istry 10, 300.
Morita, F. (1974), *Biochim. Biophys. Acta 343*, 674.
Pinkston, M. F., and Li, H. J. (1974), *Biochemistry 13*, 5227.
Schott, H. (1973a), *Angew. Chem. 85*, 263.
Schott, H. (1973b), *Angew. Chem., Int. Ed. Engl. 12*, 246.
Schott, H. (1974a), *J. Chromatogr. 96*, 79.
Schott, H. (1974b), *FEBS Lett. 41*, 203.
Schott, H., Brandstetter, F. and Bayer, E. (1973), *Makromol. Chem. 173*, 247.
Wagner, K. G., and Arfmann, H. A. (1974), *Eur. J. Biochem. 46*, 27.
Williams, E. J., Hershkovits, T. T., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, 3574.

Endonuclease II of *Escherichia coli:* DNA Reacted with 7-Bromomethyl-12-methylbenz[*a*]anthracene as a Substrate†

Dollie M. Kirtikar, Anthony Dipple,‡ and David A. Goldthwait*§

ABSTRACT: An endonuclease II preparation from *Escherichia coli* makes single-strand breaks in DNA which has been treated with the carcinogen 7-bromomethyl-12-methylbenz[*a*]anthracene. In addition, the enzyme preparation excises N^6-(12-methylbenz[*a*]anthracenyl-7-methyl)adenine and N^2-(12-methylbenz[*a*]anthracenyl-7-methyl)guanine residues from the DNA. These are released as the modified purine bases, not as purine nucleoside derivatives. The rate of release of the adenine derivative is three to four times that of the guanine derivative.

Endonuclease II is an enzyme from *Escherichia coli* which hydrolyzes phosphodiester bonds in DNA treated with alkylating agents such as methyl methanesulfonate (Friedberg and Goldthwait, 1968; Friedberg et al., 1969), dimethyl sulfate, and *N*-methyl-*N*-nitrosourea (Kirtikar and Goldthwait, 1974), as well as in DNA exposed to γ irradiation (Kirtikar et al., 1975). This activity has been purified 1600-fold (Hadi et al., 1973). In this partially purified preparation of endonuclease II, there is an activity which recognizes depurinated and depurinated reduced DNA (Hadi and Goldthwait, 1971) and this activity was originally considered to be the same enzyme as that active on alkylated DNA. Since then the activities have been separated as will be noted in the Results and Discussion sections.

This 1600-fold purified enzyme preparation is also active on DNA treated with the polycyclic aromatic carcinogen 7-bromomethyl-12-methylbenz[*a*]anthracene. This carcinogen reacts with the amino groups of the bases in DNA both in vitro and in vivo (Rayman and Dipple, 1973a,b). It is a potent carcinogen in several animal test systems (Dipple and Slade, 1970, 1971; Roe et al., 1972), it exhibits mutagenic and cytotoxic effects in mammalian cells (Huberman et al., 1971; Duncan and Brookes, 1973) and bacterial systems (Maher et al., 1974), and it is toxic to bacteriophage (Dipple and Shooter, 1974). The enzyme preparation makes phosphodiester breaks in DNA treated with this carcinogen and also releases derivatives of the purine bases.

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106 (D.M.K. and D.A.G.), and the Chester Beatty Research Institute, London, U.K. (A.D.). *Received July 11, 1975.* Supported by grants from the National Institutes of Health (CA11322), the Health Fund of Greater Cleveland, and the Cuyahoga Unit of the American Cancer Society.

‡ Present address: Frederick Cancer Research Center, Frederick, Maryland 21701.

§ National Institutes of Health Research Career Award Fellowship K6-GM-21444.

Materials and Methods

Radioactive Materials. 7-Bromo[^{14}C]methyl-12-methylbenz[*a*]anthracene (specific radioactivity 5.6 Ci/mol) and [^{3}H]-7-bromomethyl-12-methylbenz[*a*]anthracene (specific radioactivity 725 Ci/mol) were prepared as previously described (Rayman and Dipple, 1973a). [^{3}H]Thymidine- and [^{3}H]purine-labeled T_7 DNA were prepared as described previously (Kirtikar et al., 1975). [^{3}H]Thymidine-labeled T_4 DNA was prepared according to procedures of Melgar and Goldthwait (1968).

