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Characteristics of *in Vitro* Ribonucleic Acid Synthesis by Macronuclei of *Tetrahymena pyriformis**

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ABSTRACT: The objective in this work was to establish an *in vitro* system for the study of RNA synthesis in intact macronuclei isolated from normal and synchronized cultures of *Tetrahymena pyriformis* GL. A nonionic detergent, Triton X-100, was used to isolate macronuclei. Examination of these macronuclei by electron microscopy showed no cytoplasmic contamination. DNA-dependent RNA polymerase activity was retained in these preparations; the enzyme required all four ribonucleoside triphosphates, Mg^{2+} or Mn^{2+} ions, and intact DNA. No enzyme activity was observed when DNA was destroyed with deoxyribonuclease or when transcription was blocked with actinomycin D. Various factors influencing the rate of RNA synthesis in the isolated macronuclei were studied. In the presence of Mg^{2+} and KCl, it was demonstrated that the macronuclear system of *Tetrahymena* prepared from exponentially growing cultures incorporated about 5 mμmoles of labeled UTP/mg of nuclear DNA. A stimulation of activity occurs when Mn^{2+} and 200 mM ammonium sulfate are substituted for Mg^{2+} and KCl. However, analysis of base composition and nearest neighbor base frequencies indicated that the product of the RNA polymerase reaction is like DNA under both conditions, and does not resemble *Tetrahymena* ribosomal or whole cell RNA. Studies on the kinetics of RNA synthesis in isolated macronuclei showed that the newly synthesized RNA has a high turnover rate (rapid synthesis and breakdown), and that the hydrolysis of the newly synthesized RNA occurred in direct proportion to temperature and reaction time.

A ciliated protozoan, *Tetrahymena*, has several useful biochemical and morphologic properties. Like other ciliates, it is capable of exchanging nuclear materials as well as undergoing internal genetic reorganization (*e.g.*, autogamy) under defined circumstances (Beale, 1954). Its phenotypic expression can also be reversibly altered by physical factors (Lwoff, 1950). In addition, many strains can be induced to divide synchronously by physical or chemical means (Scherbaum and Zeuthen, 1954; Stone, 1968). Its evolutionary development places it close to the divergence between plant and animal forms (Holz, 1966). The cytoplasmic RNAs of *Tetrahymena pyriformis* are suggestive of a very primitive animal cell (Kumar, 1969). These various factors predict a useful role for these organisms in the biochemical analysis of gene expression and cytokinesis in eukaryotes.

We have recently been interested in RNA metabolism during the induction of division synchrony in *Tetrahymena* cultures. A variety of experiments have suggested that temperature effects on mRNA synthesis or turnover are involved (Byfield and Lee, 1970), but kinetic analysis using labeled extracellular ribonucleosides is fraught with interpretative difficulties (Nierlich, 1967). We have therefore developed a procedure to isolate substantial numbers of highly purified macronuclei in order to study the properties of the DNA-dependent RNA polymerase. The experiments reported here indicate that the enzyme from *Tetrahymena* appears similar but not identical with the equivalent enzyme activity existing in various bacterial and mammalian systems (Weiss, 1960; Hurwitz *et al.*, 1962; Chamberlin and Berg, 1962; Tsukada and Lieberman, 1964; Widnell and Tata, 1966; Nair *et al.*, 1967). In addition, some properties of the *in vitro* product, including base composition, nearest neighbor base frequency, and temperature stability *in vitro*, are described.

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Materials and Methods

Culture of Organism. T. pyriformis, strain GL, was grown axenically in a medium containing 2.0% proteose peptone (Difco), and 0.1% each of bactodextrose (Difco), sodium acetate, and dibasic potassium phosphate. Stock cultures

were maintained at 29° in immobile culture tubes containing 5 ml of culture medium. Transfers were made every 4 days by loop inoculation into fresh medium. For experimental purposes, cultures were usually grown in constant-temperature stainless steel tanks (Scherbaum and Jahn, 1964) containing 6–8 l. of vigorously shaking medium. In some experiments, cultures of 1–1.5 l. were grown in 2.5-l. low-form culture flasks with the temperature adjusted automatically in a rotating water bath.

Induction of Division Synchrony and Cell Harvesting. Two- to three-day-old stock cultures of *T. pyriformis* GL were first transferred into 100 ml of the medium in 500-ml erlenmeyer flasks and preincubated for 24–40 hr at 29°. The preincubated cultures were then transferred to the tanks containing 6–8 l. of sterile fresh medium. The cultures were maintained with aeration at 29° for 14–18 hr until the population reached 60,000–70,000 cells/ml. At this time, a portion of the culture was harvested for exponentially growing cells (log phase), and then the standard heat treatment (Scherbaum and Zeuthen, 1954) was begun. Aliquots of the synchronized culture were subsequently taken after the seventh heat shock (EHT),[1] and 20 min before synchronized division (EHT + 1 hr). The remainder of the culture was harvested 24 hr later when growth had reached the stationary phase. Cells were collected by centrifugation for 4 min at 90*g* at room temperature, and washed once with either Tris-HCl buffer (10 mM, pH 7.4 containing 2 mM $CaCl_2$ and 1.5 mM $MgCl_2$) or 0.4% NaCl at 60–90*g* for 3–4 min at room temperature.

Isolation of Nuclei. The procedure for nuclear isolation was a modification of an earlier method (Lee and Scherbaum, 1966). All procedures were carried out at 0–2° unless otherwise stated. All glassware was sterilized before use. Fresh solutions were prepared for each experiment. The nuclear isolation was performed in a previously ultraviolet-sterilized atmosphere. Cells were harvested and washed once with buffer (10 mM Tris-HCl (pH 7.4) containing 2 mM $CaCl_2$ and 1.5 mM $MgCl_2$) by centrifugation at 90*g* for 4 min at room temperature. The cells (100 ml) were then suspended in the same buffer (2×10^6 cells/ml) at room temperature and then slowly mixed with 100 ml of a solution of Triton X-100 (0.2%, v/v) and sucrose (0.5 M) made up in the same buffer. Phase microscopic observation showed complete cell lysis within 5 min, leaving intact nuclei. After slowly adding 200 ml of buffer containing 4% PVP[2] (K & K Laboratory Inc., Plainview, N. Y.) and 0.25 M sucrose, approximately 160 ml of the mixture was filtered through a cotton filter (Lee and Scherbaum, 1965), the mixture being poured evenly on the filter using a 10-ml pipet. The mixture absorbed by the filters during the filtration was squeezed back into the original flask and the combined mixture was again poured through new filters. Forty milliliters of each filtrate was then layered over 40 ml of buffer containing 0.5 M sucrose and 2% PVP in 100-ml round-bottom plastic centrifuge tubes. Centrifugation in an International centrifuge was carried out by successive stepwise 5-min accelerations to 70*g*, 250*g*, and 800*g*. The machine was then slowly decelerated. Using this technique, nuclei are packed softly at the bottom of the tubes. The nuclei obtained in this fashion are enzymatically active, show little damage, and can be isolated without sticky contamination of cytoplasmic particles on the nuclear surface. The procedure required less than 1 hr. Whole nuclei, once isolated, were washed twice by centrifugation at 500*g* for 5 min in 50 mM Tris-HCl buffer (pH 8.0) containing 3 mM $MgCl_2$ and 0.5 M sucrose. The purity of nuclear preparations was checked by phase-contrast microscopy prior to the second washing and the number of nuclei was determined using a hemocytometer. Approximately 30% of the original nuclei are recovered by this process.

RNA Polymerase Assay. STANDARD ASSAY MIXTURE. Assays of DNA-dependent RNA polymerase activity in nuclear preparations were carried out essentially according to the method of Weiss (1960). The incubation mixture contained per ml: 25 μmoles of Tris-HCl buffer (pH 8.0), 1.5 μmoles of $MgCl_2$, 1.35 μmoles of L-cysteine, 250 μmoles of sucrose, 60 μmoles each of KCl and NaCl, 0.5 μmole of ATP, 0.05 μmole each of CTP and GTP, 0.0282 μmole of [α-^{32}P]UTP (specific activity 220 mCi/mmole, Schwartz BioResearch, Inc.) or 0.02 μmole of [^{3}H]UTP (specific activity 2.5 Ci/mmole, Schwartz BioResearch, Inc.), 0.5 μmole of PEP, 50 μg of pyruvate kinase, and from 4 to 16 million nuclei.

