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# Genetic Factors in Radiation Resistance of *Bacillus subtilis*[1]

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ABSTRACT

ZAMENHOF, STEPHEN (University of California, Los Angeles), HELA BURSZTYN, T. K. RAMACHANDRA REDDY, AND PATRICE J. ZAMENHOF. Genetic factors in radiation resistance of *Bacillus subtilis*. J. Bacteriol. **90**:108–115. 1965.—A study of several wild cross-transformable strains of *Bacillus subtilis* revealed differences in the resistance of their spores to X rays. Closer study of two such strains revealed differences of the same type when vegetative cells were exposed to X rays or to ultraviolet light (UV). Cell cultures repeatedly exposed to sublethal doses of UV (with cultivation between exposures) became more resistant to UV, presumably by enrichment in a more UV-resistant mutant. A sulfanilamide-resistant mutant of one strain (vegetative cells and spores) was less resistant to ionizing radiation; this sensitivity was transferable by transformation. No difference in radiation-induced mutability could be demonstrated in any of the strains studied. It is concluded that, at least in the cases studied, (i) the differences in radiation resistance of spores of different strains are not just a result of a superimposition of a common spore resistance mechanism(s) but rather are an amplification of genetically determined resistance differences in vegetative cells of these strains; (ii) sulfanilamide-resistance locus (*p*-aminobenzoic acid overproduction locus) is one of the loci of radiation sensitivity; (iii) no evidence was obtained that the differences in radiation resistance of cells or spores can be ascribed to differences in radiation resistance of their deoxyribonucleic acid.

Despite a large amount of work on the subject of the high resistance of spores to radiation, the cause of this phenomenon is still not clear. The main object of the present work was to determine which of the following two mechanisms is of more importance for this resistance: (i) a common mechanism of high resistance in spores (such as the appearance of cystine-rich structures; Vinter, 1961) that would be superimposed on the low resistance of vegetative cells and obliterate any possible genetic differences in resistance of cells of various strains or mutants, or (ii) a mechanism that merely amplifies the resistance of vegetative cells and allows retention of these genetic differences. In the course of this work, a locus influencing sensitivity to radiation was also found, and the transfer of this sensitivity by transforming deoxyribonucleic acid (DNA) was accomplished. Enrichment in what appear to be mutants of higher resistance to ultraviolet light (UV) was also achieved by repeated irradiation and cultivation.

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## MATERIALS AND METHODS

*Bacterial strains. Bacillus subtilis* strain 23 (wild) and 168 *ind*$^-$ were those used in previous studies (Zamenhof, 1960; Ephrati-Elizur, Srinivasan, and Zamenhof, 1961). The revertants 168 *ind*$^+$ and 168 *ind*$^+$/*SA* (sulfanilamide resistant, first obtained in this laboratory by Sheldon Greer) were spontaneous mutants. Strain T168 *ind*$^+$/*SA* was a transformant of strain 168 *ind*$^+$ to sulfanilamide resistance, obtained by means of DNA extracted from strain 168 *ind*$^+$/*SA*; all the sulfanilamide-resistance strains were *p*-aminobenzoic acid (PABA) - overproducers (Zamenhof and Heldenmuth, 1963). Strains ATCC 6051, 6455, 7058, 7059, 8188, 9466, 9524, and 12432 were obtained from the American Type Culture Collection.

Vegetative cells for X-ray and UV irradiation were obtained by inoculating an overnight culture into 20-ml portions of Penassay Broth (Difco; 1 loopful of culture per 20-ml of broth). Cultures were grown at 37 C with aeration for 5 hr, centrifuged, and suspended in 20 ml of 1% saline; this suspension was centrifuged again, and again suspended in 1% saline so as to obtain $10^6$ to $10^8$ cells per milliliter.

Spores for X-ray irradiation were obtained by streaking each culture on five potato-agar or Blood Agar Base (Difco) slants and incubating for 48 hr at 37 C and for 14 days at room temperature. The spores were harvested by gently scraping and washing out each slant with 1 ml of distilled water. The suspensions were combined and heated at 80 C for 25 min to kill vegetative cells; they were then centrifuged, suspended in 5 ml of distilled water, again centrifuged, and finally suspended in 5 ml of distilled water and stored at 4 C. Before irradiation, the suspensions were diluted in 1% saline to obtain concentrations appropriate for the survival intended so that 0.1 ml of the irradiated suspension, spread on Penassay agar, yielded 100 to 500 colonies.

