NMR in Biology (Dwek, R. A., Campbell, I. D., Richards, R. E., & Williams, R. J. P., Eds.) Academic Press, London.
Theorell, H., & McKinley-McKee, J. S. (1961) *Acta Chem. Scand. 15,* 1834.
Weber, G. (1975) *Adv. Protein Chem. 29,* 1.
Williams, M. N., Poe, M., Greenfield, N. J., Hirshfield, J. M., & Hoogsteen, K. (1973) *J. Biol. Chem. 248,* 6375.
Wyman, J. (1948) *Adv. Protein Chem. 4,* 407.
Wyman, J. (1964) *Adv. Protein Chem. 19,* 223.

Properties of 3-Methyladenine-DNA Glycosylase from *Escherichia coli*†

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ABSTRACT: An *Escherichia coli* enzyme that releases 3-methyladenine and 3-ethyladenine in free form from alkylated DNA has been purified 2800-fold in 7% yield. The enzyme does not liberate several other alkylation products from DNA, including 7-methylguanine, O^6-methylguanine, 7-methyladenine, N^6-methyladenine, 7-ethylguanine, O^6-ethylguanine, and the arylalkylated purine derivatives obtained by treatment of DNA with 7-bromomethyl-12-methylbenz[*a*]anthracene. The reaction of the enzyme with alkylated DNA leads to the introduction of apurinic sites but no chain breaks (less than one incision per ten apurinic sites), and there is no detectable nuclease activity with native DNA, depurinated DNA, ultraviolet-irradiated DNA, or X-irradiated DNA as potential substrates. The enzyme is termed 3-methyladenine-DNA glycosylase. It is a small protein, M_r = 19 000, that does not require divalent metal ions, phosphate, or other cofactors in order to cleave base–sugar bonds in alkylated DNA.

When DNA is exposed to alkylating agents such as dimethyl sulfate or methyl methanesulfonate, either in vivo or in vitro, the two major alkylation products are 7-methylguanine and 3-methyladenine. The more strongly mutagenic and carcinogenic alkylating agents *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine produce the same two lesions, but in addition relatively large amounts of O^6-methylguanine and phosphotriesters are formed. Several minor alkylation products, e.g., 7-methyladenine, 3-methylguanine, 3-methylcytosine, and O^4-methylthymine, have also been detected (Lawley, 1974; Strauss et al., 1975). Further, ethylated derivatives analogous to the methylation products are obtained with the alkylating agents diethyl sulfate, ethyl methanesulfonate, or *N*-ethyl-*N*-nitrosourea (Sun and Singer, 1975). The fate in vivo of alkylated residues in DNA varies considerably between different types of lesions. Surprisingly, the major product 7-methylguanine does not seem to be actively excised from alkylated DNA in either *Escherichia coli* or mammalian cells but is slowly lost over a period of days by nonenzymatic hydrolysis (Prakash and Strauss, 1970; Lawley and Orr, 1970). On the other hand, 3-methyladenine residues are rapidly removed in both systems by an enzymatic process (Lawley and Orr, 1970; Lawley and Warren, 1976; Margison and O'Connor, 1973). O^6-Methylguanine is also actively released in *E. coli* and mammalian cells but at a considerably slower rate than 3-methyladenine (Lawley and Orr, 1970; Goth-Goldstein, 1977). Analogous results have been obtained for the removal of ethylated purines from DNA (Lawley and Warren, 1975).

These findings strongly suggest that cells exposed to alkylating agents can repair part of the alkylation damage in their DNA by excision-repair processes, and that repair enzymes exist that selectively act on alkylated DNA. The first evidence for the existence of such repair enzymes was obtained by Strauss and co-workers (Strauss, 1962; Reiter et al., 1967; Strauss and Robbins, 1968), who showed that crude cell extracts from *Bacillus subtilis* and *Micrococcus luteus* catalyze the formation of strand breaks at some, but not all, alkylated sites in methyl methanesulfonate treated DNA. While the results were interpreted to reflect the presence of an endonuclease specific for alkylated DNA, the activity was not purified. Similar results were obtained by Friedberg and Goldthwait (Friedberg and Goldthwait, 1969, Friedberg et al., 1969) with a partly purified enzyme fraction from *E. coli* termed endonuclease II. Papirmeister et al. (1970) demonstrated in this connection that *E. coli* cell extracts incise alkylated DNA at 3-methyladenine residues but not at 7-methylguanine residues, in good agreement with the data of Lawley and Orr (1970) on the relative excision rates of different alkylated purines in vivo.

The major component in early preparations of *E. coli* endonuclease II was found to be an endonuclease activity specific for apurinic sites in DNA, apparently due to an endonucleolytic function of exonuclease III, but this enzyme does not act as an endonuclease at alkylated residues in DNA (Hadi and Goldthwait, 1971; Verly et al., 1973; Weiss, 1976; Kirtikar et al., 1976; Ljungquist and Lindahl, 1977; Gossard and Verly, 1978). The endonuclease II preparations investigated by Goldthwait and co-workers have contained several additional enzymatic activities besides the endonuclease activity for apurinic sites (Kirtikar and Goldthwait, 1974; Kirtikar et al., 1975a,b, 1976), and endonuclease II was therefore recently

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redefined as an enzyme activity that releases O^6-methylguanine, 3-methyladenine, N^6-(12-methylbenz[*a*]anthracenyl-7-methyl)adenine, and N^2-(12-methylbenz[*a*]anthracenyl-7-methyl)guanine in free form from alkylated DNA and in addition cleaves phosphodiester bonds in DNA exposed to alkylating agents or ionizing radiation (Kirtikar et al., 1976).

The discovery of an enzyme that cleaves base–sugar bonds but not phosphodiester bonds in uracil-containing DNA (Lindahl, 1974), uracil-DNA glycosylase,[1] raised the obvious possibility that alkylated DNA could be attacked in the same fashion. In fact, endonuclease II preparations had been found to contain an activity that releases 3-methyladenine, and perhaps O^6-methylguanine, in free form (Kirtikar and Goldthwait, 1974). An enzyme that releases 3-methyladenine from alkylated DNA, 3-methyladenine-DNA glycosylase, was partly purified from *E. coli* cell extracts and shown to be a different enzyme from either uracil-DNA glycosylase or the major endonuclease activity for apurinic sites, as these three activities displayed different fractionation properties and heat labilities (Lindahl, 1976). In this connection, a model was proposed for the incision of deaminated or alkylated DNA by a two-step process involving enzymatic release of the damaged base residue in free form, followed by endonucleolytic attack at the apurinic/apyrimidinic site by a second enzyme, and this model was subsequently experimentally verified for uracil-containing DNA (Lindahl et al., 1977). A 3-methyladenine-DNA glycosylase has recently been purified from *M. luteus* and shown to require the simultaneous presence of an endonuclease for apurinic sites for the introduction of chain breaks in alkylated DNA (Laval, 1977). Here, we describe a 3-methyladenine-DNA glycosylase of *E. coli,* which is similar in many respects to the previously studied DNA glycosylases but differs markedly from endonuclease II.

