I. Introduction

Deoxyribonucleases, enzymes which hydrolyze specifically the inter-nucleotide bonds of polydeoxynucleotides, have been described in many bacteria. A useful general classification first introduced by Laskowski (1) divides these enzymes into exonucleases and endonucleases depending upon their mode of attack. Endonucleases attack polynucleotides at many points within the chain, generally producing only a small proportion of mononucleotides; exonucleases catalyze a stepwise attack at either the 3' or 5' terminus of a polynucleotide, producing predominantly mononucleotides. Recent detailed investigation of several of the purified bacterial deoxyribonucleases has indicated that this categorization is overly

simplified and in fact a single physically homogeneous protein may have the capacity to catalyze both the exo- and endonucleolytic cleavage of diester bonds depending upon the structure of its macromolecular substrate.

All of the bacterial deoxyribonucleases that have been examined in detail possess a specificity directed in varying degrees toward the secondary structure of the polydeoxynucleotide. With one recent exception, none of the deoxyribonucleases shows a simple base specificity whereby they attack phosphodiester bonds adjacent to a single base. However, it is now clear that several of the endonucleases may possess an extremely high order of specificity and have the capacity to recognize and attack one or a few phosphodiester bonds in polydeoxynucleotide chains composed of many thousands of internucleotide linkages.

In this review, the bacterial deoxyribonucleases to be considered will be divided into exonucleases and endonucleases. The latter category will be further subdivided into "nonspecific" endonucleases; that is, enzymes which do not show a high level of specificity for the internucleotide bond split and are, as a result, capable of degrading polynucleotides to a mixture of relatively small oligonucleotides and "specific" endonucleases, which display a high level of specificity for a given sequence within the polynucleotide chain, and as a consequence, introduce only a very few cleavages into a high molecular weight polynucleotide.

As noted above, the enzymes to be discussed are those which attack polydeoxynucleotides exclusively. Thus, nucleases from, for example, Bacillus subtilis (2–4), Serratia marcescens (5), and Staphylococcus aureus (6), which attack both RNA and DNA will not be considered. Such an enzyme from S. aureus is, however, the subject of Chapter 7 by Cotton and Hazen and Chapter 8 by Anfinsen et al. in this volume.

II. Exonucleases

The bacterial DNA exonucleases which have been purified and examined in detail are with two exceptions derived from E. coli and E. coli infected with bacteriophages; all show a strong specificity for the secondary structure of their polydeoxynucleotide substrate.

A. E. coli Exonucleases I and III

Both E. coli exonucleases I and III have been considered in a recent review (7) and will not be discussed in detail here. It is, however, worth noting again that they are prototypes of nucleases with an extreme specificity for the secondary structure of their substrate. Thus, exonuclease I attacks only single-stranded polynucleotides and exonuclease III has an equally strong preference for double-stranded structures. In both instances, once the structural requirements have been met, the enzymes initiate their attack at the 3'-hydroxyl termini sequentially liberating 5'-mononucleotides. In the case of exonuclease III, hydrolysis ceases once the bihelical structure is lost as a result of sustained exonucleolytic attack. Exonuclease I is unable to cleave dinucleotides; hence, the terminal and penultimate residues situated at the 5' end of the polynucleotide substrate remain as a residual dinucleotide. Recent experiments by Masamune and Richardson (8) have demonstrated that exonuclease III is able to initiate its attack at an internally located 3'-hydroxyl group in a DNA duplex (i.e., at a single-stranded interruption) as well as at the 3'-hydroxyl group at the end of the chain.

Finally, it should be noted that exonuclease III possesses an intrinsic DNA-phosphatase activity which specifically removes 3'-phosphoryl groups from double-stranded polynucleotides. This enzyme when acting on a 3'-phosphoryl-terminated DNA, first removes the 3'-phosphoryl group as inorganic phosphate, then proceeds as an exonuclease with the stepwise release of deoxynucleoside 5'-monophosphates (9).

Lacks and Greenberg (10) have purified an exonuclease from Diplococcus pneumoniae with properties very similar to E. coli exonuclease III. The pneumococcal enzyme acts preferentially on native DNA producing 5'-mononucleotides and single strands which are not susceptible to further attack. Like exonuclease III it shows an intrinsic 3'-phosphoryl-DNA phosphatase activity. A DNA phosphatase-exonuclease activity has also been reported in B. subtilis (11).

B. Phage λ-Induced Exonuclease

The exonuclease synthesized after induction of λ lysogens or after infection with virulent mutants of this phage has received a great deal

of attention because of its involvement in λ-recombination. The λ exonuclease has been obtained in physically homogeneous, crystalline form and is the only one of the bacterial DNases available at this level of purity (12).

