Acta 425, 1.
Berkowitz, D. N., Kakefuda, T., and Sporn, M. B. (1969), *J. Cell Biol. 42*, 851.
Bernard, O., and Brent, T. P. (1973), *Biochem. Biophys. Res. Commun. 53*, 1213.
Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973), *Arch. Biochem. Biophys. 155*, 213.
Bolden, A., Aucker, J., and Weissbach, A. (1975), *J. Virol. 16*, 1584.
Bollum, F. J. (1959), *J. Biol. Chem. 234*, 2733.
Friedman, D. L. (1974), *Biochim. Biophys. Acta 353*, 447.
Friedman, D. L., and Mueller, G. C. (1968), *Biochim. Biophys. Acta 161*, 455.
Giles, K. W., and Myers, A. (1965), *Nature (London) 206*, 93.
Knopf, K.-W., Yamada, M., and Weissbach, A. (1976), *Biochemistry 15*, 4540.
Kolber, A. (1975), *J. Virol. 15*, 305.
Krokan, H., Bjørklid, E., and Prydz, H. (1975a), *Biochemistry 14*, 4227.
Krokan, H., Cooke, L., and Prydz, H. (1975b), *Biochemistry 14*, 4233.
LaColla, P., and Weissbach, A. (1975), *J. Virol. 15*, 305.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Matsukage, A., Bohm, E. W., and Wilson, S. H. (1975), *Biochemistry 14*, 1006.
Pederson, T., and Robbins, E. (1971), *J. Cell Biol. 49*, 942.
Perry, R. P., and Kelley, D. E. (1974), *Cell 1*, 37.
Roy, P. H., and Weissbach, A. (1975), *Nucleic Acids Res. 2*, 1669.
Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinmann, R., and Roeder, R. G. (1974), *J. Biol. Chem. 249*, 5889.
Seki, S., Lemahiev, M., and Mueller, G. C. (1975), *Biochim. Biophys. Acta 378*, 333.
Spadari, S., and Weissbach, A. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72*, 503.
Stafford, D. W., and Bieber, D. (1975), *Biochim. Biophys. Acta 378*, 18.
Tseng, B. Y., and Goulian, M. (1975), *J. Mol. Biol. 99*, 317.
Van der Vliet, C., and Sussenbach, J. S. (1972), *Eur. J. Biochem. 30*, 584.
Waqar, M. A., and Huberman, J. A. (1975), *Biochim. Biophys. Acta 383*, 410.
Winnacker, E. L., Magnusson, G., and Reichard, P. (1972), *J. Mol. Biol. 72*, 523.
Yamashita, T., and Green, M. (1974), *J. Virol. 14*, 412.

Partial Purification and Characterization of a Uracil DNA *N*-Glycosidase from *Bacillus subtilis*†

Richard Cone, James Duncan, Lenore Hamilton, and Errol C. Friedberg*

ABSTRACT: A uracil specific DNA *N*-glycosidase activity has been partially purified from crude extracts of *Bacillus subtilis*. The enzyme has a molecular weight of approximately 24 000 with no subunit structure. It has no requirement for any known cofactors but is inhibited in the presence of Co^{2+}, Fe^{2+}, or Zn^{2+}. The enzyme is specific for uracil in single- and double-stranded deoxyribonucleopolymers and does not release free uracil from RNA or from poly(rU):poly(dA). In addition, neither Udr, dUMP, nor dUTP is recognized as substrate. The enzyme will attack small poly(dU) oligomers but the minimum size recognized as substrate is $(pU)_4$. This enzyme may have a role in the repair (by base excision) of uracil in DNA arising either by incorporation during DNA synthesis or by deamination of cytosine in DNA.

In recent years an enzyme activity has been identified in both prokaryote and eukaryote sources that catalyzes the removal of uracil from DNA as the free base (Carrier and Setlow, 1974; Lindahl, 1974; Friedberg et al., 1975; Lindahl, 1975; Duncan et al., 1976a; Katz et al., 1976). In previous studies from this laboratory (Friedberg et al., 1975; Duncan et al., 1976a), we showed that this activity (referred to as uracil DNA *N*-glycosidase) is present in extracts of uninfected *B. subtilis*. We also showed that, following infection of *B. subtilis* by phage PBS2 (which contains uracil instead of thymine in its DNA), the *N*-glycosidase activity is no longer present in crude extracts. This phenomenon is dependent on protein synthesis after phage infection, suggesting that the phage codes for an inhibitor of the *N*-glycosidase.

