Enzymatic Synthesis of Deoxyribonucleic Acid

II. GENERAL PROPERTIES OF THE REACTION

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In the foregoing paper (1), a procedure was described for purifying an enzyme ("polymerase") from *Escherichia coli* which catalyzes the incorporation of deoxyribonucleotides into DNA.[1] The purpose of this report is to describe the requirements for the reaction, the net synthesis of DNA, and some chemical features of the synthesized DNA. The liberation of inorganic pyrophosphate as a consequence of the reaction and studies on the participation of inorganic pyrophosphate in reversing the polymerization will also be presented.

MATERIALS

Deoxynucleoside triphosphates and "polymerase" fractions were prepared according to procedures described in the previous paper (1). Bull semen 5′-nucleotidase was prepared according to the method of Heppel and Hilmoe (2). Human semen phosphomonoesterase was prepared according to Wittenberg and Kornberg (3). Snake venom phosphodiesterase, free of phosphomonoesterase activity, was prepared by the method of Sinsheimer and Koerner (4). DNA, unless designated otherwise, refers to calf thymus DNA prepared by the Kay, Simmons, and Dounce procedure (5). A solution containing 0.5 mg. per ml. had an optical density of about 7. dTTP-2-C^{14} was synthesized as follows: 2-C^{14} thymine (4 μc. per μmole)[2] and 2-deoxyribose 1-phosphate were converted to 2-C^{14} thymidine in the presence of horse liver thymidine phosphorylase[3] (6). The thymidine was phosphorylated in the presence of ATP and a partially purified nucleoside kinase from *E. coli* (7) in order to yield thymidine 5′-phosphate. This was then converted to the triphosphate and isolated according to the directions described previously (1). dTPP*P* was prepared by the procedure described for dTPPP (1), substituting acetyl-P^{32} (8) for acetyl-P in the reaction mixture. Crystalline pancreatic DNase was a product of the Worthington Biochemical Corporation. Inorganic pyrophosphate labeled with P^{32} was prepared according to Kornberg and Pricer (9).

METHODS

"Polymerase" assays and chromatographic procedures have been described in detail in the preceding paper (1). Phosphorus was determined according to Fiske and Subbarow (10) or Lowry *et al.* (11). Deoxypentose was determined according to the procedure of Dische (12). All optical measurements in the ultraviolet region were made on the Beckman model DU spectrophotometer. Radioactivity was measured in a gas-flow apparatus having an efficiency of about 50 per cent for P^{32}.

RESULTS

Requirements of Reaction—The maximal incorporation of labeled deoxyribonucleotides into DNA was dependent on the presence of polymerized DNA, Mg^{++}, and the deoxynucleoside triphosphates of thymine, cytosine, guanine, and adenine (Table I). These results based on the incorporation of labeled dTTP (dTP*PP) were essentially the same when dCP*PP, dAP*PP, dGP*PP, or C^{14}-thymine-labeled dTTP were used instead as the labeled substrate.

Evidence for Limited Reaction—The reaction rate in the instances in which one of the deoxynucleoside triphosphates or DNA or Mg^{++} was omitted was no greater than in controls which lacked enzyme or were treated with acid at time zero. Although this meant that the rate was less than 2 per cent of the maximal, it was still true that the incorporation of about 10 nucleotide residues per DNA chain (assuming a molecular weight of 3×10^6 for individual chains of the added DNA) could have escaped detection. The sensitivity of the assay was therefore increased about 50-fold by using labeled substrates with a specific radioactivity of the order of 0.1 mc. per μmole. The background of the system was kept at a relatively low level by four precipitations of the product with thymus DNA (as carrier) from alkaline solution. Under these conditions, the incorporation of only one of the deoxynucleotides into DNA, without the addition of the other three deoxynucleoside triphosphates, was demonstrated (Table II). Although the extent of incorporation during the assay interval was only about 0.1 per cent of that observed with the complete system, if was a value that increased linearly during the incubation period, and it was not detectable when DNA was omitted.

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[1] The abbreviations used are: ATP, adenosine triphosphate; dATP or dAPPP, deoxyadenosine triphosphate; dCTP or dCPPP, deoxycytidine triphosphate; dGTP or dGPPP, deoxyguanosine triphosphate; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; P*, P^{32}-labeled phosphate; P_i, inorganic orthophosphate; "polymerase" (in order to facilitate reference, this designates the enzyme in *E. coli* responsible for DNA synthesis); PP, inorganic pyrophosphate; Tris, tris(hydroxymethyl)aminomethane buffer; dTTP or dTPPP, thymidine triphosphate.

[2] 2-C^{14} thymine (4×10^6 c.p.m. per μmole) was obtained through the generosity of Dr. Howard E. Skipper of the Southern Research Institute, Birmingham, Alabama.

[3] We are indebted to Dr. Morris Friedkin for his gift of deoxyribose 1-phosphate and thymidine phosphorylase.

