IN VITRO STUDIES OF THE SYNTHESIS OF MITOCHONDRIAL DEOXYRIBONUCLEIC ACID

Thesis by

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To Linda

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ABSTRACT

A DNA polymerase has been partially purified from the mitochondria of HeLa cells. Properties, including low levels of nuclease activity and a preference for native duplex DNA templates, were favorable for the study of *in vitro* DNA synthesis using circular duplex DNA templates. Initiation of DNA synthesis occurs predominately at single-strand scissions with covalent addition of nucleotides to the priming template strand. Centrifugation and electron microscopy have established that the template DNA strand ahead of the growing point is displaced rather than degraded. Hairpin structures are not formed in the course of DNA synthesis on duplex DNA templates. Studies with HeLa cell mitochondrial DNA template have indicated base composition-complement fidelity of the product of DNA synthesis as well as a preferential synthesis of DNA corresponding to the denser complement (in CsCl solution) of the template. This asymmetric synthesis of mitochondrial DNA appears to arise from a bias in the number of single-strand scissions sustained by the complementary strands.

DNA synthesis in isolated HeLa cell mitochondria has also been investigated. Product DNA appears in closed and nicked circular mitochondrial DNA. Preferential synthesis of DNA corresponding to the less dense complement (in CsCl solution) was observed.

The mode of action of the partially purified mitochondrial DNA polymerase and the asymmetric synthesis of mitochondrial DNA, observed *in vitro* and *in situ*, are discussed with regard to the results of recent studies of the replication of mitochondrial DNA *in vivo*.

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CHAPTER I

Properties and Mode of Action of a Partially Purified DNA Polymerase from the Mitochondria of HeLa Cells

(Introductory Statement)

Circular species of DNA have been identified as a genetic component in many systems, including the mitochondria of animal cells. Mitochondria isolated from several animal cells and tissues have been shown to incorporate deoxyribonucleotides into mitochondrial DNA in situ (1-8). Kalf and Ch'ih (9) and Meyer and Simpson (10) described the isolation and partial purification from rat liver mitochondria of a DNA polymerase with fractionation and reaction properties that differ from DNA polymerase activity associated with the cell nucleus. The mitochondria of animal cells are thus a source of DNA polymerase(s) which operate on circular duplex DNA in vivo.

Current understanding of in vivo DNA replication at the level of enzyme action is largely speculative. In vitro studies of DNA polymerase activity from animal cell mitochondria were carried out with the object of extending our understanding of the process of circular DNA replication. We have isolated and partially purified a DNA polymerase-containing fraction from sonic extracts of HeLa cell mitochondria and have investigated the mechanism of DNA synthesis on circular DNA templates.

EXPERIMENTAL PROCEDURE

Materials

Nucleic Acids and Precursors—Unlabeled nucleotides were purchased from Sigma. 5-Bromodeoxycytidine 5'-triphosphate and 5-bromodeoxyuridine 5'-triphosphate were prepared as described in "Methods." ^{3}H-dTTP was obtained from Schwarz BioResearch and New England Nuclear. Other ^{3}H-labeled nucleotides were obtained from Schwarz. Labeled and unlabeled nucleotide solutions were mixed to give samples with lower specific activity and higher concentration. The mixtures were analyzed by Dowex 1 chromatography (0.2 ml of approximately 1 mM nucleotide applied to 0.3 × 3.0-cm column; 60 ml linear gradient elution, from 10 mM Tris, pH 7.5, to 0.2 M HCl, 0.5 M LiCl) to determine the resultant specific activity and assess the purity of the nucleotide preparation. In each case, more than 85% of the applied radioactivity and optical density at the wavelength of the nucleotide's maximum absorbance were recovered as the deoxynucleoside triphosphate with constant specific activity across the peak.

Native calf thymus DNA was purchased from Sigma. T7 phage DNA was a gift of Dr. R. W. Hyman. SV40 viral DNA and SV40 intracellular DNA were prepared in the manner of Rush et al. (11). HeLa cell closed circular mitochondrial DNA was extracted from mitochondria prepared in the manner described in "Methods," omitting, however, the two buoyant sucrose gradients. ^{32}P-labeled SV40 circular DNA was a gift of Dr. R. Eason. Further samples were prepared as needed

according to Rush et al. (11). ^{14}C-thymidine-labeled ϕX174 single-stranded circular DNA was extracted from a phage preparation supplied by R. Benbow.

DNA preparations were treated with 20 mg per ml SDS[1] at room temperature for 10 min prior to banding in a CsCl equilibrium density gradient. The gradients for the isolation of closed circular DNA contained ethidium bromide (12). The dye was removed with Dowex 50 (0.3 × 3.0-cm column) in the presence of 4 M NaCl or by extraction with isoamyl alcohol. DNA samples were then dialyzed exhaustively against 10 mM Tris, 1 mM EDTA, pH 7.5. The integrity of the DNA samples was checked by analytical band velocity centrifugation.

Enzymes—Escherichia coli exonucleases I and III were stored frozen at 15,000 units per ml and 490,000 units per ml, respectively. The preparations were gifts of D. Brutlag, Stanford University.

Other Materials—Dowex 1×8, Dowex AW50×8, and BioGel P-150 were purchased from BioRad. CsCl was purchased from Harshaw Chemical Company. Bovine serum albumin, fraction V (crystalline), was from Miles Laboratories. Ethidium bromide was donated by Boots Pure Drug Co. Ltd., Nottingham, England.

Methods

Chromatography—Ion exchange resins were washed with 0.5 M NaOH, 0.5 M HCl, 0.1 M EDTA, 4 M NaCl, and then washed repeatedly with glass-distilled water to pH equivalence. Washed resins were stored in 4 M NaCl. After packing, at least 20 bed volumes were eluted from columns before application of a sample. BioGel P-150, 100 to 200 mesh,

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

was prepared by swelling in 10 mM Tris, 10 mM β-mercaptoethanol, pH 7.5, at least 8 hours before packing. One bed volume of elution buffer was passed through the column before application of the sample. The bed volume of the gel filtration columns were 100 to 200 ml and sample volume was less than 5 ml.

Spectrophotometry—Absorption spectra were recorded using a Cary Model 14 spectrophotometer. Optical density was measured as the difference in absorbance of the sample and blank solutions determined separately in the same quartz cell with an open reference beam path.

Protein concentration was assessed by absorbance at 280 nm and 260 nm using the method of Warburg and Christian (13). DNA concentration was measured at 260 nm using a value of 1.00 absorbance unit for a solution of 50 μg per ml of native duplex DNA.

Fluorescence measurements of ethidium bromide solutions were performed with quartz cells in a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation and emission wavelengths were 380 nm and 590 nm. Slit widths were varied according to the needs of each experiment. A 300 to 400 nm bandpass filter was placed between the exit slit of the excitation monochromator and the sample cell. A 430 nm cutoff filter was placed between the sample cell and the entrance of the emission monochromator. Measurements were recorded in the ratio mode to correct for fluctuations in lamp intensity.

Centrifugation—Preparative ultracentrifugation studies were performed in Beckman Model L or L2-65B centrifuges with SW 50L or SW 50.1 rotors. The CsCl self-generating density gradient system was

used for band sedimentation analysis. Samples (less than 0.2 ml) were layered through Bayol onto at least 3.0 ml of 1.4 g per ml CsCl solution prior to centrifugation at 35,000 rpm. Gradients for alkaline sedimentation velocity analysis contained 0.1 M KOH. Buoyant equilibrium CsCl density gradients were run at 35,000 rpm, 20 to 25°, for at least 36 hours. Initial solution density was 1.70 g per ml for neutral CsCl gradients, 1.74 g per ml for alkaline CsCl gradients, and 1.56 g per ml for neutral CsCl gradients containing ethidium bromide (250 μg per ml). Alkaline buoyant gradients contained 50 mM K_3PO_4 from a 0.25 M solution of K_2HPO_4 adjusted to pH 12.5 by addition of KOH. Cellulose nitrate and polyallomer tubes were used for centrifugation at neutral and alkaline pH, respectively. Velocity and buoyant gradients were punctured at the bottom of the tubes and 10-drop (about 100 μl) fractions were collected for analysis.

Liquid Scintillation Counting—Samples were applied to Whatman 3MM filter papers. Thoroughly dried filters were placed into plastic scintillation vials and covered with toluene-based cocktail containing 5.0 g per liter of 2,5-diphenyloxazole and 0.10 g per liter of 1,4-bis-2-(4-dimethyl-5-phenyloxazolyl)-benzene. The volume added was 5 ml for counting ^3H, 10 ml for ^3H and/or ^{14}C, and 15 ml for ^3H and/or ^{32}P. Counting efficiency and channel spillover in the Packard Tri-Carb liquid scintillation spectrometer were determined with standards applied to paper filters. Double-label experiments were analyzed using the ^3H channel exclusion method.

Electron Microscopy—Specimens were prepared using the formamide modification of the Kleinschmidt procedure (14). The DNA samples

were first dialyzed against 50% formamide, 100 mM Tris, 10 mM EDTA (pH 8.5). Cytochrome c was added to 50 μg per ml prior to casting on a hypophase containing 17% formamide, 10 mM Tris, 1 mM EDTA (pH 8.5). Grids were shadowed on a rotating platform 8 degrees below and 5 cm from the point of evaporation of a Pt-Pd wire. Electron micrographs were recorded on 35-mm film using the Philips 300 electron microscope. The aid and advice of Dr. P. A. Sharp and Dr. D. L. Robberson are gratefully acknowledged.

Preparation of Brominated Nucleotides—5-Bromodeoxyuridine 5'-triphosphate was prepared as described by Bessman et al. (15), by bromination of dCTP in formamide and subsequent deamination of the 5-bromodeoxycytidine 5'-triphosphate with nitrous acid. The deoxynucleoside triphosphates were isolated from reaction mixtures by adding $BaBr_2$ to 0.25 M and precipitating with ethanol. The precipitates were washed in ethanol, dried in an airstream, and resuspended in 10 mM Tris (pH 7.5). The suspended nucleotides were metasthesized to soluble potassium salts with Dowex 50. Ultraviolet absorption spectra and Dowex 1 chromatography were used to check the quality of the preparations.

Tissue Culture—HeLa cells were maintained in suspension culture using the Dulbecco modification of Eagle's phosphate medium supplemented with 5% calf serum. Spinners were innoculated at 3×10^4 cells per ml, 72 hours prior to the harvest of cells for the preparation of mitochondria. BSC-1 cells were maintained on plastic dishes and SV40 infections were carried out as described by Rush et al. (11).

Preparation of HeLa Cell Mitochondria—At the time of harvest the concentration of cells was 3 to 4×10^5 cells per ml. Cells were recovered from suspension by centrifugation in batches for 3 min each at $1400 \times g$ in the International PR-6, and in the latter part of the work at $3000 \times g$ in the Sorvall RC2 using the Szent-Gyorgyi and Blum continuous flow system. A preparation of 20-liter suspension can be processed by continuous flow in less than one hour with a normal yield of 30 to 40 ml packed cells. Cell pellets were resuspended in 150 ml TD (137 mM NaCl, 5.8 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris, pH 7.5). Examination in the phase contrast microscope did not reveal fragmentation of the cells at the higher fields used in the continuous flow harvesting operation.

Operations following cell harvest were performed at 4°. Cells were pelleted in four 50-ml conical centrifuge tubes for 5 min at $900 \times g$ in the International PR-6. After removing the supernatant, RSB (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.5) was added to 30 ml in each tube and the cells were gently resuspended with a wide bore pipette. Cells were allowed to swell in this hypotonic buffer for about 30 min. Each batch was homogenized using two strokes of the 40-ml glass Dounce homogenizer (Kontes) with tight-fitting pestle. One-third volume of 1.1 M sucrose, 10 mM Tris, 1 mM EDTA (pH 7.5) was added to the homogenate followed by an additional stroke of the homogenizer for mixing. Phase contrast microscopy confirmed greater than 90% disruption. The free nuclei appeared intact. Cells, nuclei, and large debris were removed by centrifugation three times for 5 min at $2000 \times g$, discarding the pellets each time. The resulting supernatant was layered onto

1.5 M sucrose, 10 mM Tris, 1 mM EDTA (pH 7.5) in six tubes containing 25 ml and 10 ml of the supernatant and 1.5 M sucrose stages, respectively. Centrifugation followed for 30 min in the SW 27 rotor at 25,000 rpm. Mitochondria were recovered from the interface and resuspended in 90 ml of MS buffer (210 mM mannitol, 70 mM sucrose, 10 mM Tris, 1 mM EDTA, pH 7.5). The mitochondrial sample in MS was layered onto six preformed, two-step gradients containing 10 ml of 1.1 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.5, over 10 ml of 1.5 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.5. Centrifugation followed for 30 min at 25,000 rpm as before. Mitochondria were recovered at the interface between the 1.1 and 1.5 M sucrose solutions, resuspended in MS, and pelleted for 15 min at 12,000 rpm in the Sorvall RC2-B with an SS-34 rotor. The mitochondrial pellet was resuspended in 2% SDS, 0.1 M EDTA, pH 7.5, for extraction of mitochondrial DNA, or 10 mM Tris, 10 mM β-mercaptoethanol, pH 7.5, for extraction of soluble mitochondrial proteins by sonication.

Enzyme Assays—DNA polymerase was assayed using a modification of the procedure described by Bollum (16) based on the incorporation of labeled deoxynucleoside triphosphates into acid-precipitable material retained on paper filters through a batch washing procedure. A typical reaction mixture contained 10 mM Tris (pH 7.5), 3.3 mM β-mercaptoethanol, 33 mM NaCl, 3.3 mM $MgCl_2$, 0.33 mM EDTA, 170 μM dGTP, 155 μM dCTP, 310 μM dTTP, 84 μM ^{3}H-dATP (specific activity 250 Ci per mole), 20 μg per ml of native calf thymus DNA, and enzyme protein in the range of 50 to 200 μg per ml. Prior to incubation, materials were kept at 4°. After zero, 30, and 60 min at 37°, 50-μl aliquots were withdrawn from the incubation mixture,

with the same micropipette, onto two numbered paper filters, one of which was immediately placed in a beaker containing 500 ml of cold 5% TCA, 1% sodium pyrophosphate. Thirty min after the addition of the last filter to the bath, the filters were washed with cold 5% TCA three times for 20 min and then with 95% ethanol, followed by ethyl ether and drying. Incorporation of ^{3}H-dATP was assessed in two ways. The ^{3}H-cpm retained on the washed filter divided by the specific activity of the ^{3}H-dATP was the amount of dA (nmoles) in the 50-μl aliquot rendered insoluble by action of the enzyme system. The ratio of ^{3}H-cpm on the washed and unwashed filters corresponds to the fraction of dA in the reaction which is acid-precipitable. This fraction and the initial concentration of dATP were used to calculate the total amount of dA polymerized by the enzyme system. The two methods gave the same result. Linear incorporation kinetics (Fig. 4 and Fig. 5) justified the calculation of the rate of incorporation from a single determination of the amount of nucleotide rendered acid-precipitable.

One unit of DNA polymerase activity is defined as the conversion of 1 nmole of labeled nucleotide into acid-precipitable material after 60 min at 37°. Specific activity of enzyme preparations is expressed as units per mg of protein.

Levels of deoxyribonuclease activity were so low that the Kunitz assay (17) and attempts to demonstrate the solubilization of radioactive-labeled, acid-precipitable DNA samples by the action of the enzyme system were not satisfactory. A more sensitive endonuclease assay developed by Lehman and Paoletti (18) and based on the conversion of closed circular DNA to nicked circular DNA (or to linear DNA) was used. On binding to DNA, the fluorescence of ethidium bromide is enhanced. Endonuclease action relieves a restriction on the amount of dye which can bind to closed circular DNA. The increase in fluorescence of an

ethidium bromide solution containing samples of initially closed circular DNA can be related to the extent of endonuclease action. Equal aliquots were taken at 5-min intervals from a reaction mixture containing initially closed circular SV40 DNA, and diluted 25-fold in 1 mM EDTA, 1 μg per ml ethidium bromide, pH 7.5. The fluorescence enhancement, E, is the measured increase in the fluorescence of the ethidium bromide solution after adding the sample. The fraction of closed circular molecules surviving in a given sample was calculated from the observed fluorescence enhancement using the linear relation

$$\text{Fraction SV40-I} = \frac{E_{II} - E}{E_{II} - E_{I}}$$

where E_I and E_{II} are the fluorescence enhancements of solutions of SV40-I and SV40-II, respectively, at the same concentrations as the DNA in the sample used to measure E.

RESULTS

Extraction and Partial Purification of the Mitochondrial DNA Polymerase—Freshly prepared HeLa cell mitochondria were suspended in 3 to 5 ml of 10 mM Tris, 10 mM β-mercaptoethanol (pH 7.5) and subjected to vigorous sonication. The tip of the horn of the Model S-125 Branson sonifier was immersed directly into the suspension during three 20-second bursts at level six. The 12-ml conical test tube was chilled in ice after each burst. The turbid suspension clarified. Following sonication, addition of NaCl to 1 M and extraction for up to 12 hours did not increase the yield of protein or DNA polymerase activity. Addition of salts prior to sonication greatly interfered with the extraction.

The sonicate was then centrifuged in polycarbonate tubes at 40,000 rpm for 1 hour in the Type 65 fixed angle rotor. Most of the clear, pale yellow supernatant was carefully withdrawn from the top of the tube with a pasteur pipette. The last 0.5 to 1.0 ml of fluid gave rise to visible schlieren effects when drawn into the pipette. This material and the clear gelatinous pellet were routinely discarded. Earlier experiments in which the lower phase was included in the crude extract yielded final preparations of DNA polymerase with similar levels of activity, but with significantly lower ratios of A_{280}/A_{260}, suggesting greater nucleic acid contamination.

The crude extract, designated fraction CE, was then applied to a BioGel P-150 column for gel filtration, eluting with 10 mM Tris, 10 mM β-mercaptoethanol (pH 7.5). Elution of the sample was monitored by absorbance at 280 nm. DNA polymerase can be located by assay of the

eluted fractions, but the protein is dilute at this stage and the levels of incorporation of labeled nucleotides are low. The procedure now employed is to postpone the assay until each of the fractions in which the enzyme is routinely recovered has been individually subjected to the fractional ammonium sulfate precipitation and concentration step described next. Fig. 1 shows a typical elution profile from the BioGel P-150 column and the location of DNA polymerase activity in the fractions following elution of the void volume.

Selected column fractions were raised to 35% saturated ammonium sulfate by the addition of 0.54 ml of saturated ammonium sulfate per ml of sample. After 30 min at 4° the precipitated material was removed by centrifugation at 40,000 rpm, 30 min, in the Type 65 rotor. The supernatant was raised to 50% saturated ammonium sulfate by the addition of 0.30 ml saturated ammonium sulfate per ml of sample, chilled 30 min at 4°, and the precipitate collected as before. The precipitated material was carefully resuspended in 0.5 ml of 10 mM Tris, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.5.

A typical preparation from 20 liters of suspension culture yielded 40 ml of packed cells, 1 ml of wet, purified mitochondria, about 10 mg of protein in the crude extract, and 0.5 mg of protein in fraction AS representing the pooled fractions containing DNA polymerase activity. The time required for such a preparation is about 14 hours. The DNA polymerase activity in fraction AS decays to about 50% in one week at 4°. Reactions described in the text were performed within 48 hours of a preparation of fraction AS.

An increase in the total DNA polymerase activity was observed at each step in the partial purification, presumably due to the removal of

FIG. 1. Gel filtration of supernatant fraction following centrifugation of mitochondrial sonicate. The sample (4 ml) was applied to a BioGel P-150 column having a bed volume of 170 ml. The column was pre-equilibrated with 10 mM Tris, 10 mM β-mercaptoethanol, pH 7.5, the buffer used for elution. All column operations were performed at ambient room temperature. Fractions (4.4 ml each) were collected and chilled to 4°. Elution of protein was monitored by absorbance at 280 nm (○- - -○). Aliquots from fractions 10 through 17 were assayed for DNA polymerase activity (●——●) as described in "Methods," except that the radioactive label was 6 μM ^{3}H-dTTP (7 Ci per mmole) and dATP was present at 175 μM. The DNA polymerase activity is represented by the radioactivity rendered acid-precipitable after incubation for 30 min at 37°.

^{3}H CPM x 10^{-3}
Absorbance at 280 mμ
Fraction Number
1
2
3
10
20
30
40

materials inhibiting DNA synthesis. Attempts to demonstrate DNA polymerase activity in discarded fractions were unsuccessful.

Reaction Requirements—The requirements of fractions CE and AS for DNA synthesis were assessed in a series of reactions in which certain components were omitted or replaced (Table I). Both fractions have an absolute requirement for Mg^{++}. Fraction AS has an absolute requirement for added DNA template and the four deoxynucleoside triphosphates. These requirements also hold for fraction CE, but appear to be less stringent, perhaps because of DNA and nucleotides present in the crude extract. No radioactive nucleotides were rendered acid-precipitable in reactions lacking protein.

ATP was included at 10 times the concentration of the deoxyribonucleotides to evaluate the effect of possible nucleotide degradation by phosphatases. In both fractions the presence of ATP was found to be unnecessary, if not detrimental, for DNA synthesis.

Heat-denatured calf thymus was prepared prior to use by heating the native DNA preparation at 100° for 5 min, followed by rapid chilling to 0°. This denatured DNA was a less satisfactory template for DNA synthesis with either fraction.

Native DNA Templates from Different Sources—Four native duplex DNA preparations were used as templates in otherwise identical reaction systems with fraction AS. The extent of incorporation of ^{3}H-dTTP was determined for three concentrations of each template. As seen in Fig. 2, the activity of fraction AS as a function of template concentration is the same with native calf thymus DNA, T7 phage DNA, SV40 viral DNA, and HeLa mitochondrial DNA. It should be noted that, while the reaction conditions were the same

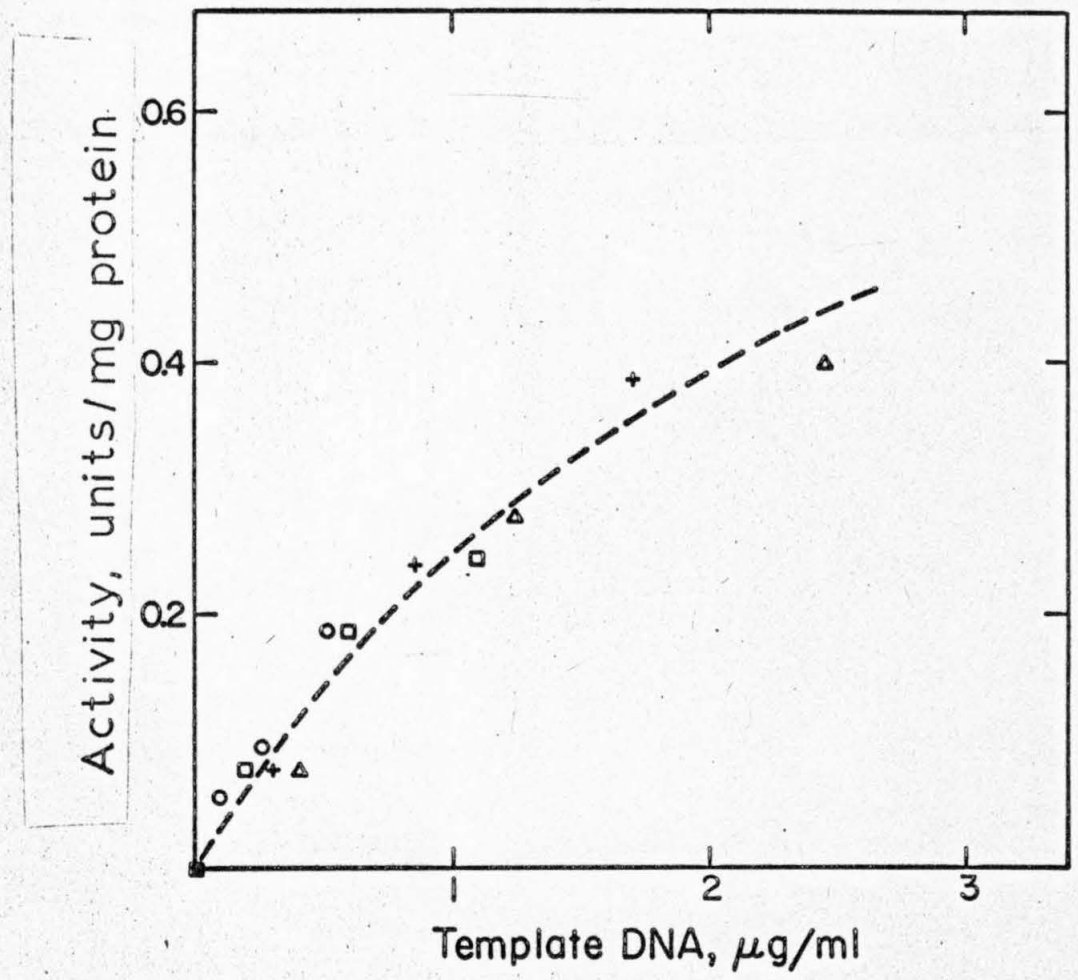
TABLE I

Reaction requirements

The complete incorporation system contains 24 μg per ml native or denatured calf thymus DNA, 210 μg per ml fraction CE protein or 33 μg per ml fraction AS protein, 2 mM β-mercaptoethanol, 10 mM Tris (pH 7.5), 4 mM $MgCl_2$, 1.5 mM ATP, 150 μM dATP, 150 μM dGTP, 150 μM dCTP, and 4.6 μM ^{3}H-dTTP, 10.35 Ci per mmole.

