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# Deoxyribonucleic Acid Nucleotidyltransferase from Landschütz Ascites-Tumour Cells

## PARTIAL PURIFICATION AND GENERAL PROPERTIES

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1. A purification procedure for DNA nucleotidyltransferase from Landschütz ascites-tumour cells is described. The enzyme can be separated from endogenous nucleic acid and from triphosphatase and deoxyribonuclease activities measurable at pH 7·5. 2. The basic properties of the nucleotidyltransferase reaction are as follows. The enzyme has optimum activity at pH 7·2–7·4. It displays an absolute requirement for DNA-primer, thermally-denatured DNA serving three to ten times as efficiently in this respect as native DNA. Maximum synthesis of polydeoxyribonucleotide occurs in the presence of all four deoxyribonucleoside 5′-triphosphates, but a limited incorporation of mononucleotide into polynucleotide is observed when the system is provided with only one triphosphate, or with various combinations of mono-, di- and tri-phosphates. The reaction requires the presence of a bivalent cation, and of those tested, $Mg^{2+}$ ions were by far the most effective. Manganous ions promoted synthesis but to a much smaller extent. Calcium ions did not support synthesis at all. At the appropriate concentrations, the univalent cations (sodium and potassium) stimulated the reaction by 25% and 125% respectively. The presence of EDTA in the reaction mixture stimulates the system five- to ten-fold. 3. The storage characteristics of the enzyme (as well as the activities of the various fractions) improve markedly if EDTA and 2-mercaptoethanol are included in the enzyme solution and in all preparative buffer solutions. 4. The enzyme loses more than 95% of its activity after heating for 1 min. at 45°. If the heating is conducted in the presence of DNA, the enzyme becomes relatively heat-resistant (presumably as a consequence of complex-formation with the DNA) and may actually display an activation effect. This is discussed in relation to a possible molecular conformation of the enzyme. 5. The product of the nucleotidyltransferase reaction is precipitable by acid or ethanol, and is susceptible to the actions of deoxyribonucleases I and II, snake-venom and spleen phosphodiesterases, and micrococcal nuclease. It forms a band in a density gradient of caesium chloride at a density similar to that of the DNA-primer. 6. By the criteria of nearest-neighbour frequency analyses, the product of the nucleotidyltransferase reaction has the characteristics to be expected of a polynucleotide synthesized in accordance with the template directions of the primer.

DNA nucleotidyltransferase (EC 2.7.7.7) from Landschütz ascites-tumour cells catalyses incorporation of radioactivity from [$\alpha$-$^{32}$P]dTTP* into polydeoxyribonucleotide in the presence of dATP, dCTP, dGTP, $Mg^{2+}$ ions and DNA-primer. We have previously described the activity of this system with respect to DNA-primer (Keir, Binnie & Smellie, 1962) and various oligodeoxyribonucleotide primers (Keir, 1962), and under conditions of inhibition by actinomycin D (Keir, Omura & Shepherd, 1963) and by certain thiol-group inhibitors (Keir & Shepherd, 1965). These experiments were conducted with partially purified preparations of the nucleotidyltransferase from which the bulk of contaminating deoxyribonuclease (EC 3.1.4.5) had been removed. In this paper we

* Abbreviations: dATP, dCTP, dGTP and dTTP, the 5′-triphosphates of deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine respectively. Monophosphates (MP) and diphosphates (DP) are represented similarly. In keeping with the original paper of Josse, Kaiser & Kornberg (1961), dinucleotide sequences derived from nearest-neighbour frequency analyses of the polynucleotide product of the nucleotidyltransferase reaction are denoted by ApC[deoxyadenylyl-(3′-5′)-deoxycytidine], GpT[deoxyguanylyl-(3′-5′)-deoxythymidine] etc.

present details of further purification of the enzyme which include a technique that effects essentially complete separation from deoxyribonuclease. Some general properties of the reaction are also described together with information on the nature of the product.

## EXPERIMENTAL

*Biological material.* Landschütz ascites-tumour cells were maintained by serial transplantation in mice (Porton strain). The ascitic fluid was harvested 6–7 days after inoculation by collection at 0° in an equal volume of 0·154M-NaCl. The cells were sedimented by centrifugation at 500*g* for 5 min., and washed twice in 10–20 vol. of 0·154M-NaCl by suspension followed by centrifugation at 500*g*. Finally, the cells were sedimented at 1500*g*, the supernatant fluid was decanted and the centrifuge tubes were thoroughly drained and their inner walls wiped with absorbent tissue to remove surplus NaCl solution.

*Partial purification of the enzyme.* All operations were performed at 0–4°. Cells from 20 to 100 mice could be dealt with satisfactorily in one operation.

Fraction 1: initial soluble extract. The washed ascites-tumour cells were suspended in 15 vol. of 1mM-EDTA–5mM-2-mercaptoethanol and homogenized in a Potter–Elvehjem homogenizer (Teflon pestle, 0·736 in. diam.; tube, 20 ml. capacity; both obtained from Sireica, Jamaica, N.Y., U.S.A.; clearance between tube and pestle, 0·005–0·007 in.), four to six passes of the pestle at 500–600 rev./min. being sufficient to ensure virtually complete cell lysis with minimal nuclear disruption. The preparation was made 0·01M with respect to potassium phosphate buffer, pH 7·5, and 0·15M with respect to KCl. Fraction 1 was the supernatant fluid obtained by centrifuging the disrupted cell preparation at 105000*g* for 1 hr. (Spinco model L ultracentrifuge, rotor no. 40).

Fraction 2: pH 5 precipitate. Fraction 1 was taken to pH 5 and the precipitated material collected as described by Keir (1962). Without delay, the precipitate was suspended in 0·2M-potassium phosphate buffer (pH 7·5)–1mM-EDTA–5mM-2-mercaptoethanol and stirred mechanically for 2–3 hr. Insoluble material was removed by centrifugation, and the resulting supernatant solution was termed fraction 2.

Fraction 3: $(NH_4)_2SO_4$ precipitate. Saturated $(NH_4)_2SO_4$ solution (adjusted to pH 7·5 with aq. $NH_3$ solution and containing 1mM-EDTA) was added to fraction 2 with mechanical stirring to give 20% saturation. After being stirred for 10 min., the preparation was centrifuged at 12000*g* for 10 min. (Servall centrifuge, rotor no. SS-34) and the sediment was discarded. Further saturated $(NH_4)_2SO_4$ solution was added to the supernatant solution with stirring to raise the degree of saturation to 45%, and after 10 min. the precipitate was sedimented by centrifugation as above. It was dissolved in 1mM-potassium phosphate buffer (pH 7·5)–1mM-EDTA–5mM-2-mercaptoethanol and dialysed for 3 hr. with constant stirring against 7 l. of the same buffer solution. The slightly opalescent, dialysed solution was termed fraction 3.

