# Inhibition of Acetoclastic Methanogenesis by Crude Oil from Bemidji, Minnesota

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## ABSTRACT

The shallow ground water at a site near Bemidji, Minnesota is contaminated with crude oil spilled from a broken pipeline in 1979. With a continued source of dissolved crude oil components, the geochemical conditions in the aquifer have evolved into aerobic, iron-reducing, and methanogenic redox zones. The methanogenic zone starts within the crude oil-contaminated region and extends more than 60 meters downgradient. The methanogenic numbers in the aquifer are low, but variable, depending on the subpopulation of methanogen. In areas close to the crude-oil source, hydrogen- and formate-utilizing methanogens are found in numbers more than one hundred times higher than acetate-utilizers. The acetate-utilizers are found only well below the non-aqueous phase oil and further downgradient. This pattern of methanogen distribution suggests that growth of acetate-utilizers is limited near the source. Laboratory results suggest that toxicity of the dissolved crude-oil is an explanation.

Serum bottle assays were conducted using crude oil in a mineral salts solution inoculated with an enriched methanogenic consortia from a creosote-contaminated site in Pensacola, Florida. Acetate, hydrogen, and formate were added and gas volume change was monitored. Hydrogen- and formate-utilization were unaffected by the crude oil whereas acetate utilization was significantly inhibited. The distribution of aquifer methanogens together with the toxicity assays form a consistent picture with the hypothesis that acetoclastic methanogenesis is inhibited in the vicinity of the oil at the Bemidji site. Because acetate degradation has been widely documented as the rate-limiting step in anaerobic waste treatment processes, it is likely that the inhibition of acetoclastic methanogenesis by the crude oil affects the overall methanogenic degradation rates of the petroleum hydrocarbon contaminants.

## INTRODUCTION

In August 1979, a crude oil pipeline near Bemidji, Minnesota (fig. 1) burst, spilling about 1,700,000 L (liters) of crude oil. Cleanup efforts removed all but an estimated 400,000 L of crude oil (Hult, 1984). The crude oil has moved through the unsaturated zone contaminating the sediments down to the water table. A plume of the water-soluble components (including benzene, toluene, ethylbenzene, xylenes, and polynuclear aromatic hydrocarbons) has formed under the crude oil. In the past 20 years, the plume has changed the geochemical conditions in the aquifer near the oil body from aerobic to iron-reducing and methanogenic (Baedecker and others, 1993; Bekins and others, 1999).

A model of the plume using a two-dimensional, multispecies reactive solute transport model with sequential aerobic and anaerobic degradation processes was successful in predicting the evolution of geochemical conditions in the plume (Essaid and others, 1995). However, the use of Monod kinetics to model microbial growth in the aquifer resulted in simulated microbial numbers that far exceeded the observed aquifer microbial numbers. One process that would act to limit the net growth is inhibition of crude-oil biodegradation due to contaminant toxicity.

Specific methanogens may be inhibited by the increased contaminant concentration near the crude oil or even by the non-aqueous phase of the crude oil itself. Several authors have found that acetoclastic methanogens are inhibited by toxic compounds [Hickey and others, 1987 (chloroform, bromoethanesulfonic acid, tricloroacetic acid, and formaldehyde); Patel and

