The Activity of Deoxyribonucleic Acid Polymerase in Some Species of Algae

By O. TH. SCHÖNHERR* and H. M. KEIR

Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, U.K.

(Received 17 March 1972)

1. The activities of DNA polymerase preparations from the algae *Euglena gracilis*, *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa*, *Anabaena variabilis* and *Anacystis nidulans* were measured. The blue-green algae *Anabaena* and *Anacystis* contain a 5–20-fold higher activity of the enzyme than do the green algae. DNA polymerases from the blue-green algae show a pH optimum of 9 and prefer a relatively low Mg^{2+} concentration (1–3 mM). DNA polymerases from the green algae, however, display a pH optimum between 7.5 and 8.5 and an optimum Mg^{2+} concentration of 8 mM. With all algae, a higher polymerase activity was obtained with denatured salmon sperm DNA as template than with native DNA. All four deoxyribonucleoside 5'-triphosphates must be present for full activity of the polymerases. 2. With one exception, the deoxyribonuclease activities in the preparations, measured under conditions of the DNA polymerase assay, are low compared with corresponding preparations from *Escherichia coli*. *Chlamydomonas* extracts contain a high deoxyribonuclease activity. 3. After purification on columns of DEAE-cellulose, the polymerase activity was linear over a wide range of protein concentrations, except for *Chlamydomonas* preparations, where the observed deviation from linearity was probably attributable to the high nuclease activity. 4. DNA polymerases from all these algae bind strongly to DNA-cellulose; 6–40-fold purifications of the enzyme were obtained by chromatography on columns of DNA-cellulose. 5. The partially purified polymerases of *Euglena* and *Anacystis* are heat-labile but become much more heat-stable when tested in the presence of DNA.

Synchronously growing cultures are very useful for the study of DNA synthesis, mitosis, cell division and other events of the cell growth-cycle. Many unicellular algae enter spontaneously into synchronous growth when cultured autotrophically with alternating periods of light and dark (Pirson & Lorenzen, 1966; Tamiya, 1966). Changes in the activity of DNA polymerase (EC 2.7.7.7) have been studied in synchronous cultures of mammalian cells (Gold & Helleiner, 1964; Madreiter *et al.*, 1971; Lindsay *et al.*, 1970) and of the green alga *Chlorella pyrenoidosa* (Schönherr & Wanka, 1971).

The purpose of the present study was to obtain information about the activities and properties of DNA polymerase from different species of algae growing non-synchronously, so that the basis could be established for long-term projects on synchronous cultures of these algae. *Euglena gracilis* was chosen because nuclei can be isolated readily from these cells (Parenti *et al.*, 1969), *Chlamydomonas reinhardtii* because of the existence in it of a gametogenesis event additional to vegetative reproduction (Kates & Jones, 1964), and *Anabaena variabilis* and *Anacystis nidulans* because they are blue-green algae and may be regarded as evolutionary intermediates between bacteria and eukaryotic cells (Echlin & Morris, 1965).

* Present address: Laboratory for Cell Biology and Histology, University of Leiden, Academisch Ziekenhuis, Rijnsburgerweg 10, Leiden, The Netherlands.

Materials and Methods

Organisms

Euglena gracilis (strain z), *Chlamydomonas reinhardtii* (strain Dangeard) and *Chlorella pyrenoidosa* were obtained from the Culture Collection of Algae and Protozoa, The Botany School, Cambridge, U.K. *Anabaena variabilis* and *Anacystis nidulans* were kindly provided by Dr. N. G. Carr.

Materials

MN 2200ff cellulose (Macherey, Nagel and Co., Düren, Germany) was used for preparing DNA-cellulose. DEAE-cellulose (Whatman DE23) was purchased from H. Reeve Angel and Co. Ltd., London E.C.4, U.K.; calf thymus DNA (type V) and salmon sperm DNA were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; deoxyribonucleoside 5'-triphosphates were from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; [3H]DNA and purified DNA polymerase from *Escherichia coli*, strains B and W3110 respectively, were gifts from Miss P. A. Costello of this Department and Dr. R. S. Hayward, Department of Molecular Biology, University of Edinburgh. The [3H]DNA was isolated by the method of Marmur (1961) after the cells had been grown in M9 medium (Clowes & Hayes, 1968) containing [3H]thymidine. The DNA polymerase was

Fraction VII of the purification method described by Richardson *et al.* (1964).

