APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1979, p. 266-273
0099-2240/79/02-0266/08$02.00/0
Vol. 37, No. 2

Deoxyribonucleic Acid Strand Breaks During Drying of *Escherichia coli* on a Hydrophobic Filter Membrane

SHOJI ASADA,* MITSUO TAKANO, AND ISAO SHIBASAKI

Department of Fermentation Technology, Osaka University, Yamada-kami, Suita-shi, Osaka 565, Japan

Received for publication 27 November 1978

Cells of *Escherichia coli* mounted on a hydrophobic filter membrane were dried under various vapor pressures. A mutant defective in deoxyribonucleic acid repair (*uvrA recA*) was more sensitive to drying at a water activity of 0.53 or below than the parent strain but not at a water activity of 0.75 and above. Sucrose gradient studies showed that single- and double-strand breaks of deoxyribonucleic acid occurred at a water activity of 0.53 or below, but no breaks could be observed at a water activity of 0.75 or above. These results were observed in all cells rehydrated with 0.03 M tris(hydroxymethyl)aminomethane-hydrochloride buffer solution at 0 or 37°C, in the presence or absence of oxygen, with saturated water vapor or with a hypertonic solution followed by a gradual dilution. Freezable water was detected in the cells only at a water activity above 0.75 by differential scanning calorimetry. Removal of unfreezable water of cells in the drying, therefore, might induce deoxyribonucleic acid strand breaks.

Many reports have suggested that any type of drying induces mutation in microorganisms. Webb (29, 30) demonstrated that drying in aerosols induced prophage and mutation in *Escherichia coli*. Freeze-drying was also found to induce mutation in *E. coli* (3) and *Saccharomyces cerevisiae* (7). Recently, Hieda (6) showed that the drying of *S. cerevisiae* on a hydrophilic filter membrane at atmospheric pressure was also mutagenic. It seems, therefore, important to study deoxyribonucleic acid (DNA) damage due to drying to better understand the mechanism of mutation. There are only a few reports on this problem. Ashwood-Smith and Grant (3) and Ohnishi et al. (17) showed that freeze-drying caused DNA strand breaks in *E. coli*. Even by freezing, which is usually accompanied by dehydration of cells, single-strand breakage of DNA has been reported in *E. coli* (1, 23) and *Salmonella typhimurium* (24).

In this study we investigated how much cell water can be removed before DNA damage occurs, and whether DNA damage is also influenced by the method of rehydration.

One problem in drying experiments is how to dry the cells. In drying cells on a hydrophilic carrier, the contact between cells and water in carrier materials is thought to retard the equilibration. To minimize this effect, drying in aerosols has been recommended by Webb (28) and Strange and Cox (22). However, the aerosol method requires special apparatus and gives samples too small for study of DNA damage. Freeze-drying does not allow precise control of water content in samples. To overcome these problems, mono- or double-layered cells were dried on a hydrophobic carrier under the control of water content of cells. Here we report that the removal of unfreezable cell water might induce DNA strand breaks.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 AB1157 and AB2480(AB1157 *uvrA recA*) were employed. These two strains were obtained from S. Kondo (9, 10). In preliminary experiments with a series of *E. coli* K-12 strains, AB2480 was found to be the most sensitive to drying.

Sample preparation. The bacteria were grown aerobically at 37°C for 20 h in EM-9 medium (containing Na_2HPO_4, 1.8%; KH_2PO_4, 0.24%; NH_4Cl, 0.2%; NaCl, 1%; $MgSO_4$, 0.05%; glucose, 0.4%; and Casamino Acids (Difco), 0.2% in deionized water, pH 7.0) and washed once with deionized water. About 10^8 bacteria in the suspension (2 to 3 ml) were placed on a hydrophobic membrane filter (FGLP 02500, Millipore Corp.) which had been steeped in ethanol and dried partially by suction on a fritted glass support just before use (the ethanol content in a membrane was less than 100 mg). A bacterium in the suspension was found to occupy ca. 2 nm^2 by a photomicroscopic study. We employed a membrane with an available area of 314 mm^2; an average of 10^8 cells was considered to cover the membrane in a mono- or double layer.

The suspension was filtered by suction in 5 s, and then the membrane filter was placed over a saturated salt solution in a vacuum desiccator under a known relative humidity (RH). Saturated solutions of KCl, NaCl, $NiCl_2 \cdot 6H_2O$, and $CaCl_2 \cdot 6H_2O$ were used for providing RHs of 85, 75, 53, and 33%, respectively. The desiccator was evacuated until the solution started to

boil at room temperature (20°C). Drying at RH 0% was conducted in vacuo (ca. 0.1 torr) with P_2O_5 and silica gel.

