DNA Polymerases from *Chlamydomonas reinhardtii*

FURTHER CHARACTERIZATION, ACTION OF INHIBITORS AND ASSOCIATED NUCLEASE ACTIVITIES

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The properties of three DNA polymerase species A, B and C, purified from *Chlamydomonas reinhardtii* were compared. DNA polymerases A and B have K_m values with respect to deoxyribonucleoside triphosphates of $19\mu\text{M}$ and $3\mu\text{M}$ respectively. DNA polymerase A is most active with activated DNA, but will also use native DNA and synthetic RNA and DNA templates with DNA primers. DNA polymerase B is also most active with activated DNA, but will use denatured DNA and synthetic DNA templates. It is inactive with RNA templates. DNA polymerase B is completely inactive in the presence of $100\mu\text{M}$ -heparin, which has no effect on DNA polymerase A activity. Heparin dissociates DNA polymerase B into subunits that are still catalytically active, but which heparin inhibited. DNA polymerase B possesses deoxyribonuclease activity that is inhibited by $5\mu\text{M}$ -heparin, suggesting that the deoxyribonuclease is an integral part of the DNA polymerase moiety. DNA polymerase A is devoid of nuclease activity. DNA polymerase C is similar to DNA polymerase B in all these properties, though it is more active with RNA primers and has greater heat-sensitivity.

It is still not possible to correlate purified eukaryotic DNA polymerases with their exact functions in DNA metabolism. Comparison of the reactions of purified DNA polymerases *in vitro* therefore becomes a more important consideration. Apart from their physical properties and general assay requirements, eukaryotic DNA polymerases have been mainly characterized with respect to their utilization of different types of synthetic template-primers, and their response to known inhibitors.

The lack of associated nuclease activities in purified preparations of mammalian DNA polymerases has facilitated their characterization with respect to their differing abilities in copying various DNA species or DNA template-RNA primers. In general, enzymes can be classified into α -, β - and γ -types by their template preferences (Bollum, 1975; Weissbach, 1975). DNA polymerase- α from HeLa cells was the only enzyme to elongate an RNA primer annealed to a natural template (Spadari & Weissbach, 1974), whereas only DNA polymerases- β will not utilize the synthetic DNA template-RNA primer. DNA polymerases- γ can be differentiated from the α - and β -activities by their ability to utilize the synthetic poly(A)·dT₁₅ at rates 5–10 times that of

deoxyribohomopolymers. Similarly, the higher eukaryotic DNA polymerases can be assigned broadly to the same three classes when their responses to different inhibitors are compared.

The mode of action of prokaryotic DNA polymerases, and *Escherichia coli* DNA polymerase I in particular, has been characterized in detail (Kornberg, 1974). It seems that the co-ordination of DNA polymerase activities with associated nuclease activities may determine the overall action of an enzyme at any time. A conditional lethal *polA* mutant of *E. coli* has been isolated and found to be defective in DNA repair and replication (Konrad & Lehman, 1974; Olivera & Bonhoeffer, 1974). The polymerizing activity is present in this mutant, but the 5':3'-exonuclease function is absent, resulting in decreased nick translation at the restrictive temperature (Uyemura & Lehman, 1976). Mutations that alter the DNA polymerase activity of T4 DNA polymerase relative to its associated 3':5'-nuclease activity, giving rise to an anti-mutator phenotype, have been well documented (Muzyczka *et al.*, 1972; Lo & Bessman, 1976. Highly purified DNA polymerases from higher eukaryotic cells, however, have been found to be devoid of nuclease activity [although the isolation of a DNA polymerase with an associated 3':5'-exonuclease has been claimed (Byrnes *et al.*, 1976)]. The presence or absence of

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associated nuclease activity therefore seems to be a fundamental difference between prokaryotic and higher eukaryotic DNA polymerases. The DNA polymerases A and B from *Chlamydomonas* were investigated with respect to the above parameters.

