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HYDROLYSIS OF 2-AMINOPURINE 2'-DEOXYRIBOSIDE IN NEUTRAL SOLUTION

The Ohio State University

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HYDROLYSIS OF 2-AMINOPURINE 2'-DEOXYRIBOSIDE IN NEUTRAL SOLUTION

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University.

By
Peter Carl Ratsep, B.Sc.

The Ohio State University
1986

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P. C. Ratsep and R. C. Pless, "Formation of 2,4-diamino-5-formamidopyrimidine from 2-aminopurine deoxyriboside", submitted for publication.


FIELD OF STUDY

BIOCHEMISTRY
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ABBREVIATIONS

A: refers to the site on a DNA molecule in which 2'-deoxyadenosine triphosphate is incorporated, i.e., to the adenine moiety in DNA.

Ade: adenine.

Ado: adenosine; 6-amino-9-(β-D-erythro-pentofuranosyl)purine.

dAdo: 2'-deoxyadenosine; 6-amino-9-(2-deoxy-β-D-erythro-pentofuranosyl)purine.

dAMP: 2'-deoxyadenosine-5'-monophosphate.

AmPur: 2-aminopurine; this abbreviation is also used in general terms to refer to those compounds which contain the 2-aminopurine moiety.


AmPurdR: 2-aminopurine 2'-deoxyriboside; 2-amino-9-(2-deoxy-β-D-erythro-pentofuranosyl)purine.

AMV: Avian myeloblastosis virus.

AP: Apurinic site.

C: refers to the site on a DNA molecule in which 2'-deoxycytidine triphosphate is incorporated, i.e., to the cytosine moiety in DNA.
CI: chemical ionization mass spectrometry.

DAFP: 2,4-diamino-5-formamidopyrimidine.

DAFPdR: 2,4-diamino-5-formamido-N^4-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimidine.

DSS: 3-(trimethylsilyl)-1-propane sulfonic acid, sodium salt.

EI: electron impact mass spectrometry.

G: refers to the site on a DNA molecule in which 2'-deoxyguanosine triphosphate is incorporated, i.e., to the guanine moiety in DNA.

gg: gauche-gauche configuration of the 5'-exocyclic group of a nucleoside or nucleotide.

gt: gauche-trans configuration of the 5'-exocyclic group of a nucleoside or nucleotide.

Guo: guanosine; 2-amino-6-hydroxy-9-(β-D-erythro-pentofuranosyl)purine.

dGuo: 2'-deoxyguanosine; 2-amino-6-hydroxy-9-(2-deoxy-β-D-erythro-pentofuranosyl)purine.

IR: infrared spectrophotometry.

Me_2SO-d_6: perdeutero-dimethyl sulfoxide.

Me_4Si: tetramethyldisilane

MS: mass spectrometry.

N: north configuration of the pentose ring.
NMR: nuclear magnetic resonance spectrometry.

dNTP: 2'-deoxynucleotide triphosphate.

S: south configuration of the pentose ring.

T: refers to the site on a DNA molecule in which 2'-deoxythymidine triphosphate is incorporated, i.e., to the thymine moiety in DNA.

TAP: 2,4,5-triaminopyrimidine.

tg: trans-gauche configuration of the 5'-exocyclic group of a nucleoside or nucleotide.

UV: ultraviolet spectrophotometry.
STATEMENT OF THE PROBLEM

The glycosidic linkage in 2-aminopurine 2'-deoxyriboside (AmPurdR) and in polydeoxyribonucleotides containing 2-aminopurine (AmPur), was found to be distinctly more labile than the glycosidic linkage in 2'-deoxyadenosine (dAdo), 2'-deoxyguanosine (dGuo), or in the adenine (A) and guanine (G) sites in DNA (Pless and Bessman, 1983). While this observation was initially used for an analytical purpose, to identify sites of high AmPur incorporation in DNA, the increased lability of the glycosidic bond of AmPur-containing compounds was of intrinsic interest in view of the mutagenic action of AmPur, which is not yet clearly understood, as well as from a mechanistic point of view.

If the relative ease of cleavage of the glycosidic linkage, observed by Pless and Bessman (1983) at basic pH (aqueous piperidine) and high temperature, is also observed under physiological conditions (neutral aqueous medium, 37°C), the increased incidence of apurinic sites in DNA which would result from initial AmPur incorporation could be of biological significance. While, from a biochemical point of view, the greatest interest attaches to the chemical characteristics of AmPur moieties incorporated in DNA, it was decided that study of the depurination reaction of AmPur-containing compounds at neutral pH
would be conducted on the simplest level, i.e. nucleosides. In view of the slow reaction rates observed for other purine nucleosides, the hydrolysis reactions were conducted at elevated temperatures. The purpose behind the studies at elevated temperatures was two-fold in nature; in the event that the reaction might be too slow to measure at 37°C, the rate at 37°C would have to be determined by extrapolation; and knowledge of the temperature dependence of the rate constant would allow calculation of the enthalpy of activation for the process responsible for the decomposition of AmPurdR. The response of AmPurdR kinetics to various parameters (salt, concentration of the buffer, pH, concentration of the nucleoside, type of buffer, solvent composition) was also examined in order to try to gain a mechanistic understanding of the increased hydrolytic lability of this nucleoside.

In the course of this work, it was found that the product distribution obtained from AmPurdR hydrolysates was not analogous to the product mixture in dAdo hydrolysates. In particular, for the major product from AmPurdR there is no equivalent compound in the dAdo hydrolysate. Identification of this AmPurdR degradation product constituted a part of the present work.
Mutagenic aspects of 2-aminopurine

The compound 2-aminopurine (AmPur) is a base-pairing analogue of adenine (Ade), and it has been recognized as a mutagen since 1959 when Freese first reported that AmPur-induced mutations in T4 phage (Freese, 1959a, 1959b). Since that time, there have been numerous studies concerning the effects of this mutagen on organisms varying from bacteria and bacteriophage to plants and mammals. An extensive review on the subject of AmPur mutagenesis has been compiled by Ronen (1979).

AmPur is incorporated into the DNA molecule (Gottschling and Freese, 1961; Ronen, 1979), primarily into the A sites (Rogan and Bessman, 1970; Pless and Bessman, 1983), where it will form an AmPur:T base pair (Freese, 1959b). This base pair (Figure 1c) is weaker than the corresponding A:T base pair (Figure 1a; Watanabe and Goodman, 1981; Scheit and Rackwitz, 1982; Goodman and Ratliff, 1984; Brennan and Gumport, 1985) or the G:C base pair (Figure 1b). AmPur has been found to primarily induce transition mutations; these are thought to be produced by a mispairing mechanism which has as an intermediate the
Figure 1. Hydrogen bonding scheme for the A:T, G:C, AmPur:T, and various proposed AmPur:C base pairs.
Ampur:C mispair. Ampur induces A:T -> G:C transitions much more frequently than G:C -> A:T transitions. This preference in the direction of transitions caused by Ampur is presumably due to the relative strengths of the hydrogen bonds of the base pairs and the 3'-> 5' exonuclease activity of the polymerase (Reha-Krantz and Bessman, 1977; Bessman and Reha-Krantz, 1977; Galas and Branscomb, 1978; Mhaskar and Goodman, 1984).

The nature of the bonding involved in the Ampur:C base pair is unclear. It was initially assumed that formation of this base pair required a tautomeric shift in either the Ampur or the C residue (Freese, 1959b). Such structures are shown in Figures 1d and 1e. However, since the formation of the Ampur:C base pair containing the rare tautomer of Ampur (Figure 1d) has been calculated to be four orders of magnitude less probable than the formation of the Ampur:C base pair involving normal tautomers (Rein and Shibata, 1983), other possible structures have been postulated; these include: an Ampur:C base pair containing only one hydrogen bond (Figure 1f), and a wobble Ampur:C base pair (Figure 1g) with two hydrogen bonds, which requires some distortion of the B-DNA backbone angles in order to realize the additional hydrogen bond (Rein and Shibata, 1983).

Based on the theoretical calculations of Rein and Shibata (1983), the probable structures can be ranked in order of decreasing probability as follows: the Ampur:C base pair with one hydrogen bond (Figure 1f) > the wobble Ampur:C base pair (Figure 1g) > the base pair formed between tautomeric Ampur and C (Figure 1d). In spite of its two hydrogen bonds, the wobble Ampur:C base pair is energetically less
favorable than the AmPur:C base pair with a single hydrogen bond, since the energy gained by the formation of the additional hydrogen bond is less than the energy required to deform the DNA backbone angles; the wobble structure requires on the order of 4-8 kcal/mol more energy to form than the corresponding Watson-Crick base pair. According to Rein's calculations, the AmPur:C base pair with a single hydrogen bond is able to account for all of the trends observed experimentally, including the misinsertion frequency and the nearest neighbor trends of misincorporation.

A different structural possibility for the AmPur:C base pair has been proposed by Goodman and Ratliff (1983). From the results of UV spectral studies of the association of various AmPur-containing DNA strands with poly(dT) or poly(dC), they concluded that the AmPur:C base pair contained two hydrogen bonds; and they speculated that this base pair would have one of two forms: it would either involve tautomeric AmPur and normal C (Figure 1d), or it would involve an AmPur:C base pair in which the N-1 position of AmPur was protonated (Figure 1h). The former base pair can be discounted according to the arguments of Rein and Shibata (1983). The latter structure is an interesting proposition; it is, indeed, likely that protonation of AmPurR and of AmPur-containing polydeoxyribonucleotides, occurs at N-1, in analogy with the conclusions of Janion and Shugar (1973) on the site of protonation in AmPur riboside (AmPurR) and in polyribonucleotides which contain AmPur. There is, however, a more likely site of protonation in the base pair, the N-3 position in the cytosine moiety. The pKa for the N-1 position of the AmPur moiety has
a value of 3.4 in the riboside (Fox et al., 1958), and a value of 4.8 in the polyribonucleotide (Janion and Shugar, 1973), while cytosine has a pKa of 4.2 in the deoxynucleoside (Fox and Shugar, 1952), and therefore, should have a pKa of about 5.6 in a polynucleotide. Thus, protonation in the AmPur:C base pair would more likely occur at the N-3 of C than at the N-1 of AmPur, as proposed by Goodman and Ratliff (1983). It is easily seen that protonation at N-3 in the cytosine moiety would also provide the possibility of formation of a second hydrogen bond in the AmPur:C base pair, without a change in the normal geometry of B-DNA (Figure 11), in a manner similar to the structure in Figure 11h, which involves protonation at N-1 of AmPur. Further studies, possibly using NMR techniques, will be required to finally determine the structure of the AmPur:C base pair.

There have been several reports that, apart from transitions, AmPur can induce other types of mutations, namely transversion and frameshift mutations (Bautz-Freese, 1961; Yanofsky et al., 1966; Ronen, 1979; Persing et al., 1981); but these results were later ascribed to spontaneous background reversions, alterations in the deoxynucleotide triphosphate pools, or else they went unexplained as to their origin (Yanofsky et al., 1966; Watanabe and Goodman, 1981; Ronen, 1979). It was speculated that if these mutations were to occur, they might be due to misinsertions of purines opposite AmPur. However, from chemical considerations and model building, it appeared that in all of the possible structural combinations of A and G with AmPur, the structures either were sterically hindered or contained no hydrogen bonds (Topal and Fresco, 1976). This lead to the general
assumption that AmPur created only transition mutations (Ronen, 1979).

Contradicting this assumption, Persing et al. (1981) have more recently presented evidence that supports the appearance of AmPur-induced transversions and frameshift mutations. In a system of various defined trpA and trpE alleles in E. coli (Yanofsky, et al., 1966; Drapeau et al., 1968; Bronson and Yanofsky, 1974), they observed, along with the expected transition mutations, a single frameshift mutation, which resulted from the deletion of an A:T base pair, and three transversion mutations, which resulted from either the G:C <-> C:G; A:T -> C:G; or A:T <-> T:A transversions. Since the models of Topal and Fresco (1976) indicated that AmPur-induced transversions could not occur via a purine-AmPur mispair, Persing et al. (1981) suggested that the transversions were the result of a misrepair process. In particular, they considered the possibility of recA dependent misrepair. To test for this possibility, they used coisogenic donors to transduce the recA+ and recA56 (inactive recA) alleles into the trpA and trpE recipient strains. Their results indicated that recA dependent misrepair was not responsible for the observed transversions and frameshift since these mutations still occurred in the recA56 strains. However, they speculated that some other form of misrepair not involving the recA protein was responsible for the observed transversion and frameshift mutations (Persing et al., 1981); in particular, they considered the mismatched-base repair system. Although this system for excision repair of mismatches has not been shown to be error-prone (Vaccaro and Siegel, 1975; Glickman et al., 1978), it utilizes DNA polymerase I, which has an
error rate app. $10^{-6}$, instead of the normal DNA polymerase III system which has an error rate of app. $10^{-7}$.

One type of potentially mutagenic lesion which has not been investigated and yet may account for the formation of AmPur-induced transversions, is the apurinic (AP) site caused by the depurination at AmPur sites in the DNA molecule. Apurinic sites in DNA are formed spontaneously and at a significant rate. For histone-free DNA, the rate is on the order of 12,000 per mammalian cell per 20 hr generation time; however, since approximately 50% of the DNA is bound to nucleohistones, a better estimate would be about 2,000 to 10,000 per cell per generation (Lindahl and Nyberg, 1972). These apurinic sites have also been found to be quite stable, the half-life being 190 hr at 37°C (Lindahl and Andersson, 1972).

Apurinic sites were recognized as being potentially mutagenic, based on correlations between mutagenesis and depurination caused by heat, acid and alkylating agents (Bautz and Freese, 1960; Bautz-Freese, 1961). However, more recent information indicates that an apurinic site could only induce a mutational event when it escaped repair by an AP endonuclease-DNA polymerase-mediated or an insertase type pathway prior to replication. The topics of mutagenesis induced by misrepair and repair enzymes are reviewed by Drake and Baltz (1976), Hartman (1980), and Lindahl (1982). Misrepair of an apurinic site in DNA may take two forms; the first occurs during replication when the polymerase copies past the apurinic sites, potentially producing a mutation; the second is caused by the induction of an error-prone repair mechanism, e.g. the SOS response found in E. coli.
In vitro, it has been demonstrated that various purified DNA polymerases could produce errors when an apurinic site was bypassed in partially depurinated homopolymer templates (Shearman and Loeb, 1979; Boiteux and Laval, 1982), in partially depurinated \( \phi X174am3 \) DNA (Kunkel et al., 1981; Schaaper et al., 1983), and in partially depurinated M13 DNA (Sagher and Strauss, 1983). Different enzymes evinced similar preferences as to the nucleotides incorporated opposite the apurinic sites, with a preference for insertion of dAMP (Sagher and Strauss, 1983; Boiteux and Laval, 1982). However, the probability for bypass of apurinic sites was different for various enzymes, the polymerases of highest fidelity showing the least capability to copy across such sites. Thus, Schaaper et al. (1983) found AMV DNA polymerase to have much higher bypass frequency than \textit{E. coli} polymerase I in an in vitro comparison using partially depurinated \( \phi X174am3 \) DNA, while in vivo experiments with the same type of DNA showed that for the highly accurate \textit{E. coli} polymerase III holoenzyme, bypass was undetectable (Schaaper and Loeb, 1981) in noninduced cells, but significant when the SOS response had been elicited.

This error-prone response has been extensively reviewed by Witkin (1976). When \textit{E. coli} is exposed to UV light or other agents which may be mutagenic (Hanawalt et al., 1979; Oishi et al., 1978), damage to its DNA occurs. The surviving cells may repair the damage by relatively error-free repair mechanisms such as photoreactivation and
the "short patch" excision repair. However, some forms of DNA damage induce another type of repair mechanism, one which is error-prone. When replication is either blocked or stalled by a potentially mutagenic lesion, the induced error-prone response will allow bypass of the lesion. Although the bypassing of the lesion allows replication to continue, it does so at the expense of its replicative fidelity. Thus, other mutations may be directly caused as a result of this response.

Although it has been demonstrated that the SOS response is not responsible for the appearance of the AmPur-induced transversions and frameshifts, two studies have provided clear evidence that under error-prone (SOS) conditions, bypass of apurinic sites is frequent and induces mutations (Schaaper and Loeb, 1981; Schaaper et al., 1983). It might be that if an error-prone repair system is induced by mismatches, i.e. AmPur:C, any unrepaired apurinic sites may be bypassed and hence, produce mutations.

Mechanistic aspects of acid catalyzed depurination of purine nucleosides

The acid-catalyzed depurination of purine nucleosides and nucleotides in aqueous medium was originally thought to be similar in mechanism to the hydrolysis of certain N-glycosyl compounds, e.g. di-c-N-methylamine glucoside (Kenner, 1957) or N-aryl-D-glucosylamines (Capon and Connett, 1965). This postulated depurination mechanism is
summarized in Figure 2: the annular oxygen of the sugar moiety is protonated; the sugar ring opens, with formation of a cationic Schiff base with the double bond located between C-1 of the sugar and N-9 of the purine; the carbinolamine is formed by the addition of water to the Schiff base; this structure ultimately decomposes producing of the free sugar and the free purine (Kenner, 1957; Dekker, 1960; Michelson and Heesing, 1961; Michelson, 1963).

