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Effect of Rehydration on Recovery, Repair, and Growth of Injured Freeze-Dried *Salmonella anatum*[1]

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From 70 to 90% of the *Salmonella anatum* cells that survived freeze-drying in nonfat milk solids were injured. After rehydration, these injured survivors failed to grow on a selective plating medium containing deoxycholate but could form colonies on a nonselective medium. In a suitable environment after rehydration, injury disappeared in most of these cells. The rate of this repair at 25 C was very rapid initially and, in a medium containing milk solids, was completed within 1 hr after rehydration. The repaired cells initiated growth about 1 hr later than normal cells and grew at a slower rate. In a medium containing milk solids, initial recovery, extent of repair of injury, initiation of growth, and rate of growth were not influenced by supplementation with extra nutrients in other rehydration media. Rehydration controlled by modifying the concentrations of lactose, sucrose, or milk solids in the rehydration medium influenced the recovery of cells and the time that growth was initiated. Glycerol failed to increase recovery. Higher numbers of cells were recovered by rehydrating at 15 to 25 C, but an earlier initiation of growth and more rapid growth were observed at 35 C.

Injury of microorganisms after exposure to freeze-drying and other environmental stresses is well documented. The various manifestations of damage in cells include an alteration in the membrane permeability, an increased sensitivity to many selective agents, an extended lag phase of growth, and an increased nutritional need (12). Injury may not lead directly to death because, in a suitable environment, damaged cells may repair and regain normal functions. The nutrient content of the medium is important for the recovery and repair of injury of these cells (1, 16). However, in the recovery of the freeze-dried organisms, rehydration is probably the most important of all factors (6). The osmolality of the rehydration medium has a marked influence on the initial recovery of the freeze-dried bacteria (3). Rehydration temperature is also important to obtain a high initial recovery (7).

This report describes the effects of rehydration conditions on the initial and subsequent recovery, repair of injury, and growth of *Salmonella anatum* freeze-dried in milk solids.

MATERIALS AND METHODS

Test organism. The test organism, *S. anatum* NF3, was isolated in our laboratory from naturally contaminated nonfat dry milk (NDM). The stock culture was transferred once per week, incubated in reconstituted sterile 10% NDM at 35 C for 24 hr, and stored at 4 C.

Freezing and drying. A 1-ml portion from the stock culture was added to 100 ml of reconstituted sterile NDM (10% solids) and incubated at 35 C for 20 to 24 hr (final population, ca. 10^9/ml). The culture was diluted 1:10 in reconstituted sterile NDM (10% solids), and 10-ml portions were placed in sterile screw-cap tubes (150 by 25 mm). Each tube was closed with a sterile cheesecloth plug. The contents were frozen by placing the tubes in a dry ice-acetone bath for 10 min (static). The tubes containing the frozen samples were immediately transferred to a lyophilizer chamber (Unitrap, The VirTis Co., Inc., Gardiner, N.Y.) and dried under vacuum (10 mμ) for 40 hr at room temperature. The samples were removed from the chamber, and the cheesecloth plugs were replaced by sterile screw caps. The samples were stored at 25 C in a forced-air incubator for 24 hr before testing. Thus, each tube contained 1-g amounts of sample (*S. anatum* freeze-dried in NDM).

Plating media. Two plating media, one nonselective and one selective, were used. The nonselective medium

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was made from xylose lysine agar base (XL agar base, Difco) supplemented with 0.5% peptone (XLP). The selective medium was prepared by adding 0.25% sodium desoxycholate (Difco) to XLP, the nonselective medium (XLDP). Both media were prepared from the XL agar base according to the manufacturer's directions. The peptone solution (10% sterilized separately) was added to both media immediately before they were dispensed into plates. The surfaces of the prepoured plates were dried by incubation at 35 C for 24 hr. If the poured plates were not used immediately, they were stored at 4 C for not more than 7 days.

Rehydration and plating. Each freeze-dried sample was rehydrated with 10 ml of a rehydrating medium. Unless otherwise mentioned, each sample was rehydrated by rapidly adding 10 ml of sterile water (in about 5 sec) at 25 C. The sample was then mixed for 1 min with a Vortex mixer (Scientific Products, Evanston, Ill.). After serial dilutions (0.31 mM phosphate buffer, KH_2PO_4), a 0.1-ml portion was surface-plated in triplicate on each type of plating medium. The first plating was done within 3 to 4 min after rehydration. The rehydrated samples were incubated at 25 C (unless otherwise specified), and subsequent samplings were made at specified intervals during the test period. The plates were incubated at 35 C for 18 to 24 hr, and the colonies were counted.

