Biochem. J. (1978) **175**, 585–594
Printed in Great Britain

Extraction and Biochemical Characterization of a Nuclear Deoxyribonucleic Acid Polymerase Activity in Bull Spermatozoa

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(*Received* 23 *January* 1978)

Bull spermatozoa heads were separated from cytoplasmic contaminants, especially mitochondria-rich middle pieces, by centrifugation through 2.4M-sucrose. DNA polymerase activity was demonstrated by incubating nuclear heads for 1h at 37°C or for 20h at room temperature in a medium containing detergent and dithiothreitol or 2-mercaptoethanol. Optimal DNA polymerase activity was detected after extraction in a medium containing 50mM-borate, pH9, 1mg of soya-bean trypsin inhibitor/ml and supplemented with either 20mM-dithiothreitol and 4% Tween 80 or 100mM-2-mercaptoethanol and 10% Tween 80. The DNA polymerase reaction was Mg^{2+}-dependent; Mn^{2+} or Ca^{2+} could not replace Mg^{2+} and all four deoxynucleoside triphosphates were required for optimal activity. The polymerase activity was pH-dependent (optimum between 8.2 and 10.5) and was a function of buffer composition and also of pH values. Optimal activity was obtained with 50mM-Na^+ or 150mM-K^+ and was partially lowered by *N*-ethylmaleimide; it was inhibited by spermidine and by salmon protamines, but was greatly stimulated by calf thymus histones. It was also resistant to actinomycin D, netropsin and ethidium bromide. The present results suggest that bull spermatozoa heads contain a β-type DNA polymerase activity.

Several enzymes that synthesize DNA *in vitro* and replicate the template present in the medium with high accuracy have been isolated from eukaryotic cells. Two of them are implicated in the duplication of chloroplastic and mitochondrial DNA, and the others (polymerases-α, -β and -γ) appear to be specifically related to the nuclear DNA metabolism of the cell. These enzymes have been isolated and their biochemical characteristics are now relatively precisely known (Craig & Keir, 1974; Fansler, 1974; Bollum, 1975; Weissbach, 1977). It is difficult to state, however, the exact role played by each of them during DNA duplication *in vivo*.

In general, great differences in DNA polymerase activities exist between one cellular type and another and the decrease in DNA polymerase activity is primarily correlated with the degree of cellular differentiation. Very little or no activity is found in cells that have accomplished their differentiation (Fansler, 1974; Chevaillier & Philippe, 1977). From this point of view, spermatozoa seem to be an exception.

Malkin (1953), Graves & Salisbury (1963, 1966), Koefoed-Johnsen *et al.* (1968) and Salisbury & Hart (1970) radioautographically demonstrated the incorporation of glycine and adenine into the nuclei of spermatozoa of several animal species. More recently, two DNA polymerase activities were extracted from ejaculated mammalian spermatozoa. Using intact bull spermatozoa, Hecht (1974) isolated a protein whose biochemical characteristics led to the conclusion that the enzyme had a mitochondrial origin. Witkin *et al.* (1975) and Witkin & Bendich (1977) extracted a complex from human spermatozoa heads which was associated with chromatin and which could synthesize DNA *in vitro*. Furthermore, we were able to demonstrate cytochemically that a DNA polymerase activity was present in all nuclei of the mouse testicular cells, especially in those of spermatozoa (Chevaillier & Philippe, 1976*a*,*b*). We obtained similar results with the spermatozoa of other species, such as the bull, newt and crab (Chevaillier & Philippe, 1977).

A more detailed study of mouse spermatozoal DNA polymerase activity revealed that only one enzyme was present in the nuclei of these cells, probably a β-type DNA polymerase (Philippe & Chevaillier, 1976).

Since the composition of spermatozoa nuclei is known to be relatively simple, containing only DNA and very few protein species, spermatozoa heads apparently constitute a satisfactory material for the rapid extraction and purification of DNA polymerase-β. The structure of these nuclei, however, is very compact and difficult to dissociate, particularly in the eutherian mammals. This is due to the presence

of cystine-containing basic proteins, which are tightly bound to the DNA of these cells (Marushige & Marushige, 1974, 1975). The various media commonly utilized for the extraction of the different DNA polymerases from somatic cells are thus unsuccessful when used on spermatozoa.

In the last several years, some authors (Calvin & Bedford, 1971; Bedford *et al.*, 1973; Marushige & Marushige, 1974, 1975; Mahi & Yanagimachi, 1975; Zirkin *et al.*, 1976; Gall & Ohsumi, 1976) tried to define better conditions for obtaining the dispersion of spermatozoa chromatin. These different methods have yielded very good results for studying the structural proteins associated with DNA. The problem is more difficult with DNA polymerase, however, since enzymic activity is partially or completely inhibited in most cases.

Before studying the biochemical characteristics of isolated nuclear DNA polymerase, it was first necessary to find a medium and incubation conditions that would enable the optimal detection of this activity. These results obtained with bull spermatozoa are described in the present paper; some properties of the enzyme are also described.

Materials and Methods

Preparation of the different biological fractions

Spermatozoa were collected from fertile bulls, frozen immediately in liquid N_2 without dilution and kept at −70°C before use.

Spermatozoa were washed with 0.14M-NaCl and centrifuged for 10min at 3000*g*. This operation was performed four times. The last pellet was suspended with a glass/Teflon Potter homogenizer in 0.25M-sucrose/1mM-EDTA, pH7.5, and sonicated with the microtip of an MSE apparatus at maximum power. Good cleavage between tails and heads was verified with a phase-contrast microscope; the time needed to obtain such a separation was generally 4×15s. The sonicated suspension was diluted with $\frac{1}{3}$ vol. of 2.4M-sucrose/1mM-EDTA, pH7.5, was then layered on top of the same 2.4M-sucrose solution and centrifuged for 1h at 47000*g* in a Beckman J-21 B centrifuge (rotor JA-20). The pellet, which contained the nuclear heads, was washed once with 1mM-EDTA, pH7.5, and was extracted for 1h at 37°C in various media; details of their compositions are given in the Results section.

