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BASIC ASPECTS OF
RADIATION ACTION ON MICROORGANISMS

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Progress Report

SUMMARY STATEMENT

The general goal of the project is to elucidate the mechanism of induction of prophage by ionizing radiation. In seeking to attain this goal a number of highly interesting new factors have shown themselves and it is clear that the induction process is part of a new response to radiation which has at least four aspects, probably different in detail. Our laboratory has been active in showing the presence of some of these, namely the inhibition of post-irradiation DNA degradation (prd), the development of radioresistance, particularly when transcription is blocked with rifampin, and the induction of the error-prone repair system with concomitant shift in the nature of mutations from square law dependence on dose to linear.

Because of the new findings and in addition because of the work of Roberts and Roberts (1975) who showed that the actual inactivation of the lambda repressors is by proteolytic cleavage, it is clear that the mechanism of induction involves a radiation-mediated derepression of genes associated with recA, lex and tif. The mechanism of induction of these is still to be elucidated, but the problem has shrunk and the means of study have greatly increased. In suggesting the direction for next year's work the new situation will become apparent. In the meantime, the laboratory has contributed in the following areas, as briefly described and with accompanying appendices:

1. Induction of lambda can take place when DNA synthesis is blocked by hydroxyurea, if the block is removed after a while (LYDERSEN & POLLARD, Appendix I; preprint).
2. Induction cannot be due to a cross-link, otherwise uvr⁻ strains would induce more readily with ionizing radiation (ior). They do not. Dose response curves for UV and ior with uvr⁺ and uvr⁻ strains have been plotted. In addition, there is no induction of inhibition of prd in

*preprints
removed*

recB⁻ strains (POLLARD & K. KELLER, ~~Appendix 2~~ preprint).

3. The induction of inducible inhibition does not depend on the presence of lambda prophage (POLLARD, KELLER, RANDALL & BOYCE, ~~Appendix 3~~ preprint).
4. The induction phenomenon causes the development of radioresistance which is more apparent if transcription is blocked with rifampin (POLLARD & ACHEY, ~~Appendix 4~~ preprint).
5. Induction of lambda shows an oxygen effect (LYDERSEN & POLLARD) but induction of radioresistance does not seem to show such an effect (POLLARD & ACHEY) (Appendices 5 and 6).
6. A cell free system for study of degradation has been worked out and the degradation of irradiated DNA can be seen. However, up to the present (the experiments are continuing) a cell free system prepared from induced cells also degrades labeled irradiated DNA. This suggests that the mechanism of inhibition may be a combination of induced protein with the cellular DNA and not the result of inhibition of a specific nuclease, such as exonuclease V. An interesting by-product of the work is the possibility that in vitro repair of DNA can be seen. More work on this is needed. (KELLER, HENRY & RAKE, Appendix 7)
7. Hydrogen peroxide acts to degrade DNA and also to induce the inhibitor of prd. However, attempts to show that previous induction of cells with UV confers resistance against hydrogen peroxide have ^{been} inconclusive. It is most likely that the inducing event does NOT protect against death due to hydrogen peroxide. This latter may be a strangulation of the respiratory system and not susceptible to inhibition by the induced proteins (KELLER & POLLARD, Appendix 8).
8. The tif mutant strains, which are temperature-inducible, show inhibition of prd and also show transcription-blocked radioresistance (RADER &

POLLARD, Appendix 9).

9. The "error-prone repair" system of mutagenesis can be shown to be inducible both with UV and ionizing radiation (PERSON, RADER & POLLARD, Appendix 10).
10. There seems to be a clear difference between the inhibition of prd and the inducible respiration halt discovered by Swenson (1970) (POLLARD, Appendix 11).

