Goldman, Y. E., Reid, G. P., Somlyo, A. P., Somlyo, A. V., Trentham, D. R., & Walker, J. W. (1986) *J. Physiol. 377*, 100P.
Gurney, A. M., & Lester, H. A. (1987) *Physiol. Rev. 67*, 583-617.
Hirata, M., Sasaguri, T., Hamachi, T., Hashimoto, T., Kukito, M., & Koga, T. (1985) *Nature 317*, 723-725.
Irvine, R. F., Brown, K. D., & Berridge, M. J. (1984) *Biochem. J. 222*, 269-272.
Irvine, R. F., Anggard, E. E., Letcher, A. J., & Downes, C. P. (1985) *Biochem. J. 229*, 505-511.
Irvine, R. F., Letcher, A. J., Heslop, J. P., & Berridge, M. J. (1986) *Nature 320*, 631-634.
Isbrandt, L. R., & Oertel, R. P. (1980) *J. Am. Chem. Soc. 102*, 3144-3148.
Kaplan, J. H., Forbush, B., III, & Hoffman, J. F. (1978) *Biochemistry 17*, 1929-1935.
Karpen, J. W., Zimmerman, A., Stryer, L., & Baylor, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1287-1291.
Lindon, J. C., Baker, D. J., Farrant, R. D., & Williams, J. M. (1986) *Biochem. J. 233*, 275-277.
Lindon, J. C., Baker, D. J., Williams, J. M., & Irvine, R. F. (1987) *Biochem. J. 244*, 591-595.
Lowry, O. H., Rosebrough, N. J., Farr, A. C., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
McCray, J. A., & Trentham, D. R. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 239-270.
McCray, J. A., Herbette, L., Kihara, T., & Trentham, D. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7237-7241.
Nerbonne, J. M. (1986) Optical Methods in Cell Physiology (de Weer, P., & Salzberg, B. M., Eds.) *Soc. Gen. Physiol. Ser. 40*, 417-445.
Nerbonne, J. M., Richard, S., Nargeot, J., & Lester, H. A. (1984) *Nature 310*, 74-76.
Ryu, S. H., Lee, S. Y., Lee, K.-Y., & Rhee, S. G. (1987) *FASEB J. 1*, 388-393.
Shibata, T., Uzawa, J., Sugiura, Y., Hayashi, K., & Takizawa, T. (1984) *Chem. Phys. Lipids 34*, 107-113.
Somlyo, A. V., Bond, M., Somlyo, A. P., & Scarpa, A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5231-5235.
Somlyo, A. P., Walker, J. W., Goldman, Y. E., Trentham, D. R., Kobayashi, S., Kitazawa, T., & Somlyo, A. V. (1988) *Philos. Trans. R. Soc. London, B 320*, 399-414.
*Spec. Publ.-Chem. Soc.* (1964) *No. 17*, 180-190.
Tsien, R. Y., & Zucker, R. S. (1986) *Biophys. J. 50*, 843-853.
Walker, J. W., & Trentham, D. R. (1988) *Biophys. J. 53*, 596a.
Walker, J. W., McCray, J. A., & Hess, G. P. (1986) *Biochemistry 25*, 1799-1805.
Walker, J. W., Somlyo, A. V., Goldman, Y. E., Somlyo, A. P., & Trentham, D. R. (1987) *Nature 327*, 249-252.
Walker, J. W., Reid, G. P., McCray, J. A., & Trentham, D. R. (1988) *J. Am. Chem. Soc. 110*, 7170-7177.
Williamson, J. R., Joseph, S. K., Coll, K. E., Thomas, A. P., Verhoeven, A., & Prentki, M. (1986) in *New Insights into Cell and Membrane Transport Processes* (Poste, G., & Cooke, S. T., Eds.) pp 217-247, Plenum Press, New York.

# Mechanism of Action of *Escherichia coli* Exonuclease III†

Yoke W. Kow*
*Department of Microbiology and Immunology, New York Medical College, Valhalla, New York 10595*
*Received July 20, 1988; Revised Manuscript Received December 27, 1988*

ABSTRACT: Exonuclease III is the major apurinic/apyrimidinic (AP) endonuclease of *Escherichia coli*, accounting for more than 80% of the total cellular AP endonuclease activity. We have shown earlier that the endonucleolytic activity of exonuclease III is able to hydrolyze the phosphodiester bond 5′ to the urea *N*-glycoside in a duplex DNA [Kow, Y. W., & Wallace, S. S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8354-8358]. Therefore, we were interested in studying the mechanism of action of the endonucleolytic activity of exonuclease III by preparing DNA containing different base lesions as well as chemically modified AP sites. When AP sites were converted to *O*-alkylhydroxylamine residues, exonuclease III was able to hydrolyze the phosphodiester bond 5′ to *O*-alkylhydroxylamine residues. The apparent $K_m$ for different *O*-alkylhydroxylamine residues was not affected by the particular *O*-alkylhydroxylamine residue substituted; however, the apparent $V_{max}$ decreased as the size of the residue increased. On the basis of a study of the substrate specificity of exonuclease III, a modification of the Weiss model for the mechanism of action of exonuclease III is presented. Furthermore, a temperature study of exonucleolytic activity of exonuclease III in the presence of $Mg^{2+}$ showed discontinuity in the Arrhenius plot. However, no discontinuity was observed when the reaction was performed in the presence of $Ca^{2+}$. Similarly, no discontinuity was observed for the endonucleolytic activity of exonuclease III, in the presence of either $Ca^{2+}$ or $Mg^{2+}$. These data suggest that, in the presence of $Mg^{2+}$, exonuclease III exists in two temperature-dependent conformations. The transition temperature for the conformation change occurred at 25 °C, and in the presence of $Ca^{2+}$, only one rate-limiting step was observed.

Apurinic/apyrimidinic (AP) sites are common intermediates in the repair of most DNA base damages (Loeb & Preston, 1986), and they are readily generated by a number of chemical and physical agents (Loeb & Preston, 1986; Teoule, 1987). Due to the instability of the N-glycosylic bond, depurination can occur at a significant rate under physiological conditions (Lindahl & Nyberg, 1972; Loeb & Preston, 1986). AP sites have been shown to be in vitro blocks to DNA synthesis

† This work was supported by National Institutes of Health Grant GM 37216.
* Address correspondence to the author at the Department of Microbiology, University of Vermont, Burlington, VT 05405.

0006-2960/89/0428-3280$01.50/0 

(Kunkel et al., 1983; Loeb & Preston, 1986) as well as mutagenic lesions (Sagher & Strauss, 1983; Loeb & Preston, 1986). They are recognized by two classes of AP endonucleases (Mosbaugh & Linn, 1980; Grossman & Grafstrom, 1982). 3′ AP endonucleases, such as endonuclease III, hydrolyze the phosphodiester bond 3′ to the AP site (class I) leaving an $\alpha,\beta$-unsaturated compound (Bailey & Verly, 1987; Kow & Wallace, 1987; Kim & Linn, 1988) at the 3′ terminus. The 3′ terminus left after the action of a class I AP endonuclease provides a poor primer binding site for DNA polymerases (Huberman & Kornberg, 1970; Mosbaugh & Linn, 1980) and thus a poor substrate for DNA repair reactions. 5′ AP endonucleases hydrolyze the phosphodiester bond 5′ to the AP sites (class II) and will remove both the baseless sugar and the $\alpha,\beta$-unsaturated compound from the 3′ terminus created by a 3′ AP endonuclease (Demple et al., 1986).

