Effect of Dichlorodifluoromethane on the Appearance, Viability, and Integrity of Escherichia coli

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Cultures of Escherichia coli H52 were treated with liquid dichlorodifluoromethane (fluorocarbon-12 [f-12]) for 2 h at 22 C and then examined microscopically. Treated cells tended to clump, and their cytoplasmic membranes were generally less dense and less uniform in appearance than those of control cells. E. coli ML30 was exposed to f-12 at a concentration of 1.25 x 10**5 saturated vapor at 22 C. Cells were examined for changes in viability (plate count), permeability (as measured by exit of a-[14C]methylglucoside or uptake of o-nitrophenyl-β-D-galactopyranoside), release of compounds absorbing at 260 nm, and lysis (changes in absorbance at 420 nm). Large losses of α-methylglucoside and of percentage of viability occurred after brief exposure to f-12. Release of compounds absorbing at 260 nm occurred more slowly than the aforementioned events, possibly because these molecules are larger than α-methylglucoside. During 1,200 min exposure to f-12, the number of survivors decreased from 10**9 to 10**5 organisms/ml, the loss of compounds absorbing at 260 nm amounted to 50%, and 32% lysis occurred. Most of these changes occurred during the first 300 min of treatment. Loss of α-methylglucoside was almost complete after 1-min exposure to f-12. These results suggest that death of the cell involves several stages, with a change of permeability occurring first, followed by leakage of compounds of increasing size and, finally, lysis.

Dichlorodifluoromethane (fluorocarbon-12 [f-12]), a common refrigerator and aerosol propel- lant, recently has been used for immersion freezing of unpackaged food (E. O. Bahas, U.S. Patent 3,603,102, 1971). Fluorocarbon-12 is one of many fluorocarbons that will form clathrate hydrates under appropriate conditions of temperature and pressure (2). Although clathrate hydrates are not yet utilized commercially, several applications have been suggested (3).

Despite extensive use, the effect of f-12 on bacteria has received little attention. Reed and Dychale (18) studied the effect of a mixture of f-12 and 1,2-dichloro-1,2-tetrafluoroethane (f-114) at a combined concentration of 33% (vol/vol) on growth of five bacteria and molds. Each sample was shaken and then held quiescently at 37 C. The mixture had no effect on growth of Pseudomonas aeruginosa or Staphylococcus aureus during a 48-hr treatment. Growth of the microaerophilic Streptococcus agalactiae and the fungi Aspergillus niger and Pa- eciomyces varioti was inhibited by the mixture, but the role of the fluorocarbons was not conclusively established.

Prior et al. (17) selected 20 bacteria, yeasts, and molds important to the food industry and studied their survival in the presence of f-12 for 48 h at 21 ± 3 C. Vapor-state f-12 caused either inactivation of the organisms or inhibition of growth. Those organisms most resistant to vapor-state f-12 were then tested against f-12 in the liquid state. Organisms selected for study were Escherichia coli, P. aeruginosa, Pseudo- monas fluorescens, Leuconostoc dextranicum, Saccharomyces cerevisiae, Streptococcus lactis, Salmonella typhimurium, S. aureus, and Bacil- lus cereus. The chemicals were far more toxic in the liquid state than in the vapor state, and in many instances no viable organisms were detected after 48 h of incubation. Fluorocarbon-12 was far less effective against spores of B. cereus than against vegetative microorganisms.

Strettton et al. (21) examined the survival of E. coli in the presence of a vapor mixture (60:40) of trichlorofluoromethane and f-12 at 25 C and observed a rate of inactivation that was much slower than that observed by Prior et al. (17). Strettton et al. (21) observed that

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these chemicals were slowly lethal to E. coli, resulting in 1.0% survivors after a 3-week treatment. Spores of B. subtilis var. niger were more resistant to the fluorocarbons than were vegetative cells of E. coli.

Thus, it is abundantly clear that fluorocarbons, under appropriate conditions, can inactivate vegetative microorganisms at significant rates. The mechanism by which fluorocarbon compounds damage microorganisms is unknown, but the lipophilic nature of these compounds led Prior et al. (17) and Stretton et al. (21) to speculate that cell lipids were in some way affected deleteriously.

