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Properties of a Cell Fraction That Repairs Damage to the Cell Division Mechanism of *Escherichia coli*

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A factor in bacterial cell extracts which induces cell division in filaments of *Escherichia coli* produced by irradiation was found to be associated with a heat-labile particulate fraction which sediments at about 100*S*. Frozen or lyophilized samples of the cell extracts were stable for considerable periods of time. Fractions purified by centrifugation contained 2% of the total protein of the extract but no measurable ribonucleic acid or deoxyribonucleic acid. The extracts were inactivated by incubation with phospholipase A, lipases, and detergents, but not by incubation with selected nucleases and proteases.

The observation of Delaporte (11) that cell division is more likely to occur in an irradiated population of *Escherichia coli* B when the cells are tightly grouped on an agar surface led us to attempt to extract from bacterial cells agents capable of repairing radiation damage in *E. coli*. We reported previously that extracts of *E. coli* can induce cell division and subsequent macrocolony formation in radiation-damaged cells of certain strains of *E. coli* (2). These extracts, as well as certain physical and chemical agents (3, 13), stimulate cytokinesis only in those mutants of *E. coli* that form long coenocytic filaments after exposure to radiation. Such filaments grow and synthesize macromolecular constituents at a normal rate for several hours after irradiation, but they do not divide and they eventually become inactive without forming macroscopic colonies. If the population of growing filaments is treated with a cytokinetic stimulating agent, a certain fraction begins to divide and produces macroscopically visible colonies.

In this paper we further characterize the division-promoting activity of bacterial cell extracts.

MATERIALS AND METHODS

Organisms. The filament-forming test organism was *E. coli* K-12 AB1899 NM, a nonmucoid streptomycin-resistant mutant derived from *Escherichia coli* K-12 AB1899. The source of division-promoting extracts was a streptomycin-sensitive strain of *Escherichia coli*, B/r (ORNL), previously described by Adler and Haskins (4). *E. coli* K-12 AB1157, the parent organism of AB1899, was used to test extracts for effects on a nonfilament-forming organism.

Media. The liquid medium was Difco Nutrient Broth (0.8%) supplemented with yeast extract (0.1%), sodium chloride (0.6%), glucose (1.0%), and 0.0033 M KH_2PO_4—K_2HPO_4, *p*H 6.8. The organisms were grown in liquid media with shaking at 37 C for 16 to 20 hr (stationary phase). For the plating medium, glucose was omitted, and 1% Difco agar and 140 μg of streptomycin per ml were added.

Irradiation of test organisms. Conditions of irradiation were essentially those described by Adler and Copeland (1). Stationary-phase cultures of AB1899 NM were diluted 1:10 with 0.067 M phosphate buffer (*p*H 6.8) and exposed to 40 kr by use of a 250 KVP, 30 ma General Electric Maxitron X-ray machine with 3-mm aluminum added filtration. The exposure for *E. coli* AB1157 was 65 kr.

Assay procedure. To conserve materials and to facilitate the assay of large numbers of samples, a small-volume assay procedure was developed to replace the 20-ml pour plates we used previously (2). Irradiated test cells were diluted 1:100, and 0.4 ml of the diluted cell suspension was added to 100 ml of agar at 45 C. Samples (1 ml) of the agar were then added to the wells of a no. 445565-46 plate (Falcon Plastics, Los Angeles, Calif.) containing 100 wells, 1.5 × 1.5 × 2 cm deep in a 10 × 10 array. The samples to be tested (usually 0.01 to 0.20 ml in volume) were added to the wells prior to the addition of the agar and cells. As soon as the agar hardened, the plates were transferred to an incubator at 37 C, and the macrocolonies were scored after 24 to 48 hr.

Results are reported in terms of "division index," which is the ratio of the number of macrocolonies in the presence of a test substance to the number of macrocolonies in the absence of the agent. Approximately 10 wells were used to determine the survival of the untreated cells, whose concentration had been adjusted to give an average of two to eight colonies per well. Extracts were tested in duplicate.

