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*ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. III. THE INCORPORATION OF PYRIMIDINE AND PURINE ANALOGUES INTO DEOXYRIBONUCLEIC ACID**

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The synthesis of DNA[1] by an enzyme from *Escherichia coli* requires the presence of the deoxynucleoside triphosphates of adenine, guanine, cytosine, and thymine, magnesium ions, and DNA.[2, 3, 4] The product contains the deoxynucleotides joined by typical 3′,5′-phosphodiester linkages and can be formed in amounts up to 20 times the initial quantity of DNA added to the reaction mixture.[2, 3] Physical measurements indicate that the properties of the synthetic product are, in all respects measured, characteristic of DNA isolated from biological sources; it has a highly ordered rigid structure and an average molecular weight of about 5 million.[5] The reaction is specific for *deoxy*nucleoside *tri*phosphates, is reversed by high concentrations of inorganic pyrophosphate, and may be formulated thus:

$$\begin{matrix} n\ \text{dAPPP} \\ + \\ n\ \text{dGPPP} \\ + \\ n\ \text{dCPPP} \\ + \\ n\ \text{TPPP} \end{matrix} + \text{DNA} \rightleftharpoons \text{DNA} - \begin{bmatrix} \text{dAP} \\ \\ \text{dGP} \\ \\ \text{dCP} \\ \\ \text{TP} \end{bmatrix}_n + 4(n)\text{PP}$$

In view of reports that the pyrimidine and purine analogues, 5-bromouracil and 8-azaguanine, are incorporated into DNA in vivo,[6–10] it was of interest to determine whether our enzyme system would also accept substrates with such "unnatural" bases. The studies to be reported show that several pyrimidine and purine analogues[11] (uracil, hypoxanthine, 5-bromouracil, 5-bromocytosine, and 5-methylcytosine) are incorporated enzymatically into DNA. In each case the analogue substitutes specifically for the base it closely resembles with respect to the hydrogen-bonding properties required in the DNA structure proposed by Watson and Crick[12, 13] (Fig. 1).

METHODS AND MATERIALS

The analytical procedures and the preparation of the deoxynucleoside triphosphates of adenine, guanine, thymine, and cytosine were carried out as reported elsewhere.[2, 3]

