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Studies on Polynucleotides

CXVIII. A FURTHER STUDY OF RIBONUCLEOTIDE INCORPORATION INTO DEOXYRIBONUCLEIC ACID CHAINS BY DEOXYRIBONUCLEIC ACID POLYMERASE I OF *ESCHERICHIA COLI**

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SUMMARY

The incorporation of ribonucleotides into DNA catalyzed by *Escherichia coli* DNA polymerase I in the presence of Mn^{++} has been studied with two synthetic DNA's of defined sequence. In general agreement with the findings of Berg *et al.* ((1963) in Symposium on Informational Macromolecules, p. 467, Academic Press, New York), CMP and GMP could be incorporated at rates comparable to their deoxy analogs. AMP was incorporated only slowly and UMP was not incorporated at all. In studies of the fidelity of incorporation, misincorporation was observed at 37° in the presence of both GTP and CTP. The misincorporation was also observed at 10° in the presence of GTP but not in the presence of CTP. The sequence G-G was found to slow down subsequent nucleotide incorporation in the presence of GTP. The DNA's containing CMP or GMP were selectively cleaved by alkali or specific ribonucleases and expected products were thus obtained.

One promising approach to determination of the nucleotide sequence of a given region in a DNA is to use the region as a template for the DNA polymerase-catalyzed elongation (3) at the 3′-hydroxyl end of a primer annealed at the appropriate site on the DNA. The nucleotide sequence of the DNA polymerase product may then be determined by using radioisotopically labeled nucleotides. This principle has been used in elucidating the sequence of the cohesive ends in the DNA of the bacteriophage λ (4, 5). The potential scope of this approach for determining sequences of longer stretches of DNA was enhanced by the discovery of Berg *et al.* (2) that ribonucleotide residues could be incorporated into DNA chains by the DNA polymerase I of *Escherichia coli* when the synthesis is carried out in the presence of manganese ions in place of magnesium ions. The ribonucleotide insertion sites would clearly provide sites for the specific cleavage of DNA chains and the method was used for a partial sequence analysis of yeast cytochrome b_2 DNA (6). Similarly, a study of the repair of exonuclease III-degraded DNA was aided by the same technique (7). Very recently, Salzer *et al.* (8) have also investigated the potential use of this method in sequence work on DNA.

No precise study has so far been carried out on the fidelity of nucleotide incorporations in the presence of Mn^{++} ions by *E. coli* DNA polymerase I. This is important to establish because manganous ions are, in general, known to bring about misincorporation of nucleotides or elicit anomalous reactions from nucleic acid-polymerizing enzymes (9, 10). Short double-stranded DNA's with defined nucleotide sequences and possessing appropriate single-stranded ends are now available from work on the synthesis of the DNA corresponding to yeast alanine tRNA (11). These have already been used in detailed studies of the DNA polymerase-catalyzed repair reactions (12). Two such DNA's, whose structures are shown in Fig. 1, have now been used in a careful evaluation of the extent and fidelity of ribonucleotide substitutions of deoxyribonucleotides. The results show that CMP is incorporated with complete fidelity at a rate similar to the deoxy analog. The incorporation of GMP was slower than that of dGMP and completeness of repair was reached only at higher temperatures. Misincorporation was encountered even at low temperatures. The incorporation of AMP was very slow and UMP was not incorporated at all. The ribonucleotide-containing DNA's were characterized after specific degradations by ribonucleases or alkali. A brief report of this work has already appeared (13).

EXPERIMENTAL PROCEDURE

Materials

Enzymes—DNA polymerase I from *E. coli* was prepared according to the method of Jovin *et al.* (1969) (14) and was a gift from Dr. K. Kleppe. T4 polynucleotide ligase and kinase were prepared from *E. coli* infected with the bacteriophage T4amN82 according to the procedure of Weiss *et al.* (15). Bacterial alkaline phosphatase, micrococcal nuclease, and spleen phosphodiesterase were obtained from Worthington Chemical Co. Pancreatic deoxyribonuclease and snake venom phosphodiesterase were obtained from Calbiochem.

Nucleoside Triphosphates—α-^{32}P-Labeled GTP, dATP, and

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dGTP were obtained from the International Chemical and Nuclear Corp. α-^{32}P-Labeled dTTP, ^{3}H-labeled GTP, and CTP were obtained from New England Nuclear Corp. α-^{32}P-Labeled dGTP was also prepared by a modification of the procedure of Wu (4) starting with dGpG. [γ-^{32}P]ATP was prepared according to the published procedure (16). Unlabeled nucleoside triphosphates were obtained from P. L. Biochemical Co. and where necessary they were further purified by paper chromatography in the solvent, isobutyric acid-1 M ammonia (100:60) followed by elution from a DEAE-Sephadex A-25 column, 1 × 7 cm, by a linear gradient from 0.1 to 0.6 M of triethylammonium bicarbonate (50 ml in each vessel).

Oligonucleotides and Polynucleotides—Syntheses of the various oligonucleotides used in the synthesis of the two duplexes have been described (17–22).

Methods

Preparation of Double-stranded DNA's—Methods for the phosphorylation of the oligonucleotides with polynucleotide kinase have been described elsewhere (23, 24).

For the joining reactions, the T4 ligase was used. The product for the synthesis of DNA-I (Fig. 1) was as described in an earlier paper (24). The general procedure for the synthesis of DNA-II was that of Gupta *et al.* (23). The details of the preparation and isolation are given in Fig. 2. A sample of the preparation was degraded to 3′-mononucleotides. As expected, most of the radioactivity was in dGp (dAp, 8 cpm; dGp, 158 cpm; dTp, 8 cpm; dCp, 9 cpm).

Assays of Repair Reactions—The reaction mixture for DNA polymerase I was modified from previous work (12), since manganese chloride gave a precipitate in phosphate buffer. The mixture contained the following components: 0.12 M Buffer A (piperazine *N*,*N*′-bis(α-ethanesulfonic acid)) (pH 6.9), manganese chloride or magnesium chloride as specified in each experiment, 10 mM dithiothreitol, 0.1 M of each of the four deoxynucleoside triphosphates (except when replaced by individual ribonucleoside triphosphates at the same concentration), and, unless otherwise stated, approximately 300 units of DNA polymerase per ml.

The concentrations of DNA's (Fig. 1) used were 100 pmoles of DNA per ml. The temperature was varied between 0 and 37°, the exact temperature in different experiments being given in the legends to figures. The total volume of the reaction mixtures was usually 50 or 100 μl.

Kinetics of Repair Reactions—Kinetic data were followed by precipitating 5-μl aliquots (containing 0.5 pmole of DNA) on Whatman No. 3MM paper discs (2.4-cm diameter) with 5% trichloroacetic acid-10 mM sodium pyrophosphate as described by Kleppe *et al.* (12).