TABLE I

Requirements for deoxynucleotide incorporation into DNA

System	*mμmoles*
Complete system	0.50
Omit dCTP, dGTP, dATP	<0.01
Omit dCTP	<0.01
Omit dGTP	<0.01
Omit dATP	<0.01
Omit Mg^{++}	<0.01
Omit DNA	<0.01
DNA pretreated with DNase	<0.01

The complete system contained 5 mμmoles of dTTP (dTP*PP, 1.5×10^6 c.p.m. per μmole), dATP, dCTP, and dGTP, 1 μmole of $MgCl_2$, 20 μmoles of glycine buffer, pH 9.2, 10 μg. of DNA, and 3 μg. of "polymerase" Fraction V in a final volume of 0.30 ml. The incubation was carried out at 37° for 30 minutes and treated as previously described for a "polymerase" assay (1).

DNase treatment of DNA was carried out in an incubation mixture containing 60 μg. of thymus DNA, 50 μmoles of Tris buffer, pH 7.5, 5 μmoles of $MgCl_2$, and 5 μg. of pancreatic DNase in a final volume of 0.50 ml. After 30 minutes at 37°, 0.02 ml. of 5 per cent bovine serum albumin and 0.05 ml. of 5 N perchloric acid were added. The precipitate was centrifuged and dissolved in dilute alkali, and neutralized. A control incubation from which DNase was omitted yielded fully active DNA.

TABLE II

Incorporation of single deoxynucleotide into DNA

Additions	μμmoles DNA-P^{32}
dCTP (DNA omitted)	0.0
dCTP	2.5
dCTP + dGTP	5.1
dCTP + dGTP + dTTP	15.7
dCTP + dGTP + dTTP + dATP	3300

The incubation mixture (0.3 ml.) contained 5 mμmoles of dCTP (dCP*PP, 7.2×10^7 c.p.m. per μmole), and 5 mμmoles of each of the other deoxynucleoside triphosphates as indicated, together with 1.0 μmole of $MgCl_2$, 20 μmoles of glycine buffer, pH 9.2, 10 μg. of DNA and 3 μg. of "polymerase" Fraction VI. The incubation was carried out at 37° for 30 minutes, and it was treated as described in the text.

Similar data to those cited here for dCP*PP were obtained when dTP*PP, dAP*PP, or dGP*PP were used.

The possibility that deoxynucleoside triphosphates omitted from the reaction were present as trace contaminants has been minimized by a number of precautions. (*a*) Each deoxynucleoside triphosphate was obtained chromatographically as a discrete homogeneous peak, and the deoxynucleotide from which it was derived had likewise been separated by chromatography. (*b*) In view of the proximity in elution of the deoxycytidine and deoxyadenosine nucleotides on the one hand, and of the thymidine and deoxyguanosine nucleotides on the other, the pyrimidine deoxynucleotides were heated at 100° for 15 minutes at pH 3 in order to destroy any traces of purine deoxynucleotides. Such heated preparations of dCP*PP and dTP*PP were incorporated at the same rate and to the same extent as the unheated ones. Heated enzyme and DNase-treated DNA were also shown not to be sources of the omitted nucleotides. (*c*)

TABLE III

Net synthesis of DNA

Experiment No.	Estimation	Control (no enzyme)	Complete	Δ μmoles
1	P^{32} incorporation	.000	.280	.280
	Optical density	.193	.458	.265
	Deoxypentose	.187	.395	.208
2	Optical density	.060	.628	.568
3	Optical density	.052	.583	.531
4	Optical density	.051	.642	.591
5	Optical density	.041	.889	.848

In Experiment 1, the incubation mixture (30 ml.) contained 0.3 ml. of dATP*PP (0.5 μmole per ml., 1.3×10^6 c.p.m. per μmole), 0.6 ml. of dGTP (0.5 μmole per ml.), 0.3 ml. of dCTP (0.5 μmole per ml.), 0.3 ml. of dTTP (0.5 μmole per ml., 0.2 ml. of potassium phosphate buffer, 1 M, pH 7.4), 0.2 ml. of $MgCl_2$ (0.1 M), 0.2 ml. of calf thymus DNA (0.5 mg. per ml.), and 12 μg. of a phosphocellulose "polymerase" fraction (1). The mixture was incubated at 37° for 180 minutes. DNA was precipitated and washed as in the assay for "polymerase" (1) with the exception that 1.5 mg. of albumin was added as "carrier", instead of DNA, for the precipitation. The final precipitate was taken up in 1.2 ml. of 0.5 N perchloric acid and heated for 15 minutes in a boiling water bath. Optical density measurements were made at 260 mμ and converted to nucleotide equivalents using a molar extinction coefficient of 8960 derived from the calculated values for an acid hydrolysate of calf thymus DNA. In the P^{32} estimation of DNA synthesis, incorporation of deoxyadenylate was multiplied by a factor based on its percentage of composition in calf thymus DNA (13). The radioactivity actually observed for the controls did not exceed the background count. In Experiments 2, 3, 4 and 5, the reaction mixture (1 ml.) contained 0.06 ml. of dCTP (5.4 μmoles per ml.), 0.03 ml. of dATP (10.4 μmoles per ml.), 0.06 ml. of dTTP (5.3 μmoles per ml.), 0.12 ml. of dGTP (2.6 μmoles per ml.), 0.06 ml. of calf thymus DNA (0.5 mg. per ml.), 0.06 ml. of potassium phosphate buffer (1 M, pH 7.4), 0.06 ml. of $MgCl_2$ (0.1 M) and 8 μg. of a refractionated diethylaminoethyl cellulose "polymerase" fraction (1). The mixture was incubated for 240 minutes at 37°. 2 M NaCl was then added in order to give a final concentration of 0.2 M, and the mixture was heated for 5 minutes at 70°. Unreacted triphosphates were removed by exhaustive dialysis against 0.2 M NaCl. When the product was isolated in this way it contained no acid-soluble material. Optical density at 260 mμ was determined, and it was converted to nucleotide equivalents using a molar extinction coefficient for DNA of 6900 (13).