In *Escherichia coli*, exonuclease III accounts for over 80% of the cellular AP endonuclease activity (Weiss, 1981). In addition to being a class II AP endonuclease, exonuclease III exhibits other nuclease activities including a 3′–5′ exonuclease activity (Richardson et al., 1964; Weiss, 1981), a 3′ phosphomonoesterase activity (Richardson & Kornberg, 1964; Weiss, 1981), a 3′ phosphodiesterase activity (Weiss, 1981; Henner et al., 1983; Demple et al., 1986), and a urea endonuclease activity (Kow & Wallace, 1985). The 3′–5′ exonuclease activity releases deoxyribose 5′-monophosphates from the 3′ terminus of duplex DNA. The activity is also capable of hydrolyzing the RNA strand from a DNA–RNA hybrid (thus acting like an RNase H) in a 3′–5′ direction (Weiss, 1981). The 3′ phosphomonoesterase activity accounts for over 90% of the total cellular phosphatase activity in *E. coli* (Richardson & Kornberg, 1964). The 3′ phosphodiesterase activity has been shown to be important in repairing the 3′ terminus resulting from oxidative cleavage of the deoxyribose moiety by bleomycin (Niwa & Moses, 1981; Henner et al., 1983) and hydrogen peroxide (Demple et al., 1986) and is capable of removing 3′-phosphoglycolate (Niwa & Moses, 1981; Henner et al., 1983) and 3′-phosphoglycoaldehyde residues (Demple et al., 1986). Recently, we have shown that exonuclease III exhibits a urea endonuclease activity, hydrolyzing the phosphodiester bond 5′ to urea residues in DNA (Kow & Wallace, 1985).

Apart from its known function as a repair nuclease, exonuclease III has been implicated in other biological functions which may or may not be directly related to its role in DNA repair. *xth* mutants of *E. coli* are unable to express a heat shock response upon abrupt temperature shift (Paek & Walker, 1986). Plumbagin- or superoxide-induced mutagenesis requires the active participation of wild-type exonuclease III (Farr et al., 1985, 1986), and *xth* mutants are extremely sensitive to killing by hydrogen peroxide (Demple et al., 1983) and long-wave UV irradiation (Sammartano & Tuveson, 1983) compared to wild type. Furthermore, exonuclease III is also important in the maintenance of certain plasmids (Bassett & Kushner, 1984).

Despite the multiple biochemical and biological activities exhibited by exonuclease III, the enzyme is a small monomeric protein having a molecular weight of 28 000 (Weiss, 1976). Exonuclease III has not been shown to possess any prosthetic group; however, all these apparently different activities require $Mg^{2+}$ (Weiss, 1981). Furthermore, there is no evidence for the presence of more than one active site in exonuclease III (Weiss, 1981). In order to explain these multiple, apparently unrelated activities of exonuclease III, Weiss (1981) proposed that the active site recognizes a space between the two DNA strands (intra-DNA space) generated by either the loss of a base (the AP endonuclease activity), the presence of a small fragmented base (the urea endonuclease activity), or simply the 3′ breathing end (the 3′ phosphomonoesterase, the 3′ phosphodiesterase, and the 3′–5′ exonuclease and RNase H activities).

Recently, *O*-methylhydroxylamine has been used for the study of the mechanism of action of some AP endonucleases (Talpaert-Borlé, 1987; Talpaert-Borlé & Liuzzi, 1983). It was found that *O*-methylhydroxylamine reacts quantitatively and rapidly with deoxyribose to give an imine adduct (Talpaert-Borlé & Liuzzi, 1983). The resultant *O*-methylhydroxylamine residue is refractory to incision by AP endonucleases from calf thymus and rat liver (Liuzzi & Talpaert-Borlé, 1985) and by pyrimidine dimer glycosylases from T4 and *Micrococcus luteus* (Liuzzi et al., 1987).

Since exonuclease III can hydrolyze the phosphodiester bond 5′ to urea residues in DNA (Kow & Wallace, 1985), we decided to examine the 5′-endonucleolytic activity of exonuclease III by studying its substrate specificity. In this paper, we show that, in contrast to mammalian AP endonucleases (Liuzzi & Talpaert-Borlé, 1985) and pyrimidine dimer glycosylases from T4 and *M. luteus* (Liuzzi et al., 1987), DNAs containing *O*-alkylhydroxylamine residues are substrates for the endonucleolytic activity of exonuclease III. Furthermore, studies on the temperature dependence of exonuclease III activities suggest that exonuclease III exists in two temperature-dependent conformations. Finally, we present an extended model to explain the mechanism of action of exonuclease III.

## Materials and Methods

*Bacteria and Bacteriophages.* PM2 phage and host *Alteromonas espejiana* were laboratory strains.

*Materials.* Poly(ethylene glycol) (PEG) 6000, dihydrothymidine, and tris(hydroxymethyl)aminomethane were obtained from Sigma Chemical Co.; agarose, ultrapure grade, was from Bio-Rad Laboratories; $^3$H-labeled thymidine triphosphate (66 Ci/mmol) was from ICN Radiochemicals.

*Enzymes.* Homogeneous preparations of exonuclease III were purchased from Pharmacia Fine Chemicals. The purity of each preparation was checked by chromatography through Mono Q (Pharmacia), where a single protein peak was observed.

*Nucleic Acids.* Poly(dA·dT) and poly(dC·dG) were obtained from Pharmacia Fine Chemicals; PM2 DNA was prepared as previously described (Kow & Wallace, 1985, 1987). $^3$H-Labeled PM2 DNA was prepared by nick translation (Maniatis et al., 1982) with $^3$H-labeled thymidine triphosphate by use of nicked PM2 as a template. The specific activity of $^3$H-labeled PM2 was $4.9 \times 10^6$ cpm/$\mu$g of DNA. Similarly, $^3$H-labeled poly(dA·[$^3$H]dT) was prepared by nick translation using $^3$H-labeled thymidine triphosphate with poly(dA·dT) as template. The specific activity of poly(dA·[$^3$H]dT) was $1 \times 10^6$ cpm/$\mu$g of DNA.