Isolation of Repaired DNA's—For further characterization

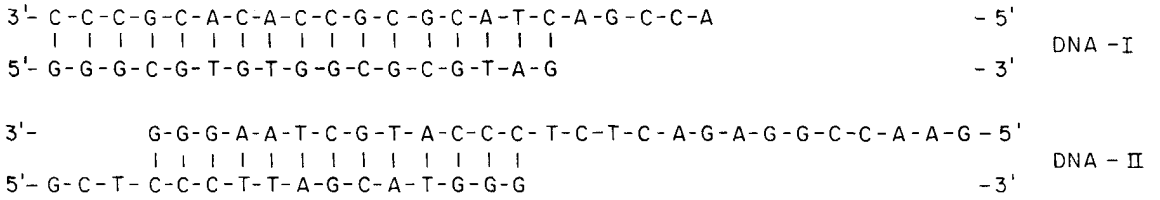

FIG. 1. Structures of the two synthetic double stranded DNA's used in the present work.

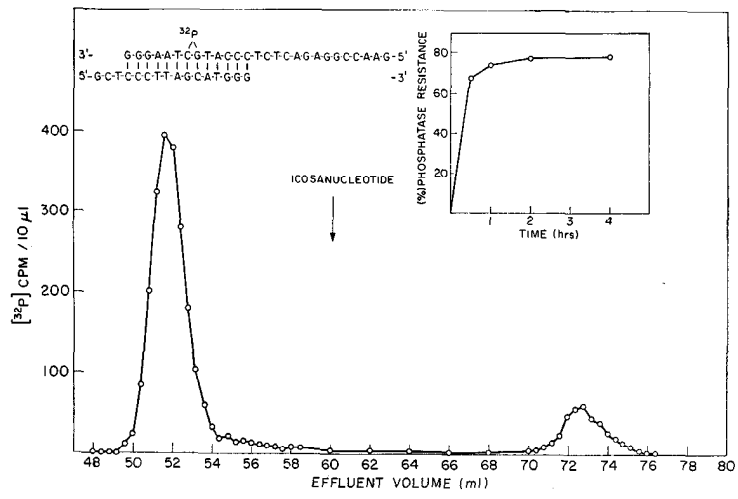

FIG. 2. (*left*). Kinetics of synthesis and separation of DNA-II. The reaction mixture contained: 1.8 nmoles of each of the icosanucleotide, the hexadecanucleotide, and the [5′-^{32}P]heptanucleotide, 40 mM Tris (pH 7.6), 0.066 mM ATP, 10 mM dithiothreitol, 10 mM $MgCl_2$, and 70 units of T4 polynucleotide ligase in a total volume of 0.3 ml. The temperature was 5°. The kinetics was followed by DEAE-cellulose paper assay described previously (19). At the end of the reaction, EDTA (pH 8.0) was added to a concentration of 0.03 M and the mixture was loaded on top of a 0.5 M agarose column (150 × 1 cm). The column was first equilibrated with 0.1 M triethylammonium bicarbonate and then run at 4° with the same solvent at a flow rate of approximately 2 ml per hour. Void volume of the column was 39.0 ml. ○——○, ^{32}P radioactivity.

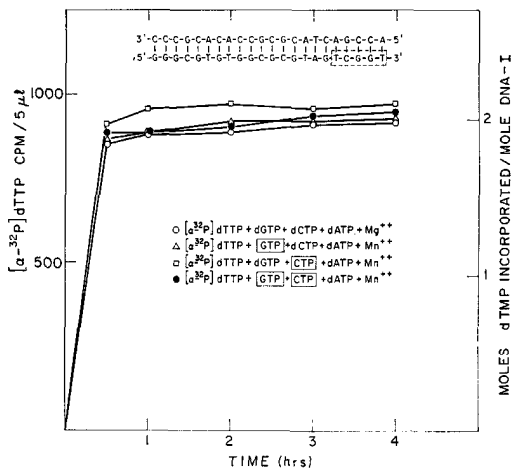

FIG. 3 (*right*). Kinetics of the repair of DNA-I using *Escherichia coli* polymerase-I. [α-^{32}P]dTTP was used as the labeled nucleotide while the other deoxyribo- and ribonucleoside triphosphates were cold. The conditions are described under "Methods" except that the temperature was 23°. In the control experiment 10 mM $MgCl_2$ was used with all four deoxyribonucleoside triphosphates (○——○). In the other experiments 1.5 mM $MnCl_2$ was used where CTP replaced dCTP (□——□), GTP replaced dGTP (△——△), and both CTP and GTP replaced dCTP and dGTP (●——●). The portion of DNA to be repaired is shown in the *dotted line* in the *inset* to the figure.

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the products of the polymerase reactions were separated from the excess nucleoside triphosphates on a column (5-ml graduated pipette) of Sephadex G-50/F (Pharmacia). The columns were first equilibrated with 0.1 M triethylammonium bicarbonate and the elution was at 25°. Before loading on the column, the reaction mixtures were made 0.05 M in EDTA and then heated to 100° for 2 min and cooled to room temperature.

Nearest Neighbor Frequency Analysis and 5′-Nucleotide Analysis—The nearest neighbor analyses were performed with the procedure described by Kleppe *et al.* (12). The 3′-nucleotides were separated in Solvent III (ammonium sulfate, 60 g-100 ml of 0.1 M sodium phosphate, pH 6.8-2 ml of 1-propanol) and also by electrophoresis in 0.05 M citrate buffer, 35 volts per cm.

5′-Nucleotide analyses were performed with the procedure described by Sgaramella *et al.* (25). The 5′-mononucleotides were separated by electrophoresis as above.

Alkaline Hydrolysis and Ribonuclease Digestions—The isolated reaction products were made to 0.3 M KOH and 5-μl aliquots were sealed in capillary tubes and incubated at 37° for various lengths of time. The solution was neutralized with an equal volume of acetic acid and the solution applied to DEAE-cellulose paper (Whatman, DE-81) followed by elution in 0.35 M ammonium formate in 7 M urea. The paper strips were scanned on a Packard model 7200 radiochromatogram scanner and the radioactivity cut out and counted in a Packard model 3380 or model 3002 scintillation counter. From previous experience in this laboratory, oligonucleotides smaller than 12 nucleotides in length moved away from the origin.

The T_1 and pancreatic digests were carried out in the same manner in 50 mM Tris, pH 7.6, 1 mM EDTA, with 24 units per ml of T_1 ribonuclease and 0.5 mg per ml of pancreatic ribonuclease.

RESULTS

Incorporation of CMP

Experiments Using DNA-I—The utilization of CTP in the repair of DNA-I was followed by using one α-^{32}P-labeled deoxyribonucleoside triphosphate and the other unlabeled nucleoside triphosphates. Fig. 3 shows the kinetics of incorporation of [α-^{32}P]dTTP (*a*) in the presence of the other three deoxynucleoside triphosphates and (*b*) in the presence of CTP and unlabeled dATP and dGTP. (The other experiments shown in Fig. 3 are referred to later.) Thus, the rate and extent of incorporation of [α-^{32}P]dTTP were virtually identical in the presence of CTP and dCTP. The nearest neighbor analysis (Table I) of the product formed showed radioactivity mostly in dGp as expected, but a small amount (about 5%) of transfer to dTp was also observed. One possibility is that this arose from misincorporation of [α-^{32}P]dTTP in place of CTP so as to give the sequence T-T-G-G-T rather than T-C-G-G-T. The fact that the extent of [α-^{32}P]dTTP incorporation in the presence of CTP was higher by a few per cent than the control in which only the deoxynucleoside triphosphates were used could be consistent with this conclusion.

