REPAIR OF X-RAY DAMAGE TO BACTERIAL DNA

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Of the lesions produced in DNA by ionizing radiation, the one most extensively studied to
date, and most closely correlated with cell killing, is the breakage of one or both strands of the
sugar-phosphate backbone. Base damage is also known to occur, although it has been little
studied. Depending on the exact site of free radical attack on the sugar moiety or phosphodiester
bond, DNA strand breaks may occur in different locations, with corresponding differences in the
size of the strand interruption and in the chemical nature of the residual end groups. These diffe-
nences call different enzymes systems into play to restore the integrity of the polynucleotide chain.
Unrepaired single-strand breaks in DNA may be lethal for the cell; cell killing is significantly in-
creased when repair is genetically or physiologically deficient or is inhibited by post-irradiation
treatment with certain chemicals.

Using selected mutants of Escherichia coli, it has been shown that essentially the same num-
bers of single-strand breaks are produced by X-rays in the presence or absence of oxygen. After
irradiation, these are rejoined to different extents by three operationally distinct repair processes,
designated Types I, II and III. Type I repair, the fastest, goes to completion in less than one
minute at 0°C. Since it preferentially rejoins breaks produced under anoxic conditions, its
effect is to leave about one-third as many single-strand breaks remaining in cells irradiated un-
der anoxic versus aerobic conditions. Preliminary evidence suggests that it may involve the
action of DNA polynucleotide ligase. Of the breaks remaining unrepaired, whether produced
anoxically or aerobically, about 90% are acted upon by the Type II system, which is also rela-
tively fast ($T_1 = 1-2$ min at room temperature), does not require growth medium, and is largely
deficient in polA mutants. Type III repair requires 30-60 min of incubation in growth medium
at 37°C. It is absent in expr mutants and also in recA mutants which are deficient in genetic re-
combination. It is capable of repairing only a small fixed number of breaks (approximately 2
breaks per single-strand genome), whether produced anoxically or aerobically. Genetic and
pharmacological evidence suggests that the recA gene is also required in an additional repair
process which does not involve DNA single-strand breaks. Several recently developed exper-
imental systems which may permit the further elucidation of these enzymatic repair processes
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1. Introduction

Many independent lines of evidence support the idea that damage to DNA caused by ionizing radiation leads to cell death in both prokaryotic and eukaryotic organisms [for reviews, see Ginoza, 1967; Kaplan, 1968]. At the level of the DNA, four factors interact to determine the ultimate response of the organism. These are:

(1) the nature and quality of the radiation (i.e., type of particle, photon or particle energy, LET);
(2) the intrinsic radiation sensitivity of the DNA target (e.g., size, base composition, strandedness);
(3) the presence of dose-modifying agents at the time of irradiation [e.g., sensitizers such as oxygen (O_2), N-ethylmaleimide (NEM), tri-acetone-amine-N-oxyl (TAN), iodine compounds; protectors such as radical scavengers, etc.]; and
(4) the ability of the organism to repair radiation damage, which is controlled by both genetic and physiological factors.

This review is concerned principally with this fourth factor—repair. Ionizing radiation produces many kinds of physical and chemical alterations in DNA: breaks in one or both polynucleotide strands and chemical alterations in the individual molecules (bases and sugars) within the strands. Accordingly, one might expect to find enzymatically distinct pathways for the repair of different kinds of damage. The response of the cell will therefore be determined not only by the initial damage, but also by whether or not the cell possesses the necessary enzymatic mechanisms to cope with the damage.

Most in vivo studies have concentrated on the production and repair of DNA single-strand breaks, probably because the technique for measuring DNA single-strand breaks is simple and reproducible. The importance of the repair of such breaks has become increasingly apparent due to the isolation of additional radiosensitive mutants deficient in this type of repair, and the recognition that physiological and pharmacological factors can concomitantly influence both repair and cell viability.

2. Nature of the damage to DNA

Early work had shown that ionizing radiation produces both physical and chemical damage to DNA irradiated in vitro. Molecular weight deter-
minations by viscosimetry and light scattering showed reductions in both single- and double-stranded molecular weight. It was also possible to detect the release of free bases and other moieties from the irradiated DNA. In much of this work, the shear sensitivity of high molecular weight DNA was either not appreciated or could not be overcome. Consequently, most of the damage seen in isolated DNA arose during the extraction procedure. In addition, since the size of the extracted DNA was relatively small, large doses of radiation (well beyond the biological range) were required to produce detectable effects.

Significant progress was made with the isolation of high molecular weight DNA from both bacteriophage and bacteria using minimal shear conditions and with the application of velocity sedimentation to measure molecular weights. McGrath and Williams [1966] successfully employed these techniques to demonstrate small numbers of single-strand breaks in E. coli DNA, and Kaplan [1966a] showed that it was also possible to measure double-strand breaks using a similar technique at neutral pH. In the following sections, the nature and biological significance of the different kinds of X-ray induced DNA damage will be reviewed.

2.1. BASE AND SUGAR DAMAGE

Studies on DNA base damage in vivo have been hindered by the chemical instability of the radiation products that have been identified in vitro. Progress in our understanding of processes for the repair of DNA damaged by ultraviolet (UV) radiation has been due largely to the chemical stability of pyrimidine dimers and their identification as biologically significant photoproducts. In the case of ionizing radiation, it is difficult to formulate models for repair when the chemical nature of the lesions remains largely unknown. In spite of this lack of knowledge, base damage has been implicated (somewhat indirectly) in the X-ray induced killing of both bacteriophage [Freifelder, 1966a] and bacteria [Howard-Flanders et al., 1966] (see also sections 6.3 and 7.1.1). The latter authors showed that the uvrA, B, C mutations, which are involved in the repair of DNA containing pyrimidine dimers, also conferred some sensitivity to ionizing radiation, although to a much lesser extent than to UV radiation.

Recently some progress has been made with the identification of a major radiolysis product of thymine in vivo and its subsequent removal from the DNA ('repair') [Hariharan and Cerutti, 1972]. This is a significant achievement and should be exploited. It remains to be seen, however, whether there is
indeed a specific mechanism for the removal of X-ray damaged bases, or whether most of the damage is not simply removed by non-specific DNA degradation. This latter possibility might explain why the uvr mutations confer only a small amount of X-ray sensitivity on the cells—despite the probability that base damage is more frequent than single-strand breaks. The current status of base damage and its repair has been reviewed recently by Cerutti [1973] and will not be further discussed.

Irradiation of DNA in aqueous solution also leads to damage to the deoxyribose moieties of the molecule [Hems, 1960; Krushinskaya and Shal'nov, 1967; Kapp and Smith, 1970a; Ullrich and Hagen, 1971]. At least some of the single-strand breaks which arise are probably a consequence of sugar damage rather than direct scission of phosphodiester bonds. This point is further discussed below (section 2.2.3). As with most X-ray-induced base damage, there is no sensitive assay for sugar damage in vivo.

2.2. DNA single-strand breaks

The experiments of McGrath and Williams [1966] were a clear demonstration, under conditions where DNA shear was minimized, that X-irradiation induces DNA single-strand breaks (and/or alkali-labile bonds, see below) in vivo. This and the further demonstration that these breaks could be repaired in a radiation resistant strain, E. coli B/r, but not in a sensitive strain, B−1, has formed the cornerstone both conceptually and technologically for much of the ensuing research on DNA single-strand breaks.

2.2.1. On the question of alkali-labile bonds

It was recognized many years ago that loss of purine bases from the DNA without scission of the phosphodiester bonds would give rise to sites which were readily hydrolysed in alkali [Tamm et al., 1953; Bayley, Brammer and Jones, 1961]. These can be distinguished from frank breaks by denaturing the DNA under neutral conditions (e.g., by the use of formamide). Unfortunately, the techniques which use whole cell lysates on the gradient are unworkable with formamide [Smith, K.C., unpublished observations]. It is therefore difficult to estimate what fraction of breaks seen under alkaline conditions actually existed as breaks within the cell and which existed simply as base damage. This could be important, as different enzymatic mechanisms might be envisaged for the recognition and repair of the two types of lesions. It has recently been reported [Ley and Setlow, 1972] that \( \sim 90\% \) of the lesions (single-strand breaks and/or alkali-labile bonds)
produced in bromouracil-substituted DNA by exposure to 313 nm radiation are rapidly repaired in both urrA6 and polA1 mutants of E. coli K-12. This suggests the existence of a mechanism for the repair of this type of damage involving an unexpected combination of enzymatic activities.

Several studies have recently addressed themselves to the alkali-labile bond question by exploiting the fact that the DNA of some bacteriophage and plasmids exists inside the cell as covalently closed double-stranded circles at some stage of replication. These closed circles are generally supercoiled and have relatively high sedimentation coefficients. A single nick in either strand will relax the supercoil and allow the molecule to revert to an open circle with a lower sedimentation coefficient. The differences in sedimentation coefficient between the closed and open circular forms are sufficiently great to allow their separation under both neutral and alkaline conditions. Hence it is possible to measure the rate of production of single-strand breaks under neutral conditions and also the total yield of breaks plus alkali-labile bonds in the same sample of DNA.

The situation which is probably most appropriate to in vivo irradiation conditions is that used by Paterson and Roozen [1972] who irradiated λdv circles in chromosome-less minicells of E. coli. They found that approximately 20% of the breaks seen under alkaline conditions were not registered under neutral conditions. Achey, Billen and Beltranena [1971], irradiated λX174 DNA in dilute aqueous solution and found that ~17% of the breaks were actually alkali-labile bonds when the DNA was irradiated frozen, and about 45% when the DNA solution was irradiated in the liquid state. For SV40 DNA irradiated in dilute solution under anoxic conditions Kessler, Bopp and Hagen [1971] found that approximately 30% of the breaks seen in alkali were not present under neutral conditions. When the DNA was irradiated dry at 10⁻² Torr, no alkali-labile bonds were formed.

The actual magnitude of the fraction of alkali-labile bonds should be determined for each experimental situation, since it has been shown by Hewitt and Marburger [1971] that, in the case of UV irradiation of bromouracil substituted DNA, this fraction is dependent on the ionic nature of the buffer, which presumably influences DNA conformation. As a working guide, a figure of 20% should be kept in mind as that fraction of the breaks seen on alkaline gradients which may not exist as frank breaks within the cell. No further explicit reference to this concept will be made, but the term 'single-strand breaks' should be read with the parenthetical 20% reservation.
2.2.2. The production of DNA strand breaks—direct or indirect effect?