Fraction 3a: DEAE-cellulose eluate. To demonstrate whether there was an absolute requirement for DNA-primer in the nucleotidyltransferase reaction, it was necessary to submit the enzyme preparation to a procedure which would remove nucleic acid. The technique adopted was similar to that described by Bollum (1960) for removal of nucleic acid from an enzyme preparation derived from calf thymus tissue. DEAE-cellulose (Whatman DE11, chloride form) was washed by decantation in 2M-NaCl and was packed in a 6 cm. × 2 cm. column. After extensive washing with 2M-NaCl, the column was equilibrated with 0·15M-potassium phosphate buffer (pH 7·5)–1mM-EDTA–5mM-2-mercaptoethanol. A portion of fraction 3 containing 35 mg. of protein was made 0·15M with respect to potassium phosphate buffer, pH 7·5, and was applied to the column. Washing was continued with the buffer with which the column was equilibrated, and the effluent solution was collected in fractions. Under these conditions, 25–50% of the applied nucleotidyltransferase was collected in a protein peak (fraction 3a) in the effluent fluid. The ratio of extinction at 280 m$\mu$ to that at 260 m$\mu$ in the effluent fractions was about 1·5, whereas it was 0·5–0·6 in fraction 3 before application to the column. This indicates that contaminating nucleic acid was retained on the column. Support for this conclusion was obtained by washing the column with 0·5M-potassium phosphate buffer, pH 7·5, which eluted a sharp peak of material with ratio $E_{280}:E_{260}$ about 0·75. Moreover, direct chemical analysis (see below) of fractions 3 and 3a confirmed that RNA and DNA had been removed from the enzyme preparation by the treatment with DEAE-cellulose.

Fraction 4: hydroxyapatite eluate. Hydroxyapatite (Hypatite C; Clarkson Chemical Co. Inc., Williamsport, Pa., U.S.A.) equilibrated with 1mM-potassium phosphate buffer (pH 7·5)–mM-EDTA–5mM-2-mercaptoethanol was packed in a 10 cm. × 1 cm. column under pressure (25 cm. Hg) applied from an air pump, and washed with the same buffer. A portion of fraction 3 containing 25–30 mg. of protein was applied to the column under pressure and elution was carried

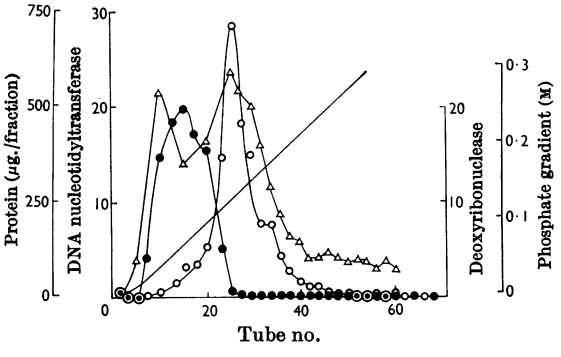


Fig. 1. Column chromatography of DNA nucleotidyltransferase on hydroxyapatite. A portion of fraction 3 (3 ml., corresponding to about 28 mg. of protein) was applied to a column (10 cm. × 1 cm.) of hydroxyapatite. Protein was eluted with a linear gradient of increasing concentration of potassium phosphate buffer, pH 7·5. ○, Activity of DNA nucleotidyltransferase in m$\mu$moles of [$^{32}$P]dTMP incorporated/fraction (0·1 ml. portions were assayed); ●, activity of deoxyribonuclease in $\mu$g. of DNA rendered acid-soluble/assay (0·25 ml. portions were assayed); △, $\mu$g. of protein/fraction.

out by using a linear gradient of potassium phosphate buffer, pH 7·5 (see Fig. 1). The mechanics of the system were such that the mixer and reservoir vessels were connected to the system without application of pressure. Elution was then started and continued under a pressure of 25 cm. Hg. The gradient was linear from 0·05 M- to 0·3 M-phosphate with a flow rate 8–10 ml./hr. Fractions (2 ml.) were collected in tubes each containing 300 $\mu$g. of thermally denatured DNA, which was included to stabilize the nucleotidyltransferase eluted from the column.

*Enzyme assays.* The basic details of the assay for DNA nucleotidyltransferase have been described by Gray *et al.* (1960) and improvements have since been reported (Keir *et al.* 1962; Keir & Shepherd, 1965). The assays were carried out in 3 ml. stoppered test tubes in a total volume of 0·25 ml. The standard assay mixture contained 5 $\mu$moles of tris–HCl buffer, pH 7·5, 0·5 $\mu$mole of potassium phosphate buffer, pH 7·5, 15 $\mu$moles of KCl, 0·1 $\mu$mole of EDTA, 1 $\mu$mole of $MgSO_4$ or $MgCl_2$, 1·25 $\mu$moles of 2-mercaptoethanol, 50–100 $\mu$g. of thermally denatured DNA, 50–75 m$\mu$moles each of dATP, dCTP, dGTP and [$\alpha$-$^{32}$P]dTTP ($3\times10^6$–$20\times10^6$ counts/min./$\mu$mole), and 15–150 $\mu$g. of protein from the DNA-nucleotidyltransferase fraction. Incubation was at 37°. Departure from these conditions for specific purposes will be described in the Results section.

Deoxyribonuclease activity was assayed by measurement of substrate (DNA) rendered acid-soluble under the incubation conditions in which the nucleotidyltransferase was assayed, with the exception that the triphosphates were omitted and the reaction mixture was scaled up fivefold (Keir, 1962).

Triphosphatase activity of the nucleotidyltransferase-containing fractions was measured under the conditions for assay of deoxyribonuclease except that DNA was omitted from the reaction mixture and dATP, dCTP, dGTP or dTTP was present at a concentration of 0·4 mM. After incubation at 37°, acid precipitation of protein was carried out as described for the deoxyribonuclease assay (Keir, 1962) and acid-soluble inorganic orthophosphate released from the individual triphosphates was estimated by the method of Allen (1940), extinctions being measured at 725 m$\mu$, the wavelength of maximal absorption of the final coloured solution.

