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Kinetic and Biochemical Analysis of Interacting Radiation Recovery
Systems in Escherichia coli

Submitted by: _____

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I. Introduction

Although a considerable amount of information is available concerning the processes by which cells repair UV-induced damage in DNA by so-called excision-resynthesis (ER) which is under the control of uvr⁺ genes, much less is known about the processes by which gamma-ray-induced damage is repaired by the metabolic reactions controlled by the rec⁺ genes.

Even less is known concerning the mechanisms by which alterations in the physiological state of the cell produce modifications in the sensitivity of the cell to DNA-damaging agents. It is now generally supposed (although certainly not proven) that some physiologically-induced changes in radiation sensitivity reflect a change in the total amount of repair which depends upon the rate of, and time available for, repair. The latter factors may be determined by a complex combination of interactions involving changes in the relative affinities for the DNA of the various enzymes involved in the repair. Furthermore, the time at which DNA replication begins after irradiation may have an important bearing on survival. The possibility that a time-dependant balance between various competing enzymatic activities (for example, synthetic and degradative) determines the extent to which successful repair occurs as determined by cell survival means that a simple correlation, or lack thereof, between the fate of certain known radiation lesions and the level of survival is not adequate to establish a causal relationship. Under these circumstances information concerning the time-course of events and how they are altered with physiologically-induced changes in sensitivity becomes a prerequisite to any understanding of cause and effect. Such knowledge when obtained would be valuable in gaining understanding of the cell's ability to maintain itself in a changing and hostile environment.

The immediate objective of the research carried out under contract AT(11-1)-1686 has been to determine the basis for the dramatic change in the radiation and drug sensitivity of certain strains of Escherichia coli when the cells are grown to the stationary phase in glucose-enriched nutrient broth (Glucose-Induced Resistance, GIR). One approach to the problem has been to investigate what relationship, if any, GIR has to other well-defined recovery processes which are genetically controlled.

These include liquid holding recovery (LHR), "shift down or shift up" recovery, split-dose recovery (SDR), low temperature reactivation (LTR), photoreactivation (PR) and host cell reactivation (HCR). Liberal use has been made of

repair-deficient substrains in these investigations. The immediate objective as described above has been used to select those aspects of the broad project objective which would have priority.

The broad objective was to pursue a systematic investigation of the interactions between the above-named recovery processes with special emphasis on GIR. The most significant information has been obtained in studying GIR to gamma-rays and to methyl methanesulfonate in genetically characterized substrains of E. coli K-12 by means of survival kinetics and the biophysical characterization of the DNA. A study of host cell reactivation of gamma-irradiated phage T1 in substrains of E. coli K-12 substrains and of GIR to Mitomycin C have also yielded results of interest. The remainder of the work which has been pursued is useful in characterizing GIR but does not always lend itself to simple explanations.

II. GIR to gamma-rays in Escherichia coli

A. Nature of GIR

The phenomenon of GIR was first found in E. coli B/r by Hollaender, Stapleton and Martin (1). Maximum GIR-G is developed only if glucose is present in the nutrient or peptone broth essentially throughout the growth cycle of the culture (i.e., lag, log and stationary phase). GIR-G is reflected primarily in an enhanced resistance at low doses which causes a 'shoulder' or 'quasi-threshold' to appear in the survival curve. The extrapolation number obtained from gamma-ray survival curves of GNB-grown E. coli B/r is greater than 20. (GNB = Nutrient broth enriched with glucose at a concentration of 0.2 to 0.4% of glucose.) The DNA content of GNB-grown E. coli B/r is approximately twice that of NB-grown cells according to our measurements. It appeared that a simple explanation of GIR-G based on an increased multiplicity of targets was not adequate. This did not rule out the possibility that GIR might partially be a reflection of an increased multiplicity of targets or that some complex interaction of multiple targets might not be involved. The work in Stapleton's laboratory (1, 2) demonstrated that the pH of the GNB-growth medium becomes acidic and that GIR is not observed if this change in pH is prevented.

E. coli B_{g-1}, which is deficient in its ability to repair DNA exhibits very little GIR. This suggested that GIR might reflect a physiologically-induced change in the amount of repair of radiation-induced damage in cellular DNA.

B. GIR in strains of E. coli K-12

Because E. coli B_{g-1} differs from B/r by at least three genetic loci involving radiation sensitivity, it appeared best to study GIR in the genetically

well-characterized substrains of E. coli K-12. A uvr⁺rec⁺lon⁺ strain of E. coli K-12 and strains mutant in various combinations of these three loci were utilized. (The uvr locus controls an essential step in excision-resynthesis (ER) repair. ER of DNA damage by UV involves at least four enzymatic reactions; nicking of 3' - 5' phosphodiester bonds, excision of the damaged nucleotides and some surrounding bases, replacement of the removed nucleotides by undamaged nucleotides, and re-joining of the 3' - 5' phosphodiester bonds between the newly polymerized portion and the old strand (3, 4). The rec loci control some aspect of what has been tentatively identified as repair by recombination after replication of DNA (5, 6). The lon locus controls the ability of cells to form cross septa after DNA replication (7).)

The survival kinetics of the various repair deficient strains of E. coli K-12 provided the first major clue as to what genetic loci were involved in GIR-G. This work was published (8) and is included as Appendix I for complete details.

The addition of glucose (0.2%) to a nutrient broth growth medium produced GIR in strains AB2497 (uvr⁺rec⁺), AB2499 (uvr⁻), and AB1886 (uvr⁻) but not in strains AB2480 (rec⁻uvr⁻), AB2463 (rec⁻), AB2470 (rec⁻), and AB1899 (lon⁻). These results may be summarized by the suggestion that GIR is exhibited by all rec⁺ strains except for the lon⁻ strains or filament formers. From these results it was suggested that GIR-G, at least in part, may be the reflection of an enhancement at low exposure of the kind of repair controlled by the rec genes.