The possibility of misincorporation induced by Mn^{++} ion was tested further in the experiment shown in Fig. 4. The incorporation of [α-^{32}P]dTTP alone into DNA-I was investigated under two conditions; in one, 10 mM Mg^{++} ions were used, while in the second Mn^{++} ions at 1.5 mM concentration were used. As is seen in Fig. 4, only 1 mole of dTMP per mole of DNA-I was incorporated when the divalent ion used was Mg^{++} while in the presence of Mn^{++} almost 2 moles of dTMP per mole of DNA-I were incorporated. The nearest neighbor analysis performed on the products obtained is included in Table II. Thus, in the product formed in presence of Mg^{++}, the transfer of ^{32}P occurred to dGp as expected, while with Mn^{++}, transfer occurred both to dTp and dGp, the ratio of radioactivity being 0.7:1.0. In addition, radioactivity was found in dCp. This undoubtedly arose from the exchange of the terminal dCMP unit at the opposite 3′ terminus of DNA-I (26).

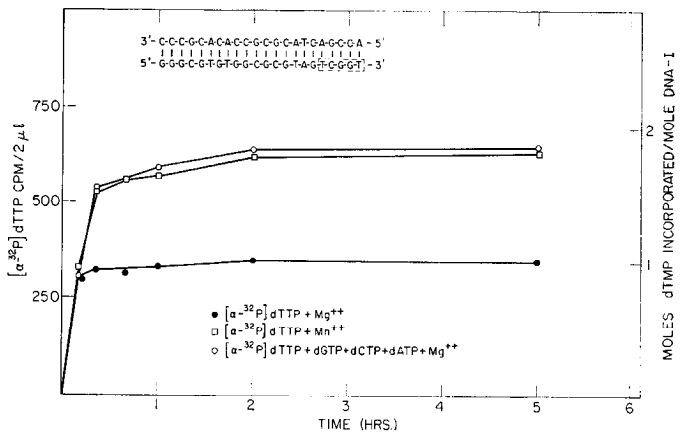

FIG. 4. Kinetics of repair of DNA-I using [α-^{32}P]dTTP alone. The conditions are as described under "Methods." The incorporation of [α-^{32}P]dTTP alone was studied in the presence of 10 mM $MgCl_2$ (●——●) and in the presence of 1.5 mM $MnCl_2$ (□——□). No other nucleoside triphosphates were present. For comparison, the incorporation of [α-^{32}P]dTTP in the presence of 10 mM $MgCl_2$ and the other unlabeled deoxyribonucleoside triphosphates is shown (○——○). The temperature of these experiments was 15°.

TABLE I

Nearest neighbor analysis of products obtained by repair of DNA-I using [α-^{32}P]dTTP as labeled nucleoside triphosphate

The conditions for repair, isolation of the products, and nearest neighbor analysis are given under "Methods." The kinetic data of the repair are shown in Fig. 3.

	Ap	Gp	Tp	Cp
	cpm	*cpm*	*cpm*	*cpm*
[α-^{32}P]dTTP + dCTP + dGTP + Mg^{++}	33	979	45	21
[α-^{32}P]dTTP + *CTP* + dGTP + Mn^{++}	129	5057	233	97
[α-^{32}P]dTTP + dCTP + *GTP* + Mn^{++}	15	1241	42	23
[α-^{32}P]dTTP + *CTP* + *GTP* + Mn^{++}	19	1637	124	28

TABLE II

Nearest neighbor analysis of products obtained by repair of DNA-I using (a) [α-^{32}P]dTTP as only nucleoside triphosphate or (b) [α-^{32}P]dGTP in presence of other nucleoside triphosphates

The conditions for repair, isolation of the products, and nearest neighbor analysis are given under "Methods." The kinetic data of repair are shown in Fig. 4 for [α-^{32}P]dTTP and Fig. 5 for [α-^{32}P]-dGTP.

	Ap	Gp	Tp	Cp
	cpm	*cpm*	*cpm*	*cpm*
a. [α-^{32}P]dTTP + Mg^{++}	19	632	21	14
[α-^{32}P]dTTP + Mn^{++}	35	962	672	184
b. [α-^{32}P]dGTP + dCTP + dTTP + Mg^{++}	26	275	25	258
[α-^{32}P]dGTP + *CTP* + dTTP + Mn^{++}	19	523	23	465

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The incorporation of CMP into DNA-I was next followed with [α-32P]dGTP in combination with CTP, dTTP, and dATP. In the control, dCTP replaced CTP and 10 mM Mg^{++} ions were used in place of Mn^{++}. The results are shown in Fig. 5. Thus, the kinetics and extent of incorporation of dGMP are essentially the same in the two experiments, 2 moles of the nucleotide being incorporated per mole of the DNA. Further, the nearest neighbor analysis performed (Table II) showed radioactivity in dGp and dCp in nearly 1:1 ratio.

In another experiment, the incorporation of CMP was tested directly by using [3H]CTP together with the other three unlabeled deoxynucleoside triphosphates. The kinetic data are shown in Fig. 6. The expected incorporation, 1 mole per mole of DNA-I was observed. The experiment also shows the incorporation of [3H]GMP (see below) as well as the additive nature of the incorporation when both [3H]GTP and [3H]CTP are provided.

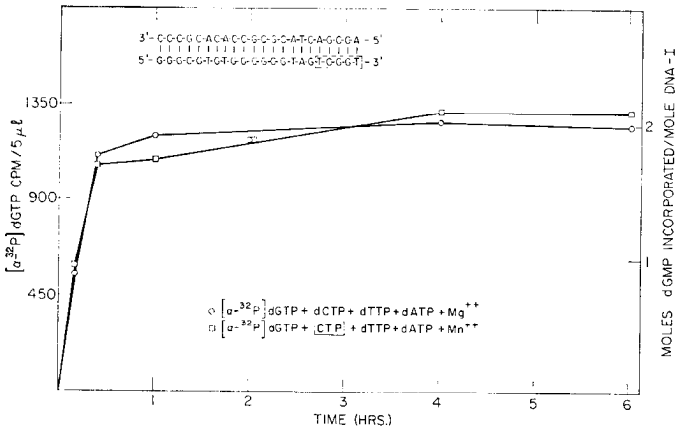

FIG. 5. Kinetics of the repair of DNA-I using [α-32P]dGTP in the presence of the other cold nucleoside triphosphates. The incorporation was studied in the presence of 10 mM $MgCl_2$ with the other deoxyribonucleoside triphosphates (○——○) and in the presence of 1.5 mM $MnCl_2$ when CTP replaced dCTP (□——□). The temperature of these experiments was 10°.

