Enzymatic Excision of 3-Methyladenine and 7-Methylguanine by a Rat Liver Nuclear Fraction†

Richard Cathcart‡ and David A. Goldthwait*

ABSTRACT: A 3-methyladenine–DNA *N*-glycosylase was identified in both nuclear and cytoplasmic extracts of rat liver. The enzyme was purified from nuclei due to a lower level of nonspecific nucleases. The enzyme was purified 100-fold by using DNA–cellulose and phosphocellulose chromatography. Instability of the enzyme prevented further purification. Several methods of assay were developed and are discussed. The molecular weight of the glycosylase was 24 000. It exhibited a preference for double-stranded alkylated DNA. The apparent K_m for 3-methyladenine residues was 2.6 nM. The enzyme was stimulated maximally by the addition of 40 mM NaCl. The addition of 1 mM EDTA had no effect on activity, but Mg^{2+} did stimulate base release. The enzyme was shown to be a glycosylase by the stoichiometric release of 3-methyladenine and the appearance of alkali-sensitive sites. There was no endonucleolytic activity on double-stranded DNA, depurinated DNA, UV-treated DNA, or γ-irradiated DNA. The phosphocellulose fraction was contaminated with an endonuclease activity specific for single-stranded DNA which could be removed by chromatography on Sephadex G-100. The enzyme preparation also released 7-methylguanine, and the rate of release was approximately 15% of that of 3-methyladenine. The relative rate of release of 3-methyladenine and 7-methylguanine was the same for both the DNA–cellulose and phosphocellulose fractions, and both activities were inactivated by heat to the same extent. Whether one or two enzymes are involved is not known. The preparation does not release 3-methylguanine or 1-methyladenine.

The repair of cellular DNA is mediated by a number of enzymes. The initial enzymatic event involved in base excision repair is the hydrolysis of the carbon–nitrogen bond between the altered purine or pyrimidine and the pentose moiety by a DNA *N*-glycosylase. The resulting apurinic or apyrimidinic site is then incised by an endonuclease with excision repair proceeding by the action of an exonuclease, DNA polymerase, and DNA ligase (Lindahl, 1976; Laval, 1977). Although this model was proposed for bacterial DNA repair, it may serve as a more general model for eucaryotic repair. Purine base insertion has recently been described and may serve as an alternative pathway for repair of apurinic sites (Deutsch & Linn, 1979a,b).

DNA glycosylases have been isolated from bacteria which recognize 3-meAd (Riazuddin & Lindahl, 1978), uracil (Lindahl et al., 1977), 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine (Chetsanga & Lindahl, 1979), and hypoxanthine (Karren & Lindahl, 1978) residues in DNA. These enzymes all have relatively small molecular weights and do not require any metal ion or cofactor for activity.

Recently a glycosylase specific for 3-meAd[1] has been partially purified from human lymphoblasts (Brent, 1979) and a glycosylase specific for uracil has been obtained from calf thymus (Talpaert-Barie et al., 1979). Neither enzyme appears to be significantly different from its bacterial counterpart, except for a slightly greater molecular weight.

We report here the partial purification of a 3-meAd–DNA glycosylase from rat liver nuclei. The enzyme exhibits several properties distinct from those of both the bacterial and human enzyme. The preparation also releases 7-methylguanine from alkylated DNA.

Experimental Procedures

Materials

Substrates. [^{3}H]Methylnitrosourea (MNU) DNA was obtained by alkylation of calf thymus DNA (Calbiochem, La Jolla, CA) (2 mg/mL in 0.02 M Ammediol buffer, pH 10) with *N*-[^{3}H]methyl-*N*-nitrosourea (1.44 Ci/mmol, New England Nuclear Corp., Boston, MA); 1 mL of the DNA solution was reacted with 0.2 mCi of [^{3}H]MNU for 15 min at 37 °C. The solution was then cooled to 0 °C, and 0.1 volume of 2.5 M sodium acetate and then 2 volumes of cold ethanol were added. The DNA was recovered on a glass rod and washed 4 times in 10-mL aliquots of ethanol and 5 times in 10-mL aliquots of ether. The alkylated DNA was suspended in 10 mL of 0.05 M Tris-HCl, pH 7.6, and dialyzed against the same buffer. The specific activity of the DNA was between 3 and 4 × 10^3 cpm/nmol of DNA nucleotide. The DNA solution was stored at −20 °C, and no significant loss of methylated bases was observed during a period of 1 month. A higher yield of alkylated bases was obtained at the higher pH. For example, at pH 7.5 and 8.0 in 0.02 M Tris-HCl the yields were 0.24 and 0.44% while at pH 9 and 10 in 0.02 M ammediol buffer the yields were 0.74 and 0.81%.

[^{3}H]Thymine-labeled *E. coli* DNA was prepared by the method of Smith (1967). [^{3}H]Thymine-labeled T7 phage DNA was purified from T7 phage grown on *E. coli* B3 in the presence of [^{3}H]thymidine (Richardson et al., 1964; Richardson, 1966).

DNA–cellulose was prepared by (Alberts et al. (1971) using calf thymus DNA (Calbiochem, La Jolla, CA.). The DNA

† From the Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106. *Received May 19, 1980; revised manuscript received September 3, 1980.* Supported by grants from the National Institutes of Public Health (CA-11322 and CA-18747) and by a contract with the Department of Energy (EY-76-S-02-2725). D.A.G. is partially supported by a National Institutes of Health Research Career Award Fellowship (K-6-6M-21444).

‡ Present address: Department of Biochemistry, University of California Berkeley, CA 94720.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; MNU, *N*-methyl-*N*-nitrosourea; EDTA, ethylenediaminetetraacetic acid; 3-meAd, 3-methyladenine; 7-meGua, 7-methylguanine; O^6-meGua, O^6-methylguanine.

0006-2960/81/0420-0273$01.00/0

content was 2.5–2.7 mg of DNA/g of cellulose.

Other Materials. 3-Methyladenine, 7-methylguanine, 7-methylguanine, and O^6-methylguanine were purchased from Cyclo Chemicals, Los Angeles, CA. All high-performance liquid chromatography solvents were glass distilled and purchased from Matheson, Coleman and Bell, Cincinnati, OH. DEAE-cellulose (DE-52) and phosphocellulose (P-11) were obtained from Whatman, Inc.

High-Performance Liquid Chromatography. The system used consisted of two Waters Model 6000A pumps, a 660 solvent programer, a 440 UV detector (254 nm), a U6K injector, a Houston Instruments recorder, and a Waters fatty acid analysis column.

