Nucleic Acid Synthesis in Intact Nuclei Isolated from Mouse Fibroblasts

Characterization of the System and Effects of Glucocorticoids

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(Received April 26, 1969)

SUMMARY

In the course of continuing investigations on the effects of glucocorticoids on nucleic acid synthesis in mouse fibroblasts, we have characterized a nuclear system capable of carrying on both DNA and RNA synthesis. Intact nuclei isolated from logarithmically growing fibroblasts can incorporate both ribonucleoside and deoxyribonucleoside triphosphates into cold acid-insoluble material under the direction of endogenous enzyme and template. The extent of DNA synthesis does not approach that proceeding in intact cells. The incorporation of both ribo- and deoxyribonucleoside triphosphates into macromolecular material is DNA template-dependent and requires the presence of all four complementary nucleotides. The DNA formed is of low molecular weight and does not sediment with bulk DNA on alkaline sucrose gradient centrifugation. Although DNA and RNA synthesis is significantly inhibited in nuclei isolated from cells treated with growth-inhibitory doses of glucocorticoids, the apparent 2-fold greater inhibition of DNA synthesis with respect to RNA synthesis as measured by thymidine and uridine incorporation in whole cells is not observed. Neither DNA nor RNA synthesis is inhibited by direct addition of very high levels of glucocorticoids to nuclear suspensions or broken whole cells. The intact nuclear system has also been examined with respect to enzymatic functions which affect the state of the nucleoside triphosphate precursors and the RNA and DNA products.

INTRODUCTION

Glucocorticoids are able to depress the rate of growth of mouse fibroblasts *in vitro*, and the growth-inhibitory potency of a series of steroids parallels their glucocorticoid potency (1–3). The biochemical basis of the growth-inhibitory effect has been studied in intact cells by Pratt and Aronow (2), and these studies suggested that an early effect was a decreased rate of DNA synthesis, as determined by inhibition of the incorporation of thymidine.

In order to study nucleic acid synthesis more directly than is possible in a whole cell system, we have developed and characterized an isolated, washed nuclear system capable of converting ribonucleoside triphosphates into RNA and deoxyribonucleoside triphosphates into DNA. Since the nuclear system utilizes endogenous polymerases and chromatin, it was hoped that control mechanisms that operate in the intact cell might be detected and further elucidated in the nuclear

This investigation was supported by Public Health Service Research Grant CA-05672 from the National Cancer Institute.

[1] Predoctoral Fellow under Public Health Service Training Grant GM322 from the Division of General Medical Sciences.

[2] Postdoctoral Fellow (No. 310) of the American Cancer Society.

system. The nuclear system described is a prospective tool for elucidating the mechanism of action of a variety of drugs affecting nucleic acid metabolism.

MATERIALS AND METHODS

Chemicals and enzymes. Thymidine-methyl-^{3}H (6.0 Ci/mmole), uridine-2-^{14}C (52 mCi/mmole), dATP-^{3}H, dTTP-^{3}H, ATP-^{3}H, CTP-^{3}H, deoxyadenosine-^{3}H, and adenosine-^{3}H were purchased from Schwarz BioResearch, Inc. Unlabeled nucleosides and nucleotides were obtained from commercial sources. In a few experiments, purified dATP-^{3}H and dCTP, free of possible contamination with dGTP or dTTP, were prepared by chromatography on Whatman No. 3 paper using an ascending system of isobutyric acid–1 N NH_4OH–0.2 M EDTA (100:60:0.8).

Highly polymerized DNA from calf thymus was bought from Calbiochem. Yeast RNA was purchased from Sigma Chemical Company. Purified *Micrococcus lysodeikticus* DNA was kindly provided by Dr. A. T. Ganesan, polynucleotide lipase prepared from *Escherichia coli* was donated by Dr. I. R. Lehman, and poly dAT was a gift from Dr. A. Kornberg, all of Stanford University. DL-β-Glycerophosphate and D-glucose 6-phosphate were obtained from Sigma Chemical Company. Glucose 6-phosphate dehydrogenase was purchased from Calbiochem.

Deoxyribonuclease I, free of ribonuclease, was purchased from Worthington Biochemical Corporation. Ribonuclease A from bovine pancreas, protease-free, came from Sigma Chemical Company. Cesium chloride, optical grade, was obtained from Harshaw Chemical Company, Cleveland. Sarkosyl was obtained from Geigy Chemical Corporation. Actinomycin D was a gift from Merck Sharp & Dohme Research Laboratories.

Cell culture. Mouse fibroblasts, L strain, were maintained on Joklik medium obtained from Schwarz BioResearch, supplemented with 10% bovine serum. Replicate groups of Roux bottles were inoculated with approximately 2×10^6 cells/70 ml of culture medium, and the cells were grown for 3 days at 37° in an atmosphere of 95% air and 5% CO_2.

Isolation and purification of nuclei. Medium was decanted from cells in the logarithmic phase of growth, and the cells were washed twice with Earle's salt solution at 37° while still adhering to the glass. The cells were then harvested by scraping in ice-cold 0.9% NaCl and collected by centrifugation in the cold at $600 \times g$. All of the following procedures were carried out at 0–4°. The whole cell pellet was suspended in a hypotonic solution of 0.01 M Tris buffer at pH 7.5 and 0.4 mM EDTA such that the final volume of suspension was approximately 5 times the volume of the cell pellet. After 5 min, the suspension was homogenized with 15 strokes of a tightly fitting pestle in a Dounce glass homogenizer. After homogenization, hypertonic buffer (1.43 M NaCl, 0.11 M KCl, 0.033 M $MgCl_2$, and 0.11 M Tris, pH 7.5) was added to bring the broken cell suspension to isotonicity. In experiments in which enzyme assays were carried out on the broken cell suspension or the supernatant from centrifugation at $600 \times g$, the broken cell suspension was not brought up to isotonicity with hypertonic buffer. Instead, 1 volume of the following hypertonic sucrose solution was added to 4 volumes of broken cell homogenate to bring the final suspension to isotonicity: 1.25 M sucrose, 0.005 M $MgCl_2$, 0.005 M $CaCl_2$, and 0.25 M Tris, at pH 7.8 for RNA polymerase assay or at pH 7.5 for DNA polymerase assay.

Optical microscopic examination of the resulting suspension revealed cell debris, nuclei, and less than 1% whole cells. The isotonic broken cell suspension was centrifuged at $600 \times g$ for 10 min and separated into a nuclear pellet and a cytoplasmic supernatant fraction. The pellet was washed twice by suspending it in approximately 10 ml of an isotonic sucrose solution (0.25 M sucrose, 0.001 M $MgCl_2$, and 0.05 M Tris, at pH 7.8 for RNA polymerase determinations or at pH 7.5 for DNA polymerase assay and in those cases

when both RNA and DNA polymerase were to be assayed in the same nuclei) and centrifugation at 600 × *g*. After the final wash, the nuclei were suspended in the same isotonic sucrose solution adjusted to the appropriate pH for the assay to be performed. The nuclear protein nitrogen concentration of the final washed nuclear suspension was between 100 and 200 μg/ml. Aliquots (0.4 ml) of this suspension were used for enzyme assay, and portions were assayed for protein and DNA.

Assays for cytoplasmic marker enzymes. Acid phosphatase and glucose 6-phosphatase were assayed in both purified nuclear suspensions and broken cell suspensions which had been brought to isotonicity with the hypertonic NaCl solution. Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was assayed by the method of Hers, Berthet, Berthet, and deDuve (4), using β-glycerophosphate as substrate. Glucose 6-phosphatase was measured by the method of Gianetto and deDuve (5). In both cases, the inorganic phosphate liberated was measured by the method of Sumner (6).

Phosphohexose isomerase was measured in nuclear suspensions and broken cell homogenates which had been brought to isotonicity with the hypertonic sucrose solution. This enzyme was measured by the method of Noltmann (7).

Assay for RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). The assay system contained the following in a final volume of 1.0 ml: 0.05 μmole of randomly labeled ATP-^{3}H (81 μCi/μmole); 1 μmole each of unlabeled GTP, CTP, and UTP; 70 μmoles of KCl; 20 μmoles of NaCl; 5 μmoles of $MgCl_2$; 10 μmoles of cysteine; 100 μmoles of Tris buffer, pH 8.0; and 0.4 ml of nuclear suspension. Any further components were added in a volume of 0.05 ml. In certain experiments, when one or two of the unlabeled triphosphates were omitted from the mixture, the amount of remaining unlabeled nucleoside 5′-triphosphate(s) was adjusted such that the total amount of unlabeled nucleoside 5′-triphosphate remained at 3 μmoles. This was done in order that any nonreplicative nucleoside incorporation which might be present would not be selectively increased. The assay mixture was incubated routinely for 10 min in a shaking water bath at 37°. The reaction was stopped by the addition of 3 ml of an ice-cold solution of 5% trichloracetic acid with 0.04 M $Na_4P_2O_7$. Pyrophosphate was included in all cold trichloracetic solutions used for washing the insoluble precipitate in order to minimize contamination due to nonspecific binding of the radioactive precursor.

The cold acid-insoluble precipitate was centrifuged at 12,000 × *g*, washed once by resuspension in cold trichloracetic acid, and recentrifuged. The pellet was suspended in cold trichloracetic acid and collected by mild suction onto 2.4-cm Whatman glass paper discs. The acid-insoluble material was washed twice with 3 ml of cold trichloracetic acid and once with cold water. Each disc was then dried and placed in a liquid scintillation counting vial, to which 10 ml of scintillator solution (4 g of 2,5-diphenyloxazole and 0.1 g of 2,2-*p*-phenylenebis[5-phenyloxazole] per liter of toluene) were added. The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. The radioactivity obtained for each sample was corrected by subtracting the radioactivity present in a sample precipitated with cold trichloracetic acid immediately after addition of the nuclei. Such zero-time values, as well as those obtained on incubation for 10 min at 0°, were less than 15% of those found after the complete 10-min incubation at 37°.

Assay for DNA polymerase (deoxynucleosidetriphosphate: DNA deoxyribonucleotidyltransferase, EC 2.7.7.7). The reaction system contained the following in a final volume of 1.0 ml: 0.01 μmole of dATP-8-^{3}H (1 mCi/μmole); 0.5 μmole each of dGTP, dCTP, and dTTP; 70 μmoles of KCl; 20 μmoles of NaCl; 5 μmoles of $MgCl_2$; 10 μmoles of cysteine; 100 μmoles of Tris buffer, pH 7.5; and 0.4 ml of nuclear suspension. In experiments

in which one or two of the unlabeled triphosphates were omitted from the assay mixture, the amount of the remaining unlabeled nucleoside 5′-triphosphate(s) was adjusted such that the total amount of unlabeled nucleoside 5′-triphosphate remained at 1.5 μmoles. The assay mixture was incubated routinely for 60 min at 37° with shaking. The incubations were terminated, and the cold acid-insoluble radioactivity was determined as described for the RNA polymerase assay. The radioactivity values obtained in samples precipitated immediately after addition of the nuclei or after incubation at 0° were approximately 10% of those found after the complete 60-min incubation at 37°.

