Properties of a DNA polymerase from purified nuclei and DNA-synthesizing complexes of human spermatozoa

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Summary. A DNA polymerase was isolated from human spermatozoa. In one procedure, spermatozoa were decapitated with detergent, the heads purified and then lysed with dithiothreitol, trypsin and deoxyribonuclease. DNA polymerase was isolated from the lysate by sedimentation through an 18% Metrizamide solution, solubilization with 0.8 M-KCl–0.5% Triton X-100 and sequential chromatography on DEAE cellulose, phosphocellulose and hydroxylapatite. Alternatively, the heads of intact spermatozoa, untreated with detergent, were lysed as above; the subsequent Metrizamide pellet fraction was isolated and further fractionated by gel filtration and buoyant density centrifugation. The enzyme in this fraction was solubilized with KCl–Triton X-100. Characterization by velocity centrifugation and phosphocellulose chromatography revealed that it possessed properties indistinguishable from those of the enzyme purified from isolated sperm nuclei. The DNA polymerase had an apparent molecular weight of 79 000–89 000, Mn^{2+} (1 mM) was the preferred divalent cation and activity was inhibited by concentrations of potassium phosphate greater than 10 mM. The synthetic template preferences of the enzyme were $dT_{12-18} \cdot \text{poly rA} > \text{poly(dA-dT)} > dT_{12-18} \cdot \text{poly dA}$; no activity was observed with $dG_{12-18} \cdot \text{poly rC}$ or dT_{10} .

Introduction

DNA-synthesizing complexes appear to be normal components of human spermatozoa. Liberated from sperm nuclei following their decondensation with dithiothreitol, these complexes were capable of the polymerization of [3H]TTP (Witkin, Korngold & Bendich, 1975) or [3H]dCTP (Bendich, Borenfreund, Witkin, Beju & Higgins, 1976) into acid-insoluble polymers in the absence of an exogenously supplied template. Analysis of the 3H -labelled product-template complex revealed that RNA was probably serving as both template and primer for the synthesis of DNA (Witkin & Bendich, 1977).

An antiserum inhibitory to the DNA polymerase component of the complex was elicited in a rabbit by immunization (Witkin & Higgins, 1978) with purified human sperm nuclei (Witkin, Evenson & Bendich, 1977b). The immunoglobulin G (IgG) fraction of this antiserum inhibited the activity of DNA polymerases partly purified from human spermatozoa and seminal fluid, as well as the reverse transcriptase (RNA-instructed DNA polymerase) of the non-oncogenic C-type baboon endogenous retrovirus (BEV) and the feline virus, RD 114. Other type C viral reverse transcriptases were unaffected (Witkin, Higgins & Bendich, 1978). In addition, IgG purified from sera of some individual male and female members of infertile marriages also inhibited the sperm-derived DNA polymerase, suggesting that the enzyme was auto-antigenic. Further studies have demonstrated that one facet of the auto-immunological consequences of

vasectomy was the production of IgG inhibitory to the seminal fluid DNA polymerase (Higgins, Witkin & Bendich, 1978), possibly because of the formation of antibodies to the sperm enzyme.

We have now analysed the properties of a DNA polymerase isolated from purified human sperm heads or the DNA-synthesizing complexes obtained from whole spermatozoa.

Materials and Methods

Semen was obtained from medical and graduate student donors. Following liquefaction at room temperature, the ejaculates were mixed with an equal volume of glycerol for storage at -20°C .

