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Studies on the Pathway of Incorporation of 2-Aminopurine into the Deoxyribonucleic Acid of *Escherichia coli*[1]

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A pathway for the incorporation of 2-aminopurine into deoxyribonucleic acid (DNA) was studied in cell-free extracts of *Escherichia coli*. It was demonstrated that the free base can be converted to the deoxynucleoside, and that the deoxynucleotide can be phosphorylated to the di- and triphosphates and then incorporated into the DNA. From a consideration of the individual reactions in crude extracts, it is likely that the rate-limiting step in this pathway is the formation of the deoxynucleotide. Of especial interest is the observation that 2-aminopurine may be viewed as an analogue of either guanine or adenine, depending on which enzymatic step is being considered. On the one hand, it resembles guanine in that it is specifically converted from the mono- to the diphosphate by guanylate kinase and not by adenylate kinase. On the other hand, it replaces adenine rather than guanine in the DNA synthesized with purified DNA polymerases. *E. coli* DNA polymerase utilizes aminopurine deoxynucleoside triphosphate as a substrate for DNA synthesis much better than does purified phage T5-induced DNA polymerase and is also much less inhibited by this analogue than the T5 enzyme. These experiments in vitro correlate with known differential effects of 2-aminopurine on *E. coli* and phage in vivo.

Incorporation of the mutagen, 2-aminopurine (AP), into bacterial deoxyribonucleic acid (DNA) has been demonstrated in *Escherichia coli* by Wacker et al. (45) and Gottschling and Freese (18), and in *Salmonella typhimurium* by Rudner (38). An interesting question concerns the enzymatic pathway(s) by which this purine, which may be considered an analogue of adenine or guanine (Fig. 1), enters the DNA. Investigation of this process can provide information on the mechanism of the mutagenesis as well as an insight into the highly efficient methods employed by bacteria and phage for assuring the integrity of their nucleic acids.

Several possible pathways of incorporation of AP into DNA are outlined in Fig. 2. The formation of 2-aminopurine ribonucleoside monophosphate (APMP) from AP and 5-phosphoribosyl 1-pyrophosphate (PRPP), catalyzed by nucleotide pyrophosphorylase (EC 2.4.2.7.8), was considered a very probable initial step, because this pathway has been demonstrated with the natural purine bases and some purine analogues. However, this reaction could not be demonstrated with AP. The research described here is consistent with a pathway of incorporation of AP into the DNA of *E. coli* as depicted.

$$\text{AP} \xrightarrow[\text{transferase}]{\text{nucleoside}} \text{APdR} \left[\xrightarrow[\text{kinase ?}]{\text{nucleoside}}\right]$$

$$\text{dAPMP} \xrightarrow[\text{kinase}]{\text{GMP}} \text{dAPDP} \xrightarrow[\text{diphosphokinase}]{\text{nucleoside}}$$

$$\text{dAPTP} \xrightarrow[\text{polymerase}]{\text{DNA}} \text{DNA-dAPMP}$$

The enzymes involved in the above scheme have all been previously identified and studied in *E. coli* except for the nucleoside kinase. The experience encountered with the latter enzyme and with the synthesis of the nucleotide in general suggests that the difficulty in forming 2-aminopurine deoxyribonucleoside monophosphate (dAPMP) may be the rate-limiting step in the incorporation of AP into *E. coli* DNA.

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MATERIALS AND METHODS

Reagents. All the nucleosides and nucleoside mono-, di-, and triphosphates were purchased from P-L Biochemicals. Purine and pyrimidine bases were products of Nutritional Biochemicals Corp. Aminopurine-2-^{3}H was from Schwarz BioResearch, Inc. Phosphopyruvate, PRPP, reduced nicotinamide adenine dinucleotide (NADH), lactic dehydrogenase (EC 1.1.1.27; type I from rabbit muscle), AP, pyruvate kinase (EC 2.7.1.40), and deoxyribonuclease (EC 3.1.4.5) were purchased from Sigma Chemical Co. The lactic dehydrogenase, an ammonium sulfate suspension, was diluted to 10 mg of protein per ml and dialyzed overnight against 100 volumes of 0.05 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.5. Dithiothreitol was purchased from Calbiochem. Dicyclohexylcarbodiimide was a product of Eastman Chemical Co., and cyanoethyl phosphate was purchased from Mann Research Laboratories.

Dowex-1-X2 and -X8 (form Cl^-; 200 to 400 mesh) resins were purchased from the J. T. Baker Chemical Co. Ag 50 W H^+, X4, minus 400 mesh, was a product of Bio Rad Laboratories. Diethylaminoethyl (DEAE)-11 cellulose was purchased from Whatman.

Two fluors were used. Bray's dioxane fluor was modified, as suggested by D. Segal, to contain 120 g of naphthalene, 8 g of 2,5-diphenyloxazole (PPO), and 200 ml of methanol and dioxane in a final volume of 2 liters. Fifteen milliliters of the fluor was miscible with at least 2 ml of water. Toluene fluor contained 2.56 g of PPO and 83 mg of *p*-bis[2-(5-phenyloxazole)]-benzene per liter.

Preparations. dAPMP or ^{3}H-dAPMP was synthesized by condensing 2-aminopurine deoxynucleoside (APdR) or ^{3}H-APdR and cyanoethyl phosphate in the presence of dicyclohexylcarbodiimide by the method of Tener (44) for the phosphorylation of deoxyadenosine. The dAPMP, purified on a column of Dowex-1-Cl^-, contained AP, deoxyribose, and phosphate in a ratio of 1.00:1.08:1.10. When incubated with purified 5'-nucleotidase (43), 98% of the organic phosphate was liberated as inorganic orthophosphate.

^{3}H-dAPTP was synthesized enzymatically from the monophosphate in a reaction mixture containing 6 mM $MgCl_2$; 0.06 M KCl; 0.06 M KPO_4, *p*H 7.5; 7.8 mM phosphopyruvate; 3 mM adenosine triphosphate (ATP); 12.5 mg of lactic dehydrogenase; 2.5 ml of nucleotide kinase (EC 2.7.4.4), fraction 12 of Fig. 5; and 1.2 mM ^{3}H-dAPMP; in a volume of 8.0 ml. After 9 hr at 37 C, the reaction mixture was heated in a boiling-water bath for 2 min, chilled, and ATP was oxidized with periodate by the procedure of Whitfeld (49). The triphosphate was separated from the mono- and diphosphates on a column of Dowex-1-Cl^- and was precipitated as the barium salt. It was metathesized with Dowex 50, NH_4^+, with a final yield of 30% based on the original dAPMP. Over 90% of the tritium migrated as the triphosphate when analyzed by paper electrophoresis in citrate at *p*H 4.9.

