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Mutants of *Diplococcus pneumoniae* that Lack Deoxyribonucleases and Other Activities Possibly Pertinent to Genetic Transformation

SANFORD LACKS

Biology Department, Brookhaven National Laboratory, Upton, New York 11973

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Mutants of *Diplococcus pneumoniae* that lacked the two major deoxyribonucleases of the cell—one an endonuclease, the other an exonuclease preferentially active on native deoxyribonucleic acid (DNA)—were obtained. The development of a method for detecting mutant colonies, based on the binding of methyl green to DNA, facilitated isolation of the mutants. Neither enzyme was essential for growth of the cells, for repair of ultraviolet damage, or for any phase of DNA-mediated transformation. Residual deoxyribonuclease activity in the double mutant corresponded to an exonuclease, approximately one-fifth as active as the major exonuclease, that attacked native and denatured DNA equally well. This activity appeared to be associated with the DNA-polymerase enzyme. A mutant that apparently lacked a cell wall lytic enzyme was also fully transformable. A mutant strain that was four times more sensitive to ultraviolet light than the wild type also transformed normally. Recipient cells of this strain were deficient in the repair of ultraviolet-irradiated transforming DNA. Mutants were found which, unlike the wild type, integrated donor markers only with high efficiency, thereby indicating that a particular cellular component that is susceptible to loss by mutation, such as an enzyme, is responsible for low integration efficiency.

Two enzymes with deoxyribonuclease activity were previously found in *Diplococcus pneumoniae* (11). One, an endonuclease, acts on both native and denatured deoxyribonucleic acid (DNA) to give 5′-P-terminated oligonucleotides. The other, an exonuclease, acts preferentially on native DNA to give 5′-nucleotides and single strands.

A role for the exonuclease was invoked in a model for DNA entry (11). According to this model, when the end of a double-stranded donor DNA molecule just penetrates the cell, the exonuclease acts on that end so as to release 5′-nucleotides from one strand and drag the other strand, pari passu, into the cell. Such a mechanism is consistent with observations on the fate of labeled DNA introduced into cells where, initially, half the DNA is converted to single strands and the other half is apparently degraded to small fragments (12). Either or both deoxyribonucleases could conceivably play a role also, at a later stage of transformation, that is, in the union of donor and host DNA that corresponds to genetic recombination. The dark repair of ultraviolet (UV)-induced lesions in DNA has also been supposed to involve the function of an exonuclease similar in specificity to the pneumococcal exonuclease (21).

To test whether either the endonuclease or exonuclease was involved in the transformation of *D. pneumoniae* or in its ability to repair UV damage, mutants that lacked the enzymes were sought. In the process of obtaining deoxyribonuclease-deficient strains, mutants deficient in other functions possibly pertinent to the mechanism of transformation were also obtained and examined.

MATERIALS AND METHODS

Bacterial strains and media. The origins of the wild-type strain of *D. pneumoniae* R6 and of the genetic markers for streptomycin resistance (*str*), sulfonamide resistance (*sul*), and inability to use maltose (*mal*) were previously described (10). The media used for growing cultures and selecting transformants with respect to the genetic markers were also described previously (10). The origin of the marker for azauracil resistance (*azu*) has also been given (5); transformants for this marker were scored with 6-azauracil at 200 μg/ml. The novobiocin resistance marker

(*nov*), scored at 10 μg/ml, and the pneumococcal strain Rx, used in the laboratory of W. Guild, were obtained from M. Fox.

A blood-agar medium for surface plating of pneumococci contained (per liter): 35 g of tryptose blood-agar base (Difco), 1 g of sucrose, and 30 ml of sterile, defibrinated sheep blood (Colorado Serum Co.).

Transformation procedure. DNA was prepared by the method of Hotchkiss (7). Cultures to be transformed were grown to an absorbancy at 650 nm of approximately 0.1, that is approximately 8×10^7 colony-forming units per ml, diluted 20-fold, and treated with DNA at 2 μg/ml for 30 min at 30 C. Addition of pancreatic deoxyribonuclease (Worthington) to 1 μg/ml terminated uptake. The cultures were then incubated for 90 min at 37 C before plating in selective medium. When deoxyribonuclease-negative transformants were sought, a more dilute culture was allowed to react with DNA for 1 hr at 30 C, after which it was incubated for 3 hr at 37 C to allow segregation of negative colony-forming units before plating.

Mutagenic treatment. Cultures were treated a number of times in succession with 1-methyl-3-nitro-1-nitrosoguanidine. A culture was initially treated with the mutagen at 20 μg/ml; however, when resistance developed after several treatments, the concentration had to be increased to 100 μg/ml. The culture, at an absorbancy at 650 nm of approximately 0.2, was treated each time for 40 min at 37 C to give ~1% survival, after which it was diluted 10-fold and allowed to grow again to an absorbancy at 650 nm of approximately 0.2. The production of mutants resistant to azauracil was used as an index of mutagenesis; after each treatment the frequency of such mutants increased by approximately 0.001. This cumulative mutagenesis gave mutant frequencies high enough to allow the selection of negative mutants by the testing of individual clones (Table 1).

UV sensitivity of strains and markers. To select a strain sensitive to UV light, 2.5-μliter samples of individual clones grown to an absorbancy at 650 nm of approximately 0.1 were spotted within squares of corresponding grids marked on each of three blood-agar plates. Before incubation, the plates were subjected, respectively, to 0-, 20-, and 60-sec irradiation with a GE germicidal lamp at a dose rate of 10 ergs per mm^2 per sec. Strain uvr-1 gave no colonies on the plate irradiated for only 20 sec, whereas all the other clones tested gave colonies even on the plate exposed for 60 sec. The original isolate of *uvr-1* also contained a *hex*$^-$ mutation. A *uvr*$^-$,*hex*$^+$ strain was constructed by transforming this isolate with wild-type DNA. Individual clones from the cross were tested for low-efficiency transformation of *nov*, which is characteristic of *hex*$^+$. One such clone was found among 108 that were tested.

The sensitivity of various strains to UV light was compared more precisely by irradiating cultures, grown to $A_{650} = 0.15$ and exposed in layers approximately 2 mm thick, with UV light at 10 ergs per mm^2 per sec. The cultures were stirred periodically, and samples were taken at intervals for the determination of surviving colony-forming units.