After extraction, the suspension was then centrifuged for 15min at 3000*g*; in most cases, a pellet and a soluble extract were obtained. In some cases, however, depending on the composition of the extraction medium, we were unable to separate a pellet from a supernatant: such a preparation was called a 'total extract'.

When two fractions were separated after extraction and centrifugation the properties of the DNA polymerase activity of these two fractions were studied; when a total extract was obtained, we looked for the presence of a DNA polymerase activity in this single fraction.

DNA polymerase assay

DNA polymerase activity was determined after incubation of the biological fraction at 37°C, generally for 30min, in the following medium: 50mM-borate/NaOH buffer, pH9.0, 100μg of soya-bean trypsin inhibitor/ml, 150mM-KCl, 15mM-$MgCl_2$, 100μM each of dATP, dGTP, dCTP, 10μM-dTTP and [*methyl*-^{3}H]dTTP (30μCi/ml; 1500c.p.m./pmol). The incubations were performed in the presence or absence of activated exogenous calf thymus DNA (400μg/ml) prepared as described by Fansler & Loeb (1974). The enzymic reaction was stopped by addition of ice-cold 5% (w/v) $HClO_4$, and the DNA polymerase activity was expressed as the amount of radioactivity incorporated into the acid-insoluble fraction collected on Whatman GF/C glass filters (de Recondo & Fichot, 1969).

dATP, dCTP, dGTP, dTTP and calf thymus DNA were from Boehringer Mannheim Corp., Mannheim, West Germany. [*methyl*-^{3}H]Thymidine 5′-triphosphate (ammonium salt) (40–60Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Nuclease assay

Nuclease activity was determined as follows: 3.5μg of linear and double-stranded SV40 viral [^{3}H]DNA (a gift from M. Méchali, I.R.S.C., Villejuif, France) was incubated for 1h at 37°C with the biological fraction in a medium containing 50mM-borate buffer, pH9.0, 150mM-KCl and 15mM-$MgCl_2$. After incubation, the reaction was stopped as above and the acid-insoluble fraction was recovered on Whatman GF/C glass filters. The difference in radioactivity of the acid-insoluble fraction before and after incubation was used as a measure of nuclease activity.

Electron microscopy

For electron-microscope observations, the nuclear head pellets unextracted or extracted under different conditions were fixed for 15min in 2.5% glutaraldehyde in 0.1M-potassium phosphate buffer, pH7.2, and then for 1h in 2% OsO_4 in the same buffer. They were then embedded in Epon/Araldite. After being stained with uranyl acetate and lead citrate, the sections were observed with an RCA electron microscope.

Results

To interpret the results unambiguously, it was first necessary to obtain pure fractions of spermatozoa heads.

As seen by phase-contrast microscopy, and more precisely on electron micrographs (Plate 1*a*), the pellets obtained after sedimentation through 2.4M-sucrose were free of cytoplasmic contaminants, particularly of mitochondria-rich middle pieces. This step was very important, since a mitochondrial DNA polymerase was isolated from intact bull spermatozoa by Hecht (1974).

Results obtained with various extraction media used by other authors (*Table* 1)

We tested those media used for extracting DNA polymerase from intact bull spermatozoa (Hecht, 1974) or from cells rich in proteolytic enzymes (Chang, 1976) or for the study of the structural proteins associated with DNA in mammalian spermatozoa (Calvin & Bedford, 1971; Marushige & Marushige, 1974, 1975).

The greatest DNA polymerase activity was detected in a modification of the medium of Calvin & Bedford (1971). Sodium dodecyl sulphate was omitted, since it denatures proteins; it, in fact, inhibited almost all of the DNA polymerase activity. The medium was supplemented with both soya-bean trypsin inhibitor, to protect the enzyme from degradation by intracellular proteinases, and Tween 80. A lower activity was detected when 50mM-Tris/HCl buffer, pH8.8, replaced 50mM-borate buffer, pH9.

It is noteworthy that only a small portion of the DNA polymerase activity of spermatozoa heads could be detected in the medium used by Hecht (1974) for intact bull spermatozoa. More satisfactory results were obtained with the various media proposed by Chang (1976) and by Marushige & Marushige (1974). The results are shown in Table 1.

In the course of our work it was noted that significant differences in DNA polymerase activity could exist among different donors or different ejaculates by the same donor (Table 2). A similar observation was made by Bendich *et al.* (1976) with human spermatozoa. Thus, to compare unambiguously the

Table 1. *Incorporation of dTTP obtained with various extraction media used by other authors*

100% DNA polymerase activity corresponded to 0.6pmol of dTTP incorporated by 3×10^8 spermatozoa heads. Abbreviation: n.d., not determined.

Reference	Composition	Activity (%)	
		Endogenous	+400μg of DNA/ml
Calvin & Bedford (1971)	50mM-Borate buffer, pH9+20mM-dithiothreitol+0.5% sodium dodecyl sulphate	n.d.	3.9
Modified media of Calvin & Bedford (1971)	50mM-Borate buffer, pH9+20mM-dithiothreitol+0.5% sodium dodecyl sulphate+10μg of soya-bean trypsin inhibitor/ml	n.d.	4.3
	50mM-Borate buffer, pH9+20mM-dithiothreitol+10μg of soya-bean trypsin inhibitor/ml	n.d.	77.7
	50mM-Tris/HCl, pH8.8+20mM-dithiothreitol+10μg of soya-bean trypsin inhibitor/ml	n.d.	27.9
	50mM-Borate buffer, pH9+20mM-dithiothreitol+10μg of soya-bean trypsin inhibitor/ml+1% Tween 80	32.5	100
Hecht (1974)	20mM-Tris/HCl buffer, pH8.1+50mM-NaCl+3mM-2-mercaptoethanol+1mM-EDTA+125mM-$(NH_4)_2SO_4$+10μg of soya-bean trypsin inhibitor/ml+20% (v/v) ethylene glycol	2.8	8.9
Chang (1976)	200mM-Phosphate buffer, pH7.4+1mM-mercaptoethanol+4mM-EDTA+1mM-phenylmethanesulphonyl fluoride	16.8	59.3
Marushige & Marushige (1974)	50mM-Tris/HCl buffer, pH8+5mM-iodoacetamide+5M-guanidinium chloride	22.7	46.6

Table 2. *Variation of dTTP incorporation as a function of spermatozoa batches*
These DNA polymerase activities were measured in the presence of 400μg of exogenous activated DNA/ml. The results were obtained with three different media. Partial extracts were obtained with the first two and a total extract with the last one.