DNA polymerase purification from sperm nuclei

In a typical experiment, 5–20 ejaculates of a single donor (about 2×10^8 spermatozoa/ejaculate) were utilized. The samples were thawed, pooled and diluted with 3 volumes of TNE buffer (0.01 M-Tris-HCl, pH 7.5, 0.15 M-NaCl, 1 mM-EDTA). All subsequent steps were at $0-4^{\circ}\text{C}$, unless otherwise noted. Spermatozoa were concentrated by centrifugation at 1500 g for 10 min, resuspended in TNE buffer and recentrifuged for 3 additional cycles to remove seminal plasma constituents. The final sperm pellet was suspended in 0.05 M-Tris-HCl, pH 7.5, to give 0.4 ml/ejaculate, and sodium lauroyl sarcosinate (Sarkosyl: Chemical Additives Co., Farmingville, New York) was added to a final concentration of 1.5%. Treatment with Sarkosyl decapitates mammalian spermatozoa (Gall, Millette & Edelman, 1975; Witkin *et al.*, 1977b) and removes the plasma, acrosomal and nuclear membranes (Witkin *et al.*, 1977b). It has been our experience that spermatozoa stored at -20°C were more resistant to this treatment than were those in freshly obtained ejaculates. However, about 98% of the spermatozoa were cleaved by vigorously mixing the spermatozoa-Sarkosyl suspension for 30 sec in a Vortex mixer (Scientific Industries Inc., Queensville, New York) followed by incubation at 37°C ; separation of heads from tails was usually complete by 15 min.

Detached sperm heads were purified according to the procedure of Calvin (1976). The suspension was brought to 9.5 ml with 1.6 M-sucrose in 0.01 M-Tris-HCl, pH 7.5, 2 mM-EDTA and layered onto a discontinuous gradient of 10.5 ml 2.2 M-sucrose and 10.5 ml 2.05 M-sucrose, both in the same Tris-EDTA buffer as the sample. Following centrifugation for 60 min at 51 000 g, sperm heads were recoverable in the pellet; the level of contamination with tails was consistently $<2\%$. The heads were resuspended (0.2 ml ejaculate) in 0.05 M-Tris-HCl, 0.02 M-dithiothreitol, plus 100 μg trypsin/ml (Worthington Biochemical, Freehold, New Jersey) and incubated at 37°C until the heads lysed and the solution became highly viscous; this usually required 10–15 min. Pancreatic trypsin inhibitor (Trasylol: Mobay Chemical Co., New York) was then added at 200 units/ml to inhibit the activity of the added trypsin. The released DNA was degraded by the addition of 5 mM-MgCl₂ and 20 μg deoxyribonuclease/ml (Worthington) followed by incubation at 37°C for 15 min. A second deoxyribonuclease treatment was usually found to be essential in order to avoid the occurrence of multiple peaks on subsequent ion-exchange chromatographic steps. The disrupted sperm head mixture was clarified by centrifugation at 16 000 g for 20 min and the DNA polymerase activity was concentrated from the resultant supernatant by sedimentation at 165 000 g for 60 min through a solution of 18% (w/v) Metrizamide (Accurate Chemical and Scientific Corp., Hicksville, New York), as previously described (Witkin, Traganos & Bendich, 1977a).

The Metrizamide pellet fraction was suspended in 1.0 ml 0.1 M-Tris-HCl, pH 7.6, 0.8 M-KCl, 0.5% Triton X-100, 5 mM-dithiothreitol and 200 units Trasylol and incubated for 30 min at 0°C to solubilize the proteins in the DNA polymerase complex. The addition of Trasylol at this and at subsequent stages of enzyme purification resulted in increased enzyme activity. The solution was diluted 40-fold with Buffer D (0.05 M-Tris-HCl, pH 7.5, 10% glycerol, 2

mm-dithiothreitol) and loaded onto a 40 ml column of DEAE cellulose (DE 52, Whatman Ltd., Maidstone, Kent, England) which had been previously equilibrated with 0.02 M-KCl in Buffer D. Two column volumes of 0.02 M-KCl in Buffer D were passed through the column and DNA polymerase was eluted by raising the KCl concentration to 0.4 M. Fractions were collected and assayed for dT₁₂₋₁₈·poly rA-templated DNA polymerase activity, as described below.

The peak activity fractions from DEAE cellulose were pooled and Trasylol was added to 200 units/ml. The solution was diluted to 0.05 M-KCl with Buffer D and loaded onto a 40 ml column of phosphocellulose (P11: Whatman), prepared as described by Burgess (1969), previously equilibrated with 0.05 M-KCl in Buffer D. After washing with 2 column volumes of 0.05 M-KCl in Buffer D, DNA polymerase was eluted with a 200 ml 0.05–0.7 M-KCl gradient in Buffer D.