*Sources.* X rays (unfiltered) were supplied by a 250-kv X-ray dual tube instrument; the sample dish was placed between the two tubes at a distance of 10 cm from each tube (180 kr per 30 min).

An electron beam was supplied by a 6 Mev linear accelerator ($10^6$ rad in approximately 5 min).

UV was supplied by an 8-w General Electric germicidal lamp G-8 at a distance of 75 cm (686 ergs per sec per $cm^2$).

*X-ray or electron beam irradiation of vegetative cells.* Amounts of 1 to 4 ml of the cell suspensions described above were irradiated in an open plastic petri dish, 5 cm in diameter. Immediately after irradiation, 0.1-ml samples of the suspension (diluted or undiluted) were spread on Penassay agar and incubated for 24 hr at 37 C.

In the case of strains 168 *ind*$^+$/*SA* and 168 *ind*$^+$, or T168 *ind*$^+$/*SA* and 168 *ind*$^+$, the strains were irradiated individually, and also as mixtures of any two strains compared; the latter procedure assures the same conditions of irradiation. To achieve this, just before irradiation equal volumes of suspensions of cells of strains to be compared were mixed, and the mixture was irradiated. The surviving colonies of each strain were scored by spreading on basal agar (Spizizen, 1958) with or without 250 $\mu$g of sulfanilamide per ml.

Because of the known tendency of vegetative cells of *B. subtilis* to lyse if not aerated, the cells (both controls and irradiated) were constantly shaken, except for the short periods corresponding to irradiation.

*X-ray or electron beam irradiation of spores.* Irradiation of spores was performed under similar conditions, with 1 ml of spore suspension (without shaking).

*UV irradiation of vegetative cells.* UV irradiation was performed essentially as described previously (Greer, 1960); 3 ml of cell suspension (see Materials and Methods) were used for each irradiation. After irradiation, 0.1-ml samples were spread on Penassay agar, and colonies were counted after 48 hr of incubation at 37 C. As indicated above, because of the known tendency of *B. subtilis* to lyse, consistent results in comparison of strains 23 and ATCC 9466 were obtained only when the two strains were irradiated simultaneously. To achieve this, just before irradiation equal volumes of suspensions of cells of these strains were mixed, and the mixture was irradiated. The surviving colonies of each strain were scored by taking advantage of the difference in colonial morphology after 48 hr of incubation in Penassay agar. All operations were performed under dim yellow light.

*Enrichment in UV-resistant mutants.* The cells were irradiated as above, with a UV dose which resulted in 8 to 12 surviving colonies per plate. All survivors were spread together on Penassay agar and incubated for 24 hr at 37 C; this growth served as inoculum for the Penassay Broth culture for next UV irradiations. This procedure was repeated four times; the UV resistance of such cultures obtained after four UV irradiations, and intermediate cultivations as above, was compared with the UV resistance of the original strain.

*Transformation.* The methods for growing cells, extraction of DNA, and transformation were described previously (Spizizen, 1958; Ephrati-Elizur et al., 1961; Zamenhof, De Giovanni-Donnelly, and Heldenmuth, 1962).

*Induced mutability.* The electron beam-irradiated spores that germinated were scored for the presence of auxotrophs (total number) by the methods previously described (Zamenhof, 1960, 1961).

*Pseudoauxotrophy.* Pseudoauxotrophy (phenotypic failure to grow on basal medium) after electron beam irradiation was scored as previously described (Zamenhof, 1960). The medium was used with or without L-alanine (50 $\mu$g/ml).

*Effect of PABA on germination.* The effect of PABA was investigated by spreading spores of strain 168 *ind*$^+$, nonirradiated or irradiated with 470 krad, on basal agar containing 100 $\mu$g of PABA per ml; plain basal agar served as control.

*Irradiation in presence of PABA.* Spores of strain 168 *ind*$^+$ were electron beam-irradiated as described above except that the saline contained 100 $\mu$g of PABA per ml.