So far, all the studies which attempt to identify the nature of the end groups left at single-strand breaks have used DNA irradiated in dilute aqueous solution. Under these conditions most of the damage is mediated by radicals produced in the water. At present, there is little information as to how much of the radiation effect on DNA in vivo is due to energy directly absorbed, and how much is due to indirect radical attack. It may be pure coincidence that the energy required to produce one DNA single-strand break is about the same as a typical energy loss event by ionizing radiation, and that this same value (\(~ 60 \text{ eV per single-strand break}\)*) is also found for dry DNA and for DNA in phage which are maximally protected against the indirect effects of radiation. By using various concentrations of radical scavengers with known rate constants, it is possible to infer which radical species is responsible for a particular event, and also the extent to which this event is caused by indirect effects. Thus Roots and Okada [1972] suggest that up to 70% of the DNA single-strand breaks are produced by OH radical action in cultured mammalian cells. Other workers [Johansen and Howard-Flanders, 1965; Sanner and Pihl, 1969] have estimated that about 50% of the lethal effect in E. coli is due to events mediated by aqueous radical species.

Whether the initial event is a radical produced directly in the DNA, or one produced in the aqueous medium which secondarily attacks the DNA, the subsequent chemical reactions leading to sugar or base damage, and possibly to strand breakage, may follow a similar course. Thus there may ultimately be little difference between the chemical nature of the DNA damage produced by direct and indirect action. Hopefully this will prove to be the case since all the available data on the chemical events leading to DNA strand breakage are for DNA irradiated in dilute aqueous solution.

2.2.3. Chemical and enzymatic analysis of end groups and damaged molecules

Figure 1 shows two adjoining deoxyribose moieties in a single DNA strand. Breaks along any of the dashed lines would lead to a single-strand break. This serves to illustrate the concept that breaks might arise with a variety of different end groups. Several studies have been published recently which attempt to identify the nature of the end groups left at single-strand breaks

* The value of 60 eV used here is the approximate modal energy value reported in the literature for production of single-strand breaks in DNA. The actual range of values is from approximately 20 eV to 100 eV. See table 2.
in X-irradiated DNA. In part they use the properties of some of the enzymes described below, and in part they use chemical methods to identify damaged or released molecules.

Using dilute aqueous solutions of calf thymus DNA, Kapp and Smith [1970a] found that for every single-strand break produced when the DNA was irradiated in the presence of $10^{-3}$ M histidine (which helps protect against indirect effects of radiation) 0.65 malonic aldehyde-like molecules (i.e., sugar damage) and 1.5 phosphomonoester groups were formed and 0.2 molecules of inorganic phosphate were liberated. In the absence of histidine these values were 0.60, 1.35, and 0.33, respectively. It was not possible to account for all these data by a simple model, but the study clearly shows that many different termini (including damaged deoxyribose molecules) may be found at the breaks.

In a similar type of study, Bopp and Hagen [1970] found approximately the same number of single-strand breaks as the total of 5'-OH plus 5'-PO$_4$ groups. There were approximately 90% 5'-PO$_4$ groups and 10% 5'-OH

![Diagram](image-url)  
Fig. 1. Possible mechanisms for the formation of single-strand breaks in DNA [from Kapp, 1970].
groups. When the irradiated DNA was treated with alkali, the yields of breaks, 5'-OH and 5'-PO₄ groups all increased by approximately 1.5-fold. They proposed that the fraction of breaks expressed only in alkali arose from sugar damage, with the alkali treatment leading to base liberation and strand breakage. However, Ullrich and Hagen [1971] found fewer alkali-labile bases than the number of alkali-labile bonds observed by Bopp and Hagen, suggesting that some alkali-labile bonds are opened without the release of bases. Ullrich and Hagen [1971] analyzed deoxyribose damage both by the thiobarbituric acid (TBA) reaction used by Kapp and Smith [1970a] and also by reaction with methylindole. This latter reaction occurs at room temperatures whereas the TBA reaction requires boiling under acidic conditions. Ullrich and Hagen [1971] found the same yield of TBA reacting material as did Kapp and Smith [1970a], but only about 2% of this yield in the methylindole reaction at room temperature. This suggests that much of the sugar damage might not result in malonic aldehyde formation at room temperature.
Figure 2 shows the distribution of reactions proposed by Ullrich and Hagen [1971] to account for their data. While this may represent only some aspects of the reactions involved, it serves to illustrate the variety of ways in which radiation can damage the DNA and lead to a single-strand break. The proposal that some of the breaks arise from the release of bases under neutral conditions also finds support in the work of Ward and Kuo [1972]. Using thymidine diphosphate as a model compound, they found that hydroxyl radical attack could lead to simultaneous release of both phosphates, thus yielding a free base. In a DNA strand, this would correspond to a missing base with phosphate groups (3' and 5') at the two ends of the interruption.

It is clear, therefore, that the term ‘single-strand break’ is merely a convenient abstraction. Not only can the breaks arise by any one of several different mechanisms, but also the breaks (or their termini) comprise a wide variety of chemical entities.

2.3. DNA double-strand breaks

At about the same time that McGrath and Williams [1966] demonstrated the repair of DNA single-strand breaks using alkaline sucrose gradients, double-strand breaks in bacteriophage and bacterial DNA were demonstrated and quantitated by sedimentation under neutral conditions [Freifelder, 1966a, b; Kaplan, 1966a]. For E. coli K-12, Kaplan [1966a; 1967] found correlations between changes in radiosensitivity and DNA sedimentation both for cells which were sensitized by incorporation of 5-bromodeoxyuridine into their DNA, and for cells which were protected by the presence of mercaptoethyamine. Further, in contrast to single-strand breaks, double-strand breaks did not seem to be repaired. However, there have been several reports which make it seem likely that some double-strand breaks can be repaired in M. radiodurans [Kitayama and Matsuyama, 1968; Lett et al., 1970; Burrell et al., 1971] and possibly also in mammalian cells [Corry and Cole, 1972]. In E. coli K-12, Tomizawa and Ogawa [1968] reported that some 32P-induced double-strand breaks (which may represent a specific kind of break) were repaired in rec+ bacteria.

The spread of values reported in the literature for the rate of double-strand breakage is so great as to preclude the possibility of predicting the rate at which such breaks are introduced into a single E. coli chromosome even though its molecular weight is accurately known. A modified technique for the analysis of native DNA from E. coli has recently been described.
[Town et al., 1973b], whereby the rate of strand breakage can easily be measured. Preliminary results suggest that after a threshold dose, a 2.8-fold greater number of double-strand breaks are produced in O₂ than in N₂. Some of these breaks appear to be rapidly induced enzymatically, however. The rate of production of primary radiation induced double-strand breaks will have to be determined in selected mutants and under conditions which strongly inhibit metabolic processes (both degradation and repair). The neutral gradient method of Town et al. [1973b] should facilitate such experiments.

3. The biological system: E. coli K-12 and its mutants

The choice of E. coli K-12 for these studies follows naturally from its central position in molecular biology. In addition to the simple handling techniques required for bacteria, E. coli K-12 offers the specific advantage of being the most thoroughly characterized of all bacteria, both genetically and biochemically.

Because of its well-characterized mating system and the existence of generalized transducing phages, the single chromosome of E. coli has been well mapped and the functions of many of its genes are understood. Since it is a haploid organism, mutations are easily expressed and mutants can be readily isolated. The ability to cross strains of E. coli by both conjugation and transduction not only allows mapping but also the construction of multiply mutant strains in order to assess the involvement and interaction of different genes in a given metabolic pathway.

3.1. MUTANTS WITH ALTERED RADIOSensitivity

Many mutants have now been isolated with altered sensitivity toward one or more agents which damage DNA: ionizing radiation, UV radiation, alkylating agents, etc. By combining knowledge of the mutant phenotypes, the biochemical nature of the gene products, if known, and the behaviour of multiply deficient strains, it is possible to estimate how many independent repair pathways there may be, and the minimal number of gene products (enzymes?) involved in a given pathway. Table 1 shows the current state of the art as far as ionizing radiation is concerned. Figure 3 shows survival curves for some of these mutants in a common genetic background.

By combining the mutants in pairs it was found that the polA exrA double mutant is considerably more sensitive than either single mutant alone
<table>
<thead>
<tr>
<th>Mutant designation</th>
<th>Map position</th>
<th>Major function affected, or relevant phenotype</th>
<th>Degree of X-ray sensitivity</th>
<th>References to original mutant and its mapping</th>
<th>References to its X-ray sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ext (lex)</td>
<td>(79)</td>
<td>X-ray sensitive, slight recombination deficiency</td>
<td>-4</td>
<td>Howard-Flanders and Boyce, 1966; Mattern et al., 1966</td>
<td>Howard-Flanders and Boyce, 1966; Mattern et al., 1966</td>
</tr>
<tr>
<td>lar</td>
<td>61</td>
<td>X-ray resistant, large cells</td>
<td>+1</td>
<td>Kvetkas et al., 1970</td>
<td>Kvetkas et al., 1970</td>
</tr>
<tr>
<td>lig</td>
<td>46.5</td>
<td>ligase</td>
<td>-2</td>
<td>Dean and Pauling, 1970; Gellert and Bullock, 1970</td>
<td>Dean and Pauling, 1970</td>
</tr>
<tr>
<td>lon</td>
<td>11</td>
<td>forms filaments after radiation</td>
<td>-2</td>
<td>Adler and Hardigree, 1964; Howard-Flanders et al., 1964</td>
<td>Adler and Hardigree, 1964</td>
</tr>
<tr>
<td>polA(res)</td>
<td>75</td>
<td>DNA polymerase I</td>
<td>-4</td>
<td>de Lucia and Cairns, 1969; Gross and Gross, 1969</td>
<td>Kato and Kondo, 1970; Town et al., 1971b</td>
</tr>
<tr>
<td>ras</td>
<td>(11)</td>
<td>UV sensitive</td>
<td>-1</td>
<td>Walker, 1969</td>
<td>Walker, 1969</td>
</tr>
<tr>
<td>recA</td>
<td>51.7</td>
<td>No detectable genetic recombination</td>
<td>-4</td>
<td>Willetts et al., 1969</td>
<td>Howard-Flanders and Theriot, 1966</td>
</tr>
<tr>
<td>recB</td>
<td>54.2</td>
<td>1% recombination; ATP dependent exo- and endonuclease</td>
<td>-3</td>
<td>Emmerson, 1968; Willetts and Mount, 1969</td>
<td>Howard-Flanders and Theriot, 1966</td>
</tr>
<tr>
<td>recC</td>
<td>54.5</td>
<td>1% recombination; ATP dependent exo- and endonuclease</td>
<td>-3</td>
<td>Emmerson, 1968; Willetts and Mount, 1969</td>
<td>Howard-Flanders and Theriot, 1966</td>
</tr>
<tr>
<td>recF</td>
<td>(72)</td>
<td>slight recombination deficiency</td>
<td>-2</td>
<td>Hortii and Clark, 1973</td>
<td>Hortii and Clark, 1973</td>
</tr>
<tr>
<td>rorA</td>
<td>54.5</td>
<td>X-ray sensitive (recombination proficient)</td>
<td>-1</td>
<td>Glickman et al., 1971</td>
<td>Glickman et al., 1971</td>
</tr>
<tr>
<td>uurA, B, C</td>
<td>80, 18, 37</td>
<td>UV sensitive</td>
<td>-1</td>
<td>Howard-Flanders et al., 1966</td>
<td>Howard-Flanders et al., 1966</td>
</tr>
<tr>
<td>uurD</td>
<td>74</td>
<td>UV sensitive</td>
<td>-1</td>
<td>Ogawa et al., 1968</td>
<td>Ogawa et al., 1968</td>
</tr>
</tbody>
</table>

1 Designations in parentheses are presumed to be allelic.
2 Positions in parentheses are not well established.
3 X-ray sensitivity is expressed on a scale of +1 to −4; 0 = wild-type, −4 being most sensitive.
[Youngs and Smith, 1973a]. This indicates that the repair pathways involving polA on the one hand and extrA on the other are at least partially distinct. It would be consistent, therefore, to expect that the polA recA and polA recB double mutants would also be more sensitive than the single mutants alone. When construction of these strains was attempted, however, both pol− rec− combinations were found to be inviable [Gross et al., 1971; Monk and Kin-

![Fig. 3. Survival curves of various radiation sensitive mutants and the wild-type strain (W3110) of E. coli K-12. Cells were grown in minimal salts medium to log phase, irradiated in phosphate buffer at room temperature in equilibrium with air and plated for survival on minimal medium agar. \( \bigcirc \), extrA polA; \( \bigcirc \), polA1; \( \square \), recA56; \( \Delta \), recB21; \( \blacktriangledown \), wild-type (data from this laboratory).]

ross, 1972]. Presumably the strains are so repair deficient that they cannot survive even in the absence of radiation.