Modified DNA Samples. Treatment of various DNA samples with hydrocarbon was carried out under the reaction conditions described by Rayman and Dipple (1973a). Samples of both T_7 and commercial salmon sperm DNAs, reacted with [^{3}H]-7-bromomethyl-12-methylbenz[*a*]anthracene [0.44 mmol of hydrocarbon per mol of DNA-P for T_7 DNA and 1.2 mmol of hydrocarbon per mol of DNA-P for salmon sperm DNA] were prepared. Salmon sperm DNA was also reacted with 7-bromo[^{14}C]methyl-12-methylbenz[*a*]anthracene to give DNA with specific activity of 0.23 μCi/mmol of DNA nucleotide. [^{3}H]Purine-labeled T_7 DNA (specific activity 1960 cpm/nmol of DNA nucleotide) was reacted with unlabeled 7-bromomethyl-12-methylbenz[*a*]anthracene at a hydrocarbon to DNA nucleotide ratio of 1:10. Other DNA samples used as substrates were prepared according to published procedures. [^{3}H]Purine-labeled T_7 DNA was treated with methyl methanesulfonate (Friedberg and Goldthwait, 1968) at a drug to DNA nucleotide ratio of 10:1 and the same labeled DNA was used to prepare depurinated reduced DNA by the procedures described by Hadi and Goldthwait (1971). [^{3}H]Purine-labeled T_7 DNA was exposed to 22.5 krad of γ irradiation from a ^{60}Co source (Kirtikar et al., 1975). [^{3}H]Thymidine-labeled T_4 DNA was irradiated with approximately 200 ergs/mm^2, a dose which should introduce approximately 60 dimers into each DNA molecule (P. Kuebler and D. Schlaes, unpublished results).

Endonuclease II Preparation. The enzyme preparation used in these studies was a 1600-fold purified fraction from *E. coli* prepared as described by Hadi et al. (1973).

Enzyme-Induced Single-Strand Breaks. These were determined by alkaline sucrose gradient analysis. Incubation mixtures (0.25 ml) contained 10–20 nmol of DNA nucleotide, 1×10^{-4} *M* β-mercaptoethanol, 1×10^{-4} *M* 8-hydroxyquinoline, 5×10^{-2} *M* Tris-HCl buffer (pH 8.0), and enzyme. After 60 min at 37°, reactions were terminated by adding EDTA and sodium dodecyl sulfate at final concentrations of 2×10^{-2} *M* and 0.25%, respectively. The samples were then incubated in alkali (0.066 *M* final concentration) at 37° for 20 min and aliquots were centrifuged through 5–20% alkaline sucrose density gradient solutions. Single-strand breaks were calculated as described previously (Kirtikar et al., 1975).

Endonuclease II Catalyzed Release of Hydrocarbon-Modified Residues from DNA. A typical reaction mixture of 0.25 ml contained 22 nmol of hydrocarbon-treated T_7 DNA nucleotides (0.44 mmol of hydrocarbon/mol of DNA-P, specific activity 425 cpm per nmol of DNA nucleotide), 1×10^{-4} *M* β-mercaptoethanol, 1×10^{-4} *M* 8-hydroxyquinoline, 5×10^{-2} *M* Tris-HCl (pH 8.0), buffer, and varying amounts of enzyme. After 30 min at 37°, the reactions were terminated by adding EDTA to a final concentration of 2×10^{-2} *M*. The samples were cooled to 0° and 50 μg of unlabeled T_4 DNA and sodium acetate to a final concentration of 0.25 *M* were added. DNA was then precipitated with two volumes of cold 95% ethanol at 0° for 60–120 min. After centrifugation, the supernatant fractions and precipitates, which were solubilized in concentrated NH_4OH and then neutralized, were counted.

Identification of the Hydrocarbon-Modified Residues Released from DNA by the Endonuclease II Preparation. Labeled and unlabeled derivatives of the purine bases were obtained for use as markers as follows. Salmon sperm DNA treated with 7-bromo[^{14}C]methyl-12-methylbenz[*a*]anthracene was enzymatically hydrolyzed (DNase I, snake venom phosphodiesterase, and alkaline phosphatase) to a mixture of nucleosides which were chromatographically separated on Sephadex LH-20 eluted with methanol as previously described (Rayman and Dipple, 1973a), to yield ^{14}C-labeled markers of N^2-(12-methylbenz[*a*]anthracenyl-7-methyl)deoxyguanosine and N^6-(12-methylbenz[*a*]anthracenyl-7-methyl)deoxyadenosine. Portions of each of these were hydrolyzed separately in 0.1 *N* HCl at 100° for 15 min and again purified on Sephadex LH-20 to yield ^{14}C-labeled markers of N^2-(12-methylbenz[*a*]anthracenyl-7-methyl)guanine and N^6-(12-methylbenz[*a*]anthracenyl-7-methyl)adenine, respectively. Unlabeled base and nucleoside derivatives were prepared in a similar manner for use as markers.

