia crassifolia L.)*, heracleum from *Heracleum sosnowskyi* Manden (Ovodov et al., 2009).

Pectic polysaccharides were isolated from the above-mentioned plants. The plant material was preliminarily treated with a formaldehyde solution to bind pigments and remove contaminants. Protopectins were destroyed by adding dilute hydrochloric acid to obtain a pH of 4.0 during rigorous stirring. Pectic polysaccharides were extracted with 0.7% (w/v) aqueous ammonium oxalate followed by precipitation with 96% ethanol. The precipitate was separated by centrifugation and dissolved in water, followed by dialysis and lyophilization. The successive fractionation of the crude pectic polysaccharides using ultrafiltration membranes with pores having transmission limits of 300 and 100 kDa, gave two fractions with different molecular masses: M >300 kDa (yield approximately 70%) and M 100 - 300 kDa (yield ca. 20%). We used for further study fraction with a molecular masse M >300 kDa.

Pectic polysaccharides with a given molecular mass were subjected to complete acid hydrolysis with 2 M trifluoroacetic acid, revealing monosaccharides as the main constituents of its sugar chains, as determined by paper chromatography and gas-liquid chromatography (Polle et al., 1999).

The isolated pectic polysaccharides have similar features in chemical structure. They contain rhamnogalacturonan-I as the primary fragment of branched macromolecule. The sugar chain of pectic polysaccharides is studied from α-1,4-D-galacturonan.

The main differences in the structure of pectic polysaccharides were in the content of galacturonic acid residues (50 to 90%) and the fine structure of the sugar side chains' branched area. As an example, Fig. 1 shows the structure of the several schemes of fragments of the sugar chain of pectic polysaccharides used.

We used nuclear human blood cells (leukocytes), nuclear-free human blood cells (platelets and red blood cells), and yeast-like fungi. The researched objects belonged to different types and were different in structure. Therefore, we selected for each object a specific cooling program adopted in cryobiology.

Leukocytes were obtained from donor-volunteers (aged 39.4±12.2; 2500 rpm, 5 minute cooling, Sorvall, USA) with their informed consent in accordance with the Law of the Russian Federation on the Donation of Blood and its Components (1993). The cells were mixed (1:1) before freezing with one of five modifications of cryoprotectant solution (n=10 for each) in a Kompoplast 300 (Russia) flexible PVC container and exposed for 20 min at normal ambient temperature. The cryopreservative components were 7% glycerol, a nontoxic concentration, and pectic polysaccharide, concentration 0.2 wt% (for the first solution modification – tanacetan, second – comaruman, third – bergenan, fourth – rauwolfia), Trilon B (0.1%) was used as an anti-clotting agent. The mixture was frozen for 15 min in an alcohol (96% ethyl alcohol) bath cooled to -20°C. The rate

Table 1. General chemical characteristic of polysaccharides used in this work

Name of the pectin/ source isolation	GalA, %	Neutral monosaccharides, %					
		Gal	Ara	Rha	Glc	Xyl	Man
Comaruman/ *Comarum palustre*	64	13.0	6.0	12.0	-	-	-
Tanacetan/ *Tanacetum vulgare*	64	8.5	8.4	5.5	1.2	0.9	0.5
Rauwolfia/ *Rauwolfia serpentina*	82	5.0	4.1	2.1	5.0	1.8	-
Bergenan/ *Bergenia classifolia*	84	3.1	2.8	1.3	2.0	0.2	1.1
Heracleum/ *Heracleum sosnowskyi*	84	2.8	2.6	1.9	-	-	-

Note: GalA – galacturonic acid, Gal – galactose, Ara – arabinose, Rha – rhamnose, Glc – glucose, Xyl – xylose, Man – mannose.