Experimental

Materials

These were described previously (Ross & Harris, 1978). In addition, ethidium bromide, novobiocin and *p*-hydroxymercuribenzoate were all from Sigma Chemical Co. Kingston upon Thames KT2 7BH, U.K. U(pU)₅, dT₁₀, poly(dA) and poly(rA) were from P-L Biochemicals, Milwaukee, WI, U.S.A., and poly(dA-dT) and poly(rA)·poly(dT) were from Miles Laboratories, Kankakee, IL, U.S.A. Heparin B.P. (mol.wt. 10000–20000) was from Evans Medical, Speke, Liverpool, U.K., and phosphonoacetic acid was from K & K Rare and Fine Chemicals, ICN Pharmaceuticals, Plainsview, NY, U.S.A. Ampholine (pH range 3.5–10.0) was from LKB, South Croydon CR2 8YD, U.K. [³H]DNA from *Bacillus subtilis* was kindly donated by S. J. Chaney of this Department.

Methods

DNA polymerase assays and glycerol-density-gradient analysis were carried out as described in the accompanying paper (Ross & Harris, 1978), with fractions (VIa) and (VIb) of the purification scheme. To ensure maximum recovery of labelled product (McLennan & Keir, 1975a,b), nitrocellulose filters instead of GF/C paper discs were used for trapping acid-insoluble material when measuring enzyme activities with synthetic template-primers. Also, when enzyme activities were measured with synthetic template-primers, assays were incubated at 26°C instead of 32°C, only 5 µg of DNA was included in the incubation mixture, and the specific radioactivity of d[³H]TTP was increased to 100 µCi/µmol. Filters were processed as described previously (Ross & Harris, 1978), except that washing with ethanol was omitted.

Preparation of template-primers. Solutions (1 mg/ml) of calf thymus DNA were made up in 50 mM-Tris/HCl, pH 7.8. DNA was denatured by heating a solution of a native DNA at 100°C for 15 min, followed by rapid cooling in an ice/water mixture. 'Activated' DNA that had been nicked with pancreatic deoxyribonuclease 1 to produce many 3'-hydroxy groups was prepared as before (Ross & Harris, 1978).

A procedure outlined by Cavalieri *et al.* (1974) and designed to produce the most active configuration of synthetic polymer mixtures was carried out. Appropriate amounts of primer and template solution (1 mg/ml in 0.015 M-NaCl/15 mM-sodium citrate,

pH 7.0) were heated together at 55°C for 15 min and then left to cool slowly to room temperature (20°C). In this way, solutions of poly (A)·dT₁₀, poly(dA)·dT₁₀, poly(A)·poly(dT) and poly(dA)·U₆ were prepared.

Deoxyribonuclease assay. Total assay volume was 280 µl and contained the following components, which made up the standard assay mixture for exonuclease I of *Chlamydomonas reinhardtii* (Tait & Harris, 1977a): 80 mM-Tris/HCl, pH 8.5, 8 µg of acetylated bovine serum albumin, 10% (w/v) glycerol, 5 mM-2-mercaptoethanol, 0.35 µg of [³H]-DNA (approx. 5000 c.p.m.), enzyme protein and either 2 mM-MgCl₂/2 mM-CaCl₂/20 mM-EDTA or 2 mM-MgCl₂ and 2 µg of ATP. After incubation at 32°C for 1 h, the assays were chilled on ice and 0.2 ml of acetylated bovine serum albumin (1.25 µg/ml) was added and finally 0.1 ml of 50% (w/v) trichloroacetic acid. The tubes were left for 20 min and then centrifuged at 1000 g_{av.} at 4°C for 45 min; 0.2 ml of supernatant was analysed in 2 ml of Triton X-100/toluene scintillation fluid (Ross & Harris, 1978).

Isoelectric focusing. Isoelectric focusing in 7–70% continuous glycerol gradients was carried out with a semi-preparative Buchler gel apparatus, the total volume of each gradient being 23 ml. Gradients contained 2 mM-2-mercaptoethanol and 4% (v/v) LKB ampholytes, pH range 3.5–10.0. Before focusing began, the enzyme was distributed throughout the gradient, and electrophoresis carried out for 20 h at 4°C at constant voltage (400 V) and with an initial current of 1.5 mA/tube. Gradients were fractionated by upward displacement with 70% (w/v) sucrose, and 0.75 ml fractions collected. The pH of each fraction was measured and 0.025 ml samples were assayed for DNA polymerase activity by using assay system 2 as described in the accompanying paper (Ross & Harris, 1978).

Results

The preparations of DNA polymerases A and B used were fractions VIA and VIB of Ross & Harris (1978). Fig. 1 shows a pI 5.0–5.5 for DNA polymerase A and pI 4.8–5.2 for DNA polymerase B, consistent with the only marginally different elution profiles of the enzymes from DEAE-cellulose (see Ross & Harris, 1978).