We have purified both the host uracil DNA *N*-glycosidase activity and the phage induced inhibitor and are studying their properties and interaction. In the present report we describe the purification and characterization of the enzyme. Studies on the inhibitor will be presented later.

Materials and Methods

1. Cells and Cell Growth. Strain SB 168 (obtained from Dr. A. T. Ganesan, Genetics Department, Stanford University) was grown in double strength nutrient broth (Difco) or in brain heart infusion broth (Difco) at 37 °C. The cells were harvested in late log or stationary phase and stored at −20 °C.

2. Enzyme Purification. (a) Crude Extract. Frozen cells

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(108 g) were thawed in 220 mL of 10 mM Tris[1]-HCl buffer (pH 8.0) at 4 °C. The thawed cell suspension was sonicated in 50–60-mL aliquots in the cold using the large probe of a Branson Biosonik III cell sonifier (Bronwill Scientific) set at maximum power. The sonicate was centrifuged at 4 °C at 12 000*g* for 10 min and the supernatant saved. The pellets were resuspended in 50 mL of 10 mM Tris-HCl buffer (pH 8.0) and resonicated and recentrifuged as described above.

(b) High-Speed Centrifugation. The pooled supernatant (crude extract) was centrifuged in a Beckman Type 60 Ti rotor at 25 000 rpm for 90 min at 4 °C. The resulting supernatant was saved.

(c) Streptomycin Sulfate Precipitation. Streptomycin sulfate powder (General Biochemicals) was slowly added to 254 mL of the high-speed supernatant fraction while stirring in the cold to give a final streptomycin concentration of 0.7%. The mixture was stirred for a further 10 min and then centrifuged at 17 300*g* for 15 min. The supernatant fraction was retrieved and dialyzed against 10 mM Tris-HCl buffer (pH 8.0). In numerous experiments, the recovery of units in the supernatant following this procedure varies between 30 and 70%. This variation is not a function of the final concentration of streptomycin sulfate between the range 0.5 and 0.9% but appears to result from a variable affinity of the enzyme for the DNA present in a given crude extract. Studies with the purified enzyme (see Results) have shown that DNA (particularly in the single-stranded form) is an inhibitor of the enzyme activity.

(d) Ammonium Sulfate Fractionation. The fraction of protein precipitable between 45 and 75% saturated ammonium sulfate was resuspended in 10 mM Tris-HCl buffer (pH 8.0) at 4 °C and dialyzed against this buffer to remove ammonium sulfate.

(e) DEAE-Cellulose Chromatography. A column measuring 60 × 2.5 cm (diameter) was packed with prewashed DE 52 (Whatman) and equilibrated with 10 mM Tris-HCl buffer (pH 8.0) at 4 °C. The column was loaded in the cold with 22.8 mL of the dialyzed ammonium sulfate fraction and washed slowly with 600 mL of 10 mM Tris-HCl buffer (pH 8.0). The column was then eluted with a linear NaCl gradient (0–0.4 M) in 1400 mL of 10 mM Tris-HCl buffer (pH 8.0) and 10-mL fractions were collected. Enzyme eluted with a peak at about 0.1 M NaCl. Active fractions were pooled and dialyzed against 10 mM KPO_4 buffer (pH 7.0) containing 30% sucrose. This procedure reduced the volume of the enzyme fraction from 345 mL to 122 mL.