Incorporation of thymidine and uridine into nucleic acids by intact cells. Tritium-labeled thymidine or ^{14}C-uridine in 0.1 ml of water was added to 70 ml of fibroblast culture 12 hr after steroid addition. After a 1-hr incubation in the presence of the radioactive precursor, the culture medium was decanted, and the adherent cells were carefully washed twice with Earle's salt solution at pH 7.2. The cells were suspended in 30 ml of 0.9% NaCl solution containing 0.03% trypsin, and 1.0 ml of the suspension was mixed with 9.0 ml of 0.9% NaCl and counted in a Coulter cell counter. Three milliliters of cell suspension were added with rapid mixing to 1.5 ml of cold 12% trichloracetic acid containing 50 μg of unlabeled thymidine and uridine per milliliter. The acid-insoluble material was collected by mild suction onto 2.4-cm Whatman glass paper discs and washed twice with 4% trichloracetic acid containing 50 μg of each unlabeled precursor per milliliter and once with cold water. The discs were dried, and the radioactivity was assayed as described above.

Chemical assays. Protein was determined according to Oyama and Eagle (8), using crystalline bovine albumin (Armour Pharmaceutical Company) as a standard. Micrograms of protein nitrogen were converted to micrograms of protein by multiplying by 6.25. DNA was determined according to Burton (9), using deoxyribose as a standard. The amount of deoxyribose-reactive material in the sample was multiplied by 2 to arrive at the actual amount of deoxyribose present, and micromoles of DNA deoxyribose were converted to micrograms of DNA by multiplying by 340. RNA was assayed by the method of Volkin and Cohn (10). Ribose was used as the standard, and the amount of ribose-reactive material in the sample was multiplied by 2 to arrive at the actual amount of ribose present. A correction for deoxyribose present was made by subtracting 1/11 of the total amount of deoxyribose in the sample from the apparent ribose content.

Isolation and electrophoresis of nucleoside phosphates. RNA or DNA polymerase assays were carried out in the usual manner and terminated by the addition of 3 ml of ice-cold 5% trichloracetic acid–0.04 M $Na_4P_2O_7$. The supernatant fluid obtained after centrifugation at 12,000 × *g* was extracted eight times with equal volumes of ethyl ether to remove the trichloracetic acid. Ten microliters of extracted supernatant were electrophoresed with appropriate nonradioactive nucleoside phosphate markers on Schleicher and Schuell 589 paper, using 0.035 M sodium citrate buffer, pH 4.0, at a potential of 1000 V over 50 cm. Markers were identified by ultraviolet light, cut out, and counted in a scintillation counter. This procedure resulted in the recovery of approximately 70% of the counts placed on the paper.

Isolation of total nuclear RNA and sucrose gradient centrifugation. Nuclei from incubations which had been terminated by addition of ice-cold Earle's salt solution and centrifuged at 600 × *g* were suspended in 5.0 ml of 0.5% Sarkosyl in 0.01 M sodium acetate, pH 5.1, with 50 μg of washed bentonite. Then 50 ml of 88% phenol were added, and the RNA was extracted three times with phenol at 60° and precipitated with cold ethanol according to Scherrer and Darnell (11). The RNA pellet was solubilized with 0.3 ml of 0.01 M sodium acetate (pH 5.1), 0.1 mM $MgCl_2$, 0.05 M NaCl, and 0.5% Sarkosyl. The 0.3-

ml solution was layered on a 25-ml linear sucrose gradient (5–20%) containing the same buffer as described above but lacking Sarkosyl.

The extracts were centrifuged for 19.8 hr in the SW 25 rotor of a Spinco model L instrument at 22,500 rpm at 12.5°. Thirty-drop fractions were collected by siphoning through a thin polyethylene tube inserted through the sucrose solution to the bottom of the gradient. Each fraction was diluted with 1 ml of water, and the absorbance at 260 mμ was determined. RNA was precipitated with cold 5% trichloracetic acid and collected by mild suction onto 2.4-cm Whatman glass paper discs. The acid-insoluble material was washed twice with 3 ml of cold 5% trichloracetic acid and once with cold water. The discs were then dried and assayed for radioactivity as previously described. Sedimentation coefficients were calculated according to Nomura, Hall, and Spiegelman (12).

DNA purification and cesium chloride gradient centrifugation. After a 1-hr incubation with dATP-^{3}H, DNA was extracted from L-cell nuclei by a slight modification (13) of the method of Marmur (14). It was then incubated for 1 hr at 37° with RNase (100 μg/ml) and 1 hr with trypsin (100 μg/ml). The final solution was dialyzed overnight against 0.15 M NaCl–0.015 M sodium citrate. The resulting purified, tritium-labeled DNA (2.6 optical density units) was brought to a final volume of 3.4 ml with 0.01 M Tris buffer, pH 8.2; 4.37 g of CsCl were added, and the DNA was centrifuged in the No. 40 rotor of a Spinco model L instrument, at 33,000 rpm for 65 hr at 12.5°, according to Flamm, Bond, and Burr (15). The bottoms of the centrifuge tubes were punctured with a needle, and 3-drop fractions were collected. Each fraction was diluted with 1 ml of water, the absorbance at 260 mμ was determined, and the cold acid-insoluble material was collected and assayed for radioactivity as described for the sucrose gradients.

Selected fractions collected from a sample of L-cell DNA centrifuged simultaneously with the tritium-labeled DNA gradient were recentrifuged in a Spinco model E analytical ultracentrifuge as described elsewhere (16). DNA densities were determined by the method of Schildkraut, Marmur, and Doty (17), using *M. lysodeikticus* DNA (density, 1.731 g/ml) as a density marker.

L-cell and sea urchin DNA, used as exogenous templates, were purified by the above method from twice-washed L-cell nuclei or from sperm collected by injection of 2 ml of 0.5 M KCl into sea urchins.

Alkaline sucrose gradient centrifugation. Nuclei were isolated and incubated as described above, except that the amount of dATP-^{3}H added was double the standard amount. The reaction was stopped by adding 1 ml of a cold buffer solution (0.15 M NaCl, 0.015 M sodium citrate, 0.01 M EDTA, 0.02 M NaN_3, 0.02 M NaF, and 0.01 M Tris, pH 7.4). Nuclei from two incubation vessels were combined and centrifuged at 600 × *g*. The nuclei were suspended in 1 ml of cold buffer identical with the first except that it contained 0.015 M NaCl and 0.0015 M sodium citrate. The suspension was adjusted to 0.1 N NaOH, and the resulting lysate was layered on top of a 5–20% sucrose gradient containing 0.1 N NaOH, 0.9 M NaCl, and 0.001 M EDTA (18) and centrifuged for 16 hr in the SW 25 rotor of a Spinco model L instrument at 21,500 rpm. Fractions were collected by puncturing the bottoms of the centrifuge tubes, and the optical density at 260 mμ was determined. DNA was precipitated with cold 5% trichloracetic acid containing 0.04 M $Na_4P_2O_7$, and the precipitate was collected by mild suction onto 2.4-cm Whatman glass paper discs. After washing twice with cold trichloracetic acid and once with cold water, the radioactivity was assayed as described above.

Assays for nuclease activity. DNase was assayed by a modification of the method of Wiernik and MacLeod (19). Whole cells or purified nuclei were suspended in distilled water for 10 min and homogenized vigorously with a tightly fitting Teflon pestle in a glass homogenizer. Portions (0.5

ml) of the homogenate were incubated with 0.75 mg of calf thymus DNA and 5 μmoles of $MgSO_4$ dissolved in 0.80 ml of 0.05 M acetate buffer, pH 7.5, at 37° for 30 or 60 min. The reaction was terminated by the addition of 0.2 ml of 25% perchloric acid. The mixture was centrifuged at 10,000 × *g* for 10 min, and the amount of DNA solubilized was estimated by determining the absorbance of the supernatant fluid at 260 mμ. The amount of optical density in the supernatant which was not the result of enzymatic digestion of the calf thymus DNA was determined with appropriate control incubations and subtracted from the total optical density present.

Acid RNase and alkaline RNase were measured by a modification of the method of MacLeod, King, and Hollander (20). Whole cell and nuclear homogenates were prepared as described for DNase, and, in addition, the homogenates were frozen and thawed three times. For acid RNase determination, 0.1 ml of whole cell or nuclear homogenate was added to 0.8 ml of a solution of yeast RNA, 1 mg/ml, in 0.05 M acetate buffer, pH 5.7. Alkaline RNase activity was determined by adding 0.1 ml of whole cell or nuclear homogenate to 0.8 ml of a solution of yeast RNA, 1 mg/ml, in 0.0075 M Tris buffer, pH 8.0. Both assay mixtures were incubated at 37° for 30 and 60 min, and the reaction was stopped by the addition of 0.2 ml of a 25% perchloric acid solution containing 0.75% uranyl acetate. The mixture was centrifuged at 10,000 × *g* for 10 min, and the amount of RNA solubilized was estimated by determining the optical density of the supernatant solution at 260 mμ.

RESULTS

Yield, purity, and polymerase content of the nuclear preparation. As determined by hemocytometer count, approximately 60% of the nuclei were recovered in the final washed nuclear suspension with the nuclear isolation procedure employed. The suspension contained 64% of the original whole cell DNA deoxyribose. The cytoplasmic fraction (i.e., the supernatant obtained after the nuclei had been centrifuged out of the broken cell suspension) contained less than 2% of the total cellular DNA deoxyribose.

The purified nuclear suspension contained 4.2 ± 0.5 (SD) μg of protein per microgram of DNA and 0.37 ± 0.05 (SD) μmole of RNA nucleotide per micromole of DNA nucleotide. On examination under the light microscope, the final nuclear suspension contained a small amount of cytoplasmic debris. Electron microscopic examination of the twice-washed nuclear suspension revealed that the nuclear membrane remained intact during the isolation procedure, as did most of the perinuclear membrane. There were occasional contaminants of granular material contiguous to the perinuclear membrane and apparently continuous with it.

