Altered Nuclear Deoxyribonucleic Acid α-Polymerases in Senescent Cultured Chick Embryo Fibroblasts†

Michael Fry* and Pnina Weisman-Shomer

ABSTRACT: DNA α-polymerase has been partially purified from nuclei of cultured chick fibroblasts and separated on phosphocellulose columns into two distinct activities designated DNA polymerases α(a) and α(b), respectively. The enzyme preparations were devoid of activities of DNA β,γ-polymerases terminal deoxyribonucleoside transferase, DNase, DNA-dependent RNA polymerase, and phosphatase. DNA polymerases α(a) and α(b) both having molecular weights of 160 000, constitute 35–50 and 65–50%, respectively, of the activity of α-polymerase in the nucleus. These enzymes differ in their requirements for maximal activity, their relative ability to copy oligo(dG)·poly(dC), their response to ribonucleoside triphosphates, and their kinetics of heat inactivation. When the properties of α polymerases derived from early or late passage cultures have been compared, no difference could be detected as a function of cell age in the specific activities of the polymerases in crude cell extracts, their chromatographic behavior on diethylaminoethylcellulose and phosphocellulose columns, and their relative abilities to utilize single deoxyribonucleoside triphosphates with activated DNA template. On the other hand, both enzymes become partially heat labile in aging cells. Also, the activity of DNA polymerase α(a) from young cells was stimulated by 2–10 mM adenosine or cytidine triphosphates, whereas the same enzyme from old cultures was inhibited by these agents. Conversely, these ribonucleoside triphosphates inhibited the activity of polymerase α(b) in young cells but slightly stimulated this enzyme derived from senescent fibroblasts. In addition, the relative ability of DNA polymerase α(a) to copy oligo(dG)·poly(dC) decreased in aged cells, whereas that of DNA polymerase α(b) increased. We have also observed significant differences in the effects of potassium chloride and *N*-ethylmaleimide on the activity of DNA polymerase α(a) from old cells as compared to young cells. These age-related alterations in the properties of the two avian DNA polymerases may reflect structural or conformational changes in these enzymes.

Normal avian cells (Hay and Strehler, 1967; Hay, 1970; Maciera-Coelho, 1972; Weisman-Shomer and Fry, 1975), as well as human diploid fibroblasts (Hayflick and Moorhead, 1961; Hayflick, 1965; Dell'orco et al., 1973), and untransformed cells of several other species (Simons, 1970; Goldstein, 1974; Stanley et al., 1975), have a limited ability to proliferate in vitro. The lifespan of normal human cells in culture is inversely proportional to the donor's age (Martin et al., 1970; Dell'orco et al., 1974), and is regarded to be proportional to the lifespan of the donor's species (Goldstein, 1974). Although the correlation between lifespans of species and the final doubling numbers of their cells is still debatable (Stanley et al., 1975), the finite proliferative capacity of untransformed cultured somatic cells is widely considered to be an expression of senescence at the cellular level (for recent reviews, see Cristofalo, 1972; Hayflick, 1975). In contrast to normal cells, numerous malignantly transformed cell lines are able to grow indefinitely under identical conditions of serial subcultivation. The correlation between transformation and unlimited growth potential suggests that the process of transformation and the loss of restricted growth potential may share common mechanisms. Elucidation of the mechanisms of aging of normal cells may therefore be important in the understanding of both aging and malignant transformation.

Orgel (1963, 1973) has suggested that cellular aging involves accumulation of altered proteins as a result of an inherent inaccuracy of the protein synthesizing machinery. Central to this hypothesis is the proposition that random errors, in the synthesis of proteins concerned with the replication of DNA and the transcription and translation of RNA, should have a self-amplifying effect on the rate of accumulation of other faulty proteins. Burnet (1974), has recently proposed that a major source of cellular deterioration during aging may be the accumulation of somatic mutations that are the product of "error prone" DNA repair and replication machinery. Synthesis of altered DNA polymerases as a result of either programmed or stochastic processes may, therefore, slow down the rate of DNA replication (Petes et al., 1974) and enhance the accumulation of somatic mutations (Fulder and Holliday, 1975). Although several studies have shown that enzymes concerned with intermediary metabolism are altered in aging cultured human fibroblasts, (Holliday and Tarrant, 1972; Goldstein and Singal, 1974; Goldstein and Moerman, 1975), the effects of cellular senescence on the properties of DNA polymerases have not yet been investigated. In this study, we report the separation of partially purified DNA α-polymerase from nuclei of cultured chick fibroblasts into two distinct activities that we designate DNA polymerases α(a) and α(b). We find that a number of characteristics of both α-polymerase activities are considerably altered in the senescent cells.

Materials and Methods

Materials. [^{3}H]Deoxyribonucleoside triphosphates were obtained from the Amersham-Searle Corp., (Amersham, England). Unlabeled deoxyribonucleoside triphosphates, ribonucleoside triphosphates, MalNEt,[1] DTT, and Triton N-101 were purchased from Sigma Chem. Co. (St. Louis, Mo.). Bo-

† From The Department of Clinical Biochemistry, Technion-Israel Institute of Technology, School of Medicine, Haifa, Israel. *Received March 15, 1976.*

[1] Abbreviations used are: MalNEt, *N*-ethylmaleimide; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; ATP, CTP, GTP, TTP, adenosine, cytidine, guanosine, and thymidine triphosphates.

vine pancreas deoxyribonuclease I and salmon sperm DNA were supplied by Worthington Biochemical Corp., (Freehold, N.J.). $(dT)_{12-18}$, $(dG)_{12-18}$, poly[d(A-T)], poly(dC), and poly(A) were purchased from Collaborative Research, Inc. (Waltham, Mass.). Diethylaminoethylcellulose (DE-52) and phosphocellulose (P-11) were the products of Whatman Co. (Maidston, Kent, England). Sephadex G-200 was supplied by Pharmacia Inc. (Uppsala, Sweden). Activated DNA was prepared by treatment with DNase I as described in a previous communication (Fry and Weissbach, 1973a). Annealing of the oligomers to their respective homopolymers was performed as previously described (Fridlender et al., 1972).

Media. Cells were grown in Dulbecco's Modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.). The medium (containing 10 g/l. of glucose) was supplemented with 10% calf serum, (Bio-Lab, Jerusalem, Israel) and contained 1.96 g/l. of sodium bicarbonate and 5×10^4 units and 5×10^4 μg/l. of penicillin and streptomycin, respectively. Cells were maintained at 37 °C as monolayers in Roux bottles containing 100 ml of growth medium/bottle. The solution was equilibrated in an atmosphere of 5% CO_2:95% air.

Growth of Cells. The cells used in this study were normal chick fibroblasts obtained from 10–11 day old white Leghorn embryos as described (Weisman-Shomer and Fry, 1975). Initially, the primary cells were seeded at a density of 3.0–4.0 $\times 10^5$ cells/cm^2. After 3 days growth, the confluent monolayer was disaggregated by treatment with 0.25% trypsin (Weisman-Shomer and Fry, 1975), and a secondary culture was inoculated at a density of 5.0×10^4 cells/cm^2. Routinely, the medium was changed every 2 days. Under our experimental conditions, young and old cell cultures achieved saturation densities of 5.0×10^5 cells/cm^2 and 3.0×10^5 cells/cm^2, respectively. Throughout the lifetime of the culture, cells were seeded at a density of 5.0×10^4 cells/cm^2. After reaching a density of 1.0×10^5 cells/cm^2, the fibroblasts were detached from the substratum, half the culture was reinoculated at a split ratio of 1:2, whereas the remaining cells were washed as described (Berkowitz et al., 1969), and stored at −70 °C until used for enzyme purification. Bimonthly tests for the detection of growth of contaminating bacteria and mycoplasma in nutrient and PPLO-agar (Difco) media, respectively, were negative. Also, cultures exposed to kanamycin, an antibiotic eliminating most PPLO strains, contained the same DNA polymerases described below. The lifespan of the fibroblasts in vitro was approximately 80 days and the total number of population doublings, 20–25. In a typical experiment, three distinct growth phases could be discerned; phase I (passages 1–4), during which the mean population doubling time was approximately 5 days and the culture contained varied cellular types; phase II (passages 5–15), in which the culture consisted exclusively of fibroblasts which doubled every 25–30 h; phase III (passages 16–22), during which the cells enlarged significantly, became irregular and vacuolated, and had a mean population doubling time of 80–90 h. All cultures tested exhibited the above pattern of growth and aging. No spontaneous transformation of the fibroblasts was ever observed.