Preparation of division-promoting extracts. Stationary-phase cultures of strain Br (ORNL) were

harvested at 4 C in a Sorvall model RC2B centrifuge with a GSA rotor (12 to 15 min at 10,000 rev/min). The pellets were washed in cold 0.067 M phosphate buffer (*p*H 6.8) and centrifuged at 4 C in a Sorvall centrifuge using the SS-34 rotor (12 to 15 min at 15,000 rev/min). This pellet was resuspended in cold 0.067 M phosphate buffer (*p*H 6.8) to give a total volume representing a 40- to 50-fold concentration with respect to the starting cultures. The concentrated suspension was passed twice through a cold French pressure cell with a 1-inch piston (American Instrument Co., Inc., Silver Spring, Md.) at 12,000 to 20,000 psi. Unbroken cells and cell walls were removed by centrifugation at 4 C at 30,000 rev/min in a Spinco no. 30 rotor for 25 min. The pellet was discarded, but the supernatant fluid, which has division-promoting activity, was saved and labeled S_1. S_1 was further fractionated by high-speed centrifugation at 4 C for 5 hr at 50,000 rev/min in a Spinco type 50 titanium rotor. The supernatant liquid was designated S_2. The pellet was resuspended in a Potter-Elvehjem homogenizer in a volume of cold 0.067 M phosphate buffer (*p*H 6.8) equal to the original volume of the S_1, and it was designated P_2.

Purification on gradients. S_1 was separated on sucrose gradients in BXV aluminum and BXV titanium zonal ultracentrifuge rotor systems (8). A 30-ml amount of freshly prepared S_1 was loaded onto a 1-liter 10 to 30% sucrose gradient (linear with volume) with a 55% sucrose cushion. The gradient contained 0.067 M potassium phosphate buffer (*p*H 6.8). Centrifuge runs were 16 to 22 hr at 4 C and 20,000 rev/min for aluminum rotors and 25,000 rev/min for titanium rotors. The gradient was collected in 40- to 50-ml samples and monitored at 260 and 280 nm by use of an Oak Ridge flow cell in a Beckman DB spectrophotometer attached to a Brown recorder (6). Samples (18 ml) from the gradient were diluted with 10 ml of 0.067 M phosphate buffer (*p*H 6.8) and centrifuged at 4 C for 18 to 20 hr at 30,000 rev/min in a Spinco no. 30 rotor. The pellet was resuspended in 2.0 ml of phosphate buffer and assayed to locate biological activity in the gradients.

To obtain further purification of the material from sucrose gradients, isopycnic banding was used by making preformed gradients of CsCl and the fraction from the sucrose gradient, essentially as described by Anderson et al. (7). Direct banding of S_1 in CsCl gradients was done as described by Fisher et al. (12), except that preformed gradients were used. Samples containing CsCl were dialyzed against phosphate buffer by use of a modified version of a block dialysis rig, described by Cho et al. (10), before assay. Fractions from the sucrose gradient were assayed directly.

Chemical analyses. Determinations of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were made on hot trichloroacetic acid-soluble extracts with the orcinol (18) and diphenylamine (9) reactions. Protein was measured on the hot trichloroacetic acid-insoluble fraction, after solubilization in 1 N NaOH, by the method of Lowry et al. (17).

Enzymes. Incubation mixtures for enzyme inactivation studies of the division-promoting extract consisted of the fraction to be tested, in 0.067 M phosphate buffer (*p*H 6.8), plus a final concentration of 100 μg/ml of Pronase, trypsin, α-amylase, deoxyribonuclease I, or ribonuclease (all of which were highly purified preparations from Worthington Biochemical Corp., Freehold, N.J.). The deoxyribonuclease incubation mixture contained 10 mM $MgSO_4$. Incubations were carried out at 25 or 37 C for 1 hr with agitation. Snake venom enzymes were incubated with the division-promoting extract in 0.125 M tris(hydroxymethyl)aminomethane (Tris)-phosphate buffer (*p*H 9.0), with (i) *Trimeresurus flavovinidis* venom (Sigma Chemical Corp., St. Louis, Mo.) at a final concentration of 62.5 μg/ml, (ii) an equal volume of a boiled preparation of this venom which retains phospholipase A activity (14), or (iii) a purified venom phospholipase A (Calbiochem, Los Angeles, Calif.), also at a concentration of 62.5 μg/ml. The venom was boiled in 0.05 M Tris-phosphate buffer (*p*H 7.4). Incubations were carried out at either 4 C for 18 hr or 37 C for 1 hr. In each case, two controls were run, in which buffer replaced either the enzyme or the division-promoting extract.