Phosphodiesterase (EC 3.1.4.1) and 5'-nucleotidase (EC 3.1.3.5) were partially purified from the venom of *Crotalus adamanteus*. Phosphodiesterase was the acetone III fraction and had no detectable nucleotidase activity (9). The 5'-nucleotidase was the ammonium sulfate II fraction (43) and had at least 1,000 times as much activity on 5'-adenosine monophosphate (AMP) as on 3'-AMP.

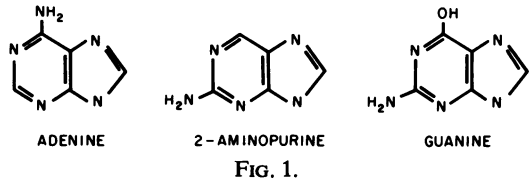

FIG. 1.

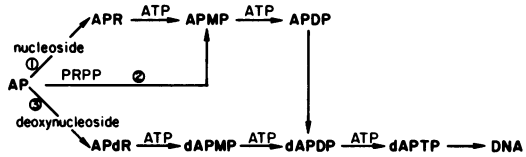

FIG. 2. *Possible pathways for the incorporation of 2-aminopurine into DNA. Abbreviations: AP, 2-aminopurine; APdR, 2-aminopurine deoxynucleoside; dAPMP, dAPDP, and dAPTP, 2-aminopurine deoxynucleoside mono- di- and triphosphate, respectively; APR; APMP, and APDP, 2-aminopurine ribonucleoside, 2-aminopurine ribonucleoside mono- and diphosphate respectively; PP, inorganic pyrophosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate.*

E. coli DNA polymerase (EC 2.7.7.7), DEAE III, and "activated" calf thymus DNA were prepared by C. D. Steuart by the method of Richardson et al. (36).

Phage T5 DNA polymerase was purified by the procedure of Steuart, Anand, and Bessman (42).

Analytical procedures. Protein was assayed by the method of Lowry et al. (28). The phosphate assay of Ames and Dubin (2) was used for inorganic and organic phosphate, and deoxyribose was measured by the method of Waravdekar and Saslaw (47) or Burton (10). Ribose was measured by the orcinol reaction by the directions of Ashwell (3).

Thin-layer chromatography was done with MN-cellulose Powder 300-PEI "for thin-layer chromatography," purchased from Brinkmann Instruments, Inc. The plate was developed in 1 M acetic acid for about 7 cm and then 0.3 M LiCl for another 7 cm.

Paper chromatography was done on Whatman no. 1 sheets in the following solvent systems: (I) water adjusted to *p*H 10 with ammonium hydroxide, run descending for 2 hr; (II) 95% ethanol:1 M ammonium acetate [*p*H 5.0, 7:3 (v/v)], run ascending overnight; and (III) isobutyric acid-concentrated ammonium hydroxide-water [66:1:33 (v/v)], run ascending overnight.

Liquid scintillation counting was done in a Packard Tri-Carb liquid spectrometer. ^{32}P was also counted in a thin-window gas-flow counter (Nuclear-Chicago Corp.).

Assay of nucleoside transferase (EC 2.4.2.6). Crude extracts were prepared from 1-liter cultures of *E. coli* grown overnight in C medium (37) at 37 C

under forced aeration. The cells were harvested at 5,000 × *g*, washed in 0.05 M sodium pyrophosphate (*p*H 6.5), suspended in 20 ml of this buffer, and disrupted by sonic oscillation. The debris was centrifuged out at 5,000 × *g*, and the supernatant fluid was used for the assay. A particle-free preparation was prepared by centrifuging the crude extract at 144,000 × *g* for 3 hr in a Spinco model L ultracentrifuge.

The incubation mixture contained 1.25 mM ^{3}H-AP (specific activity, 5 × 10^5 counts per min per μmole); 2.5 mM deoxyguanosine; 8.5 mM sodium pyrophosphate, *p*H 6.5; and enzyme in a volume of 0.12 ml. After 30 min at 37 C, the reaction was terminated by boiling for 90 sec. A 60-μliter portion was then chromatographed in solvent I for 2 hr. R_F values were as follows: adenine, 0.38; guanine, 0.40; AP, 0.47; deoxyadenosine, 0.55; deoxyguanosine, 0.65; and APdR, 0.60. Phosphorylated compounds traveled at the front. Identification of AP and its derivatives was aided by its blue fluorescence in ultraviolet light. Spots were cut out, eluted in liquid scintillation vials with 1 ml of water, and counted with 15 ml of modified Bray's dioxane fluor. A unit was defined as 1 nmole of AP(d)R formed in 30 min.

Assay of nucleoside kinase (EC 2.7.1.20). Crude extracts were prepared from bacteria washed with 5 volumes of 0.05 M KPO_4 (*p*H 7.5) and resuspended in this buffer containing 2 × 10^{-3} M dithiothreitol. The cells were broken by sonic treatment and centrifuged at low speed to remove debris. High-speed supernatant fractions were prepared by centrifuging crude extracts at 144,000 × *g* for 1 hr. These fractions were dialyzed overnight in 100 volumes of 0.05 M KPO_4 (*p*H 7.5) and 2 × 10^{-3} M dithiothreitol.

The following assay measures the nucleoside-dependent formation of adenosine diphosphate (ADP) by coupling it to the oxidation of NADH (23).

$$\text{deoxynucleoside} + \text{ATP} \rightarrow \text{deoxynucleotide} + \text{ADP}$$

$$\text{ADP} + \text{phosphopyruvate} \rightleftharpoons \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{NAD}^+$$

$$\text{deoxynucleoside} + \text{phosphopyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{deoxynucleotide} + \text{lactate} + \text{NAD}^+$$

The oxidation of NADH was followed spectrophotometrically in a Gilford recording spectrophotometer. Assay mixtures contained, in 1 ml: 20 mM Tris-maleate buffer, *p*H 5.8; 0.5 mM $MgCl_2$; 50 mM KCl; 2 mM dithiothreitol; 0.25 mM phosphopyruvate; 0.05 mM ATP; 20 μg of pyruvate kinase; 20 μg of lactic dehydrogenase; 0.2 mM NADH; 0.6 mM nucleoside; and 5 or 10 μliters of enzyme. In all cases, the rate of NADH oxidation was measured both before and after the addition of nucleoside. The difference in rate was a measure of nucleoside-dependent NADH oxidation.

