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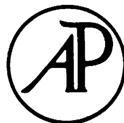

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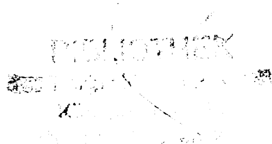

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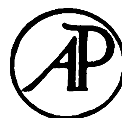

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RADIATION RESEARCH **104**, 102–108 (1985)

Enhanced Response of the *Salmonella* Mutagenicity Test to Ionizing Radiations

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ROOS, H., THOMAS, W.-H., AND KELLERER, A. M. Enhanced Response of the *Salmonella* Mutagenicity Test to Ionizing Radiations. *Radiat. Res.* **104**, 102–108 (1985).

Gamma-ray-induced reversions in the Ames *Salmonella* tester strain TA2638 have been studied for their dependence on a number of experimental parameters. It is shown that exposure to ionizing radiations soon after plating is not the procedure that yields results which correspond to those obtained in the standard utilization of the test with chemical mutagens. The ability to detect mutants is improved by irradiation 6 hr after the beginning of the incubation of the plated bacteria. This procedure has the double advantage of a markedly increased ratio of radiation-induced to spontaneous revertants and of resulting in substantial insensitivity to fluctuations in the number of bacteria initially plated. The reversion-doubling dose so obtained is 1.3 Gy; i.e., it is sufficiently small to disregard inactivation of the bacteria. © 1985 Academic Press, Inc.

INTRODUCTION

The Ames test, i.e., the test for the reversion of histidine-deficient (*his*$^-$) auxotrophic *Salmonella typhimurium* strains to histidine prototrophy, is the most widely used bacterial test for the identification of mutagenic chemicals. In spite of the desirability of a comparison of chemically induced and radiation-induced damage to DNA and the interest in assessing the interaction of the two types of damage, there have been comparatively few studies with ionizing radiations on this experimental system.

Ames noted, as early as 1972, that X rays and fission neutrons induce reversions in a number of *his*$^-$ tester strains (*1*). Later reports held that certain Ames *Salmonella* tester strains employed failed to respond to γ radiation (*2, 3*). However, Imray and MacPhee (*4*) found that the two strains TA98 and TA100 respond to γ irradiation, although doses of 200 Gy of γ rays were required to triple the spontaneous reversion rate.

A recent report by Isildar and Bakale (*5*) contained the first broad investigation of the radiation sensitivity and the mutagenic response of various *Salmonella* Ames tester strains to X rays and γ rays. It established the positive response of the majority of strains to ionizing radiations and demonstrated enhanced mutagenicity of pkM101 plasmid-containing tester strains. Mutation doubling doses from more than a few hundred Gy down to 26 Gy were found for the three plasmid-free strains, and doubling doses between 28 and 7 Gy were found for the three plasmid-containing daughter strains.

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The aim of our investigation has been to find a procedure, within the standard Ames protocol, that would yield an enhanced ratio of radiation-induced to spontaneous revertants and would make the test usable at low radiation doses which cause no substantial cell killing.

Two tester strains, TA102 and TA2638, have recently been reported to exhibit substantial mutagenicity after irradiation (*6*). The new strain TA102 responds particularly to oxidizing compounds. Its high frequency of mutation results from the enlarged number of *his*$^-$ sites, due to the insertion of the plasmid pAQ1 of which about 30 copies are contained in a bacterium. According to Levin's data the doubling dose for reversions is about 6 Gy, i.e., it is similar to the lowest doubling dose found by Isildar and Bakale. The strain TA2638 exhibits a similar response to ionizing radiation, but in contrast to TA102 it is sensitive to tetracycline because it lacks plasmid pAQ1. We have performed our investigations with the strain TA2638, because it proved to be substantially more stable than TA102. We also find the absence of the plasmid pAQ1, whose number is difficult to control, a desirable feature in a study that aims at a quantification of the role of various experimental factors.

MATERIALS AND METHODS

Bacterial strain: The tester strain TA2638 (*his*G428, rfa, pKM101) was provided by Dr. B. N. Ames, Department of Biochemistry, University of California, Berkeley.

The standard Ames-test protocol has been followed (*7*). A minor modification is the use of 25 ml glucose-agar medium, instead of the recommended amount of 30 ml. Oxoid and Difco media were used. All other chemicals were of analytical grade. Water was deionized and quartz distilled.