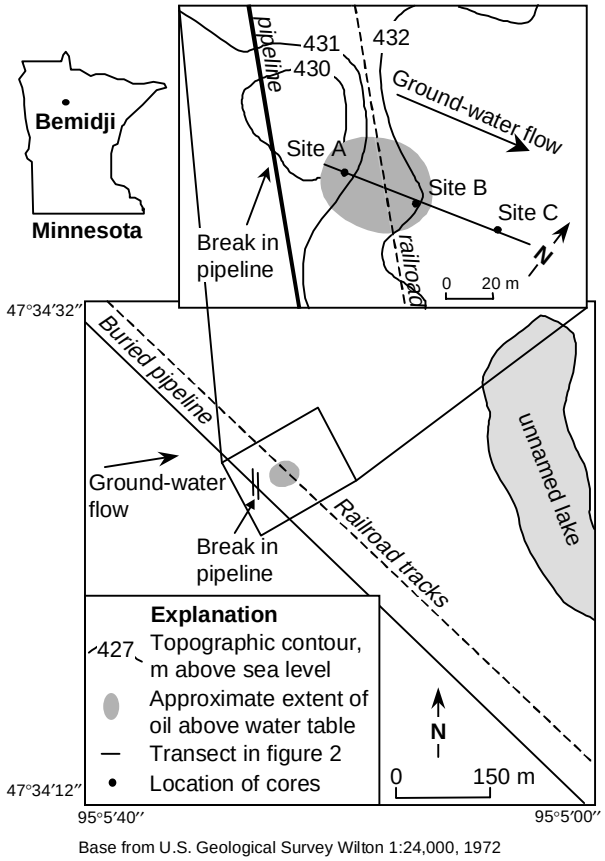


**Figure 1.** Site of 1979 crude oil spill near Bemidji, Minnesota, showing location of cores along a flow line.

others, 1991 (benzene ring compounds); Sierra-Alvarez and Lettinga, 1991 (monosubstituted benzenes, chlorobenzene, methoxybenzene, and benzaldehyde); Colleran and others, 1992 (chlorinated and fluorinated low molecular weight aliphatic and aromatic compounds); Davies-Venn and others, 1992 (chlorophenols and chloroanilines); van Beelen and Fleuren-Kemilä, 1993 (pentachlorophenol); and Donlon and others, 1995 (N-substituted aromatics)]. This is important because about 70 percent (%) of the methane produced comes from acetate fermentation by acetoclastic methanogens as opposed to carbon dioxide reduction by hydrogenophilic methanogens (Jeris and McCarty, 1965; Cappenberg, 1974; Cappenberg and Prins, 1974). Thus acetate fermentation is known to be the rate-limiting step in anaerobic wastewater-treatment processes. Parkin and Speece (1982) were able to model concentration-dependent inhibition of methanogenic degradation of a variety of toxic compounds by modeling inhibition of the acetate utilization step.

Very little research has compared the inhibition of acetoclastic methanogens to other subpopulations. Colleran and others (1992) found acetoclastic species were significantly more inhibited by halogenated aliphatics than hydrogenophilic subpopulations. Bhattacharya and others (1995) found that whereas acetoclastic methanogens were inhibited by 4-nitrophenol, hydrogenophilic methanogens were not. Bekins and others (1997) found that acetoclastic methanogens were more susceptible to toxicity of water-soluble creosote compounds than were hydrogen- or formate-utilizing methanogens.

In this paper, we present evidence that inhibition can explain some aspects of methanogenic microbial numbers at the crude-oil spill site near Bemidji, Minnesota.

## METHODS

Most Probable Number (MPN) determinations were done on sediment from cores at three sites and compared with laboratory crude oil toxicity experiments. Inhibition of methanogens was measured by an anaerobic toxicity assay (Owen and others, 1979) in which the volume of gas produced in a methanogenic microcosm with crude oil was compared to that in a microcosm without crude oil.

### Most Probable Number Determination

Most Probable Number determinations were done on sediments from three sites located within the anoxic portion of the plume, labeled A, B, and C in figure 1. Cores containing aquifer sediments and the contaminated groundwater were collected with a 2.4 m (meter) freezing drive shoe (Murphy and Herkelrath, 1996). Data from sites A, B, and C were obtained from vertical profiles consisting of 3, 1, and 2 cores, respectively. The single core from site B was collected in 1996 while the five cores from sites A and C were collected in 1997. The method was the same for each core. The core was cut with a large tubing cutter exposing the aquifer material. Under a flow of oxygen-free nitrogen gas, the first few centimeters of the core

material at the cut were removed with a sterile spatula exposing an uncontaminated surface.