Utilization of deoxyribonucleotide precursors

Both enzymes require the presence of all four deoxyribonucleoside triphosphates when activated calf thymus DNA is used as template. Omission of dGTP from the reaction mixture decreased overall activity to 15 and 20% with DNA polymerases A and B respectively (results not shown). Both enzymes displayed Michaelis-Menten kinetics when the concentrations of all four substrates were altered

Fig. 1. Isoelectric focusing of DNA polymerases A and B. Isoelectric focusing was carried out as described under 'Methods', 8 μ g of DNA polymerase A (a) and 12 μ g of polymerase B (b) being applied. $\bullet$, DNA polymerase activity; $\square$, pH. The arrow marks the position of marker haemoglobin.

Fig. 2. Activities of DNA polymerases A and B as a function of substrate concentration. The four deoxyribonucleoside triphosphates were present in equimolar amounts for each point. $\bullet$, DNA polymerase A; $\circ$, DNA polymerase B.

simultaneously, and the K_m values extrapolated from double-reciprocal plots were 19 μ M and 3 μ M for DNA polymerases A and B respectively (Fig. 2). The concentration of individual deoxyribonucleoside triphosphates were not varied independently.

Template utilization

Natural DNA templates have the advantage in that they should resemble the template *in vivo*, but since the exact nature of commercially available preparations of high-molecular-weight DNA is ill-defined, measuring enzyme activity with constant

Table 1. Activity of DNA polymerases A and B with natural DNA templates

Assays were carried out for 1 h at 32°C, each assay containing 18 μ g of calf thymus DNA. DNA polymerase A was measured in 2 mM-MgCl₂ or 0.1 mM-MnCl₂ and polymerase B in the presence of 5 mM-MgCl₂ or 0.1 mM-MnCl₂. Maximum activity in Mg²⁺ ions was 256 and 74 nmol of dTMP residues incorporated/h per mg of protein for polymerase A and B respectively. Results are expressed as percentage of the activity produced by 18 μ g of 'activated' calf thymus DNA in the presence of maximal concentration of Mg²⁺ ions for each enzyme.

Template	DNA polymerase A		DNA polymerase B	
	Mg ²⁺	Mn ²⁺	Mg ²⁺	Mn ²⁺
'Activated' DNA	100	61	100	22
Native DNA	22	22	11	2
Denatured DNA	7	5.5	41	9
None	0	0	0	0

DNA concentrations may not represent optimum conditions for each type of template utilized. From Table 1, both enzymes preferred activated calf thymus DNA to native and denatured forms in the presence of either Mg²⁺ or Mn²⁺. However, the two enzymes differed in their response to the 'strandedness' of templates, DNA polymerase A being more active with native DNA than denatured DNA, whereas DNA polymerase B utilized a denatured template more efficiently.

Table 2. *Activity of DNA polymerases with synthetic template–primers*

A portion (5 μ g) of each template–primer was used in each assay, and incubation was carried out for 1 h at 26°C. The specific radioactivity of d[Me-³H]TTP was increased to 100 μ Ci/ μ mol. Polymerase A was assayed with 2 mM-Mg²⁺ and polymerases B and C with 5 mM-Mg²⁺. All enzymes were assayed with 0.1 mM-Mn²⁺. Samples of polymerase A (0.25 μ g), polymerase B (0.8 μ g) or polymerase C (0.4 μ g) were used in each assay, and the activity produced by 5 μ g of 'activated' calf thymus DNA was 360, 264 and 348 nmol of dTMP residues incorporated/h per mg of protein for polymerases A, B, and C respectively. Results are expressed as percentage of the activity produced with 5 μ g of poly(dA)·dT₁₀ (1:1).

