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ABSTRACT: Laboratory and field studies were conducted between July and October 1999 to identify bait preference, biomarker efficacy, and bait acceptance rates for delivering an oral plague vaccine to black-tailed prairie dogs (*Cynomys ludovicianus*). Twenty juvenile captive prairie dogs were offered alfalfa baits containing either alfalfa, alfalfa and 5% molasses, or alfalfa, 5% molasses and 4% salt. Based on the results of these trials we selected a bait containing alfalfa, 7% molasses, and 1% salt for field trials to determine bait acceptance rates by free-ranging animals. The biomarkers DuPont Blue dye, iophenoxic acid, and tetracycline hydrochloride were orally administered to captive prairie dogs to determine their efficacy. Only tetracycline proved effective as a biomarker. Two field trials were conducted at separate prairie dog colonies located at the Buffalo Gap National Grassland (Pennington County, South Dakota, USA). In Trial 1, three baits containing tetracycline were distributed around each active burrow entrance and an additional bait was placed inside the burrow (1,276 baits total). In Trial 2, baits were distributed at the same density per burrow as Trial 1, but along transects spaced 10 m apart (1,744 baits total). Trapping began 3 days after bait distribution, and 30 prairie dogs then were captured at each site to determine the percentage of animals marked. In Trial 1, 67% of the prairie dogs captured had tetracycline deposits indicative of bait consumption. In Trial 2, 83% of the prairie dogs had ingested a bait. Approximately 15% of the animals in both trials ate more than one bait. Fleas (*Opisocrostis hirsutus*) were found on 64 of 70 (91%) of the prairie dogs captured during this study.

Key words: Bait consumption preference, biomarker efficiency, black-tailed prairie dog, *Cynomys ludovicanus,* fleas, *Opisocrostis hirsutus,* physiological markers.

INTRODUCTION

Plague, caused by the bacterium *Yersinia pestis,* occurs in prairie dogs (*Cynomys* spp.) throughout most of their range in North America, with devastating effects in some populations. Mortality rates near 100% in affected black-tailed (*C. ludovicianus*) and Gunnison's (*C. gunnisoni*) prairie dog colonies were reported by Rayor (1985), while mortality rates of 85% have been reported for white-tailed prairie dogs (*C. leucurus*) (Clark, 1977). Plague has been identified in 17 western states (Levy and Gage, 1999; Cully et al., 2000) and, along with various human-caused factors, has contributed to the alarming decline of these species (Wuerthner, 1997). Also, black-footed ferrets (*Mustela nigripes*) rely almost exclusively on prairie dogs for food and on prairie dog burrows for shelter (Sheets et al., 1971). Therefore, their management and recovery is tightly linked to prairie dog survival and management.

An effective means of protecting prairie dogs from plague is needed to reduce their population decline, enhance recovery potential for black-footed ferrets, and reduce the incidence of zoonotic transmission of the disease. Historically, attempts to control the spread of plague epizootics have involved population reduction of rodents through poisoning and reduction of the flea vectors with insecticides (Fitzgerald, 1978). Recent studies have evaluated the effectiveness of an insect growth regulator for flea control as an alternative to insecticides (Karhu, 1999). Advances in vaccine development have resulted in safe and efficacious vaccines that can be used for controlling diseases in wildlife, such as rabies

(Rupprecht et al., 1986; Brochier et al., 1995), and the National Wildlife Health Center (NWHC; Madison, Wisconsin, USA) is currently conducting laboratory studies to evaluate the efficacy of oral plague vaccines for prairie dogs.

As vaccine efficacy studies continue, concomitant research is needed to identify a means of delivering vaccine to free-ranging prairie dogs. Bait designs and vaccine distribution methods have been reported for a variety of species including red foxes (*Vulpes vulpes*) in Europe (Brochier et al., 1995) and Canada (Bachmann et al., 1990), raccoons (*Procyon lotor*) (Hable et al., 1991), and mongooses (*Herpestes javanicus*) (Creekmore et al., 1994). Biomarkers such as tetracycline (Linhart and Kennelly, 1967), iophenoxic acid (Fletcher et al., 1990), or DuPont Blue dye (Creekmore et al., 1994) are normally incorporated into the baits to evaluate the success of bait distribution studies or to identify animals that have consumed a vaccine-laden bait. Our investigation of encapsulated biomarkers was initiated for a variety of reasons with the primary one being the ease and convenience of use by the bait formulator and the subsequent decrease in contamination of equipment and decrease in cleanup. A protective coating that protects the contents during bait extrusion encloses encapsulated biomarkers. Coated dye particles would be essentially dust free reducing safety issues and simplifying their addition to the bait formulation as well as stabilizing the dyes. Given the variety of coating materials available, the release of dyes could be designed to occur under selected conditions within an animal depending upon the specific biomarker requirements.