Extraction medium	Spermatozoa sample	dTTP incorporated by 3×10^8 spermatozoa heads (pM)
(*a*) 50mM-Borate buffer, pH9+20mM-dithiothreitol+1% Tween 80+10μg of soya-bean trypsin inhibitor/ml	1	2.79
	2	0.253
	3	0.600
(*b*) 50mM-Borate buffer, pH9+20mM-dithiothreitol+1mg of soya-bean trypsin inhibitor/ml+4% Tween 80	1	0.310
	2	1.34
	3	1.13
	4	1.09
	5	4.51
(*c*) 50mM-Borate buffer, pH9+100mM-2-mercaptoethanol+1mg of soya-bean trypsin inhibitor/ml+4% Tween 80	1	2.13
	2	3.34
	3	3.03
	4	6.72

effects of various extraction media on spermatozoa heads, we consistently used the same batch of spermatozoa for a given series of experiments.

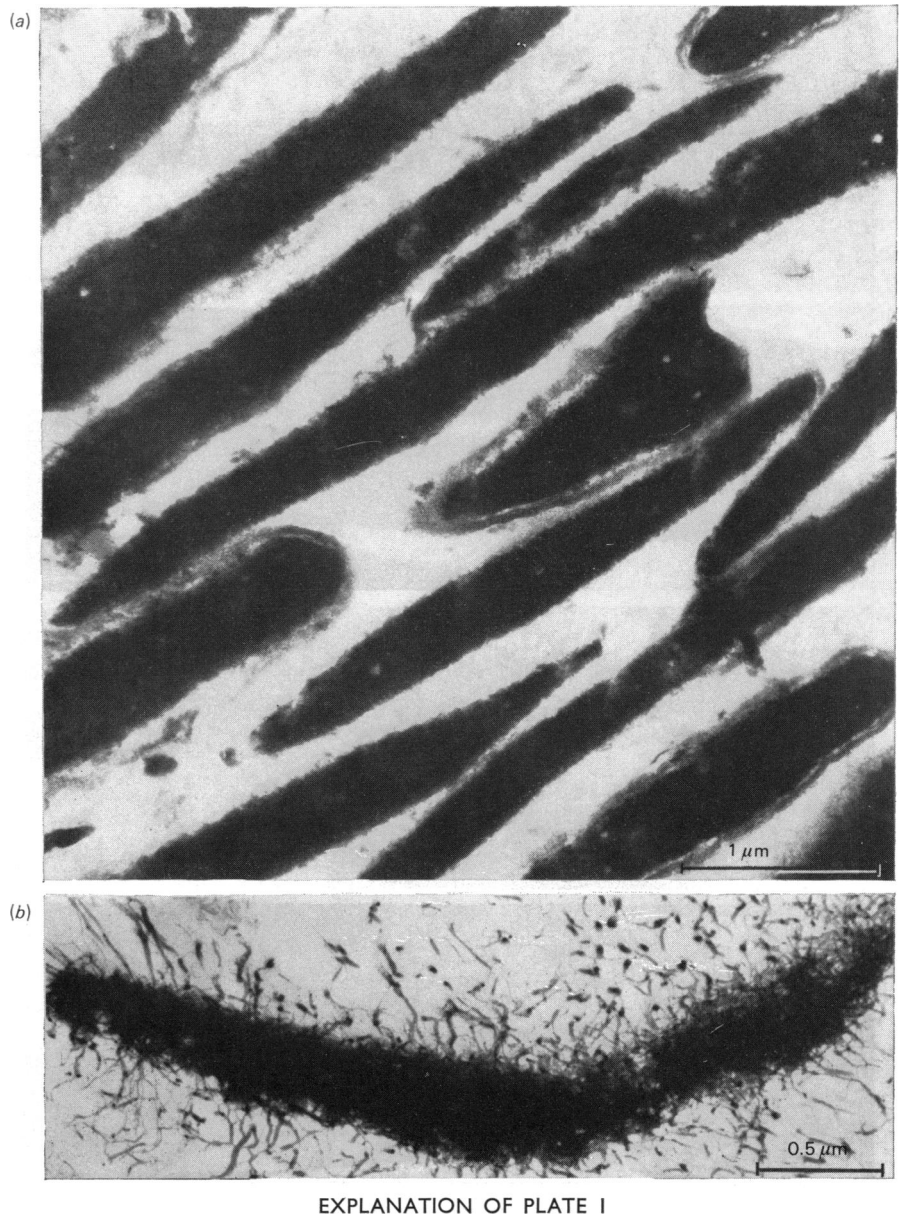
After incubation in the modified medium of Calvin & Bedford (1971), changes in spermatozoa heads could be microscopically observed. Phase-contrast observation revealed an increase in head volumes that resulted from dispersion of chromatin. This was confirmed with electron microscopy, which showed that the structural modifications of chromatin were greater at the periphery than in the central part of the nuclei, which remained practically intact. Similar results were obtained in the presence (Plate 2*a*) or absence (Plate 1*b*) of Tween 80 in the extraction medium. Decondensation of bull spermatozoa chromatin was induced by dithiothreitol, which presumably breaks the cystine bonds between the protamines, which are associated with DNA. We thus studied DNA polymerase activity as a function of dithiothreitol concentration in the extraction medium as well as the effect of replacing dithiothreitol with 2-mercaptoethanol.

Influence of dithiothreitol or 2-mercaptoethanol concentration in the extraction medium (*Table* 3)

DNA polymerase activity was measured in extracts and in pellets obtained after incubating spermatozoa heads in 50mM-borate buffer, pH9.0, containing 1mg of soya-bean trypsin inhibitor/ml, 4% (v/v) Tween 80, 400μg of activated calf thymus DNA/ml and various concentrations of dithiothreitol or 2-mercaptoethanol (Table 3). A viscous solution was obtained when spermatozoa heads were

Table 3. *Influence of dithiothreitol or 2-mercaptoethanol in the extraction medium*
Extraction was for 1h at 37°C in 50mM-borate buffer, pH9, containing 4% Tween 80 and 1mg of soya-bean trypsin inhibitor/ml. 100% DNA polymerase activity corresponded to 1.13pmol of dTTP incorporated by an extract prepared after treatment of 3×10^8 spermatozoa heads in a medium containing 20mM-dithiothreitol. Abbreviation: n.d., not determined.