DNA which had been treated with [^{3}H]-7-bromomethyl-12-methylbenz[*a*]anthracene and subsequently incubated with or without the endonuclease II preparation was separated into alcohol-soluble and alcohol-insoluble fractions as described above. The alcohol-insoluble fraction was hydrolyzed enzymatically to a mixture of nucleosides, and then hydrolyzed chemically (0.1 *N* HCl, 100°, 15 min) to convert purine nucleosides into the purine bases. Markers labeled with ^{14}C were added to this solution and to the alcohol-soluble fraction. Both of these fractions were then subjected separately to chromatography on a Sephadex LH-20 column (72 cm × 1.5 cm diameter) eluted with methanol; 5-ml fractions were collected and the radioactivity in each fraction was monitored. The fractions comprising each peak were pooled and concentrated for quantitative measurements and for further analysis. One batch of Sephadex LH-20 (3784) was found to have chromatographic properties different from those described by Rayman and Dipple (1973a), and did not allow the separation of the purine nucleoside derivatives from the purine base derivatives.

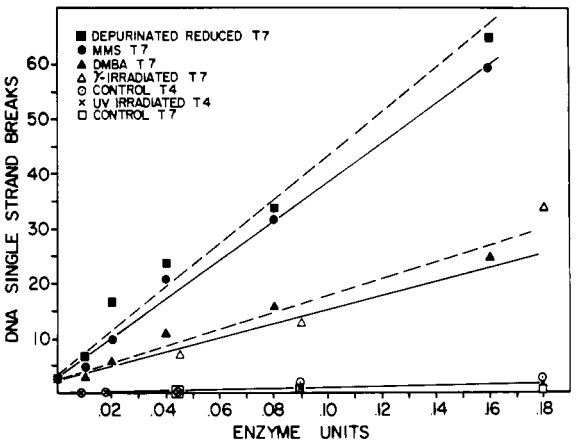

FIGURE 1: Enzyme-induced single-strand breaks in modified DNA samples. [^{3}H]Thymidine-labeled T_4 and [^{3}H]purine-labeled T_7 DNAs were used. The conditions of DNA modification and the enzyme incubation reactions are as described in Materials and Methods. Per reaction mixture, 11 nmol of depurinated and reduced [^{3}H]purine-labeled T_7 DNA, 15 nmol of methyl methanesulfonate treated [^{3}H]purine-labeled T_7 DNA, 15 nmol of 7-bromomethyl-12-methylbenz[*a*]anthracene-treated [^{3}H]purine-labeled T_7 DNA, 10 nmol of γ-irradiated [^{3}H]purine-labeled T_7 DNA, 35 nmol of untreated [^{3}H]thymidine labeled T_4 DNA, 35 nmol of uv-irradiated [^{3}H]T_4 DNA, and 10 nmol of [^{3}H]purine-labeled T_7 DNA were used.

The concentrated samples from the Sephadex LH-20 were supplemented with different mixtures of unlabeled hydrocarbon derivatives of adenine, guanine, and deoxyadenosine. The Polygram SIL N-HR/UV_{254} plates used for TLC were activated at 100° for 90 min, cooled for 30 min, and used immediately. The chromatograms were developed in spectranalyzed acetone for 90–120 min. The unlabeled derivatives were identified by uv absorption and eluted with 0.001 *M* HCl. The R_f values relative to that of N^6-(12-methylbenz[*a*]anthracenyl-7-methyl)deoxyadenosine (R_f 1.0) were 0.7 for both the guanine and deoxyguanosine products, and 0.55 for the adenine product. Thin-layer chromatographic analysis of the products released enzymatically was also done directly on the alcohol-soluble fractions after concentration.

Results

The specificity of a 1600-fold purified preparation of endonuclease II from *E. coli* (Hadi et al., 1973) for various modified DNA preparations is illustrated in Figure 1. In these experiments after the incubation with or without the enzyme, the DNA was exposed to 0.066 *N* NaOH at 37°C for 20 min. This converted any depurinated sites present into single-strand breaks (Kirtikar et al., 1975) so that only the enzyme induced breaks were assayed subsequently by alkaline sucrose gradient centrifugation. The enzyme preparation made single-strand breaks in DNA treated with 7-bromomethyl-12-methylbenz[*a*]anthracene, with methyl methanesulfonate, with γ irradiation, and in depurinated reduced DNA (a depurinated reduced site is not alkali labile). There was no significant activity on native or on uv irradiated DNA.

Table I: Enzyme-Induced Release of Radioactivity from T_7 DNA Treated with [^{3}H]-7-Bromomethyl-12-methylbenzanthracene.[a]

Enzyme Units	Percent of Total Counts Recovered	
	Alcohol-Soluble Fraction	Alcohol-Insoluble Fraction
	5	95
0.025	9	91
0.050	12	88
0.075	20	80
0.10	23	77
0.25	29	71
0.50	35	65

[a] The exact protocol is given in Materials and Methods.