The purpose of this study was to investigate the effect of f-12 on the appearance, viability, and integrity of cells of E. coli. A possibility exists that f-12 might be used to reduce the numbers of vegetative microorganisms on the surfaces of food tissues, and the aforementioned information will help determine the feasibility of this approach.

MATERIALS AND METHODS

Organisms and media. Two strains of E. coli, H52 and ML30, were used, and these cultures were supplied, respectively, by E. Marth and J. Garver of the University of Wisconsin. The studies involving microscopy and age of culture were done with E. coli H52, and all other studies were done with E. coli ML30. The change in strain is regarded of relatively minor importance since it has been shown previously that f-12 has a similar effect on several genera and species of vegetative bacteria (17). Furthermore, both strains were derived from cultures grown under conventional conditions.

Plate count agar was used to enumerate cells.

Sample containers. Aerosol cans (5.1 cm in diameter by 5.4 cm in height; 97-ml capacity) with double epoxy linings and stainless-steel caps (without dip tubes) served as sample containers.

Chemical. Dichlorodifluoromethane (CCl2F2, f-12, food grade; E. I. du Pont de Nemours and Co., Wilmington, Del.) was used in this study. This chemical was chosen because: (i) it is relatively nontoxic to humans and might therefore receive approval for food uses in addition to presently approved food-freezing applications, and (ii) it is sparingly soluble in water and moderately volatile, thereby facilitating its recovery and reuse.

Means of expressing concentration. If a toxic substance is present at equilibrium concentrations in all phases of a system, then the chemical potential of this substance will be equal in all phases. According to Ferguson (4), the chemical potential of any toxic substance that functions by a physical mechanism is closely related to its toxic potency. Since values for chemical potential are not easily obtained, Ferguson suggested that a useful approximation in the case of solutions is the term C/C*, where C is the weight of toxic substance dissolved in the liquid phase and C* is its solubility in the liquid phase at the same temperature and pressure. Since the concentration of f-12 used in this study exceeded saturation, Ferguson’s definition was modified slightly to accommodate this condition. Thus, C is defined as the weight of f-12 present in the liquid phase and C* is the solubility of f-12 in the liquid phase at the same temperature and pressure.

Based on literature values and on calculations from the equation for ideal gases it was determined that 2.83 g of f-12 was necessary to saturate (C/C* = 1.0) a closed vessel containing 10 ml of water and 87 ml of vapor space at 22 C. This value was confirmed by experiment.

For all studies except those involving microscopy and culture age, f-12 was used at a concentration of 1.25 × saturation (C/C* = 1.25). A 3.54-g weight of f-12 corresponded to 1.25 × saturation when 10 ml of culture medium was present in a 97-ml aerosol can at 22 C. Samples for microscopy and culture age were exposed to f-12 at a concentration of about 5.4 × saturation. This difference is probably of relatively little importance since, once saturation conditions are obtained, additional increases in concentration have a relatively small effect on microorganisms (17).

High pressure buret. f-12 was dispensed into aerosol cans with a buret described by Prior et al. (17).

General procedures. f-12 was added (superimposed on the normal atmosphere existing in the headspace of the can) to capped aerosol cans containing 10 ml of the cell suspension. The precision of adding f-12 was ±8% (95% confidence limits). Samples were prepared in triplicate and incubated at 22 ± 2 C while being agitated continuously on a platform shaker. The number of cells was determined by plate count before and after incubation.

Procedure for examining E. coli H52 by microscopy. Cells of E. coli H52 were grown quiescently in AC broth (Difco) for 5 h at 37 C (late logarithmic phase). This culture was then diluted with sterile AC broth to a cell density of about 4 × 10⁴ organisms/ml, as determined by absorbance.

For studies involving light microscopy, the culture was agitated in the presence of 15 g of f-12 for 2 h at 22 C and then the treated and control cells were examined with a Zeiss phase-contrast microscope.

For studies involving electron microscopy, 10 ml of the culture was placed in an aerosol can, 15 g of liquid f-12 was added, and the mixture was agitated for 5 h at 22 C. The untreated 5-h culture was used immediately as a control (no agitation or additional holding). Control and treated samples were harvested by centrifugation at 30,000 × g for 30 min and then fixed, embedded, sectioned, and stained by conventional means.