Methods

Standard Reaction Conditions. The standard enzyme assay, unless otherwise noted, contained the enzyme, 35 nmol of [^{3}H]MNU-treated DNA, 40 mM NaCl, 0.5 M sucrose, 1 mM dithiothreitol, and 0.05 M Tris-HCl, pH 7.6, in a reaction volume of 0.35 mL. All reactions were at 37 °C, and the reaction time is indicated in each legend. A unit of activity is defined as the release of 1 pmol of 3-methyladenine/min under these conditions.

Determination of 3-meAd Excision Activity. Method A: Measurement of Bases Remaining in the Substrate. After the reaction of alkylated DNA with enzyme, the DNA was precipitated at 0 °C by the addition of 0.2 mg of salmon sperm DNA, 0.1 volume of 2.5 M sodium acetate, and 2 volumes of ethanol. After centrifugation at 3000 rpm for 15 min at 0 °C, the DNA pellet was washed twice with 6 mL of ethanol and then hydrolyzed in 0.3 mL of 0.2 N HCl for 20 min at 70 °C. After neutralization with NaOH, 1 mL of 0.003 M Tris-HCl, pH 8.0, was added and the mixture passed through a DEAE-cellulose column of 1-cm^3 bed volume equilibrated with 0.03 M Tris-HCl, pH 8.0. The column was then washed with 7 mL of 0.01 M Tris-HCl, pH 8.0, and the total eluate was combined. This column step removes DNA, nucleotides, and protein which would otherwise interfere with the high-performance LC separation. After the addition of methylpurines as UV markers, an aliquot of 0.8–1.0 mL of the DEAE eluate was applied to the LC column and the methylpurines separated as described below. The peaks corresponding to each methylpurine were collected and counted for ^{3}H in a Packard Tri-Carb liquid scintillation counter. In the absence of protein, the ratio of 3-meAd to 7-meGua was 0.12 ± 0.015, and the O^6-meGua to 7-meGua ratio was 0.11 ± 0.02.

Method B: Measurement of Bases Released from the Substrate. The reaction was diluted with 1 mL of cold 0.03 M Tris-HCl, pH 8.0, and passed directly through the DEAE-cellulose column described in method A. The column was washed with 7 mL of cold 0.01 M Tris-HCl, pH 8.0. The total DEAE eluate was reduced in volume to 2–3 mL by lyophilization. LC separation of the methylpurines was achieved by the procedure described below.

Method C: Ethanol-Soluble Counts. At the end of the reaction, 0.1 volume of 2.5 M sodium acetate, 0.2 mg of carrier DNA (salmon sperm), and 2 volumes of ice cold ethanol were added. After mixing, the DNA was precipitated by centrifugation at 3000 rpm for 15 min. An aliquot of the supernatant fraction was counted for ^{3}H in a Packard Tri-Carb liquid scintillation counter.

Separation of Methylpurines by High-Performance Liquid Chromatography. Separation of 3-methyladenine (3-meAd), 7-methylguanine (7-meGua), and O^6-methylguanine (O^6-meGua) was achieved after the addition of these unlabeled bases as UV markers by the following LC program: solvent A, 10 mM KH_2PO_4: solvent B, 80:20 (v/v) methanol/water. A linear gradient of 100% solvent A to 20% solvent B over 20 min with a flow rate of 0.9 mL/min was used. A typical separation is shown in Figure 1.

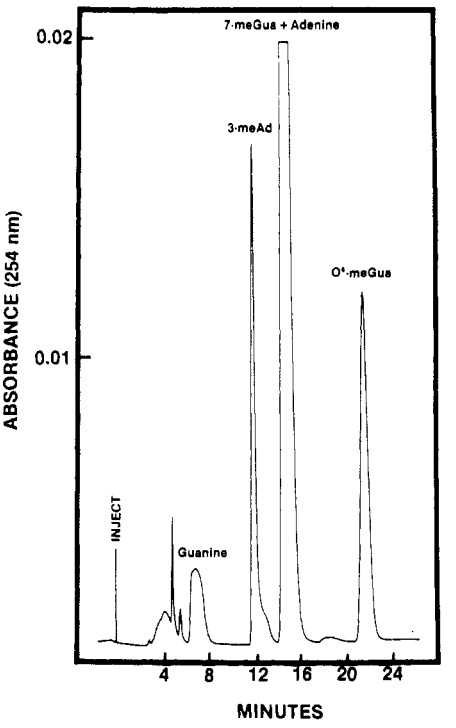

FIGURE 1: Separation of 3-meAd, 7-meGua, and O^6-meGua by high-performance liquid chromatography. The separation was achieved with a Waters fatty acid analysis column (see Methods).

Resolution of these methylpurines was also achieved with the following program: solvent A, 10 mM potassium phosphate buffer, pH 7.0; solvent B, 80:20 methanol/water. A linear gradient of 100% solvent A to 25% solvent B over 15 min with a flow rate of 1.0 mL/min was used. The retention times for the methylpurines with this condition were 3-meAd 25 min, 7-meGua 16 min, and O^6-meGua 23 min.

Determination of Endonuclease Activity. For determination of endonuclease activity, samples were centrifuged in a 70–100% formamide gradient, 4.2 mL in volume in a TV-865 vertical rotor (Du Pont/Sorvall). Each DNA sample was first dialyzed against 0.05 M Tris-HCl, pH 7.6 and then brought to 0.06% $NaDodSO_4$; 0.2 mL was then layered on a linear 70–100% formamide gradient and centrifuged for 1 h at 40000 rpm, 20 °C. Slow acceleration and deceleration were used; 10-drop fractions were collected from the bottom of the centrifuge tube. ^{3}H-Labeled λ DNA and ^{3}H-labeled SV40 form III DNA (Bethesda Research Laboratories, Bethesda, MD) were used to standardize the gradient.

