RESPONSE OF *BACILLUS SUBTILIS* SPORES TO DEHYDRATION AND UV IRRADIATION AT EXTREMELY LOW TEMPERATURES

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Abstract. Spores of *Bacillus subtilis* have been exposed to the conditions of extreme dehydration (argon/silica gel; simulated space vacuum) for up to 12 weeks at 298 K and 80 K in the dark. The inactivation has been correlated with the production of DNA-double strand-breaks. The temperature-dependence of the rate constants for inactivation or production of DNA-double strand-breaks is surprisingly low. Controls kept in the frozen state at 250 K for the same period of time showed no sign of deterioration. In another series of experiments the spores have been UV irradiated (253.7 nm) at 298 K, 200 K and 80 K after exposure to dehydrating conditions for 3 days. Fluence-effect relationships for inactivation, production of DNA-double strand-breaks and DNA-protein cross-links are presented. The corresponding F_{37}-values for inactivation and production of DNA lesions are significantly increased only at 80 K (factor of 4 to 5). The data indicate that the low temperatures that prevail in the outer parts of the Solar System or at the nightside of Mars or the Moon are not sufficiently low to crucially inhibit inactivation by dehydration. Our data place further constraints on the panspermia hypothesis.

1. Introduction

In 1985 Weber and Greenberg reported that the UV photosensitivity of *Bacillus subtilis* spores is extremely low under the simulated conditions of interstellar space (high vacuum and only a few K). Their conclusion was that spores may survive in interstellar space for millions to tens of millions of years. However, the major problems of *panspermia* are, as the authors properly state, not only the survival in interstellar space, but rather the survival during ejection from the remote life supporting source and the ultimate deposition on Earth. Matter arriving from outer space at the outskirts of the solar system would still have to travel for many, perhaps for hundred thousands of years at ambient temperatures above 50 K before reaching the Earth. During this part of their journey spores or other resistant organisms would also uninterruptedly be threatened by all kinds of space hazards including solar UV irradiation and dehydration processes.

There have been several studies on the UV sensitivity of cells or spores in aqueous environments at temperatures above –200 °C (about 70 K). Most remarkable are the early data presented by Ashwood-Smith *et al.* (1965, 1967) on the temperature dependence of the photosensitivity *of Escherichia coli* cells. These authors have reported that the photosensitivity of an aqueous suspension of *E coli* cells is lower at 21 °C than at –79 °C. Subsequently Smith and O'Leary (1967) could demonstrate that frozen *E. coli* cells are less photosensitive at –196 °C than at –79 °C and

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that the rate of photoinduced killing correlates with the production of DNA-protein cross-links, whereas the rate of thymine dimer decreases with the temperature. This latter observation is in line with the results obtained by Rahn and Hosszu (1968) who have reported that the photoinduced formation of thymine dimers in isolated *E. coli* DNA is inhibited at low temperatures. In addition to *E. coli* cells a variety of other microorganisms including spores and conidia have been investigated regarding to their photosensitivity. Spores of *Bacillus subtilis* have been reported to show a hypersensitivity at –80 °C, but their photosensitivity at –200 °C is much lower than that at 21 °C, whereas the photosensitivity of *Deinococcus radiodurans* decreases progressively at lower temperatures (Ashwood-Smith *et al.*, 1968; Ashwood-Smith and Horne, 1972). In all these cases the photosensitivity strongly decreases below –80 °C. So far the different response of cells to UV irradiation at low temperatures is not well understood. Also other DNA damages, besides DNA-protein cross-links, may significantly contribute to a loss in viability.

There are also numerous reports on survival or preservation of *frozen* cells at –200 °C to –130 °C (about 70 K to 140 K) in the dark (see the review by Mazur, 1980). No reports, however, exist on the survival or inactivation of cells by dehydration during long-term exposure to extreme dryness (including simulated space vacuum) at these low temperatures (that are also common within the Solar System), nor has the UV response of cells ever been tested under these conditions.

Recently we have analyzed the inactivation of anhydrobiotic microorganisms by dehydration during exposure to actual and simulated space vacuum at room temperature (Dose *et al.*, 1991; Dose and Gill, 1995; Dose *et al.*, 1995). Here we report the response of *Bacillus subtilis* spores to the combined effects of dehydration and UV irradiation at temperatures between room temperature and about 80 K (about –190 °C).

