AVIAN BOTULISM EPIZOOTIOLOGY ON SEWAGE OXIDATION PONDS IN UTAH[1]

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Abstract: In the microenvironment concept of avian botulism epizootiology, it is hypothesized that invertebrate carcasses may serve both as a substrate for toxin production by *Clostridium botulinum* type C and as a vehicle for toxin transmission to water birds. We field-tested that hypothesis by attempting to induce botulism in wing-clipped mallard ducks (*Anas platyrhynchos*) on sewage oxidation ponds in Utah. The experimental ponds were inoculated with *C. botulinum* spores in June 1974. Aquatic insect populations were monitored throughout the summer. Rotenone was used in August to kill insects in two ponds (one served as control), thereby providing potential substrate for clostridial growth and toxin production. Botulism was not detected among the birds even though they routinely ingested invertebrate carcasses. Samples of dead invertebrates contained no botulinum toxin. We concluded that the microenvironment concept, as it now stands, cannot always be a sufficient explanation of how type C botulism epizootics are initiated in nature. Other microbes may inhibit the growth of clostridial cells or destroy botulinum toxin.

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Avian botulism has been recognized as a significant cause of waterfowl mortality in North America for at least 60 years (Wetmore 1915). The etiology of the disease was defined nearly 50 years ago (Giltner and Couch 1930, Kalmbach 1930). The ecology of the disease, including the natural substrates used by *C. botulinum* type C for growth and toxin production and the natural modes of toxin transmission to birds, has yet to be defined clearly. Research on avian botulism has been reviewed extensively by several authors (Sciple 1953, Kalmbach 1968, Rosen 1971, Enright 1971). Studies designed to delineate the epizootiology of type C botulism have been few. Kalmbach and Gunderson (1934) originally suggested that aquatic invertebrate carcasses might serve both as a substrate for toxin production and as a vehicle for toxin transmission to birds. This hypothesis was supported and elaborated by the microenvironment concept of Bell et al. (1955:353), which stated: "(1) that *C. botulinum* type C germinates, reproduces, and synthesizes its toxin in small discrete particulate substances, possibly invertebrate carcasses; (2) that the particulate substances are in no wise dependent upon the ambient medium for nurture of the bacteria, but contain all of the requisites within them; and (3) that the toxin is probably in the bacteria which reside in the particulate materials, rather than in the form of soluble, freely diffused toxin." The microenvironment concept has become the generally accepted, but inadequately tested, working hypothesis of type C botulism epizootiology (Enright 1971).

The objective of this study was to field-test the microenvironment hypothesis of type C botulism epizootiology. We attempted to induce botulism in wing-clipped flocks of mallard ducks on sewage oxidation ponds in Utah. The ponds represented newly created aquatic habitats supporting abundant aquatic insect populations, a po-

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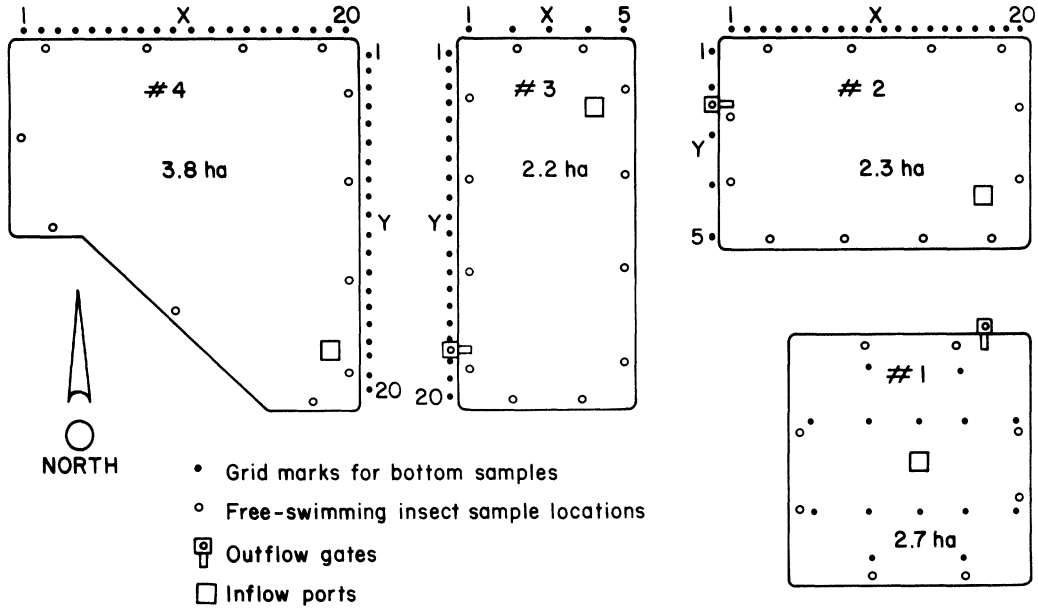