Detergents. Incubation mixtures contained the fraction to be tested and a final concentration of 1, 0.5, or 0.25% Duponol or deoxycholate. Incubations were carried out at room temperature for 1 hr. Controls contained 0.067 M phosphate buffer (*p*H 6.8) in place of the detergent.

Sonic treatment. Sonic treatment was done with a Branson model S-125 Sonifier equipped with a microtip (Branson Instruments, Inc., Danbury, Conn.), at a power setting of 2.5 d-c amp. Typical volumes used of material to be treated were 2 to 5 ml. Samples were treated in an ice-water bath to minimize temperature rises.

RESULTS

Division-promoting activity of cell extracts. In agreement with our previous report (2), the number of colonies increased as more S_1 was added to the test organisms. Figure 1 shows the response produced by an extract of average activity (lower curve) and high activity (upper curve). Different preparations of S_1 varied widely in activity and, at optimal concentrations, gave division indices ranging from 10 to 80. Under optimal conditions, the survival of the test organism was restored to that of its more radiation-resistant parent organism. This is equivalent to a maximal dose reduction factor of approximately 4. The extracts typically showed maximal stimulation of the division index at 0.05 to 0.10 ml of S_1 per ml of plating medium. The average division index and standard deviation for 10 consecutively prepared S_1 fractions was 32 ± 22. Any one extract assayed consistently, and the variation was among extracts rather than in the assay. Five separate assays of one extract gave an average division index of 54 ± 5. The extracts did not alter the radiation survival of a nonfilament-

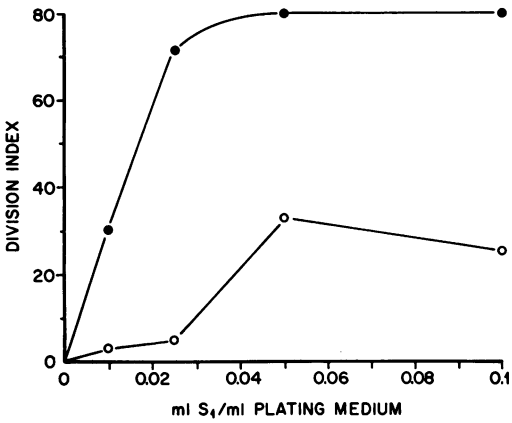

FIG. 1. *Survival of E. coli AB 1899NM exposed to X rays (40 kr), as a function of the amount of extract (S_1) added to the medium. The "division index" is the ratio of the number of colonies in the presence of the extracts to the number of colonies in the absence of the extracts. Survival at the test dose is approximately 0.5%.*

forming strain of *E. coli* (AB1157). Our assay is performed in agar gels, and we have been unable to demonstrate division-promoting activity in liquid cultures.

Centrifugal purification of division-promoting activity. Localization of the division-promoting activity from cell extracts on a sucrose gradient in the BXV zonal rotor is shown in Fig. 2. The activity was recovered as a discrete zone centered in fractions 19 and 20, just preceding the ribosomal subunits. Active fractions from the gradient were then banded in CsCl. The division-promoting activity was recovered as a single band with a buoyant density of 1.22 g/cc. A chemical analysis of the preparations at various stages of purification is presented in Table 1, along with an approximate estimate of the biological activity recovered. Data are given for a typical assay amount (0.05 ml) and for the calculated total amount in the sample. Purification was approximately 40× with respect to protein; RNA and DNA were not measurable, by the methods employed, at the later stages of purification.

Figure 3 shows the position of division-promoting activity after direct banding of S_1 on a CsCl gradient, without previous purification on a sucrose gradient. The activity was recovered at a buoyant density of 1.25 g/cc. The sample was recovered at the same density after 24 and 48 hr of centrifugation. Approximately 50% of the protein present in the S_1 was recovered in the active zone; there was no detectable RNA or DNA in the active region. Recovery of the biological activity was estimated to be 80%. Centrifugation on sucrose gradients in a Spinco SW 25.1 rotor for 24 and 48 hr indicated that the division-promoting activity is isopynic in sucrose at a buoyant density of 1.21 g/cc. Approximately 50% of the biological activity was recovered. These samples were not analyzed chemically.