Kenner (1957) stated that, in contrast to di-N-methylamine glucoside, tri-N-methylammonium glucoside is very stable in acid and decomposes by an A-1 mechanism where the rate-limiting step is the cleavage of the glycosyl linkage, ultimately producing glucose and trimethylamine. He assumed the same mechanism to be operative in the hydrolysis of benzimidazole glycosyl compounds, because of their stability in acid, and he interpreted the faster hydrolysis of purine glycosyl compounds as an indication that they did not react via the A-1 mechanism.

A revision of this mechanistic view was forced by the extensive studies of Zoltewicz et al., (1970) on the hydrolysis of guanosine (Guo), 2'-deoxyguanosine (dGuo), 2'-deoxyadenosine (dAdo), 7-methylguanosine, 7-methyl-2'-deoxyguanosine, and 1,7-dimethylguanosinium iodide. Their results indicated a depurination mechanism of the A-1 type, involving protonation of a nitrogen in the purine moiety, followed by cleavage of the N-glycosyl bond in a slow step, producing the oxycarbonium ion of the sugar and a purine tautomer; after reaction with water, the final products are the free
Figure 2. The A-2 mechanism for the hydrolysis 2'-deoxyadenosine.
sugar and the free purine (Figure 3). The major points advanced by Zoltewicz et al. (1970) against the A-2 mechanism are:

1) At 100°C, dGuo is 520 times more reactive than Guo. If the rate-limiting step were the addition of water to the charged Schiff base, the exchanging of the 2'-hydrogen for a hydroxyl group should not cause such a large difference in the rates. Admittedly, in the A-2 mechanism, the 2'-hydroxyl group should retard the formation of the charged Schiff base, but the cation, once produced, should be more reactive; this compensation of effects is expected to result in a much smaller difference between the hydrolysis rate constants for the ribonucleotides and deoxyribonucleotides than is actually observed.

2) If the rate-limiting step is the decomposition of the carbinolamine, the rates would show an inverse dependence on the acid concentration in the low pH region; this is not observed.

3) The hydrolysis of Guo, dGuo, and dAdo show a linear first-order dependence on the hydrogen ion activity at least in the regions from pH 2.3 - 4.4 (Zoltewicz et al., 1970), H0 5.54 to pH 4 (Zoltewicz et al., 1970; Zoltewicz and Clark, 1972), and pH 1.5 - 7.5 (Zoltewicz et al., 1970; Garrett and Mehta, 1972a), respectively. In order for this rate dependence to be linear, the apparent second-order rate constants for the two cases for reaction of the nucleoside with hydronium ion must be the same at pH values both above and below the pKa of the monocation. This type of behavior would be unlikely to occur in the A-2 mechanism since the rate constant for the formation of the cationic Schiff base formed in weakly acidic medium would be
Figure 3. The A-1 mechanism for the hydrolysis of 2'-deoxyadenosine.
expected to be larger than the rate constant for the cationic Schiff base formed in strongly acidic medium, where both the N-9 and the protonated N-7 carry a positive charge; the release of electrons into the furanose ring by the latter species will be greatly reduced due to the protonation of the heterocycle. Thus, the reaction rates for the monoo- and diprotonated species will not be the same in the A-2 mechanism, and the rate dependence on the hydrogen activity would not be linear. However, in the A-1 mechanism, although the dicatonic purine species is formed less readily, it is a better leaving group than the monocatonic species. Thus, it is possible to observe similar apparent second-order rate constants in the pH region where the reaction is dominated by the decomposition of the monocation and in highly acidic media where degradation occurs largely from the dication.

4) In the A-2 mechanism, the hydrolysis of 1,7-dimethylguanosinium iodide, of 7-methylguanosine, and of 7-methyl-2'-deoxyguanosine would be expected to be dependent on the pH in the dilute acid region since this pathway requires protonation of the annular oxygen. In contrast, in the A-1 mechanism a positively charged (protonated) purine moiety is essential, to act as a good leaving group. Thus, with this mechanism it would be expected that in the dilute acid region, where 1,7-dimethylguanosinium iodide, 7-methylguanosine, and 7-methyl-2'-deoxyguanosine bear a full positive charge in the aglycone, their hydrolysis rates would be independent of the pH. This is indeed observed between pH 3 and pH 5. Also, the rate constants for these
species in the pH dependent region (low pH) should be similar to that of the dication of guanosine or, in the case of 7-methyl-2'-deoxyguanosine, the dication of 2'-deoxyguanosine. Experimentally, these trends were observed; the second order rate constants (M⁻¹ sec⁻¹) for guanosine, 1,7-dimethylguanosinium iodide, and 7-methylguanosine are 1.72 x 10⁻² (100.3°C); 1.73 x 10⁻² (100.3°C); 1.78 x 10⁻² (100.6°C), respectively, while the second order rate constants for 7-methyl-2'-deoxyguanosine and 2'-deoxyguanosine are 6.03 x 10⁻² (52.4°C) and 6.98 x 10⁻² (52.6°C), respectively.

5) The isotope effect of the change of the solvent from H₂O to D₂O on the rate in a dilute acid solution is expected to be between 1.8 to 2.5 for the A-1 mechanism (Capon and Connett, 1965). Zoltewicz et al. (1970) observed an apparent solvent isotope effect of 2.4 for dAdo, and 2.0 for 7-methyl-2'-deoxyguanosine at pH = 7.72 and 7.10.

6) The positive ΔS⁰ values found in the acid-catalyzed depurination of dAdo and dGuo, +8.4 eu and +12.7 eu, respectively, are indicative of two-step, monomolecular hydrolysis reactions.

The A-1 mechanism for the acid-catalyzed hydrolysis of purine nucleosides proposed by Zoltewicz et al. (1970) has since been supported by numerous studies on the depurination of various purine nucleosides (Garrett and Mehta, 1972a; Hevesi et al., 1972; Suzuki, 1974; Romero et al., 1978), modified purine nucleosides (Garrett and Mehta, 1972a; Panzica et al., 1972), and their analogues (Lönnberg and Käppi, 1980; Lönnberg, 1980a, 1980b; Lönnberg and Käppi, 1981). This mechanism has also been invoked in the hydrolysis of pyrimidine
deoxyribosides under neutral and acid conditions (Shapiro and Kang, 1969; Shapiro and Danzig, 1972).
CHAPTER II

EXPERIMENTAL

Materials:

The chemicals were obtained from the following suppliers:
2-aminopurine, Pfaltz & Bauer, Inc.; 2'-deoxycytidine and cytosine, Sigma Chemical Co.; 2'-deoxyadenosine, adenosine, adenine, and 2'-deoxyguanosine, P-L Biochemicals; uniformly $^{14}$C-labeled 2'-deoxycytidine (5 μCi/200 μL; 480 mCi/mmol), Amersham; 2-amino-9-(8-D-ribofuranosyl)purine, Vega Biochemicals; 2,4-diamino-5-nitropyrimidine, maleic acid, and cacodylic acid (dimethylarsinic acid), Sigma Chemical Co.; potassium hydroxide, glycine, hydrochloric acid, triethylamine, disodium hydrogen phosphate dodecahydrate, and sodium dihydrogen phosphate monohydrate, Mallinckrodt; 1,4-dioxane, 2-deoxy-arabino-pentose (2-deoxy-D-ribose), Aldrich Chemical Company; sodium hydroxide, 88% formic acid, and standard phosphate buffer (pH 7.00) for pH calibration, Fisher Scientific Co.; potassium bromide (IR grade), MCB Manufacturing Chemists, Inc.

HPLC grade acetonitrile and methanol were from Burdick and Jackson Laboratories, Inc., or from J.T. Baker Chemical Co.; D$_2$O (99.5 atom
% D) was obtained from Matheson, Coleman & Bell; perdeuterodimethylsulfoxide (Me₂SO-d₆; 99.9 atom % D), D₂O (99.96 atom % D), and tetramethylsilane (Me₄Si; 99.9+% ) were purchased from Aldrich Chemical Company.

Polygram CEL 300 UV(254) for cellulose TLC was the product of Macherey-Nagel, silicagel TLC plates (60 F-254) were from EM Reagents, Amberlite XAD-4 resin was obtained from the J.T. Baker Chemical Co., and Scinti Verse II scintillation fluor was purchased from Fisher Scientific Co.

The 95% formic acid was prepared by fractional distillation of 88% formic acid. The concentration of formic acid was determined by measurement of the refractive index (Abbe refractometer, Fisher Scientific Co.) and interpolation between the reported refractive indices for 88% and 100% formic acid, 1.3666 and 1.3714, respectively (Handbook of Chemistry and Physics, CRC press, 59th ed., 1978-1979).

The enzyme, trans-N-deoxyribosylase from Lactobacillus helveticus (nucleoside: purine (pyrimidine) deoxyribosyl transferase; EC.2.4.2.6) was a gift from Dr. Maurice Bessman of the Johns Hopkins University; no information was obtained concerning the enzyme activity. The Raney nickel, W2 activity, was obtained from Dr. David Hart of this university.
Instruments

High Pressure Liquid Chromatography (HPLC):

a) The Glenco HPLC System (Glenco Scientific, Inc.) was equipped with a Glenco 5480 UV monitoring unit and a Linear chart recorder (model no. 261; Linear Instrument Corporation); the monitoring unit contained two filters to provide light in the 254- or the 280-nm region. The pathlength of the flow cell was 1.0 cm.

b) The ISCO gradient HPLC apparatus (ISCO, Inc.) was equipped with two model 2300 HPLC pumps and an ISCO V4 absorbance detector (variable wavelength) with an internal chart recorder. The ISCO V4 detector had interchangeable flow cells; the analytical flow cell had a pathlength of 0.5 cm and an illuminated volume of 3.6 µL, while the preparative flow cell had a pathlength of 0.2 cm and an illuminated volume of 5.8 µL. The ISCO HPLC apparatus was equipped with a "Chromatochart" gradient/data integration package (Interactive Microware, Inc.) which included an Apple IIe computer (Apple Computer, Inc.), a C.Itoh 8510 printer (C.Itoh Digital Products, Inc.), and a Grappler+ printer interface card (Orange Micro, inc.). All HPLC analyses were carried out on Whatman PAC columns; PAC is a cyano-amino derivatized silicagel stationary phase.

Mass Spectrometry (MS): Electron impact (EI) and chemical ionization (CI) spectra were determined using a Kratos MS-30 mass spectrometer. The carrier gas used in the CI experiment was isobutane. The mass
spectra were recorded by C. R. Weisenberger at The Ohio State University Chemical Instrument Center.

**Nuclear Magnetic Resonance (NMR):** The 200-MHz proton NMR spectra were determined using a Bruker WP 200 instrument; the 500-MHz NMR spectra were recorded on a Bruker AM 500; $^{13}$C NMR spectra were determined at 75-MHz on a CXP300 NMR spectrometer equipped with a wide-bore probe. The 200-MHz spectra were recorded by Rubio R. Punzalan; the 500-MHz spectra and the $^{13}$C spectra were obtained by Dr. C. E. Cottrell at The Ohio State University Chemical Instrument Center.

**Infrared Spectrophotometry (IR):** The samples were analyzed as KBr pellets on a Perkin-Elmer 283B Infrared Spectrophotometer.

**Ultraviolet Spectrophotometry (UV):** The UV spectra were determined in quartz cells with path lengths of 1 cm, using a Hitachi 100-80A recording spectrophotometer (Hitachi Scientific Instruments). The wavelengths were calibrated against a holmium oxide standard filter (Arthur Thomas Co.).

**Melting points:** Melting points were determined on a Thomas-Hoover (Arthur H. Thomas Co.) capillary melting point apparatus (Uni-melt), and the melting points were corrected using commercial standards (sulfanilamide: 164.5-166.5°C; sulfapyridine: 190-193°C; caffeine: 235-237.5°C).
TLC analyzer: The locations and the intensities of the radioactive species on a TLC plate were determined on a Berthold Automatic TLC-Linear Analyzer, which was equipped with an Apple IIe computer (Apple Computer, Inc.).

Scintillation counter: Radioactive samples were counted in Scintiverse II scintillation fluor on a Beckman LS 7500 scintillation counter.

Syntheses:

Synthesis of 2-amino-9-(2-deoxy-β-D-erythro-pentofuranosyl)purine (AmPurdR):

The AmPurdR was prepared by a scaled up version of the method of Pless et al. (1981). The following procedure was used in a typical preparation: 100 mg AmPur (0.74 mmol) and 606 mg 2'-deoxycytidine (2.67 mmol) were dissolved in 300 mL of 74 mM potassium malate buffer, pH 7.60. After addition of 500 μL of the enzyme solution, the mixture was incubated for app. 24 hr at 37°C. The reaction was followed by cellulose TLC using 10 mM glycine-HCl buffer, pH 10.0, for the development. The chromatographic bands were made visible using short wavelength UV light; cytosine and 2'-deoxycytidine appeared as dark bands, while AmPur and AmPurdR bands were fluorescent blue. Cytosine and 2'-deoxycytidine migrated with the solvent front, while AmPur and
AmPurdR had Rf values of 0.4 and 0.6, respectively. When the reaction appeared to be complete, the solution was concentrated to a volume of about 70 mL by rotary evaporation at 40°C, and applied to an Amberlite XAD-4 column (4.3 cm x 55.5 cm), which had been equilibrated with 1.3 L of 50 mM triethylammonium hydrogen carbonate buffer solution, pH 7.6. Fractions of 50 mL size were collected and the eluate was monitored by the UV absorption spectrum (350-200 nm) for each fraction. Cytosine, the unreacted AmPur, and 2'-deoxycytidine were eluted with 1.1 L of 250 mM triethylammonium hydrogen carbonate buffer, pH 7.6. One column-volume of degassed, deionized water was passed over the column to remove the buffer. The AmPurdR was eluted with 500 mL of water-ethanol 3:1 (v/v). The AmPurdR-containing fractions were pooled and concentrated to a small volume by rotary evaporation, and the solution was lyophilized to give 170 mg (0.68 mmol) of a fluffy, white powder. Yields were typically 70-90%; the material was pure, as judged by its 200-MHz proton NMR spectrum (Figure 4 and Table 1), by its HPLC profile, and by its UV absorption spectrum in aqueous medium.

**Synthesis of AmPurdR, uniformly 14C-labeled in the sugar moiety:**

The procedure was similar to that used in the synthesis of unlabeled AmPurdR, except that the input concentrations were altered for maximal utilization of the radioactive substrate. The incubation mixture was prepared from 20 µL of a 20 mM 2'-deoxycytidine stock solution, 90 µL of a solution which was 45 mM in AmPur and 95 mM in
Figure 4. The 200-MHz proton NMR spectrum of 2-aminopurine 2'-deoxyribose (AmpurdR) in Me₂SO-d₆, at 25°C and 32 mM nucleoside.
Table 1. 200-MHz proton NMR spectrum of AmPurdR in MeSO-d₆, 25°C, 32 mM in AmPurdR.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Chemical shift [ppm]</th>
<th>Integration</th>
<th>Multiplicity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-6</td>
<td>8.59</td>
<td>17.9</td>
<td>s</td>
</tr>
<tr>
<td>H-8</td>
<td>8.28</td>
<td>17.2</td>
<td>s</td>
</tr>
<tr>
<td>2-NH₂</td>
<td>6.52</td>
<td>33.9</td>
<td>s</td>
</tr>
<tr>
<td>1'-H</td>
<td>6.29</td>
<td>18.6</td>
<td>m</td>
</tr>
<tr>
<td>3'-OH</td>
<td>5.29</td>
<td>14.3</td>
<td>broad</td>
</tr>
<tr>
<td>5'-OH</td>
<td>4.97</td>
<td>11.9</td>
<td>broad</td>
</tr>
<tr>
<td>3'-H</td>
<td>4.38</td>
<td>18.6</td>
<td>m</td>
</tr>
<tr>
<td>4'-H</td>
<td>3.85</td>
<td>18.9</td>
<td>m</td>
</tr>
<tr>
<td>5' + 5''-H</td>
<td>3.56</td>
<td>35.5</td>
<td>m</td>
</tr>
<tr>
<td>2'-H</td>
<td>2.64</td>
<td>18.8</td>
<td>m</td>
</tr>
<tr>
<td>2''-H</td>
<td>2.26</td>
<td>18.1</td>
<td>m</td>
</tr>
</tbody>
</table>

* The assignments of the resonances are based upon the sequence of chemical shifts typically found for purine deoxyribonucleosides and upon the exchangeability of the peaks with addition of D₂O. The H-6 and H-8 protons were assigned according to the exchangeability of the H-8 proton with D₂O at high temperature.