Experimental Section

Materials. 3-Methyladenine and 3-methylguanine were purchased from Fluka AG, Switzerland, 7-methyladenine was from Vega-Fox Biochemicals, and 7-methylguanine, N^6-methyladenine, and 1-methyladenine were from Sigma. O^6-Methylguanine, synthesized according to the method of Balsiger and Montgomery (1960), was a generous gift from Dr. Magnus Elander, Department of Organic Chemistry, Royal Institute of Technology, Stockholm. 3-Ethyladenine and 7-ethylguanine were prepared from diethyl sulfate treated DNA (Sun and Singer, 1975). [^{3}H]Dimethyl sulfate (4.0 Ci/mmol), *N*-[^{3}H]methyl-*N*-nitrosourea (1.15 Ci/mmol), *N*-[^{14}C]ethyl-*N*-nitrosourea (11.5 mCi/mmol), and $^{32}P_i$ (carrier-free) were purchased from New England Nuclear Corp. 7-Bromo[^{3}H]methyl-12-methylbenz[*a*]anthracene (1.8 Ci/mmol) was a generous gift from Dr. Anthony Dipple, Frederick Cancer Research Center.

[^{3}H]Thymidine-labeled phage PM2 DNA (17 000 cpm/μg) was made according to Masamune et al. (1971). [^{14}C]Purine-labeled *Bacillus subtilis* DNA (50 000 cpm/μg) and [^{3}H]uracil-labeled phage PBS1 DNA (42 000 cpm/μg) were made as described (Lindahl and Nyberg, 1972; Lindahl et al., 1977). Nonradioactive and ^{32}P-labeled *E. coli* DNA were prepared by standard procedures (Lindahl and Nyberg, 1972), and calf thymus DNA was purchased from Worthington.

Phosphocellulose P-11 was purchased from Whatman, and Sephadex G-75 and G-50 were from Pharmacia. DNA–cellulose containing native calf thymus DNA was prepared by the method of Alberts and Herrick (1971), as modified by Riazuddin and Grossman (1977). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) was purchased from Sigma.

Alkylated DNA. Calf thymus DNA or *E. coli* DNA (1.5 mg/mL) was treated with [^{3}H]dimethyl sulfate (1 mCi/mL), *N*-[^{3}H]methyl-*N*-nitrosourea (0.5 mCi/mL), or *N*-[^{14}C]-ethyl-*N*-nitrosourea (0.05 mCi/mL) in 0.25 M potassium cacodylate, pH 7.4/10^{-3} M EDTA[2] at 37 °C for 1 h in the dark. The reaction was terminated by chilling and addition of 2 volumes of ethanol. The DNA was spooled on a glass rod, washed in 70, 80, and 90% ethanol, dissolved in 1 M NaCl/0.01 M Tris-HCl/10^{-3} M EDTA, pH 7.4, at 0 °C and dialyzed against the same buffer for 16 h, followed by an additional 3-h dialysis against buffer without NaCl. The alkylated DNA was stored in several aliquots at −70 °C. Dimethyl sulfate treated DNA preparations had a specific radioactivity of 300–400 cpm/μg, *N*-methyl-*N*-nitrosourea treated DNA 100–200 cpm/μg, and *N*-ethyl-*N*-nitrosourea treated DNA 40 cpm/μg.

[^{3}H]Dimethyl sulfate treated DNA was denatured by mixing the alkylated DNA with an equal volume of 0.3 M NaOH for 1 min at 0 °C, followed by addition of cold 1 M Tris-HCl, pH 7.0, to a final pH of 7.5–8.0. This short alkali treatment is sufficient to cause separation of the DNA strands (Davison, 1967) but does not lead to significant degradation of the alkylated purine residues, as determined by paper chromatography of an acid hydrolysate of the single-stranded DNA.

Analysis of the purine composition of radioactive alkylated DNA was performed by paper chromatography together with authentic markers after hydrolysis of the DNA in 0.1 M HCl for 16 h at 37 °C (Lawley and Thatcher, 1970).

To obtain PM2 DNA containing about one 3-methyladenine residue per covalently closed circular DNA molecule, the PM2 [^{3}H]DNA (40 μg of DNA in 0.24 mL of 0.1 M potassium phosphate, pH 7.5) was mixed with dimethyl sulfate (10 μL of a 0.025 M solution in H_2O, prepared immediately before use) and incubated for 5 min at 37 °C. The reaction mixture was then chilled to 0 °C and rapidly chromatographed on a Sephadex G-50 column (1 × 23 cm) equilibrated with 0.07 M Hepes-KOH, pH 7.5/10^{-3} M EDTA to separate the alkylated DNA from unreacted dimethyl sulfate.

Aryl-alkylated DNA was obtained by incubation of 2 mg/mL calf thymus DNA with 2 × 10^{-5} M 7-bromo[^{3}H]-methyl-12-methylbenz[*a*]anthracene in the dark under the reaction conditions of Rayman and Dipple (1973). At the end of the incubation, most of the aryl-alkylating agent was removed by repeated ether extractions, and the DNA was ethanol-precipitated, dialyzed, and stored at −70 °C as for dimethyl sulfate treated DNA. The hydrocarbon-modified DNA had a specific radioactivity of 50 cpm/μg.

DNA containing enzymatically methylated adenine residues was prepared by incubating nonradioactive *Bacillus subtilis*

[1] The base–sugar bonds in nucleic acids have traditionally been termed N-glycosidic bonds, and enzymes that hydrolyze such bonds in DNA were therefore initially called N-glycosidases (Lindahl, 1974). However, Dr. Waldo E. Cohn, Office of Biochemical Nomenclature, Oak Ridge National Laboratory, has pointed out to us that the above designations are not in agreement with the current Rules for Carbohydrate Nomenclature (1971, *Biochemistry 10,* 3983), in which a glycosidic bond is a linkage through oxygen obtained by replacement of the hydrogen atom of the hemiacetal hydroxyl group, while detachment of the entire hydroxyl group results in a glycosyl bond. Consequently, we have replaced the previously used term "glycosidase" with "glycosylase".

[2] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

DNA with *S*-adenosyl[*methyl*-^{3}H]methionine and T4 DNA methylase as described (Lindahl and Nyberg, 1972). The methylated DNA had a specific radioactivity of 12 000 cpm/μg, and analysis of an acid hydrolysate by paper chromatography in system I showed that more than 99% of the radioactive material was present in the form of N^6-methyladenine.

Irradiated, Depurinated, or Deaminated DNA. PM2 DNA containing an average of one pyrimidine dimer or one apurinic site per molecule and nitrous acid treated [^{14}C]purine-labeled *B. subtilis* DNA were prepared as described (Lindahl and Andersson, 1972; Lindahl et al., 1977). For X-irradiation, nitrogen gas was first bubbled through a solution of PM2[^{3}H]DNA (2 μg/mL) in 0.1 M potassium phosphate/0.01 M Tris-HCl, pH 7.0, for 15 min at 0 °C. The vial was immediately sealed and irradiated for 7 min at a dose rate of 1000 rd/min. A Siemens roentgen unit was the source of 190 kV x rays filtered with aluminum. The X-irradiated DNA was employed as a potential substrate both directly after irradiation and after a 4-h postirradiation incubation period at 37 °C (Kirtikar et al., 1975b).

Reagent Enzymes. E. coli endonuclease IV, 3000-fold purified and free from detectable 3-methyladenine-DNA glycosylase activity, was prepared according to Ljungquist (1977). *E. coli* alkaline phosphatase and egg-white lysozyme were purchased from Worthington and bovine carbonic anhydrase was from Sigma.

Enzyme Assays. The standard reaction mixture (0.1 mL) contains 0.07 M Hepes-KOH (pH 7.6), 10^{-3} M EDTA, 10^{-3} M 2-mercaptoethanol, 5% glycerol, 10 μg of [^{3}H]dimethyl sulfate treated DNA (4000 cpm), and a limiting amount (0.02–0.1 microunits) of enzyme. After 15 min at 37 °C, the reaction mixtures were chilled to 0 °C, and 10 μL of a 0.2% solution of heat-denatured calf thymus DNA, 10 μL of 5 M NaCl, and 300 μL of cold ethanol were added. After 10 min at 0 °C, the samples were centrifuged at 23 000*g* for 30 min, and 300 μL of each supernatant was recovered for determination of radioactivity. One unit of 3-methyladenine-DNA glycosylase is defined here as the amount of enzyme that catalyzes the release of 1 μmol of free 3-methyladenine per minute under the standard reaction conditions.