Preparation of Crude Cell Extracts and of Whole Nuclei. Frozen phase II, (passages 5–9) or senescent, (passages 18–21), chick embryo fibroblasts were suspended in 20 ml of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 15 mM $MgCl_2$ per g of cells. All subsequent steps were carried out at 0–4 °C. After 30 min in the cold, 10% Nonidet P40 (Shell) was added to a final concentration of 0.5% and the cells were broken in this buffer with a Teflon–glass homogenizer. An aliquot of the resulting crude extract was stored at −70 °C for further analysis of DNA polymerase activity. Nuclei were separated from cytoplasm by centrifugation at 1000g for 10 min. The nuclear pellet was suspended in 10 volumes of 0.32 M sucrose, 1 mM $MgCl_2$, 0.3% Triton N-101, 1 mM potassium phosphate (pH 7.0). The suspension was further Dounce homogenized and the nuclei were isolated by centrifugation. The twice washed nuclei were judged, by phase contrast microscopy at a magnification of 400-fold, to be intact and free of contaminating cytoplasmic material.

Preparation of Nuclear Extracts. Whole nuclei from 1.5–3.0 g of either early or late passage fibroblasts, were suspended in 2 ml of 0.5 M NaCl, 0.5 mM DTT, 10% glycerol, 10 mM Tris-HCl (pH 8.0). The nuclei were broken with a Teflon–glass homogenizer and sonicated for 15 s at a low no. 3 amplitude in an MSE ultrasonic disintegrator. The broken nuclear extract was centrifuged at 12 000g for 30 min and the supernatant was passed through a 2-ml column of DE-52 previously equilibrated with 0.5 M NaCl, 0.5 mM DTT, 10% glycerol, 10 mM Tris-HCl (pH 8.0). Under these conditions, nuclear DNA was retained by the DEAE-cellulose, whereas 60% of the nuclear proteins and more than 95% of the DNA polymerase activity were recovered in the buffered 0.5 M NaCl wash. The nuclear extract was dialyzed for 4 h against 1000 volumes of 0.02 M potassium phosphate (pH 7.5), 0.5 mM DTT, 10% glycerol. Approximately 60% of the polymerase activity was recovered and immediately chromatographed on a DE-52 column.

DEAE-Cellulose Column Chromatography. DE-52 column chromatography of the nuclear extract was conducted as described in a previous report (Fry and Weissbach, 1973b), except that the column was equilibrated with 0.02 M potassium phosphate (pH 7.5), 0.5 mM DTT, and 10% glycerol. Elution was performed with 12 column volumes of a linear gradient from 0.02 to 0.60 M potassium phosphate (pH 7.5), containing 0.5 mM DTT and 10% glycerol. Concentration of salt in the eluted fractions was assayed by measurements of electrical conductivity.

Phosphocellulose Column Chromatography. The peak fraction of α-polymerase activity obtained from DEAE-cellulose was pooled together, diluted, and adsorbed onto a P-11 column as described (Fry and Weissbach, 1973b). The column was equilibrated with 0.02 M potassium phosphate (pH 8.0), 0.5 mM DTT, 0.2 mM EDTa, and 20% glycerol, and eluted with 14 column volumes of a linear gradient from 0.02 to 0.40 M potassium phosphate (pH 8.0), containing 0.5 mM DTT, 0.2 mM EDTA, and 20% glycerol. In some preparations of the α-polymerase activities from either young or senescent cells, EDTA was omitted from the equilibration and elution buffers. Although higher amounts of endogeneous magnesium were bound to some of the polymerases in the absence of the chelating agents, all the properties of the different polymerases described in this paper remained unaffected by the presence or absence of EDTA during the purification procedure.

Sephadex G-200 Gel Filtration of Chick Cell DNA α-Polymerases. Aliquots (100 μl) of the pooled peak tubes of either DNA polymerase α(a) or α(b) obtained from phosphocellulose chromatography (Figure 1) were diluted to 0.3 ml in 0.1 M potassium phosphate (pH 7.5), 0.5 mM dithiothreitol, and 20% glycerol, and layered on a Sephadex G-200 (1.0 × 40.0 cm), equilibrated with the same buffer. The exclusion volume was determined by blue dextran marker and three proteins were used as markers for molecular weight: aldolase (EC 4.1.2.13), bovine serum albumin, and horse myoglobin.

Assay Conditions for Chick DNA Polymerases α(a) and

α(b) Activities. Two different reaction mixtures were used for the two distinct DNA α-polymerase activities isolated from chick fibroblast nuclei (note the different pH and magnesium concentrations required by the two activities). The assay conditions described herein were found to be optimal for enzymes derived from both phase II and phase III cell cultures. Reaction mixture A, used for the assay of the activity of DNA polymerase α(a), contained in a final volume of 100 μl: 45 μg of bovine serum albumin fraction V, 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 5 mM DTT, 50 μg of activated salmon sperm DNA or synthetic primer-template at a concentration as specified, and all four deoxyribonucleoside triphosphates at 50 μM each with one ^3H-labeled deoxyribonucleoside triphosphate at a specific activity as stated or, alternatively, chosen labeled substrates as specified. Incubation was carried out at 37 °C for periods of time as indicated under the Results. Reaction mixture B, used for the assay of the activity of DNA polymerase α(b), contained in a final volume of 100 μl: 45 μg of bovine serum albumin fraction V, 50 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$, 5 mM DTT, 50 μg of activated salmon sperm DNA or synthetic primer-template at a concentration as specified, and all four deoxyribonucleoside triphosphates at 50 μM each with one ^3H-labeled deoxyribonucleoside triphosphate at a specific activity as indicated under the Results or, alternatively, labeled substrates as specified. Incubation was carried out at 37 °C for periods of time as stated.

Assay Conditions for DNA β-Polymerase Activity. DNA β-polymerase activity (named DNA polymerase II by Brun et al., 1974) was assayed as described in a previous communication (Bolden et al., 1972), with $(dT)_{12-18 \cdot poly(A)}$ serving as primer-template and [^3H]dTTP as substrate.

Assay Conditions for Deoxyribonuclease Activity which Releases Perchloric Acid Soluble Products. This assay was conducted as described in a previous report (Fry and Weissbach, 1973a). Incubation time was 6–8 h.

Assay Conditions for DNA-Dependent RNA Polymerase Activity. These were the standard *E. coli* DNA-dependent RNA polymerase assay conditions described by Hurwitz (1963).

Results

Partial Purification of Two DNA α-Polymerase Activities from Nuclei of Early and Late Passage Cultured Chick Fibroblasts. Brun et al. (1974) have purified and characterized activities of DNA α- and β-polymerases from extracts of whole chick embryos (for nomenclature of eukaryotic DNA polymerases, see Weissbach et al., 1975). The α-polymerase, which was designated DNA polymerase I by Brun et al (1974), had a molecular weight of 148 000 and copied activated DNA but not ribohomopolymers. By contrast, both nuclear and cytoplasmic β-polymerases (named DNA polymerases II by these authors) were of lower molecular weights (50 000 and 27 000, respectively), and were able to copy activated DNA as well as ribohomopolymers (see also Stravianopoulos et al., 1972; Bolden et al., 1972).

