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Thermal Death of Fungal Conidia in Liquid Paraffin

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Abstract

The conidia of *Aspergillus niger* IAM 2045 were dispersed in liquid paraffin as well as in water, and the rates of thermal death of dried conidia suspended in liquid paraffin were determined and compared with those in water. The conidia were much more resistant to heat in liquid paraffin than in water, and the thermal death rate constant at 90°C in water was about 5×10^{14} times that in liquid paraffin. The activation energy for the thermal death in water was about 2.6 times that in liquid paraffin.

On the other hand, about 0.1 mg of water was found to be present on the surface of 1 mg of undried conidia which were just harvested from 40–60 day old slant cultures. The presence of this "surface water", though the amount was very small, markedly decreased the thermal resistance of undried conidia in liquid paraffin, and a unique survival curve which was not observed in the case of dried conidia free from the "surface water" was obtained.

Introduction

It is well known that the thermal resistance of microbial cells and spores are markedly affected by the presence of water. Dry cells are more resistant to thermal inactivation than wet cells,[1,2] and bacterial spores suspended in nonaqueous media such as oil and glycerin are also much more resistant to heat than those suspended in water.[3,4] LaRock and Severance[5,6] have recently reported that the thermal resistance of hydrocarbon-utilizing *Brevibacterium* cells suspended in liquid paraffin is much decreased by adding a small amount of water to the cellular suspension. Little is known, however, about the rates of thermal death of microbial cells in liquid paraffin and the effects of temperature on the death rates, partly because the preparation of a cell suspension dispersed in liquid paraffin is technically difficult.

On the other hand, in accordance with recent developments in the study of fermentation of hydrocarbons, practical information on the rates of thermal death of microbial cells and spores in liquid paraffin is of increasing importance from the view point of sterilization of liquid paraffin by heat.

We have found that the conidia of several fungi can be dispersed in liquid paraffin as well as in water, while bacterial spores are hardly dispersed in liquid paraffin. In this paper, the rate constant and the activation energy for the thermal death of conidia of *Aspergillus niger* IAM 2045 suspended in liquid paraffin are determined and compared with those in water. The effect of water, which is present on the surface of undried conidia, on the thermal resistance of conidia in liquid paraffin is also examined.

Materials and Methods

Test organism The conidia of *Aspergillus niger* IAM 2045 were employed throughout the experiments

since they could be dispersed in liquid paraffin as well as in water to give homogenous suspensions. The conidia were formed by incubating the mold on agar slants composed of malt extract 1.0%, glucose 0.4%, yeast extract 0.4% and agar 2.0% at 28°C for 2 days. These slant cultures were aged at room temperature for further 40–60 days to make the thermal resistance of conidia constant. The conidia were then harvested and collected in a weighing tube.

Suspending medium Liquid paraffin or 1/15 M phosphate buffer of pH 7.0 was employed. Liquid paraffin was "super heavy *n*-paraffin" supplied by Nikko Oil Chemical Co. and was composed of *n*-C_{12} 27.8%, *n*-C_{13} 44.5%, *n*-C_{14} 25.3% and *n*-C_{15} 0.5%. It was sterilized by heating at 120°C for 20 min and dehydrated with anhydrous sodium sulfate if necessary.

Preparation of conidial suspension The conidia collected in a weighing tube were dried in a desiccator, since a small amount of water was found to be present on the conidial surface, and were then suspended in sterile phosphate buffer or in liquid paraffin. The suspension was shaken on a vibrator for 10 min to disperse the conidia more homogeneously. The conidia harvested from one slant culture were ordinarily suspended in 5 ml of menstruum, and the number of conidia in the suspension ranged from 3×10^6 to 6×10^6 per ml. The conidial suspension thus prepared was tentatively designated "undiluted conidial suspension" and employed in the following experiments unless otherwise stated.

