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A DNA polymerase with unusual properties from the slime mold *Physarum polycephalum*

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Two forms of a DNA polymerase have been purified from microplasmodia of *Physarum polycephalum* by poly(ethyleneimine) precipitation and chromatography on DEAE-Sephacel, phosphocellulose, heparin Sepharose, hydroxyapatite, DNA-agarose, blue-Sepharose. They were separated from DNA polymerase α on phosphocellulose and from each other on heparin-Sepharose. Form HS1 enzyme was 30–40% pure and form HS2 enzyme 60% with regard to protein contents of the preparations. Form HS2 enzyme was generated from form HS1 enzyme on prolonged standing of enzyme preparations. The DNA polymerases were obtained as complexes of a 60-kDa protein associated with either a 135-kDa (HS1) or a 110-kDa (HS2) DNA-polymerizing polypeptide in a 1:1 molar stoichiometry. The biochemical function of the 60-kDa protein remained unknown. The complexes tended to dissociate during gradient centrifugation and during partition chromatography as well as during polyacrylamide gradient gel electrophoresis under nondenaturing conditions at high dilutions of samples. Both forms existed in plasmodia extracts, their proportions depending on several factors including those which promoted proteolysis.

The DNA polymerases resembled eucaryotic DNA polymerase β by several criteria and were functionally indistinguishable from each other. It is suggested that lower eucaryotes contain repair DNA polymerases, which are similar to those of eubacteria on a molecular mass basis.

DNA polymerases of *Physarum polycephalum* are of interest because (a) this organism is phylogenetically on the border between eucaryotes and procaryotes, and could therefore contain novel forms of DNA polymerases, and (b) *Physarum* macroplasmodia execute a naturally synchronous nuclear division (of more than 10^8 nuclei/cell) making it an ideal model for cell cycle investigations [1].

Recent studies [2–5], however, have revealed considerable difficulties in obtaining and characterizing these DNA polymerases, probably due to interfering proteolysis and uncontrollabe factors in growing *Physarum* microplasmodia. We have reinvestigated purification, during which we have monitored types of DNA polymerases as well as their molecular masses, and have used a newly developed combination of gradient gel electrophoresis with a DNA polymerase overlay assay [6]. The method is less disruptive of weak complexes than, for instance, gel permeation chromatography. We report the characterization of a DNA polymerase which does not resemble any of those of higher eucaryotes.

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Abbreviations. Ammediol, 2-amino-2-methyl-1,3-propanediol; SDS, sodium dodecyl sulfate; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; aCTP, cytosine-β-D-arabinofuranoside 5′-triphosphate; ddTTP, 2′,3′-dideoxyribosylthymine 5′-triphosphate; butylphenyl-dGTP, N^2-(*p*-*n*-butylphenyl)9-(2-deoxy-β-D-ribofuranosyl)-guanine 5′-triphosphate; ELISA, enzyme-linked immunosorbent assay.

Enzymes. Amyloglucosidase, glucan 1,4-α-glucosidase (EC 3.2.1.3); alcohol dehydrogenase ($NADP^+$) fructose-bisphosphate (EC 1.1.1.2); aldolase (EC 4.1.2.13); catalase (EC 1.11.1.6); deoxyribonuclease I, DNase I (EC 3.1.21.1); DNA nucleotidyltransferase (EC 2.7.7.7); hexokinase (EC 2.7.1.1); lysozyme (EC 3.2.1.17); pyruvate kinase (EC 2.7.1.40).

EXPERIMENTAL PROCEDURE

Materials

Microplasmodia of *Physarum polycephalum* (M_3C VII, a gift from Dr Schiebel/Martinsried, or M_3C VIII, a gift from Dr Braun/Bern) were grown in shaken cultures with semi-defined medium at 21 °C (M_3C VII) or at 27 °C (M_3C VIII), as described [7]. Cells were harvested during logarithmic growth 2 days after inoculation. They were collected on a cotton sieve (42 mesh), washed with 2–3 vol. of a solution containing 0.25 M sucrose, 10 mM EDTA pH 7.0 and stored at −60 °C for a period of several months.

Purification of DNA polymerase

Thawing microplasmodia (2.4 kg) in 3 vol. buffer A [50 mM Tris/HCl pH 7.5 containing 10 mM $MgCl_2$, 1.5 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM $PhMeSO_2F$, 3 mM sodium bisulfite, 1 μM pepstatin A, 20% (v/v) glycerol] were homogenized in a kitchen blender (60 s below 5 °C). Aqueous 10% (v/v) poly(ethyleneimine) (24 ml, pH 7.5) was added dropwise over a period of 1.5 h (0.03% final concentration).

The 8000 × *g* supernatant was dialysed against five changes of 15 l buffer B (20 mM Tris/HCl pH 7.5 containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM $PhMeSO_2F$, 3 mM sodium bisulfite, 10% glycerol). The dialysate was stirred into 2 l of DEAE-Sephacel; retained DNA polymerase was eluted in a 0–0.6 M KCl gradient in buffer B (300 ml/h).

During the following chromatography (4 °C) active fractions were always dialysed against the buffers used in the subsequent step: phosphocellulose (4 × 13.5 cm), gradient elution (0–0.4 M KCl in buffer B, 50 ml/h), active fractions

at 0.15 M KCl; heparin-Sepharose (4 × 6 cm), gradient elution (0–0.6 M KCl in buffer B, 50 ml/h), active fractions at 0.24 M KCl; hydroxyapatite (1.6 × 7 cm), gradient elution [5–500 mM potassium phosphate buffer C pH 7.5 (buffer C as buffer B with Tris/HCl omitted), 25 ml/h], active fractions at 100 mM potassium phosphate; DNA-agarose (3 × 7 cm), gradient elution (0–0.6 M KCl in buffer B, 20 ml/h), activity at 0.1 M KCl; blue-Sepharose (1 × 7 cm), gradient elution (10–700 mM potassium phosphate buffer C pH 7.5, 20 ml/h), active fractions at 210 mM potassium phosphate. Columns were washed with 3 vol. starting buffer after application of material. Gradients amounted to 8–10 column volumes. Salt concentrations were controlled by conductometry. Active fractions were concentrated with solid sucrose in the presence of 2-mercaptoethanol and stored at −60 °C in the presence of 50% glycerol in buffer B.