2. Experimental

2.1. Analysis of DNA damages

Spores of *Bacillus subtilis* strain TKJ 3412 have been used in this work. The DNA of this strain is most readily labeled with [methyl–^{14}C] thymidine (see Dose and Gill, 1995). Also the preparation of spores, the viability tests, the isolation of DNA from spores, the determination of DNA-protein cross-links by a membrane filter method, the one-dimensional pulsed-field gel electrophoresis (ODPFGE), the evaluation of DNA fractions and the determination of DNA- double strand-breaks per chromosome have been described in a previous report (Dose and Gill, 1995).

2.2. Exposure of spores

For exposure and UV irradiation, aliquots of spores were transferred to glass disks (about 5×10^5 spores per mm^2, yielding a half statistical monolayer) and predried

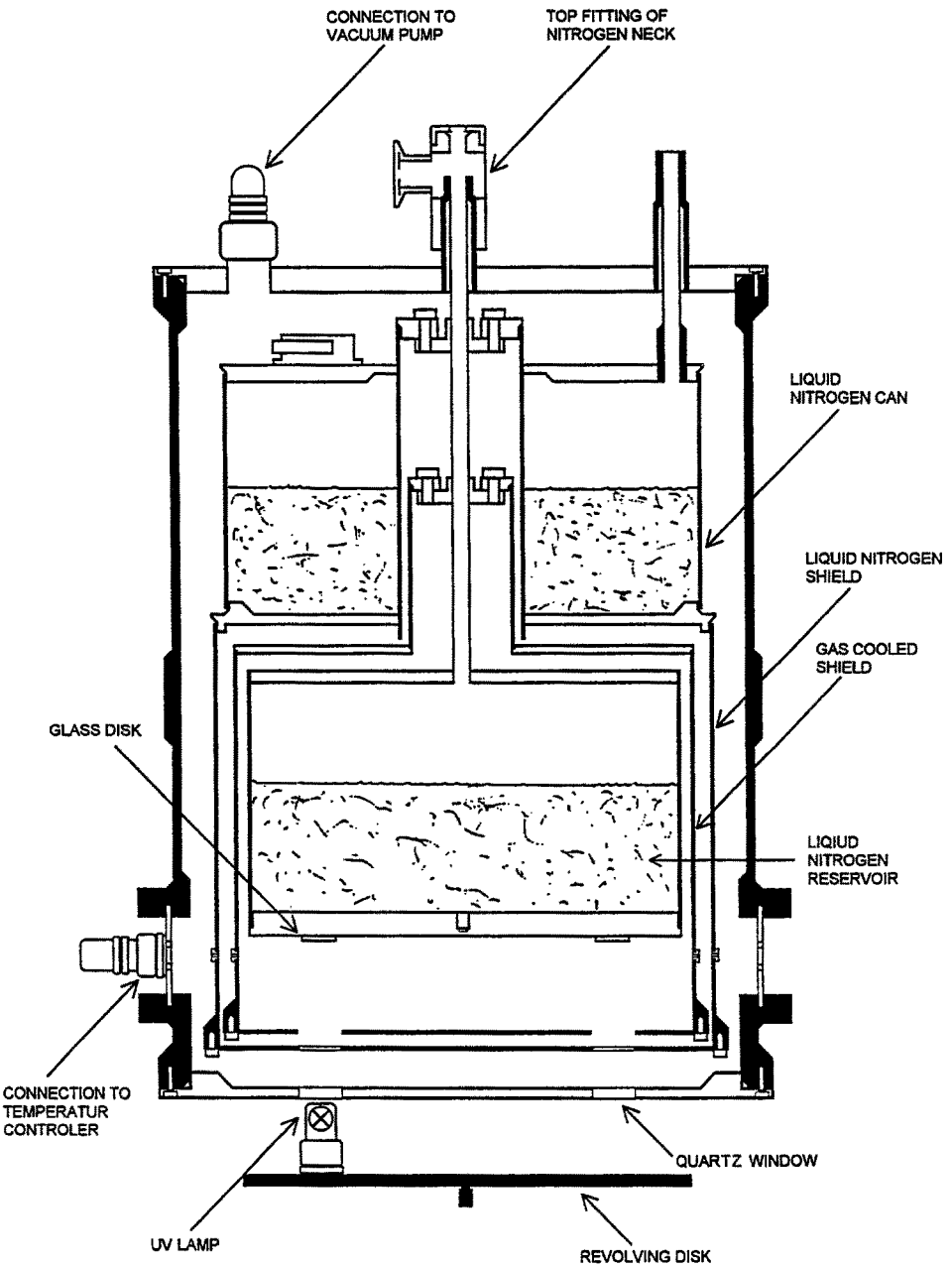

Fig. 1. Cryostat used for exposure and UV irradiation experiments at about 80 K. The glass disks carrying the statistical half monolayers of spores were attached upside down to the bottom of the liquid nitrogen reservoir. The UV irradiations were performed through quartz windows at the bottom of the cryostat. The outer diameter of the cryostat is about 284 mm.

