

Stimulation and Inhibition of Deoxyribonucleic Acid Nucleotidyltransferase by Oligodeoxyribonucleotides

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The importance of the physicochemical condition of primer DNA in the DNA nucleotidyltransferase (polymerase) system (deoxynucleoside triphosphate-DNA deoxynucleotidyltransferase, EC 2.7.7.7) from mouse Landschutz ascites-tumour cells has been emphasized by Keir, Binnie & Smellie (1962). The ability of DNA to prime the reaction was enhanced by 60% after limited pretreatment with pancreatic deoxyribonuclease, an effect which was suggested to be the consequence of increased availability of 3'-hydroxy-terminal groups for nucleophilic attack at the α -phosphate group of deoxyribonucleoside triphosphates. A more detailed investigation of the phenomenon has now been undertaken and is described in this paper, with kinetic studies of the action of DNA nucleotidyltransferase.

EXPERIMENTAL

Abbreviations. Thymidine 5'-triphosphate labelled with ^{32}P in the α -phosphate group, [^{32}P]TTP; thymidine 5'-monophosphate labelled with ^{32}P , [^{32}P]TMP. Diethyl-aminoethyl-, DEAE-. Deoxyribonuclease active in the region of pH 7 in the presence of Mg^{2+} ions and producing 5'-phosphoryl-terminal oligonucleotides, deoxyribonuclease I; deoxyribonuclease active at pH 5, not requiring added Mg^{2+} ions and producing 3'-phosphoryl-terminal oligonucleotides, deoxyribonuclease II (Laskowski, 1961).

Preparation of enzyme extracts. Soluble extracts of cells of the Landschutz ascites carcinoma were prepared as described by Keir *et al.* (1962), with the following modifications. After osmotic disruption in 20 vol. of water adjusted to pH 7 with KOH, solid KCl and 0.5M-potassium phosphate buffer, pH 7.5, were added without delay to give a solution 0.15M in KCl and 0.01M in potassium phosphate buffer, pH 7.5. The standard soluble extract was the supernatant fluid obtained by centrifuging at 105 000g for 60 min. in rotor no. 40 in the Spinco ultracentrifuge.

In a series of experiments designed to compare the deoxyribonuclease activities of certain fractions, four types of soluble extract were prepared from the same disrupted suspension: (a) prepared as described above for standard soluble extract; (b) prepared by centrifuging immediately after disruption and adding KCl and potassium phosphate buffer to the supernatant fraction; (c) prepared by adding KCl and potassium phosphate buffer, holding at 0° for 3 hr. then centrifuging; (d) prepared by holding at 0° for 3 hr., centrifuging, then adding KCl and potassium phosphate buffer to the supernatant fraction. As with the

standard soluble extract, the soluble extracts in this series had final concentrations of KCl and potassium phosphate buffer, pH 7.5, of 0.15M and 0.01M respectively, and protein concentrations of 1.5–2.0 mg./ml. For the experiment in Fig. 1 the soluble extract was prepared without delay in 0.05M-KCl-0.01M-potassium phosphate buffer, pH 7.5.

For further purification of the DNA nucleotidyltransferase activity, portions of the soluble extracts were taken to pH 5.9 by addition of 0.2N-acetic acid. After 5 min., the small precipitate which formed was removed by centrifuging at 12 000g for 10 min. in the SS-34 rotor of the Servall centrifuge. The pH of the supernatant fluid was lowered to 5.0 by further addition of 0.2N-acetic acid, and after exactly 5 min. the precipitate was collected by centrifuging at 12 000g for 10 min., dissolved in 0.15M-KCl-0.01M-potassium phosphate buffer, pH 7.5 (0.25 vol. of the soluble extract originally taken), and stored at 0°. After about 12 hr. a small precipitate formed and was removed by centrifuging. The final supernatant solution (termed the 'pH 5 precipitate' fraction) contained 55–75% of the total DNA nucleotidyltransferase activity of the soluble extract and 2–2.5 mg. of protein/ml. The other fractions had negligible activity.

The ascitic plasma was assayed for deoxyribonuclease activity after the freshly drawn ascitic fluid (made 0.075M with NaCl and 0.075M with KCl) had been centrifuged to remove all cells. The protein concentration was 12–13 mg./ml.

Enzyme assays. The assay for DNA nucleotidyltransferase was that described by Gray *et al.* (1960) with the modifications outlined in the Results section. At the end of the incubation period, the assay mixtures were cooled to 0° and 0.05 ml. of an aged pH 5 precipitate fraction was added to each mixture immediately before samples were plated on paper disks. The aged pH 5 precipitate fraction had lost all measurable DNA nucleotidyltransferase activity by storage at 0° for about 2 months, and was added to facilitate precipitation and retention of polydeoxyribonucleotide in the paper disks.

Deoxyribonuclease activity was measured in reaction mixtures identical with those used for the assay of DNA nucleotidyltransferase (see Fig. 1) except that deoxyribonucleoside triphosphates were omitted and the amounts were increased tenfold to a total volume of 2.5 ml. The samples and controls without DNA or without enzyme were incubated at 37° for 3 hr. The reaction was terminated by cooling to 0°, followed by rapid addition of 0.25 ml. of calf-thymus DNA solution (2 mg./ml.) and 0.25 ml. of bovine plasma albumin (2 mg./ml.) to facilitate subsequent sedimentation of acid-insoluble material. A portion (2 ml.) of 2.1N-HClO₄ was then added and the mixtures were kept at 0° for 10 min. before centrifuging to

remove the precipitate. The supernatant solutions were taken for measurement of E at 260 $m\mu$. The amount of Landschutz DNA rendered acid-soluble during incubation was calculated after subtraction of control values, assuming a molar extinction coefficient at 260 $m\mu$ in acid of 10 200 for the acid-soluble DNA fragments. This value is the average of the molar extinction coefficients in acid at 260 $m\mu$ of the 5'-monophosphates of deoxyadenosine, deoxycytidine, deoxyguanosine and thymidine (Burton, 1959), and is regarded as a close approximation to the true value for the acid-soluble DNA fragments, since oligonucleotides do not show hyperchromic effects at 260 $m\mu$ on hydrolysis when E values are measured at pH 2 or below (Staehelin, 1961).

Preparation of oligonucleotide fractions from deoxyribonuclease digests of DNA. (a) Oligonucleotide fractions bearing 5'-phosphoryl-terminal groups were prepared as follows: 140 mg. of Landschutz ascites-tumour DNA was dissolved in 100 ml. of water; the solution was made 0.01 M with tris-HCl buffer, pH 7.5, and 0.01 M with $MgCl_2$. A small portion was removed for measurement of E at 260 $m\mu$, and to the rest of the solution was added 0.5 ml. of a solution containing 2 mg. of crystalline pancreatic deoxyribonuclease/ml. (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) in 0.01 M-tris-HCl buffer, pH 7.5. The reaction mixture was incubated at 37° for 2 hr., during which small samples were removed, diluted with 0.01 M-tris-HCl buffer, pH 7.5-0.01 M- $MgCl_2$ for the measurement of E at 260 $m\mu$. A hyperchromic effect of 15% (relative to the DNA solution before addition of the deoxyribonuclease) was observed at the end of the 2 hr. incubation. Carboxymethylcellulose powder (300 mg.; Whatman CM 70) in the potassium form was added to adsorb deoxyribonuclease from the reaction mixture (Rosenkranz & Bendich, 1961) and the carboxymethylcellulose was then removed by centrifuging. To confirm the absence of deoxyribonuclease, 0.1 ml. of the resulting supernatant solution was added to 2.4 ml. of a solution of calf-thymus DNA (50 $\mu g./ml.$) in 0.02 M-tris-HCl buffer, pH 7.5-0.015 M- $MgCl_2$; no change in E at 260 $m\mu$ of the solution was observed in the course of 2 hr. at 37°. When a crystal of pancreatic deoxyribonuclease was added, there was a 30% increase in E . The carboxymethylcellulose supernatant fraction was then made 0.25 M with KCl and was applied to a column (15 cm. \times 2 cm.) of DEAE-Sephadex A-50, medium grade (Pharmacia, Uppsala, Sweden), equilibrated with 0.25 M-KCl-0.01 M-potassium phosphate buffer, pH 6.8. Oligonucleotides were eluted by this solution, followed by a gradient of increasing KCl concentration, the concentration of potassium phosphate buffer, pH 6.8, being 0.01 M throughout. The system incorporated two closed mixing chambers on the same level and containing 0.25 M-KCl-0.01 M-potassium phosphate buffer, pH 6.8, at the start of elution. The first mixing chamber was supplied with concentrated eluent from a reservoir that was adjusted to a suitable height to give a flow rate of 1 ml./min. Eluent from the reservoir was mixed (by magnetic stirring) in the first mixing chamber, and it flowed into the second chamber, where it was further stirred, and then passed into the column by gravitation. Periodically (see Figs. 3 and 4) the reservoir solution was changed to provide a higher KCl concentration. Fractions (7 ml.) were collected. The separation was followed by measuring E at 260 $m\mu$ (Fig. 3). Each of the pooled oligo-

