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Effect of Temperature on Spore Germination and Vegetative Cell Growth of *Clostridium botulinum*

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Spore germination and vegetative growth of *Clostridium botulinum* type E strain VH at 2 to 50°C were studied. At all of these temperatures, germination began immediately after the addition of the spores to the germination medium. Microscopic observations during germination revealed three types of spores: phase bright (ungerminated), phase variable (partially germinated), and phase dark (fully germinated). At all temperatures except 50°C, there was a pronounced lag between the initial appearance of phase-variable spores and their eventual conversion to phase-dark spores. The number of partially germinated spores increased steadily, reaching 40 to 60% by 18 to 21 h of incubation. During this time, phase-dark, fully germinated spores developed slowly and did not exceed 28% in any of the samples. At 18 to 26 h of incubation, the rate of full germination increased abruptly four-fold. There was extensive and relatively rapid germination at 2°C, the lowest temperature tested, yielding about 60% phase-variable spores by 18 h, which became phase-dark by 26 h of incubation. The optimum temperature for partial and full germination was consistently 9°C. Germination at 50°C was exceptionally rapid and was completed within 1 to 2 h, although 40% remained phase bright. Vegetative cells showed detectable growth at 6 to 41°C, with a distinct optimum at 32.5°C. No growth occurred at 50°C, and only marginal growth was observed at 6 to 14°C. The psychrophilic nature of the germination process coupled with the cold tolerance of vegetative growth appears to give *C. botulinum* type E an ecological advantage in cold climates as well as in cold-stored foods.

Clostridium botulinum type E is generally associated with cold environments. It has been isolated from several marine products in frigid areas of the world, including Alaska, Northern Japan, Canada (3), Scandinavia (2), and the Great Lakes (1). However, *C. botulinum* type E organisms have also been found in temperate and even tropical regions (8). The organism reportedly has a growth optimum of 35°C and a growth maximum of 45°C (11). On this basis, it has been designated a mesophilic organism. On the other hand, *C. botulinum* types A and B grow best at 40°C. This relatively small difference in growth optima between *C. botulinum* type E and types A and B probably has no decisive significance with respect to the natural prevalence of *C. botulinum* type E in the more frigid areas of the world. What is more significant is the ability of *C. botulinum* type E to develop at temperatures less than 10°C. These low temperatures are generally prohibitive for *C. botulinum* types A and B (4, 9, 11, 13) and therefore may give the cold-tolerant *C. botulinum* type E an ecological advantage in cold climates.

The ability of *C. botulinum* type E to grow at relatively low temperatures has been amply documented. Ohye and Scott reported that 10 strains of *C. botulinum* type E were able to grow at temperatures between 5 and 10°C, in contrast to *C. botulinum* types A and B, which did not develop at these low temperatures (11). Subsequently, Dolman et al. reported growth and toxin production by *C. botulinum* type E strain VH (Vancouver herring) at 6°C (4). Later, Schmidt et al, reported that the minimum temperatures for the growth of four strains of *C. botulinum* type E was as low as 3.3°C, although as much as 30 to 60 days at this temperature was required to produce detectable amounts of gas and toxin (*13*). The important implication from the work of Schmidt et al. is that *C. botulinum* type E is able to develop at a temperature typically used for the refrigeration of food, i.e., 3.3°C. This includes products of marine origin which are known to frequently carry spores of *C. botulinum* type E. Since Schmidt et al. raised

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the question of potential botulinal spoilage by *C. botulinum* type E in conventional refrigeration of foods, this problem has been viewed by food scientists and regulatory authorities with understandable concern.

Previous workers studying growth and potential toxic spoilage have not distinguished between the effect of temperature on vegetative cell growth and on spore germination. Any differences between these two processes in the tolerance of low temperatures may be essential for understanding and controlling potential botulinal spoilage of food refrigerated since germination is a prerequisite for vegetative growth. Therefore, it was desirable to investigate *C. botulinum* type E spore germination under comparable experimental conditions to those used first by Schmidt et al. (12, 13, 14). This report describes experimental observations concerning the temperature range and optima for spore germination as compared with those for vegetative cell growth of a typical type E organism, *C. botulinum* type E strain VH.