In several experiments, the ethanol-soluble material was further analyzed by paper chromatography, either by direct analysis of a 100-μL aliquot or after concentration by adsorption to and elution from acid-washed charcoal. The ethanol-precipitated DNA was usually discarded, but in some experiments it was resuspended in 0.1 mL of 0.1 M HCl, incubated at 37 °C for 16 h to release the purine residues, and subjected to paper chromatography.

Assays for endonuclease activity were performed by employing PM2[^{3}H]DNA containing different types of lesions in a filter-binding assay (Center et al., 1970; Riazuddin and Grossman, 1977). Standard reaction mixtures with covalently closed circular PM2 DNA (2 μg) instead of alkylated calf thymus DNA were employed. After incubation with enzyme, 2 mL of pH 12.0 buffer containing 0.1 M sodium phosphate, 0.3 M NaCl, and 0.025 M EDTA was added at 20 °C, followed by addition of 0.4 mL of 2 M Tris-HCl (pH 4.0) and filtering the mixture through nitrocellulose membrane filters. Under these conditions, only DNA that had suffered strand breaks during the initial incubation was retained. After washing of the filters with 0.3 M NaCl/0.03 M trisodium citrate, they were dried and analyzed for radioactivity.

Protein determinations on pooled enzyme fractions were made by the biuret reaction (Gornall et al., 1949) and/or by the more sensitive amido Schwarz method of Schaffner and Weissmann (1973), using bovine serum albumin as a reference.

Sucrose Gradient Centrifugation and Analytical Gel Chromatography. The structure of phage PM2[^{3}H]DNA after incubation with enzymes was analyzed by centrifugation in neutral sucrose gradients (5–20%), containing 0.02 M sodium phosphate, pH 7.0/10^{-3} M EDTA, at 40 000 rpm and 5 °C for 6 h in a Spinco SW41 rotor. Fractions were collected from the bottoms of the tubes directly into vials for scintillation counting.

The sedimentation coefficient of the purified 3-methyladenine-DNA glycosylase was estimated by cosedimentation with alkaline phosphatase (6.3 S), carbonic anhydrase (3.06 S), and lysozyme (2.11 S). The different enzymes were detected by enzyme assays after centrifugation at 45 000 rpm in a Spinco SW 50.1 rotor for 24 h and 5 °C. The sucrose gradients (5–20%) contained 0.5 M NaCl/0.05 M Tris–HCl, pH 7.4/10^{-3} M dithiothreitol.

The Stokes radius of the purified 3-methyladenine-DNA glycosylase was determined by cochromatography with the same three reference enzymes on a Sephadex G-75 column (1.1 × 100 cm), using the same buffer as in the sucrose gradient experiments with the enzymes.

Paper Chromatography. All experiments were performed by descending paper chromatography, employing Whatman 3MM paper. For analysis of slowly migrating substances, the solvent front was allowed to run off the paper. The following solvents are particularly useful for resolution of alkylated purines (Lawley and Thatcher, 1970; Sun and Singer, 1975; Lindahl, 1976): I, 2-propanol-NH_3 (specific gravity 0.88)-H_2O (7:1:2, v/v); II, methanol-concentrated HCl-H_2O (7:2:1, v/v); III, saturated ammonium sulfate solution-0.1 M potassium phosphate, pH 7.2-2-propanol (79:19:2). After drying of the chromatograms, references were localized by their ultraviolet absorption. Strips containing individual samples were then cut transversely in 1-cm pieces. Each such piece was subsequently cut into small fragments, which were transferred to a scintillation counting vial. After elution with 2 mL of H_2O at room temperature overnight, 15 mL of Aquasol (NEN Chemicals) was added and the radioactivity of the fraction determined.

Bacterial Strains. Several mutant derivative strains of *E. coli* K12 were used as sources of cell extracts. The strains AB1157 and AB1886 (*uvrA*) were from P. Howard-Flanders, BW2001 (*xthA*) and BW9101 (*xthA*) from B. Weiss, BD10 (*ung*) from B. Duncan and H. R. Warner, and 1100 (*endA*) from H. Hoffmann-Berling. The strains NH5016 (*xthA*) and AB3027 (*xthA, polA*) have been described (Ljungquist et al., 1976). All strains were grown in liquid broth with active aeration at 37 °C and were harvested in the logarithmic growth phase. Large-scale growth of *E. coli* 1100 was performed in a glucose–salts medium supplemented with 0.2% casamino acids and 0.1% yeast extract, and the harvested and washed bacteria were stored as a frozen paste at −70 °C.

Results

Enzyme Purification. A summary of the purification procedure is given in Table I. All operations were performed at 0–4 °C, unless otherwise stated, and centrifugations were carried out for 30 min at 20 000*g*.

Crude Cell Extract. E. coli 1100 (*endA*, Dürwald and Hoffmann-Berling, 1968) cells were mechanically disintegrated at −25 °C in an X-press (Edebo and Hedén, 1960). Broken cells (80 g) were thawed and gently stirred with 320 mL of 0.05 M Hepes-KOH, pH 7.8/10^{-3} M EDTA/10^{-3} M

TABLE I: Purification of 3-Methyladenine-DNA Glycosylase from 80 g of *E. coli* Cells.

Fraction	Protein (mg)	Sp act. (microunits/mg)	Total act. (milliunits)
(I) Crude extract	8100	3.3	27
(II) Ammonium sulfate	2900	6.1	18
(III) Sephadex G-75	310	34	11
(IV) Phospho-cellulose	6	670	4.0
(V) DNA-cellulose	0.2	9300	1.9

2-mercaptoethanol/5% glycerol for 30 min, and the debris was removed by centrifugation (fraction I, 315 mL).

Streptomycin Treatment and Ammonium Sulfate Fractionation. To fraction I, 315 mL of 5% streptomycin sulfate solution in the extraction buffer was slowly added under gentle stirring. After 1 h, the precipitate was removed by centrifugation. At this high final streptomycin concentration (2.5%), several contaminating enzymes such as *E. coli* exonuclease III are largely removed in the streptomycin precipitate while the 3-methyladenine-DNA glycosylase remains in the supernatant. Solid ammonium sulfate was slowly added to a final concentration of 46% saturation (1.80 M; see Wood, 1976), and the pH was kept at 7.0 to 7.4 by dropwise addition of NH_4OH. After 30 min, the precipitate was removed by centrifugation. Additional ammonium sulfate was added to the supernatant to a final concentration of 67% saturation (2.60 M), and the precipitate formed was collected by centrifugation. This material was suspended in 40 mL of buffer A (1 M NaCl/0.01 M Hepes-KOH/10^{-3} M EDTA/10^{-3} M 2-mercaptoethanol/5% glycerol, pH 7.4) and dialyzed for 4 h against buffer A (fraction II, 62 mL).

Gel Filtration. Fraction II was divided in two equal parts and chromatographed on two columns (3.8 × 105 cm each) of Sephadex G-75 equilibrated with buffer A. 3-Methyladenine-DNA glycosylase was eluted later than most of the protein, and active fractions from both columns were pooled and concentrated tenfold in an Amicon ultrafiltration cell equipped with a Diaflo PM10 membrane (fraction III, 35 mL).