C. Induction of and repair of gamma-ray-induced breaks in the DNA of GNB- and NB-grown cells.

A modification of the alkaline sucrose gradient centrifugation techniques described by McGrath and Williams (9) and by Ginsberg and Webster (10) was used to determine the extent that gamma-rays induced breaks in the DNA and to what extent the repair of such breaks was different in GNB- and NB-grown cells.

Sedimentation profiles of DNA obtained from unirradiated cells and from cells irradiated with 20 krad and incubated in nutrient broth at 37°C for 0, 5, 10, 20, 30, or 40 minutes after irradiation were obtained for strains AB1157, AB1886, SR111, and AB2463.

This initial study showed that the magnitude of the initial damage for a dose of 20 krad, as evidenced by the shift of the sedimentation profiles in the direction of lower molecular weight (Fig. 1-8), appeared to be of the same order of magnitude for all strains under both conditions of growth. The small differences which were seen either in the magnitude of the shift or in the fraction of the

- Fig. 1. Sedimentation profiles of DNA from irradiated E. coli K-12 AB1157 grown in NB. (C) unirradiated control; (0') 20 krads, no reincubation; (10') 20 krads, 10-min. reincubation; (20') 20 krads, 20-min. reincubation; (40') 20 krads, 40-min. reincubation.
- Fig. 2. Sedimentation profiles of DNA from irradiated E. coli K-12 AB1157 grown in GNB. (C) unirradiated control; (0') 20 krads, no reincubation; (5') 20 krads, 5-min. reincubation; (10') 20 krads, 10-min. reincubation; (20') 20 krads, 20-min. reincubation; (40') 20 krads, 40-min. reincubation.
- Fig. 3. Sedimentation profiles of DNA from irradiated E. coli K-12 AB1886 grown in NB. (C) unirradiated control; (0') 20 krads, no reincubation; (10') 20 krads, 10-min. reincubation; (20') 20 krads, 20-min. reincubation; (30') 20 krads, 30-min. reincubation; (40') 20 krads, 40-min. reincubation.
- Fig. 4. Sedimentation profiles of DNA from irradiated E. coli K-12 AB1886 grown in GNB. (C) unirradiated control; (0') 20 krads, no reincubation; (5') 20 krads, 5-min. reincubation; (10') 20 krads, 10-min. reincubation; (20') 20 krads, 20-min. reincubation; (40') 20 krads, 40-min. reincubation.
- Fig. 5. Sedimentation profiles of DNA from irradiated E. coli K-12 SRIII grown in NB. (C) unirradiated control; (0') 20 krads, no reincubation; (10') 20 krads, 10-min. reincubation; (20') 20 krads, 20-min. reincubation; (30') 20 krads, 30-min. reincubation; (40') 20 krads, 40-min. reincubation.
- Fig. 6. Sedimentation profiles of DNA from irradiated E. coli K-12 SRIII grown in GNB. (C) unirradiated control; (0') 20 krads, no reincubation; (5') 20 krads, 5-min. reincubation; (10') 20 krads, 10-min. reincubation; (20') 20 krads, 20-min. reincubation; (40') 20 krads, 40-min. reincubation.

Fig. 7. Sedimentation profiles of DNA from irradiated E. coli K-12 AB2480 grown in NB. (C) unirradiated control; (0') 20 krad, no reincubation; (10') 20 krad, 10-min. reincubation; (20') 20 krad, 20-min. reincubation; (30') 20 krad, 30-min. reincubation; (40') 20 krad, 40-min. reincubation.

Fig. 8. Sedimentation profiles of DNA from irradiated E. coli K-12 AB2480 grown in GNB. (C) unirradiated control; (0') 20 krad, no reincubation; (5') 20 krad, 5-min. reincubation; (10') 20 krad, 10-min. reincubation; (20') 20 krad, 20-min. reincubation; (40') 20 krad, 40-min. reincubation.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

activity involved in the shift did not seem to have a predictable pattern among the strains and could have reflected fluctuations which were not averaged out in the four gradients used to obtain the profiles. For this reason it could not be stated with complete certainty that differences in the amount of damage produced initially under the two conditions of growth are not partially responsible for the differences in the level of survival. If the differences were real, they could also reflect a different amount of repair during the period of irradiation and prior to lysis of the cells.

The significant difference between the DNA sedimentation profiles for GNB-grown Rec^+ cells and those of NB-grown Rec^+ cells appeared to be in the magnitudes of the shifts of the peaks back toward those of the unirradiated cells after a short period of post-irradiation incubation.

From the sedimentation profiles the positions of the peaks were measured and used as the distance of sedimentation in the calculation of molecular weight using Studier's (11) relationship with the equation given by McGrath and Williams (9). The ratios of the molecular weights of the DNA from GNB- and NB-grown cells given 5, 10, or 20 minutes post-irradiation incubation to that of the DNA obtained from cells immediately after irradiation are given in Table I. Because it was important to increase the reliability of the data which suggested that GNB-grown Rec^+ cells repair single-strand breaks more rapidly, a new study was initiated to reinvestigate the sedimentation behavior of DNA from irradiated cells subjected to 0, 5 and 10 minute post-irradiation incubation in NB. Table II gives the results obtained in the second study. In spite of the efforts and number of experiments performed, it has not been possible to demonstrate unequivocally that there is a significant difference in the patterns obtained for GNB- and NB-grown Rec^+ cells.

III. GIR to methyl methanesulfonate (GIR-MMS)

A. Survival kinetics

The study of the nature of the survival kinetics for MMS-treated cells is completed. Report C00-1696-20, which is a preprint of a paper accepted for publication in Journal of Molecular and General Genetics, is included with this report. Only a brief summary of the work will be given here.

GIR-MMS is present in those strains which exhibit GIR-G and is absent in those strains for which GIR-G is absent. Seventeen strains of *E. coli* K-12 and B/r have been characterized.

