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Characteristics of Deoxyribonucleic Acid Polymerase Activity in Nuclear and Supernatant Fractions of Cultured Mouse Cells

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1. DNA polymerase activity is present in both nuclear and supernatant fractions prepared from rapidly dividing L929 mouse cells. 2. Nuclear preparations are 2–5 times more active with added native DNA as template and the supernatant fractions show an equivalent preference for heat-denatured DNA. 3. Isolated nuclei can carry on limited DNA synthesis in the absence of added template but are stimulated five- to ten-fold by addition of 50 μg of native DNA per assay. 4. DNA polymerase activity can be released from intact nuclei by ultrasonic treatment or by extraction with 1.5 M-potassium chloride. 5. The activities in nuclear and supernatant fractions, with their preferred templates, respond similarly to changes in pH and Mg^{2+} and K^+ concentrations. 6. Maximal enzyme activity is approached with 40 μg of DNA per assay and activation of the DNA template by treatment with deoxyribonuclease does not decrease the amount of DNA required to reach saturation. 7. The nuclear enzyme, incubated with native DNA, is markedly inhibited by the addition of heat-denatured DNA to the assay. In contrast, the supernatant DNA polymerase activity on denatured templates is not affected by the presence of native DNA. 8. The nuclear enzyme exhibits high activity in the absence of one or more deoxyribonucleoside triphosphates but this is much diminished after partial purification of the enzyme by precipitation at pH 5 and fractionation on Sephadex G-200 columns. 9. The ^{3}H-labelled DNA products formed by Sephadex-purified nuclear and supernatant fractions, with their preferred templates, were found to be resistant to treatment with exonuclease I. Alkali-denaturation of the ^{3}H-labelled DNA products rendered them susceptible to attack by exonuclease I. 10. Analysis of the products on alkaline sucrose density gradients suggests that the newly synthesized material may not be covalently bound to the original DNA template. 11. By using their preferred templates the specific activity of supernatant fractions varies markedly with the position of the cells in the cell-cycle, but the specific activity of nuclear fractions varies only slightly.

Many of the DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7) preparations isolated from mammalian sources, e.g. calf thymus gland (Yoneda & Bollum, 1965) and ascites-tumour cells (Keir & Shepherd, 1965) show little activity with native DNA and require single-stranded DNA as a template for polymerization. However, several groups have reported the presence of enzymes that exhibit maximal activity with native DNA templates. Such an activity has been isolated from the non-histone fraction of rat liver nuclei (Patel, Howk & Wang, 1967). Mantsavinos & Munson (1966) and Iwamura, Ono & Morris (1968) have also detected in extracts of rat liver a DNA polymerase that shows greater activity with native DNA. A distinct DNA polymerase, shown to be active in the presence of native DNA, has been found in rat liver mitochondria (Kalf & Ch'ih, 1968; Meyer & Simpson, 1968). A very active DNA polymerase, requiring native DNA as template, is also known to occur in the nuclei of developing sea-urchin embryos (Loeb, Mazia & Ruby, 1967). Clarification of the differing template activities of these enzymes should lead to a better understanding of the overall mechanism of DNA replication.

We have previously reported the presence of DNA polymerase activities, with differing template requirements, in nuclear and supernatant fractions of L929 mouse cells (Lindsay & Adams, 1968). In this paper some of the general properties of these activities are described together with information on the nature of the DNA product. The variations in these activities with the position of the cells in the cell-cycle are also described.

MATERIALS AND METHODS

Growth of cells. L929 mouse cells (Sanford, Earle & Likely, 1948) were maintained in minimal essential Eagle's medium supplemented with 10% (v/v) calf serum in the absence of antibiotics. Cells and culture media were supplied by Flow Laboratories Ltd., Irvine, Ayrshire, U.K. Cultures were routinely tested for contamination by mycoplasma. The cells were propagated in monolayer cultures in flat-sided Roux flasks or in rotating Winchester bottles. Every 3–4 days the cells were harvested by treatment with trypsin, diluted in fresh medium to approx. 2×10^5 cells/ml and inoculated into fresh sterile bottles.

Cells were synchronized as described by Adams (1969). To synchronize cells by aminopterin blockage they were exposed for 16h to normal medium supplemented with aminopterin (0.2μM), adenosine (60μM), glycine (80μM) and deoxycytidine (20μM). The inhibition was reversed by addition of thymidine (0.1mM).

For isolation of cell extracts, 2–3 day-old rapidly growing cultures were used. The cells were scraped off into Earle's balanced salt solution minus glucose and centrifuged at 400*g* for 10min at 0–3°C. All subsequent operations during the isolation were carried out at 0–3°C. To remove any remaining medium from the cell pellet the cells were washed with 5vol. of 20mM-tris–HCl buffer, pH7.5, containing 0.25M-sucrose (buffered sucrose) and centrifuged as before. Disruption of the cells, suspended at $1–2\times10^7$ cells/ml in buffered sucrose, was carried out by using a Potter–Elvehjem homogenizer (Teflon pestle, 0.308 in. diam.; tube, 2ml capacity; both were obtained from Sireica, Jamaica, N.Y., U.S.A.; clearance between tube and pestle was 0.004–0.006in.).

The homogenate was centrifuged at 800*g* for 10min and the supernatant fraction, after spinning at 105000*g* for 60min (Spinco model L ultracentrifuge, rotor no. 40), was used as a source of soluble enzyme. Nuclei were washed twice more in the same volume of buffered sucrose before final suspension in the same medium as a source of nuclear DNA polymerase. The integrity and purity of nuclei were checked by fluorescent microscopy after staining with 1% (w/v) Acridine Orange.

