Biogenic Ice Nuclei. Part II: Bacterial Sources

G. VALI,[1] M. CHRISTENSEN,[2] R. W. FRESH,[2] E. L. GALYAN,[3] L. R. MAKI[3] AND R. C. SCHNELL[1,4]

University of Wyoming, Laramie 82071

(Manuscript received 24 October 1975, in revised form 31 March 1976)

ABSTRACT

Transient appearance of ice nuclei active at temperatures of −2 to −5°C has been noted to accompany the natural decay of plant leaf materials. It was shown that the development of these nuclei results from the presence of a bacterium which was identified as *Pseudomonas syringae*. These bacteria produce highly active nuclei in a variety of growth media. Evidence points to the fact that the bacterial cells themselves are the nuclei, but that nucleating capacity is a rare and changeable property of the cells. The findings raise the possibility that bacteria may play a role in atmospheric precipitation processes.

1. Introduction

In Part I of this paper data were presented which suggest that plant-derived organic materials may be major contributors to atmospheric ice nuclei in both terrestrial and marine environments. While working with terrestrial leaf-derived nuclei (LDN), occasional transient appearances of freezing nuclei active at temperatures as warm as −1.3°C were observed during the earlier stages of leaf decay (Schnell and Vali, 1972). Upon further investigation, it was discovered that the development of these active ice nuclei depended on the presence of bacteria which were subsequently isolated and grown in pure culture (Fresh, 1973). The nucleation activity associated with these bacteria was found to be a function of the intact bacterial cell wall (living or dead) and to depend on the age and growth environment of the bacterium (Maki *et al.*, 1974). These nuclei have been named bacteria-derived nuclei (BDN).

This paper summarizes present knowledge about BDN. While there is yet no demonstrated connection between the existence of BDN and atmospheric processes, the relationship of BDN to LDN, discussed in Part I, and the general concept of biosphere-atmosphere interactions make it potentially useful for atmospheric scientists to be aware of these studies. The many unknown aspects of ice formation in clouds and of the freezing of insects and plants further underscore the possible importance of these findings.

2. Methods and materials

a. Microbial isolates

Collection and storage of plant leaf and plant litter materials were the same as described in Part I. Microbes associated with these plant materials were isolated by standard microbiological techniques (Fresh, 1973). Microfungal isolates were obtained from maintained and grown surface-inoculated Martin's medium with rose bengal and streptomycin and from acidified leaf-extract agar. Bacterial isolates were obtained from dilution plates containing a mixture of Peoria #1 medium and a leaf or litter fragment suspension. All plates were incubated at room temperature for 3–5 days prior to pure culture isolation of a sample of the microbial community. Bacterial cultures selected for further study were maintained as lyophil preparations or as active cultures on Trypticose soy agar (TSA, Baltimore Biological Laboratories) and were grown both on a variety of solid media and in Koser broth (DIFCO Laboratories) bubble-aerated at room temperature.

b. Freezing nucleus content

The freezing nucleus contents of samples were measured by the method of Vali (1971). The leaf suspensions contained 10^{-6} g of solid matter for each 100 cm^3 of distilled water. Thirty 0.01 cm^3 drops of test material were placed on a controlled temperature surface and the temperature was slowly lowered from ambient temperature to −25°C. The temperatures required to freeze 10% (T_{10}), 50% (T_{50}) and 90% (T_{90}) of the drops were used in the analyses. These temperatures correspond to nucleus concentrations of 10, 70 and 250 cm^{-3}, respectively, if the assumptions made by Vali (1971) are satisfied. These assumptions are that time-dependence is only of secondary importance in the nucleation process and that each nucleus has a specific nucleation temperature. For a number of different substances empirical evidence supports the assumptions; no specific tests were made with the bacterial preparations.

[1] Department of Atmospheric Science.
[2] Department of Botany.
[3] Division of Microbiology and Veterinary Medicine.
[4] Present affiliation: Atmospheric Physics and Chemistry Laboratory, NOAA, Boulder, Colo. 80303.

