Studies of Dehydration of Cellular Water in the Freeze-Drying of Microorganisms, with Special Reference to Changes in Cell Viability*

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Abstract

Investigation of the removal of cellular water, previously unexamined in freeze-drying of microorganisms, and of its direct effect upon cell survival during the drying process have been made using some newly devised technical means. A thin layer of a highly concentrated cell suspension of *Escherichia coli* in deionized water was frozen in a specially designed ampoule and dried at different temperatures. After drying, the weight of the residual moisture content of the cells was measured and cell survival was determined by viable count following rehydration. The results thus obtained demonstrated that cell survival was reduced as the residual moisture content decreased in the final drying stage. The mechanism of cell injuries resulting from dehydration is discussed with reference to the cellular water and particularly to the unfrozen water, as reported in our previous paper.

I. Introduction

In general, it is likely that microorganisms are more resistant to freezing and drying than more highly developed plant or animal cells. Despite this, most microorganisms are apt to suffer varying degrees of injury with exposure to low temperatures.

On the other hand, the whole process of freeze-drying, which is commonly used in long-term preservation of living cells, consists of three stages; freezing, drying and reconstitution. Understanding of the mechanisms involved in the cell injuries which result from this process requires analytical investigation of

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each stage.

Many investigators have studied the effect of freezing on the cell viability of microorganisms[1]. A number of papers on drying have also been reported[2], but most of these papers are more concerned with the preservation of the materials dried than with the drying process. In the ordinary freeze-drying procedure using cell suspensions of microorganisms, the water surrounding the cells constitutes the bulk of the water withdrawn during the drying process. Sublimation of this water occupies the greater part of the whole drying procedure and this may be important if considered solely from the physical or technical point of view. The biological significance of freeze-drying of living cells, however, is presumed to lie in the process of dehydration of the intracellular water and this dehydration requires a short time in comparison with the time requires for the entire drying process. The technical difficulty involved in distinguishing one factor from among the many factors which affect the drying and rehydration processes may explain the paucity of literature on the effect of dehydration on cell viability during the drying process. There have only been a few reports and these have been concerned with measurement of cell survival in relation to the time involved in the drying process in freeze-drying of microorganisms[3,4,5]. There have been no reports of changes in cell survival rates in relation to dehydration of intracellular water during the drying process. It is very difficult to examine the dehydration of intracellular and extracellular water separately, since dehydration proceeds in the three dimensional structure of the specimen beginning with its surface, and it is presumed that the intracellular water may be removed from the superficial layer when extracellular water still remains in the deeper layer.

Clarification of the effect of dehydration of intracellular water upon cell viability during drying first requires precise determination of the cellular water content of the microorganisms involved. As reported in our previous paper[6], the cellular water content of yeast and *E. coli* cells was calculated by measuring the intercellular water content and by weighing the dry matter. Some of the cellular water was frozen by cooling to $-20°C$ or below and the amount of water frozen at these temperatures, as well as the amount remaining unfrozen, was determined by calorimetric method. In freeze-drying a cell suspension, most of the unfrozen water, which remained stable even at temperatures below $-20°C$, could be withdrawn with the ordinary freeze-drying procedure.

Various suspending media, such as protein and sugar, are generally employed as protective substances in the freeze-drying process, but they cannot be used for determining the dehydration of intracellular water during the drying process, since it is impossible to distinguish between the dehydration of cells

and that of the surrounding media. Cell suspension in pure water was used in these experiments in order to simplify the conditions affecting the removal of water from the specimen. Although it may be unsuitable from the physiological point of view, cells suspended in water are uncomplicated by any surrounding media following drying and this is very convenient.