Phosphocellulose Chromatography. A column of phosphocellulose (1.2 × 10 cm) was equilibrated by washing with 8 L of 0.01 M Tris-HCl/10^{-3} M EDTA/10^{-3} M 2-mercaptoethanol/5% glycerol, pH 6.7, at 4 °C (buffer B). Fraction III was dialyzed for 12 h against two changes of buffer B, then the dialysis bag was opened and the pH of the protein solution was adjusted to 6.7 (at 4 °C), and dialysis was continued for 2 h. The solution was then applied to the column at a flow rate of 0.5 mL/min and washed with 75 mL of buffer B. Under these conditions, 60–90% of the 3-methyladenine-DNA glycosylase activity adsorbed to the column in different preparations. The adsorbed enzyme was subsequently eluted by a linear gradient (2 × 200 mL) between buffer B and 0.25 M NaCl/0.01 M Tris-HCl/10^{-3} M EDTA/10^{-3} M 2-mercaptoethanol/5% glycerol, pH 7.6. The 3-methyladenine-DNA glycosylase activity was eluted as a single symmetrical peak at 0.10 M NaCl, before the bulk of the adsorbed protein and before uracil-DNA glycosylase, exonuclease III, and endonuclease IV, which were assayed separately. The most active fractions were pooled and concentrated fourfold by ultrafiltration (fraction IV, 10 mL).

Native DNA-Cellulose Chromatography. Fraction IV was dialyzed for 4 h against 0.01 M Tris-HCl/10^{-3} M EDTA/10^{-3} M 2-mercaptoethanol/5% glycerol, pH 7.6, at 4 °C and applied to a column of DNA-cellulose (0.7 × 5 cm) equilibrated with the same buffer. The column was washed with 5 mL of the buffer, and the enzyme was eluted with a linear gradient (2 × 10 mL) between the column buffer and the same buffer supplemented with 1 M NaCl. The enzyme was eluted as a single peak of activity at 0.5 M NaCl, and the most active fractions were pooled, dialyzed against 0.07 M Hepes-KOH, pH 7.8/10^{-3} M EDTA/2 × 10^{-4} M dithiothreitol/5% glycerol, and stored in several small aliquots at −70 °C (fraction V, 2 mL).

Fraction V contains 3-methyladenine-DNA glycosylase, 2800-fold purified in 7% yield. It is not a homogeneous enzyme, as polyacrylamide gel electrophoresis under nondenaturing conditions (Lindahl et al., 1977) revealed the presence of five protein bands, and only one of these contained enzyme activity after elution. It is not known if the material in this band was homogeneous. The enzyme activity migrated at 0.65 times the rate of bovine serum albumin, so *E. coli* 3-methyladenine-DNA glycosylase is not a basic protein.

Fraction V appears to be free from endonuclease and exonuclease activities, as detailed below, but most preparations were still contaminated by traces of uracil-DNA glycosylase. We have employed either of two methods to completely remove the latter enzyme. In the first approach, fraction V is chromatographed on a small hydroxylapatite column as described (Lindahl, 1976), and 3-methyladenine-DNA glycosylase free from uracil-DNA glycosylase is obtained in about 30% yield. In the second approach, the *E. coli* BD10 *ung* mutant is used as the source of enzyme. As uracil-DNA glycosylase does not release 3-methyladenine from DNA (Lindahl et al., 1977), the fraction V described above was employed in most experiments.

Occurrence. Extracts from several DNA repair-defective *E. coli* strains were assayed for 3-methyladenine-DNA glycosylase. All strains tested had a normal amount (80–120% of wild-type level) of this enzyme activity. The strains investigated include four exonuclease III deficient mutants, *E. coli* BW9101 (*xthA*), BW2001 (*xthA*), AB3027 (*xthA, polA*), and NH5016 (*xthA*), the UV-endonuclease deficient mutant AB1886 (*uvrA*), and the uracil-DNA glycosylase deficient[3] mutant BD10 (*ung*). These data support the previous finding that uracil-DNA glycosylase and 3-methyladenine-DNA glycosylase are two different enzymes (Lindahl, 1976). It has been claimed that strain AB3027 is defective in endonuclease II (Kirtikar et al., 1976), but this strain is clearly not deficient in 3-methyladenine-DNA glycosylase activity.

Molecular Weight. The sedimentation coefficient of 3-methyladenine-DNA glycosylase was determined by cosedimentation with three reference enzymes in a sucrose gradient to be 2.3 S. The Stokes radius was similarly determined by cochromatography with the three reference enzymes on a Sephadex G-75 column to be 20 Å. Assuming a value of $\bar{v}$ = 0.725 g/cm^3 for the partial specific volume of the protein (Martin and Ames, 1961), these data yield a molecular weight of M_r = 19 000 ± 2000 for 3-methyladenine-DNA glycosylase by the Svedberg equation (Siegel and Monty, 1966). The subunit structure of the enzyme is not known, but in view of its small size it is probably a monomer.

[3] The *E. coli* BD10 strain is not totally deficient in uracil-DNA glycosylase in our experience, but cell extracts contain a 200-fold lower level of this enzyme activity than extracts from *ung*$^+$ strains when assayed under our standard conditions (Lindahl et al., 1977). The residual activity has the same chromatographic properties on Sephadex G-75 as the wild-type enzyme.

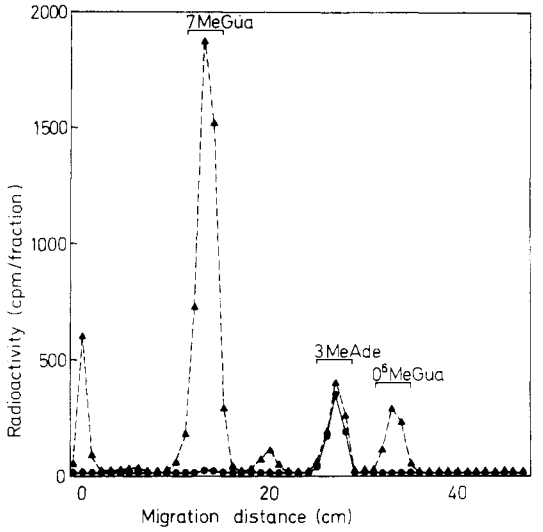

FIGURE 1: Paper chromatographic analysis in 2-propanol–concentrated NH_4OH–H_2O (7:1:2) of radioactive products released from *N*-[^{3}H]-methyl-*N*-nitrosourea-treated *E. coli* DNA by mild acidic hydrolysis (0.1 M HCl, 37 °C, 16 h) or released as ethanol-soluble material at neutral pH by 3-methyladenine-DNA glycosylase, (●) (see text for details). The authentic markers were localized as ultraviolet-absorbing material.

General Requirements. The 3-methyladenine-DNA glycosylase has a broad pH optimum at pH 7.2–7.8 and displays similar activity in either Hepes-KOH or Tris-HCl buffers. The enzyme shows no obligatory cofactor requirement, but addition of $MgCl_2$ (0.005–0.015 M) to reaction mixtures causes a slight stimulation (about 30%) over the activity observed in the presence of 10^{-3} M EDTA. No stimulatory effect was found with $CaCl_2$. Addition of 10^{-3} M P_i or 10^{-3} M ATP to reaction mixtures has no detectable effect. The enzyme contains essential sulfhydryl groups, because addition of 0.005 M *N*-ethylmaleimide to the standard reaction mixture caused 32% inhibition, while 10^{-3} M *p*-mercuribenzoate gave 93% inhibition (in the absence of mercaptoethanol). The activity is not very sensitive to addition of neutral salts, but 50% inhibition of the activity was obtained with 0.3 M KCl in the reaction mixture.