Fig. 1. Richmond sewage oxidation ponds in northern Utah used for botulism study, 1973–74.

tential source of invertebrate carcasses. Type C botulism epizootics in water birds have occurred on sewage oxidation ponds in Utah (Dodge 1972), California (Hunter 1970), and New Zealand (Martinovich et al. 1972).

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STUDY AREA

The study was conducted during the summers of 1973 and 1974 on the Richmond City sewage oxidation ponds in northern Utah. The system consists of 4 ponds with maximum surface areas ranging from 2.2 to 3.8 ha (Fig. 1). Pond 1 is the primary pond, pond 2 the secondary, pond 3 the tertiary, and pond 4 the quaternary. The system is closed and produces no effluent. Water loss by evapotranspiration exceeds inflow into the system in the summer months. The system began operation in 1973 and only pond 1 had water during that summer. A preliminary study of pond 1 (1973) determined the feasibility of the experiments that were conducted on ponds 2, 3, and 4 in 1974. In 1974, pond 2 supported no submerged or emergent vegetation. The dominant primary producers were green (Chlorophyta) and blue-green (Cyanophyta) algae. Pond 3 was characterized by submerged *Potamogeton* sp. (pondweeds); emergent *Sparganium* sp. (bur-reed), *Juncus* sp. (rush), and *Typha* sp. (cattails); and green filamentous algae of the genus *Cladophora*. Pond 4 was largely dominated by *Cladophora*.

MATERIALS AND METHODS

Water quality parameters measured were depth, temperature, pH, and dissolved oxygen. Water samples were taken with a Kem-

merer water bottle (Wildlife Supply Co., Saginaw, Mich. 48602) lowered from a boat. All measurements were made in the field at midmorning with a portable water test kit (Hach Chemical Co., Ames, Iowa 50010).

On 29 June 1974, ponds 2, 3, and 4 were inoculated with *C. botulinum* spores, derived from cultures of a strain (X220B2 type Cα) originally isolated from the liver of a duck on the Bear River Migratory Bird Refuge, Utah. The toxicity of the cultures was 2×10^5 mouse intraperitoneal (i.p.) LD_{50}/ml. Cultures were incubated for 5 days at 37 C in 32 liters of lactalysate-yeast autolysate (LYA) broth (McKee et al. 1958) and centrifuged to concentrate spores. The 800-ml yield was heat shocked at 80 C for 10 minutes to destroy vegetative cells and most toxin. The suspension contained about 4,050 viable spores/ml. Pond 4 received 300 ml and ponds 2 and 3 received 250 ml each of suspension spread over the surfaces and especially around the edges where invertebrate carcasses accumulated.

Benthic invertebrates were sampled with an Ekman dredge (Wildlife Supply Co.) that takes a 232-cm^2 sample of bottom substrate. Invertebrates were washed in a wire-bottomed bucket to remove mud and preserved in 4 percent formalin. A computer (Burroughs 6700) program randomly selected 20 *X-Y* coordinates (Fig. 1) for each set of bottom samples taken (1974). Samples of free-swimming invertebrates were taken only around the edges of the ponds (Fig. 1) by rapidly pushing a plastic bucket into the water to draw in a water sample of 7.57 liters. Samples were poured through a plankton net (80 μm mesh) and invertebrates preserved in 70 percent ethanol. Invertebrates were classified, oven-dried at 70 C for 48 hours, and weighed to the nearest 0.1 mg. Invertebrate sample means and 90 percent *t* statistic confidence intervals were calculated.