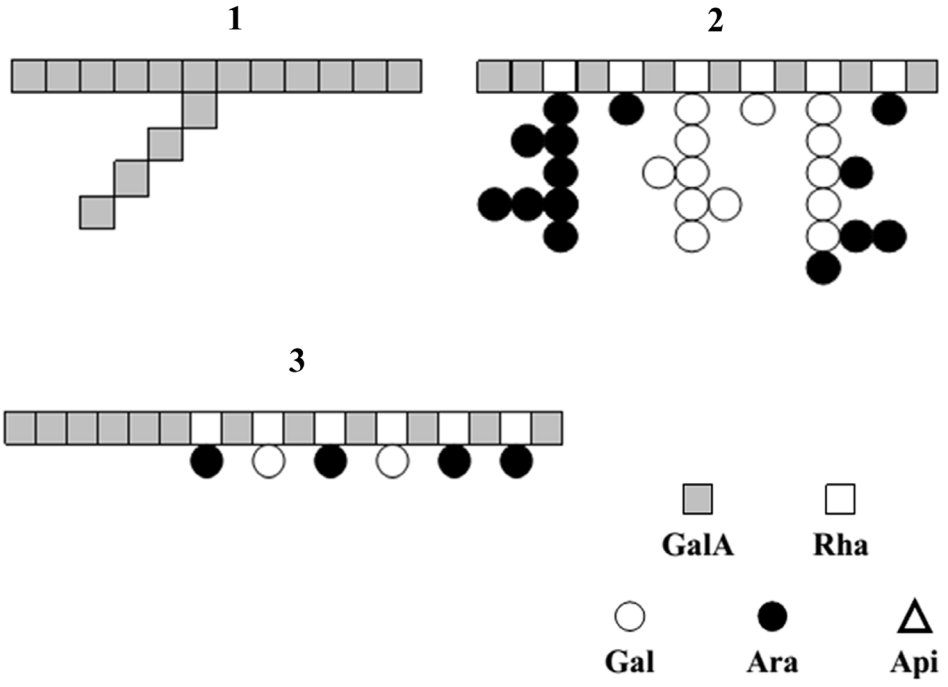

Fig. 1. Structure of the pectin sugar chain fragments:
1 – the branched galacturonan (comaruman); 2 – the poorly branched region of rhamnogalacturonan-I (bergenan); 3 – the highly branched region of rhamnogalacturonan-I (tanacetan); GalA – galacturonic acid, Rha – rhamnose, Gal – galactose, Ara – arabinose, Api – apiose.

of cooling up to eutectic point (-2°C) was 10°C/min, and afterwards 2-3°C/min. The samples were conditioned for 24 h at -20°C in an air environment of an electric freezer, warmed for 40 s in a 20-liter water bath (+38°C), while the container was being actively tilted (Svedentsov et al., 2008). The following values were assessed using light microscopy (Nikon H550S, Japan): total number of leukocytes in a Goryaev chamber; cryoresistance of different cell populations in smears immersed in May-Grünwald and

Romanowsky stains; integrity of leukocyte cell membrane in samples immersed in 1.0% eosin supravital stain solution; diffuse pink staining of cytoplasm was considered an indicator of cell membrane damage, as viable cells with an undamaged membrane are light green (Svedentsov et al., 2008). Neutrophil granule protein activity responsible for a killer effect was evaluated by lysosomal-cation test using the procedure proposed by Slavinsky and Nikitina (1999). This test method is also useful for the detection of low-temperature neutrophil damage, as lysosomal membranes are highly sensitive to freezing, and the loss of their barrier properties is the reason for possible penetration of hydrolases into the cytoplasm and the occurrence of secondary or "latent" cell damages. Neutrophil phagocytic activity was evaluated relying on a modified method proposed by Potapova et al. (1977) and using 0.08 μm inert latex particles (Sigma-Aldrich, Germany). Only the percentage of phagocytic cells was determined. The number of ingested particles (phagocytic index) for all modifications before freezing conformed to Degree 2 (11-30 particles) and to Degree 1 after freezing (up to 10 particles).

Saccharomyces cerevisiae yeast-like fungi (strain 608, SE IU RRICI, Saint-Petersburg) were grown for 48 h at 30°C on modified Saburo medium containing agar and then left to grow on liquid Saburo medium for 24 h while aerated. Then the cells were pelleted by centrifuging and resuspended in cryopreservation medium. The original number of cells was 10^8 CFU/ml. The cryoprotectant medium was normal saline solution (0.15 mol/l of NaCl + 0.01 mol/l of phosphate buffer, pH 7.4) containing no more than 7.0% of dimethylacetamide (DMAC) or 7.0% of DMAC with 0.25 Wt% pectic polysaccharide. The *Saccharomyces cerevisiae* yeast cell suspension (1 ml per sample) was frozen in Nunc (Germany, 2 ml) plastic cryotubes using a Cryoson (Germany) programmable freezer at 1, 5 and 10°C/min to -100°C. Then the tubes were transferred to liquid nitrogen (-196°C) and stored for 3 days. The samples were warmed on a water bath at 37°C. Yeast cell viability before freezing and after depreservation were assessed using Koch's Pour Plate method (Lusta and Fichte, 1990) by counting the number of macrocolonies formed on media containing agar.