Activity on Alkylated DNA. When an excess of the enzyme (0.3 or 3 microunits) was incubated with [^{3}H]dimethyl sulfate treated DNA in the standard reaction mixture, 20% of the total label was released in ethanol-soluble form. No further release of radioactivity could be obtained by increasing the enzyme concentration. Base analysis of an acid hydrolysate of the alkylated DNA employed as substrate by paper chromatography showed that 76% of the label was present as 7-methylguanine, while 21% was present as 3-methyladenine. All the enzymatically released material had chromatographic properties of authentic 3-methyladenine in three different solvent systems, while no detectable amounts of 7-methylguanine were actively released.

Similar results were obtained with DNA treated with *N*-[^{3}H]methyl-*N*-nitrosourea. This strongly mutagenic and carcinogenic alkylating agent induces the formation of significant amounts of O^6-methylguanine and phosphotriesters in DNA, in addition to 7-methylguanine and 3-methyladenine. Base analysis of an acid DNA hydrolysate in system I showed that 10% of the label remained at the origin, which is the expected position of phosphotriesters and alkylated pyrimidine nucleotides, 64% cochromatographed with 7-methylguanine, 13% with 3-methyladenine, and 10% with O^6-methylguanine (Figure 1). Treatment of this substrate with an excess of enzyme (0.3 or 3 microunits/0.1 mL) under the standard reaction conditions caused the release of 11% of the total radioactive material in ethanol-soluble form, and all this material cochromatographed with 3-methyladenine (Figure 1). Thus, there was no detectable enzymatic release of either O^6-methylguanine or 7-methylguanine. As O^6-methylguanine is actively removed from *E. coli* DNA in vivo in contrast to 7-methylguanine (Lawley and Orr, 1970) and enzymatic release of this alkylation product in free form in vitro has been reported (Kirtikar and Goldthwait, 1974), we have made several attempts to detect a glycosylase activity that would release O^6-methylguanine from DNA, but the results have been uniformly negative. Inclusion of $MgCl_2$ or several other potential cofactors in reaction mixtures did not stimulate the 3-methyladenine-DNA glycosylase to release O^6-methylguanine from DNA. Further, neither fraction I, III, or V of our enzyme preparations caused detectable release of O^6-methylguanine from either *N*-methyl-*N*-nitrosourea-treated DNA or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated DNA. We conclude that *E. coli* 3-methyladenine-DNA glycosylase does not release O^6-methylguanine from alkylated DNA.

In addition to the alkylation products described above, small amounts of 7-methyladenine and 3-methylguanine are also formed after treatment of DNA with methylating agents (Lawley, 1976). We have analyzed the ability of 3-methyladenine-DNA glycosylase to release these alkylation products by chromatographing the ethanol-soluble material obtained after incubation with the enzyme, as well as an acid hydrolysate of the ethanol precipitate recovered after the incubation, in system I followed by chromatography in system II. Neither of these two compounds is effectively released by the enzyme, and when over 90% of the 3-methyladenine in the DNA had been released less than 10% of either the 7-methyladenine or the 3-methylguanine had been enzymatically liberated. These minor alkylation products in DNA clearly are not good substrates for 3-methyladenine-DNA glycosylase, but the available data do not exclude that 3-methylguanine could be released at a very slow rate by the enzyme. It is noted that 3-methylguanine is actively released by *E. coli* in vivo, although at a considerably slower rate than 3-methyladenine, while 7-methyladenine only appears to be released by nonenzymatic hydrolysis (Lawley and Warren, 1976).

The release of 3-methyladenine from alkylated DNA is markedly dependent on the secondary structure of the DNA. While double-stranded DNA is an effective substrate, single-stranded DNA is only attacked at a very slow rate (Figure 2). These results contrast markedly with the release of uracil from DNA by uracil-DNA glycosylase, as that enzyme effectively attacks both native and denatured uracil-containing DNA but degrades the latter substrate at a two- to threefold higher rate.

Fraction V is also active on *N*-ethyl-*N*-nitrosourea-treated DNA and releases 3-ethyladenine at a similar rate as 3-methyladenine from alkylated DNA. The results obtained are quite similar to those obtained with *N*-methyl-*N*-nitrosourea-treated DNA. Thus, base analysis of an acid hydrolysate of *N*-[^{14}C]ethyl-*N*-nitrosourea-treated DNA revealed that 18% of the radioactive material chromatographed as 7-ethylguanine and 5% as 3-ethyladenine, employing authentic markers. In addition, a well-separated, fast-migrating peak of material (6%) had the expected R_f value of O^6-ethylguanine (Sun and Singer, 1975), and a large proportion of the radioactive material did not migrate in system I. It is known that the major alkylation product in *N*-ethyl-*N*-nitrosourea-treated DNA is phosphotriesters (Sun and Singer, 1975). Treatment of this substrate with fraction V (0.1–3 microunits) under the standard reaction conditions released about 4% of the total

label in ethanol-soluble form, and all this material had the chromatographic properties of 3-ethyladenine, while no release of 7-ethylguanine or O^6-ethylguanine could be detected. The activity observed on ethylated DNA comigrated with the activity on methylated DNA on further purification of fraction V by gel electrophoresis under nondenaturing conditions, and both kinds of substrates are equally effectively attacked, so we conclude that 3-methyladenine-DNA glycosylase also is active on ethylated DNA. However, as we do not know if the enzyme would also release adenine derivatives with larger alkyl groups in the 3 position, we prefer not to rename it 3-alkyladenine-DNA glycosylase at this point but retain the previously used designation of 3-methyladenine-DNA glycosylase.

When DNA is aryl alkylated by treatment with 7-bromomethyl-12-methylbenz[*a*]anthracene, the alkylated purine residues obtained are N^2-(12-methylbenz[*a*]anthracenyl-7-methyl)guanine and N^6-(12-methylbenz[*a*]anthracenyl-7-methyl)adenine, while there is no alkylation at the 3 position (Rayman and Dipple, 1973). It has been reported that such alkylated purines are actively released in the free form by treatment of aryl-alkylated DNA with purified *E. coli* endonuclease II (Kirtikar et al., 1975a). When such aryl-alkylated DNA, obtained by treatment of calf thymus DNA with small amounts of 7-bromo[^{3}H]methyl-12-methylbenz[*a*]anthracene under the conditions of Rayman and Dipple (1973), was tried here as a potential substrate for fraction V of 3-methyladenine-DNA glycosylase, no detectable radioactivity was released in ethanol-soluble form even by high concentrations of the enzyme, either in the absence or presence of 0.01 M $MgCl_2$. Similarly, fractions I or III of the enzyme also failed to act on this substrate in the expected fashion. We conclude that 3-methyladenine-DNA glycosylase does not liberate the bulky purine adducts obtained after arylalkylation of DNA. It should be noted that the active release of such adducts in vivo is dependent on the *uvr* gene products in *E. coli* (Venitt and Tarmy, 1972), so it does not seem likely that 3-methyladenine-DNA glycosylase plays any role in their removal.

N^6-Methyladenine occurs naturally as a minor base in the DNA of *E. coli*, and we have also tried DNA containing such residues as a substrate for the 3-methyladenine-DNA glycosylase. The substrate was obtained by enzymatic methylation of DNA with purified T4 DNA methylase in vitro in the presence of ^{3}H-labeled *S*-adenosylmethionine. There was no detectable release of this methylated derivative of adenine (less than 0.1% of the total amount of N^6-methyladenine) under our standard reaction conditions, employing high concentrations of 3-methyladenine-DNA glycosylase (0.3–3 microunits/0.1 mL). Moreover, no detectable release of radioactivity (less than 0.1%) occurred when native, unmodified *B. subtilis* DNA, radioactively labeled in the adenine and guanine residues, was incubated with fraction V under the standard reaction conditions.