Disruption of nuclei. (*a*) Preparation of ultrasonically treated extracts of nuclei. Ultrasonic treatment of nuclear preparations ($1–2\times10^7$ nuclei/ml) in buffered sucrose was carried out in 4–6ml batches by using a Dawe Soniprobe fitted with a brass sample-holder. Treatment was for 40s at 3A (setting 6) in two 20s bursts. Ultrasonically treated preparations were observed by fluorescent microscopy to check that nuclear disruption was complete. After centrifugation of ultrasonically treated preparations at 105000*g* for 45min the supernatant fraction was retained as a source of nuclear enzyme.

(*b*) Preparation of KCl extracts of nuclei. This was carried out essentially as described by Patel *et al.* (1967). Nuclear preparations were resuspended in 20mM-tris–HCl buffer, pH7.5, containing 1.5M-KCl and homogenized at low speed to disperse the gelatinous material. The suspension was dialysed for two 1h periods against 100vol. of 20mM-tris–HCl buffer, pH7.5, containing 0.15M-KCl; at this concentration of KCl DNA and histones recombine and precipitate out leaving 'acidic' proteins in solution. The insoluble material was sedimented by centrifugation at 10000*g* for 15min, leaving the non-histone material in the supernatant fraction. This fraction was retained as a source of DNA polymerase.

Preliminary purification of nuclear and supernatant extracts. Large-scale purification of the DNA polymerase activity in L929 cells is difficult owing to the small amounts of material available from tissue-culture cells. A seven- to eight-fold purification is achieved, however, by precipitation at pH5 and gel-filtration on Sephadex G-200. In buffer of low ionic strength the DNA polymerase activity in ultrasonically treated nuclear and supernatant extracts is eluted at or near the void volume on Sephadex G-200 and 70–100% recoveries of the enzymes are achieved after these two purification steps.

The Sephadex-purified enzyme fractions contain approx. 5% of the nuclease activity of crude cell extracts as judged by the relative ability of the two fractions to degrade double- or single-stranded ^{32}P-labelled *Escherichia coli* DNA to acid-soluble fragments. As Furlong (1966) has shown that oligonucleotides longer than 7–9 units are trichloroacetic acid-precipitable, the amount of DNA rendered acid-soluble is chiefly a reflexion of the exonuclease activity of the preparations. By using a similar procedure in the purification of DNA polymerase from Landschütz ascites-tumour cells Keir (1965) showed that precipitation at pH5 removes the bulk of the deoxyribonuclease I (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5) activity and a partial separation of the remaining nuclease activity is obtained on Sephadex G-150 columns.

Assay of DNA polymerase activity. The basic assay system was that of Shepherd & Keir (1966). Nuclear and supernatant fractions with DNA polymerase activity were incubated at 37°C for 60min in a total volume of 0.25ml with 5μmol of tris–HCl buffer, pH7.5, 2μmol of $MgCl_2$, 15μmol of KCl, 0.1μmol of EDTA, 1.5μmol of 2-mercaptoethanol, 100μg of DNA and 50nmol each of dATP, dCTP, dGTP and [*Me*-^{3}H]dTTP (50nCi/nmol) (Schwarz BioResearch Inc., Orangeburg, N.Y. U.S.A.). The reaction was terminated by the addition of 0.05ml of 2M-NaOH and the samples were incubated overnight at 37°C.

DNA was precipitated from the assay mixture by addition of 2ml of 5% (w/v) trichloroacetic acid containing Hyflo Super-Cel (20g/l) as co-precipitant. The DNA in the precipitate was thoroughly washed with 5% (w/v) trichloroacetic acid containing 0.05M-$Na_4P_2O_7$ and then dried with ethanol and ether. The DNA was dissolved by heating with 0.5ml of M-Hyamine hydroxide (Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K.) for 20min at 60°C in a counting vial. After addition of toluene-based scintillator [0.5% (w/v) 2,5-diphenyloxazole], radioactivity was determined in a liquid-scintillation spectrometer. The efficiency of counting was 20–25%.

The unit of DNA polymerase activity is defined as the amount required to catalyse incorporation of 1nmol of [*Me*-^{3}H]dTTP into acid-insoluble material in 1h at 37°C.

DNA. (*a*) DNA preparations. ^{32}P-labelled DNA was prepared from *E. coli* strain ML 308 by the method of Lehman (1960). Salmon testis DNA and *E. coli* DNA were obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

(*b*) Denaturation of DNA. DNA was dissolved in 50mM-KCl at 2mg/ml. Samples were denatured by heating at 100°C for 10min and then rapidly cooled in ice–water.

On occasion *E. coli* DNA was alkali-denatured. DNA solutions were made 0.3M with respect to NaOH and left for 10min at room temperature. The pH was readjusted to 7.5 by addition of M-HCl and a few drops of 0.8M-tris–HCl buffer, pH7.5. The solution was then dialysed for 2h against 100vol. of 50mM-KCl.

(*c*) Activation of DNA. Treatment of native DNA with small amounts of deoxyribonuclease I increases the template efficiency of the DNA in DNA polymerase assays. Activation of salmon testis DNA was carried out by a modification of the method of Aposhian & Kornberg (1962). The DNA (1mg) was exposed to 1μg of bovine pancreatic deoxyribonuclease I (Sigma Chemical Co. Ltd., St Louis, Mo., U.S.A.) in a solution (0.7ml) containing 25μmol of KCl, 5μmol of $MgCl_2$ and 50μmol of tris–HCl buffer, pH7.5. After incubation for 30min at 37°C the DNA solutions were rapidly cooled and frozen for later use in DNA polymerase assays.