The enzyme activity on depurinated reduced DNA has since been separated from an activity which hydrolyzes DNA treated with methyl methanesulfonate, γ irradiation, or 7-bromomethyl-12-methylbenz[*a*]anthracene. The different levels of enzyme activity on DNA treated with methyl methanesulfonate, 7-bromomethylbenz[*a*]anthracene, and γ irradiation, shown in Figure 1, could be due to different concentrations of substrate (that is the number of sites per DNA molecule) or to different affinities of the enzyme for the different substrates.

The activity of the 1600-fold-purified enzyme fraction on the hydrocarbon-modified DNA was investigated in greater detail. Incubation of DNA, which had been treated with [^{3}H]-7-bromomethyl-12-methylbenz[*a*]anthracene, with the endonuclease II preparation resulted in the release of bound tritium into an alcohol-soluble form (Table I). In order to determine the chemical nature of the radioactive residues excised from this hydrocarbon-modified DNA, various chromatographic procedures were employed to study both the alcohol-soluble and the alcohol-insoluble residues obtained after incubation with the enzyme preparation.

Results of chromatography of the hydrocarbon derivatives on Sephadex LH-20 are shown in Figure 2. ^{14}C markers are also indicated. In the absence of the enzyme, the alcohol-soluble fraction gave the pattern in Figure 2A. The fractions in each of the two peaks were then pooled and examined on thin-layer chromatograms (TLC) developed in acetone. This system separates the adenine derivative from the deoxyadenosine derivative and both of these from the guanine and deoxyguanosine derivatives which are not resolved. The ^{3}H and ^{14}C radioactivity from the column chromatogram peak at the position of the adenine derivative migrated only to the R_f of the adenine derivative on a thin-layer plate. Likewise, the ^{3}H and ^{14}C radioactivity from the guanine derivative peak was found only at the R_f shared by the guanine and deoxyguanosine derivatives. Since these two latter compounds are easily resolved on the Sephadex LH-20 column, it appeared that in the absence of endonuclease II, small amounts of the hydrocarbon-modified purine bases were present either initially or were liberated upon incubation under our conditions.

In the presence of the endonuclease II preparation, modified purine bases were released, now in greater quantity, into the alcohol-soluble fraction (Figure 2B). The ^{14}C-labeled markers used in this experiment were the hydrocarbon-modified adenine and deoxyadenosine derivatives. The tritium-labeled material which ran with the [^{14}C]adenine derivative marker was inseparable from this marker in the

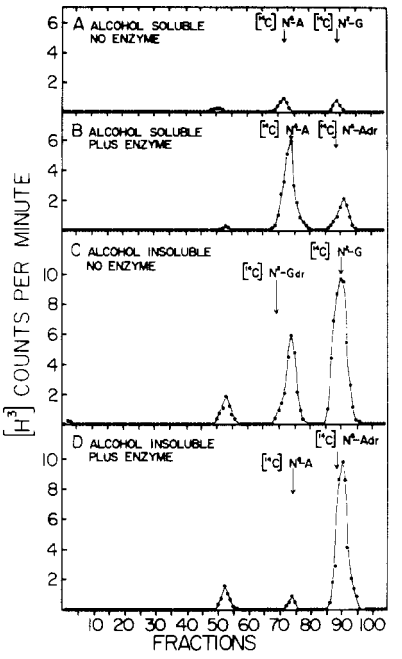

FIGURE 2: Sephadex LH-20 chromatography of alcohol soluble and insoluble fractions from [^{3}H]hydrocarbon-treated salmon sperm DNA incubated with and without enzyme. Data from experiment 2 in Table II are used. The arrows indicate the position of the peak fraction of the ^{14}C-labeled markers. N^6-A designates N^6-[12-methylbenz[*a*]anthracenyl-7-methyl]adenine, N^6-AdR designates N^6-[1-methylbenz[*a*]anthracenyl-7-methyl]deoxyadenosine, N^2-G designates [12-methylbenz[*a*]anthracenyl-7-methyl]guanine, and N^2-Gdr designates [12-methylbenz[*a*]anthracenyl-7-methyl]guanosine.

TLC system described above. The tritium-labeled material, eluted from the column adjacent to, but not coincident with the [^{14}C]deoxyadenosine derivative marker, was separated from this marker in the TLC system and was found, as expected, at the R_f of the hydrocarbon-guanine derivative. These studies demonstrate that the endonuclease II preparation recognizes the hydrocarbon-modified purines in DNA and excises them as purine derivatives rather than as nucleosides or nucleotides.