Template–primer	Polymerase A		Polymerase B		Polymerase C	
	Mg ²⁺	Mn ²⁺	Mg ²⁺	Mn ²⁺	Mg ²⁺	Mn ²⁺
Poly(dA)·dT ₁₀ (1:1)	100	32	100	1	100	0.5
Poly(dA)·dT ₁₀ (1:10)	39	29	30	0	29	0.2
Activated calf thymus DNA	49	16	9.2	0	7.5	0
Poly[d(A-T)]	15	4	9.5	0.1	11.5	0.1
Poly(rA)·poly (dT)	7	41	0	0	0	0
Poly(rA)·dT ₁₀ (1:1)	2.5	9	0.1	0	0.1	0
Poly(rA)·dT ₁₀ (1:10)	1.8	6	0.5	0	0.1	0
Poly(dA)·rU ₅ (1:1)	0	0	1.3	0	3.6	0.1
Poly(dA)	0	0	1.5	0	1.2	0
(dT ₁₀)	1	0	0.6	0	0.1	0

Table 2 shows the activity of the three enzymes with respect to different synthetic template–primers. Incubation temperature was 26°C instead of 32°C to increase the stability of the polymer mixtures, and this may explain the altered relative activity with Mg²⁺ or Mn²⁺ compared with the results in Table 1. Taking into account the nature of dTMP residues incorporated in each assay system, DNA polymerase B had 2.5-fold the activity with poly(dA)·dT₁₀ (1:1) as template–primer than with an equivalent amount of activated DNA, in terms of total incorporation of dNMP. The activity of both enzymes was decreased by two-thirds when the template:primer ratio of poly(dA):dT₁₀ was increased from 1:1 to 1:10. Substituting the oligoribonucleotide U(pU)₅ as primer with poly(dA) template abolished polymerase A activity, and only 1% of polymerase B activity remained.

Polymerase B displayed very little or no activity in the presence of ribonucleotide templates. Polymerase A, however, utilized all three ribonucleotide–template–deoxyribonucleotide–primer combinations tested, and in each case the activity recorded in 0.1 mM-Mn²⁺ was severalfold the value measured at 2 mM-Mg²⁺.

The activity of *Chlamydomonas* polymerase C in the presence of these synthetic template–primers was very similar to polymerase B, but, in addition, polymerase C utilized poly(dA)·dT₁₀ in the presence of Mn²⁺ and poly(dA)·rU₅ to a small extent.

Inhibitors

The effect of a range of known DNA polymerase inhibitors on the *Chlamydomonas* polymerases A and B was measured. For all the inhibitors listed in

Table 3. *Effect of inhibitors on DNA polymerases A and B*

Inhibitor concentration required for 50% inhibition (mM)	Polymerase	
	A	B
<i>N</i> -Ethylmaleimide	1.5	0.5
<i>p</i> -Hydroxymercuribenzoate	0.1	0.05
Novobiocin	0.7	0.8
Phosphonoacetic acid	0.7	0.15
Ethidium bromide	0.001	0.001

Table 3, the responses of the *Chlamydomonas* polymerases were similar, differing only in degree of inhibition. Both enzymes were inhibited by the thiol-blocking agents *N*-ethylmaleimide and *p*-hydroxymercuribenzoate, when assayed in the absence of β -mercaptoethanol. Polymerase B seemed slightly more sensitive than polymerase A. Since mammalian DNA polymerases- α are almost completely inhibited at 1 mM-*N*-ethylmaleimide, whereas polymerases- β enzymes retain their activity at 10 mM inhibitor, the *Chlamydomonas* DNA polymerases respond in intermediate fashion to these two extremes. Novobiocin has been shown also to be an inhibitor of high-molecular-weight eukaryotic polymerases (Sung, 1974; McLennan & Keir, 1975a), over the same concentration range used to inhibit *Chlamydomonas* polymerases A and B. By the criteria of Mao & Robishaw (1975), polymerases A and B are actually resistant to phosphonoacetic acid, a competitive inhibitor at the PP_i-binding areas of the DNA polymerase active site (Leinbach *et al.*, 1976). Their herpes simplex virus-induced

DNA polymerase was 90% inhibited at 50 μ M-phosphonoacetic acid, a concentration at which *Chlamydomonas* polymerase A retained full activity and polymerase B was inhibited by only 10%.

A significant difference was observed in the response of the *Chlamydomonas* DNA polymerases to heparin. Analogous results were obtained with dextran sulphate (results not shown). Fig. 3 shows that polymerase B was totally inhibited at a heparin concentration of only 100 μ M, whereas polymerase A retained 80% of its activity at 500 μ M inhibitor.

Fig. 3. Effect of heparin on the activity of DNA polymerases A and B

Assays were incubated at 32°C for 1 h. Results are expressed as the percentages of polymerase activity obtained by substituting water for inhibitor solution. ●, Polymerase A; ○, polymerase B.