Assay of nucleotide kinase. This assay measures the conversion of a ^{32}P-labeled deoxynucleoside monophosphate [which is hydrolyzable by semen phosphomonoesterase (EC 3.1.3.2)] to a deoxynucleoside triphosphate (which is resistant to semen phosphomonoesterase). Reaction conditions and procedures were described in detail by Bello and Bessman (5). Incubation mixtures (0.25 ml) contained: $MgCl_2$, 8 mM; Tris-Cl^-, *p*H 7.5, 80 mM; ATP, 5 mM; ^{32}P-labeled deoxynucleotide (0.5 × 10^5 to 1.5 × 10^5 counts per min per μmole), 0.64 mM; and enzyme, 0.03 to 1.0 unit. A unit of enzyme catalyzes the phosphorylation of 0.1 μmole of deoxynucleotide per hour.

An extract was prepared from stationary cells grown in C medium by sonically oscillating a suspension (1 g of packed cells in 3 ml of glycylglycine buffer, *p*H 7.4) for 10 min at 10 kc and discarding the sediment after centrifuging for 30 min at 10,000 × *g*.

Assay of purine nucleotide pyrophosphorylase. Reaction mixtures of 1 ml (containing 0.1 M Tris, *p*H 8.0; 10 mM $MgCl_2$; 1 mM adenine or AP; 1 mM PRPP; and dialyzed high-speed supernatant fraction) were as prepared for nucleoside kinase studies. After 20 min at 37 C, the mixtures were heated in a boiling-water bath, centrifuged, and loaded onto columns of Dowex-1-Cl^-, 5 cm by 0.18 cm^2. Substrate was eluted with 20 ml of 0.001 N HCl. An additional 1 ml of 0.001 N HCl was checked to insure that negligible ultraviolet-absorbing material was being eluted from the columns, and then the product was eluted in 5 ml of 0.01 N HCl. The eluates were examined in a spectrophotometer at wavelengths corresponding to their absorption maxima, and concentrations were calculated from their respective extinction coefficients. A value of 6.2 × 10^3 M^{-1} cm^{-1} at 305 nm was used for dAPMP (15). The columns were previously calibrated with authentic dAPMP.

Assay of E. coli DNA polymerase. The complete system contained 6.7 mM $MgCl_2$; 67 mM glycine, *p*H 9.3; 1.7 mM dithiothreitol; and 0.12 mM "activated" calf thymus DNA; 33 μM each of ^{3}H-dAPTP (3.93 × 10^5 counts per min per μmole), deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and *alpha*-^{32}P-deoxythymidine triphosphate (dTTP) (1.00 × 10^6 counts per min per μmole); and 0.09 unit of *E. coli* polymerase, fraction DEAE III (36) in 0.30 ml. After 1 hr of incubation at 37 C, 0.2 ml of 0.2 M sodium pyrophosphate and 0.5 ml of 7% perchloric acid were added. The mixtures were chilled, filtered on Whatman GF/C glass-fiber disks, washed, dried, and counted in toluene fluor.

Assay of phage T5 DNA polymerase. The complete system contained 7 mM $MgCl_2$; 0.2 M NH_4Cl; 67 mM Tris, *p*H 8.6; 3 mM dithiothreitol; 0.1 mM salmon sperm DNA (heated for 30 sec in boiling-water bath and quickly cooled in ice water); 33 μM ^{3}H-dAPTP, dATP, dCTP, dGTP, and ^{32}P-dTTP; and 7 units of phage T5 polymerase in 0.3 ml. The mixtures were incubated 1 hr at 37 C and treated just as the *E. coli* DNA polymerase reaction mixtures.

RESULTS

Nucleoside transferase. Extracts prepared as described above were tested for their ability to carry out the following reaction: AP + (deoxy)-nucleoside ⇌ AP(d)R + base. When deoxyguanosine was used as the deoxyribosyl donor, the rate of formation of APdR was linear for the first

20 min. The effect of enzyme concentration on reaction rate is shown in Fig. 3. Extract, heated to 100 C for 90 sec was inactive. A comparison of several ribo- and deoxyribonucleosides in effecting a transfer of their pentose moieties to AP is shown in Table 1. Purine deoxynucleosides are the most active donors, followed by purine ribonucleosides. Pyrimidine ribo- and deoxyribonucleosides are able to transfer their sugars to AP but far less effectively than the corresponding purine derivatives.

To identify the product of the reaction as APdR, the standard incubation was scaled up by a factor of 100 (12.0 ml), and, after 75 min at 37 C, protein was precipitated by the addition of perchloric acid and perchlorate was removed as

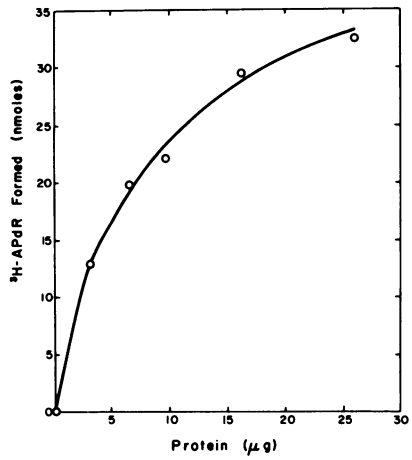

FIG. 3. *Influence of protein concentration on the amount of APdR synthesized. Standard reaction conditions of the nucleoside transferase assay were used with deoxyguanosine as the deoxyribosyl donor and the high-speed supernatant fraction as the enzyme source.*

TABLE 1. *Comparison of nucleoside donors in the nucleoside transferase reaction*[a]

Nucleoside	APdR formed
	nmoles
Deoxyguanosine	36
Deoxyadenosine	28
Adenosine	10
Guanosine	8
Thymidine	1.5
Deoxycytidine	0.2
Uridine	3
Cytidine	0.3

[a] Standard reaction conditions were used with 1.25 mM ^{3}H-2-aminopurine, 2.5 mM nucleoside, and 47 μg of high-speed supernatant fraction per ml.