*Preparation of DNA.* The method of Kay, Simmons & Dounce (1952) was followed for isolation of DNA from the sediments obtained by centrifuging the disrupted cell preparations at 105000*g* as described above. The DNA was dissolved in distilled water to give a concentration of 1·5 mg./ml. When it was to be used as primer in the standard nucleotidyltransferase assay, it was first denatured by heating for 10 min. at 100° and then rapidly cooling in an ice bath. In experiments designed to measure the priming ability of native DNA (double-stranded), the preparations were checked for absence of denatured material (single-stranded) by chromatography on columns of kieselguhr coated with methylated albumin as described by Mandell & Hershey (1960) and Sueoka & Cheng (1962). A solution of DNA (180 $\mu$g. in 2 ml. of 0·15 M-NaCl–0·015 M-sodium citrate) was applied to a column of methylated albumin on kieselguhr (3 cm. × 1 cm.) which had been thoroughly washed with 0·4 M-NaCl–0·05 M-potassium phosphate buffer, pH 6·8. DNA was eluted from the column by a linear salt gradient delivered from a reservoir and mixing vessel containing respectively 200 ml. of 2·0 M-NaCl–0·05 M-potassium phosphate buffer, pH 6·8, and 200 ml. of 0·5 M-NaCl–0·05 M-potassium phosphate buffer, pH 6·8. The gradient was applied to the column under pressure from a small peristaltic pump (Micropump type T; The Distillers Company Ltd., Epsom, Surrey). Fractions (2 ml.) were collected from the column and extinction values of each fraction were read at 260 m$\mu$. The patterns obtained showed a single peak of DNA eluted at 0·65–0·75 M-NaCl (native DNA), but no ultraviolet-absorbing material was eluted in the region of 0·9–1·0 M-NaCl where denatured DNA would be expected to emerge (Mandell & Hershey, 1960; Sueoka & Cheng, 1962). Therefore, by this criterion, the DNA preparations obtained as described above were free from single-stranded material. The sedimentation coefficient ($S_{20,w}$) of the DNA was 26 s as determined in the Spinco model E analytical ultracentrifuge.

*Isolation of the product of the DNA nucleotidyltransferase reaction.* A standard nucleotidyltransferase reaction mixture scaled up tenfold was incubated at 37° for 30 min. and placed in an ice bath. Solid NaCl was added to a final concentration of 1 M, and 2 vol. of ice-cold ethanol was then added to precipitate the DNA from the solution. The precipitate was collected by centrifugation (1500*g*, 10 min.) and was dissolved with stirring in 18 ml. of 0·15 M-NaCl–0·015 M-sodium citrate. A portion (2 ml.) of 1·5 M-NaCl–0·15 M-sodium citrate was then added and the preparation dialysed for 3 hr. against 250 ml. of 0·15 M-NaCl–0·015 M-sodium citrate. A portion of the non-diffusible material was diluted to 2 ml. with 0·15 M-NaCl–0·015 M-sodium citrate and 2·7 g. of CsCl was added. The refractive index of the resulting solution was then adjusted to 1·4 in the Abbé refractometer by addition of small amounts of water or solid CsCl. This represents a density of 1·727 g./cm.$^3$ (cf. Ifft, Voet & Vinograd, 1961). Solutions of native and of thermally-denatured DNA were similarly made up in CsCl. The three solutions (nucleotidyltransferase product, native DNA, thermally denatured DNA) were transferred to tubes in the SW 39 rotor of the Spinco model L ultracentrifuge, and each was overlaid with 1 ml. of liquid paraffin. The samples were then centrifuged for 64 hr. at 33000 rev./min. (89500*g*) and 25°. Fractions (3 drops each) were collected through a syringe needle (no. 12 hypodermic, Record fitting) inserted into the base of each tube. The fractions were diluted to 1 ml. with water and their extinction values measured at 260 m$\mu$. Duplicate portions, 0·5 ml., from the fractions from the CsCl gradient containing the radioactive nucleotidyltransferase product were plated on stainless-steel planchets and measured for $^{32}$P content in a Nuclear–Chicago D47 gas-flow counter.

*Nearest-neighbour frequency analysis.* This analysis was conducted essentially as described by Josse *et al.* (1961) but with several modifications designed to meet the requirements of the Landschütz nucleotidyltransferase reaction system. Basically, the technique consists of isolation of enzymically synthesized polydeoxyribonucleotide from four reaction mixtures each containing the four deoxyribonucleoside 5′-triphosphates, only one of which (a different one in each reaction) is labelled with $^{32}$P in the $\alpha$-phosphate position. The isolated product is then degraded to deoxyribonucleoside 3′-monophosphates by the consecutive action of micrococcal nuclease (EC 3.1.4.7) and spleen phosphodiesterase (EC 3.1.4.1), and the four mononucleotides from each reaction are separated by electrophoresis on paper and their $^{32}$P contents measured.

Each nucleotidyltransferase reaction mixture (0·27 ml.) contained 5 $\mu$moles of tris–HCl buffer, pH 7·5, 1 $\mu$mole of $MgSO_4$, 0·1 $\mu$mole of EDTA, 12·5 $\mu$moles of KCl, 80 $\mu$g. of thermally denatured Landschütz ascites-tumour cell DNA, 3 $\mu$moles of 2-mercaptoethanol, 100 $\mu$g. of protein from fraction 3 and 75 m$\mu$moles each of dATP, dCTP, dGTP and dTTP (one of the four triphosphates being labelled with $^{32}P$ in the $\alpha$-phosphate group). The specific activities of the dATP, dCTP and dGTP were $3\times10^6$–$6\times10^6$ counts/min./$\mu$mole, and of the dTTP $3\times10^6$–$20\times10^6$ counts/min./$\mu$mole. After incubation for 30 or 60 min. at 37°, the enzymically synthesized product was isolated and digested to deoxyribonucleoside 3′-monophosphates as described by Josse *et al.* (1961). The completeness of digestion to the 3′-monophosphates was checked by measuring the release of $^{32}P$ from nucleotide material by the action of alkaline phosphatase from *Escherichia coli* (Josse & Swartz, 1963). More than 94% of the nucleotide-bound $^{32}P$ was susceptible to the action of the phosphatase and therefore, by this criterion, was present as monoesterified phosphate. Each micrococcal nuclease–spleen phosphodiesterase digest was then concentrated and subjected to electrophoresis on paper for separation into the component 3′-monophosphates. Electrophoresis was carried out in a Shandon high-voltage electrophoresis apparatus on strips of Whatman 3MM paper (72 cm. × 15 cm.). The applied voltage was 2500 at a potential drop of 40 v/cm. along the paper, the buffer used was 0·05 M-ammonium formate, pH 3·5, and the running time was 2¼–2¾ hr. The nucleotide bands were located in ultraviolet light. Under these conditions, the 3′-monophosphates of deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine moved about 4, 9, 20 and 27 cm. respectively. Each band was cut out, cut into pieces of area about 1 $cm.^2$, and immersed in counting vials containing 10 ml. of liquid scintillation fluid [2 g. of 2,5-diphenyloxazole plus 100 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 473 ml. of toluene]. The $^{32}P$ content of each sample was then determined in a Nuclear–Chicago model 6725 liquid-scintillation spectrometer. Calculation of the frequencies of nearest-neighbour nucleotide sequences was performed as described by Josse *et al.* (1961).

*Miscellaneous analytical methods.* Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), DNA by the indole procedure according to Ceriotti (1955), and RNA by the orcinol reaction (Mejbaum, 1939). The standards used in these determinations were respectively bovine plasma albumin (2 mg./ml. in water; Armour Pharmaceutical Co., Eastbourne, Sussex), DNA from Landschütz ascites-tumour cells (1·5 mg./ml. in 0·1 N-NaOH; prepared according to the method of Kay *et al.* 1952) and highly polymerized RNA from yeast (1·5 mg./ml. in 0·1 N-NaOH; British Drug Houses Ltd., Poole, Dorset). The concentrations of the solutions of DNA and RNA were checked by estimation of total organically bound phosphorus by the method of Allen (1940).