In order that the properties of nuclear DNA polymerases from young and senescent chick fibroblasts may be compared, we have partially purified α-polymerases from these cells. In the course of this purification, we have observed that the nuclear α-polymerase could be separated by phosphocellulose column chromatography into two distinct activities that we designate DNA polymerases α(a) and α(b). Nuclei of either young or senescent cultured chick fibroblasts were isolated and washed as described under Materials and Methods. Using reaction mixture A, the nuclear DNA polymerase activity was approximately 30% of the total activity in the crude cell extract. As assayed, this nuclear activity consisted of both α and β polymerases (Brun et al., 1974). This was directly demonstrated using specific assay conditions for β-polymerase (data not shown). Extraction of the disrupted nuclei with 0.5 M NaCl and dialysis of the nucleic acids-free extract, as described under Materials and Methods, yielded 60% of the polymerase activity in the soluble extracts. The dialyzed extracts were chromatographed on DEAE-cellulose columns and the collected fractions were assayed for DNA polymerase activity using reaction mixture A. As shown in Figure 1A,C, the DNA α-polymerase activity from nuclei of young and senescent cells, respectively, was eluted from DE-52 as a single broad peak with a maximum at 0.07–0.09 M potassium phosphate. Although two close peaks could be discerned in some chromatograms, their separation was incomplete, and thus all the peak fractions (tubes 15–25 Figure 1A, tubes 13–24 Figure 1C) were pooled together for further purification by phosphocellulose column chromatography. The DEAE peak fractions were devoid of DNA β-polymerase activity when assayed under conditions specific for this enzyme (see Materials and Methods). Typical elution patterns from P-11 of nuclear α polymerases from early and late passage fibroblasts are depicted in Figure 1B, D, respectively. It is evident that the single DE-52 peak of DNA α-polymerase is separated on phosphocellulose into two distinct peaks, eluting at 0.18 and 0.20 M potassium phosphate. These activities were designated nuclear polymerases α(a) and α(b), respectively. In six different preparations of the nuclear DNA α-polymerases we could not trace any significant difference between young and senescent cells in respect to either the quantitative proportions between the two polymerase activities or the salt concentrations at which these activities were eluted. We found that DNA polymerase α(a) from cells at both growth phases eluted at 0.17–0.19 M potassium phosphate and constituted 35–50% of the total nuclear α-polymerase activity, whereas DNA polymerase α(b) eluted at 0.20–0.22 M potassium phosphate and constituted 65–50% of the nuclear α-polymerase content. The phosphocellulose peaks (polymerase α(a) fractions 27–29 and polymerase α(b) fractions 31–34 in Figure 1B, and polymerase α(a) fractions 25–28 and polymerase α(b) fractions 30–33 in Figure 1D) were pooled and bovine serum albumin fraction V was added to a final concentration of 1 mg/ml in order to stabilize the enzymes that were stored routinely at −70 °C. Under these conditions all nuclear polymerases were stable for at least 90 days. All the investigations concerning the requirements and properties of nuclear DNA polymerases from early and late passage chick cells were carried out using the phosphocellulose fractions of the enzymes. Table I summarizes the results of a typical purification procedure of DNA polymerases from nuclei of young and aging cells. It is apparent from our results that the specific activities of DNA polymerases in the crude extracts and isolated nuclei of phase II and phase III fibroblasts do not differ significantly. Taking into account the relative nuclear content of the two polymerases, the data presented indicate that DNA polymerases α(a) and α(b) were purified in comparison with their activity in the crude cell extract 83- and 102-fold, respectively, in young cells and 250- and 440-fold, respectively, in late passage cells. This is a minimal estimation, since the activity measured in crude extracts consisted of both α and β polymerases. Other preparations of these enzymes yielded similar degrees of purification.

Purity and General Properties of the Phosphocellulose-Purified DNA Polymerases. Under the specific assay condi-

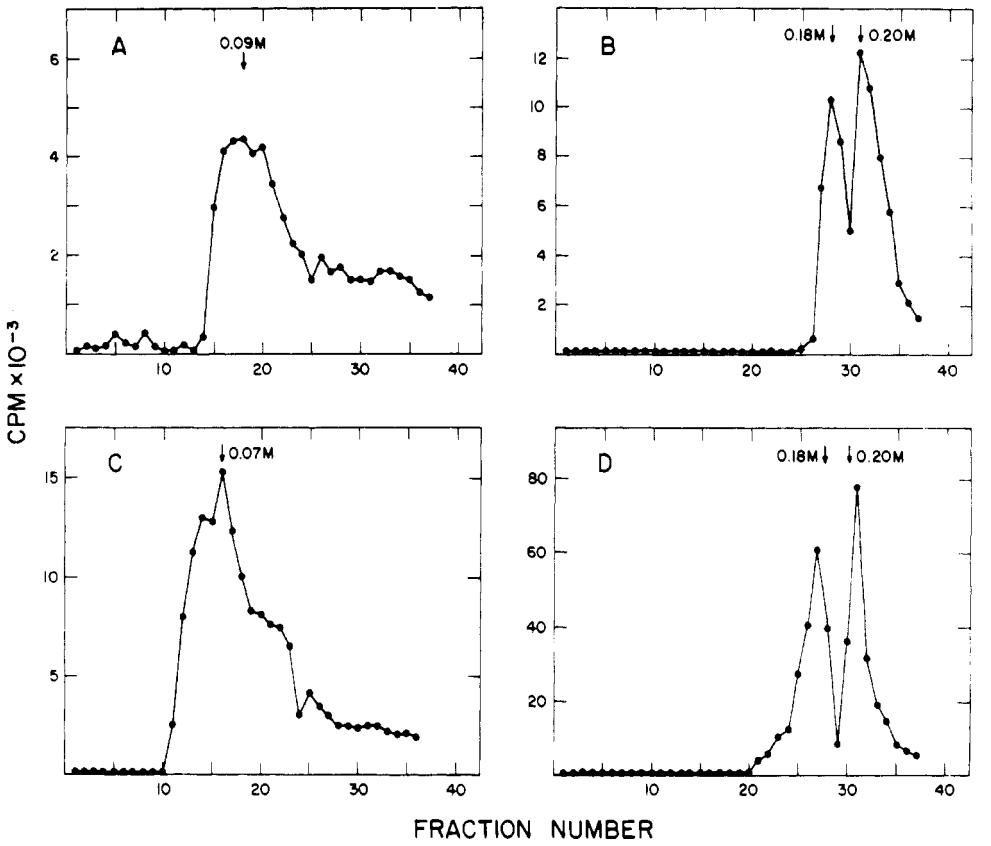

FIGURE 1: DEAE-Cellulose and phosphocellulose chromatograms of nuclear DNA polymerases derived from young or senescent cultured chick fibroblasts. A and C, DEAE-cellulose chromatograms of nuclear extracts from phase II (A) or phase III (C) cells. A 0.5 M sodium chloride extract of sonicated nuclei was prepared as described under Materials and Methods. The dialyzed extracts, containing 7.5 (A) or 13.2 mg (C) of protein, were each chromatographed on 5-ml DE-52 columns. Elution was performed as described in the text. Samples (25 μl) of each fraction (0.75 ml) were assayed for DNA polymerase activity using reaction mixture A (see Materials and Methods), activated salmon sperm DNA template and all four deoxyribonucleoside triphosphates at a specific activity of 50 cpm/pmol. The incubation time was 30 min. B and D, phosphocellulose chromatograms of the DEAE-cellulose purified DNA polymerase activities from young (B) and senescent (D) cells. The pooled peaks of the DNA polymerase activities obtained by DEAE-cellulose chromatography and containing 4.5 (B) and 9.0 mg of protein (D) were loaded onto 1- and 2-ml columns of P-11, respectively. After elution, 10 μl of each fraction (0.15 ml in B, 0.30 ml in D) were assayed for DNA polymerase activity as in A and C except that the specific activity of [^{3}H]dNTP was 50 cpm/pmol in B and 100 cpm/pmol in D. The concentrations of potassium phosphate at which each peak is eluted are indicated in the elution profiles.