* (s) singlet; (d) doublet; (m) multiplet.
potassium malate, pH 7.6, 80 μL (2.0 μCi, 4.17 nmol) of the stock solution of uniformly ^14C-labeled 2'-deoxycytidine, and 4 μL of the enzyme stock solution, to give a total reaction volume of 194 μL. The input concentrations were: 21 mM in AmPur, 45 mM in potassium malate, 2.1 mM in 2'-deoxycytidine, and the specific radioactivity of the 2'-deoxycytidine was 4.9 mCi/mmol. The solution was incubated at 37°C, and the reaction was followed by cellulose TLC, as described earlier. The radioactive bands on the chromatogram were located using the Berthold automatic TLC - Linear Analyzer. After a 5 hr incubation, the solution was applied to an Amberlite XAD-4 column, of 1.5-mL bed volume, which had been pre-equilibrated with triethylammonium hydrogen carbonate buffer. Fractions were collected manually in 1.5-mL Eppendorf snap-cap tubes, and tested for radioactivity. When elution with triethylammonium hydrogen carbonate buffer did not release any more radioactive material, the resin was washed with one column-volume of water, and then with ethanol:water 3:1 (v/v). Radioactive fractions eluting with the latter solvent were pooled, concentrated by rotary evaporation, lyophilized to a powder, and the residue was taken up in 105 μL of degassed, deionized water. The yield of ^14C-labeled AmPurdR was close to quantitative; 1.2 μCi of ^14C-labeled AmPurdR were obtained in this manner, as determined by liquid scintillation counting of an aliquot. The solution was kept at -20°C until used. The HPLC profile of this material, after 2 months of storage at -20°C, is shown in Figure 5.
Figure 5. HPLC profile for $^{14}$C-labeled ApPurd after 2 months storage at -20°C.
Preparation of 2,4-diamino-5-formamidopyrimidine (DAFP):

Isolation of Species II (DAFP) from AmPur hydrolysates:

A 100-mg sample of AmPur (0.74 mmol) was dissolved in 80 mL of degassed, deionized water, pH 7.1. Aliquots of the solution were sealed in 10-mL borosilicate glass ampules and incubated in a silicone oil bath at 100°C, along with several small samples sealed in 0.7-mL ampules. The latter samples were used to check the time course by HPLC, monitoring at 280 nm. The concentration of DAFP appeared to have reached its maximal value after 4 days. At this point, the contents of the ampules were pooled, concentrated by rotary evaporation, and applied to a column (4.3 cm x 55.5 cm) of Amberlite XAD-4. The column was eluted with water, and the chromatography was monitored by UV absorption spectroscopy. The first peak started eluting at about 150 mL and partially merged into the second peak; it was designated Species III according to its elution position in the HPLC profile. This peak had a \( \lambda_{\text{max}} \) of 267 nm; however, when the pooled fractions were subjected to rotary evaporation at a temperature of 40°C, the absorbance maximum shifted from 267 to 304 nm. The second peak was Species II (DAFP) judging by its UV absorption spectrum (\( \lambda_{\text{max}} = 287 \) nm) and by its retention time in the HPLC profile; it eluted between 480 mL and 1440 mL. The AmPur peak started at 1550 mL and ended at 3040 mL. The fractions were pooled accordingly, and the pools were concentrated by rotary evaporation and lyophilized to a powder.
For further purification, Species II (DAFP) was passed over a preparative PAC HPLC column (Whatman, M9, 9.4 mm ID x 50 cm), using the ISCO HPLC apparatus, with isocratic elution with acetonitrile-methanol 9:1 (v/v) at a flow rate of 2.0 mL/min and monitoring at 287 nm. The solvent system used in this purification was the same as that utilized in the kinetic experiments; no optimization of the solvent composition was done. Fractions were collected and those fractions which contained the central portion of the peak were pooled. The isolated yield of DAFP was 15 mg (98 μmol; 13%).

Chemical synthesis of 2,4-diamino-5-formamidopyrimidine (DAFP):

Isay (1906) reported the preparation of 2,4-diamino-5-formamidopyrimidine (DAFP) by reduction of 2,4-diamino-5-nitropyrimidine with SnCl₂ in concentrated HCl, followed by formylation in boiling 95-99% formic acid. My attempts to duplicate the first step of this procedure were unsuccessful. The compound was ultimately obtained by catalytic reduction of the diaminonitropyrimidine as described by Brown (1957), followed by formylation according to Isay (1906), as shown in Figure 6.

To a suspension of 2.01 g of 2,4-diamino-5-nitropyrimidine (13.0 mmol) in 90 mL of anhydrous methanol was added 1.2 g of Raney nickel (W2 activity), suspended in 20 mL of absolute ethanol. The vessel was evacuated and filled with hydrogen gas twice, then filled with hydrogen gas to an initial pressure of 3.0 atm. The suspension was shaken at room temperature for 3 hr; by this time, the hydrogen
Figure 6. Synthesis of 2,4-diamino-5-formamidopyrimidine.
pressure had decreased to 0.88 atm. The nickel was filtered off on paper and the purple filtrate was evaporated to dryness; 1.55 g were recovered. This corresponds to a 95% yield. The purple powder, 2,4,5-triaminopyrimidine, had a corrected melting point of 177-178.5°C (lit. 176-179°C, Isay (1906); 171-173°C, Brown (1957), probably uncorrected). In methanolic solution, this material showed an absorbance maximum at 308 nm, while in neutral and in ammoniacal aqueous medium a $\lambda_{\text{max}}$ of 303 nm and a $\lambda_{\text{min}}$ of 269 nm were observed (lit. $\lambda_{\text{max}} = 303$ nm in basic aqueous solution, Mason, 1954). In acidic aqueous medium, the spectrum showed only a shoulder at 269 nm.

A solution of 1.48 g of 2,4,5-triaminopyrimidine (11.8 mmol) in 40 mL of 95% formic acid was heated at reflux for 0.5 hr. The solution was evaporated, the residue was dissolved in 40 mL of deionized water, and the resulting solution was adjusted to pH 10 with concentrated aqueous ammonia. Upon standing overnight at 40°C, this solution deposited tan crystals, which were filtered off, air dried, and weighed: 703 mg. The filtrate was evaporated to dryness; the yellow-white powder had a mass of 1.05 g. The combined mass of these two samples was 1.75 g, corresponding to 11.4 mmol of 2,4-diamino-5-formamidopyrimidine, i.e. a yield of 96%, based on starting 2,4,5-triaminopyrimidine, and an overall yield of 88%. Both the crystals and the powder gave identical UV absorption spectra in neutral aqueous solution, with a $\lambda_{\text{max}}$ of 288 nm and a $\lambda_{\text{min}}$ of 260 nm. This matches the UV spectrum of Species II (DAFP) which was isolated from AmPur and AmPurdR.
The crystals had a corrected melting point of 226-227°C (lit. 224°C, Isay, 1906). Electron impact (EI) mass spectrometry indicated that the molecular mass of the substance was 153.067 amu, which is close to the value of 153.069 amu calculated for 2,4-diamino-5-formamidopyrimidine.

KINETICS:

Sodium cacodylate buffer was chosen for the kinetic experiments, for three reasons: cacodylate is bacteriostatic; the pH of cacodylate buffer solutions is very insensitive to temperature changes, and sodium cacodylate does not precipitate out of solution when an aliquot of the hydrolysate is prepared for HPLC analysis.

Unless otherwise specified, all of the solutions used in the kinetic experiments with unlabeled nucleosides were prepared in the following manner: 5 mg of dry AmPurdR or dAdo (20 μmol) was dissolved in degassed, deionized water, and 350 μL of a 174 mM stock buffer solution of sodium cacodylate, pH 7.0, was added. The final volume of the solution was adjusted to 4.0 mL with deionized water; and the final concentrations were 5 mM in nucleoside and 15 mM in buffer. The final concentration of the nucleoside was verified by UV absorption measurement of a suitably diluted sample. The final pH was determined with a glass electrode, standardized at pH 7.00 at 25°C with a reference phosphate buffer; pH values were estimated to have a maximum error of 0.1 pH units. There was some variance of the pH between
experiments. This variance may be due to the extent of dissolved CO$_2$ in the water; upon extensive degassing, the pH of the solution varied between pH 6.9 to 7.15. Aliquots (300 - 500 µL) of the solution were sealed in 0.7-mL borosilicate glass ampules (Wheaton Scientific) and incubated at the appropriate temperature in a thermostated heat block (Lab-Line Instruments Inc.). For the experiments conducted at 50°, 70°, 90°, and 110°C, the temperatures varied maximally by 0.5 degrees; however, for the 37°C experiment, maximal variations were ±1°C over a 239 day time period. Ampules were removed after different reaction times, frozen, and stored at -20°C until such time when the entire run could be analyzed by HPLC. Occasionally, the pH of each of the time points of an experiment was determined prior to the HPLC analysis; it was found that the pH of the samples would vary maximally by 0.1 pH units. The entire run was analyzed on the same day, in order to insure that the conditions for the analysis were constant for all the samples.

The HPLC analysis was carried out in the following manner: the samples were diluted 11-fold to a concentration of 0.45 mM nucleoside by pipetting 50 µL of the sample solution into 500 µL of the running solvent. Of each diluted solution, 15 µL was passed over a Whatman PAC precolumn followed by a Whatman 10 µm PAC column (4.6 mm I.D. x 25 cm) at a flow rate of 1.0 mL/min, using a Glenco HPLC System equipped with a UV monitoring unit. The linearity of the response of the UV monitoring unit of the Glenco HPLC apparatus was verified at both 280 and 254 nm with solutions of AmPurdR and dAdo of known
concentration. For the analysis of AmPurdR hydrolysates, the HPLC running solvent was acetonitrile-methanol 9:1 (v/v) and the eluate was monitored at 280 nm since the wavelength for the alternative filter on the Glenco system, 254 nm, coincides with the absorption minimum of AmPurdR. In the case of dAdo hydrolysates, the solvent was acetonitrile-methanol 77:23 (v/v) and the eluant was monitored at 254 nm. At least two samples of each time point were analyzed, and if the peak height for the remaining starting nucleoside differed by more than 3% for these two runs, more samples were injected until a consistent peak height was reached. The disappearance of the starting material was measured by either determining the area under the peak or by measuring the peak height. Both of these methods gave similar results.

The data generated in this manner was found to behave in a pseudo-first order manner, i.e. a straight line was observed in a plot of ln(peak height) vs. time for both dAdo and AmPurdR. The rate constant for a run was determined directly as the negative of the slope of a least-squares line through the data points. It was found that the least-squares lines generally had a correlation factor of -0.99. The reactions were followed for one to three half-lives, except for the reaction at 37°C, where the reaction was followed for 239 days out of a calculated half-life of 301 days.

In the hydrolysis of adenosine and 2-aminopurine riboside, the plot of ln(peak height) vs. time was distinctly curved. In these cases, the pseudo-first order rate constant was calculated as the
negative slope of the plot of \(\ln[(\text{peak height at time } t) - (\text{peak height at time } t + \Delta t)]\) vs. time (Guggenheim, 1926).

The Arrhenius activation energy (\(E_a\)) in a condensed phase can be determined from the slope of an Arrhenius plot of the natural logarithm of the numerical value of the rate constant versus the reciprocal of the absolute temperature. The logarithmic form of the Arrhenius equation is given by

\[
\ln k = -\frac{E_a}{RT} + \ln A \quad (1)
\]

where \(k\) is the rate constant; \(E_a\) is the energy of activation; \(R\) is 1.987 cal/mol*deg; \(T\) is the absolute temperature; and \(A\) is the pre-exponential factor. The enthalpy of activation (\(\Delta H^\ddagger\)) is calculated using the equation

\[
\Delta H^\ddagger = E_a - RT \quad (2)
\]

The entropy of activation, \(\Delta S^\ddagger\), can be calculated using the rate equation

\[
k = \left(\frac{kT}{h}\right) \times \exp(-\frac{\Delta H^\ddagger}{RT}) \times \exp\left(\frac{\Delta S^\ddagger}{R}\right) \quad (3)
\]

where \(k\) is the first order rate constant (s\(^{-1}\)); \(\kappa\) is the Boltzmann constant, \(1.381 \times 10^{-16}\) erg/deg; and, \(h\) is Planck's constant, \(6.626 \times 10^{-27}\) erg*sec.

Rearrangement of the logarithmic form of equation 3 gives

\[
\Delta S^\ddagger = R[\ln k - \ln(\frac{kT}{h}) + \frac{\Delta H^\ddagger}{RT}] \quad (4)
\]

An alternative method for the determination of the \(\Delta H^\ddagger\) and \(\Delta S^\ddagger\) directly stems from a rearrangement of the logarithmic form of equation 3

\[
\ln(\frac{kh}{kT}) = (\frac{\Delta S^\ddagger}{R}) - (\frac{\Delta H^\ddagger}{RT}) \quad (5)
\]
In a plot of \( \ln(kh/kT) \) vs. \( 1/T \), the \( \Delta H^\# \) can be determined from the slope of the line, and \( \Delta S^\# \) can be determined from the intercept.

In each of the following experiments, the assays were treated as described in the general method with the temperature held constant at 70°C, unless otherwise noted.

**Rate dependence on sodium chloride concentration:**

All of the solutions were 5 mM in AmPurR and 15 mM in sodium cacodylate, pH 7.00. The concentrations of NaCl used in these experiments were: 0 mM, 15 mM, 50 mM, and 118 mM.

**Rate dependence on sodium cacodylate concentration:**

All of the solutions were 5 mM in AmPurR. The concentrations of sodium cacodylate used in these experiments were: 15 mM (pH 6.94), 50 mM (pH 6.95), and 150 mM (pH 7.00).

**Rate dependence on pH:**

All of the solutions were 5 mM in AmPurR and 15 mM in total cacodylate concentration (i.e. cacodylate + cacodylic acid). The stock buffer solution for the run at pH 5.80 was prepared by adjusting 5.0 mL of 0.100 M cacodylic acid solution (pH 5.50) to pH 5.80. The final cacodylate concentration of this solution was 98 mM. An aliquot of this stock solution was diluted with water to 15 mM in total cacodylate concentration. The solvent for the run at pH 6.30 was
prepared from the stock 174 mM sodium cacodylate buffer (pH 7.00) by adjusting the pH with 1 M HCl. The final concentration of this buffer was 164 mM in cacodylate (pH 6.30). An aliquot of this stock solution was diluted with water to 15 mM in total cacodylate concentration. Additional data points were obtained from 70°C runs in which the pH deviated from the standard pH of 6.90.

Rate dependence on AmPurdR concentration:

The various AmPurdR solutions were prepared by the appropriate dilution of an aliquot of a 10.0 mM AmPurdR stock solution; the final AmPurdR concentrations were: 4.82 mM (pH 7.15); 0.75 mM (pH 7.06); and 0.054 mM (pH 7.10). All of the solutions were 15 mM in sodium cacodylate.

Hydrolysis rates with different buffers:

The solutions used in this experiment were 5 mM in AmPurdR, and the ionic strength was kept constant at 15 mM. The buffers used in this trial were the standard sodium cacodylate buffer, pH 6.90, and a sodium phosphate buffer, pH 6.90. The phosphate buffer was prepared by combining 55 mL of a 0.2 M Na₂HPO₄ solution and 45 mL of a 0.2 M NaH₂PO₄ solution, followed by dilution with water to a total volume of 200 mL.
Rate dependence on the solvent composition; Dioxane:water:

The solutions were prepared in the following manner:

1) **50% Dioxane:water**: 4.8 mg of AmPurdR was dissolved in 1.8 mL of degassed, deionized water and 1.8 mL of dioxane, freshly distilled from benzophenone and sodium metal under a nitrogen atmosphere. Aliquots of this solution were then sealed under a nitrogen atmosphere. The final nucleoside concentration was 5.3 mM, and the pH was not determined.

2) **80% Dioxane:water**: 5.1 mg of AmPurdR was dissolved in 0.70 mL of degassed, deionized water and 2.9 mL of freshly distilled dioxane. Aliquots of this solution were then sealed under a nitrogen atmosphere. The final nucleoside concentration was 5.6 mM, and the pH was not determined.

Kinetic analysis of AmPur degradation:

The reaction mixtures were 5.0 mM in AmPur and 15.0 mM in sodium cacodylate buffer, pH 7.10. The HPLC analysis was carried out on the ISCO HPLC system, using isocratic elution with methanol:acetonitrile 9:1 (v/v) and monitoring at 280 nm.

Kinetics of 2-deoxy-D-arabino-pentose (2-deoxy-D-ribose) hydrolysis:

The reaction mixtures were 5 mM in 2-deoxy-D-ribose and 15 mM in sodium cacodylate buffer, pH 6.95.
Kinetics of AmPurdR hydrolysis in the presence of 2-deoxy-D-arabino-pentose (2-deoxy-D-ribose):

The solution used in this experiment was 5 mM in AmPurdR, 15 mM in sodium cacodylate buffer, and 5 mM in 2-deoxy-D-ribose, pH 6.92.

Kinetics of AmPur hydrolysis in the presence of 2-deoxy-D-arabino-pentose (2-deoxy-D-ribose):

The solution used in this experiment was 5.4 mM in AmPur, 15 mM in sodium cacodylate, and 5 mM in 2-deoxy-D-ribose, pH 6.95.

Kinetics of hydrolysis of AmPur riboside and of adenosine:

The 2-amino-9-(β-D-erythro-pentofuranosyl)purine (AmPurR) solution was 5.5 mM in AmPurR and 14.5 mM in sodium cacodylate buffer, pH 6.70. The adenosine solution was 5.5 mM in Ado and 15 mM in sodium cacodylate buffer, pH 6.90.

Kinetics of formation of AmPur from 2,4-diamino-5-formamidopyrimidine (DAFP):

The 2,4-diamino-5-formamidopyrimidine (DAFP) used in this experiment was material which had been isolated from a large scale AmPur hydrolysis. The reaction mixture was 5.0 mM in DAFP, 15 mM in sodium cacodylate, pH 6.85. The HPLC analysis was carried out isocratically on the ISCO HPLC system with a solvent of methanol-acetonitrile 9:1 (v/v).
Sample Preparations

NMR sample preparations:

1) D₂O samples: In order to remove the exchangable protons, all of the samples were lyophilized one or more times with D₂O (99.5 atom % D), then one or more times with D₂O (99.96 atom % D). The powder was finally dissolved in D₂O (99.96 atom % D) and placed in a 5 mm NMR tube (Wilmad, 507-PP). No internal standard was used in the D₂O runs since some of the samples were to be re-isolated after the experiment; the peak positions were determined by setting the HOD peak to equal 4.63 ppm, relative to 3-(trimethylsilyl)-1-propane sulfonic acid, sodium salt (DSS).