*DNA Substrates.* PM2 DNA containing AP sites was prepared by heat/acid depurination according to Lindahl and Nyberg (1972) to yield approximately two AP sites per DNA molecule. The average number of apurinic sites was estimated by alkali fluorometry (Futcher & Morgan, 1979; Kowalski, 1979). PM2 DNA containing reduced AP sites was prepared by sodium cyanoborohydride reduction of AP DNA (Jorgenson et al., 1987). *O*-Alkylhydroxylamine residues were introduced into PM2 DNA by treating AP PM2 DNA (in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with 10 mM each of *O*-methyl-, *O*-ethyl-, *O*-allyl-, *O*-benzyl-, and *O*-(4-nitrobenzyl)hydroxylamine at room temperature for 1 h to produce

the respective residues at the apurinic sites (Talpaert-Borlé & Liuzzi, 1983; Kow et al., 1987). Under the conditions used, the modification was specific for apurinic sites (Talpaert-Borlé & Liuzzi, 1983; Kow et al., 1987). Excess *O*-alkylhydroxylamine was removed by extensive dialysis. PM2 DNA containing thymine glycol residues was prepared by $OsO_4$ oxidation (Kow & Wallace, 1985, 1987). PM2 DNA containing urea residues was prepared by alkali hydrolysis of thymine glycol containing PM2 DNA as described previously (Kow & Wallace, 1985, 1987). PM2 DNA containing uracil was prepared by bisulfite treatment according to Lindahl et al. (1977). PM2 DNA containing thymine dimers was prepared by UV irradiating PM2 DNA with 500 J $m^{-2}$ at 254 nm (General Electric G15T8 germicidal lamp). DNA containing formamidopyrimidine residues was prepared by alkali hydrolysis of alkylated PM2 DNA (Chetsanga & Lindahl, 1979). PM2 DNA containing dihydrothymine was prepared by in vivo incorporation of dihydrothymidine into PM2 DNA (Ide et al., 1987). The number of dihydrothymine residues per DNA molecule was estimated by determining the number of endonuclease III sensitive sites (Demple & Linn, 1980; Ide et al., 1987). β-Ureidoisobutylic acid containing PM2 DNA was prepared by alkali hydrolysis of dihydrothymine-containing PM2 DNA, under the same conditions for the hydrolysis of thymine glycol to urea residues (Kow & Wallace, 1985). The number of β-ureidoisobutylic acid residues was estimated by determining the number of endonuclease VIII sensitive sites (Melamede et al., 1987; Kow, Melamede, Ide, and Wallace, unpublished results). The presence of β-ureidoisobutylic acid residues in PM2 DNA was indicated by the change of the rate of reaction of endonuclease VIII on the substrate DNA (Kow, Melamede, Ide, and Wallace, unpublished results). Furthermore, the conversion of dihydrothymine to β-ureidoisobutylic acid residues at pH 12.0 was completed within 4 h at room temperature (Kondo & Witkop, 1968), and only a single product was observed by HPLC analysis (Ide, unpublished data). The loss of dihydrothymine was confirmed by the loss of an anti-dihydrothymine-specific antibody signal (Hubbard et al., 1989).

*Assay for Endonucleolytic Activity of Exonuclease III.* The endonucleolytic activity of exonuclease III was assayed by alkali fluorometry as previously described (Kow & Wallace, 1985, 1987). Endonucleolytic activity on *O*-alkylhydroxylamine-modified AP PM2 DNA was assayed as previously described for urea-containing PM2 DNA (Kow & Wallace, 1985). The reaction was performed at 37 °C in a standard reaction mixture (100 μL) containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, and 500 ng of substrate DNA. The reaction was started by the addition of 10 μL of an appropriately diluted exonuclease III preparation. AP endonuclease activity was estimated with the same reaction mixture, except that AP PM2 DNA was used as the substrate. The rate of reaction was estimated by agarose gel electrophoresis (Kow & Wallace, 1987). $Ca^{2+}$-dependent endonucleolytic activity on DNA containing urea and *O*-alkylhydroxylamine residues was estimated as described above except that $MgCl_2$ was replaced by $CaCl_2$. One unit of exonuclease III is defined as the amount of enzyme required to produce 1 nmol of nicks per PM2 DNA per minute.

*Assay for Exonucleolytic Activity of Exonuclease III.* The 3′–5′ exonucleolytic activity of exonuclease III was assayed under the standard reaction conditions with nick-translated $^3$H-labeled PM2 DNA or poly(dA·[$^3$H]dT) as the substrate. At appropriate times, a 10-μL aliquot of the reaction mix was taken, and 50 μL of calf thymus DNA (500 μg/mL) was added, followed by the addition of 60 μL of 10% cold trichloroacetic acid. The solution was left at 4 °C for an additional 30 min, and the precipitated DNA was removed by centrifugation in a microfuge. The acid-soluble $^3$H count was then measured by scintillation spectrometry. $Ca^{2+}$-dependent exonucleolytic activity was assayed as above except $Mg^{2+}$ was replaced by $Ca^{2+}$.

Table I: Substrate Specificity of the Endonucleolytic Activity of Exonuclease III

| *N*-glycoside in PM2 DNA | endonucleolytic activity (nicks/DNA molecule)[a] |
|---|---|
| apurinic site | 1.62 |
| reduced apurinic site | 0.84 |
| urea *N*-glycoside | 1.42 |
| *O*-methylhydroxylamine *N*-glycoside | 0.92 |
| *O*-ethylhydroxylamine *N*-glycoside | 0.75 |
| *O*-allylhydroxylamine *N*-glycoside | 0.62 |
| *O*-benzylhydroxylamine *N*-glycoside | 0.15 |
| *O*-(4-nitrobenzyl)hydroxylamine *N*-glycoside | 0.10 |
| thymine glycol *N*-glycoside | ≪0.01 |
| dihydrothymine *N*-glycoside | ≪0.01 |
| thymine dimer *N*-glycoside | ≪0.01 |
| β-ureidoisobutylic acid *N*-glycoside | ≪0.01 |
| formamidopyrimidine *N*-glycoside | ≪0.01 |

[a] The endonucleolytic actvity of exonuclease III was measured in a standard reaction mix with 1 μg of PM2 DNA containing different *N*-glycosides. The endonucleolytic activity was determined by alkali fluorometry except for DNA containing apurinic sites. In this case, the activity was determined by agarose gel electrophoresis.

*Estimation of Apparent $K_m$ and $V_{max}$ of Exonuclease III.* Apparent kinetic parameters of exonuclease III were determined by using varying DNA substrate concentrations. Appropriate time points were taken so that an approximation of the initial velocity at different DNA substrate concentrations could be estimated. Data obtained were then computer fitted by using least-squares linear regression analysis of the Lineweaver–Burk equation.

## Results

*Substrate Specificity of Exonuclease III.* Table I shows the endonucleolytic activity of exonuclease III on PM2 DNA containing a number of different residues. Only apurinic sites, reduced apurinic sites, urea, and *O*-alkylhydroxylamine residues were recognized. Others, like thymine glycol, dihydrothymine, β-ureidoisobutylic acid, formamidopyrimidine, uracil, and pyrimidine dimers, were not recognized as substrates. Figure 1 shows that the structure of these residues falls into two groups. Those that were substrates for exonuclease III have a secondary amine at the N-glycosylic bond; those lesions that were not recognized by exonuclease III have a tertiary amine at the N-glycosylic bond (with the exception of formamidopyrimidine residues).

*Apparent Kinetic Parameters of Endonucleolytic Activity of Exonuclease III on PM2 DNA Containing O-Alkylhydroxylamine Residues.* Figure 2 shows that the rate of reaction of exonuclease III with DNA containing *O*-alkylhydroxylamine residues was affected by the size of the residue, the larger the size of the residue the slower the rate of nicking at the residue. The decrease in the rate of reaction was due to a decrease in the apparent $V_{max}$ of exonuclease III on these substrates. As the size of the *O*-alkylhydroxylamine residues increased, the apparent $V_{max}$ of exonuclease III decreased. However, the apparent $K_m$ appeared to remain constant.