HYPOTHESES FOR INDUCTION AND SUGGESTIONS FOR FUTURE PROGRAM

The original suggestion by Coldthwait & Jacob (1964) that the cessation of DNA synthesis causes a rise in the concentration of a precursor that is the inducing agent, is not easy to maintain, though it cannot be excluded. It has been suggested here and also by Dr. Lorraine Gudas that degradation products (dTMP or dAMP, for example, were suggested by our group and oligonucleotides by Dr. Gudas {pers. communication}) are the inducing agents. Another quite different hypothesis is that the folded genome of the cell is acted on by the agents so as to destroy temporarily the conformation in the vicinity of one of the critical genes, such as lex or tif.

Work to test the effect of dAMP has been started, with no success at present. Since the Ca^{++} treated cells used in transfection studies are presumably penetrable by dAMP and also have just recently been shown by Silber (in Dr. Achey's laboratory, U. Fla.) to be inducible, this technique should be usable. The same could be done with oligonucleotides.

Testing the change in conformation of the DNA will take learning the technique of study of the folded genome as developed by Stonington & Pettijohn (1971). We plan to do this. Study of the effect of radiation and inducing agents on the conformation will then be undertaken.

Work done by D. Spencer in Dr. W. D. Taylor's laboratory here has shown the presence of an inducible protein, "protein X", which has a 95% overlap in the induction properties with the inhibition of prd. It is intended to include Dr. Spencer in the work for next year, and Dr. Taylor is also joining the effort as a co-principal investigator. It should be an exciting year.

In addition, it is important to sort out the similarities and differences among the pleiotropic effects observed. A recent workshop held in Gainesville, Florida brought forth evidence that there are considerable differences involving reaction to the inducing agent, need for DNA synthesis, time course of induction, and relation to cyclic AMP. Work to sort all this out in the area of our expertise cannot be wasted time.

Support for observing the ultraviolet action spectra for these various phenomena is needed. Plans for this work are beginning.

Literature cited:

- Roberts, J.W. & C.W. Roberts, PNAS 72:147 (1975).
Swenson, P.A. & R.L. Schenley, Mut. Res. 9:443 (1970).
Stonington, O.G. & D.E. Pettijohn, PNAS 68:6 (1971).
Goldthwait, D. & F. Jacob, C. R. Acad. Sci. (Paris) 259:661 (1964).

APPENDIX 6 The Oxygen Effect in the Induction of Radioresistance
E. C. Pollard and P. M. Achey

The finding that if rifampin is used to block transcription, the preliminary induction of the cells renders them radioresistant has been followed up in the laboratory of Dr. Achey (U. Fla.) where there is a very suitable irradiation arrangement for the study of the effect of various gases during irradiation.

The arrangement is that of a single cobalt element around which six tubes can be simultaneously irradiated. These can be bubbled with any gas, and in our instance controlled flows of oxygen and nitrogen were used. The pattern of the experiment is as described in a previous appendix, namely, first the pre-treatment by irradiation with oxygen or nitrogen, or not any pre-treatment, followed by a time of incubation of about 40 min, whereupon 50 µg/ml of rifampin was given. Ten minutes later the various gamma-ray doses were given, all with oxygen bubbling.

The resulting survival curves, for one dose of gamma-irradiation, 7300 rads, are shown in Fig. 1. The untreated cells show the characteristic shoulder-less and sensitive survival curve of transcription-blocked cells, while the cells pre-treated with gamma irradiation are more resistant and have a definite shoulder. However, there seems to be no difference between pre-treatment in the presence of oxygen or nitrogen. This lack of effect may be due to the particular dose used. In other experiments it was found that with lower doses the protection produced was greater in the presence of nitrogen than of oxygen. On the other hand, at lower doses there seemed to be a preponderance of effect in favor of nitrogen, while at higher doses there was a much greater oxygen effect. These findings are shown in Fig. 2.

This account is preliminary only. All of the experiments need repeating, and this is going on in Dr. Abbey's laboratory. At present we are prepared only to say that for the induction of radioresistance there seems to be no significant oxygen enhancement.