Procedure for studying the effects of f-12 on E. coli ML30. Cells of E. coli ML30 were suspended in a glucose (0.5%)-mineral salts-trace salts medium as described by Kruwich et al. (10). This medium was used to enable easy deletion of certain constituents, when desired, and to enable assessment of survival without interference from growth. Cultures were agitated at 37 C during growth and were used after
attainment of the late logarithmic phase (about 5 h; 0.7 absorbancy units at 420 nm). The cells of *E. coli* ML30 were harvested by low-speed centrifugation, washed once with cold, sterile distilled water, and finally resuspended at the desired concentration in a medium containing only mineral salts. Plate count agar (Difco) was used to enumerate the cells.

**Preparation of an *E. coli* lysate.** Cells were lysed according to the method described by Matttingly and Best (13), except that sonication was conducted for 15 min.

**Measurement of lysis.** Lysis was measured by the change in absorbance at 420 nm, following brief exposure of the sample to a low pressure to remove bubbles of f-12. Percentage of lysis was determined from the following equation: percentage of lysis = \( A - B/A - C \times 100 \), where \( A \) is absorbance of the initial untreated sample, \( B \) is absorbance of the treated or control suspension after an appropriate time lapse, and \( C \) is absorbance of a sonicated cell suspension prepared as described in the previous section.

**Measuring release of compounds absorbing at 260 nm.** Release of compounds absorbing at 260 nm was measured by centrifuging control and treated samples at 12,000 × g for 15 min, carefully removing the supernatant fluid, and measuring absorbance of this fluid at 260 nm. The amount of compounds released was expressed as a percentage of the total amount of 260-nm-absorbing compounds present in the supernatant fluid obtained from a sonically lysed, centrifuged preparation of the initial control sample.

**Measuring uptake of ONPG.** o-Nitrophenyl-β-D-galactopyranoside (ONPG) is an analogue of lactose, and the enzyme (β-galactosidase) that catalyzes hydrolysis of lactose also catalyzes hydrolysis of ONPG to yield a colored product, o-nitrophenol, that can be measured colorimetrically. Since β-galactosidase is present in *E. coli* grown under appropriate conditions, increased formation of o-nitrophenol can be used as a measure of ONPG uptake, provided β-galactosidase remains in the cell. It was determined (results not shown) that β-galactosidase was released from *E. coli* to only a minor degree under circumstances identical to those used in this study.

Lactose was substituted for glucose in the glucose-mineral salts medium to induce synthesis of β-galactosidase in the cell. Otherwise, the culture was prepared and treated with f-12 as previously described, and the test procedure for o-nitrophenol was conducted as described by Lederberg (11).

**Measuring release of MG.** Permeability changes in *E. coli* can be easily measured by following the release of α-[14C]methylglucoside (MG). Cells of *E. coli* readily take up MG (an analogue of glucose) but are unable to metabolize it. Small amounts of MG may be phosphorylated (5), but most MG is readily released when significant changes in permeability occur.

To incorporate MG, cells were washed once as described previously and then suspended in 15 ml of sterile mineral salts medium (7 to 10 °C) containing 60 μmol of MG (Amersham/Searle; specific activity, 19 μCi/mmol) and 40 μg of chloramphenicol per ml. The suspension was stirred continuously for 1 h at 22 °C to allow for incorporation of MG (33% of the labeled compound was taken up by the cells), and then it was centrifuged. The pellet thus obtained was suspended in the mineral salts medium (7 to 10 °C), and the suspension was adjusted to the desired cellular concentration. Experiments were done with this final suspension.

Aliquots of the suspension at 22 °C were placed in aerosol cans and treated with f-12 for the desired time. The f-12 was released, and cells were separated by filtration and then washed twice with cold (7 to 10 °C) mineral salts medium. Filters and cells were dried with high-intensity lamps and then placed in vials containing a scintillation fluid consisting of 0.3% 2,5-diphenyloxazole (Sigma) and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (Sigma) in toluene. Radioactivity in each vial was counted for 20 min with a scintillation spectrometer (Packard Tri-Carb series 3000; Packard Instruments Co., Downers Grove, Ill.). The counting efficiency was 72.6% in the green scaler channel using a 7% gain and window setting of 50-1,000.