The approach of constructing multiply mutants strains to delineate repair pathways is a powerful one. In yeast, there are over twenty loci controlling radiation sensitivity [Game and Cox, 1971]. By appropriate genetic crosses it has been shown that all the mutations probably fall into one of three dif-
ferent pathways. A similar analysis with the known X-ray sensitive mutants of *E. coli* K-12 (table 1) would be extremely helpful.

4. In vitro repair

4.1. DNA ENZYMES

In order to understand the enzymology of DNA repair it is necessary first to know the chemical nature of the damage. The advances in DNA enzymology in the last decade [see, e.g., Richardson, 1969] have not only indicated the versatile array of DNA enzymes which could be introduced into hypothetical repair pathways, but also provided valuable tools with which to probe the chemical nature of end groups and interruptions in polynucleotide chains. Some of these enzymes will be mentioned here briefly since they are expected to be of particular importance in the repair of DNA damaged by X-rays.

DNA polynucleotide ligase (ligase) catalyses the synthesis of a phosphodiester bond by esterification of the 5'-phosphoryl group to the 3'-hydroxyl group of adjacent nucleotides in DNA chains which have been properly aligned in a double helical structure. Ligase isolated from *E. coli* requires nicotinamide adenine dinucleotide (NAD) as a cofactor. Using synthetic polynucleotides *in vitro* it has been shown that both T4 and *E. coli* ligases are active even at low temperature [Gupta et al., 1968] and also that T4 ligase is capable of end-to-end joining of completely base-paired double-stranded molecules provided that the 5'-ends are phosphorylated and the 3'-ends carry OH termini (i.e., the same specificity as the single-strand joining reaction [Sgaramella et al., 1970]. Current evidence suggests that there is only one species of ligase in *E. coli*, and this enzyme is therefore expected to participate in the final sealing of all single-strand interruptions.

It is now recognized that there are several DNA polymerases (I, II, III) in *E. coli* [Knippers, 1970; Moses and Richardson, 1970a; Kornberg and Gefter, 1970, 1971]. All possess a 3'→5' polymerizing activity adding complementary nucleotides to a 3'-OH terminus of a partially double-stranded molecule. DNA polymerase I also possesses a 5'→3' exonuclease activity which can operate concomitantly with the polymerizing activity and is capable of excising thymine dimers *in vitro* [Kelly et al., 1969]. DNA polymerase I and II (and T4 polymerase) possess, in addition, a 3'-exonuclease activity which apparently serves an editorial function in excising mismatched bases [Brutlag and Kornberg, 1972]. It is conceivable that this function may also
operate in repair where the broken strand terminates in a damaged (and therefore non-hydrogen bonded) base. The presence or absence of this activity in DNA polymerase III has not been reported. The enzymes also differ in many other ways including sensitivity to sulphhydryl reactive agents, salt concentration, and the length of gap which they prefer to fill. Currently DNA polymerase I has a demonstrable role in several repair processes both in vitro [Kelly et al., 1969] and in vivo [Boyle et al., 1970; Town et al., 1971b]. A mutant deficient in DNA polymerase II [Campbell et al., 1972] has about the same X-ray sensitivity as the wild-type strain suggesting that DNA polymerase II plays no major role in the repair of X-ray induced damage [Youngs and Smith, 1973b]. DNA polymerase III appears to be involved in replication [Gefter et al., 1971] and in repair (see section 5.2.4).

Deoxyribonucleases of E. coli can be classified as either endonucleases or exonucleases, that is, they catalyze the hydrolysis of phosphodiester bonds either within or from the ends of a DNA strand, respectively. In general, exonucleases are more likely to be important in the repair of X-ray induced strand breaks because of their ability to remove a few (possibly damaged) nucleotides near a terminus. One nuclease with such a role is exonuclease V, the product of the recB and recC genes [Wright, Buttin and Hurwitz, 1971]. Unfortunately, the purified enzyme has so many different activities in vitro (exo- and endonuclease, ATPase, etc.) that it is difficult to predict its precise function in vivo. However, genetic evidence indicates that this enzyme is responsible for much of the X-ray induced DNA degradation in vivo [Youngs and Bernstein, 1973]. Endonucleases would be required at an early step in the excision of a damaged region of DNA, as for example in the excision of UV-induced pyrimidine dimers.

There are other enzymes which could be involved in modifications at chain termini during repair. Phosphatases hydrolyze phosphoryl end groups on DNA strands. Enzymes have been isolated with a range of activities for 3'-PO₄ and 5'-PO₄ groups both externally and internally. Polynucleotide kinase catalyzes the transfer of the γ-phosphate of a nucleoside 5'-triphosphate to the 5'-hydroxyl terminus of a polynucleotide. In vitro they work with greater efficiency at external rather than internal termini. Terminal nucleotidyl transferases have so far been isolated only from calf thymus; they catalyse the incorporation of mononucleotide units from nucleoside 5'-triphosphates into the 3'-terminal positions of DNA in a reaction which is not template directed.

So far, only a few of the above-mentioned enzymes have been shown to play a role in repair. The properties of some of the other enzymes encourage
their use in paper reaction schemes for repair. Some of these enzymes have also been particularly helpful in end-group analysis (e.g., kinase) and could serve an equally useful purpose in repair itself.

4.2. ATTEMPTS TO REPAIR BREAKS ENZYMATICALLY

Kapp and Smith [1968] found that polynucleotide ligase alone would not rejoin a detectable number of single-strand breaks produced in calf thymus DNA irradiated in dilute aqueous solution under aerobic conditions with a dose of 19.6 krad. Moroson and Anello [1968] found some rejoining of X-ray induced breaks in phage T5 DNA by a crude enzyme extract from \textit{E. coli} after a dose of 40 krad. Both of these studies employed physical measurements which necessitated using relatively large doses of radiation to produce detectable changes in molecular weight.

Using the much more sensitive assay of transformation in \textit{B. subtilis}, Gaziev, Fomenko, Zakrzhevskaya and Kuzin [1970] showed complete restoration of transforming activity by ligase alone following doses of up to 0.5 krad given to the DNA in solution. At higher doses, however, the amount of repair decreased markedly, so that no repair at all was seen after 10 krad. This is in agreement with the physical observations of Kapp and Smith [1968]. One explanation for the lack of repair at higher radiation doses is that chemically more complex lesions are probably formed. After the initial break, further radical attack may occur on the exposed bases in the region of the break. Such altered breaks might no longer be reparable by ligase alone. Radiation chemical studies show a threshold for the loss of chromophore from DNA at low doses. This has been attributed to the protection of the bases from destructive radicals as long as they remain within an intact double helix [Ward and Kuo, 1970]. It therefore appears that some ligase-rejoinable breaks are formed at low doses under aerobic conditions, but these may become modified by a subsequent part of the radiation dose and rendered non-rejoinable by ligase alone.

When DNA was irradiated under anaerobic conditions, however, up to 40% of the single-strand breaks could be directly sealed by ligase [Jacobs, Bopp and Hagen, 1972]. This differential repair of chain breaks by ligase alone, depending upon whether the DNA was irradiated in air [Kapp and Smith, 1968] or under N\textsubscript{2} [Jacobs et al., 1972], is consistent with observations on the radiation induction of DNA sugar damage. Kapp (unpublished observations) found that while the same number of DNA single-strand breaks was produced per krad whether the DNA in solution was irradiated in air.
or N₂, the amount of deoxyribose damage [TBA test, Kapp and Smith, 1970a] per krad was reduced about 10-fold under N₂.

Restoration of transforming activity and concomitant repair of single-strand breaks has been demonstrated by Laipis and Ganesan [1972a] for B. subtilis DNA. The DNA extracted from X-irradiated B. subtilis polA5 cells had a much lower transforming activity than the DNA from similarly treated pol⁺ cells.* Treatment of DNA from irradiated polA5 cells (35 krad) with purified E. coli DNA polymerase I plus ligase caused an increase in transforming activity of both single markers and linkage groups, and an increase in single-strand molecular weight (e.g., single marker survival was 36.5% after irradiation and 51% after repair; unirradiated DNA had a single-strand molecular weight of 34 x 10⁶ daltons, which was reduced to 16 x 10⁶ daltons by the radiation and increased to 22 x 10⁶ daltons upon repair). These results show that DNA polymerase I and ligase can repair single-strand breaks produced by X-rays in vivo. However, they do not directly demand the involvement of DNA polymerase I in the repair of this damage in vivo.

More direct proof of the involvement of DNA polymerase I in vivo was obtained using total cell lysates of wild-type and polA5 strains supplemented with ligase, DNA polymerase I, or both. These lysates were then incubated with DNA extracted from irradiated polA5 cells. Ligase was necessary for repair with the wild-type lysate (apparently because B. subtilis ligase is unstable in the extracts), but additional DNA polymerase I did not increase this repair further. Using the polA5 lysate, however, no repair was seen unless DNA polymerase I was added; and when DNA polymerase I and ligase were added, the same amount of repair was seen as with the supplemented wild-type lysate. The authors report that the level of repair obtained with supplemented cell lysates was slightly but consistently higher than that obtained with purified DNA polymerase I plus ligase. This may have been due to the participation of other enzymes in the cell lysate in the repair process.