Preparation and assay of exonuclease I. Exonuclease I (DEAE-cellulose fraction) was purified from ultrasonically treated extracts of *E. coli* strain B as described by Lehman (1960). The assay system was essentially as described by Lehman & Nussbaum (1964) and contained 20μmol of glycine–NaOH buffer, pH9.2, 2μmol of $MgCl_2$, 0.5μmol of 2-mercaptoethanol and 1–5μg of native or heat-denatured ^{32}P-labelled *E. coli* DNA. Incubations were carried out in a volume of 0.3ml at 37°C for various times; 0.2ml of non-radioactive 'carrier' DNA was then added (2mg of salmon testis DNA/ml of 50mM-KCl) and 0.5ml of ice-cold 0.35M-$HClO_4$. After 5min at 0°C the resulting precipitate was sedimented by centrifugation at 12000*g* for 3min. Radioactivity in the pellet was determined by using the procedures employed in the assay of DNA polymerase activity.

Neutral and alkaline sucrose density gradients. (1) Neutral gradients. After incubation the assay mixture was dialysed for 48h against 4×100vol. of 0.02M-tris–HCl buffer, pH7.5, containing 0.05M-KCl. A portion (0.15ml) of the resulting ^{3}H-labelled DNA solutions was layered on top of a 5ml sucrose density gradient (5–20% sucrose in 1M-NaCl–1mM-EDTA–20mM-tris–HCl buffer, pH7.5) and centrifuged for 10h at 24000rev./min (44000$g_{av.}$) at 4°C in a Spinco SW 39 L rotor. Fractions (2 drops) were collected, diluted to 0.5ml with water and assayed for extinction at 260nm and then for acid-precipitable radioactivity as described for DNA polymerase assays.

(2) Alkaline gradients. The procedure was similar to that used for neutral sucrose-density-gradient analysis with the following modifications. (*a*) ^{3}H-labelled DNA was alkali-denatured before layering on the gradient. (*b*) The gradient contained 0.1M-NaOH. (*c*) Centrifugation was for 10h at 32000rev./min (84000$g_{av.}$).

Measurement of DNA synthesis. Cells in Petri dishes were incubated in 3ml of medium with [^{3}H]thymidine (The Radiochemical Centre, Amersham, Bucks., U.K.). The concentration and specific radioactivity are stated in the text. After incubation, the cells, while still attached to the surface, were washed gently with balanced salts solution (3×3ml), ice-cold 5% (w/v) trichloroacetic acid (4×3ml) and ethanol and allowed to dry. They were then removed from the dish by dissolving in 1.0ml of 0.3M-NaOH and a sample (0.5ml) was evaporated to dryness in a liquid-scintillation vial. The residue was assayed for radioactivity by heating (60°C for 10min) in 0.5ml of M-Hyamine hydroxide before addition of 10ml of toluene-based scintillator. Alternatively, the acid-washed cell-sheet was covered with Ilford L4 nuclear track emulsion diluted 1:3 (v/v) with water to assay the proportion of cells labelled.

RESULTS AND DISCUSSION

The different template activities of DNA polymerase in nuclear and supernatant fractions of L929 cells are shown in Table 1. In general, the activity with isolated nuclei is two- to five-fold higher when the template is native DNA; supernatant preparations show a corresponding preference for denatured DNA. The nuclei also exhibit 10–20% of their maximal activity in the absence of added DNA. Thus intact nuclei retain to a limited extent the capacity to carry on DNA synthesis by using endogenous DNA. A similar situation occurs with nuclei from sea-urchin embryos, which are stimulated eight- to ten-fold by the addition of native DNA (Loeb *et al.* 1967). Isolated nuclei from HeLa cells also retain the capacity to incorporate deoxyribonucleoside triphosphates into nuclear DNA but, in contrast, are not activated by the presence of added native template DNA (Friedman & Mueller, 1968).

The discovery of these activities in nuclear and supernatant fractions of L929 cells led us to carry out a more detailed examination of the properties of the enzyme(s). The intranuclear location of a significant fraction of the DNA polymerase activity and its requirement for a double-stranded DNA template makes it a suitable candidate for a role in the replication process *in vivo* perhaps corresponding to the 'intact' form of DNA polymerase as proposed by Keir (1965).

Release of DNA polymerase activity from L929 nuclei. Two methods were used to release DNA polymerase from isolated nuclei. (1) After ultrasonic treatment of L929 nuclei (see the Materials and Methods section) the bulk of the DNA polymerase activity can be found in the supernatant

Table 1. *DNA polymerase activity of nuclear and supernatant fractions of L929 cells*

Nuclear and supernatant fractions were prepared from rapidly growing cells and assayed for DNA polymerase activity as described in the Materials and Methods section.

Salmon testis DNA added to incubation medium (100μg)	DNA polymerase activity (units/mg of protein)	
	Nuclear fraction	Supernatant fraction
None	0.07	0.00
Native	0.42	0.11
Heat-denatured	0.14	0.56

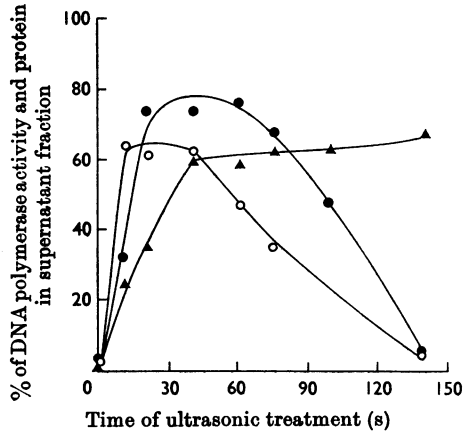

Fig. 1. Release of DNA polymerase activity from nuclei. Nuclei were disrupted for various periods of time by ultrasonic treatment (see the Materials and Methods section). Ultrasonically treated preparations were centrifuged at 105000*g* for 45min and the supernatant fractions were assayed for DNA polymerase activity and protein content. Results are expressed as a percentage of the activity found in intact nuclei with either native (●) or heat-denatured DNA (○) as template. ▲, Percentage of nuclear protein released.

fraction after centrifugation at 105000*g* for 45min. Recovery of enzymic activity (70–80%) reaches a plateau after 20s of ultrasonic treatment (Fig. 1). Treatment for more than 50s results in a decline in enzymic activity, presumably caused by denaturation of the enzyme. Under these conditions 70% of the nuclear DNA also remains in the supernatant fraction. The released enzyme still exhibits a preferential requirement for native DNA.