ALTERNATIVE ASSAY MIXTURE. In the experiments carried out for the characterization of optimum requirements of DNA-dependent RNA polymerase activity in isolated nuclei, KCl, NaCl, L-cysteine, or ATP-generating system (PEP and pyruvate kinase) were all or partly omitted from the standard assay mixture. The details of these experiments are specified in the Results.

$MnCl_2$–AMMONIUM SULFATE-ACTIVATED ENZYME REACTION MIXTURE. The incubation mixtures for the $MnCl_2$–ammonium sulfate-activated reaction (Widnell and Tata, 1966) contained per ml: 25 μmoles of Tris-HCl buffer (pH 8.0), 2 μmoles of $MnCl_2$, 400 μmoles of ammonium sulfate adjusted to pH 8.0 with NH_4OH, 0.5 μmole of ATP, 0.05 μmole each of CTP and GTP, 0.02 μmole of [^{3}H]UTP (specific activity 2.0 Ci/mmole, Schwartz BioResearch, Inc.), 250 μmoles of sucrose, and from 4 to 16 million nuclei.

Nearest Neighbor Frequency Analysis. ENZYMATIC SYNTHESIS OF RNA. The reaction mixtures used in enzymatic synthesis of RNA were the same as described above for the RNA polymerase assay (either standard assay mixture or $MnCl_2$–ammonium sulfate-activated reaction mixture) except that each reaction mixture contained 0.05 μmole each of ATP, CTP, GTP, and UTP, one of which was α-^{32}P labeled. Specific activities of [α-^{32}P]ATP, [α-^{32}P]CTP, [α-^{32}P]GTP, and [α-^{32}P]UTP were 730, 810, 360, and 500 mCi per mmole, respectively (all four nucleoside triphosphates were supplied from Schwartz BioResearch, Inc.). Each reaction mixture was incubated at 29 or 34° for 10 min, and synthesis was terminated by the addition of 10 volumes of ice-cold 0.5 N perchloric acid.

EXTRACTION OF RNA. Extraction of radioactive RNA was carried out essentially according to the methods of Weiss and Nakamoto (1961) and Nair *et al.* (1967); after termination of enzyme reaction, 1 mg of purified yeast RNA in aqueous solution at pH 7 was added to the reaction mixture

[1] Abbreviations used are: PEP, phosphenolpyruvate; PVP, polyvinylpyrrolidone; EHT, end of (synchronizing) heat treatment.

[2] We have routinely used PVP obtained from K & K Laboratories, Inc., Plainview, N. Y., for the past few years. However, the most recent purchases have been inconsistent with respect to viscosity and we have switched to PVP (K-90) supplied by Nutritional Biochemical Corp., Cleveland, Ohio. When PVP (K-90) is used, the % (w/v) PVP should be reduced to one-fourth to that described in this text.

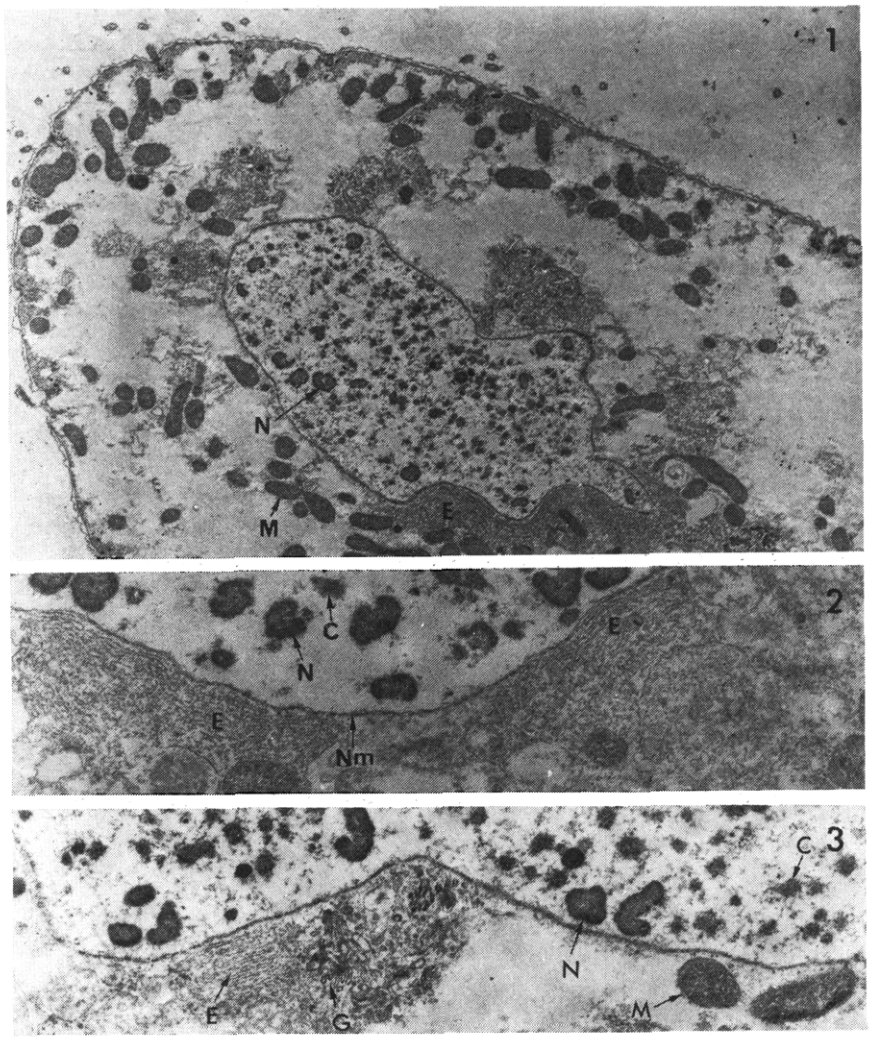

FIGURE 1: Electron micrographs of *T. pyriformis* GL showing cytoplasmic components adjacent to the nuclear envelope. C, chromatin body; E, endoplasmic reticulum; G, Golgi-like structure; M, mitochondrion; N, nucleolus; and Nm, nuclear membrane. *T. pyriformis* GL in the stationary phase of growth were fixed with 1% osmium tetroxide for 30 min, washed three times with distilled water, and stained with 2% uranyl acetate for 60 min. After quick dehydration, the samples were embeded in epon resin and sectioned at 750 Å thickness. The sections were stained with lead hydroxide and examined in a Hitachi HU 11A electron microscope (1 ×6000; 2, ×22,000; 3, ×22,000).

as carrier. After 1 hr at 0°, the precipitate was collected by centrifugation at 1500*g* for 10 min, and washed three times with 10 ml of ice-cold 0.2 N perchloric acid and twice with ethanol–ether (3:1, v/v). RNA was then extracted from the precipitate with 2 ml of 10% NaCl in 0.05 M Tris-HCl (pH 7.5) containing 0.5 mg of carrier RNA/ml at 100° for 30 min. The precipitate was reextracted for 15 min with 2 ml of the above solution and the pooled supernatants were cooled to 0°. The RNA was precipitated with two volumes of cold 95% ethanol (−20°). After 2 hr at 0°, the RNA precipitate was collected by centrifuging 10 min at 2500*g*, washed twice with cold 0.2 N perchloric acid, and once with cold 95% ethanol. The dry RNA pellet was hydrolyzed with 0.3 N KOH for 18 hr at 37°. The hydrolysate was neutralized with 6 N perchloric acid and the precipitate was removed by centrifugation.

SEPARATION OF 2′,3′-NUCLEOTIDES FROM THE PRODUCT. The hydrolyzed RNA products were then subjected to Dowex column chromatography according to the method of Katz and Comb (1963). The fractions were recorded with an automatic Isco Model 170 UV-analyzer equipped with a chart recorder. Each fraction was collected directly in a scintillation vial, lyophilized, and counted as described below.

CALCULATION OF NEAREST NEIGHBOR BASE FREQUENCY. The frequencies of the 16 possible nearest neighbor base sequences and the base composition of the radioactive RNA were calculated according to the method of Josse *et al.* (1961).

Radioactivity counting for the assays of Mg^{2+}-activated RNA polymerase activity was performed using filter paper disk procedure of Bollum (1966). To assay the $MnCl_2$–ammonium sulfate-activated RNA polymerase activity, the method of Pogo *et al.* (1966) was employed. After work-up, each disk was transferred to a scintillation vial containing 10 ml of scintillation reagent (0.6% 2,5-diphenyloxazole and 0.01% *p*-bis[2-(5-phenyloxazolyl]benzene in toluene) and counted in a Beckman CPM-200 liquid scintillation system.