The time-course for the development of GIR-MMS was found to be very similar to that observed for the development of GIR-G. That is, the glucose must be

TABLE I

Ratio of 'Peak' Molecular Weights

	$\frac{M_C}{M_{0^+}}$	$\frac{M_{5^+}}{M_{0^+}}$	$\frac{M_{10^+}}{M_{0^+}}$	$\frac{M_{20^+}}{M_{0^+}}$	$\frac{M_{30^+}}{M_{0^+}}$	$\frac{M_{40^+}}{M_{0^+}}$
AB1157 NB	3.4		2.24	2.14		2.32
AB1157 GNB	2.5	1.68	2.23	1.99		2.25
AB1886 NB	3.27		1.90	1.66	1.19	1.00
AB1886 GNB	2.03	1.79	2.29	2.03		2.10
SR111 NB	4.6		2.34	2.76	2.09	2.09
SR111 GNB	4.8	1.62	1.77	1.85		1.77
2480 NB	4.1		2.01	1.75	2.06	1.80
2480 GNB	4.3	1.68	2.01	2.01		2.32

The position of the peak obtained from the composite sedimentation profile was used as the distance, d , in the relationship $M_i/M_j = (d_i/d_j)^{2.5}$.

The subscripts on the M 's in the heading represent the time of post irradiation incubation. C represents the control.

TABLE II

	$\frac{M_C}{M_{0^+}}$	$\frac{M_{5^+}}{M_{0^+}}$	$\frac{M_{10^+}}{M_{0^+}}$
AB1157 NB	$1.94 \pm 28\%$	$1.80 \pm 27\%$	$1.86 \pm 24\%$

'Peak' molecular weights were calculated as in Table I with the exception that the 'peak' position was obtained from the results of each individual gradient, results of all equivalent gradients were averaged, and the average used to calculate the molecular weight. The error represents one standard deviation from the mean for the ratio. The averages from 12 experiments were used.

present in the medium more than 12 hours in order for maximum GIR-MMS to occur.

The effect of the temperature at the time of treatment has been studied. Very dramatic differences are seen in sensitivity (Fig. 9).

B. Induction of and repair of MMS-induced breaks in the DNA of GNB- and NB-grown cells.

In a manner similar to that described in section II. C. for investigating the repair of gamma-ray-induced breaks in DNA, the repair of MMS-induced single-strand breaks in the DNA of GNB- and NB-grown E. coli K-12 strains. AB1157, AB1886, and SR111 was examined. The results are discussed in detail in the enclosed preprint (COO-1686-23) which is being submitted for publication. Only a brief summary of the results will be given here.

Some repair of breaks is seen in all strains tested under both conditions of growth, but the amount of repair is greatest in Rec⁺ cells. Essentially all the repair that will occur takes place within the first ten minutes of post-treatment incubation. The amount of 'initial' damage in GNB-grown Rec⁺ cells seems to be less than in NB-grown Rec⁺ cells. It is not known whether this represents a difference in the rapid repair of breaks during treatment or a difference in the initial number of breaks. In addition, there is evidence that suggests that the repair of some breaks in GNB-grown Rec⁺ cells may be enhanced over that occurring in NB-grown Rec⁺ cells.

IV. Host cell reactivation (HCR) of irradiated phage in E. coli

Definitive statements concerning the nature of GIR cannot ordinarily be obtained from the survival kinetics of the cells because the intrinsic sensitivity of the cell itself may be changed by growth in the glucose-enriched medium. However, it is possible to irradiate a phage suspension and to adsorb the phages from one aliquot on NB-grown cells and the others on GNB-grown cells and to look for differences in phage survival. Differences in this case could not be attributed to differences in either radiation sensitivity or to changes in target number. With this in mind, HCR of both UV- and gamma-irradiated phage in the various substrains of E. coli B and K-12 was examined. It was found that HCR of gamma-irradiated phage was qualitatively like that of HCR of UV-irradiated phage in the various substrains. From this fact and the response of the cells themselves to irradiation, it was concluded that gamma-ray-induced base damage was probably being repaired in HCR and that phage T1 might possibly carry rec type genes to code for its own "repair by recombination" (Rec repair). This work was also published (12) and is included as Appendix II. Huskey (13) suggested that multiplicity reactivation in Rec⁺ cells should be a test for

Figure 9

The Effect of Treatment-Temperature on MMS-Survival of
E. coli K-12 AB1157 Grown to the Stationary Phase and
Treated with 0.1 M. MMS for Various Exposure Times.

- AB1157 Grown in NB, Treated at 25°C.
- AB1157 Grown in GNB, Treated at 25°C.
- ▲ AB1157 Grown in NB, Treated at 4°C.
- △ AB1157 Grown in GNB, Treated at 4°C.
- AB1157 Grown in NB, Treated at 37°C.
- AB1157 Grown in GNB, Treated at 37°C.

Each point represents the average of at least three
experiments.

SURVIVING FRACTION

the presence of a rec gene in the phage. Unfortunately our tests of multiplicity reactivation of T1 on Rec^- or Rec^+ cells gave equivocal results.

From the literature it appears that T1 undergoes multiplicity reactivation but that it can not be demonstrated as readily as for some other phages (14).

Comparison of gamma-HCR in GNB-grown cells with that in NB-grown cells shows that a small but reproducible increase in survival occurs in GNB-cultured E. coli B/r, E. coli B₉₋₁ and E. coli K-12 substrains which are Uvr^- and Rec^+ . The results with the $\text{Rec}^- \text{Uvr}^-$ substrain were equivocal because of certain difficulties in getting a good lawn with this substrain. No gamma-HCR is seen in any of these substrains when phage are irradiated initially in buffer rather than broth. Nor does growth of cells in glucose affect the survival of irradiated phage under these conditions. (See Appendix I for additional details.)

Unfortunately, in order to interpret these results in terms of enhanced repair, it is necessary to show that GNB-grown cells do not have an altered affinity for adsorbing the phage or an altered susceptibility to injection of DNA from the phage. The degree of adsorption was examined as carefully as possible but since the magnitude of the glucose effect is not large, it has not been possible to prove with certainty that altered adsorption is not involved. It does not appear to be. Furthermore, if such an explanation is offered for the effect, one would have to postulate that various combinations of the uvr and rec genes affect the cell surface in addition to playing a role in repair.