Advances in baiting and biomarker technology can be applied to designing an oral bait and a delivery protocol that is capable of delivering a plague vaccine to a large percentage of a target prairie dog population. Therefore, the objectives of this study were to (1) evaluate three bait formulations to determine which was the most palatable to prairie dogs; (2) determine the effectiveness of three biological markers for prairie dogs; (3) investigate the effect of encapsulation on biomarkers; (4) examine the effect of bait density and distribution methods on bait acceptance rates by prairie dogs and; (5) determine the percentage of free-ranging prairie dogs that consumed alfalfa baits laden with biomarkers.

METHODS

Captive animal trials

Twenty black-tailed prairie dogs were purchased in July 1999 from a private dealer located in central South Dakota (Kevin Parmely, Wessington, South Dakota, USA) and tagged with Size 1 Monel ear tags (National Band and Tag Co., Newport, Kentucky, USA). Study animals were prebreeding age individuals of both sexes that were housed in a single isolation room at the NWHC in an artificial burrow system constructed of polyvinyl chloride (PVC) pipe for tunnels and large rubber containers for burrow chambers. The burrow chambers contained welded wire cages with sliding doors. Prairie dogs were fed a commercial hamster chow (D&D Commodities Ltd., Stephen, Minnesota, USA) ad libitum for the first 5 days, and then fed a mixture of fresh vegetables for the remainder of the study.

The first phase of the captive animal trials was designed to determine which of three bait types the captive prairie dogs preferred. All three baits were manufactured from extruded alfalfa (*Medicago sativa*) pellets sold as a commerical rabbit chow (Ziegler Brothers Inc., Gardners, Pennsylvania, USA). Similar rodent chow baits were previously used to deliver biomarkers to small rodents (Creekmore et al., 1998) and this same alfalfa mixture was the first bait type we evaluated. For the second bait type, 5% molasses was added to the exterior of each pellet. The third bait type was similar to the molasses bait, except for the addition of 4% salt to the bait. All three bait types were shipped from the manufacturer in coded lots to prevent sampling bias during the bait preference trials. Prairie dogs were allowed to acclimate for 4 days prior to the start of preference testing.

In addition to the food regularly provided, the three bait types were presented free choice to the study animals for 3 days. Three containers, each holding 10 baits of one type, were placed in the isolation room. The baits were monitored until all of the baits in one container

were consumed, at which time the number and type of remaining baits were recorded. Bait containers were placed in the same location each day but the location of each bait type (left, center, right) was randomly assigned. A modification of the bait type eaten most often was used in the remaining laboratory studies, as well as the field bait trials.

The bait type selected during the bait preference trial was used to deliver biomarkers to the captive prairie dogs. The biomarkers tetracycline (TCH), DuPont Blue dye (dye), and iophenoxic acid (IA) were evaluated. In this trial, the TCH and dye were coated onto sugar beads with an Eudragit E 100 coating (Creanova Inc., Lockport, Illinois, USA) and a very thin over coating of RS 30 D (30%) (Creanova Inc.) to add water resistance during the bait formulation. The dyes and coatings were applied using a Strea-1 (Niro, Inc., Columbia, Maryland, USA) fluid bed coater with a wurster insert. Tetracycline, E 100, and talc were mixed in an ethanol:water (1:1) solution and applied to size 18/20 mesh sugar beads at a rate of 5.5 ml/min with an inlet temperature of 45 C and an outlet temperature of 36 C. The TC coated beads were then spray coated with a solution of RS 30 D (30%), triethylcitrate, talc, and water. The RS 30 D mixture was applied in the spray chamber at 5.5 ml/min with a inlet temperature of 35 C and an outlet temperature of 32 C.