Phage λ possesses its own recombination system, the "red" system (13, 14) which permits its DNA to undergo recombination in recombination deficient (rec−) hosts (15). The red system is composed of three complementation groups, two of which define the structural gene for the λ exonuclease (16). The λ exonuclease is antipodal to exonuclease III in its polarity of attack; thus, it specifically degrades double-stranded DNA, starting at the 5'-phosphoryl terminus, sequentially liberating 5'-mononucleotides. As in the case of exonuclease III, single strands are generated which are then not susceptible to further hydrolysis (17). The λ-exonuclease shows a very strong preference for termini bearing 5'-phosphoryl groups. Unlike exonuclease III, the λ-induced nuclease is unable to attack at single-stranded breaks within a DNA duplex (8). Clearly, both exonuclease III and λ exonuclease could be responsible for the production of the single-stranded regions in recombining DNA molecules postulated in most current models of genetic recombination (18).

C. E. coli EXONUCLEASE IV

Exonuclease IV is distinguished from the other DNA exonucleases of E. coli by its strong preference for relatively short chain oligonucleotide substrates (19). Native and denatured DNA are degraded at rates less than one-twentieth those observed with oligonucleotide mixtures derived from pancreatic DNase digests of DNA. At present it is not clear whether this reflects an inability of the enzyme to bind to macromolecular DNA or the relatively low concentration of DNA molecules compared to oligonucleotide chains present in the usual assay mixtures. Exonuclease IV requires magnesium ion and is optimally active at alkaline pH (pH 8–9). It produces 5'-mononucleotides exclusively, presumably by an exonucleolytic attack starting at the 3' terminus, although this latter point

BACTERIAL DEOXYRIBONUCLEASES

has not been established definitively. Koerner and his colleagues have found that exonuclease IV separates into two subfractions, termed IVa and IVb, upon gradient chromatography on columns of DEAE-cellulose (20). Though chromatographically distinct, exonucleases IVa and IVb have identical pH optima, divalent cation requirement, substrate specificity, and thermolability. The origin and significance of the two fractions are unknown.

D. PHAGE T2- AND T4-INDUCED EXONUCLEASES

An exonuclease catalytically very similar to exonuclease IV appears after infection of E. coli with phages T2 and T4 (19). Yet another catalytically indistinguishable exonuclease activity is synthesized after T2 and T4 phage infection and is closely associated with the T2- and T4-induced DNA polymerases (21, 22). An amber mutant which maps in the structural gene for the T4 DNA polymerase has been found by Nossal to induce the polymerase-associated exonuclease but not the polymerase (23). Both of the phage-induced exonucleases act optimally on mixtures of small oligonucleotides producing 5'-mononucleotides. Recent experiments by Richardson et al. (8) and by Kornberg and his colleagues (24) have demonstrated a 3' → 5' polarity of attack by the polymerase-associated exonuclease.

E. EXONUCLEASES ASSOCIATED WITH E. coli DNA POLYMERASE

The hydrolytic activity of E. coli DNA polymerase has until recently been termed exonuclease II primarily because of the uncertainty as to whether the polymerizing and hydrolytic functions of the purified enzyme were part of a single unit (25). It is now apparent as a result of studies in Kornberg's laboratory that DNA polymerase is a homogeneous protein composed of a single polypeptide chain with a single binding site for a DNA terminus and that the polymerizing and hydrolytic activities both involve a common active site (26). A detailed investigation of the

exonucleolytic activity by Deutscher and Kornberg (27) and independently by Klett et al. (28) has in fact shown that there are two exonucleolytic activities of opposite polarity intrinsic to the polymerase. One catalyzes an attack starting at 3'-hydroxyl terminus (3'→5'); the second attacks at the 5'-phosphoryl terminus (5'→3').

1. 3'→5' Exonuclease

Early studies of the nuclease activity associated with purified preparations of *E. coli* DNA polymerase indicated that it catalyzed an exonucleolytic attack on both double- and single-stranded polydeoxynucleotides beginning at the 3'-hydroxyl end, liberating deoxynucleoside 5'-monophosphates. Upon prolonged incubation, hydrolysis to mononucleotides was complete (25). It is now clear that hydrolysis of single-stranded polymers results exclusively from the 3'→5' exonuclease component of the polymerase. This activity is optimal in tris buffer at pH 8.6 and in glycine buffer at pH 9.2. It has an absolute requirement for a free 3'-hydroxyl group; 3'-phosphoryl-terminated polynucleotides are resistant (27). It therefore resembles very closely the exonuclease activity associated with the T4-induced DNA polymerase (22). Like the phage-induced enzyme, the 3'→5' exonuclease activity of the *E. coli* DNA polymerase is almost completely abolished under conditions of DNA synthesis (22, 29). Presumably a single 3'-hydroxyl terminus site serves for either polymerization or 3'→5' hydrolysis. Since the 3'→5' exonuclease activity removes nucleotide residues at the same site in the polynucleotide chain (3'-hydroxyl) at which the polymerizing activity adds nucleotides it has been suggested that this exonuclease activity represents an error-correcting mechanism whereby improperly paired nucleotides are removed in the course of synthesis (26).