One of the rehydration media (Ray's medium) had the following components: tryptone, 3.0 g; soytone (Difco), 3.0 g; yeast extract (Difco), 3.0 g; Casamino Acids (Difco), 1.0 g; sodium chloride, 1.0 g; dipotassium phosphate, 2.0 g; magnesium sulfate, 0.5 g; and water, 1 liter. The *p*H was adjusted to 7.0 before sterilizing at 121 C for 15 min. Other rehydration media used were sterile water, Brilliant Green water (0.002% dye content), lactose broth (Difco), peptone solution (0.3%), and sodium pyruvate solution (0.1%).

Calculation. The following two formulas were used to determine the percentage of injury and death: per cent injury = 1 − (counts on XLDP/counts on XLP) × 100; per cent death = 1 − (counts on XLP of treated sample/counts of XLP of untreated control) × 100.

RESULTS

Effect of freezing and freeze-drying. Freezing, freeze-drying, and subsequent storage had damaging or lethal effects on *S. anatum* cells (Table 1). The difference in counts on XLP and XLDP (measurement of injury) in the untreated control cells was less than 10%. After freezing and slow thawing (45 min at 25 C), about 60% of the surviving cells showed injury because they failed to form colonies on XLDP but not on XLP agar. The amount of death after freezing was low. After freeze-drying, about 60% of the cells were dead and, among the survivors, about 70% exhibited damage. Storage for 24 hr after freeze-drying increased the extent of death and injury. Only 10% of the original population was able to form colonies on XLP and among them about 90 to 95% were unable to grow on XLDP, indicating injury.

TABLE 1. *Effect of freezing, freeze-drying, and storage on death and injury of Salmonella anatum suspended in nonfat dry milk (10% solids)*

Determination	Per cent death	Per cent injury
Control (untreated from stationary phase)	0	10
Frozen and slowly thawed	10	60
Freeze-dried[a]		
0-hr storage	60	70
24-hr storage	90	90–95

[a] Freeze-dried samples were plated within 3 to 4 min after rehydration.

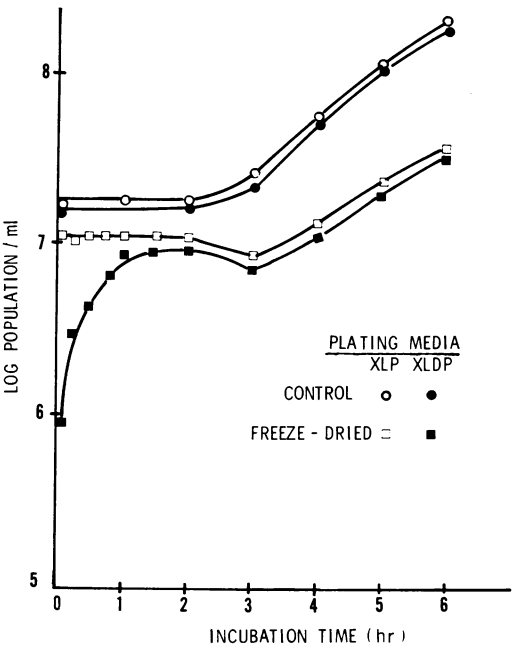

FIG. 1. *Growth curves of control (fresh) and freeze-dried Salmonella anatum at 25 C. The cells in 1 g of milk solids were rehydrated with 10 ml of sterile water and plated on xylose-lysine-peptone-agar (XLP) and XLP with 0.25% sodium deoxycholate (XLDP). For the control, a 24-hr-old culture (in 10% solids milk) at 35 C was diluted with milk to about 10^7/ml and tested at the same time.*