Fig. 4. Effect of KCl on heparin inhibition of DNA polymerase B

The heparin concentration was constant at 50 μ M, results are expressed as the percentage heparin inhibitions of uninhibited DNA polymerase in the absence of KCl. ●, Heparin inhibition; ----, polymerase B activity.

Further, the heparin inhibition of polymerase B could be abolished by increasing the KCl concentration to 200 mM (Fig. 4). Since polymerase B was also known to dissociate at KCl concentrations of 200 mM and above (Ross & Harris, 1978), it was possible to speculate that a dissociated form of polymerase B would be resistant to heparin, whereas a higher-molecular-weight form would be susceptible. That this was not so was shown by the experiment depicted in Fig. 5. The inclusion of 25 μ M-heparin in enzyme preparations loaded within glycerol gradients decreased the *s* value of the enzyme to 7.3S (Fig. 5b), intermediate between the native 8.9S (Fig. 5a) and dissociated 5.9S (Fig. 5c). This suggests that heparin dissociates the native form, the 7.9S species representing a complex of dissociated polymerase B (5.9S) and heparin. Sedimentation of the enzyme in the presence of heparin throughout 10–30% glycerol in 500 mM-KCl decreased the size of the sedimenting species to 5.9S (Fig. 5c), rather than 7.3S, a form of polymerase B that could still be inhibited by heparin in subsequent assays (results not shown).

Since the sedimentation time was 17 h, the enzyme-inhibitor complex must be quite stable at 4°C, to give the sedimentation profile of Fig. 5(b). If the stability of

Fig. 5. Effect of including heparin in glycerol gradients of DNA polymerase B

Fraction (VI) of DNA polymerase B preparations was analysed in glycerol gradients in the presence of lactate dehydrogenase as marker: (a) 10–30% glycerol containing 100 mM-KCl; (b) 10–30% glycerol containing 100 mM-KCl and 25 μ M-heparin; (c) 10–30% glycerol containing 500 mM-KCl and 25 μ M-heparin.

the enzyme-inhibitor complex were the same at 32°C as at 4°C, then preincubating enzyme and inhibitor before transferring to an assay incubation should greatly increase the observed heparin inhibition. This in fact was not the case (results not shown). A temperature-dependence of heparin inhibition can then be predicted, and experimental confirmation of this prediction is shown in Fig. 6.

Associated deoxyribonuclease activity

The deoxyribonuclease activity of purified polymerase fractions was examined over a range of pH conditions, in Tris/HCl buffer, and maximum activity for the polymerase-B-associated nuclease was pH 8.5 (pH optimum of the polymerase moiety being 7.5).

Under no circumstances was any nuclease activity detectable in purified polymerase A preparations. Only a necessarily limited range of reaction conditions were used, however, so that this observation does not preclude the existence of a contaminating or associated nuclease that has very different reaction requirements from those conditions tested.

In the presence of 2mM-Mg²⁺ ions, polymerase B will solubilize both native and denatured DNA templates, the activity being greater with denatured DNA (Table 4). Both activities were slightly stimulated by ATP. Polymerase B also showed more limited Ca²⁺-dependent nuclease activity, on denatured DNA template. The Mg²⁺-dependent nuclease activity was extremely susceptible to heparin,

Table 4. *Deoxyribonuclease activity of polymerases A and B*
Each assay contained 1.1 nmol of DNA substrate and 0.6 µg of polymerase A or polymerase B. Activity is expressed as nmol of dTMP residues rendered acid-soluble/h per mg of protein. Assays were performed in Tris/HCl buffer, pH 8.5.

DNA	Assay conditions	Specific activity	
		Poly- merase A	Poly- merase B
Native	2mM-Mg ²⁺	0	39
Native	2mM-Mg ²⁺ + 2 µg of ATP	0	52
Native	2mM-Ca ²⁺	0	0
Denatured	2mM-Mg ²⁺	0	61
Denatured	2mM-Mg ²⁺ + 2 µg of ATP	0	73
Denatured	2mM-Ca ²⁺	0	30
Denatured	2mM-Mg ²⁺ + 5 µM-heparin	—	2
Denatured	2mM-Mg ²⁺ + 50 µM-heparin	—	0

Fig. 6. *Temperature-dependence of heparin inhibition of DNA polymerase B*

Samples of DNA polymerase B were incubated in assay systems containing 100mM-KCl for 1 h at the appropriate temperature. The heparin concentration was constant at 50 µM. Results are expressed as percentage heparin inhibitions of enzyme incubated without heparin at the appropriate temperature. ----, Polymerase B; ●, heparin inhibition.