Nuclei were prepared according to [8]. In order to prepare nuclear extracts, buffer containing 28 mM magnesium acetate, 60 mM EGTA (pH 7.8), 0.5 M NaCl and 0.1% Triton X-100 was added to the nuclear pellet in an amount equal to the volume of cells, from which nuclei had been prepared. After 15 min at 0 °C and centrifugation (10 min at 12000 × *g*) the supernatant was collected for analysis.

Nucleic acids

Salmon testis DNA from Sigma was activated with DNase I [9] (5–10% acid-soluble DNA with reference to completely digested DNA). Heat-denatured DNA was prepared as described [10]. ColE amp plasmid was isolated according to [11]. Poly(rA) · $p(dT)_{12-18}$ and poly(dA) · $p(dT)_{12-18}$ (equimolar nucleotide monomers) were obtained from PL Biochemicals. Poly(dT) · $p(dA)_{10}$ and poly(dT) · $p(rA)_{10}$ were prepared by heating mixtures of corresponding oligonucleotides at base ratios of 20:1 polymer/oligomer at 60 °C followed by cooling at room temperature. For measurement of 3′-5′-exonuclease activity, DNase-I-activated calf thymus DNA was 3′-end-labelled in the presence of [^{3}H]dNTP (100 Ci/mol) and purified *Escherichia coli* DNA polymerase I [12].

Other chemicals

Nucleoside 5′-triphosphates, poly[d(A-T)], ddTTP and DNase I were obtained from Boehringer (Mannheim), ^{3}H- and ^{32}P-labelled nucleotides from Amersham. Butylphenyl-dGTP was a gift of Dr Brown (Worcester), aphidicolin of ICI. *N*-Ethylmaleimide heparin, ethidium bromide, pepstatin A, Mops and ammediol were from Sigma, electrophoretic material and buffers from Serva (Heidelberg) and chromatographic material from Pharmacia and BioRad. Marker proteins thyroglobulin (bovine), ferritin (equine), catalase (bovine), aldolase (rabbit) were from Pharmacia, bovine serum albumin, hexokinase, lysozyme, pyruvate kinase and ovalbumin from Boehringer (Mannheim) and a mixture of marker proteins for isoelectric focusing according to [13] from Serva (Heidelberg). DNA polymerase I of *E. coli* was purified as in [6].

DNA polymerase assay

The standard assay contained in 150 µl 50 mM Mops (potassium salt) pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 3 mM EDTA, 3 mM 2-mercaptoethanol, 33 µM each of dATP, dCTP, dGTP, 3 µM [^{3}H]dTTP (1 Ci/mmol), 20 µg activated DNA, 80 µg bovine serum albumin, and DNA polymerase. After a 30-min incubation at 37 °C, 10% (v/v) saturated cold trichloroacetic acid in water was added and the precipitate collected on Whatman GF/C filters, which were washed with trichloroacetic acid, then with 70% (v/v) ethanol/H_2O, dried, and counted with 20% efficiency in a toluene-based scintillation cocktail. One unit of DNA polymerase is defined as the amount of enzyme catalysing the incorporation of 1 nmol nucleotide into DNA in 60 min under assay condition. It was verified that reaction rates increased linearly as a function of amounts of enzyme. Heat-denatured DNA (20 µg//assay) was used under standard assay conditions instead of activated DNA. Other template-primer utilisation measurements were performed according to [14]. Inhibition was measured in the standard assay with effector concentrations given in the text. With *N*-ethylmalaimide the methods was that of Schiebel et al. [2, 5]. The truncated assay in the case of the specific inhibitor butylphenyl-dGTP was employed according to [15]. DNA polymerase γ was assayed as described [16].

3′,5′-Exonuclease activity was measured by following the generation of acid-soluble radioactivity from ^{3}H-labelled DNA according to [12]. Endonuclease activity was assayed by following the conversion of double-stranded superhelical plasmid ColE1 (form CCC) into nicked (OC) or linear (L) forms. The reaction mixture (50 µl) contained 0.5 µg plasmid, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris/HCl buffer pH 8 and enzyme. After 30 min at 37 °C, DNA forms were separated by electrophoresis in 0.7% agarose and stained with ethidium bromide. In control experiments with CCC-form plasmid and *Eco*RI or topoisomerase I, the positions of the L-form and of relaxed closed circular DNA, respectively, were established.

Polyacrylamide gel electrophoresis

Molecular masses of proteins under nondenaturing conditions were measured by electrophoresis in 3–8% polyacrylamide gradient gels according to [17–20] in combination with a recently developed DNA polymerase overlay gel assay [6]. Both the ammediol and Tris buffer systems were used [6] giving essentially identical results.

Molecular masses of polypeptides containing DNA polymerase activity were measured in SDS containing acrylamide 'activity gels' according to [21]. Gels were renatured for a period of 50–60 h and assayed for DNA polymerase.

Molecular masses of polypeptides were otherwise measured by electrophoresis in SDS-containing gels according to Laemmli [22]. Isoelectric focusing was carried out on Servalyt IEF-Precoates pH 3–10 under nondenaturing conditions. Marker proteins were amyloglucosidase (pI 3.8), ferritin (pI 4.4), bovine serum albumin (pI 4.7), β-lactoglobulin (pI 5.34), conalbumin (pI 5.9), horse myoglobulin (pI 7.3) and whale myoglobulin (pI 8.3).

Proteins were stained with Coomassie brilliant blue G250 as described for gels [23] and solutions [24] and with silver in polyacrylamide gels according to [25]. The degree of purity of DNA polymerase fractions was estimated from densitograms of stained protein bands in polyacrylamide gels after electrophoresis.

Velocity gradient centrifugation

Sedimentation coefficients and molecular masses were measured by velocity gradient centrifugation with reference to serum albumin (4.31 S, 68 kDa), yeast alcohol dehydrogenase

Table 1. *Purification scheme for form HS2 DNA polymerase*

Fraction	Protein	Activity	Specific activity	Purification factor	Yield
	mg	units	units/mg		%
Cell extract (2.4 kg cells)	16300	8660	0.53	1	100
Poly(ethyleneimine) precipitation and dialysis	16000	48000	3.0	5.6	560
DEAE-Sephacel	3420	14600	4.27	8.1	170
Phosphocellulose	346	4220	12.2	22	49
Heparin-Sepharose	128	4220	33	61	49
Hydroxyapatite	11.5	1403	122	225	16
DNA-agarose	1.14	1040	915	1726	12
Blue-Sepharose	0.38	690	1830	3450	8

(7.61 S, 145 kDa) and catalase (11.3 S, 240 kDa). Centrifugation was performed for 40 h at 188000 × *g* (maximal radius) and 4°C with a Hitachi preparative ultracentrifuge type 65P and a swing-out type RPS 40T rotor in 12-ml gradients of 10% sucrose/5% glycerol to 30% sucrose/10% glycerol in buffer B containing 300 mM KCl.