Rotenone (5 percent) was used to kill invertebrates in ponds 3 and 4. Laboratory simulation tests indicated that a rotenone concentration $\geqq 5$ ppm would cause severe mortality in benthic and free-swimming invertebrate populations within 24 hours. Pond 4 was treated with 113.6 liters of rotenone (Chemical Insecticide Corp., Edison, N.J. 08817) on 3 August. Pond 4 held about 12,353 m^3 of water, and the rotenone concentration attained was about 9 ppm. Pond 2 was treated with 132.5 liters of rotenone (S. B. Penick and Co., New York 10007) on 27 August. Pond 2 held about 13,573 m^3 of water, and the rotenone concentration attained was about 9.5 ppm. Pond 3 was the control. The growth and production of toxin by laboratory cultures of X220B2 were unaffected by rotenone concentrations $\leqq 20$ ppm.

Wing-clipped mallard ducks and American coots (*Fulica americana*) were placed on the ponds to insure the continual presence of susceptible water birds. As of 3 September, pond 2 supported 24 mallards and pond 3 supported 32 mallards and 20 coots. Pond 4 supported 24 mallards from 13 July until the end of August when the pond dried up. A wire-mesh fence 1 m high was erected around pond 3 to prevent birds from walking between control (3) and treatment (2 and 4) ponds.

Samples of dead invertebrates from pond 2 were tested for botulinum toxin. Two-ml volumes of each sample were removed by pipette. If entire carcasses (usually corixids) were visible in the sample, several were added to the 2.0 ml and crushed in a mortar. A 0.5-ml volume was inoculated into a tube of LYA broth, incubated at 34 C for 5 days, and diluted 1:10 in gelatin-sodium phosphate buffer prior to testing. The remainder (unincubated) was diluted 1:5 prior to testing. Each incubated and unincubated sample was injected i.p. (0.1 ml)

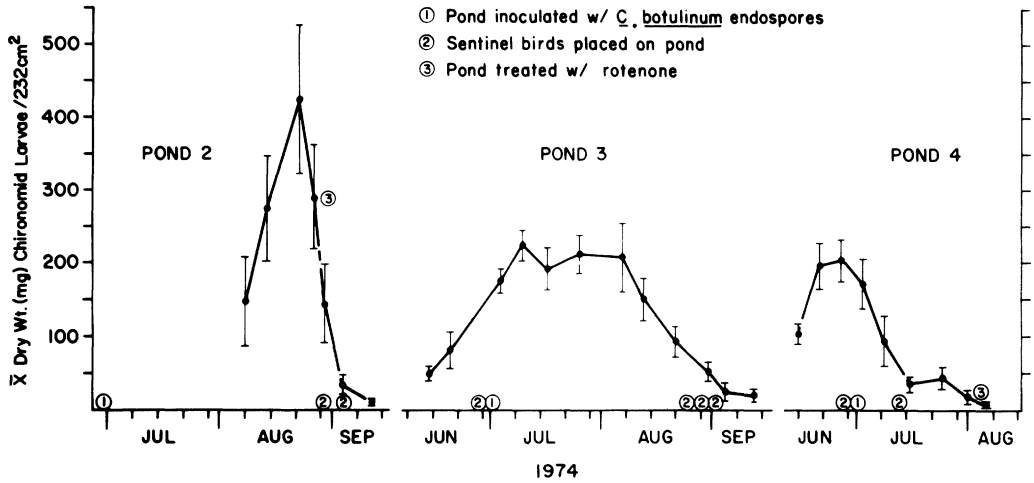

Fig. 2. Estimates of chironomid larvae biomass in Richmond sewage oxidation ponds 2, 3, and 4, northern Utah, 1974; $n = 20$ for all points, 90 percent confidence interval shown for each mean.

into two 15–20-g white mice, one of which was protected with 0.1 ml (5 IU) of type C antitoxin (Ft. Dodge Laboratories, Ft. Dodge, Iowa 50501), and both of which were protected from infection by sewage pond microbes with 0.5-mg i.p. doses of Liquamycin (Chas. Pfizer and Co., New York 10017). Test animals were observed for 5 days for signs of intoxication or death.