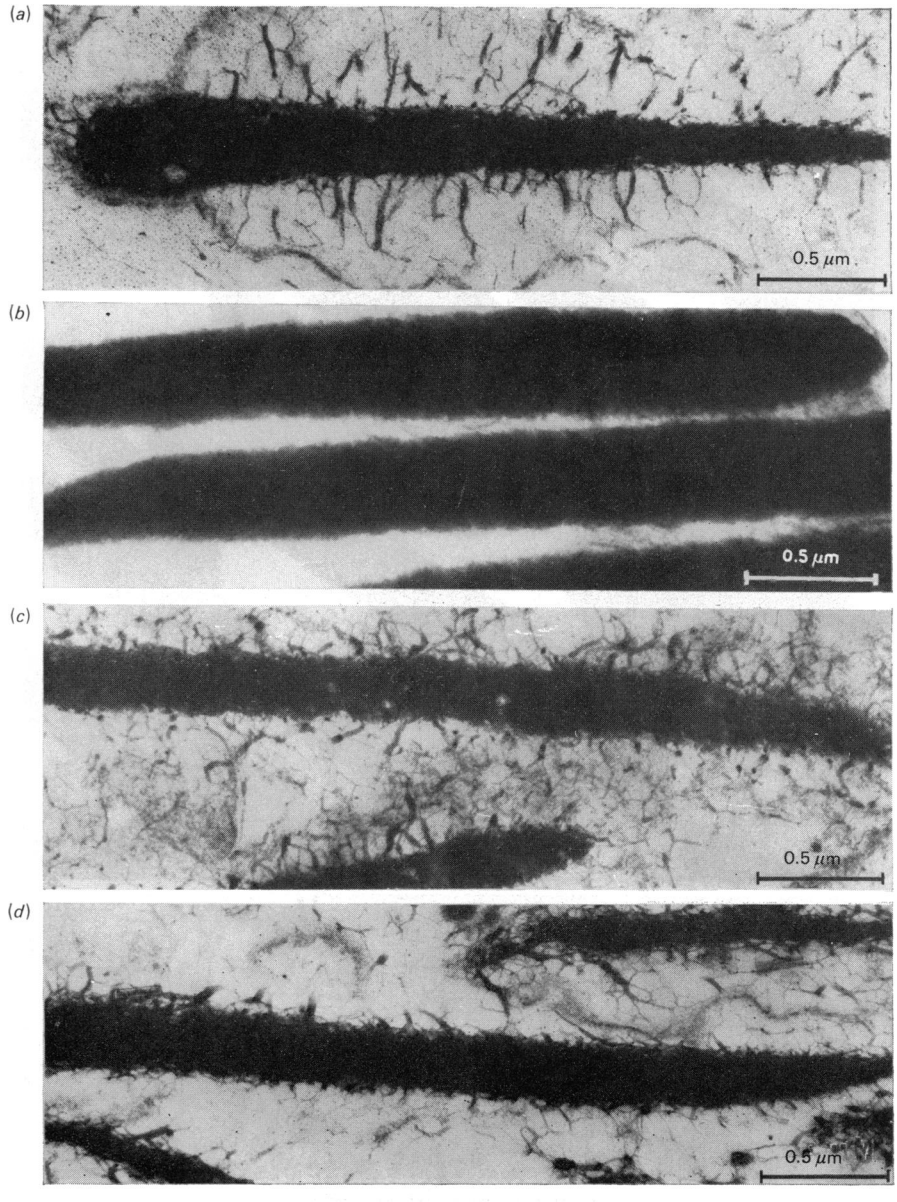
	Activity in pellet (%)		Activity in extract (%)	
	Endogenous	+400μg of DNA/ml	Endogenous	+400μg of DNA/ml
−Dithiothreitol	38.5	61.5	11	25.7
+5mM-Dithiothreitol	No pellet		117.7	167.4
+20mM-Dithiothreitol	75	132.9	29.8	100
+100mM-Dithiothreitol	n.d.		68.6	74
+1M-Dithiothreitol			8.6	21
+2mM-2-Mercaptoethanol	7.3	27.5	18.3	30
+20mM-2-Mercaptoethanol	No pellet		73.5	131
+100mM-2-Mercaptoethanol			105.3	188.5
+200mM-2-Mercaptoethanol	60.5	108.2	45	99
+1M-2-Mercaptoethanol	8	7	39	41.6



EXPLANATION OF PLATE 1

Electron micrographs of bull spermatozoa heads

(*a*) Pellet of bull spermatozoa heads obtained after centrifugation for 1 h at 40000*g* through 2.4 M-sucrose/1 mM-EDTA. (*b*) Spermatozoa heads after incubation for 20 h at room temperature in 50 mM-borate/NaOH buffer, pH 9, containing 5 mM-dithiothreitol and 100 μg of soya-bean trypsin inhibitor/ml.