Deoxynucleotides and ATP failed to substitute for the respective triphosphates in the reaction mixture, which suggests that DNA added in the reaction was not degraded enzymatically to deoxynucleotides which were then converted to triphosphates during the incubation. (*d*) Finally, the likelihood that deoxynucleoside triphosphates might be released as such from DNA by a pyrophosphorolytic reversal of the reaction was minimized by the observation that inorganic pyrophosphate levels 100 times greater than those formed in the synthetic reaction are necessary in order to produce significant levels of the triphosphates.

Net Synthesis of DNA—With a highly purified enzyme fraction, relatively free of DNase activity,[4] net synthesis of DNA,

[4] For a discussion of the DNase content of different fractions of "polymerase," see (1). The net synthesis experiment was carried out at a pH of 7.4 in order to minimize DNase action.

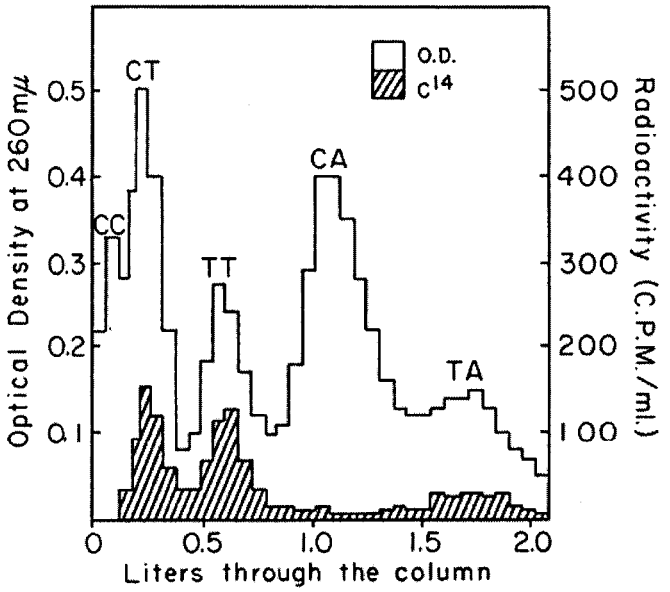

FIG. 1. Chromatography of enzymatically synthesized C^{14}-DNA (with calf thymus DNA as "carrier") after digestion with deoxyribonuclease. A reaction mixture 100 times the scale of that reported in Table I, that contained dTTP-2-C^{14} (4×10^6 c.p.m. per μmole) as the labeled substrate was incubated at 37° for 90 minutes. The reaction was stopped by the addition of "carrier" (15 mg. of calf thymus DNA) and perchloric acid (final concentration 0.5 M). The precipitated DNA was washed twice by reprecipitation from a slightly alkaline solution. The final precipitate of DNA was dissolved in dilute alkali, and it was neutralized. To this solution were added 1 mmole of Tris buffer, pH 7.5, 0.85 mmole of magnesium acetate, 185 mg. of calf thymus DNA, 1 mg. of DNase, and water to a final volume of 57 ml. The solution was incubated at 37° for 16 hours, adjusted to pH 10 with 15 N NH_4OH and chromatographed on a Dowex 1 column (acetate form, 10×1 cm.^{2}) according to the method of Sinsheimer (14). Only the dinucleotide region of the chromatogram is shown.

in amounts 2 to 20 times the amount originally present, was readily demonstrable (Table III). The results were unlike those obtained with less purified preparations, in that DNA synthesis continued progressively during a prolonged incubation period and there was no significant destruction of DNA. In the time interval of Experiment 1, the DNA content as judged by optical density, deoxypentose, and tracer measurements, was about doubled. In Experiments 2 to 5, increases of DNA by a factor of 10 to 20 were obtained, and 90 per cent or more of the DNA was derived from the deoxynucleotide substrates.

The factor, or factors, governing the ultimate synthetic ability of this system have not as yet been determined.