The extent of contamination of the nuclear preparation by cytoplasmic components was investigated by assaying the

TABLE 1

Specific activity of cytoplasmic enzyme markers in L-cell nuclear preparation

Preparation of the broken cell homogenate, purification of the nuclei, and enzyme assay procedures are described in MATERIALS AND METHODS. Specific activities are expressed per micromole of DNA deoxyribose. The activities of acid phosphatase and glucose 6-phosphatase represent the number of micromoles of inorganic phosphate formed in 120 min. Phosphohexose isomerase activity is expressed as the change in optical density at 340 mμ per minute at the initial reaction rate.

Preparation	Specific activity		
	Acid phosphatase	Glucose 6-phosphatase	Phosphohexose isomerase
Broken cell homogenate	4.86	5.78	426
Nuclear suspension	0.73	1.42	<6

specific activities of three cytoplasmic marker enzymes in both the broken cell homogenate and the purified nuclear suspension (Table 1). Less than 2% of the activity of phosphohexose isomerase, a soluble enzyme (21), was recovered in the final nuclear preparation. The specific activities of acid phosphatase and glucose 6-phosphatase in the final nuclear suspension were 16% and 24%, respectively, of those found in the broken cell homogenate. In rat liver, acid phosphatase activity is associated with the lysosomes and glucose 6-phosphatase is located in the microsomal fraction (21).

The intracellular distribution of RNA polymerase was assessed by measuring the activity of RNA polymerase in the broken cell homogenate, the cytoplasmic fraction, the crude nuclei, and the washed nuclei. To eliminate the contribution of other enzymatic functions, present in the broken cell homogenate and cytoplasmic fractions, that could transform ATP-^{3}H into cold acid-insoluble radioactivity, the incorporation of ATP-^{3}H in each fraction which was inhibited by actinomycin D was determined. The incorporation of ATP-^{3}H found in the presence of 5 μg of actinomycin D per milliliter was subtracted from the incorporation found in the absence of any inhibitor, thus yielding an assessment of the DNA template-directed, actinomycin D-sensitive ATP-^{3}H incorporation. There was no RNA polymerase activity in the cytoplasmic fraction, essentially all of the RNA polymerase activity being accounted for in the nuclei (Table 2). The addition of purified DNA to the cytoplasmic fraction did not stimulate ATP-^{3}H incorporation. The specific activity of RNA polymerase expressed on the basis of protein increases as the nuclei are washed; however, the specific activity expressed on the basis of DNA remains constant during the washing procedure.

TABLE 2

Distribution of DNA-dependent RNA polymerase in L-cells

Various cell fractions were prepared as described in MATERIALS AND METHODS. The whole cell fraction is equivalent to the isotonic broken cell homogenate. The supernatant of the 600 × *g* centrifugation is the cytoplasmic fraction, and the resuspended pellet is the crude nuclear fraction. The purified nuclei, as usual, represent the final nuclear suspension after two washes in 0.25 M sucrose solution. Incubations were carried out as described in MATERIALS AND METHODS, except that 5 μg of actinomycin D in 0.05 ml of 10% ethanol were added to duplicate samples of each cell fraction. Actinomycin D-sensitive incorporation represents the incorporation of ATP-^{3}H in the normal complete system minus the incorporation in the presence of actinomycin D. The total protein nitrogen and the total DNA deoxyribose were assayed in each fraction, and the specific activity of ATP-^{3}H incorporation is expressed with respect to both protein (left) and DNA (right). No incorporation values expressed on the basis of DNA are presented for the cytoplasm, as this fraction contained only trace amounts of DNA.

Cell fraction	Incorporation of ATP-^{3}H into cold acid-insoluble material					
	Complete system	+Actinomycin D	Actinomycin D-sensitive	Complete system	+Actinomycin D	Actinomycin D-sensitive
	$\mu\mu$mole ATP-^{3}H/μg protein N			*$\mu\mu$moles ATP-^{3}H/μmole DNA deoxyribose*		
Experiment A						
Whole cells	0.133	.047	0.086	282	99	183
Cytoplasm	0.058	0.051	0.007			
Crude nuclei	0.173	0.051	0.122	162	47	115
Purified nuclei	0.261	0.024	0.237	128	12	116
Experiment B						
Whole cells	0.088	0.029	0.059	88	33	55
Cytoplasm	0.058	0.080	0			
Crude nuclei	0.140	0.018	0.122	70	21	49
Purified nuclei	0.182	0.020	0.162	53	5	48

TABLE 3

Distribution of DNA-dependent DNA polymerase in L-cells

Incubation conditions were those described in Table 2, except that 50 μg of actinomycin D were added in these experiments, and 100 μg of heat-denatured calf thymus DNA were added to all incubations. Actinomycin D-sensitive incorporation represents the incorporation supported by the complete system containing exogenous DNA template minus the incorporation in replicate incubations containing both exogenous DNA and 50 μg of actinomycin D. The total protein nitrogen and total endogenous DNA deoxyribose were assayed in each fraction, and the specific activity of dATP-^{3}H incorporation is expressed with respect to both protein (left) and DNA (right). No incorporation values expressed on the basis of DNA are presented for the cytoplasm, as this fraction contained only trace amounts of DNA.

Cell fraction	Incorporation of dATP-^{3}H into cold acid-insoluble material					
	Complete system	+Actinomycin D	Actinomycin D-sensitive	Complete system	+Actinomycin D	Actinomycin D-sensitive
	μμmole dATP-^{3}H/μg protein N			*μμmoles dATP-^{3}H/μmole DNA deoxyribose*		
Experiment A						
Whole cells	0.071	0.007	0.064	52.7	4.9	47.8
Cytoplasm	0.083	0.008	0.075			
Crude nuclei	0.072	0.012	0.060	16.9	2.9	14.0
Purified nuclei	0.047	0.011	0.036	9.4	2.2	7.2
Experiment B						
Whole cells	0.062	0.008	0.054	49.9	5.9	44.0
Cytoplasm	0.094	0.005	0.089			
Crude nuclei	0.104	0.014	0.090	41.6	5.3	35.8
Purified nuclei	0.057	0.011	0.046	14.8	2.8	12.0

The intracellular distribution of DNA polymerase was considerably different from that of RNA polymerase. The cytoplasmic fraction of L-cells could incorporate only a very small amount of dATP-^{3}H into cold acid-insoluble material; however, the addition of purified, denatured DNA template stimulated this incorporation many fold. Therefore, heat-denatured calf thymus DNA was added to all incubation vessels in amounts which yielded maximal stimulation of dATP-^{3}H incorporation. The major portion of the DNA polymerase activity in these L-cell preparations was found in the cell cytoplasm, and only a small proportion, approximately 20%, of the total cellular enzyme activity was recovered in the purified nuclear preparations (Table 3). The enzyme activity expressed on the basis of protein was the same in the cytoplasmic and crude nuclear preparations. The fact that the specific activity of the crude nuclei, expressed on the basis of DNA content, was considerably greater than that of the purified nuclei indicates that at least a portion of the DNA polymerase is soluble and readily leached out of the nuclei on washing. If the DNA polymerase activity in the various fractions is assessed in the same manner as the actinomycin D-sensitive incorporation of ATP (i.e., without adding exogenous purified DNA to all samples), approximately 50% of the original specific enzyme activity found in the broken cell homogenate was recovered in the purified nuclear suspension.

To find out whether the dATP-^{3}H incorporation directed by endogenous DNA of the nuclear preparation proceeded inside the nucleus or was directed by extranuclear DNA polymerase and a small amount of DNA template that was extranuclear as a consequence of leakage or partial nuclear lysis, the experiment presented in Table 4 was carried out. A series of replicate incubations were stopped by cooling in ice, the assay tubes were centrifuged at 600 × *g*, the supernatant was separated from the nuclear pellet, and the cold acid-insoluble

radioactivity was determined in both the nuclear pellet and the extranuclear supernatant solution. A compact nuclear pellet could not be made without spinning the tubes at a speed high enough (i.e., $>600 \times g$) to rupture the nuclei. As a result, some nuclear material was drawn off and contaminated the supernatant fraction. The data presented in Table 4 show that almost twice as much cold acid-insoluble radioactivity from dATP-^{3}H was recovered in the nuclear pellet as from the supernatant solution. The addition of DNA to the incubation medium, however, stimulated the incorporation of dATP-^{3}H many times, and this reaction, directed by exogenous template, apparently proceeded outside the nucleus.

Assay conditions. The incorporation of both ATP-^{3}H and dATP-^{3}H remained linear with respect to the amount of nuclear suspension added, up to a concentration of 80 μg of nuclear protein nitrogen per milliliter of final incubation (equivalent to 120 μg of DNA per milliliter) (Fig. 1a and b).

The incorporation of dATP-^{3}H continued at a fairly constant rate for more than 1 hr (Fig. 1d). In many time course experiments the incorporation of dATP-^{3}H was not absolutely linear over the course of the hour, but was composed of an initial, rapid phase with a second, slower, constant rate of incorporation proceeding up to approximately 90 min. Identical experiments using dTTP-^{3}H as the labeled precursor gave the same results. ATP-^{3}H incorporation remained linear with time for 10 min and then rapidly fell off (Fig. 1c). An identical time course of incorporation was observed for CTP-^{3}H.

The incorporation of both ATP and dATP depended on the presence of a divalent cation (Fig. 1e and f). The optimum magnesium ion concentration was 5–8 mM for both ATP and dATP incorporation. The manganese ion optima were somewhat lower, 3 mM for RNA synthesis and 2 mM for DNA synthesis. Manganese caused greater stimulation of RNA synthesis, whereas magnesium and manganese equally satisfied the cation requirement for DNA synthesis. Neither reaction was stimulated by the addition of calcium up to a concentration of 9 mM.

The pH optima for RNA and DNA

TABLE 4

Site of DNA synthesis in nuclear suspensions

Replicate nuclear suspensions were incubated for 1 hr with dATP-^{3}H, and the reaction was stopped by plunging the incubation tubes into ice. All tubes were then centrifuged at 600 $\times$ *g* for 10 min. The supernatant was gently removed from one set of duplicate tubes with a Pasteur pipette, and 3 ml of cold 5% trichloracetic acid with 0.04 M $Na_4P_2O_7$ were added to the supernatant and nuclear pellet. A second set of duplicate incubations were precipitated without removing the supernatant, and these were used as the controls. All samples were washed and assayed for radioactivity in the usual manner. Where noted, 200 μg of heat-denatured calf thymus DNA were added to some of the incubations. The incubation mixtures in experiments A and B contained 55 and 53 μg of nuclear protein nitrogen, respectively.

