ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. IV. LINKAGE OF SINGLE DEOXYNUCLEOTIDES TO THE DEOXYNUCLEOSIDE ENDS OF DEOXYRIBONUCLEIC ACID

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Although the synthesis of DNA[1] by an enzyme purified from *Escherichia coli* requires four deoxynucleoside triphosphates (dAPPP, dCPPP, dGPPP, TPPP) in addition to Mg^{++} and DNA,[2, 3] nevertheless a single deoxynucleoside triphosphate can be incorporated into DNA to an extent about one-thousandth of that for the complete reaction.[3] The number of the single deoxynucleotides incorporated is of the same order as an approximation of the number of DNA chains added to the reaction mixture.

It was the purpose of this investigation to determine whether the single deoxynucleotide, incorporated in the absence of the other three deoxynucleotide substrates, was covalently linked to the DNA and, if so, where in the DNA chain. It was also of interest to establish whether only one or several deoxynucleotides were added to a DNA chain. The results show that the single deoxynucleoside triphosphate reacts with the deoxynucleoside end of a DNA chain to form a typical 3′,5′-phosphodiester linkage and that only one or a few nucleotides are added to a chain.

METHODS AND MATERIALS

Materials.—Uniformly labeled C^{14}-5′-deoxycytidylate was obtained by enzymatic hydrolysis of DNA from a *Chromatium* species grown on $C^{14}O_2$ as the sole carbon source. Deoxyribonucleoside triphosphates were prepared as described previously.[2] Micrococcal DNase[4] was generously supplied by Dr. L. Cunningham. Bull semen 5′-nucleotidase[5] and purified human semen phosphomonoesterase were kindly provided by Dr. L. A. Heppel. Calf spleen phosphodiesterase was prepared according to Hilmoe and Heppel.[6] Venom phosphodiesterase was prepared from the venom of *Crotalus adamanteus* according to Koerner and Sinsheimer[7] and was free of phosphomonoesterase. The DNA-synthesizing enzyme used in these studies (Fraction VII) was purified about 2,000-fold.[2] Calf thymus DNA was prepared according to Kay, Simmons, and Dounce.[8]

Preparation of DNA products.—Four products were prepared from $dAP^{32}PP$, $dCP^{32}PP$, $TP^{32}PP$, or $C^{14}dCPPP$, essentially as described previously,[2] except that the concentration of DNA was raised 25-fold in order to obtain greater amounts of incorporation. The reaction mixtures contained (per ml.) 6.0 mμmoles of a deoxynucleoside triphosphate; 6.7 μmoles of magnesium chloride; 67 μmoles of potassium phosphate buffer at pH 7.5; 800 μg. of calf thymus DNA; and 20 μg. of Fraction VII enzyme. The volume of these reaction mixtures ranged from 18 to 36 ml. Incubations were carried out at 37° for 2.5 hours (for $dAP^{32}PP$, $dCP^{32}PP$, and $TP^{32}PP$) or for 1 hour (for $C^{14}dCPPP$).

The reactions were stopped and the products freed of unreacted deoxynucleoside triphosphate in one of two ways: (1) acidification with an equal volume of cold 1 N perchloric acid, then alternate solubilization of the precipitated product in 0.1 N NaOH and precipitation in 0.5 N perchloric acid (when enzyme had been omitted, no radioactivity could be detected in this repeatedly precipitated product); (2) addition of sodium chloride to a final concentration of 0.2 M and heating at 70° for 10 minutes,[3] then repeated dialysis against 0.2 M sodium chloride. The radio-activity in this dialyzed product was then entirely precipitable by perchloric acid.

RESULTS

Extent and Kinetics of Reaction.—In the four DNA products prepared, the incorporation of dAP^{32}, dCP^{32}, TP^{32}, and $C^{14}dCP$ was 0.25, 0.13, 0.32, and 0.34 mμmoles, respectively, per milliliter of reaction mixture. For an assumed molecular weight of 5×10^6 for thymus DNA, these amounts correspond to an incorporation of 0.5 to 1 mole of deoxynucleotide per mole of DNA. Several factors add uncertainty to this approximation: the polydispersity of DNA, lack of information about the size and number of competent DNA molecules, and the hydrolysis of DNA to smaller molecules during the incubation.