*Effect of Calcium on Endonucleolytic Activity of Exonuclease III.* Exonuclease III required $Mg^{2+}$ for all the endonucleolytic activities observed. Monovalent cations such as

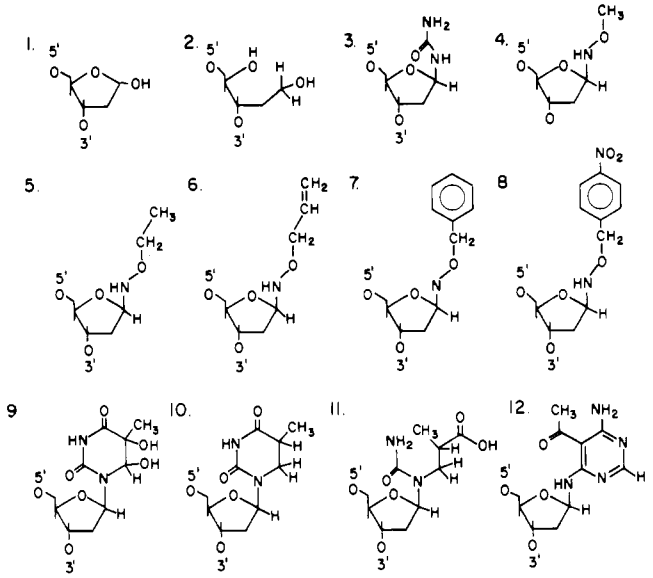

FIGURE 1: Chemical structures of DNA base lesions: (1) apurinic site; (2) reduced apurinic site; (3) urea *N*-glycoside; (4) *O*-methylhydroxylamine *N*-glycoside; (5) *O*-ethylhydroxylamine *N*-glycoside; (6) *O*-allylhydroxylamine *N*-glycoside; (7) *O*-benzylhydroxylamine *N*-glycoside; (8) *O*-(4-nitrobenzyl)hydroxylamine *N*-glycoside; (9) thymine glycol *N*-glycoside; (10) dihydrothymine *N*-glycoside; (11) β-ureidoisobutylic acid *N*-glycoside; (12) formamidopyrimidine *N*-glycoside.

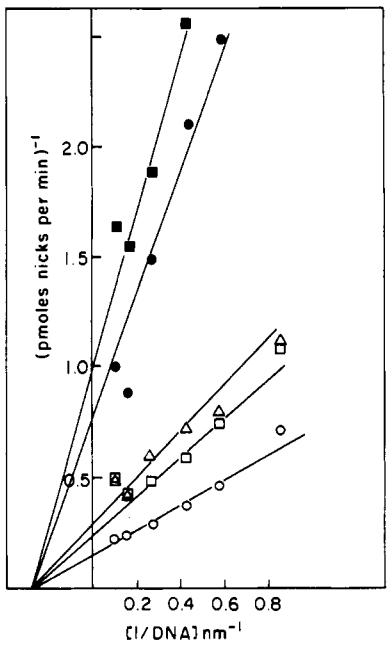


FIGURE 2: Lineweaver–Burk plot of the initial velocities of the endonucleolytic activity of exonuclease III. PM2 DNAs containing *O*-methylhydroxylamine (○), *O*-ethylhydroxylamine (□), *O*-allylhydroxylamine (△), *O*-benzylhydroxylamine (●), and *O*-(4-nitrobenzyl)hydroxylamine (■) residues were used as substrates. The reactions were performed in a standard reaction mixture (Materials and Methods) in the presence of $Mg^{2+}$ with varying DNA substrate concentrations as indicated. The reaction volume was 100 μL; the enzyme concentration used was 0.37 nM.

$K^+$ and $Na^+$ could not replace $Mg^{2+}$ at different pHs, or different ionic strengths (data not shown). However, $Ca^{2+}$ could replace $Mg^{2+}$ when AP DNA was used as a substrate; in this case the rate of reaction was only slightly diminished (Figure 3). The optimum concentration for $Mg^{2+}$ and $Ca^{2+}$ remained about the same, and the decrease in the reaction rate was due to a lower apparent $V_{max}$ on AP DNA when $Mg^{2+}$ was replaced by $Ca^{2+}$ (data not shown).

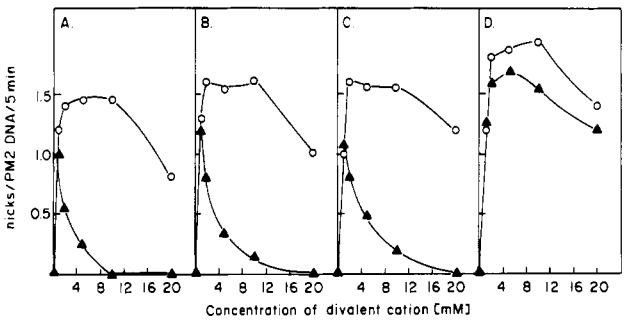


FIGURE 3: Effect of divalent cations on the endonucleolytic activity of exonuclease III. The endonucleolytic activity of exonuclease III as a function of $Mg^{2+}$ (○) or $Ca^{2+}$ (▲) concentration was assayed with PM2 DNA containing *O*-benzylhydroxylamine (panel A), *O*-methylhydroxylamine (panel B), urea (panel C), or apurinic sites (panel D) as a substrate. For panels A–C, the activity was assayed by alkali fluorometry, and for panel D, the activity was assayed by agarose gel electrophoresis. The DNA concentration used was 1 μg/100-μL reaction. The amount of exonuclease III in each panel was as follows: (A) 3.7 (○) and 17.5 nM (▲); (B) 0.37 (○) and 1.75 nM (▲); (C) 0.02 (○) and 0.1 nM (▲); (D) 0.02 (○) and 0.02 nM (▲).

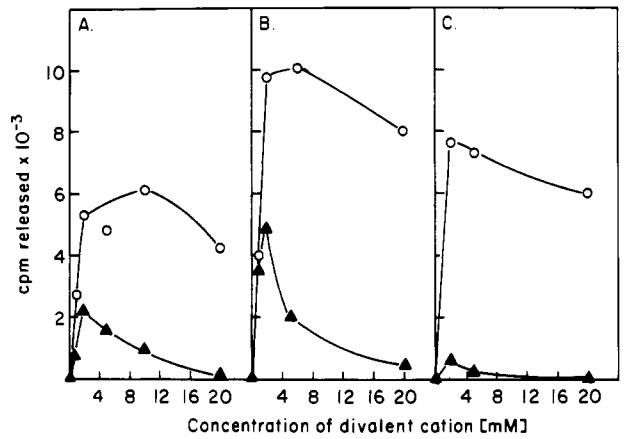


FIGURE 4: Effect of divalent cations on the exonucleolytic activity of exonuclease III. The exonucleolytic activity of exonuclease III as a function of $Mg^{2+}$ (○) or $Ca^{2+}$ (▲) concentration was assayed with $^3$H-labeled nicked PM2 DNA (panel A), poly(dG·[$^3$H]dC) (panel B), or poly(dA·[$^3$H]dT) (panel C) as a substrate. The amount of DNA used per reaction was (A) 80, (B) 80, and (C) 44 ng. For panels A and B, the rate of release of acid-soluble counts was normalized for a 10- (○) and 100-min (▲) reaction time. For panel C, the rate of release of acid-soluble counts was normalized for a 5- (○) and 50-min (▲) reaction time.