Partition chromatography

Molecular masses were measured for 100–200-µl samples by partition chromatography on Sephacryl S-200, Sephacryl S-300 and Sephadex G-200 (0.6 × 80 cm) at 4°C. Elution buffer was 0.2 M potassium phosphate (or 50 mM Tris/HCl with 200 mM KCl) pH 7.5, 1 µM pepstatin A, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol. Fractions of 600 µl were collected at a rate of 2–3 ml/h. Reference proteins were those of Fig. 4.

RESULTS

Purification of DNA polymerase

A purification protocol is summarized in Table 1. *Physarum* extracts contain the present type DNA polymerase and a type-α DNA polymerase (unpublished results). The activity of the type-α enzyme is suppressed by an inhibitor, which is removed by poly(ethyleneimine) precipitation or by chromatography on DEAE-cellulose columns. Details on this inhibitor will be published elsewhere. The 5.6-fold increase in polymerase activity at the beginning of the purification protocol is the result of removal of the inhibitor. Type-α DNA polymerase was separated from the present activity by chromatography on phosphocellulose, eluting with 0.65 M KCl; partial separation can be obtained during chromatography on DEAE-cellulose columns, the present activity eluting in a position of higher ionic strength than does type-α DNA polymerase.

A single-fraction DNA polymerase activity appears in the present protocol during chromatography on heparin-Sepharose. This fraction, HS2, is frequently accompanied by a second fraction, HS1, eluting at 0.17 M KCl. Both fractions were further purified. For fraction HS2 the results are given in Table 1. Fraction HS1 eluted from hydroxyapatite with 0.2 M potassium phosphate and on blue-Sepharose with 0.25 M potassium phosphate. At this stage, fraction HS1 had a specific activity of 1120 units/mg and resembled only 0.6% of the DNA polymerase activity of cell extracts used for this purification.

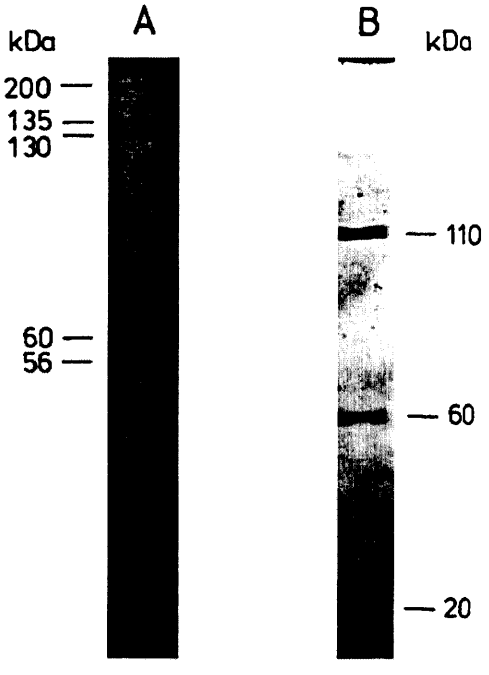

Fig. 1. *Electrophoresis under denaturing conditions in the presence SDS.* Approximately 1 µg protein per sample of blue-Sepharose fractions was applied (Table 1). Lane A, fraction HS1; the gel was prepared according to Laemmli [22]. Lane B, fraction HS2; the gel contained an 8–13% acrylamide gradient. Protein was stained with silver

Purity

The purity of prepared fractions HS1 and HS2 was examined by inspection of protein bands in polyacrylamide gels after electrophoresis in the presence of SDS (Fig. 1). Preparation HS1 contained proteins in positions of 56, 60, 130, 135 and 200 kDa, preparation HS2 proteins in positions of 20, 60 and 110 kDa. The 20-kDa and 200-kDa proteins were considered impurities because they did not comigrate with DNA polymerase activity during electrophoresis in nondenaturing polyacrylamide gels (including the overlay gel

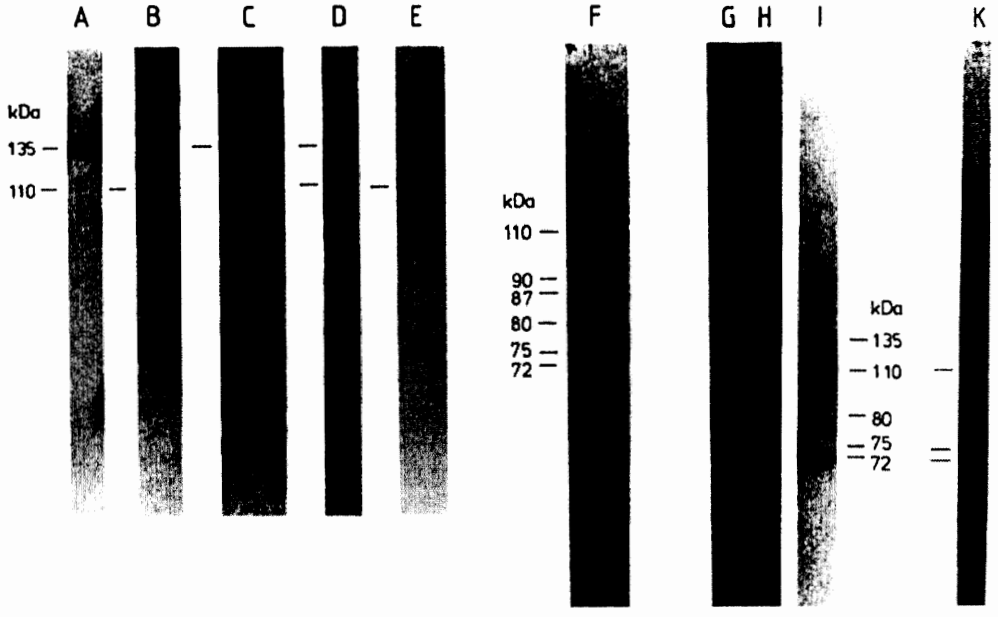

Fig. 2. *Electrophoresis in SDS/acrylamide activity gels according to a modification of [21].* Amounts of 0.1 – 0.4 unit *Physarum* DNA polymerase and 0.002 unit *E. coli* DNA polymerase I were applied. Lane A, blue-Sepharose fraction of HS1 (a preparation different from that in Fig. 1 A); lane B, blue-Sepharose fraction of HS2; lane C, extract of freshly harvested microplasmodia; lane D, a mixture of blue-Sepharose fractions of HS1 and HS2; lane E, purified DNA polymerase I of *E. coli*; lane F, an extract of frozen microplasmodia after standing for 4 weeks at 4 °C; lanes G and H, blue-Sepharose fractions of HS1 and HS2 preparations respectively after standing for 1 year at −20 °C; lane I, an extract of nuclei (0.1 M NaCl) from microplasmodia after several days at 4 °C; lane K, an extract of *E. coli* cells after 4-weeks standing at 4 °C (the activity in positions above 110 kDa is considered as an artefact due to irreversible aggregation during standing)

