Effect of Gas Hydrate Formers on Microorganisms

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Various bacteria, yeasts, and molds important to the food industry were incubated in aerosol cans containing A.C. Broth and one of the following three gas hydrate formers: propane, dichlorodifluoromethane (f-12), and 1,1-difluoro-1-chloroethane (f-142b). Most hydrate formers were tested at three concentrations: low (vapor state), intermediate (liquid state, low level), and high (liquid state, high level). Samples were continuously agitated for 48 hr at 21 ± 3°C. Changes in numbers of microorganisms were determined by plate count. With hydrate formers in the vapor state, propane was more toxic to the microorganisms tested than either f-12 or f-142b. The most resistant organisms from these trials were then tested against f-12 or f-142b in the liquid state. Hydrate formers were far more toxic in the liquid state than in the vapor state. With the exception of sporulated cultures of Bacillus cereus, all microorganisms tested were greatly reduced in numbers when agitated for 48 hr at 21°C in the presence of f-12 or f-142b.

Gas hydrates (aqueous clathrates) are icelike compounds consisting of specific kinds of molecules (hydrate formers) physically entrapped in a hydrogen-bonded matrix of water molecules. Some are stable above 0°C, provided the pressure is adequate (3). Since Davy (2) discovered gas hydrates, their physical and chemical properties have been investigated extensively (4). Some applications for gas hydrates have been suggested, but none is currently utilized commercially (1, 3; D. N. Glew, U.S. Patent 3,085,832, Oct. 16, 1962).

The effects of gas hydrates and gas hydrate formers on microorganisms are virtually unknown. Lie (M.S. Thesis, Univ. of Wisconsin, Madison, 1966) reported that trichloromonofluoromethane (f-11), a gas hydrate former, had a toxic effect on Pseudomonas striata, and she attributed this effect to the strong lipophilic characteristics of the chemical. Reed and Dychdala (5) investigated briefly the effects of a mixture of dichlorodifluoromethane (f-12) and 1,2-dichloro-1,2-tetrafluoroethane (f-114), the first being a hydrate former, on several microorganisms. They found that these chemicals had no adverse effect on growth of several aerobic bacteria, but that Streptococcus agalactiae (a microaerophile), Aspergillus niger, and Pseudomonas variotii failed to grow when these chemicals were present.

The purpose of this study was to determine the effect of several hydrate formers on the growth of microorganisms. The results will provide a necessary background for future studies involving the effect of gas hydrate crystals on microorganisms and, in addition, will help determine if these chemicals have any potential value as a means of controlling the microbial population in foods.

MATERIALS AND METHODS

Organisms and media. The bacteria, yeasts, and molds chosen for use in this study represent a broad cross-section of those common in foods. All organisms except the molds and bacterial sporeformers were prepared by growing them in sterile AC Broth (Difco) at their respective optimum temperatures. Mold spores were prepared by growing molds at 30°C on the surface of Potato Dextrose Agar (Difco) contained in mass culture bottles. Spores were harvested with sterile distilled water and held at 4°C until needed. Spores of Bacillus cereus were prepared by incubating the organism at 30°C on nutrient agar containing 10 µg of MnSO4 per ml for a time sufficient to yield 95% sporulation as indicated by the malachite green spore stain. These spores were harvested in cold, sterile distilled water and held at 4°C until needed. Spores of B. polymyxa were prepared similarly, except that Tryptic Soy Agar (Difco) served as the substrate.

Sterile AC Broth (Difco) was employed as the culture medium for all organisms during incubation in aerosol cans. Plate Count Agar (Difco) was used to enumerate all organisms except lactic acid bacteria and yeasts and molds which were enumerated with APT agar (Difco) and Potato Dextrose Agar (Difco), respectively.