*Materials.* dATP, dCTP and dGTP labelled with $^{32}P$ in the $\alpha$-phosphate group were obtained from the International Chemical and Nuclear Corp., City of Industry, Calif., U.S.A.; [$\alpha$-$^{32}P$]dTTP was synthesized as described by Gray *et al.* (1960). Non-radioactive deoxyribonucleoside 5′-mono- and 5′-tri-phosphates were purchased from Pabst Laboratories, Milwaukee, Wis., U.S.A., and from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. Deoxyribonucleoside 5′-diphosphates were purchased from British Drug Houses Ltd. Chromatographically purified micrococcal nuclease was purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A., and chromatographically purified alkaline phosphatase from *Escherichia coli* and spleen phosphodiesterase from the Sigma (London) Chemical Co. Ltd.

## RESULTS

*Partial purification of DNA nucleotidyltransferase.* The course of purification of the nucleotidyltransferase is shown in Table 1. This represents one experiment in which all fractionation steps quoted in the Experimental section were performed consecutively on an average preparation of the soluble extract. The actual value quoted for the degree of purification from the soluble extract (fraction 1) depends very largely on the condition of the cells at the time of removal from the mice (cf. Keir & Shepherd, 1965). To some extent this also affects the activities at each stage of the fraction-

Table 1. *Purification of DNA nucleotidyltransferase*

Transferase and nuclease activities were measured in the standard assay systems. Before enzyme assay, a small portion of fraction 2 and the eluates from the hydroxyapatite column were dialysed against 1 mM-potassium phosphate buffer (pH 7·5)–1 mM-EDTA–5 mM-2-mercaptoethanol to give enzyme fractions containing phosphate at a concentration low enough to be used in the standard assays. Since only one-third (3 ml.) of fraction 3 was taken for chromatography on hydroxyapatite, the recovery data for fraction 4 have been multiplied threefold. The activity of nucleotidyltransferase in fraction 4 in m$\mu$moles of [$^{32}P$]dTMP incorporated/mg. of protein is the observed value for the peak fractions emerging from the column.

| Fraction | Volume (ml.) | Total protein (mg.) | [$^{32}P$]dTMP incorporated (m$\mu$moles per mg. of protein) | [$^{32}P$]dTMP incorporated (m$\mu$moles per fraction) | Deoxyribonuclease (mg. of DNA rendered acid-soluble/fraction) | Purification of DNA nucleotidyltransferase |
|---|---|---|---|---|---|---|
| 1 | 242 | 514 | 4·43 | 2277 | 48 | 1·0 |
| 2 | 75 | 255 | 11·18 | 2851 | 21 | 2·5 |
| 3 | 9 | 85 | 18·72 | 1591 | 6 | 4·2 |
| 4 | 141 | 17 | 48·03 | 528 | 0 | 10·8 |

ation. For example, soluble extracts have been obtained with activities up to 15 mμmoles of [$^{32}$P]-dTMP incorporated/mg. of protein/hr., and activities up to 90 mμmoles/mg. of protein/hr. have been obtained for subsequent fractions. This probably reflects the delicate nature of the enzyme (Keir & Shepherd, 1965) and also variable stimulatory and inhibitory effects exerted by contaminating nucleases (Keir *et al.* 1962; Keir, 1962). Much of the deoxyribonuclease content of fraction 1 is removed during preparation of fraction 2 as it remains soluble at pH 5 (Keir, 1962), and this probably accounts for the increase of total nucleotidyltransferase activity observed after preparation of fraction 2. The degree of purification of nucleotidyltransferase at this stage depends on the protein concentration during addition of acid to fraction 1; purifications up to sixfold have been achieved with removal of up to 90% of the nuclease activity (Keir, 1962). Fraction 3a (see the Experimental section) was prepared only when it was necessary to demonstrate the requirement for DNA-primer. The yield of transferase was usually rather poor (15–30%). However, subsequent experiments (W. M. Shepherd & H. M. Keir, unpublished work) have shown that preparation of fraction 3a under different adsorption and elution conditions gives a high recovery of the transferase (84%) and a two- to three-fold purification. The activity per mg. of protein quoted in Table 1 for fraction 4 refers only to the peak transferase fractions of the hydroxyapatite eluates free from deoxyribonuclease. The progressive removal of deoxyribonuclease at the preceding stages of this experiment is also recorded in Table 1. Fractions 1, 2 and 3 were tested for ability to degrade dATP, dCTP, dGTP and dTTP under conditions similar to those obtaining in the nucleotidyltransferase assay (see the Experimental section). Only a very small amount of each triphosphate was degraded by fraction 1, and there was essentially no triphosphatase activity in fractions 2 and 3. In the experiments to be described, the activity of the nucleotidyltransferase was linear with respect to time of incubation at least up to 2 hr. at 37°, and with respect to protein concentration at least up to 150 μg. of protein per standard assay.

### *Basic features of the nucleotidyltransferase reaction*

*pH optimum.* The activity of the nucleotidyltransferase was determined at various pH values between pH 7 and 9 with tris–hydrochloric acid buffer. The activity was maximal at pH 7·2–7·4. At pH 7·0 it was 70% of maximum and at pH 9·0 it was 10% of maximum.

*Requirement for a DNA-primer.* A nucleotidyltransferase preparation (fraction 3a) from which nucleic acid had been removed by passage of a portion of fraction 3 through DEAE-cellulose was unable to catalyse incorporation of [$^{32}$P]dTMP from [$^{32}$P]dTTP into an acid-insoluble product in the absence of DNA-primer. Maximum incorporation was approached with 50 μg. of DNA primer in the assay (cf. Keir *et al.* 1962). Experiments conducted with fraction 3 (i.e. before treatment with DEAE-cellulose) gave essentially the same results, suggesting that there was no primer for the reaction in the enzyme preparation. Direct chemical analysis showed that fraction 3 contained only 3·6 μg. of DNA and 4·2 μg. of RNA/mg. of protein and that these were absent from fraction 3a. In the nucleotidyltransferase assays, fraction 3 (100–150 μg. of protein/assay) would contribute less than 0·5 μg. of DNA or RNA to the reaction, and therefore it is clear that the 0·5 μg. of DNA (which is almost certain to be present as native, double-stranded DNA in fraction 3) cannot give a significant priming effect. This may be partly due to the fact that native, double-stranded DNA is markedly inferior to thermally denatured, single-stranded DNA in serving as primer for this enzyme (Keir *et al.* 1962). The difference between the priming capacities of native and denatured DNA is observed throughout the purification procedure although the ability to accept native DNA as primer declines with increasing purification. Thus the priming capacities of native DNA in one experiment with fractions 1, 2 and 3 were 36%, 25% and 10% respectively of the values obtained with thermally denatured DNA. The explanation of this is by no means certain but one possibility arises from the fact that deoxyribonuclease is progressively removed during the fractionation procedure. Since stimulation of nucleotidyltransferase activity is effected by introduction of 3′-hydroxy terminal groups into the DNA primer by the limited action of deoxyribonuclease (Keir *et al.* 1962; Keir, 1962), it is probable that the relative capacities of native DNA to act as primer with fractions 1, 2 and 3 can be ascribed to the diminishing stimulatory effect of the endogenous deoxyribonuclease as purification proceeds. Related possible explanations will be discussed below. RNA preparations from yeast and from Krebs II ascites-tumour cells did not prime the reaction significantly (< 2% of the activity of denatured DNA).