To study the inactivation of different genetic markers, DNA was irradiated, at a concentration of 40 μg/ml in a layer 1 mm thick, with UV light at 20 ergs per mm^2 per sec. The solution was agitated mechanically and samples were withdrawn at intervals. Transforming activity in the samples was tested with different recipient strains at a DNA concentration of 2 μg/ml, which falls on the plateau portion of the concentration-response curve.

Plate assay of deoxyribonuclease phenotype. This assay permits the rapid examination of many individual clones for the presence of particular deoxyribonucleases. It is based on the binding of methyl green to highly polymerized DNA, which gives a stable color at *p*H 7.5 (8). Enzymatic degradation of the DNA releases free methyl green, which at this *p*H loses its color. Cells are plated in nutrient agar containing DNA and methyl green. Colonies appear within 30 hr, and by this time those colonies that contain the major pneumococcal deoxyribonuclease, the

TABLE 1. *Mutant frequencies after repeated treatment of D. pneumoniae with 1-methyl-3-nitro-1-nitrosoguanidine*

Strain treated	No. of treatments	Mutant phenotype	Genetic symbol	Frequency
R6	10	Azauracil-resistant	*azu*	0.026
		Endonuclease-deficient	*end*	0.004
		Exonuclease-deficient	*exo*	0.002
		Ultraviolet-sensitive	*uvr*	0.004
		Not lysed by deoxycholate	*cwl*	0.002
		Nontransformable	*ntr*	0.14
		High-efficiency integration only	*hex*	0.5
R6 end-1	4	Azauracil-resistant	*azu*	0.002
		Exonuclease-deficient	*exo*	0.001
end-5, exo-1	4	Azauracil-resistant	*azu*	0.007
		Endonuclease-positive	*end*$^+$	0.003
		Lack of residual exonuclease	—	<0.0005

endonuclease, are surrounded by a colorless zone. By 54 hr, colonies that lack the endonuclease but contain the major exonuclease are surrounded by a colorless zone. Colonies that lack both enzymes develop only a faint zone that is only generally visible at still later times. Figure 1 illustrates the assay with a mixture of (a) *end*$^+$,*exo*$^+$ and *end*$^-$,*exo*$^+$ cells in a ratio of 1:10 and (b) *end*$^-$,*exo*$^+$ and *end*$^-$,*exo*$^-$ cells also in a ratio of 1:10. At 30 hr colorless zones generated by the endonuclease are visible, and by 54 hr zones generated by the exonuclease are apparent. By the later time, zones around endonuclease-containing colonies are much larger, whereas, around the doubly negative colonies, scant decoloration has occurred.

Both in the detection of original mutants and of transformants, the following procedure was used. Methyl green, at 1 mg/ml, was dissolved in 0.02 M ammonium acetate, *p*H 4.3; this solution was thrice extracted with chloroform and then boiled to sterilize it. DNA (Calbiochem, ex salmon sperm) was sterilized by ethyl alcohol precipitation and dissolved at 2 mg/ml. Petri dishes containing basal layers of medium in 1% agar were prepared in advance. For each dish, a sample of culture containing 100 to 150 colony-