assay) as seen after electrophoresis in SDS-containing gels in the second dimension. The 60-kDa protein was common to both preparations and was found in all the other preparations obtained. The 110-kDa and 135-kDa proteins co-migrated with activity bands (Fig. 2) after electrophoresis in SDS-containing 'activity gels'. On the basis of a subunit structure (see later) containing one copy of polymerizing and one copy of 60-kDa polypeptides the preparations in Fig. 1 were estimated to be 60% (on a mass basis) HS2 and 30 – 40% HS1 DNA polymerase with regard to total protein. Specific activities of estimated pure preparations were of the order of 3000 units/mg.

Identification of DNA polymerizing polypeptides

Fractions HS1 and HS2 as well as 'mixed' preparations and an extract of freshly harvested microplasmodia all exhibited major polymerizing activities in positions of 110 kDa and 135 kDa respectively when analysed by the SDS-containing 'activity gel' method (Fig. 2). In one example (lane C) activity seems to run with a 200-kDa protein. This is considered an artefact arising from the mild dissociation condition (5 min at 37 °C instead at 100 °C used in routine molecular mass determinations according to [22]). We have observed this phenomenon previously [6]. It should be mentioned that in our hands the 'activity gel' method was unsuccessful with *Physarum* type-α DNA polymerase. We therefore consider activity bands in lane C to originate from the present DNA polymerase.

Stability

During storage at −20 °C, DNA polymerase fractions were degraded with half-lifes of several months resulting in enzymatically active proteins of molecular masses 72, 75 and 80 – 90 kDa (Fig. 2, lanes F – I). Fractions HS1 and HS2 exhibited similar patterns of activity bands suggesting that HS2 was a proteolytic degradation product of HS1. Occasionally an active band appeared also at 130 kDa and is seen in Fig. 1, lane A, after silver staining. Interestingly, degradation of DNA polymerase of *E. coli* exhibited similar patterns of activity bands (Fig. 2, lane K).

Molecular masses under nondenaturing conditions

Molecular masses were measured under nondenaturing conditions by velocity gradient centrifugation according to Martin and Ames [26] (for example of fraction HS2 in Fig. 3) and by partition chromatography (for example of fraction HS1 in Fig. 4). Values of sedimentation coefficients and molecular masses are listed in Table 2. Values of molecular masses are indistinguishable by these methods, suggesting that the hydrodynamic properties of the polymerizing polypeptides are comparable with those of 'globular' (marker) proteins.

Subunit structure

Results for molecular masses so far obtained by electrophoresis in SDS-containing denaturing polyacrylamide gels and by gradient centrifugation and partition chromatography under nondenaturing conditions are consistent with the DNA polymerase being a single polypeptide and not having a subunit structure. However, it remained possible that a relatively weak enzyme complex had been disrupted in the measurements under otherwise nondenaturing conditions. According to our experience with other weak enzyme complexes, the following observations seemed to support this possibility. (a) The activity profile after gradient centrifugation exhibited

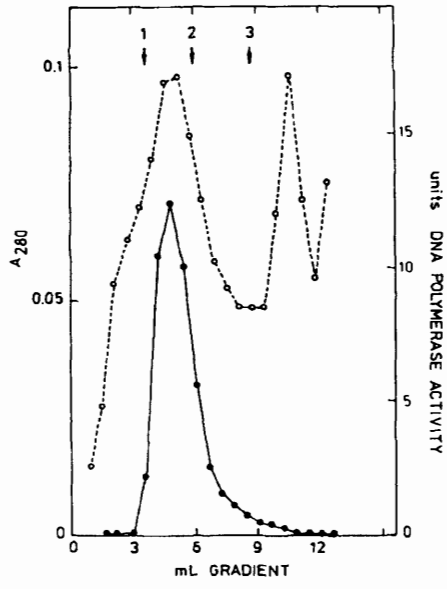

Fig. 3. *Velocity gradient centrifugation of a preparation containing form HS2 DNA polymerase.* An amount of 56 units was applied; the yield after centrifugation was 52 units. References were bovine serum albumin (1), alcohol dehydrogenase (2) and catalase (3). Symbols refer to DNA polymerase activity (●——●) and absorbance at 280 nm (○- - - -○)

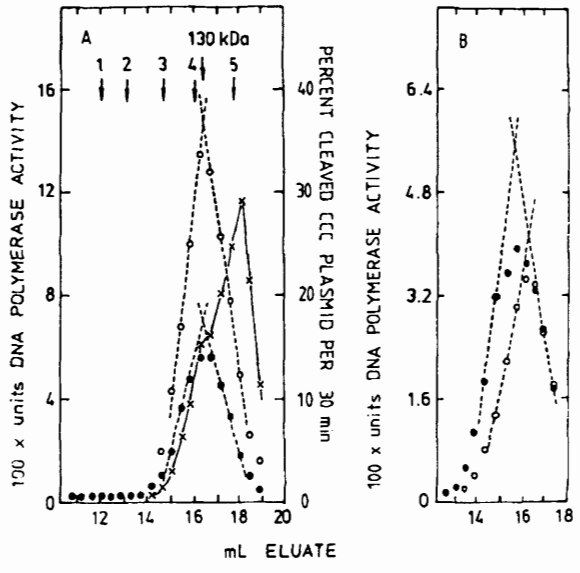

Fig. 4. *Partition chromatography on Sephacryl S-300 of a blue-Sepharose fraction containing form HS1 DNA polymerase.* Samples of 20 µl of the eluate were assayed for polymerase activity (- - - -) and samples of 1 µl for endonuclease activity (×——×). DNA polymerase activity was measured with activated (●- - - -●) or heat-denatured (○- - - -○) salmon testis DNA. (A) Blue-Sepharose fraction containing form HS1 enzyme (96 units, 60% yield after chromatography). (B) DEAE-Sephacel fraction (Table 1) containing form HS1 enzyme and DNA polymerase α (144 units, 90% yield after chromatography). References were (1) thyroglobulin (660 kDa), (2) ferritin (440 kDa), (3) catalase (240 kDa), (4) aldolase (158 kDa) and (5) bovine serum albumin (68 kDa)