Figure 1

Figure 2

APPENDIX 8 Hydrogen Peroxide
K. Keller and E. C. Pollard

It has been known for some time that ionizing radiation causes the degradation of E. coli DNA, not only directly but indirectly by damage inflicted by radicals formed as a result of the ionization of water in close proximity to the DNA. This indirect damage is presumably repaired along with the direct radiation damage (single-strand breaks) by cellular repair processes, which the latter induces. Awareness of such events led us to investigate how E. coli cells would respond to doses of radicals administered in the form of hydrogen peroxide ($H_2O_2 \longrightarrow .OH + .OH$) in the absence of ionizing radiation. Our results were positive and suggest that we might postulate a theory for the evolution of radiation response systems.

The basic procedure followed was the same in all the experiments to be described. Relevant characteristics of the cell strains used are shown in Table 1. The cells were grown in Roberts' C-minimal Medium (Roberts et al., 1955) supplemented with 0.5% glucose as carbon source and 0.2% casamino acids in the presence of 3H -thymidine to early log phase, at which time 100 $\mu g/ml$ final concentration rifampin was added. The cells were then incubated at 37°C

TABLE 1. Characteristics of Cell Strains Used

Cell Strain	Relevant Genetics	*Ior-mediated DNA Degradation	Inducible Inhibitor (requires <u>recA</u> ⁺ <u>exr</u> ⁺ (<u>lex</u> ⁺) genotype)
B/r	essentially wild type	+	+
B _s -1	<u>exr</u> ⁻	+	-
AB1157	essentially wild type	+	+
AB2463	<u>recA</u> ⁻	+	-
AB2470	** <u>recB21</u> ⁻	-	+

* Ionizing radiation-mediated DNA degradation

** Lacks the recBC nuclease, Exonuclease V, responsible for ior-mediated DNA degradation.

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for 10 min after which hydrogen peroxide was administered and incubation continued for an additional 10 min. The cells were next washed twice and resuspended in medium containing 10 µg/ml final concentration of catalase. The cells were then incubated with aeration for 120 min during which periodic samples were taken and assayed for TCA soluble materials.

Our studies showed that H_2O_2 alone is sufficient to degrade E. coli DNA and induce the inhibitor and that it does so with a genetic dependence similar to ionizing radiation.

Figure 1A shows the degradation pattern obtained using the wild type cell B/r and H_2O_2 at a concentration of 6 mM. One can observe that the amount of degradation obtained as a result of peroxide administration was much greater in the sample containing rifampin than in the sample without it. We believe that these differences were due to the fact that the sample lacking rifampin displayed inhibition of DNA degradation (this strain possesses the necessary genetic materials, namely the recA and lex genes) while the rifampin-containing sample did not (presumably transcription of the inhibitor having been blocked by the drug).

To test this belief, we next attempted to induce the inhibitor with a dose of UV (150 ergs/mm^2) given 50 min prior to rifampin administration. As can be seen in Fig. 1B, UV was very effective in inducing the inhibitor against H_2O_2 -mediated DNA degradation. Also effective in inducing the inhibitor, although not to the same extent as UV, was peroxide itself (Fig. 1B). Here the technique was to give the cells a dose of peroxide (6 mM) for 10 min 50 min prior to the rifampin treatment.

These H_2O_2 -mediated degradation responses of B/r were very similar to those obtained in ionization degradation studies although the overall amount of degradation was not as extensive.

We next studied B_{s-1} and AB2463 cells which lack the inducible inhibitor because of genetic deficiencies (refer to Table 1) hoping to observe a correlation between their known ior-mediated and heretofore unknown H₂O₂-mediated DNA degradation mechanisms. Again, a similar pattern emerged as illustrated in Fig. 2. The absence or presence of rifampin did not alter the amount of degradation obtained in these cells because the inhibition system was not in operation. When the concentration of H₂O₂ administered to these cells was changed we observed the results shown in Fig. 3. The amount of degradation obtained with B_{s-1} remained the same in the range of 3-12 mM H₂O₂ (Fig. 3A). However, AB2463 (Fig. 3B) degraded more effectively at H₂O₂ concentrations (i.e. 0.5 and 1 mM) below the experimental range. This accounts for the small amount of degradation observed in Fig. 2B.