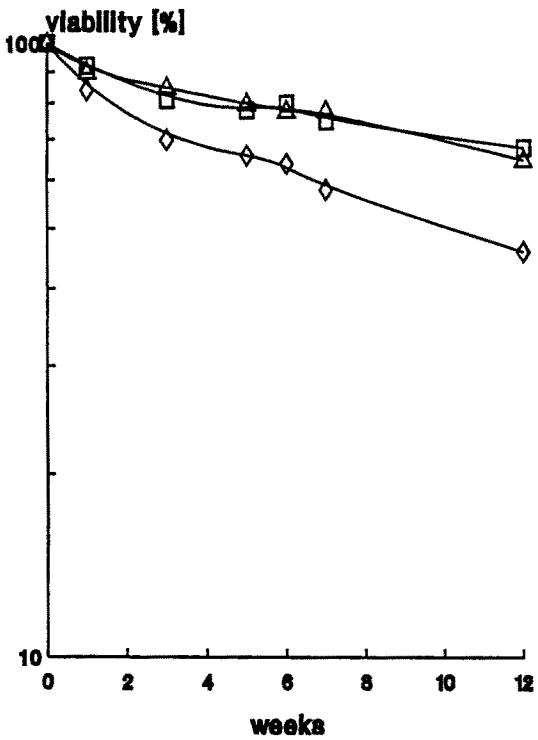

Fig. 2. Loss in viability (in the dark) during long-term exposure of spores to dehydrating conditions. Explanation of symbols: —Δ—: Exposure at 293 K under dried argon; —◇—: Exposure under vacuum at 293 K; —□—: Exposure under vacuum at 80 K. In comparison: The viability of spores kept frozen at 253 K in aqueous suspension remained at 100%.

in air (at about 70% relative humidity). The glass disks were then exposed to silica-dried argon (at 1000 mbar and below 5% relative humidity) for several weeks at 298 K (room temperature). In another series of experiments the glass disks were subsequently exposed to a dynamic pump vacuum (3×10^{-6} mbar; maintained in essence by a continuously running turbomolecular pump) at 298 K and 80 K (liquid nitrogen; see 2.3. for more details).

2.3. UV IRRADIATION OF SPORES AND UV DOSIMETRY

The UV irradiations under argon were essentially carried out as described earlier by Ashwood-Smith and Bridges (1965) with the modification that half statistical monolayers of dehydrated spores were irradiated. 200 K was maintained by methanol/dry ice and 80 K by liquid nitrogen. Vacuum exposure and subsequent UV irradiation at room temperature were carried out in a vacuum desiccator covered by a 15 mm quartz plate. Vacuum exposure and UV irradiation at the temperature of liquid nitrogen were executed in an *Infrared Detector Cryostat* (MD 1840, Oxford,UK). A cross-section of the cryostat is shown in Figure 1. The samples