The peak dT₁₂₋₁₈·poly rA activity fractions from phosphocellulose were pooled, Trasylol was added to 200 units/ml and the solution was dialysed for 16 h against 1 litre of 0.05 M-Tris-HCl, pH 7.5, in Buffer H (2 mM-dithiothreitol, 0.02 M-potassium phosphate, pH 7.5, 20% glycerol). The solution was then layered onto a 20 ml column of hydroxylapatite (Sphero-hydroxylapatite: Accurate Chemical and Scientific Corp., prepared according to the manufacturer), previously equilibrated with Buffer H. After passing 2 column volumes of Buffer H through the column, DNA polymerase was eluted with an 0.02–0.3 M-potassium phosphate gradient in Buffer H. The peak activity fractions were pooled and either utilized immediately or stored at –70°C.

Molecular weights were estimated by the method of Martin & Ames (1961). Aliquots of the DNA polymerase were layered onto 10–30% (v/v) glycerol or 15–30% (w/w) sucrose gradients in 0.1 M-Tris-HCl, pH 7.5, 0.4 M-KCl, 5 mM-dithiothreitol, subjected to centrifugation at 130 000 g for 16 h, fractionated and assayed for dT₁₂₋₁₈·poly rA templated DNA polymerase activity. Human IgG, bovine serum albumin and sperm whale myoglobin were run as molecular weight markers in parallel gradients and located by absorbance at 280 nm.

Purification of intact DNA-synthesizing complexes

We have previously devised a procedure of liberating the DNA-synthesizing complex from spermatozoa which does not involve pre-treatment with detergent (Witkin *et al.*, 1977a). Briefly, whole spermatozoa, following 3 cycles of centrifugation and suspension in TNE buffer, were treated with dithiothreitol, trypsin and deoxyribonuclease as described above for purified sperm heads. This treatment resulted in complete sperm head lysis, while the tails remained intact as viewed by phase-contrast microscopy. A 165 000 g Metrizamide pellet was obtained (see above), suspended in TNE buffer and subjected to gel filtration through an 80 ml column of Ultrogel AcA 34 (20 000–350 000 effective mol. wt range: LKB Instruments Inc., Rockville, Maryland). Fractions were eluted with TNE buffer and assayed for dT₁₂₋₁₈·poly rA-templated DNA polymerase activity (see below). The peak activity fractions, present in the void volume, were pooled and layered onto a 20 ml 25–50% (w/w) sucrose gradient in TNE buffer. After centrifugation at 165 000 g for 16 h fractions were collected, their density determined from refractive indices, and aliquots assayed for dT₁₂₋₁₈·poly rA-templated activity.

DNA polymerase assays

DNA polymerase activity was detected by measuring the polymerization of [³H]TTP or [³H]dGTP (New England Nuclear, Boston, Massachusetts) in the presence of the synthetic template-primers dT₁₂₋₁₈·poly rA, dT₁₂₋₁₈·poly dA, (dA–dT), dT₁₀ or dG₁₂₋₁₈·poly rC (P-L Biochemical, Milwaukee, Wisconsin). In a final volume of 50 µl the reactions contained 0.5 µmol dithiothreitol, 5 µg bovine serum albumin, 0.05 µmol MnCl₂, 0.08 µmol Tris-HCl, pH 7.5, 0.75 µg synthetic template-primer and either 4 nmol dTTP plus 5 µCi [³H]TTP (final sp.

act. 770 c.p.m./pmol) or 4 nmol dGTP plus 5 μ Ci [3 H]dGTP (final sp. act. 685 c.p.m./pmol); 4 nmol dATP were also added to this mixture in poly(dA-dT)-templated reactions. Incubation was at 30°C for the times indicated in the individual experiments. When measuring DNA polymerase activity in the DNA-synthesizing complex before it was solubilized with 0.1% Triton X-100 and 0.8 M-HCl, 0.1% Triton X-100 was included in the assay mixture. Reactions were terminated with 5% trichloroacetic acid-1% sodium pyrophosphate and acid-insoluble radioactivity was measured (counting error less than $\pm 4\%$) as described previously (Witkin *et al.*, 1975). Assays run in the absence of added enzyme served as controls and the amount of acid-insoluble radioactivity present in these samples (300–600 c.p.m.) has been subtracted from the experimental values.