nucleotide fractions I-IV was dialysed (Visking tubing $\frac{3}{8}$ in. inflated diam.; Hudes Merchandising Corp., London) with vigorous stirring for 30 min. against each of four to six 6 l. changes of water until chloride could no longer be detected in the diffusate. No ultraviolet-absorbing material was diffusible from fractions III and IV under these conditions, and only small amounts from fractions I and II. The fractions were concentrated by freeze-drying and were dialysed again to remove as much as possible of the remaining traces of KCl and potassium phosphate. Potassium chloride and potassium phosphate were removed completely from fraction IV by passage of portions (10 ml.) of the solution followed by water through columns (20 cm. \times 2 cm.) of Sephadex G-50, medium grade (Pharmacia, Uppsala, Sweden), with collection in 2.5-3.0 ml. fractions. Fraction III was treated in the same way but much of the oligonucleotide material in this case was contaminated with chloride and phosphate. The separations are shown in Fig. 5 (A and B). By combining eluates 5-12 in Fig. 5 (A), a preparation of oligonucleotide fraction IV free from inorganic salts was obtained and was used in the ensuing experiments designed to test its effect on DNA nucleotidyltransferase activity. Eluates 6-14 in Fig. 5 (B) were combined to give oligonucleotide fraction III, from which most of the inorganic salts had been removed. Satisfactory separation of salt from oligonucleotide material by this method was not obtained with fractions I and II of Fig. 3. In enzymic experiments with fractions I-III therefore, the content of KCl and potassium phosphate had to be taken into account.

(b) Oligonucleotide fractions bearing 3'-phosphoryl-terminal groups were prepared by the action of splenic deoxyribonuclease prepared as described by Koerner & Sinsheimer (1957*a, b*) from 600 g. of calf spleen. The final product, after chromatography on Celite 545, contained 1.1×10^6 units and was completely free from the phosphodiesterase and phosphomonoesterase activities described by the authors. A reaction mixture was set up, containing the following components in 149 ml.: 22.35 m-moles of KCl, 2.98 m-moles of ammonium acetate buffer, pH 4.5, 131 mg. of Landschutz ascites-tumour DNA and 118 000 units of splenic deoxyribonuclease. The reaction was followed by the increase in E at 260 $m\mu$; this reached 38% at the end of the 20 hr. incubation period. The solution was taken to pH 7 by addition of 2 N-KOH (about 2 ml.) and was heated for 20 min. at 80° to inactivate the enzyme. Solid KCl was added to a final concentration of 0.25 M, and the solution was applied to a column (15 cm. \times 2 cm.) of DEAE-Sephadex A-50, medium grade. Elution (Fig. 4), dialysis and concentration of the oligonucleotide fractions were carried out as in (a) above, but only fraction IV was completely separated from the remaining traces of KCl and potassium phosphate by passage through columns of Sephadex G-50 (Fig. 5). Fraction II contained oligonucleotide material, which largely coincided with the salt-containing fractions after attempts to purify it by the gel-filtration technique.

Removal of terminal phosphoryl groups from oligonucleotides. This was achieved by the action of alkaline phosphatase during incubation of the following mixture at 37° for 2 hr.: 28.4 E units (at 260 $m\mu$, pH 7) of 5'-phosphoryl-terminal oligonucleotide fraction IV, or 42.4 E units (at 260 $m\mu$, pH 7) of 3'-phosphoryl-terminal oligonucleotide fraction IV, 30 μ moles of $MgCl_2$, 30 μ moles of tris-HCl

buffer, pH 9.5, 100 μ g. of bacterial alkaline phosphatase (Nutritional Biochemicals Corp.). The total volume was 3 ml. After incubation, the mixture was heated for 10 min. at 100°. Inorganic orthophosphate was released from both the 3'- and 5'-phosphoryl-terminal oligonucleotides by this method; under the same conditions, for 2 hr. incubation, adenosine 3'-monophosphate and adenosine 5'-monophosphate at a concentration of 2 mM were converted quantitatively into adenosine and orthophosphate, and at 0.17 mM were 75% hydrolysed by the enzyme in 5 min. (Table 4). The effect of the bacterial phosphatase on the 5'-triphosphates of deoxyadenosine, deoxycytidine, deoxyguanosine and thymidine was tested in a 2.5 ml. reaction mixture of the following composition: 100 μ moles of tris-HCl buffer, pH 7.5, 10 μ moles of $MgCl_2$, 0.8 μ mole of EDTA, 100 μ moles of KCl, 0.5 μ mole of deoxyribonucleoside triphosphate and 30 μ g. of bacterial alkaline phosphatase. Inorganic orthophosphate was measured after 2 hr. at 37°. Under these conditions of pH and ionic composition,

which are closely similar to those employed in the DNA nucleotidyltransferase assay, 1.5 μ moles of inorganic orthophosphate were released from deoxyadenosine and deoxyguanosine 5'-triphosphates and 1.4 μ moles from deoxycytidine and thymidine 5'-triphosphates, indicating virtually complete conversion of the linear triphosphosphate unit into orthophosphate. This latter experiment was performed after it was found that the alkaline phosphatase was not completely destroyed by heating at pH 9.5 for 10 min. at 100° (see Results section), and that addition of dephosphorylated oligonucleotides to DNA nucleotidyltransferase assays would therefore include addition of some residual active alkaline phosphatase. This commercial preparation of bacterial alkaline phosphatase probably contains the alkaline phosphatase purified from *Escherichia coli* by Garen & Levinthal (1960), and shown by Heppel, Harkness & Hilmoe (1962) to be capable of hydrolysing deoxyribonucleoside triphosphates and of removing terminal phosphate from oligodeoxyribonucleotides.

Inorganic orthophosphate was estimated by the methods of Allen (1940) and Griswold, Humoller & McIntyre (1951). Total phosphate was estimated by the method of Allen (1940), in which extinctions were measured at the wavelength of maximal absorption of the final coloured solution, 725 μ .

Preparation of DNA. The procedures for the preparation, thermal denaturation and measurement of hyperchromicity of DNA isolated from Landschutz ascites-tumour cells and calf thymus gland were those described by Keir *et al.* (1962). On the basis of total phosphorus content of DNA solutions thus prepared, a solution at pH 7.0 giving an extinction coefficient at 260 μ of 1.0 contains approx. 45 μ g. of DNA/ml. The amounts of oligonucleotide fractions tested in the transferase assay are expressed as *E* units at 260 μ , i.e. the amount of oligonucleotide giving an extinction coefficient of 1.0 at pH 7 is 1.0 extinction unit. Hyperchromic effects resulting from thermal denaturation have been taken into account in the estimation of the amounts of DNA used for priming the DNA nucleotidyltransferase reaction.