Chemical Structure of Enzymatic DNA—The deoxynucleotides incorporated into the enzymatically synthesized DNA were linked to other deoxynucleotides by the 3′-5′-phosphodiester bridge which is characteristic of DNA when it is isolated from natural sources. dTTP-C^{14} was converted to DNA (under conditions essentially like those in Table I), and then 200 mg. of calf thymus DNA were added as carrier for the subsequent

TABLE IV

Identification of dinucleotides as deoxycytidylate-thymidylate-C^{14} and C^{14}-dithymidylate

	CT	TT
	μmoles/ml.	
Total P	1.30	0.85
P_i after phosphodiesterase	0.09	0.02
P_i after 5′-nucleotidase	0.07	0.04
P_i after semen phosphomonoesterase	0.66	0.39
P_i after phosphodiesterase + 5′-nucleotidase	1.27	0.89
P_i after phosphodiesterase + semen phosphatase	1.33	
Deoxycytidylate (after diesterase)	0.69	
Thymidylate (after diesterase)	0.72	0.81

Conditions of incubation: *phosphodiesterase*—0.5 unit of snake venom phosphodiesterase in a final volume of 0.25 ml. at pH 8.5 for 120 minutes, 37° (4); *phosphomonoesterase*—5 units of human semen phosphomonoesterase in a final volume of 0.75 ml. at pH 5 for 60 minutes, 37° (3); *5′-nucleotidase*—60 units of bull semen 5′-nucleotidase in a final volume of 0.25 ml. at pH 8.5 for 20 minutes, 37° (2). Approximately 0.02 μmole of the indicated dinucleotide was present in each incubation. P_i was determined by the micro method of Lowry, *et al.* (11).

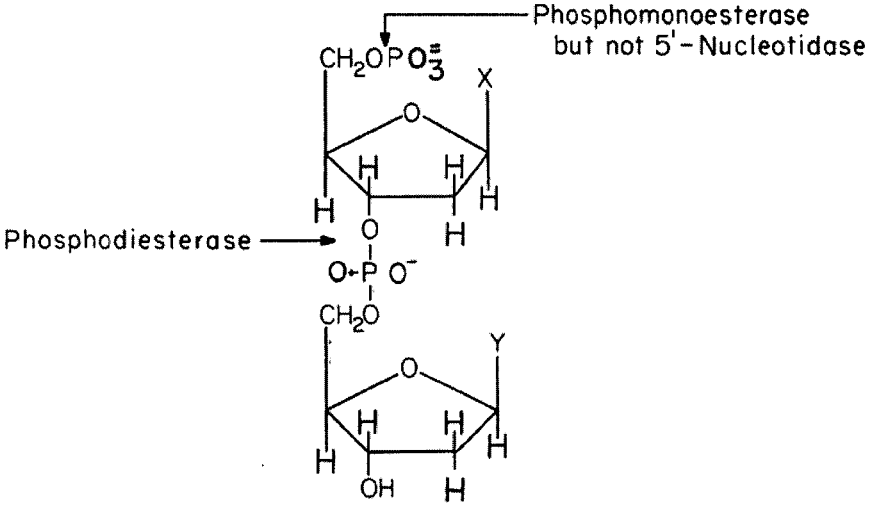

FIG. 2. Specificity of the phosphatases used in the dinucleotide analyses. The arrows indicate the linkages split by the indicated enzyme. Exposure to snake venom phosphodiesterase results in the cleavage of the dinucleotide to form two 5′-mononucleotides which are susceptible to both phosphomonoesterase and 5′-nucleotidase action.