Isolation of RNA for Sucrose Gradient Centrifugation. ISOLATION OF POLYMERIZED NUCLEAR RNA. Nuclei (28×10^6) isolated from EHT + 1-hr cells were incubated in 1 ml

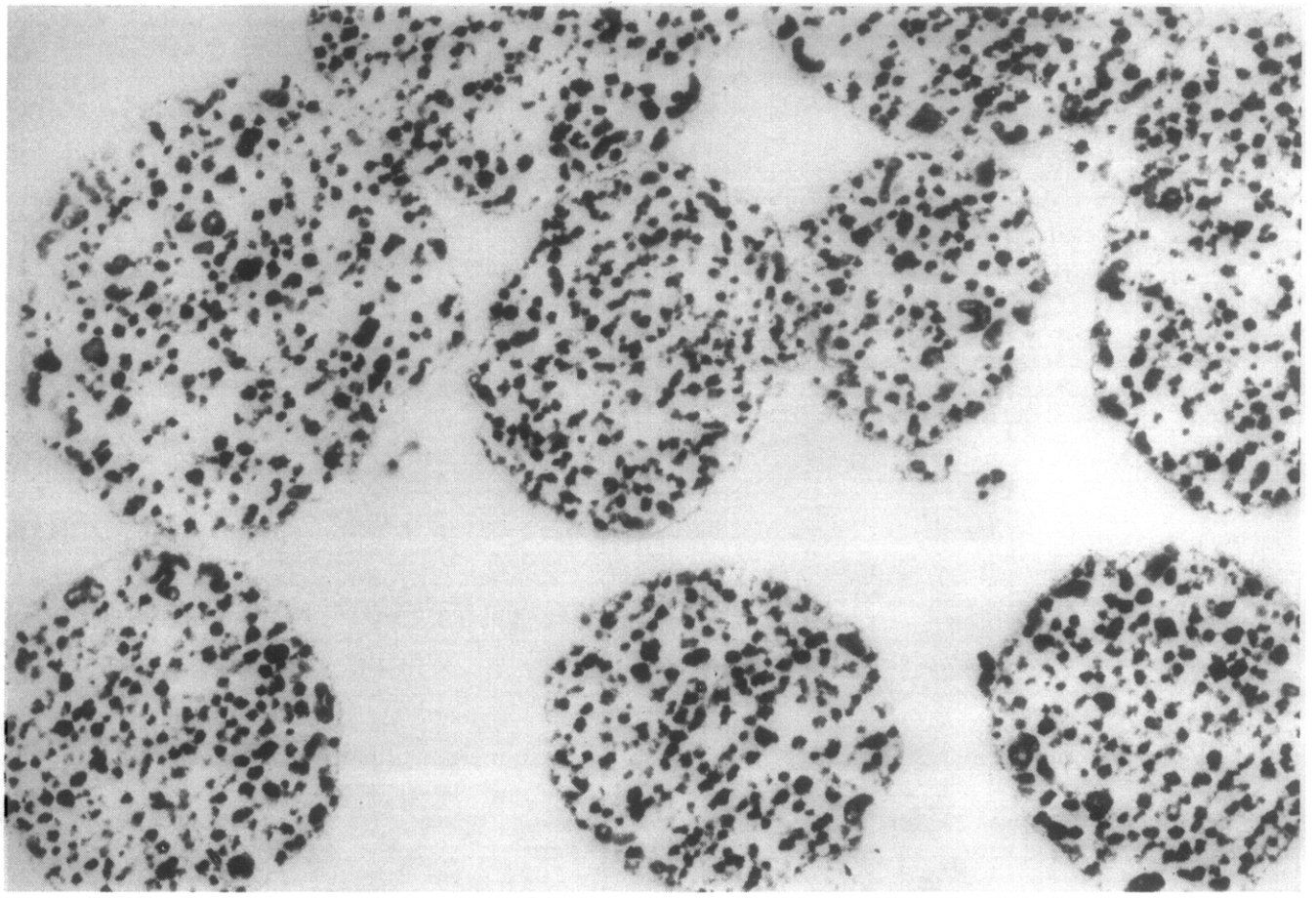

FIGURE 2: Electron micrograph of macronuclei isolated from *T. pyriformis* GL showing lack of cytoplasmic components. Nuclei were isolated from stationary-phase cells. After final purification, the nuclear pellet was fixed with glutaraldehyde (6% in phosphate buffer, pH at 7.3 and 0.5 M sucrose) and osmium tetroxide (1% aqueous solution). The subsequent procedure was the same as described in Figure 1 (×18,000).

of standard incubation mixture for RNA polymerase assay (in the Methods) except that 0.05 μmole of [α-^{32}P]ATP (630 mCi/mmole, Schwartz BioResearch, Inc.) was used as one of the labeled ribonucleoside triphosphates. After 10-min incubation at 29°, the incubation mixture was briefly chilled to 0° and centrifuged at 1500*g* for 2 min at 0°. RNA was then extracted from the nuclear pellet by a modification (Braun *et al.*, 1966) of the phenol procedure of Scherrer *et al.* (1963).

ISOLATION OF LABELED rRNA FROM WHOLE CELLS. A 100-μCi aliquot of [^{3}H]uridine ([5-^{3}H]uridine, specific activity 25 Ci/mmole, New England Nuclear Corp.) was aseptically added to 100 ml of culture medium. The medium was inoculated with 5 ml of 2-day-old stock culture and the cells were grown at 29° for 24 hr. The labeled cells were harvested and the rRNA was extracted by Method II of Kirby (1965).

Results

Isolation of Nuclei. Preliminary studies of RNA polymerase activity in nuclei with *T. pyriformis* indicated that once the nuclei were broken, RNA polymerase activity was lost. Triton X-100 (final concentration of 0.1%) can readily dissociate cytoplasmic structures (Figure 1) from macronuclear surface without affecting the morphologic integrity of the nuclei, and this procedure was used throughout these experiments. The electron micrograph of Triton-prepared nuclei (Figure 2) shows the nuclear membrane to be virtually free of contamination. No nonnuclear fragments can be seen in the space between the isolated nuclei.

Characterization of DNA-Dependent RNA Polymerase. NUCLEOTIDE DEPENDENCE AND Mg^{2+} ION REQUIREMENT. *Tetrahymena* nuclei isolated from late-log-phase cells prepared as described under the Methods catalyze the incorporation of both [^{3}H]- and [α-^{32}P]UTP into a cold trichloroacetic acid insoluble product (Tables II–VI). The maximum level of incorporation was dependent on the presence of the three other nucleoside triphosphates: ATP, CTP, and GTP (Table II). Omission of one of the nucleoside triphosphates decreased the uptake of labeled precursor to 14–16% of the complete system. Substitution of all three nucleoside triphosphates by nucleoside diphosphates yielded about 20% of the maximum labeling of the complete system. Substitution of Mg^{2+} by Ca^{2+} decreased the uptake of precursor by 95%. The addition of sodium pyrophosphate (5 mM) to the complete system inhibited the labeling almost completely (Table II). Tris buffer can be replaced by a sodium phosphate buffer without causing a significant change in the enzyme activity.

These results indicate that the incorporation of ribonucleotides into the acid-insoluble fraction occurs at the

TABLE I: DNA Content of Nuclei Isolated from Three Growth Stages of *Tetrahymena* Cultures.

Growth Stages	μg of DNA/10^6 Nuclei		Av DNA Content (Arbitrary Units)
Log phase	(13.7 ± 0.5)[a]	8.1[b]	13.2[c]
EHT	(22.8 ± 1.0)	13.9	23.7
EHT + 1 hr	(22.2 ± 0.4	13.7	25.7

[a] Isolated nuclei were washed three times with 0.14 M NaCl and 0.015 M sodium citrate and extracted three times with 0.5 N H_2SO_4. The nuclear pellets were washed with acetone following acid extraction and the DNA contents were determined by the method of Burton (1956). The values are duplicated (log, EHT + 1 hr) or triplicated (EHT) determinations. [b] DNA content reported previously (Lee and Scherbaum, 1966) was based on treatment with alkali to degrade the RNA and precipitation of the DNA together with presence of protein in acidification. DNA content was then determined from the precipitates by the method of Burton (1956). The presence of alkaline-treated protein may inhibit the modified diphenylamine reaction (Burton, 1968). In fact, the DNA content measured as previously gave only about two-thirds color development compared to that of the present determination. [c] DNA content reported by Scherbaum *et al.* (1959).

TABLE II: Requirements for RNA Synthesis in Isolated Nuclei.

