Sanfilippo, A., Morvillo, E., & Ghione, M. (1966) *J. Gen. Microbiol. 43*, 369–374.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Sutherland, J. C., Duval, J. F., & Griffen, K. P. (1978) *Biochemistry 17*, 5088–5091.
Thrum, H. (1959) *Naturwissenschaften 46*, 87.
Thrum, H., Haupt, I., Bradler, G., Zimmer, Ch., & Reinert, K. E. (1972) *Adv. Antimicrob. Antineoplast. Chemother., Proc. Int. Congr. Chemother., 7th, 1971 1*, 819.
Tinoco, I., Jr. (1955) *J. Am. Chem. Soc. 77*, 4486–4489.
Waring, M. J., Wakelin, L. P. G., & Lee, J. S. (1975) *Biochim. Biophys. Acta 407*, 200–212.
Wartell, R. M., Larson, J. E., & Wells, R. D. (1974) *J. Biol. Chem. 249*, 6719–6731.
Zimmer, Ch. (1975) *Prog. Nucleic Acid Res. Mol. Biol. 15*, 285.

Hypoxanthine in Deoxyribonucleic Acid: Generation by Heat-Induced Hydrolysis of Adenine Residues and Release in Free Form by a Deoxyribonucleic Acid Glycosylase from Calf Thymus†

Peter Karran‡ and Tomas Lindahl*

ABSTRACT: A slow conversion of adenine residues to hypoxanthine occurs in single-stranded DNA when heated in neutral aqueous buffers. The rate of this reaction at pH 7.6 and 110 °C is $k = 4 \times 10^{-8}$ s^{-1}, as determined by base analysis of heat-treated DNA that contains radioactively labeled adenine residues. It is proposed that adenine deamination is one of several forms of hydrolytic damage that may occur as spontaneous premutagenic lesions in DNA in vivo. Cell extracts from calf thymus and human fibroblasts contain a DNA glycosylase activity which specifically catalyzes the release of free hypoxanthine from DNA or polydeoxyribonucleotides that contain dIMP residues. Several properties of the purified enzyme from calf thymus are described: It has an approximate molecular weight of 31 000. No cofactors are required for activity. The enzymatic release of hypoxanthine occurs readily from double-stranded polydeoxyribonucleotides that have either thymine or cytosine residues in the complementary strand. Single-stranded polymers are 10–20-fold more slowly attacked, and there is no detectable cleavage of monomeric dIMP. Hypoxanthine is liberated from DNA directly as a free base. Thus, when poly(dI)·poly(dC) containing both [^{3}H]-dIMP and [^{32}P]dIMP residues was employed as the substrate, ^{3}H-labeled hypoxanthine but no ^{32}P-labeled material was released in ethanol-soluble form. The hypoxanthine-DNA glycosylase presumably acts in DNA repair by preventing deaminated adenine residues from being expressed as mu

DNA molecules undergo slow decay at neutral pH as a result of a number of hydrolytic reactions. One of the most important of these may be the deamination of the constituent bases. Conversion of cytosine to uracil has been shown to occur for dCMP residues in single-stranded DNA (Lindahl & Nyberg, 1974) after incubation in solution at elevated temperatures and neutral pH. A DNA repair enzyme which specifically removes uracil from DNA, uracil-DNA glycosylase, is present in extracts from both bacterial and mammalian cells (Lindahl, 1974; Friedberg et al., 1975; Sekiguchi et al., 1976). *Escherichia coli* mutants, *ung*, deficient in this enzyme have been isolated and found to exhibit an increased spontaneous mutation frequency (Duncan et al., 1978). Most of the spontaneous mutations in *ung* strains are G·C→A·T transitions (Duncan & Weiss, 1978). This is consistent with the notion that deamination of cytosine residues in DNA occurs at a significant rate in vivo and that such lesions remain unrepaired in *ung* strains. Further, the discovery of Coulondre et al. (1978) that spontaneous base substitution hot spots in *E. coli* occur at 5-methylcytosine residues further implicates cytosine deamination as relevant to mutagenesis, because the methylated derivative is converted to nonrepairable thymine residues by deamination.

Hydrolytic deamination of purines is a slower reaction than cytosine deamination (Shapiro & Klein, 1966). Nevertheless, even a very slow conversion of purine residues in DNA to deaminated forms may be of physiological significance in view of the very large amount of unique information present in a DNA molecule. In particular, heat-induced degradation at 37 °C of adenine to hypoxanthine would result in A·T→G·C transition mutations after DNA replication unless a repair mechanism exists to remove hypoxanthine from DNA. We have previously shown that *E. coli* cell extracts contain low levels of a DNA glycosylase which excises hypoxanthine from deaminated DNA and polydeoxyribonucleotides (Karran & Lindahl, 1978). Here we report that slow hydrolytic deamination of dAMP residues in DNA can be observed at neutral pH and, further, that mammalian cells contain a hypoxanthine–DNA glycosylase with properties similar to, but not identical with, those of the bacterial enzyme.

Experimental Procedures

Purines, Nucleotides, Polydeoxyribonucleotides, and DNA. Uniformly labeled [^{14}C]adenine (309 mCi/mmol), [2-^{3}H]-adenine (15 Ci/mmol), [8-^{3}H]dATP (29 Ci/mmol), and [α-^{32}P]dATP (30 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. Radioactively labeled dITP was made by deamination of labeled dATP with 3 M

† From the Department of Medical Biochemistry, Gothenburg University, 400 33 Gothenburg, Sweden. *Received June 3, 1980.* This work was supported by the Swedish Medical Research Council.
‡ Recipient of a European Molecular Biology Organization Fellowship. Present address: MRC Cell Mutation Unit, University of Sussex, Falmer, Brighton, England.