(2) DNA polymerase activity may be extracted from L929 nuclei by using the technique of Patel *et al.* (1967). Nuclei are disrupted in 20mM-tris–HCl buffer, pH7.5, containing 1.5M-KCl and the gelatinous solution is subsequently dialysed for two 1h periods against 100vol. of 20mM-tris–HCl buffer, pH7.5, containing 0.15M-KCl and 10mM-2-mercaptoethanol. As the ionic strength is lowered DNA and histones recombine and precipitate out leaving 'acidic' proteins in solution. Precipitated material is removed by spinning at 10000*g* for 15min in the 8 × 50ml rotor of an M.S.E. 18 centrifuge. By this method it was possible to obtain 50–70% recoveries of DNA polymerase activity from L929 nuclei and remove 95–98% of the nuclear DNA.

Properties of the DNA polymerase activity in nuclear and supernatant fractions of L929 cells. pH optima. Nuclear and supernatant fractions respond similarly to changes in the pH of the assay mixture. Both fractions are active in the pH range 7.0–9.0 and exhibit identical optima at pH7.5–8.0. Changes in pH do not alter the relative capacity of either fraction to utilize native or heat-denatured DNA as template. Birnie & Fox (1966), in a study of DNA polymerase activity in nuclear and supernatant extracts of primary mouse embryo cells, found significant differences between the two preparations, the pH optima being at pH7.5 and pH6.5 respectively in the presence of Mg^{2+}.

Effect of Mg^{2+}. Mg^{2+} ions are essential for DNA polymerase activity measured with either native or heat-denatured templates. The optimum concentration in both cases is 6–8mM-Mg^{2+}. This value is identical with that obtained by Gold & Helleiner (1964) for L-cell DNA polymerase activity. These authors, however, only detected activity with denatured DNA as template. No second peak of activity corresponding to the 'intact' form of the enzyme suggested by Keir (1965) could be observed. In this connection, it is of note that several DNA polymerases, found to require native DNA, have higher Mg^{2+} optima than those reported here, e.g. rat liver nuclear enzyme (16mM; Mantsavinos, 1964) and sea-urchin embryo nuclear enzyme (16mM; Loeb *et al.* 1967).

Effect of K^+. Both nuclear and supernatant fractions, with their preferred templates, are activated approximately twofold by 60mM-K^+. Similar results have been reported for the calf thymus and Landschütz ascites-tumour enzymes (Keir, 1965; Keir & Shepherd, 1965). It has been shown that the rat liver nuclear enzyme is stimulated twofold by 60mM-K^+ whereas the mitochondrial DNA polymerase is increased eightfold under equivalent conditions (Meyer & Simpson, 1968).

Effect of DNA concentration and requirement for a full complement of deoxyribonucleoside 5'-triphosphates. Isolated nuclei are active in the absence of added template (Table 1) although their activity is increased five- to ten-fold by the addition of saturating amounts of double-stranded DNA. Suspension of the nuclei in a solution containing DNA (e.g. assay mixture) causes solubilization of the DNA polymerase activity. Thus 50–70% of the activity remains in the supernatant fraction after sedimentation of the nuclei from an assay mixture (J. G. Lindsay & R. L. P. Adams, unpublished work). Supernatant fractions are inactive in the absence of added template and, in contrast with isolated nuclei, are more active with heat-denatured DNA. Maximal DNA polymerase activity in both nuclear and supernatant fractions is approached with 40μg of DNA/assay (Fig. 2). Experiments conducted with Sephadex-purified nuclear and supernatant fractions (referred to below as fractions N Seph and S Seph; see the Materials and Methods section) yield essentially the same results.

The activity of the enzyme extracted from isolated nuclei is increased if the native DNA template

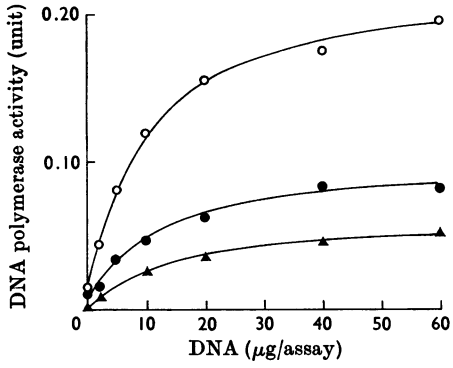

Fig. 2. Effects of increasing DNA concentration on the DNA polymerase reaction. KCl extracts of nuclei (150 μg of protein/assay) were incubated in the presence of various amounts of native (●) or activated salmon testis DNA (○) (see the Materials and Methods section). A supernatant fraction (55 μg of protein/assay) was similarly tested with a heat-denatured DNA template (▲).

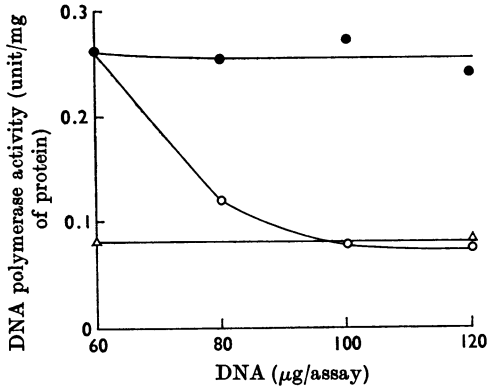

Fig. 4. Effect of addition of heat-denatured DNA on the DNA polymerase activity of KCl extracts of nuclei in the presence of saturating amounts of native DNA. KCl extracts were incubated with 60 μg of native DNA plus increasing amounts of native (●) and heat-denatured DNA (○). For comparison the activity on denatured DNA alone was also measured (△). The protein concentration used was 128 μg/assay.