trailing into the region of high molecular masses (Fig. 3). (b) Enzymatic activity after completion of partition chromatography decreased rapidly as if instability had been inferred by removal of a component. (c) A protein of 60 kDa was obvious whenever molecular masses of freshly prepared samples of HS1 and HS2 were examined by electrophoresis in SDS-containing polyacrylamide gels, whereas only traces or barely detectable amounts of this protein were seen after gradient centrifugation or partition chromatography. For clarification, an analytical method for determination of molecular masses was required that was less disruptive than gradient centrifugation and partition chromatography.

Electrophoresis under nondenaturing conditions in polyacrylamide gradient gels in combination with an overlay gel activity assay [6] was chosen to clarify the question of a DNA polymerase complex. This method has previously been reported to be less disruptive than partition chromatography [6]. Fig. 5 shows results obtained by this technique. DNA polymerase activity migrated in the position of 200 kDa in the case of preparation HS1, in the positon of 170 kDa in the case of preparation HS2 and in both positions if a mixture was examined (Fig. 5, lanes A – C). Samples of these preparations, which had been subjected to partition chromatography prior to the electrophoresis, exhibited activities in the region of 140 kDa (HS1) and 110 kDa (HS2) (Fig. 5, lanes E and G). Although the sizes of activity bands in the figure do not unambiguously allow a sharp assignment to molecular masses, activities clearly migrated in positions of lower molecular mass than in the controls (lanes D and F) and in positions that were comparable with the molecular masses of enzymatically active polypeptides seen in the SDS/acrylamide activity gels (Fig. 2A – D). In the particular example of Fig. 5, lanes D – G, proteins were allowed to migrate as far as possible to the lower edge of the gel in order to achieve maximum possible resolution. Gels of shorter migration distances and correspondingly lower resolution did not show polymerase activities in positions below 100 kDa (results not shown). This was not the case, however, for partially degraded samples as those of Fig. 2, lanes F – I, exhibiting activity in the 70 – 80-kDa region (results not shown) in accord with the smaller sizes of DNA-polymerizing polypeptides in these cases.

Table 2. *Molecular masses of form HS1 and form HS2 DNA-polymerizing polypeptides*

Method	Molecular mass of form	
	HS1	HS2
	kDa	
Electrophoresis in SDS/ acrylamide activity gels [21]	135 ± 5	110 ± 5
Gradient centrifugation [26]	130 ± 10 (s = 7.2 S)	100 ± 10 (s = 5.9 S)
Partition chromatography	130 ± 15	115 ± 10

It is conceivable that DNA polymerases exist as protein complexes which dissociate under the concentration condtions during gradient centrifugation and partition chromatography with a defined dissociation constant. If this is the case, dissociation ought to be confirmed by electrophoresis under nondenaturing conditions for protein concentrations at least of the order of the (unknown) dissociation constant. A dilution experiment was carried out in Fig. 5, lanes H – L, with a fresh preparation of the HS1 enzyme. The overlay reaction was conducted for 2 h and autoradiography for 10 days in order to provide a sensitive detection of polymerase activity for the highest dilution. The results indeed indicate a

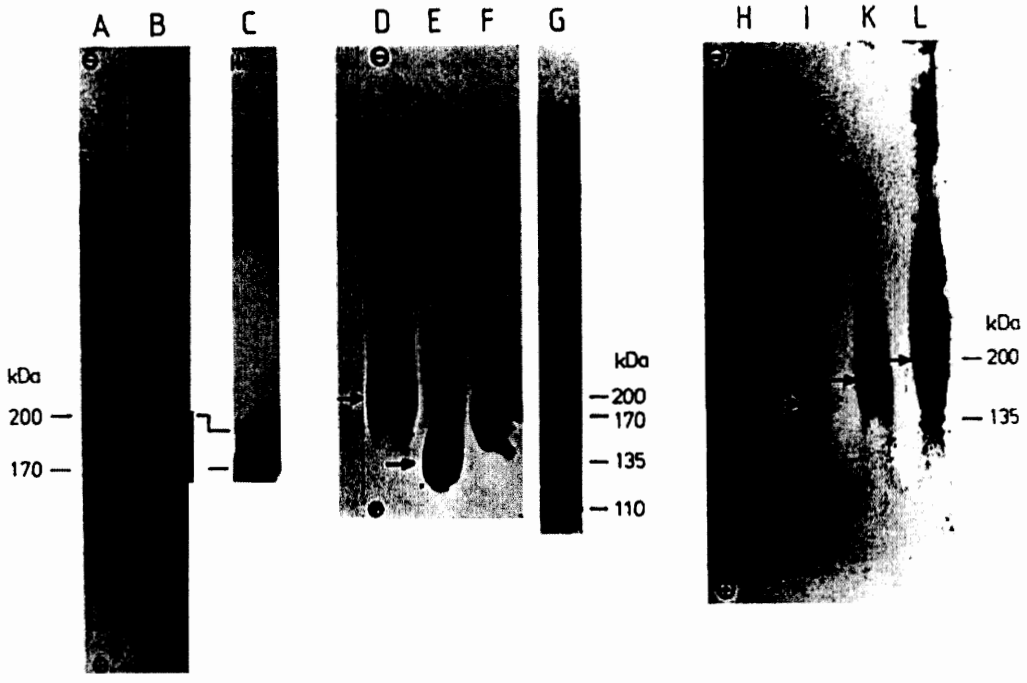

Fig. 5. *Effects of partition chromatography and sample dilution on molecular masses of DNA polymerase measured by electrophoresis in 3–8% acrylamide gradient gels under nondenaturing conditions [6] in Tris/HCl buffer.* Lanes A–C, blue-Sepharose fractions containing 0.05–0.2 unit of form HS1 (A), of form HS2 (B) and of a mixture of both forms of DNA polymerases (C); lanes D and E, 0.8 unit (D) of form HS1 enzyme before and (E) 0.05 unit after partition chromatography on Sephacryl S-300; lane F and G, 2 units (F) of form HS2 enzyme before and (G) 0.04 unit after partition chromatography; lanes H–L, blue-Sepharose fraction of 0.005 (M), 0.01 (I), 0.03 (K) and 0.06 unit (L) form HS1 enzyme. Activity bands were detected by the overlay gel assay [6]. Periods of film exposure to X-rays were 1–5 h (lanes A–G) and 10 days (lanes H–L)

shift in the position of activity at increasing dilution into the direction of the molecular masses expected if dissociation had occurred.