Reaction conditions	Activity (units/mg protein)	
	CE	AS
Complete system (native DNA)	0.08	0.74
—DNA	0.01	0.00
—native DNA + denatured DNA	0.03	0.29
—ATP	0.14	0.83
—$MgCl_2$	0.00	0.00
—dATP	0.01	0.00
—dGTP	0.01	0.00
—dCTP	0.01	0.00

FIG. 2. DNA polymerase activity with different native duplex DNA templates. Calf thymus DNA (+), T7 phage DNA (□), SV40 viral DNA (Δ), and HeLa mitochondrial DNA (○) were used at the indicated concentrations. The reaction mixtures contained 0.37 mg per ml fraction AS protein, 126 μM dATP, 126 μM dGTP, 140 μM dCTP, 65 μM ^{3}H-dTTP (250 Ci per mole), 4 mM $MgCl_2$, 10 mM Tris, pH 7.5, and 2 mM β-mercaptoethanol. The extent of DNA synthesis after 60 min at 37° was determined as described in "Methods."


Activity, units/mg protein
0.6
0.4
0.2
1
2
3
Template DNA, μg/ml

except for templates, the concentrations of NaCl and template DNA were below optimum levels for DNA synthesis.

Salt Effects—The capacity of fraction AS for DNA synthesis with native calf thymus DNA template was studied as a function of the concentration of $MgCl_2$ and NaCl present in the reaction mixture. The results shown in Fig. 3 indicate that 30 mM NaCl and 3 mM $MgCl_2$ provided maximum activity. Unless otherwise indicated, these concentrations were used in reactions described subsequently. Concentrations of NaCl higher than 70 mM were found to inhibit DNA synthesis, in distinct contrast to the optimum concentration of NaCl, 150 mM, found by Meyer and Simpson for the rat liver mitochondrial DNA polymerase (10). The replacement of NaCl and $MgCl_2$ with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of fraction AS, but to different extents. The requirement for Mg^{++} appears to be specific, as replacement by Ca^{++}, Sr^{++}, or Ba^{++} resulted in loss of activity. The results presented in Table III show that DNA synthesis in reactions containing $MgCl_2$ is inhibited by the addition of $CaCl_2$ or $SrBr_2$, but not $BaCl_2$.

Incorporation Kinetics—The incorporation of ^{3}H-dTTP into DNA by fractions CE and AS was studied with native calf thymus DNA template on prolonged incubation at 37°. Aliquots were withdrawn from reaction mixtures at selected times to determine the extent of incorporation. Fig. 4 shows the incorporation of ^{3}H-dTTP by fraction AS to be linear for at least 2.5 hours. The initial activity of fraction CE was lower and the activity reached a plateau. Other experiments of this nature have shown that nucleotide incorporation by fraction CE reached a maximum level followed by a gradual loss of acid-precipitable ^{3}H-thymidine. This is presumably due to degradative activities in fraction

FIG. 3. Effect of $MgCl_2$ and NaCl concentrations of DNA polymerase activity in fraction AS. Reaction mixtures contained 80 μg per ml fraction AS protein, 12 μg per ml native calf thymus DNA, 10 mM Tris, pH 7.5, 2 mM β-mercaptoethanol, and 0 mM NaCl (○- - - -○), 32 mM NaCl (●——●), 100 mM NaCl (Δ· · · · Δ), or 320 mM NaCl (×— · — ×). Concentration of $MgCl_2$ in the reactions is indicated by the abscissa. The concentrations of nucleotides in each reaction were 100 μM dATP, 93 μM dCTP, 100 μM dGTP, and 95 μM ^{3}H-dTTP (132 Ci per mole). The extent of DNA synthesis after 60 min at 37° was determined as described in "Methods."

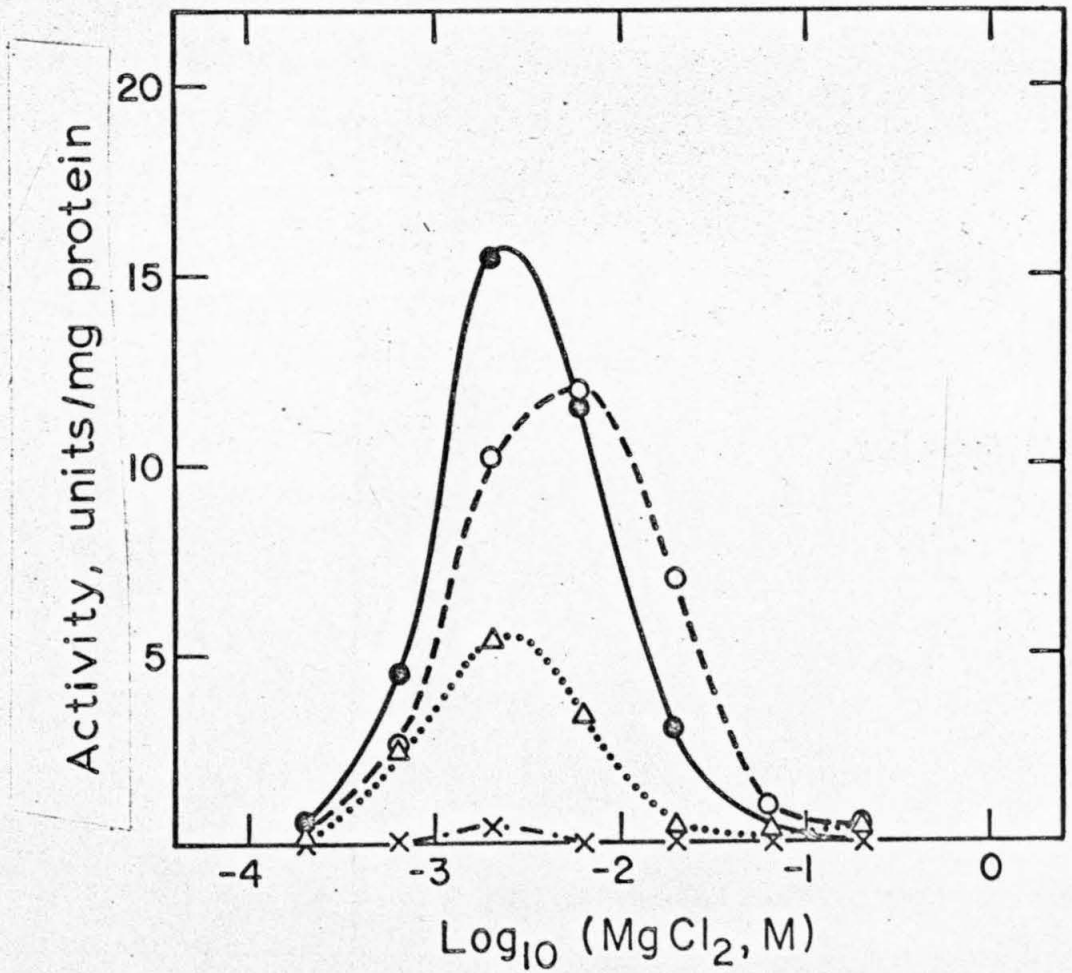

Activity, units/mg protein
20
15
10
5
-4
-3
-2
-1
0
Log10 (MgCl2, M)

TABLE II

Effects of monovalent and divalent cations

Each reaction contains 12 μg per ml native calf thymus DNA, 80 μg per ml fraction AS protein, 2 mM β-mercaptoethanol, 10 mM Tris (pH 7.5), 100 μM dATP, 92 μM dCTP, 103 μM dGTP, 95 μM ^{3}H-dTTP (0.13 Ci per mmole), and salts as specified in the table.

Monovalent cation salt (32 mM)	Divalent cation salt (2 mM)	Activity of AS
		units/mg protein
none	$MgCl_2$	8.0
LiBr	"	13.
NaCl	"	19.
NaBr	"	19.
KCl	"	20.
KBr	"	19.
RbBr	"	31.
CsCl	"	18.
NH_4Cl	"	13.
NaCl	none	0.0
"	$MgCl_2$	19.
"	$CaCl_2$	0.1
"	$SrBr_2$	0.3
"	$BaCl_2$	1.4

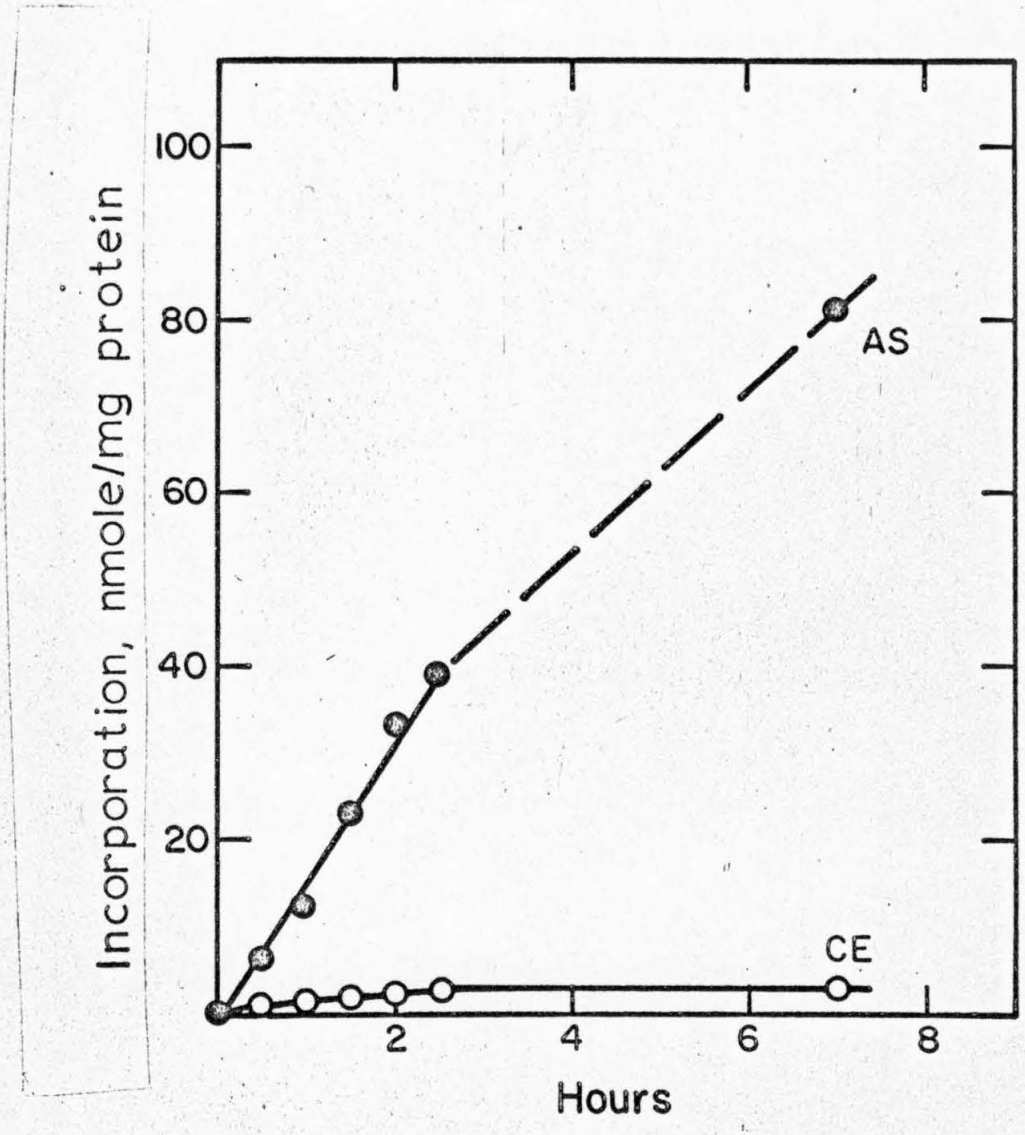
TABLE III

Activity with mixed divalent cation salts

Each reaction contains 10 μg per ml native calf thymus DNA, 67 μg per ml fraction AS protein, 1.7 mM β-mercaptoethanol, 26 mM NaCl, 84 μM dATP, 77 μM dCTP, 86 μM dGTP, 79 μM ^{3}H-dTTP (0.13 Ci per mmole), and divalent cation salts as shown in the table.

Divalent cation salt(s)	Activity of AS
	units/mg protein
None	.0
1.7 mM $MgCl_2$	13
3.4 mM $MgCl_2$	17
1.7 mM $MgCl_2$ + 1.7 mM $CaCl_2$	5
1.7 mM $MgCl_2$ + 1.7 mM $SrBr_2$	4
1.7 mM $MgCl_2$ + 1.7 mM $BaCl_2$	14

FIG. 4. Kinetics of DNA synthesis with crude extract (CE, ○—○) and partially purified fraction (AS, ●—●) from HeLa cell mitochondrial sonicate. The preparation of fractions CE and AS is described in "Results." The reaction mixtures contained 240 μg per ml of fraction CE protein or 80 μg per ml of fraction AS protein, 12 μg per ml native calf thymus DNA, 10 mM Tris, pH 7.5, 30 mM NaCl, 2 mM β-mercaptoethanol, 100 μM dATP, 100 μM dGTP, 93 μM dCTP, and 95 μM ^{3}H-dTTP (132 Ci per mole). Reaction mixtures were incubated at 37°. Aliquots were withdrawn from the reaction mixtures at the indicated times for the determination of the extent of DNA synthesis as described in "Methods." The ordinate represents DNA polymerase action in terms of the extent of incorporation of the radioactively labeled nucleotide.


Incorporation, nmole/mg protein
100
80
60
40
20
AS
CE
2
4
6
8
Hours

CE which are greatly reduced in the purification leading to fraction AS.

Circular DNA templates for DNA synthesis are an important aspect of this report. SV40 DNA was used to evaluate the dependence of incorporation kinetics on template DNA concentration. Linear incorporation kinetics over 60 min with fraction AS were observed with different concentrations of SV40 DNA template (Fig. 5). Double-reciprocal analysis of the rates of DNA synthesis at different template concentrations gave values of 2.6 nmole of dA per hour and 8 μg per ml for maximum rate of incorporation and the concentration of template DNA for half this maximum rate, respectively. The reactions contained 96 μg per ml of fraction AS protein. At high DNA template concentrations the specific activity approached 27 units per mg of protein.

Assuming the product contains 29.5% dA, in accord with the base composition of the SV40 template (19), the ratio of product DNA to initial template DNA can be calculated and used as an indication of the extent of synthesis. In the above experiment (Fig. 5) we find that after 60 min at 37° the product/template ratio is 0.11, 0.26, and 0.48 for 40, 10, and 2.5 μg per ml template, respectively.

Conservation of Template in DNA Synthesis and Deoxyribonuclease Activity of Fraction AS—Double-label experiments were performed with fraction AS to determine the extent of template degradation in the presence and absence of the nucleotides required for DNA synthesis. ^{32}P-SV40 DNA, 10^5 cpm per μg, was used as template. The reaction in which DNA synthesis was to proceed contained ^{3}H-dATP, 46,000 cpm per μg of product DNA assumed to contain 29.5% dA. The incorporation of ^{3}H-dATP into product DNA and the fractional loss of acid-precipitable template DNA were determined in the course of 60 min at 37° (Table IV). The template

FIG. 5. Kinetics of DNA synthesis with fraction AS protein and different concentrations of SV40 DNA template. Reactions contained 160 μg per ml fraction AS protein, 10 mM Tris, pH 7.5, 3 mM β-mercaptoethanol, 3 mM $MgCl_2$, 33 mM NaCl, 0.3 mM EDTA, 140 μM dGTP, 127 μM dCTP, 246 μM dTTP, and 92 μM ^{3}H-dATP (260 Ci per mole). SV40 DNA was present at 2.5 μg per ml (■—■), 10 μg per ml (▲—▲), or 40 μg per ml (●—●). Reaction mixtures were incubated at 37°. Aliquots were withdrawn from the reaction mixtures at the indicated times for the determination of the extent of DNA synthesis as described in "Methods." The ordinate refers to incorporation of the radioactively labeled nucleotide into acid-precipitable product.

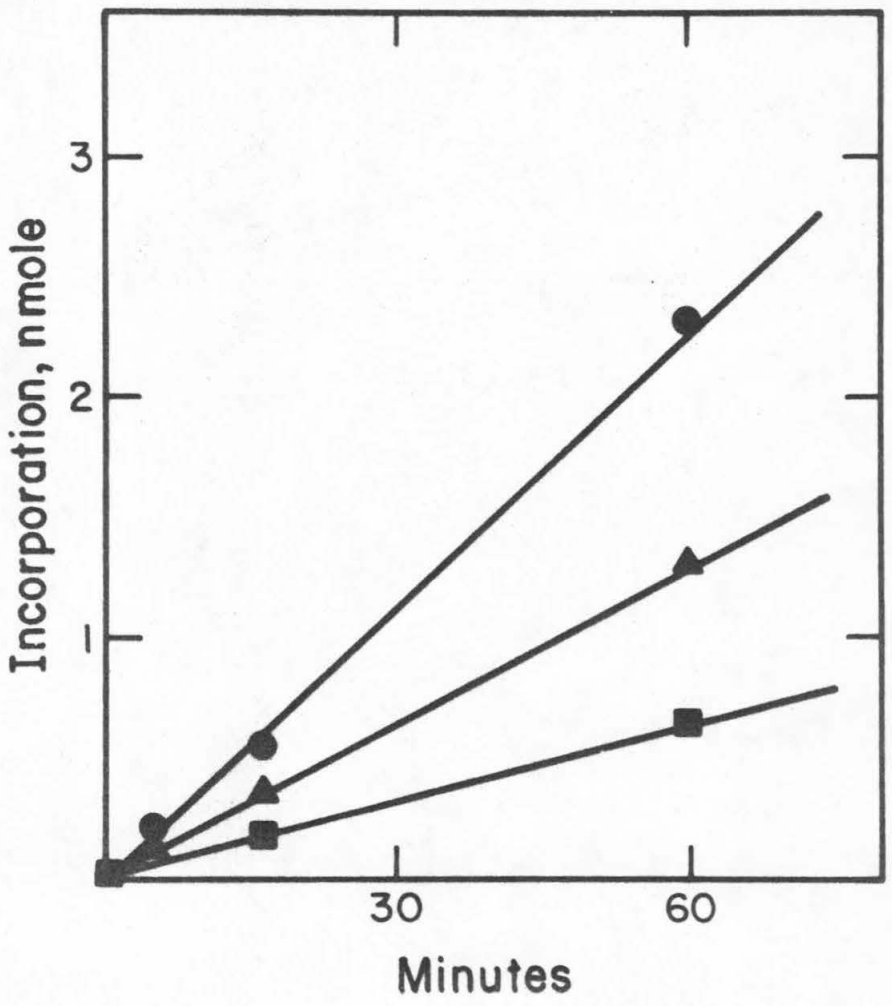

Incorporation, nmole
3
2
1
30
60
Minutes

TABLE IV

Template recovery during incubation with fraction AS

Reactions A and B contain 75 μg per ml fraction AS protein, 7 μg per ml ^{32}P-SV40-I DNA (100,000 cpm per μg), 10 mM Tris (pH 7.5), 3.3 mM β-mercaptoethanol, 33 mM NaCl, 3.3 mM $MgCl_2$, and 0.33 mM EDTA. Reaction B contains, in addition, 115 μM dGTP, 103 μM dCTP, 206 μM dTTP, and 115 μM ^{3}H-dATP (48,000 cpm per mmole).

Time of incubation	Template recovery		Product DNA in B	Product in B / Initial template
	A	B		
min	%	%	µg	%
0	98	100	0.00	0
20	101	100	0.13	6
40	100	98	0.23	11
60	101	100	0.37	18

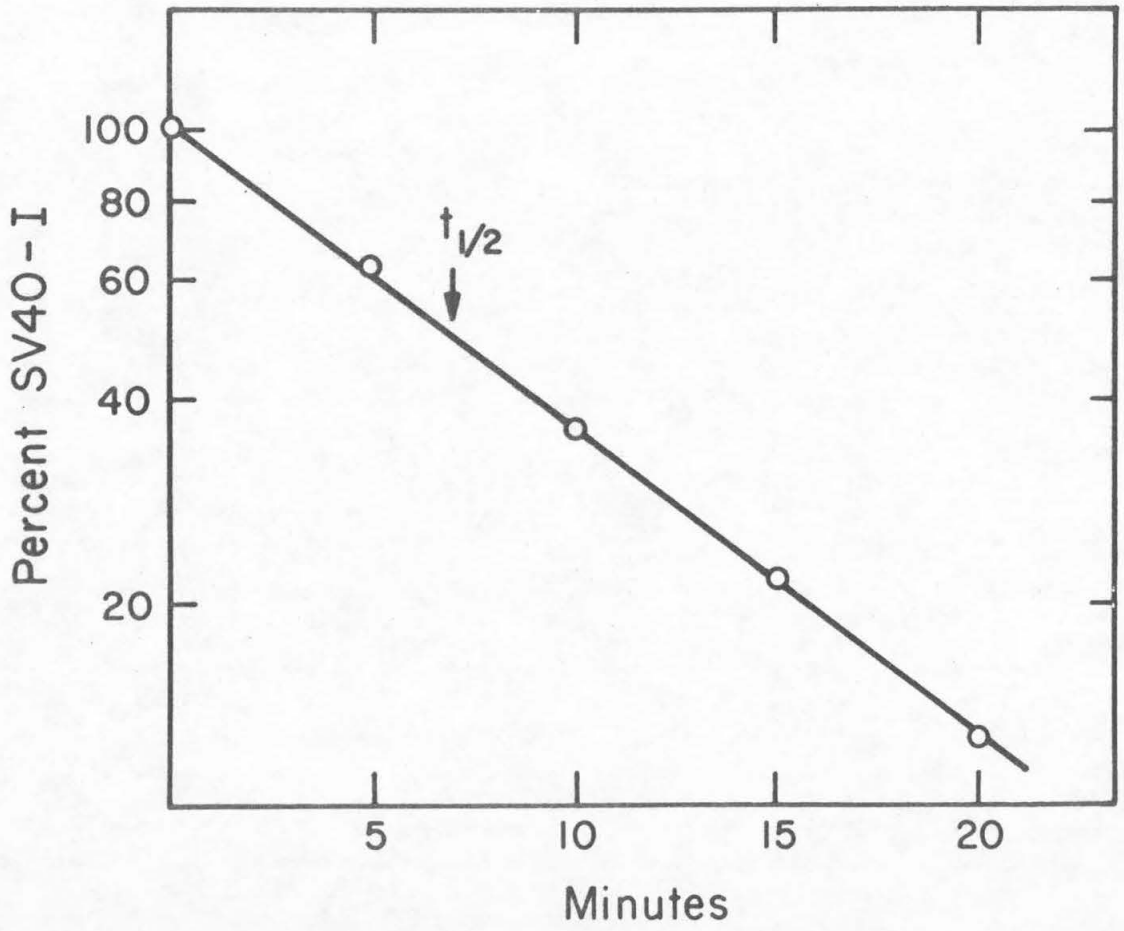
was quantitatively recoverable throughout the period of incubation. The extent of product DNA synthesis after one hour was approximately ten times greater than the error associated with the template recovery analysis.

The endonucleolytic conversion of closed circular SV40 DNA to nicked circular and/or linear DNA was followed by the fluorometric assay (18). First-order survival kinetics of SV40-I were observed during incubation with fraction AS protein. In the experiment presented in Fig. 6, the half-life of SV40-I was 7 min.

If the endonucleolytic process is considered to be a collection of randomly placed single- and/or double-strand scissions, with all molecules equally susceptible, then the average number of breaks per molecule per minute may be calculated by dividing ln 2 by the measured half-life of closed circular DNA. For the experiment shown in Fig. 6, the rate was found to be 0.10 breaks per molecule per minute. At the end of one hour there would thus be an average of 6 breaks, single- or double-strand scissions, per molecule. A similar experiment showed no significant difference in the half-life of closed circular SV40 DNA at concentrations of 33 and 66 μg per ml. This suggests a linear dependence of nuclease activity on DNA concentration. In this system circular SV40 DNA molecules up to at least 66 μg per ml sustain the same average number of breaks per molecule per minute.

Two lines of evidence show that the endonuclease action is predominately a single-strand scission process. A mixture of ^{32}P-labeled SV40-I and SV40-II was incubated at 37° in two reactions, with and without fraction AS. Sedimentation velocity analysis revealed that the DNA remained circular through both incubations, although the SV40-I was

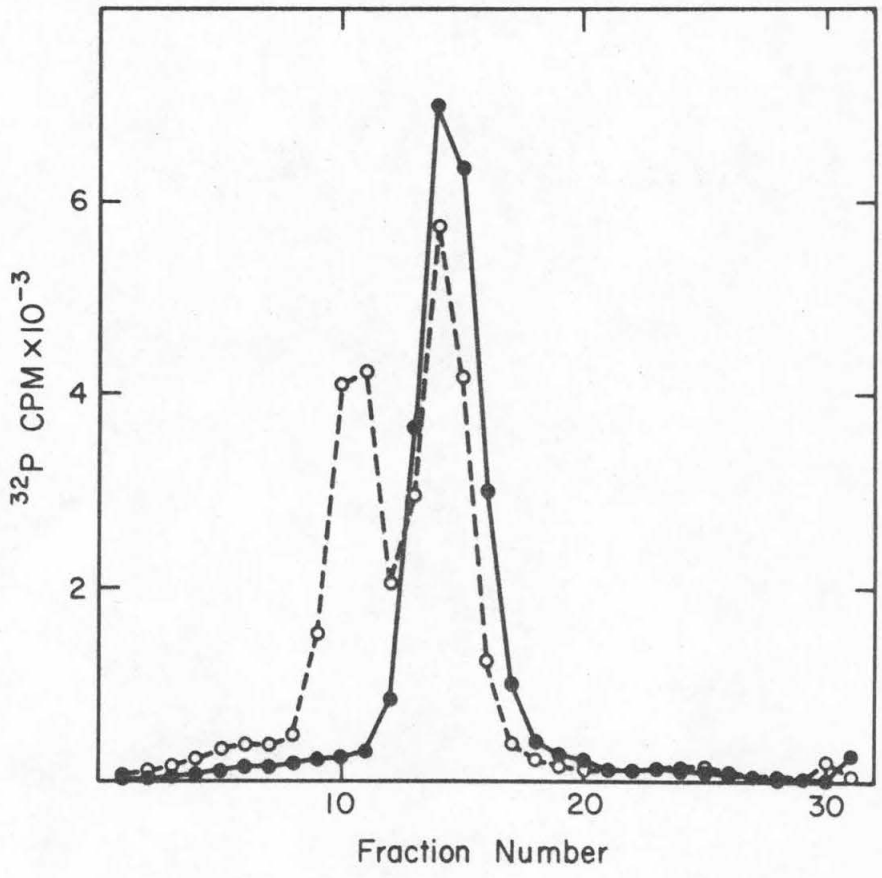
FIG. 6. Endonuclease assay with fraction AS protein and SV40 DNA template under conditions of DNA synthesis. The reaction mixture contained 102 μg per ml fraction AS protein, 8 μg per ml SV40 DNA (initially closed circular), 10 mM Tris, pH 7.5, 3 mM β-mercaptoethanol, 3 mM $MgCl_2$, 0.3 mM EDTA, 33 mM NaCl, 143 μM dATP, 133 μM dCTP, 147 μM dGTP, and 266 μM dTTP. The reaction mixture was incubated at 37°. Aliquots were withdrawn at the indicated times for determination of the fraction of SV40 DNA remaining closed circular. The fluorescence assay employed is described in "Methods." The half-life of closed circular SV40 DNA under the conditions of this experiment was 7 min.