When *O*-alkylhydroxylamine-containing DNA was used as a substrate, the endonucleolytic activity of exonuclease III showed a sharp optimum $Ca^{2+}$ requirement at 1 mM as compared to a broad optimum $Mg^{2+}$ requirement. Furthermore, the rate of reaction at the optimum $Ca^{2+}$ concentration was 2-fold lower than the rate of reaction at the optimum $Mg^{2+}$ concentration. As was observed for its endonucleolytic activity on AP DNA, the slower rate of reaction of exonuclease III in the presence of $Ca^{2+}$ was due to a reduction of the apparent $V_{max}$ of exonuclease III on DNA containing *O*-methylhydroxylamine residues (data not shown). At 10 mM $Ca^{2+}$, the endonucleolytic activity of exonuclease III on DNA containing *O*-alkylhydroxylamine was inhibited more than 90%; however, relatively little inhibition was observed for the endonucleolytic activity of exonuclease III when the reaction was performed in 10 mM $Mg^{2+}$ (Figure 3). Similar results were obtained when the endonucleolytic activity of exonuclease III was measured with urea-containing DNA (Figure 3).

*Effect of Calcium on 3′–5′ Exonucleolytic Activity of Exonuclease III.* As was observed for the endonucleolytic activity

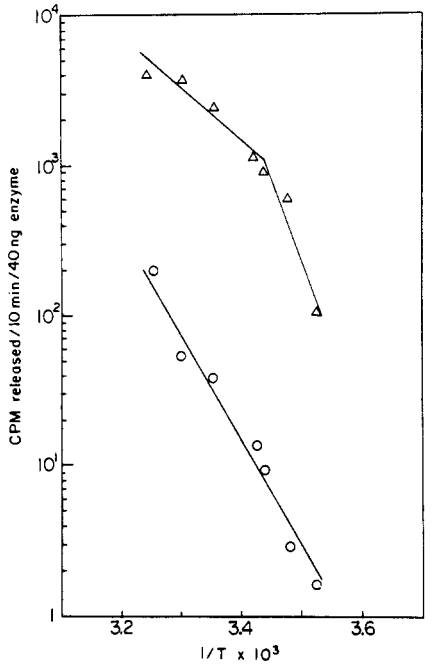


FIGURE 5: Exonucleolytic activity of exonuclease III as a function of temperature. Reactions were performed as decribed in Figure 4 at 2 mM $Mg^{2+}$ (△) or $Ca^{2+}$ (○). The temperature was varied as indicated. The DNA substrate used was poly(dA·[$^3$H]dT).

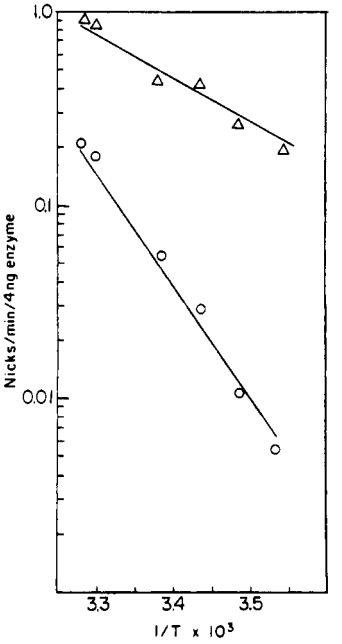


FIGURE 6: Endonucleolytic activity of exonuclease III as a function of temperature. The reaction was performed as described in Figure 3 at 1 mM $Mg^{2+}$ (△) or $Ca^{2+}$ (○). The DNA substrate used was PM2 DNA containing *O*-methylhydroxylamine residues.

of exonuclease III, its exonucleolytic activity showed a broad $Mg^{2+}$ optimum and a sharp $Ca^{2+}$ optimum (Figure 4). When poly(dA·[$^3$H]dT) was used as a substrate, the $Ca^{2+}$ optimum for the exonucleolytic activity was about 1 mM. The rate of exonucleolytic activity at optimal $Ca^{2+}$ concentration (1 mM) was about $^1/_{10}$ the rate of exonucleolytic activity at optimal $Mg^{2+}$ concentration (between 2 and 10 mM). However, when poly(dG·[$^3$H]dC) and $^3$H-labeled PM2 DNA were used as substrates, the rate of exonucleolytic activity at optimal $Ca^{2+}$ concentration was 20- and 200-fold slower, respectively, than the rate of exonucleolytic activity at optimal $Mg^{2+}$ concentration, respectively. Higher concentrations of $Ca^{2+}$ severely inhibited exonucleolytic activity with all DNA substrates tested.

*Effect of Temperature on Exonucleolytic Activity of Exonuclease III.* In the presence of $Mg^{2+}$, the Arrhenius plot of exonucleolytic activity of exonuclease III showed a biphasic behavior; however, in the presence of $Ca^{2+}$, no discontinuity was observed (Figure 5). Interestingly, the apparent activation energy for $Ca^{2+}$-dependent exonucleolytic activity was similar to that of $Mg^{2+}$-dependent exonucleolytic activity at temperatures below 25 °C. This suggests that there may be two rate-limiting steps for the $Mg^{2+}$-dependent exonucleolytic activity, with only the rate-limiting step having the higher activation energy being observed for the $Ca^{2+}$-dependent activity.

*Effect of Temperature on Endonucleolytic Activity of Exonuclease III.* Figure 6 shows the effect of temperature on the endonucleolytic activity of exonuclease III with *O*-methylhydroxylamine-containing PM2 DNA as a substrate. Exonuclease III showed a different temperature profile depending on the nature of divalent cation used. Figure 6 shows that the Arrhenius plot of log rate versus $1/T$ is linear for both $Ca^{2+}$- and $Mg^{2+}$-dependent nicking of the substrate DNA in the temperature range between 10 and 40 °C. However, the slope, which represents the activation energy of the overall reaction, is different. At optimal $Ca^{2+}$ concentration, the activation energy for the endonucleolytic activity was 2-fold higher than the activation energy for the endonucleolytic activity at optimal $Mg^{2+}$ concentration.

## Discussion

Exonuclease III exhibits two major classes of nuclease activities, namely, endonucleolytic and exonucleolytic activity. Depending on the DNA substrates used, one can study the endonucleolytic activity independently of the exonucleolytic activity or vice versa. We have shown that the endonucleolytic activity of exonuclease III was able to recognize a series of *O*-alkylhydroxylamine *N*-glycosides in duplex DNA (Table I and Figure 2). Furthermore, by using $^3$H-labeled, nicked PM2 DNA, poly(dA·[$^3$H]dT), or poly(dG·[$^3$H]dC), we were able to study the exonucleolytic activity of exonuclease III independently of the endonucleolytic activity. On the basis of the studies of substrate specificity and the effects of cations and temperature on both the endonucleolytic and exonucleolytic activities of exonuclease III, we proposed a model to explain the multiple activities of this enzyme.