These results thus suggest that the majority of the single-strand breaks can be repaired by DNA polymerase I plus ligase, and that the presence of additional enzymes (as in the cell lysates) effects the repair of only a few

* It should be noted that single-strand breaks caused by DNase inactivate transforming DNA and are therefore apparently not repaired within the recipient cell [Bodmer, 1966]. However, if such nicks were repaired by treating the DNA with E. coli or B. subtilis DNA ligase before it entered the recipient cell, both transforming activity and single-strand molecular weight were restored [Laipis, Olivera and Ganesan, 1969].
additional breaks. This in vitro situation closely resembles the Type II repair of single-strand breaks in vivo (section 5.2).

4.3. THE POTENTIAL OF IN VITRO SYSTEMS FOR THE STUDY OF DNA REPAIR

Several attempts have been made to reconstruct, in vitro, the enzymatic events which are thought to occur during DNA repair in vivo. These could be further advanced by using one or another of the methods which are now being employed for the study of DNA replication. Some of these will be reviewed briefly.

In the last few years, many attempts have been made to bridge the gap between in vitro and in vivo methods for the study of DNA replication. The advantage of in vitro work is that pure reagents can be used under carefully controlled conditions; however, levels of DNA synthesis comparable to those seen in vivo have not been achieved. On the other hand, in vivo work suffers from the disadvantage that the manipulation of enzymes and metabolites is very limited. Out of the many techniques which have made significant contributions to the art, several are worthy of consideration for studying DNA repair under controlled conditions.

The criteria which have been used for evaluating the merits of a particular in vitro system have been: (1) the rate and extent of DNA synthesis, (2) the nature of this synthesis (i.e., whether it is semiconservative, whether the product has biological activity and whether genetic markers are replicated in a sequential fashion), and (3) the response of DNA replication mutants in the in vitro system compared with their response in vivo. In evaluating a system for the study of repair it is important to bear in mind that other factors may also prove to be important [e.g., whether the repair enzymes are soluble (i.e., cytoplasmic, like DNA polymerase I) or membrane bound].

4.3.1. Concentrated lysate systems

In this system, a very dense cell suspension (5 x 10^10 cells/ml) is spread on a cellophane membrane and lysed by treatment with lysozyme and detergent followed by osmotic shock [Schaller et al., 1972]. Substrates for synthesis (e.g., deoxynucleoside triphosphates) are introduced by placing the membrane (cells uppermost) on a buffer containing the appropriate metabolites. The success of this system seems to depend on the high cell density which is thought to maintain a sufficiently high concentration of some critical factors. If liquid is added to the cell side of the membrane, or if the membrane is placed face down on the same substrate containing buffer,
the amount of synthesis is greatly reduced.

The characteristics of the system resemble in vivo synthesis in most ways, although the rate of synthesis is only about 10% of the in vivo rate and it does not continue indefinitely. However, its response to inhibitors, including irradiation with ultraviolet light, closely parallels the in vivo situation. Since DNA synthesis in this system is sensitive to added deoxyribonuclease, it indicates that exogenous enzymes have access to the DNA, and cautions that endogenous soluble enzymes may have been lost or displaced in preparing the lysate. Therefore, this might prove to be a poor system in which to study repair processes involving soluble enzymes. In most other respects, however, the system has much to offer for studying DNA metabolism in an 'open system'.

4.3.2. Permeable cell systems

4.3.2.1. Toluene-treated cells

Normally, E. coli cells are not permeable to deoxynucleoside triphosphates. However, limited treatment with toluene yields a system which may be regarded as a 'closed, in vitro system' for studying DNA replication in response to known substrates [Moses and Richardson, 1970b]. In this system, semiconservative and repair synthesis can be distinguished. The latter occurs in response to breaks in the DNA induced either by endogenous nuclease or exogenous pancreatic DNase. It occurs only in pol + cells and is not inhibited by N-ethylmaleimide, but it is inhibited by antibodies to DNA polymerase I. These results indicate that the cells have become permeable not only to triphosphates but also to larger protein molecules. Exactly what is released from the cells under these conditions has not been reported. Since DNA polymerase I-dependent repair of DNA single-strand breaks has been demonstrated in this system, it is clear that at least some of the soluble enzymes remain within the cell and are functional.

Noguti and Kada [1972] have used tolenuized cells of B. subtilis to study the repair of X-ray induced single-strand breaks and the restoration of transforming activity. They found that all four deoxynucleoside triphosphates, NAD and Mg$^{2+}$ were required for the repair process. Toluene treated cells would appear to be a good system for studying the repair of X-ray damage, at least in so far as the manipulation of precursor molecules is concerned. Permeable cells with similar properties can also be obtained by treatment with ether [Vosberg and Hoffmann-Berling, 1971].
4.3.2.2. Detergent-treated cells

Limited treatment of *B. subtilis* cells with the non-ionic detergent Brij-58 makes the cells permeable to many molecules and permits the demonstration of semiconservative DNA replication [Ganesan, 1971]. This replication satisfies many of the criteria of a good *in vitro* system, including the synthesis of biologically active DNA and the sequential replication of markers. However, during the preparation of these cells, 55% of the cellular proteins, including 95% of the DNA polymerase activity (presumably DNA polymerase I [see de Lucia and Cairns, 1969]), was lost. Thus, although the system appears promising for studying DNA replication it would only be suitable either for studying cell membrane-bound repair systems or for situations where specific enzymes could be added in order to study a particular repair reaction. This might require relatively large amounts of precious enzymes to restore *in vivo*-like concentrations. However, the feasibility of such an approach has now been demonstrated by Dr. Ann Ganesan [unpublished observations]. She has extended the Brij, permeable cell system to *E. coli*. After UV irradiation of a *uvrB* derivative of *E. coli* K-12 (which is unable to make the first dimer-specific incision in the excision repair process), the cells were made permeable to relatively large enzyme molecules by the Brij treatment. T4-induced UV specific endonuclease [Friedberg and King, 1971] was then incubated with the cells and produced incisions in the DNA in a dimer-specific manner (i.e., the number of incisions was UV dose-dependent, was reduced by photoreactivation, and was reduced during post-irradiation incubation). It has not yet been shown, however, that the added enzyme promotes the recovery of the viability of the cells. The viability of permeable cell is low, but such experiments are in prospect. This system offers the exciting possibility of being able to supplement many repair-deficient cells with appropriate exogenous enzymes and to observe the effects at the biophysical, and possibly the biological, level.

4.3.3. Phage-coded enzymes

One further approach worthy of brief mention is the use of bacteriophage to introduce missing enzymes into mutant cells and thereby stimulate repair. If bacteriophage T4 are heavily irradiated to prevent their own multiplication, they can be used to promote the survival of UV-irradiated *E. coli uvr* cells [Harm, 1968]. Once inside the cell, the phage genome can be transcribed even though replication is inhibited by a large UV dose. A suitable phage can thereby supply the missing function to the host cell. This technique offers some interesting possibilities. For example, in the case of the *polA* mutant,
which is deficient in the Type II repair of X-ray induced breaks, one could ask whether T4 DNA polymerase can supply the missing function. The T4 enzyme lacks the 5'→3' exonuclease function [Kornberg, 1969] of DNA polymerase I. Such an experiment might therefore indicate whether this particular function is involved in the Type II repair process.

5. Repair

The results to be described below have enabled us to divide the repair of DNA single-strand breaks into three operationally distinct phases. We have called these Types I, II, and III, indicating the temporal sequence in which they act. It is probable that some enzymes will play a role in more than one repair pathway while others are unique to a particular kind of repair.

The Types I, II, and III repair systems are complementary in their action, rather than exclusive. Under appropriate experimental conditions (i.e., temperature, time of lysis, mutant strain, etc.), it is possible to interrupt repair at the completion of one process, but before the next (slower) process has begun. However, in a competent cell under appropriate conditions, all of the available repair systems may begin to act the instant that damage occurs. Presumably the fastest process (Type I) will reach completion first followed by Types II and III. However, this cannot be demonstrated until we have specific and reversible inhibitors of the Types I and II mechanisms.

The repair pathways will be described in the sequence III, II, and I (that in which they were discovered), for the sake of clarity.

5.1. Type III Repair

The original experiments of McGrath and Williams [1966] demonstrated that rejoining of some single strand breaks took place when cells which had been irradiated in buffer were subsequently incubated under growth conditions. The resistant strain, E. coli B/r, differed from the sensitive mutant, B₅₋₁, at several loci (Fil, Her, Exr), but it now appears that the exr gene was responsible for this block in repair [Youngs and Smith, 1973a]. A similar type of repair was demonstrated in E. coli K-12 by Kaplan [1966a], by Morimyo et al. [1968] and by Kapp and Smith [1970b]. The latter two groups showed that this type of repair was blocked in recombination deficient (rec) mutants of E. coli K-12 (fig. 4).
Fig. 4. (a) Sedimentation patterns of DNA from irradiated B. coli K-12 AB2497 (uvr+ rec+). (A) Unirradiated; (B) 22 krad, no reincubation; (C) 22 krad, 20-min reincubation; (D) 22 krad, 30-min reincubation; (E) 22 krad, 60-min reincubation. Cells were converted to spheroplasts and lysed on top of the alkaline sucrose gradients (5 to 20% sucrose in 0.1 M NaOH). Sedimentation was performed in an SW 50.1 rotor for 105 min at 30,000 rev/min at 20 °C. The direction of sedimentation is from right to left. (b) Sedimentation patterns of DNA from irradiated B. coli K-12 SR74 (uvr+ rec A56). (○) Unirradiated; (●) 22 krad, no reincubation; (□) 22 krad, 20-min reincubation; (△) 22 krad, 40-min reincubation; (×) 22 krad, 70-min reincubation [from Kapp and Smith, 1970b].
Type III repair is defined as the slow rejoining of single-strand breaks which occurs only when cells are incubated under conditions which permit growth. After doses of 15-20 krad, it takes 40-60 min for complete repair in minimal medium at 37 °C, but less time may be required after lower doses [Ganesan and Smith, 1972]. Type III repair appears to require DNA replication of the semiconservative kind, or at least the dnaB function [Fangman and Russel, 1971], but can be finished in less time than is necessary for a complete round of DNA replication [Ganesan and Smith, 1972]. These temporal characteristics tend to exclude recombination between homologous daughter chromosomes, while a mechanism of recombination between the two parental chromosomes in the typical glucose-grown binucleate E. coli is excluded by the observation that acetate-grown cells, which possess only a single chromosome, are only slightly more sensitive than glucose-grown cells [Bridges, 1971].

5.1.1. Genetic control of Type III repair
It appears that the recA, recB, and recC gene products are all required for Type III repair. Kapp and Smith [1970b] reported that cells carrying a recA mutation were completely deficient in Type III repair, whereas cells carrying a recB or recC mutation showed some Type III rejoining, though considerably less than wild-type cells. A more detailed investigation by Youngs and Smith [1973c] has confirmed and extended these observations.