Percent SV40-I
100
80
60
40
20
$t_{1/2}$
5
10
15
20
Minutes

converted to SV40-II in the presence of fraction AS. As seen in Fig. 7 the DNA incubated with fraction AS sedimented as a sharp band corresponding to the position of SV40-II. Significant double-strand scission activity would have generated linear fragments. The sedimentation profile, although still peaked at 14 to 16 S, would have been skewed back to the meniscus. Electron microscope examination of a sample of initially closed circular SV40 DNA, incubated 60 min with fraction AS, revealed only 5% linear molecules (500 molecules counted). One might argue that the linear molecules reflect fragmentation of the more extensively nicked molecules in the sample due to proximity of single-strand scissions on opposite strands. Assuming random disposition of the breaks in the population of initially closed circular molecules, the frequency of molecules with a specified number of breaks can be predicted on the basis of the Poisson distribution. The probability that a molecule with a specified number of nicks remains circular can also be calculated with a critical number of base pairs as a variable. A molecule would not remain circular with nicks on opposite strands separated by fewer than the critical number of base pairs. An average of 6 single-strand scissions per SV40 molecule would linearize 2% of the molecules only if the critical separation is more than 1000 base pairs. This number of base pairs is unreasonably large. Nonrandom single-strand scissions, low double-strand scission activity, or interaction within the regions of DNA synthesis are alternative explanations of the low but significant frequency of linear SV40 molecules.

Buoyant Equilibrium Properties of Product DNA—The behavior of the ^{3}H-labeled product DNA in buoyant equilibrium experiments was

FIG. 7. Sedimentation analysis of a mixture of closed circular and nicked circular ^{32}P-SV40 DNA previously incubated 60 min at 37° with (O——O) or without (O- - - -O) fraction AS protein. The incubation mixtures contained 3.6 μg per ml ^{32}P-SV40 DNA (1,500 cpm per μg), 10 mM Tris, pH 7.5, 3 mM β-mercaptoethanol, 33 mM NaCl, 3 mM $MgCl_2$, 0.3 mM EDTA, 116 μM dATP, 115 μM dGTP, 103 μM dCTP, and 307 μM dTTP. One mixture also contained 140 μg per ml fraction AS protein. Following incubation EDTA was added to 50 mM, SDS added to 20 mg per ml, and the samples held at room temperature for 10 min. NaCl was then added to 1 M and the samples were chilled to 4°. After 10 min the resulting precipitate was removed by centrifugation for 10 min at 10,000 × *g*. The supernatants were kept at 4° until the time of analysis. The samples were sedimented at 20° in two 3.0-ml self-generating CsCl density gradients (initial density, 1.40 g per ml) for 3 hours at 35,000 rpm. Fractions (100 μl each) were collected from the bottom of the tubes directly onto paper filters for the subsequent analysis of radioactivity. Details of centrifugation, fractionation, batchwise acid-washing of filters, and liquid scintillation counting are described in "Methods." In this and subsequent sedimentation analyses the direction of the centrifugal field is from right to left.


^{32}P CPM x 10^{-3}
6
4
2
10
20
30
Fraction Number

compared to ^{32}P-SV40 DNA added as a marker prior to centrifugation. The buoyant density of the ^{32}P-SV40 DNA under the equilibrium conditions employed is known and the density gradient in the region of the marker DNA was calculated with data given by Vinograd and Hearst (20). The magnitude of the buoyant density differences between the DNA distributions were estimated from differences in the centers of gravity of the radioactive marker and product DNA distributions.

At the end of incubations the reaction mixtures were chilled to 4° and EDTA was added to at least 10 mM. The samples were then treated with SDS (20 mg per ml) for 10 min at room temperature, followed by addition of NaCl or CsCl to 1 M, chilling to 4°, and removal of the precipitated protein and SDS. The extent of DNA synthesis was determined from the measured incorporation of ^{3}H-labeled nucleotide.

The product DNA forms a band at the same position in a pH 7.5 CsCl density gradient as the ^{32}P-SV40 marker DNA (Fig. 8). In a CsCl density gradient containing ethidium bromide (Fig. 9), closed circular and nicked circular SV40 DNA separate and the product DNA is found in one band, coincident with nicked circular marker DNA.

Product DNA samples were prepared using ^{3}H-dATP, 5-bromodeoxyuridine 5'-triphosphate, and different concentrations of SV40 DNA template. At higher concentrations of template the total amount of product synthesized by fraction AS is greater, but the amount of product relative to the template is lower. In the CsCl density gradients shown in Fig. 10, the product DNA is shifted to higher densities as the fraction of product in the sample increases. No peak appears at the position expected (19, 25) for hybrid or fully 5-bromodeoxyuridine-labeled SV40 DNA.

FIG. 8. Distributions of ^{32}P-SV40 DNA (O- - - -O) and ^{3}H-labeled product DNA (●——●) in a CsCl equilibrium buoyant density gradient, pH 7.5. The product DNA was synthesized in a reaction mixture containing 160 μg per ml fraction AS protein, 8 μg per ml unlabeled SV40 DNA, 10 mM Tris (pH 7.5), 3 mM β-mercaptoethanol, 33 mM NaCl, 3 mM $MgCl_2$, 0.3 mM EDTA, 68 μM ^{3}H-dATP (250 Ci per mole) 137 μM dGTP, 124 μM dCTP, and 248 μM dTTP. Following incubation for 60 min at 37° the reaction was stopped by addition of EDTA to 50 mM and SDS to 20 mg per ml. After 10 min at room temperature CsCl was added to 1 M and the mixture chilled to 4°. The resulting precipitate was removed by centrifugation for 10 min at 10,000 × *g*. The supernatant was kept at 4° until the time of analysis. The sample was diluted to about 3 ml with 10 mM Tris (pH 7.5), ^{32}P-SV40 DNA (about 4000 cpm) was added as a marker and solid CsCl was added to raise the density to 1.70 g per ml. Centrifugation followed at 35,000 rpm, 20°, for 46 hours. Fractions (100 μl each) were collected from the bottom of the tube directly onto paper filters for subsequent batchwise acid precipitation of the DNA and analysis of the distributions of radioactivity in the density gradient. Details of these steps are described in "Methods." The density gradient in this and subsequent equilibrium banding experiments increases from right to left and is approximately 8 mg per ml per fraction.

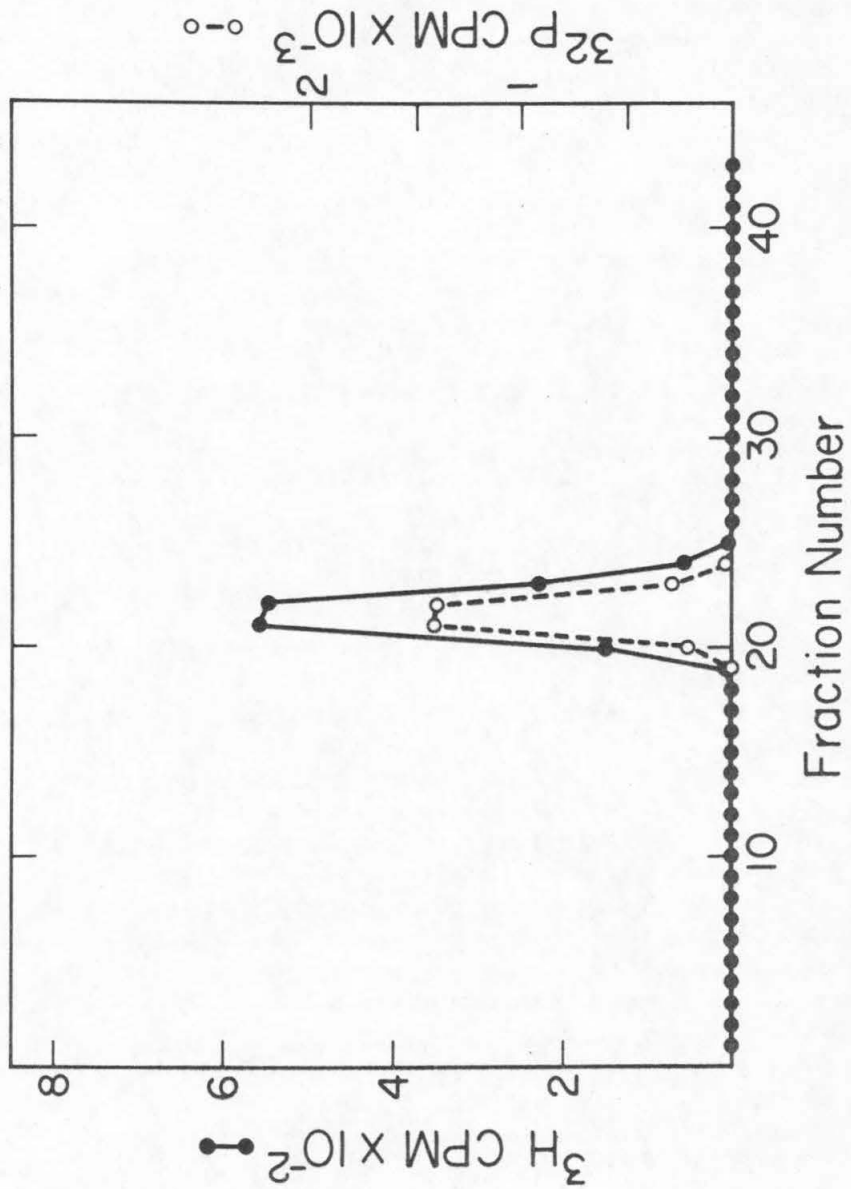

^{32}P CPM x 10^{-3}
2
1
^{3}H CPM x 10^{-2}
8
6
4
2
10
20
30
40
Fraction Number

FIG. 9. Distributions of a mixture of closed and nicked circular ^{32}P-SV40 DNA (O- - - -O) and ^{3}H-labeled product DNA (●——●) in a CsCl equilibrium buoyant density gradient (pH 7.5) containing ethidium bromide. The product DNA was synthesized in a reaction mixture containing 102 μg per ml fraction AS protein, 8 μg per ml unlabeled SV40 DNA (initially closed circular), 10 mM Tris (pH 7.5), 3 mM β-mercaptoethanol, 33 mM NaCl, 3 mM $MgCl_2$, 0.3 mM EDTA, 168 μM dATP, 171 μM dGTP, 155 μM dCTP, and 6 μM ^{3}H-dTTP (7 Ci per mmole). After 60 min incubation at 37° the reaction was stopped by addition of EDTA to 50 mM. ^{32}P-SV40 DNA (about 6000 cpm, a mixture of nicked and closed circular DNA) was added to the sample. The sample was diluted to about 3 ml with 10 mM Tris (pH 7.5), and ethidium bromide was added to 250 μg per ml. Solid CsCl was added to raise the density to 1.55 g per ml. Centrifugation, fractionation, and analysis of radioactivity distributions were the same as described in the legend for Fig. 8. The more dense band in the ^{32}P distribution corresponds to the closed circular SV40 DNA.

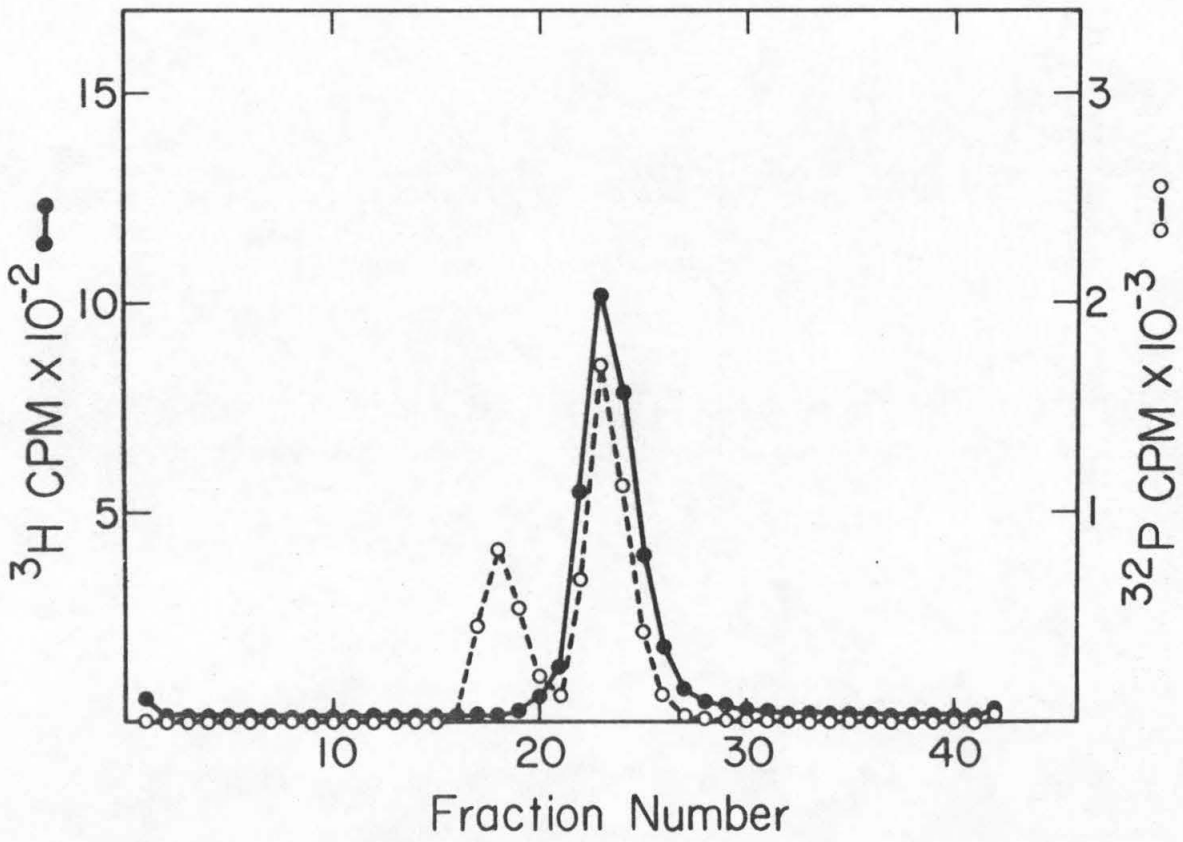
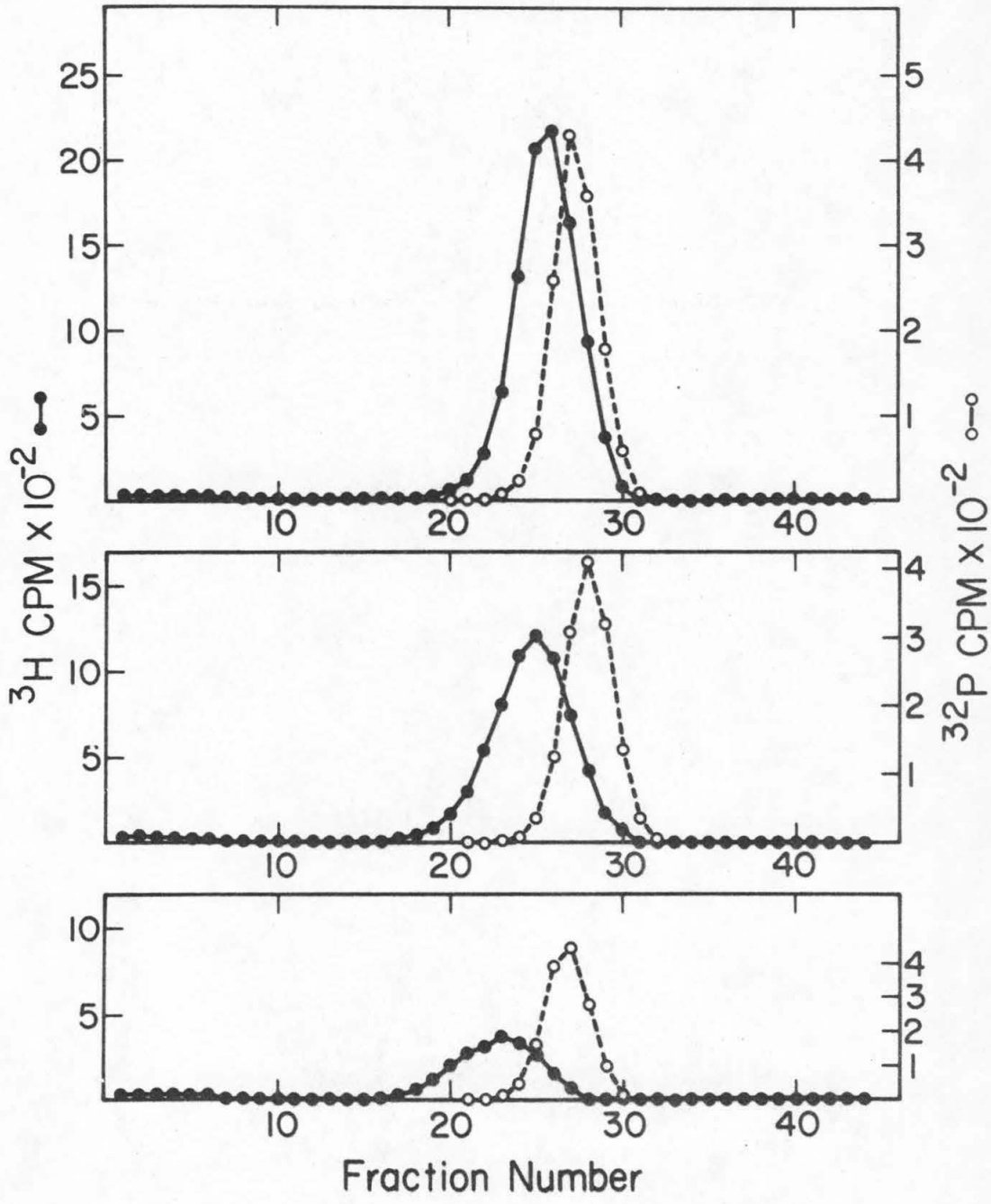

15
10
5
3
2
1
10
20
30
40
Fraction Number
^{3}H CPM X 10^{-2}
^{32}P CPM X 10^{-3}

FIG. 10. Distributions of ^{32}P-SV40 DNA (○- - - -○) and ^{3}H-labeled 5-bromodeoxyuridine containing product DNA (●——●) in CsCl equilibrium buoyant density gradients (pH 7.5). The product was synthesized in reactions containing different concentrations of unlabeled, thymidine-containing SV40 DNA template in order to obtain samples with different extents of synthesis relative to template. Template concentrations were 40 μg per ml (top), 10 μg per ml (middle), and 2.5 μg per ml (bottom). The reaction mixtures also contained 160 μg per ml fraction AS protein, 10 mM Tris (pH 7.5), 3 mM β-mercaptoethanol, 33 mM NaCl, 3 mM $MgCl_2$, 0.3 mM EDTA, 121 μM ^{3}H-dATP (260 Ci per mole), 140 μM dGTP, 127 μM dCTP, and 246 μM 5-bromodeoxyuridine 5′-triphosphate. After 60 min incubation at 37° the reactions were stopped by addition of EDTA to 50 mM. The samples were then treated with SDS as described in the legend for Fig. 8. After dilution with 10 mM Tris (pH 7.5) solid CsCl was added to raise the density to 1.72 g per ml. About 1400 cpm ^{32}P-SV40 DNA was added to each sample. Centrifugation, fractionation, and analysis of radioactivity distributions were performed as described in the legend for Fig. 8. The amount of product synthesized in each of the reactions was evaluated from the total acid-precipitable ^{3}H-radioactivity recovered in the density gradient and the specific activity of the ^{3}H-dATP as described in "Methods." The extents of synthesis [product/(product + template)] were 0.09 (top), 0.21 (middle), and 0.25 (bottom).


25
20
15
10
5
5
4
3
2
1
10
20
30
40
^{3}H CPM X 10^{-2}
^{32}P CPM X 10^{-2}
15
10
5
4
3
2
1
10
20
30
40
10
5
4
3
2
1
10
20
30
40
Fraction Number

The product DNA is apparently associated with template DNA molecules. The measured density shifts are linearly correlated with the extent of synthesis, defined as the ratio of product DNA to the sum of product and template DNA (Fig. 11). The point representing no density shift is taken from an experiment in which the extent of synthesis was 0.10, but the reaction contained dTTP instead of 5-bromodeoxyuridine 5'-triphosphate. The slope corresponds to 125 mg per ml density shift for unit extent of synthesis. The primary sources of error in this experiment were the estimation of the density gradient and the extent of synthesis. Combined, these amount to probably less than a 10% error in the value of the slope. The magnitude of the density shift relative to extent of synthesis will be discussed later.

The nature of the association of product and template DNA was examined in alkaline CsCl density gradients (Fig. 12). Under these conditions (pH 12.5) strand separation occurs for all but closed circular DNA. The latter undergoes denaturation and is about 20 mg per ml more dense than single-stranded DNA (21). Samples of product DNA were prepared using dTTP or 5-bromodeoxyuridine 5'-triphosphate, ^{3}H-dATP, and closed circular SV40 DNA template. Closed circular ^{32}P-SV40 DNA was added as a marker prior to banding in alkaline CsCl density gradients. The fraction of product DNA in the two samples was 10%. In the case of thymidine incorporation the ^{3}H-labeled product was found to be 20 mg per ml lighter than the ^{32}P-labeled marker. With incorporation of 5-bromodeoxyuridine, however, the separation of the product and marker distributions was only 1.2 mg per ml. The product distribution was skewed to higher densities in this case, but very little product was found in the region expected for fully 5-bromodeoxyuridine-labeled

FIG. 11. Linear relation between extent of synthesis with 5-bromodeoxyuridine 5′-triphosphate and the buoyant density shift relative to thymidine-containing SV40 DNA. Buoyant density differences between the ^{3}H-product DNA samples and ^{32}P-SV40 marker DNA were evaluated from the data presented in Fig. 10. The centers of gravity of the radioactivity distributions were determined in terms of fraction number. The density differences were then calculated from the separation of the ^{3}H and ^{32}P band centers and the calculated density gradient in the region of the ^{32}P-SV40 marker DNA (buoyant density of SV40 DNA, 1.694 g per ml; density gradient at 35,000 rpm, SW50L rotor, 0.090 g per cm^{4} (20); radial displacement per fraction, 0.082 cm; density gradient, 7.4 mg per ml per fraction). The extents of synthesis were given in Fig. 10. The point representing 0.00 extent of synthesis is taken from the experiment described in Fig. 8 in which dTTP was used in place of 5-bromodeoxyuridine 5′-triphosphate. In that case the ^{3}H-product DNA and ^{32}P-marker DNA distributions were coincident. The slope of the relation is 125 mg per ml density shift per unit extent of synthesis with the brominated nucleotide.