MATERIALS AND METHODS

Organism. *C. botulinum* type E strain VH (Vancouver herring) was originally obtained from C. E. Dolman and subsequently carried in the Microbial Biophysics Laboratory culture collection. The stock culture was maintained at 2 to 4°C in the form of clean spores suspended in distilled water (W. G. Murrell, personal communication).

Culture medium. The medium for sporulation and growth consisted of 5.0% Trypticase (BBL Microbiology Systems, Cockeysville, Md.) and 0.5% proteose peptone (Difco Laboratories, Detroit, Mich.) sterilized at 121°C for 20 min. A 20% (wt/vol) solution of glucose and a 10% (wt/vol) solution of sodium thioglycolate were separately autoclaved at 121°C for 15 min and added aseptically to the broth to final concentrations of 0.4 and 0.2%, respectively, just before inoculation with the organism. This medium had a pH of 6.8 and was designated TPGT broth. Freshly made TPGT broth was always used in this study.

Growth and sporulation. A stock suspension of *C. botulinum* type E strain VH was heat shocked for 13 min at 60°C as recommended by Schmidt et al. (14). A small inoculum was placed into the bottom of a screw cap tube (20 by 150 mm) containing 20 ml of sterile TPGT broth. We were careful not to shake the tube at this time, thus avoiding incorporating oxygen into the fresh medium. After overnight incubation, 2 ml of the actively growing culture was transferred to 20 ml of TPGT broth, and one more transfer was made 4 h later. Next, 10 ml of the actively growing culture was inoculated into 100 ml of TPGT broth in a 150-ml screw cap dilution bottle, which was incubated at 30°C and inverted at 18 and 23 h, as recommended previously (14) to keep the cells suspended. The gas generated by the cells was sufficient to maintain anaerobiosis during inversion. Abundant sporulation was obtained after 26 h, as determined by phase-contrast microscopy. The spores in TPGT culture were kept in a refrigerator at 4°C and used within 2 to 6 days as needed for experiments.

Germination of spores. One milliliter of the spores mentioned above was washed twice in 10 ml of distilled water by centrifugation at 3,020 × *g* for 5 min at 4°C. The final pellet was suspended in 10 ml of TPGT broth that had been equilibrated to the desired temperature before the experiment. The resulting suspension contained between 2×10^7 and 6×10^7 spores per ml. The spores were not heat shocked before germination experiments. Germination at the various temperatures was monitored by the observation with a Zeiss phase-contrast microscope of the loss of the initial phase brightness of the spores. Samples were taken at the reported time intervals, and the microscopic observations were recorded in relative percentages, with distinction made between three spore types: phase bright (ungerminated), phase variable (partially germinated), and phase dark (fully germinated). Microscopic observation of the samples was generally completed within 5 min.

Vegetative cell growth. Vegetative cell growth was achieved either from a spore inoculum or from an actively growing vegetative cell inoculum. For spore inocula, an unwashed spore suspension was heat shocked for 15 min at 60°C to inactivate possible nonsporulating contaminants as recommended previously (11) and immediately cooled. A 100-ml volume of TPGT broth was inoculated with 0.1 ml of the heat-shocked spore suspension and placed at the appropriate growth temperature.

For vegetative cell inocula, 0.1 ml of a 24-h culture obtained by incubation at 25°C as described above was used to inoculate each 100-ml bottle of TPGT broth equilibrated in advance to the desired temperature. Growth was measured by removing 20-ml samples at specified time intervals and immediately taking readings in a nephelometer (Model DRT-100; HF Instruments, Ltd., Ontario, Canada). The reference standard was 0.14 formazin turbidity units, which was supplied by HF Instruments, Ltd.

Temperature control. Spore germination and vegetative cell growth at 2 to 50°C were studied. Temperatures between 2 and 4°C were maintained in refrigerators. Temperatures between 9 and 14°C were maintained in an aluminum thermal gradient bar constructed in the Cancer Therapy Institute laboratory, and temperatures between 37 and 50°C were maintained in laboratory water baths. Under the conditions of this experiment, the temperatures were controlled to within ±0.5°C.