2. 5'→3' Exonuclease

Studies by Klett et al. (28) with a synthetic d(A-T) copolymer in which diamino purine replaced adenine at the 3' end and by Deutscher and Kornberg (27) with 3'-phosphoryl-terminated polynucleotides led to the finding that purified preparations of *E. coli* DNA polymerase possess a 5'→3' exonucleolytic activity. In the latter studies DNA preparations with 3'-phosphoryl termini introduced by the action of staphylococcal nuclease (6) and therefore insensitive to 3'→5' exonuclease action were

found to be extensively degraded by purified DNA polymerase preparations yielding 5'-mononucleotides and oligonucleotides terminating in a 3'-phosphoryl group. Detailed investigation of this phenomenon showed that the 5'→3' exonuclease activity occupies a site on the enzyme distinct from the 3'→5' exonuclease. Unlike the latter it has a strong preference for double-stranded polynucleotide substrates and displays a broad pH optimum ranging from pH 7.4 to 9.2 (27). The products of 5'→3' exonuclease action consist of dinucleotides in addition to 5'-mononucleotides. This was demonstrated in an experiment in which hydrolysis of d(pT)₃₀₀ was compared with d(pT)₃₀₀ annealed to d(pA)₄₀₀₀ and d(pT)₃₀₀ blocked at the 3' terminus by a dideoxythymidine residue also annealed to d(pA)₄₀₀₀. The dinucleotide d(pT)₂ was observed among the products of hydrolysis only in the latter two cases; it was not found when single-stranded d(pT)₃₀₀ was degraded, i.e., in the absence of 5'→3' exonuclease action. Kinetic studies showed that the rate of appearance of dinucleotides relative to mononucleotides during 5'→3' hydrolysis was not altered as a function of time (24).

In further contrast to the 3'→5' exonuclease, the 5'→3' exonuclease activity was strongly stimulated (as much as 40-fold) by simultaneous synthesis; moreover, an increased frequency of oligonucleotides was observed among the products of hydrolysis during synthesis, and oligonucleotides in lengths ranging up to the hexanucleotide appeared (30). The increase in 5'→3' exonuclease activity accompanying polymerization of nucleotides at the 3' terminus of the primer strands has been explained by postulating that the DNA polymerase binds a nicked region of a DNA duplex in its active center, thus bringing the 3'-hydroxyl- and 5'-phosphoryl-terminated chains in close alignment. The synthetic activity, by advancing the 3'-hydroxyl terminus keeps the latter adjacent to or near the 5' end and increases the rate of release of 5'-mononucleotides at this terminus. The release of oligonucleotides may be the result of the failure of the DNA polymerase to cleave terminal bonds, thereby resulting in exposure of the second or subsequent phosphodiester linkages to hydrolysis at the 5'→3' exonuclease site (24). In the case of d(pT)₃₀₀ with a tetradecoxycytidylate region at the 5' terminus annealed to d(pA)₄₀₀₀, the deoxycytidylate residues which do not hydrogen bond to the d(pA)₄₀₀₀ were found to be excised as oligonucleotides (31). Hence, the 5'→3' exonuclease is able to hydrolyze double-stranded DNA in the duplex region beyond a set of non-hydrogen-bonded residues, removing

oligonucleotides containing these residues. This property of the \(5' \rightarrow 3'\) exonuclease may play a general role in the removal of mismatched regions, for example, pyrimidine dimers. Kelly et al. (31) have, in fact, found that the \(5' \rightarrow 3'\) exonuclease can excise oligonucleotides containing thymine dimers from ultraviolet-irradiated \(d(pT)_{300}\) annealed to \(d(pA)_{4000}\) as well as from irradiated DNA. In contrast, the \(3' \rightarrow 5'\) exonuclease like \(E.\ coli\) exonuclease I and snake venom phosphodiesterase (31, 32) is blocked at the point in the \((pT)_{300}\) at which it encounters a thymine dimer.

Studies by Jovin et al. (33) had shown that acylation of the enzyme with \(N\)-carboxymethylisatoic anhydride resulted in a total destruction of polymerase activity with a concomitant 9-fold enhancement of exonuclease activity as measured at pH 7.4. More recently, Brutlag et al. (29) and Klenow and Henningsen (34) independently discovered that the \(5' \rightarrow 3'\) exonuclease activity could be dissociated from the remainder of the DNA polymerase molecule by limited proteolysis with subtilisin or trypsin. Thus, treatment of the polymerase, a single polypeptide chain of molecular weight 109,000, with these proteases led to the generation of two fragments of 76,000 and 34,000 molecular weight. The larger fragment retained the polymerizing activity and the \(3' \rightarrow 5'\) exonuclease activity; the small fragment contained the \(5' \rightarrow 3'\) exonuclease.

The 76,000 molecular weight fragment derived from the \(E.\ coli\) DNA polymerase is therefore similar to the T4-induced polymerase in that both enzymes, contain only the \(3' \rightarrow 5'\) exonuclease in addition to the polymerizing activity. The phage-induced enzyme, despite the lack of \(5' \rightarrow 3'\)- exonuclease, has a molecular weight of approximately 110,000 (22).