If one accepts these results as providing evidence concerning GIR, it is observed that the findings are also consistent with the hypothesis that glucose enhances Rec repair. In fact, the results are consistent with the idea that Rec repair of gamma-ray-induced base damage is being enhanced by the glucose and that Rec repair of single-strand breaks in phage is not affected by the glucose. This conclusion, however, is based upon Freifelder's observations that phage irradiated in buffer sustain only single and doublestrand breaks and that phage irradiated in broth additionally sustain a third type of damage which is probably some kind of base damage (15).

V. GIR to Mitomycin C (GIR-MC)

Because Mitomycin C is known to damage DNA (16), the effect of the growth medium on sensitivity to MC was also investigated. The Rec^+ strains, AB2497 and AB1886, were found to exhibit a GIR-MC, whereas AB2463 recA13, AB2480 recA13 uvrA6 and AB2462 rec12 exhibited no GIR-MC (Fig. 10a). Initially it was thought that GIR-MC and GIR-G might have a common explanation. Additional work made this appear unlikely.

Figure 10a

Mitomycin C Sensitivity of Cells Grown to the Stationary Phase

Either in GNB, NB or YET.

- E. coli K-12 AB2497 grown in GNB.
- E. coli K-12 AB2497 grown in YET.
- E. coli K-12 AB1886 grown in GNB.
- △ E. coli K-12 AB1886 grown in YET.
- ◆ E. coli K-12 AB2497 grown in NB.
- E. coli K-12 AB2463 grown in NB or GNB.
- ▽ E. coli K-12 AB1886 grown in NB.
- ◇ E. coli K-12 AB2480 grown in NB or GNB.

Following this initial study of survival kinetics studies of the MC-induced lesions in DNA by means of alkaline sucrose gradient centrifugation were initiated. The first results (Fig. 10) were disturbing. Whereas a nice DNA profile could be obtained for control cells ($\text{Rec}^+ \text{Uvr}^+$) which demonstrated that no mistakes were being made in the application of the technique, the DNA profiles for MC-treated cells were not satisfactory because the amount of radioactivity recovered (area under the curve) was so small. One might have argued that the DNA was all degraded but then more activity should have been recovered in the top portion of the gradient. MC is known to be, among other things, a crosslinking agent (16). Since the distance traveled by a molecule in a linear sucrose gradient is approximately proportional to the time of centrifugation and to the sedimentation coefficient the possibility existed that cross-linked DNA would have a sedimentation coefficient considerably higher than that of control DNA because it would not necessarily become freely single-stranded in an alkaline gradient. For this reason, the centrifugation time was cut back from 45 minutes to 10 minutes. Fig. 11 shows that MC-treated cells have some DNA which sediments more rapidly than that of the control cell. Fig. 10 and 11 show that there is also DNA which sediments more slowly. Fig. 12 shows that there is less DNA with a high sedimentation coefficient at the same exposure to MC for GNB-grown $\text{Rec}^+ \text{Uvr}^+$ cells. There is also more DNA that appears to be 'normal' in size immediately after treatment. By ninety minutes much of the "heavy" DNA in MC-treated NB-grown cells has been degraded to "small" segments (Fig. 10 and 11). This general pattern is also seen for $\text{Rec}^+ \text{uvrA6}$ and $\text{recB21} \text{Uvr}^+$ strains. In no case has there been any clear-cut indication of the return of the DNA profile to normal after a period of incubation as would be expected if repair were occurring. In the case of the $\text{recA13} \text{Uvr}^+$ mutant approximately the same kind of DNA profiles were obtained for both GNB- and NB-grown cells. It should be acknowledged here that results with MC-treated cells have not been very 'clean' and, therefore, small differences between various DNA profiles may not be significant even though gross qualitative trends are valid and reproducible.

Along with this work, additional strains of K-12 were tested for GIR-MC. Contrary to initial expectations, all strains tested except recA13 and Rec12 mutants exhibited GIR-MC. The mutants which showed GIR-MC were recC22 , rec23 , recB21 (AB2470), and recA1 . At this point it became clear that the correlation of the absence of GIR-MC with the rec loci might be fortuitous. Existing literature was reviewed to determine whether any of the rec strains used above

Figure 10

The Effect of Mitomycin C on the Sedimentation of
Tritium Labeled DNA from E. coli AB2497 Cells Grown
in Nutrient Broth. The Alkaline Sucrose Gradients
Were Run at 30,000 RPM for 45 Minutes at 20°C.

- DNA from Untreated Cells
- DNA from MC Treated Cells (1 $\mu\text{g}/\text{ml}$)
Immediately after Treatment
- △ DNA from MC Treated Cells (1 $\mu\text{g}/\text{ml}$)
after 90 Minute Incubation in NB
at 37°C.

Figure 11

The Effect of Mitomycin C on the Sedimentation of
Tritium Labeled DNA from E. coli AB2497 Cells Grown
in Nutrient Broth. The Alkaline Sucrose Gradients
Were Run at 30,000 RPM for 10 Minutes at 20°C.

- DNA from Untreated Cells
- DNA from MC Treated Cells (1 $\mu\text{g/ml}$)
Immediately after Treatment
- △ DNA from MC Treated Cells (1 $\mu\text{g/ml}$)
after 90 Minute Incubation in NB at
37°C.

Figure 12

The Effect of Mitomycin C on the Sedimentation of Tritium Labeled DNA from E. coli AB2497 Cells Grown in Glucose Nutrient Broth. The Alkaline Sucrose Gradients Were Run at 30,000 RPM for 10 Minutes at 20°C.

- DNA from Untreated Cells
- DNA from MC Treated Cells (1 µg/ml) Immediately after Treatment
- △ DNA from MC Treated Cells (1 µg/ml) after 90 Minute Incubation in NB after 37°C.

had been additionally characterized with respect to their genetic constitution. Bastarrachea and Willette (17) found that the recA13 and rec12 mutants both had lost the ability to cure K-12 of the F' factor by acridine orange and that the locus for this change could be genetically differentiated from that of recA1 and rec12. Other Rec⁻ mutants which they tested had not lost this ability.