TABLE I: Purification of Two DNA α Polymerases from Nuclei of Young and Senescent Chick Embryo Fibroblasts.[a]

	Phase II Fibroblasts				Phase III Fibroblasts			
Fraction	Vol (ml)	Total Protein (mg)	Total Act.[b] (units)	Sp Act. (units/mg of protein)	Vol (ml)	Total Protein (mg)	Total Act.[b] (units)	Sp Act. (units/mg of protein)
Crude cell extract	36.0	108.0	130.0	1.2	60.0	176.0	180.0	1.0
Whole nuclei	5.0	14.4	40.0	2.8	3.0	17.0	49.0	2.9
Nuclear extract	4.0	10.1	24.0	2.4	6.0	12.1	30.0	2.5
DEAE-Cellulose	6.5	3.4	37.0	10.9	7.5	2.8	64.0	23.0
DNA polymerase α(a) phosphocellulose	1.9	0.18	18.0	100.0	1.3	0.18	45.0	250.0
DNA polymerase α(b) phosphocellulose	2.7	0.26	32.0	123.0	1.6	0.18	80.0	444.4

[a] The purification procedure is described in detail under Materials and Methods. Reaction mixture A was used for the assay of DNA polymerase activity throughout the experiment. Using reaction mixture B, the activity of P-11 fraction of DNA polymerase α(b) increased by approximately 35%. [b] A unit of DNA polymerase activity is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphate into acid-insoluble form in 15 min at 37 °C.

tions for DNA polymerase α(a) or α(b) activities described under Materials and Methods, incorporation of labeled deoxyribonucleoside triphosphates into acid-soluble material was linear for at least 150 min for all the polymerases. We have not been able to demonstrate DNase activity in the phosphocellulose fractions of the various polymerases as measured by the solubilization of highly radioactive (>20 cpm/pmol), native or denatured DNA. All the phosphocellulose-purified DNA polymerases failed to incorporate ribonucleoside triphosphates with activated DNA as template using either DNA polymerase or DNA-dependent RNA polymerase standard assay conditions. That our α-polymerase preparations did not contain activities of DNA β,γ-polymerases or RNA-dependent DNA polymerase was also proven by the absence of [^{3}H]

dTMP incorporation when $(dT)_{12-18}$·poly(A) primer template was supplied and polymerase activity was monitored under conditions optimal for the activities of either α or β polymerases (see Materials and Methods). Also, we have failed to detect contaminating phosphatase activities in our preparations (Gerard et al., 1975), since the activity of the various polymerases remained unaffected by the addition of either increasing concentrations of deoxyribonucleoside triphosphates or glucose 6-phosphate to the reaction mixture.

The molecular weights of DNA polymerases α(a) and α(b) derived from phase II cells were determined by gel filtration on Sephadex G-200 column as described under Materials and Methods. Figure 2 shows the elution patterns of the two polymerase activities. Apparently, the two types of α-polymerase have identical molecular sizes: the measured molecular weight of both enzymes is 160 000, a value similar to that described by Brun et al., (1974) for the cytoplasmic chick DNA polymerase I (148 000 daltons). No activity was detected in the region of DNA β-polymerases (mol wt 50 000 and 27 000, Brun et al., 1974).

Comparison of the Enzymatic Requirements of the Early and Late Passage Chick Fibroblast Nuclear DNA α Polymerases. The requirements for maximal activity of the partially purified nuclear α polymerases of young and senescent cultured chick cells are presented in Table II. The presence of activated DNA in the reaction mixture is obligatory for both enzymes. Also, both polymerases require Mg^{2+} for maximal activity and in the absence of this cation the activities of the enzymes from early and late passage cells are reduced three- to fourfold. However, absence of magnesium in the reaction mixture and use of polymerase preparations containing 0.2 mM EDTA, do not abolish completely the activities of polymerases α(a) and α(b) from young and old cells. Moreover, a residual activity is apparent in all enzymes even in the presence of 2 mM EDTA in the reaction mixture. It is conceivable, therefore, that endogeneous magnesium is tightly bound to the α-polymerase molecules. Our results also indicate that Mn^{2+} is incapable of replacing Mg^{2+} and that the activity of both enzymes is greatly reduced in its presence under assay conditions for both DNA α and β polymerases. Also, the magnesium concentrations, as well as pH of the reaction mixture required for maximal activity of both enzymes, do not change as function of cell age. By contrast, we find that DNA polymerase α(a) from nuclei of senescent cells requires sulfhydryl groups to a greater extent than polymerase α(a) from young cells. As demonstrated in Table II, absence of DTT or presence of MalNEt reduce the activity of DNA polymerase α(a) derived from old cells, by 86 and 98%, respectively. However, the activity of polymerase α(a) of young cells decreases by only 38 and 28%, respectively, under similar conditions. In contrast, polymerase α(b) from either early or late passage cells is inhibited to a similar extent by the removal of DTT or the addition of MalNEt. Thus, DNA polymerase α(b) from nuclei of senescent fibroblasts does not differ much from that of young cells in its requirements for sulfhydryl groups.

Monovalent Ion Requirements of Nuclear DNA Polymerases α(a) and α(b) from Young and Senescent Cells. Various DNA polymerases are known to be stimulated by NH_4^+ and K^+ ions, (Keir, 1965; Furlong and Williams, 1965; Stravianopoulos et al., 1972; Fridlender et al., 1972; Bolden et al., 1972; Fry and Weissbach, 1973a; Brun et al., 1974). Data presented in Table II demonstrate that 2 mM $(NH_4)_2SO_4$ stimulates the activities of both DNA α polymerases from phase II cells, and to a lesser degree that of enzymes from nuclei of senescent fibroblasts. By contrast, we find that although polymerases α(b) from young and old cells are similarly stimulated by potassium ions, this cation stimulates the activity of DNA polymerase α(a) from phase II fibroblasts and inhibits the same enzyme from senescent cells. As shown in Figure 3, potassium chloride at concentrations of 20–75 mM markedly stimulates the activities of both nuclear polymerases from young cells. Higher concentrations of this salt are inhibitory for both enzymes. The response of the α polymerases from

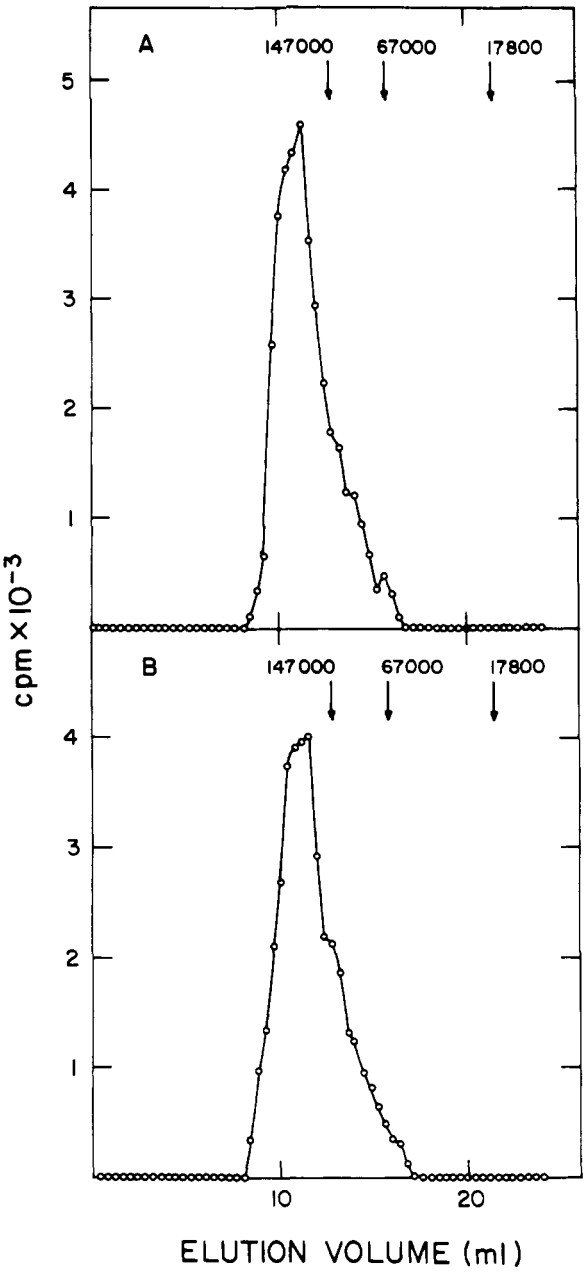

FIGURE 2: Sephadex G-200 filtration of nuclear DNA polymerases α(a) and α(b). Approximately 25 μg of protein of the phosphocellulose fraction of either polymerase α(a) or α(b) derived from nuclei of phase II cells in a final volume of 0.3 ml of 0.1 M potassium phosphate (pH 7.5), 0.5 mM dithiothreitol, 20% glycerol were layered on a Sephadex G-200 column (1.0 × 40.0 cm). The elution volume of blue dextran was 9000 μl. After elution, 50-μl aliquots of each fraction (0.4 ml) were assayed for DNA polymerase activity using the respective standard assay conditions for each enzyme. The specific activity of [^{3}H]dNTP was 100 cpm/pmol. Marker proteins of known molecular weight for calibration of the column were: aldolase (EC 4.1.2.13, mol wt. 147 000), bovine serum albumin (mol wt 67 000), and horse myoglobin (mol wt 17 800). The elution volumes of the marker proteins are indicated by arrows. A, DNA polymerase α(a); B, DNA polymerase α(b).