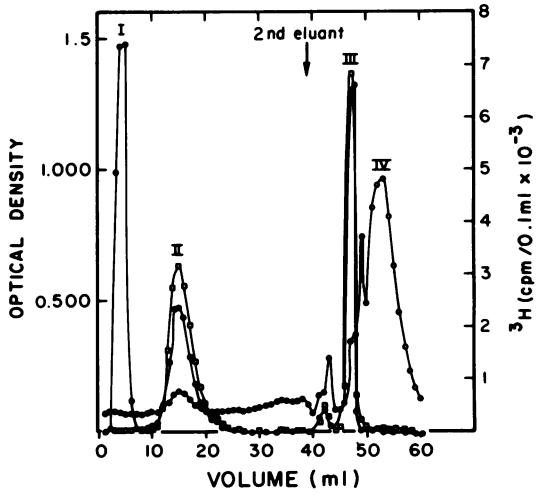

FIG. 4. *Isolation of enzymatically synthesized APdR. A reaction mixture scaled up 100-fold was treated as described in the text and chromatographed on a column of Dowex-1-formate by the method of Gottschling and Freese (18). The peaks were identified as: I, tritiated water; II, the product, APdr; III, the unreacted substrate, AP; IV, guanine and deoxyguanosine. Symbols:* ●, ^3H; □, A_{340}; ○, A_{260}.

the insoluble potassium salt by titrating with KOH. The resulting clear, protein-free filtrate was applied to an anion-exchange resin and chromatographed by the method of Gottschling and Freese (18; Fig. 4). Two major peaks containing both ^{3}H and absorbance at 304 nm were observed. Peak II was subsequently identified as the product, APdR, and peak III represented the unreacted AP. Guanine and deoxyguanosine cochromatographed at peak IV. Peak I, which exhibited no optical density in the ultraviolet and visible regions, was thought to be tritiated water. The radioactive material applied to the column, 4.35×10^6 counts/min of ^{3}H, was recovered in 100% yield.

The spectral data reported by Frederiksen (15) were very helpful in identifying the product since the free base, AP, has a distinct shoulder at 280 nm in alkali which is absent in the nucleoside. Thus, the ratio of absorbancies at 260 and 304 nm in alkali for authentic AP and APdR are 0.50 and 0.18, respectively (15), and the corresponding value for the material isolated from peak II was 0.21. Determination of deoxyribose by the method of Waravdekar and Saslaw (47) or Burton (10) revealed 1 mole of deoxypentose per 0.84 mole of AP as determined from its absorbance at 304 nm when a value of 6.8×10^3 was used as the molar extinction coefficient (15). When a sample of peak II was hydrolyzed in 0.1 N HCl at 100 C

TABLE 2. *Nucleoside-dependent oxidation of NADH*[a]

Enzyme source	Deoxyadenosine	Adenosine[b]	APdR[b]	APR[b]
Log-phase culture, high-speed supernatant fraction	2.7		0.5	3.8
Log-phase culture grown with AP, high-speed supernatant fraction	4.8		1	3.5
Stationary culture Crude extract	0.7	1.2		0.2
High-speed supernatant fraction	0.7	0.9	0.4	0.4

[a] Cells were grown and extracts were prepared as described in the text. One culture was supplemented with 1 mM AP and extracts were prepared in exactly the same way. Rates of oxidation of DPNH in the absence of added nucleosides were less than 0.1 μmole/mg of protein/hr. Abbreviations: NADH, reduced nicotinamide adenine dinucleotide; APdR, 2-aminopurine deoxynucleoside; APR, 2-aminopurine ribonucleoside; AP, 2-aminopurine.

[b] Values expressed as micromoles of nucleoside per milligram of protein per hour.

for 1 hr and then chromatographed in solvent I, more than 99% of the ^3H traveled with authentic AP, whereas before hydrolysis 100% of the material was found in the authentic APdR used as a marker. Examination of the spectrum in alkali, subsequent to acid-hydrolysis, revealed the shoulder at 280 nm characteristic of free AP.

In an analogous series of experiments, the corresponding riboside, APR, was synthesized in a reaction in which adenosine was used as the ribosyl donor. The spectral characteristics and analyses of the product were in all respects similar to the deoxyribonucleoside.

Nucleoside kinase. Synthesis of dAPMP from the corresponding nucleoside was at best a very weak reaction. Although dAPMP was not isolated from a reaction mixture, possible evidence for a nucleoside kinase was obtained by showing the AP(d)R-dependent utilization of ATP in the coupled lactic dehydrogenase, pyruvate kinase assay previously described. Data obtained with this procedure are presented in Table 2.

Although the cell extracts differed in their ability to phosphorylate nucleosides, activity was low in every case. The rate of phosphorylation of AP derivatives approached the rate with adenosine and deoxyadenosine.

Purine nucleotide pyrophosphorylase. Because the putative nucleoside kinase activity was so low, it was important to examine alternatives for the formation of AP nucleotides. Accordingly, purine nucleotide pyrophosphorylase activity was examined in our extracts. This enzyme carries out the following reaction

$$\text{purine} + \text{PRPP} \xrightarrow{Mg^{++}} \text{purine nucleotide} + \text{inorganic pyrophosphate}$$

and may be assayed by a modification of the procedure of Flaks et al. (14) described above. The results in Table 3 clearly indicate the presence of the enzyme when assayed with adenine as the substrate, but no activity toward AP could be demonstrated. Under the conditions of our assay, we would have been able to detect an activity toward AP if it were 0.5% of that with adenine. Therefore, we may conclude that the activity of adenine pyrophosphorylase is at least 200-fold greater than any activity toward AP. Portions of the reaction mixtures were analyzed by thin-layer chromatography and AMP was readily identified when adenine was used as acceptor. In incubations containing AP, no AP nucleotides were detected. From these results, formation of APMP by purine nucleotide pyrophosphorylase was considered unlikely, and further studies of this pathway were abandoned.