Text-fig. 1. Chromatography of human sperm DNA polymerase on (a) DEAE-cellulose, (b) phosphocellulose and (c) hydroxylapatite columns. (a) A 165 000 g pellet fraction isolated from human sperm nuclei was treated with 0.8 M-KCl, 0.5% Triton X-1000 and the solubilized enzyme was chromatographed on a 7.5 $\times$ 2.6 cm column. Elution was with 0.4 M-KCl. From each fraction (0.5 ml) 20 μ l were assayed using a dT₁₂₋₁₈·poly rA template. The arrow marks the start of the 0.4 M-KCl elution. (b) The pooled peak activity fractions from (a) were diluted and chromatographed on an 8.5 cm column. Elution was with 200 ml 0.05–0.5 M-KCl gradient. Fractions (1.95 ml) were collected and 20 μ l aliquots were assayed for dT₁₂₋₁₈·poly rA-templated activity. (c) The pooled peak activity fractions from (b) were dialysed and chromatographed on a 10.2 $\times$ 1.5 cm column. Elution was with 40 ml 0.02–0.3 M-KPO₄ gradient. Aliquots (20 μ l) of each 1.0 ml fraction were assayed for dT₁₂₋₁₈·poly rA activity.

Results

DNA polymerase purification from sperm nuclei

As shown in Text-fig. 1(a), most of the activity on DEAE-cellulose was coincident with the elution of 0.4 M-KCl. The small amounts of dT₁₂₋₁₈·rA activity which preceded this main peak were not directly examined further. However, no additional DNA polymerase peaks were detected when either the 0.4 M-KCl DEAE-cellulose eluant or the fractions obtained by resuspending the 165 000 g pellet in 0.4 M-KCl and passing it through the DEAE column were chromatographed on phosphocellulose.

When the pooled DNA polymerase-containing fractions from DEAE-cellulose were chromatographed on phosphocellulose with a 0.05–0.5 M-KCl gradient, there was one major peak of dT₁₂₋₁₈·poly rA-templated enzyme activity, eluting at about 0.2 M-KCl (Text-fig. 1b).

Further elution on an hydroxylapatite column with a 0.02–0.3 M-potassium phosphate gradient gave one major peak of enzyme activity, eluting at 0.07 M-potassium phosphate (Text-fig. 1c). The other 2 fractions with minor activity were not studied further.

Results of a typical fractionation are summarized in Table 1. Starting from the DEAE-cellulose pool, a 25-fold purification of the enzyme was accomplished with a 57% recovery. It was not possible to demonstrate a proportionality between enzyme activity and enzyme concentration at stages before the DEAE cellulose fraction, perhaps because of the continued presence of residual deoxyribonuclease, trypsin or seminal phosphatase. Activity was related to enzyme concentration at subsequent stages (data not shown).

Table 1. Purification of DNA polymerase from human sperm nuclei

Fraction	Protein (mg)	Activity (pmol)	Recovery (%)	Sp. act. (pmol/mg)
Metrizamide pellet	8.8	—	—	—
DEAE-Cellulose pool	4.3	218.5	100	50.8
Phosphocellulose pool	1.4	210.2	96.2	150.1
Hydroxylapatite pool	0.09	125.3	57.3	1392.2

Properties of the sperm DNA polymerase

A molecular weight estimation of the sperm DNA polymerase was obtained by subjecting the enzyme, purified through either the phosphocellulose or hydroxylapatite steps, to velocity sedimentation in a 10–30% glycerol, 0.4 M-KCl gradient and ascertaining the position of dT₁₂₋₁₈·poly rA-templated activity relative to bovine serum albumin (68 000). Assuming a globular protein, the sperm DNA polymerase was calculated to have a molecular weight of 79 000–89 000, based on 3 separate experiments using the phosphocellulose- or hydroxylapatite-purified enzyme from 3 semen samples of individual donors. A typical profile is shown in Text-fig. 2. The more rapidly sedimenting shoulder was usually present (see also Text-fig. 4a) and may represent an enzyme aggregate.