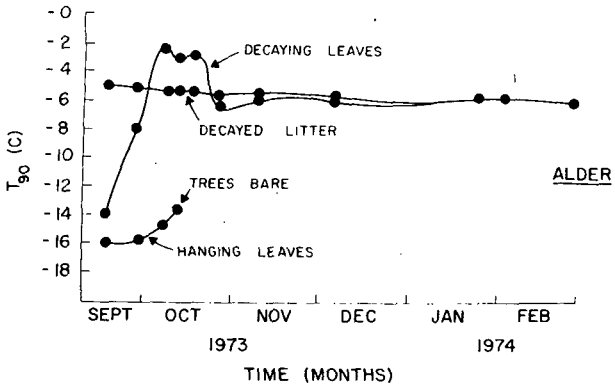

FIG. 1. Freezing nucleus production in an alder grove between 2 September 1973 and 28 February 1974 near Centennial, Wyo.

c. *Effect of physical and chemical stresses on nucleating activity*

The effect of cell disruption on nucleating activity was determined by the breaking of cells in concentrated suspensions either by sonic disruption (Model W185, Heat Systems-Ultrasonics, Inc.) or with a Sorvall RM cell fractionator equipped with a Ribi Valve (operated at 0°C and at 30 000 psi).

The effects of chemical agents were determined by adding known concentrations of the agents to bacterial cultures showing good nucleating activity, incubating them for 4 h at room temperature and subsequently measuring the activity.

The association of activity with the bacterial cells was determined by using a membrane filter (0.45 μm pore size, HA; Millipore Corp.) for filtration experiments or centrifuging for 20 min at 30 000g in a Sorvall RC-2 centrifuge (Maki *et al.*, 1974).

d. *Sizes of freezing nuclei*

Filtration tests with membrane filters (Millipore Corp.) were used to obtain measurements of the sizes of the freezing nuclei. Nuclei observed in the filtrates were assumed to be smaller than the filter pore size specified by the manufacturer. For filteration of liquids, this assumption is reasonably accurate.

3. Results

a. *Nucleus production in a natural forest*

During the earlier stages of research on LDN, it was noted that fresh leaves did not contain many freezing nuclei (none active at temperatures warmer than −15°C), but that high concentrations of nuclei (some active between −1 and −2°C) occurred in some plant leaves undergoing aerobic decomposition. Old litters were found to exhibit lesser ice nucleation activity, but that activity remained constant over periods of up to three years. To follow the course of decomposition more closely, the *in situ* production of freezing nuclei in an alder grove (*Alnus tenuifolia*) growing in the Snowy Range Mountains of Wyoming was monitored from late summer to next spring. Samples of hanging leaves, freshly fallen leaves, and old decayed leaf litter were collected at various intervals through the study period. During the winter months the overburden of snow was removed to expose the leaves beneath. Each sample was tested for ice nucleus content within 1–2 h of collection.

The observed variation of the nucleation activity of alder leaves with time is shown in Fig. 1. Hanging leaves did not at any time contain ice nuclei active at small supercoolings. Well-decayed litters exhibited a consistent T_{90} of −5°C (±1.5°C) for the duration of the experiment. In freshly fallen leaves the nucleus content rose dramatically as the leaves lost their bright yellow fall pigments and became dull brown in appearance. T_{90} temperatures between −2 and −3°C were recorded for samples collected over the interval 8–17 October. Activity dipped for subsequent samples, then stabilized by mid-November. The temperature at which stabilization occurred was the same as that for decayed litters of previous seasons.

The sizes of the freezing nuclei from decaying alder leaves at peak activity (8 October) and from litters from previous years can be inferred from the data shown in Fig. 2. Of interest is the distinct shift to colder temperatures for the alder leaf sample when nuclei >0.5 μm were removed. In contrast, nuclei from the old, well-decayed litter were found to be much smaller, as evidenced by the appreciable activity remaining after filtration through 0.02 μm pore filters. By 25 January 1974, no size distinction between decaying leaves and old litter could be made as each exhibited "old litter" characteristics; this similarity persisted throughout the remainder of the study period.

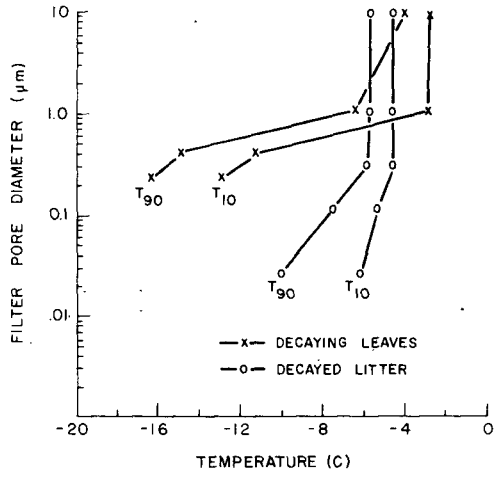

FIG. 2. Freezing temperatures for filtrates of freshly decaying leaves and of old litter from an alder grove during peak nucleus production (8 October 1973).