Results

Measurement of 3-Methyladenine N-Glycosylase in Crude Extracts. In alkylated DNA, the glycosyl bond of 3-methyladenine and 7-methylguanine is unstable at physiological pH (Lawley & Brookes, 1963). Furthermore, the secondary structure of DNA helps to stabilize this bond, as depurination at pH 5 of single-stranded DNA is 3.3 times as fast as depurination of double-stranded DNA (Lindahl & Nyberg, 1972). Preliminary experiments in this laboratory with alkylated DNA, exonuclease III, and alkaline phosphatase suggested that the glycosylic linkage in deoxynucleosides may be more unstable than that in DNA, and a number of attempts to purity a 3-methyladenine *N*-glycosylase from crude extracts resulted in the isolation of exonuclease activity. Thus, a search

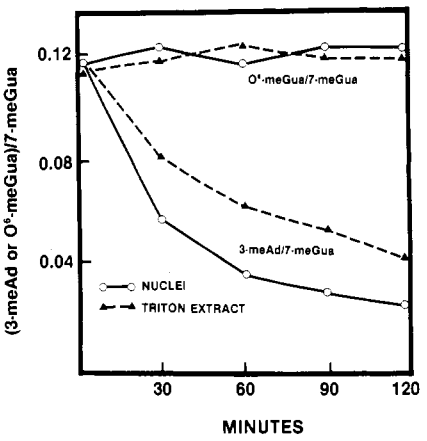

FIGURE 2: Excision of methylpurines by a rat liver nuclear suspension or extract as a function of time. The nuclear suspension was prepared as described (see Results, Large-Scale Purification); 0.1 mL of the suspension was incubated with [^{3}H]MNU DNA by using the standard reaction conditions. A Triton extract was prepared by incubating 1 mL of the nuclear suspension with 0.5 mL of Triton X-100 (10 mg/mL) in an ice bath for 30 min and then centrifuging at 10 000 rpm for 30 min; 0.1 mL of the extract was incubated as described above. The ratio of methylpurines was determined by method A (see Methods).

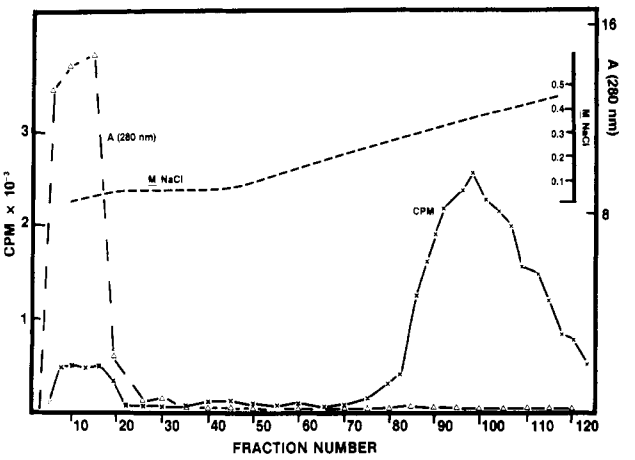

FIGURE 3: DNA–cellulose column fractionation. Fraction I was separated on double-stranded DNA–cellulose as described under Results. Activity was determined by method C (see Methods) by the incubation of 0.05 mL of each column fraction with [^{3}H]MNU DNA for 60 min.

for a 3-methyladenine *N*-glycosylase in crude extracts by using the release of radioactive base can be misleading.

Another strategy for assay is the determination of the amount of each alkylated base which remains in the substrate after the incubation. The result can be expressed as ratios of either 3-methyladenine or O^6-methylguanine to 7-methylguanine. These ratios are constant in various preparations of alkylated DNA, and a specific *N*-glycosylase which releases 3-methyladenine would alter the 3-methyladenine ratio while a nonspecific nuclease would not. In a search for glycosylases in crude extracts, it was only when this procedure (method A under Methods) was used that it was possible to identify with certainty the presence of a specific glycosylase.

An example of the use of method A is shown in Figure 2. Purified rat liver nuclei or triton extracts of nuclei were incubated with alkylated DNA for varying times, and the alkylated bases were then determined in the residual DNA (method A). The ratio of 3-meAd to 7-meGua decreases while the ratio of O^6-meGua to 7-meGua remained the same. In this case the absolute level of 7-meGua and O^6-meGua decreased during the incubation due to nonspecific nucleases. The decrease of 7-meGua can be quantitated, and from this decrease the amount of 3-meAd excised by nonspecific nucleases can be quantitated. This point will be illustrated later. Therefore, by this method it is possible to measure the loss of 3-meAd due to an enzyme specific for this base in the presence of nonspecific nucleases. The stability of the glycosidic linkage may be more of a problem with 3-methyladenine and 7-methylguanine in DNA than with the more stable glycosidic linkages of O^6-meGua and uracil.

Enzyme Purification. An enzyme which excises 3-meAd from DNA alkylated with *N*-methyl-*N*-nitrosourea was purified from rat liver nuclear extracts by using DNA–cellulose and phosphocellulose chromatography. The following purification procedure was routinely used for the preparation of enzyme.

Twenty-eight male Sprague-Dawley rats, each weighing approximately 120 g, were sacrificed by decapitation. The liver was quickly removed, minced, and washed in ice-cold buffer A (0.05 M Tris-HCl, pH 7.6, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol). The liver slices were homogenized in a total volume of 900 mL of buffer A with the use of a loose-fitting Teflon pestle. Unless otherwise noted, all purification procedures were carried out in a cold room maintained at 4 °C.

The liver homogenate was passed through two layers of cheesecloth and centrifuged at 5000 rpm for 15 min in a Sorvall GSA rotor. The pellet was suspended in a total volume of 1000 mL of buffer B (buffer A with 2.3 M sucrose, final concentration). A uniform suspension was obtained by stirring in the cold for 1 h. Nuclei were sedimented by centrifugation of the suspension for 70 min at 24 000 rpm in a Beckman Model 42.1 rotor. This is a modification of the sucrose cushion method of Blobel & Potter (1967). The resulting supernatant fraction was discarded, and the nuclear pellet was suspended in 50 mL of buffer C (0.05 M Tris-HCl, pH 7.6, 0.5 M sucrose, 1 mM dithiothreitol) by the use of a small homogenizer. Nuclei were stored at −20 °C in the buffer C solution for up to 4 months with little or no loss of activity.