Methods

Cell growth. Algae were grown aseptically in glass tubes (100 cm × 5 cm) each fitted with a water jacket for temperature control. The temperature was kept at 30°C, except for *Euglena* (25°C). The cultures were aerated with sterile air enriched with 5% CO₂. Light was provided by two fluorescent tubes ('natural' and 'warm white'). *Euglena* was grown in Hutner medium (Greenblatt & Schiff, 1959), *Chlorella* in a medium described by Wanka (1965), *Chlamydomonas* in the high-salt minimal medium described by Sueoka (1960), and *Anabaena* and *Anacystis* in medium C described by Kratz & Meyers (1955).

Cell disruption and enzyme purification. Cells in the exponential phase of growth were collected by centrifugation in the MSE 4L Mistral centrifuge (20 min at 4°C, 3100g, r_{av} 18 cm) and washed with 50 mM-potassium phosphate buffer, pH 7.5, containing 5 mM-2-mercaptoethanol and 1 mM-EDTA (buffer A). Portions of cell paste (1 ml) were then each suspended in 5 ml of buffer A. Cells were disrupted either in a French press at a pressure of 10000 lb/in² (69 MN/m²) or in a Mickle shaker with glass beads (0.2 mm diam.) by shaking for 2 min at 4°C. Solid KCl was added to a final concentration of 0.6 M and the disrupted-cell preparation was centrifuged at 4°C for 1 h at 100000g in the 10 × 10 ml fixed-angle rotor (no. 59592, r_{av} 2.55 cm) of the MSE Superspeed 65 ultracentrifuge. The supernatant fraction was either dialysed overnight against buffer A containing 0.3 M-KCl, or diluted with an equal volume of buffer A and applied to a DEAE-cellulose column (10 cm × 1 cm²) equilibrated with buffer A containing 0.3 M-KCl. Elution was carried out with buffer A containing 0.3 M-KCl. Algal DNA polymerases were freed of DNA by chromatography on columns of DEAE-cellulose. The polymerase fractions were dialysed against buffer A or passed through columns of Sephadex G-25 equilibrated against buffer A, to remove KCl. The resulting polymerase fractions, in buffer A, were applied to columns (10 cm × 1 cm²) of DNA-cellulose equilibrated against the same buffer. The column was washed with 5 ml of buffer A and then eluted with buffer A containing 0.5 M-KCl. The flow rate was 5 ml/h.

Assay of DNA polymerase. The standard assay mixture (0.25 ml) contained: heat-denatured salmon sperm DNA, 50 µg; [8-³H]dATP, 200 µM (10 mCi/mmol); dCTP, dGTP and dTTP, 200 µM each; KCl, 100 mM; 2-mercaptoethanol, 10 mM; EDTA, 4 mM; and tris-HCl buffer, 50 mM. The pH and the MgCl₂ concentration of the assay mixture were dependent on the enzyme extract used; the precise conditions are described in Table 1. After 1 h at 37°C, the re-

action mixture was cooled in an ice-bath, 50 µl of bovine serum albumin (Sigma) (2.5 mg/ml) was added, and 100 µl portions were applied to glass-fibre discs (Whatman GF/C, 2.5 cm diam.). The filters were washed three times by using suction, with ice-cold 5% (w/v) trichloroacetic acid containing 5 mM-sodium pyrophosphate, and once with ethanol. The dried discs were transferred to glass vials (scintillation grade), and the samples measured for radioactivity in the Inter technique model SL30 liquid-scintillation spectrometer. The scintillation fluid (10 ml/vial) contained 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene.

Assay of deoxyribonuclease. The incubation mixtures for nuclease assays were the same as those for DNA polymerase, except that the deoxyribonucleoside 5'-triphosphates were omitted and [³H]DNA or [³H]poly[d(A-T)] was used as substrate.