RESULTS

Temperature effects on spore germination. Three distinct types of spores were seen by phase-contrast microscopy during germination: ungerminated phase-bright spores, partially germinated phase-variable spores, and fully germinated phase-dark spores. Phase-variable spores were distinguished from phase-bright and phase-dark spores by a partial loss of refractility in the spore center and by a distinct phase-dark appearance at the spore periphery. These spores were phase variable or partially phase dark, and

in this sense they were assumed to be partially germinated. In contrast, fully germinated spores appeared uniformly phase dark at the center and the periphery. The data reported here represent one of two replicate experiments that both yielded essentially the same results.

The initial conversion of phase-bright into phase-variable spores occurred in two distinct patterns (Figs. 1 and 2). The first pattern (Fig. 1) occurred in spores incubated at 4 to 14°C. Figure 1 shows germination at 9°C, the apparent optimum for spore germination; a substantial number of spores lost their phase-bright appearance during the first 5 h, after which there was a distinct tailing of this initial germination rate. The second pattern (Fig. 2) was characterized by gradual straight-line kinetics of initial loss of phase-bright appearance. Figure 2 illustrates germination at 2°C, the lowest temperature tested in this study. Substantially similar straight-line conversion patterns of phase-bright into phase-variable spores were seen at both extremes of incubation temperatures for this organism, the low temperature of 2°C as well as the high temperatures of 37 and 50°C.

Figure 3A depicts the first observable loss of the phase-bright appearance of spores, i.e., the primary change signalling the initiation of germination. At all incubation temperatures, the initiation of germination began immediately after the addition of the spores to TPGT broth. At 4, 9, and 14°C, there was an initial rapid decline in the number of phase-bright spores, which seemed to subside after 5 to 6 h of incubation. At these three temperatures, the number of spores remaining in the phase-bright state ranged between 14 and 29% after 28 h of incubation, as compared with 81 to 91% at the beginning of the

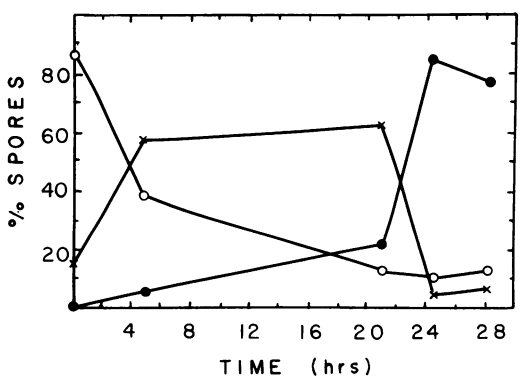

FIG. 1. Germination of spores of *C. botulinum* type E strain VH at 9°C in TPGT broth (typical two-phase germination pattern). Symbols: ○, phase-bright spores; ×, phase-variable spores; ●, phase-dark (fully germinated) spores.

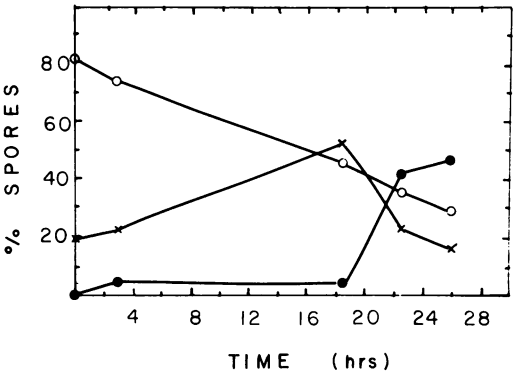

FIG. 2. Germination of spores of *C. botulinum* type E strain VH at 2°C in TPGT broth (typical gradual straight-line germination pattern). Symbols: ○, phase-bright spores; ×, phase-variable spores; ●, phase-dark (fully germinated) spores.

experiment. On the other hand, at 2 as well as 37°C, the number of phase-bright spores declined in a gradual straight-line manner over the 21- to 26-h observation period, except for a slight delay at 37°C during the first 4 h. The number of spores remaining phase-bright after 19 h was 50 to 55% for spores undergoing germination at 2 and 37°C, as compared with 80 to 84% at the start of the experiment.

At 50°C, the loss of the phase-bright appearance was also at a linear rate; however, at this high temperature, the process was extremely rapid and was completed within 2 h, with some 40% of the spores then remaining in the phase-bright state. (Although spore germination at 50°C has some interesting implications, it is clearly outside the concern of this study, and therefore will not be further discussed here.)