Platelet concentrate was obtained using the method proposed by Agranenko et al. (1985) from donor blood samples preserved in Glugicirum solution. Blood plasma containing 5.0% of DMAC or 5.0% of with 0.25 wt% pectic polysaccharide served as cryoprotectant medium. Platelets were taken in a 1:1 ratio and slowly mixed with cryoprotectant medium at normal ambient temperature and exposed for 15 min. All samples were frozen simultaneously in 6 ml plastic containers using the Cryoson (Germany) programmable freezer at the cooling rates of 10 and 20°C/min to -80°C; then the containers were immersed in liquid nitrogen (-196°C) and stored for 3 days. The samples were warmed on a water bath at 37°C. Platelet content before freezing and after depreservation were assessed using the method proposed by Ronin (1983) in a Goryaev chamber and expressed as % of the reference sample. ADP and collagen-induced platelet aggregation was evaluated by photometry in a thermostatically controlled cuvette (37±0.5°C), while constantly mixed with a magnetic stir bar, using a Colysgraph (Damon/ IEC Division, USA) blood plasma analyzer and expressed as % of the reference sample (Berkovskiy et al., 2003). Hypotonic shock response (HSR) was also evaluated by photometry (Gigout et al., 1999) using the same analyzer by continuous registration of platelet suspension optical density: the rapid optical density decrease phase related to the swelling of cells after 0.5 volume of distilled water was introduced into the platelet suspension. HSR degree was expressed as the percentage of the reference sample.

Red blood cells were isolated from donor blood by centrifuging (1 250 g 20 min), while the plasma and buffy coat were removed. The resulting red blood cell mass was flushed three times to remove damaged cells by centrifuging (1 400 g 10 min) in a 10-fold volume of normal saline solution (0.15 mol/l of NaCl + 0.01 mol/l of phosphate buffer, pH 7.4). 500 μl of condensed red blood cell mass was transferred into 5 ml of normal saline solution to obtain a red blood cell suspension. The red blood cell sus-

Table 2. Characteristics of leukocytes (M±σ) undergoing hypothermia at -20°C for 1 day in cryoprotectant solution.

Substance	n	Indicators of preservation, %				
		Amount of leukocytes	Eosin resistance	Quantity of granulocytes	Phagocytic activity of neutrophils	Content of lysosomal cationic proteins in neutrophils
Glycerol	10	86.4±13.4	75.5±8.3	50.8±11.9	cells are destroyed	81.3±13.2
+ tanacetan	5	86.6±10	86.8±6.5*	60.4±5.9*	67.2±8.5	94.3±1.4*
+ comaruman	12	90.6 ±7.9	83.1±6.6*	68.0 ±13.5*	69.1±8.7	95.9±4.4*
+ bergenan	6	89.0±6.8	80.7±3.7	70.7±6.4*	15.4±1.5	57.4±6.3*
+ rauwolfia	10	96.0±7.0	86.0±6.0*	88.0±12.0*	79.0±9.0	97.0±2.0*

Note: * Significance of difference from series with glycerol ($p<0.05$).

pension was mixed (1:1) with pectic polysaccharide solution (prepared in normal saline solution) and exposed for 20 min at normal ambient temperature. The final polysaccharide content in the sample was 0.1 and 0.2 wt%. Hypertonic stress (Schpakova et al., 1995) was induced by transferring 50 μl of red blood cell/pectic polysaccharide suspension into 1 ml of 4.0 mol/l of NaCl solution and exposing the sample for 40 min at normal ambient temperature. Then the red blood cells were pelleted by centrifuging (1 500g 3 min), and the hemoglobin content in the supernatant fluid was registered by spectrophotometry using an SF 4A spectrophotometer with a flow-through cuvette at the length of 543 nm. Free hemoglobin was expressed as the percentage relative to 100% of red blood cell hemolysis in 0.1% Triton X-100 solution. The potassium ion content in the extracellular environment was evaluated by potentiometry using a high-avidity potassium electrode as proposed by Nikolsky et al. (1974) based on a plasticized polymer valinomycin-containing membrane. The electrode was calibrated just before the test.

Statistical data manipulation involved calculation of the arithmetic mean ± standard deviation (M±δ). Wilcoxon's signed-rank test (Glanz, 1998) was applied to determine the statistical significance of differences between the groups using BIOSTAT, a computer application for medical and biological statistics.