Sample containers. Aerosol cans (200 × 214, 143-ml capacity) with a double epoxy lining and stainless-steel caps without dip tubes were used as sample containers.
# Table 1. Changes in numbers of microorganisms at 21°C as influenced by propane, dichlorodifluoromethane (F-12), and 1,1-difluoro-1-chloroethane (F-142b)\(^n\)

| Microorganism                  | Controls (no./ml) | Propane (1.0 ± 0.2 g)\(^b\) | F-12 (2.8 ± 0.3 g)\(^b\) | F-142b (1.0 ± 0.1 g)\(^b\) |
|--------------------------------|------------------|-----------------------------|--------------------------|---------------------------|
|                                | Initial (Ci)     | 48-Hr (Ca)                  | No./Ci\(^e\)             | No./Ca\(^d\)             | No./Ci\(^e\)             | No./Ca\(^d\)             | No./Ci\(^e\)             | No./Ca\(^d\)             |
| Staphylococcus aureus          | 2.7 \times 10^7  | 7.0 \times 10^4             | 0.98                     | 0.06                      | 2                         | 7.7                      | 4.1                       | 16                        |
|                                | 9.8 \times 10^6  | 1.6 \times 10^4             |                          |                          |                           |                          |                          |                           |
| Micrococcus conglomeratus      | 1.3 \times 10^4  | 4.3 \times 10^8             | 2.9 \times 10^{-2}       | 4.9 \times 10^{-4}       | 0.88                      | 1.5 \times 10^{-2}       | 1.1                       | 1.8 \times 10^{-2}       |
|                                | 3.8 \times 10^6  | 1.2 \times 10^4             |                          |                          | 33                        | 0.04                     | 110                       | 0.13                      |
| Streptococcus cremoris         | 1.7 \times 10^7  | 1.0 \times 10^9             | 0.35                     | 1.8 \times 10^{-4}       | 8.4 \times 10^{-4}        | 1.4 \times 10^{-2}       | 0.61 \times 10^{-2}       | 1.0 \times 10^{-2}       |
| S. lactis                      | 4.6 \times 10^6  | 3.8 \times 10^8             |                          |                           | 3.7 \times 10^{-2}        | 2.4                      | 7.8                       | 0.52                      |
|                                | 7.8 \times 10^5  | 1.5 \times 10^9             |                           |                           | 1.1 \times 10^{-2}        | 0.62                     | 0.23                      | 1.6                       |
| Leuconostoc citrovorum         | 6.4 \times 10^7  | 3.9 \times 10^8             | 2.2                      | 0.36                      | 9.1 \times 10^{-2}        | 0.62                     | 0.23                      | 1.6                       |
| L. dextranicum                 | 4.1 \times 10^6  | 6.2 \times 10^7             | 0.19                     | 2.2 \times 10^{-4}       | 0.56                      | 0.43 \times 10^{-1}      | 0.65                      | 0.50 \times 10^{-1}      |
| Bacillus cereus (spores)       | 6.8 \times 10^6  | 1.0 \times 10^4             | 0.22                     | 1.5                       | 1.7 \times 10^{-4}        | 2.2 \times 10^{-4}       | 0.27                      | 3.5 \times 10^{-2}       |
|                                | 5.8 \times 10^7  | 2.1 \times 10^4             | 2.2 \times 10^{-3}       | 0.62                      | 7.6 \times 10^{-2}        | 4.9 \times 10^{-4}       | 0.15                      | 1.0 \times 10^{-4}       |
| B. polymyxa (spores)           | 3.4 \times 10^6  | 4.4 \times 10^7             | 0.35                     | 0.27 \times 10^{-1}      |                           |                         |                           |                           |
| Escherichia coli               | 4.3 \times 10^6  | 4.4 \times 10^9             | 1.3 \times 10^{-3}       | 1.3 \times 10^{-4}       |                           |                         |                           |                           |
|                                | 1.3 \times 10^4  | 9.9 \times 10^7             |                           |                           | 1.7 \times 10^{-4}        | 2.2 \times 10^{-4}       | 0.27                      | 3.5 \times 10^{-3}       |
| E. intermedia                  | 8.4 \times 10^6  | 1.3 \times 10^8             | 3.8 \times 10^{-4}       | 2.5 \times 10^{-7}       | 7.6 \times 10^{-2}        | 4.9 \times 10^{-4}       | 0.15                      | 1.0 \times 10^{-4}       |
| Salmonella typhimurium          | 9.1 \times 10^7  | 1.6 \times 10^9             | 0.68                     | 3.9 \times 10^{-2}       | 7.6                       | 0.43                     | 30.8                      | 1.8                       |
| Pseudomonas aeruginosa         | 3.1 \times 10^6  | 1.5 \times 10^9             | 5.2 \times 10^{-3}       | 1.1 \times 10^{-4}       | 3.5 \times 10^{-2}        | 0.73                     | 5.2 \times 10^{-3}       | 1.1                       |
| P. fluorescens                 | 3.0 \times 10^6  | 2.4 \times 10^9             | 3.3 \times 10^{-4}       | 4.2 \times 10^{-7}       | 27                        | 3.4 \times 10^{-2}       | 73                        | 9.2 \times 10^{-2}       |
Hydrate formers. Propane (CH₂CH₂CH₃; Matheson Co., C.P. grade, minimum purity 99%), dichlorodifuoromethane (CCl₂F₂, fluorocarbon-12, Matheson Co., minimum purity 99.5%), and 1,1-difluoro-1-chloroethane (CH₂CClF₂, fluorocarbon-142b, Allied Chemical Corp., minimum purity 98%) were the hydrate formers selected for study. Each hydrate former was initially tested in the vapor state (vapor space was saturated at 21°C, but the amount was insufficient to cause condensation). Dichlorodifluoromethane (F-12) and 1,1-difluoro-1-chloroethane (F-142b), in a partially liquefied state, were further tested at two levels: (i) sufficient hydrate former was present at 21°C to saturate the vapor space and to provide a few drops of liquid condensate in the culture medium, and (ii) sufficient hydrate former was present at 21°C to saturate the vapor space and to provide liquid in an amount theoretically adequate to bind all water present if type II gas hydrate crystals were formed.