On the basis of the above results, we propose that the DNA polymerases exist as protein complexes, which dissociate into subunits in a concentration-dependent fashion. Subunits of the complexes are the DNA-polymerizing polypeptide and the 60-kDa protein that has been invariably detected in the denaturing SDS/acrylamide gels (Fig. 1). The stoichiometry of the two kinds of subunits was calculated on the basis of the photometer-scanned intensities of the corresponding bands in gels like that in Fig. 1B and was 0.96 ± 0.26 mol/mol (four determinations).

DNA polymerase complexes during purification

DNA polymerases were characterized on the basis of their molecular masses in plasmodia extracts (Fig. 6A–C) and during purification (Fig. 6D–G). Both form HS1 and HS2 enzymes were found in plasmodia extracts (DNA polymerase α migrated at short distances from the origin and required high specific radioactivities in order to be detected; unpublished results). The relative proportions of the two kinds of DNA polymerases depended on whether whole cell extracts or nuclear extracts were prepared. Preparations from plasmodia that had been stored several months at −60 °C contained only form HS2. The purification, of which samples are shown at various stages, was carried out with the extract from frozen cells (Fig. 6C–G). Our further experience was that part of the form HS1 enzyme could convert to HS2 enzyme during purification and during prolonged standing at −60 °C (not shown).

Inhibitors, template-primers and other properties of the DNA polymerases

Experiments for the classification of the present DNA polymerases were carried out on the basis of accepted criteria known for higher eucaryotes [27–29] (and references in [28]). Up to the present, we have not been able to distinguish the two forms HS1 and HS2 on the basis of these or other functional properties. Results for the inhibition of DNA-polymerizing activity are summarized in Table 3. In comparison with higher eucaryotes, the enzymes had 'β-like' properties with regard to aphidicolin, aCTP, butylphenyl-dGTP, heparin and ethidium bromide, by which it could be distinguished from DNA polymerase α of *Physarum*. However, the moderate sensitivity against ddTTP, the (moderate) sensitivity against *N*-ethylmaleimide and the sensitivity against 100 mM monovalent metal ions were atypical for a type-β DNA polymerase.

Among the template-primer acceptances, the inefficiency for poly(dT) · $p(rA)_{10}$ and the high efficiency for poly(rA) · $p(dT)_{12-18}$ (Table 4) were properties of an eucaryotic type-β DNA polymerase, although ribotemplates are known to be accepted by type-γ DNA polymerase. In order to eliminate this possibility, we followed the assay specific for type-γ DNA polymerase that contained phosphate in the reaction mixture. We observed hyperbolic inhibition as a function of phosphate concentration, namely 50% at 20 mM and 97% at 60 mM potassium phosphate. This result did not meet the criteria for DNA polymerase γ [16].

Of the enzymatic properties in Table 5, the pH optimum and the range of the Michaelis-Menten constants were compatible with properties of a type-β DNA polymerase. The value of the isoelectric point was not in agreement with that

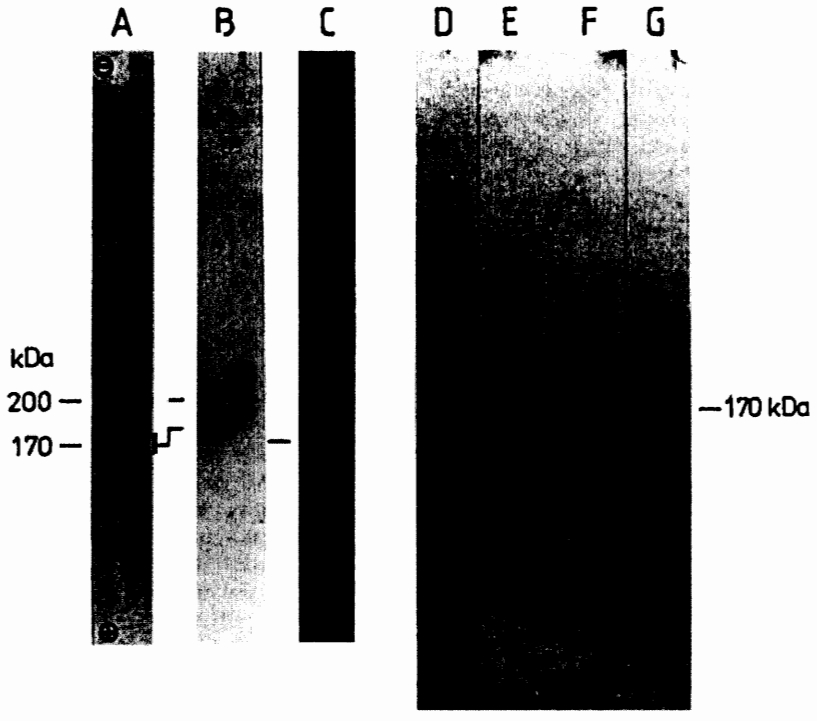

Fig. 6. *Molecular masses of DNA polymerases in extracts of plasmodia and during their purification.* Lane A, extract of freshly harvested plasmodia; lane B, extract of nuclei in the presence of 0.5% Triton X-100 and 0.5 M NaCl of freshly harvested plasmodia; lane C, extract of plasmodia, which had been stored for 6 months at −60°C; lanes D−G, fractions during the purification of DNA polymerase from an extract as in lane C; after phosphocellulose, heparin-Sepharose, DNA-agarose and blue-Sepharose, respectively. Molecular masses were measured by electrophoresis in acrylamide gradient gels (Tris buffer) according to [6]

Table 3. *Inhibition of DNA polymerases in the standard activity assay*
Form HS1 enzyme was indistinguishable from form HS2 enzyme. n.d. = not determined

Inhibitor	Concentration	Relative activity	
		DNA-polymerase α	HS1 or HS2
	mM (μg/ml)		
No inhibitor		100	100
Aphidicolin	(5)	33	100
aCTP	0.13	15	95
ddTTP	0.4	75	75
Ethidium bromide	0.01	100	25
Heparin	(0.1)	26	100
Butylphenyl-dGTP	0.0005	28	100
N-Ethylmaleimide	10	54	79
Novobiocin	0.5	n.d.	50
Spermidine	2−4	n.d.	200−400
Spermine	0.4	n.d.	50
NaCl	100	40	50
KCl	100	100	100

of a DNA polymerase β. However, when the gel was cut after electrofocusing, DNA polymerase extracted and analysed by electrophoresis in the presence of SDS according to Laemmli [22], it was found to contain the 60-kDa protein in the region of polymerase activity (not shown) suggesting that the slightly acidic pI referred to the polymerase complex rather than to the enzymatic polypeptide.