From the results obtained from our previous experiments[7,8], it was presumed that, in most cases, the cellular water in the *E. coli* cells had been extracellularly frozen, when the cell suspension was frozen at a slow rate of cooling. In those cases, the freezable water in the cells had probably been withdrawn from the cells and had frozen extracellularly. In the drying process, dehydration will remove the unfreezable water remaining in the cells after both the surrounding water and the already withdrawn, frozen cellular water have sublimated. The fact that cellular dehydration followed sublimation of extracellular ice was confirmed by observation of yeast cells with an electron microscope, equipped with a cooling device and a 16 mm cinematograph[7]. It is for these reasons that water suspension of cells has been used in the present experiment as well as in our previous experiments.

A second particular requirement for the experiment was a very thin specimen. In ordinary freeze-drying, a certain amount of liquid in the container maintains the three dimensional structure of the specimen in the frozen state. As dehydration progresses from the surface of the specimen toward the deeper layers during the drying process, there is a gradient in the temperature, drying rate and residual moisture content in each layer ranging from the surface to the bottom of the specimen. Errors may occur if the residual moisture content of the specimen, which is not uniform, is measured as a whole at certain periods during drying. Consequently, a special ampoule was designed for use in the experiment, keeping in mind the experimental necessities, with particular reference to weight weighing.

The third condition of this experiment was to obtain dried specimens retaining uniform, but different, residual moisture content. For this purpose, variations in the ambient temperature and vapor pressure in the container were examined.

II. Materials and Methods

Materials

E. coli cells from our laboratory's original strain were used as experimental material. Cells which had been cultured overnight on nutrient agar at 37°C were collected and washed three times with deionized water. These cells were then suspended in deionized water in a concentration of approximately 400 mg

(wet weight) per ml.

Containers

A specially designed glass ampoule was used. This ampoule had a thin-walled, spherical body, 3 cm in diameter, and a thicker-walled neck, 1 mm in diameter. The empty weight of the ampoule was approximately 300 mg. This ampoule was placed in a two part glass container of approximately 50 cc capacity and held in place by a wire holder placed on the bottom of the container.

Freeze-Dryer

The freeze-dryer had 20 manifolds, a cold trap, containing liquid nitrogen refrigerant, and a rotary oil pump, which maintained a vacuum of 10^{-2} mmHg in the freeze-dryer.

Freezing and Drying

0.25 ml of the cell suspension, prepared as described above, was placed in the ampoule with a small syringe. As soon as the material had been distributed in a thin, uniform layer on the inside wall of the ampoule by manual rotation, it was frozen by direct immersion in an alcohol bath maintained at −25°C. When the specimen was frozen the ampoule was transferred to the container, which had been cooled to −25°C, and placed up side down on the ampoule holder with the neck of the ampoule down. In some of the experiments ice was also placed on the bottom of the container. The experimental apparatus is shown in Fig. 1. The container was then connected to the manifold of the freeze-dryer and evacuated. Drying was performed at different temperatures to obtain dried materials retaining the desired residual moisture. To control the specimen temperature, the container was immersed in an alcohol bath in a Dewar flask maintained at constant temperatures. The time required for completion of the drying process depended upon the ambient temperature, the lower the temperature, the longer the time.

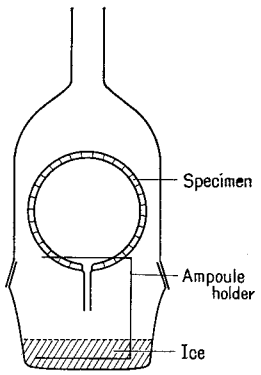

Fig. 1. Specimen and container

Measurement of Residual Moisture Content

The weight of the specimen was calculated from the weight of ampoule with and without the specimen and before and after drying. The dry matter of the cells was determined from material dried for three hours at 60°C in a vacuum of 10^{-5}mmHg. The residual moisture content was calculated by the

following formula:

$$\text{Residual moisture content} = \frac{A - B}{B} \times 100$$

where A is the weight of the specimen immediately after drying and B the weight of the dry matter. In those specimens used for viable cell counts, the dry weight was calculated from the relation between the initial weight of the specimen and the weight of the dry matter of identical specimens used as controls.