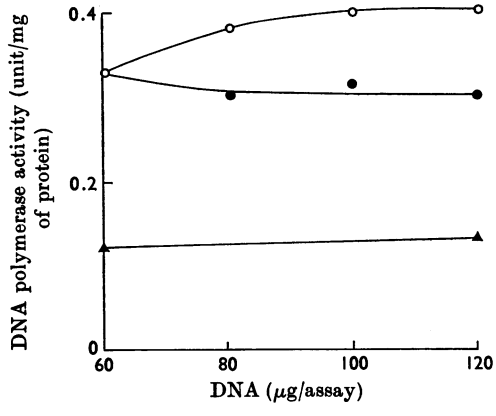

Fig. 3. Effect of the addition of native DNA on the DNA polymerase activity of supernatant fractions in the presence of saturating amounts of heat-denatured template. Supernatant preparations were incubated with a fixed amount of heat-denatured DNA (60 μg/assay) plus increasing amounts of denatured (○) and native DNA (●). For comparison, the activity on native DNA alone was also measured (▲). The protein concentration used was 178 μg/assay.

is activated by deoxyribonuclease I. A similar amount of activated DNA (i.e. 40 μg/assay) is required to saturate the enzyme (Fig. 2).

In this respect the results of incubations containing a mixture of native and denatured DNA templates are noteworthy. Thus addition of native DNA to a supernatant preparation previously saturated with denatured DNA has no effect on DNA polymerase activity (Fig. 3). However, addition of denatured DNA to a nuclear extract, saturated with native DNA, inhibits the activity to a level that would have been obtained in the absence of native DNA (Fig. 4).

In common with other nuclear preparations (Keir & Smith, 1963; Patel *et al.* 1967) the L929 nuclear DNA polymerase exhibits a high degree of activity in the absence of one or more deoxyribonucleoside triphosphates. With native DNA as template, incorporation of [^{3}H]dTTP in the absence of the other three triphosphates is 40% of that obtained under complete assay conditions, and with only dCTP omitted incorporation is 80% of the control value. The supernatant fraction, assayed with denatured DNA, has 15% of the control activity with only dTTP present and 39% in the presence of dTTP, dATP and dGTP.

The activity of fractions N Seph and S Seph, however, shows a greater dependence on the presence of a full complement of deoxyribonucleoside triphosphates. With nuclear preparations, for instance, activity with dATP, dGTP and dTTP is 19% and with dTTP alone is 10% of the control activities. The corresponding values for supernatant preparations are 16% and 7% respectively.

With the nuclear enzyme, poly d(A-T) is a much more efficient template than native salmon testis DNA. If the radioactive precursor employed, however, is [^{3}H]dCTP rather than [^{3}H]dTTP, little incorporation is detected with poly d(A-T) as

Table 2. *Template requirement of the nuclear DNA polymerase activity*

KCl extracts of nuclei were prepared as described in the Materials and Methods section and assayed for DNA polymerase activity in the presence of limiting amounts of template DNA. The radioactive triphosphate used was either [*Me*-^{3}H]thymidine 5′-triphosphate or [5-^{3}H]deoxycytidine 5′-triphosphate. The results are expressed as a percentage of the activity with 5 μg of native salmon testes DNA as template (0.3 units/mg).

Template	DNA polymerase activity	
Radioactive substrate ...	[^{3}H]dTTP	[^{3}H]dCTP
Native salmon testis DNA (5 μg)	100	100
Denatured salmon testis DNA (5 μg)	22	61
Activated salmon testis DNA (5 μg)	254	193
Poly d(A-T) (1 μg)	219	43
Poly dG:dC (5 μg)	14	74

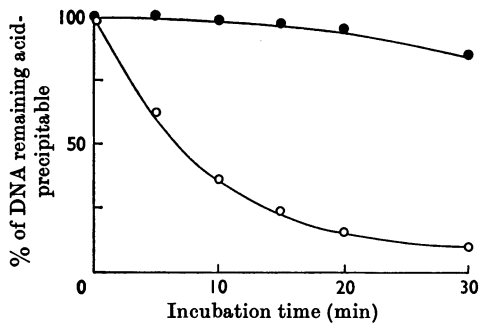

Fig. 5. Effects of exonuclease I on ^{3}H-labelled DNA. ^{3}H-labelled DNA was synthesized by using a modification of the conventional assay procedure (scaled up tenfold) and DNA polymerase preparation N Seph (see the Materials and Methods section). The incubation period was 3 h and 100 μg of native *E. coli* DNA was used as template. Approx. 3% replication of the added DNA was achieved. The reaction was terminated by cooling the sample in ice and adjusting the pH to 9.2 by the addition of 0.1 M-NaOH and a few drops of 0.5 M-glycine–NaOH buffer, pH 9.2. The assay mixture was dialysed for 48 h against 100 vol. of 50 mM-glycine–NaOH buffer, pH 9.2, containing 50 mM-KCl. Samples (0.1 ml) containing approx. 3 μg of ^{3}H-labelled DNA were withdrawn and tested for their susceptibility to exonuclease I action, as described in the Materials and Methods section. Alkali-denatured *E. coli* ^{32}P-labelled DNA (2 μg) was also present as a control. Results are expressed as the percentage of radioactive DNA remaining in acid-precipitable form. ●, ^{3}H-labelled DNA product; ○, ^{32}P-labelled control.