Repair of injury. The amount of injury of freeze-dried *S. anatum* decreased after the cells were rehydrated and incubated in a suitable environment. This repair of injury was manifested by an increase in counts on XLDP but not on XLP (Fig. 1). To observe the rate of repair and initiation of growth, a freeze-dried sample was rehydrated with 10 ml of sterile

water at 25 C and incubated at 25 C for 6 hr. For a control, a 24-hr-old fresh culture of *S. anatum* was diluted in 10% milk solids and tested similarly. In control cells, the difference in counts on the two plating media was less than 10%, and there was about a 2-hr lag before the initiation of growth. In freeze-dried samples, counts on XLP remained constant up to about 2 hr, whereas counts on XLDP increased rapidly. The rate of repair was rapid initially, and within 1 hr the injury was reduced from about 90% to about 30%. Some reduction in counts occurred after about 3 hr; growth was initiated after 4 to 5 hr of incubation. During the initial exponential stage, the freeze-dried cells exhibited a slightly lower rate of growth than the control cells. A majority of the injured cells appeared to repair rapidly after rehydration but before growth.

Rehydration media. Although milk might supply a large variety of nutrients, six different rehydration media were tested to evaluate additive effects on recovery. Each sample was rehydrated with each of six media at 25 C and incubated at 25 C for 6 hr. Averaged results from duplicate samples are presented in Fig. 2. The initial recovery (measured on XLP after rehydration) appeared relatively higher in lactose broth (Difco) and lower in water and peptone solution (0.5%). The other three media gave intermediate recovery. However, differences in recovery were not of sufficient magnitude to merit further investigation. In all six media, the amount

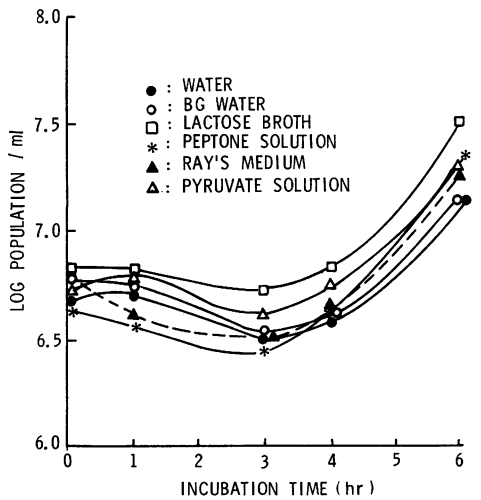

FIG. 2. *Effect of rehydration media on growth at 25 C of freeze-dried Salmonella anatum. The cells in 1 g of milk solids were rehydrated with 10 ml of each medium and plated on xylose-lysine-peptone-agar. BG water was Brilliant Green water with 0.002% dye content.*

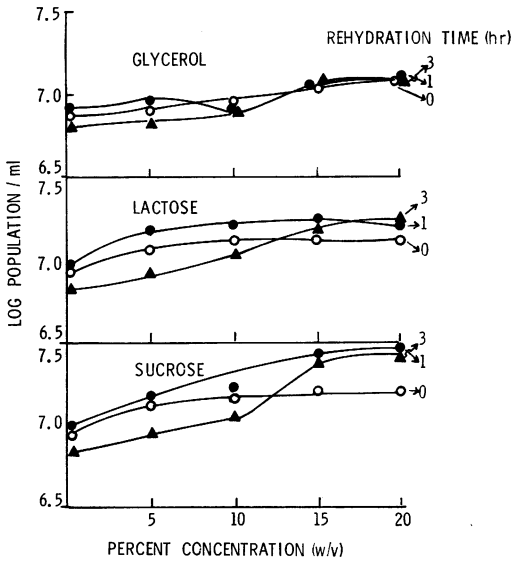

FIG. 3. *Effect of concentration of the rehydrating solution on the recovery at 25 C of freeze-dried Salmonella anatum up to 3 hr after rehydration. The cells in 1 g of milk solids were rehydrated with 10 ml of different rehydrating solutions and plated on xylose-lysine-peptone-agar.*

of injury initially was about 90%, and after 1 hr of incubation it was reduced to about 30% in all media (*data not presented*). In all six media, growth was initiated between 3 to 4 hr at 25 C after slight reduction in number at 3 hr.