A nuclear extract, fraction I, was prepared by dilution of 50 mL of nuclear suspension with 50 mL of water and incubation for 15 min at 37 °C with constant mixing. This procedure was shown to be more effective than the use of 0.3% Triton. Nuclei were then removed by centrifugation at 10 000 rpm for 30 min. The supernatant fraction was applied to a double-stranded DNA-cellulose column (2 cm × 11 cm) equilibrated with buffer C. The column was washed with 0.05 M NaCl/buffer C until the absorbance at 280 nm returned to near zero. The protein was then fractionated with a 0.04–0.6 M NaCl/buffer C gradient, 200-mL total volume. The volumes of the fractions collected during the sample application and wash were 9.7 mL and during the gradient were 1.6 mL. Figure 3 demonstrates the activity of the fractions incubated with DNA alkylated with [^{3}H]MNU. Two peaks of activity, determined by the release of ethanol soluble counts, were observed. The activity eluted by the gradient was responsible for 3-meAd excision as determined by method A. These fractions were pooled (fraction II), dialyzed against buffer D (0.02 M potassium phosphate, pH 6.8, 0.5 M sucrose, 1 mM dithiothreitol), and then applied to a phosphocellulose column (1.5 × 8.5 cm) equilibrated with the same buffer (Figure 4). Fractions of 2.0 mL were collected. After the sample was applied, the column was washed with buffer D until the absorbance returned to zero. The protein was then fractionated by a gradient of 0–0.8 M NaCl in buffer D, 150-mL total volume. The enzyme activity eluted in a fairly sharp peak at 0.15 M NaCl. No protein peak was observed,

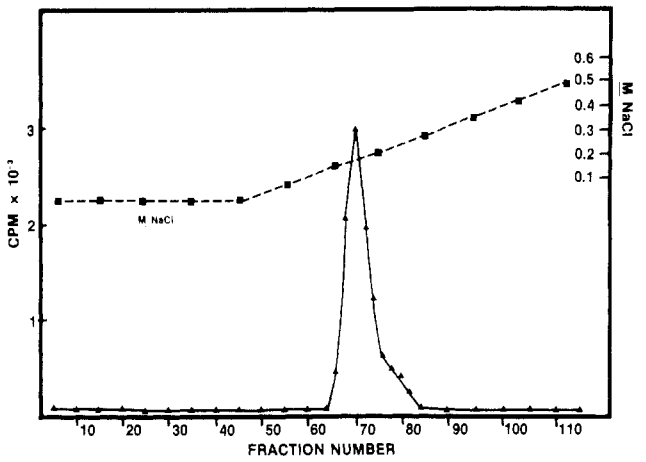

FIGURE 4: Phosphocellulose column fractionation. Fraction II was separated on phosphocellulose as described under Results. Activity was determined by method C by the incubation of 0.05 mL of each column fraction with [^{3}H]MNU DNA for 75 min. The total protein recovered in the active fractions was 224 μg.

Table I: Purification of 3-meAd Glycosylase[a]

protein fraction	specific act. (cpm)	*n*-fold purification	yield (units)	% yield
fraction I	0.085		45	100
fraction II	0.480	5.6	5.9	13
fraction III	8.22	97	1.8	4

[a] Fraction I represents the undialyzed nuclear extract, fraction II the pooled DNA cellulose enzyme, and fraction III the pooled and concentrated phosphocellulose fraction.

but when fractions 66–80 were pooled and concentrated by vacuum dialysis against 0.2 M NaCl in buffer C to a final volume of 7 mL, the total protein was found to be 12 mg. This fraction (fraction III) was stored in small aliquots at −20 °C in 0.2 M NaCl/buffer C. No loss in enzyme activity was observed for up to 4 weeks.

Data on the purification of the enzyme are shown in Table I. The specific activity of 3-meAd excision was determined for each protein fraction by utilizing the three methods discussed under Methods. It is noted that these values have varied considerably among preparations. For instance, the specific activity has varied from 2 to 16 U/mg, and the purification from 97- to 500-fold. The reason for this variability is not known.

The data used to derive the specific activity of each protein fraction are presented in Table II. Note that with either fraction II or fraction III the amount of 3-meAd excised from the DNA was approximately the same when determined by either method A or method B. Fractions II and III both release approximately 15% as much 7-meGua as 3-meAd, which was a reproducible observation and will be discussed in detail later. There was no appreciable nonspecific nuclease activity in either fraction II or fraction III, as very little 7-meGua or O^6-meGua was excised from the DNA as determined by method A. A nuclease was present in fraction II, which required Mg^{2+}, but no Mg^{2+} was included in these reactions.

The data in Table II obtained with fraction I, the nuclear extract, allow only an approximation of the enzyme units and specific activity because of contaminating nonspecific nucleases. Fraction I was shown by method A to remove 3024 cpm of 7-meGua from the DNA. This represented 6% of the total 7-meGua. Since only 169 counts of the 3024 counts were recovered as free 7-meGua (method B), most of this purine

Table II: Determination of Methylpurine Excision in Fractions I–III[a]

additions	purine excised by each method (cpm) 3-meAd A	3-meAd B	7-meGua A	7-meGua B	O^6-meGua A	O^6-meGua B
fraction I: none	1428	1038	3024	169	0	0
40 mM NaCl	2837	2706	2044	654	149	0
1 mM EDTA + 40 mM NaCl	2203	2430	0	489	0	0
fraction II: 40 mM NaCl	2090	2179	0	318	0	0
fraction III: 40 mM NaCl	1418	1575	0	269	0	0

[a] Each reaction contained 32 nmol of [^{3}H]MNU DNA (2500 cpm/nmol) and 0.35 mL of buffer C. The specific activity of methylated base was 1900 cpm/pmol. There was, therefore, 42 pmol of [^{3}H]methyl group. Fractions I (240 μg), II (40 μg), and III (1.6 μg) were added as indicated. Reactions were for 60 min at 37 °C. The amount of each methylpurine excised above the appropriate blank was determined by both methods A and B (see Methods). In the blank, the total counts of 3-meAd and 7-meGua released spontaneously were 150 and 373, respectively.

(2855 counts) was removed from the DNA by a mechanism other than a glycosylase. One likely pathway would be the degradation of the DNA by an exonuclease to mononucleotides. Since in the original substrate 3-meAd was 13% of the 7-meGua, then by this nonspecific route of degradation, 371 counts of 3-meAd should have been released from the DNA. This value was obtained by taking 13% of 2855 counts. Approximately this amount was observed as the difference between 3-meAd determined by method A which measures total excision by any pathway and 3-meAd determined by method B which measures the free base released. From this discussion, method A should show some loss of O^6-meGua with fraction I. Why this was observed only when NaCl was present is not known. None of the preparations have ever liberated free O^6-meGua.

It is emphasized that the data derived from method A are only reasonably accurate, as one is examining the loss of a small number of counts from a large number present in the DNA. Some of the observed variability in the specific activities and degrees of purification may be due to the difficulty in determining the initial activity of fraction I.