[³²P]TTP was synthesized by Mr G. Russell according to the procedure described by Gray *et al.* (1960).

RESULTS

DNA nucleotidyltransferase activity in the soluble extract of ascites-tumour cells as a function of protein concentration, for various incubation periods, is shown in Fig. 1. The depressed specific activities at low enzyme concentrations have been consistently observed and may be the consequence of the presence of an activator in the enzyme preparation (Dixon & Webb, 1958).

A comparison is presented in Fig. 2 of the time-course of DNA nucleotidyltransferase activity in three ascites-cell fractions. The specific activity of the pH 5 precipitate fraction shows a sixfold purification over the soluble extract at 3 hr. There was a lag of about 15 min. with the pH 5 precipitate fraction and 30 min. with the soluble extract before the reaction assumed linear kinetics. The disrupted preparation showed a decline in syn-

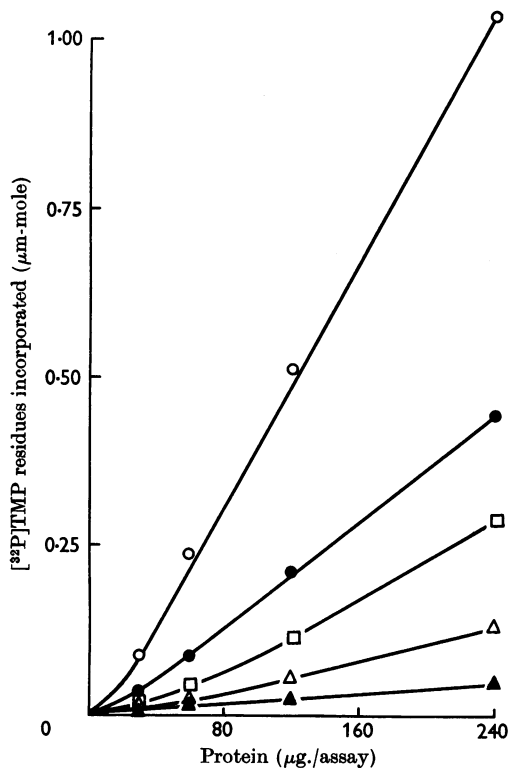


Fig. 1. DNA nucleotidyltransferase activity as a function of protein concentration. The reaction mixture contained, in a total volume of 0.25 ml.: 10 μ moles of tris-HCl buffer, pH 7.5, 9 μ moles of KCl, 0.5 μ mole of potassium phosphate buffer, pH 7.5, 0.08 μ mole of EDTA, 50 μ g. of thermally denatured Landschutz ascites-cell DNA, 1 μ mole of $MgCl_2$, 50 μ m-moles each of deoxyadenosine, deoxycytidine and deoxyguanosine 5'-triphosphates and [³²P]TTP (3.5×10^6 counts/min./ μ mole), and the indicated amounts of soluble-extract protein. Times (min.) of incubation at 37° were: 5 (▲); 10 (△); 20 (□); 30 (●); 60 (○).

thesis in the later stages of the reaction. The DNA nucleotidyltransferase and deoxyribonuclease I activities of fractions isolated after different conditions of disruption were therefore examined to determine whether release of deoxyribonuclease from subcellular particles could account for the lag phase in the synthetic reaction. The idea of leakage of the enzyme from subcellular particles seemed reasonable in the light of our findings that

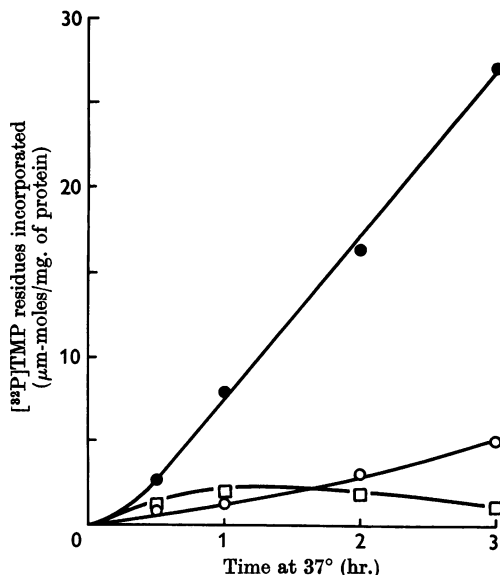


Fig. 2. Partial purification of DNA nucleotidyltransferase. Conditions of assay were as described in Fig. 1, with the following differences: specific activity of the $[^{32}\text{P}]\text{TTP}$ was 2.1×10^6 counts/min./ μmole ; assays contained $85 \mu\text{g}$. of protein from the enzyme fraction examined. \square , Disrupted cell suspension; \circ , soluble extract; \bullet , pH 5 precipitate fraction.

deoxyribonuclease I and deoxyribonuclease II activities are concentrated in these particles in Landschutz ascites-tumour cells (Keir & Aird, 1962). Two types of soluble extract were accordingly prepared: one from a disrupted suspension made 0.15M with KCl and 0.01M with potassium phosphate buffer, pH 7.5, immediately after disruption (soluble extract A), the other from a portion of the same preparation held at 0° for 3 hr., centrifuged at 105 000g and then made 0.15M with KCl and 0.01M with potassium phosphate buffer, pH 7.5 (soluble extract B). The deoxyribonuclease I activities of these and other fractions are presented in Table 1, from which it is seen that soluble extract A degraded more than twice as much DNA in 3 hr. as did soluble extract B. Most of the deoxyribonuclease I was removed, however, during preparation of the pH 5 precipitate fraction from soluble extract A. The higher deoxyribonuclease activity of soluble extract A compared with soluble extract B was paralleled by a higher DNA nucleotidyltransferase activity; the importance of this observation is emphasized by the work of Mantsavinos & Canellakis (1959), who found that addition of pancreatic deoxyribonuclease to the DNA-synthesizing system from cultured mouse lymphoblasts stimulated the reaction fivefold. The stimulatory effect of EDTA in our transferase-assay system at 0.1 mM (Smellie, 1961) is probably not directly related to an effect on deoxyribonuclease activity, since there were no outstanding differences in this respect in the presence and absence of EDTA when the soluble extracts were assayed for deoxyribonuclease. However, native DNA was degraded to only half the extent of thermally denatured DNA by the disrupted preparation and soluble extract, whereas native and thermally denatured DNA apparently served equally well as substrates for the activity of

Table 1. *Deoxyribonuclease activities of Landschutz ascites-tumour-cell fractions*

Preparation A was made 0.01M with potassium phosphate buffer, pH 7.5, and 0.15M with KCl immediately after disruption. Soluble extract A was then prepared from it without delay, and subsequently pH 5 precipitate A was prepared from soluble extract A. Preparation B (from the same batch of cells) was held at 0° for 3 hr. before centrifuging. The supernatant solution to which potassium phosphate buffer, pH 7.5, and KCl were added to 0.01M and 0.15M respectively was termed soluble extract B.

| Fraction | Percentage of DNA degraded/mg. of protein | | | DNA nucleotidyltransferase activity ($\mu\text{m-moles}$ of $[^{32}\text{P}]\text{-TMP}$ residues incorporated/mg. of protein/3 hr.) |
|--------------------|---|---------|----------------------|---|
| | Thermally denatured DNA | | Native DNA with EDTA | |
| | With EDTA | No EDTA | | |
| Ascitic plasma | 5 | 7 | — | — |
| Preparation A | 29 | 23 | 15 | — |
| Preparation B | 29 | 26 | — | — |
| Soluble extract A | 47 | 48 | 24 | 7.8 |
| Soluble extract B | 19 | 21 | — | 4.7 |
| pH 5 precipitate A | 5 | — | 5 | 29.9 |