Cell Survival

Cell survival in the original suspension of *E. coli* cells and in the dried specimens was measured by counting colonies grown in a nutrient agar plate. The dried cells were rehydrated by breaking the special ampoule in twenty times the original volume of water which was maintained at 20°C. This was then diluted to adequate cell concentration and cultured in nutrient agar. Cell survival was represented as ratio of the number of colonies counted in dried specimens to the number counted in untreated controls. Viable counts were also made on specimens, frozen for 3 hours at −25°C and thawed at 20°C.

III. Experimental Results

Drying Conditions and the Residual Moisture Content of Dried Cells

By controlling the temperature and vapor pressure during the drying process, dried cells with varying amounts of residual moisture were obtained, as shown in Table 1.

Table 1. Relation between drying conditions and the residual moisture content of dried cells

Ambient temperature	Ice	Time		Residual moisture content
20°C	−	approximately	30 min	1— 3%
〃	+	〃	〃	5— 7
−10°C	+	〃	2 hr	10—11
−20°C	−	〃	4 hr	5— 7
〃	+	〃	〃	13—18

When the drying temperature is decreased, the drying process takes longer and the cells have a higher percentage of residual moisture content. The presence of ice in the container during drying resulted in a higher residual

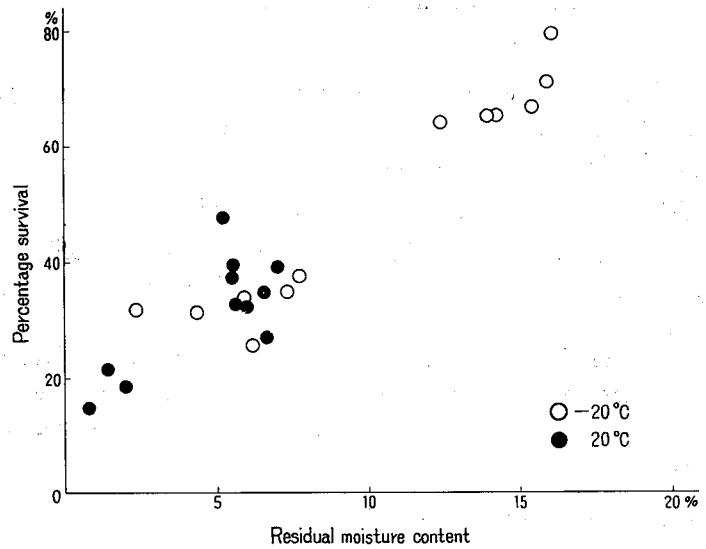

Fig. 2. Relation between residual moisture content and survival rate of *E. coli* cells dried under various conditions. (Experiment 1)

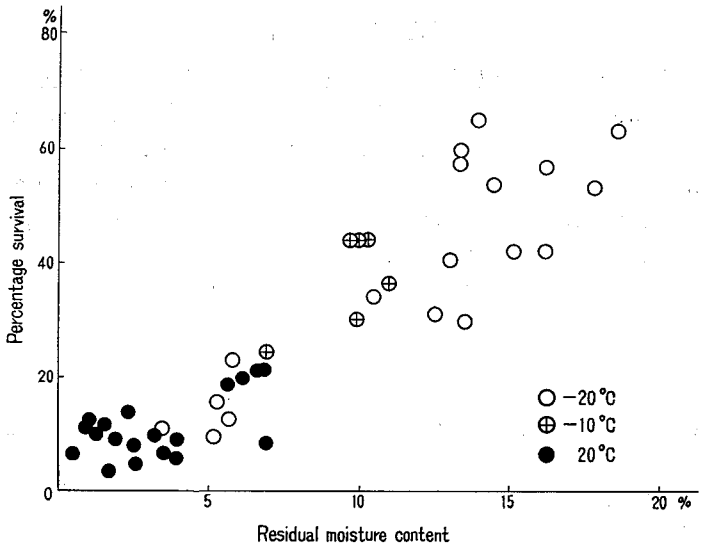

Fig. 3. Relation between residual moisture content and survival rate of *E. coli* cells dried under various conditions. (Experiment 2)

moisture content, but there was no marked difference in the drying time.