0006-2960/80/0419-6005$01.00/0

$NaNO_2$ at pH 3.8 and 25 °C for 5 h, followed by adsorption to activated charcoal, elution with 3% NH_3 in 50% ethanol, and drying under reduced pressure. Similarly, dIMP was prepared by deamination of dAMP with nitrous acid, and deoxyinosine was made from dIMP by treatment with *E. coli* alkaline phosphatase.

Polydeoxyribonucleotides were enzymatically synthesized with calf thymus terminal deoxynucleotidyltransferase prepared according to Chang & Bollum (1971). Double-stranded poly([^{3}H]dI)·poly(dC) was made by mixing equimolar amounts of poly([^{3}H]dI) [1000 cpm (pmol of nucleotide residue)$^{-1}$] and poly(dC) in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) at 20 °C. The properties of the copolymers poly(dA, [^{3}H]dI), poly(dA,[^{14}C]dX), and poly(dA,[^{3}H]dC) have been described (Karran & Lindahl, 1978). Doubly radioactively labeled poly(dI) was prepared with a mixture of [^{3}H]dITP and [^{32}P]dITP. The specific activities of the poly([^{3}H,^{32}P]dI) were 1100 cpm (pmol of nucleotide residue)$^{-1}$ for ^{3}H and 2200 cpm pmol^{-1} for ^{32}P. The polymer was mixed with an equimolar amount of nonradioactive poly(dC) before use.

The procedures used for growth of a purine-requiring strain of *Bacillus subtilis* in the presence of radioactive adenine and isolation of DNA from the bacteria were those previously described (Lindahl & Nyberg, 1972). *B. subtilis* was chosen as source of DNA because the latter does not contain the minor base N^6-methyladenine. Paper chromatographic analysis of acid hydrolysates showed that the ^{14}C-labeled DNA contained 58% of its radioactivity as adenine and 42% as guanine, while the ^{3}H-labeled DNA had 95% as adenine and 5% as guanine. The presence of radioactive guanine in the latter preparation is probably due to the fact that commercial preparations of [2-^{3}H]adenine are contaminated with [8-^{3}H]adenine, part of which may be converted in vivo to radioactively labeled guanine. The [^{3}H]DNA had a specific radioactivity of 35 000 cpm/μg and the [^{14}C]DNA had 32 000 cpm/μg. An aliquot of the [^{14}C]DNA was heat denatured, partly deaminated by treatment with 1 M nitrous acid at pH 4.2 (Schuster, 1960), dialyzed, and renatured by standard methods. This DNA contained 3.4% of its total radioactivity as hypoxanthine and 2.7% as xanthine. Calf thymus DNA was purchased from Worthington, and *E. coli* DNA was prepared by standard procedures (Lindahl & Nyberg, 1974).

Heat Treatment and Base Analysis of DNA. For measurements of heat-induced deamination of adenine residues, DNA solutions (40 μg/mL) were incubated in acid-washed, sealed glass ampules at 110 °C for various times in 0.1 M NaCl–0.01 M sodium phosphate–0.01 M sodium citrate, pH 7.4 (at 25 °C). The pH of this buffer shows little temperature dependence (pH ~7.6 at 110 °C). At the end of the incubation period, the ampules were opened at room temperature, and 0.1 volume of 1 M HCl was added and the resulting mixture incubated at 80 °C for 30 min to release purine residues in free form. To each hydrolysate was added either adenine (20 μg), guanine (4 μg), hypoxanthine (20 μg), and xanthine (10 μg) or only adenine and hypoxanthine, as markers, and the material was analyzed by descending paper chromatography. The four chromatography systems described below were useful for the analysis of the conversion of adenine to hypoxanthine. Solvent fronts were allowed to run off the papers in order to increase resolution. Migration distances are given in relation to adenine (1.00). System I: saturated ammonium sulfate–0.1 M sodium phosphate (pH 7.2)–2-propanol (79:19:2); hypoxanthine 2.8, guanine 1.7, xanthine 1.6; time 30 h. System II: H_2O–isoamyl alcohol–concentrated NH_3 (400:40:1); hypoxanthine 1.4, guanine 0.95, xanthine 1.45; time 10 h. Papers were notched when this system was used in order to reduce the flow rate of the solvent (Reeves et al., 1969). System III: 1-butanol saturated with 10% urea; hypoxanthine 0.7, guanine 0.5, xanthine 0.1; time 40 h. System IV: 1-butanol–diethylene glycol–H_2O (4:1:1); hypoxanthine 0.7, guanine 0.4, xanthine 0.4; time 30 h. After the chromatograms were dried, ultraviolet absorbing material was localized and identified. Strips containing individual samples were then cut transversely in 1-cm pieces and then recut into smaller fragments and transferred to a scintillation counting vial containing 4 mL of H_2O. After elution at room temperature overnight, 15 mL of Aquasol (NEN chemicals) was added, and the radioactivity of the fraction was determined in a liquid scintillation counter.

Enzyme Preparations. Hypoxanthine–DNA glycosylase was purified 1200-fold from calf thymus cell extracts by streptomycin treatment and ammonium sulfate fractionation, followed by chromatography on Sephadex G-75, phosphocellulose, and DNA–cellulose. Details of the purification procedure are described elsewhere (Karran, 1981). The purified enzyme shows no detectable exonuclease activity on double-stranded or single-stranded DNA. While the hypoxanthine–DNA glycosylase shows different fractionation properties from calf thymus uracil–DNA glycosylase and 3-methyladenine–DNA glycosylase, the enzyme preparation has not been completely freed from the latter two activities. All experiments were performed with the 1200-fold-purified hypoxanthine–DNA glycosylase preparation unless otherwise noted. *E. coli* hypoxanthine–DNA glycosylase (Karran & Lindahl, 1978) was purified ~800-fold by a fractionation scheme similar to that used for the calf thymus enzyme.