TABLE 1*

ULTRACENTRIFUGAL ANALYSIS OF PRODUCT

Expt. No.	Basis for Calculation	Fraction Remaining in Top Compartment (Per Cent)	Sedimentation Coefficient (S)
1........	Schlieren boundary	..	12.3
	Optical density at 260 mμ	48	12.8
	Radioactivity	48	13.2
2........	Schlieren boundary	..	12.7
	Optical density at 260 mμ	50	12.7
	Radioactivity	52	12.5
3†......	Optical density at 260 mμ	10	22.1
	Radioactivity	13	21.4

* In these experiments the product used was from a reaction with $TP^{32}PP$ as the only deoxynucleoside triphosphate. The sedimentation coefficients were measured at a relatively high concentration (optical density at 260 mμ = 7.0) in order to achieve significant measurements of radioactivity. When the same product was run at a tenfold dilution, the sedimentation coefficient was 21 S.
† In experiment 3 the product had been heated at 100° for 10 minutes. As a result of the heating, the schlieren boundaries were too diffuse to serve as a basis for calculation of a sedimentation coefficient.

A study of the time course of the reaction between DNA and a single deoxynucleoside triphosphate showed an initial rapid rate of incorporation followed by a slower rate. In a typical experiment with $dCP^{32}PP$ as the only deoxynucleoside triphosphate, the incorporation into DNA at 30, 60, and 120 minutes was 4.0, 5.0, and 7.0 $\mu\mu$moles, respectively. This slow rate of incorporation after the initial rapid reaction could be explained by the production of new, reactive DNA ends. Such degradation of the DNA, catalyzed by a contaminating nuclease, is indicated by a small decrease in viscosity during the preparation of the products. Another contaminating nuclease has the property ascribed to snake venom phosphodiesterase (see below) of removing deoxynucleotides from the deoxynucleoside ends of DNA chains.

Ultracentrifugal Analysis of the Product Showing the Incorporation of Deoxynucleotides into Molecules the Size of DNA.—In order to determine whether the incorporated radioactive deoxynucleotide possesses the same sedimentation rate as

the average DNA molecule, a product was examined in a fixed partition separation cell[9] in the ultracentrifuge. Centrifugation was stopped after about half of the DNA had sedimented out of the top compartment of the separation cell, as determined by the schlieren optical system. Then the fractions of the optical density and radioactivity remaining in the upper compartment of the cell were measured, and sedimentation coefficients were calculated from these data and from the schlieren boundaries.[10]

As shown in Table 1, the sedimentation coefficients determined from the three measurements agree. Even after the products had been heated at 100° for 10 minutes, to try to break down the structure of the macromolecules,[11] the radioactivity still sedimented at the same rate as the DNA. These data suggest that the radioactive deoxynucleotide had formed covalent linkages, rather than much weaker secondary bonds, to molecules of the same average size as DNA.

Hydrolysis of Products to 3'-Deoxynucleotides Demonstrating 3',5'-Phosphodiester Linkages between the Incorporated Deoxynucleotides and Deoxynucleoside Ends of DNA Chains.—To determine whether the incorporated P[32] deoxynucleotide is bound to DNA by a phosphodiester linkage, a method of hydrolysis was sought which would leave the P[32] with the adjacent deoxynucleotide (Fig. 1). For RNA such a result has been accomplished by hydrolysis to 2'- and 3'-ribonucleotides with alkali.[12, 13] For DNA no such reagent was available until the recent discovery and purification of a DNase from *Micrococcus pyogenes*[4] and a phosphodiesterase from calf spleen.[6] The successive action of these two enzymes has now resulted in nearly complete hydrolysis of DNA to 3'-deoxynucleotides. Koerner and Sinsheimer have obtained a similar result by combining spleen DNase and spleen phosphodiesterase.[7]

In the present experiments the progress of hydrolysis was followed by measuring phosphate released by human semen phosphomonoesterase. Micrococcal DNase in 0.01 M CaCl$_2$ and 0.1 M tris(hydroxymethyl)aminomethane at pH 8.5 hydrolyzed the products to acid-soluble fragments containing 55–65 per cent of their total phosphate in the monoester form. Spleen phosphodiesterase then added to the digest (adjusted to pH 7.0 with HCl) converted 86–94 per cent of the total phosphate to the monoester level. No inorganic phosphate (<2 per cent of the total phosphate) could be detected at the end of the digestion.