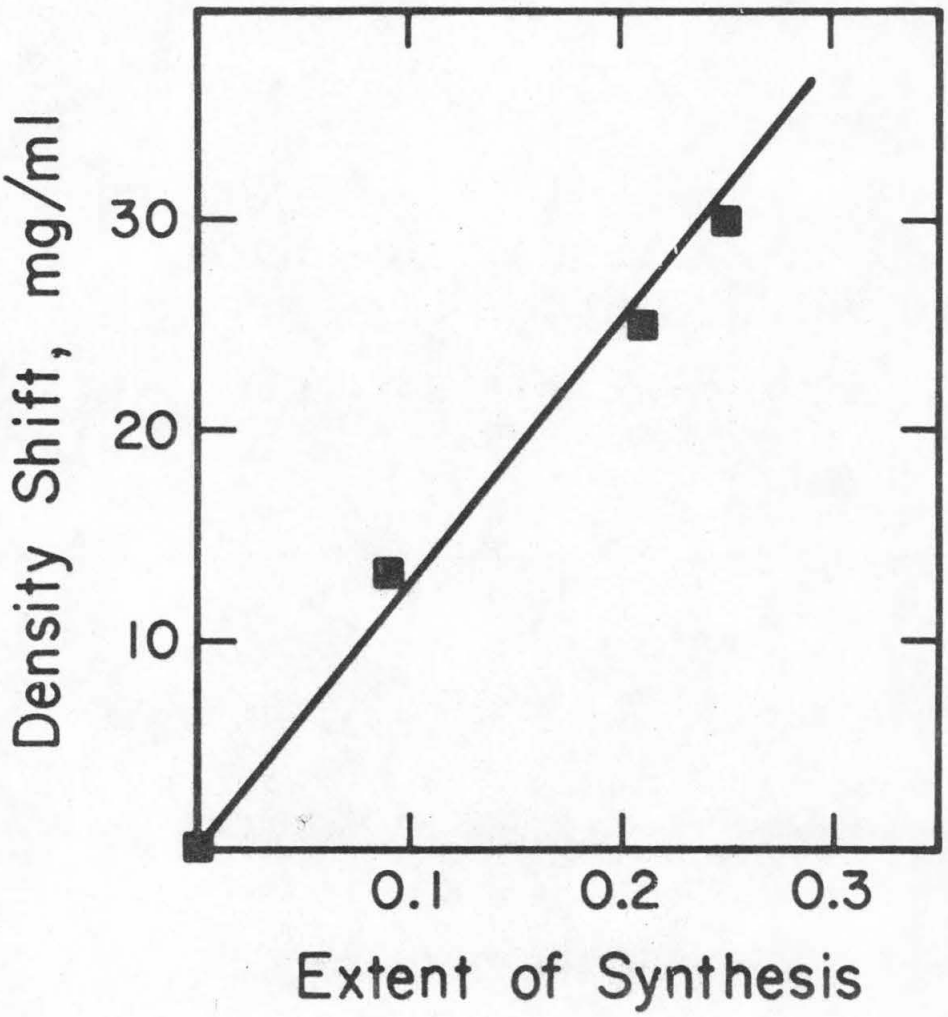
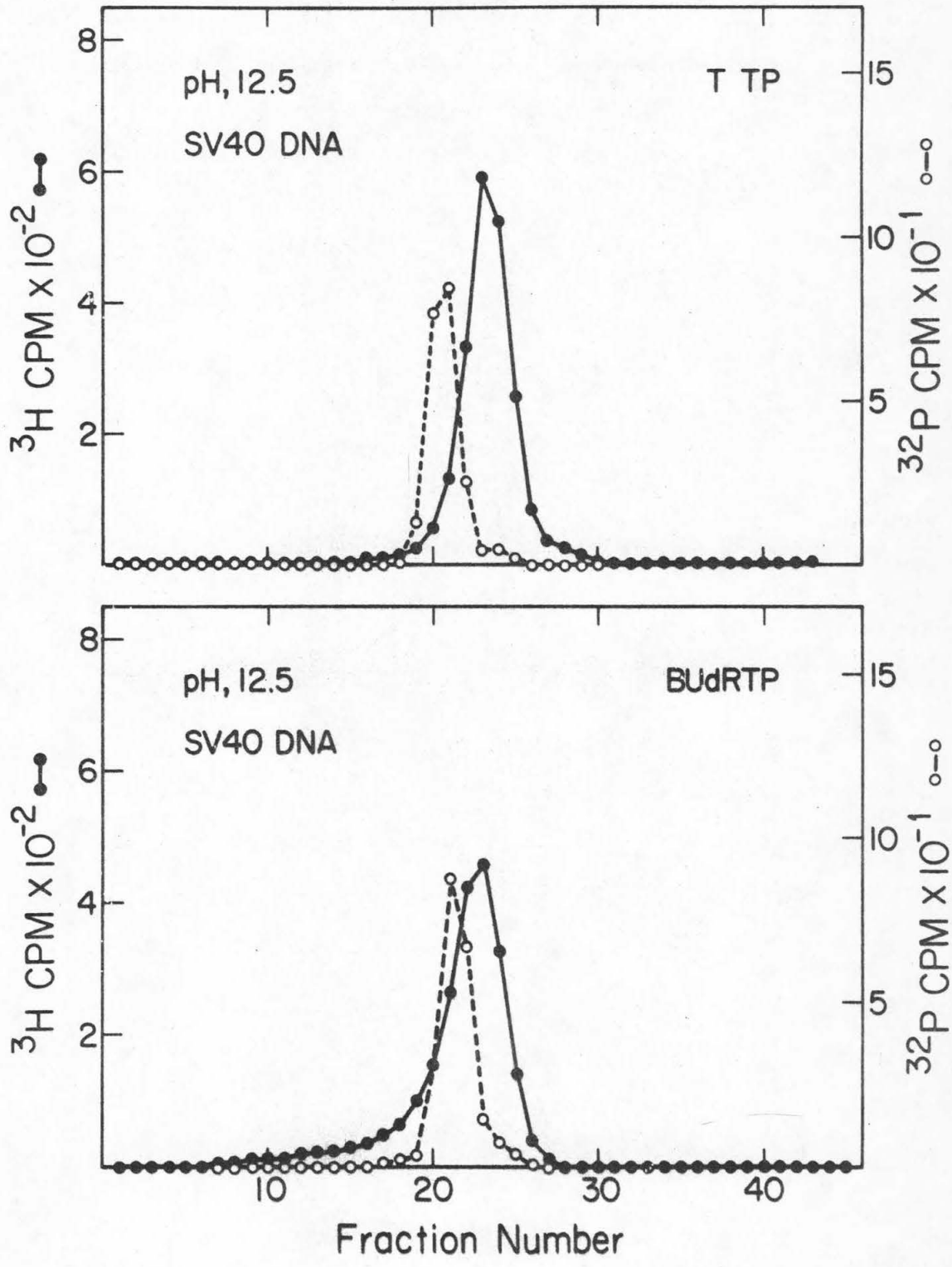

Density Shift, mg/ml
30
20
10
0.1
0.2
0.3
Extent of Synthesis

FIG. 12. Distributions of initially closed circular ^{32}P-SV40 DNA (○- - - -○) and ^{3}H-labeled product DNA samples (●——●) after buoyant equilibrium centrifugation in alkaline CsCl density gradients. The product DNA was synthesized in two reaction mixtures, one containing 309 μM dTTP (top) and the other containing 309 μM 5-bromodeoxyuridine 5′-triphosphate (bottom). Each reaction also contained 160 μg per ml fraction AS protein, 40 μg per ml SV40 DNA (unlabeled), 10 mM Tris (pH 7.5), 3 mM β-mercaptoethanol, 33 mM NaCl, 3 mM $MgCl_2$, 0.3 mM EDTA, 70 μM ^{3}H-dATP (260 Ci per mole), 175 μM dGTP, and 158 μM dCTP. After 60 min incubation at 37° the reactions were stopped by addition of EDTA to 50 mM and aliquots were removed for determination of the extent of DNA synthesis as described in "Methods." In each case the extent of synthesis [product/(product + template)] was 0.10, indicating no change in the rate of DNA synthesis with 5-bromodeoxyuridine 5′-triphosphate instead of dTTP. The samples were then treated with SDS as described in the legend for Fig. 8. 0.65 ml of 0.25 M K_3PO_4 (previously adjusted to pH 12.5 with KOH) and about 200 cpm ^{32}P-SV40 DNA (closed circular) were added to each sample. The volume of each sample was adjusted to 2.50 ml with distilled water and 3.30 g solid CsCl was added to each to raise the density to 1.74 g per ml. Centrifugation, fractionation, and analysis of radioactivity distributions were performed as described in the legend for Fig. 8. The differences in buoyant density of the ^{3}H and ^{32}P distributions were determined in the manner described in the legend for Fig. 11. The ^{3}H-labeled products were 20 mg per ml (top) and 1.2 mg per ml (bottom) less dense than the thymidine-containing closed circular ^{32}P-SV40 DNA.

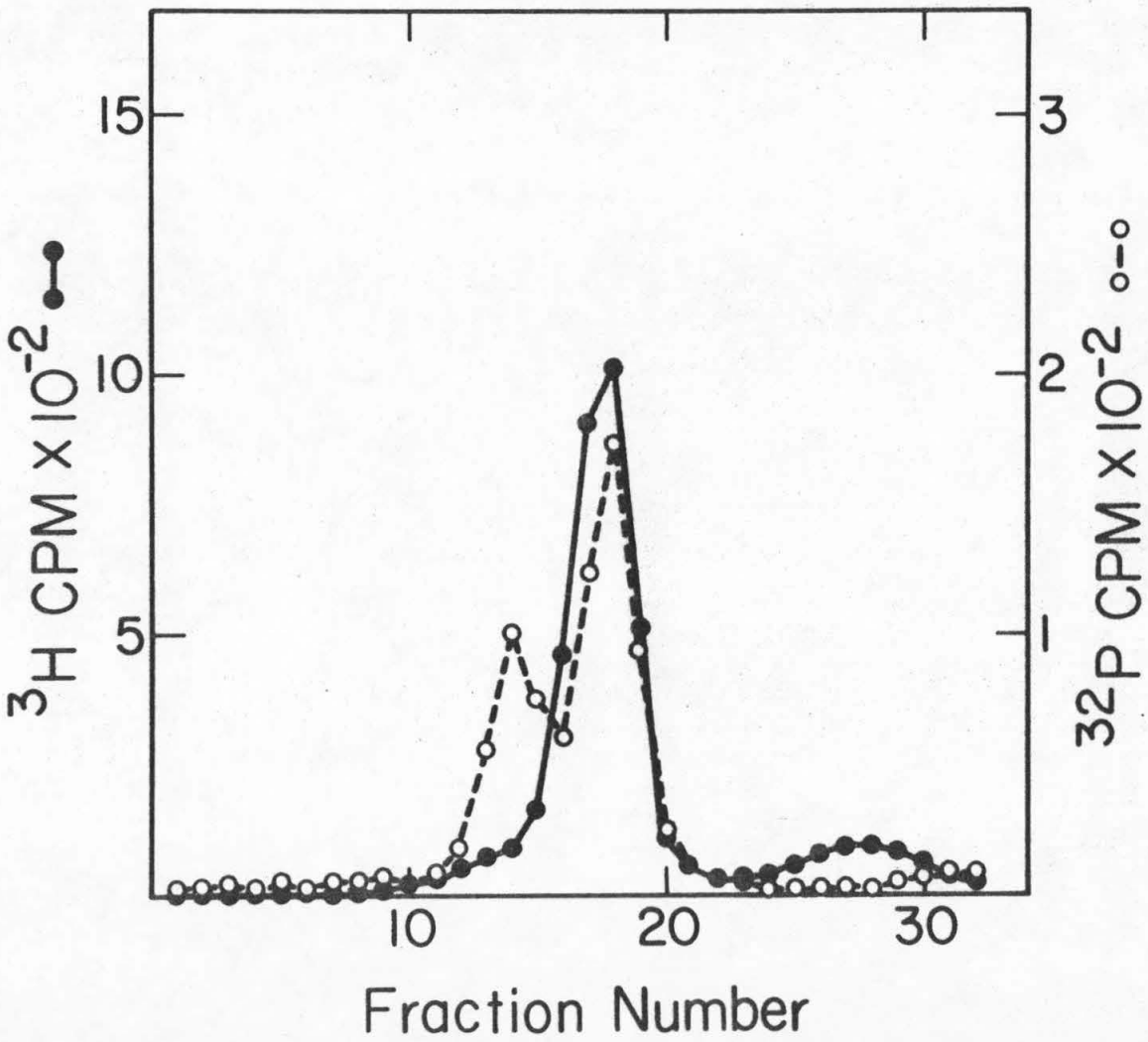

pH, 12.5
SV40 DNA
T TP
pH, 12.5
SV40 DNA
BUdRTP
^{3}H CPM X 10^{-2}
^{32}P CPM X 10^{-1}
Fraction Number

single-stranded DNA. The very small light shoulder in the ^{32}P-marker DNA distributions indicates the limited extent of alkaline hydrolysis during centrifugation. The result of this experiment suggests that most of the product DNA is covalently bound to the template DNA strands.

It was observed that omission of the SDS treatment after incubation with the normal complement of four nucleotides led to the appearance of product DNA at slightly lower density than the marker DNA, suggesting that some protein remains bound to the DNA in untreated samples at high CsCl concentrations. This effect was not observed when untreated samples were banded in gradients with ethidium bromide or adjusted to alkaline pH.

Sedimentation Properties of Product DNA—Sedimentation velocity methods were used to characterize the product DNA. The samples were prepared in the same manner as for the buoyant equilibrium experiments, with the normal complement of four nucleotides and SV40-I as template. The results of the sedimentation analysis were consistent with the introduction of regions of product DNA into circular template DNA molecules by means of covalent attachment to template DNA strands. In self-generating CsCl density gradients at pH 7.5, most of the product sedimented with and slightly ahead of the nicked circular ^{32}P-SV40 DNA marker (Fig. 13). In addition, a broad band of slowly sedimenting product, about 7 S, was sometimes observed. When ^{32}P-SV40 DNA was used as a template no ^{32}P counts appeared with the ^{3}H-labeled product DNA in this band. The slowly sedimenting material, therefore, is product DNA not associated with template. The amount of this material apparently depends in an unknown way on the preparation

FIG. 13. Band sedimentation analysis of ^{3}H-labeled product DNA (●——●) in a self-generating CsCl density gradient (pH 7.5). Product DNA was synthesized as described in the legend for Fig. 12 (top). SDS treatment, sedimentation, fractionation, and radioactivity analysis were performed as described in the legend for Fig. 7, except that the initial density of the CsCl solution was 1.45 g per ml and the centrifugation was 2.5 hours at 35,000 rpm. About 700 cpm ^{32}P-SV40 DNA (○- - - -○, a mixture of nicked and closed circular DNA) was added to the sample prior to centrifugation.


15
10
5
3
2
1
10
20
30
^{3}H CPM X 10^{-2} ●—●
^{32}P CPM X 10^{-2} o--o
Fraction Number

of the AS fraction. With some preparations, the slow band was not detected. When present, the relative amount increased with the extent of synthesis to as much as 30% of the product DNA.

Sedimentation studies were also performed in alkaline self-generating CsCl density gradients. ^{14}C-labeled ϕX174 single-stranded circular DNA, having approximately the same sedimentation velocity as circular single-stranded SV40 DNA, was used as a marker. The ^{3}H-labeled product of DNA synthesis with SV40-I template sedimented with a predominate peak in the region expected for full-length linear single strands of SV40 DNA (Fig. 14). The distribution of product DNA was skewed back to the meniscus. This sedimentation behavior is consistent with the accumulation of a limited number of single-strand scissions in the circular template molecules and with the covalent attachment of product DNA to template DNA strands.

Susceptibility of Product and Template to the Action of E. coli Exonucleases I and III—The specific reactions of E. coli exonucleases I and III make them suitable for the investigation of the structure of molecules containing both template and product DNA. An incorporation system containing ^{32}P-SV40 template, nucleotides (including ^{3}H-dATP), and fraction AS was incubated 60 min at 37°. After 20 min, it was split into three equal portions. Exonuclease I (20 units per ml) was added to one, exonuclease III (650 units per ml) to the second, while the third remained unaltered. The course of the reaction in the three cases was determined by removing aliquots for analysis of acid-precipitable product and template DNA as a function of time (Fig. 15). The template DNA was not solubilized and product DNA continued to

FIG. 14. Band sedimentation analysis of ^{3}H-labeled product DNA (●——●) in a denaturing (alkaline) self-generating CsCl density gradient. The ^{3}H-product is from the same sample used in the experiment described in the legend for Fig. 13. About 500 cpm ^{14}C-labeled $\phi X174$ phage DNA (O- - - -O) was mixed with the sample before layering onto 4.0 ml CsCl solution, density 1.50 g per ml, containing 0.10 M KOH. Centrifugation followed for 5 hours at 35,000 rpm, 20°. Fractionation and radioactivity analysis were performed as described in the legend for Fig. 7.

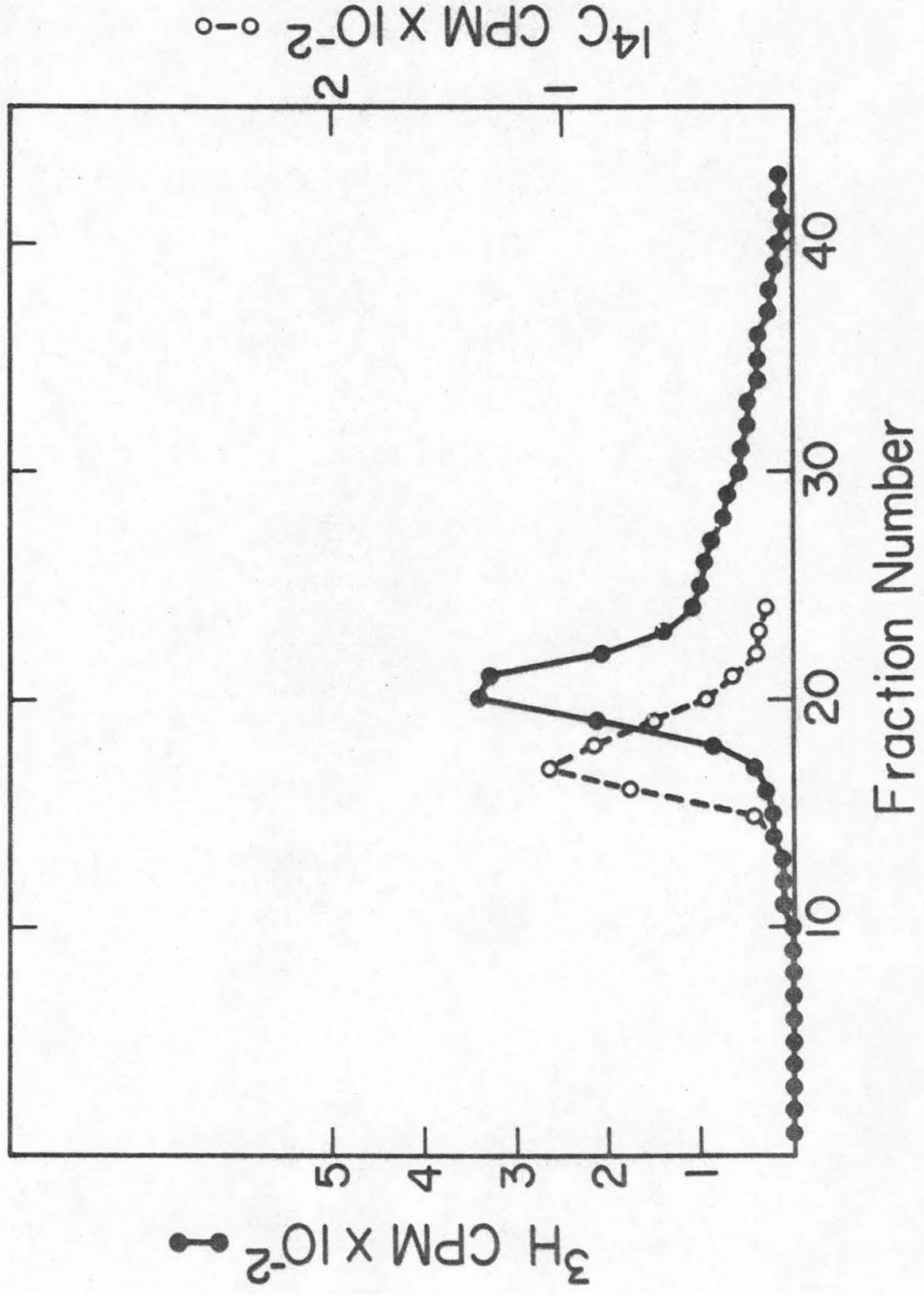
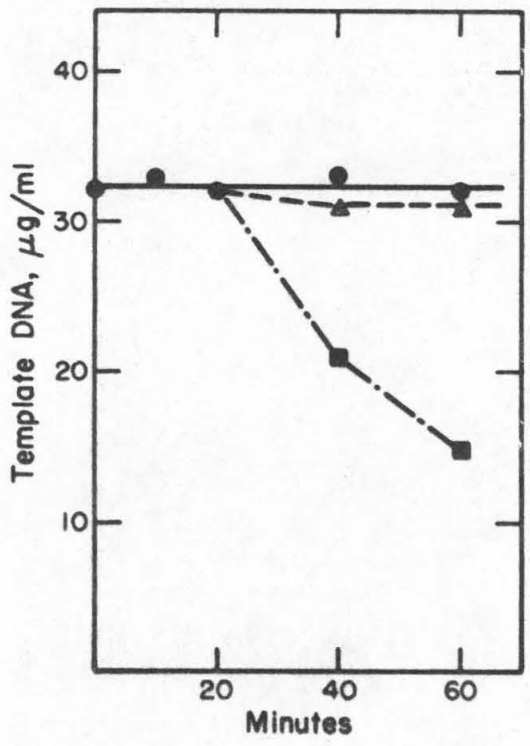
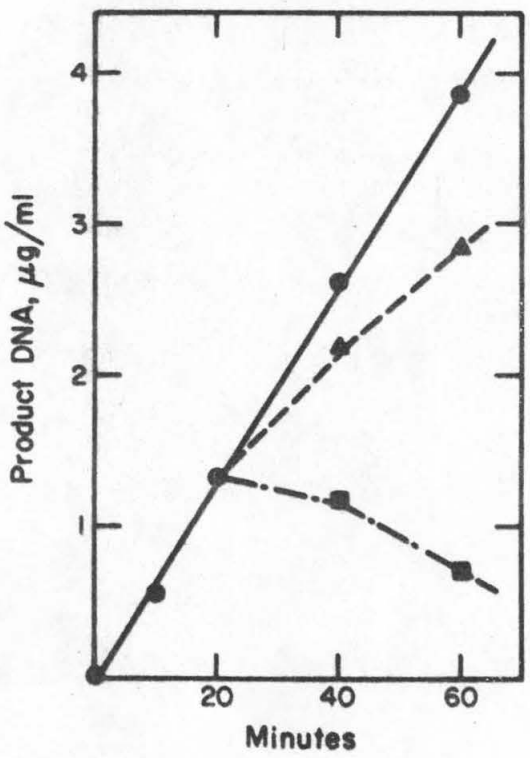

^{14}C CPM x 10^{-2} o–o
2
1
^{3}H CPM x 10^{-2} ●–●
5
4
3
2
1
10
20
30
40
Fraction Number

FIG. 15. Action of E. coli exonucleases I and III on ^{3}H-labeled product and ^{32}P-labeled SV40 template during the course of DNA synthesis with fraction AS protein. The reaction mixture contained 123 μg per ml fraction AS protein, 33 μg per ml ^{32}P-SV40 DNA (closed circular DNA, 1500 cpm per μg), 10 mM Tris, pH 7.5, 3 mM β-mercaptoethanol, 33 mM NaCl, 3 mM $MgCl_2$, 0.3 mM EDTA, 55 μM ^{3}H-dATP (260 Ci per mole), 140 μM dGTP, 127 μM dCTP, and 247 μM dTTP. At the indicated times aliquots were withdrawn for determination of acid-precipitable ^{32}P-template DNA (top) and ^{3}H-product DNA (bottom) as described in "Methods." The data are presented as μg DNA per ml calculated from the specific activity of the isotopes, an average of 330 g per mole nucleotide, and 29.5 mole percent dA in product DNA. The reaction was split into three portions after 20 min at 37°. The first (●——●) was allowed to proceed unaltered. Exonuclease I (20 units per ml) and Exonuclease III (630 units per ml) were added to the second (▲- - - -▲) and third (■—· —■) portions, respectively. Each was then followed as above for 40 more min at 37°. Note that the units of activity of exonuclease I and exonuclease III are defined under conditions differing from those in this experiment.


Template DNA, μg/ml
40
30
20
10
20
40
60
Minutes


Product DNA, μg/ml
4
3
2
1
20
40
60
Minutes

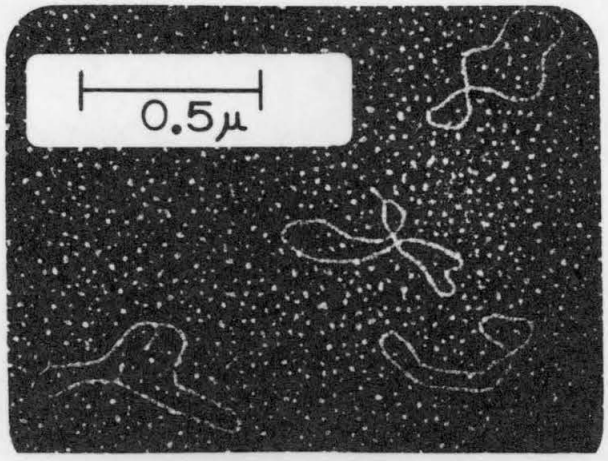
accumulate in the presence of exonuclease I; however, both template and product DNA were degraded by exonuclease III. The latter result indicates that product, as well as template, DNA are in duplex regions. The site(s) of action of the exonuclease III might be either the single-strand scissions produced by endonucleolytic activity in fraction AS or the 3'-OH end of a growing product DNA strand.

The product DNA isolated and purified from standard incorporation reactions was also treated with exonuclease I. The DNA was isolated in CsCl-ethidium bromide density gradients following treatment with SDS (20 mg per ml) as previously described. After removing the dye, the DNA containing 10% product was dialyzed against 10 mM Tris, 1 mM EDTA (pH 7.5). Exonuclease I was diluted to 300 units per ml in 10 mM Tris, 0.18 M $(NH_4)_2SO_4$, 0.1% BSA (pH 7.5). Ten μl of 1 M $MgCl_2$ and 50 μl of either the exonuclease I solution or its dilution buffer were added to 0.5 ml of the DNA sample. After 30 min at 37°, the reactions were stopped by chilling and adding 100 μl of 0.1 M EDTA (pH 7.5). Preparative velocity gradients showed no change in the sedimentation behavior of the product due to the action of exonuclease I. In another experiment the DNA was dialyzed against 50 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 7.5). The sample was denatured by heating at 90° for 5 min, then quickly chilled to 0°. Exonuclease I was diluted to 60 units per ml in 10 mM Tris, 100 mM $MgCl_2$, 0.05% BSA (pH 7.5). On incubation at 37° of a solution containing equal volumes of the denatured DNA and exonuclease I solutions, more than 95% of the ^{3}H-labeled product was solubilized in 5 min. The product DNA is, therefore, rendered susceptible to the action of exonuclease I only after denaturation.