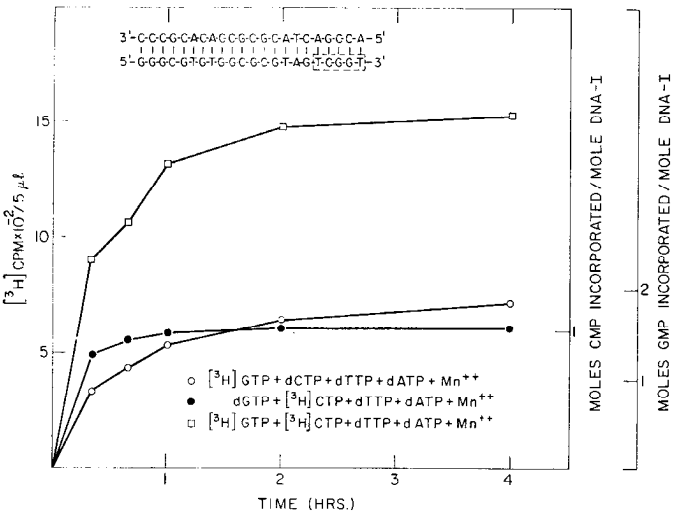

FIG. 6. Kinetics of the repair of DNA-I using [3H]GTP or [3H]CTP, or both. The conditions of repair are described under "Methods." The incorporation of CTP (●——●), GTP (○——○), and both CTP and GTP (□——□), was studied at 10° in the presence of 1.5 mM $MnCl_2$. The specific activity of the [3H]CTP was approximately two times that of the [3H]GTP resulting in the different molar ratios of incorporation. Because of this no molar ratio of incorporated nucleoside triphosphate could be determined when both labeled nucleoside triphosphates were used.

Experiments Using DNA-II—The incorporation of CTP into DNA-II was measured in the presence of [α-32P]dTTP and unlabeled dATP and dGTP; the concentration of Mn^{++} used was 0.5 mM which gave optimal repair reaction. In the control experiment, dCTP replaced CTP and Mg^{++} (10 mM) replaced Mn^{++}. The reactions were carried out at both 37 and 10°. The kinetic data are shown in Fig. 7, *a* and *b*. At both temperatures, the rate and extent of incorporation of [α-32P]dTTP were indistinguishable in the presence of dCTP or CTP. The nearest neighbor analysis of the products (Table III) showed that there was no variation in the pattern of radioactivity in the 3'-mononucleotides between the dCTP and CTP experiments at either temperature.

The incorporation of CTP into DNA-II was next measured with [α-32P]dATP or [α-32P]dGTP as the labeled nucleotide, CTP, and the two cold deoxynucleoside triphosphates. Conditions were similar to those of Fig. 7, both 37 and 10° being tested. The results are shown in Figs. 8 and 9. At 37°, signifi-

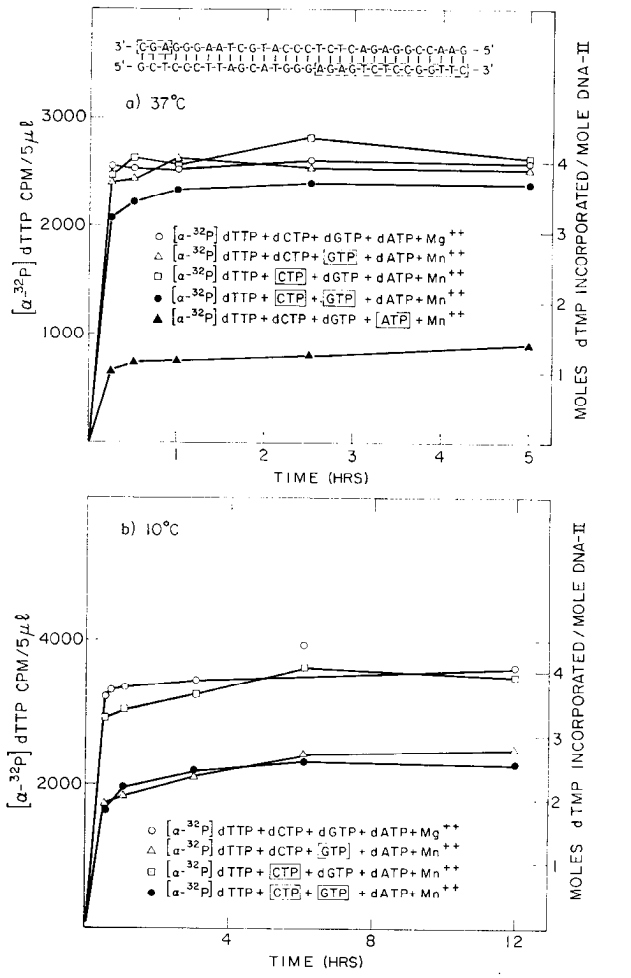

FIG. 7. Kinetics of the repair of DNA-II using [α-32P]dTTP as the labeled nucleotide and the other unlabeled deoxyribo- and ribonucleoside triphosphates. The conditions are as described under "Methods" except that the temperature was 37° in (*a*) and 10° in (*b*). In the control 10 mM $MgCl_2$ was used with all four deoxyribonucleoside triphosphates (○——○) while 0.5 mM $MnCl_2$ was used when CTP replaced dCTP (□——□), GTP replaced dGTP (△——△), ATP replaced dATP (▲——▲), and both CTP and GTP replaced dCTP and dGTP (●——●).

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TABLE III

Nearest neighbor analysis of products obtained by repair of DNA-II using [α-32P]dTTP as labeled nucleotide at 10° (a) and 37° (b)

The numbers in parentheses are the observed nucleotide ratios. The conditions for repair, isolation of the products, and nearest neighbor analysis are given under "Methods." The kinetic data of repair are shown in Fig. 7.

	Ap	Gp	Tp	Cp
	cpm	*cpm*	*cpm*	*cpm*
a. 10°				
Theoretical nucleotide ratios	(0)	(2)	(1)	(1)
[α-32P]dTTP + dCTP + dGTP + dATP + Mg^{++}	141 (0.06)	4265 (1.91)	1955 (0.88)	2231 (1.00)
[α-32P]dTTP + *CTP* + dGTP + dATP + Mn^{++}	123 (0.06)	3666 (1.89)	1616 (0.83)	1931 (1.00)
[α-32P]dTTP + dCTP + *GTP* + dATP + Mn^{++}	254 (0.14)	2456 (1.30)	629 (0.33)	1890 (1.00)
[α-32P]dTTP + *CTP* + *GTP* + dATP + Mn^{++}	176 (0.12)	1954 (1.30)	376 (0.25)	1500 (1.00)
b. 37°				
[α-32P]dTTP + dCTP + dGTP + dATP + Mg^{++}	92 (0.10)	1797 (1.92)	754 (0.81)	937 (1.00)
[α-32P]dTTP + *CTP* + dGTP + dATP + Mn^{++}	52 (0.10)	1058 (2.08)	486 (0.96)	508 (1.00)
[α-32P]dTTP + dCTP + *GTP* + dATP + Mn^{++}	156 (0.19)	1235 (1.52)	583 (0.72)	814 (1.00)
[α-32P]dTTP + *CTP* + *GTP* + dATP + Mn^{++}	139 (0.16)	1361 (1.56)	641 (0.74)	871 (1.00)
[α-32P]dTTP + dCTP + dGTP + *ATP* + Mn^{++}	106 (0.06)	3074 (1.77)	1495 (0.86)	1742 (1.00)

cantly greater incorporation of [α-32P]dATP was observed in the presence of CTP than in the control experiment with dCTP (Fig. 8*a*). The "excess" incorporation seemed to be at mispaired sites as shown by nearest neighbor analysis. Thus, as seen in Table IV, in the experiment in which only deoxynucleoside triphosphates were used, radioactivity was present only in dGp, as expected. However, when CTP was used, radioactivity was found in dAp, dCp, and dTp in addition to the expected dGp.