Table 2. *Deoxyribonuclease and deoxyribonucleic acid nucleotidyltransferase activities of Landschutz ascites-cell fractions prepared in various ways*

All fractions were prepared from the same batch of cells. The pH 5 precipitates were obtained from the correspondingly numbered soluble extracts. I, Disrupted preparation held at 0° for 10 min., centrifuged and made 0.01M with potassium phosphate buffer, pH 7.5, and 0.15M with KCl; II, preparation held at 0° for 10 min., made 0.01M with potassium phosphate buffer, pH 7.5, and 0.15M with KCl, and then centrifuged; III, as for I but held at 0° for 3 hr. instead of 10 min.; IV, as for II but held at 0° for 3 hr. instead of 10 min. The protein concentrations were the same for each fraction in both assays, 380 µg./ml. for the soluble extracts and for pH 5 precipitate III, 370 µg./ml. for pH 5 precipitate I, 340 µg./ml. for pH 5 precipitate II and 320 µg./ml. for pH 5 precipitate IV. Figures in parentheses are the specific activities multiplied by the total mg. of protein in the fraction.

| Cell fraction | Deoxyribonuclease activity (% of DNA degraded/mg. of protein/3 hr.) | DNA nucleotidyltransferase activity (µm-moles of [³² P]TMP residues incorpor- ated/mg. of protein/3 hr.) |
|-------------------|---|---|
| Soluble extract I | 11.3 (1288) | 10.3 (1174) |
| II | 30.2 (3440) | 11.1 (1265) |
| III | 4.3 (490) | 8.8 (1005) |
| IV | 22.6 (2620) | 6.9 (786) |
| pH 5 fraction I | 6.9 (191) | 26.1 (723) |
| II | 7.2 (184) | 27.0 (689) |
| III | 4.8 (137) | 24.9 (710) |
| IV | 3.9 (93) | 29.3 (703) |

the pH 5 precipitate fraction. Further experiments (Table 2) confirmed and extended these results, and suggested that the presence of 0.15M-KCl-0.01M-potassium phosphate buffer, pH 7.5, in the disrupted preparation favoured extraction of deoxyribonuclease activity into the soluble fraction. Exposure of the disrupted preparation to the KCl-phosphate buffer for 3 hr. before centrifuging did not increase the deoxyribonuclease activity of the soluble extract; on the other hand it apparently extracted an 'inhibitory factor', which depressed the specific activity of the DNA nucleotidyltransferase by about 40% and the deoxyribonuclease by 25% relative to the extract prepared without delay in the KCl-phosphate buffer. Most of the 'inhibitory factor' and deoxyribonuclease I were removed from the soluble extracts during the ensuing acid fractionation. There have been many reports of the existence in mammalian cells of an inhibitor of deoxyribonuclease I (Laskowski, 1961).

Smellie & Eason (1961) were unable to detect transferase activity in Landschutz ascites-cell nuclei isolated in non-aqueous solvents, and they suggested that the enzyme was not nuclear in origin. Our results are consistent with this observation and with the concept that the enzyme has a soluble cytoplasmic location, because variations in extraction procedure markedly influenced the yields of deoxyribonuclease and 'inhibitory factor' in the soluble extracts, but did not affect the amounts of transferase subsequently obtained from such extracts by acid precipitation (Table 2). Extraction of transferase originating in cytoplasmic particles or in nuclei might have been expected to vary with the extraction procedure, as did the

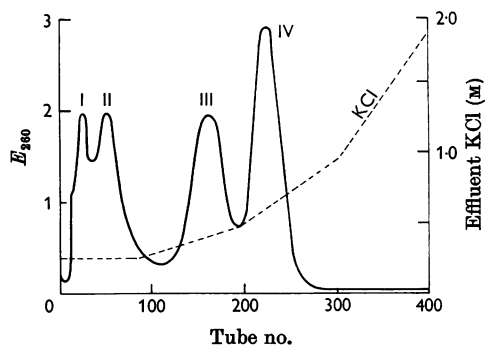


Fig. 3. Ion-exchange chromatography of components of a deoxyribonuclease I digest of DNA. Column: DEAE-Sephadex, A-50 (15 cm. x 2 cm.), equilibrated with 0.25M-KCl in 0.01M-potassium phosphate buffer, pH 6.8. Elution: tubes 1-90, 0.25M-KCl in 0.01M-potassium phosphate buffer, pH 6.8; thereafter, linear-gradient systems with 0.5M-KCl in 0.01M-potassium phosphate buffer, pH 6.8, in the reservoir at tube 91, 1.0M-KCl in 0.01M-potassium phosphate buffer, pH 6.8, at tube 191, 2.0M-KCl in 0.01M-potassium phosphate buffer, pH 6.8, at tube 301. Eluates (7 ml.) were collected at a flow rate of 1 ml./min., and were combined to give fractions I-IV as shown.

extraction of deoxyribonuclease and 'inhibitory factor'. Alternatively, the transferase may be so highly soluble that maximum yields are obtained under all extraction conditions used, no matter where it is located within the cell.

The results of ion-exchange chromatography of deoxyribonuclease digests of DNA are presented in Figs. 3 and 4. As was to be expected (cf. Laskowski, 1961), paper electrophoresis indicated that each

oligonucleotide peak was heterogeneous, and variations in ratios of E at 280–260 $m\mu$ between 0.47 and 0.59 in the pooled fractions suggested appreciable differences in gross base composition. The behaviour of the fractions on Sephadex (Fig. 5) is consistent with the expectation that shorter-chain oligonucleotides are eluted early in the ion-exchange chromatography, and longer-chain compounds in the later stages.

Measurements of the release of orthophosphate from terminal phosphoryl groups by the action of bacterial alkaline phosphatase showed that the average chain length of 5'-phosphoryl-terminal oligonucleotide fraction IV was 4.4 nucleotidyl units, and that of 3'-phosphoryl-terminal oligonucleotide fraction IV was 6.1 nucleotidyl units (Table 3).

Effect of 5'-phosphoryl-terminal oligonucleotides

Inclusion of 0.5 E unit of fractions I and II (from Fig. 3) in the DNA nucleotidyltransferase system slightly extended the lag period and reduced incorporation of [^{32}P]TMP residues at 3 hr., whereas fraction III reduced the lag and increased incorporation at 3 hr. and fraction IV, as well as substantially stimulating synthesis at 3 hr., completely eliminated the lag period (Fig. 6). The standard assay mixture (see Fig. 1) containing no oligonucleotide additions, but with 20 μg . of additional thermally denatured DNA instead (giving a

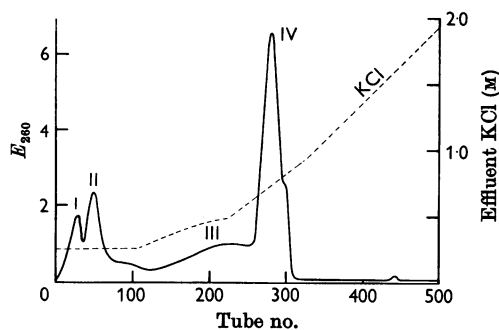


Fig. 4. Ion-exchange chromatography of the components of a deoxyribonuclease II digest of DNA. Column: DEAE-Sephadex, A-50 (15 cm. \times 2 cm.) equilibrated with 0.25 M-KCl in 0.01 M-potassium phosphate buffer, pH 6.8. Elution: tubes 1–102, 0.25 M-KCl in 0.01 M-potassium phosphate buffer, pH 6.8; thereafter, linear-gradient systems with 0.5 M-KCl in 0.01 M-potassium phosphate buffer, pH 6.8, in the reservoir at tube 103, 1.0 M-KCl in 0.01 M-potassium phosphate buffer, pH 6.8, at tube 223, 1.5 M-KCl in 0.01 M-potassium phosphate buffer, pH 6.8, at tube 308, 2.0 M-KCl in 0.01 M-potassium phosphate buffer, pH 6.8, at tube 368, and 2.0 M-KCl in 0.02 M-ammonium acetate buffer, pH 4.5, at tube 426. Eluates (7 ml.) were collected at a flow rate of 1 ml./min., and were combined to give fractions I–IV as shown.