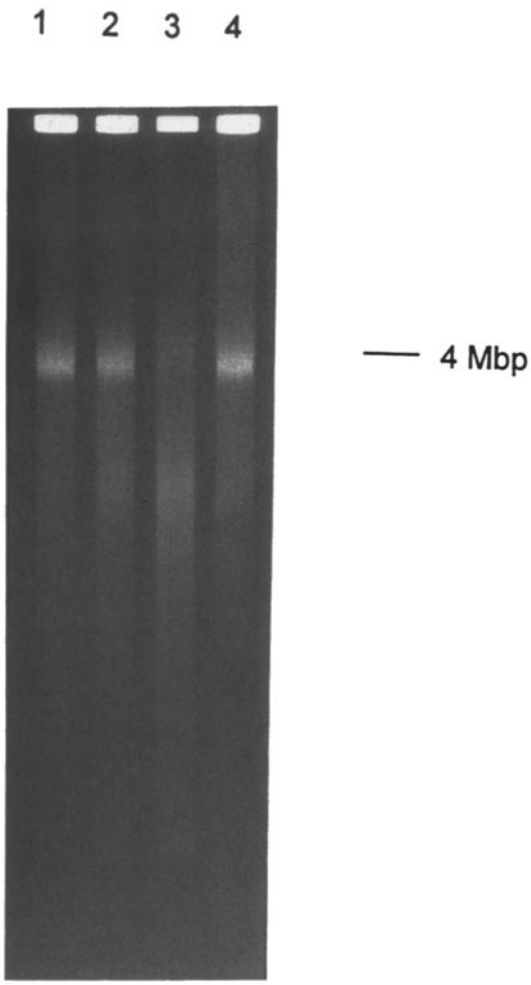

Fig. 3. ^{14}C-autoradiogram of a one-dimensional pulsed-field electrophoresis gel with DNA from differently exposed spores. Lane 1: DNA from spores kept frozen at 253 K in aqueous suspension for 12 weeks; Lane 2: DNA from spores kept at 293 K under dry argon for 12 weeks; Lane 3: DNA from spores kept at 293 K under vacuum for 12 weeks; Lane 4: DNA from spores kept at 80 K under vacuum for 12 weeks. In the course of dehydration and subsequent induction of DNA-double strand-breaks also the amount of circular DNA decreases. This circular DNA does not migrate. It is retained at the origin (black bars at the top). Its decrease cannot be visualized by the present photography, though this decrease has been measured by quantitative evaluation of the gel sections with a liquid scintillation spectrometer. The amount of the 4 Mbp fraction is controlled by its rate of formation (due to the linearization of the circular chromosome by one double strand-break) and its degradation (by additional double strand-breaks).

were attached upside down to the bottom of the lower liquid nitrogen reservoir. The UV irradiations were performed through quartz windows at the bottom of the

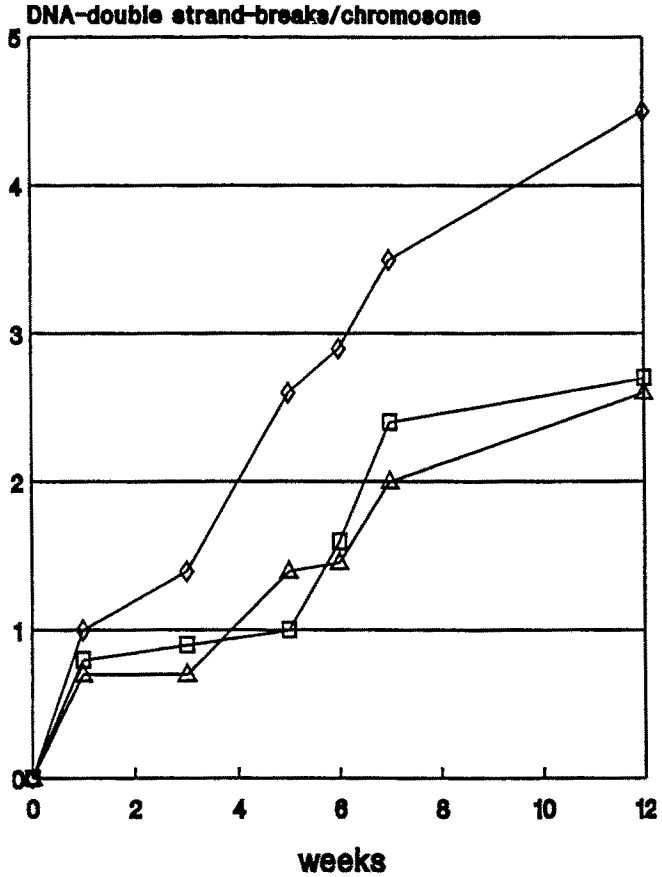

Fig. 4. Increase in the number of DNA-double strand-breaks per chromosome (in the dark) during long-term exposure to dehydrating conditions. Explanation of symbols: —△—: Exposure at 293 K under dried argon; —◇—: Exposure under vacuum at 293 K; —□—: Exposure under vacuum at 80 K.

cryostat. The temperature at the glass disks was always measured electrically by means of a calibrated resistance sensor.