It is also apparent that this enzyme excises a larger proportion of modified adenine residues from the DNA than of modified guanine residues. This was confirmed by analyzing the alcohol-insoluble fraction from the endonuclease II incubations (Figure 2C and D). The alcohol-insoluble fractions were enzymatically digested to mixtures of deoxyriboucleosides, hydrolyzed (0.1 *N* HCl, 100°, 15 min) to convert the purine deoxyribonucleoside derivatives, and then chromatographed on Sephadex LH-20. It is apparent that the loss of the adenine derivative after incubation with endonuclease II is far greater than the loss of the guanine derivative (Figure 2C and D). The first small radioactive peak eluted in these chromatograms probably corresponds to the hydrocarbon-modified deoxycytidine residues (Rayman and Dipple, 1973a).

Quantitative data from three experiments performed as above is presented in Table II. The data show good stoichiometry between the material released enzymatically from the hydrocarbon-modified DNA into the alcohol-soluble fraction and the material disappearing from the alcohol-insoluble fraction. The data also suggest that the hydrocarbon-modified pyrimidine derivative [assumed to be the first radioactive peak on the column chromatograms] is not recognized by the enzyme preparation. Again, in all experi-

Table II: Enzymatic Release of Benzanthracene Derivatives of DNA Bases.[a]

Expt	Carcinogen Substituted DNA Constituent	Total Radioactivity (cpm)					
		Alcohol Soluble			Alcohol Insoluble		
		No Enzyme	Plus Enzyme	Δ	No Enzyme	Plus Enzyme	Δ
1	N^4-Cdr[b]	1398	1302	−89	6720	6208	−512
	N^2-G	2718	9482	+6764	49870	43118	−6757
	N^6-A	3438	25861	+22423	24150	2560	−21590
2	N^4-Cdr[b]	959	914	−45	9462	9408	+146
	N^2-G	3200	9410	+6210	61864	56750	−5114
	N^6-A	3900	31683	+27783	28350	1313	−27037
3	N^4-Cdr[b]	131	135	+4	1293	1283	−10
	N^2-G	376	835	+459	8699	8214	−485
	N^6-A	292	2217	+1925	4056	2116	−1940

[a] For experiment 1, the incubation mixture of 1.0 ml contained 150 nmol of DNA nucleotide [^{3}H]hydrocarbon salmon sperm DNA (0.82 mmol of carcinogen per mol of DNA nucleotide, specific activity 500 cpm/nmol of DNA nucleotide), 1×10^{-4} *M* β-mercaptoethanol, 1×10^{-4} *M* 8-hydroxyquinoline, and 0.4 unit of enzyme. After 60 min at 37° the reaction was terminated, and an alcohol-soluble and an alcohol-insoluble fraction were obtained. The latter was digested to nucleosides (Dipple et al., 1971) and hydrolyzed to bases. The hydrolyzed sample as well as the alcohol-soluble material were supplemented with ^{14}C-labeled markers and were chromatographed on Sephadex LH-20. For experiment 2, the incubation mixture was 0.5 ml and contained 225 nmol of DNA nucleotides and 0.4 unit of enzyme. The enzyme reaction was run for 30 min at 37°. For experiment 3, the incubation mixture was 1.0 ml and contained 85 nmol of DNA and 0.4 unit of enzyme. The enzyme reaction was at 35° for 60 min. During all steps, the samples were protected from exposure to light. [b] Identified only by position of elution from the column compared to that described by Rayman and Dipple (1973a).

Table III: Enzymatic Hydrolysis of Phosphodiester Bonds in DNA Treated with 7-Bromomethyl-12-methylbenz[*a*]anthracene and Measured in Neutral Formamide and Alkaline Formaldehyde Gradients.[a]

	Single-Strand Breaks	
	Neutral Formamide Gradient	Alkaline Formaldehyde Gradient
Endonuclease II, 0.009 unit	5	5
Endonuclease II, 0.025 unit	17	11
Endonuclease II, 0.045 unit	48	55
Calf liver, apurinic enzyme, 0.06 unit	0	0
E. coli apurinic enzyme, 0.10 unit	0	0.4

[a] Incubation mixtures and conditions for the enzyme reactions are as described in Materials and Methods with some modifications; 37.5 nmol of T_7 DNA reacted with [^{3}H]-7-bromomethyl-12-methylbenz[*a*]anthracene [0.44 mmol of hydrocarbon per mol of DNA-P, specific activity 425 cpm/nmol of DNA nucleotide] was used. For neutral formamide gradient analysis, the samples were centrifuged through 70–100% formamide gradients without prior incubation in alkali. For alkaline formaldehyde centrifugation, the samples were incubated in alkali [0.066 *M* NaOH] for 20 min at 37° and aliquots were centrifuged through 5–20% alkaline sucrose solutions containing 6% formaldehyde. The endonuclease II preparation was a fraction purified approximately 3000-fold and separated from the major activities on depurinated DNA. The *E. coli* apurinic enzyme was a fraction purified 1200-fold and separated from endonuclease II (D. M. Kirtikar, unpublished). The calf liver enzyme active on depurinated DNA was a preparation purified approximately 800-fold (J. P. Kuebler, unpublished).