Since the division-promoting activity was recovered in rate zonal runs near its isopynic point in the gradient, S_1 was also subjected to differential centrifugation. The recovery of division-promoting activity in the supernatant liquid and pellet of an S_1 subjected to differential centrifugation is shown in Fig. 4. The activity sedimented in approximately 90 min.

The high-speed pellet (P_2) also showed increased activity as more material was added to the test cells, and the maximal division index was the same as for S_1 (Fig. 5). Recombination of the high-speed supernatant liquid (S_2) with P_2 indicated that the stimulation of P_2 activity by addition of S_2, which we reported earlier (2), can be seen only at selected concentrations of P_2 below the optimal division index for the extract. A similar stimulation of the division-promoting activity of particulate fractions from sucrose gradients was observed with addition of S_2.

Stability of division-promoting activity to storage and heating. Extracts (S_1) frozen at −20 C were completely stable for at least several weeks. Lyophilization of the extracts caused a 10 to 20% loss in activity, but there was no further

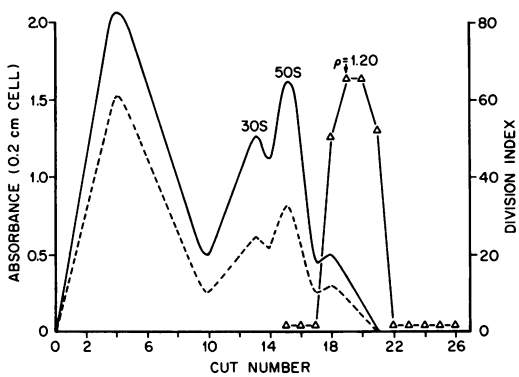

FIG. 2. *Localization of the division-promoting activity of cell extracts on a sucrose gradient in the BXV titanium rotor. The assay was done on 0.05-ml samples of material centrifuged from 18 ml of each fraction and then resuspended in 2.0 ml of phosphate buffer. The 30S and 50S ribosomal peaks are labeled for reference. Solid line, absorbancy at 260 nm; dashed line, absorbancy at 280 nm; △, division index.*

TABLE 1. *Analysis of samples containing cell division-promoting activity after purification of S_1 by sucrose gradient centrifugation, followed by banding in CsCl*

Sample	Amt of protein, RNA, and DNA/0.05 ml			Division index/0.05 ml	Amt in total sample			Activity in total sample[a]
	Protein	RNA	DNA		Protein	RNA	DNA	
	μg	μg	μg		mg	mg	mg	
S_1	955	356	82	75	573	214	49	900
Fractions 18, 19, 20, 21[b]	68	5	—	60	28.3	2.1	—	600
Fractions from CsCl	20	—	—	44	14	—	—	620

[a] Calculated number of 0.05-ml assay quantities which would yield a division index of 50.

[b] After centrifuging 18 ml of each fraction and resuspending the pooled pellets in 8.0 ml of phosphate buffer.

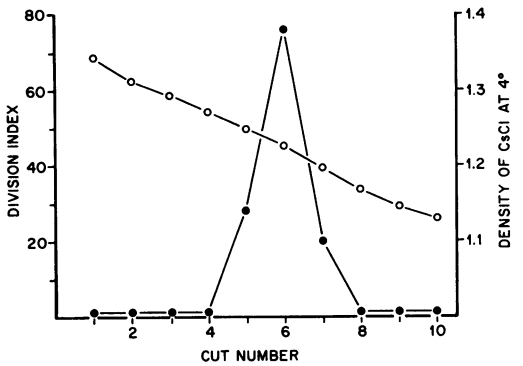

FIG. 3. *Location of division-promoting activity from cell extracts in a CsCl gradient. Samples (0.05 ml) were assayed after dialysis to remove cesium chloride. Symbols: ●, biological activity; ○, density of CsCl at 4 C.*