RESULTS

Water Quality

Although the significance of physicochemical parameters of the ambient medium to botulinum toxin production is not known, the following ranges of values were measured from 27 June to 18 September: water depth (m) ranged from 0.6 to 0.9 in pond 2, 0.2 to 0.8 in pond 3, and 0.0 to 0.7 in pond 4; surface temperature (C) ranged from 26 to 17 in pond 2, 24 to 17 in pond 3, and 23 to 17 in pond 4; pH ranged from 8.4 to 9.2 in pond 2, 8.3 to 10.0 in pond 3, and 9.0 to 9.7 in pond 4; dissolved oxygen (ppm) ranged from 1 to 15 in pond 2, 2 to 12 in pond 3, and 9 to 12 in pond 4.

Invertebrate Populations

Benthic invertebrates consisted almost entirely of Chironomidae (midge) larvae. The maximum mean dry weight of larvae ($n = 14$) observed in pond 1 (1973) was 339 ± 60.3 mg/232 cm^2 of bottom in late summer. That estimate represented a high-biomass population of chironomid larvae compared with populations observed in some Utah marshes (Jensen and Allen 1960, McKnight and Low 1969) and oxidation ponds (Dodge 1972:61, Kemmerer 1970: 74). In 1974, all three of the new ponds (2, 3, and 4) supported high-biomass populations of chironomid larvae at some point during the summer (Fig. 2). The maximum mean dry weight of larvae observed in pond 4 was 204.8 ± 29.1 mg/232 cm^2 in late June. The mean dry weight of larvae in pond 4 decreased steadily as water depth decreased. The mean dry weight of larvae in pond 3 reached a maximum of 223.6 ± 21.5 mg/232 cm^2 in early July and then declined, as in pond 4, as water depth decreased. As the water in ponds 3 and 4 evaporated, the pH increased and suspended solids (turbid-

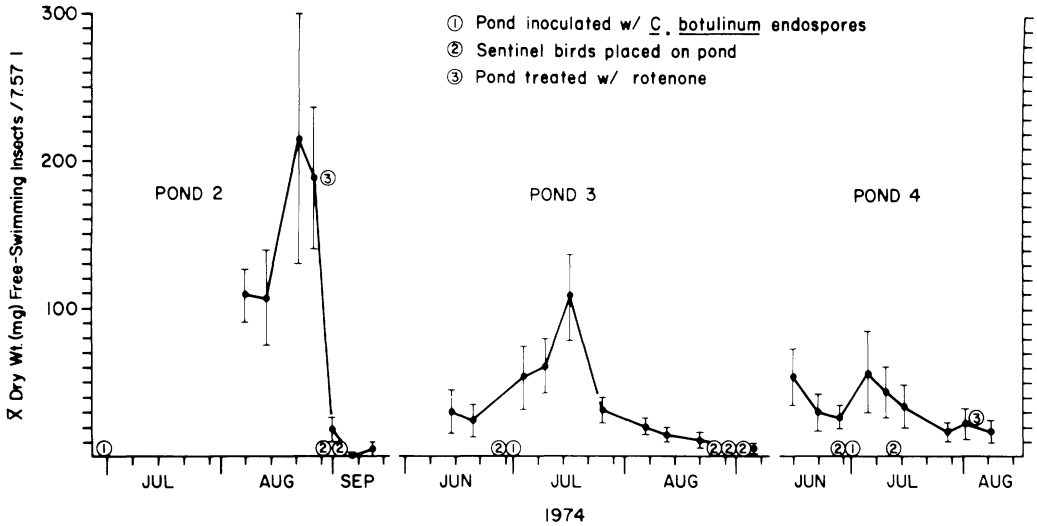

Fig. 3. Indices of free-swimming insect biomass in Richmond sewage oxidation ponds 2, 3, and 4, northern Utah, 1974; n = 12 for all points, 90 percent confidence interval shown for each mean.

ity) decreased. The larval population in pond 2 reached a maximum mean dry weight of 424 ± 101.8 mg/232 cm² in late August. The larvae were not distributed as uniformly over the bottom in pond 2 as in the other two ponds, resulting in broad confidence intervals on estimates of population biomass.