## RESULTS

*X-ray irradiation of spores of various strains.* Comparison of survival of spores of various strains at a dose 360 kr revealed differences in their resistance to X rays (Table 1). Two strains that differed considerably, strain 23 and strain ATCC 9466, were selected for more detailed study. This study (Fig. 1) revealed that (i) the inactivation curves are essentially exponential (i.e., the differences in log survival are proportional to the dose), and (ii) strain 23 is more

TABLE 1. *Comparison of survival of spores of various strains of Bacillus subtilis irradiated with X rays (360 kr)*

| Strain no. | Surviving fraction |
|---|---|
| 23 | $10^{-2}$ |
| ATCC 6051 | $2.3 \times 10^{-2}$ |
| ATCC 6455 | $4.7 \times 10^{-3}$ |
| ATCC 7058 | $4.4 \times 10^{-3}$ |
| ATCC 7059 | $6.1 \times 10^{-3}$ |
| ATCC 8188 | $2.5 \times 10^{-2}$ |
| ATCC 9466 | $5 \times 10^{-4}$ |
| ATCC 9524 | $7.5 \times 10^{-3}$ |
| ATCC 12432 | $9 \times 10^{-3}$ |

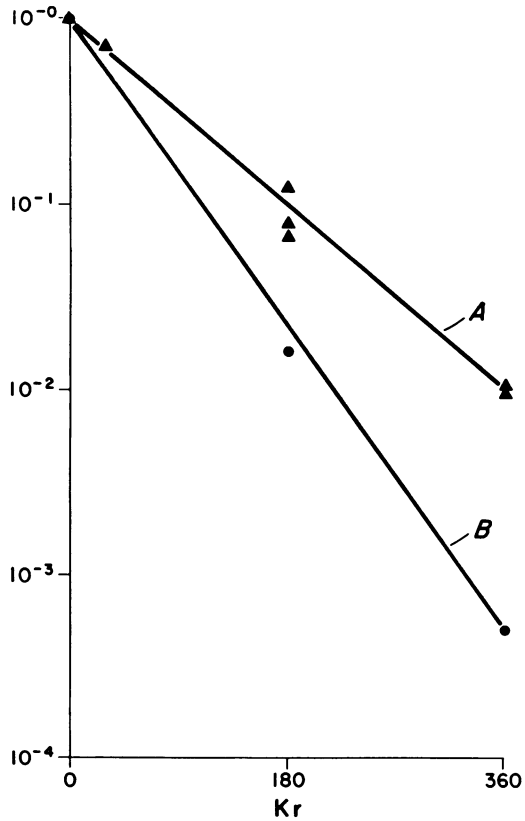


FIG. 1. *Comparison of resistance of spores of two strains of Bacillus subtilis to X-ray irradiation. Curve A, strain 23; curve B, strain ATCC 9466. The abscissa indicates dose; the ordinate, surviving fraction.*

resistant than strain ATCC 9466 at all doses studied. The ratio of survival of the two strains as a function of the survival of strain ATCC 9466 is represented in Fig. 4, which serves to compare the results of this treatment with the other treatments (see below).