FIG. 1.—Hydrolysis of product to 3'-deoxynucleotides by the combined action of micrococcal DNase and spleen phosphodiesterase. $\overset{*}{P}$ = P[32]

The resulting 3'-mononucleotides, separated by chromatography, were sensitive to semen phosphomonoesterase but were entirely resistant to 5'-nucleotidase under conditions where 5'-deoxynucleotides were completely hydrolyzed.

When products prepared from dCP[32]PP, dAP[32]PP, or TP[32]PP as the only deoxynucleoside triphosphate were hydrolyzed in this manner, each of the four 3'-deoxynucleotides of thymus DNA was found to be radioactive (experiments
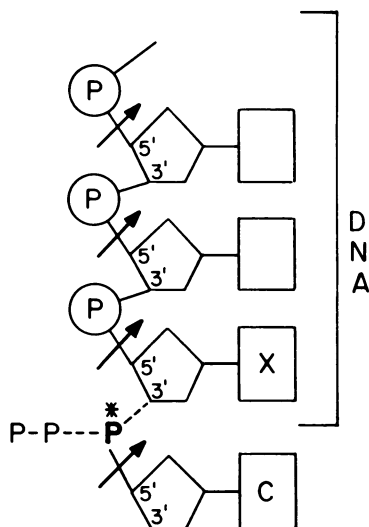
1–3, Table 2). This result shows that the single deoxynucleoside triphosphates had formed 3′,5′-phosphodiester linkages (Fig. 1) to each of the four deoxynucleotides of thymus DNA.

When a product prepared from C^{14}dCPPP as the only deoxynucleoside triphosphate was hydrolyzed to 3′-deoxynucleotides, two-thirds of the recovered radioactivity appeared in deoxycytidine and one-third in 3′-deoxycytidylate (experiment 4, Table 2). Since deoxynucleosides formed in this hydrolysis could arise only from terminal deoxynucleotides of DNA (Fig. 1), two-thirds of the C^{14}dCPPP must have been located at the deoxynucleoside ends of DNA chains. The remaining one-third probably had also reacted with ends of chains but was

TABLE 2

Hydrolysis of Products to 3′-Deoxynucleotides

Expt. No.*	Substrate for Preparation of Product	Products of Hydrolysis	Per Cent of Recovered Radioactivity†
1	dCP³²PP	3′-Deoxyadenylate	18
		3′-Deoxycytidylate	34
		3′-Deoxyguanylate	23
		3′-Thymidylate	25
2	dAP³²PP	3′-Deoxyadenylate	57
		3′-Deoxycytidylate	6
		3′-Deoxygunaylate	22
		3′-Thymidylate	15
3	TP³²PP	3′-Deoxyadenylate	17
		3′-Deoxycytidylate	10
		3′-Deoxyguanylate	21
		3′-Thymidylate	52
4	C¹⁴dCPPP	Deoxycytidine	67
		3′-Deoxycytidylate	33

* In Experiments 1, 2, 3, and 4, respectively, specific activities at the time of chromatography were 5.4×10^7, 7.5×10^7, 5.4×10^6, and 1.4×10^7 c.p.m. per μmole.
In Experiment 3 the hydrolysate was desalted by adsorption on Norit and eluted with ammoniacal ethanol, and then the products of hydrolysis were separated by paper electrophoresis (Markham and Smith, *Biochem. J.*, **52**, 552, 1952). In Experiments 1, 2, and 4 the hydrolysate was chromatographed on Dowex 1 formate columns (Cunningham, Catlin, and De Garilhe, *J. Am. Chem. Soc.*, **78**, 4642, 1956; and Sinsheimer, *J. Biol. Chem.*, **208**, 445, 1954). Deoxycytidine was determined as counts not held by a Dowex 1 formate or a Dowex 1 chloride column at pH 10. This radioactivity not held by the column contained less than 0.5 per cent of the total optical density of the 3′-deoxycytidylate and therefore could not represent 3′-deoxycytidylate unadsorbed due to overloading of the column.
On the basis of the optical density at 260 mμ, the total amounts of 3′-deoxyadenylate, 3′-deoxycytidylate, 3′-deoxyguanylate, and 3′-thymidylate recovered in experiment 1 were 30.6, 22.1, 22.9, and 30.6 μmoles, respectively. This represents 96 per cent of the optical density at 260 mμ added to the column.
† The recoveries of radioactivity after chromatography or electrophoresis were 83, 93, 78, and 83 per cent in experiments 1, 2, 3, and 4, respectively.