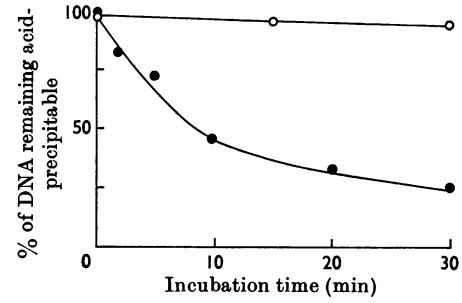

Fig. 6. Effects of exonuclease I on alkali-denatured ^{3}H-labelled DNA. ^{3}H-labelled DNA was prepared as described in the legend to Fig. 5. The DNA sample was alkali-denatured (see the Materials and Methods section) and again tested for its susceptibility to exonuclease I action (see Fig. 5). ●, Alkali-denatured ^{3}H-labelled DNA product; ○, control (exonuclease I omitted).

template (Table 2). Such results indicate that incorporation into the DNA product is to a large extent governed by the nucleotide sequence of the DNA template, i.e. replicative synthesis is occurring.

Nature of the DNA products. To examine the DNA products of L929 cell DNA polymerase, ^{3}H-labelled DNA was synthesized by using fractions N Seph and S Seph. Native or alkali-denatured DNA was used as template in these experiments. Incubations were carried out for 3 h at 37°C and the reaction was terminated by cooling the tubes in ice–water and adjusting the pH to approx. 9.2 with 0.1 M-NaOH and a few drops of 0.5 M-glycine–NaOH buffer, pH 9.2, ready for use in the assay of exonuclease I. The products were dialysed for 48 h against 4 × 100 vol. of 50 mM-glycine–NaOH buffer, pH 9.2, containing 50 mM-KCl to remove deoxyribonucleoside triphosphates. In all cases the amount of newly synthesized DNA represented only 3–7% of the original template DNA.

With native *E. coli* DNA as template, ^{3}H-labelled DNA was synthesized by using fraction N Seph and the product was tested for its susceptibility to hydrolysis by exonuclease I. Less than 5% of the ^{3}H-labelled DNA product was rendered acid-soluble although alkali-denatured *E. coli* ^{32}P-labelled DNA added to the same incubation was extensively degraded during the reaction (Fig. 5). The experiment was repeated after alkali-denatura-

tion of the ^{3}H-labelled DNA and the product was now found to be rapidly hydrolysed by exonuclease I (Fig. 6). Such results suggest strongly that the newly synthesized DNA is not present in a single-stranded form, although the possibility remains that exonuclease I action at the 3′-OH terminal of the DNA chain is prevented by a DNA polymerase molecule that is removed by treatment of the DNA at pH 13. The product of the *E. coli* DNA polymerase on native DNA templates has also been shown to be double-stranded (Kelly, Cozzarelli, Deutscher, Lehman & Kornberg, 1970).

A similar experiment was carried out on the DNA product of fraction S Seph, synthesized with alkali-denatured *E. coli* DNA as template. Treatment of the ^{3}H-labelled DNA with exonuclease I shows it also to be resistant to hydrolysis, although control single-stranded ^{32}P-labelled DNA, added before exonuclease I treatment, is rapidly rendered acid-soluble. Alkali-denaturation of the DNA product again renders it susceptible to exonuclease I action. Bollum (1963) showed that the product of the calf thymus DNA polymerase after 100% replication of a single-stranded DNA template is native DNA as judged by its behaviour on methylated albumin columns coated with kieselguhr and in aqueous polymer systems. In our experiments the extent of replication achieved is only 3–7% of the input DNA and hence the product may be a partially double-stranded structure with the newly synthesized DNA strand present in the double-stranded region of the molecule.

Analysis of the DNA products on neutral and alkaline sucrose gradients. Bollum (1966) showed that the product of calf thymus DNA polymerase, with a denatured DNA template and in the presence of initiating oligonucleotides, is native DNA. If initiator is present the newly formed DNA strand plus initiator can be separated from the DNA template whereas, in the absence of initiator, the reaction is slower and the product is a 'hairpin-like' structure with the new strand covalently attached to the DNA template.

Neutral and alkaline sucrose-density-gradient analyses of the products of L929 enzyme(s) were undertaken to determine the mode of attachment of the newly synthesized DNA to the DNA template. Fractions N Seph and S Seph were used in these experiments and the extent of replication was 0.2% of the input DNA. On neutral sucrose density gradients the DNA product formed by fraction N Seph showed an exact correspondence between the peak of extinction and acid-precipitable radioactivity (Fig. 7) indicating that, under these conditions, the newly formed strands are attached to the template DNA. In alkaline gradients, however, the bulk of acid-precipitable radioactivity sediments more slowly than indicated by the extinction profile (Fig. 8). Similar results were obtained in a study of the DNA product of fraction S Seph. Such findings suggest that at least part of the newly synthesized material is not covalently bound to the original template and is smaller in size. It has been shown, however, with the *E. coli* enzyme (Kelly *et al.* 1970) and a DNA polymerase from human KB cells (Greene & Korn, 1970) that, under conditions of limited synthesis, a covalent

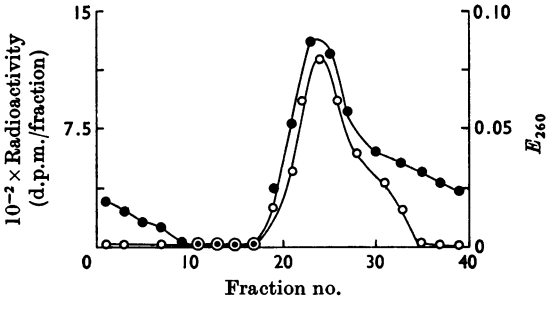

Fig. 7. Neutral sucrose-density-gradient centrifugation of the ^{3}H-labelled DNA product of preparation N Seph. The ^{3}H-labelled DNA product was synthesized as described in the legend to Fig. 5 except that 600 μg of native *E. coli* DNA was added as template. The assay mixture was dialysed for 48 h against 4 × 100 vol. of 20 mM-tris-HCl buffer, pH 7.5, containing 50 mM-KCl. Samples (0.15 ml) of the above solution were layered on top of 5–20% sucrose gradients (5 ml, containing 20 mM-tris-HCl buffer, pH 7.5, 1 M-NaCl and 1 mM-EDTA) and centrifuged for 10 h at 24000 rev./min (44000 $g_{av.}$) in the SW 39L rotor of the Spinco Model L ultracentrifuge at 0–4°C. Fractions (2 drops) were collected and assayed for E_{260} (●) and radioactivity (○) as described in the Materials and Methods section.