Several other gene products also appear to be involved in Type III repair. In some cases, direct evidence is available to show that a medium-dependent repair process is affected. In this category are exr [Youngs and Smith, 1973a], dnaB [Fangman and Russel, 1971] and ror [Glickman et al., 1971]. In other instances, experimental conditions are such that only Type III repair is relevant to the experiment, and the genetic involvement in this repair process can be inferred. On the basis of such evidence for the mutant ts7 [Dean and Pauling, 1970], DNA ligase may be inferred to be in this pathway. Current genetic evidence thus shows the involvement of seven functions in the Type III repair process. This represents a minimum estimate of the number of gene products involved.

5.1.2. Physiological factors affecting Type III repair

5.1.2.1. Drugs, metabolic inhibitors
The incubation of X-irradiated bacterial cells with certain drugs after irradiation can cause a marked increase in radiation-induced cell killing.
This post-irradiation sensitization has been shown for an impure sample of hydroxyurea [Kapp and Smith, 1971], quinacrine [Fuks and Smith, 1971], chloramphenicol (and also starvation for required amino acids or treatment with puromycin) [Ganesan and Smith, 1972], dinitrophenol [Van der Schueren, Smith and Kaplan, 1973], and acriflavin [Kapp, 1970]. These compounds irreversibly inhibit the Type III repair of DNA single-strand breaks. This irreversibility of inhibition appears to be the key to their radiation potentiating effects, since treatments such as low temperature which reversibly inhibit repair (and all metabolism) do not have any effect on viability when growth conditions are restored. Mutants which are deficient in the Type III process (recA, recB, exrA) do not show potentiation by these drugs whereas pol A strains, which are capable of Type III repair, do. This confirms genetically the observation that it is the Type III (rec and exr gene-controlled) rejoining process which is inhibited.

It is interesting to note that UV irradiation also causes X-ray potentiation by inhibiting this rec and exr gene-controlled repair process [Martignoni and Smith, 1973]. Type III repair also appears to be inhibited, and cells are sensitized, when irradiated in the presence of iodine compounds [Noguti et al., 1971].

5.1.2.2. Growth conditions

It has long been recognized that growth conditions can dramatically affect the viability of irradiated bacteria [for reviews see Stapleton, 1960; Rupert and Harm, 1966]. Two growth-induced modifications of X-ray sensitivity have recently been investigated in terms of altered capacity to repair DNA single-strand breaks [Town et al., 1971a]. Cells of the strain E. coli B/r grown to stationary phase in a peptone medium containing glucose were 3.4 times more resistant to radiation than cells grown without glucose. Log phase cells growing in complex medium (Brain Heart Infusion) were 1.7 times more sensitive to X-rays than stationary phase cells. In each case the more sensitive cells showed a reduced ability to repair single-strand breaks and greater DNA degradation during post-irradiation incubation. From the experimental conditions it appears that these growth conditions influence the Type III process in E. coli B/r. Glucose-induced resistance in E. coli K-12 (in terms of survival) has been shown to be a rec gene-dependent phenomenon [Friesen et al., 1970].

Pre-irradiation starvation for amino acids makes cells more resistant to X-rays [Ginsberg and Jagger, 1965; Billen and Bruns, 1970]. However, post-irradiation starvation for amino acids sensitizes rec+ cells to X-irradia-
tion but does not sensitize recA or recB cells [Ganesan and Smith, 1972].

Starvation of thymine-requiring bacterial cells for thymine and of purine-requiring cells for purine before irradiation increases their X-ray sensitivity [Kaplan and Howsden, 1964; Kaplan, 1966b; Ganguli and Bhattacharjee, 1966]. Sensitization by purine starvation has been shown to be dependent upon functional rec genes [Baptist et al., 1971], but a similar investigation of the genetic control of the sensitizing effect of thymine starvation has not been reported.

5.2. TYPE II REPAIR

5.2.1. DNA polymerase-deficient mutants and their X-ray response

A few years ago, de Lucia and Cairns [1969] isolated a strain of E. coli K-12 with less than 1% of the normal amount of DNA polymerase in soluble extracts. The strain showed normal viability and grew at the same rate as the parental strain. The alteration was shown to be due to an amber mutation at a single locus, designated polA1 [Gross and Gross, 1969]. Subsequent studies revealed that polA is the structural gene for DNA polymerase I, an enzyme first isolated by Kornberg [1969]. The strain showed considerable sensitivity to ultraviolet radiation and to methylmethane sulphonate. All these observations suggested that DNA polymerase I was required for DNA repair, but not for semiconservative replication.

It was obviously of interest to investigate the response of this strain to ionizing radiation. Town et al. [1971b] found that it was 3 to 5 times more sensitive to X-rays than corresponding wild-type strains (and slightly more sensitive than a recA strain). To see whether this was due to a defect similar to that in the recA cells, the ability of the polA1 strain to repair DNA single-strand breaks was examined. Unexpectedly the results showed a greater number of single-strand breaks in the polA1 strain than in the polA parent after a given dose of radiation. By modifying the irradiation and lysis techniques so that cells could be irradiated at 0°C and then lysed immediately afterwards on the alkaline gradients, Town et al. [1971b] were able to show that the same number of breaks were produced initially in polA+ and polA- cells, but that in polA+ cells, many of the breaks (~90%) were repaired in buffer at room temperature in 2 to 5 min. This is illustrated in fig. 5.

Two other polA mutants, polA3 and polA12, have also been investigated. The polA3 strain has the same radiation survival characteristics as polA1. The polA12 strain makes a temperature-sensitive enzyme which is inactivated at 42°C. This strain exhibits temperature dependent radiosensitivity
Fig. 5. Sedimentation of DNA from *E. coli* K-12 pol*+* (W3110 thy), polA1 (P3478), and pol*+* (AB2497) after aerobic irradiation with 16.3 krad followed by immediate lysis. (a) pol*+* (W3110 thy); (b) polA1 (P3478); (c) pol*+* (AB2497). The cells were irradiated in 0.05 M phosphate buffer at pH 6.9 and 25 °C or at pH 8.0 and 0 °C. The centrifugation time in (c) was reduced from 120 min to 105 min. ● Unirradiated control; ×, irradiated at pH 6.9 at 25 °C; ○, irradiated at pH 8.0, 0 °C [from Town et al., 1971b]. (Copyright 1971 by the American Association for the Advancement of Science.)

[Town, C.D., unpublished observations]. Kato and Kondo [1970] isolated a radiosensitive mutant *E. coli* B res, which was subsequently found to exhibit most of the characteristics of polA1: no DNA polymerase I, high radiosensitivity, apparently increased single-strand breaks. It has the same genetic map location as polA and is presumed to be in the same gene. A polymerase-deficient mutant of *B. subtilis* has also been isolated and found to show increased X-ray sensitivity [Laipis and Ganesan, 1972b] (see section 4.2).

5.2.2. The Type II process; definition and characteristics

On the basis of these results with polymerase-deficient strains, the Type II repair process was defined to include all the repair processes which rejoin
DNA single-strand breaks in buffer after the completion of Type I repair. In wild-type cells, this rejoining process is too rapid to follow kinetically at 37°C, or room temperature, but appears to have a half-time of 1 or 2 min in this temperature range. At 0°C, Type II repair is not completely inhibited but continues with a half-time of about 10 min for the repair of breaks produced under aerobic conditions [Town et al., 1973a].

It is inhibited by EDTA (10⁻¹ M) [Town et al., 1971b] and by preincubation with quinacrine (10⁻⁴ M) and partly inhibited by sodium cyanide [Town et al., 1972a]. However, in these experiments the chemical inhibitors were present during irradiation. It is therefore not possible to say whether they inhibit the repair process directly, or chemically alter the type of strand break produced so that it can no longer be repaired by the Type II system.

5.2.3. Genetic and enzymatic involvement

DNA polymerase I (the polA gene product) is a key enzyme in this process. In addition, DNA ligase would also be expected to participate. This is supported by (1) inhibition of Type II repair in niacin-starved cells (a treatment which inhibits ligase) [Town et al., 1972b], and (2) the in vitro demonstration by Laipis and Ganesan [1972a] (see section 4.2) that DNA polymerase I and ligase can rejoin many X-ray induced breaks without further enzymic supplement.

There is evidence to suggest that other enzymes may also be involved in the Type II process. Town et al. [1973a] showed that the Type II repair of breaks produced under anoxic conditions was faster at 0°C than the repair of breaks produced under aerobic conditions. They suggested that this might be due to the involvement of additional enzymatic step(s) in the repair of the aerobically produced breaks. Since DNA polymerase I and ligase would necessarily be involved in the repair of breaks produced under anoxia, additional enzyme(s) may be required for the repair of breaks produced under aerobic conditions.

5.2.4. An additional repair process seen in pol⁻ cells

There is also a slow rejoining process which takes place in buffer in polA1 cells. In ~40 min at room temperature, ~75% of the initial number of aerobically produced breaks are rejoined. This process differs from that seen in pol⁺ cells not only in being about 10 times slower, but also in being accompanied by considerably more DNA degradation than in the wild-type cell. This process appears to involve DNA polymerase III, since it does not occur in a dnaE mutant at the restrictive temperature [Youngs and Smith, 1973d].
Incubation of *polA1 dnaE* cells in buffer after irradiation caused a decrease in survival if the cells were held at the restrictive temperature but had no effect if the cells were held at the permissive temperature [Youngs and Smith, 1973d]. Therefore, the rejoicing process involving DNA polymerase III seems to contribute to cell survival, at least that of X-irradiated *polA1* cells.

5.3. TYPE I REPAIR

5.3.1. Definition and characteristics of the process

In order to distinguish clearly between Types I and II repair, Type I repair is defined as those rejoicing processes which occur immediately after irradiation, do not require growth conditions, and do not require DNA polymerase I. The Type I repair system rejoins about 75% of the breaks produced by irradiation under anoxic conditions, and about 25% of the breaks produced under aerobic conditions. It takes place in buffer very rapidly even at 0°C, and is complete within 1–2 min.

The existence of this repair system was demonstrated by comparing the yields of X-ray induced single-strand breaks under aerobic and anoxic conditions in untreated *polA1* cells and in cells (both *polA1* and *pol*+) which had been inactivated by either heat treatment or cold shock [Town et al., 1972a]. Heat treatment probably inactivates many enzymes in the cell, including at least one of those involved in the polymerase repair process [Rupp et al., 1970]. The cold shock causes changes in cell permeability, and ligase activity may be inhibited in these cells due to the loss of either enzyme or cofactor from the cells [Sato and Takahashi, 1969, 1970]. The results for inactivated cells are shown in fig. 6. There is a large increase in the yield of single-strand breaks observed under anoxic conditions; from 0.66 breaks per single-strand genome per krad in untreated cells to 1.85 in inactivated cells. In cells irradiated aerobically the observed yield of breaks rises from 2.1 to 2.5 breaks per single-strand genome per krad. The ratio of breaks observed in oxygen as compared with nitrogen falls from 3.2 in untreated cells to about 1.3 in heat or cold-inactivated cells [Town et al., 1972a].