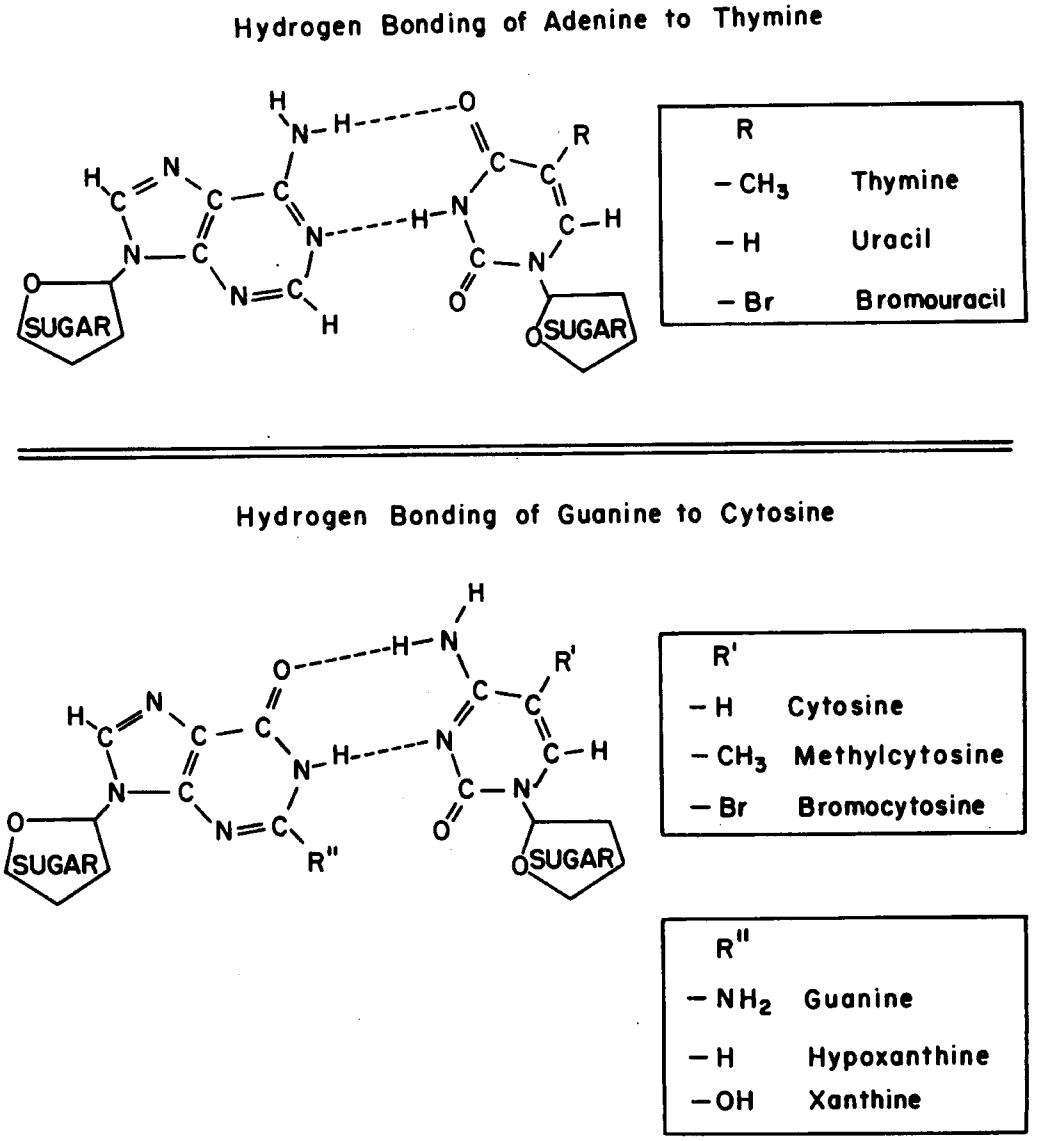

FIG. 1.

Assay for Deoxynucleotide Incorporation into DNA.—This is based on the conversion of a deoxynucleoside triphosphate (labeled with P^{32} in its innermost phosphate) to an acid-insoluble product. Complete details of this assay were provided elsewhere.[2]

Preparation of dUTP, dITP, and dXTP.—dUTP, dITP, and dXTP were prepared from dCTP, dATP, and dGTP, respectively by deamination with nitrous acid at 25° for 2 hours, according to Lohman.[14] The deaminated products were precipitated

as barium salts, converted to potassium salts by treatment with Dowex-50 K^+ at pH 5 at 0°, and purified by chromatography on Dowex-1 Cl columns.[2] The columns were washed first with enough 0.1 *M* LiCl–0.01 *N* HCl to elute completely the dCTP or dATP. dUTP or dITP was eluted in approximately 10 resin-bed volumes as a symmetrical peak by 0.20 *M* LiCl–0.02 *N* HCl and obtained in yields of approximately 60 per cent from 2 μmoles of starting material. dXTP was not chromatographed; spectrophotometric analysis after treatment with nitrous acid showed that at least 95 per cent of the dGTP had been deaminated.

Preparation of 5-Bromo dCTP and 5-Bromo dUTP.—dCTP was brominated according to the procedure of Markham,[15] by adding dropwise a solution of bromine in carbon tetrachloride to dCTP dissolved in formamide. The uptake of bromine was instantaneous; the addition was stopped when an excess had been added, as shown by persistence of a yellow color. Excess bromine was removed by adding a drop of aniline; the brominated dCTP was then precipitated as the barium salt, converted to the potassium salt, and purified by chromatography on paper.[16] 5-Bromo dUTP was prepared by deamination of 5-bromo dCTP in nitrous acid as described for the preparation of dUTP. It was purified by paper electrophoresis at pH 9.3 according to Dunn and Smith.[10]

Preparation of 5-Methyl dCTP.—5-Methyl deoxycytidylate was isolated from wheat-germ DNA according to Sinsheimer and Koerner,[17, 18] and was converted to the triphosphate by the procedure of Khorana.[19, 20] Analyses of all the analogues are reported in Table 1.