Fig. 7. *Heat denaturation of DNA polymerases B and C*
Polymerase B (75 µg) and polymerase C (50 µg) were incubated at 40°C in the presence of 1.25 mg of acetylated serum albumin/ml. At various times, samples were removed and assayed for enzyme activity at 32°C for 1 h. ●, Polymerase C; ○, polymerase B.

although *Chlamydomonas* exonuclease I (Tait & Harris, 1977a,b) and pancreatic deoxyribonuclease I were unaffected when assayed under their optimum conditions. The nuclease activity also co-sedimented with DNA polymerase B in glycerol gradients. This observation supports the view that the nuclease

activity may be present as part of the polymerase B itself, rather than as a contaminant.

DNA polymerase C

DNA polymerase C was very similar to DNA polymerase B in physical properties and in sensitivity to heparin. It may differ, however, in template utilization and was much more thermolabile than polymerase B (Fig. 7).

Discussion

The isoelectric point of both *Chlamydomonas* polymerases A and B was about 5. In this they resembled the high-molecular-weight polymerases- α of vertebrate cells (Loeb, 1974).

The K_m values of the Mg^{2+} -dNTP complexes for polymerases A and B respectively were $19\mu M$ and $3\mu M$ (Fig. 2b). Both values are within the range ($160\mu M$) quoted for mammalian DNA polymerases (Loeb, 1974) and all other lower-eukaryotic species. The K_m values of nucleotides for *Chlamydomonas* polymerases were not measured with any synthetic template-primers, nor with Mn^{2+} as bivalent cation. Generally, enzymes whose Mn^{2+} -dNTP K_m values have been measured showed a greater affinity for these substrates (Loeb, 1974; McLennan & Keir, 1975a; Stalker *et al.*, 1976).

Several workers have pointed out that the optimum bivalent cation concentration for DNA polymerases changes with the template studied (Rougeon *et al.*, 1973; Crerar & Pearlman, 1974). The utilization of different synthetic polynucleotides by most (including the *Chlamydomonas*) DNA polymerases has been measured at the optimum Mg^{2+} concentration for activated DNA. Polymerase A copied double-stranded DNA, whereas polymerase B preferred a single-stranded template, although, like the majority of other purified eukaryotic DNA polymerases, the best natural template for both enzymes was activated DNA. With synthetic polynucleotides, polymerase B activity was confined almost entirely to copying a DNA template. Polymerase A, however, displayed a more catholic range of utilized templates, preferring to copy DNA, but the enzyme showed measurable activity with RNA templates and particularly when Mn^{2+} ions were present. A limited ability to copy polyribonucleotide templates has often been cited in the characterization of purified higher-eukaryotic DNA polymerase- β and this has been used to distinguish these enzymes from polymerases- α (Bollum, 1975; Weissbach, 1975). Several of the lower-eukaryotic polymerases, however, retained the ability to copy RNA templates, whereas in other respects they resembled mammalian polymerase- α . Among these were polymerases A and B from yeast (Wintersberger, 1974), whereas the highly purified DNA polymerase from *Ustilago maydis* copied poly(dA)·dT₁₀ especially well at 37°C (Banks *et al.*,

1976). At the same time, however, polymerase B from yeast resembled the *Chlamydomonas* polymerase B in its marked preference for poly(dA)·dT₁₀ over other template-primers, as did the 7S enzyme purified from tobacco cells (Srivastava & Grace, 1974). Other lower-eukaryotic DNA polymerases also resembled polymerase B in their inability to copy RNA templates, including the *Tetrahymena* enzyme (Crerar & Pearlman, 1974), the α -like enzyme from *Dictyostelium discoideum* (Loomis *et al.*, 1976) and both high-molecular-weight enzymes from *Euglena gracilis* (McLennan & Keir, 1975b). Still other enzymes, such as the polymerase in crown-gall tumour cells of periwinkle (Gardner & Kado, 1976), copy poly[d(A-T)] efficiently, but used neither poly(dA)·dT₁₀ nor poly(rA)·dT₁₀. These differences underline difficulties encountered in trying to compare the characteristics of enzymes isolated to differing degrees of purity. Some general conclusions, however, may be made from observations enumerated here. In template-primer specificity, the *Chlamydomonas* polymerase B displayed characteristics associated with the polymerase- α from mammalian organisms, namely the exclusive copying of DNA templates. By comparison, polymerase A also had the ability to copy RNA templates, a property normally associated with polymerase- β and polymerase- γ species. This observation reinforces the point that, in general, although lower-eukaryotic DNA polymerases in many ways resemble their mammalian counterparts, they cannot be regarded as exactly comparable.