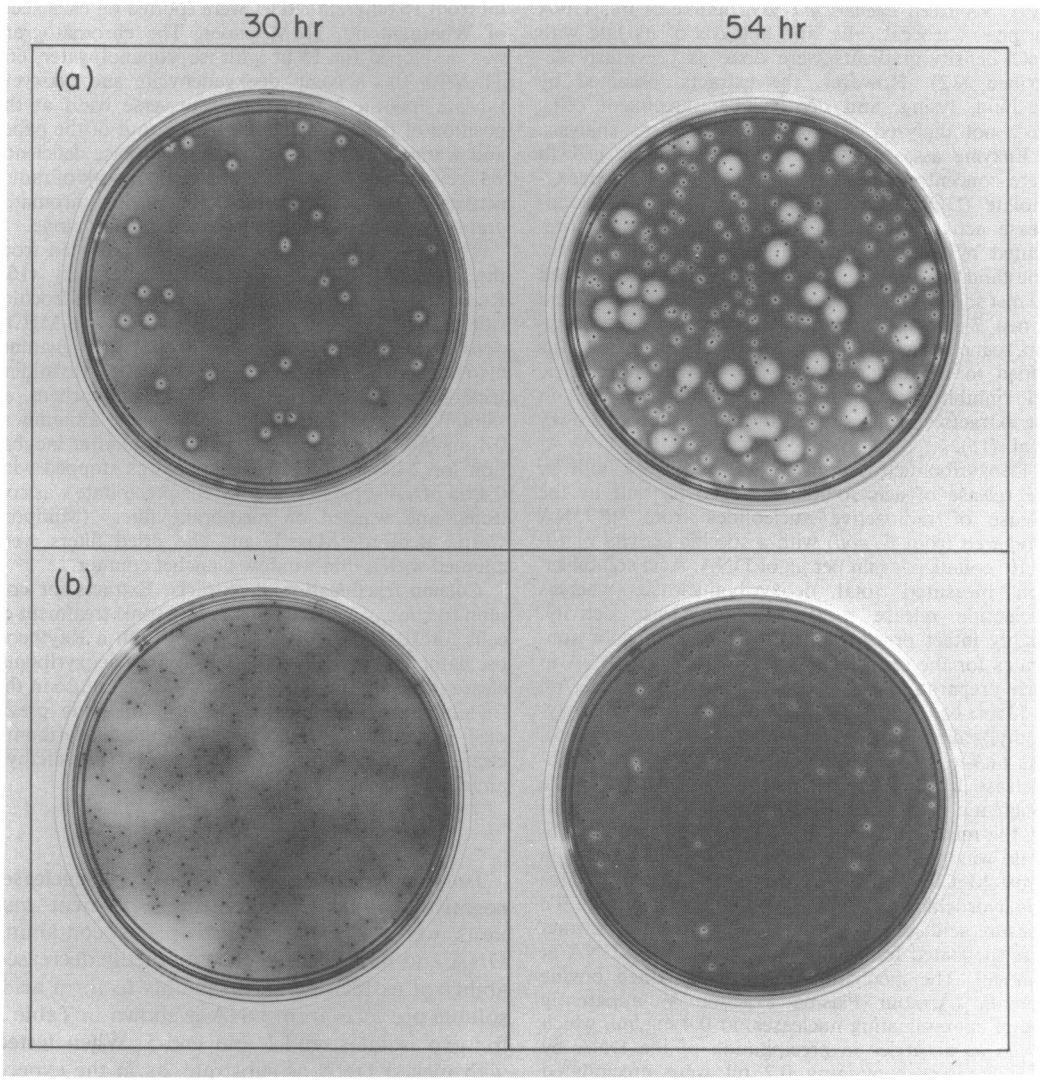

FIG. 1. *Plate assay of deoxyribonuclease phenotype. (a) Cells of end*$^+$,*exo*$^+$ *and end*$^-$,*exo*$^+$ *in a ratio of 1:10; (b) end*$^-$,*exo*$^+$ *and end*$^-$,*exo*$^-$ *cells in a ratio of 1:10. Mixtures of cells were added to nutrient agar containing DNA and methyl green. Identical plates are shown after 30 and 54 hr at 37 C. Black spots are colonies. Clear zones around the colonies result from the action of deoxyribonucleases that leach out of the colonies. Strains used were: R6(end*$^+$,*exo*$^+$*), R6end-1 (end*$^-$,*exo*$^+$*), and R6end-1, exo2 (end*$^-$,*exo*$^-$*).*

forming units was added to 4.5 ml medium. Then, 2.5 ml of DNA and 1.0 ml of methyl green were added. After bringing the mixture to 40 C, 2.0 ml of 3% agar at 48 C was added, and the total of 10 ml was plated over the basal layer. Subsequently, a covering layer of 0.75% agar in 0.15 M NaCl was superimposed and the dishes were incubated at 37 C. Platings were examined at appropriate times. Colonies without colorless zones were picked and grown up, and extracts were tested for deoxyribonuclease by in vitro methods. Occasional colonies without zones still gave a positive test for the deoxyribonuclease.

Uptake and molecular fate of DNA. Preparation of ^{32}P-DNA from *Escherichia coli*, uptake of this DNA by pneumococcal cells, and analysis of its fate with CsCl density gradients were done as previously described (12). However, the extracts, obtained by washing, lysing, and deproteinizing recipient cells, were not dialyzed before density gradient analysis.

Enzyme assays. Cells in late logarithmic growth were concentrated and lysed in 0.1% sodium deoxycholate (DOC). Gradual loss of the minor exonuclease activity at this DOC concentration was prevented by five-fold dilution immediately after lysis. The fluid used for suspension and dilution contained 50 mM sodium phosphate (*p*H 7.5), 2 mM $MgCl_2$, and 3 mM 2-mercaptoethanol. Ribonuclease (Worthington, pancreatic, 5× crystallized), at 20 μg/ml, was added to the original lysate to destroy ribonucleic acid inhibitory to endonuclease activity. Protein in the extracts was determined by the method of Lowry et al. (15).

Deoxyribonuclease activity was measured both by the release of acid-soluble radioactivity and by the release of radioactive nucleotides from ^{32}P-DNA (prepared from *E. coli*) with a specific activity of 10^3 to 10^4 counts per min per μg of DNA. Acid solubilization measured total deoxyribonuclease, whereas nucleotide release measured exonuclease activity. Highly intact preparations of DNA were poor substrates for the exonucleases (11). Therefore, DNA in such preparations, at a concentration of 100 μg/ml in 50 mM NaCl, 1 mM $MgCl_2$, and 10 mM tris(hydroxymethyl)aminomethane(Tris)-hydrochloride (*p*H 7.5), was nicked by treatment with pancreatic deoxyribonuclease I, at 30 ng/ml, for 30 min at 30 C. (The pancreatic deoxyribonuclease was destroyed by heating the mixture at 75 C for 15 min.) Denatured substrate was prepared by heating DNA at 10 μg/ml in 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, and 10 mM Tris-hydrochloride (*p*H 7.5) at 100 C for 10 min. To measure acid-soluble products, dilutions of an extract were incubated for 60 min at 30 C with ^{32}P-DNA at 5 μg/ml. The incubation mixture contained bovine albumin (Armour Plasma Fraction V, apparently free of contaminating nucleases) at 0.4 mg/ml, which served as a carrier in precipitation of the DNA by acid. Samples containing 0.2 ml were precipitated with 0.2 ml of 0.4 M perchloric acid and were centrifuged; 0.2 ml of the supernatant fluid was plated on copper planchets for counting. To measure nucleotide release, 0.05-ml samples of a reaction mixture which did not contain albumin were spotted for paper chromatography. The chromatograms were developed as previously described (11), and segments corresponding to nucleotides were excised for counting.

Before the plate assay was developed, a modification of the assay for the release of nucleotides allowed a rapid screening of single clones for exonuclease deficiency. Cultures of single colonies were grown in 2 ml of medium, and the cells were centrifuged and suspended in 0.2 ml of a solution containing 20 mM sodium phosphate (*p*H 7.5), 3 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 mM NaCl, 0.1% sodium DOC, 20 μg of ribonuclease per ml, and 3 μg of ^{32}P-DNA per ml. On incubation at 37 C, lysis occurred, and the mixture was allowed to react for 2 hr. Samples of 0.01 ml from 16 different clones were spotted on each sheet of Whatman no. 3MM paper. The chromatogram was developed for 15 hr with isopropanol-water, 66:34. With this solvent, deoxyadenylate and deoxycytidylate migrate together. A transverse band at the position of these nucleotides was cut out of the paper and scanned for radioactivity. Exonuclease deficiency was revealed by the absence of a distinct peak of radioactivity at the point corresponding to the chromatographic path of the sample from a particular clone.

The assay of DNA polymerase was adapted from the procedure used in Kornberg's laboratory (16). Reaction mixtures contained: 10 mM Tris-hydrochloride (*p*H 7.5); 3 mM 2-mercaptoethanol; 5 mM $MgCl_2$; deoxynucleoside triphosphates of adenine, guanine, cytosine, and thymine, each at 30 μM; ^{14}C-thymidine triphosphate at 0.025 μc/ml; DNA (Calbiochem, ex salmon sperm) at 40 μg/ml; and bovine albumin at 0.4 mg/ml, in a total volume of 0.4 ml. After incubation for 2 hr at 37 C, the reaction was stopped with 0.4 ml of 0.4 M perchloric acid; the precipitate was collected and washed on membrane filters (Millipore Corp., Bedford, Mass.); and the dried filters were counted with a thin-window planchet counter.