These results were interpreted as showing that the heat or cold treatments inactivate some system which normally repairs many of the breaks produced under anoxic conditions. The yield of breaks under aerobic conditions was slightly but significantly higher in physically inactivated than in untreated cells. This suggested that a small fraction of the aerobically produced breaks were also subject to some very fast repair process, most likely the same Type
Fig. 6. Production of single-strand breaks in DNA of *E. coli* K-12 inactivated by either heat treatment or cold shock, and irradiated at 0°C in phosphate buffered saline pH 7.3 in equilibrium with either air or nitrogen. ○, △, JG138 polA1, heat treated; □ JG139 pol+, heat treated; ◇, V, JG138 polA1, cold shocked. Symbols of different shapes refer to independent experiments, aerobic (open symbols) and anoxic (closed symbols) irradiations were performed with samples of the same cells in each separate experiment. The broken lines show the apparent yield of breaks in untreated polA1 cells irradiated in air or nitrogen. The solid lines are fitted through the origin for the pooled data from heat-treated and cold-shocked cells, since lines fitted to the two sets of data separately had essentially the same slope [from Town et al., 1972a].

I process.

On the assumption that the initial yield of X-ray induced DNA single-strand breaks was exactly equal in the presence or absence of oxygen, the true rate of strand breakage could be calculated as 2.8 breaks per single-strand genome per krad (or approximately 52 eV/break for energy directly absorbed by the DNA). The very fast Type I repair system appears to rejoin approximately 25% of the initial aerobic breaks and approximately 75% of the initial anoxic breaks, thus giving rise to the observed 3-fold difference in number of breaks remaining after Type I repair [Town et al., 1972a].
5.3.2. Inhibition of Type I repair

There are several ways in which the Type I system can be inhibited, as judged by an increased yield of breaks under anoxic conditions over that expected in untreated pol− cells. Pre-incubation of cells with 10−4 M quinacrine gives a 2-fold increase in anoxic breaks; irradiation in the presence of 5 × 10−4 M NEM or of 10−2 M hydroxyurea (HU) gives 3-fold and 1.6-fold increases, respectively. None of these chemicals has a significant effect on the yield of breaks under aerobic irradiation conditions [Town et al., 1972a]. It has also been reported [Ho and Ho, 1972] that irradiation of E. coli 15T− in the presence of 5 × 10−4 M parachloromercuribenzoate (PCMB) resulted in equal yields of single-strand breaks in the presence or absence of oxygen. The absolute magnitude of these yields was several-fold higher than any observed previously, but the data are qualitatively compatible with the inhibition of Type I repair by PCMB. Prestarvation of niacin-requiring cells for niacin caused a 1.9-fold increase in the level of breaks seen under anoxic conditions, with only a 1.2-fold increase under aerobic conditions [Town et al., 1972b].

It was also observed that, in polA1 cells at pH 8 or pH 9, there was a relatively greater increase in the yield of breaks measured under anoxic conditions, and thus a smaller oxygen-nitrogen breaks ratio (ONBR) than at lower pH values [Town et al., 1972a]. The higher pH may cause more local denaturation at breaks and thus inhibit repair.

5.3.3. Genetic and enzymatic factors in Type I repair

As indicated earlier (section 4.1), ligase might be expected to be involved in all single-strand break rejoining processes. Evidence for the involvement of ligase in Type I repair comes from the results with niacin-starved cells. Such starvation depletes the pools of NAD in niacin-requiring cells, and causes an inhibition of ligase, for which NAD is a required cofactor [Nozawa and Mizuno, 1969]. Under these conditions Type I repair appears to be inhibited [Town et al., 1972b]. Other indirect evidence is that cold shock, which inhibits Type I repair, may act by inhibiting the ligase activity of cells (see above). However, to this date, there is no direct evidence (such as data from ligase mutants) that ligase is involved in the Type I repair process for single-strand breaks.

Dean et al. [1969] postulated the existence of a very fast repair process (similar to Type I) in quasi-spheroplasts of M. radiodurans on the basis of a differential effect of EDTA under aerobic and anoxic conditions. On the grounds that ligase requires a divalent cation and can function at 0 °C
in vitro they postulated that ligase was involved in this repair reaction. This is compatible with what is known about Type I repair in E. coli, although EDTA does not influence Type I repair in E. coli, probably because it does not penetrate the cell [Town et al., 1972a].

There is presently no evidence for the involvement of other genes in Type I repair. The differential yield of breaks characteristic of Type I action is still seen in uvrA cells [Town et al., 1972a] and recA cells [Town, C.D., unpublished].

6. The effect of oxygen on the production and repair of DNA single-strand breaks, and on cell viability

In order to develop further the concept of several different repair systems, the extent to which each repair system contributes to the overall rejoining of single-strand breaks has been examined in some detail. At the same time the role of oxygen in modifying these repair processes has been studied in an attempt to correlate repair and cell survival. The use of oxygen as a repair-modifying agent has already been referred to in the context of the Type I repair system described above. In this section the status of the literature for the effect of oxygen on strand breakage is reviewed and the extent of Types I, II, and III repair of breaks produced in the presence or absence of oxygen are described.

6.1. PRODUCTION OF DNA SINGLE-STRAND BREAKS; THE LITERATURE

In order to understand the contribution of DNA single-strand breaks and their repair to the oxygen effect for cell survival, it becomes important to ascertain what are the rates of DNA single-strand break production when cells are irradiated in the presence or absence of oxygen under conditions in which repair is minimized, and whether breaks produced under different irradiation conditions are repaired with the same efficiency (rate and extent). The literature on DNA single-strand breakage in vivo shows a wide variation in both the efficiency of strand breakage in the presence of oxygen and in the reduction in the apparent yield of breaks in its absence (table 2). Perhaps the best indication of the effect of oxygen on the initial yield of strand breaks comes from experiments on extracellular viruses and dry DNA which are free from the effects of repair enzymes or reactive cytoplasmic sulphydryl compounds. From the data in Group I of table 2, it appears that one single-strand break occurs for every 50–60 eV of energy directly absorbed in the
<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Irradiation conditions</th>
<th>eV per break</th>
<th>Oxygen-nitrogen breaks ratio (ONBR)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GROUP I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteriophage B3 (Pseudomonas)</td>
<td>in phage, with 1 mM histidine</td>
<td>49, 49</td>
<td>1.0</td>
<td>Freifelder, 1966a, b</td>
</tr>
<tr>
<td>Bacteriophage T7 (E. coli)</td>
<td>isolated DNA, 'dry'</td>
<td>~57, ~57</td>
<td>1.0</td>
<td>Neary et al., 1970</td>
</tr>
<tr>
<td>M. radiodurans</td>
<td>in vivo, with 20 mM EDTA</td>
<td>50, 50</td>
<td>1.0</td>
<td>Dean et al., 1969</td>
</tr>
<tr>
<td>Murine lymphoma L5178Y</td>
<td>in vivo</td>
<td>66, 66</td>
<td>1.0</td>
<td>Dean et al., 1969</td>
</tr>
<tr>
<td><strong>GROUP II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteriophage T4 (E. coli)</td>
<td>in phage, with 20 mM guanylate with 100 mM thiourea</td>
<td>83, 125</td>
<td>1.5</td>
<td>Van der Schaans and Blok, 1970</td>
</tr>
<tr>
<td>Bacteriophage T7 (E. coli)</td>
<td>in phage, with 20 mM guanylate with 100 mM thioglycol</td>
<td>67, 83</td>
<td>1.25</td>
<td>Van der Schaans and Blok, 1970</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100, 143</td>
<td>1.4</td>
<td>Van der Schaans and Blok, 1970</td>
</tr>
<tr>
<td><strong>GROUP III</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. radiodurans</td>
<td>in vivo (without EDTA)</td>
<td>50, 150</td>
<td>3.0</td>
<td>Dean et al., 1969</td>
</tr>
<tr>
<td>Bacteriophage λ</td>
<td>in vivo, superinfecting E. coli K12(λ)</td>
<td>74, 207</td>
<td>2.8</td>
<td>Boyce and Tepper, 1968</td>
</tr>
<tr>
<td>Bacteriophage λ</td>
<td>in vivo, superinfecting E. coli K12(λ)</td>
<td>24, 79</td>
<td>3.3</td>
<td>Johansen et al., 1971</td>
</tr>
<tr>
<td>E. coli B/τ</td>
<td>in vivo, with 20 mM EDTA</td>
<td>87, 250</td>
<td>2.9</td>
<td>Lehner and Moroson, 1971</td>
</tr>
<tr>
<td>Mouse fibroblast (L-60)</td>
<td>in vivo</td>
<td>31, 90</td>
<td>2.9</td>
<td>Palcic and Skarsgard, 1972</td>
</tr>
<tr>
<td><strong>GROUP IV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli B/τ</td>
<td>in vivo (without EDTA)</td>
<td>~520, ~2500</td>
<td>4.5</td>
<td>Lehner and Moroson, 1971</td>
</tr>
<tr>
<td>E. coli B/τ</td>
<td>in vivo</td>
<td>~500</td>
<td>3.5–4.0</td>
<td>Achey and Whitfield, 1968</td>
</tr>
</tbody>
</table>
DNA, and that the yield of breaks is not influenced by the presence of oxygen during irradiation.

There are, however, some results from another system which suggest that although the number of strand breaks may be the same, the chemical nature of the DNA damage may be different in the presence as compared with absence of oxygen. Irradiation of aqueous solutions of calf thymus DNA under anoxic conditions results in a 10-fold reduction in sugar damage (as judged by reactivity with thiobarbituric acid) with no significant alteration in the number of DNA single-strand breaks as compared with irradiation under aerobic conditions [Kapp, D.S., unpublished observations]. While it may not be strictly valid to equate in vivo and in vitro results, a similar reduction in sugar damage in vivo by the removal of oxygen would be expected to yield breaks that were less complicated chemically and therefore more easily repaired, possibly by ligase alone (see section 4.2).

6.2. QUANTITATIVE ASPECTS OF REPAIR IN E. coli K-12

In this experimental system, an attempt has been made to measure the initial yields of DNA single-strand breaks without any enzymatic intervention, and then to follow how these breaks are repaired by the Types I, II, and III repair systems. This was done by first measuring the yield of breaks in cells which had been inactivated by heat treatment or cold shock. This is the closest approach available to date to the true initial yield of breaks.

In order to measure the capacity of each repair system separately, repair was allowed to go to completion in a step-wise fashion. By using appropriate combinations of experimental techniques and mutant strains, it was possible to determine the number of breaks remaining upon completion of one repair process before the next could begin.