TABLE 1

ANALYTIC DATA FOR ANALOGUES OF THE NATURAL DEOXYNUCLEOSIDE TRIPHOSPHATES

Analysis	dUTP	5-Bromo dUTP	5-Methyl dCTP	5-Bromo dCTP	dITP	dXTPs
Total P (moles)	3.00	3.00	3.00	3.00	3.00	3.00
Acid-labile P (moles)*	1.85	1.93	1.86	2.18	...	...
Base (moles)	1.11	0.98	1.01	...	1.07	0.94
Deoxypentose (moles)	...	...	...	...	0.91	...
λ max (mμ)†	262	279	286	299	249	263
λ 280/λ 260	0.45	1.77	3.00	4.0	0.27	0.32
λ 250/λ 260	0.72	0.62	0.46	1.10	1.62	0.72

* Determined as inorganic ortho P after 15 minutes in 1 *N* H_2SO_4 at 100°.
† Spectral measurements were at pH 1 except for dITP, which was at pH 7.

Enzymes.—Micrococcal DNase was kindly provided by Dr. Lew Cunningham. Spleen phosphodiesterase was obtained as a gift from Dr. Leon Heppel, and more was prepared according to the method of Hilmoe and Heppel.[21] Semen phosphomonoesterase was prepared according to Kornberg and Wittenberg.[22] The deoxynucleotide kinase(s) of *E. coli* was prepared as described previously.[2] The DNA-synthesizing enzyme from *E. coli* was prepared from the "polymerase" fraction VII described elsewhere[2] by refractionating with diethylaminoethylcellulose in the identical manner as fraction VII. It is referred to as "fraction VII-R."

RESULTS

Replacement of the "Natural" Bases of DNA by Their Analogues.—dUTP used in place of TTP supported DNA synthesis at 54 per cent of the rate of the control value but failed to support synthesis when used in place of dATP, dGTP, or dCTP

(Table 2). 5-Bromo-dUTP was more effective as a replacement for TTP but was unable to substitute for any of the other triphosphates. The 5-bromo and 5-methyl dCTP derivatives replaced only dCTP and were even more effective than dCTP itself in promoting DNA synthesis; evaluation of the significance of these higher rates requires further kinetic studies. dITP permitted a reduced rate of DNA synthesis in the absence of dGTP, but essentially no synthesis when any one of the other triphosphates was absent.

TABLE 2

REPLACEMENT OF NATURAL BASES BY ANALOGUES IN ENZYMATIC SYNTHESIS OF DNA

EXPT. NO.	CONTROL VALUE* (mμ MOLES)	BASE ANALOGUE USED	NATURAL BASE OMITTED (Per Cent of Control)†			
			Thymine	Adenine	Guanine	Cytosine
1	0.50	Uracil	**54**	4	6	
1*a*	0.88	Uracil				3
2	0.43	5-Bromouracil	**97**	2	4	
2*a*	0.42	5-Bromouracil				4
3	0.51	5-Bromocytosine		4	4	**118**
3*a*	0.40	5-Bromocytosine	4			
4	0.58	5-Methylcytosine		2	3	**185**
4*a*	0.52	5-Methylcytosine	2			
5	0.37	Hypoxanthine		3	**25**	5
5*a*	0.27	Hypoxanthine	4			

Control values are mμmoles of radioactive deoxynucleotide incorporated into DNA in the absence of analogue. Incubation mixtures contained in 0.3 ml., 5 mμmoles each of TTP, dATP, dCTP, and dGTP; 2 μmoles of $MgCl_2$; 20 μmoles of potassium phosphate (pH 7.4); 10 μg. of calf-thymus DNA; and 1 μg. of enzyme fraction VII-R. Experiments were performed at 37° for 30 minutes. Labeled substrates were: $dCP^{32}PP$ in Expts. 1. 2, 5*a*; $TP^{32}PP$ in Expts. 1*a*, 3, 4, 5; and $dGP^{32}PP$ in Expts. 2*a*, 3*a*, 4*a*.