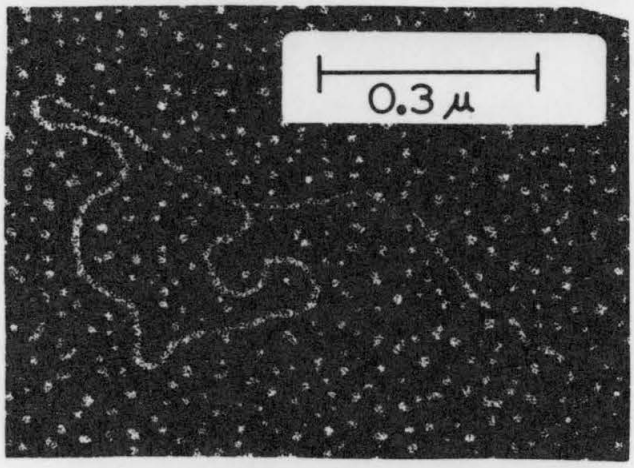
Electron Microscopy of Template-Product DNA—Basic protein film electron microscopy was used to visualize molecules containing regions of product and template DNA. The formamide system (14) was chosen for two reasons. The standard aqueous Kleinschmidt procedure (22) applied to samples of DNA from reaction mixtures usually led to aggregation of molecules in the film and a rather coarse contour of duplex DNA. This may have been due to persistence of contaminating protein in the samples, despite treatment with SDS and banding in CsCl density gradients. The formamide may have dissociated protein-DNA complexes. In addition, the formamide technique allows discrimination between single- and double-stranded regions in DNA molecules. Single-stranded DNA appears with somewhat reduced contrast from background and a more kinked contour than the thicker and smoother duplex DNA regions. The electron micrographs presented in Fig. 16 show representative molecules in a sample containing 18% product DNA in a one-hour incubation of circular SV40 template with the AS fraction. The most apparent structures are circular duplex, SV40-length molecules with single-stranded branches. About half of the circular duplex molecules have an easily visualized single-stranded branch. Molecules with more than one branch were not uncommon. Most of the branches observed were of the size seen in the molecules of Fig. 16a but occasionally much larger branches were found as in the molecules of Fig. 16, b and c. The foregoing results, together with the conclusions drawn from the centrifugation and exonuclease susceptibility studies, indicate the displacement of a template DNA strand in the course of product DNA synthesis. The length of the displaced template DNA

FIG. 16. Electron micrographs of SV40 DNA following DNA synthesis with fraction AS. The formamide spreading procedure and electron microscopy are described in "Methods." The molecules are from a sample in which the extent of synthesis after 60 min at 37° was 0.18. The reaction mixture and SDS treatment are described in the legend for Fig. 8. The DNA was banded in a buoyant CsCl density gradient containing ethidium bromide as described in the legend for Fig. 9. The fluorescent band of DNA was collected and the dye removed using the isoamyl alcohol extraction procedure as described in "Methods."

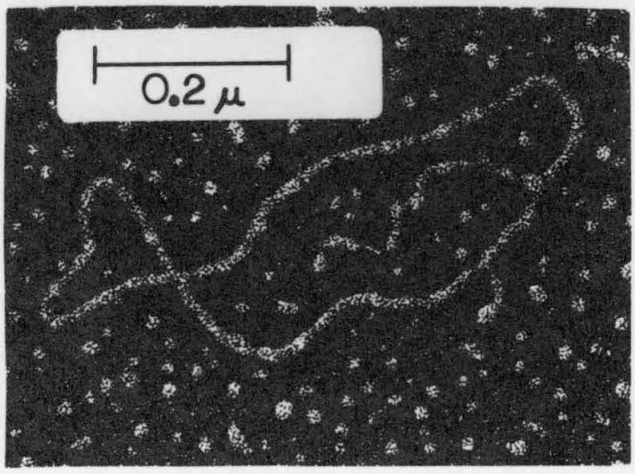
(a)



(b)



(c)



should be the same as the associated region of product DNA, since no degradation of template could be demonstrated in the course of product DNA synthesis. The more frequent smaller branches were about 2% by mass of an SV40 molecule. The longer branches shown in Fig. 16 b and c, were 20% and 10% of SV40 molecular weight, respectively. An 18% extent of synthesis would lead to an 18% increase in the average molecular weight of the SV40 molecules in the sample. The frequency of molecules with long braches was too small to significantly affect the average molecular weight. The molecules with short branches could account for an increase in the average molecular weight of only 1 to 2%. In another sample of DNA containing 6% product, the frequency of circular duplex molecules with small single-stranded branches was much lower, about 2 to 3%, which could account for only 1% of the expected increase in average molecular weight of the SV40 DNA. The rather low frequency of branched molecules and the small size of most of the branches suggest that product DNA synthesis also gives rise to short displaced template branches, too small to be visualized. Such a result is compatible with initiation of DNA synthesis at many of the single-strand scissions introduced during the incubation with fraction AS. Previous experiments indicated that approximately 10 single-stranded scissions per molecule occurred during a 60 min incubation. A 10% extent of synthesis would represent an average of about 100 nucleotides incorporated at each nick. Displaced single-stranded template DNA branches of this average length would be hard to distinguish from the contour of otherwise duplex DNA. Occasional stretches of more extensive synthesis generate branches long enough to be observed in the electron microscope.

DISCUSSION

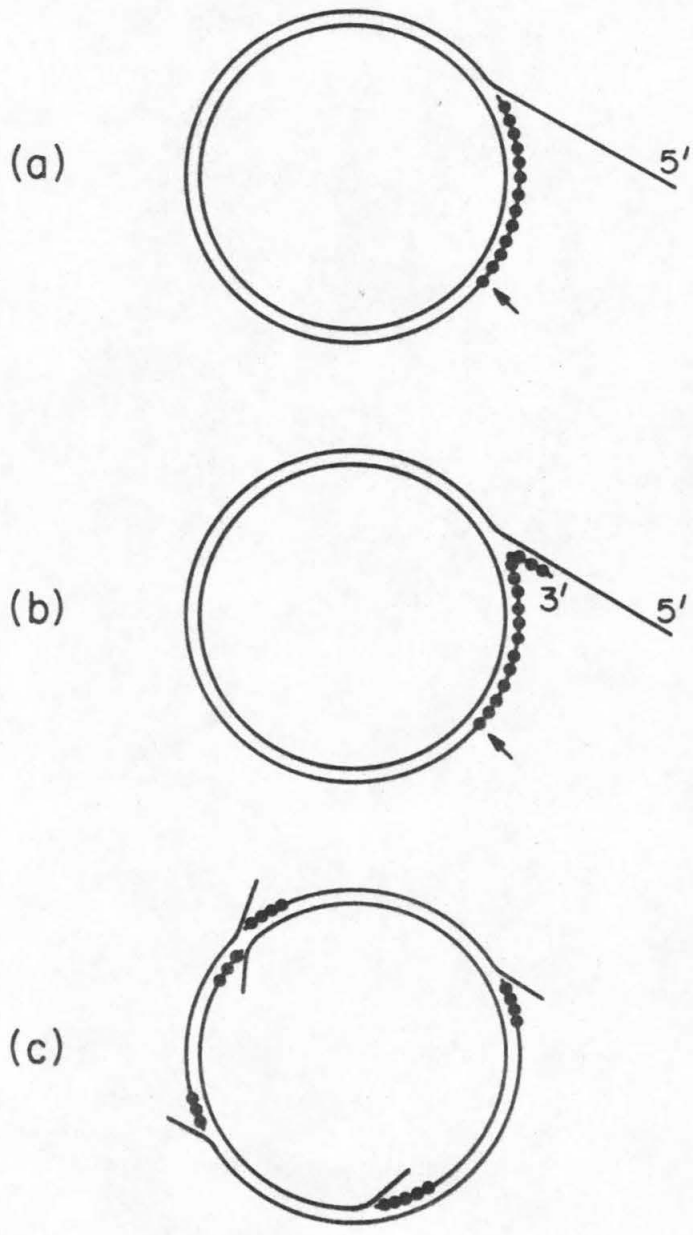
A DNA polymerase-containing fraction (AS) has been isolated and partially purified from soluble protein extracted after sonic disruption of HeLa cell mitochondria. The reaction requirements and mode of action have been characterized in studies with duplex linear and circular DNA templates. Meyer and Simpson (10) and Kalf and Ch'ih (9) have described DNA polymerase preparations from the mitochondria of rat liver, characterized the reaction requirements, and were able to distinguish the activity from that of rat liver nuclear DNA polymerase. The mitochondrial enzyme preparations observed by these two groups differed somewhat in their properties. These differences appear to be due to the different levels of purification. Meyer and Simpson (23) noted that sonication yielded higher activity of DNA polymerase than their preferred method of grinding frozen mitochondria with alumina. However, they were unable to recover the former activity from ion-exchange columns successfully employed in the purification of the latter activity. Our attempts to utilize ion-exchange columns in purification of sonic extracted DNA polymerase activity from HeLa cell mitochondria were likewise unsuccessful. It may very well be, as discussed in the following paper (29), that mitochondria contain two DNA polymerases and that the activity we have described here is functionally distinct from that described by the above investigators.

Under conditions optimized with respect to salt, Mg^{++}, and nucleotide concentrations, fraction AS can catalyze extensive synthesis of DNA without degradation of template. Buoyant equilibrium and sedimentation

velocity studies led to the conclusion that most of the product was synthesized in a covalent extension of template DNA strands. Initiation of DNA synthesis in this system is thus template-primed. The initiation sites appear to be the single-strand scissions introduced by the limited endonucleolytic activity in fraction AS. Base sequence fidelity is suggested by the requirement of all four deoxynucleotides or appropriate analogues for DNA synthesis. To maintain fidelity of base sequence, without degrading template, the template strand ahead of the point of polymerization must be displaced to allow base pairing interaction of added nucleotides with the opposing region of the complementary template strand. Electron microscopy provided evidence of such single-stranded regions in circular duplex molecules after DNA synthesis. The assignment of these single-stranded branches as displaced regions of template DNA is supported by the results of studies of the sensitivity of template and product DNA in these molecules to the action of E. coli exonucleases I and III.

The results characterize a mode of action consistent with the "rolling circle" model of Gilbert and Dressler (24). The model in Fig. 17a has a single-stranded tail with a length of about 20% of that of the duplex circle. After denaturation the product was rendered susceptible to digestion by exonuclease I. The product, therefore, does not contain short self-complementary regions at the growing point, such as in Fig. 17b. After an incubation long enough to achieve 10% synthesis, several nicks would have been introduced in most molecules. Electron microscope examination showed that the lengths and frequency of observed single-stranded branches were too small to account for localization of

FIG. 17. Possible structures resulting from DNA synthesis with fraction AS protein and circular duplex DNA template (see "Discussion").


(a)
5′
(b)
3′
5′
(c)

product in one or perhaps two sites per molecule. The formation of a pinwheel structure (Fig. 17c) accounts for all of the results in this work.

The buoyant density of a template-product complex representing the limit of unit extent of synthesis [product/(product + template)], with 5-bromodeoxyuridine 5′-triphosphate instead of dTTP, on an SV40 DNA template is the same as the buoyant density of single-stranded SV40 DNA. The density difference between such a species and thymidine-containing duplex SV40 DNA was calculated using the base composition of SV40 DNA, 41 mole percent dG + dC (19), the 0.20 g per ml density difference between the synthetic polymers dAT and dA$\overline{\text{BU}}$ (25), and the density difference between duplex and single-stranded DNA (26). We estimate the error associated with these values to be at least ±1 mole percent dG + dC, ±0.01 g per ml, and ±1 mg per ml, respectively. The density shift expected for the mode of DNA synthesis described is 126 to 142 mg per ml for unit extent of synthesis. The value determined from the slope in Fig. 11 was 125 mg per ml.

Annealing of complementary displaced strands in the samples under investigation would have maximally lowered the slope by 16 mg per ml. The slope would represent an overestimate of the density shift per unit extent of synthesis if there were large variations in the amount of product DNA among the population of template molecules, leading to different mass distributions of product and template in the density gradients. The assumption of coincident product and template distributions is supported by the electron microscopy studies which suggest that product DNA was localized in several small regions on most of the

template molecules. Molecules with long stretches of product DNA synthesis were rare.

A similar mechanism of template priming of DNA synthesis and subsequent template strand displacement was observed in recent studies with the E. coli DNA polymerase I and nicked circular duplex PM2 DNA template (27). Two important distinctions in mode of action should be noted. The HeLa mitochondrial system (fraction AS) does not show the 5′-exonucleolytic activity of the E. coli DNA polymerase and does not appear to hairpin in the course of DNA synthesis.

Current studies with fraction AS involve the use of the natural template for the system, HeLa mitochondrial DNA. In addition to evidence for base sequence fidelity, we have also observed a preference for the initiation of DNA synthesis on the heavy complement of the mitochondrial DNA. These results are described in the following publication (29). Recent studies of mitochondrial DNA replication in vivo have shown that a template strand is displaced ahead of the growing point in the first stages of replication (28). This aspect of the mode of DNA replication in vivo was likewise observed with the mitochondrial DNA polymerase in vitro.

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CHAPTER II

Asymmetric Synthesis of Mitochondrial DNA with a Partially Purified DNA Polymerase from the Mitochondria of HeLa Cells

(Introductory Statement)

In the preceding paper (1), we described the preparation and mode of action of a partially purified DNA polymerase from the mitochondria of HeLa cells. The preparation contains a low level of endonuclease activity which introduces single-strand scissions into SV40 template DNA. DNA synthesis initiates at most, if not all, of these sites with covalent attachment of product to the primer strand of the template DNA. DNA synthesis proceeds with concomitant template strand displacement and without forming hairpin structures.

We report here the results of studies of the action of this DNA polymerase preparation utilizing its natural template, HeLa cell mitochondrial DNA (mtDNA)[1]. The same mode of DNA synthesis was observed as with SV40 DNA template. We have made use of the buoyant complement separation of mtDNA in alkaline CsCl in experiments which enabled us to assess the fidelity of DNA synthesis. In these experiments we also observed a significant preference for the synthesis of H-strand DNA. This asymmetry of DNA synthesis appears to arise from asymmetric endonuclease action.

EXPERIMENTAL PROCEDURE

Materials

Materials used in this work are described in the "Materials" section of reference (1).

Methods

Preparation of ^{14}C-thymidine Labeled mtDNA—Suspension cultures of HeLa cells (2×10^4 cells per ml) were grown for 48 hr in the presence of 10 μCi per liter [2-^{14}C]-thymidine (Schwarz BioResearch, 57 Ci per mole). The cells were harvested and the mtDNA was isolated as previously described (1). The resulting closed circular mtDNA was mixed with unlabeled mtDNA to provide a sample with approximately 1000 cpm per μg of DNA. The DNA sample was dialyzed exhaustively against 10 mM Tris, 1 mM EDTA, pH 7.5.

Centrifugation in Alkaline CsCl Solutions—A stock solution of 0.25 M $K_2HPO_4 \cdot 3H_2O$, 0.25 M KOH was prepared. The pH of the solution was 12.5, determined in the manner described by Vinograd, Lebowitz, and Watson (2). The CsCl solutions used in sedimentation velocity experiments at alkaline pH contained 50 mM phosphate from this stock solution. For buoyant equilibrium density gradients the DNA sample (less than 0.3 ml) was mixed with 0.65 ml of the alkaline stock solution and distilled water was added to a volume of 2.50 ml. 3.30 g solid CsCl was added to raise the density to 1.74 g per ml. The final pH was 12.1. Centrifugation followed for at least 56 hr at 35,000 rpm, 20 to 25°. Band sedimentation was performed with a sample of DNA (less than 0.2 ml)

adjusted to 50 mM K_3PO_4 and layered onto 3.5 ml alkaline CsCl solution with a density of 1.50 g per ml. Centrifugation followed for 60 min at 60,000 rpm, 20°, in the SW65Ti rotor.

Evaluation of Radioactivity in H- and L-Strand mtDNA—The separation between the centers of H- and L-strand distributions was 5.0 fractions (100 μl each), in good agreement with the difference in buoyant density of the complements of human mtDNA, 41 mg per ml (3), and the density gradient calculated as previously described (1). This result facilitated estimation of band centers of H- and L-strand distributions.

Two methods were used to analyze the radioactivity distributions of mtDNA in alkaline CsCl buoyant density gradients. When the radioactivity at the central minimum was less than half the radioactivity at the lesser maximum, a vertical line was drawn equidistant from the visually estimated band centers. The radioactivity in the two fractions adjacent to this line was allocated to H- and L-strand DNA in the proportion of the total radioactivity of all fractions on the respective sides of the line. When the vertical line passed through, or was very near to, a fraction and the two adjacent fractions contained approximately the same amount of radioactivity, the total radioactivity in these three fractions was allocated to the H- and L-strand DNA as for the two fractions in the case above. When greater overlap of the two distributions precluded application of this method, a pair of symmetrical bands was constructed about the estimated band centers using the radioactivity distributions outside of these centers (see Fig. 3). These bands provide

overestimates of the H- and L-strand DNA because the overlap extends into the outer halves of the distributions. This overlap can be accurately estimated and subtracted if the portions of the symmetrical bands which lie in the outer halves of the opposite distributions correspond to regions of the original distribution unaffected by overlap. The sum of H- and L-strand radioactivity determined in this manner was the same (within 5%) as the total radioactivity in the original distribution. The background radioactivity was constant through gradients used for the estimation of H- and L-strand DNA.

Other Methods—Other procedures used in this work are described in the "Methods" section of reference (1).

RESULTS

Thymidine Bias in the Complementary Strands of HeLa mtDNA—^{14}C-dT labeled mtDNA was banded in an alkaline CsCl equilibrium buoyant density gradient. Fractions were collected onto paper filters for radioactivity analysis. The distribution of radioactivity is shown in Fig. 1. The radioactivity in each band was evaluated and the value of ^{14}C-dT(H/L) was found to be 1.24. This ratio is in good agreement with the ratio of the mole percent of thymidine in the two complements, determined in independent analysis of base compositions[2] (see Table II). Using the density gradient, calculated as previously described (1), the separation between the band centers corresponded to the expected (4) difference of 42 mg per ml between the buoyant densities of the H- and L-strands.

Covalent Initiation of DNA Synthesis—Two product DNA samples were prepared with ^{3}H-dATP and ^{14}C-dT labeled mtDNA template. One of the reactions contained dTTP and the other an equivalent amount of 5-bromodeoxyuridine 5′-triphosphate. The reaction conditions are described in the legend for Fig. 2. The reactions were stopped after 20 min at 37° by addition of EDTA and chilling to 4°. Before and after the incubation, aliquots were removed for acid precipitation on paper filters and radioactivity analysis. The extent of incorporation of ^{3}H-dA into product DNA was the same in each reaction and there was no corresponding loss of the ^{14}C-dT labeled template DNA. The extent of synthesis, [product/(product + template)], after 20 min was 0.04. The

Figure 1. Distribution of the complements of HeLa mtDNA in a buoyant alkaline CsCl density gradient. A sample of closed circular ^{14}C-dT labeled mtDNA was banded in an alkaline CsCl density gradient as described in "Methods." Fractions (70 μl each) were collected from the bottom of the gradient onto paper filters and dried prior to radioactivity analysis. The density increases from right to left in this and subsequent buoyant equilibrium experiments. The ^{14}C-dT(H/L) ratio was 1.24.

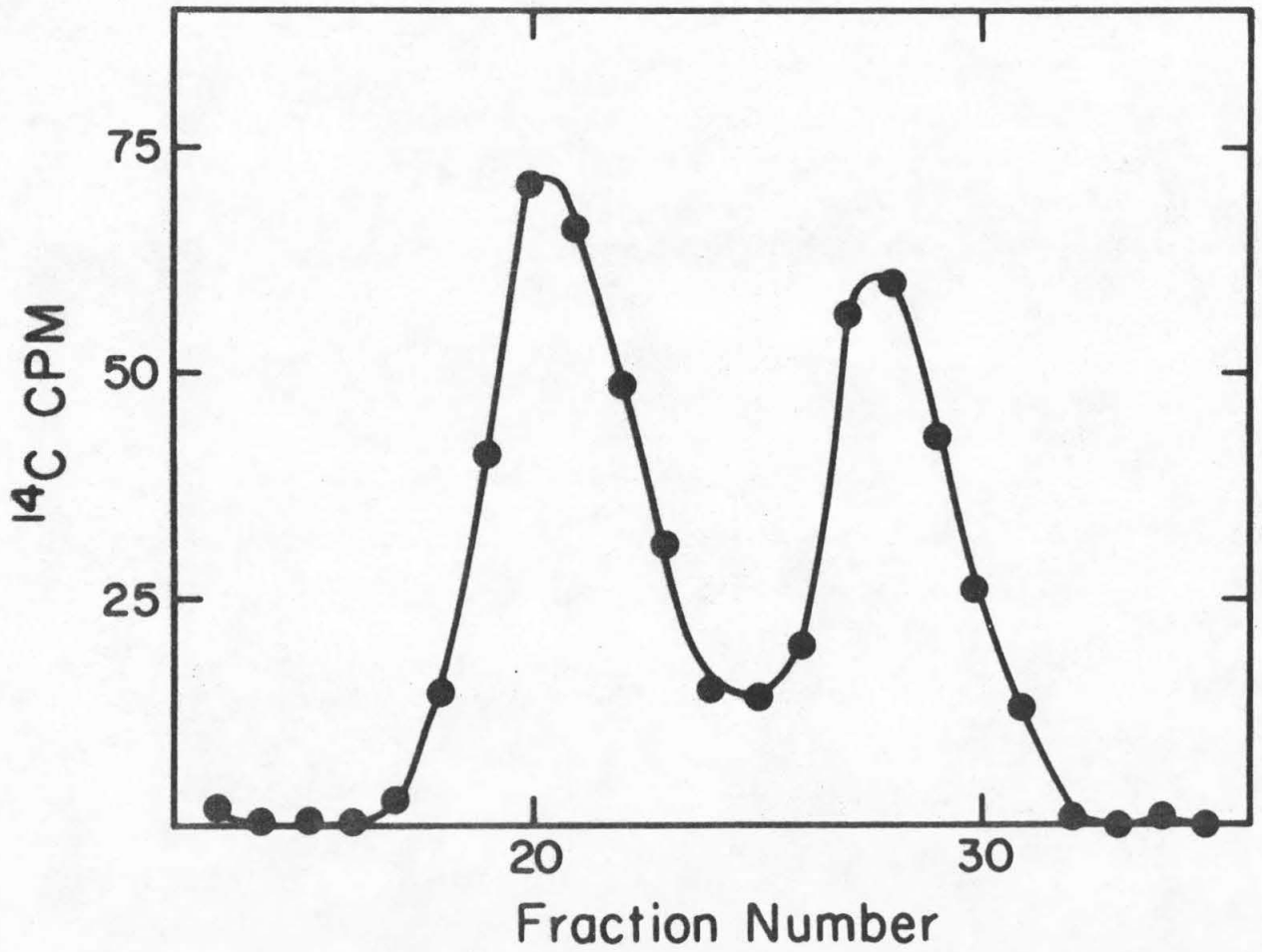
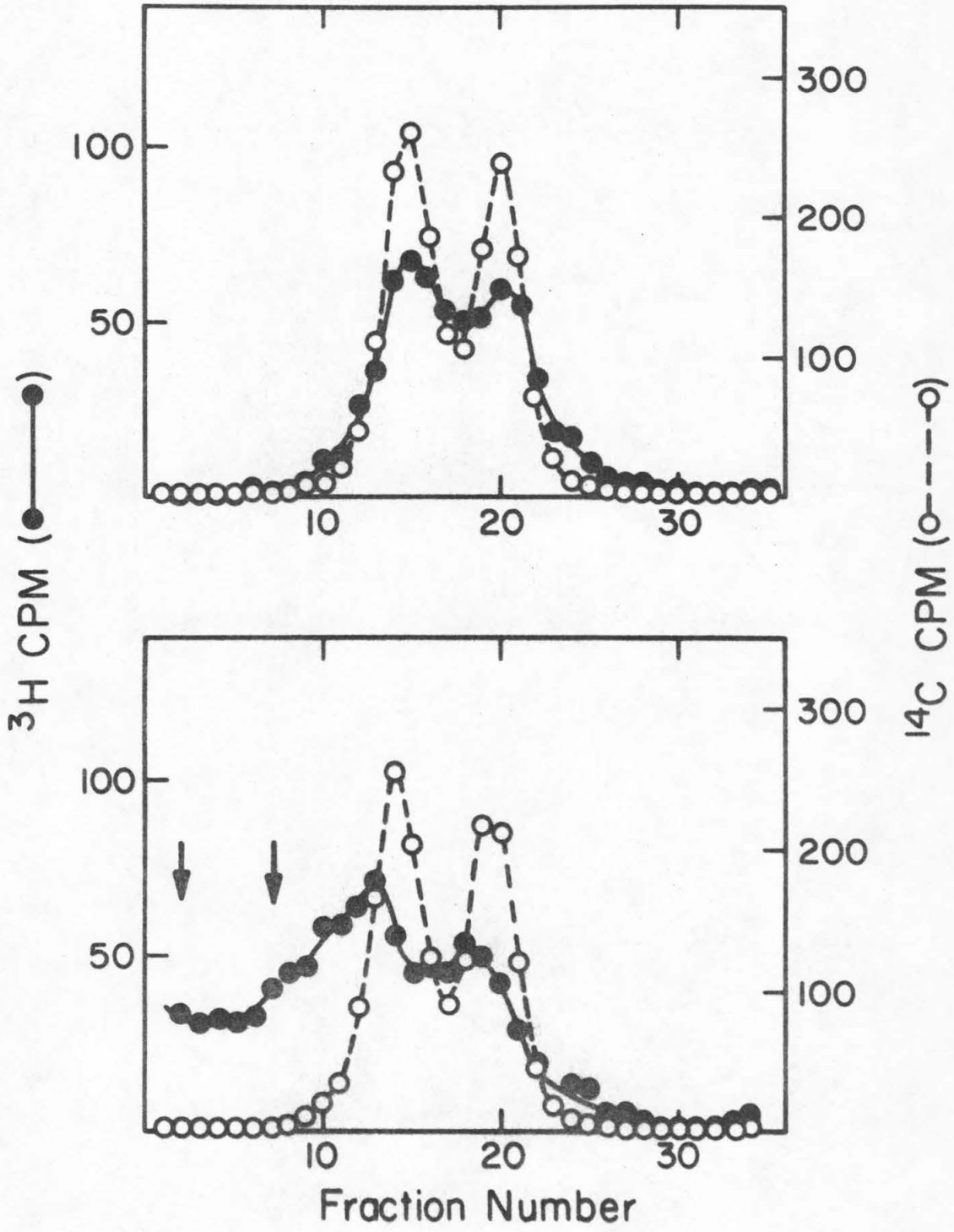

75
50
25
^{14}C CPM
20
30
Fraction Number

Figure 2. Distribution of ^{3}H-dA-labeled product DNA, containing dT (upper figure) or 5-bromodeoxyuridine (lower figure), and ^{14}C-dT labeled mtDNA template in alkaline CsCl buoyant density gradients. Product DNA was synthesized during a 20-min incubation at 37° with 103 μg per ml fraction AS protein, 23 μg per ml ^{14}C-dT labeled mtDNA, 10 mM Tris, (pH 7.5), 3 mM β-mercaptoethanol, 33mM NaCl, 3mM $MgCl_2$, 0.3 mM EDTA, 70 μM ^{3}H-dATP (260 Ci per mole), 175 μM dGTP, 158 μM, dCTP, and either 309 μM dTTP (upper figure) or 309 μM 5-bromodeoxyuridine 5′-triphosphate (lower figure). Following centrifugation as described in "Methods," fractions (100 μl each) were collected from the bottom of the gradients onto paper filters and subjected to batchwise acid washing prior to radioactivity analysis. The arrows indicate the position expected for fully 5-bromodeoxyuridine-labeled mtDNA complements (4).