These experiments have been performed as a function of dose for cells irradiated in the presence or absence of oxygen. Figure 6 shows the results for the extent of Type I repair. The inactivated cells represent the best measurements of initial yields, while the yields in untreated polA1 cells represent the final extent of Type I repair (in buffer), Type II repair being largely deficient in this strain [Town et al., 1972a]. The number of breaks remaining after Type II repair (in pol+ cells in buffer) is shown in fig. 7.

The fitted regression lines do not pass through the origin, suggesting that after low doses of X-radiation, Types I and II repair can rejoin all the single-strand breaks produced. Upon completion of the Type II process, cells can be incubated in growth medium to permit Type III repair. Results of measur-
Fig. 7. Number of DNA single-strand breaks remaining after Type II (buffer) repair in JG139 (pol+). Cells were irradiated at room temperature in phosphate buffered saline (PBS) in equilibrium with either air (open symbols) or N₂ (closed symbols) and incubated at room temperature in PBS for 15 min after irradiation. They were then lysed on gradients and the DNA analyzed by sedimentation [from Town et al., 1973b].

Fig. 8. Number of DNA single-strand breaks remaining after Type III repair in JG139 (pol+ rec+). Cells which had completed Type II repair were incubated for 60 min in minimal medium at 37 °C before being lysed and the DNA analyzed by sedimentation [from Town et al., 1973a].
Repair of X-Ray Damage to Bacterial DNA

Fig. 9. Summary of the capacity of the Types I, II, and III systems for repairing X-ray-induced DNA single-strand breaks. The initial yield was ~2.8 DNA single-strand breaks per single-strand genome (1.4 x 10^6 daltons) per krad in air or nitrogen [Town et al., 1972a]. The very fast Type I system can repair in buffer about 75% of these breaks if they were produced under nitrogen but only about 25% if produced in air [Town et al., 1972a]. The Type II system then can rapidly repair in buffer about 90% of the remaining breaks whether produced in air or nitrogen (from fig. 7). The Type III system can repair slowly in growth medium an additional two breaks per single-strand genome whether produced in air or nitrogen (from fig. 8) [Town et al., 1973a].
the initial yield of DNA single-strand breaks. Subject to certain assumptions [Town et al., 1972a] this yield appears to be 2.8 breaks per single-strand genome per krad. After anoxic irradiation, about 75% of the breaks produced are repaired by the Type I process. At doses less than about 8.3 krad, all the breaks remaining after Type I repair are rejoined by the Type II process (fig. 7). At higher doses, the slope of the line for the number of breaks not repaired by the Type II process is 10% of that for the number of breaks present before Type II repair, showing that about 90% of the breaks remaining after Type I repair are rejoined by the Type II process. Of the breaks remaining after Type II repair, a maximum of approximately 2 breaks are subject to Type III repair. If more than this number is presented to the Type III process, some of them will not be repaired. This is shown by the lowest line in fig. 9a which is displaced by ~2 breaks from the line above it, but has the same slope.

After aerobic irradiation (fig. 9b), about 25% of the initial number of breaks are repaired by the Type I process. At doses less than about 3.7 krad, all the remaining breaks are rejoined by Type II repair. After higher doses about 90% of the breaks are repaired, as after anoxic irradiation. The Type III process can repair only about 2 breaks remaining after Type II repair.

6.3. CONTRIBUTION OF REPAIR TO THE OXYGEN EFFECT FOR CELL SURVIVAL

The Type II process, which repairs about 90% of the breaks presented to it, and the Type III process, which can repair only about 2 breaks per single-strand genome, show little specificity for the repair of breaks produced in the presence or absence of oxygen. This means that if single-strand breaks are involved in the oxygen effect for cell survival (i.e., oxygen-dependent cell killing), it must be at the level of Type I repair. This system repairs three times as many of the breaks produced in the absence of oxygen as those produced in its presence, thereby leaving only 1/3 as many breaks to be repaired by the Types II and III systems in cells irradiated under anoxic conditions as compared with cells irradiated under aerobic conditions.

Thus, the effect of irradiating cells in the presence of oxygen is not to increase the number of breaks produced, but to reduce greatly the number of breaks which can be repaired by the Type I system. The Types II and III repair systems thus 'see' more breaks than they would after a similar dose under anoxic conditions. This leads to an overloading of the Types II and III repair systems at a lower dose under aerobic than anoxic conditions. It seems possible, therefore, that the oxygen-dependent cell killing is due, at
least in part, to failure to repair adequately single-strand breaks by the Types II and III systems even in repair proficient cells.*

If differential Type I repair totally determines the oxygen effect, then one would predict that cells with no Type I repair system would show no oxygen effect for cell survival. Any reduction in the oxygen enhancement ratio brought about by inhibition of the Type I system could be interpreted as indicating the extent of the involvement of Type I repair of single-strand breaks in determining the oxygen effect. These experiments must await the availability of a suitable ligase-deficient mutant.

From other evidence, however, it seems likely that only a part of the OER can be due to the differential repair of single-strand breaks. Although the Type III system does not show any preferential repair of breaks produced in the presence or absence of oxygen, recA strains (which lack the Type III

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* Sensitization of anoxic cells by NEM seems to proceed by the same mechanism since Town et al. [1972a] found that a high proportion of the DNA breaks produced in the presence of NEM were not repaired by the Type I system.

Fig. 10. X-ray survival curves for E. coli K-12 recA56 and a rec+ transductant. Cells were irradiated in PBS at room temperature in equilibrium with either air or N₂. They were diluted in PBS and plated on minimal agar. ○, recA56-Air; ●, recA56-N₂; □, rec+ -Air; ■, rec+ -N₂ [from Town et al., 1973a].
system for rejoining DNA single-strand breaks) have a lower OER than their wild-type counterparts [Rupp et al., 1970; Town et al., 1973a]. Survival curves for an otherwise isogenic rec+ and recA56 pair of strains are shown in fig. 10. The absence of recA controlled repair sensitizes anoxically irradiated cells to a greater extent than aerobically irradiated cells. Since this cannot be explained by a differential repair of DNA single-strand breaks, Town et al. [1973a] hypothesized that the recA gene was also involved in another repair system. This idea is also supported by the data on drug-induced inhibition of repair (see below, section 7.1.1.). By analogy with the recA gene-controlled system(s) for the repair of UV-induced DNA base damage [Rupp and Howard-Flanders, 1968; Smith and Meun, 1970], this second recA-dependent system could also involve the repair of X-radiation-induced base damage.

7. Inhibition of repair processes by drugs

The use of drugs to inhibit the repair of radiation damage can contribute to our understanding of these processes in several ways. If drugs with sufficient specificity can be identified they may be used to inhibit only one of the several available repair processes. Conversely the effect of a drug whose mode of action is known can tell us something about the biochemical processes involved in the repair pathway which it inhibits.

7.1. INHIBITION OF THE TYPE III REPAIR PROCESS

The class of inhibitors about which there is most information are those which inhibit the Type III process. As an example, the characteristics of 2,4-dinitrophenol (DNP) as a repair inhibitor will be described [Van der Schueren, Smith and Kaplan, 1973]. DNP at a concentration of $3 \times 10^{-3}$ M completely inhibited the Type III rejoicing process when cells were incubated in its presence after irradiation (fig. 11). In addition, if the drug was later removed from the medium, and the cells were further incubated, little rejoicing was seen, indicating that the inhibition of the repair process was largely irreversible. This post-irradiation incubation with DNP also potentiated the X-ray induced killing of these cells (fig. 12). If the cells were incubated after irradiation in growth medium without the drug, they lost their sensitivity to the drug over the same time-span that is required for the completion of Type III repair. Thus, as Type III repair progressed, the addition of the drug had a progressively smaller killing effect.
Fig. 11. Influence of dinitrophenol (DNP) on the Type III repair of DNA single-strand breaks in \textit{E. coli} K-12 (AB2497). Cells prelabelled with \(^3\)H-thymine were lysed on top of alkaline sucrose gradients and the DNA analyzed by sedimentation. The direction of sedimentation is from right to left. (a) Control; (b) X-ray 20 krad, zero time; (c) X-ray (20 krad) incubated for 90 min in supplemented minimal medium (SMM); (d) X-ray (20 krad) incubated for 90 min in SMM with \(3 \times 10^{-3} \) M DNP; (e) X-ray (20 krad) incubated for 90 min in SMM with \(3 \times 10^{-3} \) M DNP, followed by 60 min in SMM alone; (f) control, incubated for 90 min with \(3 \times 10^{-3} \) M DNP [from Van der Schueren et al., 1973].

In confirmation of the idea that the enhanced killing was due to inhibition of Type III repair, it was found that mutants which are deficient in this process \((recA, recB, exrA)\) showed no DNP-induced sensitization. However, the \textit{polA} mutant, which has a comparable radiosensitivity to the other mutants, was sensitized. This is consistent with the fact that this strain has a functional Type III repair system [Youngs and Smith, 1973a].

Several other compounds also show a similar post-irradiation sensitizing effect. They are: an impure sample of hydroxyurea [Kapp and Smith, 1971], quinacrine (a 9-amino acridine) [Fuks and Smith, 1971], chloramphenicol (or amino acid starvation) [Ganesan and Smith, 1972] and acriflavine [Kapp, 1970]. For all these compounds, data similar to those described above for DNP show that they cause an irreversible inhibition of Type III repair, a concomitant increase in cell killing in wild-type strains, and have little or no effect on \textit{rec}^- mutants.
Fig. 12. The kinetics of the sensitization of *E. coli* K-12 rec<sup>-</sup> cells (AB2497) by $3 \times 10^{-3}$ M DNP to X irradiation (20 krad). Cells were incubated for periods up to 120 min post-irradiation with or without DNP. Unirradiated cells, O; unirradiated cells treated with DNP, •; irradiated cells, △; irradiated cells treated with DNP, ▲ [from Van der Schueren et al., 1973].

7.1.1. *Comparison of the sensitizing effects of drugs and mutations: evidence for an additional repair system*

An interesting and informative comparison can be made between the radiation-sensitizing effects of drug treatment and genetic mutation.

In a wild-type (*pol<sup>+</sup>*) strain, introduction of a *recA* mutation brings about a 2.5 to 5-fold increase in X-ray sensitivity (depending on the strain). Introduction of an *exrA* mutation causes a slightly smaller sensitization (2 to 4-fold). Both the *recA* and *exrA* mutations completely block the Type III repair process for DNA single-strand breaks. On the other hand, DNP and quinacrine also appear to block the Type III process completely, but cause only a 1.5-fold sensitization. In no case (except *polA*, see below) does the final sensitivity of a drug-treated *rec<sup>+</sup>* cell approach that of a *rec<sup>-</sup>* cell, despite the fact that inhibition of rejoining is essentially complete. A possible explanation for this is that the *recA* gene is involved in more than one repair process: the Type III repair of DNA single-strand breaks, and the repair of some other type of lesion (see also section 6.3). In this hypothesis, the difference between *rec<sup>+</sup>* survival curves with and without drug would represent the contribution of Type III repair to viability, whereas the difference between
drug-treated rec\textsuperscript{+} and rec\textsuperscript{−} would indicate the contribution of the second rec gene-controlled repair process. This explanation supposes that none of the drugs so far tested acts directly on the recA gene product, but at some other level in the Type III repair pathway for single-strand breaks.