*Requirement for deoxyribonucleoside 5′-triphosphates.* The activity of fraction 3 was measured in conditions where the four triphosphates were replaced by certain combinations of mono-, di- and triphosphates. The values for $^{32}$P incorporation are given in Table 2 as percentages of incorporation in the standard assay. The four monophosphates apparently do not serve as substrates in the reaction, but, if the [$^{32}$P]dTMPis replaced by [α-$^{32}$P]dTTP,

Table 2. *Activity of DNA nucleotidyltransferase with various combinations of nucleotide precursors*

Standard conditions of assay for the nucleotidyltransferase were used, with 50 mμmoles of each mononucleotide as indicated. Protein (150 μg.) from fraction 3 was taken for each assay.

| Substrates | DNA-nucleotidyltransferase activity (% of standard assay) |
|---|---|
| dAMP, dCMP, dGMP, [$^{32}$P]dTMP | 0 |
| dADP, dCDP, dGDP, [α-$^{32}$P]dTDP | 9·2 |
| dAMP, dCMP, dGMP, [α-$^{32}$P]dTTP | 24·7 |
| dADP, dCDP, dGDP, [α-$^{32}$P]dTTP | 41·6 |
| [α-$^{32}$P]dTTP | 1·8 |

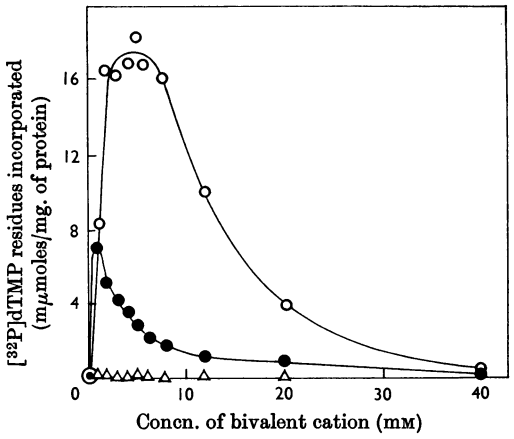


Fig. 2. Effects of certain bivalent cations on the DNA nucleotidyltransferase reaction. Fraction 3 (150 μg. of protein/assay) was assayed under standard conditions except that the bivalent cation content was varied as indicated. ○, $MgSO_4$; ●, $MnCl_2$; △, $CaCl_2$.

incorporation of label to 25% of the standard is observed. This cannot be attributed solely to the [α-$^{32}$P]dTTP because only a very low level of incorporation is observed when it is not supplemented by any other mononucleotide. A probable explanation is that fraction 3 is capable of catalysing transphosphorylation reactions between dTTP and the monophosphates and diphosphates, and among the four diphosphates, so that significant amounts of the four triphosphates would be formed and DNA synthesis catalysed by the nucleotidyltransferase would follow. This proposal is supported by the observations that when the [α-$^{32}$P]dTTP is supplemented with diphosphates the incorporation of [$^{32}$P]dTMP residues is higher than when it is supplemented with monophosphates, and that the four diphosphates together give a small but significant incorporation of [$^{32}$P]dTMP. In related experiments in which, in the standard assay, dGTP was replaced by dGMP, or dCTP by dCMP, or dATP by dAMP, or [α-$^{32}$P]dTTP by [$^{32}$P]dTMP, incorporation values expressed as percentages of the standard assay were 45·8, 50·4, 28·3 and 0·3 respectively. Clearly then synthesis proceeds optimally only when all four deoxyribonucleoside 5′-triphosphates are present.

Experiments in which the nucleotidyltransferase reaction rate was determined at various triphosphate concentrations (up to 1·2 mM total triphosphate) showed that the activity was essentially maximal at 0·6 mM. In these experiments, all four triphosphates were present in equal amounts. A triphosphate concentration of 50 to 75 mμmoles of each triphosphate per 0·25 ml., as in the standard assay, therefore allows nucleotidyltransferase activity to proceed at saturating concentrations of triphosphates.

*Bivalent cation requirement.* The nucleotidyltransferase reaction does not proceed in the absence of a bivalent cation: $Mg^{2+}$ ions are most effective in fulfilling this role (Fig. 2), optimum activity being attained at 4–8 mM; $Mn^{2+}$ ions also support synthesis but much less effectively and with a much lower optimum (2 mM). At $Mn^{2+}$ concentrations higher than 2 mM, the DNA-primer precipitates as the manganese salt and presumably cannot participate in the reaction. $Ca^{2+}$ ions do not promote synthesis at all; other evidence has shown that $Zn^{2+}$ likewise is ineffective, and, even in the presence of the optimum amount of $Mg^{2+}$, is strongly inhibitory (Keir & Shepherd, 1965).

*Response to univalent cations.* $Na^+$ and $K^+$ stimulate the nucleotidyltransferase reaction maximally at 40 and 50 mM respectively (Fig. 3); at higher concentrations, these ions inhibit incorporation and at 200 mM the inhibition exerted is over 90%.

*Effect of EDTA.* Inclusion of a small amount of EDTA in the nucleotidyltransferase reaction mixture invariably stimulates the activity of the enzyme. The degree of stimulation is usually five- to ten-fold, but occasionally enzyme preparations have been used which had virtually no activity in the absence of EDTA. The optimum range of EDTA in the reaction mixture is 0·3–0·5 mM; higher concentrations give progressively lower activities, and at 1·2 mM less than 10% of the maximum incorporation remains. Presumably the enzyme fractions and the assay reagents contain small amounts of cations that inhibit the reaction (cf. Fig. 2) and low concentrations of EDTA remove these cations by chelation without disturbing the optimum $Mg^{2+}$ concentration (cf. Williams, 1959). At higher concentrations of EDTA the $Mg^{2+}$ presumably also is chelated. The apparent sensi-

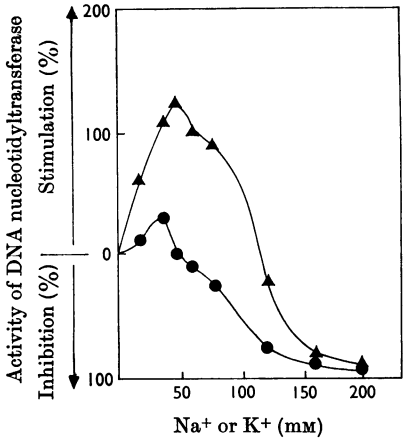


Fig. 3. Effects of $Na^+$(●) and $K^+$(▲) ions on the activity of DNA nucleotidyltransferase. The enzyme (94 $\mu$g. of fraction 3 protein) was assayed under standard conditions, the $Na^+$ or $K^+$ ion concentrations being varied as indicated.