Approximately 10 g (grams) of sediment from the center of the core was added to a 25 x 142 mm (millimeter) anaerobic isolation roll streak tube (Bellco Glass Inc., Vineland, N.J. Note: Any use of trade, product, or firm names in this paper is for descriptive purposes only and does not imply endorsement by the U.S. Government.) filled with 20.0 mL (milliliter) of pre-reduced mineral salts solution. The mineral salts were prepared as follows (per liter): 0.75 g of $KH_2PO_4$; 0.89 g of $K_2HPO_4$; 0.36 g of $MgCl_2 \cdot 6H_2O$; 0.9 g of $NH_4Cl$; 9.0 mL of trace metal solution (Zeikus, 1977); 5.0 mL of vitamin solution (Wolin and others, 1963); and 10 mg (milligram) of Tween 80® [a nonionic surfactant added to remove microbes from the sediment (Yoon and Rosson, 1990)]. The pH was adjusted to 7.0 with phosphoric acid, and the solution was then boiled, cooled, and dispensed under a stream of oxygen-free nitrogen gas. The solution was sterilized at 121°C (degrees Celsius) [100 kPa (kilopascal)] for 15 minutes. All mineral salts solutions were amended with ferrous sulfide (FeS) as a reducing agent (Brock and O'Dea, 1977) to a final concentration of 1% by volume. Oxygen-free nitrogen gas was allowed to flow over the surface of the mineral salts solution as the sediment sample was added. The tube was then sealed, mixed well, and allowed to stand for 2 hours to allow penetration of Tween 80® into the sample. The tubes were then opened and sonicated [10 watts for 30 seconds] to dislodge the bacteria into the mineral salts using a Branson Sonifier®, Model 200, with the microtip attached (Branson Ultrasonics Corporation, Danbury, Conn.) with a flow of sterile oxygen-free nitrogen gas over the surface. The sediment samples in mineral salts were stored for not more than 4 hours at 20°C before inoculation of the growth media.

Microbial concentrations in sediment samples were determined using a five-tube MPN analysis. Samples were serially diluted by orders of magnitude into dilution mineral salts solutions that were pre-reduced and anaerobically sterilized as described by Holdeman and Moore (1972). Aliquots of the dilutions were inoculated into three different media, designed to promote growth and the enumeration of acetate-, hydrogen-, and formate-utilizing methanogenic microorganisms. Acetoclastic and formate-utilizing organisms were enumerated with the addition to mineral salts of 2.5 g of sodium acetate$\cdot 3H_2O$ or 2.5 g sodium formate per liter, respectively. Hydrogen oxidizers were enumerated by aseptically pressurizing the serum bottles after inoculation with a 70:30 mix of $H_2$:$CO_2$ to 140 kPa. The serum bottles were allowed to incubate for a minimum of six weeks at room temperature. The presence of the methanogen subpopulation was established by the detection of methane using a gas chromatograph with flame ionization detection (Godsy, 1980). Subsamples of sediments from site B (fig. 2) were taken for analysis of oil content using the method described by Hess and others (1992). Values for oil content at a site near site A were determined by Dillard and others (1997).

## Anaerobic Toxicity Assays

Anaerobic toxicity assays followed an adaptation of a protocol described by Owen and others (1979). Microcosms in 120 mL serum bottles were prepared to evaluate the toxicity of crude oil to formate-, hydrogen-, and acetate-utilizing methanogens. The serum bottles contained a total of 100 mL comprised of crude oil (10% by volume), mineral salts (70%), and an enriched methanogenic consortia suspended in mineral salts (20%). The bottles were prepared in an anaerobic atmosphere and amended with ferrous sulfide (FeS) as a reducing agent (Brock and O'Dea, 1977) to a final concentration of 1%

**Table 1.** Contents of serum bottles. Number indicates number of replicates. Check mark indicates either bottle was autoclaved or crude oil was present.

| Bottle | Energy Source | Autoclaved | Crude Oil | No. |
|---|---|---|---|---|
| 1 | Acetate | | √ | 2 |
| 2 | Formate | | √ | 2 |
| 3 | Hydrogen | | √ | 2 |
| 4 | Acetate | | | 1 |
| 5 | Formate | | | 1 |
| 6 | Hydrogen | | | 1 |
| 7 | Acetate | √ | √ | 1 |
| 8 | Formate | √ | √ | 1 |
| 9 | Hydrogen | √ | √ | 1 |
| 10 | None | | √ | 2 |
| 11 | None | √ | | 1 |

