

b with 2 moles of pyridoxal-5-phosphate exhibits the kinetics of a second-order reaction. It is concluded that the formation of a Schiff base between pyridoxal-5-phosphate and protein amino groups is the rate-limiting, intermediate reaction and is followed by further rearrangement to the stable bond structure in phosphorylase.

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⁸ Na thioglycolate was selected because apophosphorylase *a* could be readily dissolved at room temperature in solutions of this sulfhydryl compound and repeatedly crystallized in the cold. Usually, crystallization of the apoenzyme did not occur when cysteine-glycerophosphate was substituted for thioglycolate.

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ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID.

V. CHEMICAL COMPOSITION OF ENZYMATICALLY SYNTHESIZED DEOXYRIBONUCLEIC ACID

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The enzymatic synthesis of DNA¹ in sufficient excess over the DNA used to prime the reaction^{2, 3} has enabled us to examine the chemical composition of the synthetic product. As has already been demonstrated, this DNA consists of deoxynucleotides linked by typical 3'-5' phosphodiester bonds, and all four of the deoxy-

nucleotides which occur naturally in DNA are present and required for its synthesis.³ The purpose of this investigation was to answer two questions: (1) Does the synthesized DNA show the equivalence of purines to pyrimidines characteristic of DNA? and (2) Does the base composition of the DNA primer influence the composition of the synthesized product?

The results to be presented answer both questions in the affirmative and further suggest that the added DNA from the very outset of the reaction is serving as a template for the synthesis of new DNA.

MATERIALS AND METHODS

Materials.—Deoxynucleoside triphosphates were synthesized by the method of Smith and Khorana.⁴ Uniformly labeled C¹⁴-deoxynucleoside triphosphates were prepared as described previously.⁵ The DNA-synthesizing enzyme was the "polymerase" fraction VII described elsewhere.² DNA of *Escherichia coli* and *Aerobacter aerogenes* was extracted from fresh or frozen cells lysed with sodium dodecyl sulfate.⁶ Precipitation of the DNA by adding two volumes of cold absolute ethanol, followed by solution in cold 0.14 M NaCl (containing 0.01 M sodium citrate) and then further deproteinization with sodium dodecyl sulfate,⁷ was carried out once or twice. Then the DNA was treated with ribonuclease (crystalline, Worthington Biochemical Corp. product, pretested for absence of deoxyribonuclease activity) and Norit to remove contaminating ribonucleic acid. After precipitating again with cold ethanol and dissolving as above, preparations were obtained which had reduced viscosities of 57 and 87 deciliters/gm for *E. coli* and *A. aerogenes* samples, respectively, and gave absorbancy values ($\alpha M \times 10^{-3}$) at 260 m μ of 6.4–6.9 based on deoxyribose. Protein contamination, as measured by the method of Lowry *et al.*⁸ was below 2 per cent; the over-all yield was over 80 per cent, measured by the Dische reaction.⁹ The DNA of *Mycobacterium phlei* was isolated by the same procedure, except that cell lysis was obtained by alumina grinding. While the *coli* and *aerogenes* products formed long white fibers on ethanol precipitation and could be quantitatively pulled out on rods, the *phlei* material had to be recovered by centrifuging. The absorbancy was 6.5×10^3 based on deoxyribose, protein content was about 5 per cent, and the reduced viscosity 24 deciliters/gm. Bacteriophage DNA was prepared from purified T₂ bacteriophage by osmotic shock,¹⁰ followed by removal of the intact bacteriophage and ghosts by centrifugation at 140,000 $\times g$. for 1 hour. Calf thymus DNA was isolated by the method of Kay, Simmons, and Dounce.⁷