The dried bacteria were detached from the membrane by mechanical shaking (Vortex) with 3 ml of 0.03 M Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer solution (pH 8.0) and kept in an ice bath. More than 90% of attached *E. coli* K-12 strains were released from the carrier by this treatment. Strains of *E. coli* B series were not used in this study because of the poor recovery from the membrane (less than 10%). The use of ethanol in this method had no effect on cell viability and DNA. Almost the same number of colonies and the same sedimentation pattern of DNA were obtained before and after filtration on the ethanol-treated membrane.

Other methods of rehydration. The dried cells were rehydrated with 3 ml of 0.03 M Tris-hydrochloride buffer solution as mentioned above or EM-9 medium at room temperature (ca. 20°C), unless otherwise stated.

To examine the effect of oxygen and osmotic shock in the rehydration, the following methods were employed.

(i) Rehydration in the absence of oxygen (Fig. 1). Pure nitrogen gas was introduced into a vacuum desiccator containing dried bacteria, which were then rehydrated with the buffer solution purged of oxygen by nitrogen gas. The procedure is described in detail in the legend to Fig. 1.

(ii) Rehydration with hypertonic solution. Two methods were employed. After rehydration with 3 ml of 20% sucrose solution, the detached bacterial suspension was placed in a cellulose tube (Visking), immersed in 3 liters of deionized water, and kept at 2°C for 3 h to dilute the hypertonic solution gradually. In the simplified method, after rehydration with 3 ml of 20% sucrose solution and subsequent dilution to 5%, 0.1 ml of the cell suspension in 5% sucrose solution was placed directly on the top of alkaline sucrose gradient and analyzed.

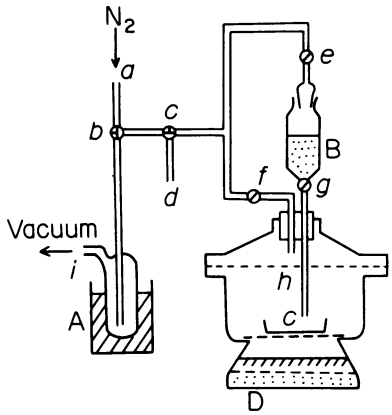

FIG. 1. *Apparatus for rehydration in the absence of air. (A) Cold trap with liquid nitrogen; (B) 0.03 M Tris-hydrochloride buffer solution (pH 8.0); (C) a filter membrane mounted with bacteria; (D) saturated salt solution or P_2O_5 for producing required a_w; (b, c) three-way cock; (e, f, g) two-way cock. First, pure nitrogen gas was passed through a, b, c, and d. Then e was closed and b and c were turned to evacuate h through the line f, c, b, i until D started to boil. After drying C, f was closed, e was opened, and b was turned to eliminate the dissolved oxygen in buffer solution by repeating the vacuumizing and the induction of pure nitrogen gas. Then f was opened and dry pure nitrogen gas was introduced into B and h. Finally, g was opened and C and B were rehydrated.*

(iii) Rehydration with saturated water vapor. Dried cells on a filter were transferred to a vacuum desiccator providing an RH of 100% and moistened for 1 h at room temperature. The remaining procedures were the same as in the control method described in above.

Determination of cell viability. Immediately after rehydration with 3 ml of Tris-hydrochloride buffer solution (pH 8.0), the cells were diluted in deionized water and plated on nutrient agar (polypeptone, 1%; meat extract, 0.5%; NaCl, 0.2%; and agar, 1%, pH 7.0). Colonies were counted after 18 to 24 h of incubation at 37°C.

Sedimentation of ^{3}H-labeled DNA in alkaline sucrose gradient. Bacteria were grown at 37°C for 20 h in EM-9 medium supplemented with 6 μCi of [*methyl*-^{3}H]thymidine (24 Ci/mmol; the Radiochemical Centre, Amersham, England) per ml and 500 μg of 2′-deoxyadenosine per ml. The suspension of treated cells was prepared as mentioned above. The alkaline sucrose gradients were prepared essentially by the method of Kato and Kondo (13), except for the addition of *N*-lauroyl sarcosine (Nakarai Chemicals Co., Ltd., Kyoto, Japan).

To 0.5 ml of the ^{3}H-labeled bacterial suspension at 0°C was added 0.05 ml of 4% ethylenediaminetetraacetic acid (EDTA), and 0.1 ml of the suspension was placed slowly onto 0.2 ml of 0.5 M NaOH containing 0.1% *N*-lauroyl sarcosine which had been layered on the top of a 4.3-ml gradient. The sucrose gradient comprised equal volumes of 5 and 20% sucrose solution containing 1 mM EDTA, 0.7 M NaCl, 0.1% *N*-lauroyl sarcosine, and NaOH (pH 12) in a Hitachi 5PE centrifuge tube (1.3 by 5.0 cm). To attain complete lysis of the cells, the tube was kept standing at 20°C for 30 min, then centrifuged in a Hitachi RPS 40T rotor at 40,000 rpm for 40 min. Four-drop fractions were collected from the bottom of the tube onto filter paper disks (Whatman no. 3, 23 mm in diameter). The disks were dried, washed twice in cold 5% trichloroacetic acid and once in cold acetone, redried, and placed in 5 ml of toluene-based scintillation cocktail for counting.