Heating procedure A 0.2 ml aliquot of "undiluted conidial suspension" was added to 19.8 ml of sterile liquid paraffin (or phosphate buffer) in a cotton-stoppered test tube (28×200 mm) which was heated in advance and kept at the test temperature in a constant-temperature water bath. Thus the temperature of the conidial suspension was raised immediately from room temperature to the test temperature, while the conidial concentration was decreased to 1/100. The conidia in the diluted suspension were then subjected to thermal inactivation at the test temperature. A 1 ml sample was removed from the test tube using a cooled pipette at the desired time intervals. The sample was immediately transferred into 9 ml of cooled sterile liquid paraffin (or phosphate buffer) with vigorous shaking.

Counting of survivors The number of survivors in the above sample was enumerated by the ordinary plate culture technique using a culture medium composed of meat extract 1.0%, glucose 0.4%, yeast extract 0.4%, glycerol 0.05% and agar 0.8% after appropriate dilutions had been made with sterile phosphate buffer. For diluting the liquid paraffin menstruum, 0.05% Emulgen 420 (polyoxyethyelene alkyl ether) was added to the phosphate buffer in order to allow dispersion of conidia and liquid paraffin in water.

The number of viable conidia in the unheated control, which was prepared by adding 0.2 ml of "undiluted conidial suspension" to 19.8 ml of liquid paraffin (or phosphate buffer) at room temperature, was also enumerated by the same method.

Results and Discussion

Amount of water present on the conidial surface It was first examined whether the condia harvested from the slant culture were free from water or not, though neither droplets nor films of water were detected on the conidial surface by microscopic observation.

Figure 1 shows a typical curve for the decrease in the weight of conidia when they were dried in a desiccator containing conc. H_2SO_4 at atmospheric pressure and room temperature. The weight of conidia became constant after 4 hr drying, and the decrease in weight was about 1.6 mg. This decrease in weight is considered to come from the removal of water from the conidial surface, because the content of free water inside of the conidia has been reported to be negligible.[7,8] It is not certain, however, whether the water is contained in the surface layer of conidia or just adheres to the surface. Therefore, the term "surface water" will hereafter represent the water present on and/or in the conidial surface.

Other experiments using 40–60 day old conidia gave similar results, and the presence of about 0.1 mg of "surface water" per 1 mg of undried conidia was confirmed.

Thermal death of conidia in liquid paraffin Thermal death of dried conidia suspended in liquid paraffin was examined at 80.3, 89.0, 92.0 and 96.1°C using the heating

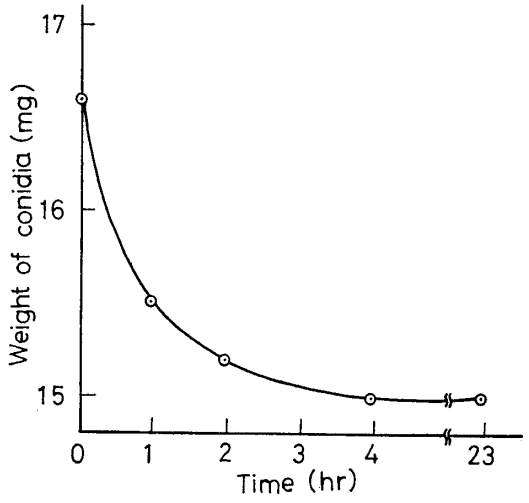

Fig. 1. Drying curve for *A. niger* conidia.
The conidia were dried in a desiccator containing conc.H_2SO_4 at atmospheric pressure and room temperature.

procedure described above, and the survival curves shown in Fig. 2 were obtained. Other than the 80.3°C experiment where the conidia were not killed, the viable counts of conidia did not decrease for a short period immediately following exposure to heat and then decreased logarithmically. This type of survival curve is rather common and has been often observed in the thermal death of microbial cells and spores in aqueous menstruum.[9,10]

Rate constants for the thermal death at each temperature were calculated from the slope of straight lines and plotted against the reciprocals of absolute temperature, $1/T$, as shown in Fig. 3, which indicates that Arrhenius' law also holds for the thermal death of *A. niger* conidia in liquid paraffin. The values of activation energy and frequency factor calculated from Fig. 3 are 70.6 kcal/mole and 1.1×10^{41} min^{-1}, respectively.