† The percentage value represents the fraction of the labeled substrate incorporated when the analogue (5 mμmoles) was used instead of a natural base. All bases, natural or analogue, were supplied as the deoxynucleoside triphosphates. Values of 5 per cent or below are near the limit of detectability and are of questionable significance

TABLE 3

INCORPORATION OF P^{32}-DEOXYURIDYLATE INTO DNA

SUBSTRATES†	P^{32}-Deoxynucleotide Incorporated (mμMoles)
$\overset{*}{U} + T + C + A + G$	0.14
Omit T	0.38
Omit C	0.01
Omit A	0.01
Omit G	0.01
$U + \overset{*}{T} + C + A + G$	0.37
Omit U	0.77

† U = dUTP; $\overset{*}{U}$ = $dUP^{32}PP$; T = TTP; $\overset{*}{T}$ = $TP^{32}PP$; C = dCTP; A = dATP; G = dGTP.

Direct Demonstration of the Incorporation of Deoxyuridylate into DNA.—Deoxyuridylate was incorporated into DNA at about half the rate of thymidylate (Table 3, compare lines 2 and 7); omission of the triphosphates of deoxycytidine, deoxyadenosine, or deoxyguanosine practically eliminated the incorporation of deoxyuridylate. With $dIP^{32}PP$ comparable results were obtained, except that deoxyinosinate was incorporated into DNA specifically in the place of deoxyguanylate and at 25 per cent the rate. However, $dXP^{32}PP$ was not incorporated into DNA. Under the same conditions in which $dGP^{32}PP$ was incorporated to the extent of 0.66 mμmole, less than 0.02 mμmole of $dXP^{32}PP$ was incorporated into an acid-insoluble product. The possibility that the chemically synthesized dXTP contained an inhibitor was ruled out by the observation that dXTP added to the normal reaction mixture had no inhibitory effect.

Demonstration of Phosphodiester Linkages between Deoxyuridylate and the Other Deoxynucleotides in Enzymatically Synthesized DNA.—DNA containing radioactive deoxyuridylate was prepared (Table 4), mixed with a 50-fold excess of

TABLE 4

DISTRIBUTION OF RADIOACTIVITY IN DEOXYNUCLEOSIDE 3'-PHOSPHATES ISOLATED FROM ENZYMATICALLY SYNTHESIZED DNA CONTAINING DEOXYURIDINE 5'-P^{32}

Isolated 3'-Deoxynucleotides	Amount* (μMoles)	Radioactivity (c.p.m. $\times 10^{-3}$)
Cytidylate	0.17	2.0
Adenylate	0.25	2.3
Guanylate	0.22	1.7
Uridylate	...	2.5
Thymidylate	0.24	0.0

* The recovery of deoxynucleotides and P^{32} from the paper electrophoresis strip was 96 per cent.

The incubation mixture for the synthesis of deoxyuridylate-containing DNA included dCTP, dATP, dGTP (75 mμmoles of each), $dUP^{32}PP$ (75 mμmoles, 3.8×10^6 c.p.m./μmole), $MgCl_2$ (30 μmoles), potassium phosphate (300 μmoles, pH 7.4), calf-thymus DNA (300 μg.), enzyme (15 μg. of Fraction VII-R), and water to a final volume of 4.5ml. After 30 minutes at 37°, the reaction mixture was chilled and 2.5 mg. of calf-thymus DNA were added as "carrier." The DNA was precipitated by the addition of perchloric acid (final concentration, 0.5 *N*) and the precipitate was dissolved in dilute NaOH. The precipitation was repeated several times until the acidic supernatant fluid contained less than 1 per cent of the radioactivity of the precipitated DNA. Details of the enzymatic digestion of the DNA and the subsequent isolation of the deoxynucleoside 3'-phosphates are in the text.

TABLE 5

SPECIFICITY OF DEOXYNUCLEOTIDE KINASE(S) OF *E. coli* TOWARD VARIOUS DEOXYNUCLEOTIDES

Expt. No.	Conditions	P^{32}-Deoxynucleotide Incorporated into DNA: With Kinase (mμMoles)	Without Kinase (mμMoles)
1	Control*	0.74	0.74
	Omit TTP	0.04	...
	Omit TTP, add thymidylate	0.83	...
	Omit TTP, add 5-bromodeoxyuridylate	0.63	0.01
	Omit TTP, add deoxyuridylate	0.04	...
2	Control	0.49	0.50
	Omit dCTP	0.02	...
	Omit dCTP, add deoxycytidylate	0.50	...
	Omit dCTP, add 5-methyldeoxycytidylate	0.02	...