EXPLANATION OF PLATE 2

Electron micrographs of bull spermatozoa heads after extraction

(*a*) Spermatozoa heads after extraction for 20h at room temperature in 50mM-borate/NaOH buffer, pH9, containing 5mM-dithiothreitol, 1% Tween 80 and 100μg of soya-bean trypsin inhibitor/ml. (*b*) Spermatozoa heads after extraction for 1h at 37°C in 50mM-borate/NaOH buffer, pH9, containing 4% Tween 80 and 100μg of soya-bean trypsin inhibitor/ml. (*c*) Spermatozoa heads after extraction for 1h at 37°C in 50mM-borate/NaOH buffer, pH9, containing 5mM-dithiothreitol and 100μg of soya-bean trypsin inhibitor/ml. (*d*) Spermatozoa heads after extraction for 20h at room temperature in 50mM-borate/NaOH buffer, pH9, containing 5mM-dithiothreitol and 100μg of soya-bean trypsin inhibitor/ml; 2M-NaCl was added 2h before the end of the extraction.

incubated in the presence of 5mM-dithiothreitol or 20–100mM-2-mercaptoethanol and it was impossible to obtain a pellet after a centrifugation at 3000*g* for 30min. The DNA polymerase activity was higher in those viscous extracts. When dithiothreitol or 2-mercaptoethanol was omitted, a decrease in DNA polymerase activity was noted, especially in the extracts. Electron micrographs of spermatozoa heads incubated without dithiothreitol or 2-mercaptoethanol (Plate 2*b*) showed that chromatin structure was intact. When dithiothreitol or 2-mercaptoethanol was present in the extraction medium at concentrations greater than 20 and 100mM respectively, a decrease in DNA polymerase activity was observed.

The results reported in Table 1 also showed that greater DNA polymerase activities were extracted when Tween 80 was present in the medium. The influence of the type of detergent on the extraction of DNA polymerase was thus investigated.

Influence of the nature of the detergent (*Table* 4)

Compared with extraction medium without detergent, the presence of 1% (v/v) Tween 80 or Nonidet P40 led to greater DNA polymerase activity in the extract. On the contrary, 1% Triton X-100 or 1.5% Sarkosyl (*N*-laurylsarcosine, sodium salt) resulted in lower enzymic activities.

At the same concentration of 4%, more DNA polymerase activity was extracted with Tween 80 than with Cemulsol NP6 or NP12 (Melle Bezons, Neuilly/Seine, France).

Influence of Tween 80 *concentration* (*Table* 5)

The influence of Tween 80 was studied in two different media, each composed of 50mM-borate buffer, pH9, and 1mg of soya-bean trypsin inhibitor/ml; one was supplemented with 20mM-dithiothreitol and the other with 100mM-2-mercaptoethanol.

Table 4. *Influence of the type of detergent in the extraction medium*

Extraction was for 1h at 37°C in 50mM-borate buffer, pH9, containing 20mM-dithiothreitol and soya-bean trypsin inhibitor (*a*, 10μg/ml; *b*, 1mg/ml). 100% activity corresponded to 2.79 (*a*) and 4.51 (*b*) pmol of dTTP incorporated by an extract prepared after treatment of 3×10^8 spermatozoa heads in a medium containing 1% (*a*) or 4% (*b*) Tween 80. Abbreviation: n.d., not determined.

	Activity in pellet (%)		Activity in extract (%)	
	Endogenous	+400μg of DNA/ml	Endogenous	+400μg of DNA/ml
(*a*)				
+1% Tween 80	10.4	32.2	n.d.	100
+1% Triton X-100	2.5	25.8	n.d.	69.9
+1% Nonidet P40	4.3	42.3	n.d.	85.3
+1.5% Sarkosyl	0	4.3	n.d.	7.2
(*b*)				
+4% Tween 80	42.6	69.4	35	100
+4% Cemulsol NP6	44	52.2	58.9	61.9
+4% Cemulsol NP12	37.3	66.4	29.8	77.6

Table 5. *Influence of Tween* 80 *concentration in the extraction medium*

Extraction was for 1h at 37°C in 50mM-borate buffer, pH9, and 1mg of soya-bean trypsin inhibitor/ml. 100% activity corresponded to 1.34pmol of dTTP incorporated by an extract prepared after treatment of 3×10^8 spermatozoa heads in a medium containing 20mM-dithiothreitol and 4% Tween 80.

	Pellet		Extract	
	Endogenous activity	+400μg of DNA/ml	Endogenous activity	+400μg of DNA/ml
+20mM-Dithiothreitol				
+0% Tween	5.98	29.49	23.94	63.63
+4% Tween	33.70	46.34	49.44	100
+10% Tween	29.26	39.68	25.05	70.50
+20% Tween	27.93	45.23	10.19	42.79
+100mM-2-Mercaptoethanol				
+0% Tween			40.57	114.85
+4% Tween	No pellet		68.73	149
+10% Tween			77.16	197.11
+20% Tween			75.60	175.16

DNA polymerase activity increased in proportion to Tween 80 concentration, reached a maximum value and then decreased. Optimal DNA polymerase activities were obtained with 4% (v/v) Tween 80 when 5mM-dithiothreitol was present in the extraction medium, and with 10% Tween 80 when dithiothreitol was replaced by 100mM-2-mercaptoethanol. This suggested that the combination of dithiothreitol or 2-mercaptoethanol with Tween 80 was more complex than a simple additive effect.

Influence of extraction time (*Table* 6)

Spermatozoa heads were incubated for various periods of time at 37°C in 50mM-borate buffer, pH9, containing 100mM-2-mercaptoethanol, 4% Tween 80 and 1 mg of soya-bean trypsin inhibitor/ml. Swelling of the heads, corresponding to chromatin decondensation, increased with increasing extraction time.

DNA polymerase activity in the pellets and extracts also increased with increasing incubation times; optimal activity was obtained after 1 h, with a decrease thereafter. Electron micrographs of spermatozoa heads incubated for 1 h at 37°C showed a decondensation of a small part of the chromatin localized at the nuclear periphery. It may thus be supposed that under these conditions of extraction only a part of the nuclear DNA polymerase in these cells was measured. When extraction time was increased to induce a more extensive decondensation of chromatin, DNA polymerase activity was decreased, probably as a result of enzyme denaturation.

Cytoenzymological observations of bull spermatozoa (results not shown) did not show any particular distribution of radioactivity at the periphery of the nuclei, so it was quite impossible to estimate the total DNA polymerase content of spermatozoa heads. This may explain why the activities measured were low, since they represented only a fraction of the total activity. When the extractions were carried out for 16–20h at room temperature (20°C), similar or slightly higher DNA polymerase activities could be obtained. Preincubation of spermatozoa heads with lysophosphatidylcholine (concentrations between 0.04 and 4mg/ml) led to no more DNA polymerase activity than in controls. Similarly, the addition of 2M-NaCl to the extraction medium, at the beginning or at the end of the incubation, led to no increase in enzyme activity.