A third mutant cell line, AB2470, was now studied. This cell is not subject to the extensive prod characteristic of the other cells studied, because it lacks the Exonuclease V enzyme (being recB21⁻) believed to be responsible for this degradation process. We therefore felt that this cell's response to H₂O₂ would be most revealing and interesting. Our results, shown in Fig. 2C, indeed were as we had predicted. No degradation above the background level could be observed. Thus it appears that the damage inflicted upon the DNA by H₂O₂ is recognized and acted upon by the same enzyme - Exonuclease V - which is in operation in ior-mediated degradation.

A basic assumption of our studies has been that the peroxide, once introduced into the cell, reacts with the environment to form hydroxyl radicals which in fact are the damaging agents. To test this assumption, we added a scavenger of .OH radicals, iodide ions, to our experimental system in the form of KI (1 mM). As Fig. 4A demonstrates, the removal of OH radicals by

the iodide ions completely inhibits the H_2O_2 -mediated DNA degradation. To assure ourselves that this was indeed the mechanism we were observing (as opposed to the killing of the cells by the KI) a parallel experiment was performed in which the degrading agent was ^{60}Co (23 krad). The results, shown in Fig. 4B, reveal that only a slight decrease in degradation is observed in the presence of KI. That any inhibition is seen is probably due to the fact that a small percentage of the overall damage originally observed is the result of $\cdot OH$ radical formation (indirect radiation damage) which is negated by the KI, resulting in less degradation.

To summarize, our investigations have revealed that hydrogen peroxide by $\cdot OH$ radical formation acts to degrade E. coli DNA and to induce the inhibitor of DNA degradation. Furthermore, H_2O_2 functions with a genetic dependence which mimics that of ionizing radiation in these capacities.

Since UV and H_2O_2 afforded protection against H_2O_2 -mediated DNA degradation by the inhibition process, we decided to investigate whether the inhibitor would also be a factor in cell survival by conferring resistance against H_2O_2 killing.

Our basic procedure was to grow cells to log phase in supplemented Roberts' C-Minimal Medium (0.5% glucose + 0.2% casamino acids + 20 $\mu g/ml$ B_6 if required). A UV dose of 150 $ergs/mm^2$ was then given to the cells for the purpose of inducing the inhibitor. After a 50-min incubation period at 37°C (which would allow for expression of the inhibitor if induced) rifampin was added to the cells at a final concentration of 50 $\mu g/ml$ and incubation continued for an additional 10 min. At the end of this incubation period, 3 mM H_2O_2 was administered to the cells and survival assayed over a 20-min time period. H_2O_2 action was stopped by immediate dilution of the samples (in unsupplemented C-minimal salts) which were then plated using the single-layer technique on nutrient agar (supplemented with B_6 if required).

It appears from our results (a portion of which are presented in Fig. 5) that the inhibitor does not protect against H_2O_2 -mediated death. The non-logarithmic survival kinetics which we obtained suggest that H_2O_2 -mediated death is not an all-or-nothing phenomenon but rather is the result of a strangulation of the respiratory system. This is in agreement with earlier investigations of this laboratory (Frey & Pollard, 1968; Pollard & Weller, 1967) which found that H_2O_2 disrupted the electron transport system of E. coli resulting in cellular death. In addition, death by strangulation of the respiratory system would not be susceptible to inhibition by an induced protein, such as the inhibitor, which is indeed what we have found.

Literature Cited:

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Frey, H. E. & E. C. Pollard, Radiat. Res. 36:59 (1968).
Pollard, E. C. & P. K. Weller, Radiat. Res. 32:417 (1967).

Figure 1. H₂O₂-mediated (6 mM) DNA degradation studies on B/r (A) with and without the drug rifampin and (B) with a dose of UV (150 ergs/mm²) and administration of H₂O₂ (6 mM) 40 min prior to rifampin treatment.

Figure 2. Hydrogen peroxide (3 mM) DNA degradation studies on (A) *B. s-1*, (B) AB2463, and (C) AB2470 (6 mM H₂O₂) with and without rifampin.