High pressure buret. The buret used for dispensing liquid or vapor-state hydrate formers into aerosol cans was similar to those available commercially, except that a membrane filter (0.22 μm pore size; Millipore Corp., Bedford, Mass.) was inserted between the buret and the aerosol cans.

Procedures. Hydrate formers were added aseptically (superimposed on the normal atmosphere existing in the head spaces of the cans) to capped aerosol cans containing 20 ml of sterile broth and a 24-hr culture of the test microorganism (number of organisms referred to as "initial or C₄"). An attempt was made to obtain the following levels of hydrate formers: propane, 2.24 g; F-12, 3.88 g, 4.25 g, and 11.37 g; F-142b, 1.68 g, 1.99 g, and 7.92 g. Controls devoid of hydrate formers were included with each treatment. The amounts of the hydrate formers actually added were determined by weighing each can before and after filling. Unintentional variations in the amounts used are shown in Tables 1, 2, and 3. Test samples were prepared in triplicate and control samples in duplicate. Avoidance of contamination during filling and sealing of the aerosol cans was verified by uninoculated controls. Samples were incubated for either 1 or 48 hr at 21 ± 3°C while being agitated continuously on a platform shaker. The numbers of bacteria and yeast were determined by plate count both prior to and after incubation.

RESULTS AND DISCUSSION

Microbial growth as influenced by hydrate formers in the vapor state. This portion of the study was designed to determine the effect of propane, F-12, and F-142b vapors on the test microorganisms. From the results as presented in Table 1, it is evident that the organisms were generally affected adversely (reduction in numbers or inhibition of growth) by all three hydrate formers when present in the vapor state. Vapors of propane were the most detrimental of the three hydrate formers. Most organisms were affected similarly by F-12 and F-142b.

| P. fragi | Achromobacter hauseri | Flavobacterium psychrophilum | Saccharomyces cerevisiae |
|---|---|---|---|
| 6.3 × 10⁻⁴ | 5.1 × 10⁻⁴ | 5.6 × 10⁻⁴ | 6.3 × 10⁻⁴ |
| 9.0 × 10⁻⁴ | 9.0 × 10⁻⁴ | 1.0 × 10⁻⁴ | 9.0 × 10⁻⁴ |
| 6.3 × 10⁻⁴ | 6.3 × 10⁻⁴ | 9.0 × 10⁻⁴ | 9.0 × 10⁻⁴ |
| 1.0 × 10⁻³ | 1.0 × 10⁻³ | 1.0 × 10⁻³ | 1.0 × 10⁻³ |
| 1.3 × 10⁻³ | 1.3 × 10⁻³ | 1.3 × 10⁻³ | 1.3 × 10⁻³ |
| 2.1 × 10⁻³ | 2.1 × 10⁻³ | 2.1 × 10⁻³ | 2.1 × 10⁻³ |
| 4.3 | 4.3 | 4.3 | 4.3 |
| 2.7 × 10⁻² | 2.7 × 10⁻² | 2.7 × 10⁻² | 2.7 × 10⁻² |
| 6.6 | 6.6 | 6.6 | 6.6 |
| 1.2 | 1.2 | 1.2 | 1.2 |
| 0.57 | 0.57 | 0.57 | 0.57 |

