most cells are not making K_1. It also seems likely that K_1-poor cells are not being sloughed into the medium although they are rich in K_2. Thus a minor subpopulation (representing approximately 20 percent of the K_2-producing cells) (Fig. 2B) must continue producing equivalent amounts of K_1 and K_2, which is followed by their death and detachment. Alternatively, there could be a delay in detachment of cells that had keratinized at an earlier time in vitro. This seems unlikely since medium from cultures as old as 10 days contained protein with the stratum corneum K_1:K_2 ratio. Neither K_1 nor K_2 proteins were found in attached cells or sloughed material from cultures of dermal fibroblasts prepared from the same donor mice by techniques previously described (*10*).

Critical for the specific identification of keratin proteins is their ability to produce filaments in vitro whose ultrastructural morphology is the same as that characteristic of filaments in situ (*4*). Figure 3 shows filaments produced when purified K_1 and K_2, isolated from epidermal cells in culture or from stratum corneum, are recombined under conditions known to generate keratin filaments but not other types of fibrous proteins (*4*). The keratin filaments formed in vitro are solid rods, 70 to 80 Å in diameter and up to 10 μm in length, have an α-type x-ray diffraction pattern (legend to Fig. 3), and contain K_1 and K_2 in the molar ratio of 1:2 or 2:1. These observations agree with the idea that a triple-chain segmented α-helical conformation constitutes the basic unit structure of keratin filaments (*4*).

The results reported here are direct proof of the production of normal keratin proteins by mouse epidermal cells in vitro. The two major protein bands obtained should therefore be useful markers in further studies of the control of differentiation in vitro. We hope that they will also be useful in studies of chemical carcinogenesis in mouse epidermal cells in vitro and in studies of the relationship between differentiation and carcinogenesis.

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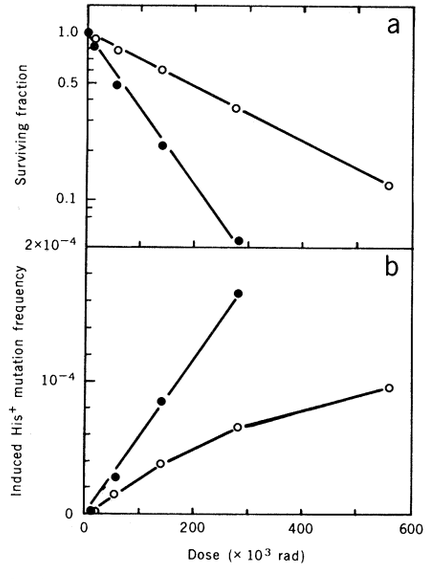
Thermorestoration of Mutagenic Radiation Damage in Bacterial Spores

Abstract. *The frequency of mutations in* Bacillus subtilis *spores irradiated anoxically with ionizing radiation decreased as a result of subsequent anoxic heating to the same extent as the increase in spore survival. This result indicates that DNA damage is the origin of the "thermorestoration" phenomenon.*

Bacterial spores exhibit a unique response to radiation. The viability of spores irradiated by ionizing radiation is reactivated by heat if anoxic conditions are used for both irradiation and heating. This phenomenon was first found in spores of *Bacillus megaterium* by Powers and his co-workers and was called "thermorestoration" (*1*). Later, Tanooka and Hutchinson (*2*) confirmed this effect in *B. subtilis* spores; however, they failed to show this effect with transforming DNA of spores possibly because of the extremely large radiation doses required to inactivate the DNA. The question of whether the thermorestoration of viability is a reflection of the reversal of DNA damage or has some other origin has long been unanswered. On the basis of the use of a newly developed mutation detection system with *B. subtilis* (*3*), I show in this report that DNA is a target molecule in the thermorestoration.

Spores of *B. subtilis* (strain HA101) carrying a suppressible mutation in the *hisB* gene were irradiated in N_2 gas with various doses of electrons and heated at 80°C for 20 minutes. Control spores were given only irradiation in N_2. Spores were then exposed to air and suspended in dis-



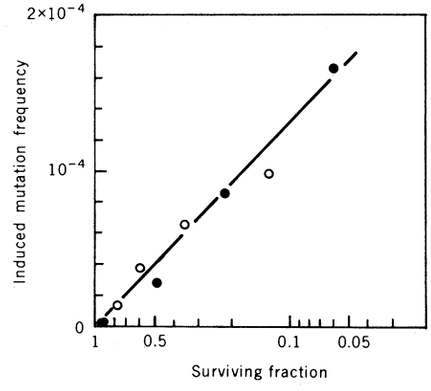


Fig. 1 (left). Effect of heat on the survival and the induced mutation frequency of irradiated spores. Dry spores of *B. subtilis* (strain HA101) (*his met leu*) were sealed in glass tubes that had been flushed with 99.999 percent N_2 gas and irradiated with 6-MeV electrons from a linear accelerator (Varian model V-7705). Spores were heated for 20 minutes at 80°C, then exposed to air, and suspended in sterile distilled water. Surviving spores (a) and His^+ mutants (b) were measured on the selective medium as described in (*3*): (○) heat, (●) no heat. Fig. 2 (right). A redrawing of Fig. 1 showing the induced His^+ mutation frequency as a function of the logarithm of the spore surviving fraction: (○) heat, (●) no heat.

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tilled water. The numbers of surviving spores and His^+ revertants were measured as described in (*3*).

The induced mutation frequency observed in irradiated spores was decreased by the heat treatment in parallel with the increase of spore survival (Fig. 1). The relation between the mutagenic damage and the lethal damage is seen more explicitly when the induced mutation frequency is plotted against the logarithm of the surviving fraction (Fig. 2). The ratio of the induced mutation frequency to the surviving fraction is unchanged whether the irradiated spores were given a heat treatment or not. This unchanged ratio indicates that DNA damage is the origin of the thermorestoration of the lethal damage as well as of the mutagenic damage.