Because of the MC-sensitivity of repair deficient strains of E. coli K-12 (18), it has been assumed that MC-induced damage is reparable, and our original hypothesis was that GIR-MC reflected an enhanced repair. On the basis of the data just discussed this hypothesis does not appear tenable, and what was assumed to be an incidental difference in the way GIR is evidenced in the MC-survival curves must be reconsidered. MC-survival curves tend to be biphasic (Fig. 10a). Classically this is interpreted to mean that there are two or more populations of cells which have different sensitivities to the damaging agent. In the case of MC-sensitivity, this must be a physiological heterogeneity because cultures obtained from colonies selected from plates for which survival is low show the same type of survival as the original stock. GIR-MC is more noticeable at low survival than at high survival as though growth in glucose causes a greater percentage of the cells to be resistant rather than causing a change in all the cells of the culture. This must be contrasted with GIR to gamma-rays (GIR-G) in which a 'shoulder' in the survival curve accounts for the major part of GIR. Thus GIR-G is most important at low doses and GIR-MC is most important at high doses. In the former case all cells (to the extent detectable in survival curves) are changed, but in the latter case GIR may merely reflect a change in the percentages of cells with the higher resistance.

From a consideration of the shape of the survival curves, it is not surprising that DNA profiles for GNB- and NB-grown Rec⁺ Uvr⁻ cells at high survival show fewer differences than at lower survival (1/4 µg/ml vs. 5 µg/ml). At low doses, the number of sensitive cells which have been killed are approximate the same leaving approximately the same number of cells surviving irrespective of their state of resistance. As the dose is increased, the cells remaining in the GNB-culture are essentially all resistant whereas this is not the case in the NB-cultures.

E. coli B/r is also GIR-MC positive. However, we have two strains of B/r which we designate 100 and 101 respectively (see section VII and Report COO-1686-9). B/r(100) is much more sensitive to both caffeine- and acriflavine-killing than is B/r(101). The difference in acriflavine-sensitivity is enormous. It turns out that B/r(100) is also much more sensitive to MC, and it shows a much larger GIR-MC than does B/r(101). However, the radiation- and

MMS- (see sections II and III) sensitivities are essentially the same for both strains. The GIR to gamma rays and to MMS are also each comparable in the two strains. Additional work uncovered yet another difference between GIR-MC and GIR-G. Work performed in our laboratory years ago had demonstrated that glucose had to be present essentially throughout the growth cycle of the culture (i.e., lag, log & stationary phase) in order to obtain maximal GIR-G. GIR-MC appears to develop within two hours if added to cells which are already in the stationary state.

There are then five lines of evidence that together are consistent with the idea that GIR-MC and GIR-G are not caused in the same way which are summarized as follows:

1. MC- and gamma-ray-survival curves for GIR positive cells are qualitatively different from each other. GIR-G involves a decrease in radiation-sensitivity at low doses for all cells, but GIR-MC may only reflect a change in the fraction of cells which are more resistant.
2. At equal doses, DNA from GNB-grown, GIR-MC positive cells does not appear to be damaged as much as DNA from NB-grown cells. In contrast, at equal doses, the initial damage to DNA in GNB- and NB-grown, GIR-G positive cells is of the same order of magnitude.
3. GIR-MC is developed within two hours after glucose is added to a stationary phase culture. GIR-G is developed fully only if glucose is present essentially throughout the growth of the culture to the stationary phase. This clearly suggests that processes by which resistance is developed are different in the two cases. (As reported in preprint C00-1585-20, GIR to methyl methanesulfonate is not developed unless it is present for a longer period of time. Thus this difference does not just reflect that one agent is a drug and the other radiation.)
4. GIR-MC is absent only in recA13 and rec12 mutants which are known to be mutant at another locus which causes the cells to be non-curable of their F' factor by acridine orange. GIR-G (and GIR-MMS) are absent in all rec⁻ strains tested. It is clearly possible that GIR-MC is fortuitously correlated with the two rec loci.
5. E. coli B/r(100) which is more sensitive to both caffeine and acriflavine than is E. coli B/r(101) is also more sensitive to MC and shows a much larger GIR-MC. The two strains have essentially the same sensitivity to gamma rays and to MMS and exhibit the same GIR-G and GIR-MMS.

These lines of evidence are all consistent with the possibility that GIR-MC involves a change in the permeability to MC or a change in the activation of MC to the active form. (In contrast, GIR-G and GIR-MMS have markedly different properties from GIR-MC and are consistent with better repair.)

VI. Split-dose recovery in Escherichia coli

Since the work of Elkind and Sutton (19), split-dose experiments in which survival of cells after a split or fractionated dose is higher than that obtained after exposure to an equivalent single or unfractionated dose have generally been interpreted as providing evidence for the presence of a recovery system in the cell. It appeared that this should be a straightforward method of obtaining additional evidence that GIR involved recovery rather than a shift in the amount or type of damage produced initially by the radiation exposure. With this in mind, split-dose recovery (SDR) was intensively studied. Under appropriate conditions SDR occurs in GNB-grown E. coli B/r, and this was interpreted initially as described above. However, a long series of control experiments demolished all justification for using the SDR experiments as supporting evidence for recovery from radiation damage even though they might be consistent with such a process. It was found that the conditioning part of the dose induces or triggers the cell to undergo some change during the holding period. This radiation-induced effect causes the cells to have a different response to subsequent irradiation. The explanations for this radiation-induced change in radiation sensitivity may be any of the alternative explanations which have been offered to explain GIR in the first place and, therefore, SDR does not provide additional insight concerning the nature of GIR but becomes a phenomenon which itself must be explained. More specifically, both GIR and SDR could be explained on the basis of changes in the repair of radiation-induced lesions, changes in the amount of lethal damage per unit exposure (for example, by change in the amount of protection against indirect effects), or by changes in the number of essential targets. These experiments are therefore, highly significant in demonstrating that SDR by itself cannot be used in the customary fashion as supporting evidence for the presence of a recovery system. Report No. COO-1686-10 describes these experiments in detail.