Repetition of the experiment of Fig. 8*a* at 10° (Fig. 8*b*) showed that the rate and extent of incorporation of [α-32P]dATP were identical in the presence of CTP and dCTP. Nearest neighbor analysis now showed the expected *specific* transfer of 32P to dGp.

The results of CTP incorporation with [α-32P]dGTP as the labeled nucleotide, which are given in Fig. 9, showed that at 37° the incorporation of [α-32P]dGTP was less in the presence of CTP than in the presence of dCTP.

The nearest neighbor analyses given in Table V showed that the fidelity of the ribonucleotide incorporation was not very good. The base ratio of dAp:dGp:dCp differed considerably from that of the control and unexpected counts appeared in dTp. The latter most likely arose from misincorporation.

A repetition of the above experiment at 10° showed that the rate and extent of incorporation of [α-32P]dGTP in the control (with dCTP, dATP, dTTP) and with CTP were essentially the same. The nearest neighbor analyses (Table V) of this experiment showed that the repair had not gone to completion in either case, but the base ratios were the same for both CTP and dCTP incorporation.

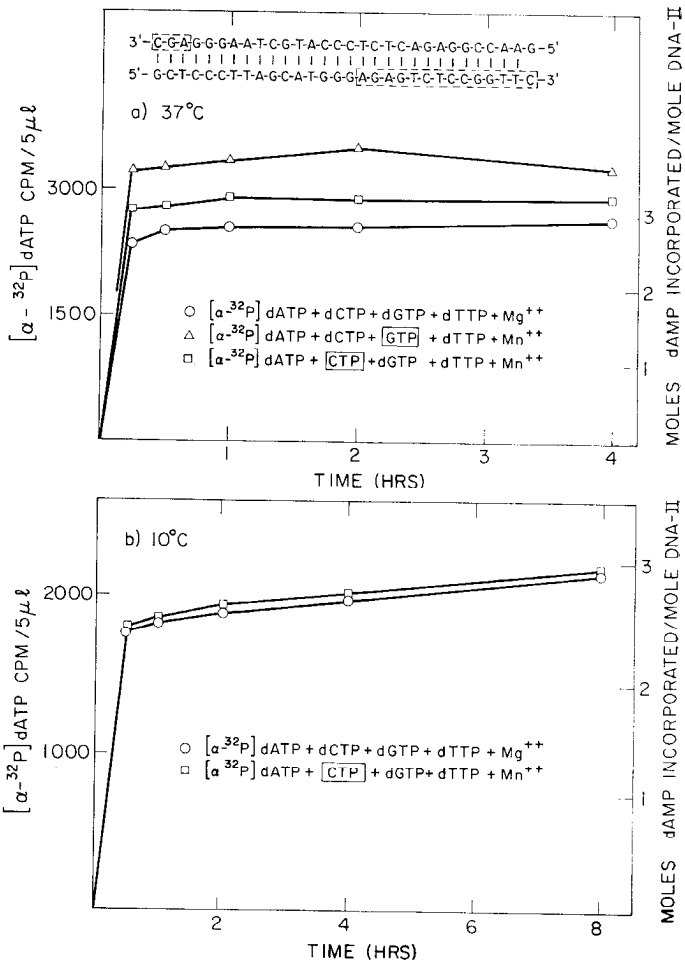

FIG. 8. Kinetics of the repair of DNA-II using [α-32P]dATP as the labeled nucleotide. The other ribo- and deoxyribonucleoside triphosphates were unlabeled and the conditions were as described under "Methods," except that the temperature was 37° in *a* and 10° in *b*. In the control experiment 10 mM $MgCl_2$ was used with all four deoxyribonucleoside triphosphates (○——○) while 0.5 mM $MnCl_2$ was used when CTP replaced dCTP (□——□) and GTP replaced dGTP (△——△).

Incorporation of GMP

Experiments Using DNA-I—The incorporation of GMP into DNA-I was first measured by using [α-32P]dTTP, GTP, dATP, and dCTP; in the control dGTP replaced GTP. The kinetics of incorporation of [α-32P]dTTP is included in Fig. 3 and, as is seen, the rate of incorporation and the plateau value (2 moles of dTMP per mole of DNA-I) were identical for dGTP and GTP. The results of the nearest neighbor analysis given in Table I show that radioactivity was found, as expected, only in Gp. While the above experiment was performed at 23°, at 10° the incorporation of [α-32P]dTTP in the presence of GTP was found to be only 60% of that in the presence of dGTP.

The incorporation of GMP into DNA-I was also followed directly by using [3H]GTP and the result is shown in Fig. 6. Slightly less than the expected 2 moles of GMP were incorporated per mole of DNA-I.

Experiments Using DNA-II—The kinetic data of incorporation of [α-32P]dTTP into DNA-II in the presence of GTP and dGTP are shown in Fig. 7. At 37° (Fig. 7*a*) the rate and extent of incorporation of [α-32P]dTTP were identical for GTP and dGTP. However, at 10°, a reduced incorporation of [α-32P]-

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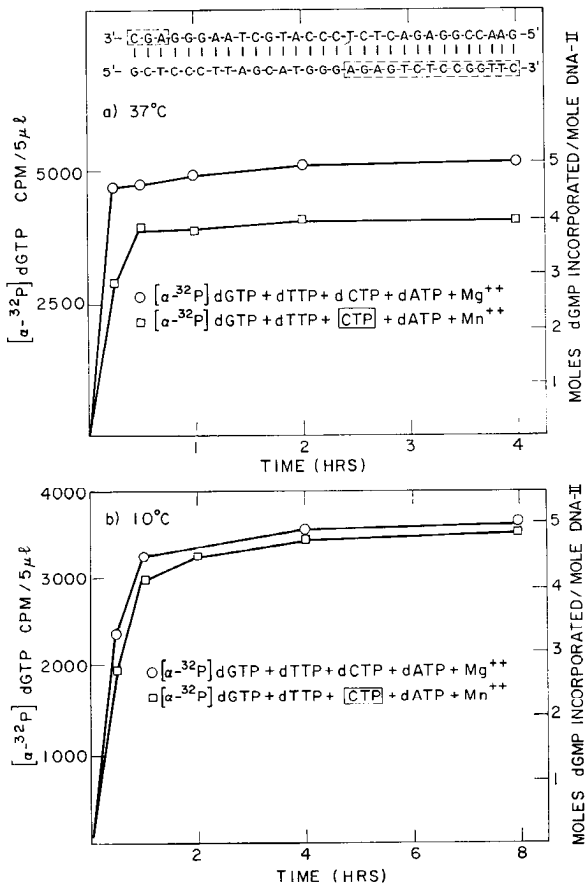

FIG. 9. Kinetics of the repair of DNA-II using [α-32P]dGTP as the labeled nucleotide and the other unlabeled ribo- and deoxyribonucleoside triphosphates. The conditions were as described under "Methods" except that the temperature was 37° in *a* and 10° in *b*. In the control experiment 10 mM $MgCl_2$ was used with all four deoxyribonucleoside triphosphates (○——○) while 0.5 mM $MnCl_2$ was used when CTP replaced dCTP (□——□).