* Incubation mixture containing all four deoxynucleoside triphosphates as described in Table 2, and also 0.25 μmole of ATP. The phosphorylation of a deoxynucleotide is measured, as in line 3 for thymidylate, by the sequence of reactions:

$$\text{Thymidylate (25 m}\mu\text{moles)} \xrightarrow[\text{ATP}]{\text{36 }\mu\text{g. of } E.\ coli \text{ kinase(s)}} \text{TTP} \xrightarrow[\text{dCTP/dGTP}]{\text{polymerizing enzyme Fraction VII-R, dAP}^{32}\text{PP}} \text{DNA-P}^{32}$$

The incorporation of $dAP^{32}PP$ is absolutely dependent on the formation of TTP from thymidylate. Phosphorylation of the other deoxynucleotides was tested under analogous conditions with the assumption that the corresponding triphosphate is in each case essential for DNA synthesis (see Table 2).

thymus DNA as "carrier," and digested first with micrococcal DNase and then with spleen phosphodiesterase. This treatment degrades the DNA to 3'-deoxynucleotides; the P^{32} of $dUP^{32}PP$ should therefore now be bound to the deoxynucleoside with which it formed phosphodiester linkages.[23] The hydrolysis with micrococcal DNase was allowed to proceed until 95 per cent of the radioactivity was converted to an acid-soluble form. Incubation with spleen phosphodiesterase rendered 90 per cent of the radioactivity sensitive to semen phosphomonoesterase. At this point, at least 80 per cent of the original DNA product had been converted

to deoxynucleoside 3′-phosphates. The preparation was desalted by adsorption on Norit and elution with ammoniacal ethanol and then subjected to electrophoresis as described by Markham and Smith[24] (Whatman 3-MM. paper, pH 3.5 in 0.02 *M* ammonium acetate buffer). The bands were eluted and identified by their spectral characteristics, and the eluates were assayed for radioactivity.

It can be seen in Table 4 that all four of the 3′-deoxynucleotide fractions were labeled with P^{32}. Thus P^{32} which originally entered the enzymatically synthesized DNA as deoxyuridylate had been in phosphodiester linkage with cytosine, adenine, guanine, and uracil deoxynucleosides. Since TTP was omitted from the reaction mixture, it might be expected that little or no radioactivity would be found in the isolated thymidine 3′-phosphate. Deoxyuridylate is not separated from thymidylate under these conditions of paper electrophoresis, and it was necessary to resolve these nucleotides by paper chromatography.[16] All the radioactivity was found in the deoxyuridylate area and less than 5 per cent was in the thymidylate region.[25]

Specificity of Deoxynucleotide Kinase(s) of E. coli toward Various Deoxynucleotides.—The data in Table 5 indicate, in confirmation of an earlier report,[26] that, while the naturally occurring deoxynucleotides such as thymidylate and deoxycytidylate are readily converted to the triphosphate level, the analogue deoxyuridylate is not. However, the result showing 5-bromo deoxyuridylate to be a favorable substrate for phosphorylation is consistent with interpretations from in vivo studies[27] that bromouracil is incorporated into DNA via a deoxynucleotide intermediate.

It is of interest to point out that 5-methyl deoxycytidylate is not converted to the triphosphate by extracts of *E. coli* (Table 5), although when supplied as the triphosphate it is incorporated into DNA (Table 2).