The problems surrounding the interpretation of the split-dose recovery (SDR) seen in E. coli B/r were not resolved even though additional efforts were made. The problems are all related to the fluctuations seen in the results. There is no doubt that in approximately 8 out of ten experiments a very marked 'SDR' is seen in NB-grown cells even though the survival curves for these cells are exponential in nature. However, in 20% of the cases the effect is not seen. On

the other hand, unirradiated controls treated in the same fashion did not ordinarily show an appreciable difference in radiation-sensitivity after 1 hour in GNB (cells taken from stationary phase). These results were presented at the Radiation Research Society meeting (20) and the chief questions raised concerning the results centered around the idea that the cells might be changing from the stationary phase to lag phase during the holding period.

Following this, experiments with unirradiated cells were again repeated. After many experiments it was found that approximately 20% of the time an increase in radiation resistance was seen. This increase, when observed, was always less than that seen in cells which first received a conditioning dose prior to the holding period. In the first control experiments cells had been removed directly from the stationary-phase-culture and placed into GNB or 10% GNB. In subsequent controls, cells were washed, resuspended in phosphate buffer for a period comparable to the irradiation time for the conditioning dose, and then placed in GNB. Under these conditions, a slight shoulder was seen in the survival curves approximately 50% of the time. In no case was this 'shoulder' as large as the one seen when the SDR experiments were positive. The combination of the variability in results together with the occasional controls which exhibited some change in radiation resistance led us to abandon these experiments even though the effect seems to exist and should be taken into account in any SDR experiments..

VII. Effect of caffeine and acriflavine on radiation and drug sensitivity.

Caffeine and acriflavine have been considered as inhibitors of DNA repair processes (see, for example, ref. 21). Most of the work done in our laboratory with caffeine and acriflavine has been reported (8) and is included in Appendix I or in the accompanying preprint C00-1696-20.. Because the effects of these drugs on GIR-G and GIR-MMS and the importance of these effects in considering the nature of GIR, a summary of the findings is given here. Two strains of B/r in our laboratory were found to have enormous differences in their sensitivity to caffeine and acriflavine (see Fig. 13 for the latter). These strains have been designated B/r 100 and B/r 101.

One of the interesting observations concerning these two strains is that their changes in radiation sensitivity at a given concentration of caffeine are the same. It should be noted that we are talking about those cells which will be capable of forming a colony at the concentration of caffeine being used. Comparable comparisons with acriflavine were not possible because of the extreme sensitivity of E. coli B/r 100. Radiation sensitivity increases

Figure 13

Colony Forming Ability of Unirradiated E. coli Cells at
Various Concentrations of Acriflavine in the Plating
Medium.

○—○ E. coli B/r (101)
△—△ E. coli B_s-1
□—□ E. coli B
●—● E. coli B/r (100)

with concentration of caffeine to the highest concentration tested (limited by the number of cells which can survive the toxicity of the caffeine). However, the cultures never become as sensitive to X-rays as E. coli B_{s-1} which lacks the E-R system. E. coli B/r 101 shows an increased X-ray sensitivity up to a concentration of 160 µg acriflavine/ml. An increase in concentration up to 240 µg/ml induced no additional change in the X-ray sensitivity. In this case also, the sensitivity never became quite as great as that of E. coli B_{s-1}.

The X-ray sensitivity of E. coli B_{s-1} appears to be affected only slightly for the concentrations tested (up to 1000 µg/ml caffeine). Its sensitivity to caffeine and acriflavine is comparable to that reported by Harm (21).

Some of the E. coli K-12 strains were also examined for the effect caffeine has on their radiation sensitivity. The pattern of response was very much like that seen in the B strains. For those strains which exhibit GIR, a given concentration of caffeine (below saturation levels) causes a decrease of greater magnitude in the survival of GNB-grown cells than that seen for NB-grown cells.

Caffeine at 0.1 and 0.2% concentrations in the agar caused the survival of UV-irradiated phage in E. coli B/r to decrease from 5×10^{-2} to 10^{-4} for a 50 sec UV exposure. The change was not quite as great at a 0.05% concentration of caffeine. If E. coli B_{s-1} is used as a host, the survival of UV-irradiated phage is changed only by a factor of three to five by a 0.2% concentration of caffeine. (Comparison is made at the same initial level of survival for phage on bacteria in the absence of caffeine.) Furthermore, the survival curve for phage in E. coli B/r subjected to 0.1 or 0.2% caffeine very nearly superimposes on the survival curve for phage in E. coli B_{s-1}. Caffeine also inhibits HCR of gamma-irradiated phage. However, the degree of inhibition of survival by caffeine is about the same whether or not phage are plated on GNB-grown or NB-grown cells.

Finally, caffeine at 2500 µg/ml causes the MMS-survival curve of GNB-grown Rec⁺ cells to be similar to that of NB-grown Rec⁺ cells plated on agar without caffeine (see Report No. COO-1686-20). These results are all consistent with the hypothesis that GIR represents an enhanced repair which is inhibited by caffeine or acriflavine. However, other interpretations are possible and are discussed in the last section.

VIII. Additional studies.

A. Introduction

A number of studies were performed that potentially might have provided

insight concerning GIR. A summary of the results of these studies is given below. The investigation of filament formation in E. coli B was originally undertaken with the idea that if filament forming tendencies could be inhibited, GIR might be observed. In that case, the absence of GIR in filament-forming strains might be fortuitous.

B. Effects of combined UV, and gamma-ray exposures

The effect of preconditioning U.V. exposures on X-ray survival and preconditioning X-rays exposures on U.V. survival were investigated for GNB-grown cells. Both 37° C and 18° C post-irradiation incubation temperatures were used. Cells were irradiated both at ice-bath temperature and at room temperature. NB-grown cells were used for comparison. The results of these experiments are somewhat equivocal because the rather large differences in the extent of synergism which are observed under different conditions have not been sufficiently reproducible to permit firm conclusions. However, as described in Appendix I, pre-exposures to UV seem to eliminate the 'shoulder' on the survival curves of GNB-grown cells before additional synergism is seen.