Nucleotide kinase. It was of interest to deter-

TABLE 3. *Assay for purine nucleotide pyrophosphorylase*[a]

Reaction conditions	Substrate	A_{260}	A_{340}	Product[b]
Complete, no enzyme	Adenine	0.21		
Complete, 10 μliters of enzyme		3.49		1.85
Complete, 20 μliters		5.43		1.42
Complete, 30 μliters		7.47		1.32
Minus PRPP[c], 30 μliters of enzyme		0.32		
Complete, no enzyme	AP		0.005	≤0.01
10 μliters			0.015	
20 μliters			0.020	
30 μliters			0.015	
Minus PRPP, 30 μliters of enzyme			0.025	

[a] Assays were done by the procedure described in the text. Extracts from log-phase cultures, 10 mg of protein per ml, were prepared according to the conditions for nucleoside kinase studies.

[b] Expressed as micromoles of product per milligram of enzyme.

[c] 5-Phosphoribosyl 1-pyrophosphate.

mine which, if any, of the nucleotide kinases present in *E. coli* was responsible for the phosphorylation of dAPMP. Consequently, a crude extract of stationary cells prepared as described previously was chromatographed on a column of DEAE cellulose under the conditions of Bessman et al. (7), which are known to resolve, partially, the four deoxynucleotide kinases. On subsequent assay of the individual fractions, the chromatographic profile depicted in Fig. 5 was obtained. The activity toward dAPMP closely followed the dGMP kinase activity and peaked in the same fraction, whereas the peak of dAMP kinase activity appeared in the previous fraction. That dAPMP kinase fractionates similarly to dGMP and *not* dAMP kinase is emphasized in Table 4 in which the ratios of activities throughout several fractions from the column are reported. As can be seen, the ratio of dAMP to dAPMP kinase decreases over 100-fold from fraction 10 through 16, whereas the ratio of dGMP to dAPMP kinase remains constant within the limits of the assay.

As a further indication that the phosphorylation of dAPMP is catalyzed by the same enzyme that phosphorylates guanine nucleotides, a sample of GMP kinase purified to homogeneity was tested with a dAPMP by using a spectrophotometric assay (32). This purified enzyme, which is highly specific for dGMP and GMP and has been shown to be incapable of phosphorylating any of the common nucleotides or several analogues, was able to catalyze the phosphorylation of dAPMP. Thus, 0.55 μmole of dAPMP was phosphorylated per min per ml of enzyme as compared to 30 μmoles of dGMP.

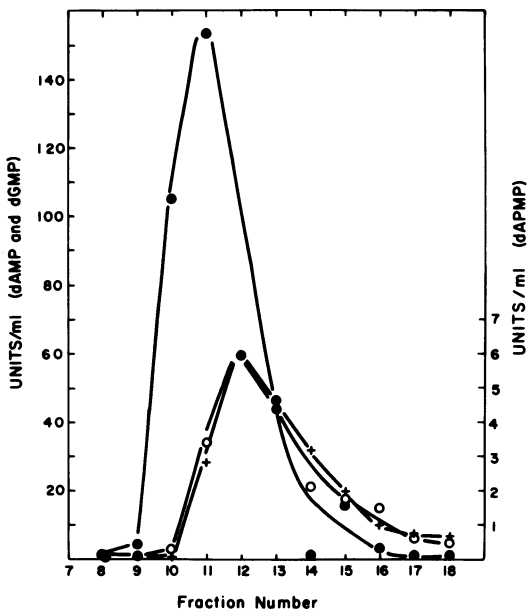

FIG. 5. *Separation of nucleotide kinase activities on DEAE cellulose. An extract of 5 g of cells (500 mg of protein), prepared as described in methods, was chromatographed on a column (11 by 1.9 cm) of DEAE cellulose under conditions described by Bessman et al. (7). Fractions (14 ml) were collected and assayed individually for dAMP, dGMP, dAPMP, dCMP, and dTMP kinases. We show here the activity towards dAMP (●), dGMP (○), and dAPMP (×). Activities toward dTMP and dCMP peaked between fractions 14 and 20 and are not shown.*

TABLE 4. *Comparison of nucleotide kinase activities in chromatographic fractions*

Fraction[a]	Substrate[b]			Ratio of dAMP to dAPMP	Ratio of dGMP to dAPMP
	dAMP	dGMP	dAPMP		
10	105	3	0.2	525	15
11	153	34	2.8	55	12
12	100	59	5.9	17	10
13	46	44	4.4	10	10
14	18	30	3.2	6	9
15	9	18	2.0	5	9
16	3	12	1.0	3	12

[a] Values for the individual fractions were taken from the data used to construct Fig. 5.

[b] Expressed as units per milliliter. Abbreviations: dAMP, deoxyadenosine monophosphate; dGMP, deoxyguanosine monophosphate; and dAPMP, 2-aminopurine deoxynucleoside monophosphate.

Nucleoside diphosphate kinase (EC 2.7.4.6). Purified guanylate kinase of *E. coli*, free of nucleoside diphosphate kinase, produces diphosphates exclusively (32). No triphosphates are formed unless nucleoside diphosphate kinase is added to the reaction mixture or less purified fractions of the enzyme containing endogenous diphosphate kinase as an impurity are employed. The fractions of GMP kinase from the column in Fig. 5 did, in fact, contain nucleoside diphosphate kinase because analysis of the nucleotides formed during the reaction of the enzyme with dAPMP in the presence of ATP and Mg^{2+} revealed the synthesis of both dAPDP and dAPTP. When portions of a standard incubation mixture for nucleotide kinase were removed during the course of the reactions and chromatographed in solvent III, the data revealed in Fig. 6 were obtained. It can be seen that concomitant with the disappearance of dAPMP, there is a formation of both dAPDP and dAPTP. The sum of the latter derivatives was essentially equivalent to the decrease in dAPMP, indicating the relative absence of side reactions. There was enough nucleoside diphosphate kinase present in the enzyme fraction to convert about

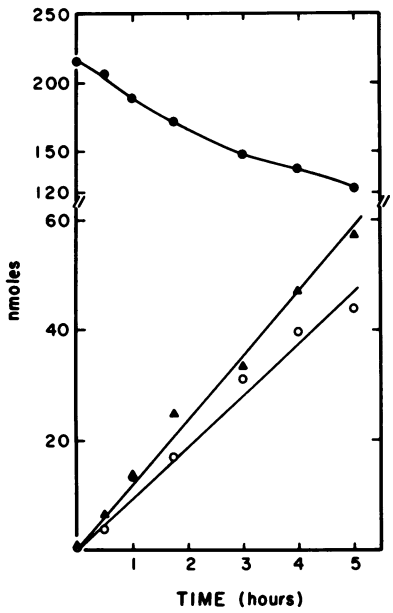

FIG. 6. *Enzymatic synthesis of dAPDP and dAPTP. Samples of a standard incubation mixture for nucleotide kinase were chromatographed in solvent III. Symbols: ●, dAPMP; ▲, dAPDP; and ○, dAPTP.*

one-half of the dAPDP to the triphosphate at any time.