Components	Incorp of [^{3}H]UTP (cpm/7 × 10^5 Nuclei, 10 min at 29°)[b]
Complete system[a]	9202
Minus ATP	1302
Minus CTP	1260
Minus GTP	1472
Minus $MgCl_2$ plus $CaCl_2$ (1.5 mM)	468
Minus ATP, CTP, GTP plus ADP, CDP, GDP	1853
Plus sodium pyrophosphate (5 mM)	220

[a] Complete system (0.2 ml) contains 18.7 × 10^5 nuclei isolated from late-log-phase cells, and the standard assay mixture was described under the Methods except that the NaCl, the ATP-generating system, and cysteine have been excluded from the mixture. [b] Radioactivity in duplicated 75-μl aliquots were counted by filter paper disk procedure as described in the Methods.

triphosphate level and requires all four ribonucleoside triphosphates. The reaction apparently proceeds by the elimination of PP_i.

EFFECT OF INHIBITORS. The incorporation of both [^{3}H]- and [α-^{32}P]UTP in the presence of three other nucleoside triphosphates was inhibited by DNase, RNase, or actinomycin D (Tables IV and V). The enzyme activity is inhibited by 99% when the DNA in the nuclei is destroyed by DNase (Table V). In addition, no polymerized products remained when RNase was added to the reaction mixture. Therefore, the enzyme reaction requires DNA and the product is RNase sensitive. Radiochromatography of the hydrolysis products showed that the labeled product contains all four nucleotides linked by internucleotide bonds.

OTHER FACTORS INFLUENCING RNA SYNTHESIS. Potassium chloride greatly enhances the uptake of both [^{3}H]- and [α-^{32}P]UTP into cold trichloroacetic acid insoluble fraction by isolated nuclei. Sodium chloride was somewhat less effective. Table VI shows the effect of various concentrations of KCl and NaCl on the RNA polymerase assay system. It was found that KCl and NaCl could stimulate the Mg^{2+}-requiring RNA polymerase reaction up to 356% of control. Maximum activity was observed at a concentration of 60 mM KCl and 60 mM NaCl (Table VI, expt 2), and the addition of L-cysteine to that salt combination further enhanced the enzyme activity to 404% of control (Table VI, expt 2). From these results the standard assay mixture for the subsequent enzyme assay experiments was developed (see Methods).

An ATP-generating system (pyruvate kinase and PEP) enhances the incorporation of [^{3}H]UTP into RNA by about threefold in comparison to the control (Table VI, expt 1). The experiments carried out in the presence or absence of an ATP-generating system (comparing the data in Tables II and III) suggests the presence of CDP–kinase and GDP–kinase activities in the *Tetrahymena* nuclei. The nucleotide dependence was less prominent when the ATP-generating system was included (Table III) than when it was excluded (Table II). A marked stimulation of enzyme activity occurs when Mn^{2+} and ammonium sulfate are substituted for Mg^{2+} and KCl (Table VI, expt 1).

OPTIMUM pH. The optimum pH for [α-^{32}P]UTP incorporation into RNA was 8.9; activity fell off rapidly below pH 7.8 (Figure 3).

TABLE III: Activity of Kinases in Isolated Nuclei.

Components	Incorp of [α-^{32}P]UTP (cpm/2.3 × 10^5 Nuclei, 10 min at 29°)[b]
Complete system[a]	21,033
Minus ATP	14,787
Minis CTP	6,388
Minus GTP	6,515
Minus ATP, CTP, GTP plus ADP, CDP, GDP	15,402

[a] Complete system (0.2 ml) contains 6.14 × 10^5 nuclei isolated from late-log-phase cells, and the standard assay mixture was described under the Methods except that cysteine was omitted. [b] Radioactivity counted as described in Table II.

TABLE IV: Inhibition of RNA Synthesis in Isolated Nuclei by Actinomycin D.

Components	Incorp of [α-^{32}P]UTP (cpm/2.3 × 10^5 Nuclei, 10 min at 29°)[b]
Complete system[a]	21,033
Plus actinomycin D (0.02 μg/ml)	11,374
Plus actinomycin D (0.2 μg/ml)	5,964
Plus actinomycin D (1μg/ml)	3,941
Plus actinomycin/D (2 μg/ml)	2,394
Plus actinomycin D (20 μg/ml)	192

[a] Complete system (0.2 ml) contains 6.14 × 10^5 nuclei isolated from late-log-phase cells, and standard assay mixture was described under the Methods except that cysteine has been excluded from the mixture. [b] Radioactivity counted as described in Table II.

RNA Polymerase Activity in Various Growth Stages. The rate of RNA synthesis per nucleus was dependent on the growth stage of the cells from which the nuclei were isolated. The maximum net UTP incorporation into RNA was achieved after incubation for 8–10 min at 29°. One million nuclei from log-phase cells incorporated 65 μμmoles of UTP, EHT nuclei incorporated 104 μμmoles, and EHT + 1-hr

TABLE V: Inhibition of RNA Synthesis in Isolated Nuclei by Deoxyribonuclease and Ribonuclease.

Components	Incorp of [^{3}H]UTP (cpm/3.3 × 10^5 Nuclei, 10 min at 29°)[c]
Complete system[a]	13,970
DNase (50 μg/ml) added at time zero	4,704
Nuclei preincubated[b] with DNase	195
RNase (100 μg/ml) added at time zero	0

[a] Complete system (0.2 ml) contains 8.8 × 10^5 nuclei isolated from late-log-phase cells, and the standard assay mixture was described under the Methods. [b] Nuclei were preincubated with DNase (10 μg) in 0.2 ml of standard assay mixture except that the [^{3}H]UTP was excluded. [^{3}H]UTP was added after 10-min preincubation and the incubation was continued 10 more min. Deoxyribonuclease (DNase) was obtained from Worthington Biochemical Corp. (electrophoretically purified DNase I). Ribonuclease (RNase) was obtained from California Biochemical Corp. (pancreatic RNase, A grade). [c] Radioactivity counted as described in Table II.

TABLE VI: Factors Influencing Increase of RNA Synthesis in Isolated Nuclei.

Components	Incorp of [^{3}H]UTP (cpm/7 × 10^5 Nuclei, 10 min at 29°)[b]
Experiment 1	
Complete system[a]	4,351
Plus KCl (30 mM)	7,451
Plus NaCl (30 mM)	6,783
Plus KCl (60 mM)	9,202
Plus NaCl (60 mM)	8,561
Plus KCl (90 mM)	12,945
Plus NaCl (90 mM)	12,722
Plus KCl (120 mM)	12,689
Plus NaCl (120 mM)	13,339
Plus KCl (60 mM) and ATP-generating system	27,486
Plus NaCl (60 mM) and ATP-generating system	24,905
Plus $(NH_4)_2SO_4$ (200 mM)[c]	27,437
Plus $(NH_4)_2SO_4$ (200 mM)[c] and $MnCl_2$ (0.5 mM)[d]	36,275

	Incorp of [α-^{32}P]UTP (cpm/2.3 × 10^5 Nuclei, 10 min at 29°)[f]
Experiment 2	
Complete system[e]	5,910
Plus KCl (30 mM) and NaCl (90 mM)	13,352
Plus KCl (60 mM) and NaCl (60 mM)	21,033
Plus KCl (90 mM) and NaCl (30 mM)	19,315
Plus KCl (120 mM)	19,771
Plus NaCl (120 mM)	17,448
Plus KCl (240 mM)	17,758
Plus NaCl (240 mM)	10,857
Plus KCl (60 mM), NaCl (60 mM), and L-cysteine (1.35 mM)	23,864
Plus KCl (60 mM), NaCl (60 mM), and NaF (5 mM)	18,251
Plus KCl (60 mM), NaCl (60 mM), and $MnCl_2$ (0.5 mM)	14,470

[a] Complete system (0.2 ml) contains 18.7 × 10^5 nuclei isolated from late-log-phase cells, and the standard assay mixture was described under the Methods except that NaCl, KCl, cysteine, and the ATP-generating system have been excluded from the mixture. [b] Radioactivity counted as described in the Table II. [c] Ammonium sulfate was added without adjusting pH. [d] $MgCl_2$ was replaced by $MnCl_2$. [e] Complete system (0.2 ml) contains 6.14 × 10^5 nuclei isolated from late-log-phase cells, and the standard assay mixture was described under the Methods except that KCl, NaCl, and cysteine have been excluded from the mixture. [f] Radioactivity counted as described in the Table II.