For all experiments, cultures were started with 0.4 ml freshly thawed bacterial suspensions from frozen permanent stocks. The cultures were then grown for 8 hr, i.e., to the end of the exponential phase. Subsequently, aliquots were taken and, where necessary, were diluted or concentrated by centrifugation. For both mutagenicity experiments and determination of the number of viable bacteria, aliquots of 0.1 ml were mixed with top agar and poured onto minimal glucose-agar plates (for details see (*7*)).

Individual experiments were always performed with bacteria from the same liquid culture. Where several culture flasks were required, their contents were pooled.

For the *his*$^-$ → *his*$^+$ reversion experiments the bacteria were washed twice with freshly prepared sterile saline. After resuspension in saline, 0.1-ml aliquots were mixed with top agar containing 0.5 m*M* D-biotin and the desired amount of L-histidine. The standard amount of histidine was 0.1 μmole per plate. The total incubation time was 48 hr.

The irradiations were performed with ^{60}Co γ rays at room temperature with absorbed-dose rate 0.17 Gy/min. The earliest exposures—subsequently termed irradiations immediately after plating—were begun 30 min after pouring the top agar.

In experiments with numerous plates subjected to different treatments, successive platings had to be appropriately timed. To avoid repeated shaking which could cause some continued growth, the necessary plateau-phase samples were drawn simultaneously and were then kept in individual vials at room temperature until plating. In those experiments where the number of plated bacteria was critical, additional assessments of the concentrations of viable bacteria were made for the actual times when the samples were utilized.

To adhere to the standard protocol we have in the present investigation irradiated the bacteria on the agar. In the previous studies with ionizing radiations, the exposures were performed soon after plating (*4*, *5*). This timing may have seemed to be analogous to the standard protocol with chemicals. In fact, however, the procedure fails to account for the difference between the instantaneous character of the irradiation and the continued presence of the chemical. A short-term irradiation soon after plating leads to a poor ratio between radiation-induced and spontaneous revertants because it has a direct effect only on the plated bacteria and not on their descendants. Experimental studies were therefore performed to identify more suitable procedures.

RESULTS

Growth Characteristics after Plating

When chemical mutagens are examined in the Ames test, they can affect the bacteria during their entire growth phase on agar until the histidine is exhausted. A determination of the growth characteristics and of the sensitive phase for mutagenicity is therefore not required. For a short-term exposure with ionizing radiation, on the other hand, the number of bacteria at the time of irradiation is an important parameter. Timing is therefore critical and requires, as will be seen, a knowledge of the growth after plating.

To determine the number of viable bacteria at various times, plates with an initial number of about $7 \cdot 10^8$ bacteria were incubated. At specified times samples of 2-cm^2 area were taken from the agar and were, after homogenization and dilution, distributed on five plates with nutrient medium. From the counts of colonies the numbers of viable bacteria on the original plates were then inferred. These numbers are represented in Fig. 1. Standard errors are given for the sets of five plates. They do not account for fluctuations between samples, and similar considerations apply to standard errors given in some of the subsequent figures.

After a lag phase of about 2 hr the bacteria begin to divide and, with the standard histidine supplement of 0.1 μmole per plate, a maximum number of about $6 \cdot 10^9$ viable bacteria per plate is reached after about 8 hr. The total number of bacteria increases under this condition by a factor of about 8; i.e., even with a relatively high number of bacteria plated, most of the unreverted bacteria originate on the plate.

Dependence of the Number of Reversions on the Amount of Histidine

When irradiation is performed immediately after plating and with the usual amount of histidine, the subsequent period of growth, which corresponds to about three generations, may be longer than required for the expression of induced reversions. Less histidine would then be sufficient to yield the same, or nearly the same, number of

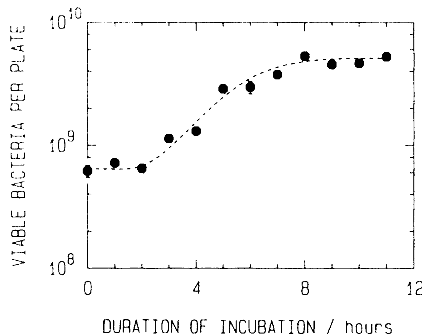

FIG. 1. Mean number of viable bacteria (6.3×10^8) per plate at specified times after the beginning of incubation. The data are mean values for five plates prepared from one 2-cm^2 sample. In this and subsequent figures standard errors, where not given, are less than the size of the symbols. Dashed lines are inserted for better readability of the diagrams; they have no mathematical significance.