TABLE II: Requirements of Nuclear DNA α Polymerases from Young and Senescent Cultured Chick Cells.[a]

	Incorporation (%)			
	DNA α(a) Polymerase		DNA α(b) Polymerase	
	Phase II Cells	Phase III Cells	Phase II Cells	Phase III Cells
Complete mixture	100.0	100.0	100.0	100.0
−Activated DNA	0.0	0.0	0.0	0.0
$-Mg^{2+}$	30.0	29.3	25.0	24.3
$-Mg^{2+}$ + 2 mM EDTA	12.5	7.3	6.5	4.3
$-Mg^{2+}$ + 1 mM Mn^{2+} [b]	17.3		31.1	
−DTT[c]	61.8	14.2	26.7	35.4
−DTT + 0.05 mM MalNEt	71.5	1.7	26.0	14.0
+2 mM $(NH_4)_2SO_4$	136.5	124.0	195.0	120.0

[a] Protein; 0.5–0.7 μg of the phosphocellulose fraction of DNA polymerases α(a) and α(b) from phase II or phase III cells in medium containing 0.2 mM EDTA were assayed under their respective standard assay conditions as described under Materials and Methods. The complete reaction mixture contained activated DNA template and all four deoxyribonucleoside triphosphates at a specific activity of 50 cpm/pmol. One hundred percent incorporation was 95 and 145 pmol of dNTP incorporated in 30 min for DNA polymerase α(a) from nuclei of phase II and phase III cells, respectively, and 103 and 205 pmol of dNTP incorporated in 30 min for DNA polymerase α(b) from nuclei of phase II and phase III cells, respectively. [b] Using reaction mixture for the assay of DNA β-polymerase activity (see Materials and Methods), the activity of both enzymes was less than 5% of that obtained with reaction mixtures A or B. The optimal concentration of Mn^{2+} for the activities of both polymerases was 1 mM. [c] No exogenous dithiothreitol added. Due to the presence of 0.5 mM dithiothreitol in the enzyme solution, its final concentration in the reaction mixture was 0.025 mM.

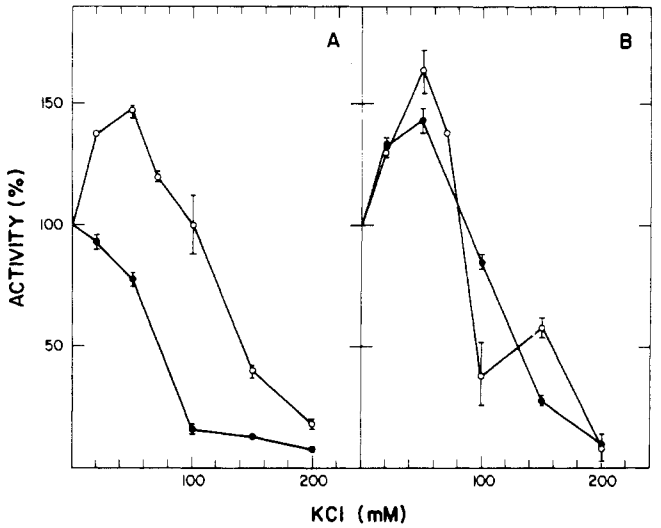

FIGURE 3: The effect of potassium chloride on the activity of nuclear DNA polymerase α(a) and α(b) from young and senescent cultured chick fibroblasts. The activity of 0.5 μg of protein of the phosphocellulose fraction of each enzyme was assayed in the presence of varying concentrations of potassium chloride using the standard assay conditions described under Materials and Methods. Activated salmon sperm DNA served as template and the specific activity of all four deoxyribonucleoside triphsphates was 50 cpm/pmol. One hundred percent activity was in the range of 40–157 pmol of dNTP incorporated in 30 min. A, DNA polymerase α(a); B, DNA polymerase α(b). (O—O) Enzyme derived from phase II cells. (●—●) Enzyme derived from phase III cells.

young cultures to potassium chloride is very similar to that described by Brun et al. (1974) for the cytoplasmic DNA polymerase I of chick cells. Whereas the response of DNA polymerase α(b) toward potassium chloride remains almost unaltered in aging cells, DNA polymerase α(a) from nuclei of senescent fibroblasts is clearly inhibited by 20–75 mM potassium chloride (Figure 3A). The modified response of senescent DNA polymerase α(a) toward K^+ ions may reflect structural or conformational changes in this enzyme, (see Discussion).

Response of Nuclear DNA Polymerases α(a) and α(b) from Early and Late Passage Cells toward Ribonucleoside Triphosphate. Most eukaryotic DNA polymerases are effectively inhibited by ATP (Fry and Weissbach, 1973a). We find, however, that although DNA polymerase α(b) from nuclei of young chick cells is inhibited 15–50% by 1–5 mM ATP, respectively (Figure 4B), DNA polymerase α(a) from the same source is stimulated by ATP at the same range of concentrations up to 150% (Figure 4A). Similar results are obtained by the addition of CTP to the reaction mixtures of DNA polymerases α(a) and α(b) from early passage cells (Figure 4C, D, respectively). The response of DNA polymerases of senescent cells toward ATP and CTP clearly differs from that of enzymes from young fibroblasts: DNA polymerase α(a) from phase III cultures is slightly inhibited—and certainly not stimulated—by 1–5 mM ATP (Figure 4A) and although this enzyme is 10% stimulated by 1–2 mM CTP, higher concentrations of this ribonucleoside triphosphate are inhibitory (Figure 4C). In contrast, DNA polymerase α(b), which is clearly inhibited by 1–5 mM ATP or CTP in early passage cells, is slightly stimulated by 1–2 mM of both ribonucleoside triphosphates and inhibited to a lesser degree by higher concentrations of these reagents (Figure 4B, D).

The observed stimulation of α polymerases by ribonucleoside triphosphates could be attributed to interaction of these substrates with contaminating phosphatase (Gerard et al., 1975). Thus, ATP and CTP could have protected the deoxyribonucleoside triphosphate substrates against the hydrolytic activity. However, as mentioned, we could not detect such phosphatase activity in any of the polymerase preparations. Also, all the α-polymerase preparations tested were devoid of DNA-dependent RNA polymerase. We tend, therefore, to interpret the stimulatory effect of ATP and CTP as directly related to the α polymerases rather than to a nonpolymerase component.

Heat Inactivation of DNA Polymerases from Nuclei of Young and Aging Cultured Fibroblasts. Several authors have described thermolabile fractions in numerous enzymes derived from senescent cultured human fibroblasts (Holliday and Tarrant, 1972) and in enzymes from cells of human subjects