In a polA1 strain an interesting situation arises. As indicated above, this strain possesses a Type III repair system. Since a recA polA double mutant is inviable [Gross et al., 1971] it is more convenient to block repair genetically by introducing the exrA mutation, which leaves the double mutant still viable [Youngs and Smith, 1973a]. The exrA polA1 double mutant has a $D_0$ of approximately 0.6 krad compared with 0.85 krad for the polA1 single mutant. Treatment of irradiated polA1 cells with DNP also reduces the $D_0$ to approximately 0.6 krad [Van der Schueren et al., 1973]. Thus, in the polA1 background, DNP appears capable of blocking Type III repair as completely and effectively as an exrA mutation. Since the exrA mutation sensitizes the wild-type strain by a factor of 1.9 but only sensitizes the polA strain by a factor of 1.3 [Youngs and Smith, 1973a], this suggests that the exrA (and presumably recA) genes are involved in a second repair system, in addition to the Type III system for the repair of DNA single-strand breaks. A corollary to this argument is that since the polA1 strain is sensitized equally by the exrA mutation and by DNP, it must lack this second recA and exrA gene-dependent repair system. Thus the polA gene must be involved, along with recA and exrA, in the hypothetical second repair system.

This is an excellent example of how data from mutants and chemical repair inhibitors can be integrated to give more information about repair systems; in this case, this approach has yielded strong indirect evidence for a new repair system. Although the rec and exr gene functions appear to be involved in both this hypothetical new repair process and in the Type III repair of DNA single-strand breaks, it is not unreasonable to suppose that mutants can be found which are blocked in one repair pathway but not the other. A possible example of such a mutant is rorA [Glickman et al., 1971]. This strain is UV resistant, recombination proficient, about 1.5 times more X-ray sensitive than its wild-type counterpart, and appears to have no Type III rejoining process. In view of the UV-resistance of rorA, and its moderate X-ray sensitivity, it seems likely that its only repair deficiency is in the Type III process. There are also mutants which are very UV-sensitive but show only a little X-ray sensitivity (e.g., ras, uvrD). These would be good candidates for mutants blocked in the rec gene-mediated repair of X-ray induced base damage, while possessing normal Type III repair of single-strand breaks. The response of these mutants to metabolic inhibitors and their ability to
repair single-strand breaks is obviously of considerable interest, and is currently being investigated in this laboratory.

7.2. INHIBITION OF THE TYPE II REPAIR PROCESS

Type II repair was partially inhibited at 0°C, although there was less inhibition of the repair of breaks produced under anoxic conditions. Sodium cyanide (10^{-3} M) at 0°C inhibited Type II repair [Town et al., 1973a], as did 10^{-1} M EDTA [Town et al., 1971b]. The presence of quinacrine (2 \times 10^{-4} M) during X-irradiation (after a 15 min pre-incubation) also inhibited Type II repair [Town et al., 1972a]. However, none of these treatments has been tested to see whether it also sensitizes the cells to radiation-induced killing.

Cold shock and heat treatment inhibit Type II repair [Town, C.D., unpublished observations]. Heat treatment of some strains has been shown to sensitize them to radiation [Bridges et al., 1969]. Niacin starvation before irradiation also inhibits Type II repair [Town, C.D., unpublished observation] and sensitizes the cells to radiation [Town et al., 1972b]. However, in neither of these two cases has it been shown that the radiation sensitization is due specifically to inhibition of the Type II repair pathway, as the treatments employed can also inhibit other repair pathways.

7.3. INHIBITION OF THE TYPE I REPAIR PROCESS

It was the specific purpose of the heat-treatment and cold shock experiments to investigate whether or not there were repair processes independent of the Type II and Type III repair functions. Therefore, the argument that these treatments inhibit Type I repair is somewhat circular. The radiation sensitivity of repair-inactivated cells was not investigated, but a reduction in OER in these cells would be predicted provided that the inactivation of repair enzymes had occurred equally both in the killed cells and in those surviving the treatment.

Several chemicals, when present during irradiation, also appeared to inhibit this repair process. In E. coli these were NEM, HU, and quinacrine [Town et al., 1972a] and PCMB [Ho and Ho, 1972]; in M. radiodurans, EDTA [Dean et al., 1969]. Since these compounds were present during irradiation, it is not possible to say whether they acted directly by inhibiting the repair mechanism or indirectly by altering the chemical nature of the breaks produced so that they were no longer a substrate for the Type I process. Oxygen should also be considered to inhibit the Type I process by the same indi-
rect mechanism. Prestarvation of niacin-requiring cells for niacin also inhibited the Type I repair process [Town et al., 1972b], as discussed above (section 5.3.3).

Of these treatments, NEM and HU have been shown to sensitize anoxic cells specifically; quinacrine, PCMB and EDTA have not been tested in this regard. Niacin starvation sensitizes cells irradiated under both aerobic and anoxic conditions.

8. Summary and conclusions

8.1. diversity of DNA damage: its implications for repair systems

This review has concentrated upon the repair of X-ray induced DNA single-strand breaks. It should be clear from the preceding text that there is a broad spectrum of chemical products within this single category of lesion. Evidence that this is so comes both from the direct chemical and enzymatic analyses of breaks in vitro, and is also inferred from the demonstration of several distinct processes for the repair of DNA single-strand breaks in vivo.

In vitro evidence would suggest that the diversity of base damage produced by ionizing radiation may be even greater, although the instability of many of these products has thus far prevented their identification in vivo. Double-strand breaks are presumably also produced with a variety of end groups.

The existence of repair processes can be postulated on genetic grounds long before the actual lesion has been identified, as for example in the case of the second rec, exr and pol gene-mediated system discussed in sections 6.3 and 7.1.1. Since the presence of sensitizers or protectors during irradiation can qualitatively alter the DNA damage, one may also expect to find that the importance of different repair pathways may be modified by the irradiation conditions. One example of this is the way that the presence of oxygen during irradiation dramatically alters the relative proportions of single-strand breaks repaired by the Type I and II systems.

8.2. importance of DNA single-strand break repair

8.2.1. Repair deficient mutants

That single-strand breaks can contribute to lethality is demonstrated by the fact that mutants which are deficient in one or another process for their repair (e.g., polA or recA) are very much more radiosensitive than the corresponding repair-proficient strains. It is difficult to see how, if
replication could proceed past an un repaired single-strand break at all, it would not give rise to a broken daughter chromosome. On this basis, one un repaired single-strand break in each strand of the chromosome would presumably lead to cell death. Assuming a strand break efficiency of approximately 50 eV/break [which is close to the extrapolated value of 2.8 breaks/ krad/single-strand genome measured by Town et al., 1972a] this would give a \( D_0 \) of 0.36 krad. Assuming that other damage (base, sugar, etc.) contributes equally to lethality, then the \( D_0 \) for a totally repair-deficient strain would be approximately 0.18 krad. This value is several-fold lower than the \( D_0 \) of 0.6 krad for the exrA polA mutant, the most sensitive strain described to date. This discrepancy is unlikely to be due simply to errors in measuring the number of single-strand breaks. The more probable explanations (which are not exclusive) are either that the un repaired lesions have a probability less than one of leading to lethality or that a totally repair deficient mutant either has not been or cannot be isolated [e.g., the polA recA mutant is inviable; Gross et al., 1971].

8.2.2. Metabolically inhibited cells

By the same argument as used for repair-deficient mutants, un repaired single-strand breaks appear to cause cell death in cells where repair has been irreversibly inhibited by a pharmacological agent. The many examples of this are cited above (section 7.1) and the correlations appear convincing. Other physiological treatments (e.g., growth conditions) which bring about radiation sensitization and a concomitant reduction in repair ability also support this notion. The argument is further strengthened by the observation that, at least in some conditions, cell killing does not occur when repair is inhibited in a reversible manner (e.g., by low temperature).

8.2.3. Repair proficient cells

The previous two sections have substantiated the idea that DNA singlestrand breaks can cause lethality in cells which are impaired in their repair capacity. The question which remains to be answered is: do single-strand breaks contribute to cell lethality in cells which are genetically and physiologically repair proficient; and if so, to what extent? In an effort to clarify this question, Town et al. [1972a; 1973a, b] investigated the effects of oxygen in modifying both repair and cell survival. Subject to the qualifications discussed in section 6.3 the implication of their work is that the higher survival of cells irradiated under anoxic conditions is due (at least in part) to the fact that breaks produced under such conditions can be more readily
and more extensively repaired. The same initial number of single-strand breaks produced a greater killing under aerobic conditions presumably because fewer breaks were repaired by the Type I process, thus overloading the Type II and III repair systems. Overloading of the Type II and III repair systems also occurs after irradiation under anoxic conditions, but only after much higher X-ray doses.

These experiments alone do not give any information about the relative contribution of single-strand breaks to lethality, except that it is greater in repair-deficient mutants and in repair-proficient cells irradiated under aerobic conditions. Other information such as the relative importance of double-strand breaks is needed to complete the picture.

8.3. PROSPECTS IN REPAIR

8.3.1. DNA single-strand breaks

What has been identified so far is only a very basic skeleton upon which repair pathways can be built. The identification of the pathways rests on the fact that they are major repair pathways under these conditions. Under different irradiation conditions one may expect to find that other repair mechanisms predominate. In addition, any type of repair as now identified can probably be subdivided on the basis of greater enzymatic specificity within the class. One can thus expect to find branched, divergent and convergent pathways. Normally minor pathways may also assume greater significance in particular situations. An example of this is the slow, dnaE-controlled repair process which takes place in polA1 cells in buffer (see section 5.2.4.). This activity was not recognized in pol+ cells because of the much greater speed of DNA polymerase I in this buffer repair process.

8.3.2. Biological activity of repaired DNA

This is an area of research which has so far received too little attention. It is not sufficient to demonstrate that DNA breaks have been repaired. The biological activity of the repaired molecule (i.e., the accuracy of the repair process) is equally important. This question can be answered by using a transformation system. Examples of this are in the in vitro repair work of Laipis and Ganesan [1972a] and Noguti and Kada [1972] both using B. subtilis. Transformation is much more difficult in E. coli although reportedly possible [Avadhani, Mehta and Rege, 1969]. Other ways may also be found using phage, episomal or conjugally transferred DNA to test the biological accuracy of the repair processes. This aspect of the study of repair processes should not be overlooked.
8.3.3. Other lesions

As suggested above, there may be additional repair processes both for known kinds of damage and for unsuspected lesions. The isolation of new radiation-sensitive mutants and the assignment of these and the existing mutants to repair pathways is an intriguing prospect.

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