C. Photoreaction and Liquid Holding Recovery

Photoreactivation of GNB-grown and NB-grown cells suggest that the effect of glucose on the radioresistance is additive to the repair obtained by photoreactivation. U.V.-irradiated GNB cells have the same survival on nutrient agar plates containing 500 µg caffeine/ml as NB-grown cells have on nutrient agar without caffeine. Photoreactivation in all these cases appears to be additive.

No significant liquid holding recovery was observed for E. coli B/r.

D. Effect of 5BUdR (5-bromodeoxyuridine) Incorporation on GIR

Cells were grown in media with various concentrations of 5BUdR up to 500 µg/ml. In keeping with the findings of Orce et al (22), the radiosensitivity of the cells increased with increasing concentration of 5BUdR up to 300 µg/ml. No further sensitization was observed at 500 µg/ml. This sensitization is chiefly reflected by the disappearance of the shoulder on the survival curves for GNB-grown cells. The radiosensitivity of NB-grown cells was not changed by 300 µg/ml. It should be noted that the degree of incorporation was not established. If GNB-grown cells which have been cultured in the presence of 300 µg 5BUdR/ml are plated on plates containing 500 µg caffeine/ml, the survival is decreased as though the treatments had independent modes of action in determining sensitivity. This work was not pursued because it is now believed by comparison with work on U.V.-irradiation (23) that the

photoproducts which produce the biochemical lesions are different from those produced when thymine is present. The complexities of introducing a new type of lesion were preferably avoided.

E. Microcolony Formation versus Filament Formation in E. coli B

In order to investigate the kinetic correlation of survival with filament formation, "microcolony survival" curves were obtained for a variety of conditions. Cells were examined under the microscope after post-irradiation growth on agar covered slides. Because the microscopic fields become cluttered with filaments at high doses, the "microcolony survival" could not be followed below 1% survival. Microcolony survival curves were obtained by counting the number of microcolonies and the number of filaments present on the agar (slide) after 3 to 6 hours of post-irradiation incubation at the appropriate temperature. The ratio of microcolonies to the sum of microcolonies and filaments is called the fractional survival. These curves were compared with the conventional macroscopic colony survival. The results are summarized by the statement that within experimental variations the microcolony survival curves for all conditions are identical with the macroscopic colony survival curves. This holds for photoreactivation, various post-irradiation incubation temperatures, and post-irradiation exposure to caffeine or pantoic lactone. It was found that post-irradiation incubation of the cells at 42° C in the presence of pantoic lactone yielded a radiation resistance which approached that of E. coli B/r grown in nutrient broth. In spite of this, the one to one correlation between the "microcolony survival" and the macrocolony survival remained unchanged.

F. Dimer Excision in GNB-grown Cells

The method of Boyce and Howard-Flanders (24) was used essentially as described, except for the counting procedure, to look for differences in thymine dimer excision. Cells were grown in NB and GNB in the presence of approximately 200 μ C/ml thymidine, and 250 μ g/ml adenosine, and 0.5 μ g/ml thiamine. Cells in stationary phase were washed and irradiated with a dose of UV, incubated in a nutrient medium for one hour, washed and resuspended, which resulted in survivals which differed by a factor of a thousand. DNA was precipitated with TCA. This was followed by hydrolysis with tetrafluoroacetic acid. The digest was concentrated and spotted on Whatman #1 filter paper and developed with n-butanol/acetic acid/H₂O. Strips were cut and eluted in water and counted in Bray's solution by a liquid scintillation counting system. In the experiments that were performed, the thymine dimer to thymine

ratio for GNB-grown cells was approximately 1.3 times as great as that for NB-grown cells. There is a large uncertainty in that value because the thymine dimer peak is small, and therefore the area under the peak is a very sensitive function of the value that is chosen for the background and for the width of the peak. This method for correlating survival with differences in the fate of the radiation-induced lesion would be useful only if large differences in the thymine dimer/thymine ratios had been observed. Furthermore, the other work which has been performed strongly suggests that the major effect of cell growth in GNB is on REC repair. For these two reasons, this work was not pursued any further for this set of conditions.

G. Post-irradiation DNA Degradation and GIR

The post-irradiation degradation of DNA in *E. coli* B/r and *E. coli* B_s-1 was examined quite extensively in a partial attempt to correlate physiological parameters with the radiation sensitivities of the cells. Special attention was directed to the effect of glucose (i.e., GIR), caffeine, acriflavine, and low temperature reactivation (LTR) on DNA degradation. Two observations summarize the chief findings of these studies. First, all of the treatments mentioned inhibit the post-irradiation degradation of DNA, although the magnitude of the inhibition varies with the treatment and with the strain being studied. Second, no correlation has been found between the extent or rate of DNA degradation and the absolute radiation sensitivity of the various strains under the conditions tested. It would appear that the relationship between DNA degradation and survival is at a secondary level.

Post-irradiation DNA degradation was also examined in *E. coli* K-12 substrains AB2497, AB2463 recA13, AB1896 uvrA6, and AB2480 recA13 uvrA6. DNA degradation was followed after three different doses and up to 5 hours after irradiation. Except in the case of AB2463 at 5 and 60 KR, and AB2480 at 20 KR, growth of cells in GNB decreased the total amount of DNA degraded in five hours. The magnitude of the difference varied in an unpredictable manner both with dose and strain. It will be seen by comparison of GIR in these cells (Section II) that no obvious correlation is possible. The early rate of degradation also varied in both cases (GNB or NB-grown cells) with exposure and with the strain. If one uses the per cent degradation sixty minutes after irradiation as a measure of the early rate of degradation the following correlations can be made. In GNB-grown cells with a rec⁺ locus, the early rate of degradation is less at low doses than in NB-grown cells, but increases with dose up to 50 KR where it reaches a rate approximately twice that observed in NB-grown cells. In Rec⁻ Uvr⁺ cells, the early rate is greatest at low doses and is

smaller at the higher doses. Very little difference is observed in the DNA degradation rates for GNB- or NB-grown cells. The $\text{Uvr}^- \text{Rec}^-$ substrain exhibits a very low DNA degradation rate at all three doses when the cells are grown in GNB and an increase in rate with dose in NB-grown cells.