A similar process was used to spray coat DuPont dye onto size 16/18 mesh sugar beads. Dye, E 100, and talc were added to an ethanol:water:acetone (7:3:7) solution and applied at 10ml/minute with an inlet temperature of 40 C and an outlet temperature of 30 C. The RS 30 D coating was then applied as previously described. Tetracycline and dye were incorporated into baits at a rate of 25 and 10 mg per bait respectively. Tetracycline and dye were microencapsulated at the USDA National Wildlife Research Center (Ft. Collins, Colorado, USA) and shipped to Ziegler Brothers Inc. for incorporation into the alfalfa bait matrix.

Bait production techniques require that a minimum of 9.1 kg or approximately 2,600 baits be manufactured for each bait/biomarker combination to assure an even distribution of the microencapsulated biomarkers within the bait matrix. Due to the small scale of the IA evaluation (four prairie dogs) it was not economically feasible to incorporate IA into baits. Therefore, 2 mg IA was administered to each of four prairie dogs in a 1 ml corn oil solution (Fletcher et al., 1990). Before administration of the IA, the four animals were anesthetized using isoflurane gas, and 1 ml of blood was collected from the anterior vena cava. Serum was

harvested pretreatment and saved frozen for use as IA controls. The IA/corn oil solution was then administered via a 20-gauge metal ball end stomach feeding tube (Popper and Sons, New Hyde Park, New York, USA). All prairie dogs were fasted for 12 hr, then placed in individual $25.4 \times 25.4 \times 40.6$ cm pet kennels. Sixteen prairie dogs, including the four animals administered IA, were randomly assigned to the treatment group, and four animals were assigned to the control group. All 16 treatment animals were offered two baits, one each containing TC or dye, and the four control prairie dogs were offered two baits containing no biomarkers. Five prairie dogs (four treatment, including one animal fed IA, and one control) were anesthetized with isoflurane gas and euthanized in a CO_2 chamber (Custer and Franson, 1988) on days 3 and 6. The five prairie dogs sampled on days 14 and 21 post ingestion were anesthetized with either 0.4 ml ketamine administered intramuscular or ketamine/acepromazine (Fort Dodge Laboratories, Fort Dodge, Iowa, USA) and euthanized via heart puncture with 0.1 ml Euthasol[R] (Delmarva Laboratories, Inc., Midlothian, Virginia, USA). A blood sample was collected via anterior vina cava puncture from those animals fed IA, and serum was submitted for serum iodine analysis (University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania, USA). Abdominal, femur marrow, and mandibular fat were grossly inspected in all animals for the robin's egg blue color characteristic of DuPont dye deposition (Creekmore et al., 1994). Mandibles were removed from the euthanized prairie dogs and stored at -4 C. A 180 μm thick section was taken from a mandible from each prairie dog using an Isomet[R] low speed saw (Buehler Ltd., Lake Bluff, Illinois, USA). Sections were mounted on slides in glycerin, covered with 0.17 mm cover slips, and stored at -4 C. Prepared slides were viewed using a microscope emitting UV light with a wavelength of 365 nm (Buyske et al., 1960). Samples containing TC deposits in teeth and bones emitted a yellow-gold fluorescence at a wavelength of 560 nm (Buyske et al., 1960).

Field trials

Field trials were conducted during September 1999 at two prairie dog towns located at Buffalo Gap National Grassland (Wall, South Dakota, USA). These colonies were selected as study sites to evaluate bait acceptance and to determine the optimal bait density and method of bait distribution for delivering alfalfa baits to prairie dogs. Colony SC 126, located within the Conata Basin (43°43.885′N, 102°12.531′W), is

a 7.28 ha prairie dog town that contained 319 (43.8/ha) active burrow entrances. Colony SC 134, a 4.05 ha town with 436 active burrow entrances (107.7/ha), is located approximately 800 m southwest of SC 126 (43°43.721′N, 102°12.695′W). Even though colony sizes and burrow densities differed between the two study sites, we elected to distribute baits at a rate of four baits per active burrow entrance for both trials.