Osmotic environment during rehydration. The influences of sucrose, lactose, and glycerol in 0 to 20% rehydration solutions were evaluated on freeze-dried samples (Fig. 3). Relatively higher recoveries were obtained by rehydrating with increasing concentrations of glycerol (0 hr). Essentially no change in apparent count was observed up to 3 hr in glycerol solutions. Rehydration with 5 to 20% lactose solutions resulted in initial recoveries higher than those with water alone. After 1 hr, there was an increase in apparent counts at all concentrations. However, at 3 hr, a reduction in counts occurred in 0 to 10% lactose solutions. The population levels remained almost unchanged at 3 hr in 15 and 20% lactose samples. Rehydration with sucrose solutions resulted in responses similar to those observed with lactose solutions.

Data on the amount of initial injury, rate of repair of injury, and growth after rehydrating the freeze-dried *S. anatum* in glycerol, lactose, and sucrose solutions are presented in Fig. 4. Numbers of colonies on XLDP immediately after rehydration were low compared to those on XLP, indicating injured cells. Numbers on

XLDP were lower in 20% than in 0 and 10% lactose and sucrose solutions. In all three rehydration media at all concentrations, counts on XLDP increased, indicating repair. In 0 and 10% concentrations of all three rehydration media, the cells showed initiation of growth after 3 hr. At 20% concentrations, growth started after 4 hr in lactose and sucrose and did not occur in glycerol solution at 8 hr. The rate of growth in 10% glycerol solution was also much slower than in 10% lactose and sucrose solutions.

The effects of nonfat milk solid concentrations in the rehydration medium on recovery, repair of injury, and growth also were determined (Fig. 5). A 9.1% milk solids concentration was minimal and obtained by rehydrating one sample (1 g of milk solids) in 10 ml of sterile water. To obtain 16.7% milk solids, each of the two such samples was rehydrated with 5 ml of water and then combined within 1 min. Similarly, to obtain 28.6% milk solids, each of the four such samples was rehydrated with 2.5 ml of water and combined. The numbers were adjusted to an equivalent 9.1% solids level. Initial recoveries (counts on XLP) at all three milk solids concentrations were similar, but counts in 16.7 and 28.6% solids were higher after 1 and 3 hr. Also at 3 hr, a reduction in apparent population was observed in 9.1% but not in 16.7 and 28.6% milk solids. The numbers on XLDP increased rapidly shortly after rehydration. The cells started dividing in 9.1% milk solids after 3 hr and in 16.7% milk solids after 4 hr. No growth was observed in 28.6% milk solids during the 6-hr test period. The 9.1% milk solids sample here was equivalent to 0% of glycerol, lactose, and sucrose described previously.

Rehydration temperature. The effects of rehydration and subsequent incubation temperatures on initial recovery, repair of injury, and growth were studied. Each freeze-dried sample was rehydrated with sterile water pretempered at 15 to 45 C, incubated at the respective temperature, and tested up to 6 hr. The initial recovery (XLP counts at 0 hr) was the same at 15 and 25 C and higher than those obtained at 35 and 45 C (Fig. 6). No apparent differences in amount of injury initially and 1 hr after rehydration were observed at the four temperatures (*data not shown*). At about 2 hr after rehydration, there was a reduction in population at 25, 35, and 45 C but not at 15 C (Fig. 6). Initiation of growth occurred after 2 hr at 35 and 45 C and after 3 hr at 25 C. No growth was observed at 15 C within 6 hr. The growth rate was higher at 35 C than at 45 or 25 C. Rehydration of the freeze-dried cells at 15 and 25 C produced higher recoveries. Repair of injury was unaffected by the test tem-