The spores were UV irradiated after 3 days of exposure to the indicated conditions with a Philips low pressure mercury lamp (92,5% of the light energy within the range 100 to 380 nm is UV light of the wavelength 253.7 nm). The UV actinometry was performed according to Johns (1968). The method involves monitoring the decrease in extinction at 265 nm that is caused by the photoaddition of water to the 5, 6 double bond of 1, 3-dimethyluracil. The quantum yields published by Rahn and Sellin (1979) were used for calculating the fluence rates. The fluence rates applied during our experiments were about 1 Wm^{-2}.

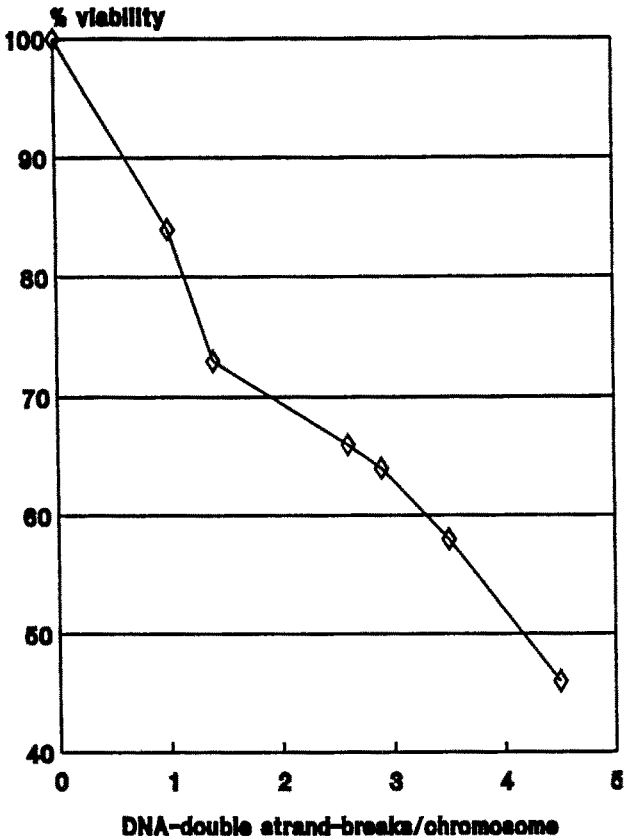

Fig. 5. The viability of spores decreases reciprocal to the occurrence of DNA-double strand-breaks analyzable by one-dimensional pulsed-field gel electrophoresis. A correlation for exposure at 293 K under vacuum is shown here. Congruent curves (within an error of ±15%) have been obtained both for exposure at 80 K under vacuum and at 293 K under argon.

3. Results and Discussion

3.1. Inactivation by Exposure to Extremely Dry Conditions in the Dark

The loss in viability of spores during exposure to dehydrating conditions is shown in Figure 2. At about 80 K under vacuum the loss in viability is only inhibited by about 40% in comparison to room temperature. Incidentally, there is no significant difference between the response to argon exposure at room temperature and vacuum exposure at 80 K. This temperature-induced inhibition of inactivation under *dehydrating* conditions, however, is to be distinguished from the preservation of *frozen* biological materials at the temperature of liquid nitrogen, a technique that allows preservation of cells or tissues for long periods of time (see, e. g., Mazur,

1980, 1976). The low temperature preservation is largely achieved by the inhibition of diffusion-controlled bimolecular reactions. Under our present conditions, however, the dryness-induced intramolecular dehydration reactions evidently proceed *within* the DNA-double helix. These reactions are not diffusion-controlled and exhibit a relatively small temperature-dependence of rate constants.

Earlier we have demonstrated that dehydration processes also induce cross-links between DNA and proteins in cells, conidia and spores (Dose *et al.*, 1991, Bieger-Dose *et al.*, 1992, Dose *et al.*, 1992). This cross-linking effect is relatively small in the case of spores (Dose and Gill, 1995): Isolated DNA from fresh spores exhibits about one statistical DNA-protein cross-link per Mbp. In the case of DNA from spores that have been exposed to dry argon or vacuum for about three weeks this amount is maximally increased by a factor of two or three. In the course of our present studies we could neither observe any further increase, nor state any significant temperature effect (between 80 K and 293 K) during exposure times of up to 12 weeks.