Column fractionation of extracts. Extracts for column fractionation were prepared by sonic treatment of cells for 10 min at maximal power with a Raytheon oscillator. Extracts made by DOC lysis of deoxyribonuclease-deficient mutants were too viscous to use in the fractionation procedure. Otherwise, methods previously described for fractionation of the deoxyribonucleases on columns of Sephadex G-200 and diethylaminoethyl-cellulose were used (11).

RESULTS AND DISCUSSION

Isolation and transfer of deoxyribonuclease-negative mutations. Endonuclease-deficient mutants were selected on agar plates containing DNA and methyl green. The markedly decreased ability of extracts of such mutants to form acid-soluble products from DNA is shown in Table 2 for two isolates, end-1 and end-5. When tested with nicked DNA as substrate, as in the experiment shown in Table 2, *end*$^-$ extracts showed ~15% of normal activity. With intact DNA as substrate, this proportion was usually lower. Virtually all of the remaining activity is the result of exonuclease action, since the amount of DNA

TABLE 2. *Deoxyribonuclease (DNase) activities of wild and mutant strains of D. pneumoniae*

DNase genotype	Strain	Specific activity of DNase[a]	
		Acid soluble products	Mononucleotides
end$^+$, *exo*$^+$	R6	118	15
	hex-1	108	11
end$^-$, *exo*$^+$	hex-1, end-1	12	10
	R6 end-1	15	12
	end-1	16	14
	end-5	16	11
end$^+$, *exo*$^-$	exo-1	67	3
end$^-$, *exo*$^-$	end-5, exo-1	2	1
	hex-1, end-1, exo-1	3	2
	R6 end-1, exo-1	3	2
	R6 end-1, exo-2	3	2
	R6 end-1, exo-3	3	2
	R6 end-1, exo-4	3	3
	R6 end-1, exo-5	2	1

[a] Expressed as micrograms of DNA released per hour per milligram of protein.

rendered acid-soluble corresponds to the amount of nucleotides released.

A strain, exo-1, deficient in exonuclease but normal in endonuclease, was obtained by screening individual clones for exonuclease activity. Although this strain rendered DNA acid soluble almost as fast as the wild type, only about one-fifth as many nucleotides were produced. Strains carrying this mutation appear to be devoid of the phosphatase-exonuclease. It will be shown later that the residual exonuclease activity is due to another enzyme.

When DNA from *end*$^-$ mutants was transferred into wild-type cells, *end*$^-$ transformants could be selected by the plate assay technique. In this way *end-1* was transferred into R6, to separate the deoxyribonuclease-negative mutation from possible ancillary mutations in the original mutant strain. Similarly, the *exo-1* mutation was passed into various *end*$^-$ strains. Here, too, the plate assay technique permitted selection of the doubly negative transformants. Other *end*$^-$, *exo*$^-$ strains (those containing mutations *exo-2* through *-5*) were obtained as independent mutations of strain R6end-1. In all of the *end*$^-$, *exo*$^-$ strains, deoxyribonuclease activity was reduced to the level of the residual exonuclease.

Data on the transformation frequencies of deoxyribonuclease markers are given in Table 3. The frequencies of transformation were compared with *nov*, a marker normally showing low integration efficiency, and *str*, a marker showing high integration efficiency. The *end-1* marker was integrated with high efficiency in the wild-type recipient. This indicates that the *end-1* mutation occurred at a single site, or at most, in a short region of the gene (10). The *end*$^+$ marker corresponding to the *end-1* mutation also was integrated with high efficiency; this is shown by the second cross listed in Table 3. The same cross shows that the marker for exonuclease deficiency, *exo-1*, was integrated with a low efficiency, similar to *nov*. With a *hex*$^-$ recipient, in which all single-site markers show high integration efficiency, the *exo-1* marker also shows high efficiency. Low efficiency in a *hex*$^+$ host and high efficiency in a *hex*$^-$ host can only be characteristic of a single-site marker, not of a multisite marker such as a deletion. Therefore, it appears that loss of function in either the *end* or *exo* genes can occur by single-site mutations in those genes.

In the following examination of the physiological roles of the deoxyribonucleases governed by the *end* and *exo* genes, the conclusions are justified only if, as assumed, the absence of activity in vitro indicates the absence of activity in vivo. Furthermore, reference to the deoxyribonuclease governed by the *exo* gene as the major exonuclease implies only that it is the major source of exonucleolytic activity under the particular conditions of assay.

TABLE 3. *Transformation with deoxyribonuclease markers*

Cross[a]	Donor marker	Transformation frequency[b]	Ratio to *str*
end-1, *exo*$^+$ DNA → *end*$^+$, *exo*$^+$, *hex*$^+$ cells	*nov*	0.046	0.1
	str	0.46	(1.0)
	end-1	0.61	1.3
end$^+$, *exo-1* DNA → *end-1*, *exo*$^+$, *hex*$^+$ cells	*nov*	0.029	0.1
	str	0.21	(1.0)
	exo-1	0.029	0.1
	end$^+$	0.20	1.0
end$^+$, *exo-1* DNA → *end-5*, *exo*$^+$, *hex*$^-$ cells	*nov*	0.24	0.9
	str	0.27	(1.0)
	exo-1	0.23	0.9
	end$^+$	0.17	0.6

[a] Donor DNA contained novobiocin resistance (*nov*) and streptomycin resistance (*str*) markers in every case.

[b] Ratio, in percentage, of transformants to total colony-forming units.

Properties of deoxyribonuclease-negative strains. (i) *Growth.* Neither the endonuclease nor the major exonuclease appears to be essential for growth, since the strain R6end-1,*exo-2*, which lacks both enzymes, grows normally (Fig. 2). Some of the mutants that were isolated, such as *exo-1*, did grow more slowly. Although the slower growth rate accompanied the *exo-1* mutation when it was transferred to the normally growing R6end-1 strain, such growth deficiencies probably result from independent mutations induced by the strong mutagenic treatment. The absence of endonuclease in cells of *E. coli* also does not appreciably impair their growth rate (2).

(ii) *UV resistance.* A strain that lacks both the endonuclease and the major exonuclease is just as resistant to UV irradiation as the wild type (Fig. 3). Repair of UV damage must occur in these strains, since other mutants, such as uvr-1, do exhibit heightened sensitivity to UV. However, neither deoxyribonuclease seems to be essential for the repair process in *D. pneumoniae*. That the exonuclease is not implicated in repair is particularly interesting because it is a phosphatase-exonuclease analogous to exonuclease III of *E. coli*. A proposed scheme for UV repair in *E. coli* suggested that exonuclease III might participate in the repair process by stripping down one strand of DNA after excision of a pyrimidine dimer (21). If repair in *E. coli* and *D. pneumoniae* are identical in mechanism, either the stripping down does not occur, or it is not essential for repair, or another enzyme performs this function.