Sedimentation of ^{3}H-labeled DNA in neutral sucrose gradient. Neutral sedimentation analysis was carried out by similar experimental procedures to those of Kitayama and Matsuyama (14). To a centrifuged pellet of bacteria, 0.8 ml of 14% sucrose, 0.1 ml of EDTA (2.7 mg/ml), and 0.1 ml of lysozyme (2 mg/ml) were added, and the suspension was kept at room temperature for 10 min. A 0.1-ml portion of the protoplast suspension was placed on the top of a 4.4-ml neutral sucrose gradient, onto which 0.1 ml of 0.5% sodium dodecyl sulfate and 0.5% *N*-lauroyl sarcosine had been layered. The neutral sucrose gradient com-

prised equal volumes of 5 and 20% sucrose containing 0.3 M Tris-hydrochloride, 10 mM EDTA, 1 M NaCl, and 0.1 M *N*-lauroyl sarcosine (pH 7.6). After keeping the tube at 5°C for 30 min, centrifugation was carried out at 30,000 rpm for 40 min at 5°C. The remaining procedures were the same as those for the alkaline sucrose gradient sedimentation.

Analysis of the sedimentation data. To estimate the change in molecular weight of DNA due to drying, bacteriophage T4 (IFO 20004) was used as a molecular weight marker. ^{3}H-labeled phage particles prepared by the method of Hirose (8) were layered directly onto the alkaline sucrose gradients to release the DNA (18). After centrifugation of samples, the acid-precipitable radioactivity was measured as described above.

For convenience, all the experimental data were normalized to gradients of unit length with the meniscus as origin. The activity in each fraction was expressed as a percentage of the total radioactivity found on the gradient. If we assume that drying causes random strand breakage of DNA, it is possible to estimate the molecular weight of DNA and the number of breaks. The method of Town et al. (26) was used for calculation. Thus, the number of breaks per single-strand genome (n) was calculated by $n = 6[(D_1/D_2)^{2.63} - 1]$, where D_1 and D_2 are relative sedimentation distances of DNA from untreated and dried *E. coli*, respectively. The molecular weight of DNA was calculated by $D_1/D_2 = (M_1/M_2)^{0.38}$, where D_1 and D_2 are relative sedimentation distances of DNAs of molecular weights M_1 and M_2, respectively. We used the value of 6×10^7 per single-strand genome (27) as the molecular weight of T4 phage DNA.

Unfreezable water content and the critical a_w. Stationary-phase cells of *E. coli* K-12 were harvested by centrifugation, and the washed cells were partially dried on a filter paper. Samples of various water activities (a_w) were prepared in two different ways, desorption and adsorption. For the desorption samples, lots of about 30 mg (dry weight) of cells were transferred to aluminum pans for differential scanning calorimetry. The pans were equilibrated at various RHs by the method described above. For the adsorption samples, the cells were first freeze-dried, then equilibrated at various RHs. It took about 24 h to attain more than 90% of the water equilibrium. Samples with water content of more than 0.4 g of water per g of dry cells (a_w 0.9) were prepared by partially drying wet cells or by adding water to the freeze-dried cells.

A sample and the reference (powdered silver) in the aluminum pans were placed in sample holders of a differential scanning calorimeter (DSC, Rigaku Denki Co., Ltd., Tokyo; low-temperature model). They were cooled at an average rate of 5°C/min, maintained at −100°C for 10 min, and rewarmed at a constant rate of 5°C/min. From the thermogram obtained during the rewarming process, the heat of fusion or amount of freezable water in each sample was obtained.

After the DSC analysis, the moisture content of each sample was estimated by the weight decrease in the sample placed in a desiccator with P_2O_5 in vacuo at room temperature for 24 h. The adsorption or desorption isotherm of *E. coli* cells was obtained from the relationship between the a_w and the water content in each sample. The unfreezable water content of undried bacteria was estimated from data on the freezable water and the water content in dried cells by the method of Simatos et al. (21).

The critical a_w, which we define here as the a_w at which a sample has full unfreezable water but no freezable water, was estimated from the unfreezable water content and the isotherm. Besides *E. coli* cells, pressed bakers' yeast (Oriental Yeast Co., Ltd., Tokyo), skim milk (Difco Laboratories), and egg albumin (Wako Pure Chemicals) were used as reference samples for the estimation of the unfreezable water content and the critical a_w.

RESULTS

Loss of viability in the course of drying. To examine the possibility of DNA damage in the course of drying by our method, we compared the survival curve of AB1157 (*uvrA*$^+$ *recA*$^+$) with that of AB2480 (*uvrA recA*). The DNA repair-deficient mutant, AB2480, was more sensitive to drying at an RH of 53% or below than the parent strain (AB1157) but not at an RH of 75% or above, suggesting that DNA damage might begin to occur at an a_w of 0.75 to 0.53 in the cells (Fig. 2).