TABLE IV

Nearest neighbor analysis of products obtained by repair of DNA-II using [α-32P]dATP as labeled nucleotide at 10° (a) and 37° (b)

The conditions for repair, isolation of the products, and nearest neighbor analysis are given under "Methods." The kinetic data of repair are shown in Fig. 8. Only Gp was expected in all of these experiments.

	Ap	Gp	Tp	Cp
	cpm	*cpm*	*cpm*	*cpm*
a. 10°				
[α-32P]dATP + dCTP + dGTP + dTTP + Mg++	75	2333	76	53
[α-32P]dATP + *CTP* + dGTP + dTTP + Mn++	18	4478	26	23
[α-32P]dATP + dCTP + *GTP* + dTTP + Mn++	388	1732	92	440
b. 37°				
[α-32P]dATP + dCTP + dGTP + dTTP + Mg++	72	1944	47	43
[α-32P]dATP + *CTP* + dGTP + dTTP + Mn++	152	3840	199	568
[α-32P]dATP + dCTP + *GTP* + dTTP + Mn++	168	4522	643	47

TABLE V

Nearest neighbor analysis of products obtained by repair of DNA-II using [α-32P]dGTP as labeled nucleotide at 10° (a) and 37° (b)

The numbers in parentheses are the observed nucleotide ratios. The conditions for repair, isolation of the products, and nearest neighbor analysis are given under "Methods." The kinetic data of repair are shown in Fig. 9.

	Ap	Gp	Tp	Cp
	cpm	*cpm*	*cpm*	*cpm*
a. 10°				
Theoretical nucleotide ratios	(3)	(1)	(0)	(1)
[α-32P]dGTP + dCTP + dTTP + dATP + Mg++	2108 (3.75)	571 (1.01)	48 (0.09)	562 (1.00)
[α-32P]dGTP + *CTP* + dTTP + dATP + Mn++	2163 (4.00)	585 (1.08)	56 (0.10)	540 (1.00)
b. 37°				
[α-32P]dGTP + dCTP + dTTP + dATP + Mg++	770 (3.37)	229 (1.00)	22 (0.10)	215 (0.94)
[α-32P]dGTP + *CTP* + dTTP + dATP + Mn++	830 (3.43)	242 (1.00)	108 (0.45)	83 (0.34)

dTTP in the presence of GTP was observed relative to dGTP. The nearest neighbor analysis of the products are shown in Table III. As is seen, the pattern for dAp:dGp:dCp was the same for both GTP and dGTP for the product formed at 37° while the product obtained at 10° showed a divergence indicating that only 30% repair had occurred beyond the G-G sequence. It thus appears that ribonucleotide addition to a G-G sequence is more difficult than addition to a C-C sequence at low temperature.

Fig. 8 shows the incorporation of [α-32P]dATP into DNA-II in the presence of GTP and of dGTP. At 37° the incorporation of [α-32P]dATP in the presence of GTP exceeded that in the presence of dGTP and the nearest neighbor analysis of the product confirmed that incorporation of [32P]dAMP in additional mispaired sites had taken place. At 10°, some misincorporation of dAMP was still observed in the presence of GTP (Table IV).

In a further experiment, the extent of incorporation of GMP in the presence of dTTP, dATP, and dCTP was studied as a function of temperature by using [α-32P]GTP. The results are given in Table VI. At plateau values, the incorporation at low temperatures was below that expected for complete repair; at 37°, the incorporation rose up to the theoretical. The findings were consistent with the lower value of [α-32P]dTTP incorporation obtained above at 10° in the presence of GTP. Nearest neighbor analyses for the reactions with [α-32P]GTP at 20 and 37° are given in Table VI, Part b. The distribution of radioactivity in 3′-nucleotides is largely as was expected for faithful repair of DNA-II.

Simultaneous Incorporation of CMP and GMP—The kinetic data of incorporation of CTP and GTP as followed by the use of [α-32P]dTTP are shown in Fig. 3. The expected incorporation of 2 moles of dTMP per mole of DNA-I was observed and the nearest neighbor analysis of the product obtained showed (Table I) radioactivity mainly in dGp, as expected. The incorporation of [3H]CTP and [3H]GTP into DNA-I was also studied (Fig. 6). Degradation of the product to 5′-nucleotides (Table VII) gave the expected 1.71:1 ratio of GMP to CMP in the mononucleotides.

The simultaneous incorporation of CMP and GMP into

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TABLE VI

Extent of [α-32P]GTP incorporation into DNA-II at different temperatures and nearest neighbor analysis of some of products

The numbers in parentheses are the observed nucleotide ratios. The conditions for repair, isolation of the products, and nearest neighbor analysis are given under "Methods." The theoretical value for moles of GMP per mole DNA-II is 5.0.

a. Extent of reaction

Temperature	Amount GMP/mole DNA-II
	moles
5°	2.4
10	3.1
15	3.1
20	3.7
37	5.0

b. Nearest neighbor analyses

	Ap	Gp	Tp	Cp
	cpm	*cpm*	*cpm*	*cpm*
Theoretical molar ratios	(3)	(1)	(0)	(1)
20°	1638	491	60	455
	(3.34)	(1.00)	(0.12)	(0.93)
37	1850	650	42	578
	(2.85)	(1.00)	(0.06)	(0.89)

TABLE VII

Nearest neighbor analysis of repair replication of DNA-I using [3H]CTP and [3H]GTP

The conditions for the repair, isolation of the products, and their degradation to 5′-nucleotides are described under "Methods." The numbers in parentheses are the molar ratios calculated from the specific activities of the nucleoside triphosphates. The kinetic data of the repair are shown in Fig. 6.

	pA	pG	pT	pC
	cpm	*cpm*	*cpm*	*cpm*
Theoretical molar ratios	(0)	(2)	(0)	(1)
[3H]CTP + [3H]GTP	39	1263	72	1271
		(1.71)		(1.00)

DNA-II was also followed by using [α-32P]dTTP. The results of experiments carried out at 37 and 10° are shown in Fig. 7, *a* and *b*. At 37°, the rate and extent of incorporation were about 10% lower than those in the control (all the four deoxynucleoside triphosphates). At 10°, however, the rate and extent were very much less, reaching about the same value as when GTP alone was present. The nearest neighbor analyses (Table III) were again consistent with incomplete repair at 10° and complete repair at 37° whenever GTP is present.