Assay Procedure. The standard reaction mixture (100 μL) contains 70 mM Hepes–KOH (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 0.15 μg (3×10^5 cpm) of poly([^{3}H]dI)·poly(dC), and a limiting amount of enzyme. After 30 min at 37 °C each reaction mixture was chilled to 0 °C, and 2 μL of 0.05 M hypoxanthine, 10 μL of heat-denatured calf thymus DNA (2 mg/mL), 10 μL of 2 M NaCl, and 300 μL of ethanol (−20 °C) was added. After 20 min at −20 °C, the samples were centrifuged at 15000*g* for 15 min and 300 μL of supernatant was recovered for analysis. When the purified enzymes from calf thymus of *E. coli* were employed, the radioactivity of the supernatant was usually determined directly in 5 mL of dioxane-based scintillation fluid, since control chromatography experiments established that more than 98% of the released material was present in the form of free hypoxanthine in these cases. In assays of crude cell extracts, released hypoxanthine was purified from the ethanol supernatants by paper chromatography prior to determinations of radioactivity (Karran & Lindahl, 1978). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the release of 1 μmol of free hypoxanthine/min under the standard assay conditions.

Size of Enzyme. The sedimentation coefficient and Stokes radius of the enzyme were determined according to Siegel & Monty (1966), using the same experimental conditions and reference proteins as employed in studies on 3-methyladenine–DNA glycosylase (Riazuddin & Lindahl, 1978).

Results

Heat Treatment of DNA Containing Radioactively Labeled Adenine Residues. The rate of deamination of cytosine to uracil in DNA has been determined previously by heat treatment of neutral solutions of DNA containing radioactive cytosine residues, followed by hydrolysis and analysis of the

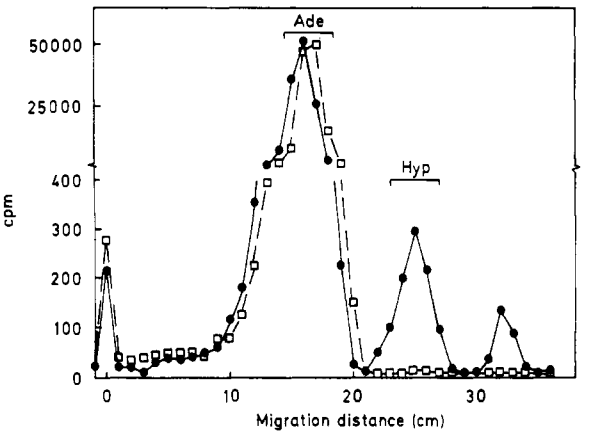

FIGURE 1: Heat-induced deamination of [2-^{3}H]adenine-labeled *B. subtilis* DNA. A DNA solution was incubated for 72 h at 110 °C and pH 7.6 and then hydrolyzed in 0.1 M HCl for 30 min at 80 °C to release free purines, which were analyzed by paper chromatography (●). The material was chromatographed in system II for 10 h together with authentic markers. A control hydrolysate of DNA not incubated at 110 °C was also analyzed (□), and the two chromatographic profiles have been superimposed.

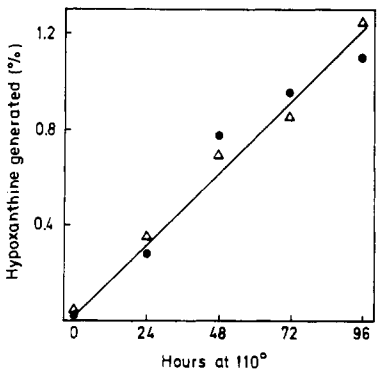

FIGURE 2: Rate of generation of hypoxanthine residues in *B. subtilis* DNA at 110 °C and pH 7.6. (●) [2-^{3}H]Adenine-labeled DNA, analyzed in chromatography system III; (Δ) [^{14}C]purine-labeled DNA, analyzed in system II. The hypoxanthine generated has been determined as the percent of adenine in DNA.

hydrolysate using paper chromatography (Lindahl & Nyberg, 1974). In a similar approach, the rate of deamination of radioactive adenine residues in DNA has now been investigated. Adenine residues in DNA were found to be converted slowly to hypoxanthine at pH 7.6 (Figure 1). The identity of the major new peak of radioactivity which appeared in hydrolysates of heated DNA as hypoxanthine was confirmed by the demonstration that this material cochromatographed with authentic hypoxanthine in four different solvent systems. The deamination of adenine to hypoxanthine in DNA proceeds at a linear rate for at least 4 days at 110 °C, and similar results were obtained with [2-^{3}H]adenine-labeled DNA and with [^{14}C]DNA labeled both in adenine and guanine residues (Figure 2). The rate constant for deamination of adenine in (single-stranded) DNA at 110 °C and pH 7.6 is $k = 4 \times 10^{-8}$ s^{-1}. Under the same conditions, cytosine residues are deaminated to uracil at a rate 40 times faster (Lindahl & Nyberg, 1974). Thus, the hydrolytic deamination of adenine residues in DNA is a much slower reaction than either depurination (Lindahl & Nyberg, 1972) or cytosine deamination at neutral pH. For this reason, it is not possible to determine the rate of adenine deamination in double-stranded DNA by prolonged incubation of DNA solutions at temperatures below the t_m, because significant depurination with accompanying chain breakage and generation of single-stranded sequences would occur before detectable amounts of hypoxanthine were formed.

Since a hypoxanthine–deoxyribose bond is slightly weaker than an adenine–deoxyribose bond in DNA (Shapiro & Chargaff, 1966), some of the deaminated adenine residues were released from the DNA by depurination during the longest incubations. In the standard procedure, the whole DNA solution was analyzed for the presence of hypoxanthine after heat treatment. However, in a control experiment the DNA itself was analyzed for its hypoxanthine content. That is, adenine-labeled DNA was heated for 2 days at 110 °C, chilled, and precipitated with a volume of cold ethanol, and the precipitate hydrolyzed in 0.1 M HCl and analyzed for the presence of hypoxanthine. This material contained a distinct peak of hypoxanthine (0.3% of the radioactivity that was present as adenine), showing that deamination of adenine did occur in DNA in situ. Moreover, the mild acid hydrolysis procedure used to release purines from DNA did not generate detectable amounts of hypoxanthine from adenine residues (Figure 1).