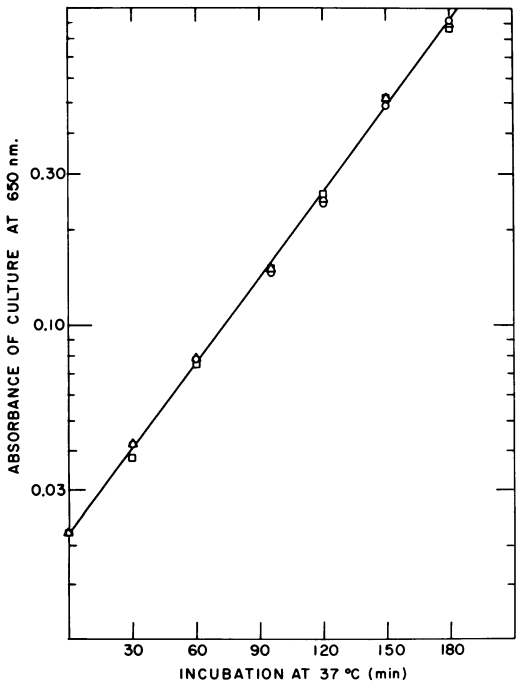

FIG. 2. *Growth of deoxyribonuclease-deficient strains. Mutant and wild strains were inoculated into a semisynthetic medium composed essentially of casein hydrolysate, vitamins, and minerals with sucrose as an energy source. Turbidity measures on samples of the cultures were begun when the absorbance passed 0.020. Symbols:* □, *R6(end⁺,exo⁺);* △, *R6end-1 (end⁻, exo⁺);* ○, *R6end-1,exo-2 (end⁻,exo⁻).*

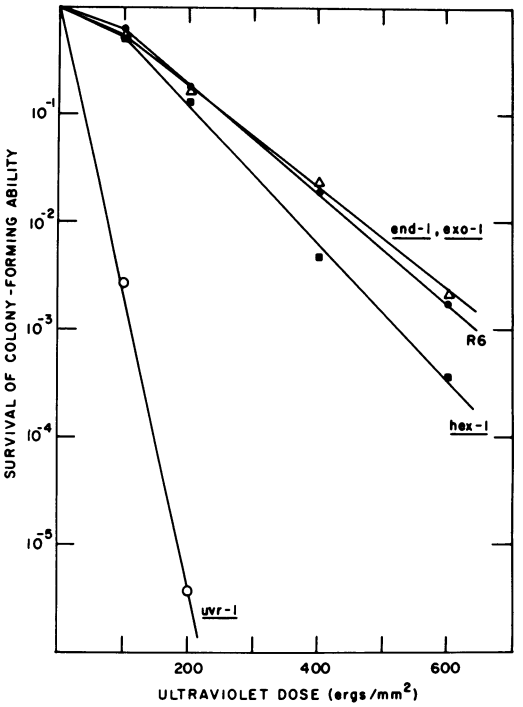

FIG. 3. *Ultraviolet sensitivity of mutant strains. The wild-type strain, R6 (●), and ultraviolet-sensitive mutant, uvr-1 (○), a mutant that gives only high integration efficiency, hex-1 (■), and a mutant lacking both major deoxyribonucleases, end-1,exo-1 (△) were tested for survival after ultraviolet irradiation as described.*

(iii) *Transformability.* Neither the endonuclease nor the major exonuclease is essential for transformation of *D. pneumoniae*. Four out of eight endonuclease-negative mutants could be transformed normally. The strain in which the *exo-1* mutation first appeared was not transformable, but when this mutation was transferred to *end⁻* strains, the doubly defective (*end⁻,exo⁻*) strains were transformable. Four independent *exo⁻* mutations in the R6end-1 strain also did not impair transformability. Nontransformability of some of the mutants probably resulted from mutations independent of the deoxyribonuclease deficiency, since the nontransformable character is found in an appreciable portion, ~15%, of a highly mutagenized population. Since cells lacking both deoxyribonucleases are normally trans-

formed with a variety of genetic markers, neither enzyme can be essential for either the uptake of DNA or its subsequent integration into the recipient cell genome.

Fate of donor DNA in an end⁻,exo⁻ recipient. Normal uptake of DNA by the double mutant end-5,exo-1 was demonstrated with ^{32}P-labeled DNA from *E. coli*. The fate of the newly entered DNA in the mutant, as analyzed by equilibrium centrifugation in a CsCl gradient (Fig. 4), was the same as its fate in wild-type cells (12). About half the introduced radioactivity was in single-stranded DNA. The other half of the radioactivity was divided about equally between low-molecular-weight fragments, which are distributed throughout the CsCl gradient, and double-stranded DNA at the density of native *D. pneumoniae* DNA. Occurrence of radioactivity at this position, rather than at the position of greater density corresponding to native *E. coli* DNA, is evidence that it represents de novo synthesis, presumably also from fragments. Therefore, the degradation of donor DNA and its conversion to single strands do not depend on either the endonuclease or the major exonuclease, because both enzymes are lacking in this strain.

The major exonuclease had been an attractive candidate for the enzyme involved in the degradation of incoming DNA, since its action in vitro gives equal amounts of single-stranded DNA and mononucleotide fragments (11). Furthermore, the action of this enzyme could account for the entry of DNA. Although the evidence now rules out such functions for the major exonuclease, it is conceivable that they are carried out by the enzyme responsible for the residual exonuclease activity observed in the deoxyribonuclease-deficient mutants.

Nature of residual exonuclease. Gel filtration of an extract of strain R6end-1,exo-1 showed that the residual deoxyribonuclease activity resided in an enzyme distinct from both the endonuclease and the major exonuclease. The profiles of deoxyribonuclease activity and protein eluted from a column of Sephadex G-200 are shown in Fig. 5. Activity of deoxyribonuclease was measured with both native and denatured DNA as substrate. An enzyme that attacks only denatured DNA emerges with the peak of high molecular weight or aggregated protein that is excluded from the gel. This enzyme was not studied further. Another peak of activity toward denatured DNA corresponds with the only significant peak of activity toward native DNA. Thus the residual activity observed in *end⁻,exo⁻* strains appears to represent a single enzyme that is about equally active on native and denatured DNA. The specificity of this exonuclease is therefore distinct from the major exonuclease, which acts preferentially on native DNA (11).