Incorporation of ATP and UTP—The incorporation of AMP was studied only at 37° with [α-32P]TTP as the labeled nucleotide. It can be seen in Fig. 7*a* that the extent of incorporation was only 20% of the control value obtained with dATP. Surprisingly, a plateau was reached even at this low extent of reaction. The nearest neighbor analysis (Table III) gave a radioactivity ratio which was very close to that of the control with deoxynucleotides. This indicated that even if a large part of the DNA was not being repaired, the part which was repaired was repaired with good fidelity.

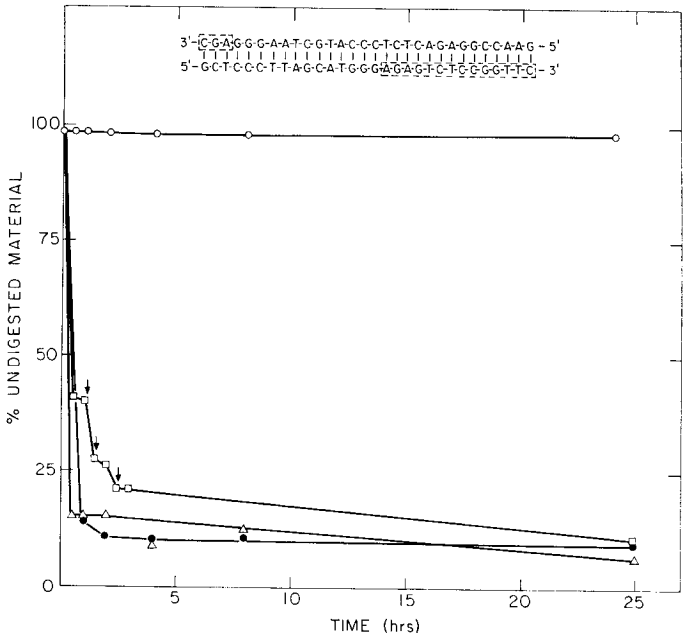

FIG. 10. Kinetics of the enzymatic and alkaline hydrolysis of ribonucleotide containing DNA-II labeled with [α-32P]dTTP. The conditions are described under "Methods." The control experiment was DNA-II repaired with all four deoxyribonucleoside triphosphates in the presence of pancreatic RNase (○——○). DNA-II repaired to contain GMP in place of dGMP was incubated with T1 RNase (□——□). At certain times (*arrow*), the reaction mixture was boiled for 2 min and more enzyme was added. DNA-II repaired to contain CMP in place of dCMP was incubated with pancreatic RNase (△——△) and with 0.3 N KOH (●——●).

The incorporation of UMP was studied with [α-32P]dGTP as the label. If no dTTP or UTP are given to the repair reaction, 3 moles of GMP per mole of duplex should be incorporated. Slightly more than this was observed, but absolutely no stimulation of incorporation was produced when UTP was added to a reaction mixture lacking dTTP. Hence, UTP is not incorporated under our reaction conditions and this is in agreement with Berg *et al.* (2).

Selective Cleavage of Mixed Polymers

Degradation by Specific RNases—DNA-I and DNA-II were repaired by using [α-32P]dTTP, CTP, or GTP and the two other deoxyribonucleoside triphosphates or by using all the four deoxynucleoside triphosphates. The repaired, selectively labeled DNA's were separated from the excess of nucleoside triphosphates by chromatography on Sephadex G-50 columns. The kinetic data of degradation of a number of preparations by appropriate nucleases and by alkali are shown in Fig. 10. In the control, with DNA-II repaired with only deoxynucleoside triphosphates, no degradation was observed with pancreatic RNase, nor was any digestion detected with T_1-RNase or alkali up to 25 hours. DNA-II containing CMP was degraded rapidly by pancreatic RNase, 85% degradation occurring within 30 min. Degradation of DNA-II containing GMP by T_1-RNase was more sluggish with a plateau at 60% digestion being reached in 1 hour. The extent of degradation was increased by heating, chilling, and a further addition of the enzyme.

Degradation by Alkali—The use of KOH and piperidine was studied as recommended by Bock (27). Piperidine (10% aqueous solution) at 100° was found to degrade DNA-II consisting

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TABLE VIII

Extent of hydrolysis of ribonucleotide containing repair products to small oligonucleotides by 0.3 M KOH after 16 hours at 37°

Experimental conditions for the alkaline hydrolysis and chromatography on DEAE-cellulose paper are described under "Methods."

Label	Ribonucleotide	Percentage of counts moving from origin	Percentage of expected to move from origin
		%	%
a. [α-^{32}P]dTTP	None	2.2	0
[α-^{32}P]dTTP	CMP	48.5	50
[α-^{32}P]dTTP	GMP	90.0	100
b. [α-^{32}P]dGTP	None	5.1	0
[α-^{32}P]dGTP	CMP	38.8	40
c. [α-^{32}P]dATP	None	5.8	0
[α-^{32}P]dATP	CMP	6.5	0
[α-^{32}P]dATP	GMP	4.5	0

Summarized in Table VIII are the extents of hydrolysis found for various ribonucleotide-containing DNA's. The values are actually the extent of hydrolysis of labeled material to a size which moved away from the origin of a DEAE-cellulose paper strip when elution was performed with 0.4 M ammonium formate in 7 M urea. It can be seen that in all cases the observed values are very close to those expected. The higher values seen in some cases may have been inherent in the assay system since incomplete neutralization would have resulted in applying alkaline solutions to the DEAE-cellulose paper strips and this would have retarded the mobility of the radioactivity.

Characterization of Hydrolysis Products—The products from a number of reactions were characterized by electrophoresis in two dimensions (28) followed by degradation to 3′-nucleotides or partial spleen phosphodiesterase digestion. For example DNA-II was labeled with either [α-^{32}P]dGTP or [α-^{32}P]dTTP in the presence of CTP. Alkaline hydrolysis followed by electrophoresis of the repaired duplexes gave the expected products in both cases (Fig. 11). Thus, DNA-II labeled with [α-^{32}P]dTTP gave two products. One was a long oligonucleotide which gave