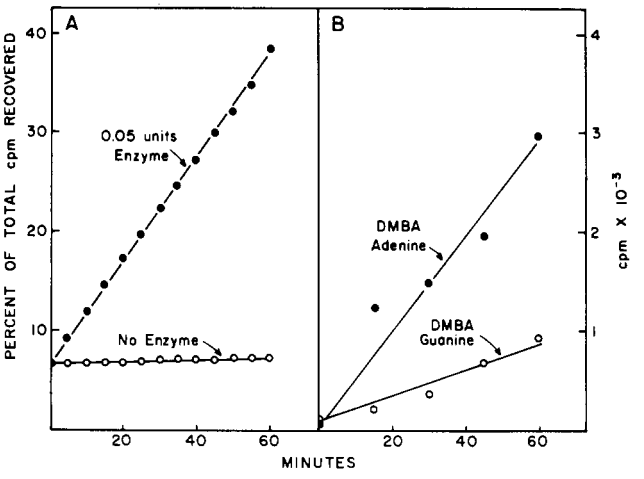

FIGURE 3: Kinetics of release of [^{3}H]hydrocarbon modified nucleic acid products with and without endonuclease II. (A) Release into alcohol-soluble fraction. The reaction mixtures and conditions are as described in Materials and Methods. Per 0.25 ml of reaction mixture, 75 nmol of [^{3}H]hydrocarbon treated salmon sperm DNA nucleotides and 0.05 unit of enzyme preparation were used. The incubation was at 37° and at indicated intervals, the reactions were terminated with NaEDTA at a final concentration of 0.01 *M*. The alcohol-soluble and insoluble material were separated (see Materials and Methods) and counted. Total cpm recovered accounts for both alcohol-soluble and insoluble counts per sample. (B) Rate of release of modified purines. The alcohol-soluble material, separated as above, was concentrated by evaporation to a small volume [0.025 ml] and after supplementing with unlabeled N^2-[12-methylbenz[*a*]anthracenyl-7-methyl]guanine and N^6-[12-methylbenz[*a*]anthracenyl-7-methyl]adenine, subjected to TLC chromatography developed in acetone. The radioactivity in areas identified under uv light was determined.

ments the extent of removal of modified adenine residues from the DNA was considerably greater than the extent of removal of modified guanine residues.

Experiments were then undertaken to examine the kinetics of release of these purine products (Figure 3). The rate of endonuclease II catalyzed release of radioactivity into the alcohol-soluble fraction was found to be linear over a 60-min period (Figure 3A). The blank value is elevated to zero time and increases only slightly during the incubation. This suggests that the nonenzymatic release of the purine derivatives shown in Figure 2A occurred primarily before the incubation. The rate of release of each modified purine was then studied by separating these two products in the alcohol-soluble fraction by the TLC system (Figure 3B). The radioactive adenine derivative appeared in the alcohol-soluble fraction about 3–4 times more rapidly than did the radioactive guanine derivative.

Since the enzyme preparation released the hydrocarbon derivatives of the purine bases, and since in the experiments which showed phosphodiester bond hydrolysis of the hydrocarbon treated DNA (Figure 1), all reaction mixtures were exposed to alkali, it was logical to propose that the enzyme activity released only purine base derivatives and that the breakage of phosphodiester bonds occurred because of the β elimination reaction due to the alkali. A second reason for phosphodiester bond hydrolysis after enzymatic release of the purine base derivatives was the presence of the depurination enzyme in the preparation. In Table III evidence is presented to rule out both of these possibilities. To avoid treatment with alkali the reaction mixtures were analyzed in formamide gradients. To avoid the activity of the enzyme which is specific for depurinated DNA, a fraction was used which had been separated cleanly from the major peak of activity on depurinated DNA, and which was active on DNA treated with methyl methanesulfonate, 7-bromomethyl-12-methylbenz[*a*]anthracene, or γ irradiation (D. M. Kirtikar, unpublished). The data indicate that in the presence of increasing amounts of this enzyme fraction, there are increasing numbers of single-strand breaks. This fraction also releases the purine base derivatives (D. M. Kirtikar, unpublished). Thus, enzymatic depurination followed by alkali-catalyzed β elimination is ruled out. Phosphodiester bond hydrolysis by the enzyme which is specific for depurinated DNA is also ruled out. However, the enzyme specific for methyl methanesulfonate treated DNA, etc., has a very low activity on depurinated DNA and this is present at various stages of purification. This activity is approximately 10% of the activity on methyl methanesulfonate treated DNA (D. M. Kirtikar, unpublished). Therefore a two-step reaction with depurination followed by enzymatic hydrolysis of depurinated DNA due to this enzyme fraction cannot be ruled out. The chromatographic behavior of this minor activity on depurinated DNA follows very closely the behavior of the major activity on methyl methanesulfonate treated DNA. It is probable that these activities are in the same protein, but as yet this has not been proven.