Figure 3B shows the intermediate step in spore germination, i.e., the appearance of phase-variable or partially germinated spores. At all incubation temperatures except 50°C, a maximum number of partially germinated spores was observed at 18 to 20 h. During longer incubation, the number of partially germinated spores generally declined rapidly because of conversion to fully germinated spores. In comparison, at 50°C, maximum partial germination occurred very rapidly (within 1 to 2 h) (Fig. 3B, C).

Figure 3C shows the final step in spore germination, i.e., the appearance of phase-dark fully germinated spores. At all incubation temperatures except 50°C, fully germinated spores did not exceed 28% during the initial 18 to 21 h of incubation. Thereafter, however, the number of fully germinated spores increased by four-fold. The maximum number of fully germinated spores in a duplicate set of experiments was

consistently at 9°C, which thus appears to be the optimal temperature for the final step in spore germination. It is interesting that at 2°C, the lowest temperature tested in this study, there was rather extensive (and rapid) germination to both phase-variable and phase-dark spores (Figs. 2 and 3C). The occurrence of germination of *C. botulinum* type E spores at 2°C has important implications for the low-temperature storage of certain foods susceptible to type E botulinal spoilage since germination is a prerequisite for growth. The slight decline in all cases (Fig. 3C) in the number of phase-dark spores seen after the maximum was reached suggests that some spores had become vegetative cells and were no longer microscopically scored as germinated spores.

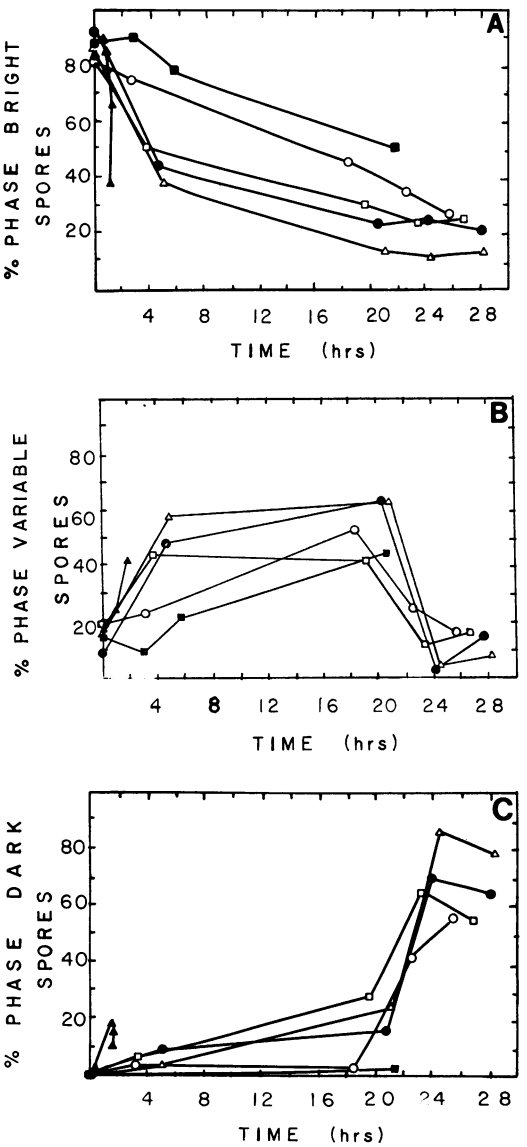

FIG. 3. Effect of incubation temperature from 2 to 50°C on the germination of *C. botulinum* type E strain VH spores. (A) Percentage of phase-bright spores. (B) Percentage of phase-variable spores. (C) Percentage of phase-dark (fully germinated) spores. Incubation temperatures were 2°C (○), 4°C (□), 9°C (△), 14°C (●), 37°C (■), and 50°C (▲). The first, second, and final stages of germination are represented by A, B, and C, respectively.

Temperature optimum for vegetative cell growth. Table 1 summarizes the nephelometric turbitity reading of vegetative cell cultures of *C. botulinum* type E strain VH incubated for 18 and 23 h at 6 to 50°C. Detectable growth was observed at all of these temperatures except 50°C. The optimum for growth was consistently at 32.5°C. This was true whether the cultures were started from a spore inoculum or from a vegetative cell inoculum. Extending the time of incubation from 18 h to 23 h resulted in considerable additional vegetative growth at all incubation temperatures between 25 and 41°C, with the growth optimum remaining at 32.5°C. However, little or no increase in cell growth was detected at the extremes of incubation temperatures, i.e., between 6 and 14°C and at 50°C.