The free-swimming invertebrate populations of the ponds were dominated by Corixidae (water boatmen), by far the most abundant group, and Notonectidae (back swimmers) were second in abundance. Ephemeroptera (mayfly nymphs) were numerous in pond 1 (1973) and in ponds 3 and 4 (1974). Aquatic Coleoptera were present but not abundant. Dytiscidae (predaceous water beetles), Hydrophilidae (water scavenger beetles), and Gyrinidae (whirligig beetles) were present in pond 1 (1973) and ponds 3 and 4 (1974). A few Odonata (dragonflies and damselflies) nymphs were taken from ponds 3 and 4. Data for free-swimming insects (Fig. 3) were not true population biomass estimates, because only the pond edges (Fig. 1) were sampled; however, these data did provide indices that showed major trends in population biomass. The maximum mean dry weight of free-swimming insects ($n = 8$) observed in pond 1 (1973) was 174.8 ± 48.6 mg/7.57 liters of water in late August. The maximum mean dry weights of free-swimming insects ($n = 12$) observed in ponds 2, 3, and 4 were: 215.2 ± 86 mg/7.57 liters in late August in pond 2, 197.6 ± 29.5 mg/7.57 liters in mid-July in pond 3, and 56.7 ± 27.9 mg/7.57 liters in early July in pond 4.

Pond 4 supported low-biomass populations of benthic and free-swimming insects (Figs. 2, 3) at the time of rotenone treatment (3 August). The ratio of dead/living larvae in the last set of bottom samples from pond 4 (5 August) indicated that about 30 percent of the larvae were killed by the rotenone. Not more than 10.4 ± 9.7 mg (dry weight) of dead larvae/232 cm² of bottom were provided as potential bacterial substrate. The effect, if any, of the rotenone upon free-swimming insects was not obvi-

ous. Filamentous algae in pond 4 tended to hold the rotenone in localized pockets. Pond 2 supported high-biomass populations of benthic and free-swimming insects at the time of treatment (27 August). The result of the treatment was a rapid and massive die-off of both benthic (Fig. 2) and free-swimming (Fig. 3) insects. The mean dry weight of larvae in pond 2 on 26 August was 289.8 ± 72 mg/232 cm^2 of bottom, decreasing to 143.9 ± 53.8 mg/232 cm^2 by 29 August, 2 days after treatment, and to 33.8 ± 12.7 mg/232 cm^2 by 3 September, 1 week after treatment. About 255.9 ± 73.3 mg (dry weight) of dead larvae/232 cm^2 of bottom were provided as potential bacterial substrate. Many dead larvae were observed floating on the surface of pond 2. The declines in population biomass of larvae in ponds 3 and 4 (Fig. 2) did not represent the death of the larvae but their removal due to metamorphosis and emergence. This was true also of some free-swimming forms (e.g., mayfly nymphs). The free-swimming insect population of pond 2 consisted almost entirely of corixids, and these were virtually eliminated by the rotenone. The mean dry weight of free-swimming insects in pond 2 on 26 August was 188.4 ± 48.6 mg/7.57 liters of water, decreasing to 17.9 ± 8.8 mg/7.57 liters of water by 31 August, 4 days after treatment. About 170.5 ± 49.4 mg (dry weight) of dead corixids/7.57 liters of water were provided as potential bacterial substrate. Large numbers of dead corixids were observed floating on the surface of pond 2.

Sentinel Birds

As of 25 August 1973, pond 1 supported 78 mallards and 24 coots. Botulism was not observed in these birds. In 1974, all mallards on pond 4 were moved to the other ponds when pond 4 dried up about the end of August. It is not certain that ducks on pond 4 ingested invertebrate carcasses. However, the 24 mallards on pond 2 were observed to feed routinely upon the large quantities of invertebrate carcasses provided by the rotenone treatment, and they continued to do so as long as carcasses were available. As water depth in pond 2 increased, invertebrate carcasses eventually sank and became unavailable to the ducks. An anchor dragged along the bottom around the edges of the pond on 16 September and again on 20 October raised large quantities of invertebrate carcasses that the ducks ingested readily. Examination of two mallards sacrificed on pond 2, one on 10 September and one on 3 October, confirmed the visual observations that the ducks were ingesting invertebrate carcasses. The esophagi of both contained the cases of dead chironomid larvae and fragments of corixid wings and exoskeletons. The esophagus of a mallard sacrificed on pond 3 on 10 September was full of sago pondweed (*Potamogeton pectinatus*). Botulism was not observed in any of the birds on any pond in 1974.