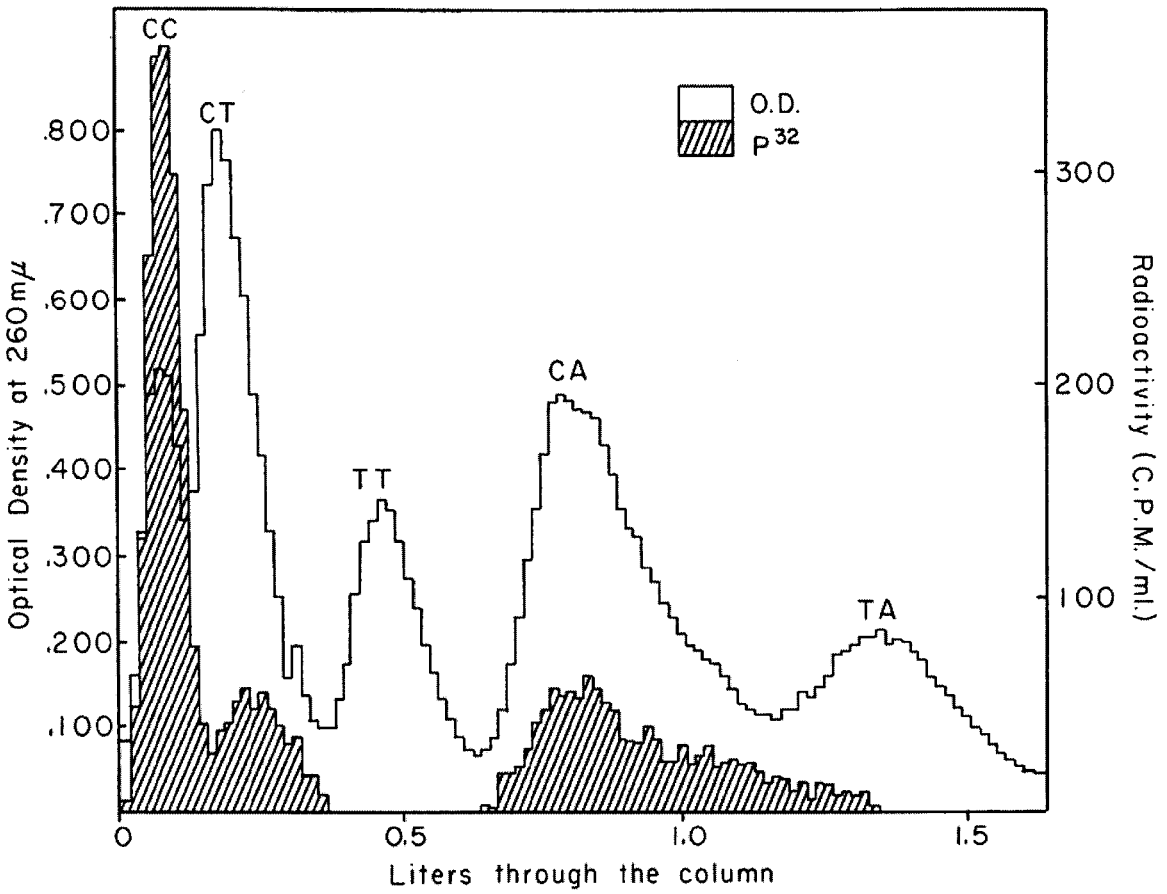

FIG. 3. Chromatography of enzymatically synthesized P^{32}DNA (with calf thymus DNA as "carrier") after digestion with deoxyribonuclease. The experimental procedure was the same as that reported in Fig. 1, except that dTTP was omitted from the reaction mixture, and dATP, dCTP, and dGTP were all labeled in the 5′-phosphate group (1.2×10^8 c.p.m. per μmole). Only the dinucleotide region of the chromatogram is shown.

procedures of isolation and enzymatic degradation. The DNA was isolated by precipitation with acid, digested with crystalline pancreatic DNase (1 mg. in 57 ml. for 16 hours at 37°), and then chromatographed on a column of Dowex 1 acetate according to Sinsheimer's procedure (14). The products of digestion consisted of about 6 per cent mononucleotides, about 15 per cent dinucleotides, and the remaining percentage consisted of higher oligonucleotides. In the dinucleotide areas of the chromatogram, two radioactive zones were selected, which, judged by their spectral characteristics and specific radioactivities, were considered to contain a deoxycytidylate-thymidylate-C^{14} dinucleotide and a C^{14}-dithymidylate dinucleotide (Fig. 1). These zones, designated CT and TT, respectively, were rechromatographed on Dowex 1 acetate, and symmetrical peaks were obtained in which the specific radioactivity (counts per minute per optical density unit) remained constant.

Chemical and enzymatic analyses of the CT and TT fractions verified them as dinucleotides of the expected composition, with internucleotide linkages consisting of 3′-5′-phosphodiester bridges (Table IV). All the phosphate was esterified, and essentially none of it was liberated by snake venom phosphodiesterase or bull semen 5′-nucleotidase. Approximately half of the phosphate was liberated by human semen phosphomonoesterase, and all of it was liberated by 5′-nucleotidase, or phosphomonoesterase acting in combination with phosphodiesterase (Fig. 2). Equivalent amounts of deoxycytidylate and thymidylate were isolated by chromatography on Dowex 1 chloride columns after phosphodiesterase action on the CT fraction, and only thymidylate was recovered from the TT fraction after similar treatment. All the radioactivity of the CT fraction was found in the isolated thymidylate.

In another experiment in which dTTP was omitted and the other triphosphates were strongly labeled with P^{32} (as in Table II), the small amount of DNA synthesized was shown to contain little or no radioactive thymidylate. On the other hand, dinucleotides in the DNase digest that contained deoxycytidylate or deoxyadenylate were highly radioactive (Fig. 3). No statement can be made about deoxyguanylate since the chromatogram was not carried far enough to elute dinucleotides that contain deoxyguanylate.

Liberation of Inorganic Pyrophosphate—The incorporation of a deoxynucleotide into DNA is attended by the release of inorganic pyrophosphate. In an experiment using dTPP*P*-2-C^{14} in the presence of the other three non-labeled deoxynucleoside triphosphates,[5] the incorporation of thymidylate

[5] dTPP*P*-2-C^{14} was simulated by mixing dTPPP-2 C^{14} with dTPP*P*. In this experiment, the radioactivity contributed by the C^{14} was 6.8×10^5 c.p.m. per μmole, and that contributed by the P^{32} was 7.05×10^6 c.p.m. per μmole.

TABLE V

Liberation of inorganic pyrophosphate

Estimation	Control*	Experimental	Δ
	mμmoles	*mμmoles*	*mμmoles*
DNA-C^{14} incorporation	1	25	24
P*P* released	2	22	20
P^*_i released	7	11	4

* dATP was omitted.