followed by another C^{14}-deoxycytidylate to produce terminal "runs" of C^{14}-deoxycytidylate. This possibility is supported by the disproportionately high radioactivity found in 3′-P³²-deoxycytidylate in experiment 1 of Table 2. Also in products prepared from dAP³²PP or TP³²PP, "runs" of the incorporated deoxynucleotide are indicated similarly by experiments 2 and 3 of Table 2. It cannot be decided from these data whether these "runs" occur frequently and are very short or whether they occur rarely and are long.

Kinetics of Hydrolysis of Products to 5′-Deoxynucleotides Demonstrating the Location of Incorporated Deoxynucleotides at the Deoxynucleoside Ends of DNA Chains.—To confirm that the incorporated radioactive deoxynucleotides are located at the deoxynucleoside ends of DNA chains, a reagent was sought which would hydrolyze the products stepwise from the deoxynucleoside ends so as to

release all the radioactivity at a time when few of the total deoxynucleotides had been released. Laskowski *et al.*[14] and Boman[15] have suggested that snake venom phosphodiesterase attacks DNA stepwise from one end. Recently, Khorana *et al.*[16] have shown that this phosphodiesterase hydrolyzes synthetic thymidine oligonucleotides stepwise from the deoxynucleoside end, and with thymidine oligonucleotides bearing a 3′-acetyl group at the deoxynucleoside end the first product to appear was the 3′-acetyl derivative of 5′-thymidylate. Singer, Hilmoe, and Heppel[17] have obtained a similar result with oligoribonucleotides.

When the products were treated with venom phosphodiesterase, nearly all (>90 per cent) of the radioactivity was liberated as acid-soluble deoxynucleotides at a time when less than 3 per cent of the deoxynucleotides had been released, judged by the appearance of acid-soluble ultraviolet absorbing material (Fig. 2). Such a result was obtained for products prepared from dAP³²PP, TP³²PP, or C¹⁴dCPPP. This finding provides independent evidence that the radioactivity in these products is located at or near the deoxynucleoside ends of DNA chains. It may be inferred from these kinetic studies that the phosphodiesterase does not remain attached to a DNA molecule, degrading it sequentially to completion, but that, instead, other DNA molecules compete favorably with the polydeoxynucleotide product of the enzyme action.
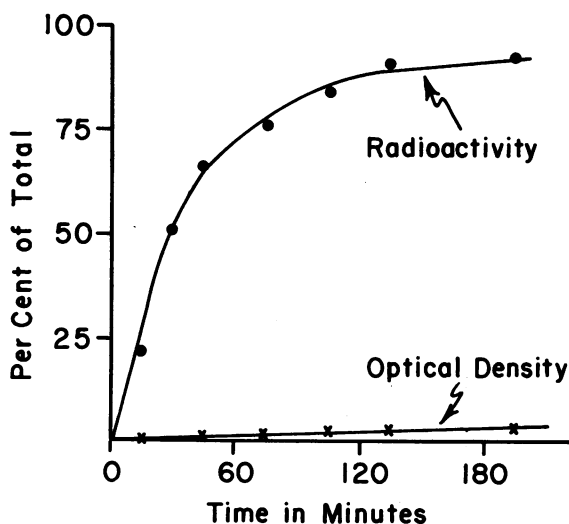

FIG. 2.—Action of venom phosphodiesterase on product. The product used in this experiment was from a reaction with C¹⁴dCPPP as the only deoxynucleoside triphosphate. The reaction mixture contained 3 mg. of product, 2 units[7] of snake venom phosphodiesterase, 50 μmoles of MgCl₂, and 200 μmoles of tris(hydroxymethyl)aminomethane at pH 8.5 in 5.0 ml. At the times indicated, 0.5-ml. aliquots were removed, 0.25 mg. of DNA was added as carrier, and the reactions were stopped by the addition of 0.5 ml. of 1 N cold perchloric acid. Radioactivity and optical density at 260 mμ were measured in the supernatant fractions. At 200 minutes, 50 more units of diesterase were added, which resulted in total release of both radioactivity and optical density in less than 3.5 hours.