The preferred divalent cation for the dT₁₂₋₁₈·poly rA-templated reaction was Mn²⁺ with an optimum at 1 mM; substitution of Mg²⁺ at its optimal concentration of 2 mM resulted in 5-fold less activity (Table 2). The enzyme was not inhibited by 0.01 M-potassium phosphate but phosphate concentrations of 0.02 M and above were progressively inhibitory (Table 2).

The synthetic template-primer preferences of the purified sperm DNA polymerase are shown in Table 3. For comparison, values obtained with substitution of bovine DNA polymerase γ (Worthington Biochemical) for the sperm enzyme are also given. The preferred template for both enzymes was dT₁₂₋₁₈·poly rA; substitution of dT₁₂₋₁₈·poly dA led to a 4-fold (sperm enzyme) or 7-fold (DNA polymerase γ) decrease in activity. The sperm enzyme was more effective than

Text fig. 2. Velocity centrifugation of human sperm DNA polymerase. An aliquot (0.2 ml) of the peak activity fraction from phosphocellulose was diluted with 0.1 ml gradient buffer (see 'Materials and Methods') and sedimented in a 10–30% glycerol gradient. Aliquots (20 μ l) of each 0.3 ml fraction were assayed using a dT_{12–18}·poly rA template. The arrow marks the position of bovine serum albumin (mol. wt 68 000) run in a parallel gradient.

the bovine enzyme in poly(dA–dT)-templated reactions. Neither enzyme efficiently utilized dT₁₀ or dG_{12–18}·poly rC.

Isolation of DNA-synthesizing complex

A single major peak was obtained (Text-fig. 3a) in gel filtration fractions corresponding to the void volume (determined using blue dextran). Endogenous activity consistently co-eluted in these same fractions (data not shown). After equilibrium centrifugation in a sucrose gradient, peak activity was found at a density of 1.19 g/ml (Text-fig. 3b), corresponding with our previously reported density for endogenous activity (Witkin *et al.*, 1977a).

Characterization of the DNA polymerase in the DNA-synthesizing complex

The size of the enzyme in the 1.19 g/ml density fraction was estimated by velocity sedimentation in a 10–30% sucrose gradient. There was a major peak in a fraction corresponding to a protein of 79 000 molecular weight, and this peak and its shoulder (Text-fig. 4a) were similar

Table 2. Effect of PO_4^{-3} , Mg^{2+} and Mn^{2+} on human sperm DNA polymerase

Exp.	Addition	Conc. (mM)	Incorporation (pmol [^3H]TTP)
I	None	—	2.3
	K_3PO_4	10	2.6
	K_3PO_4	20	1.8
	K_3PO_4	30	1.8
	K_3PO_4	50	0.9
	K_3PO_4	100	0.8
II	None	—	0
	MnCl_2	0.2	0.1
	MnCl_2	0.5	0.4
	MnCl_2	1.0	1.2
	MnCl_2	2.0	0.4
	MnCl_2	5.0	0.2
	MgCl_2	1	0.06
	MgCl_2	2	0.3
	MgCl_2	5	0.2
	MgCl_2	7.5	0.03
	MgCl_2	10	0.03

Aliquots of phosphocellulose-purified sperm DNA polymerase were assayed in the presence of increasing concentrations of K_3PO_4 , MnCl_2 or MgCl_2 , using a dT_{12-18} ·poly rA template. The incorporation of [^3H]TTP into acid-insoluble product is per 50 μl reaction incubated at 37°C for 30 min.