Fraction	Incorporation of dATP-^{3}H			
	Complete system		+Denatured DNA	
	μμmoles	*% control*	*μμmoles*	*% control*
Experiment A				
Control	1.33	100	14.63	100
Nuclear pellet	0.72	54	2.91	20
Supernatant	0.40	30	10.30	70
Experiment B				
Control	1.55	100		
Nuclear pellet	0.93	60	1.51	
Supernatant	0.51	33	12.85	

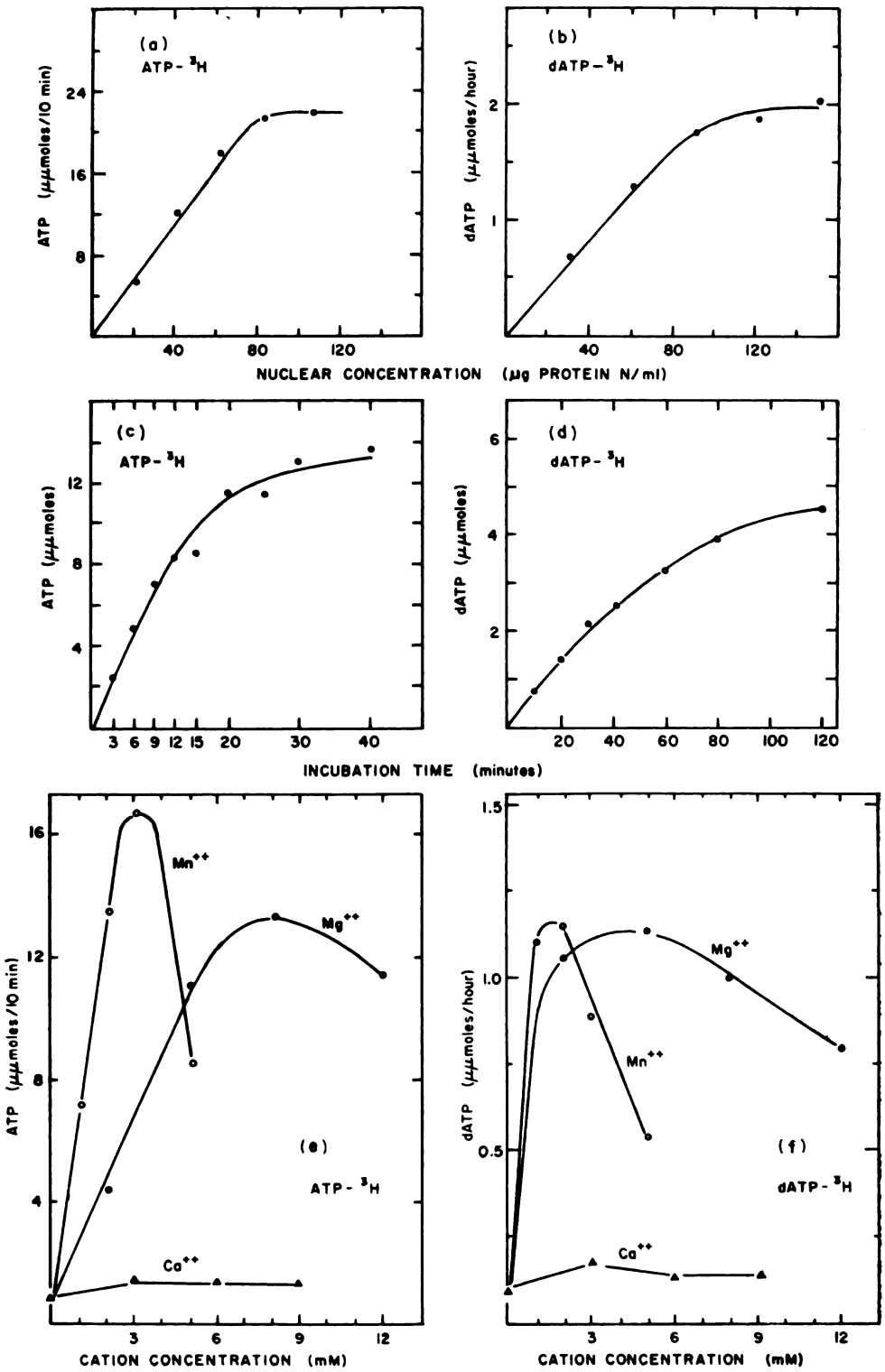

FIG. 1. *Incorporation of ATP-³H and dATP-³H into cold acid-insoluble material by L-cell nuclear suspensions as a function of nuclear concentration, time, and divalent cation concentration*

Incubation conditions were those described in MATERIALS AND METHODS. a. Incorporation of ATP-³H

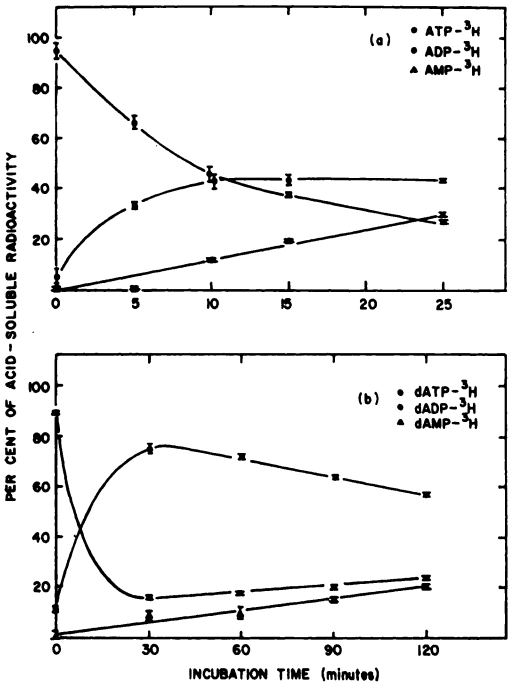

FIG. 2. *Conversion of tritium-labeled ATP (a) and dATP (b) to nucleoside mono- and diphosphates by L-cell nuclei*

Nuclear isolation, incubation conditions, and isolation of cold acid-soluble radioactivity were carried out as described in MATERIALS AND METHODS. Each value represents the mean and standard error of four separate nuclear incubations.

synthesis are not shown. There was a rather sharp optimum at pH 7.6 for dATP incorporation, whereas the optimum for ATP incorporation was broad, with a maximum around pH 8.0.

Enzyme functions affecting state of precursor. In order to determine the amount of breakdown of the ribo- and deoxyribonucleoside triphosphate precursors by enzymes present in the nuclear suspension, tritium-labeled ATP or dATP was added to the conventional reaction mixture, and after varying intervals the reaction was terminated. The nucleoside mono-, di-, and triphosphates were isolated from the acid-soluble supernatant by electrophoresis, and the amount of radioactivity in each of the phosphorylated nucleoside fractions was assayed. The results of four separate such experiments are expressed in terms of percentage of radioactivity recovered on the chromatogram in Fig. 2. There was extensive breakdown of both ATP and dATP to the mono- and diphosphorylated forms. No radioactivity was recovered from the nucleoside region of the electrophoretogram in either case. During the 10-min reaction time, 55% of the ATP was metabolized. The recovery of ADP and AMP from the supernatant fraction increased to 45% and 10% of the total radioactivity, respectively, by 10 min, at which time the ADP concentration had reached equilibrium. As Fig. 2b shows, 80% of the dATP was metabolized to the diphosphate and monophosphate forms earlier than 30 min after initiation of the reaction. In the absence of added nuclei, there was no breakdown of either ATP or dATP on incubation at 37°.

To determine whether nuclei contained extensive kinase activity capable of phosphorylating ribo- and deoxyribonucleosides to the triphosphate precursor form required for polynucleotide synthesis, tritium-labeled adenosine and deoxyadenosine were added to nuclear suspensions in the same amount and at the same specific activity as tritium-labeled ATP and dATP added to control vessels. Under standard incubation conditions, adenosine was incorporated 30% as well as ATP, and deoxyadenosine 25% as well as dATP (Table 5).

To find out whether the nucleoside tri-

during 10 min. b. Incorporation of dATP-³H during 60 min. c. Incorporation of ATP-³H; 27 μg of nuclear protein nitrogen were added to each incubation vessel. d. Incorporation of dATP-³H; 81 μg of nuclear protein nitrogen were added to each incubation vessel. e. Incorporation of ATP-³H during 10 min. f. Incorporation of dATP-³H during 60 min. In experiments e and f, nuclei were isolated as described in MATERIALS AND METHODS except that the nuclear pellet was suspended after the final wash in 0.25 M sucrose–0.05 M Tris without $MgCl_2$. Aqueous divalent salt solution (0.05 ml) was added to 1.0 ml of the usual incubation mixture without $MgCl_2$.

TABLE 5

Effect of addition of nucleosides and nucleotides on incorporation of ATP-^{3}H and dATP-^{3}H into cold acid-insoluble material

Incubation conditions were those described under MATERIALS AND METHODS. Unlabeled nucleosides and nucleoside phosphates were added in 0.05 ml of aqueous solution such that 1.0 μmole was added to each incubation in experiment A and 0.50 μmole in experiment B. In experiment C, adenosine-^{3}H and ATP-^{3}H were added to replicate nuclear incubations at the same concentration, 0.05 μmole/ml, and at the same specific activity, 81 mCi/μmole. In experiment D, the concentration of dATP-^{3}H or deoxyadenosine-^{3}H was 0.01 μmole/ml, and the specific activity was 1 mCi/μmole. The respective amounts of nuclear protein nitrogen and substrate incorporated in the controls for each experiment were: experiment A, 78 μg and 10.0 μμmoles; experiment B, 66 μg and 2.9 μμmoles; experiment C, 93 μg and 15.4 μμmoles; experiment D, 40 μg and 3.7 μμmoles.

Labeled precursor and added nucleoside or nucleotide	Incorporation of labeled nucleotide triphosphate
	% control
A. ATP-^{3}H	
None	100
Adenosine	89
AMP	55
ADP	30
ATP	8
B. dATP-^{3}H	
None	100
Deoxyadenosine	83
dAMP	40
dADP	9
dATP	8
C. ATP-^{3}H	
None	100
Adenosine-^{3}H	
None	29
D. dATP-^{3}H	
None	100
Deoxyadenosine-^{3}H	
None	26

phosphate entered the nucleus in the triphosphate, diphosphate, monophosphate, or nucleoside form, the incorporation of ATP-^{3}H and dATP-^{3}H was challenged by adding 50 times as much unlabeled nucleoside or nucleoside phosphate (Table 5). None of the ribonucleoside phosphates could compete with ATP incorporation as effectively as ATP itself. The incorporation of dATP, however, was inhibited equally well by dADP and dATP.