Deamination of deoxyadenosine to deoxyinosine occurs readily in 1 M KOH at 100 °C, while dAMP is more resistant to deamination under those conditions and deamination of free adenine is not detected (Jones et al., 1966). The slow deamination of adenine residues in DNA does not appear to exhibit a strong pH dependence. The rate of adenine deamination in DNA at 110 °C was only ~2-fold faster in a borate-containing buffer at pH 9.5 than at pH 7.6. Thus, the reaction at pH 7 probably is not alkali catalyzed. Similar results have been obtained previously for the pH dependence of cytosine deamination in DNA (Lindahl & Nyberg, 1974).

In addition to hypoxanthine, a second, minor degradation product of adenine appeared in hydrolysates of heated DNA (Figure 1). This compound, which was present in 3–4-fold smaller amounts than hypoxanthine, did not cochromatograph with two adenine derivatives whose imidazole ring had been opened (4,6-diamino-5-formamidopyrimidine or 4,5,6-triaminopyrimidine), and its structure is presently unknown. Further, no systematic attempt to demonstrate and measure the rate of deamination of DNA guanine residues to xanthine has been made in these studies. However, from data obtained with [^{14}C]DNA containing both radioactive adenine and guanine in chromatography systems II and III, and with [^{3}H]DNA in system II, it would appear that at neutral pH, guanine deamination in DNA is an even slower reaction than adenine deamination.

Hypoxanthine–DNA Glycosylase in Mammalian Cells. We have previously described a hypoxanthine–DNA glycosylase from *E. coli* which specifically excises hypoxanthine from DNA and from polydeoxyribonucleotides that contain dIMP residues (Karran & Lindahl, 1978). Cell extracts from calf thymus have been shown to contain somewhat higher levels of such an activity (0.2 microunit/mg of protein) than the *E. coli* extracts. Further, we have observed that crude cell extracts of human fibroblasts also contain a hypoxanthine–DNA glycosylase (~0.1 microunit/mg of protein) with general properties very similar to those of the calf thymus enzyme. The bovine hypoxanthine–DNA glycosylase, though not homogeneous, has been purified 1200-fold by conventional procedures. Several of its properties are described below.

Molecular Weight and General Requirements of the Calf Thymus Enzyme. The sedimentation coefficient of hypoxanthine–DNA glycosylase from calf thymus was determined by cosedimentation in sucrose gradients with alkaline phosphatase (6.3 S), carbonic anhydrase (3.06 S), and lysozyme (2.11 S). The value obtained for hypoxanthine–DNA glyco-

Table I: Activity of Calf Thymus Hypoxanthine-DNA Glycosylase under Different Assay Conditions

	pmol of hypoxanthine released
standard reaction mixture (with 0.025 microunits of enzyme)	0.72
plus 1.0 mM ATP	0.60
plus 1.0 mM P_i	0.74
plus 5.0 mM $MgCl_2$	0.52
plus 20 mM $MgCl_2$	0.10
plus 10 mM $CaCl_2$	0.02
plus 5.0 μg mL^{-1} *E. coli* tRNA	0.70
plus calf thymus DNA, 1.1 μg ml^{-1}	0.52
plus calf thymus DNA, 2.2 μg ml^{-1}	0.28
plus *E. coli* DNA, 2 μg ml^{-1}	0.35
plus 1.5 mM caffeine	0.70
plus 1.5 mM adenine	0.70
plus 1.0 mM hypoxanthine	0.67
plus 3.0 mM hypoxanthine	0.65
plus 0.1 mM deoxyinosine	0.74
plus 0.15 mM xanthine	0.63

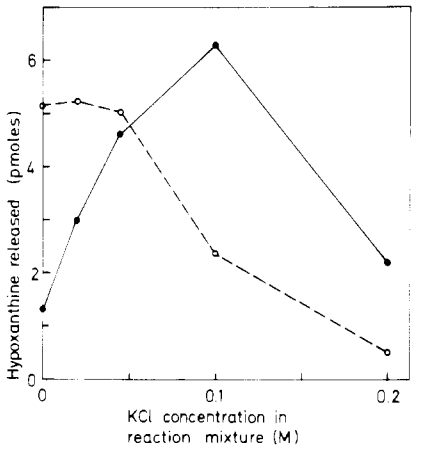

FIGURE 3: Effect of KCl on the activities of hypoxanthine–DNA glycosylases from calf thymus and *E. coli*. The enzymes were assayed in the standard reaction mixture supplemented with various concentrations of KCl. (●) Calf thymus enzyme (0.05 microunit); (○) *E. coli* enzyme (0.2 microunit).

sylase was 3.25 S. The Stokes radius of the enzyme, estimated from gel chromatography experiments, was 2.4 nm. These data may be used to calculate an approximate molecular weight of the protein (Siegel & Monty, 1966) which for the native enzyme is 31 000 ± 3000. The *E. coli* hypoxanthine–DNA glycosylase has a molecular weight of ~30 000 (Karran & Lindahl, 1978), so the bacterial and mammalian enzymes are similar in size.