Table 3. Comparison of synthetic template-primer utilization by human sperm DNA polymerase and bovine DNA polymerase γ

Template primer	Incorporation (pmol [^3H]TTP/ 50 μl reaction)	
	Human sperm polymerase	Bovine DNA polymerase γ
dT_{12-18} ·poly rA	2.1	2.0
dT_{12-18} ·poly dA	0.5	0.3
poly (dA–dT)	1.0	0.5
dG_{12-18} ·poly rC	0.014	0.014
dT_{10}	<0.05	<0.05

The sperm head DNA polymerase, purified through the glycerol gradient centrifugation step, and 0.25 μg fetal calf liver DNA polymerase γ were incubated with the various template primers plus the [^3H]TTP or [^3H]dGTP reaction mixture (see 'Materials and Methods') for 30 min at 37°C and the acid-insoluble radioactivity was determined. Duplicate analyses varied by less than 10%. The KCl concentration in all reactions was 160 mM.

to that found with DNA polymerase purified from isolated sperm nuclei (Text-fig. 2). Chromatography of this activity peak on phosphocellulose (Text-fig. 4b) yielded a major peak of dT_{12-18} ·poly rA activity eluting at 0.2 M-KCl, similar to the elution profile of the enzyme isolated from sperm nuclei (Text-fig. 1b).

Text-fig. 3. Gel filtration and buoyant density of human sperm DNA-synthesizing complex. (a) A 165 000 g pellet fraction isolated from enzyme-disrupted sperm heads was subjected to gel filtration on a 73 × 1.2 cm Ultrogel AcA 34 column. The elution rate was 14 ml/h; 1.2 ml fractions were collected and 20 μ l aliquots were assayed using a dT₁₂₋₁₈·poly rA template. (b) The pooled peak activity fractions from (a) were centrifuged to equilibrium in a 20 ml 25–50% sucrose gradient; 0.9 ml fractions were collected, density was determined from refractive indices, and 20 μ l aliquots were assayed for dT₁₂₋₁₈·poly rA-templated activity.

Discussion

A major question arising from these studies is the origin of the sperm DNA polymerase. The improbability of semen-derived somatic cells, or mycoplasma or extracellular viruses as the source of the enzyme has already been discussed (Witkin *et al.*, 1975). A possible sperm mitochondrial origin of the DNA polymerase is more difficult to rule out, especially in light of indications that the mitochondrial DNA polymerase is, in fact, DNA polymerase γ (Bolden, Noy & Weissbach, 1977; Beriazioni, Scovassi & Brun, 1977; Hübscher, Kuenzle & Spadari, 1977). Bolden *et al.* (1977) demonstrated that 20% of the DNA polymerase γ present in HeLa cells was associated with mitochondria, while the remainder was distributed between the nucleus and cytoplasm. If a similar situation exists for spermatozoa, then the intracellular localization of the enzyme reported here may have to be made solely by morphological criteria. Accordingly, the identification of an enzyme with identical properties both from detergent-treated purified

Text-fig. 4. Velocity centrifugation and phosphocellulose chromatography of DNA polymerase from the sperm DNA-synthesizing complex. (a) The peak activity fractions from the sucrose gradient (Text-fig. 3b) were concentrated by centrifugation and DNA polymerase was solubilized by the addition of 0.8 M-KCl, 0.5% Triton X-100. The enzyme was sedimented in a 5 ml 15–30% sucrose gradient. Aliquots (20 μ l) of each 0.17 ml fraction were assayed using a dT_{12–18}·poly rA template. The arrows mark the position of human immunoglobulin G (mol. wt 160 000), bovine serum albumin (68 000) and sperm whale myoglobin (17 800) run in parallel gradients. (b) The pooled peak activity fractions from (a) were chromatographed on a 10 $\times$ 0.9 cm column. Elution was with 100 ml of a 0.05–0.5 M-KCl gradient; 1.5 ml fractions were collected and 20 μ l aliquots were assayed using a dT_{12–18}·poly rA template.

sperm heads and from enzymically disrupted spermatozoa suggests a nuclear location for the DNA polymerase.