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Sodium Channel Inactivation in Squid Axon is Removed by High Internal *p*H or Tyrosine-Specific Reagents

Abstract. *In squid axon, internal alkalinization from* p*H 7.1 to* p*H 10.2 results in a reversible decrease of the maximum inward current and the steady state sodium channel inactivation. Similar effects were observed after treatment of the axon with tetranitromethane or after iodination with lactoperoxidase. These results suggest that a tyrosine residue is an essential component of the inactivation process in this nerve.*

The molecular basis of excitability is poorly understood. Several lines of evidence suggest that sodium channels are sparsely distributed in the lipid membrane (*1*). Moreover, the sodium inactivation process appears to involve some proteinaceous component since internal perfusion of axons with proteolytic enzymes removes the inactivation (*2–4*). Armstrong and Bezanilla have proposed a model in which the inactivation process results from the plugging of open sodium channels by a mobile protein. This protein molecule is thought to be tethered to the inner surface of the membrane on one end and free to plug open sodium channels on the other end (*5*). Rojas and Rudy showed that after removal of the inactivation process with proteolytic enzymes, inactivation-like behavior could be reinstated by the addition of tetraethylammonium ion derivatives (*4*). They suggested that the plug of the Armstrong-Bezanilla model bears a positive charge on an amino acid residue of arginine or lysine. The last suggestion recently received additional support from the observation that glyoxal and diacetyl trimer, amino and guanidino group reagents blocked inactivation (*6*). In the experiments described here we tested the charged-tethered-plug model by varying the *p*H and titrating the specific amino acid groups involved in the inactivation process. Using the observed *pK* as a guide we then applied certain group-specific reagents to further confirm the presence of those amino acid residues.

Squid giant axons were provided by the Marine Biological Laboratory, Woods Hole, Massachusetts. Axons were dissected, cannulated, and perfused for 3 minutes with 1 mg of pronase grade B (Calbiochem) per milliliter of standard internal perfusate to remove the axoplasm. This procedure does not itself remove much of the inactivation (*2*). The axons were then internally perfused with 500 m*M* CsF, 5 m*M* NaF, and 20 m*M* buffer, or with 400 m*M* KF, 100 m*M* tetraethylammonium bromide (TEABr), 5 m*M* NaF, and 20 m*M* buffer. Both solutions blocked the voltage-dependent outward potassium currents. The voltage-dependent sodium currents could then be studied uncontaminated by potassium currents. The buffers used were Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pK_a 7.5 at 20°C; Bicine [*N,N*-bis(2-hydroxyethyl)-glycine], pK_a 8.3 at 20°C; CHES (cyclohexylaminoethanesulfonic acid), pK_a 9.5 at 25°C; and CAPS [3-(cyclohexylamino)-propanesulfonic acid], pK_a 10.4 at 20°C. In some experiments phenol red was added to the internal perfusate to ensure that the axon was adequately perfused and buffered. Experiments were run at temperatures between 0° to 10°C. The temperature in any given experiment was constant to within 0.2°C. Appropriate temperature corrections for the *p*H were made (*7*). The external perfusate was Woods Hole seawater at *p*H 8.2. Axons were voltage clamped by means of an axial wire piggyback electrode in an air-gap configuration (*8*). Series resistance compensation was employed.

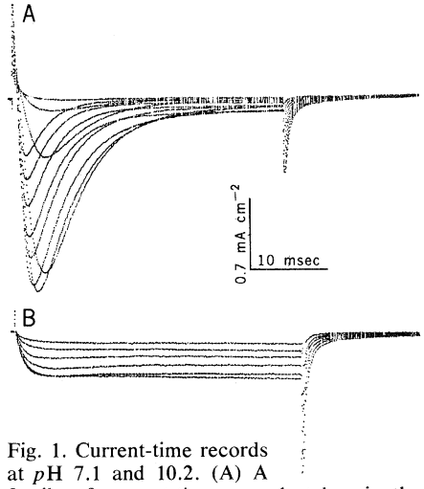

Fig. 1. Current-time records at *p*H 7.1 and 10.2. (A) A family of current-time records taken in the control solution containing 500 m*M* CsF, 5 m*M* NaF, and 20 m*M* Hepes buffer. The temperature-corrected *p*H was 7.1. The potential steps were −51, −40, −33, −23, −14, 10, 18, 28, 35. (B) The effect of increasing the *p*H to 10.2. The buffer was 20 m*M* CAPS. The number of voltage steps has been reduced for clarity (−40, −22, −13, −6, 2, 12). The holding potential was −60 mV with a conditioning pulse to −100 mV for 35 msec. The temperature was 3.7°C.

When the internal *p*H was raised from 7.1 to 10.2 a dramatic alteration of the sodium current was noted. Figure 1 illustrates two families of current-time records collected under voltage clamp conditions at normal and high *p*H. Two alterations by the *p*H 10.2 internal perfusate are noteworthy. First, the peak inward current was reduced by about 75 percent. Second, the sodium inactivation process was abolished, leaving the sodium current in an activated state at the end of the 45-msec pulse.

To further characterize the blockage of inactivation we applied a conditioning pulse of 70-msec duration and of varying magnitude immediately before the test pulse. The ratio of the peak inward current of the test pulse to the maximum peak inward current of the test pulse for large negative conditioning steps was plotted against the voltage of the conditioning pulse (Fig. 2). While the magnitude of the peak inward current of the test pulse was reversibly decreased by 50 percent at *p*H 9.9, the current during the test pulse was much less sensitive to the

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