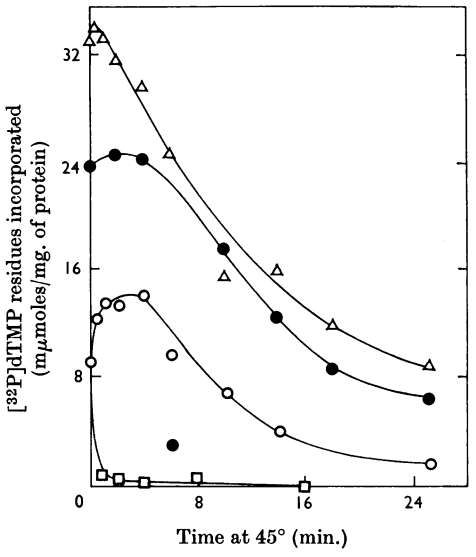


Fig. 4. Thermostability of DNA nucleotidyltransferase. The enzyme fractions were incubated at 45° for the indicated times at pH 7·5 and then cooled to 0°. The other components of the nucleotidyltransferase reaction mixture were then added, and incubation at 37° followed, for assay of nucleotidyltransferase under standard conditions. The preliminary incubations at 45° were conducted for each time-point in the enzyme-assay tubes with fractions 1 (112 $\mu$g. of protein/tube, ○), 2 (99 $\mu$g. of protein/tube, ●) and 3 (161 $\mu$g. of protein/tube, △) in the presence of denatured DNA (75 $\mu$g./tube). For the ensuing assay at 37°, a further portion (75 $\mu$g.) of denatured DNA was added to each tube. In a concurrent experiment, samples of fraction 1 (112 $\mu$g. of protein/tube, □) were heated at 45° in the absence of DNA and then assayed for nucleotidyltransferase in the presence of 150 $\mu$g. of DNA.

tivity of the enzyme to the presence of certain cations, and its stimulation by EDTA, provide the basis for including EDTA at 1 mM in all buffer solutions during extraction and purification of the enzyme, and at 0·4 mM in the reaction mixture. Some aspects of this effect of EDTA have been discussed previously in relation to the presence of thiol groups in the DNA nucleotidyltransferase (Keir & Shepherd, 1965). Early experiments on the stability of the enzyme during storage at 0° in the absence of EDTA revealed a progressive loss of activity (75–80% loss after 16 days). Addition of either EDTA or 2-mercaptoethanol markedly improved both the stability of the enzyme during storage and the activities of the various fractions obtained during enzyme purification. These considerations led to the inclusion of 2-mercaptoethanol in the preparative buffer solutions at 5 mM and in the enzyme assay mixture at 1–2 mM.

### *Thermostability of the nucleotidyltransferase*

Incubation of the enzyme at 45° in a buffer solution at pH 7·5 followed by assay in the standard system at 37° revealed that the enzyme was very thermolabile. However, a relatively high degree of resistance to heating was conferred on the enzyme when the prior incubation at 45° was performed in the presence of DNA. The thermostabilities of fractions 1, 2 and 3 in the presence of denatured DNA and of fraction 1 in the absence of DNA are shown in Fig. 4. An interesting feature of these results is the marked activation effect observed with fraction 1 after 2–5 min. heating at 45° in the presence of DNA; this effect is less marked in fraction 2 and is almost non-existent in fraction 3. Omission of DNA from the enzyme solution during the prior heating results in loss of almost all activity within 1 min. Other experiments have shown that native DNA also has the ability to protect the enzyme during heating at 45°.

### *Nature of the product of the nucleotidyltransferase reaction*

*Density-gradient analysis.* The sedimentation properties of the primer and of the product of the nucleotidyltransferase reaction were investigated by buoyant density ultracentrifugation in caesium chloride solution (Meselson, Stahl & Vinograd, 1957). The plan of the experiment was to form three

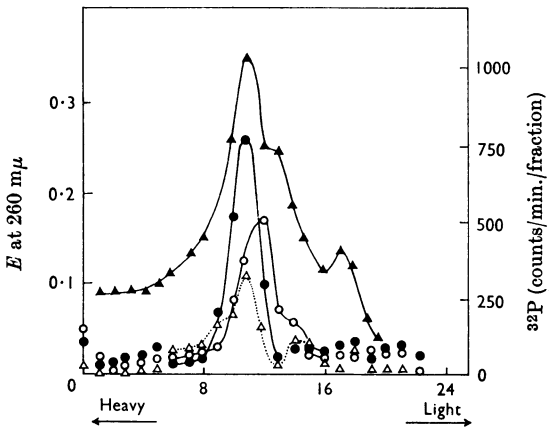


Fig. 5. Equilibrium density gradient centrifugation of DNA and of the product of the DNA nucleotidyltransferase reaction in CsCl solution. Native DNA (○), thermally denatured DNA (●) and nucleotidyltransferase reaction product (△) were prepared, centrifuged to equilibrium in CsCl solution, and collected from the CsCl gradients as described in the text. The collected fractions were measured for extinction at 260 mμ and, with the nucleotidyltransferase reaction product, for $^{32}P$ content (▲) arising from incorporation of [$^{32}P$]dTMP units.

density gradients of caesium chloride under the same conditions of centrifugation, the first containing native DNA, the second thermally denatured DNA and the third $^{32}P$-labelled product isolated from the nucleotidyltransferase reaction mixture (Fig. 5). After centrifuging, fractions were collected from each centrifuge tube (see the Experimental section); it was observed that the native and denatured DNA preparations formed bands roughly at the middle of the gradient, the denatured form being located slightly nearer the bottom of the tube owing to its higher density (Schildkraut, Marmur & Doty, 1961). Both the product DNA and the radioactivity banded at the same point as the denatured DNA; it may be significant that the curve representing radioactivity has a 'shoulder' on the side of lower density, suggesting the possible presence of a radioactive product of density corresponding to native DNA.