Our DNA polymerases preferred heat-denatured DNA over activated DNA, among DNA polymerases an unusual property, by which it could be distinguished from *Physarum* DNA polymerase α (Fig. 4B).

Table 4. *Template-primer utilization*
Form HS1 enzyme was indistinguishable from form HS2 enzyme. Assay conditions for synthetic polymers and for activated DNA as reference in these cases were as in [14]

Template-primer	Relative activity
Activated salmon testis DNA	100
Heat-denatured salmon testis DNA	240
Poly d(AT)	150
Poly(dT) · $p(dA)_{10}$	70
Poly(dT) · $p(rA)_{10}$	7
Poly(rA) · $p(dT)_{12-18}$	2400

Table 5. *Enzymatic properties*
Form HS1 enzyme was indistinguishable from form HS2 enzyme

Parameter	Value
Isoelectric point (DNA polymerase complex)	pH 5.2
pH optimum	pH 8−9
Mg^{2+} optimum	5.5 mM
half maxima	3.5 mM and 9.0 mM
Mn^{2+} optimum	3.5 mM
K_m values in the presence of activated or heat-denatured salmon testis DNA	
dATP	0.3 μM
dCTP	0.5 μM
dGTP	0.8 μM
dTTP	0.4 μM
DNA, activated or heat-denatured	120 μg/ml

Preparations did not contain detectable 3′-5′-exonuclease activity corresponding to less than 1 pmol nucleotide liberated per h^{-1} (unit DNA polymerase activity)$^{-1}$. They contained, however, endonucleolytic activity producing single-stranded nicks in circular closed plasmid DNA. Most of the activity could be separated from the polymerase activity by passing the preparations over a Sephacryl S-300 column (Fig. 4A). It cannot be ruled out, at present, that the 60-kDa protein of the complex contained the endonuclease activity.

DISCUSSION

DNA polymerases of lower eucaryotes such as *Physarum polycephalum* are difficult to purify because of interfering proteolytic activities and, perhaps, unusual properties. Analysis by the method of SDS/acrylamide activity gels according to [21] revealed an accumulation of DNA polymerase activities in positions of low molecular mass on standing of plasmodia extracts and also of purified samples of DNA polymerases. Patterns of molecular masses were similar as those obtained on standing of DNA polymerase I of *E. coli* and confirmed previous findings [30] concerning certain structural homologies that were thought to be conserved among DNA polymerases. Species of DNA polymerases in plasmodia extracts were also examined by electrophoresis in polyacrylamide gradients under nondenaturing conditions and visualization by the overlay assay method [6]. Two activities were found migrating with molecular masses of 170 kDa and 200 kDa. The properties of activity in these bands depended on the kind of extracted material, whether whole plasmodia or nuclei or whether freshly harvested or frozen plasmodia were extracted. Especially extracts from frozen plasmodia contained a high proportion of the 170-kDa polymerase. The two bands generated by the overlay gel assay were previously assigned to molecular masses of 200 and 240 kDa and one of them (240 kDa) to DNA polymerase α [6]. Re-determination of molecular masses revealed that these bands reffered to those of 170 and 200 kDa. The availability of monoclonal antibodies against DNA polymerase α and the analysis of eluates of the separation gels by ELISA ruled out that either band resembled DNA polymerase α (indeed this polymerase migrated in the position of considerably higher molecular mass, unpublished results).

The DNA polymerases of molecular mass 170 kDa and 200 kDa were separated from DNA polymerase α by passage over phosphocellulose and purified to approximately 60% and 40% homogeneity. They were separated by chromatography on heparin-Sepharose into fractions HS1 (200 kDa) and HS2 (170 kDa). The purification was followed by electrophoresis under nondenaturing conditions together with the overlay gel assay [6]. This method revealed also that form HS1 could convert to form HS2 polymerase after prolonged standing of preparations.

The composition of purified fractions HS1 and HS2 was analysed by SDS/acrylamide gel electrophoresis according to Laemmli [22] and by SDS/acrylamide activity gel electrophoresis [21]. The results can be summarized as indicating that form HS1 (200-kDa) DNA polymerase was a complex of a 135-kDa DNA-polymerizing subunit and of a 60-kDa protein. The HS2 (170-kDa) DNA polymerase was a complex of a 110-kDa DNA polymerizing subunit and a 60-kDa protein.

The tendency of the polymerase complexes to dissociate into subunits became evident during gradient centrifugation and partition chromatography while at comparable concentrations dissociation was not apparent during preparative purification and during electrophoresis in polyacrylamide gradient gels under nondenaturing conditions. A gross value of the dissociation constant is estimated on the basis of the results from the dilution experiment in Fig. 5H – L. Bands corresponding to a dissociation of the complex emerged at sample concentrations of the order of 2×10^{-4} units/μl. By taking into account a specific activity of 3000 units/mg and a molecular mass of the complex of 200 kDa, the concentration is of the order of 0.1 – 1.0 nM. According to standard rules that dissociation is recognized at concentrations of the complex of the order of its dissociation constant, this value appears to be an appropriate estimate. Complexes of this high affinity display slow rates of dissociation. On thermodynamic and kinetic bases and among other arguments (pressure effects [31], material-specific effects of chromatographic material), it was conceivable that a high degree of dissociation occurred during the 40-h gradient centrifugation and during partition chromatography on long columns but not in the case of chromatography on relatively short preparative columns and not under the conditions of electrophoresis (see also [6]).