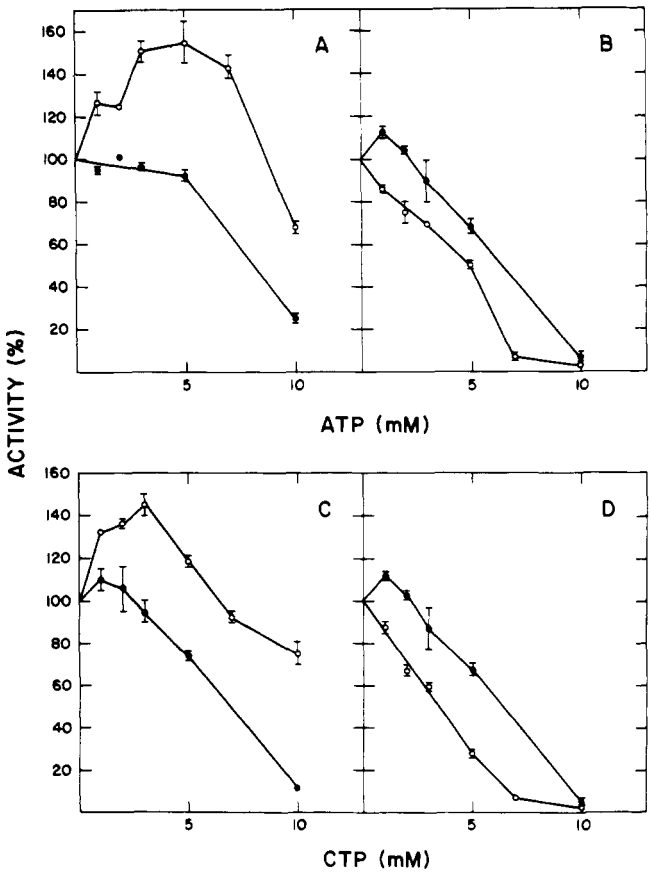

FIGURE 4: The effect of ribonucleoside triphosphate on the activity of nuclear DNA polymerases α(a) and α(b) from phase II and phase III cultured cells. The activity of 0.5 μg of protein of the phosphocellulose fraction of each enzyme was assayed in the presence of varying concentrations of ATP and CTP using the standard assay conditions as specified under Materials and Methods. Native nicked salmon sperm DNA served as template and the specific activity of all four deoxyribonucleoside triphosphates was 50 cpm/pmol. One hundred percent activity was in the range of 42–173 pmol of dNTP incorporated in 30 min. A and C, activity of DNA polymerase α(a) in the presence of varying concentrations of ATP and CTP, respectively. B and D, activity of DNA polymerase α(b) in the presence of varying concentrations of ATP and CTP, respectively. (O—O) Polymerase from young cells. (●—●) Polymerase from senescent cells.

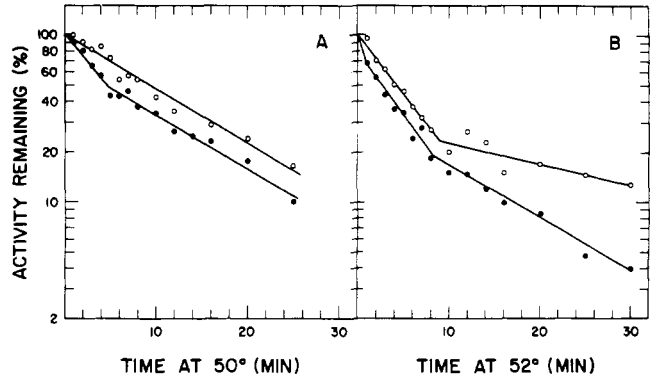

FIGURE 5: Heat inactivation of nuclear DNA polymerase α(a) and α(b) from young and senescent cultured chick fibroblasts. The phosphocellulose fractions of the enzymes were simultaneously incubated at the indicated temperatures in the presence of 2 mg/ml of bovine serum albumin fraction V. At various intervals, a sample of 0.5 μg of protein of each polymerase was removed into standard reaction mixture and assayed for DNA polymerase activity as described under Materials and Methods. Activated salmon sperm DNA served as template and the specific activity of all four deoxyribonucleoside triphosphates was 150 cpm/pmol. One hundred percent activity was in the range of 43–147 pmol of dNTP incorporated in 30 min. A, DNA polymerase α(a); B, DNA polymerase α(b). (O—O) Enzyme derived from phase II cells. (●—●) Enzyme derived from phase III cells.

with Werner's Syndrome, a hereditary disease of accelerated aging (Goldstein and Singal, 1974; Goldstein and Moerman, 1975). We provide evidence herein showing that nuclear DNA polymerases α(a) and α(b) from aging cultured chick cells contain a thermolabile fraction. As shown in Figure 5A, DNA polymerase α(a) from nuclei of young fibroblasts is inactivated at 50 °C with a first-order kinetics having a half-life of 10 min. By contrast, this enzyme derived from senescent cells possesses a thermolabile fraction that constitutes 30% of the total activity and that decays with a half-life of 90 s. DNA polymerase α(b) is inactivated very slowly at 50 °C and its pattern of heat inactivation was therefore examined at 52 °C. Figure 5B shows that this enzyme contains two fractions in early passage cells. The first part, constituting 65–70% of the total activity, decays with a half-life of 150 s, whereas the more stable fraction is inactivated with a half-life of 26 min. Both fractions of polymerase α(b) become partly or completely heat labile in senescent cells. In the first fraction, 30% of the enzyme has a half-life of less than 1 min at 52 °C. The more stable part of this polymerase becomes completely heat labile and decays with a half-life of 20 min. Interestingly, at a temperature of 48 °C and in the presence of 5 mg/ml of bovine serum albumin fraction V, both α-polymerase activities decayed very slowly and no thermolabile fractions could be discerned in enzymes from old cells. Choice of a sufficiently high temperature and relatively low concentration of stabilizing protein are therefore essential for the detection of the thermolabile fractions of these enzymes.

Template Utilization by DNA Polymerases α(a) and α(b) from Nuclei of Phase II and Phase III Fibroblasts. The relative abilities of the two nuclear DNA polymerases derived from young and senescent cells to utilize various natural and synthetic templates are compared and are shown in Table III. It is evident from the data presented that polymerase α(a) from either phase II or phase III cultures is unable to copy native or denatured DNA. Similarly, polymerase α(b) from both young and old cells copies native DNA at a very low efficiency and fails completely to utilize denatured DNA. It is also clear that both enzymes of young and aging cells are unable to utilize oligo(dG) in the absence of an added template strand. A similar result was obtained with the synthetic primer oligo(dT), (data not shown) It is therefore clear that all enzymes used were free of terminal deoxyribonucleotidyl transferase activity. All DNA polymerases examined were devoid of DNA γ,β-polymerases and RNA-dependent DNA polymerase activities, since the synthetic primer template $(dT)_{12-18}$·poly(A) was not utilized by any enzyme under assay conditions for either DNA β-polymerase or DNA α-polymerase activity. DNA polymerase α(b) from both early and late passage cells is able to utilize poly[d(A-T)] at a low efficiency. In contrast, whereas polymerase α(a) from young cells copies this template, the enzyme from old cells fails to utilize it. The generally low efficiency of utilization of poly[d(A-T)] stands in contrast to its highly efficient utilization by the previously described cytoplasmic α-polymerase (Brun et al., 1974). A more significant difference between the two polymerases and between enzymes from young and senescent cells is revealed in their relative abilities to utilize $(dG)_{12-18}$·poly(dC) as primer-template. DNA polymerase α(a) from phase II cells copies this synthetic template threefold more efficiently than DNA polymerase α(b) from the same cell culture. However, whereas the relative efficiency with which polymerase α(a) copies $(dG)_{12-18}$·po-

TABLE III: Template Utilization by Nuclear DNA α Polymerases from Young and Senescent Cultured Chick Cells.[a]

Template	^{3}H Substrate	Incorporation (%)			
		DNA Polymerase α(a)		DNA Polymerase α(b)	
		Phase II Cells	Phase III Cells	Phase II Cells	Phase III Cells
Native nicked salmon sperm DNA	dNTP[b]	100.0	100.0	100.0	100.0
Native salmon sperm DNA	dNTP	0.0	0.0	3.0	1.1
Denatured salmon sperm DNA	dNTP	0.0	0.0	0.0	0.0
$(dG)_{12-18}$	dGTP	0.0	0.0	0.0	0.0
poly[d(A-T)]	dTTP + dATP	5.1	0.0	3.0	2.2
$(dG)_{12-18}$·poly(dC)	dGTP	143.4	77.0	42.0	68.2
$(dT)_{12-18}$·poly(A)	dTTP	0.0	0.0	0.0	0.0

[a] The activities of the phosphocellulose fractions of DNA polymerases α(a) and α(b) from young or senescent cells were assayed under their respective standard assay conditions. Copying of activated DNA was taken as 100% activity. Utilizing this template, 0.5 μg of DNA polymerase α(a) from phase II or phase III cells incorporated 87 and 135 pmol of dNTP in 30 min, respectively. Similarly, 0.7 μg of DNA polymerase α(b) from phase II and phase III cells incorporated 117 and 192 pmol of dNTP in 30 min, respectively. The specific activity of all four dNTP substrates was 50 cpm/pmol and that of the single deoxyribonucleoside triphosphates was 300 cpm/pmol. All DNA templates were added at a concentration of 50 μg/100 μl of assay mixture. The concentration of the synthetic primer template was 5–10 μg/100 μl of assay mixture. Under these conditions, all enzymes were saturated by their templates. [b] dNTP refers to all four deoxyribonucleoside triphosphates.