Although the enzyme was purified from nuclei, 3-meAd excision activity has been detected in the cytoplasmic fraction. The specific activity was, however, very low, and the level of nonspecific nucleases high. Nuclei were used as a source for enzyme to avoid some of the potential problems discussed above.

Further Purification. Attempts to purify the enzyme further were not very successful. Sephadex G-100 was used, although this resulted in a large loss in activity and a very unstable preparation. The inclusion of phenylmethanesulfonyl fluoride or bovine serum albumin in the enzyme buffers had no effect on either the yield or stability of fraction III. Further purification of fraction III by single-stranded DNA–cellulose, DEAE-cellulose, Sephacryl S-200, or hydroxylapatite resulted in the loss of enzyme activity.

Requirements for Optimum Activity. Sodium or potassium ions were required for maximal activity, and 40 mM NaCl was optimal (Table III). In the absence of added sodium chloride, the activity was 19% of the maximum activity. Potassium could substitute for sodium. EDTA up to 1.1 mM final concentration had no effect on the activity, but Mg^{2+} was found to stimulate in the presence of 40 mM NaCl. At the optimal Mg^{2+} concentration of 1.83 mM, the activity increased

Table III: Agents Which Influence 3-Methyladenine DNA Glycosylase Activity[a]

addition	concentration (mM)	% of control activity
none (control)		100
NaCl	0	20
	14	58
	43	100
	71	78
	143	58
$MgCl_2$	1.83	159
	3.66	135
	7.31	109
	9.10	79
$MnCl_2$	3.66	86
	9.10	60
EDTA	0.23	95
	0.57	100
	1.14	97
N-ethylmaleimide	0.40	43
	1.60	0
spermidine	0.08	103
	0.20	124
	0.80	126
	1.60	153
	2.40	153

[a] Each reaction contained 0.028 unit of fraction III, and the incubation was for 30 min. NaCl at 40 mM concentration was added to all reactions except those where NaCl was added as indicated. Method B was used to determine activity.

by about 50%. Higher concentrations of Mg^{2+} inhibited. In contrast, Mn^{2+} at 1.6 mM produced a 50% decrease in activity. Finally, the enzyme exhibited a broad pH optimum between 7.5 and 8.0 in Tris-HCl buffer. Eighty percent of the maximum activity was observed at pH 7.0 and 8.2. The enzyme activity was determined by measuring the free 3-meAd liberated (method B).

Molecular Weight. The molecular weight of the enzyme as determined by chromatography on Sephadex G-100 was 24000 ± 1000. This value has been confirmed for both Fractions II and III. The Sephadex G-100 columns were standardized with ovalbumin, chymotrypsinogen A, and cytochrome *c*. On a $NaDodSO_4$–polyacrylamide gel, fraction III showed five faint bands.

Nuclease Contaminations. Fraction II was found to be contaminated with a Mg^{2+}-dependent nuclease which acted on double-stranded DNA. This enzyme was removed by the phosphocellulose column. Fraction III did not contain any exonuclease activity, or endonuclease activity for native or depurinated T7 DNA when tested with or without Mg^{2+}.

Fraction III did contain a Mg^{2+}-dependent endonuclease activity specific for single-stranded T7 DNA as indicated in Figure 5. Increasing amounts of enzyme led to an increase in the number of single-stranded breaks. With the G-100 enzyme fraction, 0.004 unit did not result in any detectable endonuclease activity against single-stranded T7 DNA, whereas the same number of units of fraction III contained the activity.

The Sephadex G-100 fractionation of either fraction II or III resulted in a 30–50% loss in total glycosylase activity. Furthermore, when this enzyme was pooled and concentrated by vacuum dialysis, even with 0.1% bovine serum albumin, it was quite unstable, and lost 50% of the remaining activity after freezing and thawing.

Mechanism of Action. Good stoichiometry was observed between the amount of free 3-meAd released (method B) and the amount of 3-meAd lost from the substrate (method A), as noted in Table II.

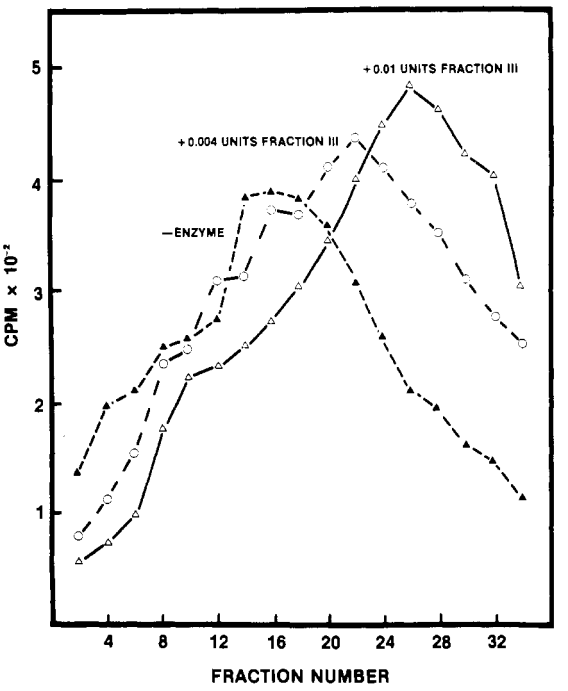

FIGURE 5: Single-strand endonuclease activity of fraction III. ^3H-Labeled T7 DNA was denatured by heating at 95 °C for 5 min and then cooled rapidly. Each reaction contained the indicated units of fraction III, 40 mM NaCl, 3.66 mM $MgCl_2$, and 12 nmol of ^3H-labeled DNA (4141 cpm/nmol) in 0.35 mL of buffer C. Reactions were for 60 min at 37 °C. Enconuclease activity was determined as described in Methods.

Table IV: Single-Stranded DNA Breaks Introduced into Alkylated T7 DNA by Fraction III[a]

reaction conditions					
DNA	enzyme	0.2 N NaOH	number-average mol wt	single-strand breaks per molecule of DNA	single-strand breaks per total pmol of DNA (pmol)
native	–	–	9.2×10^6		
native	+	–	10.7×10^6		
MMS	–	–	9.6×10^6		
MMS	+	–	6.5×10^6	0.66	0.27
MMS	–	+	1.5×10^6		
MMS	+	+	0.8×10^6	0.92	2.65

[a] Each reaction contained 12.7 nmol of either MMS DNA or native DNA nucleotide labeled with [^3H]thymidine (5480 cpm/nmol), 0.045 unit of Fraction III, and 40 mM NaCl in 0.35 mL of buffer C. The reactions were for 60 min at 37 °C. Samples were then incubated either with or without 0.2 N NaOH for 30 min at 37 °C, neutralized, and then dialyzed against 0.05 M Tris-HCl, pH 7.6–0.06% $NaDodSO_4$ and centrifuged in a 70–100% formamide gradient as described in Methods. In the calculations the top three fractions in the gradient were not counted due to the large error in this part of the gradient.