Figure 3. Effect of H_2O_2 concentration on the DNA degradation patterns of (A) B_{s-1} and (B) AB2463.

Figure 4. Effect of KI (1 mM) on (A) H₂O₂-mediated and (B) ior-mediated DNA degradation of *B8-1*.

Figure 5. H_2O_2 (3 mM) survival curves on (A) B/r and (B) AB1157 given a dose of UV (150 ergs/mm^2) 50 min prior to rifampin ($50 \text{ } \mu\text{g/ml}$) treatment.

APPENDIX 9 Inhibition of Postirradiation DNA Degradation and Development of Radioresistance

E. C. Pollard and M. Rader

The tif mutant strains discovered by Castellazzi et al. (1972) have been of great importance in uncovering the nature of inducible phenomena. Witkin (1974) has used them to show that error-prone repair is a factor in UV mutagenesis. We, accordingly, have investigated whether the temperature inducibility of these strains also induces inhibition of the postirradiation DNA degradation and whether radioresistance develops after heat treatment.

The strains used, WP44s tif-1 and WP44s tif-1 sfi, were kindly provided by Dr. Witkin. The first has the property of developing filaments after the temperature has been raised above 41°, particularly in the presence of 70 µg/ml of adenine. The latter has a suppressor of filamentation "sfi", not yet mapped, which prevents, to a large extent, the filamentation. Both strains were used.

Postirradiation degradation

The cells were labeled with ³H-thymidine in the presence of deoxyadenosine. They were then divided into two parts. One was kept at the permissive temperature of 34° and the other raised to 43° for 45 minutes. Rifampin was then added to prevent further transcription, ten minutes allowed for this to act, and then a dose of 30 krad given. The cells were then incubated at 34° for the times shown and sampled into 7% trichloroacetic acid (TCA). After 30 min in the cold, with 5 mg/ml bovine serum albumin present to give a precipitate, they were spun down and the supernatant counted in a Tri-Carb scintillation counter. The degraded DNA is soluble and appears in the supernatant. It can be seen that in the unheated case, shown as the upper line in Fig. 1, there is some degradation. This strain of E. coli is remarkable for the low degree

of degradation seen; nevertheless it is apparent. In contrast the cells which have been induced by heating at 43° show very little or no DNA degradation. This is quite apparent in the tif-1 sfi case and rather less so in the tif-1 without the filament suppression.

Induced radioresistance

The pattern of experimentation has been previously described in Appendix 4. Applied to these strains, with and without thermal induction, it can be seen that there is quite clear radioresistance in the tif-1 strain; the heating seems to produce as much radioresistance as induction with 2J/m² UV. The tif-1 sfi strain is not so clearly resistant, though resistance does show. It is not improbable that these pleiotropic effects of the same genes are really somewhat different in that the filament-forming tif-1 gains resistance while the suppressed strain gains less, while for the inhibition of degradation the relative behavior is opposite.

Elucidating the varied pleiotropic responses is one important task for the immediate future.

Literature cited:

- Castellazzi, M., J. George & G. Buttin, Mol. Gen. Genet. 119:139 (1972).
Witkin, E., PNAS 71:1930 (1974).

Figure 1

Figure 2

Figure 3

APPENDIX 10 Evidence for an Induced Mutagenic System

S. Person, E. C. Pollard and M. Rader

Ultraviolet light induced revertants are found to follow a relationship which fits with the revertants per survivor being proportional to the square of the dose. An explanation for this would be a two-step requirement, the first step being the eliciting of the mutagenic system by induction. This system is quite probably a DNA polymerase which will accept a dimer and possibly other defects in the DNA, and yet continue to make DNA. The dimer in the template is read equivocally and the result is an unusual abundance of single base-change events resulting in mutations.

Evidence for this type of "error-prone" or "S.O.S." repair has been found by Radman (1974), using the ϕ X174 system, where it is found that a heavily irradiated phage will not generate the full RF duplex, but that the DNA stops at the dimer next to the initiation point. However, if the infection is allowed to occur in a cell that has received UV light and been incubated for 45 min, a much more nearly complete RF is formed.