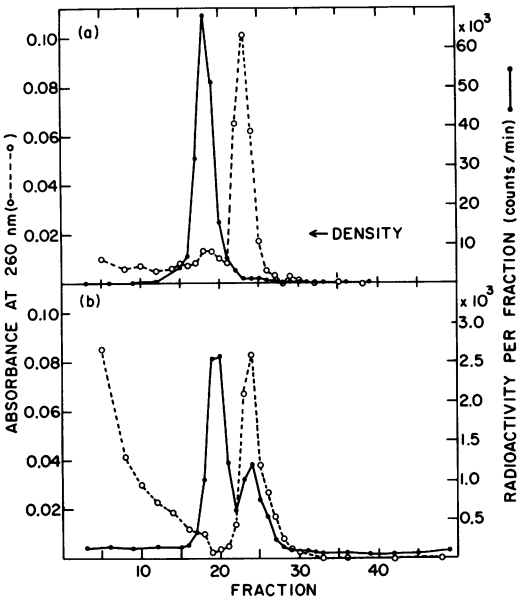

FIG. 4. *Fate of donor ^{32}P-DNA in a deoxyribonuclease-deficient recipient. (a) Control mixture of 1 μg of heat-denatured ^{32}P-DNA from E. coli and 29 μg of native DNA from D. pneumoniae. (b) Extract of cells of strain end-5,exo-1 washed and lysed immediately after uptake of E. coli ^{32}P-DNA. The cells were treated with this DNA for 10 min at 30 C. Fractionation was accomplished by equilibrium centrifugation in a density gradient of CsCl buffered at pH 11.0.*

The position at which the residual exonuclease emerged, before the peak of protein retained by the gel, also showed it to be distinct from the major exonuclease. The latter enzyme and the endonuclease both were eluted after the protein peak. The slight amount of deoxyribonuclease activity in the latter position on fractionation of an extract from a strain carrying both the *end-1* and *exo-1* mutations shows that these mutations reduce the endonuclease to <0.1% and the major exonuclease to <1% of their activities in a wild-type strain. Fractionation on diethylaminoethyl-cellulose columns also showed that the residual exonuclease was distinct from the major exonuclease. At *p*H 7.5, the former enzyme was eluted at an NaCl concentration of 0.18 M, whereas the latter enzyme was eluted at 0.10 M. Fractions were analyzed, also, for DNA polymerase activity. With both the gel filtration (Fig. 5) and the diethylaminoethyl-cellulose fractions, the peak of polymerase activity corresponded to the peak of residual exonuclease. This result suggests that the residual exonuclease activity is inherent to the DNA polymerase molecule. A similar exonuclease

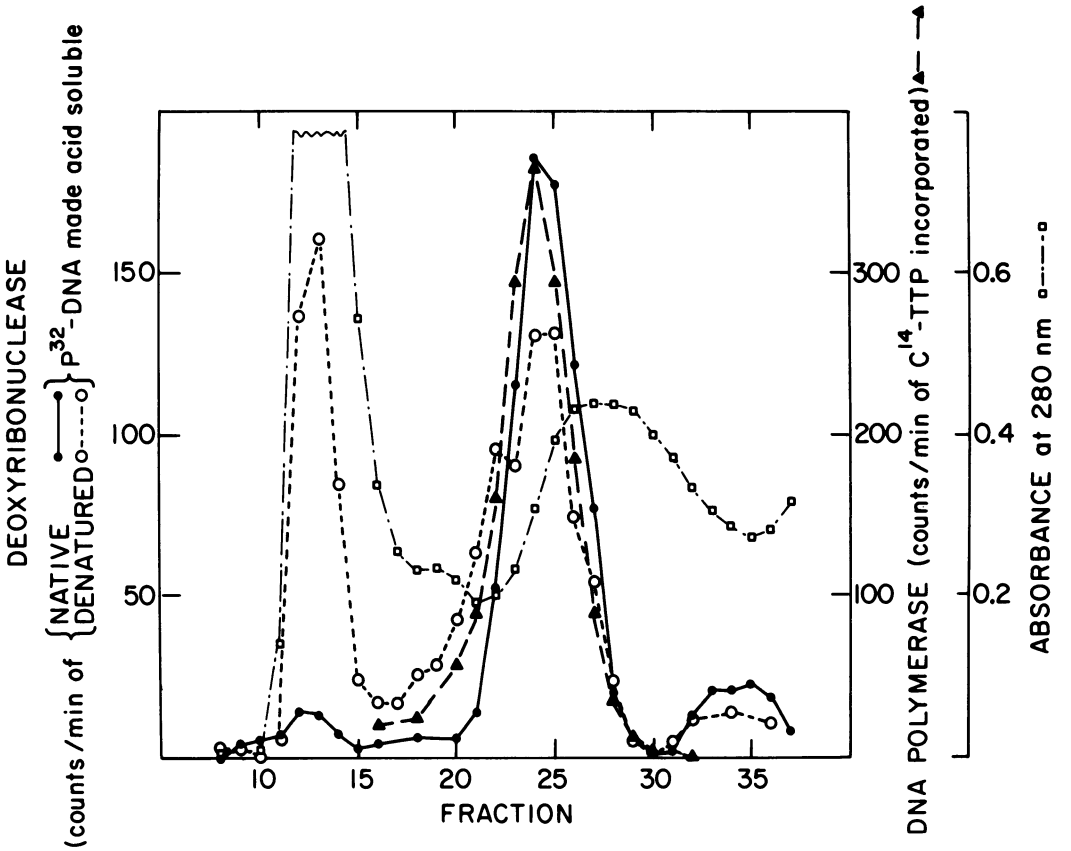

FIG. 5. *Fractionation of an extract of deoxyribonuclease-deficient (end⁻, exo⁻) pneumococci by gel filtration on Sephadex G-200. The extract was prepared by sonically disrupting cells of strain R6end-1, exo-1, treating the crude extract with pancreatic ribonuclease, and centrifuging to remove insoluble matter. A 2-ml amount of extract, containing ~40 mg of protein, was applied to a column (1.9 by 50 cm) and eluted with buffer containing 0.01 M Tris-hydrochloride (pH 7.5), 0.003 M 2-mercaptoethanol, and 0.5 M NaCl. Fractions (4 ml) were collected and assayed for deoxyribonuclease activity with both native and heat-denatured substrate and for DNA polymerase. Absorbance at 280 nm gives an indication of protein concentration in the fractions, but contaminating nucleic acids contribute to this absorbance, particularly in the early and late parts of the profile.*

activity was found associated with the DNA polymerase of *E. coli* (18).

