SHORT COMMUNICATIONS

Growth Inhibition of *Vibrio cholerae* by d-Camphor

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INTRODUCTION

Camphor is widely used as a preservative in pharmaceuticals and cosmetics, a weak anti-septic, an insect repellent and a counter irritant. Camphor has mutagenic effects on *Pasteurella pestis* (Won, 1950) and *Escherichia coli* (Ogg & Zelle, 1957; Zelle & Ogg, 1957; Ogg & Humphrey, 1963; Kvetkas, Krisch & Zelle, 1970), producing large cells. Inhibition by camphor of growth of *E. coli* was first reported by Ogg & Zelle (1957) and studied in some detail by Cardullo & Gilroy (1973).

Camphor is used in the treatment of serious diarrhoea and is also effective against cholera. However, no experimental analysis of its action on the organisms has been reported. We report the effect of camphor on *Vibrio cholerae*.

METHODS

Organism. *Vibrio cholerae* INABA569B, obtained from Dr N. K. Dutta, Haffkine Institute, Bombay, was used.

Test procedure. Bacteria were grown in 20 ml amounts of syncase medium (Finkelstein *et al.* 1966) in 100 ml Erlenmeyer flasks at 37 °C in a shaker bath (120 rev./min). Normal aerobic growth of *V. cholerae* in this medium is characterized by a 2 h lag period followed by a 3.5 h exponential-growth phase. d-Camphor (I.P., Aurora Pharmaceuticals, India) was used after sublimating it three times in the laboratory. Stock solution of camphor was prepared by adding 0.5 g d-camphor to 1 ml of 100% ethanol in a sterile test tube. Varying volumes of this stock solution were incorporated into the growth medium in the early exponential phase at 2.5 h, to give the desired concentrations of d-camphor as indicated. In each experiment, an ethanol control was included to determine its effect on growth of the vibrios. Growth was estimated by measurement of E_{680} in a photoelectric colorimeter. To ensure proportionality between extinction and bacterial concentrations, vibrio cultures were diluted with syncase medium to an extinction of less than 0.4. The extinction of the culture was then obtained by multiplying the measured extinction (after dilution) by the dilution factor. Viability was determined by counting the number of colony-forming units after overnight incubation at 37 °C of peptone agar plates on the surface of which had been spread 0.1 ml volumes of 10^5 to 10^7 times diluted bacterial cultures.

Protein and nucleic acid determinations were carried out on 10 ml samples of the cultures removed at suitable intervals and chilled rapidly. Bacteria were harvested by centrifugation (10000g), washed twice in 0.15 M-NaCl adjusted with 1 M-NaOH to pH 7.6, and then suspended in 3 ml of 0.5 M-perchloric acid and left for 30 min in an ice bath. The insoluble

Fig. 1. Effect of adding d-camphor at early-exponential phase on aerobic growth of *V. cholerae* in syncase medium, d-Camphor concentrations ($\mu\text{g/ml}$): ●, 0 (ethanol control); □, 700; ○, 950; △, 1200. Bacteria were grown at 37 °C in a shaker bath; d-camphor was added at 2.5 h and the growth was measured by (a) extinction at 680 nm, and (b) colony-forming units.

materials were collected by centrifugation and suspended in 2.5 ml of 0.5 M-perchloric acid at 80 °C for about 30 min. The acid-insoluble material was collected by centrifugation. DNA and RNA in the acid-soluble fraction was estimated colorimetrically, with diphenylamine (Burton, 1956) and orcinol (Aswell, 1957) respectively, using DNA (from calf-thymus gland; BDH) and RNA (from yeast; BDH) as the standards. Protein was estimated on the hot perchloric acid-insoluble material, digested overnight in 1 M-NaOH at 37 °C, by the method of Lowry *et al.* (1951) with crystalline serum albumin as the standard.

To study the release of intracellular materials after d-camphor treatment, 10 ml samples of the cultures were filtered through membrane filters (BAC-T-FLEX type B-6; Schleicher and Schull Co., Keene, New Hampshire, U.S.A.). The extinctions of these solutions were measured at 260 nm in a Beckman DU spectrophotometer with a blank of the medium. Protein in the filtrates was estimated by the method of Lowry *et al.* (1951) and carbohydrate by the method of Dubois *et al.* (1956) with glucose as the standard.

RESULTS AND DISCUSSION

Addition of d-camphor to growing *V. cholerae* cultures in the early-exponential phase significantly inhibited growth of the vibrios as measured by E_{680} ; higher concentrations of d-camphor inhibited more strongly (Fig. 1a). At the level of 1200 $\mu\text{g/ml}$, d-camphor completely inhibited growth of the vibrios. With 700 μg d-camphor/ml, the number of viable cells increased at a reduced rate for 2 h after camphor addition and was thereafter constant for the rest of the period of study (2.5 h); with 950 μg d-camphor/ml, the viable count did not increase throughout the period of study (4.5 h after addition of camphor) (Fig. 1b). The increase in extinction during this period at these d-camphor concentrations might be due to the increasing size of the d-camphor-treated cells, which was revealed by optical microscopy of untreated and camphor-treated cells (700 $\mu\text{g/ml}$). Similar results were obtained by Cardullo & Gilroy (1973) with *E. coli*. Treatment with 1200 μg d-camphor/ml resulted in slight decrease in the viable count of the vibrios (Fig. 1b).

Five hundred μg d-camphor/ml, added similarly, produced no significant effect on the growth of the vibrios; nor did the concentrations of ethanol used as solvent for the incorporation of d-camphor into the culture medium. No alteration of pH was observed when d-camphor was included in the culture medium at 1200 $\mu\text{g/ml}$.

With 950 μg d-camphor/ml, there was no further increase in the amounts of protein, RNA and DNA in *V. cholerae* cells, and this closely reflects the pattern of growth as revealed by viable cell numbers. At higher concentrations of d-camphor (1200 $\mu\text{g}/\text{ml}$), the amounts of nucleic acid and protein in vibrio samples taken 3 h after d-camphor addition were substantially less than the values at 2.5 h, indicating a release of the macromolecular constituents to the extracellular medium, either after their degradation into smaller units or otherwise. Cell-free filtrates of samples treated with 1200 μg d-camphor/ml contained about 17 % more protein and 20 % more carbohydrate than controls treated with ethanol. There was also considerable leakage of 260 nm-absorbing materials from the vibrios after d-camphor treatment (1200 $\mu\text{g}/\text{ml}$). Samples taken at intervals of 10 min did not enable us to determine which aspect of metabolism was being affected first. However, inclusion of d-camphor in the growth medium significantly inhibited vibrio growth, presumably by interfering with some basic metabolic function of the vibrios.

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