Although inconclusive, there are some biochemical data which suggests that the DNA polymerase described here is not from mitochondria. The inhibition of the sperm enzyme by 0.1 M-phosphate is consistent with similar properties of DNA polymerase γ obtained from rat liver and HeLa cells; in contrast, the mitochondrial DNA polymerase γ from rat liver and HeLa cells was unaffected by 0.1 M-phosphate (Bolden *et al.*, 1977). In addition, the endogenous DNA polymerase reaction of mitochondria purified from rat liver (D'Agostino, Lowry & Kalf, 1975) or chick embryo fibroblasts (D'Agostino & Nass, 1976) was inhibited by dithiothreitol or B-mercaptoethanol, while dithiothreitol was non-inhibitory to DNA synthesis by the sperm DNA-synthesizing complex (unpublished observations).

Our bovine DNA polymerase γ preparation was present in a 0.2 mg/ml solution purported by the supplier to contain 2 units of activity/ml; 1 unit is defined as the polymerization of 1 nmol dTTP by incubation at 37°C for 60 min in the presence of dT₁₂₋₁₈ · poly rA. In the experiment described in Table 3, 1.25 μ l of this DNA polymerase γ -containing solution catalysed the incorporation of 2 pmol dTTP (0.004 units) in a 30-min reaction. This is slightly better than the expected calculated value of 0.0025 enzyme units per 2 pmol dTMP polymerized. Our assay system is thus shown to be efficient for measuring this enzyme activity and a minimum estimate (assuming 100% recovery at each stage of purification) for the number of DNA polymerase γ -equivalents/spermatozoon can be calculated. An aliquot of the glycerol gradient-purified sperm DNA polymerase from approximately 2×10^9 spermatozoa also possessed 0.04 units (2 pmol) of activity (Table 3), i.e. 5.8×10^{-10} units/spermatozoon or 1.2×10^{-1} units/ejaculate. Similar calculations for DNA polymerase γ , utilizing the data of Bolden *et al.* (1967) and estimating 3×10^8 cells or 3×10^{11} mitochondria (A. Bendich, personal communication), give an estimate for rat liver of approximately 6×10^{-10} units of enzyme in the non-mitochondrial fraction per cell.

The results of Bolden *et al.* (1977) also show that there are approximately 2.8×10^{-14} units of enzyme/mitochondrion. If a human spermatozoon contains 30 mitochondria (Lung, 1974) and if our head preparations were contaminated with mitochondria at a level of 2%, then there would be 4×10^6 tails or 1.2×10^8 mitochondria in the head preparation per ejaculate. If mitochondrial DNA polymerase γ activity was similar in spermatozoa and in rat liver cells, 3.3×10^{-6} units of DNA polymerase γ activity/ejaculate could be expected. However, our minimum estimate for the enzyme activity in our sperm preparation was over 100 000 times greater than this value (see above), and the majority, at least, of the sperm DNA polymerase is not therefore of mitochondrial origin.

The DNA polymerase obtained by two fractionation procedures following rupture of the sperm nucleus appears to be the enzyme present in the DNA-synthesizing complex (Witkin *et al.*, 1975; Witkin & Bendich, 1977; Witkin *et al.*, 1977a). DNA polymerase γ has been found associated with other endogenous DNA-synthesizing components from human lymphoid cells (Yoshida & Cavalieri, 1977), rat brain (Witkin & Schumaker, 1977) and adenovirus-infected cells (Ito, Arens & Green, 1975; Brison, Kedingner & Wilhelm, 1977) and it may therefore be involved in specific DNA synthesis. The retention of this enzyme by the highly specialized male gametes, which have lost cytoplasm and achieved a condensed nucleus during spermatogenesis, suggests that the enzyme might have a function after fertilization. Further characterization of the sperm DNA-synthesizing complex might offer clues as to the role, if any, these enzymes play in DNA synthesis during embryogenesis and, by inference, in DNA replication in general.

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