Although the reason why the survival change occurs in two steps is unknown, the same result has been reported for drying in aerosols by Webb (28). With our drying methods, it is very difficult to confirm the time required to reach equilibrium with environmental RH because of the small amount of cells on a membrane. In an

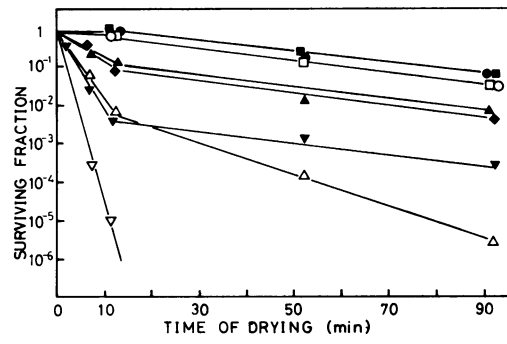

FIG. 2. *Survival of E. coli K-12 AB1157 (uvrA*$^+$ *recA*$^+$*) and AB2480 (uvrA recA) after drying at various RHs. About 10*^{8} *cells were mounted on a hydrophobic membrane filter, dried at a given RH, and rehydrated with 0.03 M Tris-hydrochloride buffer (pH 8.0). Cell suspensions were then diluted with deionized water and plated on nutrient agar. Colonies were counted after 24 h of incubation at 37°C. Each value is an average of duplicate determinations; counting errors between two determinations were less than 28%. Solid and open symbols indicate responses of E. coli K-12 AB1157 (uvrA*$^+$ *recA*$^+$*) and AB2480 (uvrA recA), respectively. Symbols: □, RH 85%; ○, RH 75%; △, RH 53%; ◇, RH 33%; ▽, RH 0%.*

analogous experiment with a greater amount of cells (ca. 20 mg, dry weight), 10 to 15 min were found sufficient to attain more than 99% of the equilibrium by a gravitational method. Thus, the direct effects of dehydration in the pre-equilibrium step seem to occur in the early period (0 to 12 min) rather than later.

The DNA damage suggested by the above result was confirmed by the following experiments.

Single- and double-strand breaks of DNA in dried *E. coli*. As shown in Fig. 3 (dried for 12 min) and Fig. 4 (dried for 92 min), DNA single-strand breaks were observed at an a_w of 0.53 and below, but not at an a_w of 0.75 and above. Figure 4 shows almost the same sedimentation patterns as those in Fig. 3, indicating that the breakage of DNA occurred mainly from 0 to 12 min in the course of drying and increased little after 12 min. As with the viability loss (Fig. 2), the DNA damage in the first 12 min was a_w dependent (Fig. 3), whereas the small increase from 12 to 90 min was a_w independent (Fig. 4).

From the results shown in Fig. 3, the number of breaks produced by drying was calculated by the method of Town et al. (26) and is presented in Fig. 5. The molecular weight of the control single-stranded DNA was about 5×10^8, similar to that reported by Town et al. (26) for an *E. coli* K-12 strain. Figure 5 shows that there is a close relationship between the number of breaks and a_w levels of 0.75 and below (or residual water content of 15% and below).

Since more than 10 breaks per single-strand genome were observed at an a_w of 0, double-strand breaks were also assumed to occur on drying. This was confirmed by neutral sucrose density gradient sedimentation, as shown in Fig. 6. On the other hand, no double-strand breaks have been detected in vitro (2), indicating that mechanisms causing the DNA damage differ in vitro and in vivo.

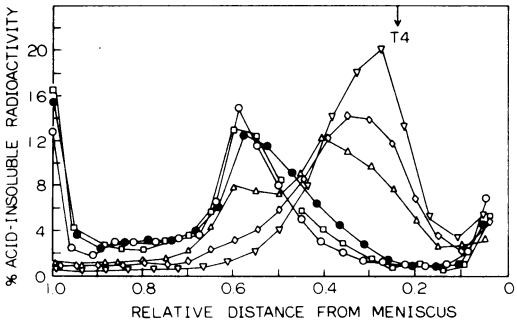

FIG. 3. *Sedimentation in alkaline sucrose gradient of DNA from dried E. coli K-12 AB1157 (uvrA⁺ recA⁺). ³H-labeled cells were dried to a given a_w at the corresponding RH for 12 min, then rehydrated with 0.03 M Tris-hydrochloride buffer (pH 8.0), as described in the legend to Fig. 2. Cells were directly lysed on top of the alkaline sucrose gradient without spheroplast formation and centrifuged as described in the text. The direction of sedimentation is from right to left. Vertical arrow indicates the position of DNA from T4 phage. Symbols: ●, a_w 1.0 (not dried); □, a_w 0.85; ○, a_w 0.75; △, a_w 0.53; ◇, a_w 0.33; ▽, a_w 0.*