The reaction mixture was the same as that described in Table I, except that dTPP*P*-2-C^{14} was used as the labeled substrate, and the incubation was performed at 10 times the scale (total volume, 3 ml.). After 60 minutes at 37°, the reaction mixture was chilled and 0.50 ml. of DNA (0.5 mg. per ml.) was added, followed by 3 ml. of perchloric acid (1 N) and 5 ml. of water. The mixture was centrifuged; the supernatant fluid was decanted and saved, and the precipitate was washed twice by precipitation from alkaline solution, it was plated, and its radioactivity was determined. The supernatant fluid and the two wash liquids were combined (final volume, 38 ml.), treated with 3.8 ml. of Norit (20 per cent packed volume) in order to remove unreacted substrate, centrifuged, and the supernatant fluid was neutralized. To it were added 10 μmoles of P_i and 5 μmoles of PP (both pH 7) as "carrier", and the solution was chromatographed on a Dowex 1 column (chloride form, 10 × 1 cm.²) (12). P_i was determined according to Fiske and Subbarow (10), and PP as P_i after hydrolysis in 1 N H_2SO_4 for 15 minutes at 100°. The values cited have not been corrected for those obtained with enzyme-less or zero-time controls.

approximated the release of labeled inorganic pyrophosphate. 20 mμmoles of inorganic pyrophosphate were isolated from the acid-soluble fraction by chromatography on a Dowex 1 chloride column, and 24 mμmoles of thymidylate were found in the DNA fraction (Table V). The relationship between pyrophosphate release and deoxynucleotide incorporation with the other deoxynucleoside triphosphates is being studied.

Reversal of Reaction—PP in the reaction mixture at the same concentration as the triphosphate substrates (1.6×10^{-5} M) had no effect on the reaction rate. However, at much higher concentrations (3×10^{-3} M) the synthetic rate was inhibited by 50 per cent, and PP was incorporated into the deoxynucleoside triphosphates (Table VI). The rate of this PP exchange with the triphosphates was of the same magnitude as the rate of incorporation of the deoxynucleotides into DNA in the absence of PP. Thus, assay of the enzyme fraction cited in Table VI for "polymerase" indicated the incorporation of 900 mμmoles of deoxynucleotide into DNA per ml. of enzyme in 30 minutes, whereas the exchange rate of PP was 1600. The exchange of PP and the triphosphates was dependent on the presence of polymerized DNA. It was not detectable when a pancreatic DNase digest of DNA replaced the DNA or when P_i was substituted for PP. The inference that the conversion of P*P* to a form adsorbable by Norit represents its incorporation into the triphosphates was verified by isolation of the four triphosphates by ion exchange chromatography. The percentages of composition (in respect to the total radioactivity) recovered after chromatography were: dTTP, 31; dATP, 33; dCTP, 22; and dGTP, 8. The low recovery in the dGTP fraction may be due

TABLE VI

Evidence for reversal of reaction

System	P^{32}-deoxynucleoside triphosphate*
	mμmoles
Complete system	3.47
Omit DNA	<0.02
Omit enzyme	<0.02
Omit dATP	2.34
Omit dATP, dTTP	2.14
Omit dATP, dTTP, dGTP	1.34
Omit dATP, dTTP, dGTP, dCTP	0.20
Replace P*P* with P^*_i	<0.02
Replace DNA with DNase-treated DNA	<0.02

* These values are corrected for a zero-time value of 0.05 mμmole.

The complete system contained 5 mμmoles each of dATP, dTTP, dGTP and dCTP, 1 μmole of P*P* (5×10^5 c.p.m. per μmole), 10 μg. of DNA, 2 μmoles of $MgCl_2$, 20 μmoles of Tris buffer, pH 7.5, and 0.8 μg. of "polymerase" Fraction VII in a final volume of 0.3 ml. After incubation at 37° for 30 minutes, the mixture was chilled and cold solutions of albumin (0.05 ml. of 10 mg. per ml.), perchloric acid (0.25 ml. of 1 N), and "carrier" PP (0.5 ml. of 0.1 M, pH 7) were added. The mixture was centrifuged, the precipitate discarded, and the supernatant fluid was treated first with 0.05 ml. of an acid-washed Norit suspension (25 per cent packed volume) and then with 2 ml. of cold water. The Norit precipitate collected by centrifugation was washed three times with 2.5 ml. portions of cold water (containing 1 drop of 1 N perchloric acid), suspended in 0.5 ml. of 50 per cent ethanol (containing 0.03 N NH_4OH), and then transferred to a planchet where it was dried for radioactivity determination.

TABLE VII

Relative dependence of synthetic and pyrophosphorolytic reactions on deoxynucleoside triphosphates

	Synthetic reaction	Pyrophosphorolytic reaction
	%	%
Complete system	100.0	100.0
Omit dATP	0.48	67.0
Omit dATP, dTTP	0.15	61.5
Omit dATP, dTTP, dGTP	0.07	38.6
Omit dATP, dTTP, dGTP, dCTP		5.8
Omit DNA	<0.01	<0.6

The data for the synthetic and pyrophosphorolytic reactions are derived from Tables II and VI, respectively.

to its partial destruction by an enzyme known to be present in the "polymerase" fraction used in this experiment (15). All these considerations have led to the assumption that this PP substrate exchange is a reversal of the synthetic reaction.