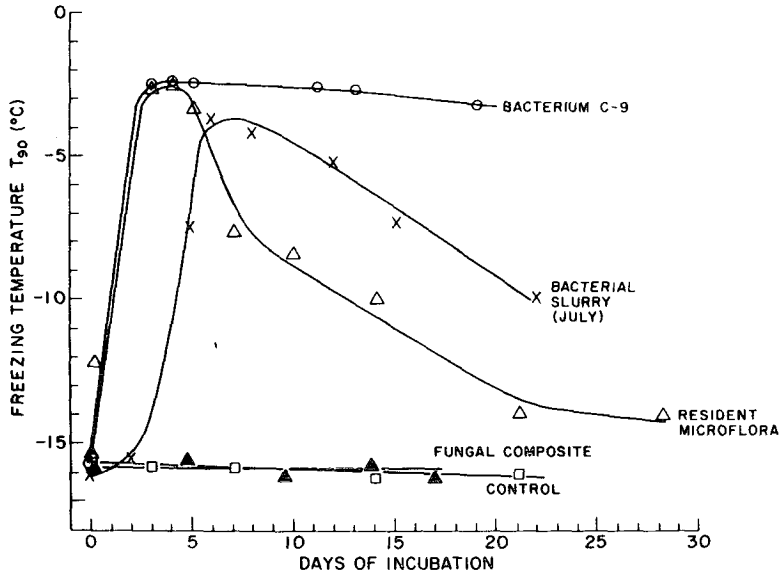

FIG. 3. Development of freezing nuclei in a natural leaf slurry (resident microflora), in sterilized leaves (control), and in sterile leaf slurries inoculated as shown.

b. Isolation of Pseudomonas syringae

In an attempt to isolate the component of the microflora which is responsible for ice-nucleus production, bacteria and microfungi were isolated from a laboratory leaf slurry at peak activity (aerobic, with native microflora; $T_{90} = -2.6$°C) and these were then systematically re-introduced to sterilized leaves. Fig. 3 summarizes the results of these experiments.

The evolution of nucleating ability in leaves decaying in the laboratory with the entire resident microflora present was characterized by a fairly sudden raise in nucleation temperatures followed by a slow decrease. The two major microbial constituents of the resident microflora, bacteria and microfungi, were next reintroduced as composites to sterilized leaves. As the figure shows, no nucleating activity developed with the fungi but the bacteria produced a similar, but somewhat slower, pattern of evolution to that observed with the entire resident microflora. Following this, 50 bacterial isolates were tested separately. Only one of the 50 isolates (designated as C-9) produced active nuclei. As shown in Fig. 3, the nucleus content in the C-9 pure culture slurry increased rapidly and then remained unchanged. It was concluded from this that nucleating ability in the decaying leaves is produced by bacterium C-9, or at least requires the presence of C-9 (Fresh, 1973). The loss of activity after the peak with the resident microflora and with the bacterial composite is speculated to be evidence for a decline of the population of C-9 bacteria due to competition from other bacteria and a degradation of C-9 cells. The reason for the slower development of activity with the bacterial composite than with the resident microflora or with C-9 is not known.

Similar tests were conducted over several months. With the entire resident microflora present the June and July samples behaved as the curve in Fig. 3 indicates, but the August and September samples followed closely the pattern shown in Fig. 3 for C-9, i.e., practically no decline after peak activity had been reached. This variation over a period of months supports the suggestion that it is a competition from other bacteria or a degradation of nucleating ability mediated by other bacteria which was responsible for the nucleus content decreases.