RESULTS

The analysis of the effects of cryoprotectant solutions during freezing and storage of leukocytes in a state of suspended animation (-20°C) demonstrated (Table 2) that the presence of such pectic polysaccharides as tanacetan (Fig. 2), comaruman (Fig. 3) and rauwolfia (Fig. 4) helps boost glycerol activity and retain a high-level functional and morphological capacity in nuclear blood cells.

Saccharomyces cerevisiae yeast-like fungi retained more of its colony-forming ability when frozen (-196°C) in a DMAC cryoprotectant environment, if the environment contains pectic polysaccharides (Table 3).

Pectic polysaccharides added to the cryoprotectant environment during platelet cooling to -196°C at the rate of 10°C/min and 20°C/min in a DMAC protective environment considerably improved the viability of the said cells. For instance, tanacetan, comaruman and bergenan helped platelets retain their aggregation ability, while heracleum, tanacetan and rauwolfia increased their resistance to hypotonic shock (Table 4).

Pectic polysaccharides were found (Table 5) to have an antihemolytic effect; however they do not affect the loss of potassium ions by red blood cells

Table 3. Effect of pectic polysaccharides on colony-forming ability of yeasts *Saccharomyces cerevisiae* at different cooling rates (M±σ; n=6).

Substance	Rate of cooling, °C/min		
	1,0	5,0	10,0
dimethylacetamide	39.0 ± 3.3	73 ± 4.6	37 ± 4.9
+ heracleum	58.5 ± 3.7 *	92 ± 5.3 *	43 ± 6.5
+ tanacetan	60.7 ± 4.1 *	87 ± 5.1 *	59 ± 5.2 *
+ comaruman	58.5 ± 5.4 *	85 ± 4.2 *	49 ± 7.0 *
+ bergenan	61.3 ± 5.2 *	79 ± 6.7	43 ± 6.6
+ rauwolfia	74.8 ± 4.7 *	71 ± 5.2	32 ± 5.1

Note: * Significance of difference from series with DMAC ($p<0.05$).

Table 4. Effect of pectic polysaccharides on the safety of platelets (in % of normal) at different rates of cooling down to -196°C (M±σ; n=7).

Substance	Rate of cooling, °C/min	Amount of cells	Aggregation activity	Reaction on hypotonic shock
dimethylacetamide	10	65.3 ± 6.3	58.5 ± 3.7	59.1 ± 5.2
	20	77.6 ± 5.1	60.7 ± 4.1	55.4 ± 4.7
+ heracleum	10	67.3 ± 4.3	58.5 ± 5.4	65.8 ± 4.6 *
	20	82.1 ± 4.5	61.3 ± 5.2	53.7 ± 3.9
+ tanacetan	10	73.2 ± 5.2 *	74.8 ± 4.7 *	58.8 ± 3.7
	20	80.7 ± 5.1	78.9 ± 7.7*	64.7± 4.8*
+ comaruman	10	69.6 ± 4.0	64.2 ± 3.3 *	63.2 ± 5.6
	20	79.7 ± 4.2	69.6 ± 5.1*	55.6 ± 5.1
+ bergenan	10	75.5 ± 3.9 *	75.3 ± 3.9 *	53.7 ± 4.9
	20	81.4 ± 4.3	81.1 ± 6.5*	56.8 ± 4.4
+ rauwolfia	10	55.6 ± 5.5 *	45.4 ± 5.5	69.0 ± 6.1 *
	20	64.6 ± 6.9 *	53.8 ± 8.0	67.4 ± 5.8 *

Note: * Significance of difference from series with DMAC ($p<0.05$).

Table 5. Erythrocytes hemolysis and potassium content in the presence of pectic polysaccharides (M±σ; n=10).

Substance	Concentration weight %	Hemolysis % of control	potassium content out of cells, mg/l
heracleum	0.1	63.6 ± 3.1	10.8 ± 1.0
	0.25	59.2 ± 4.0*	10.9 ± 0.8
tanacetan	0.1	68.7 ± 2.9	11.1 ± 0.9
	0.25	54.8 ± 5.8*	11.1± 0.9
comaruman	0.1	73.1± 6.2	11.0 ±1.1
	0.25	61.5 ± 4.3*	10.9 ± 0.8
bergenan	0.1	70.8 ± 3.7	10.7 ± 0.9
	0.25	72.4 ± 3.1	10.5 ± 0.8
rauwolfia	0.1	85.6 ± 5.9	11.2 ± 1.0
	0.25	79.6 ± 4.0*	11.2 ±1.2

Note: * Significance of difference with the index using 0.1 wt% of the material ($p<0.05$).