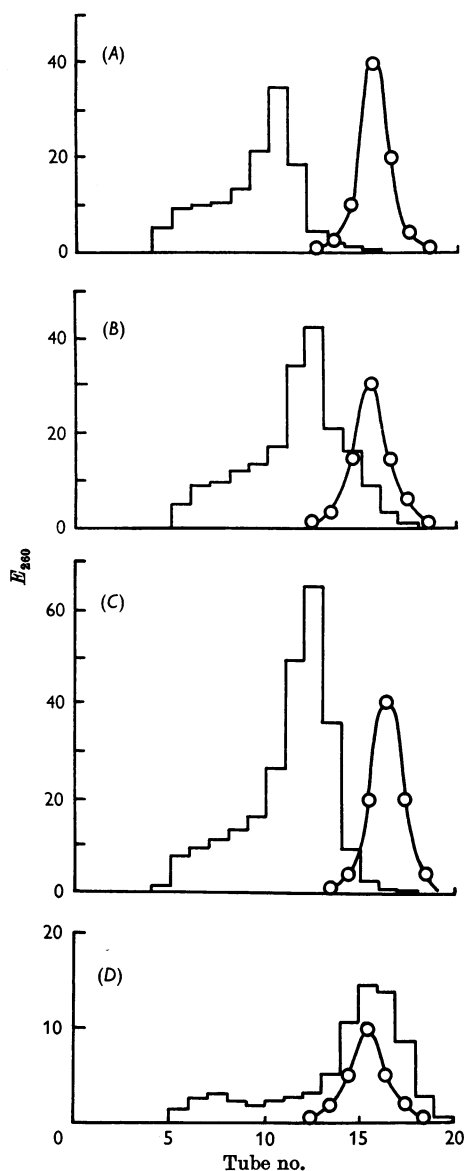


Fig. 5. Gel-filtration of oligonucleotide fractions on columns of Sephadex. Columns: Sephadex G-50, 20 cm. \times 2 cm. for (A) and (B), 21 cm. \times 2 cm. for (C) and (D). Samples (10 ml.) were applied and washed through with water, 2.5–3.0 ml. fractions being collected at a flow rate of 0.5 ml./min. (A) Fraction IV from a pancreatic-deoxyribonuclease digest of DNA (Fig. 3). (B) Fraction III from the same digest. (C) Fraction IV from a splenic-deoxyribonuclease digest of DNA (Fig. 4). (D) Fraction II from the same digest. \circ , Positions and approximate relative amounts of chloride and orthophosphate in the eluates. Fractions 5–12 of (A) and 5–13 of (C) were combined and used respectively as sources of 5'-phosphoryl-terminal and 3'-phosphoryl-terminal oligonucleotides in the ensuing experiments.

Table 3. *Estimation of average chain length of oligonucleotide fractions*

Terminal phosphorus released by the action of bacterial alkaline phosphatase was estimated by the method of Griswold *et al.* (1951) and also by the method of Allen (1940). Total phosphorus in the fractions was estimated by the method of Allen (1940) after digestion with HClO_4 . E values at 260 $m\mu$ were 14.2 for the 5'-phosphoryl fraction and 21.2 for the 3'-phosphoryl fraction.

| Oligonucleotide fraction | Terminal phosphorus ($\mu\text{g./ml. of fraction}$) | Total phosphorus ($\mu\text{g./ml. of fraction}$) | Ratio total/terminal phosphorus |
|---|--|---|---------------------------------|
| 5'-Phosphoryl-terminal fraction IV (Fig. 3) | 13 | 57 | 4.4 |
| 3'-Phosphoryl-terminal fraction IV (Fig. 4) | 13 | 79.5 | 6.1 |

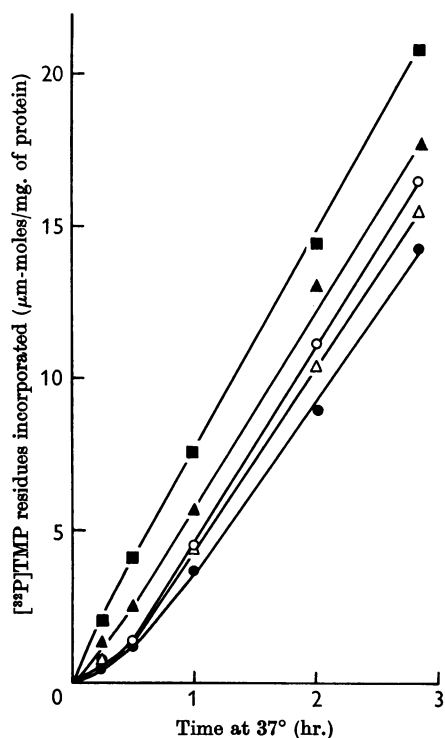


Fig. 6. Effects of 5'-phosphoryl-terminal oligonucleotide fractions on DNA nucleotidyltransferase activity. Conditions of assay were as described in Fig. 1 with the following differences: specific activity of the $[^{32}\text{P}]\text{TTP}$ was 5.3×10^6 counts/min./ μmole ; enzyme source was 87 $\mu\text{g.}$ of pH 5 precipitate-fraction protein; 0.5 E unit of oligonucleotide fraction was added as shown: \circ , no addition; \bullet , fraction I; \triangle , fraction II; \blacktriangle , fraction III; \blacksquare , fraction IV.

total of 70 $\mu\text{g.}$), produced an incorporation curve similar to that of the standard assay but with a slightly extended lag period. When the experiment was performed with 0.5 E unit of any one of the four oligonucleotide fractions but without the standard 50 $\mu\text{g.}$ of thermally denatured DNA in the reaction mixture, the highest incorporation value obtained was only 0.36 $\mu\text{m-mole}$ of $[^{32}\text{P}]\text{TTP}$ residues/mg. of protein/3 hr., with fraction IV.

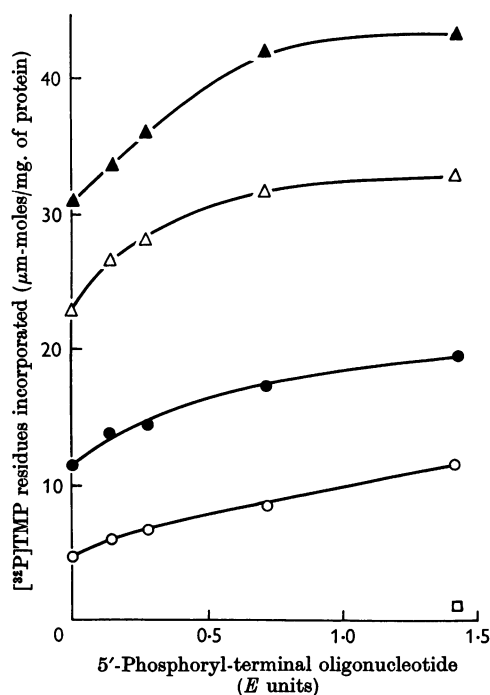


Fig. 7. Stimulation of DNA nucleotidyltransferase activity by a 5'-phosphoryl-terminal oligonucleotide fraction. Conditions of assay were as described in Fig. 1 with the following differences: specific activity of the $[^{32}\text{P}]\text{TTP}$ was 1.65×10^6 counts/min./ μmole ; enzyme source was 125 $\mu\text{g.}$ of pH 5 precipitate-fraction protein; the indicated amounts of 5'-phosphoryl-terminal oligonucleotide fraction IV (in E units) were included as shown. Incubations were carried out for varying times (min.) at 37°: \circ , 30; \bullet , 60; \triangle , 120; \blacktriangle , 180; \square , 180 with no DNA primer present.