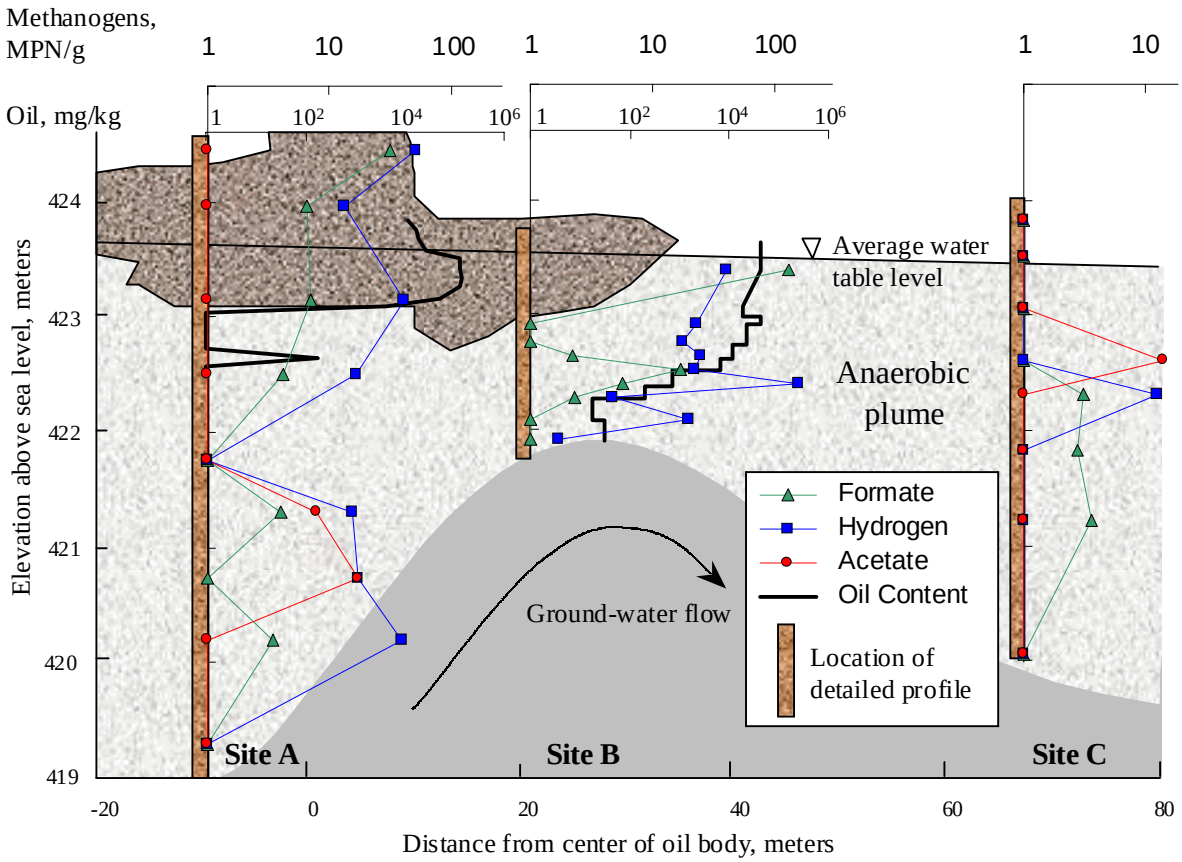


**Figure 2.** Formate-, hydrogen-, and acetate-utilizing microbial numbers [upper scale in graphs, shown in MPN/g (Most Probable Number per g)] from cores taken at sites A, B, and C, along with oil content [lower scale, shown in mg/kg (milligrams per kilogram of sediment)] at sites A and B. The dark brown area denotes greater than 10% oil saturation of pore space. Oil content for site A was determined by Dillard and others (1997). The light gray area corresponds to the anaerobic area of the plume (Bekins and others, 1999).

by volume. The bottles were sealed with solid butyl rubber stoppers and aluminum crimp seals. They were stored on their sides to reduce the possibility of gas leakage. Change of gas volume in the headspace was measured using a horizontal manometer.

Formate, hydrogen, or acetate were added to the bottles (table1). The acetate and formate microcosms contained, respectively, 110 mg/L (milligrams per liter) acetate or 330 mg/L formate in mineral salts. The headspace of the formate and acetate microcosms were flushed with oxygen-free nitrogen and carbon dioxide at a 80:20 ratio by volume. The hydrogen microcosms were flushed with 80% hydrogen and 20% carbon dioxide. At the start of the experiment, each microcosm was brought to equilibrium with atmospheric pressure using oxygen-free nitrogen.