If the exonuclease activity of the DNA polymerase is responsible for the entry of DNA and its conversion into single strands in transformation, then it may not be feasible to obtain mutants lacking the enzyme. If the DNA polymerase is essential, most such mutations would be lethal. One attempt to obtain mutants lacking residual exonuclease, by selection on agar containing DNA and methyl green with incubation for 5 days, was not successful (*see* Table 1). The inability to obtain strains lacking the activity makes it difficult to test the hypothesis that the residual exonuclease plays a role in DNA entry.

Mutations affecting other functions possibly related to transformation. (i) *Cell wall lysis.* Wild-type cells of *D. pneumoniae* rapidly lyse in the presence of 0.1% DOC. As a result of solubilization of cell walls, the turbidity of a suspension clears within a few minutes. Cell wall solubilization appears to be catalyzed by an autolytic enzyme, the action of which is triggered somehow by DOC treatment (1). Among the clones individually tested for the absence of exonuclease, one mutant was found that did not lyse in the presence of DOC.

The mutant, called cwl-1, was killed by DOC just as fast as the wild strain. The data in Fig. 6 suggest that the mutant strain fails to lyse because it lacks a cell wall lytic enzyme that is present in the wild strain. Extracts of the wild strain served as a source of the enzyme in lysing DOC-treated mutant cells. Until the cwl-1 cells were treated

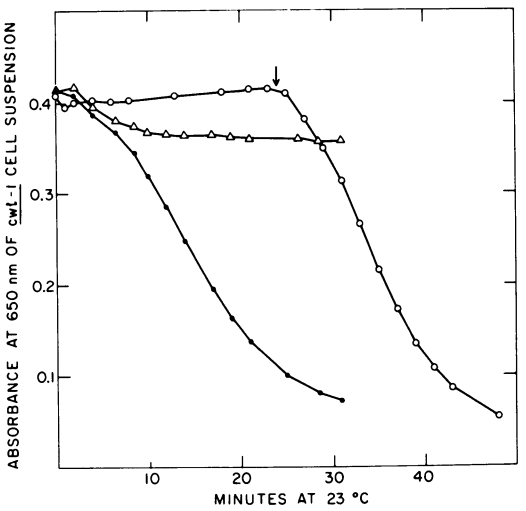

FIG. 6. *Absence of a cell wall lytic enzyme activity in strain cwl-1. The culture of cwl-1 cells was grown in medium to A_{650} = 0.4 and divided into 2-ml portions in spectrophotometer cuvettes held at 23 C. The wild-type extract was prepared by lysing a suspension of cwl+ cells in 0.05 M sodium phosphate buffer, pH 7.5, containing pancreatic ribonuclease at 20 μg per ml, 2 mM $MgCl_2$, 3 mM 2-mercaptoethanol, and 0.1% deoxycholic acid (DOC), present as the sodium salt. The crude lysate was dialyzed to remove oligonucleotides and DOC and then centrifuged to remove insoluble material. The protein content of the final extract was 4.4 mg per ml; the amount of extract added to a cuvette contained 22 μg of protein. DOC was added to a concentration of 0.1%. Symbols: ●, wild-type extract and DOC both added at zero time; △, DOC only at zero time; ○, wild-type extract at zero time and DOC at the time marked by the arrow.*

with DOC, however, the lytic enzyme did not attack their walls. A plausible view of the lytic phenomenon is that DOC, which is a mild detergent, removes some material, perhaps lipid in nature, from the cell wall, thereby exposing it to attack by the lytic enzyme.

Cells of cwl-1 behave like wild-type cells grown in the presence of ethanolamine instead of choline (23), in that they are killed but fail to lyse in the presence of DOC. Unlike ethanolamine-grown cells, however, the cwl-1 strain is normally transformable. It has been thought that partial lysis or weakening of the cell wall might be essential for initiating entry of donor DNA in transformation (11, 22, 24). This may still be true, but the behavior of cwl-1 seems to exclude a role for the autolytic enzyme in the process.

(ii) *Integration efficiency.* In typical laboratory strains of *D. pneumoniae*, different donor markers are each integrated with a characteristic efficiency (4, 10). For markers that correspond to mutations at single sites, integration efficiencies vary over a 20-fold range. Mutations that give different efficiencies occur within a single locus, apparently independent of position in the locus, but dependent rather on the nature of the base change corresponding to the mutation (10). An example of differences in integration efficiency, between a novobiocin-resistance marker and a streptomycin-resistance marker in the transformation of strain R6, is shown in Table 4. In this strain, the *nov* marker is low efficiency.

Guild and Fox (*personal communication*) found that the same *nov* marker that transforms R6 with low efficiency will transform Rx, the strain used in Guild's laboratory, with high efficiency (Table 4). Markers in the amylomaltase locus that gave low-efficiency integration in the transformation of mutants derived from R6 gave high-efficiency integration after the corresponding mutations were introduced into Rx. This confirmed a suggestion by Fox (*personal communication*) that the integration of all markers with high efficiency was characteristic of the recipient strain. Similar strains, of genotype designated *hex*, were isolated by individually testing clones of an NG-treated culture for transformation with normally high- and low-efficiency markers. *Hex* mutants appear to have lost the ability to give low-efficiency transformation, an ability that is a property of the wild-type strain. The hex^+ character was transferred back into one such mutant with a frequency sufficiently high to indicate that mutation of a single gene caused loss of the property in question.