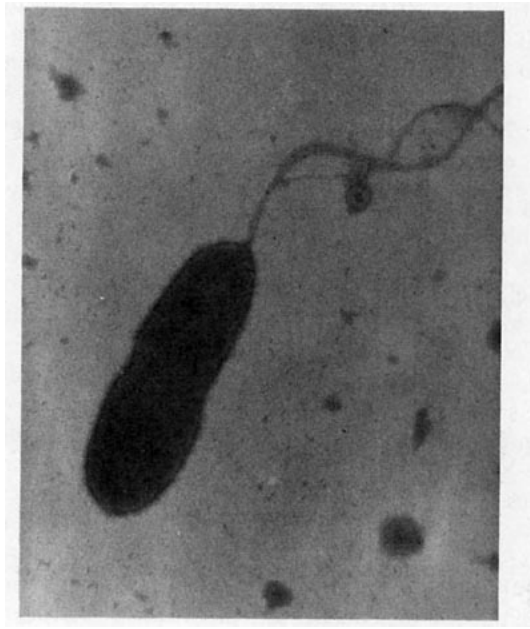

FIG. 4. Electronmicrograph of a cell of C-9. Length of the cell body is 2 μm.

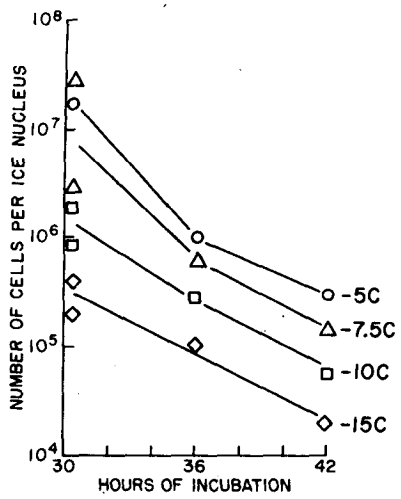

FIG. 5. Ratios of cells to nuclei as a function of incubation time. At 30 h two independent determinations were made.

Bacterium C-9 has been identified as a strain of *Pseudomonas syringae*, a widely-distributed plant pathogen (Maki *et al.*, 1974). Cells of C-9 are about 2 μm long and 0.8 μm wide. An electron micrograph of a cell of C-9 is shown in Fig. 4.

By growing cultures of C-9 in different media it was shown that, in addition to natural tree leaves, active nuclei can be produced by C-9 also from glucose, citrate and sucrose media.

A known culture of *Pseudomonas syringae* (provided by Dr. D. J. Hagedorn of the Department of Plant Pathology, University of Wisconsin) was tested and has been found to produce closely similar nucleation activity to that of C-9. This lends support to the identification of C-9 and the association of freezing nuclei with *Pseudomonas syringae* in general, rather than just with the particular strain isolated in our experiments.

c. *Bacterial numbers relative to freezing nucleus concentrations*

It was of interest to determine the ratio of the numbers of bacteria to the numbers of freezing nuclei, as a measure of nucleating "efficiency." For this purpose, Koser citrate broth was inoculated with C-9 bacteria and the growth of the culture was monitored by counts of TSA platings at about 4–6 h intervals. Nucleating ability was determined for the samples at the same times. The results of these tests are shown in Fig. 5, expressed as the ratio of the number of cells to the number of ice nuclei at different temperatures. These ratios decreased as the culture aged, indicating that the development of ice nucleating ability is not a random property of some proportion of the total numbers of cells, as in that case the ratio would remain constant, independent of the increase in the total number of cells. As the numbers in Fig. 5 reveal, cell concentrations were very much higher than nucleus concentrations. If it should prove that the ice nuclei are *not* the cell bodies themselves (as argued in the previous section), then the data in Fig. 5 simply show that nucleus concentrations in the culture increase at a faster rate than cell concentrations.

An alternate way of comparing bacteria and freezing nucleus counts is to make stepwise dilutions of a suspension. The results of such a test, starting with a sample of very high concentrations, is shown in Fig. 6. It appears that there is very little change in nucleation temperatures for samples containing in excess of 10^7 bacteria cm^{-3}. Using the results obtained with the more dilute suspensions, the computed ratios of cells to nuclei at the different temperatures turn out to be quite similar to the values given in Fig. 5 for 42 h of incubation.

Various attempts to improve the nuclei-to-cell ratio by altering growth conditions have proven to be unsuccessful to date.

d. *BDN related to intact bacterial cell walls*

As reported by Maki *et al.* (1974), nucleating activity seems to be associated with intact bacterial cells and is not an extracellular by-product of the bacteria.