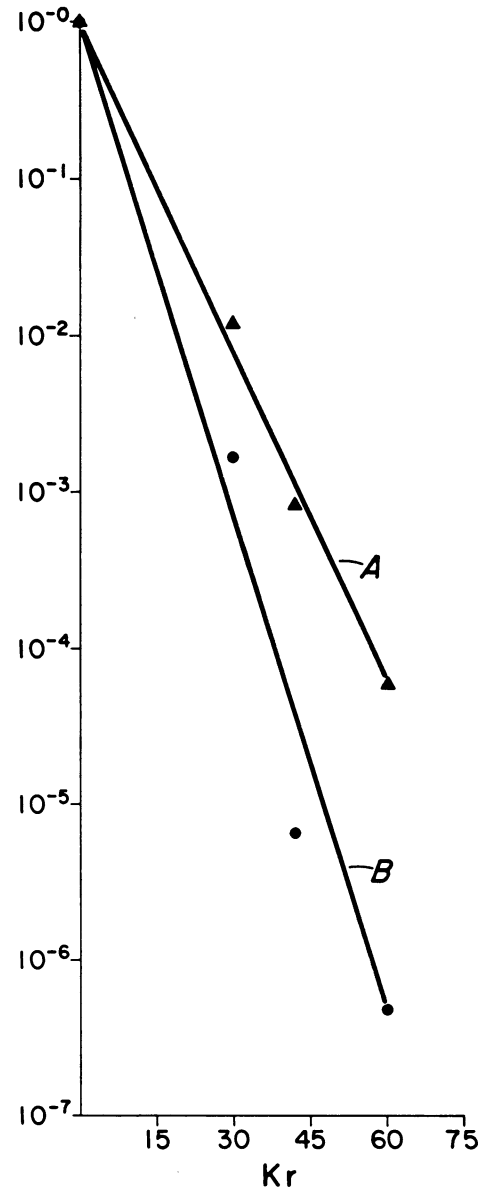


FIG. 2. *Comparison of resistance of vegetative cells of two strains of Bacillus subtilis to X-ray irradiation. Symbols as in Fig. 1.*

*X-ray irradiation of vegetative cells of strains 23 and ATCC 9466.* Differences found for vegetative cells (Fig. 2 and 4) paralleled differences found for spores; however, the differences in dose for a given survival were much smaller (Fig. 2).

*UV irradiation of vegetative cells of strains 23 and ATCC 9466.* The survival curves (Fig. 3) were concave, i.e., different from the exponential

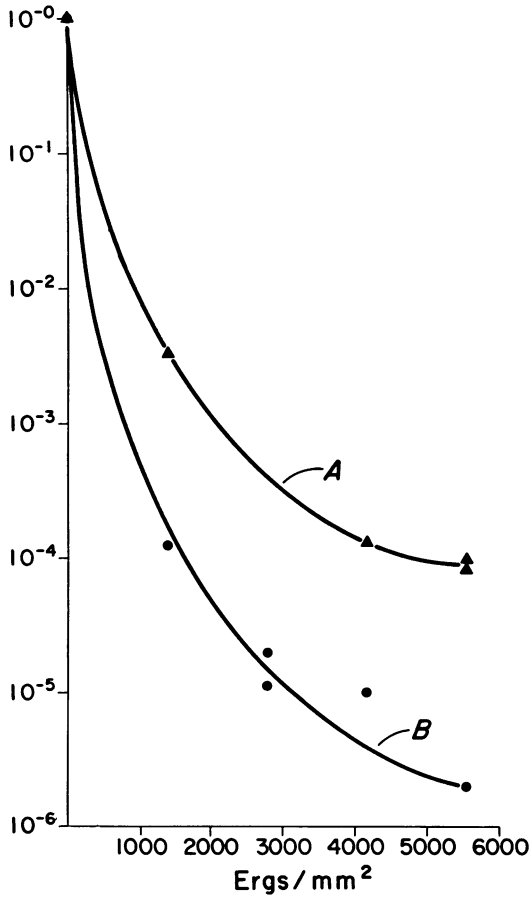


FIG. 3. *Comparison of resistance of two strains of Bacillus subtilis to UV irradiation. Symbols as in Fig. 1.*