F. \textit{B. subtilis} \textbf{Phage SP-3-Induced Exonuclease}

Trilling and Aposhian have partially purified a DNase from extracts of \textit{B. subtilis} infected with phage SP-3 (35). This enzyme requires magnesium ion and shows optimal activity between pH 7.8 and 8.9 in Tris buffers. It is highly specific for denatured DNA and appears to catalyze a unique type of exonucleolytic attack beginning at the \(5'\) end of the chain which sequentially releases dinucleotides. Neither mono-

nucleotides nor oligonucleotides of intermediate size between dinucleotides and the polymeric DNA substrate (with the exception of a few percent trinucleotides) is observed at any time during hydrolysis.

G. *E. coli* ATP-DEPENDENT DNASE

An ATP-dependent DNase has been partially purified from extracts of *E. coli* by Oishi (36) and Barbour and Clark (37). It shows an absolute requirement for magnesium or manganese ion and has a broad pH optimum ranging from pH 7.5 to 9.5. The partially purified enzyme preferentially degrades native DNA (including glucosylated T4 DNA) and has an almost absolute requirement for ATP or dATP. Current preparations of the enzyme are also active on denatured DNA; however, there is only a slight stimulation of hydrolysis by added ATP. This latter activity may therefore represent some contamination with exonuclease I. The mode of attack is stated to be exonucleolytic.

A particularly interesting feature of the ATP-dependent DNase is its possible involvement in genetic recombination. Thus, certain recombination deficient strains of *E. coli* with the recB– and recC– phenotypes (15) appear to lack this enzyme.

III. ENDONUCLEASES

A. NONSPECIFIC ENDONUCLEASES

1. *E. coli* Endonuclease I

The existence of a deoxyribonuclease in *E. coli* bound to an inhibitory RNA was first suggested by Kozloff (38) who found that the DNase activity of freshly prepared extracts could be markedly enhanced by pretreatment with ribonuclease. The enzyme was subsequently purified and freed of inhibitor (39). The purified enzyme termed endonuclease I could, in turn, be competitively inhibited by a variety of RNA's including transfer RNA, and *K*₁ values as low as 10⁻⁸ M (nucleotide) have been observed (40). Examination of various purified RNA species and synthetic polyribonucleotides for their inhibitory activity has led

to the suggestion that some ordered conformation is required for a polynucleotide to be an effective inhibitor (7).

endonuclease I has a pH optimum ranging from pH 7.5 to 8.5 and absolutely requires magnesium or manganese ion for activity. It degrades native DNA at a rate sevenfold greater than denatured DNA. Attack is purely endonucleolytic yielding oligonucleotides terminated by 5'-phosphoryl groups. The enzyme shows little, if any, base specificity and limit digests produced after exhaustive digestion of DNA contain a mixture of oligonucleotides with an average chain length of 7 residues. Analysis of the oligonucleotides has shown an almost random distribution of nucleotides at the 3' and 5' termini.

Studier (41) and independently Bernardi and Cordonnier (42) have found that endonuclease I cleaves both strands of a DNA double helix at or near the same level. Paoletti et al. (43) have examined this question in greater detail employing a fluorometric method based on the increase in the amount of ethidium bromide bound to closed circular duplex DNA upon the introduction of a single-stranded break (44). In contrast to pancreatic DNase in which there was a linear increase in fluorescence, nonlinear kinetics were observed with endonuclease I. Further investigation of this phenomenon led to the suggestion that endonuclease I possesses a significant exonucleolytic component with the result that approximately 400 nucleotides are removed at each endonucleolytic scission introduced by the enzyme. Consequently, endonuclease I does not promote a clean double-stranded break but rather causes a "shattering" at the point of cleavage, leading to the liberation of small oligonucleotides, presumably by an exonucleolytic mode of attack.

2. Streptococcal and Pneumococcal Endonucleases

A deoxyribonuclease termed streptodornase, optimally active at pH 7.0 in the presence of magnesium ion, has been partially purified from culture fluids of Streptococcus pyogenes (45). This enzyme yields a distribution of products from DNA very similar to that seen with E. coli endonuclease I: Only traces of mono- and dinucleotides are found, the majority of products being rather large oligonucleotides terminated by

41. F. W. Studier, JMB 11, 373 (1965).
5'-phosphate groups. Like E. coli endonuclease I, streptococcal endonuclease is inhibited by RNA.

A careful study of various strains of Streptococcus by Wannamaker has demonstrated that group A streptococci produce three different DNases (A, B, and C) which can be distinguished by their electrophoretic and immunological properties and by their pH optima (46). The relative amount of each enzyme produced appears to be a function of strain variation. Streptodornase has been identified with DNase A.

Lacks and Greenberg have partially purified an endonuclease from Diplococcus pneumoniae in conjunction with the exonuclease cited in Section II,A (10). This enzyme is active on both native and denatured DNA and produces 5'-phosphoryl-terminated oligonucleotides.

3. Phage T5-Induced DNase

A deoxyribonuclease not normally present in E. coli is rapidly synthesized after infection with phage T5 (47). This DNase appears at approximately the same time as the other "early" phage-specific enzymes (DNA polymerase and deoxynucleotide kinase, etc.) induced following infection with this bacteriophage.