The loss in viability of spores by dehydration largely depends on the increase in the number of analyzable DNA-double strand-breaks per chromosome (Dose and Gill, 1995). In comparison to exposure under vacuum at 293 K the relative amount of DNA-double strand-breaks appearing after exposure at 80 K is only about 50%. Figure 3 shows an electrophoresis gel (autoradiogram) depicting the fractionation of chromosomal DNA by double strand-breaks under different exposure conditions. From the data obtained by evaluating these gels the number of DNA-double strand-breaks per chromosome has been calculated (Dose and Gill, 1995). Figure 4 shows the increase of DNA-double strand-breaks in the course of long-term exposure at room temperature and at 80 K. A correlation between DNA-double strand-breaks and loss in viability is suggested by Figure 5. Only the data for vacuum exposure at room temperature are shown. The corresponding curves for argon exposure at room temperature and vacuum exposure at 80 K are congruent within an error range of ±15%, though they end at about 70% survival.

3.2. Inactivation by the combined effects of dehydration and UV light

Figure 6 shows the fluence effect curves for loss in viability of *B. subtilis* spores by UV irradiation under argon or vacuum at 298 K and 80 K. The fluence effect curve for inactivation under argon at 200 K almost matches the corresponding curve for 298 K. The curve taken at 200 K is therefore not shown here for simplicity.

The resulting F_{37}-values are summarized in Table I.

Figures 7 and 8 demonstrate that both the relative yields for the formation of DNA-double strand-breaks and those for the production of DNA-protein cross-links are significantly lowered if the UV irradiation is carried out at the temperature of liquid nitrogen. The differences are not conspicuous for fluences under 100 Jm^{-2}, because of a relatively high experimental error. However, they become very pro-

TABLE I

F_{37}-values [Jm^{-2}] for the UV inactivation (at 253.7 nm) of *B. subtilis* spores under various conditions

Temperature [K]	Vacuum (3×10^{-6} mbar) F_{37}-values	Dry argon F_{37}-values
298 K	50	48
200 K	not determined	42
80 K	230	220

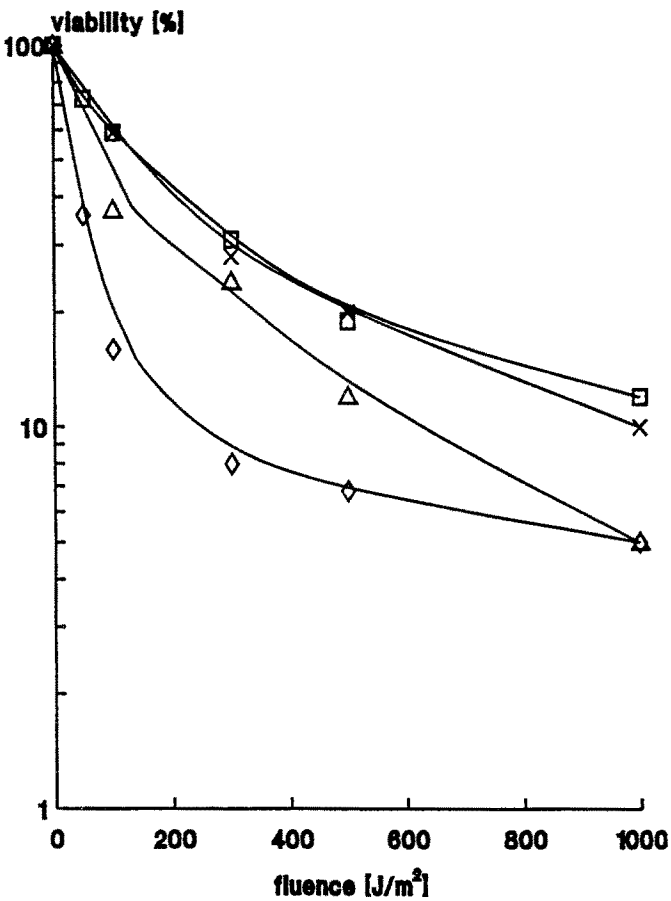

Fig. 6. Fluence effect curves for the loss in viability by irradiation of spores with UV light of 254 nm after exposure to dry argon or vacuum for 3 days at 80 K or 293 K. The UV irradiations were carried out under the conditions of exposure. Explanation of symbols: —△—: Dry argon at 293 K; —×—: Dry argon at 80 K; —◇—: Vacuum at 293 K; —□—: Vacuum at 80 K.