Thermal death of conidia in water Thermal death of dried conidia was also examined in phosphate buffer at 47.4, 48.7 and 49.9°C by the same heating procedure as above, and the survival curves obtained (Fig. 4) were compared with those in liquid paraf-

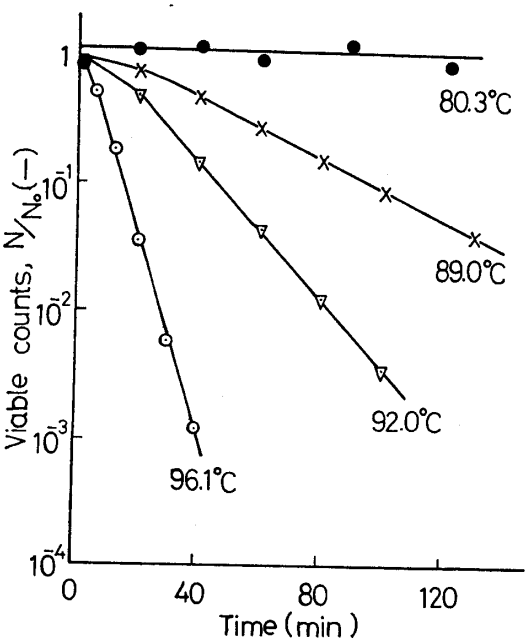

Fig. 2. Survival curves for dried conidia suspended in liquid paraffin.
The temperature was raised immediately as described in the text. The viable count of conidia is given by N/No, where N is the number of survivors and No is the number of viable conidia in the unheated control.

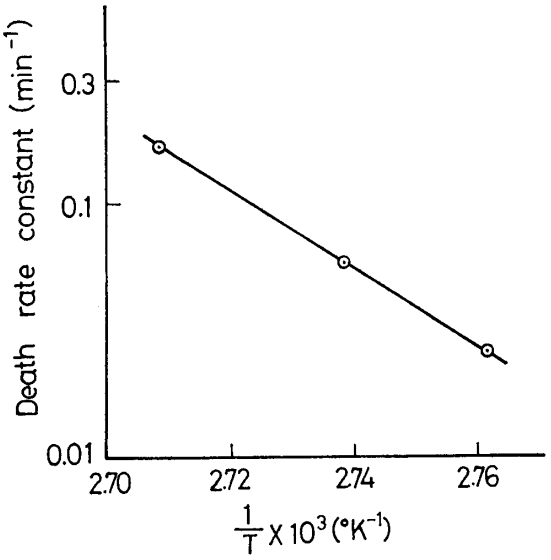

Fig. 3. Correlation of death rate constant in liquid paraffin with temperature.

fin (Fig. 2). Little difference was observed in the shape of the survival curves between Fig. 2 and Fig. 4. It should be noted, however, that the condidia were killed at much lower temperature in water than in liquid paraffin. Moreover, the activation energy for the thermal death in water, which is calculated from the results in Fig. 4, is 182 kcal/mole. This value is about 2.6 times that in liquid paraffin. These data are summarized in Table 1, which demonstrates that the fungal conidia are much more resistant to heat in liquid paraffin than in water.