The radioactive count (counts per minute) of the MG remaining in the sample was expressed as a percentage of the count of the initial control sample, and this value was used to indicate changes in permeability.

**RESULTS AND DISCUSSION**

**Age of *E. coli* H52 and resistance to f-12.** The purpose of this study was to determine the resistance of *E. coli* H52, harvested during various phases of growth, to inactivation by f-12. Cultures were harvested after 5 (late logarithmic phase), 14 (stationary phase), and 72 h (late stationary phase) of growth at 37 °C, the absorbance (550 nm) was adjusted to 0.8, and 20-ml quantities were agitated (120 oscillations/min) in the presence of f-12 (in excess of saturation) for various times up to 24 h at 22 °C. Control samples were treated similarly, except f-12 was absent. Results in Fig. 1 show that f-12 inactivated young cells more rapidly than old cells. However, it is possible that prolonged incubation in the presence of f-12 (beyond 24 h) would have resulted in essentially the same degree of inactivation in all instances.

For all subsequent experiments, only cells from the late logarithmic phase of growth (least resistant) were used. This was done in the belief that use of these cells would facilitate detection of small differences in treatment. The late logarithmic phase was identified on the basis of absorbance measurements (0.7 absorbance), and usually corresponded to 5 h of growth at 37 °C.

**Effect of f-12 on the microscopic (phase-contrast) appearance of *E. coli* H52.** Control
cells (Fig. 2A) were evenly dispersed throughout the field, and nearly every cell possessed a uniformly dark cytoplasm. Cells treated with f-12 (Fig. 2B) were extensively clumped and the cytoplasms of clumped cells were generally light and grainy, suggesting substantial damage and perhaps lysis. The cause of clumping is unknown, but it is possible that released cytoplasmic substances contributed to this change. Most treated unclumped cells possessed dark cytoplasms, characteristic of normal cells. Rings of tiny droplets surrounded some cells and their nature is unknown.

Other workers have treated microorganisms with organic chemicals and have observed the consequences by means of phase-contrast microscopy. Middleton (14) treated cells of S. cerevisiae with dichlorofluoromethane (f-21), and no lysis was observed by phase-contrast microscopy. Resistance to lysis probably occurred because f-21 was present at a concentration less than saturation. Jackson and De Moss (8) used phase-contrast microscopy to examine cells of E. coli after exposure to 5% (vol/vol) toluene, and they failed to observe any change in morphology.

**Effect of f-12 on the microscopic appearance (electron microscope) of E. coli H52.** The purpose of these studies was to observe in detail any morphological effects of f-12 on cells of E. coli. The hope was that electron microscopy would show subtle changes in cell morphology that preceded lysis.

Some differences were observed between treated and control cells, but the results were not sufficiently important to warrant presentation of photomicrographs. Generally the treated cells had cytoplasms that were less dense and less uniform than those of control cells. The abnormal appearance of the treated cells may have resulted from lysis, since broken cell walls were clearly evident in some instances.

Since electron microscopy failed to clarify events preceding lysis, other techniques were sought for this purpose.

**Effect of f-12 on the permeability of E. coli ML30: uptake of ONPG.** Data in Fig. 3 indicate that ONPG is taken up more rapidly by E. coli treated with f-12 than by control cells, suggesting that f-12 causes a substantial increase in the permeability of E. coli.

**Effect of f-12 on the permeability of E. coli ML30: release of MG.** Cells of E. coli containing MG were treated with f-12 as described previously. After an exposure of only 1 min, about 95% of the MG had leaked from the cells (Fig. 4). Leakage of MG from control cells (no f-12) occurred to only a slight extent during a 60-min period.

Results of the two preceding experiments clearly indicate that the permeability of E. coli was increased greatly by exposure to f-12. In subsequent experiments, changes in permeability were measured only by leakage of MG. Reasons for selecting this test over the ONPG test were: (i) the MG test is more precise, (ii) the MG test appeared to be more sensitive to changes in permeability, and (iii) a subsequent experiment (data not shown) indicated that β-galactosidase is partially inhibited by f-12, thus bringing into question the accuracy of the ONPG test.