The calf thymus hypoxanthine–DNA glycosylase has a broad pH optimum between 7.2 and 7.8. There are no apparent obligatory cofactor requirements, and the enzyme is fully active in the presence of 1–5 mM EDTA. Table I shows the effect of the addition of various compounds to the standard reaction mixture. No promotion of activity is detected in the presence of ATP, phosphate, or Mg^{2+}. Higher concentrations of Mg^{2+} or Ca^{2+} are inhibitory. The enzyme is not detectably inhibited by the addition of tRNA to reaction mixtures but is markedly inhibited by low concentrations of native DNA under the standard reaction conditions, possibly because of binding of the enzyme to the DNA. These properties of the calf thymus enzyme are similar to those of the *E. coli* enzyme previously described (Karran & Lindahl, 1978). However, the two enzymes respond differently on addition of KCl to the reaction mixtures. As shown in Figure 3, the activity of the calf thymus hypoxanthine–DNA glycosylase is 4-fold stimulated by the addition of 0.1 M KCl, whereas the *E. coli* enzyme shows no such stimulation and is 2-fold inhibited. Thus, while the same standard assay conditions have been defined for both the *E. coli* and calf thymus hypoxanthine–DNA glycosylases, it is advantageous to include 0.1 M KCl in assay mixtures for mammalian hypoxanthine–DNA glycosylase.

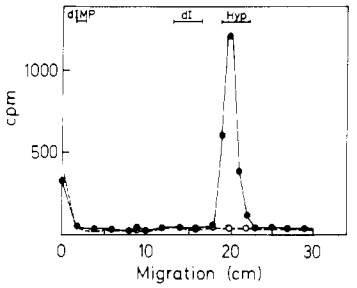

FIGURE 4: Chromatographic analysis of the ethanol-soluble material released from poly([^{3}H]dI)·poly(dC) by calf thymus hypoxanthine–DNA glycosylase. Standard reaction mixtures (100 μL each) were incubated with or without 0.12 microunit of enzyme. After ethanol precipitation and centrifugation, the ethanol-soluble fractions were supplemented with 25 μg each of hypoxanthine, deoxyinosine, and dIMP, concentrated to ~50 μL under reduced pressure, and applied to Whatman 3MM paper. The samples were then fractionated by descending chromatography in isobutyric acid–H_2O–0.1 M Na_2EDTA–toluene–concentrated NH_3 (66:23:2:20:2) for 16 h. Marker substances were localized as ultraviolet-absorbing material. Radioactive material was determined as described under Experimental Procedures. (●) Reaction mixture with added enzyme; (○) no enzyme added.

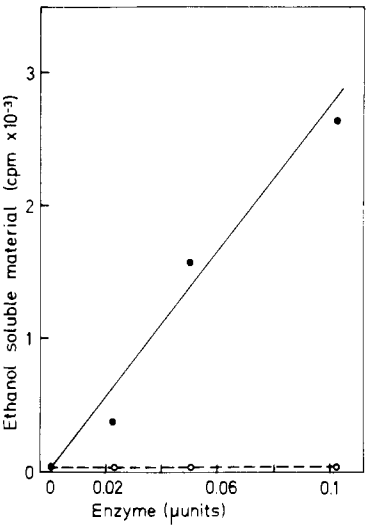

FIGURE 5: Radioactive material released by calf thymus hypoxanthine–DNA glycosylase from a doubly labeled polymer. Poly(dI)·poly(dC), containing [^{3}H]hypoxanthine and ^{32}P residues in the poly(dI) strand, was employed as the substrate under the standard reaction conditions. Each assay mixture contained 260 pmol of dIMP residues in polymeric form, and increasing amounts of enzyme were added. After 30 min at 37 °C, the ethanol-soluble radioactive material was determined. (●) ^{3}H; (○) ^{32}P.

Release of Free Hypoxanthine from DNA. The hypoxanthine that is enzymatically liberated from poly(dI)·poly(dC) is released as the free base, and no detectable excision of either dIMP or deoxyinosine is observed (Figure 4). These data indicate that the enzyme acts by catalyzing the direct cleavage of the base–sugar bonds of dIMP residues in the polymer. This was shown more directly by employing a doubly radioactively labeled poly(dI)·poly(dC) substrate that contained ^{3}H in the hypoxanthine residues and ^{32}P in the poly(dI) chain. The calf thymus enzyme released ^{3}H-labeled material efficiently from this substrate, with no detectable concomitant

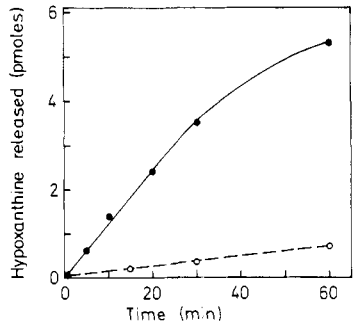

FIGURE 6: Kinetics of enzymic release of hypoxanthine from poly([^{3}H]dI), with or without a complementary poly(dC) chain. Reaction mixtures containing 300 pmol of dIMP residues, either double-stranded poly([^{3}H]dI)·poly(dC) or single-stranded poly([^{3}H]dI), and 0.12 microunit of enzyme/100 μL in each case were incubated for various times under the standard assay conditions. (●) Double-stranded substrate; (○) single-stranded substrate.

release of ^{32}P (Figure 5). A similar experimental approach, employing a doubly labeled DNA substrate, has been taken previously by Duncan et al. (1976) and Linsley et al. (1977) to show that uracil–DNA glycosylase catalyzes the release of free uracil but no phosphate from DNA containing dUMP residues.

Substrate Specificity. Calf thymus hypoxanthine–DNA glycosylase has properties similar to those of the *E. coli* enzyme with regard to its substrate specificity. Thus, the calf thymus enzyme shows a marked but not absolute preference for double-stranded substrates. It catalyzes the release of hypoxanthine 10–15 times more efficiently from poly(dI)·poly(dC) than from poly(dI) (Figure 6). Under the assay conditions employed here, poly(dI) would be expected to occur in a single-stranded conformation (Inman, 1964). The two substrates, poly(dA,[^{3}H]dI)·poly(dT) and poly([^{3}H]dI)·poly(dC), were attacked by the calf thymus enzyme at about the same rate, while hypoxanthine was released 10 times more slowly from single-stranded poly(dA,[^{3}H]dI). These experiments demonstrate that the calf thymus hypoxanthine–DNA glycosylase can act on a dIMP residue hydrogen bonded to either a dCMP or a TMP residue in the complementary chain. Moreover, the relative activities on the various polymers remained the same during enzyme purification and may be ascribed to a single enzyme.