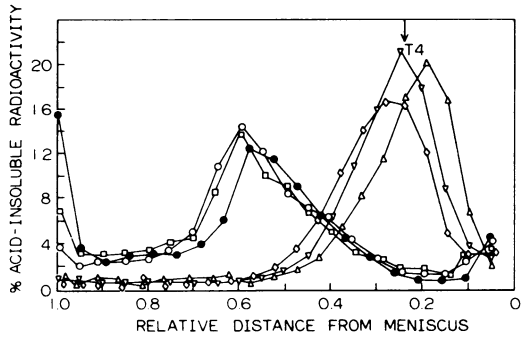

FIG. 4. *Sedimentation in alkaline sucrose gradient of DNA from dried E. coli K-12 AB1157 (uvrA⁺ recA⁺). ³H-labeled cells were dried to a given a_w at the corresponding RH for 92 min. Rehydration, lysis, and sedimentation were performed as described in the legend to Fig. 3. The direction of sedimentation is from right to left. Vertical arrow indicates the position of DNA from T4 phage. Symbols: ●, a_w 1.0 (not dried); □, a_w 0.85; ○, a_w 0.75; △, a_w 0.53; ◇, a_w 0.33; ▽, a_w 0.*

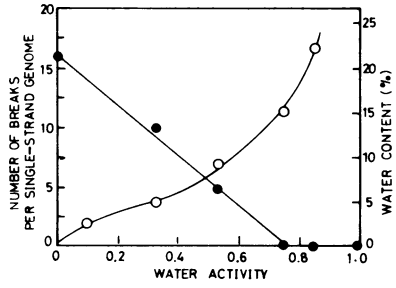

FIG. 5. *Relationship between the number of breaks per single-strand genome caused by drying and a_w level of cells. From the results shown in Fig. 3, the number of breaks was calculated by the method of Town et al. (26). Water content of cells at each a_w was measured as the difference in weight between dried cells and cells equilibrated at each RH. Solid and open circles indicate the number of breaks and water content of cells at a given a_w level, respectively.*

Effect of rehydration on DNA breakage. From the above results, it seems obvious that DNA strand breakage is induced not only by freeze-drying (3, 17) but also by usual drying at a_w 0.75 to 0.53. However, it is not clear whether the DNA damage was dictated exclusively by drying or was also influenced by other processes

in the assay, including rehydration. In the method described above, we exposed the dried cells to air before the rehydrating them with a solution. As Strange and Cox (22) and Israeli et al. (11) reported, oxygen is thought to be toxic to dried bacteria. The direct dehydration with a dilute solution might also have an osmotic effect on the dried cells. Furthermore, the extent of DNA breakage might be influenced by some enzymatic reactions—deoxyribonucleases released or activated intracellularly as suggested in freeze-dried cells (3)—in the step of rehydration. In the method described above, we rehydrated the dried cells at room temperature (ca. 20°C) and cooled them to 0°C soon after the cells were detached from the membrane. Therefore, other methods of rehydration—under nitrogen atmosphere, with a hypertonic solution followed by a gradual dilution under saturated water vapor pressure, or with 0.03 M Tris-hydrochloride buffer (pH 8.0) solution at 0°C and 37°C—were also employed, and the results (Table 1) were compared with those of previous methods.

Except with the water vapor method, no significant differences were observed in the average molecular weight of DNA in bacteria rehydrated by these methods. Rehydration with saturated water vapor produced a further small decrease in the molecular weight for unknown reasons. These new rehydration methods, which were expected to preclude or minimize oxygen effects, osmotic effects, or enzymatic effects, did not protect bacteria from DNA damage.

TABLE 1. *Effect of rehydration on the decrease in DNA molecular weight of E. coli AB2480 after drying*[a]

Rehydration[b]	DNA in rehydrated bacteria[c]
Not dried	$(5.4 \pm 0.26) \times 10^8$
Tris-hydrochloride buffer solution (0.03 M, pH 8.0, control method) at room temp (ca. 20°C)	$(2.3 \pm 0.21) \times 10^8$
Tris-hydrochloride buffer solution at 0°C	2.2×10^8
Tris-hydrochloride buffer solution at 37°C	2.2×10^8
EM-9 medium	2.2×10^8
Oxygen-free Tris-hydrochloride buffer solution (0.03 M, pH 8.0)	2.0×10^8
20% Sucrose and diluted to 5%	2.6×10^8
20% Sucrose and diluted in cellulose tube	2.6×10^8
Saturated water vapor	1.7×10^{8d}

[a] Dried at RH 0% for 12 min.
[b] See text for experimental details.
[c] Values indicate average molecular weight ± standard deviation from mean value.
[d] Value was significantly different at $P < 0.05$ from value obtained by control method.