*Mechanism of Action of Exonuclease III: A Modified Weiss Model.* Despite the fact that many of the biological and biochemical properties of exonuclease III are known, its mechanism of action is not fully understood (Weiss, 1981). The ingenious model put forward by Weiss to explain the different enzymatic activities of exonuclease III fails, however, to explain the following observations: Exonuclease activity is blocked at protruding 3′ termini when the terminus is longer than four nucleotides (Henikoff, 1984). Exonuclease III only releases mononucleotides from the 3′ terminus of duplex DNA, and no di- or trinucleotides have ever been observed (Weiss, 1981). Exonuclease III is unable to excise pyrimidine dimers from the 3′ end of duplex DNA (Royer-Pokora et al., 1981), nor is it able to incise at mismatches and hairpins (Weiss, 1981).

In this study we have shown that the DNA substrates recognized by the endonucleolytic activity of exonuclease III, such as urea and *O*-alkylhydroxylamine residues, have a secondary amine at the $N_1$–$C_{1'}$ glycosylic linkage. These lesions also have disrupted base-pairing ability. In contrast, DNA substrates containing base damages such as thymine glycol

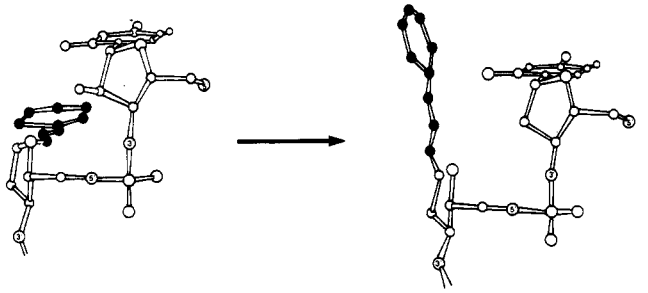

FIGURE 7: Schematic illustration to show the generation of an inter-DNA strand space during the endonucleolytic interaction of exonuclease III with a modified DNA base. The lesion depicted in the scheme is *O*-benzylhydroxylamine *N*-glycoside. It is assumed that the secondary amine at the N-glycosylic bond facilitates the enzyme-assisted ring opening of the deoxyribose moiety at the site of attachment of the lesion. As ring opening progresses, the $N_1$–$C_{1'}$ glycosylic bond becomes a double bond, the imine bond. During this process, the *O*-benzylhydroxylamine residue rotates from a $\beta$-conformation to a conformation which is coplanar with the deoxyribose moiety. An inter-DNA strand space is thus generated in the microenvironment 5′ to the lesion. Enzyme-catalyzed hydrolysis of the phosphodiester bond 5′ to the lesion can then occur.

or $\beta$-ureidoisobutylic acid residues were not incised by exonuclease III. These lesions have a tertiary amine at the $N_1$–$C_{1'}$ glycosylic linkage. Therefore, it appears that there are at least two factors that determine the substrate susceptibility of each potential lesion, the presence of a secondary amine at the $N_1$–$C_{1'}$ glycosylic linkage and the absence of base pairing. An exception to this rule was the formamidopyrimidine residue, which has the N-glycosylic linkage present as a secondary amine but was not incised by exonuclease III. However, the inability of exonuclease III to nick at DNA containing formamidopyrimidine may be due to the possible retention of its base-pairing ability.

The presence of a secondary amine at the N-glycosylic linkage might facilitate the ring opening of the deoxyribose moiety at the site of attachment of the damaged base. Upon ring opening of the sugar, the damaged base would shift from the $\beta$-conformation to one which is coplanar with the deoxyribose moiety (Figure 7). This change in the stereochemistry of the lesion would result in a substantial increase in the inter-DNA strand space (the space around the phosphodiester linkage 5′ to the lesion where cleavage occurs), thus providing the space necessary for exonuclease III to hydrolyze the 5′ phosphodiester bond as originally proposed by Weiss (1981). As long as the lesion of interest is able to undergo such a ring-opening process, the increase in space in the microenvironment 5′ to the damage should be independent of the nature of the damage. This model is consistent with the fact that DNA containing *O*-alkylhydroxylamine residues was a substrate for exonuclease III. However, if a hydrogen is absent from the N-glycosylic linkage of the lesion (such as thymine glycol), ring opening of the deoxyribose moiety would not be favored, since the process would result in the formation of an unstable iminium intermediate. This is consistent with the observations that DNA containing lesions such as thymine glycols, pyrimidine dimers, dihydrothymine, or $\beta$-ureidoisobutylic acid was not a substrate for exonuclease III.

Despite the fact that exonuclease III exhibits multiple enzymatic activities (Weiss, 1981), the molecular weight of the enzyme is relatively small (Weiss, 1976). Furthermore, Yajko and Weiss (1975) have shown that point mutations in the *xth* structural gene led to a concomitant loss of both the endonucleolytic and exonucleolytic activities, suggesting that both the endonucleolytic and exonucleolytic activities may be sharing the same active site. Therefore, it has been assumed

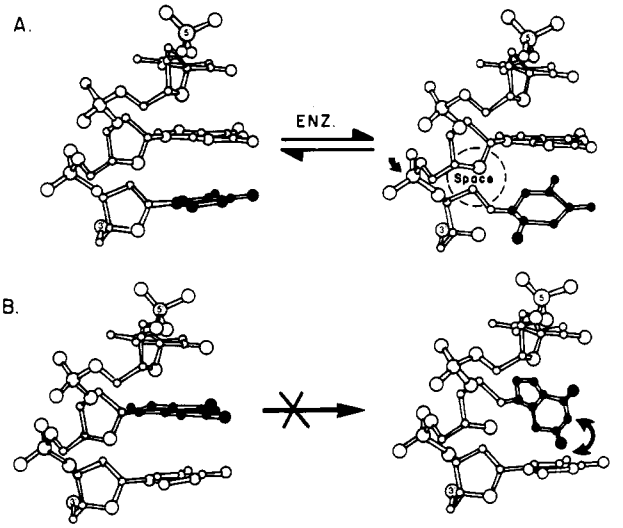


FIGURE 8: Schematic illustration to show the steric hindrance exerted by the nucleotide 3′ to the lesion. Panel A shows that the exonucleolytic activity of exonuclease III will catalyze the ring opening of the deoxyribose ring of the terminal 3′-nucleotide. Upon ring opening, the iminium intermediate is assumed to be stabilized by the enzyme. The space 5′ to the terminal nucleotide would then allow exonuclease III to catalyze the hydrolysis of the phosphodiester bond 5′ to the terminal nucleotide (arrow). The process is then repeated to exhibit the observed exonucleolytic reaction. Panel B shows that the enzyme is unable to assist the ring opening of the deoxyribose moiety at an internal lesion or nucleotide. Since ring opening of deoxyribose moiety requires the base or lesion to become coplanar with the deoxyribose, the base 3′ to the rotation would exert tremendous steric hindrance (double-headed arrow), thus preventing the reaction from occurring.

that the exonucleolytic and endonucleolytic activities work by the same mechanism. Weiss has suggested that the kinetic breathing of the nucleotide at the 3′ terminus of duplex DNA would provide the required space for exonucleolytic action (Weiss, 1981). Since the N-glycosylic linkage of the nucleotide at the 3′ terminus is present as a tertiary amine, the above model predicts that the active site of exonuclease III should be able to stabilize the iminium intermediate upon ring opening of the deoxyribose moiety, thus generating inter-DNA strand space 5′ to the lesion. Thus, exonuclease III should be able to remove pyrimidine dimers from the 3′ terminus of duplex DNA and should also be able to release oligonucleotides larger than mononucleotides. However, such reactions are never observed. Furthermore, exonuclease III cannot incise at mismatches, hairpins, or base damages such as thymine glycols. Therefore, additional factors must be operating to account for exonuclease III action, and the following is consistent with the data. As ring opening of the deoxyribose occurs, the base 3′ to the cleavage site would exert steric hindrance and prevent the enzyme-assisted stabilization of the iminium intermediate (Figure 8). Thus, only the release of the terminal 3′-nucleotide of duplex DNA would occur, for it is only in this position that the ring-opening process would encounter minimum steric hindrance. This would explain why base damages such as pyrimidine dimers would not be released by exonuclease III since the steric hindrance from the nucleotide 3′ to the lesion would prevent the ring-opening process. That steric hindrance may be a factor in the exonuclease III reaction is also supported by the observation that, as the size of the *O*-alkylhydroxylamine residue increased, the apparent $V_{max}$ for the endonucleolytic reaction decreased (Figure 2).