Effect of "surface water" on thermal death in liquid paraffin As the thermal resistance of conidia was found to be markedly decreased in the aqueous menstruum, the effect of "surface water" of conidia on the thermal death in liquid paraffin was investigated. In this experiment undried conidia just harvested from the slant culture were employed. These conidia were suspended in liquid paraffin and exposed to heat at 55.4, 60.3, 67.7, 73.5, 80.1 and 85.5°C by the same heating procedure as above. The

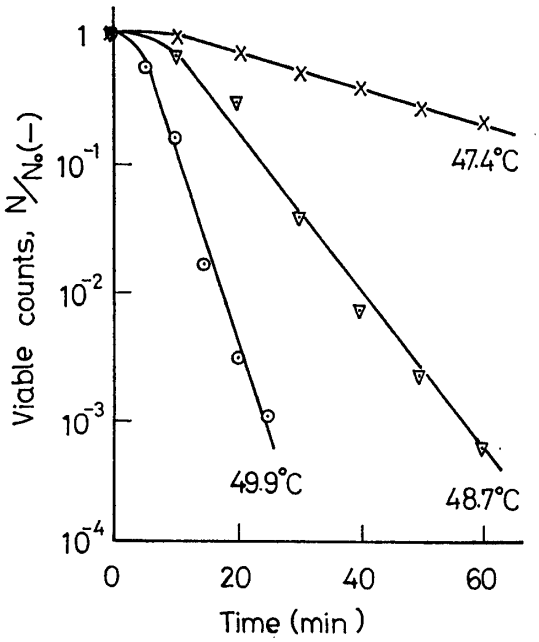

Fig. 4. Survival curves for dried conidia suspended in water.
The conidia were suspended in 1/15 M phosphate buffer of pH 7.0. The temperature was raised immediately as described in the text.

Table 1. Rates of thermal death of *A. niger* conidia in water and in liquid paraffin.

Suspending medium	Thermal death rate const. (min^{-1})			Activation energy (kcal/mole)
Phosphate	47.4°C	48.7°C	49.9°C	182
buffer	0.038	0.13	0.35	
Liquid	89.0°C	92.0°C	96.1°C	70.6
paraffin	0.026	0.059	0.17	

Dried conidia were employed. The temperature was raised immediately as described in the text.

survival curves, the reproducibilities of which were confirmed by repeated experiments, were unique as shown in Fig. 5. The viable count of conidia decreased sharply immediately after exposure to heat in all cases except that of 55.4°C, where the count did not decrease but increased slightly.[9] After that, further decrease was not observed in the viable count except in the 85.5°C curve, and the higher the test temperature was, the less the conidia survived.

It is possible that the decrease in viable counts may stem not from the thermal death but from the flocculation of conidia caused by heating, since the viable counts are enumerated by the colonial counts grown on culture plates. Such flocculation, however, was not found in the heat treated conidial suspensions, and the decrease in colonial counts was confirmed to correspond to the thermal death of conidia.

The very remarkable feature of the survival curves noted above may be elucidated by the following considerations. The amount of "surface water" in 0.2 ml of "undiluted suspension of undried conidia", which is added to 19.8 ml of liquid paraffin, is 0.07 mg at the most, whereas the solubility of water in 20 ml of liquid paraffin calculated by the

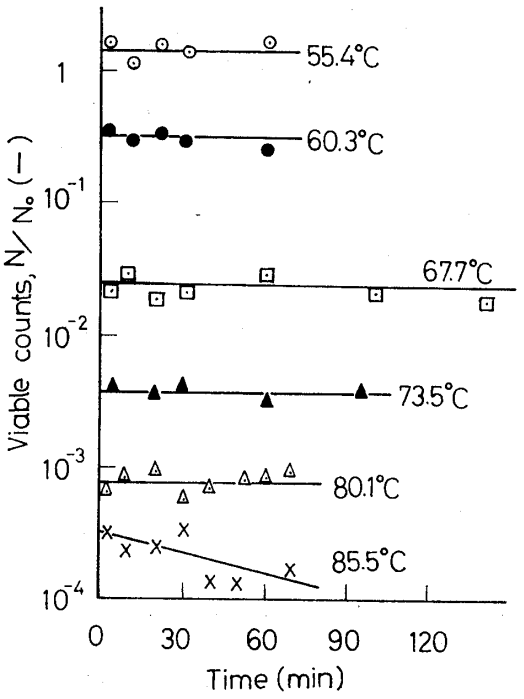

Fig. 5. Survival curves for undried conidia suspended in liquid paraffin.
The temperature was raised immediately as described in the text.