Freeze-Thawed Cells as Controls

The number of viable cells in the original suspension was approximately 10^{11} per ml. Control material, frozen at -25°C and thawed in water at 20°C, showed approximately 90% survival.

Residual Moisture Content and Survival of Dried Cells

The relation between the residual moisture content of cells dried under various drying conditions and the survival rate of those cells is illustrated in Figs. 2 and 3.

As these figures illustrate, there is a tendency for the survival rate of the dried cells to decrease as the residual moisture content is reduced. Residual moisture content and cell survival have a positive interdependency which is nearly linear.

Comparison of Drying Temperatures

As is shown in Figs. 2 and 3, this relation between the residual moisture content and the cell survival rate was not altered by variation in the drying temperature. Cells retaining the same residual moisture content had approximately the same survival rate when dried at 20°C or at -20°C.

IV. Discussion

As previously described in the introductory chapter of this paper, the relationship between the residual moisture content and cell survival in freeze-drying of microorganisms has been studied by many investigators. The majority of these investigations were concerned with changes in dried materials during storage and there have been few investigations of changes during the drying process. Even in these few cases, cell survival was only examined during certain periods of the drying process. There have been no reports of the effect of dehydration of cellular water on cell survival during the drying process.

The experiments reported here were designed to investigate this subject of changes during the drying process from several aspects.

1) Precise determination of the amount of dehydration of the cellular water was made by using cell suspension in pure water alone. If protective substances, such as protein or sugar, are used, it is more difficult to distinguish between the intracellular and extracellular water.

2) The specimens were frozen and dried in thin layers to retain uniform moisture content. If thicker layered specimens are used, a moisture gradient is produced in the dried specimen.

3) Highly concentrated cell suspensions were used to prevent the loss of cells through the pump which sometimes occurs when water suspensions are dried.
4) A specially designed ampoule was used for convenience in measuring the residual moisture content.
5) The change in cell survival rates during the drying process has previously been examined in relation to time under identical drying condition[3,4,5], but the methods used made it difficult to obtain dried specimens retaining different but uniform residual moisture content. In ordinary freeze-drying, drying is completed in a relatively short time following sublimation of the frozen portion of the specimen and it is difficult to obtain dried cells having a wide range of residual moisture content. In the present experiments, the drying temperature and the vapor pressure in the container were controlled so that the relation between the residual moisture content and the cell survival rate could be investigated.

Although several attempts were made, it was impossible to obtain dried material with a residual moisture content greater than 20%. If the specimen had a high moisture content, it often melted after drying. It was presumed that such specimens had still some small amount of ice in the undried portion. As it is difficult to determine precisely when the drying process is completed, specimens which melted after drying were omitted from the data given.

In our previous experiments[6], the water content of *E. coli* cells was measured by WHITE's method. Cellular water and dry matter were 66 and 34% of the total cell weight, respectively. In subsequent experiments, Wood's calorimetric method was used and the frozen and unfrozen water content of *E. coli* cells were estimated to be 60 and 6%, or 180 and 18%, if converted to percentage to dry matter.

In the present work, the residual moisture content of the dried specimens ranged from approximately 18%, which just corresponds to the percentage of unfrozen water, to 0%, Therefore, it can be said that the part of the drying process discussed in this paper is that part concerned with the dehydration of the unfrozen water of the cells after the drying of the frozen water.

The cell suspensions used here consisted of 40 weight % cells and 60 weight % surrounding water. During drying from the frozen state, removal of the surrounding water (pure ice) was presumed to precede that of the cellular water, as was observed in the cinematograph taken with the electron microscope using the incorporated cooling device[7]. Intracellular water is removed after extracellular water.