*Conformation of Exonuclease III Is both Temperature and Cation Dependent.* It has been previously shown (Weiss, 1981) that all the enzymatic activities of exonuclease III require $Mg^{2+}$. When AP DNA was used as a substrate, the reactivity

of exonuclease III showed a broad optimum for both the $Ca^{2+}$ and $Mg^{2+}$ requirement (Figure 3D). Furthermore, $Ca^{2+}$ replaced $Mg^{2+}$ with equal efficiency (Figure 3D). However, for other substrates, such as DNA containing urea or *O*-alkylhydroxylamine residues, the enzyme showed a sharp optimum concentration of $Ca^{2+}$ around 1 mM. In these cases, the replacement of $Mg^{2+}$ by $Ca^{2+}$ in the endonucleolytic reaction resulted in a decrease in the $V_{max}$ (Figure 3A,B). In contrast to earlier observations (Weiss, 1981), the exonucleolytic activity of exonuclease III exhibited an optimum $Ca^{2+}$ concentration at 1 mM. Depending on the nature of DNA substrate used, the 3′–5′ exonucleolytic activity was affected much more by the replacement of $Mg^{2+}$ with $Ca^{2+}$ than the endonucleolytic activity (Figure 4). When poly(dA·[$^3$H]dT) was used as a substrate, the optimum exonucleolytic activity was 10-fold lower with $Ca^{2+}$ compared to the rate observed in the presence of $Mg^{2+}$. However, with $^3$H-labeled PM2 DNA, the optimum exonucleolytic rate was more than 100-fold lower with $Ca^{2+}$ than with $Mg^{2+}$. Exonuclease III exhibited a very sharp $Ca^{2+}$ optimum for both its endonucleolytic and exonucleolytic activities; higher $Ca^{2+}$ concentration severely inhibited both activities. This is in agreement with earlier observations which showed that the exonucleolytic activity of exonuclease III was specifically inhibited while little effect was observed for the AP endonucleolytic activity (Weiss, 1981).

The observed discontinuity in the Arrhenius plot for the exonucleolytic activity of exonuclease III in the presence of $Mg^{2+}$ suggests that there may be two rate-limiting steps in the exonuclease III catalysis (Figure 5). The transition temperature ($T_m$) where the discontinuity occurred was around 25 °C (Figure 5), which is similar to that reported recently (Henikoff, 1987). Interestingly, in the presence of $Ca^{2+}$ no discontinuity was observed. These data suggest that, in the presence of $Mg^{2+}$, exonuclease III might exist in two conformational forms (Londesborough, 1980) characterized by the difference in their activation energy ($E_a$) for the particular reaction catalyzed. However, only one conformation below the $T_m$ was observed in the presence of $Ca^{2+}$.

On the basis of these considerations, if the exonucleolytic activity of exonuclease III exhibits two temperature-dependent conformations in the presence of $Mg^{2+}$, one would have expected to observe a similar discontinuity in the Arrhenius plot for the endonucleolytic activity. However, this did not occur (Figure 6). In the presence of $Mg^{2+}$, only the low $E_a$ form was observed. The difference in the temperature dependency of these two enzymatic activities of exonuclease III can be explained by the difference in the method of measurement of these activities. For the assay of endonucleolytic activity, only the initial nicking event is measured (cleavage of the phosphodiester bond); subsequent nicking events on the same DNA molecule are not detected. The assay for exonucleolytic activity measures a combination of the phosphodiesterase activity and the procession of the enzyme on the DNA substrate. It has been shown that exonuclease III exhibits an interesting dependency on temperature (Donelson & Wu, 1972; Wu et al., 1976; Thomas & Olivera, 1978), becoming highly processive at temperatures below 20 °C (Wu et al., 1976) and distributive at higher temperatures (Thomas & Olivera, 1978). At temperatures below 5 °C, exonuclease III binds tightly to the DNA substrate, and the enzyme–DNA substrate complex can be isolated by gel filtration (Donelson & Wu, 1972). Furthermore, the enzyme could only excise about six nucleotides from the 3′ terminus of duplex DNA (Donelson & Wu, 1972). This suggests that at temperatures below 20 °C the movement or procession of exonuclease III on the DNA backbone becomes a rate-limiting step.

Taken together, the data suggest that at temperatures below 25 °C the high $E_a$ conformer of exonuclease III binds tightly to the DNA substrate and is highly processive on the DNA backbone. Therefore, the higher activation energy required for this conformer may reflect the energy required for the dissociation/association of exonuclease III with the DNA substrate (or the linear processivity of the enzyme on the DNA substrate). At higher temperatures, the conformation of exonuclease III changes to one that is distributive, and association/dissociation with the DNA substrate is no longer rate limiting, and the rate-limiting step becomes the cleavage of the phosphodiester bond. Since only the initial nicking event was measured for the endonucleolytic activity, the rate-limiting step reflects the activation energy of the hydrolysis of the phosphodiester bond catalyzed by exonuclease III. In the presence of $Ca^{2+}$, exonuclease III is assumed to lock into only the high $E_a$ form, since no discontinuity was observed in the Arrhenius plot for both the endonucleolytic and the exonucleolytic activities.

## ACKNOWLEDGMENTS

I am grateful to Drs. Susan Wallace, Robert Melamede, and Hiroshi Ide for their valuable discussions.