Lack of Activity on Irradiated, Depurinated, or Deaminated DNA. The ability of fraction V to introduce chain breaks in different types of damaged Dna was investigated by using modified covalently closed circular PM2 DNA as a substrate in a convenient filter-binding assay (Center et al., 1970; Riazuddin and Grossman, 1977). Several different concentrations of the enzyme (0.1–3 microunits/mL) were employed. In these experiments, there was no detectable cleavage of unmodified PM2 DNA (less than 0.1 strand break per molecule) even with the highest concentrations of enzyme. Similarly, ultraviolet-irradiated DNA, which became sensitized to a UV-endonuclease from *M. luteus* (Lindahl et al., 1977), remained resistant to 3-methyladenine-DNA glycosylase in this assay. X-irradiation of the DNA caused a substantial part (30% directly after incubation, 50% after a 4-h postirradiation incubation at 37 °C) of the DNA to be converted to a nicked circular form, as judged from the greatly increased background of filter-bound DNA from reaction mixtures without enzyme, but there was no detectable enzyme-catalyzed chain cleavage (less than 0.2 chain breaks per molecule introduced by 3 microunits of fraction V under standard reaction conditions) of the remaining covalently closed circular, irradiated DNA, when tried as a substrate either directly after irradiation or after a 4-h postirradiation incubation at 37 °C. However, less purified fractions of the enzyme did attack the X-irradiated DNA under the same conditions. Part of this activity may be ascribed to endonucleases acting at apurinic/apyrimidinic sites, but in addition an endonuclease activity that apparently recognizes a different lesion in X-irradiated DNA was present and showed similar chromatographic properties as the 3-methyladenine-DNA glycosylase. This endonuclease, which will be described in greater detail elsewhere (S. Riazuddin, in preparation), eluted from phosphocellulose at only a slightly higher ionic strength than the glycosylase but was completely removed by the DNA-cellulose chromatography step.

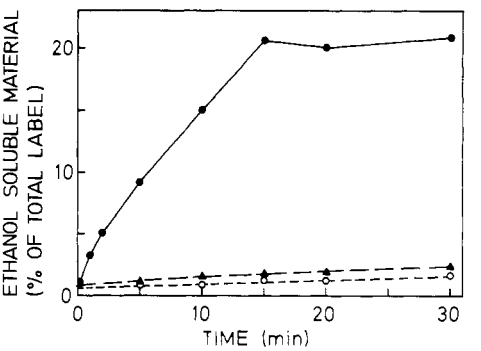

FIGURE 2: Kinetics of release of 3-methyladenine from [^{3}H]dimethyl sulfate treated calf thymus DNA by 3-methyladenine-DNA glycosylase. The reaction mixture contained 1 microunit of enzyme/mL and either double-stranded alkylated DNA (●–●) or single-stranded alkylated DNA (▲- -▲) as substrate. Controls incubated without enzyme (○- -○) containing single-stranded alkylated DNA are shown, and similar data were obtained with double-stranded alkylated DNA.

No radioactive material (<0.1%) was released from DNA, labeled in the purine residues and partly deaminated by nitrous acid treatment, by 3-methyladenine-DNA glycosylase (3 microunits in 0.1 mL). Consequently, the enzyme does not release hypoxanthine or xanthine in free form from deaminated DNA.

K_M Value and Mechanism of Action. The amount of 3-methyladenine-DNA glycosylase activity in *E. coli* cell extracts appears low; for comparison, it is noted that the level of uracil-DNA glycosylase activity is about 300-fold higher in crude extracts. However, variation of the assay conditions, or addition of presumptive cofactors to extracts or partially purified enzyme preparations, have failed to increase the amounts of 3-methyladenine-DNA glycosylase activity detected, with the exception of the very slight stimulation obtained by addition of Mg^{2+} to reaction mixtures. Further, the K_M value for 3-methyl-dAMP residues in DNA is quite low, 6×10^{-9} M, as determined by varying the concentration of alkylated DNA containing a known amount of modified adenine residues in the standard reaction mixture, so the amount of substrate in the assay is not a limiting factor. Similar low K_M values have been observed for other enzymes active in DNA repair, e.g., the *M. luteus* endonucleases that selectively act at pyrimidine dimers in DNA (Riazuddin and Grossman, 1977). The low K_M value observed here implies that DNA adenine residues al-

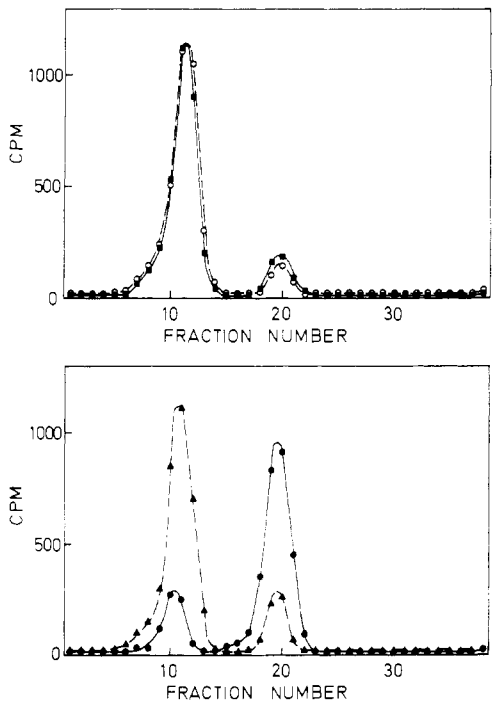

FIGURE 3: Introduction of chain scissions in dimethyl sulfate treated PM2 [^{3}H]DNA by the concerted action of *E. coli* 3-methyladenine-DNA glycosylase and endonuclease IV. The DNA was incubated under standard reaction conditions without enzyme (○), with 3-methyladenine-DNA glycosylase (■), with endonuclease IV (▲), or with both enzymes (●), and the different reaction mixtures were then analyzed for the presence of strand breaks by neutral sucrose gradient centrifugation. The direction of sedimentation is from right to left.

kylated in the 3 position are the "true" substrates of the enzyme. The low activity of the enzyme could then simply be due to the fact that very few enzyme molecules may be present per cell or that the enzyme might have a low turnover number. There is presently no direct information on these points, as the enzyme has not been purified to homogeneity.

When $^{32}P_i$ (10^{-5} M, 10^8 cpm/μmol) was included in standard reaction mixtures, less than 0.1 phosphate residue was found to be incorporated into DNA for each 3-methyladenine residue enzymatically released. These data are analogous to those previously obtained with uracil-DNA glycosylase (Lindahl et al., 1977) and show that 3-methyladenine-DNA glycosylase acts by a hydrolytic, not a phosphorolytic, mechanism.

Fraction V is free from detectable DNA exonuclease activity, as less than 0.1% of the radioactivity of [^{32}P]DNA was released by 3 microunits of the enzyme under the standard assay conditions, in the presence or absence of 0.01 M $MgCl_2$.

Product Inhibition. The 3-methyladenine-DNA glycosylase is increasingly inhibited if reaction mixtures are supplemented with free 3-methyladenine, and 50% inhibition is observed with 8×10^{-4} M 3-methyladenine. On the other hand, 2×10^{-3} M 3-methylguanine or 5×10^{-3} M of either O^6-methylguanine or 7-methyladenine caused less than 10% inhibition, in agreement with the notion that such residues in DNA are not effective substrates for the enzyme. 3-Methylguanine was not studied at higher concentrations because of its poor solubility. Free adenine, 1-methyladenine, 7-methylguanine, caffeine, or uracil, at a concentration of 5×10^{-3} M, also failed to inhibit 3-methyladenine-DNA glycosylase.