DNA polymerase. Two techniques were used to measure the incorporation of dAPMP into DNA. Direct incorporation of the analogue was measured by following the uptake of ^{3}H into an acid-insoluble product when ^{3}H-dAPTP was used as substrate. A second technique, described by Bessman et al. (8), measures the ability of dAPTP to replace one of the normal triphosphates in the standard incubation mixture. Since optimal DNA synthesis requires the presence of all four deoxynucleoside triphosphates, the rate of DNA synthesis under standard conditions may be compared to incubations in which each of the individual nucleotides has been omitted or replaced by dAPTP. The ability of dAPTP to restore DNA synthesis in the absence of one of the normal triphosphates may be taken as an indication that dAPTP acts as an analogue of the particular triphosphate omitted. By using both these assays, the synthesis of DNA by purified *E. coli* DNA polymerase was measured, and as a comparison, purified T5-induced DNA polymerase was also studied. The results of these experiments are recorded in Table 5. ^{3}H-dAPMP is incorporated into DNA by both the *E. coli* and phage enzymes and it specifically replaces dATP. Omission of dCTP, dGTP, or dTTP reduced the rate of DNA synthesis to barely detectable levels, indicating that dAPTP could not replace these nucleotides. Omission of dATP, however, actually increased the incorporation of dAPTP with *E. coli* polymerase, showing an antagonism between these two nucleotides. The indirect assay supports these results, because the incorporation of ^{32}P-dTMP fell off markedly with the omission of any of the deoxynucleoside triphosphates except dAPTP. When dAPTP was omitted, the rate of dTMP incorporation was actually *increased*, indicating that dAPTP inhibits the reaction. This is even more striking with the T5 enzyme where the incorporation of ^{32}P-dTMP increased over fourfold on omission of dAPTP. When both dAPTP and each of the nucleotides was omitted in pairs, there was no effect over and above the omission of a single triphosphate except in the case of dATP. Here, omission of both nucleotides reduced the incorporation of dTMP more than omission of dATP alone. Thus, it may be inferred that dAPTP can partially replace dATP in the reaction.

The inhibition of DNA synthesis by dAPTP is emphasized in Fig. 7a, in which it can be seen that increasing the concentration of dAPTP along with the other substrates leads to an initial increase then a marked decrease in the rate of incorporation of ^{32}P-dGMP. This is to be compared to the curve showing the effect of varying the concentration of substrates in the absence of dAPTP where a normal increase in incorporation of ^{32}P-dGMP followed by a plateau at saturation is evident. The inhibition by dAPTP can be overcome by increasing the concentration of dATP (Fig. 7b). Here, the rate of ^{32}P-dGMP incorporation is stimulated, whereas there is a concomitant decrease in the incorporation of ^{3}H-dAPMP as the dATP concentration is raised, thus further documenting the antagonism between dAPTP and dATP.

DISCUSSION

Only trace quantities of AP are found in the DNA of microorganisms grown in the presence of relatively large amounts of the analogue. Thus, Gottschling and Freese reported finding about one AP residue for every 1,000 bases (18), and in experiments not reported here, we have found similar values. Our investigations on the pathway of incorporation of AP into DNA suggest that the rate-limiting step in the series of reactions leading from the free base to DNA is the synthesis of the nucleotide. Although it seemed plausible that crude extracts of *E. coli* would catalyze the condensation of AP with PRPP, the nucleotide pyrophosphorylase(s) present in the extract failed to convert AP to a nucleotide at a measurable rate. The same extracts which effected a rapid synthesis

of AMP from adenine and PRPP were inactive with AP. Our assay would have been able to detect the synthesis of APMP at a rate 0.5% of that of AMP. Examination of reports on the specificity of nucleotide pyrophosphorylases from partially purified extracts of yeast (22, 30), monkey liver (24), human red cells (25), or *Salmonella* (20) reveals that some of the enzymes can utilize 2,6-diaminopurine, but none of those tested could use AP as a substrate. The only report of a nucleotide pyrophosphorylase capable of utilizing AP is by Frederiksen (15), who indicated that extracts of Ehrlich ascites cells catalyzed the formation of APMP from PRPP and AP at a rate of about 2.6% of that of adenine. However, whole cells incubated with AP were found to be devoid of ribo- or deoxyribonucleotide derivatives in their acid-soluble fraction, although it was clear that AP was transported into the cell.

If our inability to detect the direct formation of APMP by a pyrophosphorolytic reaction is not due to a deficiency in our assay procedure, other routes to AP nucleotides, such as the conversion of AP to the nucleoside and thence to the nucleotide, must be operative. A rapid conversion of AP to APdR was observed in extracts supplemented with purine deoxyribonucleosides. This suggests that the *trans-N*-glycosidase (EC 2.4.2.6) first described by MacNutt (29) is responsible for the formation of AP deoxynucleosides in *E. coli*. The observation that ribonucleosides can serve as donors to form APR indicates that ribosyltransferases (EC 2.4.2.5) are also present. The possibility also exists that AP nucleosides are synthesized from pentose phosphates in a reaction catalyzed by nucleoside phosphorylase (EC 2.4.2.1) by the following scheme

(deoxy)guanosine + inorganic orthophosphate $\rightleftharpoons$
(deoxy)ribose-1-P + guanine