2) Me₂SO-d₆ samples: The samples were dried for several hours under a vacuum in a drying pistol using ethanol as the refluxing liquid (b.p. 78°C). If the experiment required the removal of the exchangable protons, the sample was lyophilized repeatedly from D₂O (99.5 atom % D), as described above. The samples were then dissolved in Me₂SO-d₆ (99.9 Atom % D), and transferred to a 5 mm NMR tubes. Peak positions are reported relative to tetramethylsilane (Me₄Si) as an internal standard.

Preparation of DAFP and Ampur for IR:

The KBr was dried overnight in an oven at 200°C, then cooled in a desiccator. The AmPur was dried for several hours under vacuum at 78°C. The DAFP was air-dried.
Preparation of samples for mass spectral analysis:

The DAFP isolated from AmPur was not specially treated in any way. The chemically synthesized DAFP was dried for seven hours under a vacuum at 78°C. A 200-MHz proton NMR spectrum of the material dried in this way indicated that the material had not dehydrated under these conditions.
CHAPTER III

RESULTS

Hydrolysis of dAdo and of AmPurdR at pH 6.9:

Dependence on temperature:

Kinetic experiments were carried out at 50°C, 70°C, 90°C, and 110°C for dAdo hydrolysis, and at 37°C, 50°C, 70°C, 90°C, 110°C, and 111°C for AmPurdR hydrolysis. In the case of dAdo, no kinetic experiment was carried out at 37°C, due to the extremely long half-life (1,490 days) expected for the reaction at this temperature. This half-life was extrapolated from rate data at higher temperatures.

In the hydrolysis experiments involving dAdo, it was expected that only two UV absorbing peaks would appear. At all of the temperatures investigated, the HPLC profiles in fact showed only two peaks which absorbed at 254 nm, one due to the unreacted nucleoside and the other due to the free purine, adenine (Ade). Figure 7 shows representative HPLC profiles for different reaction times in the hydrolysis of dAdo at 50°C and at 110°C. The identities of the species were confirmed by their retention times and their UV spectra in acidic, basic, and neutral aqueous media. At pH 2, pH 7, and pH 12, the putative deoxyadenosine had \( \lambda_{\text{max}} \) values of 258, 260, and 261 nm, and
Figure 7. HPLC profiles for the hydrolysis of 2'-deoxyadenosine at 50°C and 110°C.
\( \lambda_{\text{max}} \) values of 229, 227, and 239 nm, respectively; while the spectrum of the putative adenine at these pH values had maxima at 263, 260, and 269 nm and minima at 229, 227, and 239 nm, respectively, in good agreement with published spectra for deoxyadenosine and adenine.

In weakly acidic up to highly acidic media, hydrolysis of dAdo is thought to occur via the A-1 mechanism (Zoltewicz et al., 1970; Hevesi et al., 1972; Garrett and Mehta, 1972a). It was assumed that the depurination of dAdo would also follow the A-1 pathway at the pH values used in my experiments. It was expected that under these conditions, the disappearance of the starting material would exhibit pseudo-first order behavior. In fact, plots of ln(peak height) or ln(peak area) vs. time were linear for all of the temperatures investigated. An example is shown in Figure 8 which refers to the hydrolysis of dAdo at 110°C at pH 6.70. Similar rate constants were obtained, regardless of whether ln(peak height) or ln(peak area) were plotted vs. time. The rate constants were determined from the slopes of these plots, and the results are listed in Table 2.

Unlike the dAdo case, the depurination reaction for AmPurdR gave quite different product distributions over the temperature range covered (110°, 90°, 70°, 50°, and 37°C). It was expected that only two UV absorbing peaks would appear in the HPLC profile, as was observed in the dAdo case. However, the number of peaks varied with the temperature from two peaks at low temperatures to three (or four) peaks at higher temperatures. At 37°C (Figure 9), the first peak to elute corresponded to unreacted AmPurdR, judging by the elution volume
Figure 8. Pseudo-first order kinetic plot of $\ln$ (peak height) vs. time for the hydrolysis of 2'-deoxyadenosine at 70°C.
Table 2. Temperature dependence of the pseudo-first order rate constants for the hydrolysis of dAdo and AmPurdR.

<table>
<thead>
<tr>
<th>T(°C)</th>
<th>pH</th>
<th>half-life</th>
<th>k (s⁻¹)</th>
<th>r*</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>6.95</td>
<td>301 days</td>
<td>2.7 x 10⁻⁸</td>
<td>-0.992</td>
</tr>
<tr>
<td>70</td>
<td>6.98</td>
<td>41.4 days</td>
<td>2.0 x 10⁻⁷</td>
<td>-0.997</td>
</tr>
<tr>
<td>90</td>
<td>6.80</td>
<td>5.2 days</td>
<td>2.02 x 10⁻⁶</td>
<td>-0.984</td>
</tr>
<tr>
<td>110</td>
<td>6.70</td>
<td>23 hr</td>
<td>8.37 x 10⁻⁶</td>
<td>-0.996</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>T(°C)</th>
<th>pH</th>
<th>half-life</th>
<th>k (s⁻¹)</th>
<th>r*</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>6.70</td>
<td>316 days</td>
<td>2.5 x 10⁻⁸</td>
<td>-0.989</td>
</tr>
<tr>
<td>50</td>
<td>6.90</td>
<td>50.4 days</td>
<td>1.6 x 10⁻⁷</td>
<td>-0.998</td>
</tr>
<tr>
<td>70</td>
<td>6.90</td>
<td>8.2 days</td>
<td>(9.1 ± 0.6) x 10⁻⁷</td>
<td>-0.991</td>
</tr>
<tr>
<td>90</td>
<td>6.90</td>
<td>28 hr</td>
<td>6.84 x 10⁻⁶</td>
<td>-0.999</td>
</tr>
<tr>
<td>110</td>
<td>6.70</td>
<td>5.6 hr</td>
<td>3.44 x 10⁻⁵</td>
<td>-0.994</td>
</tr>
<tr>
<td>111</td>
<td>6.80</td>
<td>5.3 hr</td>
<td>3.61 x 10⁻⁵</td>
<td>-0.997</td>
</tr>
</tbody>
</table>

* r = correlation coefficient.
Figure 9. HPLC profiles for the hydrolysis of AmPurdR at 37°C and at 50°C.
and the UV spectrum of this peak, but the second peak to be eluted (designated as Species II) was clearly distinct from AmPur by both criteria. At intermediate temperatures (50°C and 70°C; Figures 9 and 10), Species II formed first followed by the formation of another broad peak which eluted after Species II (designated as Species III). As was the case with Species II, this third peak was distinct from AmPur by the criterion of elution volume. After long reaction times, a fourth peak emerged; this peak was assigned to AmPur based on its UV spectrum and elution volume. At 90°C (Figure 10), Species II appears first followed by AmPur; Species III was not seen in these HPLC profiles, suggesting that this species is not very stable at high temperatures. At 111°C (Figure 11), both Species II and AmPur were present at early reaction times, with Species II appearing as the larger peak at 280 nm; at later times (22 hr), the AmPur peak became larger than the Species II peak. Based on the relative order of appearance, it was hypothesized that AmPurdR produced Species II, which in turn can be converted to AmPur in a slow reaction.

As in the case of dAdo hydrolysis, the plots of ln(peak height) vs. time were linear, indicating that the depurination reaction of AmPurdR follows pseudo-first order kinetics. An example of such a plot, for AmPurdR hydrolysis at 111°C is shown in Figure 12. The rate constants were determined from the slopes of these plots, and the results are listed in Table 2.
Figure 10. HPLC profiles for the hydrolysis of AmPurdR at 70°C and at 90°C.
Figure 11. HPLC profile for the hydrolysis of AmPurdR at 111°C.
Figure 12. Pseudo-first order kinetic plot of \( \ln(\text{peak height/cm}) \) vs. time for the hydrolysis of AmPurdR at 111°C.
The UV spectra for Species II (Figure 13) were clearly different from those of AmPurdR and AmPur (Figure 14). From fractions collected from the HPLC column, the UV spectral characteristics were determined; they are listed in Table 3. The spectral shifts observed in acid and in base were fully reversible by the addition of base and acid, respectively.

Table 3. UV Spectral Data of Species II.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\lambda_{\text{min}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$CN-MeOH (9:1)</td>
<td>287</td>
<td>261</td>
</tr>
<tr>
<td>H$_2$O pH 7</td>
<td>287</td>
<td>261</td>
</tr>
<tr>
<td>0.1 M HCl</td>
<td>272</td>
<td>261</td>
</tr>
<tr>
<td>0.1 M NaOH</td>
<td>296</td>
<td>274</td>
</tr>
</tbody>
</table>

Hydrolysis of AmPurdR in neutral solution at 70°C: Effects of various parameters.

The effect of several parameters was examined in comparison to the following standard conditions: 5 mM nucleoside, 15 mM aqueous cacodylate buffer, pH 6.90, and 70°C. One parameter was varied at a time, and the results are presented in Table 4.
Figure 13. Ultraviolet absorption spectra of Species II at various pH values.
Figure 14. Ultraviolet absorption spectra of AmPur and AmPurdR at various pH values.
Table 4. Pseudo-first order rate constants for the hydrolysis of AmPurdR, 70°C, in dependence on various parameters.

<table>
<thead>
<tr>
<th>Parameter examined</th>
<th>pH</th>
<th>k (s⁻¹)</th>
<th>r*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of NaCl added</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.00</td>
<td>1.1 x 10⁻⁶</td>
<td>-0.988</td>
</tr>
<tr>
<td>15 mM NaCl</td>
<td>6.98</td>
<td>1.17 x 10⁻⁶</td>
<td>-0.987</td>
</tr>
<tr>
<td>118 mM NaCl</td>
<td>7.00</td>
<td>9.5 x 10⁻⁷</td>
<td>-0.998</td>
</tr>
<tr>
<td>Cacodylate Buffer Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 mM buffer</td>
<td>6.94</td>
<td>9.0 x 10⁻⁷</td>
<td>-0.997</td>
</tr>
<tr>
<td>50 mM buffer</td>
<td>6.95</td>
<td>8.1 x 10⁻⁷</td>
<td>-0.987</td>
</tr>
<tr>
<td>150 mM buffer</td>
<td>7.00</td>
<td>7.8 x 10⁻⁷</td>
<td>-0.997</td>
</tr>
<tr>
<td>Input Concentration of AmPurdR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.8 mM AmPurdR</td>
<td>7.15</td>
<td>6.8 x 10⁻⁷</td>
<td>-0.993</td>
</tr>
<tr>
<td>0.75 mM AmPurdR</td>
<td>7.06</td>
<td>5.7 x 10⁻⁷</td>
<td>-0.999</td>
</tr>
<tr>
<td>0.054 mM AmPurdR</td>
<td>7.10</td>
<td>3.7 x 10⁻⁷</td>
<td>-0.982</td>
</tr>
<tr>
<td>15 mM Phosphate Buffer</td>
<td>6.90</td>
<td>2.6 x 10⁻⁶</td>
<td>-0.988</td>
</tr>
<tr>
<td>Dioxane-Water 1:1 (v/v)</td>
<td>---</td>
<td>8 x 10⁻⁸</td>
<td>-0.34</td>
</tr>
<tr>
<td>Dioxane-Water 4:1 (v/v)</td>
<td>---</td>
<td>1.93 x 10⁻⁶</td>
<td>-1.01</td>
</tr>
<tr>
<td>Addition of 5 mM deoxyribose</td>
<td>6.92</td>
<td>1.24 x 10⁻⁶</td>
<td>-0.990</td>
</tr>
</tbody>
</table>

* r = correlation coefficient.
Effect of added sodium chloride:

The pseudo-first order rate constant for the disappearance of AmPurdR was reduced by 4% upon addition of NaCl to 15 mM and by 15% upon addition of NaCl to 118 mM (Table 4). The product distribution in the HPLC profile was unaffected by the addition of sodium chloride.

Effect of change in cacodylate buffer concentration:

When the sodium cacodylate concentration was increased 10-fold, from 15 to 150 mM, the rate decreased by 14% (Table 4). The product distribution in the HPLC profile was unaffected by the increased concentration of the buffer.

Dependence on nucleoside concentration:

The pseudo-first order rate constant for the hydrolysis of AmPurdR at 70°C appeared to be only slightly dependent on the nucleoside concentration; there was a 15% decrease in the rate with a 6.4-fold decrease in the concentration; and, there was a 46% reduction in the rate when there was an 89-fold reduction in the concentration (Table 4).

When the rates are corrected for the pH differences in the runs, the adjusted values are as follows: a 6.4-fold decrease in the concentration leads to a 23% decrease in the rate, while a 89-fold decrease in the concentration leads to a 49% reduction in the rate.
In order to determine the order of the reaction with respect to the nucleoside concentration, a plot of log (k) vs. log ([initial nucleoside]) was generated, using the rate constants which were adjusted for the pH variance (Figure 15). The plot was linear, with a correlation factor (r) of 1.000 and a slope of 0.15, indicating that the order of the reaction was 1.15 in nucleoside. Thus, the first-order treatment of the kinetics data is a reasonable approximation.

**Effect of phosphate buffer:**

When the standard sodium cacodylate buffer is replaced by sodium phosphate buffer of identical pH and ionic strength, the hydrolysis rate of AmPurdR is increased by a factor of 2.6 (Table 4). Not only was the rate faster than in the cacodylate case, but the product distribution was different (Figure 16). The profile indicated a considerable increase in the peak which is associated with Species III. This assignment is based upon the retention time alone.

**Effect of aqueous dioxane as a solvent:**

The reaction of AmPurdR in unbuffered dioxane-water 1:1 (v/v) at 70°C was followed only to 10% consumption of the starting material, and the pseudo-first order rate constant calculated from the data can only indicated an order of magnitude. The reactions is about 10-fold slower in this solvent than in neutral aqueous solutions (Table 4). In this experiment, no UV absorbing products eluted on HPLC column after AmPurdR.
Figure 15. Pseudo-first order rate constant for hydrolysis of AmPurR as a function of initial nucleoside concentration.
Figure 16. HPLC profile for the hydrolysis of AmPurdR in 15 mM sodium phosphate buffer, pH 6.9.
In dioxane-water 4:1 (v/v), the degradation of the nucleoside was about twice as fast as in water. In this case, again, no UV active products were observed eluting from the HPLC column after AmPurdR, although the reaction was followed for 1.6 half-lives.

It is unlikely that the rate of degradation of AmPurdR in these experiments was affected significantly by peroxides present in the reaction mixture. The dioxane used was freshly distilled from sodium, the water was degassed under vacuum, and the solutions were sealed in ampules under nitrogen. Even if this gas were to contain a considerable proportion of oxygen (e.g. 1%), the total amount of oxygen available would be far less than equivalent to the amount of nucleoside present, due to the small gas space in the ampule. Also, the rate of degradation of AmPurdR observed in dioxane-water (1:1) was very low, indicating either that peroxides were absent or that they did not attack the nucleoside.

Control experiment: kinetics of hydrolysis of 2-deoxy-D-ribose:

It has been observed that at elevated temperatures and at pH values between 2 and 0.2, 2-deoxy-D-ribose degrades to 5-methyl-3(2H)-furanone (Zoltewicz et al., 1970); this product has a $\lambda_{\text{max}}$ at 261 nm. Although it was unlikely that this degradation product would form under the reaction conditions used in this work, a control experiment was performed. A solution of 5 mM 2-deoxy-D-ribose in 15 mM sodium cacodylate buffer, pH 6.92, was incubated at 70°C. Hydrolysates obtained with reaction times up to 18 days were analyzed by HPLC,
monitoring at 280 nm: no UV absorbing products were detected.

**AmPurdR hydrolysis in the presence of 5 mM 2-deoxy-D-ribose:**

In order to determine if the presence of the free sugar plays a role in the kinetic scheme, 2-deoxy-D-ribose was incubated with AmPurdR and AmPur. The rate constant of a 5 mM AmPurdR reaction mixture, which was 5 mM in 2-deoxy-D-ribose, increased by a factor of 1.3 over the expected rate constant (Table 4). The HPLC product distribution for the reaction was different from that observed in a normal AmPurdR hydrolysis; a single broad peak eluted at the positions associated with Species II and III (Figure 17). While a high concentration of 2-deoxy-D-ribose clearly affects the product distribution of the reaction, the rate of disappearance of AmPurdR is changed only slightly by the presence of free sugar. Thus, in the standard hydrolysis mixture, reaction of the starting nucleoside with the small amounts of free deoxyribose initially formed, cannot be important.

**Rate dependence on pH:**

In the A-1 mechanism, the cleavage of the N-glycosyl bond occurs from the monocationic or dicationic species without the direct involvement of water in the transition state; thus, the slope of a \( \log(k) \) vs. pH (or \( H_0 \)) plot should be close to -1 at pH values distinctly higher or near to the pKa for the monocationic and dicationic species (Zucker-Hammett hypothesis; Zucker and Hammett,
Figure 17. HPLC profile for the hydrolysis of AmPurdR in the presence of 5 mM 2-deoxy-D-ribose.
1939). Experimentally, the slopes vary from -0.95 to -1.03 (Zoltewicz et al., 1970; Hevesi et al., 1972) for dAdo. When the pseudo-first order rate constants (Table 5) for the pH dependence of AmPurdR are plotted in the above fashion, the slope has a value of -0.43 (Figure 18).