Wickin (1974) has shown that cells which carry the tif mutation, when raised to 42° in the presence of adenine, change their response to UV, the mutations to tryptophan independence being now both more in number at low doses and also linear with dose and not following the square law.

Evidence has been found in our laboratory for the induction by UV of this mutagenic system. The evidence consists of the observations that giving a preliminary dose of UV causes the cells to move from a dose squared to a linear dependence for revertants, and also that an increased number of mutants is found. This can be understood mathematically as follows. If the induction event, as is found for such processes, is all-or-nothing, then the probability of a hit that induces, for a dose D, is

$$(1 - e^{-D/D_1})$$

(1)

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where D_1 is a parameter that expresses the inducibility. When $D = D_1$ there is a 63% probability of induction.

If now we suppose that the probability of mutation in these induced cells is proportional to the dose, with a proportionality factor, m , then the number of mutations should be

$$mD(1 - e^{-D/D_1}). \quad (2)$$

If D is less than D_1 , then to a first approximation the exponential is $1 - D/D_1$ and the overall relationship states that the mutations should obey the relation

$$\text{mutations} = \frac{mD^2}{D_1}; \quad (3)$$

this is the square law dependence on dose mentioned previously.

Now if the cells are previously induced by some agent, for example a previous dose of UV, and allowed to incubate to develop the induced mutagenic system, the additional mutants should show a linear behavior. It is unlikely that the inducing dose is 100% inducing, but that instead a fraction x can be induced. Since induction is an all-or-nothing phenomenon, the fraction not induced is then $1 - x$. Thus the overall mutation response to a dose D should be, to a first approximation,

$$R/S = xmD + (1 - x)\frac{m}{D_1}D^2.$$

The first derivative of this is $xm + 2D(1 - x)m/D_1$. Thus at the zero dose origin there should be a finite slope if the induction process is at work and a zero slope if it is not.

Figure 1 shows plots of some experimental data for leucine revertants in strain WU3610-89, which is uvr⁻. It can be seen that for four pre-doses as shown, the initial slope is not zero, as in contrast to the case of no pre-dose. Indeed, the fraction induced in the case of the 0.6 and 0.9 J/m²

pre-dose must be quite high. The time allowed for development of the mutagenic enzyme (if that be what it is) was 15 min, which is reasonable in terms of the other inducible phenomena.

Similar findings are obtained if the induction is performed with ionizing radiation. This is still to be considered preliminary work, but we feel no doubt about the reality of an induced mutagenic system, and that it can be induced with radiation.

Literature cited:

Radman, M., Molecular and environmental aspects of mutagenesis, in press.
Witkin, E., PNAS 71:1930 (1974).

APPENDIX 7 The Cell Free System

K. Keller, F. Henry and A. Rake

For many years our laboratory has been studying the process of post-irradiation DNA degradation (prd) in E. coli, first observed by Stuy (1960). Since that time it has been established that the amount of DNA degradation obtained depends on the genotype of the cell, being quite extensive in recA⁻ and lex (exr⁻) strains while less so in cells that possess the recA and lex gene products. In cells of the recA⁺lex⁺ genotype it is possible, under certain conditions such as UV irradiation, to induce a process (the possible result of cellular repair activities) which decreases the amount of prd obtained. We have extensively studied this degradation reduction phenomenon which we call the inducible inhibitor of prd.

Recently it became apparent to us that our knowledge of the processes of degradation and inhibition could be greatly increased if a cell free (in vitro) system could be developed over which we would have greater manipulative powers than we have had in the past with in vivo systems. Development of our system was based upon our belief that the recBC nuclease - Endonuclease V - is responsible for the extensive prd observed in vivo, both by ourselves (Pollard & Keller) and other independent investigators (Emmerson, 1968; Youngs & Bernstein, 1973). This recBC nuclease has been purified to near homogeneity and characterized by Goldmark & Linn (1972). They have reported the optimum conditions under which Exonuclease V functions and it is these conditions that we have adapted and modified to form an environment under which cell free degradation and inhibition could proceed. Development of this cell free system has been difficult, and to date, while we are able to observe in vitro DNA degradation, we have not yet been able to detect the inhibition process in our system. It is possible that the inhibition acts on the DNA and not on the enzyme system.