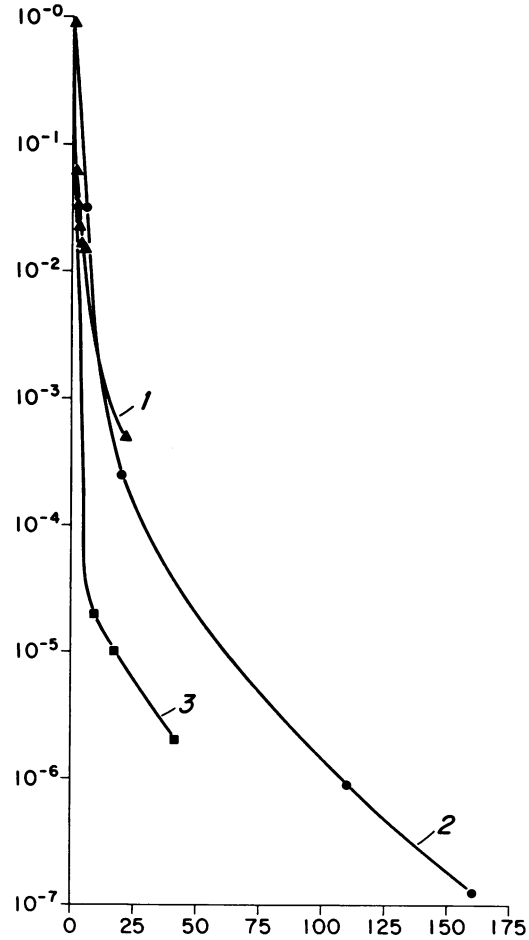


FIG. 4. *Comparison of resistance of two strains of Bacillus subtilis to irradiation. Curve 1, spores, X rays; curve 2, vegetative cells, X rays; curve 3, vegetative cells, UV. The abscissa indicates the ratio of survival of strain 23 over strain ATCC 9466; the ordinate, surviving fraction of strain ATCC 9466.*

curves obtained for X-ray irradiation of vegetative cells and spores; these results suggest different mechanisms of inactivation or repair. Nevertheless, for UV also, strain 23 was more resistant (Fig. 3 and 4).

When cultures of strain 23 were repeatedly exposed to sublethal doses of UV, with cultivation between exposures (see Materials and Methods), they became more resistant to this irradiation (Table 2).

*X-ray and electron beam irradiation of strains sensitive or resistant to sulfanilamide.* When the sulfanilamide-sensitive strain 168 *ind*$^+$, its sulfanilamide-resistant mutant *168 ind*$^+$/*SA*, and the parent strain transformed to sulfanilamide resistance (T168 *ind*$^+$/*SA*) by DNA from strain 168 *ind*$^+$/*SA* were irradiated by X rays or an electron beam, the sulfanilamide-sensitive strain was found to be more resistant to radiation than either sulfanilamide-resistant strain (Table 3).

TABLE 2. *Survival of vegetative cells of Bacillus subtilis strain 23 after repeated exposure to UV**

| Dose (ergs/mm$^2$) | No. of previous exposures to UV | |
|---|---|---|
| | 0 | 4 |
| 6,180 | $4.3 \times 10^{-6}$ | $4 \times 10^{-5}$ |
| 6,860 | $7.7 \times 10^{-7}$ | $10^{-5}$ |

* With intermediate cultivation. Results expressed as surviving fraction.

These results were obtained for both spores and vegetative cells.

*Transformation.* DNA extracted from the wild strain ATCC 9466 (donor) transformed strain

TABLE 3. *Survival of vegetative cells and spores of sulfanilamide-sensitive and sulfanilamide-resistant strains of Bacillus subtilis on X-ray and electron beam irradiation*

| Dose (kr)* | Vegetative cells | | | | | Spores | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Strain | | | Ratio of | | Dose | Strain | | Ratio of | |
| | 168 *ind*$^+$ | 168 *ind*$^+$/*SA* | T168 *ind*$^+$/*SA* | Survivals | *K*† | (krad)‡ | 168 *ind*$^+$ | T168 *ind*$^+$/*SA* | Survivals | *K*† |
| 60 | $4.75 \times 10^{-5}$ | — | $7.7 \times 10^{-6}$ | 6.2 | 0.846 | 470 | $7.4 \times 10^{-3}$ | $1.6 \times 10^{-3}$ | 4.6 | 0.764 |
| 60 | $1.43 \times 10^{-4}$ | — | $5 \times 10^{-5}$ | 2.9 | 0.894 | 940 | $5.4 \times 10^{-5}$ | $6.5 \times 10^{-6}$ | 8.3 | 0.821 |
| 72 | $1.25 \times 10^{-5}$ | $2.5 \times 10^{-6}$ | — | 5.0 | 0.874 | | | | | |

* X rays.

† Inactivation constant (Tallentine and Powers, 1963).

‡ Electron beam; average of six experiments. The survival of strain 23 did not differ significantly from the survival of strain 168 ind$^+$.

168 *ind*$^-$ (receptor) to 168 *ind*$^+$ with the same frequency as DNA from strain 168 *ind*$^+$ as donor. Strain ATCC 9466 was not active as a receptor in transformation to sulfanilamide resistance when DNA of strain 168 *ind*$^+$/*SA* was used as a donor.

*Induced mutability.* The proportion of total auxotrophs induced by a dose of 470 krad in all four strains tested was essentially the same (Table 4).