DISCUSSION

Temperature ranges and optima for spore germination and vegetative cell growth. In this study, the spores were not heat shocked before the germination experiments. In laboratories heat shocking is frequently used to activate

TABLE 1. Nephelometric turbidity measurements of vegetative growth of *C. botulinum* type E strain VH[a]

Incubation temp (°C)	Nephelometric turbidity units	
	18-h incubation	23-h incubation
6.0	85	100
9.0	100	85
14.0	120	120
25.0	1,000	2,500
30.0	3,600	6,150
32.5	9,100	10,000
35.0	3,400	6,700
37.0	2,050	4,300
41.0	185	1,200
50.0	69	56

[a] Organism was grown in freshly prepared TPGT broth and equilibrated to the respective incubation temperature before inoculation with a vegetative cell inoculum. The initial nephelometric turbidity unit readings of the cultures were essentially the same as that of the uninoculated (control) TPGT broth, i.e., 70 ± 15 nephelometric turbidity units. The incubation temperatures were controlled within ±0.5°C.

spores. Only rarely would *C. botulinum* type E spores under natural environmental conditions receive a heat treatment. Such a situation may conceivably arise when foods are pasteurized or smoked. However, foods that are refrigerated to prevent spoilage caused by *C. botulinum* type E may or may not be pasteurized or smoked. Furthermore, there is no agreement upon what activates type E spores; be it mild heat, cold, or aging. For these reasons, we decided not to heat shock the spores before the germination experiments.

Perhaps the most important discovery that we made was that the spores germinated rather extensively at the low temperature of 2°C although no growth was detected within the time frame of this study. It seems equally important that the temperature optima for spore germination and those for vegetative growth were substantially different. This discovery represents a new understanding of the temperature relationships in strain VH of *C. botulinum*. Evaluation of the cardinal temperatures for spore germination and for growth is summarized in Fig. 4. The temperature optima for both partial and full spore germination was between 4 and 14°C, with a distinct maximum at 9°C. Germination rates in the moderate temperature range between 25 and 37°C were substantially slower than those at lower temperatures. Since these optima are below 15°C, the germination process of strain VH may be designated psychrophilic or cryophilic (10), which is distinct from vegetative cell growth with a maximum at 32.5°C, and is clearly mesophilic.

The differences in germination patterns at various temperatures (cf. Figs. 1 and 2) seem to imply that either different combinations of germination enzymes are involved at different temperature ranges or that perhaps basically the same enzymes are active at all temperatures, but that the kinetics of each individual germination enzyme is affected differently by various temperatures. Although germination enzymes are favored by relatively low temperatures, the activities of these enzymes span an exceedingly wide range from 2 to 50°C. At both extremes, this range greatly exceeds that permissible for the vegetative growth of this organism. In fact, at 50°C, this range seems to exceed the heat tolerance of *C. botulinum* type E spores. Germination data at 50°C are marginal to this study since our concern was essentially with cold-storage conditions. These high temperatures were included for experimental perspective only. Germination at 50°C was exceptionally rapid (2 h) although only 40% of the spores became phase dark. Germination in this case may have proceeded concurrently with heat inactivation; or at best, the cells were heat killed immediately after germination. Rapid germination at 60°C has also been observed (6) for strain PA3679h. In the absence of any prospect of vegetative outgrowth, the significance of germination at these high temperatures is problematic, to say the least.

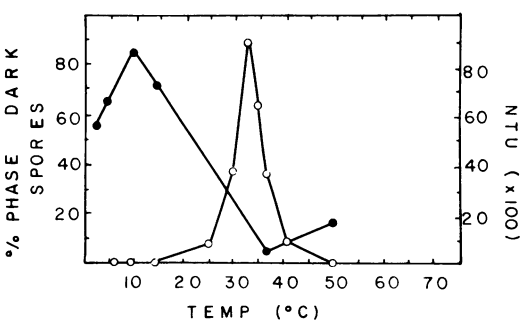

FIG. 4. Effect of incubation temperature upon spore germination (●) and vegetative cell growth (○) of *C. botulinum* type E strain VH. Spore germination was recorded at 24 h, and vegetative cell growth was recorded at 23 h (both at the respective incubation temperatures). The medium for germination and growth was TPGT broth. NTU, Nephelometric turbidity units.