The stimulatory effect of addition of increasing amounts of fraction IV is shown in Fig. 7. Enhancement of the synthetic reaction by 1.4 E units of fraction IV varied from 142% at 30 min. to 37% at 3 hr. However, addition of this amount of fraction IV to an assay mixture containing no thermally denatured DNA gave the low incorporation of 0.8 $\mu\text{m-mole}$ of $[^{32}\text{P}]\text{TTP}$ residues/mg. of protein/3 hr.

From the data in Fig. 7, 0.71 *E* unit of the 5'-phosphoryl-terminal oligonucleotide in the presence of 50 μg . of thermally denatured DNA brought about incorporation of 5.25 μm -moles of [^{32}P]TMP residues in 3 hr. Assuming that identical amounts of deoxyadenylyl, deoxycyidylyl and deoxyguanylyl residues were also incorporated from the 5'-triphosphates of deoxyadenosine, deoxycytidine and deoxyguanosine, and that the average molecular weight of a deoxyribonucleotidyl residue is 327, it can be calculated that 6.9 μg . of polydeoxyribonucleotide was synthesized. 0.71 *E* unit of the oligonucleotide contained 2.85 μg . of P, which is equivalent to 30 μg . of oligonucleotide. If it is then assumed that the total polydeoxyribonucleotide incorporated into the product, on the DNA primer as template, was 30 μg . from oligonucleotides (of 4.4 nucleotidyl units average chain length) plus 6.9 μg . synthesized from triphosphates, giving 36.9 μg ., then overall incorporation was equivalent to 74 % of the DNA primer.

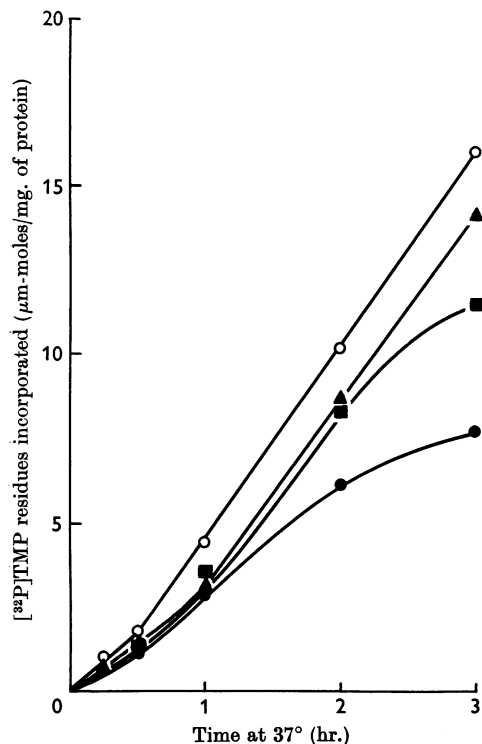


Fig. 8. Effect of 3'-phosphoryl-terminal oligonucleotide fractions on DNA nucleotidyltransferase activity. Conditions of assay were as described in Fig. 1 with the following differences: specific activity of the [^{32}P]TTP was 3.6×10^6 counts/min./ μmole ; enzyme source was 75 μg . of pH 5 precipitate protein; 0.5 *E* unit of oligonucleotide fraction was included as indicated: \circ , no addition; \bullet , fraction I; \blacksquare , fraction II; \blacktriangle , fraction IV.

Effect of 3'-phosphoryl-terminal oligonucleotides

In contrast with the 5'-phosphoryl derivatives, the 3'-phosphoryl-terminal oligonucleotides extended the lag period and reduced the overall synthesis at 3 hr. (Fig. 8). The shorter-chain fractions were more effective than equal amounts of the longer-chain compounds in these respects. The inhibitions could not be attributed to contaminating KCl and potassium phosphate from the DEAE-Sephadex columns as the concentrations of these salts in the assay mixtures arising from the oligonucleotide fractions were less than 1 mM and not sufficient to influence the activity of DNA nucleotidyltransferase (Smellie, 1961). Inhibition of the reaction increased progressively with increased amounts of fraction IV in the assay mixture (Fig. 9), and for 2.1 *E* units of the oligonucleotide fraction inhibition ranged from 58 % in 30 min. incubation to 51 % at 3 hr. If 2.1 *E* units of thermally denatured DNA (in addition to the standard 50 μg .) were added instead of 2.1 *E* units of the oligonucleotide fraction, an inhibition of 2 % was obtained. If the supplementary DNA was increased

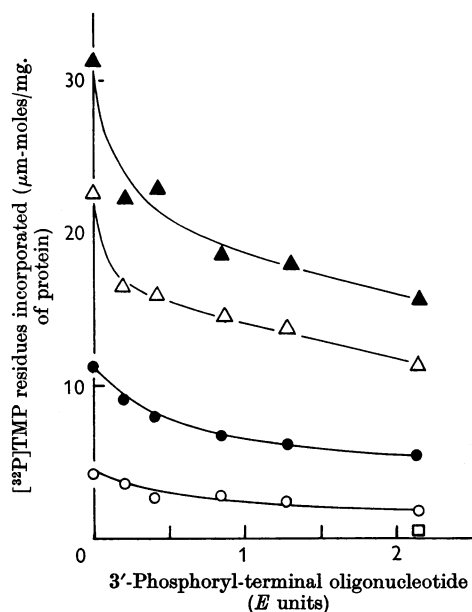


Fig. 9. Inhibition of DNA nucleotidyltransferase activity by a 3'-phosphoryl-terminal oligonucleotide fraction. Conditions of assay were as described in Fig. 1 with the following differences: specific activity of the [^{32}P]TTP was 1.65×10^6 counts/min./ μmole ; enzyme source was 125 μg . of pH 5 precipitate protein; indicated amounts (in *E* units) of 3'-phosphoryl-terminal oligonucleotide fraction IV were included. Incubations were at 37° for the following times (min.): \circ , 30; \bullet , 60; \triangle , 120; \blacktriangle , 180; \square , 180 with no DNA primer present.

to a total of 6.5 *E* units the inhibition was 27%, still well below the effect obtained with 2.1 *E* units of the oligonucleotide fraction. Incorporation of [³²P]TTP residues in the presence of this amount of the oligonucleotide but in the complete absence of thermally denatured DNA was less than 0.3 μmole/mg. of protein/3 hr.

Effects of removal of terminal phosphoryl groups from oligonucleotides by bacterial alkaline phosphatase

Removal of the 3'-phosphoryl-terminal groups from oligonucleotide fraction IV was accompanied by a loss of the inhibitory activity associated with this fraction (Fig. 10). The previously observed lag phase was also eliminated. In contrast, removal of the 5'-phosphoryl-terminal group from the activating oligonucleotide fraction resulted in a 55%

depression of priming activity, but there was no concomitant introduction of a lag period such as that displayed by 3'-phosphoryl-terminal oligonucleotides or with thermally denatured DNA alone. The two oligonucleotide fractions thus freed of their terminal phosphoryl groups permit priming of the reaction to a similar extent, as might be predicted from the availability of 3'-hydroxy-terminal groups in them. There was a 22% priming superiority of the dephosphorylated oligonucleotide derived from the 3'-phosphoryl fraction over that of an equal amount (measured by total phosphorus content of the dephosphorylated compound) of that derived from the 5'-phosphoryl fraction (Fig. 11). This may be attributed to the