Trial 1 involved distribution of baits around active burrow entrances. Five prairie dogs captured prior to bait distribution were euthanized and sampled for the presence of TC. Prairie dogs were captured using MSI Model 24 Tru-Catch traps (MSI Inc., Belle Fourche, South Dakota) baited with Hubbard sweet feed mix (Hubbard Inc, Mankato, Kansas, USA). Traps were not prebaited prior to capture attempts. To determine bait acceptance rates, three alfalfa baits, each containing 25 mg TC were distributed in a circle 1 m in diameter around each active den entrance, and an additional bait was placed within each entrance. Bait densities were calculated based on the number of active prairie dog burrows present, and baited, on the study site. The number of baits remaining at each of 10 randomly selected den entrances was recorded at 24 hr intervals for 3 days.

Three days after bait distribution, 30 prairie dogs were captured as previously described, anesthetized with ketamine, and euthanized with Euthasol[R]. Non-target species were collected concurrently to determine bait ingestion by those species. Small rodents were captured with Sherman live traps (H.B. Sherman Traps Inc., Tallahassee, Florida, USA), baited with Hubbard sweetfeed mix and euthanized with Euthasol[R]. Nontarget species, such as small birds, that were unlikely to have eaten an alfalfa type bait were released when captured. Prairie dogs and non-target rodents were necropsied as previously described to determine if they had ingested baits containing TC. Prairie dog carcasses were frozen individually in plastic bags, then combed for fleas and ticks; all ectoparasites were sent in 70% ETOH to the U.S. Department of Agriculture, National Veterinary Services Laboratory (NVSL; Ames, Iowa, USA) for identification. Representative ectoparasite specimens also were deposited with the NVSL under accession numbers 22134, 22240, 22241, 22242 (fleas), 22137, 22144, 22239 (ticks), 22285, 22280, 22345, and 22351 (lice). Carcasses were returned to the NWHC for incineration.

Trial 2 investigated the effectiveness of bait distribution along transect lines. Baits containing 25 mg TC were distributed along transect lines spaced 10 m apart. Sixteen baits were identified and monitored every 24 h for 3 days to evaluate bait uptake. Thirty prairie dogs were captured and sampled as described in Trial 1. Prairie dogs with mandibles indicating ingestion of baits containing tetracycline were used to estimate the percentage of animals that ingested baits.

Comparisons of marked prairie dogs between the two study sites were made using a chi-square test executed on SAS (SAS Institute, Inc., 1989). Results were considered significant when $P \leq 0.05$.

RESULTS

Captive animal trials

The rabbit chow bait containing 5% molasses (48%) was chosen slightly more often than the plain rabbit chow (46%). The bait containing 5% molasses and 4% salt was only chosen 6% of the time. Based on these results, the bait formulation produced for subsequent laboratory and field trials consisted of rabbit chow bait containing 7% molasses and 1% salt.

Biomarker encapsulation worked better for TC than the dye. The TC-coated beads yielded 709 g (716 g possible) with a 13.1% dye loading. The dye beads yielded 1,815 g (1,904 g possible) but the dye loading was 1.6% instead of the 4.0% target.

Twenty-five mg of TC and 10 mg of DuPont dye contained in separate baits were presented to individually housed prairie dogs. Fourteen of 16 treatment animals and four of four control animals ate both baits. Prairie dog 5 ate 100% of the dye bait and 75% of the TC bait, while animal number 3 only ate 10% of the dye bait and none of the TC bait.

Tetracycline effectively marked all 15 animals that consumed all or part of a TC-laden bait. Prairie dog 3 failed to eat the bait containing TC and consequently was not marked. The four control animals that were fed baits containing no biomarkers were all negative for TC.

DuPont Blue dye failed to mark prairie dogs past day seven of the trial. Two of three prairie dogs fed dye laden baits had dye-marked abdominal fat at day 3 post ingestion. Prairie dog 3 ate only 10% of

the dye bait and was not marked. Three of four prairie dogs had abdominal fat marked with dye on day 7, and none of the animals tested on days 14 and 21 were marked by the dye.

Two mg of IA administered in a corn oil solution failed to illicit an increased serum iodine level in the four animals tested.