The T5-induced DNase has been purified nearly to the point of physical homogeneity. It has a pH optimum of pH 9.3 in glycine buffer and absolutely requires a divalent cation (magnesium or manganese) for activity. The mode of attack by the enzyme appears to be both endonucleolytic and exonucleolytic, yielding ultimately a mixture of 5'-phosphoryl-terminated mononucleotides and oligonucleotides of average chain length 4–5. Although the enzyme attacks both native and denatured DNA at approximately the same rate, the proportion of mononucleotides produced from denatured DNA at the limit of digestion is only about one-fourth that found with native DNA.

4. M. luteus ATP-Dependent Endonuclease

In 1964, Tsuda and Strauss discovered a DNase activity in crude extracts of Micrococcus lysodeikticus (later renamed Micrococcus luteus) which required a nucleoside di- or triphosphate for activity (48). This enzyme has recently been purified extensively (2400-fold) and examined in detail by Takagi and his colleagues (49). It has an alkaline pH

optimum (pH 9.4) and requires a divalent cation, preferably magnesium ion for activity. Double-stranded DNA is degraded at a rate 40-fold greater than denatured DNA. The mode of attack is endonucleolytic yielding 5′-phosphoryl-terminated oligonucleotides with an average chain length of 5.5 residues at the limit of digestion.

A kinetic analysis of DNA degradation by the M. luteus endonuclease by means of sucrose density gradient centrifugation of the products as hydrolysis proceeded showed that the DNA added initially disappeared and was replaced by very slowly sedimenting material. Products of intermediate size were not detectable. Takagi and his colleagues interpreted these findings in terms of a one-by-one type of degradation in which the products of the initial hydrolysis possess a higher affinity for the enzyme than the undegraded DNA substrate, with the result that a DNA molecule initially attacked is degraded to completion before attack of a second molecule is begun.

The role of the nucleoside triphosphate in the hydrolysis of DNA has not yet been clarified. ATP and dATP are the most effective nucleotides and only slight activity (10% or less) is observed with the other triphosphates; nucleoside diphosphates are inactive. The rate of DNA hydrolysis is proportional to the ATP concentration and the ATP is converted to ADP and inorganic phosphate in the course of the reaction. Three moles of ATP are consumed for each phosphodiester bond cleaved, indicating a complex mechanism of participation of ATP in the endonucleolytic reaction. Preliminary experiments by Takagi and his colleagues indicate that the purified enzyme catalyzes an exchange of ADP with ATP in the absence of DNA, suggesting that a phospho enzyme may be an intermediate.

B. SPECIFIC ENDONUCLEASES

During the past three years endonucleases have been discovered which possess a specificity which is considerably more refined than that shown by the nuclease considered thus far. Typically, these enzymes catalyze the cleavage of one or, at most, a few phosphodiester bonds in a DNA molecule composed of many thousands of nucleotide residues. In no instance has the basis for this remarkable specificity been established. However, in the case of the E. coli “restriction” enzymes the presence or absence of a methyl group on a specific deoxyadenylate or deoxycytidylate residue may be involved.

The limited attack catalyzed by the specific endonucleases obviously poses formidable assay problems which have in several cases been re-
solved by novel and ingenious methods. These are indicated where applicable.

1. *E. coli* Restriction Endonucleases

The *E. coli* restriction endonucleases are involved in the phenomenon of host-controlled modification and restriction whereby bacterial cells of one strain are able to destroy DNA from cells of foreign strains (50). As noted above, the current working hypothesis is that resistance to a given restriction endonuclease is conferred by specific methylation at nucleotide sequences that would otherwise be vulnerable to that enzyme. The restriction enzymes have been assayed either by measuring the decrease in sedimentation coefficient of a homogeneous DNA preparation (usually phage λ) (51) or more simply by measuring the inactivation of infectious DNA (phage λ or fd) (52), both of which are sensitive to the introduction of one or a few phosphodiester bond scissions. One of the restriction endonucleases, called endonuclease R·K, has been purified approximately 5000-fold from *E. coli* strain K by Yuan and Meselson (51). This enzyme cleaves double-stranded DNA synthesized in other *E. coli* strains but is totally without effect on DNA synthesized in strain K itself. In addition to magnesium ion the enzyme specifically requires ATP and S-adenosylmethionine; its pH optimum lies in the range pH 7.5–8.0. Several very similar nucleases with different specificities but with the same unusual cofactor requirements have also been described. An enzyme partially purified from *E. coli* lysogenic for phage P1 attacks DNA from *E. coli* lacking P1 (51), and another enzyme from *E. coli* strain B attacks DNA from strains other than B (52). There has also been a brief report of a restriction endonuclease in *Hemophilus influenzae* (53).