The DNA polymerases displayed features of an eucaryotic type-β DNA polymerase. However, a main obstacle for a classification of that kind was the high molecular mass (110 and 135 kDa) in comparison to eucaryotic DNA polymerase β (30 – 45 kDa). In this regard they resemble eubacterial 'repair' DNA polymerases (110 kDa for *E. coli* DNA polymerase I). Rabbit anti-(DNA polymerase I) antibodies do not cross-react with the *Physarum* polymerases, however (Zabel, personal communication). Their biological role as 'repair' DNA polymerases is likely in view of their relatively low (10 – 20%) fraction of total DNA-polymerizing activity in growing plasmodia and of their constant concentration level during the *Physarum* cell cycle (H. Fischer, unpublished results as part of his thesis). 'Repair' DNA polymerases of high molecular mass may be considered more common in lower eucaryotes, as a type-β enzyme of 70 kDa has been reported for *Dictyostelium discoideum* [32]. It would be of interest to see whether the described tendency to form complexes exists among this kind of DNA polymerases.

Among the properties of the DNA polymerases listed in Tables 3 – 5 the following properties also seem worth mentioning. (a) The enzymes display an unusually high activity for heat-denatured DNA. It has to be established whether this property could be related to the action of the 60-kDa protein and whether it has a biological role. (b) Spermidine at intermediate concentrations activated DNA polymerization. Spermine, and spermidine at concentrations above 6 mM inhibited DNA synthesis. The effects of the polyamines are considered as of some biological significance. Indeed, spermine stimulated DNA synthesis in isolated nuclei of *Physarum* [33 – 35] and apparently neutralized an inhibitor of DNA polymerase α [2] (and our own results). Ornithine decarboxylase of *Physarum*, the first enzyme in the polyamine biosynthetic pathway, has been reported to increase stepwise in the S-phase of the cell cycle [36]. However, since polyamines interact with nucleic acids and with many other sites *in vivo*, any conclusions are premature at present.

Several forms of DNA polymerases from *Physarum* have been reported by Schiebel and coworkers [2, 3, 5]. The form described by the sedimentation coefficient 5.5 S [3] and as form DE-III enzyme [5] is probably identical with the HS2 DNA-synthesizing polypeptide, and their form C enzyme [2] with the HS1 DNA-polymerizing polypeptide. The results of the present investigation confirm and extend many of the previous findings.

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REFERENCES

1. Holt, E. C. (1980) in *Growth and differentiation in* Physarum polycephalum (Dove, W. F. & Rusch, H. P., eds) pp. 9–63, University Press, Princeton, NJ.
2. Baer, A. & Schiebel, W. (1978) *Eur. J. Biochem. 86*, 77–84.
3. Zänker, K. S. & Schiebel, W. (1978) *Biochem. J. 171*, 445–451.
4. Choundhry, M. K. & Cox, R. A. (1979) in *Current research on* Physarum, *Publications of the University of Innsbruck*, vol. 120 (Sachsenmaier, W., ed.) pp. 71–76, University of Innsbruck.
5. Schiebel, W. & Raffael, A. (1980) *FEBS Lett. 121*, 81–85.
6. Holler, E., Fischer, H. & Simek, H. (1985) *Eur. J. Biochem. 151*, 311–317.
7. Daniel, J. W. & Baldwin, H. H. (1964) *Methods Cell Physiol. 1*, 9–41.
8. Brewer, E. N. & Busacca, P. A. (1979) *Biochem. Biophys. Res. Commun. 91*, 1352–1357.
9. Loeb, L. A. (1969) *J. Biol. Chem. 244*, 1672–1681.
10. Albert, B. & Herrick, G. (1971) *Methods Enzymol. 21*, 198–217.
11. Burkardt, H. J., Mattes, R., Schmid, K. & Schmitt, R. (1978) *Mol. Gen. Genet. 166*, 75–84.
12. Richardson, C. C., Lehman, I. R. & Kornberg, A. (1964) *J. Biol. Chem. 239*, 251–258.
13. Radola, B. J. (1983) *Biochim. Biophys. Acta 295*, 411–428.
14. Masaki, S. & Yoshida, S. (1978) *Biochim. Biophys. Acta 521*, 74–78.
15. Khan, N. N., Wright, G. E., Dudyez, L. W. & Brown, N. C. (1984) *Nucleic Acids Res. 12*, 3695–3706.
16. Knopf, K.-W., Yamada, M. & Weissbach, A. (1976) *Biochemistry 15*, 4540–4548.
17. Andersson, L.-O., Bor, H. & Mikaelsson, M. (1972) *FEBS Lett. 20*, 199–202.
18. Lorentz, K. (1976) *Anal. Biochem. 76*, 214–220.
19. Lasky, M. (1978) in *Electrophoresis '78* (Catsimpoolas, N., ed.) pp. 195–210, Elsevier/North-Holland, Amsterdam.
20. Lambin, P. & Fine, J. M. (1979) *Anal. Biochem. 98*, 160–168.
21. Spanos, A., Sedwick, S. G., Yarranton, G. T., Hübscher, U. & Banks, G. R. (1981) *Nucleic Acids Res. 9*, 1825–1839.
22. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680–685.
23. Maurer, H. R. (1968) *Disk Elektrophorese*, pp. 44–46, Walter de Gruyter & Co., Berlin.
24. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
25. Heukeshoven, J. & Dernick, R. (1985) in *Elektrophoresis forum '85* (Radola, B. J., ed.) pp. 108–118, Technische Universität München.
26. Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.
27. Scovassis, A. I., Plevani, P. & Bertazzoni, U. (1980) *Trends Biochem. Sci. 5*, 335–337.
28. Hübscher, U. (1983) *Experientia (Basel) 39*, 1–25.
29. Falaschi, A. & Spadari, S. (1977) *Nato Adv. Study Inst. Ser. A, 17*, 487–515.
30. Hübscher, U., Spanos, A., Albert, W., Grummt, F. & Banks, G. R. (1981) *Proc. Natl Acad. Sci. USA 78*, 6771–6775.
31. Jaenicke, R. & Rudolph, R. (1980) in *Protein folding* (Jaenicke, R., ed.) pp. 529–530, Elsevier/North-Holland Biomedical Press, Amsterdam.
32. Baril, E. F., Scheiner, C. & Pederson, T. (1980) *Proc. Natl Acad. Sci. USA 77*, 3317–3321.
33. Brewer, E. N. & Rusch, H. P. (1966) *Biochem. Biophys. Res. Commun. 25*, 579–584.
34. Schiebel, W. & Schneck, U. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 335*, 1515–1525.
35. Brewer, E. N. & Ting, P. (1975) *J. Cell Physiol. 86*, 459–470.
36. Mitchell, J. L. A. & Rusch, H. P. (1973) *Biochim. Biophys. Acta 297*, 503–516.