100
50
300
200
100
10
20
30
^{3}H CPM (●)
^{14}C CPM (○)
Fraction Number

specific activity of the DNA polymerase in fraction AS under these conditions was 18 units per mg protein and was about the same as we had observed with other preparations of fraction AS in reactions with SV40 DNA template under otherwise identical conditions (1). This is consistent with the displacement of a template strand in the course of DNA synthesis as described previously (1).

The two samples were banded in alkaline CsCl buoyant density gradients. As seen in Fig. 2 (upper figure) the product from the reaction containing dTTP appears in two bands coincident with the H- and L-strands of the template DNA. In Fig. 2 (lower figure) the distribution of product DNA from the second reaction also has two maxima, but each is shifted slightly towards the dense side of the respective H- and L-strand bands of the template DNA. The distribution of product DNA is skewed towards the dense region of the gradient, but no peaks occur in the positions expected (4) for fully 5-bromodeoxyuridine labeled product DNA strands (indicated by the two arrows). This result indicates that most of the product is covalently bonded to template DNA strands. Material in the dense region of the gradient (lower figure) may have arisen through fragmentation of strands, containing stretches of product and template DNA, by endonucleolytic action during the incubation or hydrolysis during alkaline centrifugation. The complement separation of closed circular HeLa mtDNA in alkaline CsCl, Fig. 1, derives from its alkaline sensitivity. However, we cannot exclude the possible non-covalent initiation of the synthesis of a minor fraction of the product, as suggested in the recent mtDNA replication studies of Kasamatsu, Robberson and Vinograd (5).

The ^{14}C-dT(H/L) ratios of the template DNA were 1.25 and 1.23 in the upper and lower figures respectively. These are in good agreement with the earlier determination of the thymidine bias in HeLa mtDNA, Fig. 1.

Fidelity and Asymmetry of DNA Synthesis — The mode of action of the DNA polymerase activity in fraction AS and the complement separation of mtDNA in alkaline CsCl density gradients suggested the following test of the fidelity of DNA synthesis. If the product of DNA synthesis is complementary to the opposed template strand, and if the regions of DNA synthesis are distributed in a representative manner, the relative amount of a radioactive nucleotide incorporated into product attached to H- and L-strand DNA should reflect the strand bias of that nucleotide in mtDNA.

Three samples were prepared in reactions under the same conditions except that the ^{3}H-label in the product DNA appeared in dT, dA, or dC. The samples were banded in alkaline CsCl density gradients. Fractions were collected from the bottom of each gradient onto paper filters for analysis of the distributions of acid precipitable radio-activity, Fig. 3. The radioactivity in the H- and L-strand bands was evaluated as described earlier, Table I. These data show a significant asymmetric synthesis of mtDNA, which we express numerically as the ratio of nucleotides incorporated into H-strand relative to L-strand product DNA. The ^{3}H-dT(H/L) for the product DNA in this experiment, 1.84, and the ^{14}C-dT(H/L) value of 1.24, observed previously for the uniformly labeled closed circular mtDNA,

Figure 3. Distribution of radioactivity with ^{3}H-dT, ^{3}H-dA, and ^{3}H-dC labeled product DNA in alkaline CsCl buoyant density gradients. Three reactions contained 180 μg per ml fraction AS protein, 6 μg per ml HeLa mtDNA, 10 mM Tris (pH 7.5), 3 mM β-mercaptoethanol, 33 mM NaCl, 3 mM $MgCl_2$, 0.3 mM EDTA. Nucleotides included were 155 μM dATP (replaced by 157 μM H-dATP, 260 Ci per mole, center figure), 161 μM dGTP, 146 μM dCTP (replaced by 150 μM ^{3}H-dCTP Ci per mole, lower figure), and 148 μM dTTP (replaced by 149 μM ^{3}H-dTTP, 260 Ci per mole, upper figure). Samples were incubated 30 min at 37° and the reactions were then stopped by addition of EDTA to 50 mM. Centrifugation in alkaline CsCl buoyant density gradients followed as described in "Methods". Fractions (100 μl each) were collected from the bottoms of the gradients onto paper filters and subjected to batchwise acid precipitation prior to radioactivity analysis.

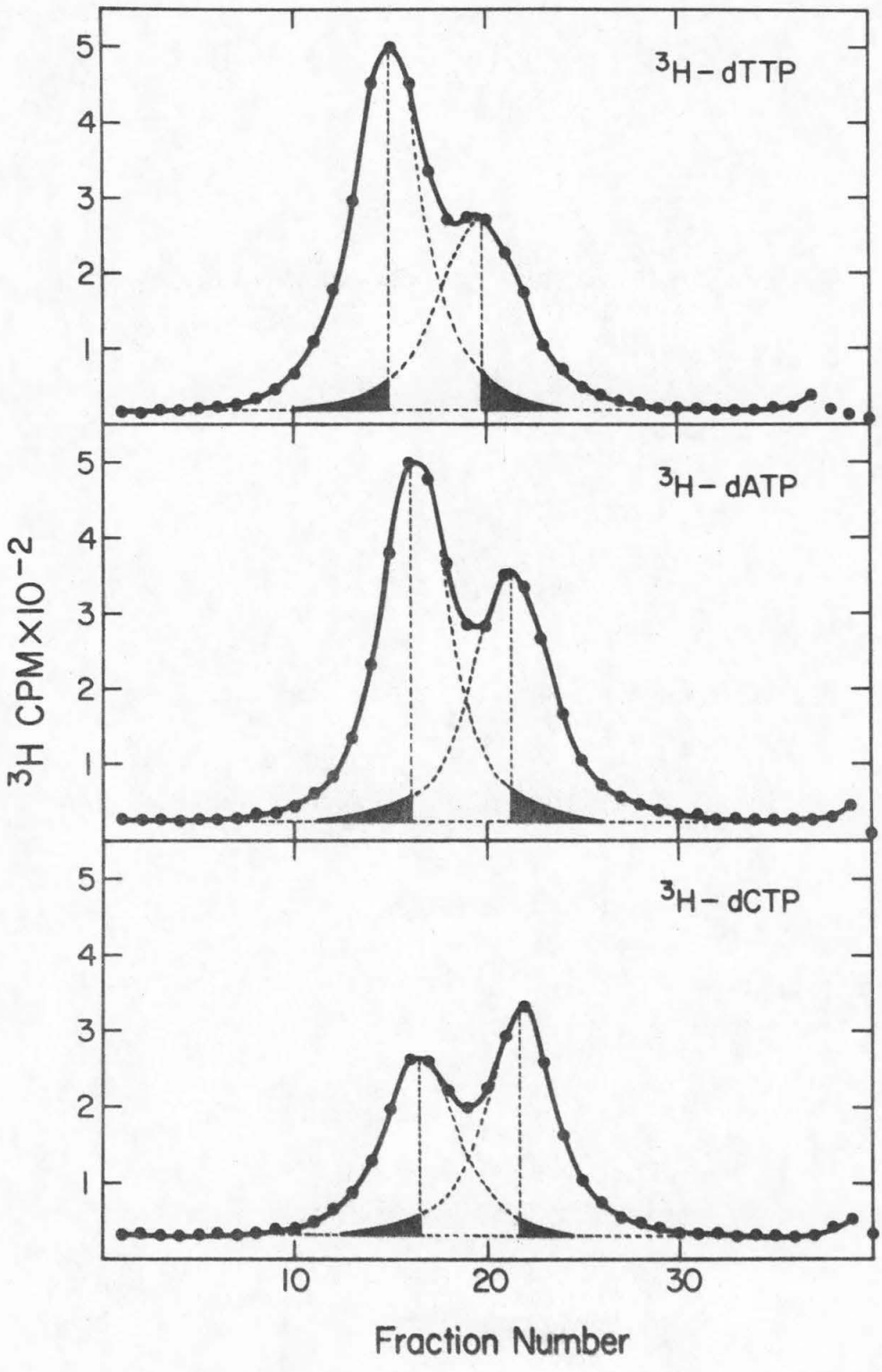

^{3}H – dTTP
^{3}H – dATP
^{3}H – dCTP
^{3}H CPM X 10^{-2}
Fraction Number
10
20
30
1
2
3
4
5

TABLE I

Analysis of radioactivity in H- and L-strand product DNA

The distributions presented in Fig. 3 were analyzed in the manner described in "Methods" to determine the relative amount of H- and L-strand radioactivity in the product DNA of each sample. The subscripts s and c refer to evaluations using the symmetrical distributions before and after correction for overlap, respectively.

Nucleotide	$(H + L)$ cpm $\times 10^{-3}$	$(H_s + L_s)$ cpm $\times 10^{-3}$	$(H_c + L_c)$ cpm $\times 10^{-3}$	H_s/L_s	H_c/L_c
^{3}H-dT	3.59	3.88	3.62	1.74	1.84
^{3}H-dA	3.73	4.08	3.81	1.18	1.19
^{3}H-dC	2.43	2.60	2.45	0.74	0.68

give a value of 1.48 for the asymmetry. The distributions of ^{3}H-dA and ^{3}H-dT labeled product likewise indicate the preferential synthesis of H-strand DNA. Complementarity, in the case of unbiased synthesis of H- and L-strand DNA, requires that ^{3}H-dA(H/L) be the reciprocal of ^{3}H-dT(H/L). This reciprocal relation is obtained by dividing each observed ratio by 1.48. Two methods of evaluating the magnitude of the asymmetry of DNA synthesis thus give the same result. Ratios corresponding to equimolar quantities of H- and L-strand product DNA, (H/L)*, were determined: dT(H/L)*, 1.24; dA(H/L)*, 0.81; dC(H/L)*, 0.46; dG(H/L)*, 2.08. The latter value was calculated as the reciprocal of dC(H/L).

The above results were used to calculate the base compositions of product DNA attached to H- and L-strands. The values of (H/L)* correspond to equimolar quantities of H- and L-strand DNA and are thus representative of the biases expected in duplex mtDNA. The buoyant density of human mtDNA in CsCl corresponds to a dG-dC content of 46 mole percent (6). The relative amount of each nucleotide in H- and L-strand product DNA is readily calculated with the (H/L)* ratios and the mole percent of each nucleotide in duplex mtDNA, Table II. The agreement between the calculated base compositions of product DNA and the results of independent analysis of the base compositions of mtDNA complements indicates that the DNA synthesized with the DNA polymerase in fraction AS is indeed complementary to the mtDNA template.

The asymmetric synthesis of mtDNA in other experiments with different preparations of fraction AS was in the range of 1.2 -

TABLE II

Base composition of HeLa mtDNA complements and the product of DNA synthesis with HeLa mtDNA template

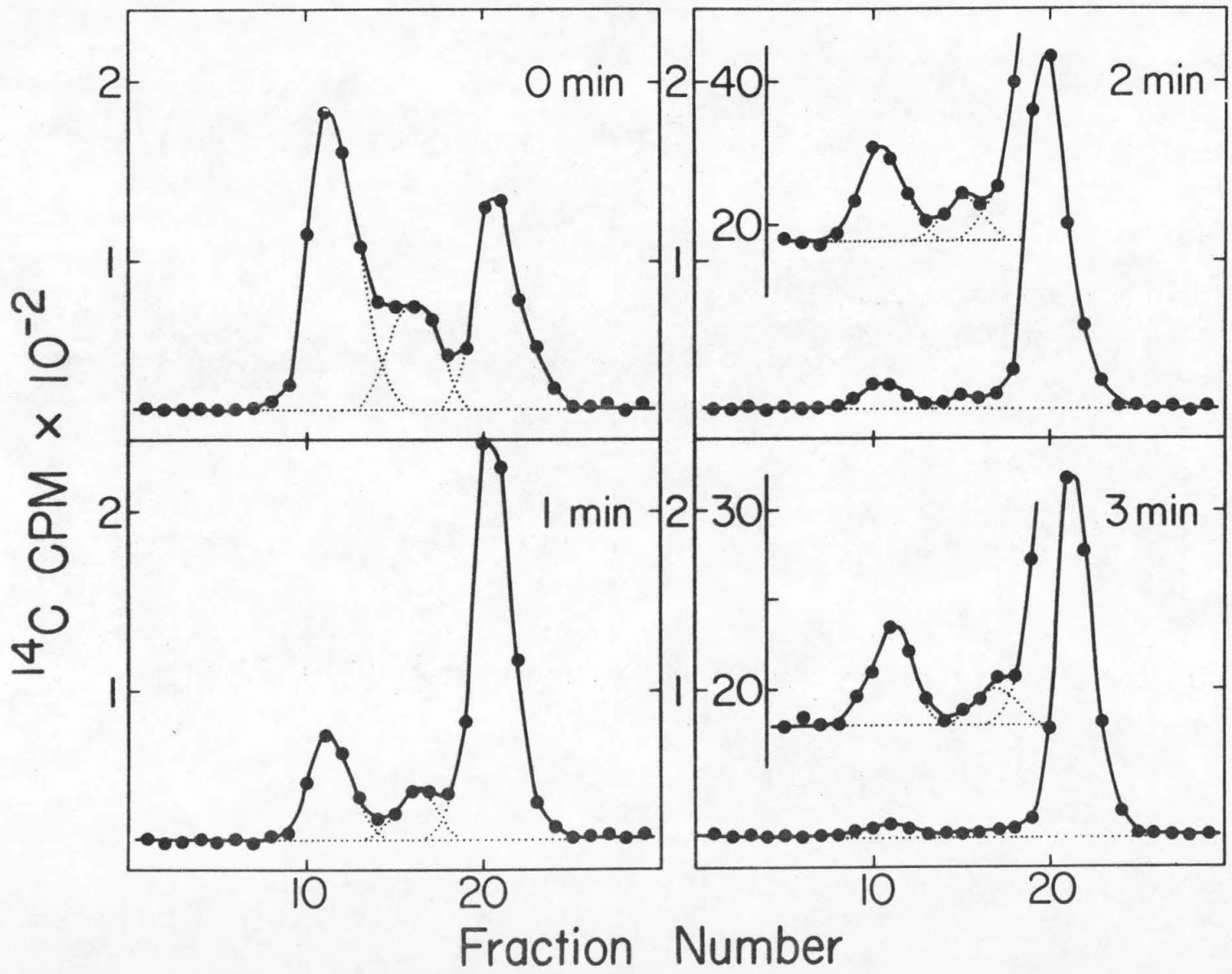
The base composition analysis of template mtDNA was performed by W. M. Brown of this laboratory using hydrolysates of the purified complements of uniformly ^{32}P-labeled HeLa mtDNA. The base composition analysis of product DNA was based on the data presented in Table I and the dG-dC centent of duplex mtDNA determined from its buoyant density in CsCl. The error associated with the values presented in the table is about ±1 mole percent.

Nucleotide	Mole percent in each complement			
	H-strand		L-strand	
	Template	Product	Product	Template
dA	25	24	30	31
dT	31	30	24	24
dG	30	31	15	13
dC	14	15	31	32

to 1.5-fold more H-strand than L-strand product DNA. The predominate mode of action of the DNA polymerase in fraction AS involves covalent initiation at single strand scissions. A strand bias in the action of endonuclease(s) present in fraction AS could account for, as described in the following section, the asymmetric DNA synthesis. Correspondingly, variation in the biased endonucleolytic activity from preparation to preparation could account for the variations of the asymmetry of DNA synthesis noted above.

Asymmetric Endonuclease Action— The endonuclease activity in fraction AS was examined by following the conversion of closed circular mtDNA to nicked mtDNA in the course of DNA synthesis. A reaction mixture containing closed circular ^{14}C-labeled mtDNA, fraction AS protein and the standard complement of four nucleotides was incubated at 37°. Aliquots withdrawn at 1 min intervals were diluted 10-fold with cold 50 mM EDTA. The samples were later banded in CsCl density gradients containing ethidium bromide. Fractions were collected from the bottom of the gradients onto paper filters for radioactivity analysis, Fig. 4. The fraction of closed circular DNA remaining in each sample was evaluated as the sum of the radioactivity in the lower band and half the radioactivity in the intermediate band, divided by the total radioactivity in the gradient. The intermediate bands in these gradients contained partially nicked catenated oligomers of mtDNA (7). These were treated as species in which half the submolecules were nicked. The presence of partially nicked catenated oligomers suggests that the endonucleolytic action is predominately a single strand scission process, as previously observed in experiments with

Figure 4. Distributions of ^{14}C-dT labeled mtDNA in CsCl buoyant density gradients containing ethidium bromide following incubation with fraction AS. The initial reaction mixture contained 130 μg per ml fraction AS protein, 23 μg per ml closed circular ^{14}C-dT labeled mtDNA, 10 mM Tris, pH 7.5, 3 mM β-mercaptoethanol, 33 mM NaCl, 3 mM $MgCl_2$, 0.3 mM EDTA, 309 μM dTTP, 168 μM dATP, 175 μM dGTP, and 158 μM dCTP. The reaction was placed in a 37° water bath and aliquots (about 100 μl each) were then removed at 1 min intervals and diluted 10-fold with cold 50 mM EDTA. Centrifugation of the samples in CsCl density gradients containing ethidium bromide was performed as previously described (1). The fraction of closed circular DNA remaining in each sample was determined and the results were used in the analysis of endonuclease activity in Fig. 5. The inserts in the two right hand figures present the data with an expanded ordinate scale.


0 min
2 min
1 min
3 min
^{14}C CPM x 10^{-2}
Fraction Number
10
20
40
30

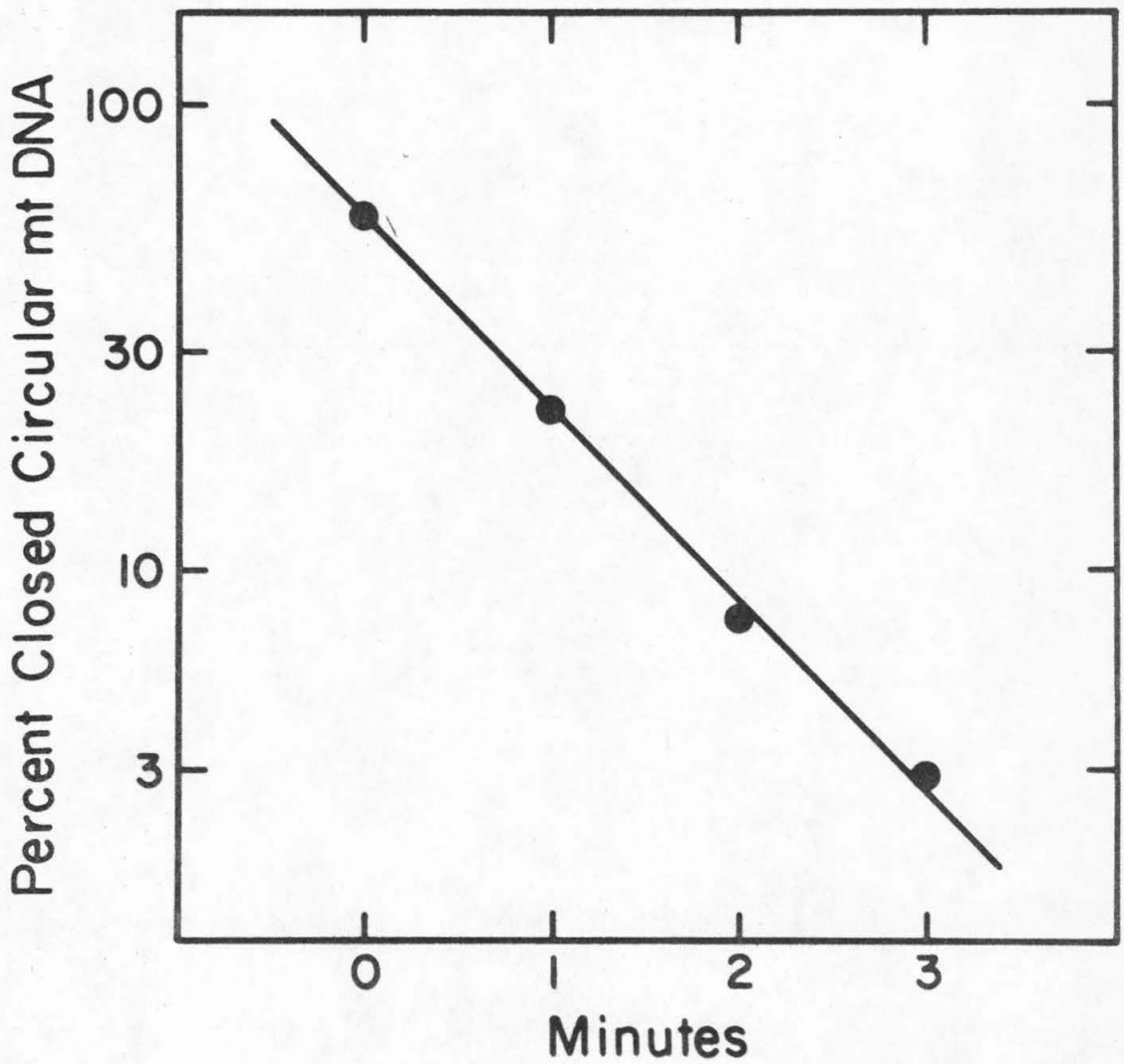
SV40 DNA (1). Double strand scissions would have linearized some submolecules in catenated oligomers and released products which would have appeared only in the upper or lower bands. The first order survival kinetics of the closed circular mtDNA are presented in Fig. 5. The half-life for closed circular mtDNA was 0.71 min which corresponds to a rate of 1.0 scission per monomer per minute.

Single strand scissions, introduced by endonuclease activity in fraction AS, appear to be the sites for the covalent initiation of DNA synthesis. A bias in the number of scissions sustained by the complementary strands of mtDNA would account for, or contribute to the asymmetry of DNA synthesis observed with this template. To examine this possibility we performed the following experiment.

Closed circular mtDNA, labeled with ^{14}C-thymidine, was incubated 15 min at 37° with fraction AS protein and nucleotides including ^{3}H-dATP. The reaction was stopped by addition of EDTA. The sample was denatured prior to alkaline band sedimentation analysis as described in "Methods." A sample of closed circular mtDNA was denatured in the same manner for sedimentation in a parallel gradient. If the average number of scissions sustained by the mtDNA complements differ, then we expect that the average molecular weight of the separated single strand fragments of H- and L-strand DNA will differ accordingly. The preferential synthesis of H-strand DNA leads to the prediction that the H-strand fragments will sediment more slowly, in a denaturing solvent, than the L-strand fragments.

Following centrifugation, fractions were collected from the bottom of the gradients into polyallomer tubes. Aliquots were withdrawn

Figure 5. Survival kinetics of closed circular mtDNA during incubation with fraction AS. The fraction of closed circular DNA in each of the four samples described in the legend for Fig. 4 was determined. The linear decay kinetics presented above show that the closed circular mtDNA molecules have a half-life of 0.71 min under conditions of DNA synthesis.


Percent Closed Circular mt DNA
100
30
10
3
0
1
2
3
Minutes

from each fraction onto paper filters for radioactivity analysis. Limited nicking, resulting from exposure of mtDNA to alkaline conditions, is indicated by the results with the control sample, Fig. 6 (upper figure). Most of the closed circular DNA in the sample is found at the bottom of the gradient. The slower peak corresponds to full length single strands of HeLa mtDNA, 21-22s[3], resulting from denaturation of molecules nicked prior to and during centrifugation. The arrow (upper figure) indicates the position of a sample of ϕX174 phage DNA, about 15s (ref. 8), run in a separate tube in the same rotor. The fraction of closed circular mtDNA recovered, about 60%, suggests an average of 0.5 scission per monomer introduced by alkaline hydrolysis in the course of this analysis.

The sedimentation distribution of the sample of mtDNA incubated with fraction AS is shown in Fig. 6 (lower figure). We estimate from the center of gravity of the template DNA radioactivity distribution that the weight average sedimentation coefficient is 40 to 50% of that for the full length strands. The weight average molecular weight was calculated (9) to be 10 to 18% of the intact strands. This result corresponds (10) to an average of 6 to 10 scissions per strand. The independent endonuclease assay, Fig. 5, predicts an average of 7 to 8 scissions per strand after 15 min incubation.

Fractions from the gradient containing the mtDNA incubated with fraction AS were pooled as indicated in Fig. 6 (lower figure) to give two samples representing larger (F) and smaller (S) fragments. These samples were banded in alkaline CsCl equilibrium buoyant density gradients to assess the relative amount of H- and L-strand DNA in each, Fig. 7. The values of ^{14}C-dT(H/L) were 1.15 and

Figure 6. Alkaline sedimentation velocity profiles of closed circular mtDNA (upper figure) and a sample of mtDNA used as a template for DNA synthesis with fraction AS (lower figure). The reaction mixture for incubation of the latter sample was the same as described in the legend for Fig. 4, except that ^{3}H-dATP (17 Ci per mole) was used and was present at 6 μM. The reaction was stopped by addition of EDTA to 50 mM. Sedimentation analysis was performed as described in "Methods". Fractions were collected (310 μl each) in polyallomer tubes and aliquots were removed for determination of the radioactivity distributions. The distribution of the sample incubated with fraction AS (lower figure) was divided into fast (F) and slow (S) fractions by pooling the remaining portions of fractions 7 through 10 and fractions 11 and 12 respectively. Sedimentation is from right to left.