Electron micrographs showed no differences between spermatozoa heads extracted for 1 h (Plate 2*c*) or 20 h (Plate 1*b*) at room temperature in 50mM-borate buffer, pH9, containing 5mM-dithiothreitol, 1% Tween 80 and 100 μg of soya-bean trypsin inhibitor/ml, in the presence (Plate 2*d*) or absence (Plate 2*c*) of 2M-NaCl.

Conditions for the extraction of optimal DNA polymerase activity thus being determined, some properties of the enzyme were then investigated. This study was done on 'total extracts' or pellets obtained after a partial extraction of the nuclear heads.

When dATP, dCTP and dGTP were omitted from the reaction, dTTP incorporation in the acid-insoluble fraction was considerably decreased (38% of controls).

Influence of the type and the concentration of bivalent cation (*Fig.* 1)

The enzymic reaction was Mg^{2+}-dependent: radioactivity incorporated in the absence of Mg^{2+} was similar to 'sticky counts', i.e. to the radioactivity recovered in the acid-insoluble fraction in the absence of the biological fraction in the incubation medium. Optimal activities were obtained with 20mM- and 10mM-Mg^{2+} respectively when the reaction was performed with or without 400 μg of exogenous activated DNA/ml; this was verified in several experiments and was the only case where a difference was observed between reactions performed in the presence or absence of exogenous DNA.

The replacement of Mg^{2+} by Mn^{2+} led to a considerable decrease in DNA polymerase activity, at all concentrations tested. When Ca^{2+} was present, the

Table 6. *Influence of extraction time*

Extraction was done at 37°C in 50mM-borate buffer, pH9, containing 100mM-2-mercaptoethanol, 4% Tween 80 and 1 mg of soya-bean trypsin inhibitor/ml. 100% activity corresponded to 1.09 pmol of dTTP incorporated by an extract after treatment of 3×10^8 spermatozoa heads during 60 min.

	Activity in pellet (%)		Activity in extract (%)	
Time (min)	Endogenous	+400 μg of DNA/ml	Endogenous	+400 μg of DNA/ml
0	9.96	21.72	1.78	7.73
15	13.09	24.69	11.60	27.97
30	11.61	24.25	10.71	32.43
60	No pellet		46.12	100
120	No pellet		30.65	70.23

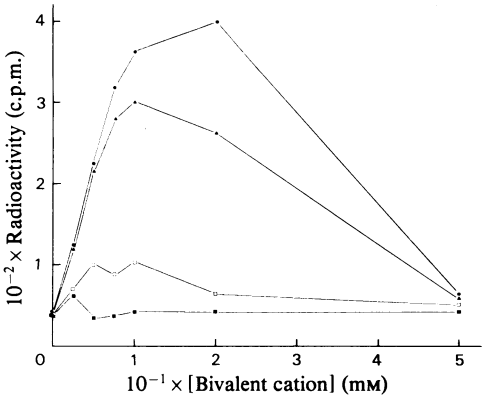


Fig. 1. *Influence of the type and the concentration of bivalent cation*
Details are given in the text. ■, Mn^{2+}; □, Ca^{2+}; ▲, Mg^{2+} (endogenous activity); ●, Mg^{2+} (+activated DNA).

activities measured were slightly higher than in the presence of Mn^{2+}, but were still low, being only twice the 'sticky counts'. The same results were obtained regardless of the presence of exogenous DNA.

Influence of the type and the concentration of univalent cation (*Fig.* 2)

Maximal DNA polymerase activities measured in the presence of K^+ (between 100 and 200mM) or Na^+ (50mM) were of the same order. In the presence of high concentrations (0.5M) or in the absence of univalent cations, DNA polymerase activity decreased, but was consistently detectable.

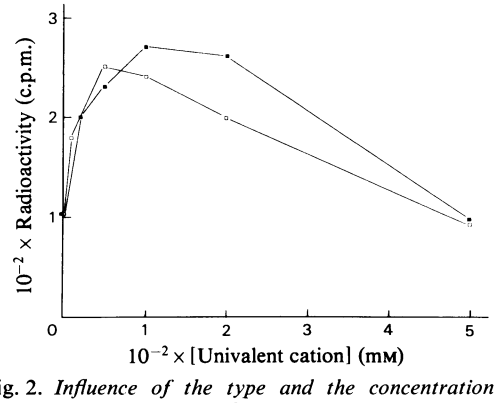


Fig. 2. *Influence of the type and the concentration of univalent cation*
Details are given in the text. □, NaCl; ■, KCl.

Influence of pH and buffer composition (*Fig.* 3)

Optimal pH was a function of buffer composition [pH7.5 with 20mM-phosphate buffer; pH8.8 with 50mM-Tris/HCl; pH9.1 with 50mM-borate/NaOH buffer; pH9.5 with 50mM-glycine/NaOH; between pH8.2 and 9.6 with 50mM-ammediol (2-amino-2-methylpropane-1,3-diol)/HCl buffer].

The shape of the pH–activity curves was also a function of the buffer. Optimal activity was detected either in a relatively narrow (glycine/NaOH) or wide (ammediol) pH range and the value of maximal activity was also dependent on buffer composition. The highest DNA polymerase activities were detected in the presence of 50mM-borate buffer, pH9.1, or 50mM-glycine/NaOH, pH9.5. Only a small fraction of enzymic activity was detected in the presence of 20mM-phosphate buffer, pH7.5.

Action of various DNA polymerase inhibitors and of various anti-metabolites interfering with nucleic acid and protein syntheses (*Table* 7)

The sensitivity to thiol-group-blocking agents was studied with *N*-ethylmaleimide. The fractions were incubated at 0°C with 1mM- or 10mM-*N*-ethylmaleimide for 5min; incubation was than performed at 37°C in the complete medium without dithiothreitol or 2-mercaptoethanol. DNA polymerase activity in the presence of *N*-ethylmaleimide decreased (70% for 1mM; 45% for 10mM), compared with controls.

Enzymic activity was resistant to ethidium bromide: 80 or 55% of the initial activity was measured in the presence of 25μM- or 100μM-ethidium bromide respectively.