TABLE VII: $MnCl_2$–Ammonium Sulfate-Activated RNA Polymerase Reaction in Isolated Nuclei.

Experimental Conditions[a]		Incorp of [^{3}H]-UTP (cpm/4 × 10^5 Nuclei, 10 min at 29°)[c]
pH	$(NH_4)_2SO_4$[b] (%)	
	Experiment 1	
7.4	10	3160
8.0	5	5765
8.0	10	3530
8.0	15	1280
8.9	10	7204
8.9	10 ($MnCl_2$ was replaced by 2 mM $MgCl_2$)	2553

Experimental Conditions[d]				Incorp of [^{3}H]UTP
pH	Temp (°C)	Time (min)	$(NH_4)_2SO_4$ (%)[e]	(cpm/3 × 10^5 Nuclei)[f]
		Experiment 2		
8.9	29	10	5	3527
8.9	29	20	5	5100
8.9	29	30	5	5247
8.9	34	10	5	5205
8.9	34	100	5	5915
8.9	29	10	1	1358
8.9	29	10	10	2752
8.9	29	10	15	1817
8.0	29	10	5	4407
8.5	29	10	5	4520
9.2	29	10	5	3842

[a] Incubation mixture (0.2 ml) contains 10.7 × 10^5 nuclei isolated from EHT + 1-hr cells, and $MnCl_2$–ammonium sulfate-activated enzyme reaction mixture described under the Methods except that the pH was adjusted with 25 mM Tris-HCl buffer as specified in each experiment. [b] Final concentration of ammonium sulfate in the incubation mixture, pH adjusted with concentrated NH_4OH prior to addition. [c] Radioactivity counted as described in the Methods. [d] Incubation mixture (0.2 ml) contains 8 × 10^5 nuclei isolated from EHT + 1-hr cells, and $MnCl_2$–ammonium sulfate-activated enzyme reaction mixture was as described under the Methods except that the pH was adjusted with 25 mM Tris-HCl buffer as specified in each experiment. [e] Final concentration of ammonium sulfate in the incubation mixture, pH adjusted with concentrated NH_4OH prior to addition. [f] Radioactivity counted as described in the Methods.

nuclei incorporated 187 μμmoles. The DNA content per one million nuclei isolated from these three growth stages is listed in Table I. The maximum net incorporation of [α-^{32}P]UTP/mg of nuclear DNA, calculated from the data in Figure 4, was approximately 5 mμmoles in log nuclei, 4 mμmoles in EHT nuclei, and 8 mμmoles in EHT + 1-hr nuclei.

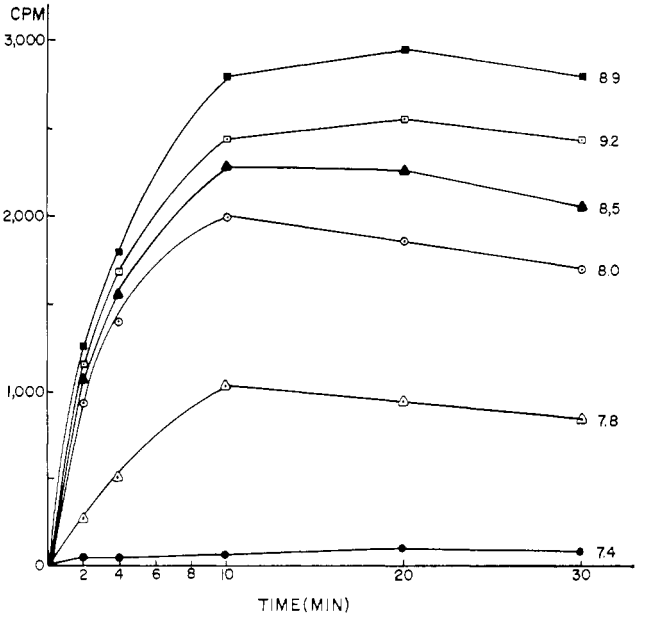

FIGURE 3: Kinetics of RNA synthesis by isolated macronuclei as a function of pH. Reactions were carried out in the standard assay mixture (described under the Methods) containing [α-^{32}P]UTP and 1.5 × 10^6 nuclei/ml, isolated from EHT + 1-hr cells. The pH in each reaction mixture was adjusted from 7.4 to 9.2 prior to the final nuclear washing process. Duplicated 75-μl aliquots were taken at the time indicated, and the radoactivity in the aliquots was counted as described in the Methods.

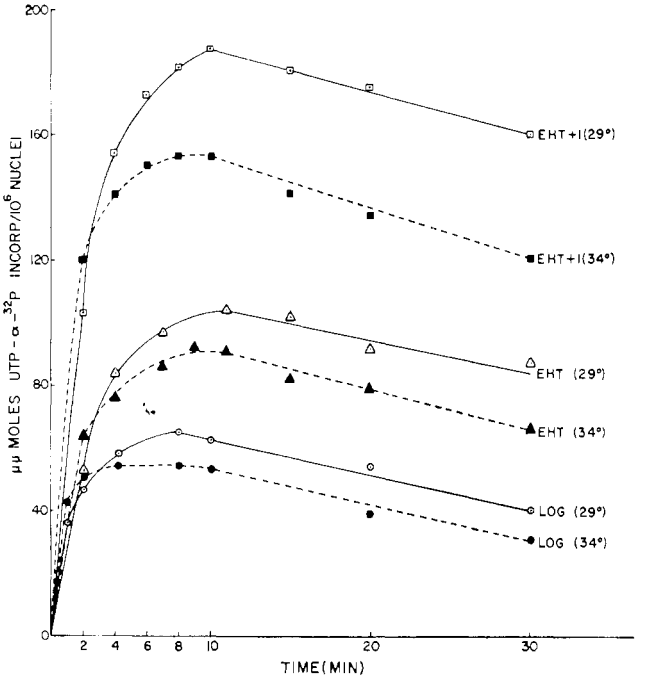

FIGURE 4: Kinetics of RNA synthesis in macronuclei isolated from three growth stages as a function of temperature. The incubation mixture contained per milliliter of the standard assay mixture as described under Methods, in which labeled precursor was [α-^{32}P]-UTP (0.0282 μmole; 2.25 × 10^8 cpm/μmole). Nuclei were isolated from three growth stages for the three experiments (log phase, EHT, and EHT + 1 hr). In each experiment, 30 × 10^6 nuclei were resuspended in 3 ml of ice-cold incubation mixture as described above, and divided into two equal portions; one-half was incubated at 29° (——), the other at 34° (------). Radioactivity was counted as described in Figure 3.

TABLE VIII: Analysis of Nearest Neighbor Frequencies (NNF) for the Product of RNA Polymerase in Nuclei Isolated from Log, EHT, and EHT + 1-hr Cells.

Labeled Nucleoside Triphosphate	Expt	% of Total ^{32}P cpm Recovered in 2′,3′-Nucleoside Monophosphate			
		AMP	CMP	GMP	UMP
Experimental Result: ^{32}P Content of Isolated Mononucleotides[a]					
[α-^{32}P]ATP	a	43.6	14.3	16.0	26.1
	b	41.8	16.2	16.4	25.6
	c	41.5	17.1	15.8	25.6
	d	42.9	16.3	15.6	25.2
[α-^{32}P]CTP	a	29.8	16.9	14.1	39.2
	b	30.1	17.1	16.2	36.6
	c	32.0	16.7	14.3	37.0
	d	30.5	15.8	14.7	39.0
[α-^{32}P]GTP	a	37.7	12.6	14.7	35.0
	b	37.4	12.7	15.5	34.4
	c	36.5	13.9	14.7	34.9
	d	38.5	12.4	13.9	35.2
[α-^{32}P]UTP	a	32.2	18.7	11.9	37.2
	b	33.1	18.6	12.1	36.2
	c	32.8	18.8	11.0	37.4
	d	33.0	18.4	11.8	36.8

Labeled Nucleoside Triphosphate	Expt[c]	Isolated 2′,3′-Nucleoside Monophosphate			
		AP	CP	GP	UP
Calculated Result: Nearest Neighbor Frequencies[b]					
		APA	CPA	GPA	UPA
[α-^{32}P]ATP	a	0.160	0.053	0.059	0.096
	b	0.152	0.059	0.060	0.093
	c	0.151	0.062	0.057	0.093
	d	0.159	0.060	0.058	0.093
		APC	CPC	GPC	UPC
[α-^{32}P]CTP	a	0.048	0.027	0.023	0.062
	b	0.050	0.028	0.027	0.061
	c	0.055	0.029	0.025	0.063
	d	0.050	0.026	0.024	0.064
		APG	CPG	GPG	UPG
[α-^{32}P]GTP	a	0.053	0.018	0.021	0.049
	b	0.056	0.019	0.023	0.051
	c	0.051	0.019	0.020	0.048
	d	0.054	0.017	0.019	0.049
		APU	CPU	GPU	UPU
[α-^{32}P]UTP	a	0.107	0.062	0.039	0.123
	b	0.106	0.060	0.039	0.116
	c	0.107	0.062	0.036	0.122
	d	0.108	0.060	0.039	0.120