formula of Black *et al.*[11] is 3 mg at 20°C and 7.6 mg at 80°C, respectively. Therefore, the "surface water" may dissolve in the liquid paraffin immediately after the addition of "undiluted conidial suspension", and the response of undried conidia to heat after the "surface water" is eliminated may be the same as that of the dried conidia shown in Fig. 2 in which no thermal death is observed at test temperatures below 80°C or so. If so, it is very probable that some of the conidia are killed during a very short period before the "surface water" is eliminated, because of the decreased thermal resistance in the presence of water, and after that no thermal death takes place. However, as is clear from the fact that no thermal death is observed at 54.4°C, the rate of thermal death in the presence of "surface water" is much slower than that in aqueous menstruum.

The above assumption that the thermal resistance of undried conidia after the "surface water" has completely dissolved into liquid paraffin is the same as that of dried conidia is also supported by the fact that the rate of thermal death obtained from the slope of the 85.5°C curve in Fig. 5 is not so different from that of dried conidia calculated from the results in Table 1. That is, the rate constant for the former is 0.016 min^{-1}, while that for the latter is 0.01 min^{-1}.

Thermal death of conidia in liquid paraffin when temperature is raised gradually In the preceding experiments, the temperature of test conidial suspension was raised immediately from room temperature to the test temperature. In the practical procedures for heat sterilization, however, the temperature in most cases is not raised immediately but gradually. Therefore, the response of dried and undried conidia to heat was examined by raising the temperature of test suspension gradually. A 0.2 ml aliquot of "undiluted conidial suspension" was added to 19.8 ml of liquid paraffin in a test tube at room temperature to obtain the same diluted conidial suspension as in the preceding experiments. The test tube was placed on a shaker immersed in a water bath, and the temperature of the conidial suspension was gradually raised to the test temperature by heating the water bath at a rate which was slow enough to minimize the difference of temperature between the outside and the inside of the test tube. The temperature of the water bath was kept constant after the test temperature was attained, and the conidia were subjected to thermal inactivation at that temperature with continuous shaking.

A typical time course for the temperature raise and a survival curve for dried conidia under the above heating procedure are shown in Fig. 6. It took about 80 min to raise the temperature from 45°C to 88.6°C, the test temperature, but no decrease was observed in the viable count during this period. Thermal death, however, began to take place when the temperature came up to 88.6°C, and the viable count decreased logarithmically after the test temperature was attained. The survival curves for dried conidia at 85.9, 91.2 and 94°C are also shown in Fig. 7, in which broken and solid lines give the viable counts of conidia before and after each test temperature was attained, respectively. Time courses for the temperature rise in these experiments were very similar to that in Fig. 6. In these survival curves, the viable counts also decreased logarithmically during the exposure to heat at each test temperature. It may be reasonable that the viable counts do not decrease before each test temperature is attained, except 94°C curve in Fig. 7, since the rate constant for the thermal death is only 4.6×10^{-2} min^{-1} even at 91.2°C.