The stoichiometry of base release and alkali-sensitive sites produced by the enzyme was then determined. The phosphodiester bonds at depurinated sites are stable at neutral pH but are hydrolyzed at alkaline pH (Tamm et al., 1953). Because of the slight contamination by the nonspecific single-stranded endonuclease, it was necessary to distinguish between single-strand breaks made by this enzyme and single-strand breaks generated from depurinated sites. Alkylated T7 DNA was incubated with and without enzyme and the aliquots were centrifuged in formamide gradients at neutral pH. From number-average molecular weight determinations, the total number of enzyme-induced single-strand breaks per reaction mixture was calculated. Other aliquots were exposed to alkali and then centrifuged in formamide gradients. In this case, the total number of enzyme-induced single-strand breaks

plus alkali-sensitive sites could be determined. The difference represented the total number of alkali-sensitive sites.

Table IV presents the experimental conditions, the number-average molecular weights of the DNA, and the number of single-strand breaks.

The number of single-strand breaks introduced into alkylated DNA was determined from the molecular weight of native DNA treated with enzyme but not exposed to alkali. The addition of the enzyme fraction shifted the observed molecular weight of native DNA from 9.2×10^6 to 10.7×10^6. The reason for this slight shift is now known, but it was a reproducible observation. The enzyme was responsible for 0.66 single-strand break per alkylated DNA molecule on the basis of a shift in molecular weight from 10.7×10^6 to 6.5×10^6. Native DNA plus enzyme rather than MMS DNA minus enzyme was used in the calculation because of the effect of added protein. From the total amount of DNA in the reaction with a molecular weight of 10.7×10^6, the concentration of enzyme-induced DNA single-strand breaks was determined to be 0.27 pmol. This endonuclease activity was probably due to the single-strand endonuclease known to be a contaminant in fraction III, which had slight activity even in the presence of EDTA.

Alkali treatment of alkylated DNA decreased the molecular weight to 1.5×10^6, and incubation with enzyme prior to alkali treatment decreased the molecular weight from 1.5 to 0.8×10^6, or 0.92 break per DNA moleculae. This corresponded to 2.65 pmol of alkali-sensitive sites created by the enzyme. Since there were 0.27 pmol of nonalkali sensitive breaks introduced into the alkylated T7 DNA by the enzyme, the 2.38 pmol of depurinated sites was produced by the enzyme.

When the amount of enzyme used in these experiments was reacted with a saturating amount of [^{3}H]MNU DNA, 217 pmol of 3-meAd was liberated. This 2.7 pmol of base released enzymatically corresponds well with the 2.4 pmol of alkali-sensitive sites. The enzyme is thus a 3-meAd DNA *N*-glycosylase and does not have an associated phosphodiesterase activity.

K_m Determination. The apparent K_m was determined by varying the amount of alkylated DNA in the reaction and measuring the release of 3-meAd by HPLC. A K_m of 2.63 nM was obtained for 3-meAd. A value of 6 nM was reported for the *E. coli* 3-meAd glycosylase by Riazuddin & Lindahl (1978).

Effect of Purines and Methylated Purines on Enzyme Activity. 3-meAd up to 1 mM final concentration had no effect on enzyme activity. Other purines tested at concentrations around 1 mM, such as 7-meGua, O^6-meGua, adenine, and caffeine, also had no effect on the activity.

Substrate Specificity. The enzyme preparation does not release 3-methylguanine or 1-methyladenine. The lower limit of detection of a methylated band was determined to be 0.026 pmol. There may be a low level of activity with 7-methyladenine in DNA, but from the data, this is uncertain.

The enzyme demonstrated a preference for double-stranded alkylated DNA (Figure 6). The rate of release from single-stranded DNA was only 20% that from double-stranded DNA. No endonuclease activity was observed on double-stranded DNA or depurinated DNA, or UV-irradiated DNA. For UV irradiation, T7 DNA was irradiated with a fluence of 1000 erg/nm^2. This would correspond to approximately 6500 dimers for 10^7 bases (Schlaes et al., 1972). No activity was observed with γ-irradiated DNA. T7 DNA was irradiated under nitrogen with 22.5 krad from a ^{60}Co γ-ray source, then incubated under nitrogen at 37 °C for 4 h (Kirtikar et al., 1975) prior to reaction with enzyme. No evidence for the production of alkali-sensitive sites was obtained under these conditions.

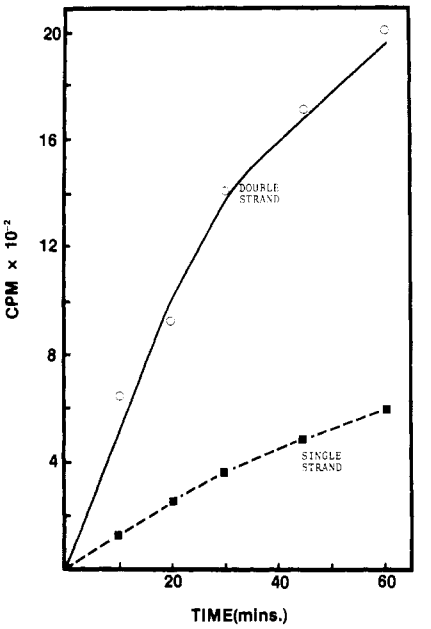

FIGURE 6: Relative rate of release of 3-meAd from double-stranded and single-stranded DNA. [^{3}H]MNU DNA was single-stranded by increasing the pH to 13 for 15 min in an ice bath and then neutralizing to pH 7.6. Reactions contained 0.033 unit of fraction III and were for the specified time. Enzyme activity was determined by method B.