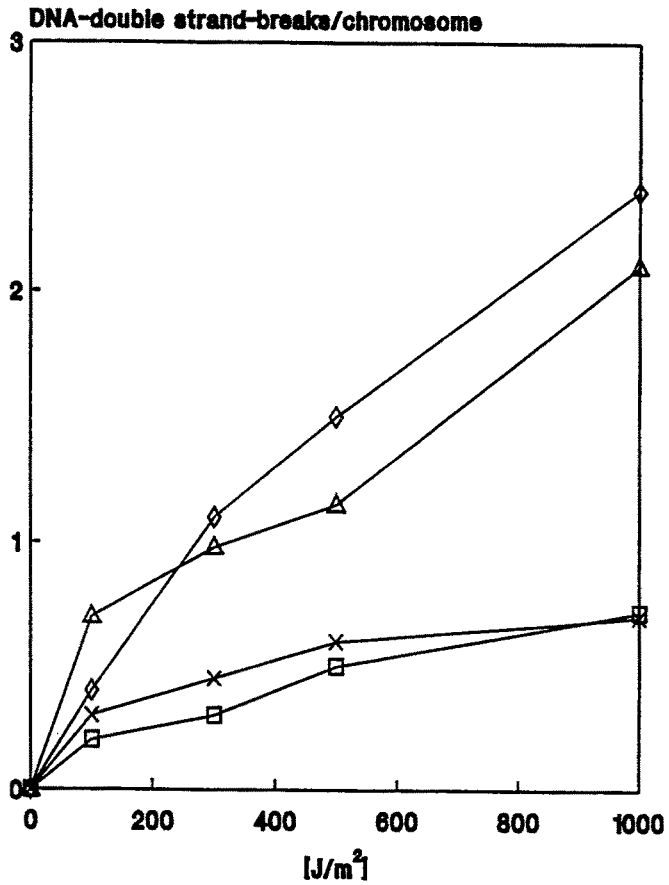

Fig. 7. Fluence effect curves for the production of DNA-double strand-breaks per chromosome by irradiation of spores with UV light of 254 nm after exposure to dry argon or vacuum for 3 days at 80 K or 293 K. The UV irradiations were carried out under the conditions of exposure. Explanation of symbols: —Δ—: Dry argon at 293 K; —×—: Dry argon at 80 K; —◇—: Vacuum at 293 K; —□—: Vacuum at 80 K.

nounced for higher fluences. The data suggest that both the temperature-dependent inhibition of double strand-break and cross-link formation are responsible for the higher UV resistance at 80K. These results appear to contradict earlier data presented by Smith and O'Leary (1967) on the UV irradiation of *frozen* (aqueous) *E. coli* cells. The latter authors reported both an increase in UV photosensitivity and in cross-link formation at 77 K. They explained this effect on the basis of freezing phenomena. In the case of *dehydrated* spores, however, such phenomena do not exist because of the absence of freezable water. The general inhibition of chemical reactions, including radical reactions, at lower temperatures is likely responsible for the higher UV resistance of dried spores at 80 K.