Nucleoside triphosphate requirements for RNA and DNA synthesis. Table 6 shows that the nuclear incorporation of ATP-^{3}H depended on the presence of all four ribonucleoside triphosphates. Omission of any one of the three unlabeled ribonucleoside triphosphates reduced the incorporation of ATP-^{3}H by approximately 80%. Actinomycin D at 5 μg/ml inhibited the complete system by 87%, but had less effect in the absence of one or two unlabeled ribonucleoside triphosphates. The unlabeled deoxyribonucleoside triphosphates could not substitute for the ribonucleoside triphosphates.

The incorporation of dATP-^{3}H into DNA by L-cell nuclei was also dependent on the presence of all four deoxyribonucleoside triphosphates (Table 6). The incorporation of dATP-^{3}H in the absence of one or two deoxyribonucleoside triphosphates was 45%, and in the absence of all three unlabeled triphosphates 20%, of the incorporation supported by the complete system. The complete reaction was inhibited 72% by actinomycin D (20 μg/ml), and the reactions proceeding in the absence of one, two, or three unlabeled deoxyribonucleoside triphosphates were similarly inhibited. Ribonucleoside triphosphates could not effectively replace the deoxyribonucleoside triphosphates.

To determine whether the limited reaction proceeding in the absence of one or more unlabeled deoxyribonucleoside triphosphates was supported by small amounts of the omitted nucleoside triphosphates present as impurities in the nucleoside triphosphates added, the following experiment was performed. dATP-^{3}H and dCTP were purified by chromatography as described in MATERIALS AND METHODS; in the absence of three nucleoside triphosphates, both purified and unpurified preparations of dATP-^{3}H were incorporated to the same extent relative to the complete system. The relative incorporation of purified dATP-^{3}H in the

TABLE 6

Nucleoside triphosphate requirements for RNA and DNA synthesis

Incubation conditions were those described under MATERIALS AND METHODS, except as noted. In the case of the omission of one or two unlabeled nucleoside triphosphates, the amounts of the remaining unlabeled nucleoside triphosphates were adjusted such that the total amount of unlabeled nucleoside triphosphates was the same as in the complete system. Actinomycin D was added dissolved in 0.05 ml of 10% ethanol in the ATP-^{3}H incorporation studies, and in 40% ethanol in the case of dATP-^{3}H. An equal amount of ethanol was added to all other incubations. Each incubation vessel contained 52 μg of nuclear protein nitrogen.

	Incorporation of ATP-^{3}H			Incorporation of dATP-^{3}H	
Components	−Actinomycin D	+Actinomycin D (5 μg/ml)	Components	−Actinomycin D	+Actinomycin D (20 μg/ml)
	μμmoles			*μμmoles*	
Complete system	10.52	1.43	Complete system	1.17	0.33
−UTP	1.13	0.63	−dTTP	0.43	0.18
−GTP	1.59	1.20	−dGTP	0.70	0.19
−CTP	2.65	0.76	−dCTP	0.52	0.18
−UTP, GTP	1.30	1.15	−dTTP, dGTP	0.54	0.15
−UTP, CTP	0.91	0.48	−dTTP, dCTP	0.53	0.14
−GTP, CTP	1.74	1.35	−dGTP, dCTP	0.53	0.18
−UTP, GTP, CTP	2.37	2.65	−dTTP, dGTP, dCTP	0.23	0.08
+dGTP, dCTP, dTTP[a]	1.11	0.50	+GTP, CTP, UTP[b]	0.37	0.10

[a] Instead of ribonucleoside triphosphates.

[b] Instead of deoxyribonucleoside triphosphates.

absence of dGTP and dTTP was the same whether either purified or unpurified dCTP was added.

Evidence for template requirement for incorporation of ATP or dATP into cold acid-insoluble material. The addition of DNase (100 μg/ml) resulted in 75% inhibition of ATP-^{3}H incorporation. After a 5-min preliminary incubation of the nuclei with DNase, RNA synthesis was inhibited more than 90%. That this inhibition was due to the action of DNase in digesting the template and did not result from digestion of the product is supported by the data in Table 7, which show that only 10% of the RNA product was digested during a 30-min incubation with the same DNase preparation. Addition of purified DNA template to the nuclear suspension did not stimulate RNA synthesis as it did DNA synthesis. Complete sonic disruption of the nuclei obliterated the RNA-synthesizing capacity of the preparation, and the activity could not be restored by addition of purified exogenous DNA.

In addition to the fact that ATP incorporation is DNase-sensitive, the sensitivity of RNA synthesis to low concentrations of actinomycin D indicates that the observed ATP incorporation was template-directed (22). The incorporation of ATP into acid-insoluble material was inhibited more than 70% by only a 0.25 μg/ml concentration of actinomycin D and was maximally inhibited at a concentration of 2 μg/ml (Fig. 3). DNA synthesis was also inhibited by actinomycin D; however, the inhibition at 2 μg/ml was only 50%. A maximum of 80% inhibition of DNA synthesis was achieved at 20 μg of actinomycin D per milliliter.

The incorporation of dATP-^{3}H into DNA was stimulated many fold by the addition of purified DNA to the incubation mixture (Table 4 and Fig. 4). Heat-denatured DNA was more effective than native DNA, and the maximum stimulation of DNA synthesis achieved by addition of denatured DNA was greater than that attained with native DNA. Highly polym-

TABLE 7

Effects of acid, base, and enzyme hydrolysis on cold acid-insoluble products of ATP-^{3}H and dATP-^{3}H incorporation

Incubation of the nuclei was carried out as described under MATERIALS AND METHODS. The washed, cold acid-insoluble precipitate was incubated for 18 hr at 37° with 0.3 M KOH or for 15 min at 90° with 6% perchloracetic acid, and the amount of radioactivity remaining in cold acid-insoluble form was assayed. In the case of RNase or DNase treatment of the product, the incorporation of ATP-^{3}H or dATP-^{3}H was stopped by heating at 60° for 5 min. After cooling, 200 μg of RNase or DNase were added, and the samples were incubated for 30 min at 37°. The reaction was stopped by addition of cold trichloracetic acid, and the samples were washed and assayed for radioactivity in the usual manner. Each incubation contained 32 μg of nuclear protein nitrogen.

Treatment of product	AMP-^{3}H remaining in acid-insoluble form	dAMP-^{3}H remaining in acid-insoluble form
	μμmoles	*μμmole*
None	6.2	0.95
Alkaline hydrolysis	0	0.92
Hot acid hydrolysis	0	0
RNase	0	0.68
DNase	5.4	0.16

erized synthetic poly dAT was a more active template than either the native or denatured form of any DNA yet tried (Fig. 4). Purified, highly polymerized DNAs from several sources (L-cell, calf thymus, and sea urchin sperm) all stimulated dATP incorporation.

Analysis of products of ATP and dATP incorporation. To ascertain that the radioactive product formed on incorporation of ATP-^{3}H was indeed RNA and that measured by the incorporation of dATP-^{3}H was DNA, the experiment presented in Table 7 was carried out. The cold acid-insoluble radioactivity derived from a 10-min incubation with ATP-^{3}H was completely hydrolyzed by 0.3 M KOH, was completely solubilized by a 30-min incubation with RNase, and was not affected by a 30-min incubation with DNase. The product of a 60-min incorporation of dATP-^{3}H was not hydrolyzed by 0.3 M KOH, was completely hydrolyzed by hot trichloracetic acid, and was slightly digested by the purified RNase preparation and extensively digested by a 30-min incubation with DNase.

The labeled RNA produced on incorporation of ATP-^{3}H was further characterized by sucrose density centrifugation (Fig. 5).

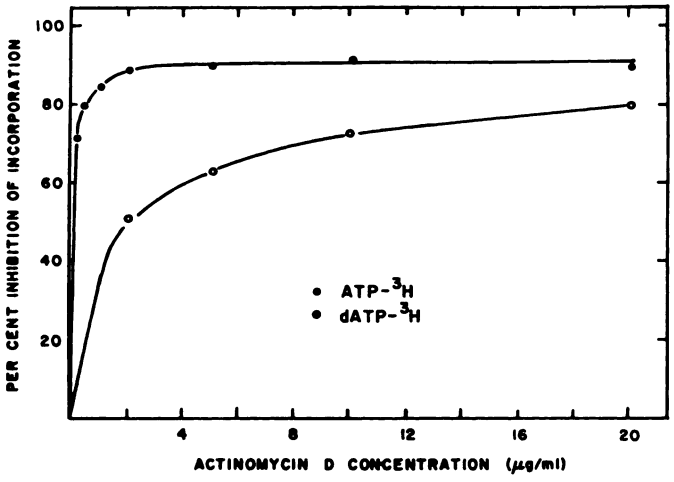

FIG. 3. *Inhibition by actinomycin D of ATP-^{3}H and dATP-^{3}H incorporation into cold acid-insoluble material by L-cell nuclei*

Actinomycin D was added dissolved in 0.05 ml of aqueous ethanol, and equivalent amounts of ethanol were added to all control incubations.

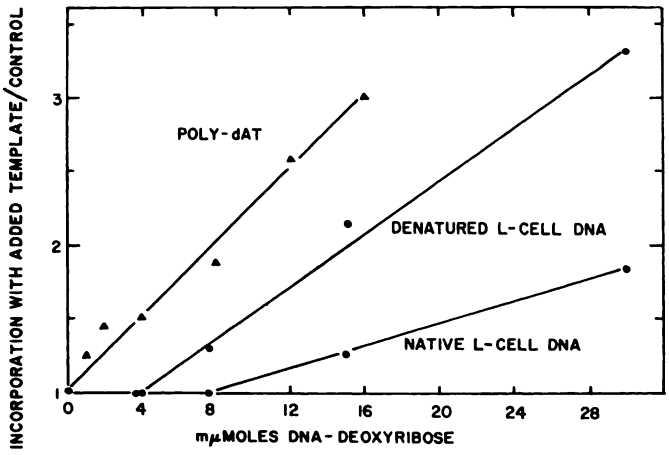

FIG. 4. *Comparison of the efficacy of native DNA, denatured DNA, and poly dAT as templates for dATP incorporation by an L-cell nuclear suspension*

Native and heat-denatured L-cell DNA were added to the incubation in 0.05 ml of 0.01 M KCl. Native, highly polymerized L-cell DNA, purified from washed L-cell nuclei as described under MATERIALS AND METHODS, was heated to 100° for 10 min and quickly cooled in ice. There was a 30% increase in absorbance at 260 mμ on heat denaturation. Poly dAT was added dissolved in 0.05 ml of 0.02 M KCl–0.01 M Tris, pH 8.0. Each incubation mixture contained 85 μg of nuclear protein nitrogen.

The optical density sedimentation profile revealed very little 28 S and 18 S material in comparison with the light RNA peak. The radioactive RNA product sedimented largely in the range from 15 to 30 S. Only a very small fraction of the newly formed RNA remained with the light material.