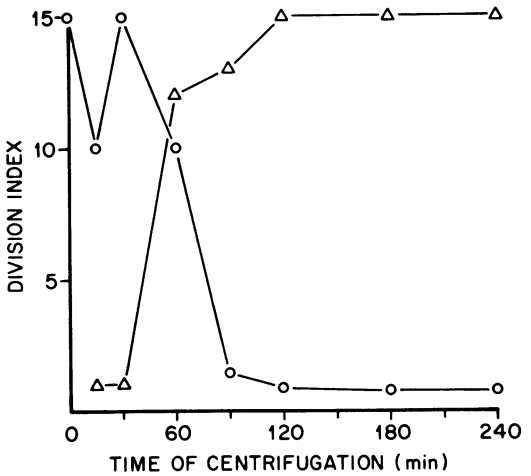

FIG. 4. *Distribution of the division-promoting activity between the supernatant liquid (○) and pellet (△) of an S_1 subjected to differential ceutrifugation.*

loss in activity with storage for several weeks. Extracts stored at 4 C lost 80 to 90% of their activity in 1 week. The stability of the particulate fractions was comparable.

Both S_1 and the particulate fractions isolated from it by centrifugation were completely inactivated by heating for 1 min at 100 C. The division-promoting activity in all preparations was stable at 25 and 37 C for at least 90 min.

Sensitivity of the division-promoting activity of extracts to selected enzymes. The following enzymes did not affect the division-promoting activity of S_1, P_2, or material isolated from sucrose gradients (1-hr incubation at 37 C and an enzyme concentration of 100 μg/ml): Pronase, trypsin, chymotrypsin, ribonuclease, deoxyribonuclease, lysozyme, and α-amylase. *Trimeresurus flavovinidis* venom and phospholipase A preparations purified from the venom (*see* Materials and Methods section for conditions) completely inactivated the division-promoting activity of S_1 and P_2, whether incubation was for 18 hr at 4 C or 1 hr at 37 C. Incubation of S_1 with lipase D or pancreatic lipase at a concentration of 100 μg/ml for 1 hr at 37 C substantially decreased the division-promoting activity of the extracts (Fig. 6). The enzymes tested had little or no effect on survival when added directly to the irradiated test organisms.

Sensitivity of the division-promoting activity to selected detergents and sonic treatment. Both deoxycholate and Duponol inactivated the division-promoting activity of S_1 and P_2 (Table 2). The detergents alone had no effect on the survival of the irradiated test organism.

The division-promoting activity of the extracts was gradually inactivated by sonic treatment, but after a 15-min treatment more than 50% of the division-promoting activity was retained. The activity in sonically treated samples was completely sedimented after 120 min of centrifugation at 40,000 rev/min.

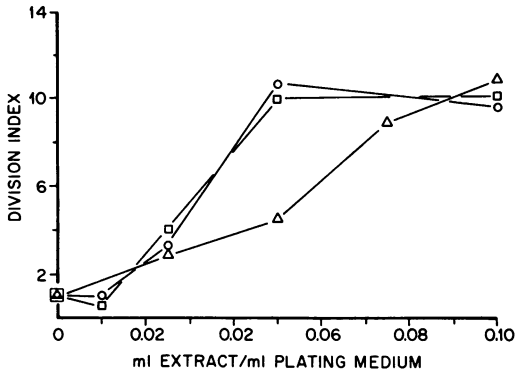

FIG. 5. *Comparison of the effect of increasing amounts of extract (S_1), ○, high-speed pellet (P_2), △, and reconstituted $S_1(P_2 + S_2)$, □, on the survival of irradiated E. coli.*

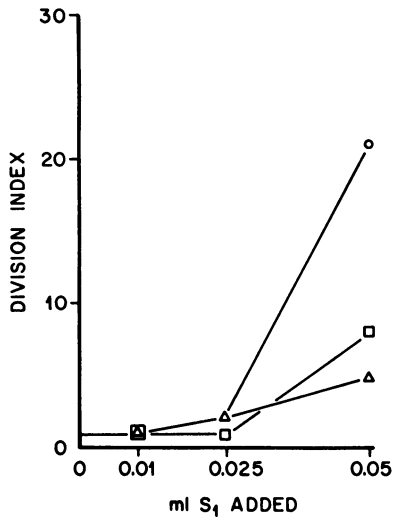

FIG. 6. *Effects of lipases on the division-promoting activity of S_1 extracts. Symbols: ○, control; △, pancreatic lipase; □, lipase D.*

TABLE 2. *Effect of various concentrations of detergents on the division-promoting activity of S_1 and P_2*

Detergent	Division index	
	S_1, 0.05 ml	P_2, 0.10 ml
Duponol		
0%	33	35
0.25%	1	1
0.5%	1	1
1.0%	1	1
DOC		
0%	33	35
0.25%	5	2
0.5%	2	1
1.0%	1	1

[a] The extracts were incubated for 1 hr (20 C) in the detergents and then applied to the irradiated test organism.