[a] Procedure for the experiment has been described under the Methods. Expt a: NNF for the product of RNA polymerase in nuclei isolated from log-phase cells (enzyme reaction at 29°, 10 min; average of two to four experiments). Expt b: NNF for the product of RNA polymerase in nuclei isolated from log-phase cells (enzyme reaction at 34°, 10 min; average of two to three experiments). Expt c: NNF for the product of RNA polymerase in nuclei isolated from EHT cells (enzyme reaction at 29°, 10 min; average of two to three experiments). Expt d: NNF for the product of RNA polymerase in nuclei isolated from EHT + 1-hr cells (enzyme reaction at 29°, 10 min; average of two to six experiments). The recovery of the total ^{32}P counts per minute in the 2′,3′-nucleoside monophosphate following analysis averaged 99% of the total ^{32}P input (Lee, 1969). [b] In each experiment, the base incorporating factors for ATP, CTP, GTP, and UTP are as following: (expt a), 0.3679, 0.1593, 0.1416, 0.3312; (expt b), 0.3641, 0.1660, 0.1485, 0.3214; (expt c), 0.3634, 0.1714, 0.1382, 0.3270; (expt d), 0.3703, 0.1636, 0.1397, 0.3264, respectively. [c] The nearest neighbor frequencies were calculated according to Josse *et al.* (1961).

The initial rate of RNA synthesis (Figure 4) is almost linear for the first 2 min. Log-phase nuclei incorporated 3.6 mμmoles of [α-^{32}P]UTP/mg of nuclear DNA after 2-min incubation, EHT nuclei incorporated, 2.4 mμmoles, and EHT + 1-hr nuclei incorporated, 4.9 mμmoles. The net rate incorporation per isolated nucleus is therefore not simply dependent on the amount of DNA present in the nucleus. These results suggest that the isolated nuclei retain not only the ability to synthesize RNA but may also have retained nuclear control mechanisms as well.

Characterization of Rapidly Labeled Nuclear RNA. NEAREST NEIGHBOR FREQUENCY ANALYSIS. Nuclei were isolated from three different growth stages (log phase, EHT, EHT + 1 hr) and nearest neighbor frequency anlyses were performed, using the four [α-^{32}P]ribonucleoside triphosphates (see Methods). To evaluate the effect of synchronizing temperature on the nature of the RNA products, nuclei isolated from log-phase cells were incubated at 29 and 34° for 10 min. In addition, to determine whether the nature of RNA products vary during the different growth stages, nuclei isolated from EHT and EHT + 1-hr cells were incubated at 29° for 10 min each. The nearest neighbor frequency analyses of the products from both experiments are shown in Table VIII.

SUCROSE DENSITY GRADIENT CENTRIFUGATION. An aliquot of phenol-extracted RNA was analyzed by sucrose gradient centrifugation; the newly labeled nuclear RNA was found to be of low molecular weight (Figure 5), having a sedimentation rate approximately equal to tRNA. The specific radioactivity along the gradient was relatively constant, however, which suggests that degradation had occurred during either the extraction procedure or the 10-min incubation period.

The rate of [α-^{32}P]UTP incorporation into nuclear RNA

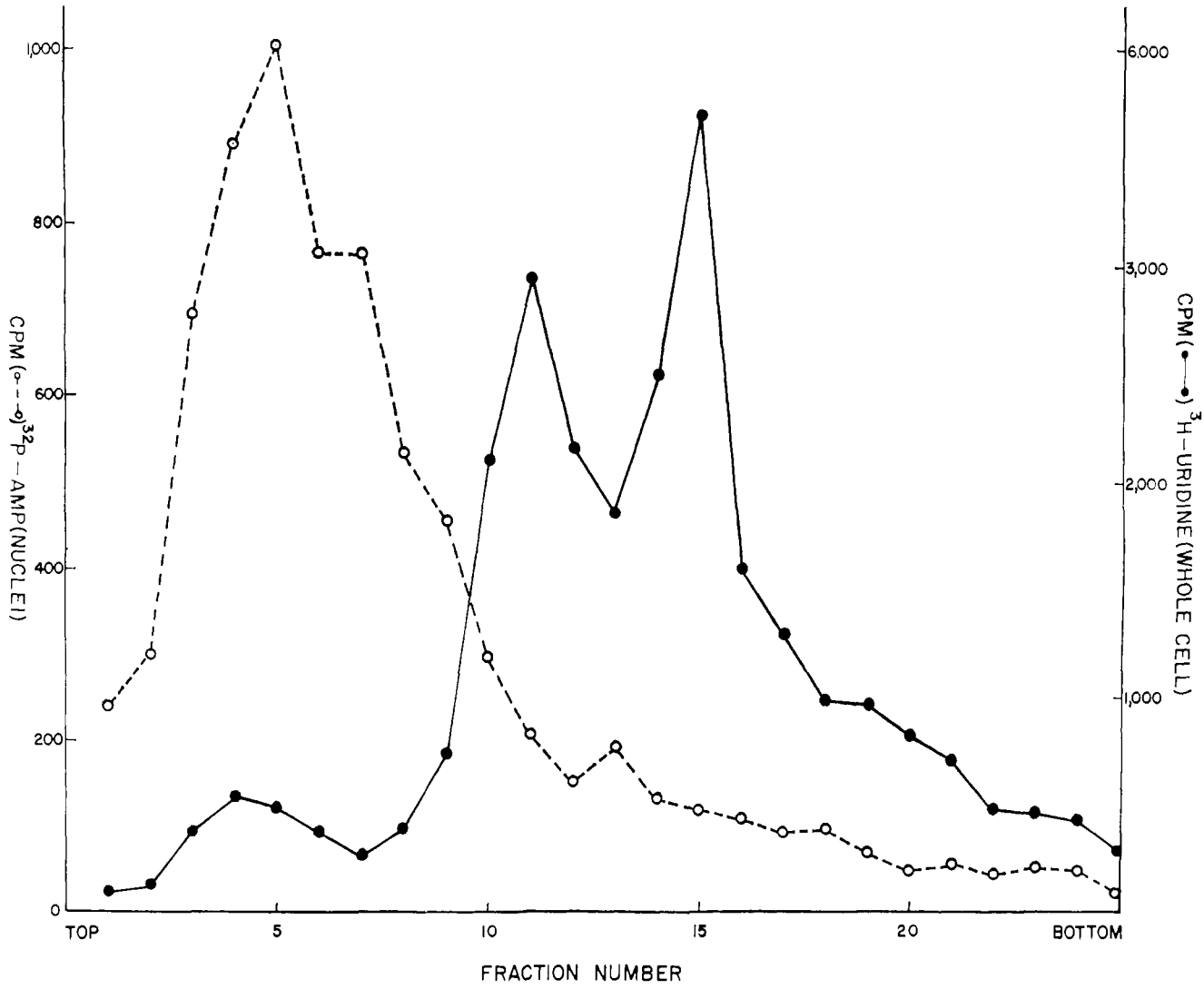

FIGURE 5: Sucrose gradient sedimentation profile of RNA synthesized by isolated macronuclei. Nuclei isolated from EHT + 1-hr cells were labeled with [α-^{32}P]ATP for 10 min using the incubation mixture described under the section of nearest neighbor frequency analysis in the Methods. RNA was prepared from nuclei and whole cells as described in the Methods. A solution of phenol-extracted nuclear RNA (approximately 2 absorbancy units at 260 mμ) was combined with RNA prepared from whole cells labeled with [^{3}H]uridine for 24 hr (approximately 10 absorbancy units at 260 mμ). The latter served as a marker. They were then layered over 25 ml of a 5–25% linear sucrose gradient which was buffered to pH 5.1 with 0.01 M sodium acetate. The gradient was centrifuged for 16 hr at 5°. After centrifugation the gradient was fractionated into 1-ml samples by means of an ISCO automatic fractionator. The samples were prepared for counting by the addition of 1 ml of 10% cold trichloroacetic acid containing 0.1 mg of purified yeast RNA as a carrier, washed with 5% trichloroacetic acid on Millipore filter (0.45 μ), and counted in a Beckman scintillation system using 10 ml of scintillation reagent as described in the Methods. Two large peaks obtained from whole cell RNA at the heavier density correspond to the 17S and 25.4S RNA fractions (Kumar, 1969).

was almost linear for the first 2 min of incubation; the uptake of precursor continued thereafter but at a slower rate for about 10 min (Figure 4). Decay in acid-insoluble counts occurred in all experiments and suggests that degradation proceeded coincident with incorporation.