After incubation with [³H]DNA from *Esch. coli* as substrate, 50 µl of calf thymus DNA (2.5 mg/ml) and 200 µl of ice-cold 7% (w/v) HClO₄ were added successively. After centrifugation for 10 min at 5000g, 100 µl samples of the supernatant fluid were added to a mixture (10 ml) of Triton X-100 and the toluene-based scintillator fluid described above (1:2, v/v), and counted for radioactivity in the Inter technique liquid-scintillation spectrometer.

When poly[d(A-T)] was used as substrate, 50 µl of calf thymus DNA (2.5 mg/ml) was added after the incubation and 100 µl samples were applied to Whatman GF/C glass-fibre discs (2.5 cm diam.). The discs were washed with ice-cold 5% (w/v) trichloroacetic acid, ethanol and ether, and dried. They were then measured for radioactivity as described above.

Other methods. Protein was determined as described by Lowry *et al.* (1951), with highly purified bovine serum albumin as a standard. DNA-cellulose (400 µg of DNA/g of cellulose) was prepared with native DNA from calf thymus by the method of Alberts *et al.* (1968). [³H]Poly[d(A-T)] was prepared as described by Schachman *et al.* (1960) and denatured DNA was obtained by heating a solution of DNA (1 mg/ml in 10 mM-KCl) for 5 min at 100°C, followed by rapid cooling in an ice-bath.

Results

Disrupted preparations from all the species of algae tested displayed a much lower activity of DNA polymerase (measured by incorporation of [³H]-dAMP residues into acid-insoluble product) than did the corresponding 100000g supernatant fractions. Because the incorporation rate catalysed by dialysed supernatant fractions was linear over a much wider range of protein concentrations than with non-dialysed preparations, dialysis was carried out as a routine. When the disrupted preparation from

Fig. 1. Effect of increasing concentrations of dialysed supernatant fractions from *Euglena* on incorporation of [^3H]dAMP from [^3H]dATP into acid-insoluble material

The supernatant fractions were obtained by centrifuging disrupted *Euglena* cell preparations at 100000g or 1h in the presence (●) or in the absence (▲) of 0.6M-KCl. The former supernatant fraction was also tested for its ability to catalyse incorporation of [^3H]dAMP residues after it had been purified by passage through a column of DEAE-cellulose (○).

Euglena contained KCl at 0.6M or more during centrifugation, the supernatant fraction subsequently obtained showed a much higher activity of DNA polymerase although the KCl concentration in the incubation mixtures was kept at 100mM (Fig. 1). This could be attributed to precipitation of (and consequent sedimentation of) nucleoprotein complexes containing DNA polymerase in the absence of the high salt concentration. Clear-cut effects of this kind were not obtained with preparations from the other algae, but the same procedure was nevertheless adopted as a routine for all species examined. It is of course recognized that the situation regarding the formation of nucleoprotein complexes is by no means a simple one, and it cannot be resolved completely in the context of these observations without further extensive experimentation.

Chromatography of the supernatant fractions on columns of DEAE-cellulose gave enzyme preparations that displayed an enhanced polymerase activity. Under the conditions of chromatography used, nucleic acid was retained on the column and almost all of the protein was eluted. The E_{280}/E_{260} ratio of the eluate increased to greater than 1.0 during elution.

The activities of DNA polymerase and deoxyribonuclease from the various algae were measured after purification by DEAE-cellulose chromatography. The results are shown in Table 1. These activities are readily reproducible; moreover, the template specificity of the polymerase activity and the substrate specificity of the deoxyribonuclease activity can be measured more reliably in the absence of endogenous DNA (retained on the column). The activity of DNA polymerase preparations from *Euglena*, *Chlorella* or *Chlamydomonas* is much lower than that obtained from the blue-green algae *Anabaena* and *Anacystis*. The activity in comparable extracts from *Esch. coli*, however, is higher than that derived from the blue-green algae. When enzyme activity was expressed as total nucleotide incorporated into acid-insoluble material, all the algal polymerase preparations tested showed higher incorporation of nucleotide into acid-insoluble material in the presence of denatured DNA than with native DNA or poly[d(A-T)] as template.