Unfreezable water content and the critical a_w. Previously, we found a_w 0.75 to be a critical level of cell water for the viability and DNA of *E. coli* in the course of drying. Therefore, we investigated the relationship between the a_w and the properties of cell water. Unfreezable water in biological materials is generally considered to contribute to the maintenance of macromolecules. Table 2 shows unfreezable water contents measured by differential scanning calorimetry and the corresponding critical water activity of *E. coli* K-12 and other samples. Every biological sample in the drying process lost the freezable water at a different level of water content. However, coincidences of the critical water activities of all samples were obtained at around a_w 0.75 from the unfreezable water contents and the desorption or adsorption isotherms. In other words, drying in each sample was thought to remove the freezable water in the course of a_w 1.0 to 0.75, then the unfreezable water in less than a_w 0.75, as long as the quasi-equilibrium of water was maintained in the sample. Consequently, the trigger for strand breakage of DNA in *E. coli* by drying seems to be the loss of unfreezable water.

DISCUSSION

The first problem we examined is how much cellular water should be removed to induce DNA damage. Cellular water is expressed in this report in terms of a_w, rather than water content, since it is considered a preferable indicator for the following reasons: (i) it is easy to prepare a small amount of sample at a given a_w, and (ii) the a_w is a closer direct indicator of the activity of water in various samples.

TABLE 2. *Unfreezable water content of various samples measured by differential scanning calorimetry and the critical a_w*

Sample	Unfreezable water content (g/g, dry wt)	Critical a_w[a]
S. cerevisiae in adsorption process	0.18	0.75
S. cerevisiae in desorption process	0.24	0.75
E. coli K-12 AB1157 in desorption process	0.23	0.75
Egg albumin in desorption process	0.28	0.80
Skim milk in adsorption process	0.10	0.80
Skim milk in desorption process	0.13	0.80

[a] See text for definition of critical a_w.

For this purpose, a hydrophobic filter membrane was used as a carrier material which could contain a minimum amount of water to equilibrate cells quickly with a given RH. Pretreatment of the membrane with ethanol was required to filter the cell suspension. Such treatment, in which cells came in contact with ethanol in a concentration of less than 5% for less than 5 s at room temperature, had no effect on the sedimentation pattern of DNA.

The DNA repair-deficient mutant (*uvrA recA*) showed a higher sensitivity to drying at a_w 0.53 and below than at a_w 0.75 and above (Fig. 2). DNA single-strand breaks were also observed only at a_w 0.53 and below (Fig. 3 and 4), indicating that DNA damage did occur at a_w 0.75 to 0.53 in the course of drying. This is supported by the fact that the sensitivity of bacteria (28, 31) and yeast (5) to ultraviolet light undergoes a drastic change at around a_w 0.75. The occurrence of mutation at a_w 0.75 to 0.53 (6, 30) might be associated with the DNA damage described above.

The DNA damage due to drying did not depend on the rate of drying but on the final equilibrium moisture content. This was supported by the following facts. (i) Hieda (6) found that drying of *S. cerevisiae* at atmospheric pressure was mutagenic to the same extent as in vacuo. (ii) Drying of the cell suspension with 6% (wt/vol) inositol did not decrease the number of breaks of DNA, although the polyol retarded the moisture equilibrium (unpublished data).

The next problem is whether the DNA damage is dictated exclusively by drying or is influenced by the following processes for assay of viability and DNA damage. Osmotic shock, oxygen-induced radicals, or enzymatic action after rehydration might cause such breaks in DNA. These possibilities, however, can be excluded by the results in Table 2 and the alkaline sucrose gradient method used in this study. As described in Materials and Methods, DNA strand breaks were examined soon after rehydration and subsequent rapid alkaline treatment. Furthermore, the incubation of rehydrated cells of *E. coli* AB1157 (wild type) at 37°C for 30 min did not decrease the molecular weight of damaged DNA but increased or repaired it to some extent (unpublished data). It seems, therefore, unlikely that enzymatic action in the rehydration step causes or increases DNA breaks significantly as suggested in freeze-dried cells (3).

The last problem is why drying causes DNA strand breaks. It seems unlikely that significant chemical or biochemical reactions are associated with a_w-dependent DNA breakage during drying. Amino-carbonyl reactions, for example, which are known to occur often in intermediately moistured materials, do not relate linearly with a_w but reach a maximum at a_w 0.8 to 0.4 (20). Enzymatic breakage of DNA cannot be supposed to take place at a very low a_w (0.53 or below).