(f) Phosphocellulose Chromatography. A column measuring 30 × 2.5 cm (diameter) was packed with prewashed phosphocellulose (Cellex-P, Bio-Rad) and equilibrated with 10 mM KPO_4 buffer pH 7.0 at 4 °C. The concentrated DEAE-cellulose fraction was loaded onto the column in the cold and eluted with a linear NaCl gradient (0–0.9 M) in 1000 mL of 10 mM KPO_4 buffer (pH 7.0), after washing the loaded column with 120 mL of phosphate buffer. Fractions (10 mL) were collected and assayed for enzyme activity. A small amount of enzyme eluted in the void volume coincident with a large peak of protein. the bulk of the enzyme eluted as a peak at about 0.37 M NaCl. Active fractions were pooled and dialyzed against 10 mM KPO_4 buffer (pH 7.0). The dialyzed enzyme (860 mL) was concentrated in an Amicon ultrafiltration unit (180 mL capacity) using a PM 10 Diaflow membrane filter. When the volume was reduced to 21.5 mL, ultrafiltration was stopped, and the membrane filter was rinsed for 10 min in 5 mL of 10 mM KPO_4 buffer. The final volume of the concentrated enzyme was 26.5 mL which was then dialyzed against 10 mM Tris-HCl buffer (pH 8.0).

3. Polynucleotide Substrates. The following preparations of polynucleotide were used in the study. [^{14}C]thymine-labeled colE_1 DNA and [^{3}H]thymine-labeled SV40 DNA were generously provided by Dr. Ann Ganesan, Department of Biology, Stanford University. [^{3}H]Thymine-labeled *E. coli* DNA was prepared from *E. coli* by the procedure of Thomas and Abelson (1966). [^{3}H]Uracil-labeled PBS2 DNA was prepared as described previously (Friedberg et al., 1975). The identical procedure was used for the preparation of [^{3}H]adenine-labeled PBS2 DNA, except that 0.5 mCi of [^{3}H]adenine was added as the source of isotope instead of uridine. Apurinic SV40 DNA was prepared by the procedure of Gates and Linn (1977). This procedure produces an average of 1.5 apurinic sites/PM2 DNA molecule. [^{3}H]Poly(dU) was prepared as described previously (Duncan et al., 1976a). An aliquot of the polymer was electrophoresed in a 1.5-mm slab gel consisting of 4% acrylamide and 0.05% agarose and shown to be about 120 bases long on the average. [^{3}H]Uracil-labeled tRNA was a gift from Dr. Mandy Gillum, Department of Pathology, Stanford University. [^{3}H]Poly(rU):poly(dA) was obtained from Biogenics Research. Poly(rA):poly(rU), poly(rA), poly(dA), poly(rC), and poly(rG, rI) were obtained from Miles Laboratories. Calf thymus DNA was purchased from General Biochemicals. Poly(dG) and poly(dC) were obtained from Collaborative Research.

4. Preparation and Characterization of Oligonucleotides from [^{3}H]Poly(dU). A random distribution of small oligomers was prepared by incubating [^{3}H]poly(dU) with DNase I. The incubation mixture (3.5 mL) contained 30 nmol (as nucleotide) of polymer, 8 mM $MgCl_2$, 2 mM $CaCl_2$, 50 mM potassium phosphate buffer (pH 7.5), and 1 mg of DNase I (Worthington Biochemicals). At various times during incubation at 37 °C, small aliquots were removed and precipitated with the addition of carrier bovine serum albumin and cold trichloroacetic acid (5% final concentration). The formation of acid-soluble radioactivity was monitored until all polymer was rendered acid soluble. The entire incubation took 8.5 h and, after 4 h, a further 0.5 mg of DNase I was added. Following the incubation 0.5 mL of 0.1 M EDTA was added and the volume of the mixture brought to 20 mL with 50 mM potassium phosphate buffer at pH 7.0. Oligonucleotides of varying sizes were fractionated by DEAE-cellulose chromatography according to the procedure of Bollum (1968). Figure 1 shows the elution profile of an ordered series of oligomers expected from the random degradation of the polymer. The identity of peak I was established as dUMP by thin-layer chromatography with an authentic marker using the procedure of Weimann and Randerath (1963). Peaks 1–9 were individually pooled and concentrated by elution from DEAE-cellulose with the volatile solvent ammonium carbonate (Rushizky and Sober, 1962) and evaporating at 47 °C.