Briefly our experimental procedure is as follows: 1) cells are grown in Roberts' C-Minimal Medium (1955), supplemented with 0.5% glucose as carbon source and 0.2% casamino acids until they reach a concentration of about 2×10^8 cells/ml; 2) at this time they are spun down and concentrated 10 times in our "cell free medium" which consists of 0.05 M glycylglycine-NaOH (at pH 7 or 9 depending on experimental design), 0.01 M $MgCl_2$, 0.025 M dithiothreitol or dithioerythritol (maintain -SH groups in reduced state), and 20 μ M ATP; 3) the cells are next sonicated on ice using a Sonic 300 Dismembrator (Aretak Systems Corp.) for 60 sec with a large tip at a setting of 80; 4) the drug rifampin is now added to the cell free system at a final concentration of 50 μ g/ml; 5) at the end of a 10-min incubation period at 37°C γ -irradiated, 3 H-thymidine labeled E. coli DNA purified by the Marmur method (1968) (hereafter called *DNA) is added to the cell free system at approximately 2000 cpm/ml cell free medium; and 6) immediately the degradation profile is begun with samples taken over a 200-min time period and assayed for TCA soluble material.

In conjunction with the preceding technique, alkaline sedimentation profiles of the purified DNA subjected to cell free degradation were determined. Samples taken at the indicated incubation times were held at ice temperature for the duration of the experiment. The standard 5% to 18% alkaline sucrose gradients (pH 12, 1.0 M NaCl, 3 mM EDTA) including the 0.1 ml, pH 13 "lysing layer" were loaded with 0.2 ml sample, held at room temperature for 30 min, then centrifuged at 30K RPM for 240 min at 20°C in a Beckman SW 50.1 rotor. Seven drop fractions were collected from the bottom and 3 H (and 14 C) thymidine activity assayed in toluene-Triton X scintillation fluid containing 19% water.

The first in vitro degradation experiment we attempted was performed using *DNA which had been irradiated at total of 39 krads and a B_{5-1} cell free

system at two values of pH, 7 and 9 (pH 7 being comparable to our in vivo situation and pH 9 being the optimum pH for Exonuclease V activity). The results of this experiment, shown in Fig. 1, were very encouraging to us; it was the first time we were able to observe extensive (up to 70%) DNA degradation in vitro. This extensive pattern was seen only at Exonuclease V's optimum, pH 9, the amount of degradation obtained at pH 7 being much less (about 30%). It therefore became part of our standard procedure to run our cell free system at pH 9.

Alkaline sucrose gradients of *DNA degraded at pH 7 in the above experiment are shown in Fig. 2. The gradient assay sensitively detected breakdown in the larger, faster sedimenting pieces. The *DNA profiles for samples showing 5, 12 and 18% degradation are clearly differentiated (Fig. 2b). The amount of degradation was not measured by this method as a particular number of fractions due to mixing and diffusion. Even so, the increase in slowly sedimenting pieces with incubation supported the degradation data.

We were next interested in observing the effect that an increased γ -irradiation dose of the *DNA would have on the degradation pattern in the B_{s-1} cell free system. As Fig. 3 shows, there is not a significant difference in the amount of DNA degraded at a dose of 117 krad from that obtained at a dose 3 times less.

Alkaline sucrose gradients of control and 39 krad x-irradiated *DNA, degraded at pH 9 (Fig. 4) reveal extensive breakdown. Clearly, degradation proceeds more rapidly in the irradiated *DNA (Fig. 4b) than in the unirradiated DNA (Fig. 4a). The double-peaked profile of the 80-min unirradiated incubation suggests that larger pieces are being reduced to pieces half the size plus fragments.