Toxicity Tests

None of the 14 samples of dead invertebrates collected between 9 September and 19 November on pond 2 was toxic for mice either before or after incubation. The absence of toxin accounted for the fact that no ducks contracted botulism even though they routinely ingested invertebrate carcasses.

DISCUSSION

The preliminary study of pond 1 (1973) demonstrated the feasibility of testing the microenvironment hypothesis of type C botulism epizootiology on the Richmond sewage oxidation ponds. The 1973 study showed (1) that ponds 2, 3, and 4 could be expected to support abundant populations

of benthic and free-swimming aquatic insects in 1974, (2) the approximate numbers of samples required to monitor invertebrate populations so that valid statistical comparisons could be made if required, and (3) that the ponds could be expected to support waterfowl flocks. The 1974 experiments on ponds 2, 3, and 4 constituted the actual field test of the hypothesis.

The microenvironment concept hypothesizes that the following conditions are necessary for the initiation of a type C botulism epizootic in water birds: (1) the etiologic agent (viable, toxigenic type C cells) must be present in the environment, (2) a suitable substrate (e.g., invertebrate carcasses) must be available for clostridial growth and toxin production, and (3) a susceptible population of water birds must be present and must ingest the toxin, which is probably contained in the bacterial substrate. This study attempted to satisfy these conditions during the critical time of year, late summer and early fall, when botulism epizootics characteristically occur. The inoculation of ponds 2, 3, and 4 with *C. botulinum* spores was done primarily to insure that a negative result (no botulism in the birds) could not be attributed to an absence of the etiologic agent. It seems likely, however, that any aquatic habitat in northern Utah, an area where type C botulism is enzootic, would have a natural population of *C. botulinum* type C. Data from pond 2 demonstrated that even when conditions (1) and (3) were satisfied, the provision of large quantities of invertebrate carcasses as a potential bacterial substrate was not sufficient to cause type C botulism in the birds. This result indicates that, although these conditions may be necessary for the initiation of a type C botulism epizootic in Utah marshes, they probably do not represent all necessary conditions. If they did, we would have observed some cases of botulism in the birds on pond 4 (low treatment) and some larger number of cases in the birds on pond 2 (high treatment). The problem appears to be one of defining the additional condition(s) necessary for the initiation of an epizootic.

The toxicity tests of invertebrate carcasses demonstrated that the absence of botulism in the birds on pond 2 was due to the absence of toxin in the dead invertebrates ingested by the birds. Preliminary results of ongoing research at the Bear River Research Station (Moulton 1975) indicate that other microbes present in the sewage ponds may have an inhibitory effect upon the toxicity of cultures of the highly toxigenic X220B2 cells, even when large numbers of cells are involved under ideal growth conditions. If this is true, it is not difficult to imagine how *C. botulinum* cells might be inhibited in their growth or production of toxin (or both) by other microbes in a sewage oxidation pond (or marsh) where smaller numbers of clostridial cells might be involved under less than ideal growth conditions.

The concept that other microbes may inhibit the survival of clostridial cells or toxin is not new. Sherman et al. (1927) reported that human intestinal bacteria could destroy botulinum toxin. Quortrup and Holt (1941) stated that aerobic bacteria are capable of rapidly destroying preformed toxin and that aerobic microbes have much stronger detoxifying properties than have anaerobes. Quortrup and Sudheimer (1943) reported that the detoxifying action of *Escherichia coli* upon botulinum toxin could be overcome by *Pseudomonas aeruginosa,* which could outgrow *E. coli* and allow *C. botulinum* to produce toxin. Hunter (1970) stated that one possible cause of subsidence of botulism epizootics might be microbial action upon toxin. Jensen and Allen (1960), in a discussion of the evidence

required to prove the validity of the microenvironment concept on the Bear River Refuge, stated that either the abundance of invertebrate carcasses must increase or environmental changes favoring an increased rate of toxin production must occur prior to the initiation of a botulism epizootic. The environmental change required to favor an increased rate of toxin production in nature may be the alleviation of the normal inhibitory effects of other microbes upon clostridial growth and toxin production. This aspect of botulism research (microbial ecology) has been largely overlooked for the past 30 years.

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