*Pseudoauxotrophy.* The recovery of irradiated spores on basal medium with and without L-alanine (as compared with Penassay medium) was not lower for the radiation-sensitive strain T168 *ind*$^+$/*SA* than for strain 168 *ind*$^+$ (Table 5). In the presence of L-alanine in basal medium, the recovery was close to 100% in all cases. The addition of 50 μg of L-alanine per ml to Penassay agar did not increase the recovery.

*Effect of PABA on germination.* Excess PABA did not essentially change the recovery of irradiated spores on basal agar.

*Irradiation in the presence of PABA.* The presence of PABA during irradiation did not reduce the survival. Instead, this presence resulted in a 2- to 2.5-fold higher survival than in the case of irradiation in saline alone (irradiation doses, 470 and 940 krad).

TABLE 4. *Mutants* produced by electron beam irradiation† of spores of strains of Bacillus subtilis differing in their radiation sensitivity*

| Strain | Per cent mutants‡ |
|---|---|
| 23 | 3.2 |
| ATCC 9466 | 2.3 |
| 168 *ind*$^+$ | 2.2 |
| T168 *ind*$^+$/*SA* | 2.5 |

* Total number of auxotrophs (average of three experiments). For experimental details see Zamenhof (1960).

† Dose, 470 krad.

‡ As per cent survivors. The figures for non-irradiated controls were 0.2 to 0.3% for strain 23 and 0.1% for strain 168 *ind*$^+$. The radiation resistance of spores of all auxotrophs scored (mixed) was not significantly different from that of spores of the parental strain ("reconstruction experiment") when irradiated together at 470 krad.

TABLE 5. *Recovery* of nonirradiated and irradiated† spores of Bacillus subtilis on basal and on Penassay agar*

| L-Alanine | 168 *ind*$^+$ | | T168 *ind*$^+$/*SA* | |
|---|---|---|---|---|
| | Non-irradiated | Irradiated | Non-irradiated | Irradiated |
| μg/ml | | | | |
| 0 | 38 | 24 | 18 | 33 |
| 50 | 97 | 91 | 81 | 90 |

* Assuming recovery on Penassay agar as 100%. (Average of three experiments.)

† Dose, 470 krad.

## DISCUSSION

In discussing the survival of vegetative cells and of spores, one must first admit that in these two cases the term may describe different phenomena. In the case of spores, survival involves not only the factor essential in both cases (e.g., the genome) but also that not involved in survival of vegetative cells as such, namely the mechanism of spore germination. In addition, survival in each case may be differently influenced by conditions of recovery, such as temperature (Powers, 1961) or presence of nutrients (Stapleton, Sbarra, and Hollaender, 1955). It was, therefore, not expected to find that the genetic differences between strains which confer marked differences in survival (resistance to radiation) in the spore

stage, are operative in a parallel manner in the vegetative-cell stage. For the two wild strains studied, this difference also holds for UV irradiation (vegetative cells), despite the fact that in this case the mechanisms of injury and repair appear to be of a different nature (Beukers and Berends, 1961; Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). Parallel studies (Chiasson and Zamenhof, *in preparation*) indicate that the spores of the two strains also exhibit differences of the same type in their resistance to heat. It is, therefore, tempting to speculate that the most sensitive substance essential for survival in all cases is the substance susceptible to UV, namely, DNA. More detailed consideration, however, reveals that this is unlikely (*see also* Alexander and Bacq, 1961).

The radiation-sensitive units in spores appear to be dispersed uniformly throughout the internal region of the spore, and not concentrated in a compact nucleus (Davis, 1954). In the present case, although the two wild strains may differ by several genes, the gross chemical structure of their DNA is not very different, as evidenced by their cross-transformability. Thus, no gross differences in resistance of their DNA should be expected. This fact is even more pertinent in the case of strains 168 *ind*$^+$ and 168 *ind*$^+$/*SA* or T168 *ind*$^+$/*SA*, where the genetic difference appears to be in only one site of one gene unlikely to affect DNA repair. The lack of differences in the ionizing radiation-induced mutability of all strains tested (Table 4) also speaks against the concept that the differences in survival are due to general differences in DNA structure or repair. The nature of another substance(s) that could be involved must at present remain a matter of speculation. Whatever their nature, their radioresistance, already different in vegetative cells, is greatly amplified by the protection offered by sporulation: for the two wild strains, the difference in dose for a surviving fraction of $10^{-2}$ is 9 kr for the vegetative cells and 130 kr for the spores (Fig. 1 and 2). However, the improvement of resistance by sporulation appears to be similar for both strains (ratios of doses required for any particular survival of spores and cells being 11 and 12, from Fig. 1 and 2), suggesting the same mechanism of protection by sporulation.