Active microcosms were compared to controls to determine inhibition. Each active bottle containing crude oil was made in duplicate. Positive controls were made with acetate, hydrogen, and formate and no crude oil. Negative controls consisted of just the methanogenic consortium in mineral salts with the crude oil (in duplicate) as well as autoclaved bottles with acetate, hydrogen, and formate and the crude oil. The headspace was brought to atmospheric pressure with each measurement and any consumption of gas was replaced with nitrogen. All volume data were adjusted to standard temperature and pressure. Volumes from hydrogen microcosms were further normalized to the headspace volume to account for differing amounts of initial hydrogen mass.

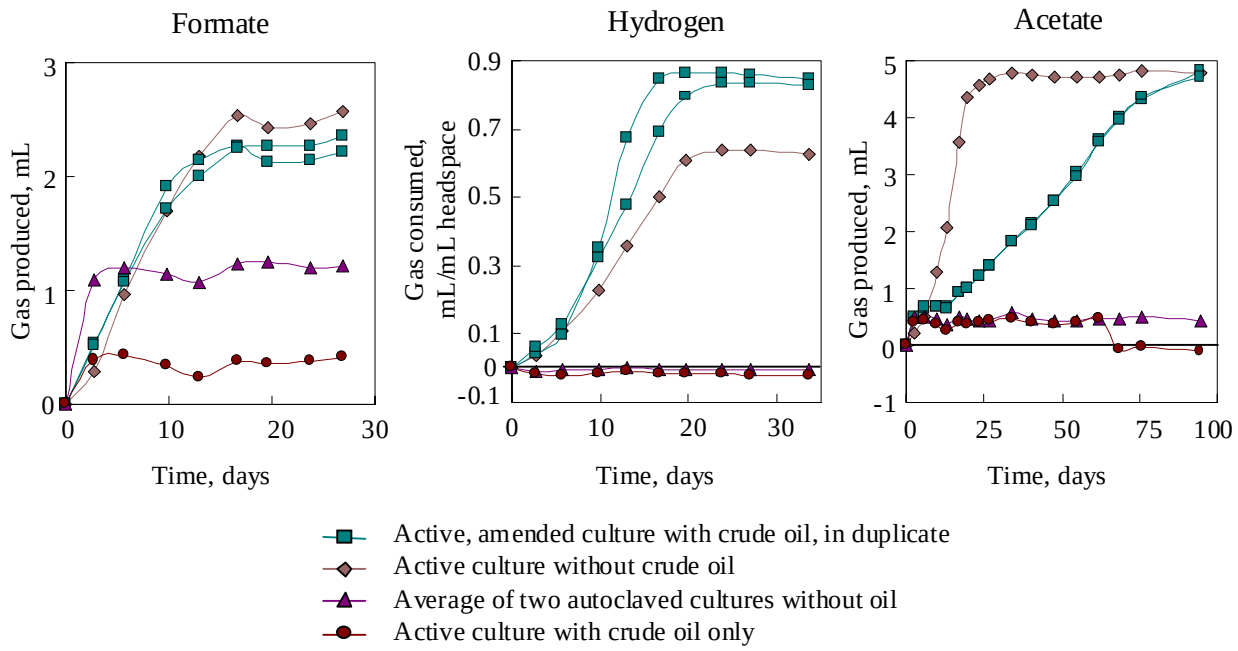


**Figure 3.** Gas production or consumption in microcosms fed with formate, hydrogen, or acetate.

## RESULTS

Most Probable Numbers from the site reveal that acetoclastic methanogens are not found near the oil body. In contrast, hydrogenophilic and formate-utilizers are found in similar numbers both within the oil body and outside it in the aqueous plume. The anaerobic toxicity assays show that the acetoclasts are inhibited significantly by the crude oil whereas the hydrogenophiles and formate-utilizers are not (figs. 2 and 3).