TABLE IV: Utilization of All Four Vs. Single Deoxyribonucleoside Triphosphates by Nuclear DNA α Polymerases from Young and Senescent Chick Cells.[a]

Substrate	DNA Polymerase α(a)				DNA Polymerase α(b)			
	Phase II Cells		Phase III Cells		Phase II Cells		Phase III Cells	
	pmol	%	pmol	%	pmol	%	pmol	%
[^{3}H]dATP + dNTP	42.05	100.00	125.00	100.00	122.80	100.00	152.70	100.00
[^{3}H]dATP	1.45	3.50	4.20	3.40	3.90	3.20	2.72	1.80
[^{3}H]dTTP + dNTP	46.30	100.00	85.60	100.00	85.75	100.00	125.40	100.00
[^{3}H]dTTP	1.55	3.30	2.90	3.40	3.75	4.40	3.70	2.90
[^{3}H]dGTP + dNTP	64.00	100.00	237.70	100.00	212.30	100.00	287.50	100.00
[^{3}H]dGTP	4.00	6.90	9.40	4.00	10.95	5.20	11.00	3.80
[^{3}H]dCTP + dNTP	11.05	100.00	35.00	100.00	32.95	100.00	42.30	100.00
[^{3}H]dCTP	0.65	5.90	1.90	5.40	1.25	3.80	3.20	7.50

[a] All four polymerases (0.5 μg of protein of the phosphocellulose fraction of each enzyme) were incubated for 30 min under their respective standard assay conditions with activated salmon sperm DNA as template as described under Materials and Methods. The specific activities of the substrates were 50 cpm/pmol for the single deoxyribonucleoside triphosphates. Each determination was performed in duplicate.

ly(dC) decreases twofold in senescent cells, that of polymerase α(b) increases by more than 1.5-fold in phase III fibroblasts.

Utilization of Single Deoxyribonucleoside Triphosphates with Activated DNA as Template by DNA Polymerase α(a) and α(b) Derived from Nuclei of Young and Senescent Fibroblasts. Most eukaryotic DNA polymerases, though requiring all four deoxyribonucleoside triphosphates for maximal activity, still show significant incorporation in the presence of only 1, 2, or 3 of the nucleotides with an activated DNA template (Schlabach et al., 1971; Chang and Bollum, 1972a; Sedwick et al., 1972; Fry and Weissbach, 1973a). In contrast to the "relaxed" requirements for the four deoxyribonucleoside triphosphates of most known vertebrate DNA polymerases, *E. coli* DNA polymerase I (Fry and Weissbach, 1973b) and HeLa cell mitochondrial DNA polymerase (Fry and Weissbach, 1973a) incorporate single deoxyribonucleoside triphosphates at a significantly low rate. We have compared the relative abilities of DNA polymerases α(a) and α(b) from nuclei of young and aging chick cells to incorporate single deoxyribonucleoside triphosphates with activated DNA serving as template. It is evident from the data presented in Table IV that there is no significant difference in the ability of DNA polymerases derived from nuclei of phase II and phase III cells to utilize single nucleotides. Our results also show that polymerase α(b) from both young and old cultures and polymerase α(a) from senescent cells demonstrate similar relative efficiencies of incorporation of the various labeled deoxyribonucleoside triphosphates when all four substrates are supplied: these relative efficiencies are dGTP > dATP > dTTP > dCTP. By contrast, the relative efficiencies of incorporation of the four deoxyribonucleoside triphosphates by polymerase α(a) from young cells are dGTP > dTTP > dATP > dCTP.

Direct Measurement of the Infidelity of Nuclear DNA Polymerases from Young and Aging Cells in Polynucleotide Replication. Measurement of the rate of nucleotide misincorporation in the copying of polynucleotides by isolated DNA polymerases allows a direct measurement of the accuracy of DNA synthesis in vitro (Springgate and Loeb, 1973; Battula and Loeb, 1974). We have used this technique in order to inquire whether the copying of homopolymers by the DNA polymerases from senescent cells is less exact than that by po-

lymerases from early passage cells. In two parallel sets of experiments, polymerases α(a) and α(b) from phase II or phase III cultures were assayed under their respective reaction conditions for their ability to incorporate [^{3}H]dATP or [^{3}H]dGTP using oligo(dG)·poly(dC) as primer-template. The ratio [^{3}H]dATP/[H]dGTP incorporated was taken as the frequency of error. The lowest frequency of error that we could have measured under our experimental conditions was approximately 1:5000. Both polymerases from either young or senescent cells failed to incorporate [^{3}H]dATP into acid-insoluble material. Therefore, under our experimental conditions, we could not detect any errors in the copying of oligo(dG)·poly(dC) by polymerases derived from either young or old cultures.

Discussion

In this communication we describe the isolation of α-polymerase activity from nuclei of young and senescent cultured chick fibroblasts and its separation on phosphocellulose columns into two distinct activities that we name nuclear DNA polymerases α(a) and α(b). Generally, the two nuclear α polymerases resemble the previously described cytoplasmic avian α-polymerase (Brun et al., 1974). However, in several respects, such as the pH and concentrations of magnesium required for maximal activity of the two enzymes, their degree of susceptibility to oxidation of sulfhydryl groups and relative abilities to copy synthetic templates, polymerases α(a) and α(b) differ from each other, as well as from the chick cytoplasmic DNA polymerase.

Both nuclear DNA polymerases are classified as α activities by virtue of their high molecular weight, (160 000), their inhibition by MalNEt, their inability to copy oligo(dT)·poly(A), and the apparent absence of nuclease activity in the polymerase preparations (Weissbach et al., 1975). These enzymes differ in their chromatographic behavior on phosphocellulose columns (Figure 1), they respond differently to ribonucleoside triphosphates (Figure 4), and copy oligo(dG)·poly(dC) at different relative efficiencies (Table III). Also, the two activities differ in their patterns of heat inactivation which demonstrate that whereas polymerase α(a) from young cells is homogenous, polymerase α(b) from these cells consists of two different fractions (Figure 5). We do not know, therefore, at the present stage, what kinship exists between these two enzymes. A possible way to investigate the interrelationship between the two nuclear α polymerases would be to analyze their subunit structure or to study their antigenic relation as reported for the multiple mammalian DNA polymerases (Chang and Bollum, 1972b; Spadari and Weissbach, 1974; Mordoh and Fridlender, 1975).

The central observation of this study is that nuclear DNA polymerases α(a) and α(b) are altered in senescent cultured chick fibroblasts. Our results show that some of the requirements for maximal activity of both enzymes as well as several of their physical and catalytic properties change in senescent cells. A conspicuous alteration in the two polymerases is the increased heat lability of enzymes from late passage cells (Figure 5). It has been shown that wholly or partly thermolabile enzymes appear in tissues of aging mouse (Wulf and Cutler, 1975), in senescent cultures of *Neurospora* (Lewis and Holliday, 1970), in aging human diploid cell cultures (Holliday and Tarrant, 1972), and in genetically determined prematurely aging cultured human cells (Goldstein and Singal, 1974). The increased heat lability of enzymes from senescent cells has been interpreted as reflecting the accumulation of random amino acid substitutions in these proteins (Holliday and Tarrant, 1972). We cannot exclude, however, other possibilities such as age-related posttranslational modifications of the polymerases or appearance of new heat-labile isoenzymes of DNA polymerases in aging cells. Another remarkable difference between DNA polymerases from early and late passage cultures is their different response to ribonucleoside triphosphates (Figure 3). Whereas DNA polymerase α(a) from phase II cells is stimulated by ATP or CTP, it is clearly inhibited by these nucleotides in senescent cells. The opposite situation prevails for DNA polymerase α(b); enzyme from young cells is inhibited by ATP or CTP, whereas polymerase α(b) from aging fibroblasts is slightly stimulated by these reagents.