Field trials

Four baits were distributed at each of 319 active burrows (175 baits/ha) at colony SC 126. Thirty percent of the baits from 10 monitored burrows had been taken after 24 hr. After 48 and 72 hr, 40% and 45% of the baits had been removed, respectively. Seventy percent of the monitored burrows had at least one bait removed after 72 hr. Trapping was initiated 3 days after bait distribution. Thirty prairie dogs (20 males, 10 females) were captured in 93 trap days at colony SC 126. Evidence of the biomarker TC was present in the mandibles of 20 (67%) of the prairie dogs captured, and multiple TC deposits were seen in the incisors of three of these animals indicating ingestion of at least one bait on more than 1 day. None of the five control prairie dogs captured prior to bait distribution had TC deposits in the mandibles.

At colony SC 134, 1,744 baits were placed along 16 transects (431 baits/ha) spaced 10 m apart. Monitoring of 16 flagged baits indicated that 15 baits were present 24 hr after bait distribution, and 14 baits were present at 48 and 72 hr post distribution. Trapping was initiated 3 days after bait distribution. Thirty prairie dogs (16 males, 24 females) were captured in 93 trap days. Twenty-five (83%) prairie dogs had mandibular TC deposits indicative of bait ingestion. Tetracycline deposits in the incisors indicate that four of these animals ate at least one bait on more than one day. None of the five control prairie dogs captured prior to bait distribution had TC marked mandibles. No nontarget small rodents were captured at SC 126 in 90 trap nights. The one deer mouse (*Peromyscus maniculatus*) captured at SC 134

in 90 trap nights was TC positive. No significant difference ($\chi^2 = 2.22$, 1 df, $P = 0.14$) in bait ingestion rates was noted between the two study sites.

Fleas were found on 64 (91%) of the 70 prairie dogs sampled. The mean number ($\pm$SD) of fleas for animals captured at SC 126 was 10.0 ± 8.96 (Range = 0–39) compared to a mean of 5.2 ± 4.46 fleas (Range = 0–18) per animal from SC 134. Fleas were collected from 30 of 35 prairie dogs captured at SC 134. *Opisocrostis hirsutus* was identified from 30 animals, and the sucking louse *Linognathoides cynomyis* was found on four prairie dogs. Thirty-four of 35 prairie dogs captured at SC 126 had *O. hirsutus* fleas. No *L. cynomyis* were noted on these animals but *Ixodes sculptus* ticks were found on three prairie dogs.

DISCUSSION

We conclude that modified alfalfa bait is an effective vehicle for delivering the proposed plague vaccine to prairie dogs. Bait acceptance rates of 67% and 83% are acceptable and probably would have been even higher if trapping had started 7 to 10 days after bait distribution rather than 3 days post distribution. The proportion of a prairie dog population that would need to be vaccinated to have a significant impact on the spread of plague is unknown. However, Voigt et al. (1985) suggested that immunization of 60% of the red fox (*Vulpes vulpes*) population in Ontario (Canada) would be effective in eradicating rabies, and vaccination rates as low as 50% have been considered effective in suppressing fox rabies in Europe (Bachmann et al., 1990). Obviously, care should be taken when comparing diseases with different hosts, population densities, and modes of transmission. But reduction of the fatality rate in prairie dogs due to plague from 99% to 30% or less would be beneficial in certain sensitive areas.

The bait selected for the field trials contained more molasses than those tested in the laboratory trials even though there was no significant difference between baits

containing molasses and plain alfalfa baits. The authors felt that the dried molasses coating on the bait would provide a limited amount of protection from precipitation.

The percentage of prairie dogs that had eaten a bait increased during trapping efforts at site SC 126. Sixty percent ($n = 10$) of the prairie dogs trapped on day 1 had eaten a bait compared to 63% ($n = 8$) and 75% ($n = 12$) on days 2 and 3 respectively. Similar results were seen at site SC 134 where the percentage of prairie dogs that had eaten baits on days 1–3 of trapping was 77% ($n = 13$), 81% ($n = 11$) and 100% ($n = 6$), respectively. Also, tetracycline deposits in the incisors of study animals indicate that approximately 15% of the marked prairie dogs ate baits on more than 1 day.

The failure of DuPont Blue dye to effectively mark prairie dogs was probably in part caused by the difficulty encountered in adhering the dye to the microspheres. The decrease in dye loading was likely caused by the limited solubility of the dye in the solvent mixture and the inability to keep the dye particles in suspension in the spray solution due to the particle size and density. Another factor may have been that the coatings may not have released the largely water insoluble dye in an appropriate manner within the animal. However, the successful deposition of TC in the laboratory and field shows potential for the encapsulation process. The selective release of biomarkers utilizing encapsulation, while only partially successful in this study, seems to warrant further study. For example, biomarkers for scat would best be treated with an enteric coating where release would only occur in the lower GI tract. A biomarker that is systemic in uptake would best be delivered using a controlled release coating; while a dye such as the DuPont dye used in this study would probably work well using a quick release coating such as the E 100 but without the RS 30 D overcoating.