Over the pH range examined, the product distribution did not change appreciably with the acidity of the solution. This is seen in Figure 19, which shows elution profiles for hydrolysates obtained as pH 5.8 in comparison to elution profiles (already shown in Figure 11) for AmPurdR hydrolysis at pH 6.9 and 70°C.

Hydrolysis of adenosine and of 2-aminopurine riboside:

These reactions were carried out in neutral aqueous solution at 110°C. For both ribosides, the plot of ln(peak height) vs. time was distinctly curved. On the assumption that this reflected a non-zero value for the peak height at infinite time, the data were analyzed by the method of Guggenheim (1926), giving straight lines, from the slope of which were calculated the pseudo-first order rate constants listed in Table 6. Comparison to the rate values in Table 2 shows that under the conditions of the experiment, adenosine is about six times less reactive than the corresponding deoxyriboside, and 2-aminopurine riboside reacts about 2.5 times more slowly than AmPurdR.
Table 5. Dependence of the rate constant of AmPurdR hydrolysis on pH, 70°C.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k$ (s$^{-1}$)</th>
<th>$r^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.80</td>
<td>$2.36 \times 10^{-6}$</td>
<td>-0.993</td>
</tr>
<tr>
<td>6.30</td>
<td>$1.63 \times 10^{-6}$</td>
<td>-0.990</td>
</tr>
<tr>
<td>6.90</td>
<td>$9.8 \times 10^{-7}$</td>
<td>-0.991</td>
</tr>
<tr>
<td>6.94</td>
<td>$9.0 \times 10^{-7}$</td>
<td>-0.997</td>
</tr>
<tr>
<td>7.00</td>
<td>$1.1 \times 10^{-6}$</td>
<td>-0.988</td>
</tr>
<tr>
<td>7.15</td>
<td>$6.8 \times 10^{-7}$</td>
<td>-0.993</td>
</tr>
</tbody>
</table>

$r^*$ is the correlation coefficient.

Table 6. Pseudo-first order rate constants for the hydrolysis of adenosine and of 2-aminopurine riboside at 110°C.

<table>
<thead>
<tr>
<th>nucleoside</th>
<th>pH</th>
<th>$k$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>adenosine</td>
<td>6.90</td>
<td>$1.35 \times 10^{-6}$</td>
</tr>
<tr>
<td>2-aminopurine riboside</td>
<td>6.98</td>
<td>$1.36 \times 10^{-5}$</td>
</tr>
</tbody>
</table>
Figure 18. Pseudo-first order rate constant for hydrolysis of AmPurdR as a function of pH.
Figure 19. HPLC profiles for the hydrolysis of AmPurdR at pH 5.8 and at pH 6.9.
Identification of Species II:

Reaction of $^{14}$C-labeled AmPurdR:

In order to determine whether Species II is a modified nucleoside, AmPurdR, uniformly $^{14}$C-labeled in the sugar moiety, was subjected to hydrolysis at 90°C. The HPLC profile, in terms of absorbance at 280 nm and radioactivity, for the 5 day sample is shown in Figure 20. The absorbance tracing shows sharp bands at 4.0 mL and 4.4 mL; these are caused by the change in refractive index as the loading solvent passes through the optical cell, and they are prominent in this case because of the higher detector sensitivity used because of the small amount of sample. Bands eluting at 5.9 mL, 7.5 mL, 8.2 mL, and 10.4 mL, are interpreted as AmPurdR, Species II, Species III, and AmPur, respectively.

When the radioactivity and the HPLC profiles are compared, a large number of counts are located with the peak associated with AmPurdR, as is expected. However, the other three peaks, Species II, Species III, and AmPur, are not associated with any distinct radioactive bands. The broad distribution of radioactivity seen in this area is probably due to elution of various decomposition products of deoxyribose in this region.

A sample of this hydrolysate was chromatographed on a silicagel thin-layer plate in chloroform-methanol (7:3), along with a sample of unlabeled Species II previously isolated by HPLC. The radioactivity distribution was determined using the Berthold TLC-Linear Analyzer.
Figure 20. HPLC profile for the hydrolysis of $^{14}$C-labeled AmPurdR.
The chromatographic position of Species II ($R_f = 0.19$) was found to be free of radioactivity. Thus, Species II is not nucleosidic in nature, and must be a modified heterocycle.

At the time of these experiments, the starting labeled nucleoside had been stored as a frozen aqueous solution for six weeks since its synthesis. The HPLC profile for this material at the time of these experiments is shown in Figure 5. Small amounts of UV absorbing materials are seen at the elution positions of Species I and of AmPur. These are possibly due to a small extent of radiolysis during storage.

**Kinetics of AmPur hydrolysis:**

In order to determine whether Species II was an intermediate in the depurination of AmPurdR or whether it was a product of AmPur decomposition, the hydrolysis of AmPur was carried out under the standard conditions, 5 mM AmPur and 15 mM sodium cacodylate, pH 7.1. Judging by the HPLC profiles (Figure 21), a minor product is formed prior to the appearance of Species II. This minor product is always present in small amounts, and the elution position of this product corresponds to Species III, which, as stated earlier, had appeared in the HPLC profiles of some AmPurdR hydrolysates.

The hydrolysis was followed only to the point of 10% decrease in the starting material; thus, the rate constant for this reaction, $4 \times 10^{-8} \text{ s}^{-1}$, must be taken as an approximate value. Nonetheless, its low value indicates that AmPur cannot be the precursor of Species II since the rate of the conversion of AmPur to Species II is about
Figure 21. HPLC profiles for the decomposition of AmPur.
20-fold slower than the rate for AmPurdR disappearance under the same conditions, and AmPur does not accumulate appreciably during the early stages of AmPurdR hydrolysis.

Kinetics of the formation of AmPur from Species II:

In order to test the hypothesis that Species II was the precursor for the formation of AmPur, the hydrolysis of Species II was examined under the standard conditions. The HPLC profiles (Figure 22) showed conversion of Species II to AmPur, with a first-order rate constant of $8.6 \times 10^{-7}$ s$^{-1}$. This reaction is thus about 20 times as fast as the reverse reaction.

Identification of Species II by spectroscopic techniques:

Speculation on the structure of Species II:

A summary of the data on Species II, described in the Kinetics section, is listed below.

1) Species II is a relatively stable structure at weakly acidic to neutral pH values since it appears even at elevated temperatures (Figures 9, 10, and 11).

2) The UV spectra of Species II are different from those of AmPurdR and AmPur (Figures 13 and 14). This indicates that Species II has a heterocycle which is different from AmPur.
Figure 22. HPLC profiles for the decomposition of Species II.
3) Species II is not a nucleoside as determined by the $^{14}$C-labeling experiment (Figure 20).

4) Species II appears not to be the deamination product of AmPur, 2-oxopurine, since Species II and AmPur are interconvertible.

This interconvertibility of AmPur and Species II in hot aqueous solution suggests that the two might be related by processes of hydration and dehydration. Covalent hydration of pteridines has been well documented, e.g. for 2-aminopteridine (Figure 23d, 23e; Albert and McCormack, 1966). Purines appear to much more resistant to hydration, at least under the mild conditions employed in the reported experiments (Albert, 1967). In the case of 8-aza-2-aminopurine (2-amino-$\gamma$-triazolo[4,5-d]pyrimidine), however, hydration across the N(1)-C(6) double bond to form 8-aza-1,6-dihydro-6-hydroxy-2-aminopurine has been demonstrated (Figure 23f, 23g; Albert, 1966; Albert and Pendergast, 1972). The probable sites for the hydration of AmPur (Figure 23a) are across the C(6)-N(1) bond (Figure 23b) or across the C(8)-N(7) bond (Figure 23c).

The following series of experiments was performed to determine the structure of Species II.

a) Mass Spectra of Species II:

The electron impact (EI) spectrum gave an m/e value of 153 for the molecular ion peak (Figure 24). The EI technique can sometimes cause the loss of water from a compound; thus, the less energetic technique
Fig. 23. Illustration of various observed, and potential covalent water adducts of AmPur, 8-aza-2-aminopurine, and 2-aminopteridine.
Figure 24. Electron Impact mass spectrum of Species II obtained from AmPur.
of chemical ionization (CI; isobutane carrier gas) was also employed (Figure 25). The M+H peak in the CI spectrum had an m/e value of 154, confirming that Species II indeed has a molecular weight of 153 g/mol. The CI mass spectrum shows only a very small peak at m/e = 136, indicating that the sample was essentially free of AmPur. Thus, the band seen at m/e = 135 in the EI mass spectrum must have arisen from the decomposition of the compound in the mass spectrometer.

From an examination of the EI mass spectrum, there appears to be two fragmentation pathways, the peaks of which have the following tentative assignments:

1) Along the major pathway: the m/e peak of 125 corresponds to the loss of CO from M+; the loss of a hydrogen radical from 125 or loss of HCO from M+ produces the peak at 124; the m/e peak of 97 results from the loss of HCN from the 124 peak; the loss of H₂NCN from the 124 peak results in the formation of the 82 peak; the loss of HCN, or of H₂NCN from the 97 peak results in the formation of the peaks at 70, and 55, respectively; the peak at m/e = 55 can also result from loss of HCN from 82.

2) Along the minor pathway: the m/e peak of 135 correspond to the loss of H₂O from M+; the peak at 108 results from the loss of HCN from the 135 peak; the peak at 81 results from the loss of HCN from the 108 peak; the peak at 54 results from the loss of HCN from the 81 peak.
Figure 25. Chemical Ionization mass spectrum of Species II.
b) Infrared (IR) spectrophotometry:

The IR spectrum of Species II (Figure 26b) was taken to obtain information on the functional groups present in the molecule. The products of water addition across the N(1)-C(6) or the N(7)-C(8) double bond in 2-aminopurine (Figures 23b and 23c) should show IR absorption bands at the hydroxyl group stretching frequencies. However, the bands for the unassociated OH stretch (sharp; 3600 cm⁻¹) and the associated OH stretch (broad; 3400 cm⁻¹) did not appear in the spectrum. A highly diagnostic band in the IR spectrum of Species II, not seen in the spectrum of AmPur (Figure 26a), occurs at 1670 cm⁻¹; this is the region for the Amide I stretching frequency.

The presence of a formyl group was already indicated by the appearance of a peak at (M - CO) in the EI mass spectrum (Figure 24). One can consider two structures containing an amide group, which could arise from water addition to AmPur: 2,4-diamino-5-formimidopyrimidine (DAFP) and 2,5-diamino-4-formimidopyrimidine. In fact, the IR spectrum for a related compound, 2-chloro-4-amino-5-formimidopyrimidine, has been published (The Coblentz Society, 1971, Spectrum No. 7378) and the spectrum is very similar to the Species II spectrum.

c) Ultraviolet (UV) spectrophotometry:

The UV spectra at acidic, neutral, and basic pH were determined for Species II; the results are shown in Table 3 and Figure 13. Based on the close similarity of the IR spectra of
Figure 26. Infrared spectra of AmPur and Species II.
2-chloro-4-amino-5-formamidopyrimidine and Species II, the literature was searched for data concerning the UV spectral characteristics of DAFP. Roth and Streitz (1969) reported the \( \lambda_{\text{max}} \) values for the neutral and monocationic species of DAFP in aqueous solution:

**neutral species**

\[
\begin{align*}
\lambda_{\text{max}} &= 222 \text{ nm} \quad (\varepsilon = 8.40 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}) \\
\lambda_{\text{max}} &= 287 \text{ nm} \quad (\varepsilon = 5.50 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1})
\end{align*}
\]

**monocationic species**

\[
\begin{align*}
\lambda_{\text{max}} &= 208 \text{ nm} \quad (\varepsilon = 22.2 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}) \\
\lambda_{\text{max}} &= 270 \text{ nm} \quad (\varepsilon = 3.70 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1})
\end{align*}
\]

They also reported a thermodynamic pKa value of 6.03 \pm 0.03 (20°C) for DAFP. Their values for both species agree well with the observed values for Species II (Table 3).

d) **NMR analysis of Species II:**

Samples of Species II were obtained by hydrolysis of both AmPur and AmPurdR. Species II isolated from an AmPur hydrolysate was very pure, judging from the analytical HPLC profile. The proton NMR spectrum of this sample, taken in Me\(_2\)SO-\(d_6\), is shown in Figure 27. Those of the major bands which were found to be exchangeable with D\(_2\)O are marked by an (X). The chemical shifts and the coupling constants of the main bands are listed in Table 7.

The spectrum shows ten bands between 10 ppm and 5 ppm, while six bands are expected for a covalent hydrate of 2-aminopurine and five bands are expected for a diaminoformamidopyrimidine. The absorbances
Figure 27. 500-MHz proton NMR of Species II in Me$_2$SO-d$_6$. 
Table 7. 500-MHz proton NMR spectrum of Species II, obtained by hydrolysis of AmPur, measured in Me$_2$SO-d$_6$ at 25°C and 50 mM sample concentration.

<table>
<thead>
<tr>
<th>Assignment*</th>
<th>Chemical shift [ppm]</th>
<th>Relative peak area</th>
<th>Multiplicity*</th>
<th>J(1H-1H) [Hz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(0)C-NH (trans)</td>
<td>9.11</td>
<td>2.73</td>
<td>s?</td>
<td>?</td>
</tr>
<tr>
<td>H(0)C-NH (cis)</td>
<td>8.75</td>
<td>1.00</td>
<td>d</td>
<td>11.1</td>
</tr>
<tr>
<td>H(0)C- (trans)</td>
<td>8.13</td>
<td>2.58</td>
<td>d</td>
<td>1.4</td>
</tr>
<tr>
<td>H(0)C- (cis)</td>
<td>7.95</td>
<td>0.92</td>
<td>d</td>
<td>11.1</td>
</tr>
<tr>
<td>H-6 (trans)</td>
<td>7.69</td>
<td>1.97</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>H-6 (cis)</td>
<td>7.56</td>
<td>0.79</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>4-NH$_2$ (cis)</td>
<td>6.30</td>
<td>2.32</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>4-NH$_2$ (trans)</td>
<td>6.22</td>
<td>5.93</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>2-NH$_2$ (cis)</td>
<td>5.92</td>
<td>2.53</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>2-NH$_2$ (trans)</td>
<td>5.85</td>
<td>5.93</td>
<td>s</td>
<td>---</td>
</tr>
</tbody>
</table>

* cis and trans refer to the conformation of the amide bond; (s) singlet, (d) doublet; the "?" indicates that this resonance is observed as a singlet, it is presumed to be a doublet.
appears as pairs with similar shifts; each pair consists of a stronger and a weaker band, with an intensity ratio of about 2.5 to 1. This allows two interpretations: either the sample contained two species which are very close in structure and co-migrate both on XAD-4 column chromatography and in the HPLC system, e.g. 2,4-diamino-5-formaminopyrimidine (DAFP) and 2,5-diamino-4-formamidopyrimidine; or the sample contains a single species which can occur in two defined conformations which interconvert slowly on the NMR time scale. The latter description would fit both DAFP and 2,5-diamino-4-formamidopyrimidine. In these two molecules, the formamido group can exist in two defined planar configurations, defined as cis and trans according to the geometric relationship between the carbonyl oxygen in the formamido group and the amide hydrogen (Figure 28).

\[ \text{trans} \]
\[ \text{cis} \]

Figure 28. The trans and cis conformations of 2,4-diamino-5-formamidopyrimidine (DAFP).

N-Substituted formamides do, in fact, give two sets of absorbances in their proton NMR spectra, as reviewed by Stewart and Siddall (1970). This is due to the fact that in the different conformations
the anisotropy of the carbonyl group will differently affect the chemical shifts of the formyl proton and the protons in the N-substituent. The ratio of the peak intensities reflects the equilibrium ratio of the two conformations, the more intense peaks are associated with the more stable conformation.

Support for the assumption that Species II is one of the formamidopyrimidines stems from a characteristic aspect of the cis and trans conformations: the three-bond coupling between the formyl hydrogen and amide hydrogen is very different for the two conformations. The coupling constants for the cis conformation are much larger than those for the trans conformation, e.g. for formanilide the $J_{\text{cis}(^1\text{H}^-^1\text{H})}$ has a value of 11.0 Hz and $J_{\text{trans}(^1\text{H}^-^1\text{H})}$ is equal to 2.0 Hz (Bourn et al., 1964). In Figure 27, the resonances at 9.11 ppm and 8.75 ppm can be assigned to the amido hydrogen, while the resonances at 8.13 ppm and 7.94 ppm can be assigned to the formyl proton. The peaks at 8.75 and 7.94 ppm appear to be related because of their similar intensities and their identical coupling constants (11.1 Hz), while the resonances at 9.11 ppm and 8.13 ppm are inferred to be coupled with a coupling constant of 1.6 Hz. The peak at 9.11 ppm appeared as a singlet, presumably due to the small coupling constant and the quadrupolar effects of the amido nitrogen.