Our hypothesis is that DNA strand breakage is induced by the removal of unfreezable water which maintains the normal structure of native DNA. Nei et al. (16) reported that bacterial cells contain 0.18 g of unfreezable water per g of dry cells and that removal of unfreezable water induced cellular injury in the course of freeze-drying. The critical a_w, that at which cells have full unfreezable water but no freezable water, was found here to be around a_w 0.75 for all biological samples (Table 2), which corresponds with the amount of water reported by Nei et al. (16). Koga et al. (15) studied several physical properties of cell water in partially dried *S. cerevisiae* and found that the water content at which every property changed was around 10% on the wet basis, which corresponds to a_w 0.75 in their data of the sorption isotherm. This result and our value of the critical water activity indicate that microorganisms might lose all their free water at a_w 0.75 in the course of drying, and further dehydration should bring a reversible or irreversible conformational change in the macromolecules including DNA. Falk et al. (4) found in their in vitro experiment, using optical techniques, that DNA films undergo a "reversible" conformational change from the native form to a disordered form as the a_w is lowered below 0.75. They suggested that such a change of DNA is caused by the removal of hydrated water molecules, which maintains the base stacking of DNA contributing to the stability of the normal DNA structure (the B form).

If we assume that such a conformational change in DNA also occurs in vivo, it might result in physical stress in the cellular DNA which causes the "irreversible" strand breakage. DNA is generally known to be partially attached to the cytoplasmic membrane of cells (19), and this attachment may be a cause of the physical stress that is accompanied by the conformational change of DNA. Ando and Fukada (2) reported that drying in vitro (at 10^{-4} torr for 1 h) caused a reduction in molecular weight and molecular size of 10 to 20% with little alteration in the double-strand character. In vivo, however, a decrease in molecular weight of DNA of about 60% and double-strand breaks were observed (Table 1 and Fig. 6). This difference seems to suggest that the mechanism producing DNA strand breaks differs in vivo and in vitro. Our hypothesis will explain why very few strand

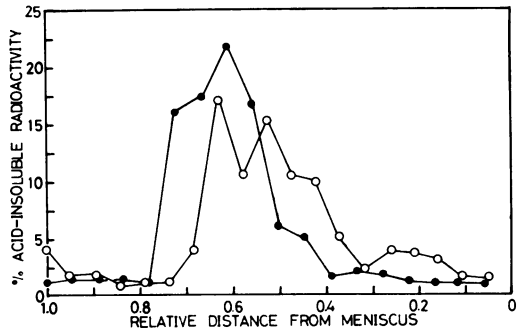

FIG. 6. *Sedimentation in neutral sucrose gradient of DNA from dried E. coli K-12 AB1157 (uvrA⁺ recA⁺). ³H-labeled cells were dried at an RH of 0% for 12 min and then rehydrated with 0.03 M Tris-hydrochloride buffer solution (pH 8.0) as described in the legend to Fig. 2. Cells were converted to spheroplasts, placed on top of the neutral sucrose gradient, and centrifuged as described in the text. The direction of sedimentation is from right to left. Symbols: ●, control cells (not dried); ○, cells dried under RH 0% for 12 min.*

breaks of DNA were observed after drying in vitro (2) and why only bacteriophage are more stable to genetic change due to drying than other microorganisms having a cytoplasmic membrane (6). Further in vitro studies, using a membrane-DNA complex system, are required to test the hypothesis.

ACKNOWLEDGMENTS

We are grateful to S. Kondo and T. Iijima for providing some strains of *E. coli* K-12 and bacteriophage T4 used in this study. We are also indebted to T. Shinobu for his valuable technical assistance.