Enzymatic Formation of Apurinic Sites in Alkylated DNA. Uracil-DNA glycosylase introduces apyrimidinic sites but no chain breaks in DNA containing uracil residues (Lindahl et al., 1977), and Laval (1977) has recently shown that *M. luteus* 3-methyladenine-DNA glycosylase acts by the formation of apurinic sites but no strand incisions in DNA. On the other hand, it has been reported that *E. coli* endonuclease II cleaves phosphodiester bonds at the same time as it releases alkylated purines from DNA (Kirtikar and Goldthwait, 1974; Kirtikar et al., 1976). Here, we have briefly treated covalently closed circular PM2 [^{3}H]DNA with 10^{-3} M dimethyl sulfate to introduce about one 3-methyladenine residue (and five 7-methylguanine residues) per DNA molecule. The alkylated DNA was then either exposed to 3-methyladenine-DNA glycosylase (10 microunits/mL), to *E. coli* endonuclease IV (600 units/mL), or to both enzymes. *E. coli* endonuclease IV cleaves DNA specifically at apurinic and apyrimidinic sites but does not release 3-methyladenine from alkylated DNA (Ljungquist, 1977). The DNA was then immediately analyzed by neutral sucrose gradient centrifugation to separate covalently closed and nicked circular DNA from each other. The results are shown in Figure 3. In the absence of added enzymes, the alkylated PM2 DNA contained 0.08 strand break per molecule, assuming a Poisson distribution, while DNA exposed to 3-methyladenine-DNA glycosylase had 0.12 strand break per molecule and DNA exposed to endonuclease IV had 0.17 strand break per molecule, respectively. However, the DNA exposed to both enzymes contained 1.6 strand breaks per molecule. These data show that both enzymes are required for effective introduction of strand breaks in DNA and that 3-methyladenine DNA-glycosylase introduces less than 0.1 incision in alkylated DNA for each 3-methyladenine residue released. These data are similar to those previously obtained with *E. coli* uracil-DNA glycosylase and *M. luteus* 3-methyladenine-DNA glycosylase (Lindahl et al., 1977; Laval, 1977).

When PM2 DNA containing about one apurinic site per molecule, introduced by pH 5 treatment, but no alkylated residues was instead used as a substrate in the same type of experiment, endonuclease IV introduced 0.8 strand incision by itself. 3-Methyladenine-DNA glycosylase had no significant activity (less than 0.1 strand break per molecule formed), and the combination of both enzymes was not more effective than endonuclease IV by itself, as 0.8 strand break per DNA molecule was still obtained in this case.

Discussion

The *E. coli* 3-methyladenine-DNA glycosylase described here resembles the previously described uracil-DNA glycosylase in several respects, although they are two different proteins. Both enzymes are of relatively low molecular weight, do not require divalent metal ions for activity, and recognize only one particular type of abnormal nucleotide residue in DNA, dUMP residues vs. 3-methyl-dAMP and 3-ethyl-dAMP residues, with great selectivity. Thus, unusual DNA nucleotides of related structure, in the present case 7-methyl-dAMP, 7-methyl-dGMP, N^6-methyl-dAMP, O^6-methyl-dGMP, and the corresponding ethylated derivatives, are not substrates, while 3-methyl-dGMP residues are cleaved very slowly, if at all. These findings clearly suggest that additional DNA glycosylases exist, which like the already described enzymes may be small, highly specialized enzymes with little versatility in recognizing and removing different kinds of damaged bases from DNA. This type of repair clearly differs from that depending on the *uvr* genes of *E. coli*, in which several gene products apparently interact to remove a variety of chemically

very different types of bulky lesions from DNA, e.g., pyrimidine dimers, cross-linked residues, and aryl-alkylated purines.

A 3-methyladenine-DNA glycosylase from *M. luteus* has recently been purified and characterized (Laval, 1977), and that enzyme and the one described here have very similar properties. Both enzymes bind tightly to DNA–cellulose and DNA–agarose columns, and they only introduce chain breaks in alkylated DNA if a DNA endonuclease for apurinic sites is simultaneously present. A preliminary report of a human DNA glycosylase acting on alkylated DNA in a similar fashion has also appeared (Brent, 1977). On the other hand, the *E. coli* 3-methyladenine-DNA glycosylase differs in many respects from the endonuclease II described by Goldthwait and coworkers (Kirtikar et al., 1976), although both activities release free 3-methyladenine from DNA. It is presently unclear to us if 3-methyladenine-DNA glycosylase and endonuclease II are simply two different enzymes, or if endonuclease II preparations contain several different enzymes including 3-methyladenine-DNA glycosylase. Some of the more important differences between these activities are as follows: (a) endonuclease II has a molecular weight of 33 000 determined by gel filtration of native enzyme and 34 500 determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, while native 3-methyladenine-DNA glycosylase has a molecular weight of 19 000 ± 2000; (b) endonuclease II releases O^6-methylguanine in free form from DNA, while 3-methyladenine-DNA glycosylase does not catalyze this reaction; (c) endonuclease II releases the bulky aryl-alkylated purine residues obtained by 7-bromomethyl-12-methylbenz[*a*]anthracene treatment from DNA, while 3-methyladenine-DNA glycosylase does not catalyze this reaction either; (d) endonuclease II incises X-irradiated DNA, while 3-methyladenine-DNA glycosylase has no associated nuclease activity; (e) endonuclease II is reported to be missing in the *E. coli* strain AB3027 (*xthA, polA*), while extracts of this strain have normal 3-methyladenine-DNA glycosylase activity; (f) endonuclease II, but not 3-methyladenine-DNA glycosylase, is strongly inhibited by EDTA. We have not been able to detect an enzyme with the described properties of endonuclease II during the course of these studies, and further work is clearly required to resolve these differences.

The alkyl group of 3-methyladenine and 3-ethyladenine is located in the minor groove of the Watson–Crick double helix, while the methyl group of 7-methylguanine, O^6-methylguanine, or the naturally occurring derivative N^6-methyladenine is found in the major groove. One possible role of the enzyme described here is to keep the minor groove of double helical DNA free from alkyl groups, introduced by alkylating agents or perhaps enzymatically. In this regard, it is noted that the 3-methyladenine–deoxyribose bond is labile in vitro at neutral pH but still has a half-life of about 30 h at 37 °C (Lawley and Orr, 1970; Margison and O'Connor, 1973). This corresponds to several cell generations for rapidly growing *E. coli* cells, so it is not unreasonable that a repair enzyme would be required to speed up the process if 3-methyladenine residues cannot be well tolerated in DNA. While a 3-methyl group on adenine will not interfere directly with DNA base pairing, there is presently very little information available on the degree of accuracy with which cellular polymerases copy a DNA template containing 3-methyladenine residues. Studies on *E. coli* mutants with a defective 3-methyladenine-DNA glycosylase should help to clarify the physiological roles of the enzyme, and two such *E. coli* strains, sensitive to alkylating agents in vivo, have now been isolated (P. Karran, T. Lindahl, I. Ofsteng, and E. Seeberg, in preparation).

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References

Alberts, B., and Herrick, G. (1971), *Methods Enzymol. 21D,* 198–217.

Balsiger, R. W., and Montgomery, J. A. (1960), *J. Org. Chem. 25,* 1573–1575.

Brent, T. P. (1977), *Nucleic Acids Res. 4,* 2445–2454.

Center, M. S., Studier, F. W., and Richardson, C. C. (1970), *Proc. Natl. Acad. Sci. U.S.A. 65,* 242–248.