Methods.—The enzymatic synthesis of DNA and the isolation of the synthesized material were achieved as follows: The incubation mixtures (3.0 ml.) contained 1.0 μ mole each of dCTP, dATP, dTTP, and dGTP, 200 μ moles of potassium phosphate buffer (pH 7.4), 20 μ moles of MgCl₂, 36 μ g. of "polymerase" fraction VII, and 100 μ g. of the appropriate DNA primer. After incubation for 240 minutes at 37°, the mixtures were treated with 4 M NaCl to give a final concentration of 0.2 M and then heated for 5 minutes at 70°. The mixtures were dialyzed against several changes of 0.2 M NaCl to remove unreacted deoxynucleoside triphosphates, followed by dialysis against distilled water to remove the NaCl. They were concentrated to a small volume and then hydrolyzed with formic acid. The base composition of the various primers and products was determined by the method of Wyatt and Cohen.¹¹

The recoveries of bases from the paper chromatograms ranged from 96 to 101 per cent.

The incorporation of C^{14} -labeled deoxynucleoside triphosphates into DNA was measured as reported previously.²

RESULTS

The Base Composition of Enzymatically Synthesized DNA Resembles That of Its Primer.—In each enzymatically synthesized DNA sample the content of adenine approximated the amount of thymine, and the guanine content was about the same as the cytosine, so that the total amount of purine in each sample was the same as the total amount of pyrimidine (i. e., $A = T$; $G = C$; $A + G = T + C$) (Table 1).

TABLE 1*

PURINE AND PYRIMIDINE COMPOSITION OF ENZYMATICALLY SYNTHESIZED DNA

DNA	No. of Analyses	A	T	G	C	A + T G + C	
<i>M. phlei</i> { primer	3	0.65	0.66	1.35	1.34	0.49 (0.48-0.49)	1.01 (0.98-1.04)
product	3	0.66	0.80	1.17	1.34	0.59 (0.57-0.63)	0.85 (0.78-0.88)
<i>A. aerogenes</i> { primer	1	0.90	0.90	1.10	1.10	0.82	1.00
product	3	1.02	1.00	0.97	1.01	1.03 (0.96-1.13)	0.99 (0.95-1.01)
<i>E. coli</i> { primer	2	1.00	0.97	0.98	1.05	0.97 (0.96-0.99)	0.98 (0.97-0.99)
product	2	1.04	1.00	0.97	0.98	1.02 (0.96-1.07)	1.01 (0.96-1.06)
Calf thymus { primer	2	1.14	1.05	0.90	0.85	1.25 (1.24-1.26)	1.05 (1.03-1.08)
product	6	1.19	1.19	0.81	0.83	1.46 (1.22-1.67)	0.99 (0.82-1.04)
T_2 bacteriophage { primer	2	1.31	1.32	0.67	0.70	1.92 (1.86-1.97)	0.98 (0.95-1.01)
product	2	1.33	1.29	0.69	0.70	1.90 (1.82-1.98)	1.02 (1.01-1.03)
Synthetic A-T copolymer†	1	1.99	1.93	<0.05	<0.05	>40	1.05

* A, T, G, and C refer, respectively, to adenine, thymine, guanine, and cytosine except that C in the case of T_2 bacteriophage primer refers to hydroxymethylcytosine. The values given represent averages of the number of analyses indicated. The figures in parentheses represent the range of values obtained.

† A copolymer of deoxyadenylate and thymidylate is formed by the "polymerase" system in the absence of added DNA after a lag period of 3-6 hours. Once formed and isolated, this A-T polymer initiates the synthesis of new polymer without any time lag and, despite the presence of all four deoxynucleoside triphosphates in the incubation mixture, contains exclusively A and T. These and further details will be reported at a future date by H. K. Schachman and the authors.

Furthermore, the ratio $A + T/G + C$ in the enzymatic product was always close to that in the corresponding primer; primer values ranged from 0.49 for *M. phlei* to greater than 40 for an enzymatically synthesized copolymer of deoxyadenylate and thymidylate. It should be pointed out that the net amount of DNA synthesized in each case was at least ten times the primer DNA added, except in the *M. phlei* experiments, where it was only fourfold. Thus in all but the *M. phlei* samples, over 90 per cent of the bases were derived from the nucleotide substrates for the reaction.