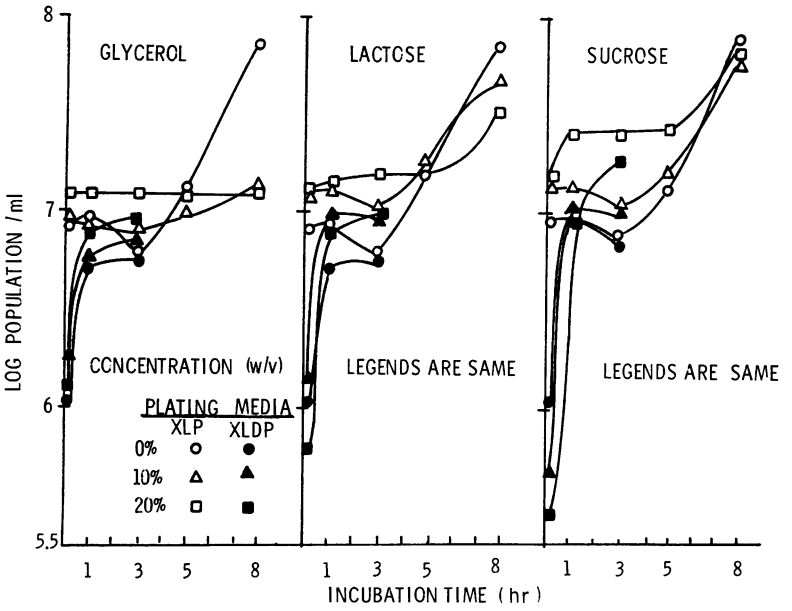

FIG. 4. *Effect of concentration of rehydrating solutions on recovery (at 0 hr), repair of injury (up to 3 hr), and growth (up to 8 hr) at 25 C of freeze-dried Salmonella anatum. The cells in 1 g of milk solids were rehydrated with 10 ml of different rehydrating solutions and plated on xylose-lysine-peptone-agar (XLP) and XLP with 0.25% sodium deoxycholate (XLDP). To demonstrate repair of injury, measurement on XLDP is shown up to 3 hr.*

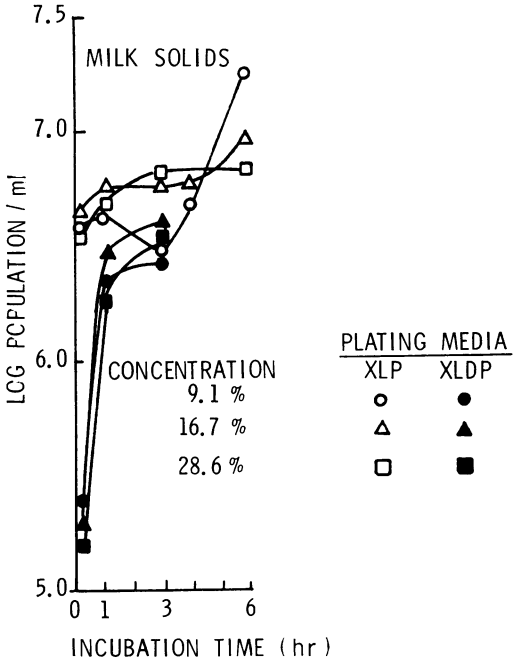

FIG. 5. *Effect of concentration of milk solids in the rehydrating solutions on the recovery (at 0 hr), repair of injury (up to 3 hr), and growth (up to 6 hr) at 25 C of freeze-dried Salmonella anatum. The samples were plated on xylose-lysine-peptone-agar (XLP) and XLP with 0.25% sodium deoxycholate (XLDP). To demonstrate repair of injury, measurement on XLDP is shown up to 3 hr.*

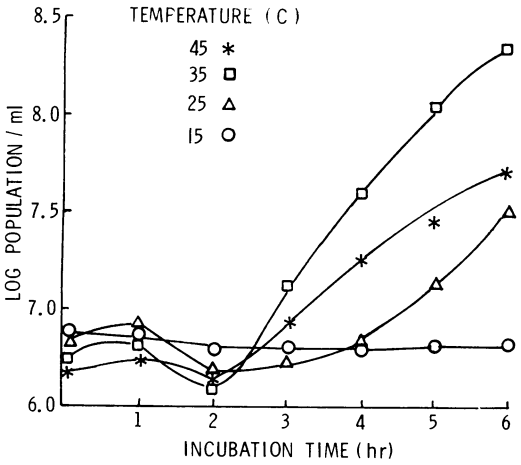

FIG. 6. *Effect of temperature on growth of freeze-dried Salmonella anatum. The cells in 1 g of milk solids were rehydrated with 10 ml of pretempered water and plated on xylose-lysine-peptone-agar.*

peratures, but the cells started to grow at 35 and 45 C earlier than at 25 C.

DISCUSSION

Freeze-drying and subsequent storage of *S. anatum* cells in milk solids resulted in death and injury. Damage of cells after freezing and freeze-drying has been demonstrated by other workers (12, 13). A major portion of these injured cells repaired after rehydration. The rate of repair was rapid, and within 30 to 60 min the majority of cells repaired. Similar rates of injury repair during the initial stage of incubation have been observed in thermally stressed *Staphylococcus aureus* (9).