Davison, P. F. (1967), *Biopolymers 5,* 715–721.

Dürwald, H., and Hoffmann-Berling, H. (1968), *J. Mol. Biol. 34,* 331–346.

Edebo, L., and Hedén, C. G. (1960), *J. Biochem. Microbiol. Technol. Eng. 2,* 113–120.

Friedberg, E., and Goldthwait, D. A. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 934–940.

Friedberg, E., Hadi, S. M., and Goldthwait, D. A. (1969), *J. Biol. Chem. 244,* 5879–5889.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177,* 751–766.

Gossard, F., and Verly, W. G. (1978), *Eur. J. Biochem., 82,* 321–332.

Goth-Goldstein, R. (1977), *Nature (London) 267,* 81–82.

Hadi, S. M., and Goldthwait, D. A. (1971), *Biochemistry 10,* 4986–4994.

Kirtikar, D. M., Cathcart, G. R., and Goldthwait, D. A. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 4324–4328.

Kirtikar, D. M., Dipple, A., and Goldthwait, D. A. (1975a), *Biochemistry 14,* 5548–5553.

Kirtikar, D. M., and Goldthwait, D. A. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2022–2026.

Kirtikar, D. M., Slaughter, J., and Goldthwait, D. A. (1975b), *biochemistry 14,* 1235–1244.

Laval, J. (1977), *Nature (London) 269,* 829–832.

Lawley, P. D. (1974), *Mutat. Res. 23,* 283–295.

Lawley, P. D. (1975), Screening Tests in Chemical Carcinogenesis, Montesano, R., Bartsch, H., and Tomatis, L., Ed., Lyon, France, Int. Agency Res. Cancer, pp 181–210.

Lawley, P. D., and Orr, D. J. (1970), *Chem.-Biol. Interact. 2,* 154–157.

Lawley, P. D., and Thatcher, C. J. (1970), *Biochem. J. 116,* 693–707.

Lawley, P. D., and Warren, W. (1975), *Chem.-Biol. Interact. 11,* 55–57.

Lawley, P. D., and Warren, W. (1976), *Chem.-Biol. Interact. 12,* 211–220.

Lindahl, T. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3649–3653.

Lindahl, T. (1976), *Nature (London) 259,* 64–66.

Lindahl, T., and Andersson, A. (1972), *Biochemistry 11,* 3618–3623.

Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B., and Sperens, B. (1977), *J. Biol. Chem. 252,* 3286–3294.

Lindahl, T., and Nyberg, B. (1972), *Biochemistry 11,* 3610–3618.

Ljungquist, S. (1977), *J. Biol. Chem. 252,* 2808–2814.

Ljungquist, S., and Lindahl, T. (1977), *Nucleic Acids Res. 4,* 2871–1879.

Margison, G. P., and O'Connor, P. J. (1973), *Biochim. Biophys. Acta 331,* 349-356.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372-1379.
Masamune, Y., Fleischman, R. A., and Richardson, C. C. (1971), *J. Biol. Chem. 246,* 2680-2691.
Papirmeister, B., Dorsey, J. K., Davison, C. L., and Gross, C. L. (1970), *Fed. Proc., FED Am. Soc. Exp. Biol. 29,* 727.
Prakash, L., and Strauss, B. (1970), *J. Bacteriol. 102,* 760-766.
Rayman, M. P., and Dipple, A. (1973), *Biochemistry 12,* 1202-1207.
Reiter, H., Strauss, B., Robbins, M., and Marone, R. (1967), *J. Bacteriol. 93,* 1056-1062.
Riazuddin, S., and Grossman, L. (1977), *J. Biol. Chem. 252,* 6280-6286.
Schaffner, W., and Weissmann, C. (1973), *Anal. Biochem. 56,* 502-514.
Siegel, L. M., and Monty, K. J. (1966), *Biochim. Biophys. Acta 112,* 346-362.
Strauss, B. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 1670-1675.
Strauss, B., and Robbins, M. (1968), *Biochim. Biophys. Acta 161,* 68-75.
Strauss, B., Scudiero, D., and Henderson, E. (1975), Molecular Mechanisms for Repair of DNA, Part A, Hanawalt, P. C., and Setlow, R. B., Ed., New York, N.Y., Plenum Press, pp 13-24.
Sun, L., and Singer, B. (1975), *Biochemistry 14,* 1795-1802.
Venitt, S., and Tarmy, E. M. (1972), *Biochim. Biophys. Acta 287,* 38-51.
Verly, W. G., Paquette, Y., and Thibodeau, L. (1973), *Nature (London), New Biol. 244,* 67-69.
Weiss, B. (1976), *J. Biol. Chem. 251,* 1896-1901.
Wood, W. I. (1976), *Anal. Biochem. 73,* 250-257.

Studies of Virus Structure by Laser-Raman Spectroscopy. Turnip Yellow Mosaic Virus and Capsids†

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ABSTRACT: Laser-Raman spectroscopy of the turnip yellow mosaic virus (TYMV) and its capsid indicate the following features of the structure and assembly of the virion. The secondary structure of coat-protein molecules in TYMV is comprised of 9 ± 5% α-helix, 43 ± 6% β-sheet, and 48 ± 6% irregular conformation and is not altered by the removal of the RNA from the capsid. Introduction of as many as 200 chain scissions per RNA molecule also does not affect the overall secondary structure of the encapsulated RNA, which is 77 ± 5% in the A-helix form. Tryptophan and cysteine residues of the coat protein appear to be in contact with the solvent, while only one of three tyrosines per coat protein is available for hydrogen bonding of its *p*-hydroxyl group with H_2O molecules. Both cytosine and adenine residues of TYMV RNA are protonated in substantial numbers near pH 4.5, suggesting elevation of their respective pK_a values within the virion. The Raman data are consistent with chemical evidence favoring interaction between protonated bases of RNA and amino acid side chains of coat protein in TYMV.

TYMV[1] is composed of 180 identical coat-protein molecules which reside in an icosahedral shell or capsid. The capsid contains one molecule of RNA which appears to be in contact with the protein. The RNA is rich in cytosine bases (22.4% A, 17.2% G, 38.3% C, and 22.1% U) and tends to slowly degrade in the isolated virion. RNA-free capsids can be produced by treatment of TYMV with alkali (Kaper, 1975).

The coat-protein molecule contains 189 amino acid residues in known sequence (Kaper, 1975). Empirical methods predict a secondary structure which contains 16% α-helix, 41% β-sheet, and 43% irregular structure (Turano et al., 1976). The distribution of amino acid residues along the chain partially segregates into hydrophobic and hydrophilic regions. The stability of the capsid and protein structure has been analyzed in terms of hydrophobic interactions and specific interactions involving the 13 acidic residues (Glu plus Asp), the 10 basic residues (Lys plus Arg), and the 4 Cys, 3 Tyr, and 2 Trp residues. The stabilizing interactions in the virion may include hydrogen bonding between the OH groups of Tyr and $-CO_2^-$ groups of Glu or Asp and, at lower pH values, specific hydrogen bonds between $-CO_2H$ and protonated cytosine residues (Kaper, 1975).

The Raman spectrum of a virus contains bands or "lines" due to light scattered in transitions involving the vibrations of molecular subgroups. The frequencies and intensities of these lines are influenced by the secondary structure of the macromolecule, hydrogen-bonding interactions, and the environment of the molecular subgroups in question. Therefore, the Raman spectrum potentially contains considerable information about structure and interactions (Thomas et al., 1976; Hartman et

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[1] Abbreviations used are: TYMV, turnip yellow mosaic virus; rRNA, ribonucleic acid from ribosomes; $\Delta\sigma$, spectral slit width.

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