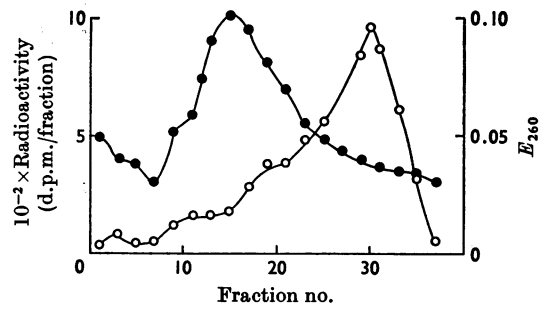

Fig. 8. Alkaline sucrose-density-gradient centrifugation of the ^{3}H-labelled DNA product of preparation N Seph. The procedure was similar to that described for Fig. 7 with the following modifications: (1), DNA samples were alkali-denatured before gradient analysis; (2), the gradient contained 0.1 M-NaOH; (3) centrifugation was for 10 h at 32000 rev./min (84000 $g_{av.}$). ●, E_{260}; ○, acid-precipitable radioactivity.

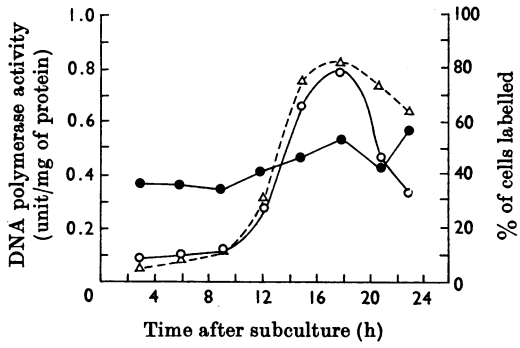

Fig. 9. Nuclear DNA polymerase activity in L929 cells. Cells were grown to stationary phase under conditions of frequent medium replenishment, and were harvested by treatment with trypsin and diluted into Petri dishes ($1-2\times10^5$ cells/ml) at zero time. They were harvested by scraping at various times thereafter and nuclear preparations were prepared and assayed for DNA polymerase activity as described in the Materials and Methods section. The '% of cells labelled' values are the results of a radioautographic analysis carried out by incubating parallel dishes with [^{3}H]thymidine (3.3μCi/10nmol per ml) for 1h before acid-fixation (see the Materials and Methods section). The values for DNA polymerase activity (nmol of [^{3}H]dTTP incorporated/h per mg of protein) are with a native (●) or a heat-denatured (○) DNA template. △, % of cells labelled.

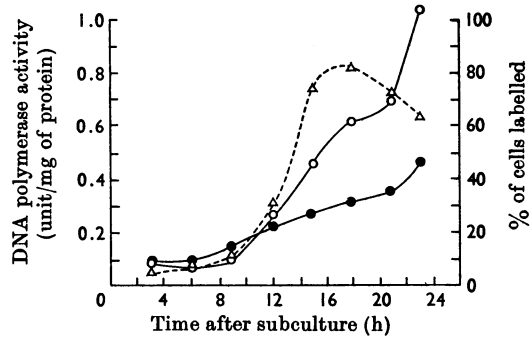

Fig. 10. Supernatant DNA polymerase activity in L929 cells. Preparation and conditions were as described in the legend to Fig. 10 and the Materials and Methods section.

attachment is formed between the 3′ end of the template and the 5′ end of the product. In view of these findings it may be that the separation found in our case represents preferential breakdown of the product DNA by contaminating endonuclease activity.

DNA polymerase activity in released stationary cells. When L929 mouse cells are allowed to grow to high density under conditions of frequent medium replenishment, cell division ceases and the rate of DNA synthesis falls to very low values (J. G. Lindsay & R. L. P. Adams, unpublished work). On subculturing such stationary cultures there is a wave of DNA synthesis, which does not start for at least 12h and which reaches a maximum at about 18h after subculture (Adams, 1969).

In the period after subculture before the onset of DNA synthesis the predominant DNA polymerase activity that can be detected is in the nuclear fraction, and this preparation shows a fourfold greater activity with a native than with a denatured DNA template. The level of this activity shows little change over the 24h period after subculture (Fig. 9). In contrast, the activity of the nuclear enzyme with denatured DNA shows an eightfold increase, reaching a maximum at about 18h. This increase closely follows the increase in the number of cells making DNA (Fig. 9). At the peak of activity the nuclear fraction shows 40% more activity with a denatured than with a native DNA template.

The DNA polymerase activity of supernatant fractions rises as the cells enter the S phase, but there is a reproducible plateau at about the time of the maximum rate of DNA synthesis, followed by a further increase in activity as the proportion of cells in the S phase falls (Fig. 10). Supernatant fractions show twice the activity with a denatured as with a native DNA template 20h after subculture. This system thus shows a noteworthy parallel with regenerating rat liver (Iwamura *et al.* 1968).

The increases in the activities of the nuclear and supernatant enzymes (with a denatured DNA template) are not dependent on DNA synthesis, as they occur in the presence of hydroxyurea (Table 3), aminopterin or 5mM-thymidine (see below). However, addition of actinomycin D (0.1μg/ml) at 11h after subculture prevents the rises in activity and also inhibits the increase in the rate of DNA synthesis measured at 18h (Table 3). This concentration of actinomycin D has no immediate effect on the rate of DNA synthesis but inhibits RNA synthesis to 30% of control values. Similar results have been found with primary rabbit kidney cells by Adams, Abrams & Lieberman (1966).