Optimum polymerase activity was found at a higher pH value for the blue-green algae than for the green algae (Table 1). Further, the blue-green algae showed a much lower optimum concentration (1–3 mM) for Mg^{2+} in the polymerase assay than did the green algae (8 mM). Mn^{2+} could replace Mg^{2+} , but gave only 50% of the optimum activity of the enzyme shown by Mg^{2+} . By contrast, Ca^{2+} failed to promote polymerase activity in all the preparations examined.

If one or more of the deoxyribonucleoside 5'-triphosphates were omitted from polymerase assays containing denatured DNA as template, the incorporation of [^3H]dAMP residues fell to less than 5% of the value obtained in the presence of all four triphosphates. It can therefore be concluded that most of the algal DNA polymerase activity, as measured in the standard assay system, was catalysing template-directed synthesis of polydeoxyribonucleotide, as distinct from non-specific incorporation at the template 3'-termini.

DNA polymerase activities could be measured with satisfactory reproducibility in all the algae except *Chlamydomonas*. The incorporation catalysed by the latter preparations was linear with respect to protein content in the assay only over low ranges of protein concentration. This is explicable, at least in part, by the high deoxyribonuclease activity in the *Chlamydomonas* enzyme preparations (Table 1). In the corresponding preparations from the other algae the deoxyribonuclease activity acting on DNA was low relative to preparations from *Chlamydomonas* and *Esch. coli*. The observations were qualitatively similar when poly[d(A-T)] was used as substrate, but the effects were quantitatively rather different (Table 1).

Bacterial DNA polymerases are known to bind to DNA-cellulose (Alberts *et al.*, 1968; Litman, 1968). An investigation was carried out to determine

Table 1. *DNA polymerase and deoxyribonuclease activities in fractions obtained from various algae after purification on columns of DEAE-cellulose*

DNA polymerase activity was measured by using salmon sperm DNA or poly[d(A-T)] as template. Deoxyribonuclease activity was measured by using either [³H]DNA from *Esch. coli* or [³H]poly[d(A-T)] as substrate.

Species	DNA polymerase activity (nmol of [³ H]dAMP residues incorporated/h per mg of protein)			DNA polymerase optima		Deoxyribonuclease activity (nmol of nucleotide rendered acid-soluble/h per mg of protein)	
	Denatured DNA	Native DNA	Poly[d(A-T)]	[Mg ²⁺] (mM)	pH	Denatured DNA	Poly[d(A-T)]
<i>Euglena</i>	2.6	1.6	1.8	8	7.5	27	8
<i>Chlamydomonas</i>	1.0	<0.1	1.6	8	8.5	371	85
<i>Chlorella</i>	2.3	<0.2	<0.1	8	8.5	74	<2
<i>Anabaena</i>	12.0	10.8	19.2	1	9.0	<5	<2
<i>Anacystis</i>	39.0	15.6	31.2	3	9.0	26	9
<i>Esch. coli</i>	123.0	—	—	6	9.0	169	268

Table 2. *Partial purification of DNA polymerase from Anacystis nidulans*

Cell paste (6g) was suspended in buffer A (70ml) and homogenized and centrifuged as described in the Materials and Methods section. Chromatography was carried out on a column of DEAE-cellulose (30cm × 4cm²). The eluted polymerase peak was dialysed overnight against buffer A and then submitted to chromatography on a column of DNA-cellulose (10cm × 1cm²).