*Nearest-neighbour frequency analysis of reaction products.* The technique of Josse *et al.* (1961) was followed for determination of dinucleotide frequencies in the reaction product. Four nucleotidyltransferase reaction mixtures were set up with respectively [$\alpha$-$^{32}P$]dATP, [$\alpha$-$^{32}P$]dCTP, [$\alpha$-$^{32}P$]dGTP and [$\alpha$-$^{32}P$]dTTP as the radioactive precursor, each in the presence of the other three, but non-radioactive, triphosphates (see the Experimental section). After incubation, the product DNA was isolated and degraded to $^{32}P$-labelled deoxyribonucleoside 3′-monophosphates by the successive action of micrococcal nuclease and spleen phosphodiesterase. The four monophosphates from each reaction mixture were separated by high-voltage electrophoresis, cut out as bands from the paper-electrophoresis strips and measured for content of radioactive phosphorus. The data derived from this experiment therefore consist of the distributions of $^{32}P$ among the four 3′-monophosphates in four incorporation reactions each of which involves one $^{32}P$-labelled triphosphate and three non-radioactive triphosphates as precursors. For example, in the reaction in which dATP was radioactive and dCTP, dGTP and dTTP were non-radioactive, all radioactivity would be incorporated as deoxyadenosine 5′-monophosphate. Enzymic degradation of the product to 3′-monophosphates results in transfer of the $^{32}P$ moiety of the incorporated deoxyadenosine 5′-monophosphate to the next nucleoside residue in the polydeoxyribonucleotide sequence, i.e. to the nearest neighbour, so that the $^{32}P$ content of each 3′-monophosphate represents the frequency of its occurrence next to the deoxyadenosine residue. The dinucleotides involved in this case are therefore ApA, CpA, GpA and TpA. Four frequencies are similarly derived from each of the reaction mixtures involving [$\alpha$-$^{32}P$]dCTP, [$\alpha$-$^{32}P$]dGTP and [$\alpha$-$^{32}P$]dTTP, giving a total of 16 dinucleotide frequencies. Finally, the frequencies of the dinucleotide sequences are then algebraically weighted so that each is expressed as a decimal proportion of 1 (cf. Josse *et al.* 1961). The results for synthesis of DNA by the Landschütz DNA nucleotidyltransferase are presented in Table 3. The data obtained by Swartz, Trautner & Kornberg (1962) using the *Escherichia coli* nucleotidyltransferase primed by an ascites-tumour cell DNA are included in the Table for comparison. Chemical analysis of Landschütz ascites-tumour cell DNA (Keir *et al.* 1963) has shown that the ratio (adenine + thymine): (guanine + cytosine) is 1·33; the ratio (1·35) obtained from the nearest-neighbour frequency analysis (Table 3) is in excellent agreement with this value. Clearly, there are differences between the two sets of data in Table 3. It is not possible to decide at present whether these have arisen for technical reasons or because two different tumour systems were examined, but the differences are small and it seems reasonable to conclude that the Landschütz ascites-tumour cell nucleotidyltransferase is capable of synthesizing a polydeoxyribonucleotide product that reflects the nucleotide composition of the primer. Further, the close agreement of frequencies between the pairs of dinucleotides as presented in

Table 3. *Nearest-neighbour frequencies of mouse ascites-tumour cell DNA*

The chemically determined ratio (adenine+thymine):(guanine+cytosine) for DNA from Landschütz ascites-tumour cells is 1·33 (Keir *et al.* 1963), and that for DNA from cells of another mouse ascites-tumour is 1·38 (Swartz *et al.* 1962).

| | Dinucleotide frequency | |
|---|---|---|
| Dinucleotide sequence | Landschütz nucleotidyltransferase (present paper) | *E. coli* nucleotidyltransferase (Swartz *et al.* 1962) |
| ApA, TpT | 0·083, 0·086 | 0·084, 0·100 |
| CpA, TpG | 0·074, 0·073 | 0·074, 0·074 |
| GpA, TpC | 0·057, 0·065 | 0·059, 0·068 |
| CpT, ApG | 0·066, 0·064 | 0·072, 0·072 |
| GpT, ApC | 0·063, 0·059 | 0·056, 0·050 |
| GpG, CpC | 0·056, 0·053 | 0·048, 0·052 |
| TpA | 0·069 | 0·062 |
| ApT | 0·077 | 0·078 |
| CpG | 0·019 | 0·011 |
| GpC | 0·036 | 0·039 |
| | Ratio* 1·35 | Ratio* 1·43 |

* (Adenine+thymine):(guanine+cytosine).

Table 3 indicates that polymerization has taken place in accordance with the product's having an antiparallel structure (cf. Josse *et al.* 1961).

## DISCUSSION

The purification procedure described here for DNA nucleotidyltransferase is simple and rapid for fractions 1, 2, 3 and 3a; preparation of fraction 4 is somewhat more tedious, but is of considerable importance, for it effects complete separation of the bulk of the nucleotidyltransferase from contaminating deoxyribonuclease. Indeed, a significant conclusion based on the data in Fig. 1 is that the activity of the nucleotidyltransferase is not dependent on concurrent nuclease action under the assay conditions used *in vitro*. Clearly, it would be advantageous to scale up preparation of fraction 4 to obtain larger quantities of the transferase free from nuclease. Exploratory experiments in this direction have shown that operation of larger hydroxyapatite columns (20cm. × 2·8cm.), under pressure as described in the Experimental section, is unsatisfactory, but that the same columns operated under gravity flow promise to give results qualitatively the same as those obtained with the 10cm. × 1cm. columns. Most of the experiments reported here were performed with the nucleotidyltransferase of fraction 3, since most of the deoxyribonuclease and all of the triphosphatase activities had been removed at this stage (Table 1).

Throughout many purification experiments, and under the standard assay conditions, the average range of activities from fraction 2 to fraction 4 was 8–60mμmoles of [$^{32}$P]dTMP residues incorporated/mg. of protein/hr. Assuming that equal amounts of the monophosphates of deoxyadenosine, deoxycytidine and deoxyguanosine were concomitantly incorporated into the product, then the range of activities becomes 32–240mμmoles/mg. of protein, and since there was usually 150μg. of protein from the appropriate enzyme fraction added to each assay, the range of nucleotide incorporation is 4·8–36mμmoles (or 1·6–11·9μg.)/assay. Thus with 50μg. of DNA-primer present in the assay mixture, synthesis of product amounting to 3·2–23·8% of the primer may be achieved in 1hr. The enzyme therefore probably has the potential to carry the reaction further, e.g. to the level of synthesis of product amounting to 100% of the primer added; incubation periods in excess of 1hr. would, of course, be necessary. However, prolonged incubation has the disadvantage that nucleotidyltransferase activity is gradually lost. Preincubation of the enzyme in the presence of DNA has shown that there is a gradual reduction of activity (up to 50%) after 2hr. at 37° (cf. Keir & Shepherd, 1965). This must account in some measure for the deviation from linearity of activity with time observed in many nucleotidyltransferase reactions incubated for periods beyond 2–3hr. (Shepherd, 1965), and indicates that the maximum possible extent of synthesis might best be assessed by periodic supplementation with fresh enzyme during incubation over prolonged periods. Moreover, it is clear that the most satisfactory approach to this problem would be to use only nucleotidyltransferase preparations free from deoxyribonuclease activity, namely fraction 4, because continuous attack by nuclease on primer and product (no matter how limited it