In addition to the release of hypoxanthine from polydeoxyribonucleotides, the calf thymus enzyme also catalyzes the liberation of free hypoxanthine from DNA that has been partly deaminated by nitrous acid treatment. Under conditions where 0.4 pmol of hypoxanthine was enzymically released from the DNA, no simultaneous liberation of xanthine (<0.03 pmol), adenine, or guanine was found to occur. The inability of the enzyme to release xanthine from dXMP residues in polymeric form was further tested by using the xanthine-containing copolymer poly(dA,[^{3}H]dX)·poly(dT) as a potential substrate in the standard assay. No detectable release of xanthine was observed (<2 pmol of xanthine liberated by 2 microunits of hypoxanthine–DNA glycosylase in 30 min). Similarly, the calf thymus enzyme did not appear to be able to act in the correction of mismatched base pairs (other than those produced with hypoxanthine), because free cytosine was not liberated to a detectable extent (<0.01 pmol released by 0.5 microunit of enzyme) from a poly(dA,[^{3}H]dC)·poly(dT) polymer under the standard reaction conditions.

The calf thymus hypoxanthine–DNA glycosylase shows no detectable ability to catalyze the cleavage of monomeric dIMP. Less than 0.05 pmol of free hypoxanthine was liberated from 120 pmol of [^{3}H]dIMP by 0.5 microunit of enzyme under the standard reaction conditions, as determined by paper chromatography. This observation supports the argument that hypoxanthine is released directly from poly(dI) or deaminated DNA without the intermediate formation of monomeric dIMP (Figure 4) and that the enzyme is specific for dIMP residues in polymers.

K_m Value and Lack of Product Inhibition. The K_m of the calf thymus hypoxanthine–DNA glycosylase for dIMP residues was determined by varying the concentration of the poly([^{3}H]dI)·poly(dC) double-helical polymer in the standard reaction mixture (25–400 pmol of dIMP residues added/100 μL of reaction mixture). From a double-reciprocal plot, the K_m of the enzyme for dIMP residues in this polymer was estimated to be 9×10^{-7} M. In the same fashion, the K_m value for dIMP residues in the poly(dA,[^{3}H]dI)·poly(dT) polymer was found to be 5×10^{-7} M.

Uracil–DNA glycosylase and 3-methyladenine–DNA glycosylase are inhibited by high concentrations of the free base that is being liberated. In contrast, no detectable product inhibition was observed in experiments with the calf thymus hypoxanthine–DNA glycosylase where the standard reaction mixture was supplemented with 1–3 mM hypoxanthine (Table I). Further, no inhibition of enzyme activity by deoxyinosine, adenine, xanthine, or caffeine was observed (Table I).

Discussion

It is shown here that deamination of adenine residues to hypoxanthine in DNA occurs at a slow but detectable rate at pH 7.6. The experimental protocol adopted was similar to that employed previously to study cytosine deamination in DNA (Lindahl & Nyberg, 1974). Under the conditions used (single-stranded DNA, high temperature) the deamination of adenine residues was 40 times slower than that of cytosine. Making the reasonable assumption that both types of deamination reactions are associated with similar activation energies, it may be predicted that adenine deamination occurs at lower temperatures but at a lower rate than cytosine deamination. Compared to single-stranded DNA, deamination of cytosine is strongly suppressed in double-stranded DNA; it may occur primarily in vivo in regions of single-stranded DNA generated during replication and transcription. It is not known if adenine residues are similarly protected in double-stranded DNA against hydrolytic deamination. In any case, the occasional and rare spontaneous conversion of an adenine residue to a base with different coding properties probably cannot be ignored by living cells. The existence of an enzyme that specifically catalyzes the cleavage of dIMP residues in DNA also indicates that the hydrolytic deamination reaction occurs in vivo. The enzyme, hypoxanthine–DNA glycosylase, has been observed previously in *E. coli* (Karran & Lindahl, 1978), but this study presents the first evidence for its occurrence in mammalian cells.

Little is presently known about pathways other than heat-induced hydrolysis for the introduction of hypoxanthine residues into DNA at neutral pH. It has been reported that trace amounts of hypoxanthine may be recovered from adenine solutions exposed to ultraviolet light (Kland & Johnson, 1957) and ionizing radiation (Ponnamperuma et al., 1961), but it remains unclear if hypoxanthine occurs as a minor radiation-induced lesion in DNA. Adenine residues, as well as guanine and cytosine, are readily deaminated by nitrous acid treatment at low pH (Schuster, 1960). While this reagent is very useful for deamination of purine residues in experimental work on mutagenesis, it seems unlikely that the reaction of DNA with nitrous acid is of any great relevance under normal physiological conditions. Further, it is not known if enzymes

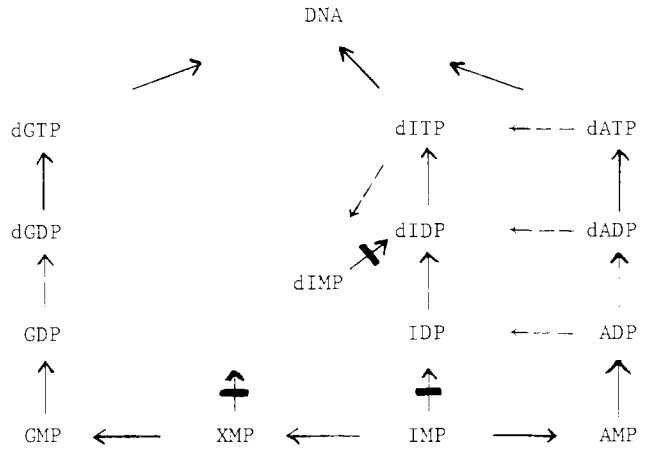

FIGURE 7: Scheme for incorporation of purine nucleotides into DNA. Solid arrows show major pathways, while dashed arrows indicate infrequent reactions. Arrows with a crossed bar show forbidden pathways. Several of the steps represent reversible reactions, but this is not indicated in this diagram.

such as adenosine deaminase or the enzyme that converts certain AMP residues to IMP in tRNA precursor molecules might occasionally act by mistake on a dAMP residue in DNA. Considering the specificity of most enzymes, this appears unlikely.