Thus, for Species II, as obtained from the hydrolysis of AmPur, mass spectrometry, NMR spectrometry, and IR spectroscopy pointed to a diaminiformamidopyrimidine, and UV spectroscopy showed good agreement with the spectral data published for DAFP (Roth and Strelitz, 1969).
Therefore, DAFP was synthesized by formylation of 2,4,5-triaminopyrimidine. The chemical shifts for the proton NMR spectrum of this material are listed in Table 8. It is seen that the chemical shifts for this sample closely agree with the chemical shifts for the material obtained by hydrolysis of AmPur (Table 7). The greatest difference in the chemical shifts was 0.1 ppm. Table 7 and 8 list assignments for the various bands in the proton NMR spectrum of DAFP. These are based on the considerations of chemical shifts, peak areas, coupling constants, and D$_2$O exchange experiments. Of the two sets of amino resonances, the upfield one was ascribed to the 2-NH$_2$ group, because this group is more distant from the formamido moiety than is the 4-NH$_2$ group and should, therefore, show the smaller difference in chemical shifts for the cis and trans conformations of the amide group.

DAFP synthesized from 2,4,5-triaminopyrimidine and Species II, as obtained by hydrolysis of AmPur, also closely agree in their EI mass spectra, as seen in a comparison of Figures 29 and 24. Therefore, it was concluded that Species II and DAFP are the same compound.

While it has thus far been assumed that the structure of DAFP prepared by formylation of 2,4,5-triaminopyrimidine is, in fact, 2,4-diamino-5-formamidopyrimidine, this had not been rigorously proven prior to the present work. Isay (1906) reasoned that the formylation occurred at the 5-amino group of the triaminopyrimidine based on the ability of the compound to form AmPur by ring closure and on its basicity in aqueous solution. The former observation argues against
### Table 8. 500-MHz proton NMR spectrum of DAFP, synthesized from 2,4,5-triaminopyrimidine, measured in Me$_2$SO-d$_6$ at 25°C and 50 mM sample concentration.

<table>
<thead>
<tr>
<th>Assignment*</th>
<th>Chemical shift [ppm]</th>
<th>Relative peak area</th>
<th>Multiplicity*</th>
<th>$J(^1$H,$^1$H) [Hz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(O)C-NH (trans)</td>
<td>9.01</td>
<td>2.6</td>
<td>s?</td>
<td>?</td>
</tr>
<tr>
<td>H(O)C-NH (cis)</td>
<td>8.74</td>
<td>1.0</td>
<td>d</td>
<td>11.1</td>
</tr>
<tr>
<td>H(O)C- (trans)</td>
<td>8.14</td>
<td>5.8</td>
<td>d</td>
<td>1.4</td>
</tr>
<tr>
<td>H(O)C- (cis)</td>
<td>7.95</td>
<td>1.1</td>
<td>d</td>
<td>11.1</td>
</tr>
<tr>
<td>H-6 (trans)</td>
<td>7.68</td>
<td>2.7</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>H-6 (cis)</td>
<td>7.58</td>
<td>1.0</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>4-NH$_2$ (cis)</td>
<td>6.34</td>
<td>2.3</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>4-NH$_2$ (trans)</td>
<td>6.22</td>
<td>5.6</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>2-NH$_2$ (cis)</td>
<td>5.97</td>
<td>7.4</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>2-NH$_2$ (trans)</td>
<td>5.94</td>
<td>7.4</td>
<td>s</td>
<td>---</td>
</tr>
</tbody>
</table>

* cis and trans refer to the conformation of the amide bond; (s) singlet, (d) doublet; the "?" indicates that this resonance is observed as a singlet, it is presumed to be a doublet.
Figure 29. Electron Impact mass spectrum of DAFP synthesized from 2,4-diamino-5-nitropyrimidine.
the formylation of the 2-amino group; the latter argues for formylation at the 5-amino group since formylation at this position does not interfere with protonation at N-1 or N-3 being resonance-stabilized by participation of the free pair of electrons on the N-1, N-3, or 4-amino nitrogen atoms, while this effect would lapse in the event of formylation of the 4-amino group. In addition, the 5-amino group of 2,4,5-triaminopyrimidine should be the best nucleophile of the three amino groups, because the resonance structures involving its free pairs of electrons do not place the negative charge on the ring nitrogens. In the related case of 2-methylthio-4,5-diaminopyrimidine, formylation has been proven to occur exclusively at the 5-amino position (Brown, 1955). However, to ascertain the site of formylation in 2,4,5-triaminopyrimidine, the $^{13}$C NMR spectra of this compound and of the DAFP synthesized from it, were compared.

The proton decoupled spectrum of 2,4,5-triaminopyrimidine in Me$_2$SO-$d_6$ (Figure 30) exhibited four peaks, at 157.17, 155.61, 140.37, and 118.07 ppm. These peaks were assigned to C-2, C-4, C-6, and C-5, respectively, based on the fact the C-6 resonance appears split in the $^1$H-$^{13}$C coupled spectrum and on the typical order of the chemical shifts of pyrimidine carbons (Lauterbur, 1965; Mathias and Gil, 1965; Pugmire and Grant, 1968). Nine peaks are seen in the proton decoupled spectrum of DAFP (Figure 31), due to the presence of both the cis and the trans isomers. Since the trans isomer is predominant, as judged by the proton NMR spectrum, the five intense peaks were attributed to the trans isomer (Table 9). The assignment of the formyl carbon and
Figure 30. 75-MHz $^{13}$C proton-decoupled NMR spectrum of 2,4,5-triaminopyrimidine in Me$_2$SO-$d_6$. 
Figure 31. 75-MHz $^{13}$C proton-decoupled NMR spectrum of 2,4-diamino-5-formamidopyrimidine (DAFP) in Me$_2$SO-d$_6$. 
Table 9. 75-MHz $^{13}$C NMR of 2,4,5-triaminopyrimidine (TAP) and 2,4-diamino-5-formamidopyrimidine (DAFP) in Me$_2$SO-$d_6$, chemical shifts from Me$_4$Si.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>TAP [ppm]</th>
<th>trans [ppm]</th>
<th>cis [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>157.17</td>
<td>160.52</td>
<td>161.49</td>
</tr>
<tr>
<td>C-4</td>
<td>155.61</td>
<td>159.31</td>
<td>160.27</td>
</tr>
<tr>
<td>C-6</td>
<td>140.37</td>
<td>150.67</td>
<td>150.67</td>
</tr>
<tr>
<td>C-5</td>
<td>118.07</td>
<td>106.17</td>
<td>106.87</td>
</tr>
<tr>
<td>formyl</td>
<td>--------</td>
<td>163.54</td>
<td>163.45</td>
</tr>
</tbody>
</table>

Table 10. $^{13}$C NMR of aniline and formanilide, chemical shifts from Me$_4$Si.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>aniline [ppm]</th>
<th>trans [ppm]</th>
<th>cis [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2, C-6</td>
<td>115.1</td>
<td>118.7</td>
<td>120.3</td>
</tr>
<tr>
<td>C-3, C-5</td>
<td>129.2</td>
<td>128.9</td>
<td>129.3</td>
</tr>
<tr>
<td>C-4</td>
<td>118.2</td>
<td>125.1</td>
<td>124.7</td>
</tr>
<tr>
<td>C-1</td>
<td>146.7</td>
<td>137.0</td>
<td>137.2</td>
</tr>
<tr>
<td>formyl</td>
<td>--------</td>
<td>163.3</td>
<td>160.1</td>
</tr>
</tbody>
</table>
C-6 carbon bands was confirmed by their splitting in the \(^1H-\(^{13}C\) coupled spectrum. Upon formylation, all of the peaks were shifted downfield except for the resonances assigned to the C-5 carbon, which was shifted 11.9 ppm upfield. Similar behavior is observed when aniline is N-formylated. The \(^{13}C\) chemical shifts for aniline and for formamidilide in CDCl\(_3\) (Sadtler Index, 1979, Spectrum Nos. 6001C and 1420C, respectively) are listed in Table 10. It is seen that the band which shifts distinctly upfield upon N-formylation is the resonance for C-1, i.e. the carbons bearing the amino group which is being formylated, while all other bands shift downfield or remain virtually unchanged. On the basis of this comparison, it is concluded that formylation of 2,4,5-triaminopyrimidine takes place at the 5-amino position. Thus, Species II is 2,4-diamino-5-formamidopyrimidine.

Hydrolysis of AmPur gave, after Amberlite XAD-4 column chromatography, a highly pure sample of Species II. In contrast, in two separate preparations from AmPurdR, the Species II peak obtained from Amberlite XAD-4 column chromatography contained large amounts of Species III, as judged by HPLC. This mixture was partially resolved by preparative HPLC, and fractions were pooled to give two samples: Sample A was enriched in Species II and Sample B was enriched in Species III. The 500-MHz proton NMR spectra for these two samples, taken in Me\(_2\)SO-d\(_6\), are shown in Figures 32 and 33, respectively.

Clearly, each of these samples contains a highly complex mixture of compounds. In particular, there is a large number of bands in the region from app. 5.5 ppm to 1 ppm; these are believed to arise from
Figure 32. 500-MHz proton NMR spectrum of Sample A in Me₂SO-d₆.
Figure 33. 500-MHz proton NMR spectrum of Sample B in Me$_2$SO-d$_6$. 
breakdown products of the sugar moiety, which are known to approximately comigrate with Species II in the HPLC system, as seen from the radioactivity distribution in the HPLC profile of the hydrolysate of AmPurdR_{14}C-labeled in the sugar moiety (Figure 20).

Chemical shifts for the main bands to the downfield side of 5.5 ppm for Samples A and B are compared to the chemical shifts of DAFP (Table 8) and Species II (Table 7) in Table 11. This region of the NMR spectrum was expanded for both Samples A and B in Figures 34 and 35, respectively. It is seen that ten major bands in Sample A closely coincide with the bands observed for DAFP and Species II; these resonances are marked by (X)'s. An analogous set of weaker resonances are also seen in the spectrum for Sample A (Figure 34); these peaks are associated with Species III and are marked by circles. This latter set appears with stronger intensities in the spectrum for Sample B, which also shows, more weakly, the DAFP resonances.

The peaks clearly identified with Species III have been tentatively assigned to certain features and are listed in Table 12. Species III appears to be a structure in which the imidazole ring has opened, due to the appearance of resonances which can be assigned to formyl and amido protons. It was considered that Species III might be 2,5-diamino-4-formamidopyrimidine. However, it is difficult to explain the large difference in the chemical shift of H-6 for the cis and trans conformers. In 2,5-diamino-4-formamidopyrimidine, this proton is too distant from the carbonyl group to be either strongly shielded or deshielded by it. Second, only one of the two expected
Table 11. Comparison of Species II in Samples A and B with Species II (from AmPur) and DAFP (from 2,4,5-triaminopyrimidine); 500-MHz proton NMR spectra in Me$_2$SO-$_d_6$, 25°C.

<table>
<thead>
<tr>
<th>Assignment*</th>
<th>Samples</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H(0)C-NH (trans)</td>
<td>9.13</td>
<td>9.16</td>
<td>9.11</td>
<td>9.01</td>
</tr>
<tr>
<td>H(0)C-NH (cis)</td>
<td>8.95</td>
<td>8.78</td>
<td>8.75</td>
<td>8.74</td>
</tr>
<tr>
<td>H(0)C- (trans)</td>
<td>8.13</td>
<td>8.13</td>
<td>8.13</td>
<td>8.14</td>
</tr>
<tr>
<td>H(0)C- (cis)</td>
<td>7.85</td>
<td>7.95</td>
<td>7.95</td>
<td>7.95</td>
</tr>
<tr>
<td>H-6 (trans)</td>
<td>7.69</td>
<td>7.70</td>
<td>7.69</td>
<td>7.68</td>
</tr>
<tr>
<td>H-6 (cis)</td>
<td>7.57</td>
<td>7.57</td>
<td>7.57</td>
<td>7.58</td>
</tr>
<tr>
<td>4-NH$_2$ (cis)</td>
<td>6.30</td>
<td>6.30</td>
<td>6.30</td>
<td>6.34</td>
</tr>
<tr>
<td>4-NH$_2$ (trans)</td>
<td>6.18</td>
<td>6.19</td>
<td>6.22</td>
<td>6.22</td>
</tr>
<tr>
<td>2-NH$_2$ (cis)</td>
<td>5.91</td>
<td>5.91</td>
<td>5.92</td>
<td>5.97</td>
</tr>
<tr>
<td>2-NH$_2$ (trans)</td>
<td>5.84</td>
<td>5.84</td>
<td>5.85</td>
<td>5.94</td>
</tr>
</tbody>
</table>

* (c) refers to the cis conformation; (t) refers to the trans conformation.
Figure 34. Expanded view of the 5.0 to 10 ppm region of Figure 32. The peaks which are marked with an (X) are attributed to Species II, while the (0) belong to the peaks attributed to Species III.
Figure 35. Expanded view of the 5.0 to 10 ppm region of Figure 33. The peaks which are marked with an (X) are attributed to Species II, while the (O) belong to the peaks attributed to Species III.
Table 12. Comparison of Species III in Samples A and B: 500-MHz proton NMR spectra in Me₂SO-δ₆, 25°C.

<table>
<thead>
<tr>
<th>Tentative Assignment*</th>
<th>ppm A</th>
<th>ppm B</th>
<th>Multiplicity*</th>
<th>J(¹H-¹H) (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(O)C-NH (trans)</td>
<td>9.23</td>
<td>9.23</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>H(O)C-NH (cis)</td>
<td>8.85</td>
<td>8.86</td>
<td>d 12.0</td>
<td></td>
</tr>
<tr>
<td>H(O)C- (trans)</td>
<td>8.14</td>
<td>8.14</td>
<td>d 1.5</td>
<td></td>
</tr>
<tr>
<td>H(O)C- (cis)</td>
<td>7.99</td>
<td>7.98</td>
<td>d 11.0</td>
<td></td>
</tr>
<tr>
<td>H-6 (trans)</td>
<td>7.78</td>
<td>7.78</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>H-6 (cis)</td>
<td>7.66</td>
<td>7.66</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>4-NH₂ (cis)</td>
<td>6.45</td>
<td>6.46</td>
<td>s</td>
<td>---</td>
</tr>
<tr>
<td>4-NH₂ (trans)</td>
<td>6.34</td>
<td>6.36</td>
<td>s</td>
<td>---</td>
</tr>
</tbody>
</table>

* cis and trans refer to the conformation of the amide bond; (s) singlet, (d) doublet.
amino groups is easily identified in the spectrum. The 5-amino group would be expected to be further upfield than the other two amino groups, but no resonances upfield of the observed amino group can be clearly assigned to the 5-amino group. Thus, at the present stage, Species III cannot be conclusively assigned to the structure 2,5-diamino-4-formamidopyrimidine. It is clear that Species III cannot be the glycosylated DAFP, i.e. 2,4-diamino-5-formamido-N^4-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimidine (DAFPdR). Although the NMR data, at the current level of resolution, might be consistent with this structure, it is inconsistent with the data from the radiolabeling experiment, which showed no defined radioactive band eluting in HPLC at the position of Species III (Figure 20). Further NMR data is required on purified Species III in order to determine the a structure of this species.

Two attempts were made to isolate Species III from large scale hydrolyses of AmPur (Methods section). Fractions containing Species III were collected from XAD-4 column chromatography of the AmPur hydrolysates; these fractions had absorbance maxima at 267 nm. In the first attempt, when the pooled fractions were concentrated by rotary evaporation at a temperature of 40°C, the λ_max shifted from 267 to 304 nm, indicating that this material formed AmPur under these conditions. In the second attempt, the bulk solution was lyophilized to a powder in order to prevent the conversion of Species III to AmPur. Although the resultant powder did contain Species III judging by its UV spectrum in water, the mass spectral analysis of this powder
showed that it consisted largely of salt.
Kinetics of dAdo hydrolysis

Linear plots of ln(peak height) vs. time were obtained for the depurination of dAdo at near neutral pH values, indicating that the kinetics were pseudo-first order (Figure 7), as had also been observed in earlier studies on dAdo hydrolysis, conducted in acidic solution (Zoltewicz et al., 1970; Garrett and Mehta, 1972a; Hevesi et al., 1972).

Zoltewicz et al. (1970) calculated a value of 23.3 ± 0.5 kcal/mol for the enthalpy of activation (ΔH*) for the depurination of dAdo in aqueous medium at moderate to high acid concentrations. Under similar conditions, this result was confirmed by Hevesi et al. (1972) and by Garrett and Mehta (1972a), who reported ΔH* values of 23.7 kcal/mol and 23.2 kcal/mol, respectively. In the present work, the enthalpy of activation for dAdo hydrolysis was determined at pH 6.8, and found to have the same value, 23.3 kcal/mol.

For the entropy of activation (ΔS*), Zoltewicz et al. (1970) report +8.4 ± 1.3 eu. Examination of their data shows that the
computation of this entropy was based on the second-order rate constant for the reaction of nucleoside with hydronium ion, measured in mol L⁻¹ sec⁻¹; the standard state for the entropy of activation is thus understood to be 1 M acid (Lalder, 1965). The identical value of 8.4 eu was also reported by Hevesi et al. (1972), while the data of Garrett and Mehta allow a determination of a ΔS⁺ value of 7.7 eu, again for a standard state of 1 M acid.