Initial control values are means derived from duplicate plates prepared from one sample. The 48-hr control values are means derived from duplicate plates prepared from three samples. No viable organisms were detected.

*Values less than 10 indicate a reduction in viable microorganisms caused by the treatment, whereas values in excess of 1.0 indicate that some growth occurred. Values greater than 1.0 indicate either a reduction in viable numbers (determined from values in column headed by no. [C₄]) or growth which is less than in the incubated control, whereas values in excess of 1.0 indicate that growth in the treated sample exceeded that of the incubated control.
Table 2. Changes in numbers of microorganisms at 21°C as influenced by dichlorodifluoromethane (f-12)<sup>a</sup>

| Microorganism                  | Controls (no./ml) | Fractional change in numbers after 48 hr |     |       |
|--------------------------------|------------------|-----------------------------------------|-----|-------|
|                               | Initial (C<sub>i</sub>) | 48-Hr (C<sub>a</sub>) | No. / C<sub>i</sub><sup>d</sup> | No. / C<sub>a</sub><sup>d</sup> | No. / C<sub>i</sub><sup>d</sup> | No. / C<sub>a</sub><sup>d</sup> |
| **Escherichia coli**           | 5.0 × 10<sup>7</sup>  | 1.7 × 10<sup>8</sup>  | 2.7 × 10<sup>-3</sup> | 1.3 × 10<sup>-4</sup> | 0.3 × 10<sup>-4</sup> | 8.8 × 10<sup>-6</sup> |
| **Pseudomonas fluorescens**    | 4.8 × 10<sup>8</sup>  | 9.9 × 10<sup>7</sup>  | 5.7 × 10<sup>-4</sup> | 4.4 × 10<sup>-7</sup> | NO            | NO            |
| **P. aeruginosa**              | 1.8 × 10<sup>9</sup>  | 9.7 × 10<sup>9</sup>  | 1.6 × 10<sup>-4</sup> | 3.5 × 10<sup>-8</sup> | 2.0 × 10<sup>-7</sup> | 3.8 × 10<sup>-9</sup> |
| **Leuconostoc dextranicum**    | 6.8 × 10<sup>7</sup>  | 1.1 × 10<sup>10</sup> | NO            | NO            | NO            | NO            |
| **Saccharomyces cerevisiae**   | 8.4 × 10<sup>6</sup>  | 1.1 × 10<sup>9</sup>  | NO            | NO            | NO            | NO            |
| **Streptococcus lactis**       | 3.8 × 10<sup>5</sup>  | 9.0 × 10<sup>7</sup>  | NO            | NO            | N            | NO            |
| **Salmonella typhimurium**     | 1.3 × 10<sup>7</sup>  | 2.6 × 10<sup>4</sup>  | NO            | NO            | N            | NO            |
| **Staphylococcus aureus**      | 9.0 × 10<sup>7</sup>  | 1.2 × 10<sup>9</sup>  | 1.6 × 10<sup>-6</sup> | 1.2 × 10<sup>-6</sup> | NO            | NO            |
| **Bacillus cereus**            | 2.5 × 10<sup>6</sup>  | 3.4 × 10<sup>4</sup>  | 1.9 × 10<sup>-4</sup> | 1.4 × 10<sup>-4</sup> | NO            | NO            |
| (spores)                       | 1.4 × 10<sup>7</sup>  | 1.4 × 10<sup>3</sup>  | 3.6 × 10<sup>-3</sup> | 36            | 10            |               |

<sup>a</sup> Initial control values are means of duplicate plates prepared from one sample. The 48 hr control values are means derived from duplicate plates prepared from two samples, and all other values are means derived from duplicate plates prepared from three samples. NO, no viable organisms were detected.