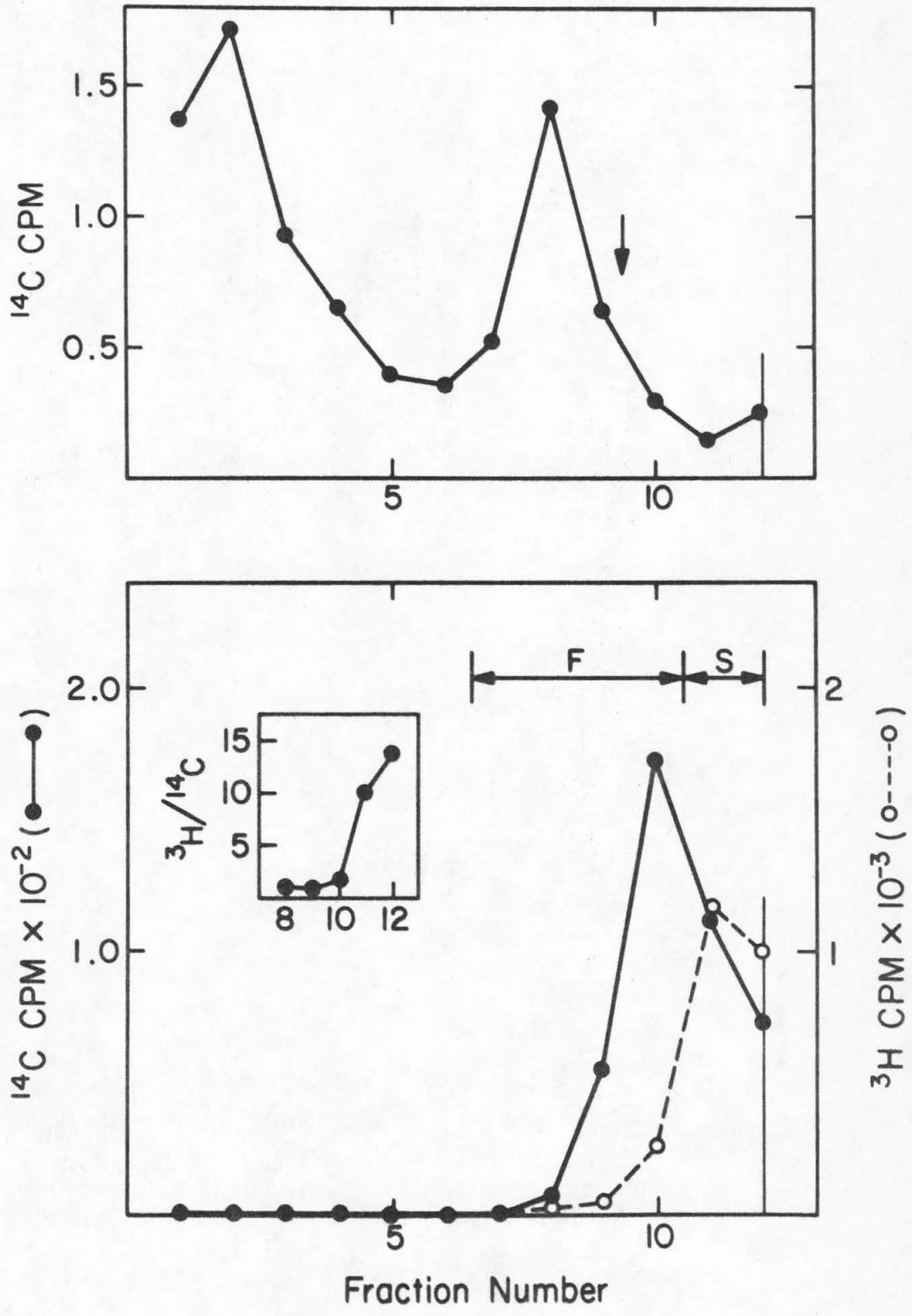
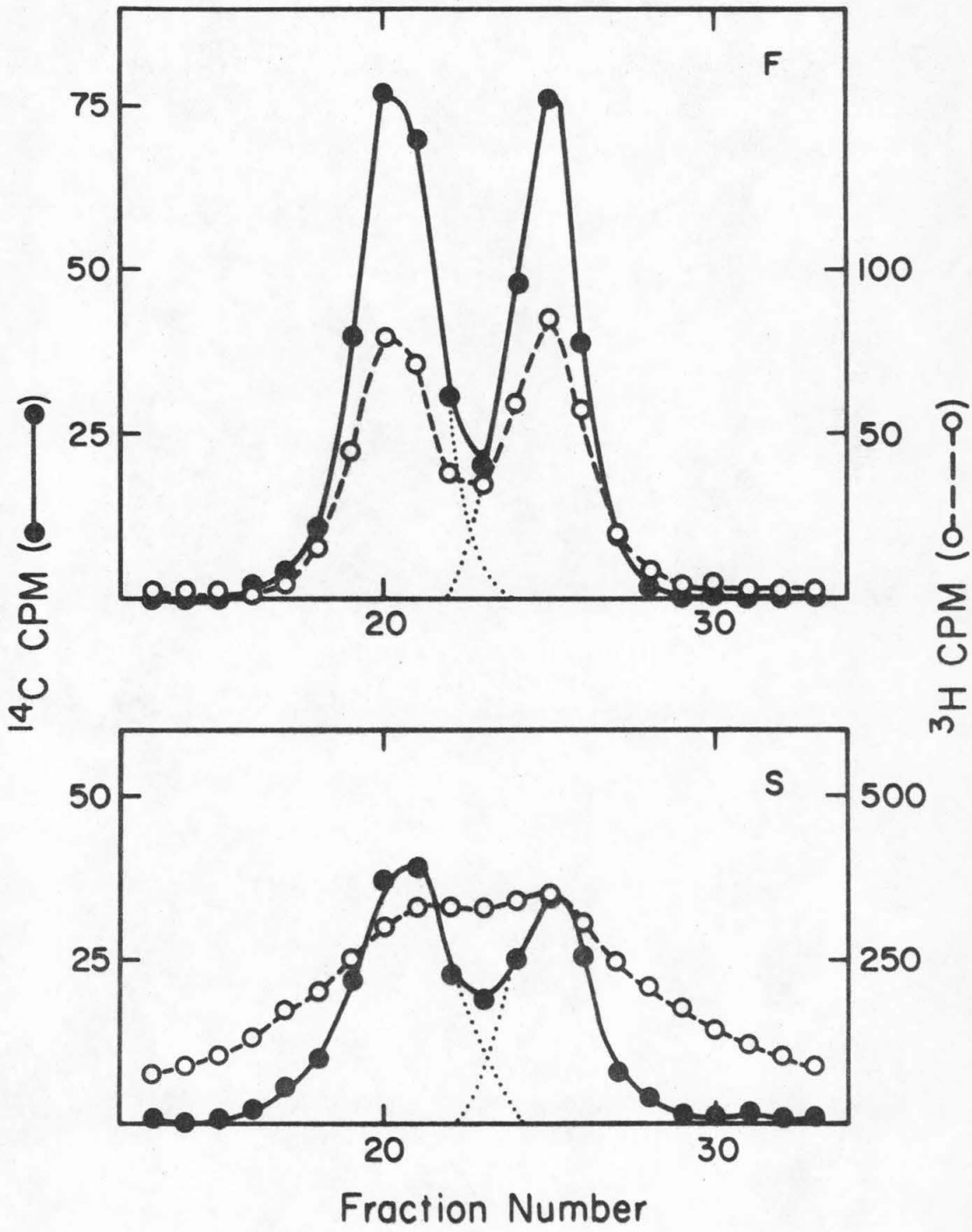

1.5
1.0
0.5
^{14}C CPM
5
10
F
S
2.0
1.0
15
10
5
^{3}H/^{14}C
8
10
12
2
1
^{14}C CPM x 10^{-2} (●——●)
^{3}H CPM x 10^{-3} (o---o)
5
10
Fraction Number

Figure 7. Distribution of larger (F, upper figure) and smaller (S, lower figure) fragments of mtDNA, previously incubated with fraction AS, in alkaline CsCl buoyant density gradients. Fractions F and S are described in the legend for Fig. 6. Centrifugation was performed as described in "Methods". Fractions were collected for radioactivity analysis as described in the legend for Fig. 2.


F
S
75
50
25
100
50
500
250
20
30
^{14}C CPM (●)
^{3}H CPM (o---o)
Fraction Number

1.41 for the template mtDNA in the fast (F) and slow (S) samples respectively. This result indicates a significant bias in the endonuclease activity on the two complements, since the value expected for molar equivalence of H- and L-strand DNA was earlier shown to be 1.24, Fig. 1. When the ^{14}C-dT(H/L) values are averaged in proportion to the total radioactivity in the two fractions, the resulting value is 1.25, for the total sample before fractionation. The observed bias indicates, as predicted, that the H-strand sustained more scissions than the L-strand.

A precise evaluation of the magnitude of the biased endonuclease action is not possible because of the coarse fractionation. We can crudely estimate the average size of the H- and L-strand DNA fragments from the average sedimentation coefficient of the total DNA in the pooled fractions of the fast and slow samples, 14s and 4.5s respectively, and the molar ratios of H- to L-strand DNA in each, 0.93 and 1.14 respectively. The latter values were calculated by dividing the observed ^{14}C-dT(H/L) ratios by 1.24. The ratio of the resultant average molecular weights, 0.89 (H- to L-strand) sets a lower limit for the nicking bias, 1.13-fold more scissions in the H-strands than in the L-strands. This would be the expected lower limit of the asymmetric, preferential synthesis of H-strand DNA in the total sample. The ratios of H-strand to L-strand product DNA were estimated in the fast and slow fractions from the values of ^{3}H-dA(H/L) and the value previously obtained for dA(H/L)*. The ^{3}H-dA(H/L) was 0.95 in the product DNA in the fast sample. While the value of ^{3}H-dA(H/L) in the slow sample cannot be measured because of the

extensive overlap, it is still safe to estimate an upper limit of 1.0. The value of 0.95 and the latter upper limit of 1.0 allow us to set an upper limit of 1.23 for the asymmetry in the total product DNA. While the lower limit of the nicking bias, 1.13, and the upper limit of the asymmetric synthesis, 1.23, are consistent with the proposed role of asymmetric endonuclease activity, we cannot conclude that this activity is the sole reason for asymmetric DNA synthesis. It should be noted, however, that if the preferential nicking of the H-strand takes place early in the incubation period, the overall nicking bias observed at later times might be less than the value for the resulting asymmetry of DNA synthesis.

DISCUSSION

DNA synthesis catalyzed by the mitochondrial DNA polymerase in fraction AS has been investigated with the natural template, HeLa cell mtDNA. The specific activity of the DNA polymerase was the same with mtDNA as with SV40 DNA template. Density shift experiments demonstrated that most of the product was synthesized in a covalent extension of template DNA strands, as previously observed with SV40 DNA template (1). The base compositions of the product strands were found to be the same as in the template strands to which the product DNA was attached. If hairpin events had occurred in the course of DNA synthesis, the base compositions of product DNA, attached to either complement, would have more closely resembled that of duplex mtDNA. Hairpin structures were absent in the product synthesized with SV40 DNA template as determined by E. coli exonuclease I digestion experiments and electron microscopy. The base composition experiment also revealed a significant preference for the synthesis of H-strand mtDNA. It was shown that the residual endonucleolytic activity in fraction AS gave rise to more scissions in the H- than in the L-strand, thereby providing more sites for initiation of DNA synthesis on the H-strand. However, we cannot exclude at this time that other factors, such as different average lengths of regions of H- and L-strand product DNA, might also contribute to the asymmetric synthesis of mtDNA.

Kalf and Ch'ih (11) and Meyer and Simpson (12) have described the properties of partially purified DNA polymerase from rat liver mitochondria. It is now believed (13) that these investigators have

studied the same enzyme and that the differences between the observed reaction properties were due to the different levels of purification. The preparative procedure involved disruption of mitochondria by freeze-thawing, pelleting of insoluble material, ammonium sulfate precipitations, and DEAE-cellulose chromatography (11, 12). We have been unable to recover the DNA polymerase activity in fraction AS, derived from sonicated HeLa cell mitochondria, from such ion-exchange columns (1). Meyer and Simpson have more recently noted that sonication of rat liver mitochondria led to greater specific activity of DNA polymerase in the extracts but the activity was lost on subsequent ion-exchange chromatography (13). The rat liver mitochondrial DNA polymerase is stimulated by high salt concentrations. The optimum cation concentrations described by Meyer and Simpson (12, 13) were about 8 mM Mg^{++} and 150 mM Na^{+}, and a 7- to 10-fold stimulation occurred on adding the Na^{+}. With fraction AS 3 mM $MgCl_2$ was optimum and there was only a 60% enhancement in the presence of the optimum concentration of NaCl, 30 mM. At 100 mM NaCl the reaction was inhibited 70% relative to optimum conditions. The preference for native or denatured DNA templates is an area of major discrepancy in the work of the two groups studying the rat liver mitochondrial DNA polymerase. The most highly purified preparation, described by Meyer and Simpson (13), does show a significant preference for denatured DNA templates. The DNA polymerase in fraction AS shows a preference for native over heat-denatured calf thymus DNA template (1). These observations suggest that the activity which we have studied with fraction AS from sonicated HeLa cell mitochondria may be functionally

distinct from the partially purified DNA polymerase extracted by freeze-thawing of rat liver mitochondria.

The intriguing notion of two functionally distinct DNA polymerases in mammalian mitochondria is also inferred from the results of recent in vivo studies of mtDNA replication in mouse cells (5, 14). These studies have indicated that the parental H-strand is displaced in the course of H-strand DNA synthesis and that the L-strand is synthesized on the resulting single-stranded regions in the replicative intermediates. Structures similar to mouse cell replicative intermediates have also been observed in HeLa cell mtDNA[4]. The DNA polymerase in fraction AS may be the enzyme associated with the synthesis of that complement of HeLa mtDNA which is made in vivo with template strand displacement. This suggestion is in accord with the observed mode of action of fraction AS and with the decreased activity of the DNA polymerase on denatured DNA template. However, the ability of the DNA polymerase to effect DNA synthesis on well-defined, partially single-stranded templates, without template strand displacement has not yet been investigated. The preference of the rat liver mitochondrial DNA polymerase for denatured templates suggests that it may be associated, in vivo, with DNA synthesis on displaced, single-stranded regions in the replicative intermediates.

(Footnotes)

[1]The abbreviation used is: mtDNA, mitochondrial DNA. H-strand and L-strand refer to the complementary strands of mtDNA with greater or lesser buoyant density in alkaline CsCl, respectively. (H/L) refers to the ratio of the radioactivity appearing in the H-strand band to the radioactivity in the L-strand band in alkaline CsCl density gradients. The radioactive nucleotide in the DNA sample for which this ratio is evaluated is identified as: ^{14}C-dT(H/L), ^{3}H-dC(H/L), etc. Fraction AS is the HeLa cell mitochondria as previously described (1).

[2]W. M. Brown, unpublished results.

[3]C. A. Smith, unpublished results.

[4]P. J. Flory, Jr., unpublished results.

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CHAPTER III

Preliminary Investigations of DNA Synthesis in Isolated Mitochondria of HeLa Cells

INTRODUCTION

Borst (1,2) has thoroughly reviewed the results of studies of the incorporation of deoxyribonucleotides into DNA by mitochondria isolated from a variety of organisms. Rat liver and chick liver mitochondria have been the most extensively studied systems. The comparison of results obtained by different investigators is hindered by discrepencies attributable, in all likelihood, to different methods of preparation and incubation of mitochondria. However, the following general observations can be made. DNA synthesis in isolated mitochondria is dependent on the presence of magnesium ion and four deoxyribonucleoside triphosphates. The incorporation of nucleotides into DNA is unaffected by the presence of pancreatic DNase and does not require the addition of exogenous DNA template. The maximum rate of DNA synthesis observed with liver mitochondria is from 3 to 10 pmole total nucleotide incorporated per hour per mg mitochondrial protein (1,3). This corresponds to approximately 0.3 to 1.0 % net synthesis of mtDNA[1] per hour, based on the amount of endogenous mtDNA per mg mitochondrial protein (1). Rat liver mitochondria sustain linear incorporation of nucleotides for up to two hours (4), whereas the chick liver mitochondrial

system shows a sharp decline in the rate of nucleotide incorporation after 10 to 15 minutes (3,5).

The identity of newly synthesized DNA as mtDNA was suggested in studies of its buoyant density in CsCl and supported by its ability to renature under defined conditions (6). Ter Schegget and Borst (3) have found that most of the product synthesized by chick liver mitochondria cosedimented with closed circular mtDNA in neutral sucrose gradients and banded with closed circular mtDNA in CsCl density gradients containing ethidium bromide (3, see also ref. 5). The remaining fraction of the product cosedimented with nicked circular mtDNA. Karol and Simpson showed that rat liver mitochondria can incorporate 5-bromodeoxyuridine into DNA when dTTP is replaced with 5-bromodeoxyuridine 5'-triphosphate in the reaction mixture. This led to a shift in the buoyant distribution of product DNA, in neutral CsCl density gradients, to an average density greater than the buoyant density of thymidine-containing mtDNA. They believed the observed density shift to be too large for the incorporation to have resulted from a repair process (7). Ter Schegget and Borst (8) have used 5-bromodeoxyuridine to study the DNA synthesized by chick liver mitochondria. In neutral CsCl density gradients, the product DNA was not shifted to greater density in the case of 5-bromodeoxyuridine incorporation, in contrast the results of Karol and Simpson above. However, the

extent of synthesis was much less than that obtained by the latter investigators. They did observe that sonication led to fragments containing product DNA which banded at the expected hybrid position in a CsCl density gradient. Ter Schegget and Borst also observed that lower band product[2] was released as small fragments, under denaturing conditions, which sedimented with a peak at about 10 s. On release of the product DNA, the parental mtDNA still sedimented as closed circular mtDNA. This result and subsequent experiments (9) suggested that the product DNA was associated with closed circular mtDNA as a small, hydrogen bonded, template displacing strand. The proposed structure is the same as the mtDNA species containing D-loops, recently characterized in studies of mouse cell mtDNA by Kasamatsu, Robberson, and Vinograd (10).

The *in situ* synthesis observed by Ter Schegget and Borst appears to involve extension of pre-existing displacing strands of D-loop molecules by about 10% (9). Further synthesis would require a swivel to allow unwinding of the parental strands in the course of DNA synthesis. The topological restriction associated with DNA synthesis on closed circular DNA may account for the striking decrease in the rate of DNA synthesis early in the incubation period. The rat liver mitochondrial system studied by Simpson and co-workers shows linear incorporation kinetics for up to two hours, but the

fraction of product appearing in closed circular mtDNA has not been determined in this system.

I have undertaken a preliminary investigation of DNA synthesis in HeLa cell mitochondria. Mitochondria from this rapidly dividing cell line were expected to sustain higher rates of <u>in situ</u> DNA synthesis than liver mitochondria. Gentle homogenization techniques, applicable to HeLa cells, afford quantitative rupture of the cells without fragmentation of nuclei. This is an important advantage in the preparation of mitochondria free of nuclear contamination. Furthermore this system allows radioactive labeling of the endogenous mtDNA, a distinct advantage in light of the small quantities of mtDNA that are generally available.

The HeLa mitochondrial system sustains linear kinetics of nucleotide incorporation into DNA for at least one hour. The rate of DNA synthesis is 10 to 40 times greater than reported in studies of liver mitochondria. The product associated with closed circular mtDNA does not accumulate after about 10 min incubation at 37°, whereas the amount of product associated with nicked mtDNA continues to increase. The product DNA was found to undergo the buoyant complement separation expected (11) of mtDNA in alkaline CsCl density gradients. Furthermore a significant bias for the synthesis of L-strand DNA was observed in the product associated with nicked mtDNA. These results are particularly

intriguing in light of recent *in vivo* studies of the replication of mtDNA in mouse cells (10, 12).

METHODS

Preparation of HeLa Cell Mitochondria --- Purified HeLa cell mitochondria (13) were resuspended in Mod. MS buffer (0.21 M mannitol, 70 mM sucrose, 50 mM Tris, pH 7.5, 25 mM KCl, and 2.5 mM $MgCl_2$) and pelleted 10 min at 10,000 x g. The pellet was resuspended in Mod. MS buffer to a final volume corresponding to 0.5 ml per ml packed cells from which the mitochondria were isolated. Extraction of mtDNA from mitochondria prepared in this manner yields 2 to 3 μg mtDNA per ml of the suspension.

Reaction Mixture for in situ DNA Synthesis --- 0.5 ml of the mitochondrial suspension in Mod. MS buffer was mixed with 0.5 ml of a solution containing 0.1 mg per ml dATP, 0.1 mg per ml dGTP, 0.1 mg per ml dCTP, 1.0 mg per ml ATP, 50 mM Tris, pH 7.5, 3 mM $MgCl_2$, and 10 mM β-mercaptoethanol. 50 μl of 1 mg per ml DNase (Sigma, DN-C), 1 mg per ml RNase (Sigma), 50 mM Tris, pH 7.5, and 25 μl ^{3}H-dTTP (1.8 nmole, 7 Ci per mole) were then added. The mixtures were incubated at 37° in test tubes (0.5 inch x 4 inch) with vigorous stirring. The tubes were not covered during the incubations.

Isolation of mtDNA Following Incubation --- Reactions, 1.1 ml, were stopped by adding 1 to 2 volumes of cold 50 mM EDTA. SDS was added to 20 mg per ml and after

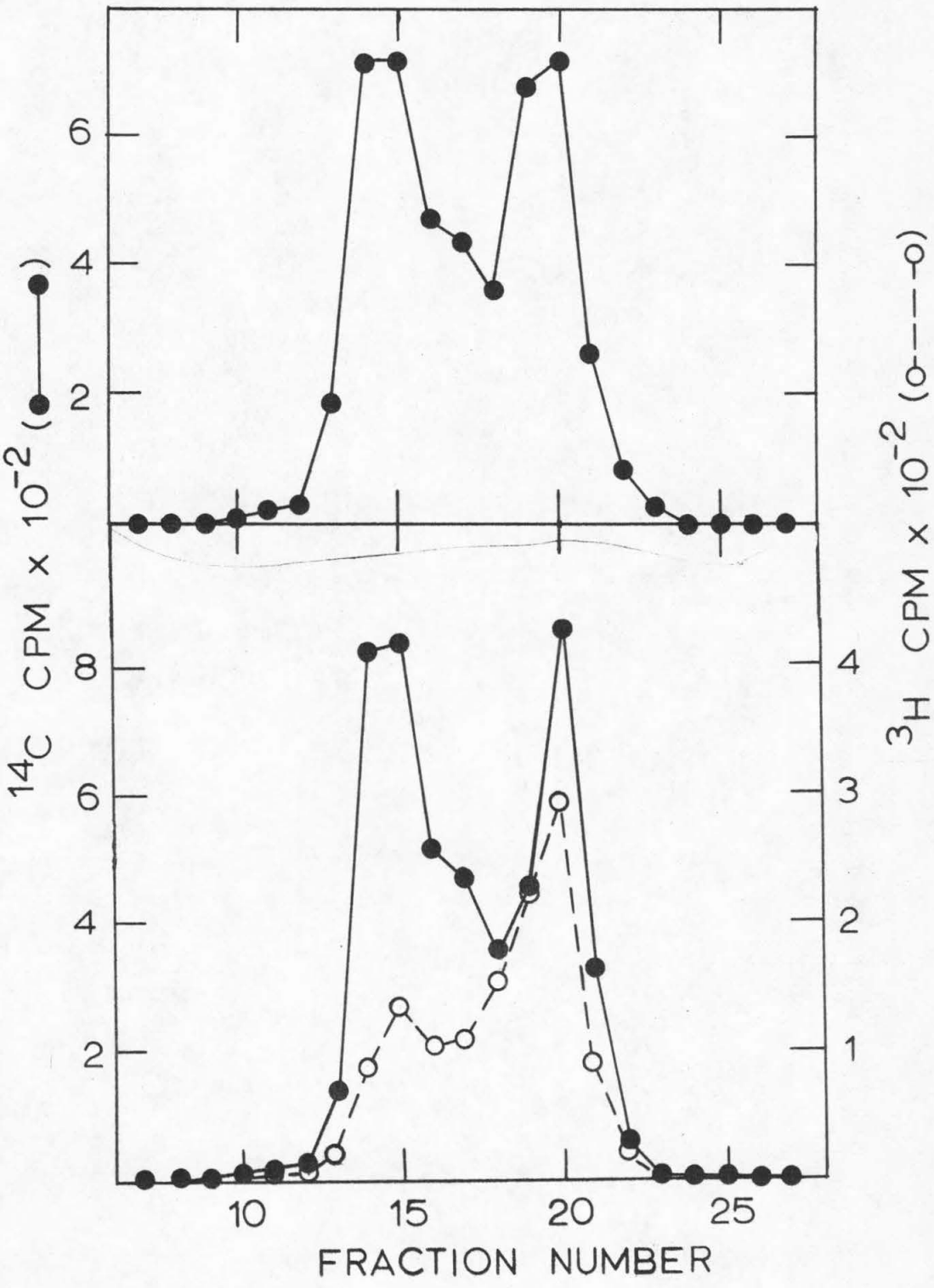
10 min at room temperature, solid CsCl was added, 1.0 g per ml solution, to raise the density to 1.60 g per ml. The sample was chilled to 4°. Centrifugation followed for 10 min at 10,000 x g. The clear solution was recovered from beneath the resulting pellicle. Ethidium bromide (stock solution 5 mg per ml) was added to a final concentration of 250 μg per ml. The final density was 1.55 g per ml. Samples were banded at 35,000 rpm, 20°, for 36 to 48 hr in an SW50.1 rotor.