RNA Polymerase Activity Stimulated by $MnCl_2$–Ammonium Sulfate in Isolated Nuclei. OPTIMUM CONCENTRATION OF AMMONIUM SULFATE. Table VII shows that RNA polymerase activity in isolated *Tetrahymena* nuclei was generally increased by the addition of 5% (200 mM) ammonium sulfate to the assay mixture (which also included 2 mM $MnCl_2$ and 250 mM sucrose buffered with 10 mM Tris-HCl). Higher ammonium sulfate concentration (10% or 400 mM) consistently gave less stimulation. When Mn^{2+} was replaced by Mg^{2+} the rate of [^{3}H]UTP incorporation into RNA was reduced to one-third (Table VII, expt 1). The rate of [^{3}H]UTP incorporation into RNA was greater at 34° than at 29°. Moreover, variation from the pH 7.4 to 9.2 did not significantly affect the uptake of the precursor into RNA at the optimum concentration of ammonium sulfate. The rate of RNA polymerase activity was greater at 34° than at 29° in the presence of $MnCl_2$ and ammonium sulfate and decreased progressively at both temperatures (Table VII).

Nature of RNA Synthesized upon the Addition of Ammonium Sulfate. To evaluate whether the RNA produced by the Mg^{2+}–NaCl–KCl-activated RNA polymerase reaction (described in the preceding section) differs significantly from that produced

TABLE IX: Comparison of Nearest Neighbor Frequency Analysis on the Products of Mg^{2+}-Activated and Mn^{2+}-Activated RNA Polymerase Reactions in Nuclei Isolated from Log-Phase Cells.[a]

Labeled Nucleoside Triphosphate	RNA Polymerase Reaction	Expt	% of Total ^{32}P cpm Recov in 2′,3′-Nucleoside Monophosphate				AMP + UMP / CMP + GMP
			AMP	CMP	GMP	UMP	
[α-^{32}P]UTP	Mg^{2+} activated	1	31.1	19.2	11.9	37.8	2.2
		2	33.2	19.5	10.4	36.9	2.3
		3	32.4	18.4	12.3	36.9	2.3
		4	32.1	17.8	13.0	37.1	2.3
	Mn^{2+} activated	1	34.3	19.1	10.4	36.2	2.4
		2	31.6	19.5	12.6	36.3	2.1
[α-^{32}P]GTP	Mg^{2+} activated	1	37.8	12.4	14.3	35.5	2.8
		2	37.6	12.9	15.1	34.4	2.6
	Mn^{2+} activated	1	39.6	9.7	16.5	34.2	2.8
		2	41.2	10.9	15.2	32.7	2.8

[a] Enzyme reactions were carried out at 29° for 10 min, other conditions were described in the Methods.

by the Mn^{2+}–ammonium sulfate-activated RNA polymerase reaction, a nearest neighbor frequency analysis of the RNA produced by the latter was carried out using [α-^{32}P]UTP or [α-^{32}P]GTP as the labeled ribonucleoside triphosphates. Table IX shows a comparison of the two RNA products. It is evident that RNA produced by the two different reaction mixtures was quite similar.

Discussion

Macronuclei isolated from *T. pyriformis* contain an enzyme that is similar in many respects to mammalian DNA-dependent RNA polymerase. The reaction depends on the presence of DNA, Mg^{2+}, and all four nucleoside triphosphates (Tables II and V). It is inhibited by the antibiotic actinomycin D. Pyrophosphate inhibits the enzyme (Furth *et al.*, 1962), while orthophosphate has little effect (Tables II and IV). The enzyme is also resistant to rifampicin both *in vivo* and *in vitro* (Byfield *et al.*, 1970), another property similar to the mammalian enzyme.

The product of the enzyme reaction can be characterized as RNA on the basis of physical, chemical, and enzymatic criteria. It is not attacked by DNase but is digested by RNase and alkali. Alkaline digestion yields the expected four ribonucleotides and reveals that the labeled nucleotides have been incorporated nonterminally, *i.e.*, within the molecule (Table VIII). The cell-free macronuclear system prepared from *T. pyriformis* thus retains an enzymatic activity catalyzing a DNA-directed RNA synthesis similar but not identical (see later) with that found in higher animals, plants, or bacterial cells (*cf.* Hurwitz and August, 1963; Davidson, 1965; Tata, 1966).

Stimulatory Effects of Salts on the RNA Polymerase Activity. DNA-dependent RNA polymerase activity in rat liver nuclei was first demonstrated by Weiss (1960). His aggregate enzyme assay system has usually been used to characterize the RNA polymerase activity in isolated mammalian nuclei (*cf.* Tata, 1966). Recently, Widnell and Tata (1964, 1966) have reported two distinct RNA polymerase activities in isolated liver nuclei. The RNA product in the presence of Mg^{2+} and low ionic strength resembles rRNA, while that synthesized in the presence of Mn^{2+} and high ionic strength ammonium sulfate (0.4 M, ionic strength of 1.6) more closely resembles DNA-like RNA. Stimulation of mammalian nuclear RNA polymerase activity by addition of ammonium sulfate was first described by Goldberg (1961).

The conclusions of Widnall and Tata (1966) have been supported by other investigators (Pogo *et al.*, 1966; Liao *et al.*, 1966; Maul and Hamilton, 1967; Nair *et al.*, 1967). According to autoradiographic observation of isolated rat liver nuclei, Mg^{2+} stimulates primarily nucleolar RNA synthesis, whereas Mn^{2+} at high ionic strengths stimulates uridine incorporation in extranucleolar chromosomal regions (Maul and Hamilton, 1967). Nearest neighbor frequency analysis of RNA produced under the two conditions led Nair *et al.* (1967) to similar conclusions. Using a DNA–RNA hybridization technique, Mandel and Chambon (1969) have shown that RNAs synthesized *in vitro* at high ionic strength by rat liver nuclear RNA polymerase were also present *in vivo* in rat liver nucleus. However, only a fraction of the RNAs synthesized *in vitro* was found both in the nucleus and cytoplasm *in vivo*. Roeder and Rutter (1969) have recently suggested that the varying effects of Mg^{2+} and Mn^{2+} and ionic strength on the specificity and localization of RNA synthesis in eukaryotic cells are attributable to the selective ionic effects on at least two distinct RNA polymerase species. In *Tetrahymena*, the RNA polymerase activity of isolated macronuclei is stimulated by relatively low concentrations of KCl and NaCl (60 mM each, ionic strength of 0.12) when the reaction mixture contains Mg^{2+} (Table VI). However, the nearest neighbor frequency analysis of RNA polymerized under these conditions appears similar to the RNA produced in the presence of Mn^{2+} and high ionic strength of ammonium

sulfate (Table IX). Since we have studied the enzyme activity of detergent-isolated nuclei, the possibility that RNA polymerase activity producing a ribosomal-like RNA may have been selectively removed or inactivated by this type of isolation procedure cannot be entirely ruled out. In addition, the synthesis and processing of *Tetrahymena* rRNA precursors is known to be very rapid (Leick, 1969) and it seems likely that rRNA precursors in eukaryotes can undergo turnover (Byfield and Scherbaum, 1968; Darnell, 1969). In the absence of net accumulation *in vitro*, nearest neighbor frequency analysis might therefore fail to detect their formation in the presence of simultaneous synthesis of large amounts of DNA-like RNA. Futher experiments will be required to evaluate these possibilities.