<sup>b</sup> The amount of hydrate former was sufficient to provide a few drops of liquid in excess of that needed to saturate the vapor space and the medium.

<sup>c</sup> The amount of hydrate former was sufficient to bind theoretically all of the water present if hydrate crystals had been formed.

<sup>d</sup> Values less than 1.0 indicate a reduction in viable microorganisms caused by the treatment whereas values in excess of 1.0 indicate that some growth occurred in spite of the treatment.

<sup>e</sup> Values less than 1.0 indicate either a reduction in viable numbers (determined from value in appropriate column headed by "no. / C<sub>i</sub>") or growth which is less than in the incubated control, whereas values in excess of 1.0 indicate that growth in the treated sample exceeded that of the incubated control.

Of the 18 organisms tested, *S. aureus*, *Micrococcus conglomeratus*, *S. lactis*, *Leuconostoc citrovorum*, *L. dextranicum*, *Salmonella typhimurium*, and spores of *B. cereus* and *B. polymyxa* resisted substantial reductions in viable numbers during 48 hr of incubation in the presence of propane vapors. [These samples contained populations of viable organisms at least equal to 0.1 (10%) of their respective initial populations (column 4, Table 1)].

Of the above eight organisms which were most resistant to propane vapors, none grew as well in the presence of propane as they did in its absence (all fractions less than 1.0 in column 5 of Table 1). Furthermore, only *L. citrovorum* and spores of *B. cereus* grew sufficiently well in the presence of propane vapors for 48 hr so that their viable numbers were in excess of 0.1 (10%), the numbers present in the 48 hr controls.

In the presence of vapors of f-12 for 48 hr, 11 of the 18 organisms resisted a substantial reduction in viable numbers (fractions greater than 0.1 in column 6 of Table 1), and seven organisms (*S. aureus*, *M. conglomeratus*, *L. dextranicum*, *S. typhimurium*, *P. aeruginosa*, *Saccharomyces cerevisiae*, and spores of *B. cereus*) grew essentially as well in the presence of f-12 as they did in its absence (fractions greater than ca. 0.1 in column 7 of Table 1).

In the presence of vapors of f-142b for 48 hr, 16 of the 18 organisms resisted a substantial reduction in viable numbers, but only 7 of these 16 organisms (*S. aureus*, *S. lactis*, *L. dextranicum*, *S. typhimurium*, *P. aeruginosa*, spores of *B. cereus*, and *S. cerevisiae*) grew essentially as well in the presence of f-142b as they did in its absence.

It is interesting that *L. citrovorum* exhibited considerable tolerance to vapors of propane but little tolerance to the generally less-detrimental vapors of f-12 and f-142b. The reason for this behavior is not known.

Note also should be made of the poor growth of
Table 3. Changes in numbers of microorganisms at 21°C as influenced by 1,1-difluoro-1-chloroethane (f-142b) a

| Microorganism                  | Controls (no./ml) | Fractional change in numbers after 48 hr no./ml (treated) | Fractional change in numbers after 48 hr no./ml (control) |
|--------------------------------|-------------------|----------------------------------------------------------|----------------------------------------------------------|
|                                | Initial (C₀)      | 48-Hr (C₄)                                               | Low level of liquid f-142b (2.0 ± 0.4 g)b                  | High level of liquid f-142b (7.7 ± 0.8 g)c                  |
| Escherichia coli...            | 4.8 X 10^7        | 9.9 X 10^7                                               | NO                                                      | NO                                                      |
| Pseudomonas fluorescens       | 1.8 X 10^6        | 9.7 X 10^6                                               | 1.9 X 10^-7                                             | 3.6 X 10^-9                                             |
| P. aeruginosa                 | 6.8 X 10^7        | 1.1 X 10^8                                               | NO                                                      | NO                                                      |
| Leuconostoc dextranicum       | 8.4 X 10^4        | 1.1 X 10^9                                               | 8.8 X 10^-4                                             | 6.7 X 10^-6                                             |
| Saccharomyces cerevisiae      | 3.8 X 10^6        | 9.0 X 10^7                                               | NO                                                      | NO                                                      |
| Streptococcus lactis         | 1.3 X 10^7        | 2.6 X 10^6                                               | NO                                                      | NO                                                      |
| Salmonella typhimurium        | 9.0 X 10^4        | 1.2 X 10^8                                               | 3.3 X 10^-5                                             | 2.5 X 10^-6                                             |
| Staphylococcus aureus         | 2.5 X 10^4        | 3.4 X 10^6                                               | 1.9 X 10^-4                                             | 1.4 X 10^-4                                             |
| Bacillus cereus...            | 1.4 X 10^7        | 1.4 X 10^3                                               | 4.1 X 10^-3                                             | 4.1                                                   |