Actinomycin D and netropsin (congocidin; Rhône-Poulenc, Vitry/Seine, France) had no effect on DNA

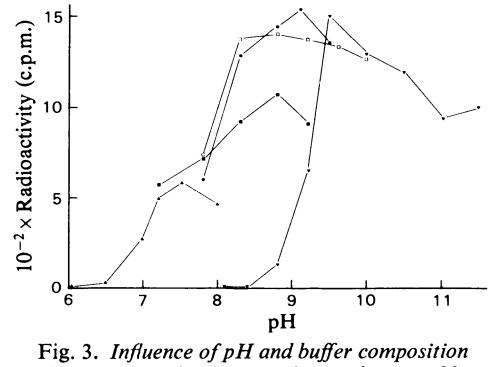


Fig. 3. *Influence of pH and buffer composition*
Details are given in the Results section. ▲, 20mM-Phosphate buffer; ■, 50mM-Tris/HCl buffer; □, 50mM-ammediol buffer; ●, 50mM-borate buffer; ▼, 50mM-glycine/NaOH buffer.

Table 7. *Influence of various inhibitors on nuclear DNA polymerase activity of bull spermatozoa*
Details are given in the corresponding paragraph of the Results section.

	Activity (%)	
	Endogenous	+400 μg of activated DNA/ml
Control	100	100
+*N*-Ethylmaleimide		
1 mM	72.5	74
10 mM	44.5	40
+Ethidium bromide		
10 μM	97.5	95.5
25 μM	80.5	81
100 μM	58	55.5
+Actinomycin D		
5 μg/ml	93	98
50 μg/ml	77	97
+Netropsin		
5 μg/ml	72	100
50 μg/ml	72	77

polymerase activity. Even at a concentration as high as 50 μg/ml, at least 72% of control activity remained.

Spermidine was inhibitory. At a concentration of 5 mM, less than 60% of the initial activity was measured and complete inhibition was observed at a concentration of 50 mM.

Salmon protamines also led to a considerable decrease in endogenous DNA polymerase activity: 35% of control activity was measured in the presence of 125 μg/ml and 8% with 250 μg/ml.

On the contrary, calf thymus histones used at the same concentrations as protamines have a stimulatory effect, which was higher when exogenous DNA was present during the incubation. The presence of 125 μg of histones/ml of medium led to a 25% stimulation of endogenous DNA polymerase activity, and a 60% increase was observed when exogenous activated DNA was present. This stimulation increased to 90% when the histone concentration was 250 μg/ml.

No significant effect was observed with ATP at concentrations lower than 1 mM (90% of the control activity), but inhibition appeared with higher concentrations (30% of residual activity with 3 mM-ATP). The effect of different alcohols (ethanol, propan-2-ol, methanol) in the incubation medium was also tested at concentrations as high as 25% (v/v). Inhibition was noted in all cases, and it increased with increasing alcohol concentration (25, 67 or 100% inhibitions were obtained with alcohol concentrations of 1, 5 and 25%).

The thermal stability of the DNA polymerase activity was also determined. The biological fraction was preincubated at 50 or 80°C for 10 min and the reaction was then carried out at 37°C in the complete medium with or without exogenous DNA: 50 and 80% of DNA polymerase activity was lost after preincubation at 50 and 80°C respectively.

Kinetically, the extent of enzymic reaction was proportional to incubation time for the first 30 min. A plateau was reached after 60 min. The very low nuclease activity in the biological fractions was also noticeable. Between 84 and 91% of the labelled DNA used for detection (see the Materials and Methods section) remained acid-insoluble after an incubation for 1 h at 37°C.

Discussion and Conclusion

We attempted to find the most satisfactory conditions for demonstrating the majority of DNA polymerase activity in bull spermatozoa heads and also to define some biochemical characteristics of the enzyme. The extraction medium of Calvin & Bedford (1971) used to study nuclear structural proteins associated with mammalian spermatozoa DNA served as a basis in the search for the optimal conditions for DNA polymerase extraction, so several modifications of that medium were made. They are described and discussed in detail in the Results section. In brief, optimal DNA polymerase activity was detected when bull spermatozoa heads were extracted for 1 h at 37°C or 20 h at room temperature in a medium composed of 50 mM-borate buffer, pH 9, 1 mg of soya-bean trypsin inhibitor/ml and supplemented with 20 mM-dithiothreitol and 4% Tween 80 or with 100 mM-2-mercaptoethanol and 10% Tween 80.

The enzymic activity is strongly dependent on the presence of Mg^{2+} in the incubation medium: optimal activities were obtained with 20 mM- and 10 mM-Mg^{2+} respectively when incubations were performed with or without exogenous DNA. Replacement of Mg^{2+} by Mn^{2+} or Ca^{2+} dramatically decreased DNA polymerase activity, particularly with Mn^{2+}. Similar results were reported by Baril *et al.* (1973) with polymerase-α and -β activities when activated DNA was used as template. An inhibition of DNA polymerase-β when Mg^{2+} was replaced by Mn^{2+} was also described by Berger *et al.* (1971) and by Chang & Bollum (1972); however, the loss of activity was not so great as in the preceding case. Similar activities of spermatozoa DNA polymerase were detected with 50 mM-Na^+ and 150 mM-K^+. Spermatozoa DNA polymerase activity was decreased in the absence of univalent cations as well as in the presence of high concentrations (0.5 M), which are known to inhibit DNA polymerase-α almost completely. In the present

case, only a small decrease was noted. The optimal concentration of K^+ was similar to that described by Hecht (1974) for the enzyme extracted from intact bull spermatozoa, but the shape of the curve was quite different: for K^+ concentrations lower than 100mM or higher than 250mM, DNA polymerase activity was inhibited and the shapes of the curves were similar to those reported by Stalker *et al.* (1976) for Novikoff hepatoma DNA polymerase-β.

Activity depended on pH and buffer composition. Maximal activities were obtained between pH8.2 and 10.5 and depended on the buffer. Activity was greatly decreased in the presence of 20mM-phosphate buffer, which is consistent with other observations on DNA polymerase-β.