It is known that, if the cells are slowly cooled, only extracellular water freezes. The cells shrink and intracellular ice formation does not occur even

at very low temperatures[9,10]. In this case, it is presumed that the greater part of the cellular water is withdrawn from the cells and extracellularly frozen. This added extracellular water, now pure ice, is then sublimed with the original extracellular water. In the case illustrated in Fig. 4, the two together total 84 weight % of the entire suspension. Most of the freeze-drying process consists of removal of extracellular water.

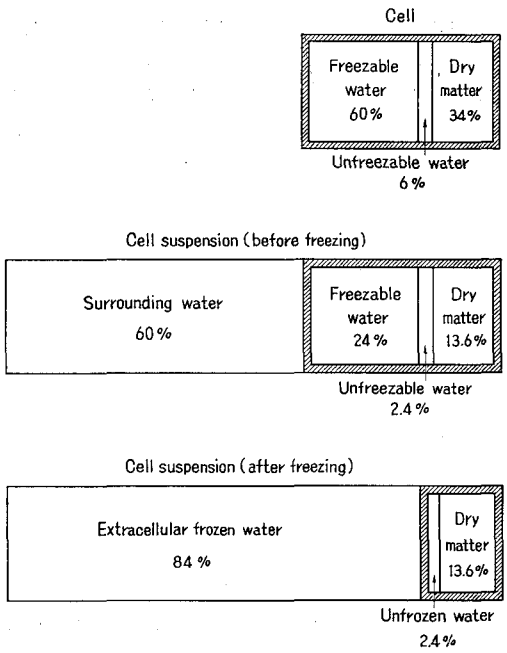

Fig. 4. Phase relations in slow freezing of *E. coli* cells suspended in water

The sublimation of this extracellular ice has extremely slight or no influence on cell viability, as is proven by the following evidence. The cell survival rate in dried specimens containing a residual moisture content of approximately 20% was almost the same as that of the simply frozen and thawed cells. The survival rate of both was approximately 90 to 80%.

The most important result obtained in the present work is that, as residual moisture content was reduced from 18%, just corresponding to the unfreezable water content, to 0%, cell survival decreased. In other words, a decrease in cell survival must result from dehydration of cellular unfrozen water in the final stage of freeze-drying. It is therefore presumed that the unfreezable water of cells plays an important role in cell viability.

In our previous papers[10,11,14], we reported that the percentage survival of coli cells rapidly decreased when dehydration reduced the residual moisture content below 18%. This is the percentage of unfreezable water in the cells, as shown in Fig. 5. But, in those experiments, the conditions for drying and

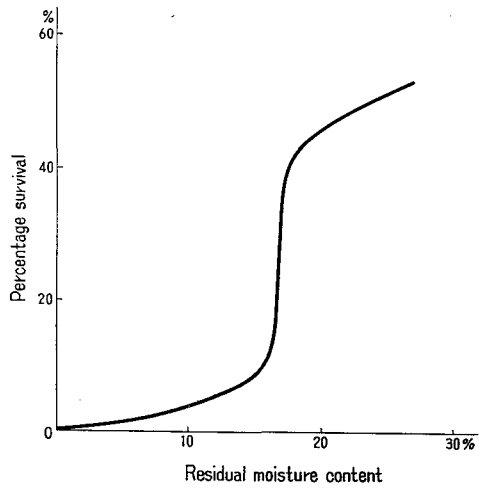

Fig. 5. Relation between residual moisture content and survival rate of freeze-dried *E. coli* cells, previously reported[11]

rehydration were somewhat different from those in the present work. The difference in the survival curves in Figs. 2, 3 and 5, may result from the differences in experimental conditions.