The Composition of the Product Is Established Early in the Reaction.—The base ratios of the synthesized product were essentially the same, regardless of whether it was isolated early in the reaction or after synthesis had stopped. Thus with calf thymus DNA as primer, the $A + T/G + C$ was found to be 1.34 after a threefold net increase in DNA and 1.42 after net synthesis had exceeded eight fold.

To determine whether this result held even at the earliest stages of reaction, isotopically marked substrates were used to distinguish the newly synthesized DNA. In such an experiment, two identical reaction mixtures were set up as described under "Methods," one containing C^{14} -dCTP and the other C^{14} -dTTP. The amount of enzyme and length of incubation period were adjusted to permit an increase of

DNA as low as 2 per cent relative to the DNA added as primer. Isotope incorporation into DNA was determined as described previously for the assay of "polymerase."² The T/C ratio of the product was found to be similar to that of the primer when the net increases in DNA ranged from 2 to 500 per cent (Table 2).

TABLE 2*

EFFECT OF EXTENT OF NET SYNTHESIS ON COMPOSITION OF ENZYMATICALLY SYNTHESIZED DNA

Primer	Exp. No.	Time (Hours)	Increase in DNA (Per Cent)	T Incorporated (m μ Moles)	C Incorporated (m μ Moles)	T/C
<i>M. phlei</i> DNA { primer product	1		2	0.047	0.11	0.49
	2	1	35	0.97	2.05	0.47
		2	86	2.13	5.03	0.43
		4	163	4.20	9.54	0.44
		6	...	10.25	9.88	1.04
		7	...	24.20	12.10	2.00
Calf thymus DNA { primer product	3		8	0.40	0.28	1.24
	4	0.5	63	3.08	2.43	1.27
		1	197	9.10	7.72	1.20
		3	534	31.60	20.60	1.53
<i>A. aerogenes</i> DNA { primer product	5	0.5	18	0.74	0.93	0.82
		1.0	56	2.59	2.89	0.91
		3.0	344	15.00	17.75	0.84
		4.0	406	16.62	21.00	0.80

* Duplicate reaction mixtures were used as described under "Methods," with the exception that C¹⁴ dTTP was used in one vessel and C¹⁴ dCTP was used in the second. Aliquots were removed at the intervals indicated and assayed for the incorporation of isotope into DNA.

It may be noted in Table 1 that the value for the ratio A + T/G + C was slightly higher than that for the primer in some cases; lower ratios in the product have never been observed. It seems most probable that these divergencies between primer and product values are due to contamination with the deoxyadenylate-thymidylate (A-T) copolymer which is produced after 3-6-hour lag periods (see n.† to Table 1). For example, when DNA synthesis with *M. phlei* DNA as primer was examined over an extended reaction time interval (Table 2), the T/C ratio of the product remained the same as that of the primer for 4 hours, after which time the synthesis of the A-T copolymer seemed to predominate.

Distortion of the Relative Concentrations of the Deoxynucleoside Triphosphates Does Not Affect the Base Ratio of the Enzymatic Product.—While the results in Tables 1 and 2 were obtained with equimolar concentrations of the deoxynucleoside triphosphates in the synthetic reaction, similar results were also observed with markedly distorted substrate concentrations (Table 3). The starting concentration of dTTP

TABLE 3*

EFFECT OF RELATIVE SUBSTRATE CONCENTRATIONS ON COMPOSITION OF SYNTHETIC DNA

SUBSTRATES (RELATIVE MOLAR CONCENTRATION)				NET SYNTHESIS	PRODUCTS	
dCTP	dGTP	dTTP	dATP		A + T G + C	A + G C + T
1.0	1.0	1.0	1.0	11x	1.98	1.00
1.0	1.0	0.2	1.0	6x	1.82	1.04
1.0	1.0	0.2	0.2	6x	1.82	0.97
T ₂ bacteriophage DNA as primer					1.92	0.98

* The reaction mixtures were the same as described under "Methods," except that 1.5 or 0.3 μ mole of the substrates were used as noted above and 50 μ g. of T₂ bacteriophage DNA was used as primer.

or of both dTTP and dATP was lowered to one-fifth the level of the other triphosphates. As the reaction proceeded, the disparity in relative concentrations was exaggerated, and presumably the extent of DNA synthesis was curtailed by the exhaustion of the limiting substrate. Nevertheless, the product synthesized maintained the same $A + T/G + C$ ratio as the primer, and the purine content was equivalent to the pyrimidine content.