The product formed on incubation of nuclei with dATP-^{3}H was analyzed by cesium chloride density gradient centrifugation (Fig. 6). The radioactivity representing the newly synthesized material sedimented with the same average density as purified L-cell DNA, which consists of a

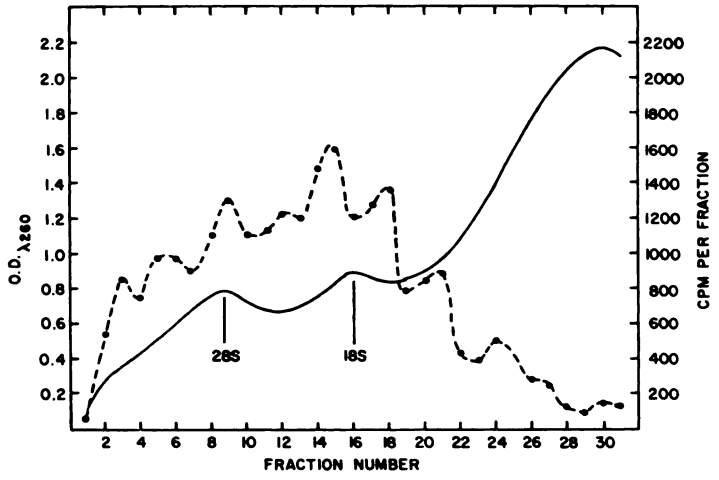

FIG. 5. *Sucrose density gradient analysis of the product formed on incubation of an L-cell nuclear suspension with ATP-^{3}H*

RNA was purified from nuclei which had been incubated for 10 min with ATP-^{3}H and centrifuged in a 5–20% sucrose gradient as described under MATERIALS AND METHODS. ——, Optical density at 260 mμ; ●– – –●, counts per minute recovered per fraction.

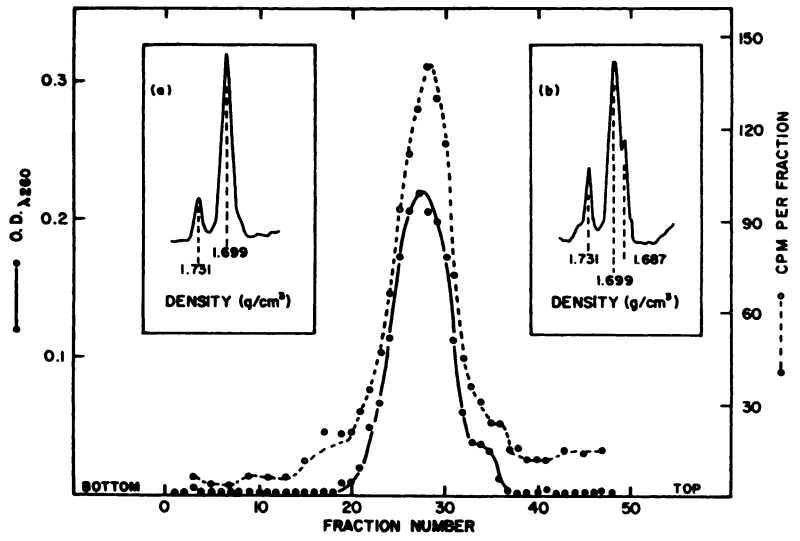

FIG. 6. *Cesium chloride equilibrium gradient analysis of the product formed on incubation of an L-cell nuclear suspension with dATP-³H*

DNA was purified from nuclei which had been incubated for 60 min with dATP-³H and centrifuged in cesium chloride as described in MATERIALS AND METHODS. Insets represent optical densitometer tracings of the recentrifugation of fractions from the light and dense sides of the DNA peak as described in the text.

main peak with a density of 1.699 g/ml and a satellite band representing approximately 5% of the total nuclear DNA (23, 24), with a density of 1.687 g/ml. A second gradient was run in parallel with that shown in Fig. 6, and fractions corresponding to Nos. 25 and 33–34 were recentrifuged in an analytical ultracentrifuge. Figure 6a is a microdensitometer tracing of the recentrifugation of fraction 25, exhibiting a single optical density peak at 1.699 g/ml. Inset b, representing the recentrifugation

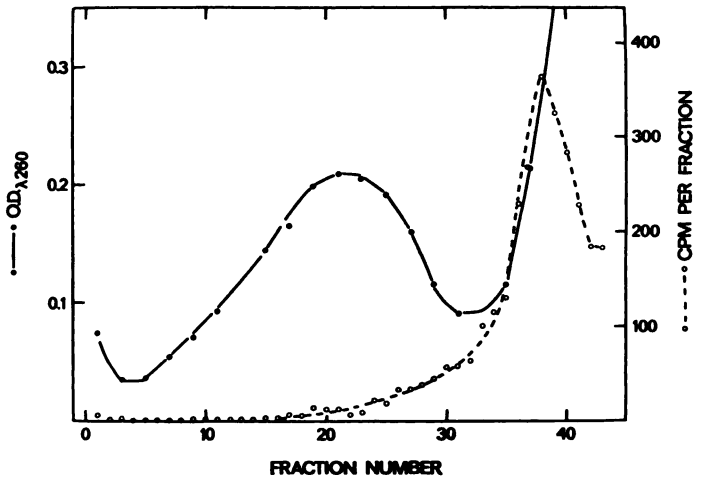

FIG. 7. *Alkaline sucrose density gradient analysis of the product formed on incubation of an L-cell nuclear suspension with dATP-³H*

Nuclei were incubated with dATP-³H for 60 min, after which they were centrifuged at 600 × *g*, lysed with 0.1 N NaOH, and centrifuged in a 5–20% alkaline sucrose gradient as described in MATERIALS AND METHODS.

of pooled fractions 33 and 34, shows the presence of a large amount of satellite DNA with a density of 1.687 g/ml. The satellite peak was enriched several times relative to its presence in total nuclear DNA (16). The radioactivity was recovered with the satellite band as well as with the main band. Labeled DNA was purified and analyzed in the same manner after incubation of nuclei with dCTP-^{3}H, with identical results.

Newly synthesized DNA was also analyzed on an alkaline sucrose density gradient (Fig. 7). dATP-^{3}H incorporation into DNA resulted in a small molecular weight product that formed a band at the upper portion of the gradient tube, whereas bulk nuclear DNA settled in the center of the gradient. Addition of NAD did not change the banding pattern of the tritium-labeled light material, nor did the addition of bacterial ligase. Addition of ATP stimulated the total reaction (measured by incorporation of dATP-^{3}H) by 20–30% and increased the molecular weight of some of the radioactive product. This effect is being investigated further.

RNase and DNase activities in L-cell nuclear preparations. The specific activity (expressed on the basis of protein nitrogen) of acid RNase in the nuclear preparation was very low, approximately 10% of that of the broken cell suspension. The activity of alkaline RNase in the nuclear preparation was approximately 30% of the enzyme activity assayed in the broken cell suspension. The specific activities of DNase were similar in the purified nuclear preparation and the broken cell suspension.

Stability of the product RNA and DNA in the nuclear preparation at 37°. To determine the rate of breakdown of RNA and DNA synthesized by the nuclear preparation, a large excess of unlabeled ATP or dATP was added to standard incubations in progress, and the cold acid-insoluble radioactivity was assayed at various time intervals thereafter. The amount of cold acid-insoluble radioactivity per incubation declined slowly after the addition of excess unlabeled nucleoside triphosphate in both the ATP-^{3}H and dATP-^{3}H incorporation mixtures (Fig. 8). Approximately 10% of the radioactivity

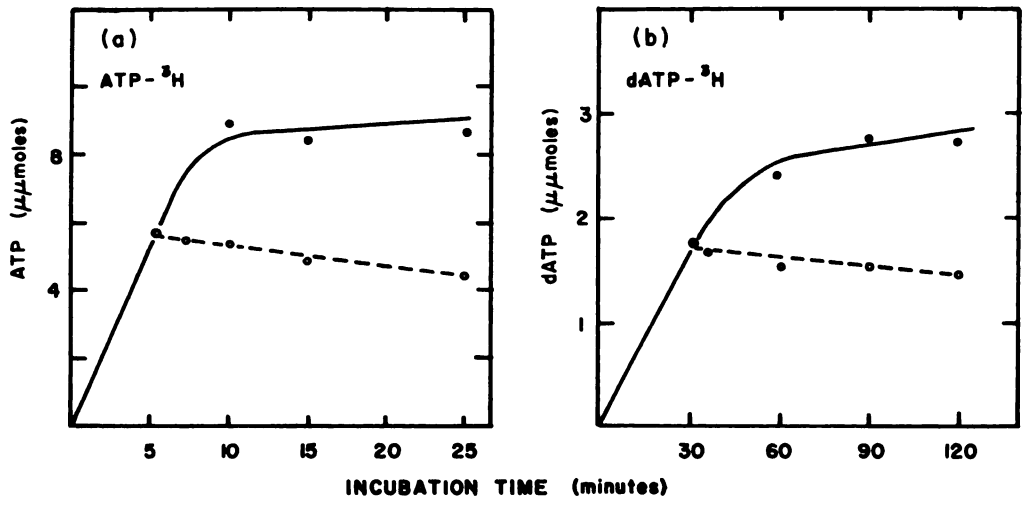

FIG. 8. *Stability of the product formed on incubation of L-cell nuclei with ATP-^{3}H (a) or dATP-^{3}H (b)*

One micromole of unlabeled ATP (a) or 0.5 μmole of dATP (b) was added at 5 min and 30 min, respectively, to incubations in progress. At various time intervals thereafter, duplicate control samples and duplicate unlabeled nucleoside triphosphate chase incubations were precipitated with cold trichloracetic acid, and the radioactivity was assayed in the usual manner. ●——●, Control incubations; O- - -O, incubations with added unlabeled nucleoside triphosphate. Each incubation mixture contained 44 and 66 μg of nuclear protein nitrogen in experiments a and b, respectively.

from ATP-^{3}H incorporation into the macromolecular form was solubilized in 10 min of incubation at 37° (the duration of linear RNA synthesis). The rate of digestion of the DNA product was considerably slower: approximately 10%/hr.

Effect of direct addition of fluocinolone acetonide on nuclear RNA and DNA synthesis. Fluocinolone acetonide, at 5×10^{-7} M and at 5×10^{-5} M (the latter concentration is 500 times the maximum growth-inhibitory dose in the intact cell), was added to crude broken cell preparations and to isolated, washed nuclei. There were no detectable effects on either RNA or DNA synthesis at either concentration (Table 8).