RESULTS

Appearance of Product in mtDNA --- HeLa cells were grown in the presence of [2-^{14}C]-thymidine as described previously (14). Mitochondria were prepared as described in "Methods". Two reactions were incubated 20 min at 37°. One of the reactions lacked dCTP and ^{3}H-dTTP. The DNA was extracted from the mitochondria and banded in CsCl density gradients containing ethidium bromide. Fractions were collected from the bottom of the gradients onto paper filters for batchwise acid washing and radioactivity analysis, Fig. 1. The distributions of the parental, ^{14}C-labeled mtDNA were the same in each sample. ^{3}H-labeled product DNA, Fig. 1 (lower figure), was found in the upper and lower bands as well as through the intermediate regions of the gradient. This result suggests that product DNA appears in closed circular as well as nicked mtDNA. The product DNA in the intermediate regions of the gradient may be associated with partially nicked catenated oligomers of HeLa mtDNA (15). The total ^{3}H-radioactivity in the gradient indicates that about 0.05 μg DNA (27 mole percent dT) was synthesized during the incubation. This represents 5 % of the endogenous mtDNA in the reaction.

Several similar experiments have been performed which

Fig. 1. Distributions of ^{14}C-labeled endogenous (●——●) and ^{3}H-labeled product (O---O) mtDNA in CsCl buoyant density gradients containing ethidium bromide. Two reaction mixtures, one lacking dCTP and ^{3}H-dTTP (upper figure), were incubated 20 min at 37°. The mtDNA was extracted from each and banded in the CsCl-ethidium bromide density gradients as described in "Methods". Fractions (100 μl each) were collected from the bottom of the gradients onto paper filters for batchwise acid washing and radioactivity analysis. The density increases from right to left. Fractions above and below the regions illustrated contained no radioactivity above background.


6
4
2
8
6
4
2
4
3
2
1
10
15
20
25
FRACTION NUMBER
^{14}C CPM x 10^{-2} (●——●)
^{3}H CPM x 10^{-2} (o——o)

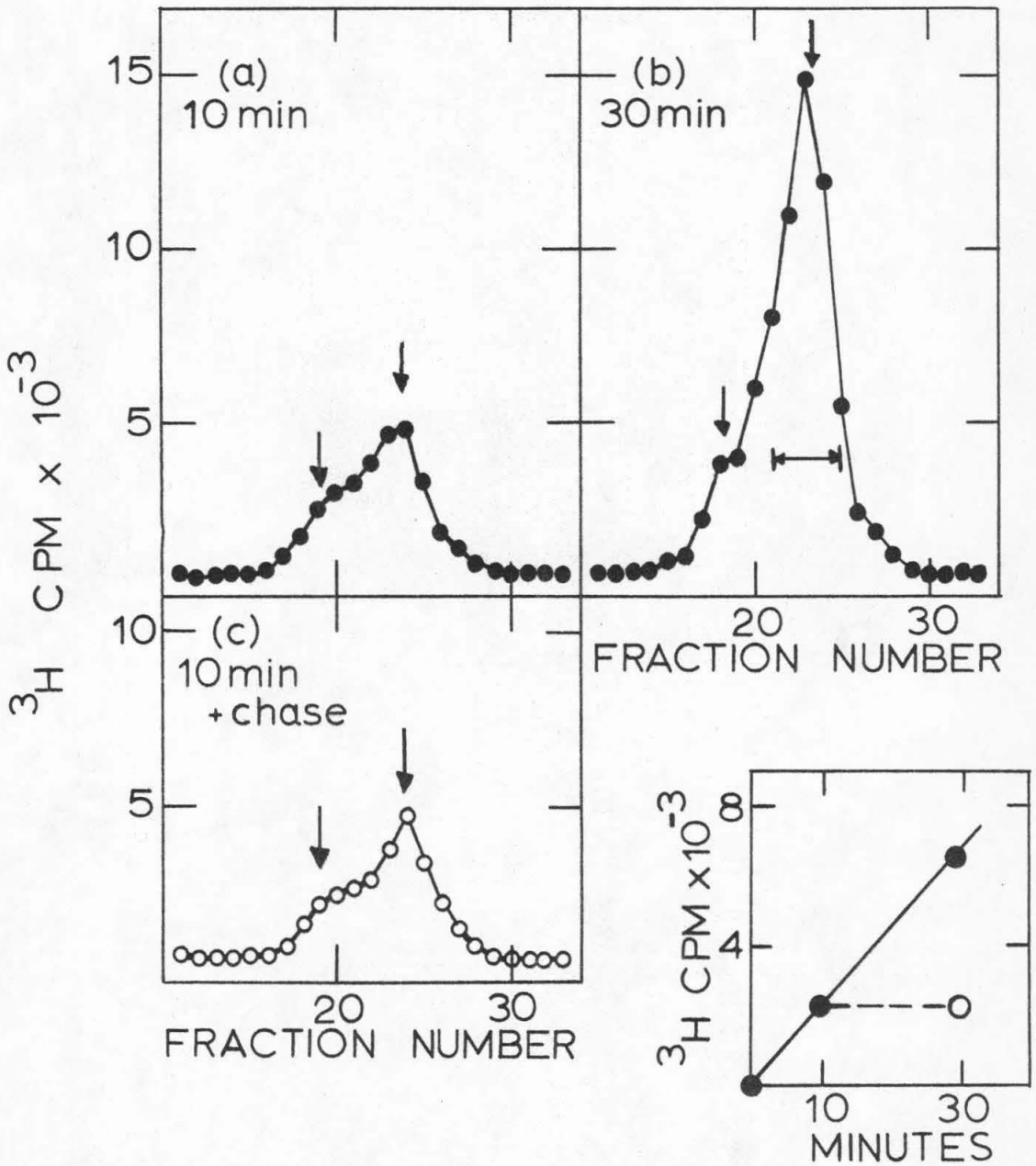
have led to the following observations. The incorporation of radioactive nucleotide into total product DNA was linear up to at least one hour. The rate of DNA synthesis in standard reaction mixtures was from 0.1 to 0.2 μg product DNA per hour at 37°. This corresponds to about 10 to 20% net synthesis of mtDNA per hour. When samples of DNA were isolated at intervals through 60 min incubation, no change was observed in the ratio of lower band to upper band fluorescence intensity in the CsCl density gradients containing ethidium bromide. This suggests that there is no extensive conversion of closed circular mtDNA to nicked species during the incubation. More upper band product DNA is synthesized than lower band product DNA. The ratio of product to endogenous mtDNA was significantly greater in the upper band than in the lower band, since there was generally 2 to 8 times more closed circular DNA than nicked DNA in the gradients.

Upper and Lower Band Product DNA --- Three reaction mixtures were prepared as described in "Methods," except that the reactions contained 0.74 nmole ^{3}H-dTTP (17 Ci per mole). After 10 min at 37°, one reaction was stopped, the second reaction was left to continue, and 20 μl 3.7 mM dTTP was added to the third. The amount of dTTP added to the third reaction diluted the specific activity of the ^{3}H-dTTP 100-fold. The second and third reactions were stopped after a total of 30 min at 37°. The DNA in each reaction mixture was isolated by SDS extraction and banding

in CsCl density gradients containing ethidium bromide. Fractions were collected from the bottom of the gradients and aliquots were removed onto paper filters for radioactivity analysis, Fig. 2. The arrows indicate the centers of the upper and lower bands of the unlabeled parental mtDNA. The lower right hand figure presents the time course of radioactive product synthesis. The rate of DNA synthesiswas linear and the chase with unlabeled dTTP appears to have been effective. The distributions of product DNA after 10 and 30 minutes of incorporation of ^{3}H-dTTP (upper figures) suggest that little or no lower band product was made after 10 minutes, whereas upper band product DNA continued to accumulate. The product DNA synthesized during the first 10 minutes had the same distribution in the gradient as observed with the product DNA from the sample chased for 20 minutes with the unlabeled dTTP.

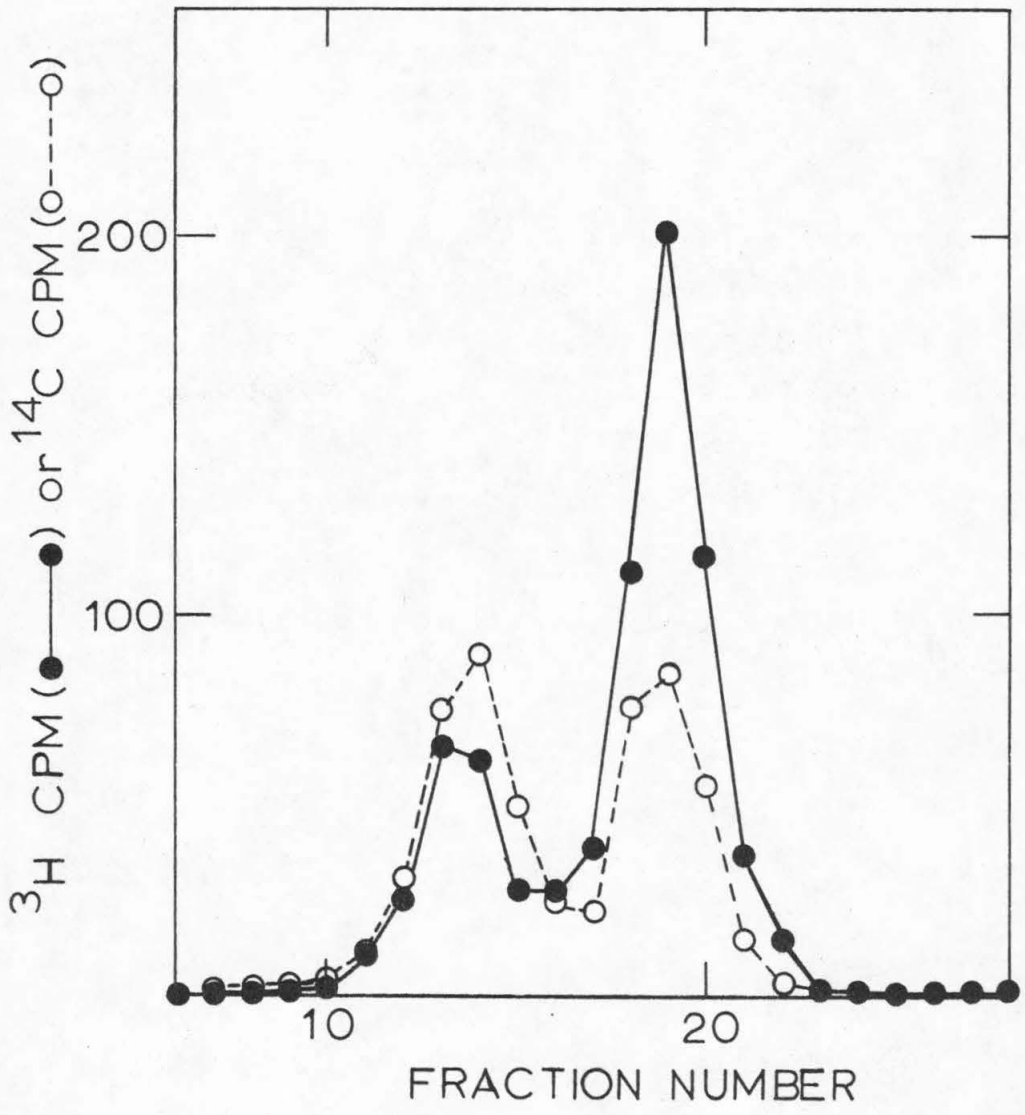
Alkaline Complement Separation of Product DNA --- The identification of upper band product DNA as mtDNA rested upon the assumption of efficient DNase action during the incubations. The continued accumulation of upper band product, described above, raised the possibility of extramitochondrial DNA synthesis leading to products which would band with nicked mtDNA in the CsCl density gradients containing ethidium bromide. Fractions from the upper band, Fig. 2 (b), were pooled as indicated. The ethidium bromide was removed by extraction with isoamyl alcohol.

Fig. 2. Distributions of product DNA in CsCl density gradients containing ethidium bromide. Three reaction mixtures were prepared and incubated at 37°. The first reaction, (a), was stopped after 10 min and the other two reactions were stopped after 30 min. The specific activity of the ^{3}H-dTTP in the third reaction, (c), was diluted 100-fold after the first 10 min of incubation by addition of unlabeled dTTP. The mtDNA was extracted and banded in CsCl density gradients containing ethidium bromide. Fractions (100 μl each) were collected and aliquots (50 μl each) were withdrawn onto paper filters for batchwise acid washing and radioactivity analysis. The lower right hand figure presents the total ^{3}H radioactivity recovered in the individual gradients as a function of the time of incubation. The "chased" sample (c) is shown with the open circle and dashed line. The pairs of arrows indicate the positions of the closed and nicked circular mtDNA in the gradients. The density increases from right to left in each gradient. The fractions in the interval indicated in Fig. 2 (b) were pooled to provide a sample of upper band DNA for the experiment in Fig. 3.


(a)
10 min
(b)
30 min
(c)
10 min
+ chase
15
10
5
10
5
20
30
FRACTION NUMBER
20
30
FRACTION NUMBER
^{3}H CPM x 10^{-3}
8
4
10
30
MINUTES
^{3}H CPM x 10^{-3}

The DNA was then banded in an alkaline CsCl buoyant equilibrium density gradient as previously described (14). Fractions were collected from the bottom of the gradient onto paper filters for batchwise acid washing and analysis of radioactivity, Fig. 3. Two bands of product DNA were observed, coincident with the separated complements of a sample of ^{14}C-dT labeled mtDNA, added as a marker prior to centrifugation. This result lends strong support to the identification of upper band product as mtDNA. Furthermore, the experiment revealed that the upper band product contained significantly more L-strand than H-strand DNA. The extent of this asymmetric synthesis was analyzed as previously described (14). The value of dT(H/L)* for duplex HeLa mtDNA is 1.24. The value of ^{3}H-dT(H/L) observed in the product DNA, Fig. 3, was 0.34. This is equivalent to 3.6 times more L-strand than H-strand DNA in the product associated with nicked mtDNA.

Fig. 3. Distributions of product DNA (●——●) and ^{14}C-labeled mtDNA marker (O--O) in an alkaline CsCl buoyant density gradient. The ethidium bromide was removed from the sample of upper band product DNA from the gradient in Fig. 2 (b). The sample was then mixed with ^{14}C-labeled mtDNA and banded in a CsCl density gradient containing 50 mM K_3PO_4. The initial pH and density of the gradient were 12.1 and 1.74 g per ml respectively. Fractions (100 µl each) were collected onto paper filters for batchwise acid washing and radioactivity analysis. The density increases from right to left.


^{3}H CPM (●——●) or ^{14}C CPM (o- - - -o)
200
100
10
20
FRACTION NUMBER

DISCUSSION

DNA synthesis in isolated HeLa cell mitochondria has been investigated. The rate of DNA synthesis, relative to the amount of endogenous mtDNA, was found to be 10 to 40 times greater than the rates reported by investigators working with rat and chick liver mitochondria (1). The division time of HeLa cells is about 20 hours. In "non-dividing" systems such as rat liver, heart and kidney, mtDNA turnover accounts for the bulk of mtDNA synthesis. Turnover of mtDNA in these systems is characterized by half-lives of from 5 to 10 days (16, 17). Thus it is not surprising that isolated HeLa cell mitochondria show a much higher capacity for <u>in situ</u> DNA synthesis than mitochondria from such animal tissues.

The DNA synthesized by isolated HeLa cell mitochondria appeared in closed circular and nicked mtDNA. While the overall incorporation of deoxyribonucleotides into DNA was linear for up to one hour, the amount of lower band product did not increase after the first 10 minutes. In the chick liver mitochondrial system, DNA synthesis proceeds at the maximum rate for only 10 to 15 minutes, but most of the product synthesized during this period is associated with closed circular mtDNA (3). The nature of the lower

band product synthesized by HeLa cell mitochondria has not been investigated, although it seems likely that the results of such studies will parallel those obtained by Borst and co-workers for the lower band product made by chick liver mitochondria (3,8,9). Lower band product probably represents a relatively short extension of the displacing piece of H-strand DNA in D-loop molecules (9,10).

The nature of the upper band product, synthesized by HeLa cell mitochondria, was studied. The alkaline buoyant complement separation of upper band product demonstrated, in a manner not previously reported, that the product is indeed mtDNA. The experiment also revealed a significant bias for the synthesis of L-strand DNA. The preferential synthesis of upper band product DNA in this system does not appear to be due to endonuclease action relieving the topological restriction of H-strand synthesis in closed circular mtDNA. The observed asymmetry of DNA synthesis may be due to greater opportunity for L-strand synthesis in the population of parental mtDNA molecules (more sites or larger potential stretches of L-strand synthesis), a higher rate of L-strand than H-strand DNA synthesis (the latter involving concomitant template strand displacement), or conditions of incubation which are unfavorable for the continued synthesis of H-strand product in nicked mtDNA.

A major result of *in vivo* studies of mtDNA replication in mouse cells (division time about 20 hours), was the rather high frequency of observed replicative intermediates (10,12). Investigation of closed circular chick liver mtDNA revealed a similarly high frequency, about 30 %, of D-loop containing molecules (9). In this system, however, the frequency of more extensively replicated species, expected in upper band DNA (12), has not been documented. One might expect, with the low level of mtDNA synthesis in liver, that the frequency of replicative intermediates in upper band mtDNA would be rather low. I suspect that the replicative intermediates, present in the endogenous upper band fraction of mtDNA, contain the sites for continued synthesis of DNA in isolated mitochondria. There would then be relatively few molecules available for continuing synthesis of upper band DNA in isolated chick liver mitochondria. With very low endonucleolytic action *in situ*, overall DNA synthesis in isolated chick liver mitochondria would be expected to become very slow, or stop entirely, when lower band DNA synthesis reached the point of topological restriction.

(Footnotes)

[1]The abbreviation is mtDNA, mitochondrial DNA. When the product of DNA synthesis in isolated mitochondria is compared with mtDNA, it is understood that the mtDNA is that of the same species as the incubated mitochondria.

[2]Lower and upper band product refer to newly synthesized DNA which bands with closed and nicked circular mtDNA, respectively, in buoyant CsCl density gradients containing ethidium bromide.

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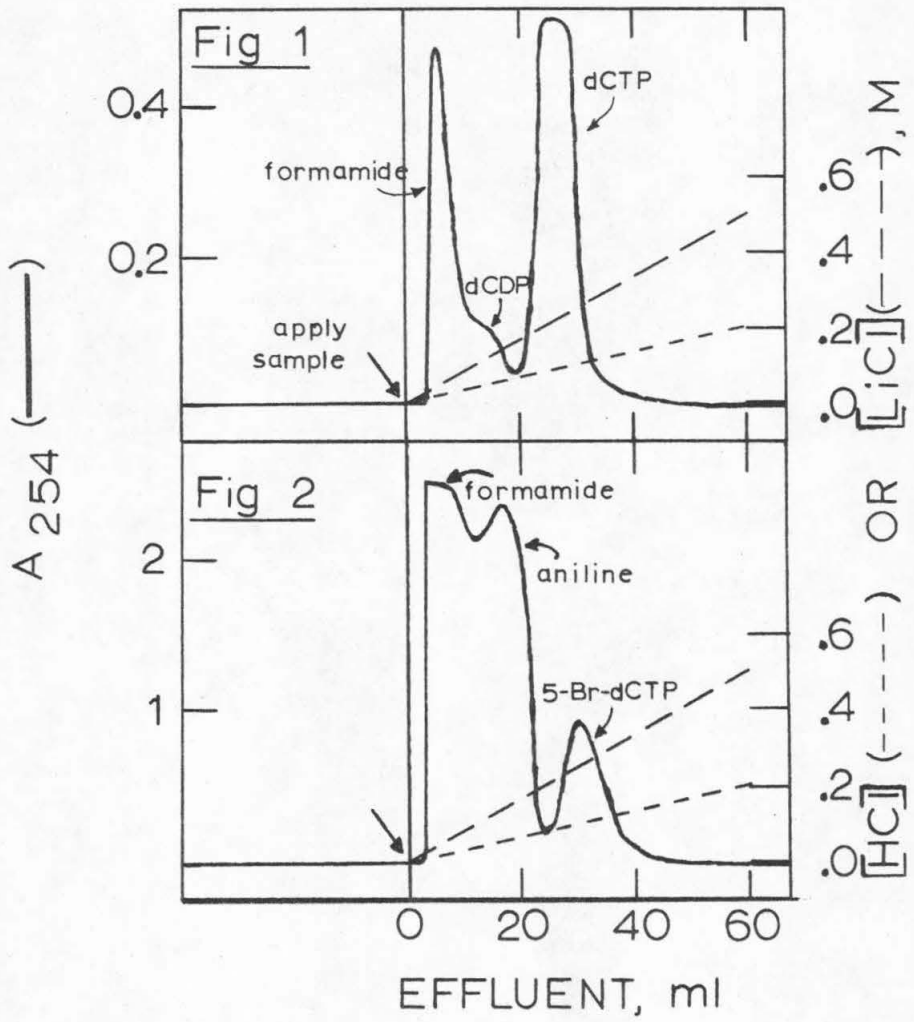
APPENDIX

Preparation of Brominated Pyrimidine Deoxyribonucleotides

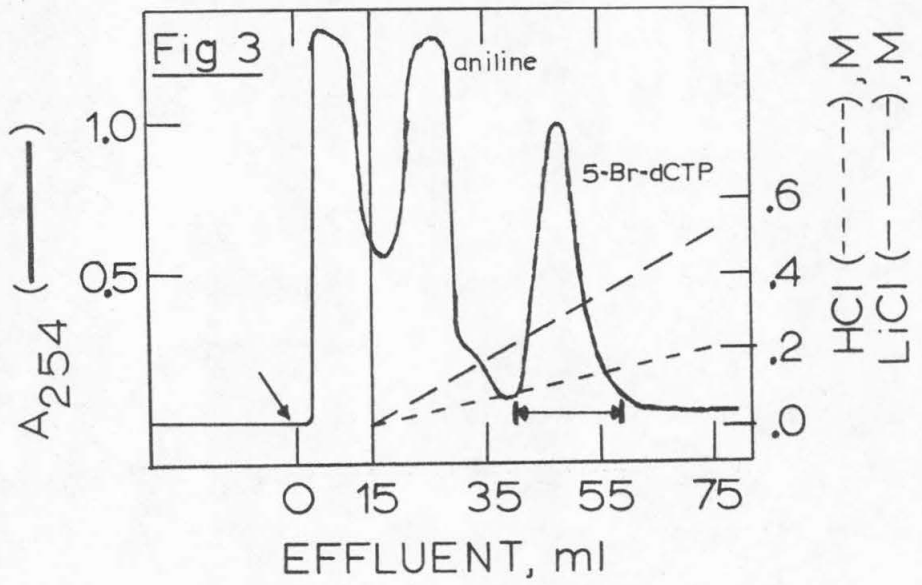
The synthesis of 5-Br-dUTP from dCTP, through the intermediate 5-Br-dCTP, was described by Bessman et al. (1). The introduction of Dowex 1 chromatography has facilitated their procedure.

dCTP was brominated by the procedure of Markham, described by Bessman et al. (1). Br_2 in CCl_4 was added dropwise to dCTP, 10 mg in 1.0 ml formamide, until the bromine color persisted. One drop of aniline was added to react with the excess Br_2. Distilled water was then added dropwise until the mixture became turbid. After chilling to 4°, the CCl_4 was removed by centrifugation. The supernatant was applied to a small Dowex 1 x 8 column (0.3 x 3.0 cm) followed by a 60 ml linear gradient gradient from distilled water to 0.2 M HCl, 0.5 M LiCl. The effluent was passed through a flow cell to monitor ultraviolet absorption. The Isco Model UA-2 ultraviolet analyzer was used to record the elution profile. Fractions were collected, 2 ml each. Fig. 1 shows the elution profile of dCTP in formamide. A small amount of dCDP was indicated. Fig. 2 shows the elution of a reaction mixture following bromination of dCTP.



The components eluted from the column were identified by ultraviolet absorption spectra of fractions corresponding to the indicated bands.

Passage of 15 ml distilled water before the gradient improved the separation of 5-Br-dCTP from aniline, Fig. 3.



Fractions corresponding to the interval indicated in Fig. 3 were pooled. The 5-Br-dCTP was precipitated by adding $BaBr_2$ to 0.25 M, 2 volumes of ethanol, and chilling to 4°. The precipitate was washed in ethanol, dried in an airstream, and resuspended in distilled water. Dowex 50, K^+ form, was used to metasthesize the suspended nucleotide to the soluble potassium salt. A sample was diluted 100-fold in 0.10 M HCl and the ultraviolet absorption spectrum was recorded. The data are presented below. The values in parentheses are from Bessman et al. (1).

5-Br-dCTP	λ_{max} = 299 nm	(299)
	A_{280}/A_{260} = 4.12	(4.0)
	A_{250}/A_{260} = 0.95	(1.10)

Deamination of 5-Br-dCTP leads to 5-Br-dUTP. The procedure used is that of Lohman (2). 2.0 ml 60 % $NaNO_2$ and 1.0 ml glacial acetic acid were added to 3.5 ml of purified 5-Br-dCTP (A_{299} = 125). After 3 hr at room temperature, the reaction was neutralized with NaOH. The 5-Br-dUTP was precipitated as the barium salt and metasthesized to the potassium salt as described for 5-Br-dCTP earlier. The data from the ultraviolet absorption spectrum (in 0.10 M HCl) are given below.

5-Br-dUTP	λ_{max} = 279 nm	(279)
	A_{280}/A_{260} = 1.83	(1.77)
	A_{250}/A_{260} = 0.52	(0.62)

The estimated yields of 5-Br-dCTP and 5-Br-dUTP, relative to the initial quantity of dCTP, were 86 % and 70 % respectively.

These brominated analogues of natural nucleotides were used in density transfer experiments with the product of *in vitro* DNA synthesis catalyzed by the HeLa cell mitochondrial DNA polymerase. No change in the rate of DNA synthesis was observed in reactions in which dCTP, dTTP, or both were replaced with the corresponding brominated analogue or analogues.

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