Fraction	Volume (ml)	Total polymerase activity (nmol of [³ H]dAMP residues incorporated)	Protein (mg/ml)	Specific activity (nmol of [³ H]dAMP residues incorporated/mg of protein)
100000g super- natant	66	10400	4.4	36
DEAE-cellulose	100	11300	2.4	47
DNA-cellulose	5	8900	3.0	590

whether the algal DNA polymerases display this same property. All the algal polymerases could be bound to and were subsequently eluted from the DNA-cellulose with buffer A containing 0.5M-KCl. Depending on the polymerase preparation under investigation, purifications of 6–40-fold were obtained by this method. By using a gradient of increasing KCl concentration from 0 to 0.5M, polymerase fractions of even higher specific activity could be obtained. Table 2 shows the course of purification of the enzyme from an extract of *Anacystis*. The partially purified enzyme was stable for several months at –60°C in the presence of 20% (v/v) ethylene glycol. At 4°C, the enzyme was stable only for a few days.

Heat-inactivation curves for the purified enzymes from *Euglena* and *Anacystis* are shown in Fig. 2. In the absence of DNA, the enzymes were almost totally inactivated by heating for 5–10min at 45°C. The presence of DNA during the heating period conferred a marked degree of stability on the enzyme;

this protective effect was especially strong with the *Anacystis* enzyme. Initial enzyme extracts containing endogenous DNA showed stability characteristics similar to those of the purified enzymes heated in the presence of DNA. The enzymes from the other algae showed similar heat-inactivation behaviour.

Discussion

Information on the characterization of DNA polymerase in plant systems is scanty (Tewari & Wildman, 1967; Spencer & Whitfield, 1967; Stout & Arens, 1970; Wever & Takats, 1970). Attempts to detect and characterize this enzyme activity in higher plants such as *Lemna* have so far met with little success (J. Bates & H. M. Keir, unpublished work). However, we show here that DNA polymerase of several unicellular green and blue-green algae can be extracted with 0.6M-KCl, and its activity demonstrated with reproducible precision in dialysed

Fig. 2. Heat-inactivation curves of partially purified preparations of DNA polymerase from *Euglena* and *Anacystis*

During heating at 45°C, the mixtures contained the components of the standard assay mixture for polymerase except that the deoxyribonucleoside 5'-triphosphates were omitted, and DNA was either present (○) or absent (●). After the heating at 45°C, the deoxyribonucleoside 5'-triphosphates and DNA were added to give the standard assay concentrations and incubation at 37°C, carried out for 1 h as in the standard assay. (a) *Euglena*; (b) *Anacystis*.

100000g supernatant fractions. Moreover, subsequent partial purification on columns of DEAE-cellulose gives a polymerase preparation that displays linearity of activity with respect to protein concentration over a wide range.

The specific activity of the enzyme in extracts from the green algae was similar to that of the enzyme derived from several lines of mammalian cells grown in culture (Keir, 1965; Greene & Korn, 1970). The polymerase activity of the blue-green algae, *Anabaena*

and *Anacystis*, was intermediate between the corresponding activities of the green algae and bacteria. This observation is compatible with the view that the blue-green algae are evolutionary intermediates between the bacteria and eukaryotic cells.

Measurement of the activity of DNA polymerase in extracts from *Chlamydomonas* cells was difficult; this is very probably attributable to the relatively high deoxyribonuclease activity in these extracts. The values for deoxyribonuclease activity in extracts of algae prepared by our methods do not, of course, necessarily indicate the total deoxyribonuclease content of the cell because (a) the nuclease activities were measured under polymerase assay conditions and not under conditions optimum for maximal nuclease action; (b) deoxyribonuclease activity in the ultracentrifugal sediment fraction, which might contain most of the cell deoxyribonuclease (Schönherr *et al.*, 1970), was not measured in these experiments. Therefore it is not possible to compare our results directly with those of Norton & Roth (1967), who measured deoxyribonuclease activity in a number of blue-green and higher algae.

The results presented here indicate that studies with synchronized cultures of the algal cells might well be feasible. However, strong variations of deoxyribonuclease activities during the cell cycle could interfere seriously with measurement of DNA polymerase and polynucleotide ligase activities in initial or partially purified preparations. Thus, there is a danger that the observations from such studies are open to erroneous interpretation. In *Chlorella*, for example, there are marked fluctuations of deoxyribonuclease in cell homogenates throughout the cell cycle (Schönherr *et al.*, 1970), although the nuclease activity in 100000g supernatant fractions did not adversely affect the DNA polymerase reaction (Schönherr & Wanka, 1971). The problem is more acute in synchronized cultures of *Chlamydomonas* in which the soluble fractions contain relatively high activities of deoxyribonuclease. Nevertheless, the present study has established the basis for further experimentation on enzymes of DNA synthesis during synchronous cell growth.