In general, because of the large number of factors involved (Powers, Webb, and Ehret, 1960; Alper, 1963), the "resistance-repair" mechanisms are likely to be different for different cases. In the case of the two genetically different wild strains studied, the "resistance-repair" mechanisms tend in the same direction for all injurious agents studied. In the other cases, such as in the case of strains 168 *ind*$^+$ (or 168 *ind*$^-$) and 168 *ind*$^+$/*SA* (Chiasson and Zamenhof, *in preparation*), or the radiation-resistant cocci (Anderson et al., 1956; Davis, Silverman, and Masurovsky, 1963), the resistance to irradiation and to heat are not concomitant.

Studies have been made on the location of various genes conferring resistance to UV radiation (Adler and Copeland, 1962; Howard-Flanders et al., 1962; Rörsch, Edelman, and Cohen, 1963) and to ionizing radiation (Adler and Hardigree, 1963) in *Escherichia coli*. In the present work, one locus controlling sensitivity to ionizing radiation in *B. subtilis* has been recognized and mapped without difficulty, because it is the locus for sulfanilamide-resistance and for the causal overproduction of PABA (Zamenhof and Heldenmuth, 1963). The identity of all three features was confirmed by transformation. Undoubtedly, many loci controlling sensitivity to ionizing radiation can be discovered through the study of various mutants. A small increase in antibiotic resistance of some of the UV-resistant mutants of *E. coli* has been reported (Witkin, 1947; Szybalski and Bryson, 1952). However, even in the simple case studied here, the elucidation of the cause of a change in radiosensitivity in both vegetative cells and spores is difficult. The number of sensitive targets ($n$) for spores of *B. subtilis* appears to be one [Woese, 1959; see also the simple exponential curve (Fig. 1) in the present work]. It is very unlikely that $n$ could decrease by a single mutation to sulfanilamide resistance. The recoveries of irradiated spores on basal media as compared with full media (see "pseudoauxotrophy" in case of heat treatment; Zamenhof, 1960), were almost complete in all cases, provided L-alanine, which is needed for germination (Hills, 1949), was added (Table 5). Thus, lower recovery of irradiated cells on basal media, demonstrable in irradiated vegetative cells (*E. coli*; Stapleton et al., 1955), does not occur in spores of *B. subtilis* just as it does not occur in the case of heat (Zamenhof, 1960), possibly because of special enzyme protection.

Since the recovery of the sulfanilamide-resistant strain was essentially not lower than the recovery of the parent strain, the former has no additional nutritional requirements for germination.

PABA appears to stimulate sporulation (Ordal, 1957) and might conceivably counteract germination. However, in the present work, the addition of an excess of PABA did not affect the germination of a strain that normally does not produce this excess. In addition, irradiation in the presence of PABA did not reveal an increased sensitivity. On the contrary, PABA exerted a 2- to 2.5-fold protective effect, possibly by acting as H donor

for peroxides, thus competing with (protecting) the biologically important oxidizable substances. Thus, overproduction of PABA could not be proved to be responsible for increased radiation sensitivity. This sensitivity could be caused by altered enzymes (Wolf and Hotchkiss, 1963) or altered permeability (Pato and Brown, 1963) in the sulfanilamide-resistant strains, but no evidence of this is available at present.

The finding that in certain cases the differences in radiation sensitivity in vegetative cells parallel the differences in spores may be useful for isolation of radiation-resistant spores by sublethal irradiation of vegetative cells; the sublethal irradiation of spores themselves may require extreme doses.

Although the shapes of the survival curves (Fig. 3) suggest the appearance of UV-resistant mutants, attempts to isolate such mutants by sublethal irradiation were not successful; however, a certain enrichment in what appear to be UV-resistant cells has been achieved (Table 2).

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