DISCUSSION

A close correspondence of adenine to thymine and of guanine to cytosine has been established as characteristic of DNA isolated from all natural sources^{12, 13} and is a key element in the Watson and Crick structure for DNA.¹⁴ Such equivalence of purines to pyrimidines was observed in our synthesized DNA, regardless of the relative concentrations of the substrates present in the reaction mixture or of the extent of the reaction. From these findings, as well as earlier observations on the replacement of the naturally occurring deoxynucleotide substrates by related analogues,¹⁵ it is clear that the relationship of adenine to thymine and of guanine to cytosine is an inherent feature of DNA synthesis by the "polymerase" enzyme of *E. coli*. However, these results alone do not implicate the added DNA as a template in the process, nor do they explain why all four of the deoxynucleotides are essential for a significant amount of DNA synthesis.

A crucial and sensitive test of the function of the added DNA in the reaction was the use of a variety of DNA samples which differ widely in their content of adenine and thymine relative to guanine and cytosine. Here the nice agreement found between the base composition of the added DNA and the synthesized DNA is decisive evidence for the participation of the added DNA in the reaction; a most plausible interpretation is that the primer DNA provides a template. There are implications of the template hypothesis that can and should be tested. One consequence of replication would be the reproduction of the transforming activity inherent in DNA samples from *Hemophilus influenzae*¹⁶ and *Diplococcus pneumoniae*.¹⁷ In preliminary experiments in which *H. influenzae* or *D. pneumoniae* DNA with the marker for streptomycin resistance was used as primer, there was no increase in transforming activity despite chemically determined increases of six- to eightfold in DNA content. Such experiments have, however, been complicated by the persistence of sufficient deoxyribonuclease activity in the most purified "polymerase" preparations to cause destruction of 30–90 per cent of the transforming activity added initially. More definitive experiments must therefore await an enzyme preparation in which such DNase activity has been completely eliminated. Another result of replication of DNA chains would be the influence of the molecular size of the template on the size of the product. Earlier studies¹⁸ have, in fact, shown that the molecular size of the product synthesized with calf thymus DNA as primer is very close to that of its primer. Similar experiments should also be attempted with a monodisperse primer of smaller size.

It should be pointed out that T₂ bacteriophage DNA, which contains hydroxymethylcytosine, supports extensive DNA synthesis, although this base is not provided as a substrate. However, the base required for pairing with the hydroxymethylcytosine of the phage DNA is guanine, and the guanine may then pair with the cytosine that is provided in the enzymatic reaction.

SUMMARY

Analysis of the purine and pyrimidine composition of DNA, enzymatically synthesized in the presence of a variety of DNA primers, reveals that the content of adenine residues is equal to that of thymine and that the number of guanine residues equals the cytosine residues. These results agree with the composition data for DNA samples isolated from nature and with the requirements of the DNA structure proposed by Watson and Crick.

The ratio of the number of adenine-thymine pairs to the number of guanine-cytosine pairs in an enzymatically synthesized sample reflects the ratio present in the primer used in its synthesis; the ratios in the primers tested varied from 0.5 to >40.

Distortion of the relative concentrations of the deoxynucleotide substrates in the reaction mixture or variation of the extent of enzymatic synthesis from 2 to more than 1,000 per cent had no effect on the base composition of the DNA product.

These results suggest that the enzymatic synthesis of DNA by the "polymerase" of *E. coli* represents the replication of a DNA template.

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¹ The abbreviations used in this report are dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; dTTP, thymidine triphosphate; DNA, deoxyribonucleic acid.

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