Rate constants for the thermal death at each test temperature were calculated from Fig. 6 and Fig. 7 and compared with those in liquid paraffin given in Table 1 by plotting them against the reciprocals of absolute temperature. As shown in Fig. 8, the data points for the former as well as for the latter are distributed on the same straight line, the slope of which gives the activation energy of 70.6 kcal/mole. That is, the response of dried conidia to heat when the temperature is gradually raised is the same as that when the temperature is

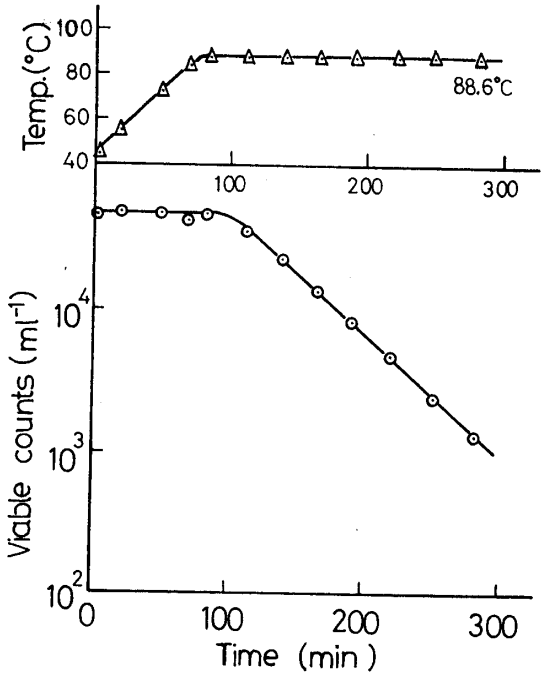

Fig. 6. A time course for temperature rise and a survival curve for dried conidia.
The conidia were suspended in liquid paraffin, and the temperature of the suspension was raised gradually as described in the text.

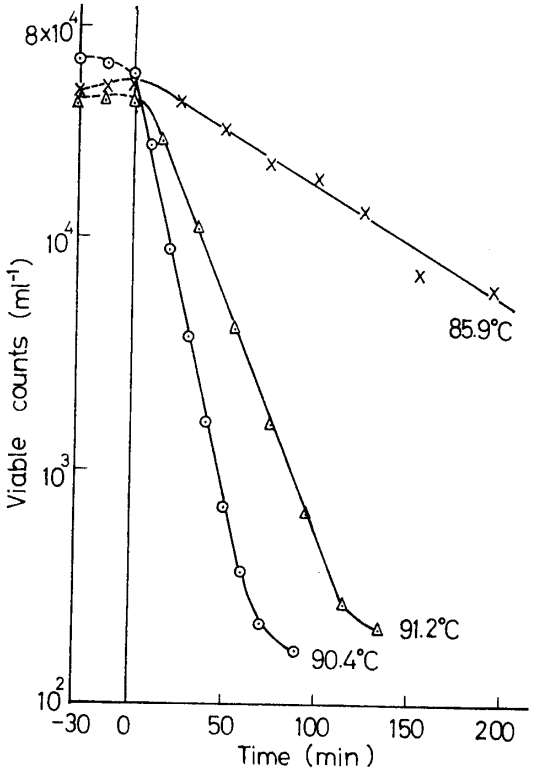

Fig. 7. Survival curves for dried conidia suspended in liquid paraffin.
The temperature was raised gradually as described in the text. Broken and solid lines give the viable counts of conidia before and after each test temperature is attained.

raised immediately.

Similar experiments were also carried out using undried conidia, and the survival curves shown in Fig. 9 were obtained. It is notable that a survival curve such as in Fig. 5 was not obtained in this case, though undried conidia were employed as in the case of Fig. 5. The features of survival curves in Fig. 9 are very similar to those in Fig. 7 where dried conidia

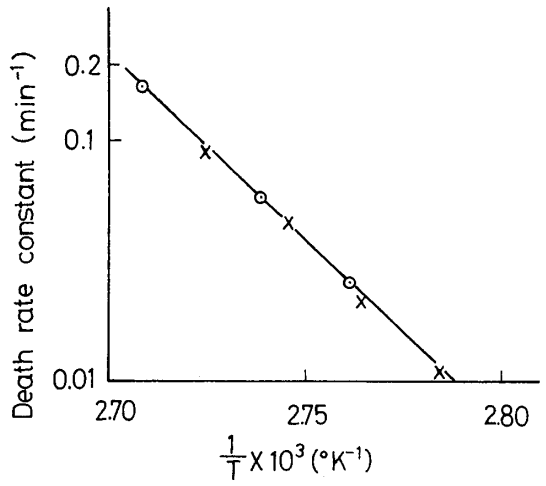

Fig. 8. Arrhenius type plots for thermal death rate constants of dried conidia in liquid paraffin.
⊙ and ×: rate constant for the thermal death when the temperature is raised immediately and gradually, respectively.