Meselson and Yuan have carried out a detailed examination of the mode of attack of λ·C DNA (i.e., DNA from phage λ grown on *E. coli* strain C and therefore lacking the K enzyme modification) by purified endonuclease R·K (51). Sucrose density gradient analysis of the products formed after treatment of λ·C DNA with the endonuclease has shown that they consist of duplexes containing little or no single-stranded DNA and no single chain breaks. The products sediment in sucrose gradients at or near the position at which quarter molecules of λ-DNA would sediment. Thus, the λ·C DNA appears to undergo double-stranded

cleavage at fixed sites along its length. By examining the action of the enzyme on the twisted circular form of λ·C DNA (54), Meselson and Yuan have also been able to demonstrate that single-stranded scission precedes cleavage of the duplex. The occurrence of single chain scissions early in the reaction taken together with the paucity of single chain breaks in the limit product indicate that the enzyme first cleaves only one chain and then a few seconds later breaks the complementary chain at a point directly or nearly opposite to the initial break. A similar result was obtained by Linn and Arbor with the restriction endonucleases partially purified from E. coli and E. coli lysogenic for P1 (58). It has not been determined whether a given enzyme molecule remains bound to the DNA, catalyzing breaks in both chains, or whether the two chains are attacked independently. In the case of endonuclease R·K acting on phage λ DNA it has been established that duplexes in which only one of the two chains is modified (presumably by the appropriate methylation) are not attacked at all. Thus, heteroduplexes are resistant even to single chain scissions and are therefore modified at every site of potential attack.

The role of ATP and S-adenosylmethionine in the reaction remains an intriguing but as yet unresolved question. Recently Yuan and Meselson have reported that in the presence of magnesium ion, ATP and S-adenosylmethionine the R·K endonuclease forms a specific complex with its DNA substrate (55). Complex formation is, however, observed at ATP concentrations ($4 \times 10^{-8} M$) at which nucleolytic activity is not detectable. This result suggests that ATP may be involved in at least two steps: (1) formation of a nonhydrolytic complex at low ATP levels and (2) formation of more stable (or more numerous) complexes and nucleolytic action at higher concentrations of ATP. The S-adenosylmethionine requirement for complex formation is in the same concentration range as observed for restriction.

2. E. coli Endonuclease II

Endonuclease II of E. coli was first recognized by Friedberg and Goldthwait as an activity in extracts of E. coli mutants lacking endonuclease I, that specifically attacked double-stranded DNA alkylated with the monofunctional alkylating agent methyl methane sulfonate (56). It was subsequently found that the partially purified enzyme could in fact

introduce a limited number of single-stranded breaks in nonalkylated du-
plex DNA, approximately 3–4 per single strand. The activities on the alkyl-
ated and nonalkylated DNA appear to be associated with the same
protein; however, this point has not yet been definitely settled. Endo-
nuclease II has a broad pH optimum ranging from pH 8.0 to 9.0. It
has no absolute requirement for a divalent cation but is stimulated by
added magnesium or manganese ion. Unlike endonuclease I, it is not
inhibited by RNA. Goldthwait and his co-workers have assayed endo-
nuclease II by measuring the release of ³H-thymidine-labeled fragments
from a suspension of polyacrylamide gel containing alkylated T4 DNA
(57). Most (>90%) of the radioactivity released from the gel is acid-
precipitable, hence, still macromolecular.

With lightly alkylated DNA, endonuclease II makes predominantly
single-stranded breaks suggesting that the enzyme can hydrolyze a phos-
phodiester bond at or near an alkylated base in a native DNA molecule
with no single-stranded breaks in this region. With more extensively
alkylated DNA, double-stranded breaks predominate.

Friedberg et al. (56) pointed out that since alkylation of DNA occurs
principally at the N-7 position of guanine and the N-3 position of
adenine (58) these chemical modifications might be expected to result
in electron rearrangements in the purine rings that may affect hydrogen
bonding, base-stacking, or both. Thus, alkylation might produce a dis-
tortion in the secondary structure of DNA, and it is this distorted struc-
ture which is the substrate for endonuclease II. Since the enzyme makes
a limited number of single-stranded breaks in native DNA, it is pos-
sible that a similar type of conformational distortion exists in nonalkyl-
ated DNA.

The role of endonuclease II in vivo is not known. Assay of recombi-
nation defective mutants of E. coli and mutants abnormally sensitive to
ultraviolet irradiation and to treatment with methyl methane sulfonate
showed them all to possess normal levels of the enzyme (56).

3. Phage T7-Induced Endonuclease

Center et al. (59) have purified extensively (1000-fold) an endo-
nuclease induced after infection of E. coli with phage T7. This enzyme
attacks native DNA to yield products with a molecular weight of ap-
proximately $2 \times 10^4$ and denatured DNA to produce fragments having

57. E. Melgar and D. A. Goldthwait, JBC 243, 4401 (1968).
a molecular weight of $10^4$ or less. The fragments have not been further characterized. The endonuclease activity on native DNA was assayed by measuring the conversion of $^3$H-labeled $\phi$X-174 RF I (60) to a form which could be trapped on nitrocellulose membrane filters after heating at 100°. Thus, covalently closed duplex DNA is not retained on these filters, whereas the single strands produced by heating the circular duplexes that have suffered a single-stranded break are retained. The action of the enzyme on single-stranded DNA was followed by measuring the conversion of single-stranded circular $\phi$X 174 DNA (61) to a form susceptible to E. coli exonuclease I (62).