5. Other Materials. [5-^{3}H]Deoxyuridine, [5-^{3}H]uridine, [5-^{3}H]adenosine, [5-^{3}H]deoxyuridine monophosphate, and [*methyl*-^{3}H]thymidine were purchased from Schwarz/Mann and New England Nuclear Corp. [^{3}H]-5′-Deoxyuridine triphosphate was obtained from Amersham/Searle. Unlabeled bases, deoxynucleosides, and deoxynucleoside triphosphates were obtained from Calbiochem and Sigma Chemical Co.

6. Enzyme Assays. (a) Uracil DNA *N*-Glycosidase Activity. The routine assay (0.15 mL) contained [^{3}H]poly(dU) (0.075 to 0.23 nmol as nucleotide), 10 mM Tris-HCl buffer

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; UV, ultraviolet.

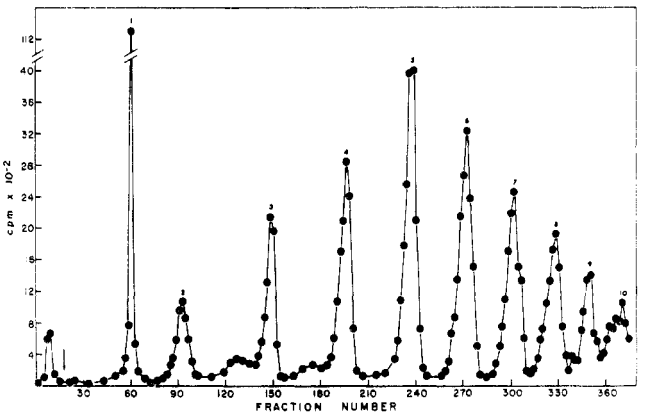

FIGURE 1: Elution profile of oligomers of [^{3}H]poly(dU) from DEAE-cellulose. A DEAE-cellulose column measuring 24.5 × 0.9 cm (internal diameter) was packed with DE-52 (Whatman) previously washed according to the procedure of Bollum (1968). The column was loaded with DNase I digest of [^{3}H]poly(dU) (see Materials and Methods) and eluted as described by Bollum (1968) in a total volume of 2 L. Fractions (3.3 mL) were collected and radioactivity was measured in 0.6-mL aliquots.

(pH 8.0), 25 mM NaCl, 1–10 mM EDTA, and 0.1% bovine serum albumin. Incubations were at 37 °C and were initiated by the addition of polymer. During the addition of polymer, care was taken to avoid direct contact of the aliquot with the sidewall of the incubation tube, since this resulted in random sticking of polymer to the tube. In all experiments, control tubes containing all components of the incubation except enzyme were included. Incubations were usually for 20 min and were terminated by the successive addition of 1% bovine serum albumin (75 μL) and 20% cold trichloroacetic acid (75 μL). Tubes were kept on ice for 5 min and then centrifuged in a GLC-1 table top centrifuge (Sorvall Instruments) at 2000 rpm for 5 min. An aliquot (150 μL) of the acid-soluble fraction was placed in a liquid scintillation vial to which was added 10 mL of a mixture of Omnifluor (New England Nuclear) in toluene base and Triton X-100, in a ratio of 2:1 (v/v). Radioactivity was measured in a Beckman Model L-230 or L-250 liquid scintillation spectrometer. It was previously shown that all acid-soluble radioactivity released from [^{3}H]poly(dU) in the presence of EDTA is in the form of uracil. This was confirmed in initial experiments in this study. A unit of activity is defined as the amount of enzyme that liberates 1 pmol of uracil from [^{3}H]poly(dU) during a 20-min incubation at 37 °C.

In some experiments, uracil DNA *N*-glycosidase activity was measured by the identification of free uracil as a product of the reaction. In these instances uracil was identified by thin-layer chromatography using either polyethylenimine thin layer and water as the solvent, or cellulose thin layer and ethyl acetate–1-propanol–water as the solvent as previously described (Friedberg et al., 1975).

(b) Nonspecific Endonuclease Activity. Incubation mixtures (50 μL) contained [^{14}C]thymine-labeled colE$_1$ DNA (0.3 nmol as nucleotide), 10 mM Tris-HCl buffer (pH 8.0), 25 mM NaCl, 10 mM EDTA or $MgCl_2$, and 100 units of fraction VI. Incubation was for 15 min at 37 °C, following which the entire incubation mixture was subjected to electrophoresis in agarose gels to separate form I from form II and linear DNA molecules, using a modification (P. Seawell and A. Ganesan, personal communication) of the procedure of Helling et al. (1974).