a Initial control values are means of duplicate plates prepared from one sample. The 48-hr control values are means derived from duplicate plates prepared from two samples, and all other values are means derived from duplicate plates prepared from three samples. NO, no viable organisms were detected.

b The amount of hydrate former was sufficient to provide a few drops of liquid in excess of that needed to saturate the vapor space and the medium.

c The amount of hydrate former was sufficient to bind theoretically all of the water present if hydrate crystals had been formed.

d Values less than 1.0 indicate a reduction in viable microorganisms caused by the treatment, whereas values in excess of 1.0 indicate that some growth occurred in spite of the treatment.

e Values less than 1.0 indicate either a reduction in viable numbers (determined from value in appropriate column headed “no./C₀”) or growth which is less than in the incubated control, whereas values in excess of 1.0 indicate that growth in the treated sample exceeded that of the incubated control.

The control samples containing spores of B. cereus and B. polymyxa, because these results have a bearing on the inconsistent values reported for the treated spore samples.

The resistance of the various microorganisms to vapor-state hydrate formers was compared statistically with microbial characteristics such as Gram reaction, sporulation, capsulation, shape, surface-to-volume ratios, and optimum temperature of growth as reported in Bergey's Manual. Significant correlations were lacking in all instances.

Growth of molds (Penicillium notatum and A. flavus) was greater in the absence of hydrate formers than in their presence, and growth was retarded more by propane than by either f-12 or f-142b (data not shown).

Microbial growth as influenced by hydrate formers in the liquid state. The purpose of this series of experiments was to determine the effect of liquid hydrate formers on various microorganisms. Only f-12 and f-142b were selected for use because the major purpose at this point in the study was to find hydrate formers with minimal detrimental effect on organisms so that future studies involving hydrate formation could be more easily interpreted. Hydrate formation was avoided by maintaining the temperature above the decomposition temperatures of the respective hydrates. Those microorganisms showing either substantial resistance to vapor-state hydrate formers (Table 1) or organisms of general importance in foods were selected for study.

Comparison of the data in Tables 2 and 3 indicates that liquid f-12 and f-142b had similar effects on the organisms tested.

Treatment with liquid f-12 for 48 hr (Table 2) substantially reduced the number of viable organ-
The level of liquid f-142b (Table 3) had no important effect on survival of the test organisms, except for *S. aureus* whose survival was poorer in the presence of the higher level of liquid f-142b.

Additional samples essentially identical to those listed in Tables 2 and 3 were prepared and incubated for 1 hr in the presence of liquid f-12 and f-142b. These data are not shown because the results were similar to those obtained with 48 hr of incubation and shown in Tables 2 and 3, except for some organisms, most notably *L. dextranicum*, *S. cerevisiae*, *S. lactis*, and *S. aureus*, which exhibited markedly better survival and growth after the 1-hr exposure than they did after the 48-hr treatment.

A point of considerable importance is the relationship between the physical state of the hydrate former and its effect on survival of microorganisms. The results obtained with *L. dextranicum* in the presence of f-12 are typical (Fig. 1). The presence of liquid hydrate former is obviously associated with a great reduction in survival.

Based on the results reported here and on other unpublished results, it appears that the toxic action of these hydrate formers: (i) does not involve exclusion of oxygen; (ii) requires their presence in a liquid state, the amount not being very important; and (iii) requires agitation, suggesting that interaction between the water-insoluble hydrate formers and lipid components of the microorganisms may be involved.

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**FIG. 1.** Survival of *Leuconostoc dextranicum* as influenced by exposure to dichlorodifluoromethane for 48 hr.