Studies with conditional lethal mutants of phage T7 have shown that gene 3 is the structural gene for the DNase (T7 contains at least 19 distinct complementation groups) (63). In the restrictive host, gene 3 mutants synthesize only limited amounts of phage DNA (64). Inasmuch as they are also defective in carrying out the degradation of host DNA, the gene 3 endonuclease may be the enzyme required for this function. The purified endonuclease produces little acid-soluble material; thus, an additional activity is presumably required for the complete degradation of host DNA. Center et al. suggested that gene 6 specifies an exonuclease which acts at the breaks produced by the gene 3 endonuclease (59).

As noted above amber mutants in gene 3 produce only limited amounts of DNA under restrictive conditions. It is therefore clear that degradation of host DNA is required for normal T7 DNA synthesis and phage production, a result which is to be anticipated in light of the finding that T7 derives most of its nucleotides from host nucleic acids (65).

Center et al. have observed that the purified T7 endonuclease can hydrolyze T7 DNA in in vitro (59). Hence, it is not known how T7 infection results in the selective degradation of E. coli DNA in vivo.

4. Phage T4-Induced Endonucleases II and IV

Sadowski and Hurwitz have described two endonucleases synthesized in T4 phage-infected E. coli which they have named T4 endonucleases II and IV (66).

10. BACTERIAL DEOXYRIBONUCLEASES

a. **T4 Endonuclease II.** T4 endonuclease II has been purified approximately 300-fold (66). The partially purified enzyme has a broad alkaline pH optimum (pH 8.4–10.1) and shows an absolute requirement for magnesium ion. Two methods have been used for the assay of the enzyme. One measures the discharge of $^3$H-AMP from the *E. coli* DNA ligase–AMP complex on reaction of the complex with DNA pretreated with endonuclease II. Since ligase–AMP repairs single-stranded breaks in duplex DNA that have apposing 3'-hydroxyl and 5'-phosphoryl termini (67), the discharge of AMP is a direct measure of the number of breaks introduced into the DNA by T4 endonuclease II. The second assay estimates the number of 3'-hydroxyl termini formed by measuring the increase in $^{14}$C-labeled λ DNA which becomes susceptible to *E. coli* exonuclease I after thermal denaturation of the nicked DNA. Since exonuclease I acts exonucleolytically from the 3'-hydroxyl terminus of denatured DNA (62), the amount of acid-soluble $^{14}$C formed is an estimate of the number of 3'-hydroxyl termini generated by T4 endonuclease II action.

The enzyme introduces predominantly single-stranded breaks into native DNA, although at high enzyme concentrations some double-stranded breakage occurs. Purified preparations of the endonuclease do, however, show some activity on denatured DNA, possibly resulting from contamination with T4 endonuclease IV (see below). As noted above, the single-stranded breaks produced by T4 endonuclease II bear 3'-hydroxyl and 5'-phosphoryl termini. The enzyme makes a limited number of breaks in duplex DNA and the average length of the limit product from phage λ DNA, as determined by sucrose density gradient centrifugation, is about 1000 residues. As in the case of *E. coli* endonuclease II, the basis for the very limited extent of hydrolysis of DNA is not known. Sadowski and Hurwitz have examined the nucleotide residues at the 5'-phosphoryl termini by means of the polynucleotide kinase reaction and found all four deoxynucleotides to be present. There did, however, appear to be a significantly higher proportion of deoxyguanylate and deoxycytidylylate residues at these termini. A limited base specificity of this kind cannot, however, account for the apparent high degree of specificity actually observed.

T4 endonuclease II appears to differ from *E. coli* endonuclease II in several respects, the most striking difference being the inability of the phage-induced enzyme to attack either glucosylated or nonglucosylated T4 DNA. Friedberg *et al.* have also found that whereas the activity of

extracts of infected and uninfected cells showed similar activities when tested with alkylated DNA, there was a marked increase in relative endonucleolytic activity on unalkylated DNA in the infected cell extract, suggesting that the phage-induced enzyme either has no activity or very little activity on alkylated DNA (56).

Recently, two groups have independently isolated non-lethal mutants of T4 that are incapable of degrading the host E. coli DNA and may therefore be defective in T4 endonuclease II (68, 69).

b. T4 Endonuclease IV. T4 endonuclease IV has been purified approximately 150-fold (66). It has a pH optimum which ranges from pH 8.4 to 9.2 and an absolute requirement for magnesium or cobalt ion. Endonuclease IV was assayed by measuring the conversion of single-stranded circular fd DNA (70), labeled with 14C to an exonuclease I-sensitive form. The partially purified enzyme has a strong preference for denatured DNA; however, it does attack native λ DNA at a significant rate (one-tenth that of fd). Like T4 endonuclease II, endonuclease IV shows only limited activity, producing oligonucleotides 150 residues long; it does not form measurable amounts of acid-soluble material. The oligonucleotide products contain 3'-hydroxyl and 5'-phosphoryl termini; the latter bear deoxycytidylate residues exclusively. T4 endonuclease IV therefore has an absolute base specificity and is unique among the DNA endonucleases which have thus far been described. Some other, as yet undetermined feature of its mechanism must, however, be responsible for the limited extent of attack observed. Again, like T4 endonuclease II it does not hydrolyze T4 DNA, whether glucosylated or not.