AP + (deoxy)ribose-1-P $\rightleftharpoons$
AP(d)R + inorganic orthophosphate

deoxyguanosine + AP $\rightleftharpoons$ AP(d)R + guanine

The interconversion of nucleosides by crude extracts of *E. coli* has been investigated by Koch (21), and his studies show that these reactions take place with all natural purine and pyrimidine bases. Indeed, Ott and Werkman (34) suggest that the transfer of purine and pyrimidine bases between nucleosides in *E. coli* is in reality a result of coupled nucleoside phosphorylase reactions. Abrams et al., (1) and Pinto and Touster (35) have provided evidence that a single enzyme in Ehrlich ascites cells catalyzes both base transfer and phosphorolytic reactions. The latter authors have shown that one activity

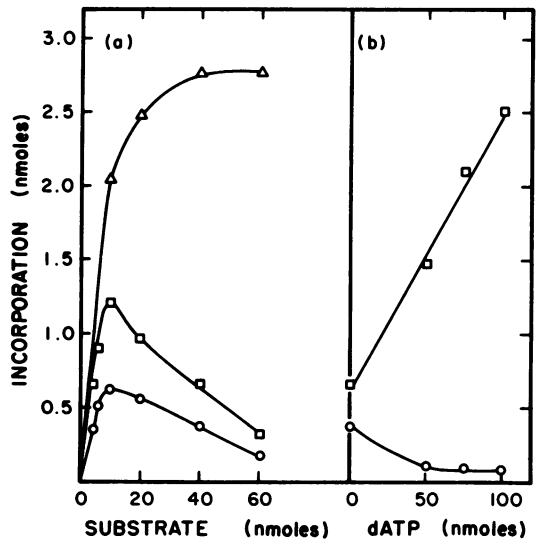

FIG. 7. *Inhibition of E. coli DNA polymerase by dAPTP and reversal by dATP. (a) Standard reaction mixtures were assayed as described in the text except that nucleotide concentrations were varied as indicated. The concentrations of all triphosphates were equal. When ^{3}H-dAPTP was included, dATP was omitted. Symbols: △, incorporation of ^{32}P-dGMP in the absence of ^{3}H-dAPTP; □, incorporation of ^{32}P-dGMP in the presence of ^{3}H-dAPTP; ○, incorporation of ^{3}H-dAPMP. (b) Standard incubations using 40 nmoles of each of the triphosphates were assayed. Symbols: □, incorporation of ^{32}P-dGMP; ○, incorporation of ^{3}H-dAPMP.*

can be converted to the other depending on the presence or absence of reducing agents such as mercaptoethanol. Thus, it is evident that, although extracts of *E. coli* can convert AP to a nucleoside in the presence of the common nucleosides acting as ribosyl donors, it will be necessary to purify the enzyme(s) catalyzing this reaction before a mechanism can be presented.

The next step in the pathway, the formation of the nucleotide, (d) APMP, by the phosphorylation of the corresponding nucleoside was at best a very weak reaction. We were unable to accumulate any of the nucleotide in our reaction mixtures and our only evidence for its formation was the AP(d)R-dependent utilization of ATP. The phosphorylation of nucleosides has not been extensively studied. Evidence for the phosphorylation of uridine (11, 40), cytidine (40), and adenosine (23, 27, 39) has been presented. Kornberg and Pricer showed that adenosine kinase (EC 2.7.1.20) of yeast can utilize 2,6-diaminopurine as a substrate, but guanosine or deoxyadenosine are not phosphorylated. Lindberg

et al., (27) have partially purified an adenosine kinase from rabbit liver which is active on several analogues of adenosine but has a K_m for deoxyadenosine three orders of magnitude higher than for the ribonucleoside. It has no measurable activity on APdR. Thymidine kinase (EC 2.7.1.21) has been purified from *E. coli* (33) but comparatively little is known about the phosphorylation of other nucleosides and still less about deoxynucleosides from this source. Although the AP(d)R-dependent disappearance of ATP is suggestive of a direct phosphorylation of the (deoxy)nucleoside, confidence in the authenticity of this reaction should be tempered with caution until such a reaction can be demonstrated unequivocally in a purified system in which the stoichiometry of the reaction can be demonstrated. This is especially true since Ginsburg (17) has shown that the apparent phosphorylation of deoxyadenosine in extracts of *L. plantarum*, as measured by the nucleoside-dependent conversion of ATP to ADP, is in reality the sequential action of a nucleosidase and deoxyribokinase leading to the formation of 2-deoxyribose-5-phosphate.

The next steps in the pathway were readily demonstrated. The interesting question of whether APdR resembled dAMP or dGMP in respect to nucleotide kinases was answered by studying the reaction in a crude extract and corroborating the finding with a purified enzyme. It was important to investigate crude extracts from which no intracellular proteins were removed because of the possiblity that more than one enzyme could catalyze the phosphorylation. From an inspection of the chromatographic profile (Fig. 5) and analysis of the individual fractions it was obvious that GMP kinase (EC 2.7.4.3) and not AMP kinase (EC 2.7.4.3) was the enzyme involved. This hypothesis was confirmed with a substantially pure enzyme from *E. coli* which was highly specific for guanine nucleotides (32).

An alternate route to dAPDP is depicted as pathway (1) in Fig. 2. Again, this would involve phosphorylation of the ribonucleoside to the nucleotide or direct formation of the nucleotide by condensation of AP with PRPP. As described earlier, these two reactions have not been satisfactorily demonstrated. If APMP were synthesized by this route, its phosphorylation to APDP should provide no special problem to the cell because GMP kinase does not distinguish between ribose or deoxyribose. The resulting APDP might then be reduced to dAPDP by the system described by Larrsen and Reichard for *E. coli* (26). We have not investigated whether APDP can be reduced by ribonucleotide reductase.

At the outset of this work, we felt that the next step in the reaction, the conversion of (d)APDP to the triphosphate should present no problem to the cell because it has been observed that ATP:nucleoside diphosphate phosphotransferase (EC 2.7.4.6) is a relatively nonspecific enzyme. A good example of the ability of the *E. coli* enzyme to phosphorylate "foreign" deoxynucleoside diphosphates was seen by Bello and Bessman (4) who demonstrated the phosphorylation of 5-hydroxymethyl-dCDP to the triphosphate in uninfected *E. coli*. This substrate is not present in normal *E. coli* and appears only after infection with the T-even bacteriophages (13). Thus, it was not surprising to find that dAPDP was converted to dAPTP in these extracts. The K_m for dAPDP must be fairly low because a substantial percentage of dAPTP was formed early in the incubation at times when only small amounts of dAPMP had been converted to dAPDP (Fig. 6).