DISCUSSION

In the Watson and Crick model of DNA,[12, 13] two polynucleotide strands are coiled about a central axis and are held together by hydrogen bonds through specific pairings between thymine and adenine and between guanine and cytosine. Our data demonstrate that deoxyuridylate and 5-bromo deoxyuridylate are incorporated into enzymatically synthesized DNA and that they specifically replace thymidylate. In a like manner deoxycytidylate may be replaced by the 5-methyl or 5-bromo derivatives, and deoxyguanylate may be replaced by deoxyinosinate. Substitution of a hydrogen or a bromine atom for a methyl group in the 5-position of thymine still maintains the keto grouping in the 6-position and an available H in the 1-position for hydrogen-bonding with the 6-amino and 1-N groups, respectively, of adenine (Fig. 1). Since the methyl group in thymine plays no assigned role in the hydrogen-bonding of thymine to adenine, it is not surprising that the other uracil derivatives replace thymine so well in the enzymatic synthesis of DNA. The results obtained with the cytosine analogues may be regarded in a similar way. Although hypoxanthine lacks the amino group at the 2-position in guanine, the other two substituents responsible for the hydrogen-bonding of guanine to cytosine remain. It is possible that the substantially lower rate of deoxyinosinate incorporation into DNA as compared with deoxyguanylate may be due to the absence of the third hydrogen bond with the keto group of cytosine.[28] At present no definitive explanation can be given for the failure of deoxyxanthylate to be incorporated into DNA in place of deoxyguanylate.

The specific replacement of the purines and pyrimidines of DNA by analogues with similar hydrogen-bonding capacities is in agreement with the specific base pairing in the Watson and Crick model. However, it would be premature to state a priori that a given analogue will be incorporated into DNA simply from a consideration of its capacity to form specific hydrogen bonds with the bases in DNA. The specificity of the enzyme toward a given substrate must be considered, as well as the unexplored influence of the DNA primer. For these reasons it is not feasible at this time to attribute different rates of incorporation of dUTP, dITP, and the natural deoxynucleoside triphosphates to conditions controlled simply by hydrogen-bonding characteristics of base pairs.

It should be emphasized that the results in this paper are only approximations to reaction rates and do not provide any indication as to how the analogues support extensive net synthesis of DNA. Further studies of the reaction kinetics with the analogues are necessary, as are investigations of the primer capacity of the DNA containing the analogue.

Since deoxyuridylate replaces thymidylate so readily in the enzymatic synthesis of DNA, it is pertinent to inquire why uracil has never been observed as a component of DNA in nature. The answer appears to lie in an observation previously noted[26] and further documented here that there may not be any mechanism for phosphorylating deoxyuridylate to the triphosphate form. While high levels of kinase activity for the conversion of adenine, guanine, thymine, and cytosine deoxynucleotides to the respective triphosphates are evident in extracts of *E. coli*, we have not yet been able to demonstrate comparable activity toward deoxyuridylate. It is of further interest that 5-bromodeoxyuridylate is readily converted to the triphosphate by *E. coli* preparations and is thus consistent with the known capacity of *E. coli* cells to incorporate 5-bromouracil into its DNA.[9, 10]

SUMMARY

The deoxyribonucleoside triphosphates of analogues of the pyrimidine and purine bases were prepared chemically and tested as substrates for the enzyme which polymerizes deoxyribonucleotides to deoxyribonucleic acid.

Uracil and 5-bromouracil were incorporated into deoxyribonucleic acid specifically in place of thymine; 5-methyl- and 5-bromocytosine in place of cytosine; and hypoxanthine in place of guanine. Xanthine was not incorporated into deoxyribonucleic acid. Analysis of the uracil-containing product demonstrated that the uracil deoxyribotide was bound in 3′-5′ phosphodiester linkage with each of the deoxyribotides of adenine, guanine, cytosine, and uracil.

The specific replacement of the natural bases by these analogues offers additional support for the base-pairing relationships in the double helix proposed by Watson and Crick for the structure of deoxyribonucleic acid.

The existence of kinases in *E. coli* which phosphorylate 5-bromodeoxyuridylate to the triphosphate and the apparent absence of such kinases for the phosphorylation of deoxyuridylate are in keeping with the fact that cells incorporate 5-bromouracil, but not uracil, into DNA.

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[1] The abbreviations used in this report are: ATP, adenosine triphosphate; c.p.m., counts per minute; dATP or dAPPP, deoxyadenosine triphosphate; dCTP or dCPPP, deoxycytidine triphosphate; dGTP or dGPPP, deoxyguanosine triphosphate; TTP or TPPP, thymidine triphosphate; dITP or dIPPP, deoxyinosine triphosphate; dUTP or dUPPP, deoxyuridine triphosphate; dXTP or dXPPP, deoxyxanthosine triphosphate; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; PP, inorganic pyrophosphate.

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