We have previously made the argument that DNA contains thymine instead of uracil because cells must be able to recognize specifically and remove from DNA the deaminated cytosine residues generated by spontaneous hydrolysis (Lindahl et al., 1977). The same argument could be extended to purines (Coulondre et al., 1978). That is, a hypothetical DNA molecule which contained adenine and hypoxanthine as its two purine bases might not be adequate for preservation of genetic information. Although it might exhibit reasonably satisfactory base-pairing properties, it would have the inherent disadvantage that deaminated adenine residues might not be easily recognized and repaired. IMP is present in cells as a precursor for AMP and GMP but is not further phosphorylated and incorporated into nucleic acids. No kinase for IMP and dIMP seems to exist (Oeschger & Bessman, 1966; Kammen & Spengler, 1970; Carson et al., 1977). The restricted substrate specificity of nucleoside monophosphate kinases may be of crucial importance in the exclusion of unusual base residues from DNA. If enzymatic conversion of IMP to IDP occurred in vivo, the IDP could be efficiently reduced to dIDP by ribonucleotide reductase, converted to dITP by nucleoside diphosphokinase, and used for DNA synthesis instead of dGTP by DNA polymerase. Instead, IMP is converted by two enzymatic steps to GMP, presumably in order to generate a purine derivative more useful for DNA replication (Figure 7).

While dITP probably should not exist in living cells, traces of this compound may nevertheless occur in vivo as a result of deamination of dATP or some other unusual chemical reaction. Recent studies with a cell-free system from *E. coli* that catalyzes DNA replication in vitro (Thomas et al., 1978) have shown that a dITPase, analogous to dUTPase, does not seem to exist for the rapid and specific degradation of this nonconventional deoxynucleoside triphosphate. Thus, dIMP residues are incorporated into DNA in the place of dGMP residues when dITP is added to such a cell-free system. Although these dIMP residues remain for longer time periods in DNA than dUMP residues, presumably because of the much lower level of hypoxanthine–DNA glycosylase activity than of uracil–DNA glycosylase activity in cell extracts, they are nevertheless specifically excised soon after replication.

The general properties and substrate specificity of the calf thymus hypoxanthine–DNA glycosylase described here are similar to those of the corresponding *E. coli* enzyme (Karran & Lindahl, 1978; Oeda et al., 1978). Both enzymes efficiently remove hypoxanthine residues that lie in complementary positions to either cytosine or thymine. The properties of uracil–DNA glycosylase and 3-methyladenine–DNA glycosylase isolated from mammalian cells (Talpaert-Borlé et al., 1979; Ishiwata & Oikawa, 1979; Brent, 1979) also appear quite similar to their bacterial counterparts (Lindahl et al., 1977; Riazuddin & Lindahl, 1978). It seems likely that all these enzymes serve in DNA repair by initiating a base excision–repair event at a damaged or misincorporated residue. There is presently no evidence for any other role of hypoxanthine–DNA glycosylase, and no mutant cells deficient in the enzyme have so far been isolated. In this regard, cell extracts from human fibroblasts of a number of patients with diseases associated with defective DNA repair, e.g., ataxia-telangiectasia, contained levels of hypoxanthine–DNA glycosylase activity similar to those found in extracts from normal fibroblasts (P. Karran, T. Lindahl, and S. Söderhäll, unpublished observations). A further search in bacteria or human cells for enzyme-deficient mutants should be initiated, since such mutants would allow more precise information to be obtained on the contribution of DNA hypoxanthine residues to spontaneous mutagenesis or cellular sensitivity to deaminating agents.

References

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

Carson, D. A., Kaye, J., & Seegmiller, J. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5677–5681.

Chang, L. M. S., & Bollum, F. J. (1971) *J. Biol. Chem. 246*, 909–916.

Coulondre, C., Miller, J. H., Farabaugh, P. J., & Gilbert, W. (1978) *Nature (London) 274*, 775–780.

Duncan, B. K., & Weiss, B. (1978) in *DNA Repair Mechanisms* (Hanawalt, P. C., Friedberg, E. C., & Fox, C. F., Eds.) pp 183–186, Academic Press, New York.

Duncan, B. K., Rockstroh, P. A., & Warner, H. R. (1978) *J. Bacteriol. 134*, 1039–1045.

Duncan, J., Hamilton, L., & Friedberg, E. C. (1976) *J. Virol. 19*, 338–345.

Friedberg, E. C., Ganesan, A. K., & Minton, K. (1975) *J. Virol. 16*, 315–321.

Ishiwata, K., & Oikawa, A. (1979) *Biochim. Biophys. Acta 563*, 375–384.

Jones, A. S., Mian, A. M., & Walker, R. T. (1966) *J. Chem. Soc. C*, 692–695.

Kammen, H. O., & Spengler, S. J. (1970) *Biochim. Biophys. Acta 213*, 352–364.

Karran, P. (1981) in *DNA Repair. A Laboratory Manual of Research Procedures* (Friedberg, E. C., & Hanawalt, P. C., Eds.) Marcel Dekker, New York (in press).

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

Kland, M. J., & Johnson, L. A. (1957) *J. Am. Chem. Soc. 79*, 6187–6192.