DISCUSSION

The division-promoting activity of cell extracts is particle-associated. Based on the position of this activity in sucrose gradients, a reasonable estimate of the sedimentation coefficient for the active particulate material is $\geq 100S$. A more accurate estimate is difficult, since the active material is approaching its isopycnic banding density. Calculation of the sedimentation coefficient for the active particulate material from differential centrifugation (*see* Fig. 4) gives a value of 80*S*, assuming that the activity sediments in 90 min. For a spherical particle with density of 1.22 and a sedimentation coefficient of 100*S*, the Stokes equation yields a diameter of approximately 30 nm. Preliminary electron microscopy (kindly performed by Warren Harris) on fractions from sucrose gradients and on fractions from sucrose gradients rebanded in CsCl did not reveal a unique class of particles in regions of the gradients with division-promoting activity.

Both chemical analysis of purified active fractions (Table 1) which showed no detectable nucleic acid and the insensitivity of the division-promoting activity to nucleases suggest that nucleic acids are not the active material. Our purified material still contained approximately 2% of the total protein in the extract but was insensitive to several proteases.

The low density of the division-promoting activity and its sensitivity to phospholipases, lipases, and detergents suggest that it may be membrane-associated. For several reasons—e.g., the assay is performed routinely in agar gels and intact cells have division-promoting activity (2)—it seems reasonable to suppose that the active material is a "diffusable substance." Since our division-promoting activity is associated with a fairly large particle, the active material may be a product (breakdown or synthetic) of the particle. We have, however, been unable to solubilize the division-promoting activity of the extracts, and treatment with detergents destroys the biological activity. These results and the sensitivity of the preparation to lipases and phospholipases are consistent with at least three interpretations: (i) the intact particle is required to produce the active material; (ii) the active material is protected from inactivation by endogenous enzymes while associated with the membrane; and (iii) the active material itself is labile to lipases and lipid-disrupting agents.

Since we have no evidence bearing directly on the mode of action of the particle, it is possible that its action is indirect and does not act on the final steps in cell division. Our suggestion that it may act on the cell division mechanism in radiation-induced filaments is based on the following observations. (i) The enhanced radiation sensitivity of the test organism is directly correlated with filament production (3). (ii) The addition of optimal amounts of extract restores the survival of the test organism to that of the nonfilament-forming parent organism. (iii) The extract has no effect on the survival of the irradiated parent organism. (iv) The extract is still effective when added to populations of irradiated cells regrown as long as 2 hr after filament formation has already begun. (v) Microscopic measurements of filaments in agar gels show that concentrations of the extract which produce maximal recovery do not alter the rate of growth (elongation) in the filaments but rather result in cytokinesis at the ends of growing filaments (Fisher et al., *unpublished data*). This is in contrast to agents like photoprotecting light (15) and chloramphenicol (5) which promote recovery in *E. coli* B and have in common the ability to inhibit growth and protein synthesis.

Korganonkar and Raut (16) described a cell-free bacterial extract which increases survival of ultraviolet-irradiated *E. coli* cells. This material does not appear identical to our extracts. The chemical analysis and enzymatic sensitivity of the two preparations differ markedly. Korganonkar and Raut (16) isolated the activity from their extracts in a nucleoprotein fraction which lost its biological activity after treatment with ribonuclease or deoxyribonuclease. The division-promoting activity from our extracts is insensitive to nucleases and is recovered in fractions which contain no measurable nucleic acids. Although our work has been primarily on X-irradiated cells, we have also demonstrated recovery with our extracts in ultraviolet-treated cells (2).

The most interesting aspect of division-promoting activity is its possible role in the normal control of cell division. One direct approach to this problem is the correlation of division-promoting activity with the growth stage of the donor organism. The variation in the division-promoting activity of different extracts makes this approach unattractive at present. However, if the active agent(s) in the extracts could be identified, it should then be possible to investigate such a correlation.

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