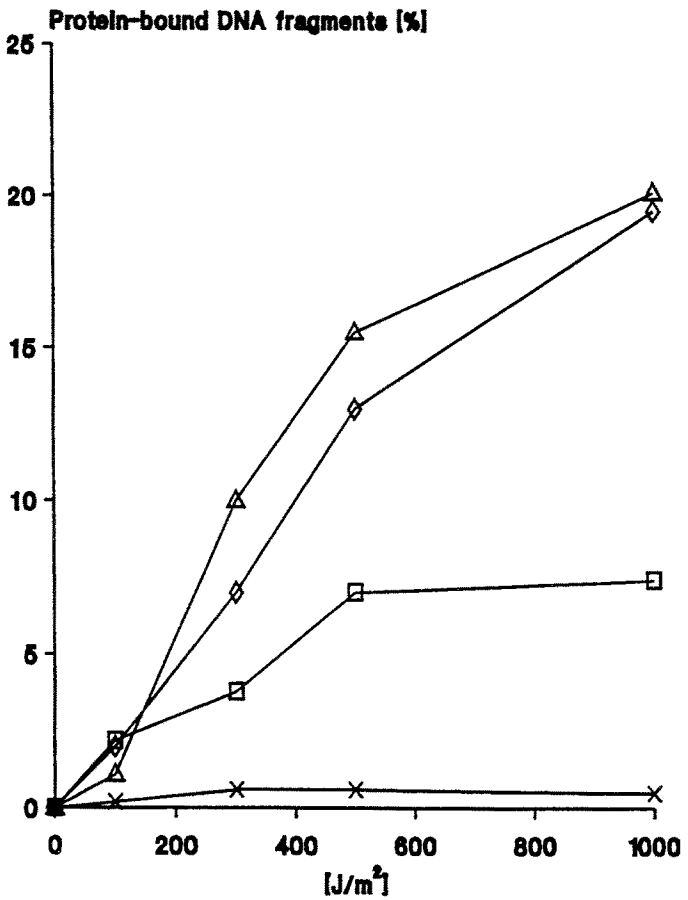

Fig. 8. Fluence effect curves for the increase in the relative amount of chromosomal ~50 kbp DNA fragments cross-linked to proteins by irradiation of spores with UV light of 254 nm. The total amount of chromosomal DNA is 100%. The cross-linking of chromosomal DNA to proteins is a primary UV effect. During extraction of the DNA from spores the DNA is ruptured by mechanical stress. The resulting DNA fragments have an average size of about 50 kbp. Fragments covalently cross-linked to proteins can be separated from 'free' DNA fragments by the membrane filter method mentioned in the text. The UV irradiations were carried out after exposure to dry argon or vacuum for 3 days at 80 K or 293 K as specified. Explanation of symbols: —Δ—: Dry argon at 293 K; —×—: Dry argon at 80 K; —◇—: Vacuum at 293 K; —□—: Vacuum at 80 K.

4. Conclusions

The present data on the inactivation of *Bacillus subtilis* spores by exposure to dehydrating conditions (dry argon or vacuum) reveal that the exposure time for 50% inactivation at room temperature is about 9 weeks and at 80 K presumably still about 20 weeks. With respect to exobiology the implication is that unprotected spores would be completely inactivated within a few decades both, if exposed to space vacuum at room temperature (Dose and Gill, 1995) or at the extremely low

temperatures found at the outskirts of the Solar System. The F_{37}-values for UV inactivation of dehydrated spores at 80 K are only increased by a factor of ~4. Solar radiation would therefore continue to be a lethal threat to spores at 80 K. These data seriously restrict the survival chances of unprotected spores while being transported through the Solar System and therefore further limit the validity of the *panspermia* thesis (Arrhenius, 1913; Weber and Greenberg, 1985). In view of our present data appreciable chances of survival could only be maintained by inhibiting any dehydration or decomposition process. This could be achieved by embedding spores or other cells in huge icy masses at temperatures below 140 K. This is the critical temperature for freezing out all liquid water. Below this temperature the rate of all diffusion-controlled chemical reactions is practically zero. (Mazur, 1980). The size of such icy masses would have to be comparable to the size of the Halley comet, because evaporation processes would lead to huge losses of matter during the long journey from a remote planet. In addition, sufficient shielding from cosmic radiations is to be provided by a thick cover of matter. The source of such a cosmic 'iceberg' could be seen in the glaciers or permafrost areas of another life-bearing planet. Terrestrial glaciers provide various habitats for life. A well-investigated example is represented by the cryoconite holes (Wharton *et al.*, 1985). Also in permafrost viable microorganisms have been detected (Gilichinsky *et al.*, 1993). A cosmic body (comet or meteorite) may have collided with the life-bearing ice cover of a remote planet several billion years ago and lifted off huge amounts of frozen masses. A fraction of these icy masses may have finally reached the early Earth and implanted life here. We realize, however, that the probability of such a life transfer process is extremely small. Moreover, we see no significant advantage in moving the origin of life to a remote planet. The timescale for chemical evolution and subsequent development of primitive procaryotes on an ancient planet could have been a few times larger than the 200 to 400 Myr possibly available for the evolution of microbial life on Earth. On the other hand, however, an upper limit for such a timescale is set by the age of the Universe (probably 10,000 to 15,000 Myr) and the time required to produce an early planetary system being sufficiently rich in bioelements (probably several 1,000 Myr). Nevertheless, the time available for the evolution of first procaryotes on an ancient planet could have reached the order of 1,000 Myr. This advantage, however, is largely counterbalanced by the long time required for transportation of spores through space (several Myr or more) and by the general risks of such a transport.

After examining all these imponderables we realize that our data put further constraints on the panspermia thesis and therefore encourage the search for the origin of life on Earth.

Acknowledgements

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