LITERATURE CITED

1. **Alur, M. D., and N. Grecz.** 1975. Mechanism of injury of *Escherichia coli* by freezing and thawing. Biochem. Biophys. Res. Commun. **62**:308-312.
2. **Ando, Y., and E. Fukada.** 1974. Effects of drying on deoxyribonucleic acids. J. Radiat. Res. **15**:212-218.
3. **Ashwood-Smith, M. J., and E. Grant.** 1976. Mutation induction in bacteria by freeze-drying. Cryobiology **13**: 206-213.
4. **Falk, M., K. A. Hartman, Jr., and R. C. Lord.** 1963. Hydration of deoxyribonucleic-acid, III. A spectroscopic study of the effect of hydration on the structure of deoxyribonucleic acid. J. Am. Chem. Soc. **85**:391-394.
5. **Hieda, K.** 1971. Effects of dehydration on the sensitivity of yeast to ultraviolet light. Mutat. Res. **12**:365-380.
6. **Hieda, K.** 1977. Effects of dehydration on the genetics of yeast, p. 79-95. *In* T. Hasegawa (ed.), Adaptation and regulation in yeast. (In Japanese.) University of Tokyo Press, Tokyo.
7. **Hieda, K., and T. Ito.** 1974. Induction of genetic change by drying in yeast, p. 71-78. *In* T. Nei (ed.), Proceedings of the Symposium on Freeze-Drying of Biological Materials. International Institute of Refrigeration, Paris.
8. **Hirose, S.** 1973. Preparation of bacterial and phage DNA, p. 4-8. *In* A. Ishikawa, R. Okazaki, Y. Kyogoku, and S. Nishimura (ed.), Methods in nucleic acid research. (In Japanese.) Kyoritsu Shuppan Pub., Tokyo.
9. **Howard-Flanders, P., and R. P. Boyce.** 1966. DNA repair and genetic recombination: studied on mutants of *Escherichia coli* defective in these processes. Radiat. Res. Suppl. **6**:156-184.
10. **Howard-Flanders, P., and L. Theriot.** 1966. Mutants of *Escherichia coli* K-12 defective in DNA repair and in genetic recombination. Genetics **53**:1137-1150.
11. **Israeli, E., A. Kohn, and J. Gitelman.** 1975. The molecular nature of damage by oxygen to freeze-dried *Escherichia coli*. Cryobiology **12**:15-25.
12. **Kaplan, R. W., and C. Kaplan.** 1956. Influence of water content on UV-induced s-mutation and killing in *Serratia*. Exp. Cell Res. **11**:378-392.
13. **Kato, T., and S. Kondo.** 1970. Genetic and molecular characteristics of X-ray-sensitive mutants of *Escherichia coli* defective in repair synthesis. J. Bacteriol. **104**:871-881.
14. **Kitayama, S., and A. Matsuyama.** 1971. Double-strand scissions in DNA of Gamma-irradiated *Micrococcus radiodurans* and their repair during post-irradiation incubation. Agric. Biol. Chem. **35**:644-652.
15. **Koga, S., A. Echigo, and K. Nunomura.** 1966. Physical properties of cellular water in partially dried *Saccharomyces cerevisiae*. Biophys. J. **6**:665-674.
16. **Nei, T., H. Souzu, and T. Araki.** 1965. Relationship between water content and survival of cells during the drying process, p. 13-19. *In* T. Nei (ed.), Freezing and drying of cells. (In Japanese.) Science Council of Japan, Tokyo.
17. **Ohnishi, T., Y. Tanaka, M. Yoh, Y. Takeda, and T. Miwatani.** 1977. Deoxyribonucleic acid strand breaks during freeze-drying and their repair in *Escherichia coli*. J. Bacteriol. **130**:1393-1396.
18. **Rupp, W. D., and P. Howard-Flanders.** 1968. Discontinuities in the DNA synthesized in an excision-defective strain of *Escherichia coli* following ultraviolet irradiation. J. Mol. Biol. **31**:291-304.
19. **Ryter, A.** 1968. Association of the nucleus and the membrane of bacteria: a morphological study. Bacteriol. Rev. **32**:39-54.
20. **Scott, W. J.** 1960. A mechanism causing death during storage of dried microorganisms, p. 188-202. *In* A. S. Park and A. U. Smith (ed.), Recent research in freezing and drying. Blackwell Scientific, Oxford.
21. **Simatos, D., M. Faure, E. Bonjour, and M. Couach.** 1975. The physical state of water at low temperatures in plasma with different water contents as studied by differential thermal analysis and differential scanning calorimetry. Cryobiology **12**:202-208.
22. **Strange, R. E., and C. S. Cox.** 1976. Survival of dried and airbone bacteria, p. 111-154. *In* T. R. G. Gray and J. R. Postgate (ed.), The survival of vegetative microbes. Cambridge University Press, Cambridge.
23. **Swartz, H. M.** 1971. Effect of oxygen on freezing damage. II. Physical-chemical effect. Cryobiology **8**:255-264.
24. **Takano, M., A. J. Sinskey, and D. Baraldi.** 1974. DNA damage in *Salmonella typhimurium* LT-2 by a combination of freezing and nutrition, p. 61-70. *In* T. Nei (ed.), Proceedings of the Symposium on Freeze-Drying of Biological Materials. International Institute of Refrigeration, Paris.
25. **Takano, M., and G. Terui.** 1969. Correlation of dehydration with death of microbial cells in the secondary stage of freeze-drying, p. 131-142. *In* T. Nei (ed.), Freezing and drying of microorganisms. University of Tokyo Press, Tokyo.
26. **Town, C. D., K. C. Smith, and H. S. Kaplan.** 1971. Production and repair of radiochemical damage in *Escherichia coli* deoxyribonucleic acid: its modification by culture conditions and relation to survival. J. Bacteriol. **105**:127-135.
27. **Veatch, W., and S. Okada.** 1969. Radiation-induced

breaks of DNA in cultured mammalian cells. Biophys. J. **9**:330–346.

28. **Webb, S. J.** 1965. The role of bound water in the maintenance of the integrity of a cell or virus, p. 146–171. *In* Bound water in biological integrity. Charles C Thomas, Publisher, Springfield, Ill.
29. **Webb, S. J.** 1967. Mutation of bacterial cells by controlled desiccation. Nature (London) **213**:1137–1139.
30. **Webb, S. J., and M. D. Dumasia.** 1967. The induction of lambda prophages by controlled desiccation. Can. J. Microbiol. **13**:33–43.
31. **Webb, S. J., and C. C. Tai.** 1968. Lethal and mutagenic action of 3200–4000 Å light. Can. J. Microbiol. **14**:727–735.