Kinetics of RNA Synthesis. The kinetics of RNA synthesis differed markedly under two different conditions, *viz.*, in the presence of Mn^{2+} and high ionic strength of ammonium sulfate (Table VII), and in the presence of Mg^{2+} and low ionic strength of KCl and NaCl (Figure 4). However, it was consistently observed that the high ionic strength of ammonium sulfate (0.2–0.4 M, ionic strength of 0.8–1.6) produces immediate disruption of macronuclei at 0° and forms a highly viscous solution. Pogo *et al.* (1967) have also observed that 0.4 M ammonium sulfate produced extensive changes in the organization of rat liver chromosomes. Such a complete disruption of nuclear structure by high ammonium sulfate concentrations may not only lead to the changes in template efficiency through the removal of histones as suggested by Widnell and Tata (1966), but also dissociate other chromosomal components. At high salt concentrations, bacterial RNA polymerase is known to be dissociated into subunits (Richardson, 1966; Stevens *et al.*, 1966; Pettijohn and Kamiya, 1967). Another possibility is that the high salt concentrations may affect the activity of endogenous nuclear degradative enzyme(s) (Liau *et al.*, 1968). For these reasons the two different kinetic experiments probably cannot be compared directly.

RNA Synthesis by Macronuclei from Different Growth Stages. The capacity for RNA synthesis of macronuclei isolated from various growth stages of *Tetrahymena*, has been studied in the Mg^{2+} and KCl system. Under these conditions, the macronuclear system of *Tetrahymena* prepared from normal log growth is capable of a maximum incorporation of about 5 mμmoles of labeled UTP per mg of nuclear DNA (Figure 4). The activity of *Tetrahymena* macronuclear RNA polymerase appears to be considerably higher than that reported for liver nuclei (1–2 mμmoles) and equal to the activity reported for nuclei isolated from actively growing ascites tumor (5 mμmoles) cells (Hurlbert, 1965). However, with respect to the kinetics of RNA synthesis, the macronuclear system of *Tetrahymena* in the presence of Mg^{2+} and KCl is strikingly similar to Weiss's (1960) system prepared from liver nuclei: The initial rate of RNA synthesis is rapid, and the reaction is linear for about 2 min. However, the synthesis of RNA virtually ceases after 10 min (Figure 4). This comparison of the different growth stages is based on DNA determinations which differed slightly from our previous methods as noted in Table I. It should be noted clearly, however, that our previous determinations and the present data show a 71 and 66% increase, respectively, in DNA content during the synchronization treatment. The "excess DNA" (Hjelm and Zeuthen, 1967) which continues to be proposed to exist in heat-synchronized *Tetrahymena* (Westergaard and Pearlman, 1969) has never been confirmed using purified macronuclear preparations. The slight decline in DNA content in the 60 min following the last heat shock (Lee and Scherbaum, 1966) has been confirmed (Table I) and may represent degradation secondary to increased DNase activity (Holm, 1966). Degradation during this period had been suggested previously on the basis of histologic (Scherbaum *et al.*, 1958) and more recently from autoradiographic (Hjelm and Zeuthen, 1967) data. The physiologic basis for increased *in vitro* ribonucleotide incorporation at EHT + 1 hr is not known. Since actinomycin D will not inhibit synchronous cell division when added at this time (Lazarus *et al.*, 1964), this increased activity appears to have no direct relevance to synchronized cell division.

Hydrolysis of Rapidly Labeled Nuclear RNA. The diminished net incorporation observed beyond the 10-min incubation period is due to hydrolysis of the product (Byfield and Lee, 1970). The enzyme(s) involved in nuclear RNA hydrolysis are unknown. RNase may be responsible for the degradation of nuclear RNA, both *in vivo* (*cf.* Shugar and Sierakowska, 1967) and *in vitro* (Hymer and Kuff, 1964; Maul and Hamilton, 1967; Nair *et al.*, 1967). Lazarus and Scherbaum (1967) isolated and characterized three components of the RNase activity from *T. pyriformis;* each has an acidic pH optimum, is an endonuclease, and has nucleolytic activity stimulated linearly as a function of temperature in the biological range. We have found that the degradation of the RNA product decreases as the pH is increased (Figure 3), and that the degradation increases with temperature (Figure 4). These results are consistent with degradation of the newly formed RNA in the macronuclear system by RNase.

Nature of the Polymerized RNA. RNA produced under various conditions was characterized using sucrose density gradient centrifugation, gel electrophoresis, nearest neighbor frequency analysis, and hybridization. Sucrose density gradient centrifugation analysis and acrylamide gel electrophoresis of the RNA synthesized in the polymerase reaction was not found to be helpful because of the marked breakdown of RNA during incubation. On the other hand, nearest neighbor frequency analysis permitted a comparison of the base composition of the RNA synthesized in the nuclear system under various conditions with known species of RNA existing in intact cells.

According to the principle upon which the nearest neighbor analysis of Josse *et al.* (1961) is based, this procedure should give the composition of the newly formed RNA. To evaluate whether the nature of the newly polymerized RNA synthesized in isolated nuclei resembles DNA or rRNA, the results from these nearest neighbor frequency analysis were compared with other data available in the literature (Table X). Although the base composition of newly formed RNA in isolated nuclei showed slightly higher CMP and slightly lower UMP, the composition of the newly polymerized RNA bears obvious relationship to the composition of *Tetrahymena* DNA, but not to purified *Tetrahymena* rRNA or *Tetrahymena* whole cell RNA. This suggests that the rapidly labeled RNA isolated in this study is not ribosomal precursor RNA. In organisms other than bacteria, rapidly labeled RNA (whether from isolated nuclei or whole tissue) resembles DNA more closely in its base composition than it does rRNA and is considered to represent mRNA (Hiatt, 1962; Sporn and Dingman, 1963; Lang and Sekeris, 1964; Girard *et al.*, 1965). However, messenger function has not always been proven. Furthermore, *T.*

TABLE X: Comparative Values Obtained for RNA Base Composition.

Source	% of 2′,3′-Nucleoside Monophosphate				Reference
	AMP	CMP	GMP	UMP	
Nuclei, log (29°)	36.8	16.0	14.2	33.0	This study (nearest neighbor frequency analysis)
Nuclei, log (34°)	36.4	16.6	14.9	32.1	
Nuclei, EHT	36.4	17.2	13.8	32.6	
Nuclei, EHT + 1 hr	37.1	16.3	14.0	32.6	
Complementary RNA (*cf.* Nair *et al.*, 1967)	36.7	15.0	14.3	34.0	Calculated from Brunk's (1967) data for *T. pyriformis* GL
	36.8	14.4	14.4	34.4	
rRNA (*T. pyriformis*)	31.9	15.7	27.9	24.5	Lyttleton (1963)
	30.9	17.7	25.7	25.7	
Whole cell RNA (*T. pyriformis*)					
Log phase	30.9	19.3	19.8	30.0	Scherbaum (1957)
EHT	29.8	21.4	20.4	28.4	
EHT + 1 hr	30.4	19.8	21.9	27.9	

pyriformis is a eukaryote and may well have nuclear RNA lacking a template function (Harris, 1968). We have shown elsewhere (Byfield and Lee, 1970) that the newly synthesized RNA in the *T. pyriformis* macronucleus has a high turnover (rapid synthesis and breakdown) and the experiments reported here indicate a DNA-like base composition.

Relevance of These Studies to Temperature-Induced Division Synchrony. In the preceeding discussion, we have suggested that the rapidly labeled RNA produced in the macronuclear RNA polymerase reactions may be messenger-like RNA, because its base composition is similar to DNA and its rapid synthesis and destruction. Previous experiments indicate that pulse-labeled *Tetrahymena* nuclear RNA has a half-life of 8 min at 34° and 22 min at 29° for strain GL macronuclei (Byfield and Lee, 1970). These results are in accord with the earlier indirect *in vivo* observations from this laboratory, which showed that *in vivo* pulse-labeled RNA is temperature labile (Byfield and Scherbaum, 1966) and that the unstable RNA probably includes cytoplasmic messengers (Byfield *et al.*, 1969). The DNA-like base composition of the RNA labeled during pulses adds further support to the proposal that mRNA may be involved in temperature-induced division synchrony.

The effect of synchronizing temperature on RNA polymerase appears to be slightly stimulatory (Table VII). This indicates that synchronizing temperature (equivalent to a single heat shift) does not exert any significant acute effect at the level of transcription. The results of nearest neighbor frequency analysis (Table VIII) suggest that the overall heat synchronization procedure does not produce any detectable qualitative effect on genetic transcription either, since the RNA synthesized in the nuclei isolated from either normal log growth or heat-synchronized cultures of *Tetrahymena* appears remarkably similar in its dinucleotide frequencies and base composition.

The current observations are therefore in agreement with earlier observations obtained in this laboratory, and tend to support our original interpretation of the basic synchrony mechanism (Byfield and Scherbaum, 1966, 1967). The latter proposes that the basic effect of each synchronizing temperature shift is to dissociate the pathways of messenger synthesis from those of translation by causing hydrolysis of the template RNA without translation.

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