We are grateful to the Medical Research Council for financial support, to the European Molecular Biology Organization for providing a Fellowship to O. Th. S., and to Mr. Lawrence Morrice for assistance with the growth of algal cultures.

References

- Alberts, B. M., Amodio, F. J., Jenkins, M., Gutmann, E. D. & Ferris, F. L. (1968) *Cold Spring Harbor Symp. Quant. Biol.* 33, 289–305
- Clowes, R. C. & Hayes, W. (eds.) (1968) *Experiments in Microbial Genetics*, p. 187, Blackwell Scientific Publications, Oxford

- Echlin, P. & Morris, I. (1965) *Biol. Rev. Cambridge Phil. Soc.* **40**, 143–187
- Gold, M. & Helleiner, C. W. (1964) *Biochim. Biophys. Acta* **80**, 193–203
- Greenblatt, C. L. & Schiff, J. A. (1959) *J. Protozool.* **6**, 23–28
- Greene, R. & Korn, D. (1970) *J. Biol. Chem.* **245**, 254–261
- Kates, J. R. & Jones, R. F. (1964) *J. Cell. Comp. Physiol.* **63**, 157–163
- Keir, H. M. (1965) *Progr. Nucl. Acid Res. Mol. Biol.* **4**, 81–128
- Kratz, W. A. & Meyers, J. (1955) *Amer. J. Bot.* **42**, 282–287
- Lindsay, J. G., Berryman, S. & Adams, R. L. P. (1970) *Biochem. J.* **119**, 839–848
- Litman, R. M. (1968) *J. Biol. Chem.* **243**, 6222–6233
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Madreiter, H., Kaden, P. & Mittermayer, C. (1971) *Eur. J. Biochem.* **18**, 369–375
- Marmur, J. (1961) *J. Mol. Biol.* **3**, 208–218
- Norton, J. & Roth, J. S. (1967) *Comp. Biochem. Physiol.* **23**, 361–371
- Parenti, F., Brawerman, G., Preston, J. F. & Eisenstadt, J. M. (1969) *Biochim. Biophys. Acta* **195**, 234–243
- Pirson, A. & Lorenzen, H. (1966) *Annu. Rev. Plant Physiol.* **17**, 439–458
- Richardson, C. C., Schildkraut, C. L., Aposhian, H. V. & Kornberg, A. (1964) *J. Biol. Chem.* **239**, 222–232
- Schachman, H. K., Adler, J., Radding, C. M., Lehman, I. R. & Kornberg, A. (1960) *J. Biol. Chem.* **235**, 3242–3249
- Schönherr, O. Th. & Wanka, F. (1971) *Biochim. Biophys. Acta* **232**, 83–93
- Schönherr, O. Th., Wanka, F. & Kuypers, Ch. M. A. (1970) *Biochim. Biophys. Acta* **224**, 74–79
- Spencer, D. & Whitfield, P. R. (1967) *Biochem. Biophys. Res. Commun.* **28**, 538–542
- Stout, E. R. & Arens, M. Q. (1970) *Biochim. Biophys. Acta* **213**, 90–100
- Sueoka, N. (1960) *Proc. Nat. Acad. Sci. U.S.* **46**, 83–91
- Tamiya, H. (1966) *Annu. Rev. Plant Physiol.* **17**, 1–26
- Tewari, K. K. & Wildman, S. G. (1967) *Proc. Nat. Acad. Sci. U.S.* **58**, 689–696
- Wanka, F. (1965) *Arch. Mikrobiol.* **52**, 305–318
- Wever, G. & Takats, S. T. (1970) *Biochim. Biophys. Acta* **199**, 8–17