The modified response of α polymerases from senescent cells toward ribonucleoside triphosphates can be interpreted in several says. First, ATP and CTP might exert their effect on the various polymerases through interaction with varying amounts of nonpolymerase contaminants present in the enzyme preparations. Due to the limited purification of the nuclear DNA polymerases, such possibility cannot be ruled out. However, we have failed to detect activities of phosphatase, DNA-dependent RNA polymerase, and DNase in the polymerase fractions. The data presented also show that ATP and CTP stimulate polymerase α(a) from young cells to a much higher degree than both polymerases α(a) and α(b) from senescent fibroblasts. Thus, the possibility that, as a result of an insufficient resolution between the two polymerases, the various enzyme preparations consisted of different mixtures of the two activities, cannot satisfactorily explain our results. Although the observed inhibitory effects of ATP and CTP could be attributed to their ability to chelate magnesium ions, it is improbable that these ribonucleoside triphosphates stimulate the α polymerases through chelation, since all assays were performed in the presence of optimal concentrations of magnesium. Lastly, assuming a direct interaction between ribonucleoside triphosphates, which are structurally similar to deoxyribonucleoside triphosphates and the α polymerases, the age-related altered response of enzymes from senescent cells to these reagents may reflect structural or conformational changes in the enzymic protein. Changes of this type can also explain other altered requirements and properties of DNA polymerases from old cells such as the modified response of DNA polymerase α(a) to blocking of sulfhydryl groups and to addition of potassium chloride (Table II and Figure 3, respectively).

Several investigators have reported considerable changes in proteins from aging animals or cultured cells. The presence of several catalytically inactive but antigenically reactive enzymatic proteins in tissues of aging animals of various species has been reported (Gershon and Gershon, 1970; Zeelon et al., 1973). In addition, the appearance of heat-labile enzymes (Holliday and Tarrant, 1972; Goldstein and Moerman, 1975) and loss of reactivity of HL-A antigens in senescent cultured human fibroblasts (Goldstein and Singal, 1972, 1974) have been demonstrated. Although some of the cited data are still debatable (Brautbar et al., 1972; Pendergrass et al., 1976), it appears that numerous proteins are altered in aging cells. We have presented in this paper data suggesting that nuclear DNA polymerases are altered in chick fibroblasts aging in vitro. Structural or conformational alterations in these vital enzymes could cause the experimentally observed decreased rates of DNA replication and repair and the accumulation of somatic mutations in senescent cultured cells, (Epstein et al., 1974; Petes et al., 1974; Fulder and Holliday, 1975). We have attempted to assess in vitro the relative catalytic activities of DNA polymerases from early and late passage fibroblasts.

Although there is some difference in the relative efficiencies with which both partially purified enzymes from young or senescent cells copy the synthetic templates poly[d(A-T)] and oligo(dG)·poly(dC), we could find no difference in the specific activities of the enzymes in the crude cell extracts of young and old cultures, neither could we detect a significant difference in the abilities of the various polymerases to utilize single deoxyribonucleoside triphosphates with activated DNA serving as template. We have also failed in detecting altered fidelity of polynucleotide replication by α polymerases from senescent cultures. However, our measurements of the frequency of error in the copying of oligo(dG)·poly(dC) were of low sensitivity and additional and more accurate experiments should be conducted.

Lastly, it should be remarked that, although the molecular basis for the observed age-related alterations in the avian nuclear DNA α polymerases is not clear, several possible explanations for this phenomenon can be offered. A trivial interpretation would assume that changes in the amount of non-polymerase contaminants that affect the polymerase activity are responsible for the described alterations. Such assumption can hardly explain the complex nature of the changing response of both polymerases to ribonucleoside triphosphates, monovalent ions, blocking of sulfhydryl groups, and elevated temperature. Another possibility is that the modified properties of both polymerases might reflect alterations of the enzymatic proteins as recently described for DNA α-polymerase from acute lymphoblastic leukemia cells (Mordoh and Fridlender, 1975). It could be that such enzymatic alterations stem from the accumulation of random translational errors as originally suggested by Orgel (1963, 1973), or are the result of age-related posttranslational modifications such as protein cleavage, phosphorylation, carbamylation, acetylation, etc. A third possibility is that the DNA polymerases of senescent cells are new isoenzymes and are the product of genes distinctly different from those coding for DNA polymerases of young cells. We are now in the process of examining these various possibilities.

Acknowledgments

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Synthesis and Properties of Poly(O^6-methylguanylic acid) and Poly(O^6-ethylguanylic acid)†

Jitendra R. Mehta and David B. Ludlum*,‡

ABSTRACT: The nucleotide analogues, O^6-methyl- and O^6-ethylguanosine diphosphate, have been synthesized and polymerized to high-molecular-weight homopolymers with polynucleotide phosphorylase. The ultraviolet spectra of these polymers show marked hypochromicity, which suggests that they possess considerable secondary structures. Graphs of optical density vs. temperature in 0.15 M NaCl indicate that cooperative melting occurs for both polymers, and that the secondary structure of poly(O^6-methylguanosine monophosphate) is somewhat more stable than that of poly(O^6-ethylguanosine monophosphate). Mixing experiments show that these analogue polymers no longer form helical structures with poly(C), nor do they form helices with poly(U). We would conclude from these data that environmental mutagens and carcinogens which react at the O^6 position of guanine not only disrupt normal base-pairing relationships, but may also affect the secondary structure of nucleic acids.

Many environmental mutagens and chemical carcinogens are known to react covalently with nucleic acids. Individual bases are modified and, in some cases, this leads to an alteration in the informational content of the nucleic acid that contains them. Evidence for these effects has been obtained previously in model template systems (Ludlum and Wilhelm, 1968; Singer and Fraenkel-Conrat, 1970; Ludlum, 1970; Gerchman and Ludlum, 1975; Ludlum, 1975).

It is apparent that these base changes could be causally related to the carcinogenic process. As an essential step in establishing this link, the molecular effects of each base modification must be elucidated. In most cases, the sites of base substitutions have been identified and good reviews of this work have been published recently by several authors (Sarma et al., 1975; Singer, 1975; Lawley, 1972). Current evidence seems to indicate that attack on the O^6 position of guanine is a particularly important molecular event (Loveless, 1969).

A base modification such as the O^6 alkylation of guanine could produce a change in the informational content of a nucleic acid in at least two ways. The specific base-pairing properties of the nucleotide could be altered, or the secondary structure of the macromolecule itself could be changed.

In order to investigate which of these mechanisms might be operating in the case of O^6-alkylguanines, we have synthesized and studied the physical properties of poly(O^6-methyl-) and poly(O^6-ethylguanylic acid). The synthesis of poly(O^6-methylguanylic acid) has been described briefly in a previous communication (Gerchman et al., 1972), and the properties of this base have also been investigated in templates for RNA polymerase (Gerchman and Ludlum, 1973). These studies have shown that the presence of O^6-methyl-GMP[1] in a template leads to misincorporation, and the investigations reported here are designed to help explain the mechanism by which this occurs.

Experimental Section

Synthesis of Monomers. O^6-Methylguanosine was prepared from 6-chloro-2-aminopurine riboside (Waldhof, West Germany) as described previously (Gerchman et al., 1972). Following our earlier procedure, the nucleoside was phosphorylated to m^6-GMP with carrot phosphotransferase. Then m^6-GDP was synthesized by converting the monophosphate to the imidazolidate and displacing the imidazole groups with inorganic phosphate (Gerchman et al., 1972). The corresponding ethyl compounds were synthesized in an analogous fashion from 6-chloro-2-aminopurine riboside and sodium ethoxide as starting materials. All derivatives were chromatographically pure in the systems described below.

Chromatography and Paper Electrophoresis. Paper chromatography was carried out on Whatman no. 1 paper in the following solvent systems: (A) methanol–concentrated HCl–water (8/1/1, v/v); (B) isobutyric acid–concentrated ammonium hydroxide–water (66/1/33, v/v); (C) isopropyl alcohol–concentrated ammonium hydroxide–water (7/1/2,

† From the Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201. *Received November 20, 1975.* This work was supported by Research Grants CA 15696 and CA 16299 from the National Institutes of Health, United States Public Health Service and by American Cancer Society Grants MD 74-19 and NP-74 B.

‡ Present address: Department of Pharmacology and Experimental Therapeutics, Albany Medical College, Albany, N.Y. 12208.

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; GMP, GDP, guanosine mono- and diphosphates; m^6-GMP, e^6-GMP, 6-methyl- and 6-ethylguanosine monophosphates.