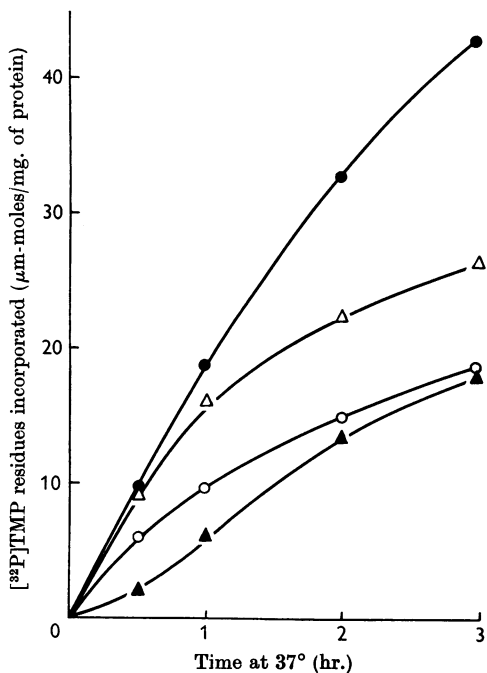


Fig. 10 Effects of removal of terminal phosphoryl groups from stimulatory and inhibitory oligonucleotides on DNA nucleotidyltransferase. Conditions of assay were as described in Fig. 1 with the following differences: specific activity of the [³²P]TTP was 1.65×10^6 counts/min./μmole; enzyme source was 125 μg. of pH 5 precipitate protein; in addition to the standard amount of tris-HCl buffer, pH 7.5, 1 μmole of tris-HCl buffer, pH 9.5, was present; ●, 1.0 *E* unit of 5'-phosphoryl-terminal oligonucleotide fraction IV; ○, 1.0 *E* unit of 5'-phosphoryl-terminal oligonucleotide fraction IV after treatment with bacterial alkaline phosphatase; ▲, 1.33 *E* units of 3'-phosphoryl-terminal fraction IV; △, 1.33 *E* units of 3'-phosphoryl-terminal oligonucleotide fraction IV after treatment with bacterial alkaline phosphatase.

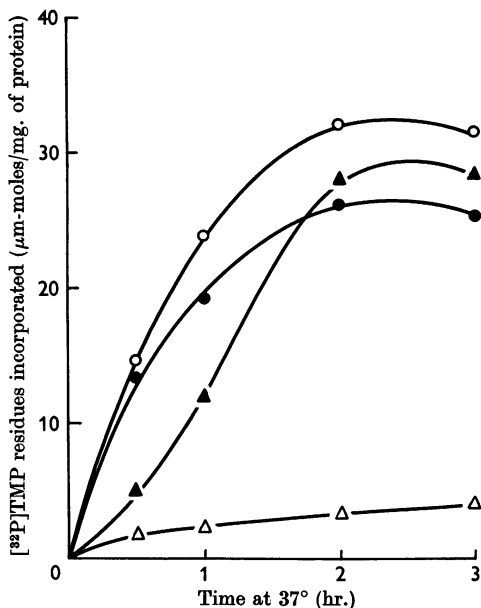


Fig. 11. Comparison of the effects on DNA nucleotidyltransferase of pretreatment of oligonucleotides and DNA primer with alkaline phosphatase. The assay mixture contained, in a total volume of 0.25 ml.: 10 μmoles of tris-HCl buffer, pH 7.5, 9 μmoles of KCl, 0.5 μmole of potassium phosphate buffer, pH 7.5, 0.08 μmole of EDTA, 50 μmoles each of deoxyadenosine, deoxycytidine and deoxyguanosine 5'-triphosphates and [³²P]TTP (4.5×10^6 counts/min./μmole), 50 μg. of thermally denatured Landschutz ascites-cell DNA, and 80 μg. of pH 5 precipitate protein. Also included was 0.1 ml. of an incubated and then heated (10 min. at 100°) bacterial alkaline-phosphatase reaction mixture (see text) containing 1.0 *E* unit of oligonucleotide fraction from the 5'-phosphoryl-terminal fraction IV (●), or 1.0 *E* unit of oligonucleotide from 3'-phosphoryl-terminal oligonucleotide fraction IV (○), or no oligonucleotide (△), or no oligonucleotide and no phosphatase (▲). 1.0 *E* unit of each dephosphorylated oligonucleotide fraction was equivalent to 2.7 μg. of diesterified phosphate in the compound.

Table 4. *Observations on the heat stability of bacterial alkaline phosphatase*

Reaction mixtures contained the following components in 3 ml.: 0.5 μ mole of AMP, 30 μ moles of $MgCl_2$, 30 μ moles of tris-HCl buffer, pH 9.5, and 100 μ g. of bacterial alkaline phosphatase. In Expt. I incubation was started immediately after addition of the phosphatase. In Expt. II, the reaction mixture, before addition of substrate, was heated for 10 min. at 100°, cooled quickly to 37° and then incubated in the presence of substrate. Phosphatase action was terminated by cooling to 0° followed by addition of $HClO_4$ as in the Allen (1940) method of phosphorus estimation.

| Substrate | Time at 37° (min.) | Inorganic phosphate released (% of theoretical maximum) |
|----------------------------|--------------------|---|
| Adenosine 3'-monophosphate | 5 | 76 |
| | 10 | 83 |
| Adenosine 5'-monophosphate | 5 | 74 |
| | 10 | 88 |
| Adenosine 3'-monophosphate | 5 | 15 |
| | 10 | 32 |
| Adenosine 5'-monophosphate | 5 | 16 |
| | 10 | 34 |

greater average chain length of the former oligonucleotide, which, for a given amount of enzyme, would permit synthesis of more long-chain polynucleotide than would the same amount (in *E* units) of the shorter-chain derivative, and would therefore favour retention of more radioactive acid-insoluble polynucleotide in the nucleotidyltransferase assay.

It was found that heating the alkaline-phosphatase reaction mixtures after incubation did not destroy all the phosphatase activity (Table 4). The presence of heat-treated alkaline phosphatase in the standard assay for nucleotidyltransferase resulted in drastic inhibition of synthesis; however, substantial synthesis with elimination of the lag phase was obtained in the accompanying experiments with dephosphorylated oligonucleotides (Fig. 11).

DISCUSSION

Precise measurement of DNA nucleotidyltransferase activity in soluble fractions prepared from Landschutz ascites-tumour cells is technically difficult owing to the relatively small amount of synthesis that takes place, to the wide variety in chain length of polynucleotide material which might theoretically be produced, and because acid-soluble short-chain oligonucleotides synthesized during incubation would not be detected.

The inhibitory effect (Fig. 6) of short-chain 5'-phosphoryl-terminal oligonucleotides might be attributed to the acid-solubility of slightly longer

oligonucleotides synthesized from them during the early phase of incubation. On the other hand, the stimulation promoted by an equal amount (in *E* units) of longer-chain oligonucleotides is probably due to the smaller number of 3'-hydroxy-terminal groups, diminished competition for the active site of the nucleotidyltransferase and to consequent earlier synthesis of acid-insoluble polydeoxyribonucleotide. The lag phase observed in the absence of stimulatory oligonucleotides (Figs. 2, 6 and 11) probably arises from the initial requirement for formation of suitable terminal groups in the DNA primer by action of the endogenous deoxyribonuclease I (Tables 1 and 2). It is unlikely that the DNA primer serves merely to co-precipitate oligonucleotides that are synthesized from the oligonucleotide additions and which would otherwise be acid-soluble, because primer added at the end of the incubation period does not alter the low incorporation observed with oligonucleotides tested alone as primers (Fig. 7). Moreover, exposure of DNA to a low pH destroys its hydrogen-bonded configuration (Jordan, 1960), so that, during acid precipitation in the assay used here, it is expected that small acid-soluble oligonucleotides hydrogen-bonded to the DNA primer are released into solution and only oligonucleotides long enough to be acid-insoluble are retained for measurement of radioactivity.