A major difference between *Chlamydomonas* DNA polymerases A and B was the effect of heparin on the two activities, polymerase A being quite resistant, whereas polymerase B was completely inhibited at low concentrations of inhibitor. The effect of heparin on polymerase B was to dissociate the enzyme molecule after, or concomitant with, binding of the inhibitor (Fig. 5). The difference in molecular weight between polymerase B dissociated by salt (Fig. 5c) and the inhibitor-enzyme complex was about 40000. Since the molecular weight of commercial heparin is in the range 10000–20000, it is reasonable to suggest that the inhibitor-enzyme complex contained probably two bound inhibitor molecules.

The exact mechanism whereby heparin brought about dissociation of DNA polymerase B was not discovered. One possibility was that heparin, being a linear anionic polysaccharide, associated with a 100000-mol.wt. form of polymerase B to cover an area on the surface of the enzyme containing, not only the active site, but also a part involved in enzyme subunit interactions. The inhibition of RNA polymerase (Walter *et al.*, 1967) and *E. coli* DNA polymerase I synthesis in isolated mammalian nuclei (Kraemer & Coffrey, 1970) by heparin has been

recorded. In the latter case, the effect of the inhibitor was to compete with the DNA template for binding sites on the polymerase molecule.

de Recondo & Abadiebat (1976) have reported the salt-mediated dissociation of a high-molecular-weight heparin-inhibited DNA polymerase from regenerating rat liver, which displayed many of the properties of a mammalian polymerase- α , into a low-molecular-weight DNA polymerase- β form, resistant to heparin. Lazarus & Kitron (1974) have also published evidence that a high-molecular-weight DNA-synthesizing activity from BHK cells, inhibited by heparin, was also dissociated into smaller subunits, which were partially resistant to the compound. The dissociation of *Chlamydomonas* polymerase B by heparin therefore is not exactly analogous to its effect on polymerases from higher eukaryotes.

The most likely site for the interaction of heparin and polymerase B was the active site of the enzyme, where a reasonable role for the inhibitor would be the occupation of binding sites normally held by template DNA. The specificity of the inhibitor towards only one of the *Chlamydomonas* DNA polymerases is intriguing, and, in this context, the utilization of a denatured DNA template over a native one by polymerase B in contrast with the preference for a native template by polymerase A may be relevant. The association of an anionic polysaccharide with the functional area of only one of the enzymes suggests that the architecture of the active sites of polymerases A and B differed.

Chlamydomonas polymerase B also displayed an Mg^{2+} -dependent nuclease with activity greatest with denatured DNA. It is noteworthy that the nuclease activities associated with yeast DNA polymerase B (Wintersberger & Wintersberger, 1970), the *Tetrahymena* enzyme (Crerar & Pearlman, 1974), polymerase B from *Euglena gracilis* (McLennan & Keir, 1975b) and the *Ustilago maydis* DNA polymerase (Banks *et al.*, 1976) all displayed greatest activity when measured with denatured DNA in the presence of Mg^{2+} ions. The 3':5'-nuclease of the *Ustilago* DNA polymerase undergoes the same salt-induced change in sedimentation coefficient as the polymerase moiety (Banks & Yarranton, 1976). The sensitivity of the *Chlamydomonas* polymerase B nuclease activity to heparin, at concentrations one-tenth of those that left *Chlamydomonas* exonuclease I and pancreatic deoxyribonuclease I unaffected, gave indirect evidence of a genuine association of the two activities. Yeast, *Euglena* and *Chlamydomonas* are similar in that these organisms contain (at least) one high-molecular-weight DNA polymerase with associated nuclease activity and another DNA polymerase without. In this respect, again, lower eukaryotes present an intermediate position between bacterial and mammalian DNA polymerases. The significance of this observation will not be fully understood until

a greater understanding of the mechanisms of DNA synthesis is obtained.

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