Heating of bacterial suspensions to 65°C for 5 min destroyed the most active freezing nuclei indicating heat-lability typical of cells or of complex organic materials.

Centrifugation for 20 min at 30 000*g* removed the nuclei from the supernatant fluid, but the activity could be shown to have remained associated with the sediment. The same result was obtained by filtration tests. These tests showed the activity resides with particles which are at least similar in size to the cells.

Further indications that the activity is cell-associated were the observations that cetyl-pyridinium chloride,

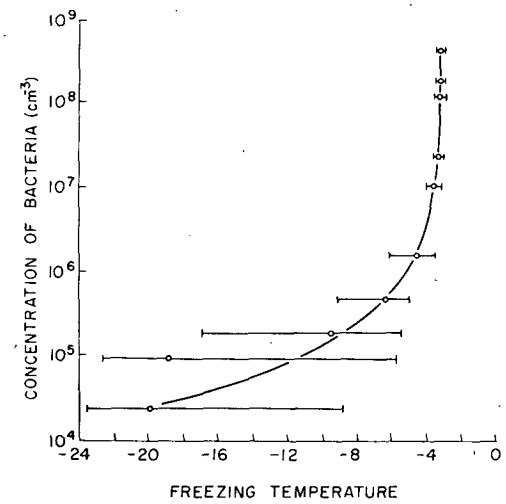

FIG. 6. Freezing temperatures and cell concentrations in successive dilutions of a sample of high original activity. Bars indicate the T_{10} to T_{90} ranges and circles indicate T_{50}.

dyes and physical disruption destroyed ice nucleating activity. Congo red, which does not combine with the cell wall, did not affect the activity. Certain antibiotics, notably streptomycin and polymycin B, which killed the bacteria without destroying their cell walls, did not diminish activity at low supercoolings.

e. Nucleus production with other species of leaves and bacteria

During the course of investigation of the worldwide abundance of LDN (cf. Part I), a number of fresh leaf samples also were collected. For some of these samples nucleus content was monitored as decay of the leaves progressed under laboratory conditions (the leaves were kept in loosely covered containers and moisture was added periodically). From such tests a number of plant species were identified whose leaves developed high transient nucleus concentrations ($T_{10} > -3$°C). The duration of high activity was less than two weeks for all samples. A list of the samples for which high nucleating activity was observed is given in Table 1. Since the sampling times (with respect to season) and the locations and microhabitat conditions varied among the many specimens taken, the only point that can be made from Table 1 is that there certainly are a number of different plant species which exhibit a potential for development of ice nuclei similar to that demonstrated in detail for *Alnus tenuifolia.*

It is also difficult to gauge fully at this time the uniqueness of *Pseudomonas syringae* in ice nucleus producing capacity. C-9 originally was identified as the only bacterial taxon producing active ice nuclei among 50 different isolates tested. This hinted at a great degree of specificity. To examine the point further, *Pseudomonas aeruginosa* and seven species in other genera were tested; all were found to be inactive (Maki *et al.*, 1974). These tests confirm the uniqueness of *Pseudomonas syringae* in its ice nucleating ability.

4. Discussion

The evidence is quite persuasive for the production of highly active freezing nuclei by, or in association with, a species of bacteria. Growth of bacterium C-9 (*Pseudomonas syringae*) both on sterilized natural substrate (alder leaves) and in sterilized laboratory culture media resulted in production of large numbers of active nuclei. The relatively wide range of utilizable growth media and the specific tests described above give strong indications that the bacterial cells themselves form the ice nuclei. The field evidence, while not broad, also strongly indicates that development of active nuclei is a widespread natural phenomenon especially in areas inhabited by trees and shrubs. It may well be that the same or similar processes also occur with other vegetative matter and with other microbial species. That the process is not universal is demonstrated by the absence of activity with several plant/bacteria combinations already tested; however, these represent but a miniscule fraction of the possibilities.

TABLE 1. Plant species exhibiting nucleation activity warmer than -3.0°C (T_{10}) during leaf decay.