Discussion

Endonuclease II of *E. coli* is active on DNA treated with methyl methanesulfonate (Friedberg et al., 1969), dimethyl sulfate, methylnitrosourea (Kirtikar and Goldthwait, 1974), 7-bromomethyl-12-methylbenz[*a*]anthracene, and also DNA exposed to γ irradiation (Kirtikar et al., 1975). The enzyme fraction active on these substrates has recently been separated (D. M. Kirtikar, unpublished) from an activity on depurinated DNA which was originally thought to also be endonuclease II (Hadi and Goldthwait, 1971) and which was similar to the activity observed by Verly and Paquette (1972) and by Ljungquist and Lindahl (1974). Endonuclease II is not active on DNA treated with uv irradiation (Figure 1 and Minton, K. and Friedberg, E., unpublished experiments).

The enzyme preparation used for all but one of these experiments was the 1600-fold purified fraction (Hadi et al., 1973) in which the activity for depurinated DNA was not separated from the activity on the other substrates. The 1600-fold purified enzyme preparation as well as the fraction separated from the major activity on depurinated DNA both release base derivatives and cleave phosphodiester bonds in DNA treated with 7-bromomethyl-12-methylbenz[*a*]anthracene. The mechanism of this reaction is not understood as yet. Preliminary results from the purification of the fraction active on hydrocarbon or methyl methanesulfonate treated DNA indicate that this enzyme fraction has a low level of activity on depurinated DNA which is constant through purification. The level is low enough so that it is unlikely that one protein hydrolyzes the glycosidic linkage and then the substrate dissociates and reassociates with a second protein active on depurinated DNA. However, further work will be required to demonstrate that a single enzyme molecule carries out both reactions in a sequential manner. The release of purine bases by this enzyme preparation from DNA methylated by reaction with dimethyl sulfate or methyl methanesulfonate has also been observed (Kirtikar and Goldthwait, 1974). Methylation of DNA by dimethyl sulfate or methyl methanesulfonate results in the formation of 3-methyladenine and 7-methylguanine (Lawley and Orr, 1970). The enzyme preparation recognizes and releases only 3-methyladenine (Kirtikar and Goldthwait, 1974). Methylnitrosourea reacts with DNA to give a series of methylated bases, the major ones of which are O^6-methylguanine, 3-methyladenine, and 7-methylguanine (Loveless, 1969; Lawley and Shah, 1972). The enzyme releases both O^6-methylguanine, a derivative responsible for altered base pairing (Gerchman and Ludlum, 1973), and 3-methyladenine (Kirtikar and Goldthwait, 1974). The studies reported here indicate that the enzyme can also recognize and release N^6-(12-methylbenz[*a*]anthracenyl-7-methyl)adenine and N^2-(12-methylbenz[*a*]anthracenyl-7-methyl)guanine but not N^4-(12-methylbenz[*a*]anthracenyl-7-methyl)cytosine. Thus, to date the enzyme preparation seems to have a specificity for derivatives of purines.

With methylnitrosourea, correlations of in vitro (Kirtikar and Goldthwait, 1974) and in vivo (Lawley and Orr, 1970) activity in *E. coli* are reasonably good. In both cases, O^6-methylguanine and 3-methyladenine are removed from the DNA. In in vivo studies in *E. coli* treated with 7-bromomethylbenz[*a*]anthracene, removal of 70% of the derivatives of adenine, guanine, and cytosine has been observed by Venitt and Tarmy (1972). However, they found that the removal of the guanine derivative was more rapid than the adenine and cytosine derivatives, and that the removal in UVRA$^-$ and UVRA$^-$, Exr$^-$ strains was decreased to 10 and 0%, respectively. In the light of the in vitro findings reported in this paper, and the presence of endonuclease II in extracts of UVRA mutants (Friedberg et al., 1969), the marked decrease in the in vivo removal by the mutant is difficult to understand.