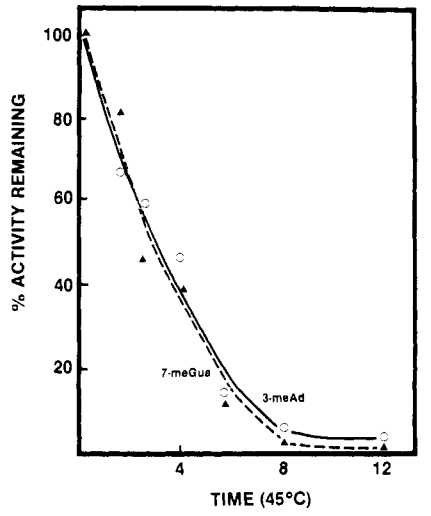

FIGURE 7: Heat inactivation of enzyme activities which excise 3-meAd and 7-meGua. Fraction III was incubated at 45 °C for the indicated time. Each reaction contained 0.018 unit of enzyme and was for 30 min. Each activity was determined by method B.

The enzyme did not release 7-methylguanine from the synthetic polymer (dG·dC) alkylated with dimethyl sulfate. This may be due to the Z structure of this polymer.

7-meGua Activity. A second *N*-glycosylase activity responsible for the release of 7-meGua was observed in the preparation. This activity was a consistent observation, and for several preparations the amount of 7-meGua released was 15 ± 4% of the 3-meAd released. This ratio of 3-meAd to 7-meGua released did not change from fraction II to fraction III. Both activities, as shown in Figure 7, possessed similar heat labilities. This has been observed with two preparations. At present it is not possible to state definitively whether the activity which released 7-meGua is due to the same enzyme or a different enzyme. The marked instability of fraction III

Table V: Properties of the Rat Liver, Human Lymphoblast, and *E. coli* 3-meAd Glycosylases

property	rat liver nuclei	human lymphoblast	*E. coli*
purification	100-fold (4% yield)	25-fold (5% yield)	2800-fold (7% yield)
glycosylase activity	3-meAd (7-meGua)	3-meAd (7-meGua)	3-meAd (7-meAd, 3-meGua)
nuclease activity	single-strand endonuclease, fraction III, removed by G-100	endonuclease specific for depurinated sites	none reported
requirements	Na^{2+} or K^{2+} stimulated by Mg^{2+} (50%); active in 1 mM EDTA	none, active in 1 mM EDTA	none, stimulated by Mg^{2+} (30%); active in 1 mM EDTA
essential sulfhydryl	yes	not tested	yes
purine inhibitors	none	not tested	3-meAd (50% inhibition with 8×10^{-4} M)
K_m	2.6×10^{-9} M	not done	6×10^{-9} M
pH optimum	7.5-8.0	7.5-8.5	7.2-7.8
DNA specificity	double stranded	double stranded	double stranded
mol wt	24 000	34 000	19 000

on further purification has blocked attempts to separate these activities.

Discussion

An enzyme which releases 3-meAd from DNA alkylated with *N*-methyl-*N*-nitrosourea was purified from rat liver nuclei. The protein, extracted from nuclei in a soluble form, was purified 100-fold by DNA-cellulose and phosphocellulose chromatography. Further attempts at purification of the enzyme were unsuccessful due to the instability of the protein. The preparation is heterogenous, as judged by $NaDodSO_4$–polyacrylamide gels.

The enzyme acts as a 3-meAd–DNA *N*-glycosylase on the basis of the following experimental evidence: (i) 3-meAd is released from alkylated DNA; (ii) the amount of 3-meAd removed from the DNA is equal to the free 3-meAd recovered from the reaction; (iii) the enzyme generates alkali-sensitive sites in alkylated DNA, the number being approximately equal to the number of 3-meAd residues released.

The only nuclease contaminant detected in fraction III was a magnesium-stimulated single-stranded DNA endonuclease. This activity was removed from the glycosylase by Sephadex G-100 chromatography, but this step resulted in a large loss in glycosylase activity. The instability of the purified enzyme prevented further purification.

The enzyme is similar in many respects to the *E. coli* enzyme purified by Riazuddin & Lindahl (1978). A comparison of these two enzymes and the human lymphoblast glycosylase reported by Brent (1979) is presented in Table V. All three enzymes are relatively small in size and have a preference for double-stranded DNA. Both the rat liver and *E. coli* enzymes have very low K_m values, which have also been observed for other enzymes active in DNA repair. All three enzymes were active in 1 mM EDTA, but both the bacterial and rat liver activities were stimulated by Mg^{2+}. In contrast, we have demonstrated with the rat liver enzyme a requirement for either Na^+ or K^+ for maximum activity. None was required for either of the other enzymes.

We have consistently observed the enzymatic release of 7-meGua with our preparations. This activity, which represents approximately 15% of the 3-meAd released, demonstrates a heat lability similar to that of the 3-meAd activity. The two activities also copurify from fraction II to fraction III, and neither 3-meAd nor 7-meGua inhibits either activity. Attempts to separate these activities by further purification were not successful because of the instability of the enzyme preparation. For the lymphoblast enzyme, Brent (1979) presented data demonstrating the enzymatic loss of 7-meGua, and this loss represented 10% of the 3-meAd excised. It is possible that both mammalian 3-meAd glycosylases also remove 7-meGua at a much lower rate than 3-meAd.

This lower rate of removal of 7-meGua would be consistent with several in vivo observations. Margison et al. (1973, 1976) have presented evidence for the in vivo loss of 7-meGua in both rat liver and hamster liver. However, the rate of removal of 3-meAd appears to be much faster than that of 7-meGua (Margison & O'Connor, 1973). More recently, Warren et al. (1979) have shown that with Chinese hamster cells, 3-methyladenine was very rapidly excised. They also observed the excision of 7-meGua, but at a reduced rate.

Further work on the enzymology of the mammalian DNA *N*-glycosylases should help clarify whether one protein is responsible for both 3-meAd and 7-meGua excision.

In our initial search for the enzyme activity in various tissue extracts, such as placenta and calf liver, a high level of release of both 3-meAd and 7-meGua was observed. However, no enzyme was ever identified which acted as a specific *N*-glycosylase. This led to the development of the present methods of assay which, on the basis of our observations with the bacterial exonuclease and alkaline phosphatase, could circumvent the problem of nonspecific nucleases.

References

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

Blobel, G., & Potter, V. R. (1967) *J. Mol. Biol. 26*, 279–292.

Brent, T. P. (1979) *Biochemistry 18*, 911–916.

Chetsanga, C., & Lindahl, T. (1979) *Nucleic Acids Res. 6*, 3673–3684.

Deutsch, W. A., & Linn, S. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 141–144.

Deutsch, W. A., & Linn, S. (1979b) *J. Biol. Chem. 254*, 12099–12103.

Karran, P., & Lindahl, T. (1978) *J. Biol. Chem. 253*, 5877–5879.