DISCUSSION

In these experiments a reaction has been observed in which a DNA molecule (containing about 20,000 deoxynucleotides) adds at its deoxynucleoside end one or a very few molecules of a single deoxynucleoside triphosphate to form a 3′,5′-phosphodiester bond (Fig. 1). Several alternative mechanisms appear to have been eliminated.

1. A non-covalent binding of the deoxynucleoside triphosphate to the DNA is excluded by the demonstrated phosphodiester linkage between the incorporated deoxynucleotide and the three other deoxynucleotides of DNA.

2. Pyrophosphorolysis of the added DNA to supply the missing three deoxynucleoside triphosphates, followed by *de novo* synthesis of DNA chains, seemed from the start an unlikely hypothesis for explaining the reaction between DNA and

a single deoxynucleoside triphosphate, because levels of inorganic pyrophosphate 100 times greater than those formed in the reaction are necessary to produce significant pyrophosphorolysis.[3] This possibility was eliminated by the finding that the incorporated deoxynucleotide is located exclusively at or near the ends of DNA chains.

3. *De novo* formation of chains containing exclusively the single deoxynucleotide is eliminated by the demonstrated phosphodiester linkage between the single deoxynucleoside triphosphate and each of the three other deoxynucleotides of thymus DNA.

4. Reaction of the single P^{32}-deoxynucleoside triphosphate with the non-nucleoside end of the DNA chain[18] would not have resulted in the appearance of P^{32} in the three other deoxynucleotides of DNA when the product was hydrolyzed to 3'-deoxynucleotides.

The basis of a sensitive tool for analyzing the number and kinds of end-groups of a homogeneous DNA could be furnished by reacting such a DNA sample with a single deoxynucleoside triphosphate and then hydrolyzing the product to 3'-deoxynucleotides. Since nucleases which contaminate the purified enzyme could produce new ends or remove deoxynucleotides added in the reaction, extension of these studies in this direction must await further purification of the enzyme.

What is the pertinence of this reaction between DNA and a single deoxynucleoside triphosphate to the extensive DNA synthesis observed when all four deoxynucleoside triphosphates are present? It appears likely that it is the same highly purified *E. coli* enzyme which is responsible for both reactions. One possibility is that the reaction between DNA and a single deoxynucleoside triphosphate may actually be the first step in the over-all process of enzymatic replication of the DNA primer; then the terminal replicating chain, formed when four deoxynucleoside triphosphates are present, would loop back, in order to orient itself along the primer chain. It is conceivable that such loops could be produced by the rotation of a very few deoxynucleotide units and could be dissipated at a later time by nuclease action. Another possibility is that the reaction between DNA and a single deoxynucleoside triphosphate is merely part of the extension of the shorter of two unequally long chains of a DNA double helix and is not directly related to the replication of intact DNA molecules. In this event replication of DNA would not involve a covalent binding between the primer and the replicating chain; instead, the deoxynucleoside triphosphates would first form hydrogen bonds to corresponding deoxynucleotides of the primer chain and would then polymerize. It is evident that more must be learned about the chemistry of the DNA primer itself as well as about these enzymatic reactions before these questions of mechanism can be answered.

SUMMARY

1. The reaction between deoxyribonucleic acid (DNA) and a single radioactive deoxynucleoside triphosphate resulted in a product whose radioactivity sediments at the same rate as the average DNA molecules; this finding indicates that the deoxynucleotide had become incorporated into molecules which have the same size as DNA.

2. Hydrolysis of the product to 3'-deoxynucleotides demonstrated the formation

of 3',5'-phosphodiester linkages between the deoxynucleoside end of a DNA chain and the added deoxynucleotide. These studies further showed that only one or a very few molecules of the single deoxynucleoside triphosphate reacted with the end of each chain.

3. The kinetics of hydrolysis of the product by snake venom phosphodiesterase, involving a sequential liberation of deoxynucleotides from the deoxynucleoside end of DNA chains, provided independent evidence that the deoxynucleoside triphosphate reacted with the deoxynucleoside end of a DNA chain.

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[1] Abbreviations used: DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; dAPPP, deoxyadenosine triphosphate; dCPPP, deoxycytidine triphosphate; dGPPP, deoxyguanosine triphosphate; TPPP, thymidine triphosphate.

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