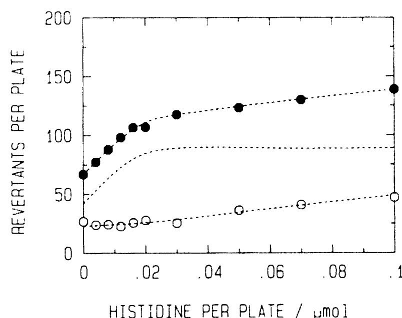

FIG. 2. Dependence of the number of revertants on the amount of histidine added per plate. The upper curve gives the mean number of revertants for groups of 12 plates exposed immediately after plating to 9.5 Gy of ^{60}Co γ rays. Spontaneous revertants are represented by the lower curve. The intermediate curve gives the differences.

radiation-induced revertant colonies. However, reducing the amount of histidine would lower the number of spontaneous reversions on the plate.

To quantify this dependence, experiments were performed with a fixed dose of 9.5 Gy of ^{60}Co γ rays applied immediately after plating, but with a variable amount of histidine. Figure 2 gives the total number of revertants for the irradiated plates (upper curve) and the number of spontaneous revertants at the specified amounts of histidine (lower curve). The intermediate curve represents the difference between the irradiated and the unirradiated plates. The difference is meaningful because there is, as shown in Fig. 3, little inactivation of the bacteria at doses up to 10 Gy. One may therefore disregard corrections for inactivation, and one can interpret the intermediate curve as the number of radiation-induced revertants.

From the observed dependence one concludes that an addition of about 0.02 μmole of histidine per plate is sufficient for expression of the radiation-induced reversions, when about $4 \cdot 10^8$ bacteria are plated and exposed. Any further increase of histidine leaves the number of radiation-induced revertants unchanged, while it enhances the number of spontaneous revertants. Accordingly, the ratio of the number of radiation-

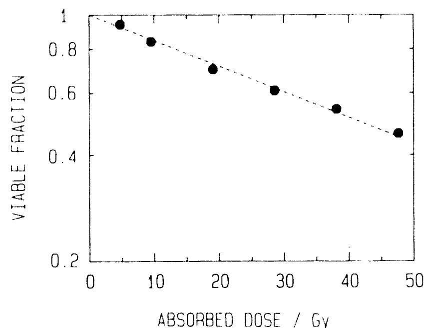

FIG. 3. The fraction of viable bacteria after exposure to different doses of ^{60}Co γ rays immediately after plating. The individual points are mean values from sets of at least nine plates. In this experiment the dose rates were varied to keep the duration of the exposure constant at 55 min.

induced revertants to spontaneous revertants is largest when about 0.02 μmole of histidine is added per plate.

Reducing the amount of histidine could therefore be a method to increase the ratio of induced to spontaneous revertants in studies with ionizing radiations. However, it is not certain that irradiation soon after plating, i.e., during the initial lag phase on agar, is the optimal procedure. It is furthermore desirable to find a method that increases the response to ionizing radiations without departure from the standard protocol. Additional investigations have therefore been performed with the fixed conventional amount of histidine but with variable timing of the irradiation.

Reversion Rates after Delayed Irradiation

When bacteria plated with the standard amount of histidine are incubated, their number remains constant during the initial lag phase (see Fig. 1). Subsequently the number of bacteria increases. As the total amount of histidine is more than sufficient to permit expression of initially induced reversions, one would expect increased reversion rates with delayed irradiations, which affect more bacteria. The panels of Fig. 4 show the resulting dependence of numbers of revertants on timing of the exposure from two experiments. The essential finding is that one attains a maximum ratio of the total number of revertants to spontaneous revertants for an irradiation delayed by about 6 hr. The maximum ratio is approximately 9 in both experiments. This is substantially higher than the value of about 4 reached in the experiments with a reduced amount of histidine.