Iophenoxic acid was administered to four prairie dogs in an effort to identify a biomarker that did not require euthanizing the animal for detection. However, elevated serum iodine values, indicative of IA ingestion, were not seen in the four animals tested in this study. Similar results were reported by Larson et al. (1981) in studies where 5 mg of IA were orally administered to Richardson's ground squirrels (*Spermophilus richardsoni*).

The failure of both DuPont dye and IA as biomarkers could potentially impact future bait trials if prairie dogs are designated a protected species. The need to euthanize the target animals to determine bait acceptance rates when using TC as a biomarker may become unfeasible. Other options such as using antigenic compounds as biomarkers are being considered.

Population estimates of target species are used to determine a baiting density that will maximize bait uptake while minimizing cost. The number of active burrows has been used as an index of population density in white-tailed prairie dogs (Forrest et al., 1985) but this technique failed to reflect short-term fluctuations in populations (Menkens and Anderson, 1993). Biggins et al. (1993) reported a correlation between counts of active white-tailed ($r^2 = 88.4\%$) and black-tailed ($r^2 = 42.5\%$) prairie dog burrows and estimates of prairie dog density, but Severson and Plumb (1998) found no significant relationship between mark-recapture estimates and active burrow counts. In our study, baits needed to be distributed in a manner that allowed comparison between colonies of different size and density of active burrows. We felt that efforts to determine a population index through mark-recapture studies might confound bait acceptance results by affecting capture rates following bait distribution. Therefore, we assumed that no short-term population fluctuation had occurred at either site and that the number of prairie dogs per burrow entrance was approximately the same between sites. Colony SC 126 contained 43.8 burrows/ha compared to 107.7 burrows/ha for colony SC 134, and at a bait

distribution rate of four baits per burrow, the number of baits per ha was much higher for SC 134 (430.6) than SC 126 (175.3). However, the burrows were more dispersed at SC 126 and baits were only distributed at burrow entrances, so the discrepancy between the two sites in the number of baits per ha is misleading.

The low disappearance rate for flagged baits at site SC 134 was not indicative of the percentage of prairie dogs that had eaten baits. This was probably due to prairie dogs avoiding baits that were marked with a surveyors flag 1 m away.

Trapping efforts for nontarget small rodents were largely unsuccessful. This was due to the fact that we only placed baits in areas of low vegetation that were obviously used by prairie dogs and subsequently attempted to trap nontarget rodents in these same areas. Small rodents have been implicated as possible reservoirs of plague (Thomas et al., 1988), and vaccine delivery to these species may be an important factor in limiting the effects of plague within a prairie dog colony. In future trials, bait distribution strategies should include baiting a buffer area of taller vegetation surrounding the active colony.

The flea, *O. hirsutus,* has been identified as an efficient plague vector in black-tailed prairie dogs in the plains east of the Rocky Mountains, in Gunnison's prairie dogs in the southern Rocky Mountains, and in white-tailed prairie dogs from Wyoming, northwestern Colorado, and northeastern Utah (Barnes, 1993). To date, *Yersinia pestis* has not been isolated from any species in South Dakota, but plague antibodies were found in carnivores sampled from Fall River county in 1995 (Levy and Gage, 1999), and the presence, relative abundance, and proven vector capability of *O. hirsutus* indicate that in South Dakota, the potential exists for plague to have a significant impact on black-tailed prairie dogs and associated species such as the black-footed ferret, if the disease becomes established, or if environmental conditions become temporarily suitable for plague

epizootics to spread into these areas from plague-enzootic regions in Wyoming.

Additional research is needed to better identify the most efficient and cost-effective strategies for distributing baits designed to deliver a plague vaccine as concomitant work continues on vaccine testing. Recent attempts to designate the black-tailed prairie dog as a threatened species underscore the impact of plague on this species and highlight the need to develop a strategy to halt the decline.

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