The occurrence of strains that lack the property suggests that low integration efficiency might be due to a positive, gene-determined factor, such as an enzyme. This contradicts the hypothesis that low integration efficiency is due simply to preferential binding of certain base combinations (10). It supports the sort of hypothesis advanced by Ephrussi-Taylor (3), in which an enzyme recognizes and excises mismatched bases. The high frequency of *hex* mutants in the NG-treated population (Table 1) suggests that loss of the hex^+ mechanism may have survival value for cells

TABLE 4. *Donor marker integration efficiencies in different recipient strains*

Recipient strain	*hex* genotype	Transformants per ml		Ratio *nov/str*
		nov	*str*	
R6	hex^+	3.1×10^4	3.8×10^5	0.11
R6 end-1, exo-2	hex^+	2.0×10^4	1.5×10^5	0.13
hex-1	hex^-	3.4×10^5	3.0×10^5	1.1
Rx	hex^-	1.1×10^5	1.0×10^5	1.1

when, on account of a high mutation rate, the frequency of mismatched bases in their DNA is exceptionally high. If excision of DNA in the region of mismatched bases is involved in the *hex*$^+$ mechanism, the enzyme missing in the *hex* strains could be a deoxyribonuclease of appropriate specificity. Neither of the two major pneumococcal deoxyribonucleases, however, is essential for the mechanism (Table 4). Furthermore, since the UV resistance of *hex* strains is about normal (Fig. 3), the low efficiency mechanism does not depend on those agents of scission and excision which would function in the repair of UV damage.

(iii) *Ultraviolet sensitivity.* A UV-sensitive mutant was obtained by directly testing the survival of irradiated samples of individual clones. The mutation to sensitivity, called *uvr-1*, occurred in a strain that was also mutant with respect to the *hex* character; but a *hex*$^+$ strain was obtained by transforming this double mutant with wild-type DNA. Since the *hex*$^+$ transformant retained the *uvr-1* character, the two characters were due to independent mutations. The sensitivity of the *uvr-1* strain, as measured by the exponential decline in survival, is four times that of the wild type (Fig. 3). If the resistance of the wild strain is due to repair of potentially lethal UV damage, then at least 75% of such damage is reparable. Loss of this substantial repair mechanism, however, does not impair the transformability of the cells (*see* Table 5).

The genetic transforming activity of markers in UV-irradiated donor DNA was lost more rapidly when recipient cells were *uvr*$^-$ than when they were *uvr*$^+$ (Table 5). In *D. pneumoniae*, as in *Haemophilus influenzae* (19), survival of marker activity is inversely proportional to the square of the UV dose. When the data were appropriately plotted, high-efficiency markers appeared about four times more sensitive to UV when tested with a *uvr*$^-$ recipient. This factor corresponds to that found for cell survival. It indicates that donor DNA is repaired in the recipient cell and that about 75% of the lesions that eliminate transformants in *uvr-1* cells are repaired in *uvr*$^+$ cells. Since newly introduced DNA in *Diplococcus pneumoniae* is single-stranded (12), and therefore not susceptible to repair by a thymine dimer excision mechanism, donor DNA repair presumably occurs after integration of donor strands with host DNA. Low-efficiency markers, however, were barely more sensitive when tested in *uvr*$^-$ than when tested in *uvr*$^+$ strains (Table 5). In *uvr*$^+$ strains, however, low-efficiency markers are much more sensitive to UV, to begin with, than are high-efficiency markers.

The high UV sensitivity of markers with low integration efficiency has been found for a variety of genetic loci (4, 6, 9, 14). A most reasonable hypothesis to explain both their high sensitivity in the normal recipient and their failure to increase in sensitivity in a *uvr* recipient is that cellular repair of UV damage does not normally intervene with low-efficiency markers. The sensitivity of such markers, therefore, would be independent of the presence or absence of a repair system. This hypothesis has been advanced to explain the differential sensitivity to UV of markers in *H*

TABLE 5. *Ultraviolet inactivation of donor markers tested with different recipient strains*

Recipient strain (genotype)	Donor marker	Integration efficiency[a]	Transformants per ml		Fractional survival
			Unirradiated	Irradiated[b]	
R6 (*uvr*$^+$, *hex*$^+$)	*nov*	0.037	2.37×10^4	2.63×10^3	0.011
	str	0.63	4.02×10^5	5.73×10^4	0.14
	sul	(1.00)	6.44×10^5	1.86×10^5	0.29
uvr-1 (*uvr*$^-$, *hex*$^+$)	*nov*	0.062	1.03×10^4	0.55×10^3	0.005
	str	0.74	1.24×10^5	4.86×10^3	0.039
	sul	(1.00)	1.67×10^5	9.50×10^3	0.057
E7 (*uvr*$^+$, *hex*$^+$, *mal*$^-$)	*azu*	0.060	1.12×10^4	1.50×10^3	0.013
	mal$^+$	0.071	1.32×10^4	1.50×10^3	0.011
	str	0.38	7.13×10^4	1.52×10^4	0.21
	sul	(1.00)	1.86×10^5	5.88×10^4	0.32
uvr-1-E7 (*uvr*$^-$, *hex*$^+$, *mal*$^-$)	*mal*$^+$	0.17	1.46×10^4	2.53×10^3	0.017
	sul	(1.00)	8.80×10^4	7.25×10^3	0.082
RxE7 (*uvr*$^+$, *hex*$^-$, *mal*$^-$)	*azu*	1.30	3.15×10^4	1.13×10^4	0.36
	mal$^+$	1.15	2.79×10^4	9.05×10^3	0.32
	str^r	(1.00)	2.42×10^4	5.55×10^3	0.23

[a] Ratio to *sul* transformants with unirradiated DNA, except for RxE7, where ratio is taken with respect to *str*.

[b] Irradiated with a dose of 7.2×10^3 ergs per mm^2.

influenzae and their differential response to inhibition of repair by acriflavine (17). Results with repair-deficient mutants of *H. influenzae* (20), however, do not seem to be fully consistent with the hypothesis. In *D. pneumoniae*, the UV response of a single-site marker is clearly related to its efficiency of integration and not to its intrinsic nature. Thus, when the mechanism for producing low-efficiency integration is absent, as in a *hex* strain, markers that ordinarily are integrated with low efficiency are integrated with high efficiency, and their resistance to UV inactivation is maximal (Table 5).

Conclusions. The analysis of deficient mutants rules out a role for either of the two major pneumococcal deoxyribonucleases in the process of transformation, in the phenomenon of low integration efficiency in transformation, and in the repair of UV damage. Furthermore, defects in any one of these processes do not interfere with the other processes. A possible role in transformation for the deoxyribonuclease activity associated with the DNA polymerase or for deoxyribonucleases of yet undefined specificity (M. Kohoutova, *personal communication*) remains open. The failure so far to relate known enzymes to known functions points up the multiplicity of reactions that DNA can undergo within a living cell.

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