**Registry No.** $Mg^{2+}$, 7439-95-4; $Ca^{2+}$, 7440-70-2; exonuclease III, 9037-44-9.

## REFERENCES

Bailey, V., & Verly, W. G. (1987) *Biochem. J. 242*, 565–572.
Bassett, C. L., & Kushner, S. R. (1984) *J. Bacteriol. 157*, 661–664.
Chetsanga, C. J., & Lindahl, T. (1979) *Nucleic Acids Res. 6*, 3673–3684.
Demple, B., & Linn, S. (1980) *Nature (London) 287*, 203–208.
Demple, B., Halbrook, J., & Linn, S. (1983) *J. Bacteriol. 153*, 1079–1082.
Demple, B., Johnson, A., & Fung, D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7731–7735.
Donelson, J. C., & Wu, R. (1972) *J. Biol. Chem. 247*, 4661–4668.
Farr, S. B., Natvig, D. O., & Kagoma, T. (1985) *J. Bacteriol. 164*, 1309–1316.
Farr, S. B., D'Ari, R., & Touati, D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8268–8272.
Futcher, S. D., & Morgan, A. R. (1979) *Can. J. Biochem. 57*, 932–938.
Grossman, L., & Grafstrom, R. (1982) *Biochemie 64*, 577–580.
Henikoff, S. (1984) *Gene 28*, 351–359.
Henikoff, S. (1987) *Methods Enzymol. 155*, 156–165.
Henner, W. D., Grunberg, S. M., & Haseltine, W. D. (1983) *J. Biol. Chem. 258*, 15198–15205.
Hubbard, K., Ide, H., & Wallace, S. S. (1989) *Biochemistry* (in press).
Huberman, J. A., & Kornberg, A. (1970) *J. Biol. Chem. 245*, 5326–5334.
Ide, H., Melamede, R. J., Kow, Y. W., & Wallace, S. S. (1987) in *Anticarcinogenesis and Radiation Protection* (Cerutti, P. A., Nygaard, O. F., & Simic, M. G., Eds.) pp 145–150, Plenum Press, New York and London.
Jorgenson, T., Kow, Y. W., Wallace, S. S., & Henner, W. H. (1987) *Biochemistry 26*, 6436–6443.
Kim, J., & Lin, S. (1988) *Nucleic Acids Res. 16*, 1135–1141.
Kondo, Y., & Witkop, B. (1968) *J. Am. Chem. Soc. 90*, 764–770.

Kow, Y. W., & Wallace, S. S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8354–8358.
Kow, Y. W., & Wallace, S. S. (1987) *Biochemistry 26*, 8200–8206.
Kow, Y. W., Ide, H., & Wallace, S. S. (1987) in *Anticarcinogenesis and Radiation Protection* (Cerutti, P. A., Nygaard, O. F., & Simic, M. G., Eds.) pp 135–138, Plenum Press, New York and London.
Kowalski, D. (1979) *Anal. Biochem. 93*, 346–354.
Kunkel, T. A., Schaaper, R. M., & Loeb, L. A. (1983) *Biochemistry 22*, 2378–2384.
Lindahl, T., & Nyberg, B. (1972) *Biochemistry 11*, 3610–3618.
Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B., & Sperens, B. (1977) *J. Biol. Chem. 252*, 3286–3294.
Liuzzi, M., & Talpaert-Borlē, M. (1985) *J. Biol. Chem. 260*, 5252–5258.
Liuzzi, M., Weinfeld, M., & Paterson, M. C. (1987) *Biochemistry 26*, 3315–3321.
Loeb, L. A., & Preston, B. D. (1980) *Annu. Rev. Genet. 20*, 201–230.
Londesborough, J. (1980) *Eur. J. Biochem. 105*, 211–215.
Maniatis, F., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Melamede, R. J., Kow, Y. W., & Wallace, S. S. (1987) in *Anticarcinogenesis and Radiation Protection* (Cerutti, P. A., Nygaard, O. F., & Simic, M. G., Eds.) pp 139–144, Plenum Press, New York and London.
Mosbaugh, D. W., & Linn, S. (1980) *J. Biol. Chem. 255*, 11743–11752.
Niwa, O., & Moses, R. E. (1981) *Biochemistry 20*, 238–344.
Paek, K. H., & Walker, G. C. (1986) *J. Bacteriol. 165*, 763–770.
Povirk, L., & Goldberg, I. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3182–3186.
Richardson, C. C., & Kornberg, A. (1964) *J. Biol. Chem. 239*, 242–250.
Royer-Pokora, B., Gordon, L. K., & Haseltine, W. A. (1981) *Nucleic Acids Res. 9*, 4594–4609.
Sammartano, L. J., & Tuveson, R. W. (1983) *J. Bacteriol. 156*, 904–906.
Sagher, D., & Strauss, B. (1983) *Nucleic Acids Res. 13*, 4285–4298.
Seeberg, H., & Steinum, A. (1980) *J. Bacteriol. 141*, 1424–1427.
Talpaert-Borlē, M. (1987) *Mutat. Res. 181*, 45–56.
Talpaert-Borlē, M., & Liuzzi, M. (1983) *Biochim. Biophys. Acta 740*, 410–416.
Taylor, A. F., & Weiss, B. (1982) *J. Bacteriol. 151*, 351–357.
Teoule, R. (1987) *Int. J. Radiat. Biol. 51*, 573–589.
Thomas, K. R., & Olivera, B. M. (1978) *J. Biol. Chem. 253*, 424–429.
Warner, H. R., Demple, B., Deutsch, W. D., Kane, C. M., & Linn, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4602–4606.
Weiss, B. (1976) *J. Biol. Chem. 251*, 1896–1901.
Weiss, B. (1981) *Enzymes (3rd Ed.) 14*, 203–231.
Weiss, B., & Grossman, L. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol. 60*, 1–34.
Wu, R., Ruben, G., Siegel, B., Jay, E., Spielman, P., & Tu, C. D. (1976) *Biochemistry 15*, 734–740.
Yajko, D. M., & Weiss, B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 688–692.

# The Location of DNA in Complexes of recA Protein with Double-Stranded DNA. A Neutron Scattering Study†

Elisabeth DiCapua,‡ Manfred Schnarr,§ and Peter A. Timmins*,‡
*Institut Laue-Langevin, 156X, 38042 Grenoble Cedex, France, and Institut de Biologie Moléculaire et Cellulaire, 15, rue Descartes, 67000 Strasbourg, France*

*Received July 7, 1988; Revised Manuscript Received November 29, 1988*

ABSTRACT: Purified recA protein is found as rodlike homopolymers, and it forms filamentous complexes with double-stranded DNA that are stable in the presence of ATPγS, a nonhydrolyzable analogue of ATP. The structure of these filaments has been described in some detail by electron microscopy. Here we confirm the mass per length of 6.5 recA/100 Å in solution by small-angle neutron scattering and extend the analysis to homopolymers of recA protein, finding a mass per length of about 7 recA/100 Å and a radial mass distribution (cross-sectional radius of gyration) significantly different for the two filaments. The models proposed so far for the structure of the complex have placed the DNA in the center of the filament. Here we verify this assumption using small-angle neutron scattering to locate the DNA in the complexes, exploiting the contrast variation method in $D_2O/H_2O$ mixtures. Model calculations show that the natural contrast difference between DNA and protein is not sufficient to locate the DNA (which accounts for only 4.7% of the mass in the complex). When deuterated DNA is used, the contrast difference is enhanced, and model calculations and experiment then converge, indicating that the DNA is indeed near the axis of the complex.

Homologous recombination in *Escherichia coli* is dependent on the *recA* gene product. In vitro, the purified protein is able to perform strand exchange. This reaction was found to be initiated by the covering of one of the DNA partners to form a filamentous structure, the presynaptic complex, which will subsequently interact with the second DNA molecule [reviewed in Howard-Flanders et al. (1984)].

The presynaptic complex is formed in the presence of ATP on single-stranded DNA (Flory et al., 1984); it is known to

† This project was supported by the European Molecular Biology Organization (long-term fellowship to E.D.C.).
‡ Institut Laue-Langevin.
§ Institut de Biologie Moléculaire et Cellulaire.

0006-2960/89/0428-3287$01.50/0 