In a comparison of the synthetic and pyrophosphorolytic reactions, a noteworthy feature is the effect of omitting one or more of the deoxynucleoside triphosphates from the reaction mixture (Table VII). It is apparent that although synthesis is reduced by two or three orders of magnitude, the reversal is affected relatively little except when the triphosphates are omitted entirely. Under the latter condition, there is a distinct

TABLE VIII

Reversal of reaction in presence of enzymatically synthesized, P^{32}-DNA

Additions	P^{32}-deoxynucleoside triphosphate
	mμmoles
DNA	.03
DNA + PP	0.38
DNA + PP + triphosphates	0.27
DNA + P*P* + triphosphates	2.84

The reaction mixture (0.3 ml.) contained 2 μmoles of $MgCl_2$, 2 μmoles of Tris buffer, pH 7.5, 0.8 μg. of "polymerase" Fraction VII, and where indicated, P^{32}-DNA, 10 μg. (6×10^5 c.p.m. per μmole of nucleotide); PP, 1 μmole; P*P*, 1 μmole (5×10^6 c.p.m. per μmole); triphosphates, 5 mμmoles each of dATP, dCTP, dGTP, and dTTP. The experimental conditions and procedure have been described under Table VI.

but only slight reaction, suggesting a slow or limited pyrophosphorolysis of the calf thymus DNA.

The behavior of enzymatically synthesized DNA is quite similar in this regard. Using P^{32}-labeled, enzymatically prepared DNA, the release of acid-soluble material adsorbable on Norit, as determined by radioactivity measurements, was relatively slight and of the same order as that observed with thymus DNA (Table VIII). This was true whether or not the triphosphates were present. Identification of the P^{32}-labeled material released from the DNA has not been attempted and its significance is still uncertain. The enzymatically prepared DNA sample also served for the exchange of PP^{32} with the triphosphates (last line of Table VIII).

DISCUSSION

Studies of bacterial genetics by means of transformation and bacteriophages, as well as the earlier cytochemical findings, make it appear that DNA is the molecular unit which in most cells is responsible for their hereditary properties. According to this concept, the structural details of the DNA molecule carry the information for producing the biochemical features of the cell and must be reproduced during cell division. The elucidation of the chemistry of DNA biosynthesis is thus necessary for a clear understanding of genetic processes.

We have described in this report procedures which have yielded a protein fraction that is over 2000 times more active in the synthesis of DNA than the protein in the cell-free extract. As a result of purifying the enzyme, certain characteristics of the system have become evident which are of considerable interest because of their plausibility in relation to genetic knowledge.

For the synthesis of significant amounts of DNA, it has been found essential to provide the enzyme with DNA and the triphosphates of each of the four deoxynucleosides which commonly occur in DNA. These findings immediately suggest the hypothesis that the added DNA is serving as a primer and template and that extensive synthesis of DNA chains is possible only when all the complementary deoxynucleotides are available for polymerization. This speculation is attractive because it is consistent with what would be expected if this enzyme were responsible for the synthesis of a self-duplicating molecule. It should be emphasized that direct proof of the precise functions of DNA in the enzyme system requires much information which is now lacking and is being sought.

The exchange of inorganic pyrophosphate with the deoxynucleoside triphosphates appears to be of great significance to the mechanism of the synthetic reaction, but a clear interpretation is not at hand. These results can be regarded as indicating that whereas pyrophosphorolysis of a long, tightly coiled, hydrogen-bonded molecule is slow and difficult, single nucleotides, or short runs of them, added to the DNA chain during the course of synthesis are readily pyrophosphorolyzed.

The question of the specificity of the nucleotide substrates has been investigated only partially. Thymidine diphosphate has been noted to be inert in replacing the triphosphate (7), and the diphosphates of deoxycytidine and deoxyguanosine have also been found totally inactive in place of the respective triphosphates.[6] ATP, even at high concentrations, fails to substitute for deoxyadenosine triphosphate, and when labeled with C^{14} it is not incorporated to a detectable extent.[6] Cytidine triphosphate labeled with P^{32} (Cytidine-P*PP) is not incorporated.[6] Comparable studies with other ribonucleotides are contemplated. The ability of deoxyuridine triphosphate to replace thymidine triphosphate and to be incorporated into DNA has been mentioned and commented upon (1).

With regard to the type of DNA which can serve in the synthetic reaction, we have pointed out that DNA derived from animal or microbial cells is able to support DNA synthesis, but that acid or pancreatic DNase-treatment of the DNA renders it totally inactive. It is of interest to mention current studies showing that DNA may be activated several-fold either by an *E. coli* enzyme fraction separated from the "polymerase" late in the fractionation or by minute amounts of pancreatic DNase. With 0.2 unit or more of the most purified enzyme fractions, the reaction rate becomes a function of the DNA concentration and under such conditions preincubation of calf thymus or T2-phage DNA with pancreatic DNase[7] increases the reaction rate up to 3-fold.