Nucleic acid synthesis in whole cells and isolated nuclei 12 hr after exposure to fluocinolone acetonide. Cells growing *in vitro* were treated with 5×10^{-7} M fluocinolone acetonide, and radioactive thymidine and uridine incorporation into whole cells was measured 12 hr later. It has been determined (2) that at this time interval, at this dose of drug, the inhibition of thymidine incorporation in intact cells is twice that of uridine. As expected, there was marked inhibition of thymidine incorporation and a smaller effect on uridine incorporation (Table 9). Nuclei were isolated from other cultures treated identically, and RNA and DNA synthesis from labeled nucleoside triphosphates was measured. DNA synthesis (per microgram of protein nitrogen) was inhibited to only a small extent, and similar inhibition of RNA synthesis was observed (Table 9). Both the control and treated cultures had very similar amounts of total cells, nuclei, and nuclear protein. By 44 hr after treatment, however, the treated group had only 32% of the number of cells per culture in the control group, as expected.

DISCUSSION

In studies on nucleic acid synthesis in intact nuclei, it is necessary to take into account the activity of enzymes present, other than the polymerases, which can directly affect the observed rate of nucleoside triphosphate uptake. These include the nucleases, which may degrade either the template or the product, and the phosphatases and kinases, which affect the levels of the nucleotide substrates. The intact L-cell nuclear preparation contained some nuclease activity, which might conceivably interfere with the detection and estimation of polymerase activity. However, the RNase and DNase present in the purified nuclear preparation solubilized less than 10% of the newly synthesized polynucleotide during the standard incu-

TABLE 8

Effects of direct addition of fluocinolone acetonide on subcellular preparations of mouse fibroblasts

Nuclei and broken cells were prepared and incubated with dATP-^{3}H and ATP-^{3}H as described in MATERIALS AND METHODS. The steroid, dissolved in ethanol, was added to replicate incubation vessels, and the appropriate amount of vehicle (maximum, 5 μl) was added to the controls. Each broken cell incubation contained 52 μg of protein nitrogen; nuclear incubations contained 60 μg of protein nitrogen.

Labeled precursor	Concentration of fluocinolone acetonide	Broken cells		Nuclei	
		Incorporation	Ratio of treated to control	Incorporation	Ratio of treated to control
	M	*μμmoles*		*μμmoles*	
dATP-^{3}H	0	1.57		1.88	
	5×10^{-7}	1.52	0.97	2.03	1.08
	5×10^{-5}	1.37	0.87	2.17	1.15
ATP-^{3}H	0	9.4		16.4	
	5×10^{-7}	9.7	1.03	16.0	0.98
	5×10^{-5}	8.9	0.95	15.2	0.93

TABLE 9

Effects on cell growth and nucleic acid synthesis in whole cells and isolated nuclei of mouse fibroblasts after 12 hr of treatment with 5×10^{-7} M fluocinolone acetonide

The table summarizes the data from three experiments. Fluocinolone acetonide was added to replicate cultures during the logarithmic phase of growth. In each experiment the number of cells per culture was approximately the same at the time of steroid addition. Twelve hours after treatment, either 0.55 mμmole of thymidine-methyl-^{3}H (6.0 Ci/mole) or 9.6 mμmoles of uridine-2-^{14}C (52 mCi/mmole) were added to triplicate control and treated cultures for 1 hr at 37°. At the end of the incubation, the cells were counted and assayed for radioactivity as described under MATERIALS AND METHODS. Incorporation of uridine was examined in only two of the three experiments. The cells in the remainder of the replicate cultures were harvested, and the nuclei were isolated and assayed for DNA and RNA polymerase activity as described under MATERIALS AND METHODS. In each experiment, two independent nuclear suspensions were prepared from both treated and control cells. *n* refers to the number of individual cultures analyzed in the case of cell count, thymidine incorporation, and uridine incorporation, and to the individual nuclear preparations with respect to dATP-^{3}H and ATP-^{3}H incorporation. Results are expressed as mean ± standard error. Forty-four hours after steroid measurement, the number of cells per treated culture was 32 ± 2% that of the control cultures in the three experiments.

	Whole cells			Nuclei	
Preparation	Cells/culture (n = 15)	Thymidine (n = 9)	Uridine (n = 6)	dATP (n = 6)	ATP (n = 6)
	$\times 10^{-5}$	(cpm/culture) $\times 10^{-4}$		cpm/μg protein N	
Control	4.45 ± 0.26	5.21 ± 0.20	1.47 ± 0.05	9.5 ± 0.5	7.2 ± 0.6
Treated	4.14 ± 0.22[a]	2.21 ± 0.21[b]	1.00 ± 0.04[b]	6.9 ± 0.4[b]	5.4 ± 0.2[b]
Inhibition	7%	58%	32%	27%	25%

[a] Not significantly different from the control.

[b] Significantly different from the control ($p < 0.01$).

bation interval. We also examined the capacity of intact, washed nuclei to degrade ATP and dATP, but not the other complementary nucleoside triphosphates. Both ATP and dATP were rapidly degraded to the nucleoside monophosphates and diphosphates. No radioactivity from dATP-^{3}H was recovered as the nucleoside, even after extended periods of incubation, indicating either a low level or a complete absence of 5′-nucleotidase activity. Adenosine and deoxyadenosine were about one-fourth as active as substrates for nucleic acid synthesis as their corresponding triphosphates. The L-cell nuclei therefore have nucleoside kinase, nucleoside monophosphate kinase, and nucleoside diphosphate kinase activities. The addition of an ATP-regenerating system to the nuclear suspension did not enhance the rate of incorporation of labeled nucleoside triphosphate into RNA. This observation contrasts with the rat liver nuclear system described by MacGregor and Mahler (25), in which a phosphoenolpyruvate–pyruvate kinase system stimulated the incorporation of GTP-^{3}H into RNA.

Since Weiss first demonstrated that RNA polymerase activity was present in a DNA-protein complex of nuclear origin (26), the RNA polymerase activity in the complex or in intact nuclei has been well characterized. In spite of the extensive studies done on RNA polymerase activity in isolated nuclei (25, 27–29), we thought it useful to characterize RNA synthesis in addition to DNA synthesis in isolated L-cell nuclei. The intact nuclear system thus could be employed in the rapid identification of the site of action of growth-inhibitory agents which exert differential inhibition on the rate of synthesis of DNA as opposed to that of RNA [e.g., actinomycin D (30), miracil D (31), mitomycin C (32)].

The activity of RNA polymerase in our

system averaged around 900 $\mu\mu$moles of ATP-^{3}H incorporated in 10 min/mg of DNA. The activity of liver nuclei has been reported as 926 $\mu\mu$moles of ATP-^{3}H incorporated in 15 min/mg of DNA (33) and 700 $\mu\mu$moles of GTP-^{3}H incorporated in 10 min/mg of DNA (25). The RNA polymerase activity of L-cell nuclei is more than twice that reported for heart, uterine (33), and lymphocyte nuclei (34).

Studies on the subcellular distribution of RNA polymerase revealed that all of the activity is present in the nuclei, or that any soluble polymerase is rapidly inactivated during the nuclear isolation. This observation is consistent with the presence of RNA polymerase activity in the DNA-protein complex described by Weiss (26), and with the assignment of RNA polymerase to the chromatin space of the cell (21). Some rat liver nuclear RNA polymerase has been shown to exist in a soluble form (35, 36), but in our studies the addition of exogenous DNA to any of the different cellular fractions of the mouse fibroblast did not result in any increase in ATP incorporation.

As shown in Fig. 5, the RNA produced by fibroblast nuclei had an intermediate sedimentation velocity (15–30 S), and very little radioactivity accompanied low molecular weight RNA. These results are similar to those obtained by MacGregor and Mahler (25) with RNA synthesized *in vitro* at 23° by suspensions of rat liver nuclei. Similar experiments carried out by Widnell and Tata (28) yielded somewhat different results, indicating that rat liver nuclei synthesize mainly RNA that sediments more slowly than 18 S RNA. The primary reason for the differences in molecular weight of the RNAs synthesized in the various systems may be the different levels of ribonuclease activity present. Mouse fibroblast nuclei have relatively low ribonuclease levels, as evidenced by the stability of the newly synthesized RNA, and therefore, even though the incubation was carried out at 37°, RNA with a reasonably high molecular weight was obtained. In rat liver nuclei incubated at 23°, the ribonuclease activity is probably considerably less than at 37°, the incubation temperature used by Widnell and Tata. It has been stressed by Nair, Rabinowitz, and Tu (37) that the RNA synthesized by the Mg^{++}-activated reaction undergoes marked degradation from high to low molecular weight material.

Although the ability of isolated mammalian nuclei to synthesize DNA has been demonstrated by several investigators, the DNA polymerase reaction in isolated nuclei has never been characterized to an extent that would allow the system to be useful in studying the effects of agents on DNA synthesis. Instead, the very low activity recovered in isolated nuclei and the ready solubility of the bulk of the enzyme have resulted in a concentration on purification and characterization of the soluble enzymes (38–43). There are several arguments for preferring the use of isolated nuclei in studies of the regulation of mammalian DNA synthesis instead of the purified, soluble enzyme.

In contrast to purified systems, the intact nucleus provides an environment which more closely approximates that of the whole cell while allowing considerable manipulation of the reaction conditions. The fact that the polymerase is not purified may be an advantage, as has been suggested by Keir (44). The use of nuclei allows the DNA template to be studied in its natural state as a combination of DNA, RNA, histones, and non-histone proteins. An intact nuclear membrane is highly desirable in a system in which drug effects on DNA synthesis are studied, because considerable indirect evidence has indicated that membrane components play a role in DNA synthesis (45–47). Finally, it is becoming clear that auxiliary enzymes such as polynucleotide ligase (48) and deoxyribonuclease (49) may be necessary for normal DNA synthesis to occur. The existence of these enzymes in a partially purified DNA polymerase–membrane complex in bacteria supports the idea that these enzymes will be retained in isolated nuclei (50). In fact, polynucleotide ligase in bone marrow cells appears to be predominantly a nuclear enzyme (51).

Although DNA polymerase is soluble,

there is evidence that the DNA polymerase retained in isolated nuclei may be a better measure of the rate of DNA synthesis *in vivo* than the activity of the soluble enzyme. Our own results confirm the fact that the enzyme is very soluble. A large percentage of the activity is easily leached out of the nuclei by washing in sucrose solution, but continued washing of the purified nuclei results in very little additional loss of DNA polymerase, an observation basically in agreement with the findings of Bazill and Philpot (52) with calf thymocyte nuclei. Also, DNA polymerase is not as readily extracted from the nuclei during washing as is a soluble enzyme such as phosphohexose isomerase. These data indicate that a portion of the DNA polymerase in the cell may be firmly bound in the nucleus. The small amount of particulate DNA polymerase may have considerable biological significance, because the enzyme appears to become bound to particulate material during periods of DNA synthesis and then released in soluble form when DNA synthesis is not taking place (40, 53). The inhibition of DNA synthesis by porfiromycin also results in an increase in soluble DNA polymerase (54). These experiments suggest that DNA polymerase which is actively synthesizing DNA exists in bound particulate form.