Kirtikar, D. M., Slaughter, J., & Goldthwait, D. A. (1975) *Biochemistry 14*, 1235–1244.

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

Lawley, P. D., & Brookes, P. (1963) *Biochem. J. 80*, 127–138.

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

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.

Margison, G. P., Capps, M. J., O'Connon, P. J., & Craig, A. W. (1973) *Chem.-Biol. Interact. 6*, 119–124.

Margison, G. P., Margison, J. M., & Monotesano, P. (1976) *Biochem. J. 157*, 627–634.

Margison, G. P., & O'Connor, P. J. (1973) *Biochim. Biophys. Acta 331*, 349–356.

Riazuddin, S., & Lindahl, T. (1978) *Biochemistry 17*, 2110-2118.
Richardson, C. C. (1966) *J. Mol. Biol. 15*, 49-61.
Richardson, C. C., Inman, R. B., & Kornberg, A. (1964) *J. Mol. Biol. 9*, 46-69.
Schlaes, D. M., Anderson, J. A., & Barbour, S. D. (1972) *J. Bacteriol. 111*, 723-730.
Smith, M. G. (1967) *Methods Enzymol. 12*, 545-550.
Talpaert-Borle, M., Clerici, L., & Campagnari, J. (1979) *J. Biol. Chem. 254*, 6387-6391.
Tamm, C., Shapiro, H. S., Lipshitz, R., & Chargaff, E. (1953) *J. Biol. Chem. 203*, 673-688.
Warren, W., Crathorn, A. R., & Shooter, K. V. (1979) *Biochim. Biophys. Acta 563*, 82-88.

Phosphorus-31 Nuclear Magnetic Resonance of fd Virus†

J. A. DiVerdi and S. J. Opella*

ABSTRACT: ^{31}P NMR experiments on the filamentous bacteriophage fd are used to characterize the viral DNA. Because fd is a 16.4×10^6 dalton rod-shaped particle, methods of high-resolution solid-state NMR including cross polarization, proton decoupling, and magic angle sample spinning are utilized. The ^{31}P chemical shielding tensor of solid fd is indistinguishable from that of single-stranded or double-stranded DNA in the absence of proteins; therefore the ^{31}P chemical shift does not show evidence of structural changes in DNA upon incorporation into the virus. fd in solution has a very broad ^{31}P resonance line width. The line width is due to static chemical shift anisotropy that is not motionally averaged, as shown by the generation of sidebands with magic angle sample spinning and a linear dependence of line width on magnetic field strength. These results indicate that DNA packaged inside fd is immobilized by the coat proteins.

fd is a filamentous virus that infects *Escherichia coli* (Marvin & Hohn, 1969). The virus is a protein–DNA complex with no associated membrane components. The particle weight is 16.4×10^6 daltons, 88% of which is from 2700 copies of the 5000-dalton major coat protein (Newman et al., 1977), 10% is from the 6400 nucleotides of the circle of single-stranded DNA (Beck et al., 1978), and 2% is from about 5 copies of a minor coat protein located at one end of the filament (Day & Wiseman, 1978). In solution, fd is a 900 by 9 nm rod with somewhat smaller diameter in the absence of water (Newman et al., 1977; Marvin et al., 1974). fd is similar to other single-stranded DNA bacteriophages, such as M13, Pf1, and Xf, in life cycle as well as structure (Marvin & Hohn, 1969).

There is a substantial amount of experimental evidence that filamentous bacteriophages have their DNA extended lengthwise within a tubular chamber made from the major coat-protein subunits (Marvin & Wachtel, 1975). Simple design principles are expected for biological supramolecular structures like viruses, yet there are significant problems outstanding in the description of filamentous viruses especially with regard to how the DNA is packed inside the coat-protein shell. X-ray diffraction data combined with molecular model building have shown that the coat-protein subunits are arranged in an overlapping helical array (Marvin et al., 1974). While the details of the coat-protein helix are under active investigation (Marvin, 1978; Makowski & Caspar, 1978), there is little doubt that the protein shell of these viruses is highly symmetrical.

The difficulties with understanding the architecture of fd as a nucleoprotein complex start at the most basic level since a circle of DNA is packed in a cylinder with a length to diameter ratio of around 300:1. The X-ray diffraction results that have been interpreted to give the model for the coat-protein arrangement do not have intensity recognizable as from the DNA; therefore there is no diffraction data on how the DNA is arranged in the virus interior or how the nucleotides interact with the amino acids of the coat protein (Marvin et al., 1974). There is no evidence of base pairing of the phage DNA (Beck et al., 1978). A particularly glaring piece of data about fd is the nonintegral ratio (2.3:1) of nucleotides to coat protein (Newman et al., 1977) which is difficult to reconcile with most plausible models of symmetrical DNA–protein interactions and differs from the integral ratios found for other filamentous viruses (Day & Wiseman, 1978). There are few spectroscopic means of separating the nucleotide and aromatic amino acid chromophores, although laser Raman spectroscopy does indicate that the DNA is not in the A form (Thomas & Murphy, 1975). Day and co-workers (Day & Wiseman, 1978; Day et al., 1979) have had to rely almost exclusively on physicochemical characterization of the virus particles to propose models for the packing of DNA in the filamentous viruses. Photochemical cross-linking experiments indicate that a small part of fd DNA exists in a hairpin structure, and this may fix the location of the DNA relative to one end of the particle (Shen et al., 1979); however, this approach gives no indication of overall packing arrangements of the DNA.

There are several possible explanations for the apparent lack of symmetry between the nucleotides and coat-protein subunits of fd and the absence of diffraction intensity from DNA in oriented fibers of fd. These include the DNA having significant motional freedom within the confines of the coat-protein shell or the DNA being disordered relative to the coat proteins without specific DNA–protein interactions. It is also possible that specific and rigid nucleotide–amino acid interactions exist but are complex, and the diffraction from the relatively large mass of highly ordered coat proteins simply overwhelms that from the DNA with lower symmetry.

NMR spectroscopy of the filamentous viruses and their coat proteins and DNA can contribute to a description of their structure and dynamics (Cross et al., 1979; Cross & Opella,

† From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received March 28, 1980.* This work was supported by grants from the American Cancer Society (NP-225A), the National Institutes of Health (GM-24266), and the National Science Foundation (PCM-05598). S.J.O. is a fellow of the A. P. Sloan Foundation (1980–1982).

0006-2960/81/0420-0280$01.00/0