DNA polymerase activity in synchronized cells. Taking advantage of the small proportion of cells in the S phase shortly after subculture of stationary cells a single block in DNA synthesis imposed from 8 to 24h results in more than 90% of the cells being synchronized near the beginning of the S phase (Adams, 1969). Such a block was imposed by aminopterin and was reversed by addition of thymidine (0.1mM). The changes in the DNA polymerase activities of nuclear preparations after release of

Table 3. *Effect of inhibitors on induction of DNA polymerase measured with a denatured DNA template*

Cells were grown and enzymes assayed as described in the legend to Figs. 9 and 10 and in the Materials and Methods section. The rate of DNA synthesis was measured by following the incorporation of [^{3}H]thymidine (0.66 μCi/5 nmol per ml) into acid- and ethanol-insoluble material after a 1 h incubation from 10 to 11 or 17 to 18 h after subculture as described in the Materials and Methods section.

Time after subculture (h)	Addition at 11 h	DNA polymerase activity (unit/mg of protein)		Rate of DNA synthesis (d.p.m. in [^{3}H]thymidine incorporated/h per 10^3 cells)
		Supernatant fraction	Nuclear fraction	
11	—	0.10	0.15	1.6
18	—	0.66	0.41	7.8
18	Hydroxyurea (2 mM)	0.63	0.41	1.0
18	Actinomycin D (0.1 μg/ml)	0.15	0.16	1.6

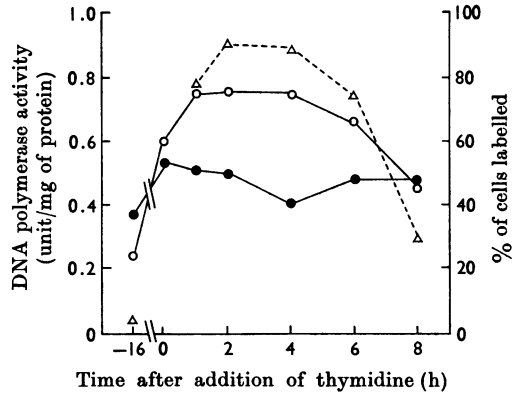

Fig. 11. Nuclear DNA polymerase activity in synchronized L929 cells. At 8 h after subculture of stationary cells DNA synthesis was inhibited by addition of aminopterin (see the Materials and Methods section). The inhibition was reversed 16 h later and cells harvested and assayed as described in the Materials and Methods section. The '% of cells labelled' (△) values are the results of a radioautographic analysis carried out by incubating dishes with [^{3}H]thymidine (10 μCi/dish, final concentration 0.1 mM) for 1 h before acid-fixation (see the Materials and Methods section). The values for DNA polymerase activity (nmol of [^{3}H]dTTP/h incorporated per mg of protein) are with a native (●) or with a heat-denatured (○) DNA template. Similar results were obtained in several other experiments with aminopterin-synchronized cells, and in one experiment with cells synchronized by treatment with excess of thymidine.

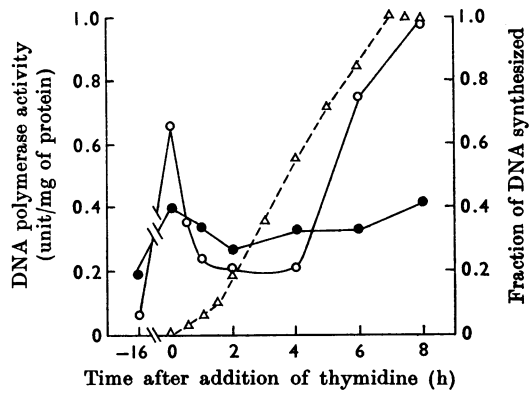

Fig. 12. Supernatant DNA polymerase activity in synchronized L929 cells. Preparation and conditions were as described in the legend to Fig. 11 and the Materials and Methods section. The fraction of DNA synthesized (△) was obtained by addition at zero time of [^{3}H]thymidine (5 μCi/dish at a final concentration of 0.1 mM). Dishes were fixed at various times thereafter and the radioactivity incorporated into DNA was assayed as described in the Materials and Methods section. The results are expressed as a fraction of the incorporation at 7½ h after reversal, a value corresponding to the synthesis of 9.3 pg of DNA/cell. ●, Native DNA template; ○, heat-denatured DNA template.

an aminopterin block are shown in Fig. 11, from which it can be seen that the activity with a denatured DNA template has risen during the block. Moreover, the nuclear preparations preferentially use denatured DNA as template from 0 to 6 h after reversal of the block, a situation parallel to that pertaining in Fig. 9 from 14 to 20 h. There is no significant change in the activity of nuclear preparations when native DNA is the template.

Immediately after reversal of the block in DNA synthesis there is a sharp fall in the DNA polymerase activity of supernatant fractions measured with denatured DNA (Fig. 12). The extent of this fall is not consistent but varies markedly from a 30% to an 80% fall in activity. Minimum activity is found between 2 and 4 h after reversal, after which the activity rises again. Similar findings have been reported by Littlefield, McGovern & Margeson (1963) and Gold & Helleiner (1964).

One possible explanation for the persistence of enzymic activity in the nucleus at a time when DNA synthesis is no longer occurring is that the binding of the polymerase in some way stabilizes the enzyme. Stabilization of the DNA polymerase from ascites-tumour cells in the presence of DNA was reported by Shepherd & Keir (1966) and we have obtained similar results with the enzyme from L929 cell nuclei.

The constant specific activity of the nuclear native primed enzyme throughout the cell-cycle may reflect a limited number of binding sites that are always maintained saturated. The fall in the activity of the supernatant enzyme at the time of peak DNA synthesis (Fig. 12) may reflect the inactivation of this enzyme or its binding in the nucleus to sites liberated by nuclear enzyme inactivated during DNA synthesis.

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