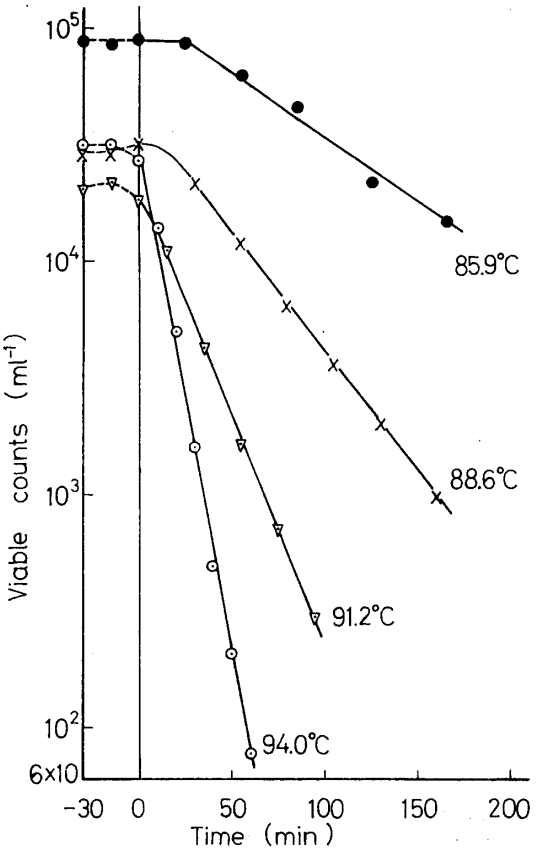

Fig. 9. Survival curves for undried conidia suspended in liquid paraffin.
The temperature was raised gradually as described in the text. Broken and solid lines give the viable counts of conidia before and after each test temperature is attained.

are employed, and the values of rate constants for thermal death at each test temperature, as shown in Table 2, are also nearly equal to those calculated from Fig. 7. That is, no difference is found in the response to heat between dried and undried conidia when the temperature of test suspension is raised gradually. This may be explained in the same way as in the preceding discussion. The amount of "surface water" of undried conidia introduced into 20 ml of liquid paraffin is only 0.07 mg and very small compared with the solubility of water in liquid paraffin at room temperature. Therefore, the "surface water" may completely dissolve into liquid paraffin before the temperature at which the thermal

Table 2. Rate constants for thermal death of dried and undried conidia in liquid paraffin.

Conidia	Thermal death rate constant $\times 10^2$, (min^{-1})			
	85.9°C	88.6°C	91.2°C	94.0°C
Dried	1.1	2.1	4.6	8.9
Undried	1.2	2.4	4.6	10.0

The temperature was raised gradually as described in the text.

death of conidia takes place is attained. The response of undried conidia to heat, after the "surface water" is thus eliminated from the conidial surface, may be the same as that of dried conidia, since it was confirmed by an additional experiment using water-saturated liquid paraffin that the water once dissolved into liquid paraffin has no more influence on the thermal resistance of conidia.

The above findings suggest practical procedures for the sterilization of liquid paraffin by heat. It is recommended not only that the liquid paraffin is saturated with water at the desired temperature for the sterilization but also that a small amount of free water is present on the conidial surface. On the other hand, an instantaneous elevation of temperature may be also effective in killing the natural contaminants present in liquid paraffin, since some of them may have "surface water" as in the case of fungal conidia employed in this experiment.

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