In most of the previous work by several investigators[4,12], a higher percentage of survival of freeze-dried cells was obtained when the drying was carried out at lower temperatures. It was thought that low temperatures favored cell survival during the drying process. In those studies, however, with the exception of that of OBAYASHI[4], there were no reported measurements of residual moisture content. As shown in our present experiment, cell survival, determined immediately after drying, depends upon the residual moisture content. In particular, dried cells containing almost the same residual moisture content showed the same percentage of survival when dried at different temperatures; furthermore, percentage survival was different in the cells of different residual moisture content dried at the same temperature. It may be concluded that the high percentage of survival in specimens dried at lower temperatures is probably due to high residual moisture content, not to the drying temperature itself.

In problems concerning freeze-drying temperature, a careful distinction must be made between specimen temperature and ambient temperature. The relation

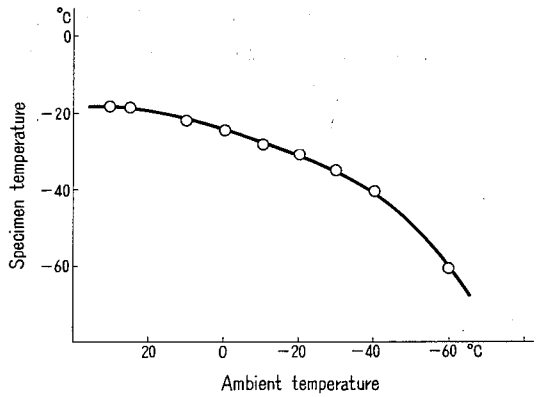

Fig. 6. Relation between specimen temperature and ambient temperature

between these temperatures is illustrated in Fig. 6, as reported in our previous paper[13]. In ordinary freeze-drying, the specimen temperature is lower than the ambient temperature, depending upon the sublimation rate of ice in the specimen during the drying process. Although the thinness of the specimens used in the present experiments prevented measurement of the specimen temperatures, changes in specimen temperature during the drying process are presumed to follow the curves in Fig. 7. Even at ambient temperatures as different as +20

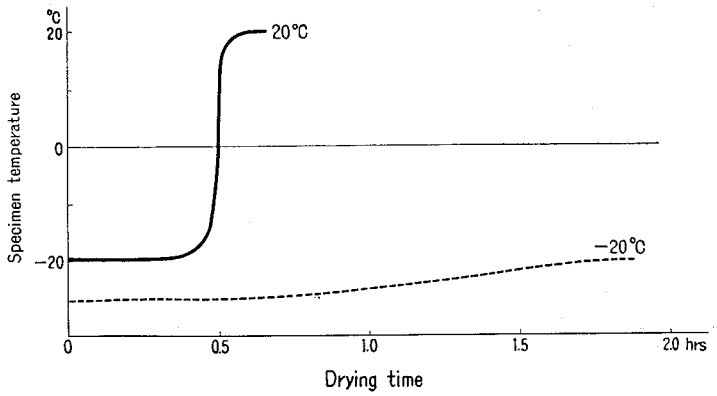

Fig. 7. Schematic curves of specimen temperatures during drying at 20 and −20°C

and −20°C, there is only a slight difference in specimen temperatures during sublimation of the ice. Provided the removal of such freezable water does not affect cell survival as described above, it can be said that drying of the unfreezable water is the most important part of the freeze-drying of living cells,

although unfreezable water forms a very small percentage of the total water in the specimen. During evaporation of this small amount of water in the final stage of freeze-drying, the specimen temperature rises to the ambient temperature. Again comparing drying temperatures of +20 and −20°C, the rate of drying in the final stage may be greater in the former case than in the latter. But, considering the fact that there is no marked difference in survival between cells dried at +20 and −20°C, it may be concluded that such differences in either drying rate or temperature do not affect cell survival.

In the present experiment, all of the dried specimens were rehydrated for viable counts in the same way. If there is any difference in the rehydration process, depending upon the residual moisture content of the dried materials, some improvement in experimental procedure should be made in further research.

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