In the present work, the plot of ln(kh/kT) vs. 1/T (Figure 36), based on the pseudo-first order rate constants for dAdo hydrolysis at pH 6.8, which are listed in Table 2, gave an intercept from which the entropy of activation was calculated at -21.2 eu. For comparison with the reports cited above, one can convert this value for the entropy of activation to the form based on second-order rate constants; to this end, all pseudo-first order rate constants would be divided by 10⁻⁶.8 M, and a renewed plotting would then give an intercept higher by the term 2.303 x 6.8 and a ΔS⁺ value higher by the term 2.303 x 6.8 x R, viz. +10.2 eu. This close agreement with the ΔS⁺ values reported by Zoltewicz et al. (1970), by Hevesi et al. (1972), and by Garrett and Mehta (1972a), may appear surprising, because their values were determined under experimental conditions where the breakdown of the dication is significant or even dominant, while the hydrolysis of dAdo at pH 6.8 likely proceeds from the monocation. However, hydrolysis of both the monocation and the dication are thought to occur by the A-1 mechanism (Zoltewicz et al., 1972), and the ΔS⁺ values should be similar in the two cases.
Figure 36. Plot of $\ln(kh/kT)$ vs. $1/T$ for the hydrolysis of dAdo and of AmPurdR. The data for dAdo are given by the symbol (x) and the solid line, whereas the data for AmPurdR are given by (o) and a dashed line. The lines were drawn from a least-squares analysis of the data points. The correlation factors ($r$) for the two lines are: dAdo, $r = -0.998$; and AmPurdR, $r = -0.999$. 
Although it was presumed from the enthalpy and entropy of activation that the hydrolysis of dAdo at pH 6.8 primarily follows the A-1 mechanism, additional evidence for this conclusion can be obtained from the pH dependence of the depurination reaction. While the pH dependence of the hydrolysis rate of dAdo was not examined in my experiments, Garrett and Mehta (1972a) have reported rate constants for this reaction over the pH range from pH 3.8 to 7.5, at 80°C. When their data was used in a plot of log(k) vs. pH (figure 5 of Garrett and Mehta, 1972b), between the pH values of 3.8 to 6.2, the slope of a least-squares line is -1.06. This relationship between observed rate constant and pH also holds for other purine nucleosides in the acidic range; it is taken as evidence that the reaction proceeds from a cationic species, presumably from the monocation. However, between the pH values of 6.2 and 7.5, the slope, as defined by these two points, has a value of only -0.81. This change in slope must reflect the fact that at pH 7.5 the observed rate constant is larger than accounted for by the decomposition of the cations alone. The extent of the effect can be estimated by redrawing the pH profile of Garrett and Mehta (1972b); in Figure 37, the observed rate constant at pH 7.5 is about 1.5-fold as high as the expected rate constant. The apparent increase in rate constant may reflect that, at pH values approaching neutrality, the observed rate constant is a composite quantity arising from a combination of several separate depurination reactions, not just from the A-1 mechanism as observed at lower pH values. These alternative mechanisms are the uncatalyzed reaction, which depurinates
Figure 37. Replot of the pH-rate profile for dAdo hydrolysis from Garrett and Mehta. The solid line represents the line as drawn by Garrett and Mehta, the dashed line represents a least-squares line drawn using the data of Garrett and Mehta (1972a) from pH 3.8 to pH 6.2.
via the neutral species, and the alkaline solvolysis reaction. The latter has been studied in the pH range of 11 to 13 (Garrett and Mehta, 1972b), where the plot of log(k) vs. pH has a slope of +1. Between the acidic branch of this plot (pH < 7.5) and the alkaline branch (pH > 11), the plot must be strongly curved, but no experimental points were determined by Garrett and Mehta for this intervening pH range. An estimate of the contribution of the alkaline solvolysis to the rate of depurination in neutral solution can be obtained by extrapolation of the alkaline branch of the log(k)-pH profile of Garrett and Mehta (1972b) to lower pH values. This process gives an estimate of $1 \times 10^{-9}$ s$^{-1}$ for the pseudo-first order rate constant for alkaline hydrolysis at pH 7.5, and $5 \times 10^{-11}$ s$^{-1}$ at pH 6.2. Thus, this process contributes only negligibly the nucleoside degradation at pH 7.5, and the deviation in the curve seen by Garrett and Mehta (1972b) at this pH likely reflects the uncatalyzed reaction.

Even at pH 7.5, this deviation from the rate behavior expected for breakdown from the cationic state, is moderate. Thus, for the experiments carried out at pH 6.8 in the present work, the interpretation of the reaction as depurination of the dAdo cation is warranted.

There is one aspect in the kinetic behavior of dAdo seen in the present work which differs considerably from previous observations. It has been reported that dGuo depurinates about 500 times faster than Guo at 100°C and at low pH (Zoltewicz et al., 1970), and that dAdo depurinates about 1000 times faster than Ado at 80°C in 0.1 M HCl.
(Garrett and Mehta, 1972a). The present results obtained at 110°C and neutral aqueous solution show for both AmPur and adenine a less than 10-fold decrease in the hydrolysis rate when the riboside is compared to the deoxyriboside. It is possible that the mechanism of dAdo depurination at neutral pH, probably proceeding from the monocation, differs in some important aspects from the A-1 mechanism as documented in solutions of high acidity, where reaction of the dication should predominate. In this context, it is interesting to note the speculation of Shapiro (1969) that depurination may proceed with intervening hydration of the aglycone. This idea was attacked by Zoltewicz et al. (1970), but has not been definitively refuted.

**Kinetics of AmPurdR hydrolysis**

At the start of this investigation, the depurination of AmPurdR was expected to follow the A-1 mechanism. However, the product distribution observed in this reaction indicated that a different hydrolysis pathway was operative.

Plots of ln(peak height) vs. time were linear, indicating that pseudo-first order kinetics are followed in the depurination of AmPurdR (Figure 12). This observation was supported by the minimal effect of the nucleoside concentration on the rate. The plot of ln(kh/kT) vs. 1/T (Figure 36), using the observed rate constants (Table 2), was linear; the slope and the intercept of the line were used for a formal determination of the values for $\Delta H^*$ and $\Delta S^*$. 
22.2 kcal/mol and -21.5 eu, respectively; here, $\Delta S^\ddagger$ is evaluated from the pseudo-first order rate constants. These values are similar to the kinetic parameters calculated for the dAdo hydrolysis. However, the complex product mixture obtained and the nonintegral slope of the plot of $\log(k) \text{ vs. } pH$ make it appear likely that the observed rate constant is, in a composite way, dependent on several processes; thus, these values are of very limited use for any mechanistic conclusions. The analysis of products and of the effects of various parameters on the kinetics will give some indication of the mechanism involved in the depurination of AmPurdR.

The effect of salt concentration on the rate of hydrolysis of AmPurdR was slight, a decrease of pseudo-first order rate constant by 15% attendant on a ten-fold increase in the ionic strength. Given the error estimated for the determination of the reaction rates (7%), this salt effect is marginal. If this result is significant, it indicates that charge dispersion occurs as the transition state is approached.

When the buffer concentration was changed from 15 to 150 mM, the hydrolysis rate constant decreased by 14%. This change, if significant, is accounted for by the effect of ionic strength. Therefore, the rate is unaffected by changes in the concentration of cacodylic acid and cacodylate, and the reaction thus shows neither general acid nor general base catalysis.

In the hydrolysis of most purine nucleosides and their analogues, at acid concentrations above the $pK_a$ of the monocation, the observed slope of a plot of $\log(k) \text{ vs. } pH$ is close to -1. In the case of
AmPurdR hydrolysis, however, this plot has an average slope of -0.43 in the region between pH 5.8 and 7.1 (Figure 18). This excludes, for the pH range examined in this study, the possibility that the reaction proceeds by a simple A-1 mechanism from the monocationic form of AmPurdR. It is possible, however, that AmPurdR hydrolysis may follow an A-1 mechanism at much lower pH values.

One case has been described where the slope of the plot of log(k) vs. pH for degradation of a purine nucleoside significantly deviates from the value of -1.0 in the weakly acidic pH range. This was observed for 9-(β-D-ribofuranosyl)purine (Lönnberg and Lehikoinen, 1982). At pH and H₀ values lower than the pKₐ for the monocation (2.01 at 25°C; app. 1.6 at 90°C), the slope for the plots of log(k) vs. pH and of log(k) vs. H₀ has a value of -1.0, indicative of reaction largely from the dicationic species; however, at pH values larger than the pKₐ, the slope of this plot steadily decreases in steepness, with a value of about -0.4 around pH 4, the highest pH examined.

Lönnberg and Lehikoinen (1982) also observe a change in the product distribution with the change in the pH dependence. In highly acidic media, where the slope of the log(k) vs. pH plot is -1, the reaction follows the typical A-1 mechanism; the products of the reaction are purine and ribose. However, at the higher pH values, where the plot of log(k) vs. pH has a slope of approximately -0.4, a different product distribution is observed; in addition to purine and ribose, 4-amino-5-formamidoxyrimidine and 4,5-diaminopyrimidine are
obtained. The formamido compound must have arisen from the reaction of the imidazole ring in purine riboside, since it was determined that purine itself was not degraded under the conditions used in the experiments. Since this change in mechanism occurs above the pKa of the monocation, Lönnberg and Lehikoinen believe that the second mechanism involves the monocationic species while the dication uses the A-1 mechanism. They tentatively propose the following mechanism: attack of hydroxide ion at C-8 of the monocationic species followed by the opening of the imidazole ring and detachment of the glycosyl group. This type of mechanism resembles, in part, the alkaline hydrolysis of dAdo (Garrett and Mehta, 1972b). Lönnberg and Lehikoinen (1982) do not comment on how the sugar is cleaved from the heterocycle.

An important part of this study of the hydrolysis of AmPurdR was the identification of the major products of the reaction. Column chromatography on an Amberlite XAD-4 resin, using water as the eluant, resolved Species III, Species II, AmPur, and AmPurdR. Species II was identified as 2,4-diamino-5-formamidopyrimidine (DAFP). Comparison of the NMR spectra of the mixed fractions enriched in Species II and in Species III, respectively, showed for Species III a set of resonances analogous to those seen for Species II. It is possible that Species III is 2,5-diamino-4-formamidopyrimidine. This product could be formed by hydration and opening of the imidazole ring in AmPurdR; alternatively, DAFP could undergo ring closure, or AmPur can be hydrated to form 2-amino-7,8-dihydro-8-hydroxypurine, which could
then form either AmPur, 2,5-diamino-4-formamidopyrimidine, or DAFP at different rates.

While hydrolysis of dAdo leads to formation of adenine as the only UV absorbing product, in the case of AmPurdR the free base was, in most cases, only a minor component of the product mixture. The major product was DAFP, and its appearance precedes the formation of AmPur. At 37°C, no formation of AmPur was detected; at intermediate temperatures the HPLC profiles showed DAFP in clear preponderance over AmPur, particularly at the earlier reaction times. Only at 110°C and 111°C were reaction mixtures obtained which contained more AmPur than DAFP (in terms of OD(280) units). While it was shown that AmPur hydrolysis can produce DAFP, the large amounts of DAFP seen in the AmPurdR hydrolysates cannot have arisen by this pathway; the slow hydration-dehydration equilibrium between DAFP and AmPur clearly favors the latter, as seen from the pseudo-first order rate constants of roughly $4 \times 10^{-8}$ s$^{-1}$ for hydrolysis of AmPur, and $8.6 \times 10^{-7}$ s$^{-1}$ for hydrolysis of DAFP, at 70°C.

From the analysis of the products and the pH dependence, the following tentative mechanism for the hydrolysis of AmPurdR is proposed (Figure 38). The first step is a reversible protonation of the heterocycle. The thermodynamic site of protonation appears to be N-1 (Janion and Shugar, 1973), however, this site may not be the protonation site responsible for the observed hydrolysis. A consideration of the bonding scheme in the 2-aminopurine moiety indicates the addition of water to C-8 should be much more strongly
Figure 38. Proposed mechanism for the hydrolysis of AmPurR.
favored by protonation at N-7 than by protonation at N-1 or N-3. In
the second step, the protonated nucleoside is attacked by water at C-8
forming 2-amino-7,8-dihydro-8-hydroxy-9-(2-deoxy-β-D-erythro-
pentofuranosyl)purine. This reaction is assumed to be reversible, in
analogy to the observation made with the free bases, where conversion
of DAFP to AmPur was documented in this work. This step is followed
by the opening of the imidazole ring and the elimination of the sugar
moiety, yielding 2-deoxy-D-arabino-pentose (2-deoxy-D-ribose) and
2,4-diamino-5-formamidopyrimidine (DAFP). The latter product can
undergo ring closure to form 2-amino-7,8-dihydro-8-hydroxypurine,
which can dehydrate to give AmPur, or reopen to form DAFP or
2,5-diamino-4-formamidopyrimidine.

At the present time, it is not known how the sugar moiety is
removed from the heterocycle. Conceivably, the N-glycosyl bond is
cleaved via a Schiff base intermediate. After formation of 2,4-
diamino-5-formamido-N⁴-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimidine
(DAFPdR), the N-4 nitrogen (formerly designated N-9) is no longer an
amidino nitrogen atom, and the electron distribution in this
nucleoside is more closely akin to that in N-aryl glycosylamines,
which are understood to hydrolyze via an A-2 mechanism (Capon and
Connett, 1965). The change from an amidino-type nitrogen to a
secondary amino nitrogen will allow the release of electrons into the
furanose ring; thus, the formation of the Schiff base is no longer
unfavorable. Addition of water to the Schiff base will produce the
carbinolamine, and ultimately the free sugar and DAFP. For Schiff
base hydrolysis at acidic pH, the decomposition of the carbinolamine was found to be rate-limiting, while at neutral to basic pH, the rate-determining step is the hydration of the Schiff base (Cordes and Jencks, 1963). Thus, the rate-determining step in the hydrolysis of AmpurdR is presumably the addition of water to the Schiff base intermediate.

Although the exact mechanism cannot be deduced from the present data, there are indications that the depurination follows the A-2 mechanism. The appearance of a Schiff base intermediate could explain the fact that the plot of log(k) vs. pH shows deviation from the normal A-1 behavior; this deviation indicates that the mechanism is more complex than the unimolecular cleavage of the glycosyl linkage, and that at least one of the steps prior to the rate-determining step requires protonation. There are two steps which require protonation, the protonation of N-7 prior to addition of water across the C(8)-N(7) double bond, and the protonation of the annular oxygen, which is required for the formation of the Schiff base. The former step has an absolute requirement on the acid concentration; however, the latter step may be independent of the pH of the solution. As depicted in Figure 39, the formation of the Schiff base may occur via a concerted proton transfer of the hydrogen of the 8-hydroxyl group to the annular oxygen. This intermediate is not unreasonable considering the acidic nature of the 8-hydroxyl hydrogen. Alternately, the formation of the Schiff base may follow a step-wise mechanism, in which the imidazole ring of the hydrated AmpurdR opens to form DAFpdR, protonation of the
Figure 39. Postulated mechanism for the cleavage of the N-glycosidic bond via a Schiff base intermediate.
annular oxygen in DAFPdR will allow the formation of a cationic Schiff base.

The lack of general acid catalysis appears to argue against formation of the Schiff base (Capon and Connett, 1965); in the case of AmPurdR hydrolysis, however, general acid or base catalysis may not be necessary for the addition of water to the Schiff base intermediate or for the decomposition of the carbinolamine. As shown in Figure 39, the N-3 nitrogen atom may act as the general base by the removal of a proton from the attacking water molecule, as well as the removal of the proton from the C-1 hydroxyl group in the carbinolamine, via a six-membered transition state.

Further experiments are necessary to determine if in fact the reaction proceeds through a Schiff base intermediate; these would include an examination of the configuration of the starting material remaining after intermediate reaction times to determine whether any anomerization of the glycosyl linkage occurred, and more extensive experiments involving the variations of the buffer concentrations. The deuterium isotope effect might not be helpful since there are several steps in which water addition or proton exchange will occur, leading to difficulties in interpreting the results.

Discussion of AmPur mutagenesis

One of the initial goals of this research was to determine if the greater hydrolytic lability of AmPurdR compared to dAdo, initially observed by Pless and Bessman (1983) in basic solutions at 90°C, also
obtained under physiological conditions, i.e. neutral pH at 37°C. Pseudo-first order rate constants of degradation in neutral aqueous solution were determined for both AmPurdR and dAdo at 110°C, 90°C, 70°C, and 50°C; at each temperature, AmPurdR was found to be approximately five-fold more labile than dAdo. In the case of AmPurdR, the hydrolysis rate at 37°C was determined directly, although the reaction was very slow, with a half-life of 301 days. A plot of \( \ln(kh/kT) \) vs. \( 1/T \) for AmPurdR hydrolysis gave a straight line from 37°C to 110°C. Similarly, a linear plot was obtained when plotting \( \ln(kh/kT) \) vs. \( 1/T \) for the hydrolysis of dAdo, from 50°C to 110°C (Figure 36). This plot was extrapolated to 37°C, to give a rate constant of \( 5.4 \times 10^{-9} \text{ s}^{-1} \); corresponding to a half-life of 1,490 days. From this extrapolated value, it can be concluded that at 37°C, AmPurdR is about five times as labile as dAdo. If, in comparison to adenine, the glycosyl linkage to AmPur exhibits a similar increased propensity for cleavage in a polynucleotide as it does in the nucleoside, the several-fold increase in the depurination rate when an A site in DNA is occupied by AmPur should be of biological importance.