Results similar to those described above have been observed by other investigators using toluene. Toluene is commonly used to increase the permeability of cells for biochemical studies (7). Jackson and De Moss (8) allowed cells of E. coli to incorporate [14C]thiomethylgalactoside, exposed them to toluene at a concentration of 5% (vol/vol), and observed nearly complete loss of [14C]thiomethylgalactoside within 1 min. At a lower concentration of 0.15% (vol/vol) toluene, all the [14C]thiomethylgalactoside was lost after a 5-min exposure period. They suggested that toluene destroyed the galactose permease system.

Bacteria also undergo increases in cell permeability when exposed to butanol (16), ethanol (9), or phenethyl alcohol (20).

**Studies on the viability and integrity of E. coli treated with f-12.** The purpose of these studies was to compare four tests for assessing the consequences of f-12 on viability and integrity of E. coli. Cells of E. coli were treated with f-12 as described previously, and the changes in viability and integrity were measured by the
Fig. 2. Effect of f-12 on the microscopic (phase-contrast) appearance of cells of E. coli H52. (A) Control. (B) Treated (f-12 present in excess of saturation; agitation for 2 h at 120 oscillations/min; temperature, 22 °C; culture age, 5 h at 37 °C; magnification, 1,472×; bar length, 10 μm).

Amount of MG and ultraviolet (UV)-absorbing materials released, the degree of lysis, and by plate counts. Figure 5A shows that the number of survivors in the treated samples decreased from 10⁸ to 10⁴ organisms/ml during the first 200 min and then decreased another 10-fold during the remaining 1,000 min. The number of survivors in the control samples decreased to only 5
Fig. 3. Uptake of ONPG by E. coli ML30 treated with f-12 (f-12 concentration, 1.25x; temperature, 22°C; agitation, 120 oscillations/min; culture age, 5 h at 37°C). Each symbol indicates the range of three observations.

Fig. 4. Rate of release of MG from E. coli ML30 treated with f-12 (f-12 concentration, 1.25x; temperature, 22°C; agitation, 120 oscillations/min; culture age, 5 h at 37°C). Each symbol indicates the range of three observations.

× 10^6 organisms/ml after 1,200 min. During treatment for 1,200 min, cells also incurred a 50% loss of UV compounds and 32% lysis, with most of these changes occurring during the first 300 min. Controls after 1,200 min exhibited 12.7% loss of UV compounds and 19.2% lysis.

Data in Fig. 5B indicate changes in E. coli during the first 60 min of exposure to f-12. Decreases in viability and losses of MG both occurred rapidly and yielded curves that were quite similar, suggesting that there might be a relationship between the two events. Release of UV compounds occurred more slowly than the changes in viability and the loss of MG. The probable reason is that the UV materials are considerably larger molecules than MG, thus necessitating a greater change in permeability before they can be released. Alternatively, partial degradation of the UV compounds might be necessary before their release. As would be expected, lysis occurred more slowly than any of the other changes. As stated in the legend to Fig. 5, changes in control samples were small after 60 min.

Many investigators have considered the relationship between loss of viability and leakage of cellular material. A direct relationship has been reported between loss of viability and rate of leakage of UV materials from E. coli during freeze stress (12) and heat stress (19). On the other hand, work by Moss and Speck (15) and Allwood and Russell (1) suggest that such relationships exist but are coincidental. Furthermore, Hugo (6) showed that leakage of cellular material resulting from treatment with toxic chemicals does not necessarily indicate a fatal process, since this type of damage is sometimes repairable.
The results reported here indicate that f-12 can cause marked changes in the permeability of cells of *E. coli* and that death is often associated with these changes. These results appear to contradict several earlier studies involving chemicals similar to f-12. However, the apparent discrepancies generally disappear when the concentration of chemical used is taken into account. In this study a chemical concentration in excess of saturation was used, whereas studies that have yielded less dramatic results generally have involved chemical concentrations well below saturation.

Results of this study suggest that treatment of food with liquid f-12 may hold some promise as a means of preservation. If surface microorganisms behave like *E. coli* in this study, it should be possible to markedly reduce the population of microorganisms on the surfaces of foods, and thereby extend their keeping qualities. Since f-12 is sparingly soluble in water and reasonably volatile, it could be easily removed after the treatment so as to avoid toxic effects when the food is eventually consumed and to enable reuse. It is not known how f-12 would affect the sensory properties of the food, so this aspect needs investigation.

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