H. Low temperature reactivation in E. coli

Irradiated cells were plated and incubated at various selected temperatures for sixteen hours following which they were placed at 37°C until the colonies were developed. It was found that the type of response to variations in the post-irradiation temperatures for GNB-grown and NB-grown E. coli B/r was qualitatively the same but the absolute increase in survival is greater for GNB-grown cells. The time of incubation at 18° required for maximal increase in survival was the same for cells grown under the two conditions, and this maximum was reached in 12 hours. Furthermore, the maximum increase was observed at 18°C for E. coli B/r. [This agrees with the earlier work of Stapleton et al (25)]. E. coli B_{s-1} does not show such a response.

The substrains of E. coli K-12 were examined for LTR. The strains AB2497 and AB2463 appeared to show a small LTR (a difference of a factor of four at 10^{-4} survival which was too small a difference to pursue.

I. Factors which might lead to variations in survival curves

The use of survival kinetics in characterizing the response of bacteria to radiation is always made difficult by the variations seen in the survival curves of repeat cultures of cells under what are supposed to be equivalent conditions. Every effort was made to record various parameters which might possibly affect the survival of the cells. Factors which were investigated for correlation with variations in survival of cells exposed to a given dose include variations in the size of the inoculum to start the culture, the period of growth, the pH of the final growth medium, the temperature at the time of irradiation, the dilution procedures after irradiation, the initial temperature of the assay plates, the stock materials from which growth media are made, and the age of the stock slant. Variations in these factors within the range of normal operations cannot be correlated with the variations in the survival.

IX. Conclusions

The explanation of a change in radiation- or drug-sensitivity which is caused by a change in the growth conditions of a cell prior to treatment is always difficult to obtain because so many possible explanations exist. The possibilities generally speaking, are 1) a change in the effectiveness of the agent in producing damage, 2) a change in the number of essential targets, and 3) a change in the ability of the cell to recover from the damage.

The work performed under this contract has provided considerable insight concerning the nature of glucose-induced resistance to gamma radiation and to certain drugs. It must be admitted that none of the three alternatives mentioned above has been entirely eliminated as a possibility. However, if one assumes any one of the alternatives, the work that has been performed has to be interpreted in rather specific ways. A brief summary of the chief consequences assuming each of the alternatives in turn is instructive. If it is assumed that GIR reflects a change in the effectiveness of the agents, one must explain the necessity for a full complement of rec⁺ genes in order for GIR to be observed. One must also explain why a posttreatment exposure to caffeine or acriflavine abolishes GIR. The similarity between GIR-G and GIR-MMS must also be explained. In addition, such an assumption has implications concerning the effect of single-strand breaks on viability. For example, one might postulate that all of the rec mutants have an altered cell wall and that single-strand breaks are not important to survival or that the differences observed are not large enough to be reflected in measurable differences in survival. The alteration in the cell wall would be such that growth in GNB could not change the permeability of the Rec⁻ strains but could decrease the permeability of Rec⁺ strains. As a consequence MMS could not as readily get into GNB Rec⁺ grown cells and some protective agent against gamma-ray-induced damage could not get out. The finding that the MMS associated with a GNB-grown Rec⁺ cell is greater than that associated with an NB-grown cell would have to be explained. Since the amount of MMS per unit weight of cells remained the same, it is possible to suppose that cell weight is proportional to cell surface area rather than to the volume. In that case the MMS adsorbed to but not getting into the cell could increase with cell size without inflicting damage in the DNA. Furthermore, one would have to assume that caffeine and acriflavine are not acting on repair processes but are lethal to cells which have sustained prior damage by drugs or radiation. That is, caffeine and acriflavine would be more toxic for a damaged cell than for a healthy cell. Finally, one would have to postulate that

a reasonably high exposure to either gamma-rays or MMS would destroy the relatively impermeable state of GNB-grown Rec⁺ cells since GIR reflects a higher resistance only at low exposures.

As pointed out in section II.A., the extrapolation numbers for the survival curves are larger than can be accounted for on the basis of the increased amount (two-fold) of DNA in GNB-grown cells. In order to explain our results on the basis of an increased multiplicity of targets, one would either have to conclude that DNA is not the 'sensitive' target or that there is some kind of 'rescue' between the slightly increased number of targets. That is, a doubling in the number of genomes could increase the extrapolation number by more than a factor of two if there were some kind of rescue of viable genomes by recombination between DNA molecules. Such rescue, not unreasonably, could involve rec gene products. However, enhanced survival, in this case, would really be the consequence of a repair or rescue operation and belongs to the third category. The effect of caffeine or acriflavine could now reflect an inhibition of rescue operations.

Finally, one can assume that GIR reflects repair or recovery process controlled by rec genes which becomes saturated at higher doses. Each of the previous sections has outlined the manner in which results were consistent with this hypothesis and will not be repeated here.

In the case of gamma-ray-induced single-strand breaks, the analysis of the sedimentation profiles indicated that no statistically significant difference in the repair of breaks in DNA of NB-grown and GNB-grown Rec⁺ cells could be seen. In the case of MMS-induced breaks, one measure of 'molecular weights' suggested that a significant difference in the repair of DNA in the GNB- and NB-grown Rec⁺ cells. The other measure suggested there was no difference. Overall, the correlation of GIR with an enhanced repair of single-strand breaks is weak. The possibility remains, however, that single-strand breaks are not the only lethal lesions and that the production and repair of single-strand breaks only partially determines survival. Appendix I, ~~Appendix II~~ ^{removed, removed} and section IV discuss results of survival kinetics which strongly suggest that GIR may be the consequence of an enhanced repair of a second type of lesion, perhaps base damage. Final resolution of these questions may have to await the identification of other types of lethal lesions.

Reprints

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