Temperature optimum for vegetative cell growth. When testing 10 strains of *C. botulinum* type E, including strain VH, in neopeptone-glucose-yeast extract medium, Ohye and Scott came to the conclusion that the growth optimum was about 35°C. (11). Since our temperature points were spaced more closely than those of Ohye and Scott, our data could be interpreted as being in essential agreement with their findings, and perhaps the 32.5°C optimum that we observed is more accurate than the 35°C that they reported. Furthermore, Ohye and Scott indicated that growth was unlikely at temperatures above 45°C. Our results essentially confirm their conclusion. No growth was detected at 50°C and very little growth was found at 41°C (Table 1).

Lag period between partial and full spore germination. An important discovery deserving emphasis is that there were two distinct stages in the germination process of *C. botulinum* type E strain VH, i.e., partial germination and full germination (Fig. 1, 3B, and 3C). The occurrence under suboptimal germination conditions of partial germination (the core remaining refractile) was previously reported for strain PA3679h, at germination temperatures above 45°C in L-alanine or in the presence of D-alanine by Uehara and Frank (Bacteriol. Proc., p. 36, 1965) and subsequently in the presence of nitrite or sodium chloride by Duncan and Foster (5). Bimodal germination behavior was also recog-

nized for the spores of *Bacillus cereus* by Hashimoto et al. (7); in this case, certain chemical inhibitors could block stage two of the germination process.

The reason for the lag between partial and full germination is not immediately apparent. Generally, spore germination is viewed as a triggered process running its entire course without explicit inflection between stages one and two. Partial germination seems to occur whenever conditions are suboptimal for germination, including suboptimal temperatures, inadequate media, or the presence of inhibitors. In our study, the low temperature may have induced the lag, or perhaps TPGT broth may be an inadequate medium lacking either nutrient(s) or containing an inhibitor(s). Although the formulation of an optimal germination medium was beyond the scope of our study, it is noteworthy that unspecified strains of *C. botulinum* type E germinate best in the presence of lactate and carbonate (Y. Ando et al., Rep. Hokkaido Inst. Public Health, p. 12–19, 1970), which are absent from TPGT broth. The use of TPGT broth is justified here by our objective to expand the initial work of Schmidt et al. (12, 13, 14), who first warned of possible *C. botulinum* type E botulinal spoilage of food in cold storage. Our study emphasized the effect of low temperatures on germination, and we simply adapted the methods of these workers, even though we realized that a number of alternative methods and new challenges have come to light that deserve additional investigation.

Practical significance. It is important to know the temperature limits for germination since spore germination is required before outgrowth can occur (12). The occurrence of germination is also an index of physiological activity. Any vegetative growth of *C. botulinum* type E, no matter how slow, signals potential danger for the consumer. The situation becomes especially critical if it is inadvertantly aggravated by a short period of temperature abuse. In this context, the question logically arises: do spores that germinate at these low temperatures grow when shifted to an abusive temperature or do they lyse after outgrowth, as was observed with strain PA3679h germinating under adverse conditions (5)? Unfortunately, within the time frame of our experiment, these questions could not be resolved. However, from our data, it is clear that germination (a prerequisite for growth) does occur at 2°C, and from our observations, germination is, in fact, most rapid in the low temperature range. Therefore, these data reemphasize, from a different point of view, the original warning of the potential danger of *C. botulinum* type E activity in refrigerated foods (12, 13, 14).

General validity. In an attempt to extrapolate the validity of our observations to other strains of *C. botulinum* type E, reference is made to the report of Ohye and Scott (11). On the basis of a study of 10 strains, they concluded that *C. botulinum* type E constitutes a rather homogenous group with essentially identical reactions toward temperature. There is some degree of confidence, therefore, that our findings about strain VH may have general validity with respect to other type E strains. Whether this reasoning applies to spore germination and the characteristic temperature optima observed in this study remains, for the moment at least, an intriguing possibility which may lead to future investigations of this subject.

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