Thus, we had found that the DNA degradation process could proceed in the B_{s-1} cell free system (and presumably other cell free systems as well) and most importantly that its pattern was reproducible with a maximum of about 65-70%.

In order to study the inhibition process it became necessary to employ a cell which fulfilled the genetic requirements (namely, recA⁺lex⁺exr⁺ genotype) of the inducible inhibitor which B_{s-1} did not fulfill, being exr⁻. Thus, our attempts to observe inhibition in vitro were performed on B/r cell free systems (B/r being recA⁺ and exr⁺).

UV irradiation, as previously mentioned, is an effective inducing agent of the inhibitor. Therefore a dose of 150 ergs/mm² of UV was given the B/r cells 50 min prior to their being spun down and concentrated in the cell free medium (refer to basic procedure). This 50-min incubation period at 37°C would allow the inhibitor, if induced, to become expressed. Figure 5 illustrates the results obtained when such a procedure was followed under our standard experimental conditions (pH 9, *DNA irradiated 117 krad). No inhibition of prd was observed; all samples, including those given the inducing dose of UV, showed an extensive degradation pattern (up to 75%) similar to the pattern obtained with B_{s-1}.

Feeling that perhaps our inhibitor could not function at pH9 we lowered the pH of our system to 7. Again we noted no change in the degradation pattern (Fig. 6).

With the purpose of trying to understand more fully what was happening in our cell free system (i.e, why the inducible inhibitor could not be demonstrated, although all we knew from our in vivo work told us it should be present) our next experiment monitored not only the degradation of the exogenously added *DNA but also the degradation of the DNA native to the system

(endogenous DNA) by use of another radioactive label, ^{14}C -thymidine. The results, shown in Fig. 7, fail to clarify the situation; they show that the inhibitor is not functioning to reduce the amount of degradation of either the exogenous or endogenous *DNA.

The double-label alkaline sucrose sedimentation profiles (Fig. 8) show that exogenous *DNA was broken down during a 180-min incubation. The striking feature of this data was that endogenous DNA got larger during incubation while the exogenous DNA in the sample was degraded. The double-label technique made this observation difficult to discredit. It is a temptation to speculate that endogenous DNA may have acted as substrate for the repair enzymes in this cell free system. A second feature was that the *DNA at all times had a larger average piece-size than did the endogenous DNA.

We have not yet discovered why we are unable to observe the process of inhibition in our B/r cell free system, even though it is so readily observable in vivo. This fact in itself is most interesting. One possibility has been mentioned, namely that inhibition occurs because the DNA is covered by a protein (possibly the "protein X" of Inouye (1969) and Kazanis, Spencer & Ginoza {submitted}) which acts to inhibit. This would be removed in the Marmur procedure.

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FIGURE 1. B_{s-1} cell free DNA degradation study at pH 7 and pH 9 with *DNA γ -irradiated a total of 39 krad.

Figure 2: Alkaline sucrose sedimentation profiles of exogenous DNA degraded at pH 7.0. (a) unirradiated, (b) 39 krad x-rays. (o) 0 min, (Δ) 20 min, (□) 40 min postirradiation incubation.

Figure 3: B_{s-1} cell free DNA degradation study at pH 9 with *DNA being irradiated with various doses of γ -rays.

Figure 4. Alkaline sedimentation profiles of purified DNA degraded in cell free system at pH 9.
 a. Unirradiated; b. irradiated with 39 krad x-rays.
 o, 0 min; Δ , 40 min; $\square$, 80 min incubation in cell free system.

Figure 5. B/r cell free DNA degradation study with and without UV inducing dose of 150 ergs/mm^2 at pH 9 with *DNA being irradiated a total of 117 krad.

Figure 6. B/r cell free DNA degradation study with and without UV inducing dose of 150 ergs/mm² at pH 7 with *DNA being γ-irradiated a total of 117 krad.

Figure 7. In vitro DNA degradation study using B/r cell free system at pH 9 monitoring both exogenous and endogenous *DNA (both irradiated with 117 krads) degradation.

Figure 8. Alkaline sedimentation profiles of double label degradation experiment