The freeze-dried *S. anatum* cells, upon rehydration and subsequent incubation at 25 C, showed a lag phase that was about 1 hr longer than the fresh control cells. Also, the rate of growth of the rehydrated freeze-dried cells was lower than the fresh cells. Observance of an extended lag phase is consistent with other reports on freeze-dried *Escherichia coli* (12), on freeze-injured *Aerobacter aerogenes* (10), and on thermally stressed *S. aureus* (5). This extended lag has been associated with metabolic damage of the cells. During the lag, the injured cells repair the damage and synthesize protein, ribonucleic acid, and deoxyribonucleic acid necessary for subsequent growth (12).

Increased or modified nutritional requirements that permit high recovery (maximal populations on nonselective medium) and that permit repair of injury of the freeze-dried cells have been detailed by several workers (1, 4, 12). Similarly, requirements for some biologically active peptides in restoring injured frozen *E. coli* (8) and for yeast extract in recovering heat-damaged *E. coli* have been reported (11). However, in the present studies, media containing various nutrients had little or no influence on the initial recovery, repair, or growth. The milk may have supplied all necessary nutrients to the injured cells and thus masked any effects of various added nutrients. The presence of selective agents such as Brilliant Green dye (0.002%) in one of the rehydrating media did not interfere with recovery, repair, or growth of the freeze-dried cells. This could be due to the relatively low available concentration of the dye resulting from its binding with the milk proteins (15).

Rehydration of the freeze-dried *S. anatum* in solutions containing glycerol, lactose, sucrose, or milk solids resulted in recoveries higher than when the cells were rehydrated with water. Under the test conditions used here, a sample that was rehydrated with water contained about 9 to 10% milk solids. In general, higher recoveries were obtained by increasing the concentrations of the solutes (Fig. 3–5). Leach and Scott (6)

also reported higher recoveries by rehydrating freeze-dried bacteria in glycerol or sucrose solution. They observed optimum recoveries with solutions of about 0.96 to 0.98 water activity (a_w). For sucrose, this a_w was obtained at about 1 molal concentration. For glycerol, an optimum concentration was not detected, but a relatively greater viability was obtained in a 29% glycerol solution at 0.92 a_w (6). In the present study, 20% sucrose and lactose solutions give higher recoveries of *S. anatum* than at 20% glycerol. Choate and Alexander (3) obtained optimum recoveries of freeze-dried *Spirillum atlanticum* by rehydrating in a 24% sucrose solution. They speculated that sucrose, acting as an osmotic buffer, regulated the rehydration of these freeze-dried cells and thus reduced the rehydration damage. Therefore, in restoring the water to the dry cells, there should be some finite rate of rehydration at which damage to the cells could be minimized (3). Rehydration in a solution of high osmotic pressure probably controls the rehydration rate.

The results presented here indicate that some structure(s) necessary for the cell integrity may be damaged in freeze-drying. By controlling the rate of hydration, the disruption of the cells may be reduced. The component responsible for maintaining the cell structure is the cell wall and may be involved in the freeze-drying injury. The site of the lesion in freeze-damaged *E. coli* has been reported by Bretz and Kocka (2) to be the cell wall.

A relatively low initial recovery of freeze-dried cells after rehydration with water may have been due to disruption and death of many injured cells during the rehydration process. Although this environment has about 9 to 10% milk solids, the concentration may not have been sufficient for maximal recovery.

Rehydration temperatures of 15 and 25 C gave initial recoveries higher than 35 and 45 C. Similar results have also been reported by Leach and Scott (6) and Speck and Myers (14). The beneficial effect of low-temperature rehydration may be related to a slower rate of hydration of the cells; however, the repair process did not appear to be affected by temperature within the range tested.

The rehydration process appears to be very important for recovery of cells freeze-dried in milk solids. A higher recovery may be obtained with an environment of high osmotic pressure that may control the rate of hydration. A low-temperature rehydration may also be useful in obtaining high recovery. Under the present test conditions, the composition of the rehydration medium did not appear to have a significant effect on recovery, repair of injury, and growth.

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