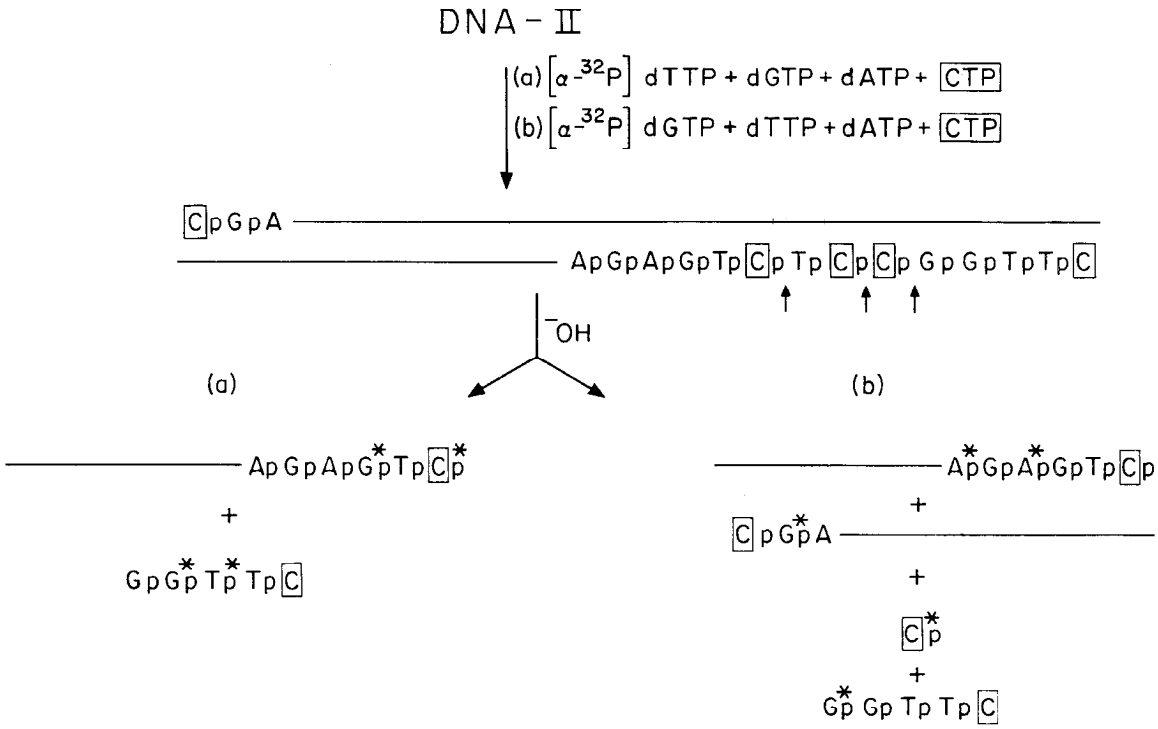

FIG. 11. Derivation of radioactive products from alkaline hydrolysis of DNA-II containing CMP and labeled with either [α-^{32}P]dTTP (*a*) or [α-^{32}P]dGTP (*b*). The symbol $\overset{*}{p}$ denotes ^{32}P.

only of deoxynucleotides to the extent of 57% in 20 hours. Consistent with this was the observation that DNA-II containing CMP was degraded more extensively than expected. The use of piperidine was therefore abandoned in favor of KOH which gave only the expected results.[1]

Included in Fig. 10 is the kinetics of hydrolysis of DNA-II containing CMP by 0.3 N KOH at 37°. Similarly fast degradation was observed for DNA-II containing GMP (data not shown). Complete degradation of both DNA's occurred at as low as 0.1 M KOH in 16 hours at 37°. Concentrated ammonia brought about only partial degradation of CMP and GMP containing DNA-II in 16 hours at 37°.

[1] Salzer *et al.* (8) have used a 10% piperidine solution at 50° for 16 hours for degradation at the ribonucleotide sites.

a dGp:Cp ratio of 1.14:1. The second product moved from the origin in the second dimension and gave a dGp:dTp ratio of 1.25:1, consistent with GpGpTpTpC.

DNA-II labeled with [α-^{32}P]dGTP gave three radioactive spots. One was a long oligonucleotide which did not move in the second dimension of electrophoresis and which gave only dAp upon nearest neighbor analysis. A second spot coincided with Cp. The third spot was GpGpTpTpC giving only dGp on both nearest neighbor analysis and partial spleen phosphodiesterase digestion.

Similarly, DNA-II repaired with [α-^{32}P]dATP in the presence of CTP and DNA-I repaired with [α-^{32}P]dTTP in the presence of CTP were characterized by analysis of the products formed by alkaline hydrolysis.

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DISCUSSION

The incorporation of ribonucleotides by *E. coli* DNA polymerase I into DNA was first described by Berg *et al.* (2) in 1963. Since then, surprisingly, only a limited number of reports have appeared (6–8) on this potentially useful approach to sequence analysis of DNA. In particular, no careful analysis of the fidelity of incorporation of ribonucleotides by *E. coli* DNA polymerase I in the presence of manganous ions has been reported. Such an analysis was undertaken in the present work with DNA templates of defined sequence. Similar DNA templates were previously used in a study of the DNA polymerase I-catalyzed repair reactions and conditions were established for complete and accurate repair with only deoxyribonucleoside 5′-triphosphates (12).

In the present work, maximum rates and extents of incorporation in the presence of ribonucleoside triphosphates were observed with 0.5 to 1.5 mM manganous ion concentrations. This range of concentration includes values used in the previous work; 1 mM (6, 7) and 0.67 mM (8). Of the four ribonucleotides, the incorporation of CMP was almost as fast as that of dCMP while GMP was incorporated to a lesser extent than dGMP at 10°. At 37°, however, GMP was incorporated at the same rate as dGMP. The incorporation of AMP occurred to only a very limited extent and UMP was not incorporated at all under the conditions used. The over-all results differ slightly from those of Berg *et al.* (2) who found more facile incorporation of GMP than CMP.

The most significant aspect of the present work has been the study of fidelity of incorporation and of minimizing any misincorporations. In the presence of [α-^{32}P]dTTP alone, Mn^{++} and DNA-I, almost 2 moles of dTMP were incorporated while in the presence of Mg^{++} ions only 1 mole of dTMP was incorporated. Thus Mn^{++} ions caused the formation of a T·G base pair instead of a C·G base pair. Further, misincorporations were detected when GTP incorporation was performed both at 10 and 37° (thus, [α-^{32}P]dATP was incorporated in place of GTP). Also, in the event that 2 or more consecutive guanine residues were to be incorporated, the incorporation of GMP failed to occur beyond this point. This would account for the lower plateau values obtained in the present work and in the original work of Berg *et al.* (2). Wu *et al.* (29), who have applied the same technique to the sequencing of the cohesive ends of the bacteriophage λ, have obtained results very similar to those reported here. They found that where 3 guanine or cytidine residues must be incorporated, DNA polymerase I is unable to complete the repair at low temperature. This is analogous to the incomplete repair of DNA-I and DNA-II at 10° in the presence of GTP.

As expected, alkaline treatment provided a specific means of cleavage of deoxyribopolynucleotide chains at the site of ribonucleotide residues. The expected products were formed and characterized. The use of specific RNases was also investigated. T_1 RNase was less effective than pancreatic RNase. The latter gave complete degradation within 2 hours while the former required several heat cycles and enzyme additions for complete hydrolysis.

As a tool for DNA sequencing, the method would appear to give complete fidelity only for CMP incorporation at 10°. The repair-synthesis catalyzed by DNA polymerase I has already proven useful in deriving the sequence of the cohesive ends of DNA. Combined with the limited ribonucleotide insertion, sequencing of longer segments of DNA should be feasible.

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