The final step in the pathway, incorporation of dAPTP into DNA, was studied with purified enzymes, because our experiences in several other studies on the in vitro synthesis of DNA have indicated that crude extracts are not suitable due to a myriad of complicating side reactions leading to destruction and interconversion of substrates, modification of templates and primers and degradation of products. An examination of Fig. 8 reveals that AP can form two hydrogen bonds with thymine and one with cytosine. As Freese pointed out, this would suggest that AP could substitute for adenine and guanine in DNA, and since it can form two bonds with thymine and only one with cytosine it should prefer to pair with thymine. An interesting confirmation of the duplicity of AP was provided by the experiments of Wacker et al. (46), who synthesized polyribonucleotides of AP and used them as synthetic messengers for protein synthesis in the system of Nirenberg and Matthaei (31). From the pattern of incorporation of amino acids into polypeptides using the poly AP message, they showed that the homopolymer functionally appeared to be a copolymer of adenine and guanine but having properties more resembling polyadenylate than polyguanylate. Our results partially substantiate these predictions but invoke another parameter as important in determining the incorporation of a base analogue into DNA. It can be seen in Table 5 that AP was incorporated into DNA in place of adenine, but it was not an effective replacement for guanine. Thus, something other than mere base-pairing characteristics must be invoked, and enzyme specificity seems to be a

likely candidate. This view is supported not only by the incorporation data but by the differential effect of AP on the two enzymes studied. First, AP substitutes much better for adenine in the reaction catalyzed by *E. coli* DNA polymerase than the reaction catalyzed by the T5-induced enzyme. Secondly, the two enzymes have much different sensitivities to inhibition by AP. Inclusion of dAPTP in the standard reaction decreased DNA synthesis as measured by ^{32}P-dTMP incorporation 79% with the T5 enzyme, whereas *E. coli* polymerase was inhibited about 3%. These results may provide an explanation to Gottschling and Freese's observations (18) that AP has a marked effect on the physiology of phage infection, and that only about one-tenth the amount of AP is incorporated into phage DNA compared to that of the bacterium. The possibility that DNA polymerase itself is involved in determining base sequence and not solely the template was first indicated by the work of Speyer in his studies of temperature-sensitive mutants of T4 phage (41) and later elegantly shown by Hall and Lehman (19) with purified mutationally altered T4-induced DNA polymerase. Our results showing a differential response of the two purified enzymes to dAPTP are in accord with the view that the normal enzyme itself plays some role in the selection of bases. However a more complete study involving different templates, varied substrate compositions and detailed kinetic analysis is necessary to establish this in respect to the incorporation of AP. An interesting question, difficult to approach from genetic analysis, is whether a given base analogue causes a transition by mispairing during incorporation or during subsequent replication. Depending on the mechanism, we should expect an AT → GC transition (incorporation error) or GC → AT transition (replication error; see reference 16). Since we find substantial replacement of dATP by dAPTP and essentially no replacement of dGTP by dAPTP, we predict that the mutagenic effect of AP is exerted primarily during replication as suggested from base pairing considerations by Freese (16) and not during incorporation. It will be interesting to construct a polydeoxyribonucleotide containing AP residues to test this hypothesis.

Recently, Cairns has described a mutant of *E. coli* which contains little, if any, extractable DNA polymerase activity (12). On this basis he has suggested that there must be another system responsible for DNA replication, and that DNA polymerase is chiefly involved in the repair of DNA. Our results with purified DNA polymerase from *E. coli* and T5-infected cells must be viewed with this in mind. It may be significant, however, that our observations in vitro with the purified "repair" enzymes are compatible with results obtained in vivo.

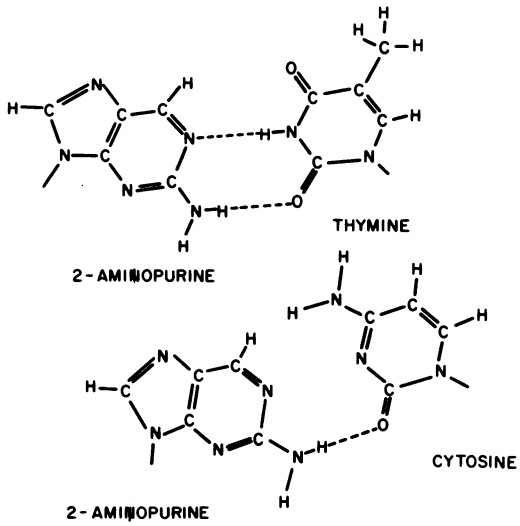

FIG. 8. *Hydrogen bonding of AP with thymine and cytosine (see reference 16).*

TABLE 5. *Substitution by H-dAPTP in the DNA polymerase reaction*[a]

Conditions	*E. coli* polymerase		T5 polymerase	
	Incorporation of ^{3}H-dAPMP	Incorporation of ^{32}P-dTMP	Incorporation of ^{3}H-dAPMP	Incorporation of ^{32}P-dTMP
	μmoles	*μmoles*	*μmoles*	*μmoles*
Complete	0.20	2.09	0.06	0.40
omit enzyme	<0.01	0.01	<0.01	0.01
omit ^{3}H-dAPTP		2.16		1.89
omit dATP	0.48	0.78	0.06	0.12
omit ^{3}H-dAPTP and dATP		0.14		0.07
omit dCTP	0.01	0.16	<0.01	0.10
omit dCTP and ^{3}H-dAPTP		0.17		0.09
omit dGTP	0.02	0.29	<0.01	0.10
omit dGTP and ^{3}H-dAPTP		0.17		0.14
omit ^{2}P-dTTP	0.01		<0.01	

[a] Reaction mixtures contained 33 μM ^{3}H-dAPTP (3.93×10^{5} counts per min per μmole) where indicated in addition to the normal constituents for the assay of DNA polymerase. Abbreviations: dAPTP, 2-aminopurine deoxynucleoside triphosphate; dTMP, deoxythymidine monophosphate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytosine triphosphate; dGTP, deoxyguanosine triphosphate; and dTTP, deoxythymidine triphosphate.

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