(c) UV Endonuclease Activity. The assay was the same as that described for nonspecific endonuclease activity except that the DNA was previously irradiated at 20 J/m^2 with a 15-W General Electric lamp. Dose-rate was measured with an International Light Co. germicidal photometer (IL 254). In addition, 1000 rather than 100 units of enzyme was included in the incubation.

(d) Exonuclease Activity. Incubation mixtures (0.15 mL) contained either native or alkali-denatured [^{3}H]thymine-labeled *E. coli* DNA (3 nmol), 10 mM EDTA or 2 mM $MgCl_2$, 25 mM NaCl, 0.1% bovine serum albumin, 10 mM Tris-HCl buffer (pH 8.0), and 1000 units of enzyme. Incubation was at 37 °C for 30 min following which reactions were terminated by the addition of carrier bovine serum albumin and trichloroacetic acid as described above. The acid-soluble fraction was harvested and radioactivity measured as described above.

(e) Apurinic Endonuclease Activity. Two types of enzyme assay were used. In one, incubation mixtures (0.15 mL) contained [^{3}H]adenine-labeled or [^{3}H]uracil-labeled PBS2 DNA (4.5 nmol), 10 mM EDTA, 0.1% bovine serum albumin, 25 mM NaCl, 10 mM Tris-HCl buffer (pH 8.0), and 500 units of fraction VI. Reactions were terminated and acid-soluble radioactivity was measured as described above. The inclusion of the uracil-labeled DNA provided an indirect measure of the extent of uracil removal from adenine-labeled PBS 2 DNA during the incubation. The second assay utilized incubations (0.05 mL) that contained depurinated [^{3}H]thymine-labeled form I SV40 DNA (0.75 nmol) or an equal amount of native SV40 DNA, 0.1% bovine serum albumin, 10 mM EDTA, 25 mM NaCl, 50 mM Tris-HCl buffer (pH 7.5), and 151 units of enzyme (fraction VI). Incubation was for 30 min at 37 °C following which the entire reaction volume was subject to electrophoresis on agarose gel and the fraction of form I DNA molecules remaining was determined as described above.

(f) DNA Polymerase Activity. DNA polymerase activity was measured by the procedure of Sedgwick et al. (1972). Reaction mixtures (0.25 mL) contained salmon sperm DNA (600 nmol as nucleotide) as a template-primer. Also present in the incubation were the usual deoxynucleoside triphosphates each at 5 μM, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 50 μg of bovine serum albumin, 10 mM Tris-HCl buffer (pH 9.2), [^{3}H]TTP (0.04 Ci/mmol), and 1000 units of fraction VI. Incubations were for 10 min at 37 °C.

(g) Alkaline Phosphatase Activity. Alkaline phosphatase was assayed by the procedure described in the Worthington Biochemical Corp. Manual Assay Procedure using *p*-nitrophenyl phosphate as the substrate. Incubation mixtures (1.0 mL) contained 0.05 M monoethanolamine (pH 11.3), 2 mg/mL *p*-nitrophenyl phosphate, and 500 units of fraction VI.

7. Molecular Weight Determinations. (a) Sedimentation Velocity in Sucrose Gradients. Fraction VI (50 000 units) was layered onto a 5.5-mL 5–20% sucrose gradient in 10 mM Tris-HCl buffer (pH 7.5), containing either 0.3 M NaCl or no added salt. A gradient without NaCl was loaded with 0.1 mg of grade A hemoglobin (mol wt 68 000; *S* value 4.2) (Calbiochem) as a marker protein. The gradients were centrifuged at 4 °C in a Beckman SW 50.1 rotor at 49 000 rpm for 28 h. Fractions (10 drops) were collected through the bottom of the tubes and assayed for *N*-glycosidase activity. The position of the hemoglobin marker was determined by reading the OD_{416} of individual fractions.