Although apurinic sites are primarily blocking lesions (Schaaper and Loeb, 1981; Boiteux and Laval, 1982), it has been demonstrated that the apurinic sites are mutagenic \textit{in vitro} (Shearman and Loeb, 1979; Boiteux and Laval, 1982; Kunkel \textit{et al.}, 1981), and potentially mutagenic \textit{in vivo} under conditions of reduced replicative fidelity (Schaaper and Loeb, 1981; Schaaper \textit{et al.}, 1983). The latter event can cause transversion mutations, as well as transition mutations.
Although it has not been demonstrated that AmPur can induce an error-prone repair mechanism, it has been suggested (Persing et al., 1981) that some form of misrepair is responsible for the appearance of AmPur-induced transversion and frameshift mutations. An error-prone repair mechanism, in conjunction with an increased number of apurinic sites, could be the source of the transversions observed by Persing et al. (1981).

Another potential mutagenic lesion may result from the unusual depurination mechanism of AmPurdR. In this process, 2,4-diamino-5-formamido-N⁴-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimidine (DAFPdR) is a possible intermediate. This compound was not isolated nor identified in the present work, indicating that, if it was formed, DAFPdR was relatively short lived in solution. However, it is not known how stable this structure would be when it is located within a DNA molecule. If the depurination occurs via a Schiff base intermediate, the stability of DAFPdR within a DNA molecule might increase due to lower solvent accessibility of the N-glycosyl bond.

Similar formamidopyrimidine lesions have been documented in natural DNA. In alkylated DNA, the imidazole ring of 7-methylguanine residues can open (Haines et al., 1962; Lawley and Brooks, 1961; Jones and Robins, 1962); and, in γ-irradiated DNA, the attack of hydroxyl radicals at the C-8 position of A residues will cause formation of the formamidopyrimidine product (van Hemmen and Bleichrodt, 1971). These types of lesion are detrimental to the cell since it has been demonstrated that these molecules will inhibit DNA replication.
(Boiteux and Laval, 1983). A repair enzyme (formamidopyrimidine-DNA glycosylase) has been isolated which removes these lesions (Chetsanga et al., 1981; Margison and Pegg, 1981; Breimer, 1984), with release of the formamidopyrimidine and production of an apurinic site. This apurinic site is then removed via an AP endonuclease-DNA polymerase repair mechanism.

It is worth considering the potential mispairing capabilities of the three formamidopyrimidines listed above. Figure 40a shows the structure which can be drawn for a base pair between a DAFP moiety and an opposite cytosine moiety. This possibility is of interest in the context of the transition mutations mediated by AmPur. If the positions are adjusted to give linear hydrogen bond geometry and lengths of 2.8 Å for the H-bonds involving the N-4 and N-3 positions of cytosine, the third H-bond, at O-2 of cytosine, will be compressed and will give a clearly angled H-bond geometry. A more serious problem, which is largely independent of minor adjustments in H-bond lengths, is the position of the two sugar moieties with respect to each other: the distance between the two 1'-carbons is less than half the normal value for this distance in B-DNA. The base pair may also require the change in sugar pucker from the S conformation (2'-endo) to the N conformation (3'-endo) which is usually attendant on a change from the anti to the syn conformation of the purine base around the glycosidic bond. Thus, the DNA backbone would have to undergo considerable alterations to accommodate the hydrogen bonds. It is expected that this distortion of the dihedral angles would be too
Figure 40. Geometry of putative base pairs formed between a DAFP moieties and cytosine or adenine moieties in DNA. In each case, A and B indicate the positions of the corresponding 1'-carbons. C indicates, for B-DNA, the normal position of the 1'-carbon of the nucleotide moiety forming a Watson-Crick base pair with the base which is attached at A.
costly in terms of energy, even though X-ray crystallographic studies of the self-complementary dodecamer d-CGCGAATTCCG indicate that within B-DNA there is considerable variability in local geometry (Wing et al., 1980; Drew et al., 1981; Drew and Dickerson, 1981; Dickerson and Drew, 1981a, 1981b).

Another potentially mutagenic base pair is shown in Figure 40b. This structure could explain the AmPur-mediated A:T <-> T:A transversions reported by Persing et al. (1981), but here again, the relative position of the bases required to form two H-bonds forces a large reduction of the distance between the two DNA strands.

It has been reported by Boiteux and Laval (1983) that the formamidopyrimidine derivative obtained from the 7-methylguanine sites in poly(G-C) does not form mispairs with A or with T. An examination of the potential base pairs to A (Figure 41) shows that it can form only one hydrogen bond, the second hydrogen bond being precluded by the alkylmethylation. Similarly, it is expected that the ring-opened product obtained from an adenine site, i.e. the 4,6-diamino-5-formamidopyrimidine moiety, will not form mispairs with A or with C: as Figure 42 shows, steric interference between an amino hydrogen and the formyl hydrogen will prevent the formamido group from assuming the required coplanarity with the potential partner. Thus, in the latter two cases considerations beyond deformation of the DNA backbone militate against base pair formation.

The above arguments are based on simple consideration of geometry in the plane of the potential base pair. Computer simulation of the
Figure 42. Geometry of a putative base pair formed between a 4,6-diamino-5-formamidopyrimidine moiety and a cytosine moiety in DNA. A and B indicate the positions of the corresponding 1'-carbons. C indicates, for B-DNA, the normal position of the 1'-carbon of the nucleotide moiety forming a Watson-Crick base pair with the base which is attached at A.

Figure 41. Geometry of a putative base pair formed between the ring-opening product of a 7-methylguanine moiety in DNA and an adenine moiety. A and B indicate the positions of the corresponding 1'-carbons. C indicates, for B-DNA, the normal position of the 1'-carbon of the nucleotide moiety forming a Watson-Crick base pair with the base which is attached at A.
proposed mispairs in double-stranded DNA might provide insight into
the question whether these base pairs are energetically favorable
enough to be significant. Considerable work would be required to
experimentally test the possibility of formation of these base pairs.
The most direct evidence for the mispairing of the formamidopyrimidine
would come from NMR studies of oligomer duplexes containing the
potential mispair. The major problems expected for such a study would
be: the uncertainties involved in choosing a sequence which could
stabilize the mispair, the difficulties of the actual synthesis of the
oligomer containing the formamidopyrimidine, and the potential
hydrolytic lability of the formamidopyrimidine moiety under the
conditions of the experiment.
Conformational analysis of AmPurdR, dAdo, and dGuo

Several NMR techniques have been developed for the determination of nucleoside, nucleotide, and oligonucleotide conformation in solution. The conformational analysis of a nucleoside addresses three fundamental structural questions: the conformation of the furanose ring, the conformation of the exocyclic group about the C(4')-C(5') bond, and the syn or anti orientation of the heterocyclic base with respect to the sugar. For an accurate description of the conformation, all three pieces of information must be determined; however, since all of the structural components are interrelated, the structure can be approximated from the knowledge of the sugar pucker and the exocyclic group conformation.

The furanose ring generally has a non-planar, puckered structure, which exists in two ranges of conformations (Figure 43; Sundaralingam, 1969). For a simple treatment, these conformational ranges are generally divided in two groups, termed as N (north) and S (south; Altona and Sundaralingam, 1972). Classically, the various conformations were designated by the exo/endo description (Jardetzky, 126
Fig. 43. Illustration of the sugar pucker conformations associated with the N and S conformational types.
1960), however, this nomenclature has been replaced by the E and T notation (IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN), Recommendations 1980). The population of sugar conformers is usually calculated using the method of Altona and Sundaralingam (1973), in which the measured vicinal proton-proton coupling constants in the sugar moiety are interpolated between the corresponding coupling constants expected for the pure conformers (100% N and 100% S).

The average conformation of the exocyclic group about the C(4')-C(5') bond is described in terms of contributions from three defined rotomers, gauche-gauche (gg); gauche-trans (gt); and, trans-gauche (tg) (Figure 44). The populations of the rotomers are determined from the proton-proton coupling constants $J(4'-5')$ and $J(4'-5'\text{''})$ using empirical equations (Blackburn et al., 1970; Lee and Sarma, 1976; Stolarski et al., 1980). The conformation of the exocyclic group is a useful piece of information, since it has been shown that the conformation affects the chemical shifts of the sugar protons (Stolarski et al., 1980).

There are several NMR techniques available for the determination of the syn and anti populations of nucleosides. These include the chemical shift method (Dudycz et al., 1979; Stolarski et al., 1980), examination of the vicinal $^{13}\text{C}-^1\text{H}$ coupling constants between C-2 and H-1' in pyrimidine nucleosides (Lemieux et al., 1972; Schweizer et al., 1973), and C-8 and H-1' in purine nucleosides (Stolarski et al., 1980), and NOE measurements between H-8 and H-1' in purine
Figure 44. Illustration of the conformations of the 5'-exocyclic group, and of the syn and anti conformations for a purine nucleoside. The dashed lines indicate the amount of deviation from the normal rotomers due to oxygen-oxygen repulsion (Remin and Shugar, 1972).
nucleosides and H-6 and H-1' in pyrimidine nucleosides (Schirmer et al., 1972; Davis and Hart, 1972; Zens et al., 1976). There are considerable discrepancies in the evaluation of the data as determined by the different techniques. The first method compares the chemical shifts of the sugar protons in the nucleoside of interest with the chemical shifts observed in related model nucleosides which are locked in to either the syn or the anti conformation. When this method is used in conjunction with the heteronuclear coupling constants technique, the population distribution between the syn and anti forms can be determined and, in addition, some information about the preferred angles of the pure syn and anti forms is obtained. The accuracy of determinations of the syn and anti populations using NOE measurements has been questioned by Stolarski et al. (1980). Although the NOE measurements are accurate, the interpretation of the measurements can be fraught with errors depending on the theoretical model used. There can be considerable differences between nucleosides in the angles preferred for the syn and anti conformations and the preferred sugar conformations. The accuracy of the model is dependent on the assumptions used to assign the parameters for the glycosidic angle and the sugar conformation, as well as the simplification used in the calculations (e.g. the correlation times). Thus, in order to obtain a realistic notion of the conformation about the glycosidic bond, a number of methods should be used.
The 500-MHz proton NMR spectra in D$_2$O for the three nucleosides, dAdo, dGuo, and AmPurdR, are shown in Figures 45-47, respectively. The $^1$H-$^1$H coupling constants and the chemical shifts for each resonance are given in Table 13. The evaluation of the furanose conformation follows interpolation of the measured vicinal coupling constants between the coupling constants for the extreme conformations (100% N and 100% S) given by Altona and Sundaralingam (1973). The results are presented in Table 14. Following the method of Altona and Sundaralingam (1973), the sum of $J(1'2') + J(1'2'')$ was used to assign the fraction of the pseudo-rotational population in the S-type configuration, these values are: 72%, 73%, and 73%, for AmPurdR, dAdo, and dGuo, respectively. The other coupling constants gave similar values for the percentage of the S, except for $J(1'2'')$ and for the term $J(1'2') + J(2''3')$, because of the small difference between the coupling constants for the two extreme conformations in these two parameters. The conclusion is that the conformer distributions, in terms of sugar pucker, is very similar in AmPurdR, dAdo, and dGuo, with a decided preference for the S conformation.

The preferred conformation of the exocyclic group about the C(4')-C(5') bond was determined using the empirical equation of Lee and Sarma (1976) and the proton-proton coupling constants $J(4'-5')$ and $J(4'-5'')$ in Table 13. The equation of Lee and Sarma (1976) has the form:

$$\% \text{ gg} = \frac{(13.7 - \Sigma) \times 100}{9.7}$$

(6)
Figure 45. 500-MHz proton NMR spectrum of 2'-deoxyadenosine in D$_2$O.
Figure 46. 500-MHz proton NMR spectrum of 2'-deoxyguanosine in D$_2$O.
Figure 47. 500-MHz proton NMR spectrum of 2-aminopurine 2'-deoxyriboside in D$_2$O.
Table 13. 500-MHz \(^1\)H NMR spectra in D\(_2\)O, 25°C, 50 mM nucleoside.

Chemical shifts in ppm, relative to DSS:

<table>
<thead>
<tr>
<th></th>
<th>HOD</th>
<th>H-1'</th>
<th>H-2'</th>
<th>H-2''</th>
<th>H-3'</th>
<th>H-4'</th>
<th>H-5'</th>
<th>H-5''</th>
<th>H-2</th>
<th>H-6</th>
<th>H-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmPurdR</td>
<td>4.63</td>
<td>6.18</td>
<td>2.65</td>
<td>2.33</td>
<td>4.46</td>
<td>3.96</td>
<td>3.65</td>
<td>3.94</td>
<td>8.34</td>
<td>8.04</td>
<td></td>
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<tr>
<td>dAdo</td>
<td>4.63</td>
<td>6.23</td>
<td>2.62</td>
<td>2.37</td>
<td>4.45</td>
<td>4.00</td>
<td>3.66</td>
<td>3.66</td>
<td>7.95</td>
<td>8.07</td>
<td></td>
</tr>
<tr>
<td>dGuo</td>
<td>4.63</td>
<td>6.19</td>
<td>2.68</td>
<td>2.41</td>
<td>4.52</td>
<td>4.03</td>
<td>3.72</td>
<td>3.66</td>
<td>7.95</td>
<td>8.07</td>
<td>7.87</td>
</tr>
</tbody>
</table>

Coupling constants in Hz:

<table>
<thead>
<tr>
<th></th>
<th>J_{1'2'}</th>
<th>J_{1'2''}</th>
<th>J_{2'2''}</th>
<th>J_{2'3'}</th>
<th>J_{2''3'}</th>
<th>J_{3'4'}</th>
<th>J_{4'5'}</th>
<th>J_{4'5''}</th>
<th>J_{5'5''}</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmPurdR</td>
<td>7.1</td>
<td>6.5</td>
<td>14.0</td>
<td>6.6</td>
<td>3.4</td>
<td>3.1</td>
<td>3.5</td>
<td>4.5</td>
<td>12.5</td>
</tr>
<tr>
<td>dAdo</td>
<td>7.5</td>
<td>6.2</td>
<td>13.9</td>
<td>6.4</td>
<td>3.3</td>
<td>3.1</td>
<td>3.2</td>
<td>4.2</td>
<td>12.6</td>
</tr>
<tr>
<td>dGuo</td>
<td>7.3</td>
<td>6.4</td>
<td>14.1</td>
<td>6.4</td>
<td>3.6</td>
<td>3.5</td>
<td>3.6</td>
<td>4.8</td>
<td>12.5</td>
</tr>
</tbody>
</table>
Table 14. Conformational analysis of the furanose ring.

<table>
<thead>
<tr>
<th></th>
<th>N type calcd.</th>
<th>S type calcd.</th>
<th>AmPurdR J (Hz)</th>
<th>%S</th>
<th>dAdo J (Hz)</th>
<th>%S</th>
<th>dGuo J (Hz)</th>
<th>%S</th>
</tr>
</thead>
<tbody>
<tr>
<td>J₁₂¹</td>
<td>0.3</td>
<td>10.4</td>
<td>7.1</td>
<td>67</td>
<td>7.5</td>
<td>71</td>
<td>7.3</td>
<td>69</td>
</tr>
<tr>
<td>J₁₂²</td>
<td>6.8</td>
<td>5.7</td>
<td>6.5</td>
<td>27</td>
<td>6.2</td>
<td>54</td>
<td>6.4</td>
<td>36</td>
</tr>
<tr>
<td>J₁₂¹ + J₁₂²</td>
<td>7.1</td>
<td>16.1</td>
<td>13.6</td>
<td>72</td>
<td>13.7</td>
<td>73</td>
<td>13.7</td>
<td>73</td>
</tr>
<tr>
<td>J₂³¹</td>
<td>5.0</td>
<td>5.7</td>
<td>6.6</td>
<td>--</td>
<td>6.4</td>
<td>--</td>
<td>6.4</td>
<td>--</td>
</tr>
<tr>
<td>J₂³²</td>
<td>11.3</td>
<td>0.4</td>
<td>3.4</td>
<td>72</td>
<td>3.3</td>
<td>73</td>
<td>3.6</td>
<td>71</td>
</tr>
<tr>
<td>J₁₂¹ + J₂³¹</td>
<td>11.6</td>
<td>10.8</td>
<td>10.5</td>
<td>--</td>
<td>10.8</td>
<td>100</td>
<td>10.9</td>
<td>88</td>
</tr>
<tr>
<td>J₃⁴¹</td>
<td>10.1</td>
<td>0.2</td>
<td>3.1</td>
<td>71</td>
<td>3.1</td>
<td>71</td>
<td>3.6</td>
<td>66</td>
</tr>
</tbody>
</table>
where Σ is the sum of \( J(4'-5') \) and \( J(4'-5'') \). The values of \( \%gg \) and \( \%(tg + gt) \) are: for dA do, 65% and 35%; for dGuo, 55% and 45%; and for AmPurdR, 59% and 41%. The data indicates that the conformation of the exocyclic group of AmPurdR lies between those of dA do and dGuo. This may indicate that AmPurdR has a slightly higher preference for the \textit{syn} conformation than does dA do, but AmPurdR has a somewhat higher \textit{anti} conformational population than does dGuo.

The results derived from the coupling constants and the exocyclic conformation indicate that in solution, AmPurdR, dA do, and dGuo prefer the \textit{S} conformation and the \( \%gg \) conformation of the exocyclic group. The \textit{syn} and \textit{anti} populations have not been directly obtained from NOE or heteronuclear coupling constants; the \textit{anti} conformation is generally associated with the \textit{S} conformation of the sugar. Thus, one can infer on the basis of the available data that AmPurdR prefers the \textit{anti} conformation, and prefers a conformation which is intermediate between dA do and dGuo.
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