One of the disadvantages of the nuclear system is the low DNA polymerase activity observed in mammalian nuclei. In rat thymus nuclei (52) the DNA polymerase activity has been reported as 44 $\mu\mu$moles of nucleotide incorporated per hour per milligram of DNA, which is similar to our observation of an average activity of 18 $\mu\mu$moles of dATP-^{3}H incorporated per hour per milligram of DNA in L-cell nuclei. Nuclei isolated from sea urchin embryos (55) are reported to be much more efficient, with an activity of approximately 800 $\mu\mu$moles of nucleotide incorporated per hour per milligram of DNA, directed solely by endogenous primer. This higher activity might be expected, however, since sea urchin embryo cells divide in synchrony every 100 min whereas L-cells have a generation time of 18 hr and divide randomly.

The activity observed by us is equivalent to 6 $\mu\mu$moles of dATP-^{3}H incorporated per hour per micromole of endogenous DNA deoxyribose, or approximately 24 $\mu\mu$moles of nucleotide incorporated per micromole of DNA deoxyribose, and represents an extent of synthesis of only 0.0024% of the endogenous template. Since the doubling time of the cells under these growth conditions is 18 hr, a culture after 1 hr in the logarithmic phase of growth should synthesize an amount of new DNA equivalent to 1/18, or 5.6%, of the total DNA in the culture. The reason for the large discrepancy between predicted and observed extents of synthesis is not known. The small extent of synthesis cannot be attributed merely to loss of polymerase activity from the nucleus, as the addition of cytoplasm containing high polymerase activity to the washed nuclei did not result in an increase in DNA synthesis. In addition, we have tried several more gentle methods of cell disruption, without increasing the extent of DNA synthesis.

Although most DNA polymerase preparations have exhibited a preference for denatured DNA over native DNA as primer, several exceptions to this observation have been published (56–59). When we added exogenous primer to a nuclear suspension, the bulk, if not all, of the resulting increased incorporation of tritium label was found outside the nuclei. Therefore, we probably measured a reaction involving soluble DNA polymerase which had leached out of the nuclei and the extranuclear purified DNA template. Under these conditions, denatured DNA was a more efficient template than native DNA. This observation does not necessarily mean that the DNA polymerase as it functions inside the nucleus has a structural preference for denatured over native DNA as a template. In the nuclear system examined in this paper dAT copolymer was more active as a primer than either native or denatured DNA; however, it was not 20 times more active, as has been reported for purified polymerases from *Escherichia coli* and *Bacillus subtilis* (60). The observation that exogenous DNA stimulates nuclear

dATP incorporation contrasts with data obtained by Friedman and Mueller (61), who observed no stimulation of DNA synthesis upon addition of exogenous DNA to isolated nuclei of HeLa cells. The reason for this discrepancy is unclear.

An enzyme which incorporates ribo- or deoxyribonucleotides into terminal positions of DNA has been identified in calf thymus nuclei and Landschutz ascites tumor cells (62, 63). A similar cytoplasmic terminal addition enzyme has been recognized in calf thymus (64). The incorporation of any single nucleotide is not stimulated by addition of the complementary nucleotides in these terminal addition reactions. Because L-cell nuclear dATP incorporation is inhibited 80% by withdrawal of the three complementary nucleotides, most of the incorporation of dATP during a 60-min incubation cannot be accounted for by terminal nucleotidyltransferase activity. Furthermore, 70–80% of nuclear incorporation in L-cells is inhibited by actinomycin D and cannot represent terminal nucleotidyltransferase activity, as this reaction is not inhibited by actinomycin D at concentrations which inhibit the replicative enzyme almost completely (63). In addition, the cytoplasmic terminal addition enzyme is basically a dATP polymerase, while L-cell nuclei readily incorporate both tritium-labeled dTTP and dATP.

dATP incorporated into DNA by terminal addition or repair reactions (65) is added to a pre-existing long polynucleotide chain, and the resulting product would be expected to have a high molecular weight. Alkaline sucrose gradient sedimentation, however, indicated that the newly formed DNA in isolated nuclei consisted entirely of low molecular weight material. It is unlikely that this was due to the breakdown of DNA of high molecular weight by nucleases, because the average molecular weight increases with the time of incubation. While this observation is not consistent with terminal addition or repair reactions, it agrees quite well with *in vivo* data concerning normal replication. Newly synthesized DNA in viruses, bacteria, HeLa cells, and regenerating liver apparently has a low molecular weight, in contrast to bulk DNA (18, 66, 67). This suggests that the nuclei support replicative DNA synthesis.

It is disturbing that little of the DNA product was converted to DNA with a higher molecular weight in the 1-hr incubation, because in intact HeLa cells (66), liver cells (67), and L-cells 1-hr pulse-labeling with radioactive thymidine results predominantly in DNA with a high molecular weight, equal to that of bulk DNA. This may be explained by the low extent of synthesis discussed above. In a cell culture with a generation time of 18 hr, the extent of synthesis, 0.0024%, observed in a 1-hr incubation in isolated nuclei would require much less than 1 min. In this short time all of the DNA formed would have a low molecular weight. It is also possible that the isolated nuclei were unable to convert the DNA to a high molecular weight form and that this factor limited the extent of synthesis. We are presently exploring conditions necessary for this conversion in isolated nuclei.

The incorporation of dATP into cold acid-insoluble material by L-cell nuclei depended on the presence of all four complementary deoxyribonucleoside triphosphates, was DNA template-dependent, and resulted in a macromolecular product which behaved like DNA and had the same buoyant density as the endogenous nuclear DNA template. These findings suggest that incorporation of dATP is the expression of DNA polymerase activity in the nuclear preparation. Although it is not possible to define the type of DNA polymerase reaction precisely, the low molecular weight of the DNA synthesized suggests that it is similar to the reaction which occurs in normal replication. It is quite likely that the DNA synthesis observed in isolated nuclei is similar to that seen in a broken cell preparation containing intact nuclei, which has been characterized and described by Friedman and Mueller (61) as a model system for replication.

The treatment of mouse fibroblasts with glucocorticoids resulted in profound inhibition of DNA and RNA synthesis as measured by thymidine and uridine incorporation, with no inhibition of leucine uptake (2).[3] As the inhibition of thymidine incorporation was twice that of uridine, the hormone would seem to affect primarily DNA synthesis. The inhibition of thymidine and uridine incorporation apparently did not result from changes in the pool size of the precursors. The inhibition of DNA synthesis was not attributable to inhibition of the conversion of thymidine to dTTP or to increased breakdown of DNA, and could not be reversed by the addition of deoxyribonucleosides to the culture medium. From this evidence it was postulated that the glucocorticoid might affect the amount or activity of DNA polymerase or the priming ability of the DNA template.

The present study indicates that glucocorticoids do not directly affect the activity of DNA or RNA polymerase or the template activity of DNA if added directly to isolated nuclei. This is not surprising, in view of the fact that there is a lag period of 6 hr before any effect on thymidine incorporation is observed in whole cells, indicating some intermediate steps between the addition of the steroid and the inhibition of DNA synthesis. It is unlikely that metabolism of the steroid is one of the steps involved in this delay, because fluocinoline acetonide is not metabolized by L-cells (68). The possibility remains that the lag period may be explained by the time needed to alter the level or amount of DNA polymerase or another factor which influences nucleic acid synthesis.

To test this hypothesis, nuclei were isolated from L-cells that had been treated for 12 hr with fluocinolone acetonide, a procedure that results in maximal inhibition of both uridine and thymidine incorporation in the whole cell, with the characteristic differential effect. The inhibition of RNA polymerase activity in these isolated nuclei was of approximately the same magnitude as the inhibition of uridine incorporation seen in the intact cell, and thus the administration of glucocorticoids appears to result in depression of either the activity of RNA polymerase or that of the template. Similar results have been obtained in thymocytes after cortisol treatment (69, 70).

DNA polymerase activity in the nuclei was also inhibited, but to only half the extent of the inhibition of thymidine incorporation in intact cells, and no differential between inhibition of RNA and DNA synthesis was found in the nuclear preparation. This result suggests that the inhibition of thymidine incorporation is quite complex and may be due to several factors rather than to simple inhibition of DNA polymerase or DNA template activity. Small decreases in the pool sizes of substrate in the whole cell or alterations in the transport of the precursor into the cell may be partially responsible. A second possibility is that glucocorticoids might affect a factor important in DNA synthesis, but not properly assayed in the isolated nuclei. An example in this respect is polynucleotide ligase, since, as mentioned before, there is apparently little conversion of newly synthesized DNA to high molecular weight DNA under the assay conditions described in this study. Thus, if glucocorticoids affected the levels of ligase in the cell, little impairment of DNA polymerase activity would be seen in the isolated nuclei.

A possibility not entirely ruled out is the alteration of the cellular population by glucocorticoids so that fewer cells are in the synthesis phase of the cell cycle. This is supported by the fact that the amount of DNA per L-cell increases during treatment with cortisol (3). This would be consistent with a block in the G_2 phase of the cell cycle just prior to cell division. The lag phase of 6 hr before any inhibition is observed and the 12 hr required for the development of maximal inhibition are

[3] It is absolutely necessary that the L-cell cultures be free of contamination with *Mycoplasma* for this effect of glucocorticoids to be demonstrated.

also consistent with this hypothesis. Evidence has been obtained that cortisol alters the cell cycle of squamous epithelial cells of the mouse forestomach (71). If this hypothesis is true, it is still quite difficult to explain the lack of correlation between the inhibition of thymidine incorporation in whole cells and the inhibition of DNA polymerase activity in isolated nuclei.

The data obtained for fibroblasts as well as for thymocytes and lymphatic cells are inadequate to support the idea that alterations of nucleic acid synthesis are primary effects; in fact, the lag period suggests that these alterations are secondary to other, earlier biochemical lesions. As mentioned before, an effect on transport mechanisms may be important. In thymocytes an early effect appears to be decreased transport of nucleic acid and protein precursors into the cell (72). The same type of process may diminish the energy available to the cell by inhibiting the uptake of glucose, as has been demonstrated in the thymocyte (73). Whether alterations of nucleic acid metabolism are primary or secondary responses to glucocorticoid administration, they are important molecular events underlying the growth-inhibitory effects of these steroids.

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