It is thus believed that the action of these stimulatory oligonucleotides is to form hydrogen bonds with single-stranded regions of the DNA primer and to provide priming centres (the 3'-hydroxyl groups), from which polymerization of nucleotidyl units can be initiated along the adjacent DNA single strand according to its base sequence. Without this DNA template the rate of synthesis is low, whereas the reaction with the template but without oligonucleotides to start the synthesis exhibits a lag during which deoxyribonuclease I builds up a supply of suitable end groups in the primer molecules. The reaction eventually slows, probably because of excessive deoxyribonuclease activity but also because of maximum possible replication of single-stranded regions in the thermally denatured DNA primer, for the primer is regarded as a complex collapsed structure containing some double-stranded regions (Doty, Boedtger, Fresco, Hasekorn & Litt, 1959), which presumably are unable to participate in the synthetic reaction. Data in Fig. 7 indicate that many stimulatory oligonucleotide units can act simultaneously in the synthetic process by providing nucleophilic centres for the single-stranded regions, and that a saturation point for the number of oligonucleotide molecules is reached. It is therefore inferred that these oligonucleotides are incorporated into the product and that they occupy a proportion of the template, allowing the remainder of it to

be utilized for polymerization of nucleotidyl units from triphosphates. Single-stranded regions of the DNA primer could well present a complementary base sequence to the oligonucleotides, for these were prepared enzymically from DNA from the same source.

The product synthesized from triphosphates and oligonucleotides amounted to 74% of the DNA primer after 3 hr. incubation (Fig. 7). At this stage the reaction was approaching completion (this is inferred by plotting the specific activities against incubation time in the reaction containing 0.71 *E* unit of oligonucleotide). Much of the remaining 26% of the primer could then be regarded as sections that were double-stranded at the outset and which did not participate in the reaction. If, as is suggested above, the oligonucleotides found specific sites for hydrogen-bonding on the template, then the reaction would represent extensive replication of the primer.

The most efficient primer would theoretically be the single-stranded DNA from bacteriophage ϕ X174 (Sinsheimer, 1959), and this has been shown to be so in our Landschutz ascites-cell extracts (Keir *et al.* 1962).

The inhibitory effect of 3'-phosphoryl-terminal oligonucleotides becomes readily understandable on the basis of their inability to accept nucleotidyl units from triphosphates and also of competition for the active site of the nucleotidyltransferase with 3'-hydroxyl groups in the DNA primer.

Removal of terminal phosphoryl groups from oligonucleotides by the action of bacterial alkaline phosphatase abolishes the inhibition associated with 3'-phosphoryl-terminal groups and diminishes a proportion of the stimulation induced by 5'-phosphoryl-terminal oligonucleotides (Figs. 10 and 11). Comparison of the stimulatory effects of dephosphorylated oligonucleotides of two different average chain lengths, 4.4 and 6.1 nucleotidyl units, reveals (Fig. 11) that the longer-chain fraction gives a slightly better synthetic effect than an equal amount of the shorter oligonucleotide. The incorporation curves in these experiments may have been altered owing to incomplete inactivation of the alkaline phosphatase (Table 4). However, despite inhibition by the phosphatase alone, substantial stimulation and synthesis are observed with the dephosphorylated oligonucleotides, which probably contained alkaline phosphatase. The inhibition by heat-treated alkaline phosphatase may be due to removal of stimulatory 5'-phosphoryl-terminal groups in the DNA primer, but the absence of an early lag phase suggests that removal of a few 3'-phosphoryl groups likely to be in the primer also produced an early but small stimulation. It is also possible that the deoxyribonuclease I in the pH 5 precipitate

fraction may be inhibited by the action of the phosphatase, since Vanecko & Laskowski (1961) have indicated that deoxyribonuclease I is influenced by the nature of the terminal groups in the substrate. Thus the incorporation curve for DNA primer plus heated alkaline phosphatase may actually represent a greatly extended lag effect.

Stimulation of polydeoxyribonucleotide synthesis by deoxyribonuclease I has been observed in other mammalian systems (Mantsavinos & Canellakis, 1959; Sarkar, 1961), and the partially purified DNA nucleotidyltransferase from calf thymus gland (Bollum, 1960*a*) was found to contain appreciable deoxyribonuclease activity although the relationship between the degradative and synthetic activities was not fully investigated. However, the calf-thymus enzyme seems to utilize chemically synthesized members of the homologous series of thymidylate polymers as primers (Bollum, 1960*b*) and it would be interesting to know whether these oligonucleotides act by accepting nucleotidyl units from triphosphates or by serving as templates or in both ways.

It would seem that the group of enzymes responsible for production of polydeoxyribonucleotides bearing 5'-phosphoryl- or 3'-phosphoryl-terminal groups and for removal of these groups from polydeoxyribonucleotide may be essential components of the system controlling the biosynthesis of DNA. In this respect, they might well be included in the pattern of regulatory mechanisms of DNA metabolism described by Davidson (1961).

SUMMARY

1. Soluble extracts prepared directly from osmotically disrupted suspensions of Landschutz ascites-tumour cells contained less deoxyribonuclease activity than extracts prepared in the presence of potassium chloride-phosphate buffer. Increased deoxyribonuclease activities were accompanied by increased deoxyribonucleic acid nucleotidyltransferase activities. Prolonged extraction procedures yielded preparations containing a factor that diminished these increased activities of deoxyribonuclease and nucleotidyltransferase.

2. Acid fractionation removed much of the deoxyribonuclease activity from the soluble extracts and purified the preparation sixfold with respect to the nucleotidyltransferase.

3. The lag phase observed under standard conditions of assay of the transferase in soluble extracts and in the partially purified fraction was eliminated by addition of certain 5'-phosphoryl-terminal oligonucleotides from a pancreatic-deoxyribonuclease digest of deoxyribonucleic acid; also, the extent of synthesis of polydeoxyribo-

nucleotide was increased, and, assuming that the product was derived from oligonucleotides and deoxyribonucleoside triphosphates, the synthesis amounted to 74% of the deoxyribonucleic acid primer.

4. The nucleotidyltransferase was inhibited and the lag phase extended by 3'-phosphoryl-terminal oligonucleotides from a splenic-deoxyribonuclease digest of deoxyribonucleic acid.

5. The compounds obtained by removal of terminal phosphoryl groups from the 3'- and 5'-phosphoryl-terminal oligonucleotides by bacterial alkaline phosphatase abolished the lag phase, and they supported synthesis at a level similar to that observed under standard assay conditions.

6. It was concluded that 5'-phosphoryl-terminal oligonucleotides associate by specific hydrogen-bonding with single-stranded regions of the deoxyribonucleic acid primer and stimulate synthesis of polydeoxyribonucleotide by providing 3'-hydroxyl groups to initiate polymerization along the adjacent single-stranded template. The 5'-phosphoryl-terminal group on these oligonucleotides is partly responsible for the stimulation observed. Inhibition by 3'-phosphoryl-terminal oligonucleotides is attributable to competition with 3'-hydroxyl groups formed on the deoxyribonucleic acid primer by deoxyribonuclease during the nucleotidyltransferase assay, and the lag phase observed under standard conditions is a period of production of stimulatory end groups in the deoxyribonucleic acid primer.

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The Degradation of Acid-Soluble Collagen by Rat-Liver Preparations

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In the development of normal connective tissue, the mature collagen fibres are stable and, metabolically, relatively inert (Neuberger, Perrone & Slack, 1951). In certain physiological and pathological conditions, however, a rapid turnover of collagen has been demonstrated. Thus Harkness &

Harkness (1954) showed that the collagen of the involuting uterus was rapidly resorbed, and Jackson (1957) showed that, although connective tissue growth was stimulated by carrageenin, the newly formed granulation tissue was metabolically unstable and was rapidly degraded, being replaced