The combined action of T4 endonucleases II and IV and an exonuclease which Sadowski and Hurwitz have identified in extracts of T4-infected cells can result in the double-stranded breakage of duplex DNA. Presumably, endonuclease II introduces a single-stranded break in native DNA and the exonuclease removes mononucleotides from the internal 3'-hydroxyl termini exposing a region of single-stranded DNA on the opposite strand. This region is then cleaved by endonuclease IV to yield double-stranded fragments of DNA. Because of the inability of T4 endonucleases II and IV to attack T4 DNA, it is possible that these enzymes together with the "T4 exonuclease" promote the initial stages

of breakdown of host DNA following T4 infection. Warren and Bose have, in fact, reported that in the initial phase of host DNA breakdown, *E. coli* DNA is degraded to fragments of molecular weight approximately $1 \times 10^6$ in a process in which single-stranded breaks occur first, followed by double-stranded cleavage (71). The double-stranded DNA fragments are then presumably degraded to acid-soluble oligonucleotides and ultimately mononucleotides, which can then be used in the synthesis of T4 DNA.

5. *M. luteus* "UV Repair Enzymes"

In 1962, Strauss described an activity in extracts of *Micrococcus lyeodeikticus* (*M. luteus*) which preferentially inactivated UV-irradiated *B. subtilis* transforming DNA (72). It was subsequently shown by Carrier and Setlow that such extracts were able specifically to excise thymine-thymine and thymine-cytosine dimers from the irradiated DNA (73). Fractionation of the *M. luteus* extracts by Nakayama et al. demonstrated that two chromatographically separable protein fractions were required for pyrimidine dimer excision, one of which they suggested was an endonuclease that introduced a phosphodiester bond cleavage at or near the pyrimidine dimer and the second of which released the dimer as part of an acid-soluble oligonucleotide (74). The two activities have been purified and examined in detail by Takagi et al. (75) and independently by Grossman and his colleagues (32, 76). The purified enzymes acting together do in fact have the capacity to excise quantitatively thymine dimers from UV-irradiated DNA. The first enzyme in the sequence has been purified approximately 5000-fold; it is of relatively low molecular weight (14,000–15,000 based on Sephadex gel filtration). The pH optimum of the enzyme is pH 6.5–7.5 and it is stimulated by, but it is not dependent upon, added magnesium ion. The purified enzyme is entirely specific for UV-irradiated, double-stranded DNA and is free of the ATP-dependent endonuclease of *M. luteus*. The UV endonuclease introduces a single-stranded break in close proximity to a

thymine dimer leaving a 3'-phosphoryl terminus. Nucleotides are not released during this incision step. The subsequent thymine dimer excision is carried out by the second enzyme, an exonuclease also purified extensively (1500-fold) by Grossman et al. This enzyme, whose activity is dependent upon added magnesium ion, acts on unirradiated denatured DNA releasing 5'-mononucleotides by an exonucleolytic mechanism starting at either the 3' or 5' terminus. Native DNA is resistant to the action of the exonuclease; however, the enzyme will attack irradiated native DNA that has been pretreated with the endonuclease in the 5'→3' direction liberating an average of 6 nucleotides per endonucleolytic break. The digestion products consist of mono-, di-, and trinucleotides; and the thymine dimers are contained in the trinucleotide fragments. Purified preparations of the exonuclease are devoid of DNA polymerase activity, suggesting that the UV exonuclease is not a 5'→3' exonuclease component of the M. luteus DNA polymerase.

It would appear from these studies that the excision of thymine, and more generally pyrimidine dimers may be a two-step process. The initial single-stranded incision is probably dependent upon the presence of a distorted area in the DNA duplex resulting from the formation of a thymine dimer. Once the initial break has been introduced, a short single-stranded region containing the photoproduct results which is then susceptible to the action of the exonuclease.

Grossman et al. have isolated a mutant of M. luteus by nitrosoguanidine mutagenesis which is abnormally sensitive to UV and X-irradiation and also shows a reduced capacity to support the replication of UV-irradiated phages (the hcr- phenotype). Extracts of this mutant have a correspondingly low level of UV endonuclease activity (76). Takagi et al. have transformed the mutant with DNA derived from UV-resistant cells and found that the UV-resistant transformants displayed the same level of sensitivity to UV irradiation as the wild type; however, the UV endonuclease activity in the extract remained at the same low level observed in the original mutant strain (75). All attempts to isolate a revertant have thus far been unsuccessful, suggesting that the mutant bacterium may harbor a double mutation. Thus, while it appears that the UV endonuclease may be involved in the repair of UV damage in vivo, this point has not been firmly established.