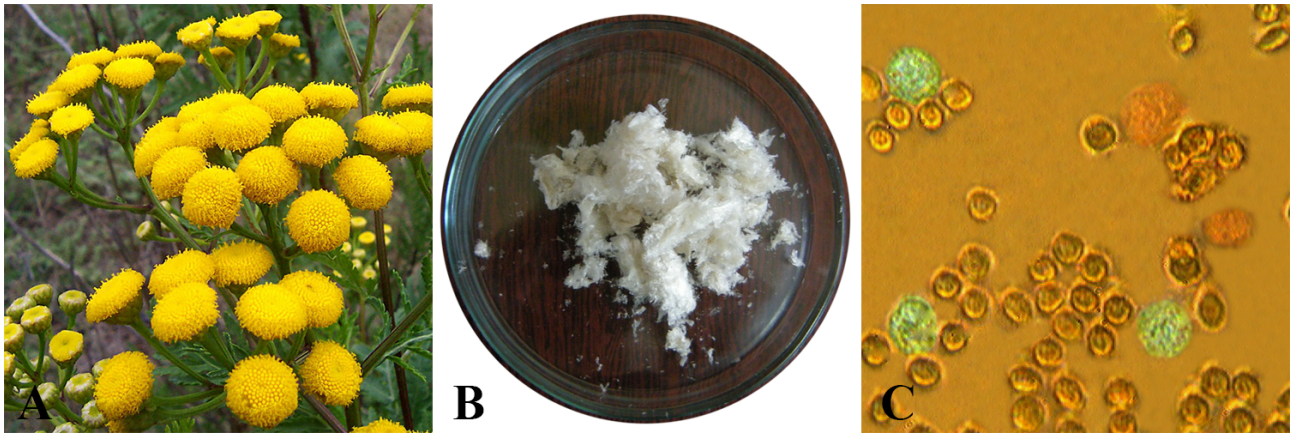

Fig. 2. *Tanacetum vulgare* (A), isolated from its pectic polysaccharide tanacetan (B), Human leukocytes are resistant to dye eosin (blue) and the damaged membrane (red) after warming (C).

Fig. 3. *Comarum palustre* (A), isolated from its pectic polysaccharide comaruman (B), human leukocytes with active lysosomal proteins after warming (C).

Fig. 4. *Rauwolfia serpentina* (A), isolated from its pectic polysaccharide rauwolfia (B), human neutrophils with the latex particles after warming (C).

as a result of hypertonic shock (potassium ion loss for native cells is 0.24 mg/l and 11 mg/l in a state of shock).

Therefore, pectic polysaccharides incorporated in preservative solutions considerably increase the effect of metrical cryoprotectant. The data obtained in this study demonstrate that this substance class has potential for use in biological object cryopreservation technology.

DISCUSSION

Every life form on our planet has its own strategy of resistance to cold. A body either "avoids" freezing by stabilizing the supercooled state by removing all possible nucleating agents to withstand freezing up to -20°C, or increases its freezing resistance by activating the process of synthesis of polyols, sugars, antifreeze proteins and other substances to lower the lethal temperature considerably. Ice forms only in intercellular spaces in most plants at the natural temperature decrease rate; when the ambient temperature is -2°C a process of oligosaccharide to cryoprotectant monosaccharide hydrolysis is activated. As it gets colder, simple proteins form complex aggregates with carbohydrates in the cells of hardening plants. These aggregates help reduce the ice crystallization temperature and remove water at low ambient temperatures. The difficulty of isolating pectins and pectic polysaccharides from plants has prevented the use of their cryoprotectant ability in practical cryobiology for a long time. Current technology advancement makes it possible to extract this group of natural cryoprotectants and develop new methods of bio object cryopreservation in the environment of these substances.

The data obtained demonstrate that the pectic polysaccharides researched are capable of enhancing the cryoprotectant effect of the primary protector and increasing red blood cell osmotic resistance, most likely owing to their chemical structure and intricate macromolecule branching. Pectic polysaccharide proved to be effective on diverse test objects (leukocytes, platelets, yeast-like fungi) at varying cooling rates (1, 2, 5, 10°C/min), storage temperatures (-20°C, -196°C) and for different primary cryoprotectants (glycerol, DMAC). A further study of the use of this substance group as part of cryoprotectant environments for plant and animal cell storage proves relevant.

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