may be) must interfere with the priming capacity of the polydeoxyribonucleotide present, through reaction of 3′-hydroxy-terminal groups (Keir, 1962). In this context it is important to emphasize that DNA nucleotidyltransferase from calf thymus gland has been separated from endogenous nucleases (Yoneda & Bollum, 1965), and that it apparently accepts only single-stranded DNA as primer, catalysing synthesis of polydeoxyribonucleotide in a complementary manner to give a double-stranded product; the reaction ceases at this stage since the double-stranded product is not capable of serving as primer (Bollum, 1963). Whether the Landschütz nucleotidyltransferase behaves in the same way has not yet been determined, for while fractions 1–3a can utilize native DNA as primer, the extent to which the latter primes decreases in direct proportion with the decrease of deoxyribonuclease content of each fraction (Table 1). It may therefore be that the nuclease is responsible for converting the native DNA into a priming form. Other mammalian systems may have a bearing on this problem. Furlong & Williams (1965) have purified DNA nucleotidyltransferase from Walker 256 carcinosarcoma of the rat to the extent that it has very low endonuclease activity. The preparation can use native DNA as primer with an efficiency up to 55% of that obtained with denatured DNA. Mantsavinos (1964) found that partially purified DNA nucleotidyltransferase from regenerating rat liver utilizes native and denatured DNA as primers with roughly equivalent efficiencies. Sarkar, Mukundan & Devi (1963) have suggested that deoxyribonuclease may function in the synthesis of DNA by promoting conversion of DNA into an effective primer, but there is more recent evidence that a factor other than deoxyribonuclease is responsible for the formation of effective primer from native DNA (de Recondo, Frayssinet & Le Breton, 1964; de Recondo, 1964). The question of priming by native DNA therefore seems to be settled only with the calf thymus nucleotidyltransferase and remains unanswered with the other systems. Experiments are now in progress with a view to obtaining a definitive solution with the nuclease-free nucleotidyltransferase from Landschütz ascites-tumour cells.

The heat-inactivation data for DNA nucleotidyltransferase very clearly demonstrate (Fig. 4) the delicate nature of the enzyme; indeed, retention of activity during heating in the absence of DNA is negligible after 1 min. at 45°. It therefore seems that to allow significant survival of activity during heat-treatment, the enzyme must form an association with DNA. This observation provided the basis for including DNA in the hydroxyapatite eluates (see the Experimental section). The apparent activation effects observed after heating fractions 1 and 2 for about 5 min. and fraction 3 for 1 min. are difficult to explain. One possibility is that the activation can be ascribed to the action of deoxyribonuclease on the DNA during heating at 45°, creating additional priming centres (3′-hydroxy-terminal groups), which stimulate the ensuing nucleotidyltransferase reaction (Keir *et al.* 1962; Keir, 1962). Consistent with this view are the observations that deoxyribonuclease is progressively removed as purification proceeds (Table 1) and that the activation effect diminishes in parallel.

Another possibility that merits consideration arises from speculation on the nature of the nucleotidyltransferase molecule. If the enzyme possessed a sub-unit (e.g. oligomeric) structure that collapsed on heating to give the individual sub-units (e.g. monomers), then an activation effect might be expected, provided that (i) the monomers retained enzymic activity, and (ii) interdependence of action of monomers in the intact oligomeric nucleotidyltransferase catalysed synthesis of polynucleotide at a rate lower than that obtained with the dissociated monomers. This model has additional attractions, and is compatible with the supposition that there is a requirement for two binding sites for DNA on the enzyme, one for each component strand of the double helix, so that replication of DNA *in vivo* proceeds by the concerted action of two catalytic centres located on one enzyme molecule. The implication is that the existence of two sites is not inconsistent with their occurrence on two individual monomer units. The possibility has been discussed in detail by Keir (1965). According to this postulate, the progressive loss of ability to accept native DNA as primer, and the progressive disappearance of the activation effect on going from fraction 1 to fraction 3 (Fig. 4), are attributed to destruction of the oligomeric conformation of the enzyme during exposure to acid and ammonium sulphate, giving predominantly the monomer units. The postulate is supported by observations on the heat-stability characteristics of DNA nucleotidyltransferase from mammalian cells infected with herpes simplex virus (Keir, Hay, Morrison & Subak-Sharpe, 1966). In that system, the control cell nucleotidyltransferase behaves similarly to the Landschütz enzyme described here, but the infected cell nucleotidyltransferase preparation does not display a major activation phenomenon until after 30–40 min. preincubation at 50°. At this time, the control enzyme has lost all activity. Since the nuclease contents of the control and infected cell preparations were essentially the same, it would seem that the marked difference between the control and infected samples cannot be attributed to nuclease action on the DNA-primer but that it could, conceivably, be the consequence of loss of an oligomeric conformation of nucleotidyltransferase.

The action of another factor (enzymic or otherwise) cannot, of course, be precluded. These points may bear a close relationship to the priming capacities of native and denatured DNA discussed above, if it is assumed that only an intact oligomeric nucleotidyltransferase will accept native DNA. The postulate also would explain the wide variations of priming with native DNA found in other purified systems (Bollum, 1963; Mantsavinos, 1964; Furlong & Williams, 1965).

The precise structure of the product of the Landschütz nucleotidyltransferase reaction is still not completely defined, but there are some features that give a strong indication of its general nature. The product is acid-precipitable, ethanol-precipitable, susceptible to the action of deoxyribonucleases I and II (EC 3.1.4.5 and 3.1.4.6), snake-venom phosphodiesterase (EC 3.1.4.1), spleen phosphodiesterase and micrococcal nuclease (cf. also Smellie *et al.* 1960). By the criterion of forming a band in a density gradient of caesium chloride, it is macromolecular and of density similar to that of the DNA-primer. Whether it is double-stranded is less clear, but the implications of the nearest-neighbour frequency analysis (Table 3) have a strong bearing on this particular point. The equivalences of adenine with thymine and of guanine with cytosine in the product indicate that incorporation of nucleotides has proceeded by base-pairing according to the classical concept of Watson & Crick (1953). Moreover, the observation that the ratio (adenine + thymine):(guanine + cytosine) determined by the nearest-neighbour frequency analysis of the product is in very close agreement with that ratio determined by direct chemical analysis of the primer (Keir *et al.* 1963) suggests that the nucleotidyltransferase has catalysed a complementary synthesis along the single strands of DNA-primer provided. Since the single strands were obtained by thermal-denaturation of Landschütz DNA, equal amounts of the two complementary strands must have been present in the primer preparation. One can therefore conclude from the data (Table 3) that the synthesis has proceeded along both types of strand in accordance with the base sequence of the original DNA, and that the product probably has a double-stranded conformation with component strands of opposite polarity (Josse & Swartz, 1963).

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