DNA polymerase activity of spermatozoa heads was also found to be sensitive to *N*-ethylmaleimide, which is considered to be a specific inhibitor of DNA polymerase-α. It is now well established (Rossignol *et al.*, 1972; Craig & Keir, 1974; Bollum, 1975; Weissbach, 1977) that 1mM-*N*-ethylmaleimide greatly inhibits DNA polymerase-α. The activity of DNA polymerase-β has been reported to be resistant to *N*-ethylmaleimide (Bollum, 1975), even at a concentration as high as 10mM. Several authors, however, have described a partial inhibition of the enzyme which depended on the concentration of *N*-ethylmaleimide and also on the origin of the biological material: 1mM-*N*-ethylmaleimide led to a 90% loss of DNA polymerase activity when DNA synthesis was studied *in vitro* in isolated nuclei from chick-embryo fibroblast cell cultures (Hallick & Namba, 1974). Chiu & Sung (1972) isolated a nuclear DNA polymerase from rat liver whose activity was decreased to 60% by 1.3mM-*N*-ethylmaleimide. The nuclear DNA polymerase isolated from mouse fibroblasts by Adams *et al.* (1973) was also sensitive to *N*-ethylmaleimide, the degree of inhibition depending on the template used (36% inhibition with native DNA and 83% with denatured DNA). Two nuclear DNA polymerases with different sensitivities to 1mM-*N*-ethylmaleimide (96 and 23% inhibition) were purified from rat ascites hepatoma cells (Tsuruo & Ukita, 1974). Poulson *et al.* (1974) isolated a nuclear DNA polymerase from rat intestinal mucosa whose activity was decreased to 50% by 2.5mM-*N*-ethylmaleimide. Craig & Keir (1975) also described a partial inhibition of the nuclear DNA polymerase of BHK-21/C13 cells by *N*-ethylmaleimide: they observed a 12 or 44% inhibition with 1mM- or 5mM-*N*-ethylmaleimide respectively. Novikoff hepatoma DNA polymerase-β has been shown to be partially sensitive to *N*-ethylmaleimide (28% inhibition with 4mM-*N*-ethylmaleimide) (Stalker *et al.*, 1976). Similar results were reported by Dube *et al.* (1977) with DNA polymerase-β of different biological origins. These results clearly show that DNA polymerase-β is sensitive to *N*-ethylmaleimide, at least partially, even at a concentration as low as 1mM.

The nuclear DNA polymerase of bull spermatozoa is highly resistant to ethidium bromide. Similar results were also described by Sedwick *et al.* (1972) and by Berger *et al.* (1971) with the DNA polymerase-β isolated from cultivated KB cells or from rat liver respectively. Kornberg (1974) reported that DNA polymerase-α had a greater sensitivity to ethidium bromide, and Hecht (1974) described a strong inhibition (more than 80%) of mitochondrial DNA polymerase by 16μM-ethidium bromide.

The nuclear DNA polymerase of bull spermatozoa was not affected by actinomycin D in the incubation medium. This is in agreement with the results obtained by Witkin *et al.* (1975) with nuclear DNA polymerase from human spermatozoa and is similar to our own results obtained with mouse spermatozoa (Philippe & Chevaillier, 1976). The cytoenzymological study of the mouse nuclear DNA polymerase revealed that 40μg of actinomycin/ml led to an inhibition of approx. 35%.

We found that enzymic activity was greatly decreased by spermidine and was increased when histones were present in the incubation medium. The stimulatory effect of histones seemed to be specific and not attributable to the basic character of the protein, since other basic proteins had either no effect (cytochrome *c*) or were strongly inhibitory (salmon protamines). The heating of biological fractions at 50°C for 10min led to a 50% loss of DNA polymerase activity. This is probably due to enzyme denaturation, since similar results were obtained when the incubations were performed with or without exogenous DNA. Similar losses of activity have been reported after the heating of DNA polymerase-β (Kornberg, 1974; Dube *et al.*, 1977); on the contrary, these authors reported contradictory results about the heat-sensitivity of DNA polymerase-α.

The activity detected in the presence of a single deoxyribonucleotide remained relatively high (38%). Similar values were given for DNA polymerase-β by Craig & Keir (1974) and by Bollum (1975), especially when activated DNA was used as template. We also obtained similar results with isolated nuclei from mouse liver (Philippe *et al.*, 1976). As in the latter case, we observed a consistent increase in DNA polymerase activity when exogenous DNA was added to the incubation medium. This can be explained only by an exchange of a portion of the enzyme, initially bound to chromatin, with exogenous DNA. However, the enzyme seemed to be tightly bound to its endogenous template in bull spermatozoa nuclei: the addition of 2M-NaCl did not increase the extraction of the enzyme. This seems to be a characteristic of spermatozoa, since the addition of 1M-NaCl to the medium led to the extraction of almost all nuclear DNA polymerase

in other systems studied. Contrary to the results reported by Hecht (1974) for intact bull spermatozoa, we always detected an endogenous DNA polymerase in extracts prepared by the addition of 2 M-NaCl to the medium.

The results described here lead to the conclusion that the enzyme contained in bull spermatozoa heads is a DNA polymerase-β, and are consistent with our cytoenzymological results with mouse spermatozoa. Some differences exist, however, particularly the resistance of the nuclear bull enzyme to netropsin, the inhibitory effects of alcohols and ATP, and also the partial sensitivity to *N*-ethylmaleimide. We believe that these differences are probably due more to the preparation methods of the biological material than to differences actually existing between these two mammalian spermatozoa nuclear DNA polymerases.

The following paper (Philippe & Chevaillier, 1978) gives more precise biochemical characteristics, especially the sedimentation coefficient, of this bull spermatozoa nuclear DNA polymerase.

We thank Dr. Jondet (Centre d'Insémination, Rennes), who kindly provided bull spermatozoa. We are very grateful to Dr. A.-M. de Recondo and her group (I.R.S.C., Villejuif) for advice and discussion in the course of this work, and to the Société Rhône-Poulenc for the synthesis of netropsin. We also thank D. Tesson for the preparation of the manuscript. This work was supported by the Centre National de la Recherche Scientifique (ATP 'Chromatine' no. 2888) and the Fondation pour la Recherche Médicale Française.

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