### Vertical Profiles of Methanogens

Vertical profiles of numbers of methanogens and oil concentrations through the plume of dissolved crude-oil compounds are shown in figure 2. The figure shows the microbial numbers of three subpopulations of methanogens at each location, along with oil content on sediments from sites A and B. Acetate-utilizing methanogens were found under the crude oil body at site A but only at a vertical distance of greater than 1 meter below the oil. At the center site, B, the anaerobic part of the plume is very narrow due to upwelling of oxygenated water below a low-permeability horizon. At this location there is apparently no niche sufficiently distant from the oil for the acetate-utilizers to occupy. At the down-gradient site where there is no non-aqueous oil, acetate utilizers are again present. Hydrogen- and formate-utilizers are found both in the vicinity of the oil and also downgradient. All MPNs were below 100/g sediment. At site A, there were relatively high numbers of hydrogenophiles and formate-utilizers starting in the unsaturated zone in the oil. They remained high until about 1 meter below the oil where they dropped to less than 1/g. They were found again over the next 1.5 m, but hydrogenophiles were found in greater abundance in this interval than formate-utilizers. At site B, there was a peak of formate-utilizers in the oil and another at 422.5 m near the center of the aqueous plume. The hydrogenophiles were relatively high throughout the core. The hydrogen- and formate-utilizers do not seem to be affect by the oil. Site C contained all three subpopulations of methanogens, but at very low numbers (note the scale on the site C plot).

### Anaerobic Toxicity Assays

The results from the toxicity experiment are shown in figure 3. Formate-utilizing methanogens produced 2.5 mL of gas after 26 days. The microcosms with crude oil closely paralleled the active control indicating no detectable toxicity

effect. After the first reading, gas in the killed control increased. Since the microcosm remained inactive for the duration of the experiment, the likely source is due to an experimental error, possibly incomplete equilibrium with atmospheric pressure at the start of the experiment.

When hydrogen is utilized by methanogens, the net effect is a reduction of the gas volume in the headspace. The rates of hydrogen utilization in the microcosms with crude oil were similar to the active control indicating no discernable toxicity effect. Surprisingly, the hydrogen-utilizing methanogens used about 20% more gas in the presence of crude oil than in its absence. This indicates a slightly greater level of activity in the presence of the crude oil. The difference may be due to an initial excess of hydrogen in the headspace. Because hydrogen and carbon dioxide were added in stoichiometric amounts (table 2), if the hydrogen:carbon dioxide ratio in the headspace is too low, carbon dioxide-limiting conditions will occur when the as the hydrogen is exhausted. The microcosms with crude oil may have produced enough carbon dioxide from the degradation of organics to eliminate this limitation. Further studies including tighter controls of the headspace gas are planned to verify the possibility of carbon dioxide limitation.

The behavior of the acetate microcosms contrasts sharply with that of the hydrogen and formate microcosms. Production of methane and carbon dioxide in the acetate microcosms was affected significantly by the crude oil. All active microcosms produced 4.8 mL of gas. However, while the microcosm without crude oil took 30 days, the microcosms with crude oil took 100 days to reach completion.

**Table 2.** Reactions and energy yields (kilojoules per mole) for acetate fermentation and carbon dioxide reduction using formate and hydrogen.

| |
|---|
| Acetate |
| $CH_3COOH \rightarrow CH_4 + CO_2$ |
| –28.3 kJ/mol acetate |
| Hydrogen |
| $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ |
| –32.7 kJ/mol hydrogen |
| Formate |
| $4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$ |
| –36.1 kJ/mol formate |

## DISCUSSION AND CONCLUSIONS

Serum-bottle toxicity assays using crude oil and enriched methanogenic cultures with formate, hydrogen, and acetate showed that formate- and hydrogen-utilization were unaffected by the crude oil whereas acetate utilization was significantly inhibited. This result is consistent with observed numbers of methanogens found in the aquifer. Microbial numbers at the site indicate that formate- and hydrogen-utilizing methanogens are present near the non-aqueous oil whereas acetoclastic methanogens are found only at distances greater than 1 meter from the oil.

Anaerobic-toxicity experiments using water-soluble components from creosote also showed that acetoclastic methanogens were more susceptible to inhibition than the others were. This observed inhibition may be due to the lower energy yield (table 2) from the degradation of acetate. The energy gained from acetate fermentation may be inadequate to compensate for the toxicity near the oil. Methanogens from the two carbon dioxide reduction pathways may get just enough energy to overcome the toxic effects of the oil (Bekins and others, 1997).

Because the inhibition of acetoclastic methanogenesis by the crude oil would affect the overall methanogenic degradation rates of the petroleum hydrocarbon contaminants, it is important to identify sources and extent of inhibition.

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