(b) Gel Filtration. A column of dimensions 54.5 cm × 0.9 cm (internal diameter) was packed with swollen Sephadex G-100 (40–120 μm particle size) (Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) plus 0.3 M NaCl, under

TABLE I: Purification of Uracil DNA *N*-Glycosidase from *B. subtilis*.

Fraction	Volume (mL)	Protein (mg)	Units	Spec act. (units/mg of protein)
I. Crude	260.0	7871	1.67×10^6	2.13×10^2
II. High-speed supernatant	254.0	6561	1.81×10^6	2.77×10^2
III. Streptomycin sulfate	241.0	3285	9.09×10^5	2.76×10^2
IV. Ammonium sulfate	22.8	456	6.52×10^5	1.43×10^3
V. DEAE-Cellulose	345.0	25.7	3.69×10^5	1.43×10^4
VI. Phosphocellulose	26.5	1.5	2.24×10^5	1.49×10^5

a pressure head of 20 cm. The flow rate of the column was 0.15 mL/min. The column was calibrated both before and after enzyme was filtered using bovine serum albumin, ovalbumin, chymotrypsinogen, and pancreatic ribonuclease as molecular weight markers and blue dextran and phenol red as column volume markers. The position of the protein markers was determined by measuring protein by OD_{280} and in some experiments by the Lowry procedure (Lowry et al., 1951) in individual fractions (0.36 mL). Enzyme (fraction VI) (165 000 units) was loaded onto the column in 0.165 mL and eluted with 50 mM Tris-HCl buffer (pH 8.0), containing 0.3 N NaCl. Individual fractions were assayed for *N*-glycosidase activity using 5 μL of each fraction in a total reaction volume of 0.15 mL. The enzyme and protein markers were also filtered (in separate experiments) through the Sephadex column equilibrated in 50 mM Tris-HCl buffer (pH 8.0) without added NaCl. The Stokes radius for the *N*-glycosidase was calculated according to the procedure of Ackers (1964). The parameter "*r*" was calculated for the Sephadex column based on the known Stokes radii of the protein markers used. Molecular weight was determined (i) by comparison of the gel filtration profile relative to the known standards and (ii) from the *S* value and Stokes radius using the formula mw $= kas$, where a = Stokes radius and s = sedimentation coefficient (Martin and Ames, 1961).

8. Polyacrylamide Gel Electrophoresis. An aliquot of fraction VI containing 500 μg of protein was lyophilized and resuspended in 0.1 mL of 50 mM Tris-HCl buffer (pH 8.0) plus 2% sodium dodecyl sulfate and 5% β-mercaptoethanol. This was electrophoresed in a polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate as described by Forger et al. (1976). Marker proteins were run simultaneously. Staining, destaining, and scanning of the gel were performed as described by Forger et al. (1976).

9. K_m of N-Glycosidase for [^{3}H]Poly(dU). The K_m of the purified enzyme for poly(dU) was measured from a Lineweaver-Burk (1934) reciprocal plot of [S] and V_0 determined experimentally from time curves run at different substrate concentrations. All incubations (0.15 mL) contained 40 units of enzyme (fraction VI) and between 0.116 and 0.693 nmol of polymer (as nucleotide). Incubations were between 4 and 30 min at 37 °C.

10. Sedimentation of Polymer in Sucrose Gradients. Incubations (0.15 mL) contained 1.8 nmol of [^{3}H]poly(dU) and 3000 units of enzyme (fraction VI). Incubation was at 37 °C for 15 min at which time PBS2 phage induced uracil DNA *N*-glycosidase inhibitor (J. Duncan, R. Cone, and E. C. Friedberg, manuscript in preparation) was added to stop the reaction. Measurement of the total acid-soluble radioactivity in an aliquot of the incubation revealed that 33% of the uracil was removed from the polymer. A 50-μL aliquot of the reaction mixture was layered onto either a neutral (pH 7.0) or alkaline (pH 12.0) 5–20% sucrose gradient and sedimented in a Beckman SW56 rotor at 51 000 rpm for 18 h at 21 °C. Control samples containing incubated polymer without added enzyme were sedimented at the same time. Eight-drop fractions were collected from the bottom of each tube directly into liquid scintillation vials. To each was added 0.5 mL of water plus 10 mL of scintillation mixture and radioactivity was measured as described above.