Repair of the DNA of some eucaryotic systems treated with 7-bromomethylbenz[*a*]anthracene has been studied. In nondividing human lymphocytes treated with [^{3}H]-7-bromomethylbenz[*a*]anthracene, unscheduled DNA synthesis proceeded for 12 hr with removal of approximately 15% of the radioactivity. The adenine derivative was removed preferentially (Lieberman and Dipple, 1972). Also, in lymphocytes from patients with xeroderma pigmentosum, the induction of unscheduled DNA synthesis by 7-bromomethylbenz[*a*]anthracene was 10% of that observed in lymphocytes from normal individuals (Slor, 1973). These findings are consistent with the properties of an endonuclease isolated from rat liver by Van Lancker and Tomura (1974). These authors found that this enzyme recognizes thymine dimers, and DNA treated in vivo with acetylaminofluorene or in vitro with 7-bromomethylbenz[*a*]anthracene (Maher et al., 1974). Because of the properties of this enzyme, it

seemed desirable to again rule out the possibility that the endonuclease II of *E. coli* was active on uv irradiated DNA. Since this experiment was negative, it is concluded that the enzyme from *E. coli* has substrate requirements different from those of the rat liver enzyme.

The complexities involved in the mechanisms of action of the wide range of agents which exhibit toxic, mutagenic, or carcinogenic properties are becoming more apparent. These biological consequences may result from different types of damage introduced into the DNA even by a single agent and the effectiveness of the damage may be modified by more than one repair system. A precise understanding of the enzymes involved in these repair systems will be required before the details of the biological actions of many of these agents will be understood.

References

Dipple, A., Brookes, P., Mackintosh, D. S., and Rayman, M. P. (1971), *Biochemistry 10,* 4323.

Dipple, A., Lawley, P. D., and Brookes, P. (1968), *Eur. J. Cancer 4,* 439.

Dipple, A., and Shooter, K. V. (1974), *Biochim. Biophys. Acta 374,* 392.

Dipple, A., and Slade, T. A. (1970), *Eur. J. Cancer 6,* 417.

Dipple, A., and Slade, T. A. (1971), *Eur. J. Cancer 7,* 473.

Duncan, M. E., and Brookes, P. (1973), *Mutat. Res. 21,* 107.

Friedberg, E., and Goldthwait, D. A. (1968), *Proc. Natl. Acad. Sci. U.S.A. 62,* 934.

Friedberg, E., Hadi, S. M., and Goldthwait, D. A. (1969), *J. Biol. Chem. 244,* 5879.

Gerchman, L. L., and Ludlum, D. B. (1973), *Biochim. Biophys. Acta 308,* 310.

Hadi, S. M., and Goldthwait, D. A. (1971), *Biochemistry 10,* 4986.

Hadi, S. M., Kirtikar, D. M., and Goldthwait, D. A. (1973), *Biochemistry 12,* 2747.

Huberman, E., Aspiras, L., Heidelberger, D., Grover, P. L., and Sims, P. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3195.

Kirtikar, D. M., and Goldthwait, D. A. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2022.

Kirtikar, D. M., Slaughter, J., and Goldthwait, D. A. (1975), *Biochemistry 14,* 1235.

Lawley, P. D., and Orr, D. J. (1970), *Chem. Biol. Interact. 5,* 286.

Lawley, P. D., and Shah, S. A. (1972), *Biochem. J. 128,* 117.

Lieberman, M., and Dipple, A. (1972), *Cancer Res. 32,* 1855.

Ljungquist, S., and Lindahl, T. (1974), *J. Biol. Chem. 249,* 1530.

Loveless, A. (1969), *Nature (London) 223,* 206.

Maher, V. M., Donville, D., Tomura, T., and Van Lancker, J. L. (1974), *Mutat. Res. 23,* 113.

Melgar, E., and Goldthwait, D. A. (1968), *J. Biol. Chem. 243,* 4401.

Rayman, M. P., and Dipple, A. (1973a), *Biochemistry 12,* 1202.

Rayman, M. P., and Dipple, A. (1973b), *Biochemistry 12,* 1538.

Roe, F. J. C., Dipple, A., and Mitchley, B. C. V. (1972), *Br. J. Cancer 26,* 461.

Slor, H. (1973), *Mutat. Res. 19,* 231.

Van Lancker, J. L., and Tomura, T. (1974), *Biochim. Biophys. Acta 353,* 99.

Venitt, S., and Tarmy, E. M. (1972), *Biochim. Biophys. Acta 287,* 38.

Verly, W. J., and Paquette, Y. (1972), *Can. J. Biochem. 50,* 217.