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

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

Lindahl, T., & Nyberg, B. (1974) *Biochemistry 13*, 3405–3410.

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

Linsley, W. S., Penhoet, E. E., & Linn, S. (1977) *J. Biol. Chem. 252*, 1235–1242.
Oeda, K., Shimizu, K., & Sekiguchi, M. (1978) *J. Biochem. (Tokyo) 84*, 1165–1169.
Oeschger, M. P., & Bessman, M. J. (1966) *J. Biol. Chem. 241*, 5452–5460.
Ponnamperuma, C., Lemmon, R. M., Bennett, E. L., & Calvin, M. (1961) *Science 134*, 113.
Reeves, W. J., Seid, A. S., & Greenberg, D. M. (1969) *Anal. Biochem. 30*, 474–477.
Riazuddin, S., & Lindahl, T. (1978) *Biochemistry 17*, 2110–2118.
Schuster, H. (1960) *Z. Naturforsch., B 15*, 298–304.
Sekiguchi, M., Hayakawa, H., Makino, F., Tanaka, K., & Okada, Y. (1976) *Biochem. Biophys. Res. Commun. 73*, 293–299.
Shapiro, H. S., & Chargaff, E. (1966) *Biochemistry 5*, 3012–3018.
Shapiro, R., & Klein, R. S. (1966) *Biochemistry 5*, 2358–2362.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.
Talpaert-Borlé, M., Clerici, L., & Campagnari, F. (1979) *J. Biol. Chem. 254*, 6387–6391.
Thomas, K. R., Manlapaz-Ramos, P., Lundquist, R., & Olivera, B. M. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 231–237.

Studies on the Mechanism of Membrane Fusion: Kinetics of Calcium Ion Induced Fusion of Phosphatidylserine Vesicles Followed by a New Assay for Mixing of Aqueous Vesicle Contents†

Jan Wilschut,‡ Nejat Düzgüneş,§ Robert Fraley,⊥ and Demetrios Papahadjopoulos*

ABSTRACT: We describe an assay for following the mixing of aqueous contents during fusion of phospholipid vesicles. Terbium is encapsulated as the $Tb(citrate)_3^{6-}$ chelation complex in one population of vesicles, dipicolinic acid (DPA) in another. Vesicle fusion results in the formation of the fluorescent $Tb(DPA)_3^{3-}$ chelation complex. The presence of EDTA (0.1 mM) and Ca^{2+} (>1 mM) prevents the formation of the Tb/DPA complex in the external medium. We have studied the Ca^{2+}-induced fusion of small or large unilamellar vesicles (SUV or LUV, respectively) composed of phosphatidylserine (PS). In addition, vesicle aggregation was monitored by light scattering, and release of vesicle contents was followed by carboxyfluorescein (CF) fluorescence enhancement. The addition of Ca^{2+} induced an immediate enhancement in Tb fluorescence with both SUV and LUV, which occurs on the same time scale as aggregation but much faster than the release of CF. The release of contents from LUV occurs with a considerable delay. It is estimated that the initial fusion of SUV is accompanied by 10% leakage of the internal volume per fusion event; in contrast, fusion of LUV is essentially nonleaky. Massive release of vesicle contents appears to be a secondary phenomenon related to the collapse of fused vesicles. The initial rate and the extent of Tb fluorescence enhancement are markedly dependent on the Ca^{2+} concentration. Threshold Ca^{2+} concentrations are 1.2 and 2.4 mM for SUV and LUV, respectively. At saturating Ca^{2+} concentrations (>10 mM), the rate of fusion of LUV is slightly lower than that of SUV at the same vesicle concentration. At any Ca^{2+} concentration, the rates of both SUV and LUV fusion are consistent with vesicle aggregation being rate limiting. When measured at a subsaturating Ca^{2+} concentration, fusion is essentially second order over a wide range of relatively low vesicle concentrations, whereas at higher vesicle concentrations the order is decreased. This suggests that at high vesicle concentrations (and at relatively low Ca^{2+} concentrations) aggregation may proceed faster than fusion.

The molecular mechanism of biological membrane fusion has received a great deal of attention in a number of recent investigations. The majority of these studies dealt with the application of artificial membrane systems as simplified models for biological membranes. In particular, the divalent cation induced aggregation and fusion of negatively charged phospholipid bilayers have been studied extensively, since they show several characteristics that are similar to those of biological membrane fusion (Papahadjopoulos et al., 1977, 1978; Papahadjopoulos, 1978).

It has been shown previously that Ca^{2+} induces massive aggregation of sonicated phosphatidylserine (PS)[1] vesicles (Lansman & Haynes, 1975; Portis et al., 1979; Düzgüneş & Ohki, 1977), release of vesicle contents (Papahadjopoulos & Bangham, 1966; Papahadjopoulos et al., 1977; Portis et al., 1979), and formation of large cochleate structures (Papahadjopoulos et al., 1975). These structural reorganizations occur at a threshold Ca^{2+} concentration of about 1 mM; at this concentration, the binding of Ca^{2+} to PS approaches a level corresponding to the stoichiometric ratio of one Ca^{2+} per two

† From the Cancer Research Institute and the Department of Pharmacology, School of Medicine, University of California, San Francisco, California 94143. *Received April 24, 1980.* This investigation was supported by Research Grant GM-26359 from the National Institutes of Health and by a fellowship grant (J.W.) from The Netherlands Organization for the Advancement of Pure Research (ZWO).

‡ Present address: Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands.
§ Recipient of National Institutes of Health Fellowship CA-06190-01 from the National Cancer Institute.
⊥ Recipient of National Institutes of Health Fellowship GM-07167-01.

[1] Abbreviations used: PS, phosphatidylserine; DPA, dipicolinic acid; CF, carboxyfluorescein; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DSC, differential scanning calorimetry; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles.

0006-2960/80/0419-6011$01.00/0