For the delayed irradiation the initial number of bacteria plated is far less critical than for irradiations immediately after plating. While the yields of revertants from the two experiments vary for the early irradiations, they are in substantial agreement for delays of about 6 hr. A further experiment was performed to determine the dependence of the yield of revertants on the number of bacteria plated for immediate and delayed irradiation. As shown in Fig. 5, the yield of revertants is nearly independent

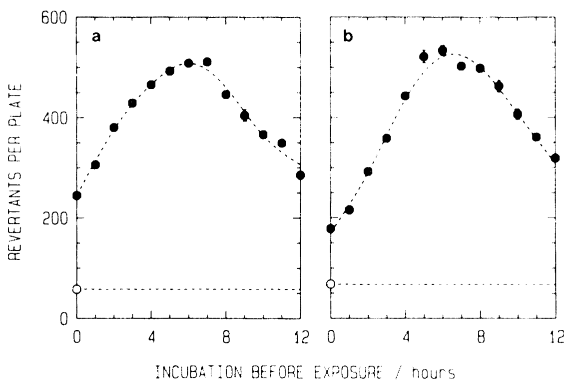

FIG. 4. Dependence of the yield of revertants on timing of the exposure to 9.5 Gy of ^{60}Co γ rays. The two panels show the results for two separate experiments with somewhat different numbers of bacteria plated: (a) 6.3×10^8; (b) 3.2×10^8. The yields differ for the immediate exposures and the exposures with short preincubation; they are similar in the two experiments when the exposures are delayed by about 6 hr.

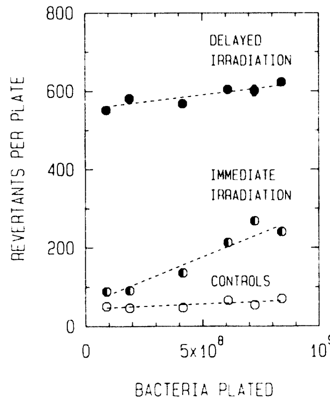

FIG. 5. The dependence of the yield of revertants on the number of bacteria plated for immediate exposure to 9.5 Gy of ^{60}Co γ rays (intermediate curve) and for exposures delayed by 6 hr (upper curve). The bottom curve gives the number of spontaneous revertants.

of the number of bacteria plated for the delayed irradiation, while there is a marked dependence for the immediate irradiation.

Figure 6 shows a dose-effect relationship obtained with ^{60}Co γ rays. One derives from this dose-effect relation a doubling dose for the reversions of 1.3 Gy, i.e., a value considerably below the doubling dose of 6 Gy obtained in the experiments of Levin *et al.* (*6*) with the same strain, and even further below the doubling doses reported in studies with other strains. At such low doses one can disregard inactivation of the bacteria (see Fig. 3).

CONCLUSION

In earlier studies of the effects of ionizing radiations on Ames *Salmonella* tester strains the irradiations have been performed soon after plating of the bacteria (*4, 5*). This procedure may have been chosen in ostensible analogy to the immediate addition of chemicals in the usual application of the test. However, it does not account for the

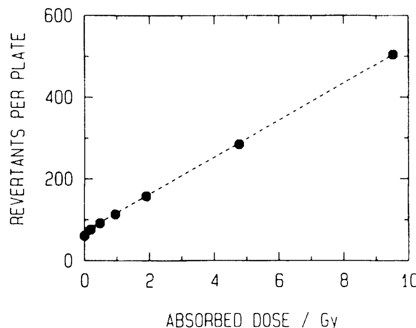

FIG. 6. The number of revertants per plate as a function of the ^{60}Co γ-ray dose for irradiations 6 hr after the beginning of incubation. The dose rate is 0.17 Gy/min; 5.3×10^8 bacteria plated. The slope of the curve is 46/Gy; the doubling dose is 1.3 Gy.

difference between the instantaneous character of the irradiation and the continued presence of chemical mutagens. Although the kinetics of the process have not been studied in detail, it is evident that—at least for stable chemicals—most of the reversions arise several hours after plating when the number of bacteria has considerably increased. Disregarding the more complicated possibility of a protracted exposure during incubation, a delayed irradiation therefore corresponds best to the standard protocol of the Ames test.

In the present study it has been shown that irradiation 6 hr after plating has two substantial advantages. As in the test with sufficiently persistent and nontoxic chemicals, the yield of reversions is insensitive to variations of the number of bacteria initially plated. Furthermore, the delayed irradiation increases the ratio of radiation-induced to spontaneous reversions. In the strain TA2638, which is particularly responsive to ionizing radiations, the doubling dose for reversions has, in this way, been reduced to 1.3 Gy of ^{60}Co γ rays. This removes the need to apply inactivation corrections to the observed mutation rates, and it should facilitate studies of the relative mutagenic efficiency of different radiations and of the combined effect of chemicals and ionizing radiations.

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