Species	Location
Caragana arborescens	Alberta, Canada
Populus balsamifera	Alberta, Canada
Unidentified grass mixture	Alberta, Canada
Alnus tenuifolia	Wyoming, USA
Populus tremuloides	Wyoming, USA
Populus canadensis	Wyoming, USA
Unidentified grass mixture	Colorado, USA
Quercus macrocarpa	Illinois, USA
Fagus grandifolia	Virginia, USA
Fagus sp.	Siberia, USSR
Bambusa sp.	Kyushu, Japan

There is as yet no definite tie between the BDN discussed here and the LDN and ODN discussed in Part I. With all of these sources, the concomitance of biological matter and ice nucleating ability is clear. Beyond that there are only some suggestive parallels. The closest connection is between the BDN and the LDN associated with alder leaves. It is tempting to suggest that the BDN are in some way precursors to the LDN, the highly active transient nuclei changing to the somewhat less active but stable variety. The validity of this suggestion will have to be tested by further experiments.

The widespread presence of microbes in the atmosphere is well-demonstrated and this certainly includes *Pseudomonas syringae* and C-9, even if there is yet no specific experimental proof to that effect. Gregory (1967) speaks of "microbial cloud systems": "It contains vegetative cells of protozoa, bacteria, fungi, mosses, ferns, ... this microbial soup bathes man, animals and crops for good or ill."

It is also known that large numbers of living and dead microorganisms can be found in rain, hail and snow (Gregory, 1967; Parker, 1968; Parker and Barscom, 1970; Mandrioli *et al.*, 1973). It was customarily assumed that these microorganisms were simply swept out by the hydrometeors, although Soulage (1957) found microbes at the centers of artificially grown snow crystals, suggesting that they possibly served as ice nuclei. Dingle (1966) showed evidence that pollen can become condensation nuclei. In a different vein, based on studies of microorganisms and of their organic by-products in the atmosphere and in rainfall, Parker (1970) hypothesized that some microorganisms may be spending their entire life cycles within the humid, nutrient-rich confines of some cloud systems. This same suggestion had been put forth earlier by Miguel (1878).

Demonstration of the possibility that ice nuclei which are of great importance in atmospheric processes

may be created as a result of biological processes is a new aspect of the relationship between the atmosphere and the biosphere. Heretofore this relationship has been viewed primarily as a case of the atmosphere defining the environment for the biosphere and providing for the transport of seeds, spores, pollen and microbes. Only in a climatological sense was the influence of the biosphere on the atmosphere recognized, through modification of the albedo, as moisture supply, and as a factor in boundary-layer friction. That microscopic-scale microbial ice nuclei can perhaps modify precipitation processes, affect climate, etc., is a fascinating new manifestation of the interdependences of our natural world.

REFERENCES

Dingle, A. N., 1966: Pollens as condensation nuclei. *J. Rech. Atmos.*, **2**, 231–237.

Fresh, R. W., 1973: Microbial production of freezing nuclei from decomposing tree leaves. Rep. AR106, Dept. Atmos. Res., University of Wyoming, 15 pp. [Copies available from Department of Atmospheric Science, University of Wyoming].

Gregory, P. H., 1961: *The Microbiology of the Atmosphere.* Leonard Hill, London, 251 pp.

——, 1967: Atmospheric microbial cloud systems. *Sci. Progr. Oxford*, **55**, 613–628.

Maki, L. R., E. L. Galyan, M. C. Chien and D. R. Caldwell, 1974: Ice nucleation induced by "Pseudomonas syringae." *Appl. Microbiol.*, **28**, 456–459.

Miguel, P., 1878–99: In *The Microbiology of the Atmosphere*, (1961) by P. H. Gregory, Leonard Hill, London, England, 251 pp.

Mandrioli, P., G. K. Puppi, N. Bagni and F. Prodi, 1973: Distribution of micro-organisms in hailstones. *Nature*, **246**, 416–417.

Parker, B. C., 1968: Rain as a source of vitamin B. *Nature*, **219**, 617–618.

——, 1970: Life in the sky. *Nat. History*, **79**, 54–59.

——, and G. Barscom, 1970: Biological and chemical significance of surface monolayers in aquatic ecosystems. *Bioscience*, **20**, 87–93.

Schnell, R. C., and G. Vali, 1972: Atmospheric ice nuclei from decomposing vegetation. *Nature*, **236**, 163–165.

Soulage, G. 1957: Les noyaux de congelation de l'atmosphere. *Ann. Geophys.* **13**, 103–134.

Vali, G., 1971: Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids. *J. Atmos. Sci.*, **28**, 402–409.