Concerning the nature of the enzymatically synthesized DNA, it has been shown to consist of deoxynucleotides linked by typical 3′-5′ phosphodiester bonds. Furthermore, it has physical properties essentially the same as those that are considered characteristic of native preparations of DNA.[8] More thorough chemical and physical characterization of the DNA is clearly necessary, including tests of biological activity.[9]

[6] Unpublished results.

[7] Amounts of the DNase were used which produce a 60 to 85 per cent inactivation of the *Haemophilus influenzae* transforming factor.

[8] Ultracentrifugal analysis of material, of which more than 90 per cent originated from the deoxynucleoside triphosphates, showed polydisperse material with average sedimentation coefficients ranging from 20 S to 30 S. The viscosities of such preparations were slightly less than those of thymus DNA with values for different preparations that ranged from 15 to 30 (gm. per 100 ml.)$^{-1}$. These data indicate a molecular weight of the order of 5×10^6. A preliminary report of these findings by Schachman *et al.* is in *Federation Proc.* (in press).

[9] Current studies with *Haemophilus influenzae* DNA show considerable destruction of the activity of the transforming factor upon incubation with the purified "polymerase." This residue of DNase activity must be eliminated in order to make definitive and quantitative studies possible.

Of great interest is the question as to whether or not a phosphodiester linkage exists between the added DNA and the synthesized DNA. An experimental approach to this problem seems apparent in the use of a substrate with a base that is chemically distinct from any of those found in the added DNA. Thus, if deoxyuridine triphosphate were used in place of thymidine triphosphate in a reaction mixture, the demonstration of a dinucleotide of deoxyuridylate and thymidylate (in a DNase digest of the isolated DNA) would indicate such an interaction of a substrate with an added DNA chain.

SUMMARY

Net synthesis of deoxyribonucleic acid (DNA) has been achieved with an enzyme purified from *Escherichia coli* in excess of 2000-fold. For this synthesis, highly polymerized DNA, 4 deoxynucleoside triphosphates (adenine, guanine, cytosine, and thymine) and Mg^{++} are required. Omission of any one of the 4 triphosphates reduces the rate of incorporation to about 0.5 per cent of the maximal value; omission of DNA abolishes it almost completely (less than 0.05 per cent).

Newly synthesized DNA was produced in amounts that exceeded by 10 times the quantity of DNA added. Analyses of the dinucleotides obtained from DNA digests of the enzymatically synthesized DNA showed them to contain 3′-5′-phosphodiester linkages characteristic of DNA isolated from natural sources.

The synthesis of DNA is accompanied by a release of inorganic pyrophosphate equal to the amount of nucleotide incorporated. Inorganic pyrophosphate in high concentrations inhibits the synthetic reaction. P^{32}-labeled pyrophosphate exchanged with the terminal pyrophosphate group of the deoxynucleoside triphosphates only in the presence of DNA, and at a rate commensurate with the synthetic rate. In contrast to the synthetic reaction, the omission of one of the triphosphates reduced the rate of pyrophosphorolysis by 30 per cent instead of by 99.5 per cent. These results indicate an active pyrophosphorolysis under conditions where polymerization is very limited or abortive.

REFERENCES

1. Lehman, I. R., Bessman, M. J., Simms, E. S., and Kornberg, A., *J. Biol. Chem.*, **233**, 163 (1958).
2. Heppel, L. A., and Hilmoe, R. J., *J. Biol. Chem.*, **188**, 665 (1951).
3. Wittenberg, J., and Kornberg, A., *J. Biol. Chem.*, **202**, 431 (1953).
4. Sinsheimer, R. L., and Koerner, J. F., *J. Biol. Chem.*, **198**, 293 (1952).
5. Kay, E. R. M., Simmons, N. S., and Dounce, A. L., *J. Am. Chem. Soc.*, **74**, 1724 (1952).
6. Friedkin, M., and Roberts, D., *J. Biol. Chem.*, **207**, 257 (1954).
7. Kornberg, A., In W. D. McElroy, and B. Glass (Editors), *The chemical basis of heredity*, Johns Hopkins Press, Baltimore, 1957, p. 579.
8. Kornberg, A., Kornberg, S. R., and Simms, E. S., *Biochim. et Biophys. Acta*, **20**, 215 (1956).
9. Kornberg, A., and Pricer, W. E., *J. Biol. Chem.*, **191**, 535 (1951).
10. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
11. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M., and Farr, A. L., *J. Biol. Chem.*, **207**, 1 (1954).
12. Dische, Z., In E. Chargaff, and J. N. Davidson (Editors), *The nucleic acids, Vol. I*, Academic Press, Inc., New York, 1955, p. 285.
13. Chargaff, E., Vischer, E., Doniger, R., Green, C., and Misani, F., *J. Biol. Chem.*, **177**, 405 (1949).
14. Sinsheimer, R. L., *J. Biol. Chem.*, **208**, 445 (1954).
15. Kornberg, S. R., Lehman, I. R., Bessman, M. J., Simms, E. S., and Kornberg, A., *J. Biol. Chem.*, **233**, 159 (1958).