Results

Enzyme Purification. The purification of the enzyme is indicated in Table I. Analysis of the purified enzyme by polyacrylamide gel electrophoresis under reducing and denaturing conditions reveals a single major band at a molecular weight of approximately 24 000; however, the preparation is not homogeneous. No significant contamination by a variety of other relevant enzymes tested was detected in fraction VI. Thus when 1000 units of enzyme was incubated with 300 pmol (as nucleotide) of [^{14}C]thymine-labeled form I colE_1 DNA, no endonucleolytic cleavage of the DNA was observed. In addition, no acid-soluble radioactivity was observed when 1000 units of enzyme were incubated with 6 nmol of [^{3}H]thymine-labeled native or alkali-denatured *E. coli* DNA in the presence of either 10 mM EDTA or 2 mM $MgCl_2$. These experiments indicate the absence of detectable nonspecific endonuclease or exonuclease activity. Endonuclease activity active on ultraviolet-irradiated DNA was tested by incubating form I colE_1 DNA irradiated with 20 J/m^2 of 254-nm ultraviolet light, with fraction VI. No reduction in the fraction of form I DNA molecules was detected. The presence of apurinic endonuclease contamination was also tested. Firstly [^{3}H]adenine-labeled PBS2 DNA was incubated with enzyme under conditions that resulted in the loss of 50% of the uracil residues as determined from an incubation with an identical concentration of [^{3}H]uracil-labeled PBS2 DNA. Since uracil comprises approximately 33% of the bases in PBS2 DNA, one out of every six phosphodiester bonds (on the average) was sensitive to attack by apurinic/apyrimidinic endonuclease; yet, less than 0.2% of the adenine label was rendered acid soluble in the presence of 1.0 mM EDTA. In an independent experiment, when [^{3}H]thymine-labeled form I SV40 DNA containing on the average 0.75 apurinic site/DNA molecule was incubated with enzyme in EDTA (10 mM), 88% of the DNA molecules retained the closed circular conformation. No DNA polymerase or alkaline phosphatase activity was detected in 1000 units of fraction VI.

Enzyme Characterization. It was previously shown that the *N*-glycosidase activity from *B. subtilis* releases free uracil from either [^{3}H]uracil-labeled PBS2 DNA or [^{3}H]poly(dU) (Friedberg et al., 1975; Duncan et al., 1976a). The present studies were carried out using labeled polymer as substrate in most instances. Since the enzyme has no requirement for divalent cation and is not inhibited in EDTA (see below), the enzyme was assayed in the presence of 1.0 mM EDTA routinely. Figure 2 shows that the activity is linear with respect to both protein concentration and time of incubation. All further experiments described were carried out within the linear range of the assay.

Optimal Assay Conditions. The enzyme has a broad pH optimum between 7.3 and 7.8 when assayed in 50 mM Tris-HCl buffer. At pH 8.4 the activity is 78% of that at pH 7.5. The

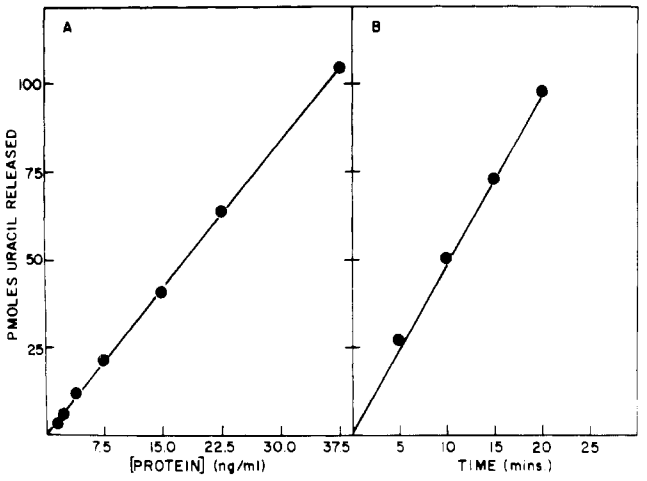

FIGURE 2: Linear relationship of enzyme activity to protein concentration and time of incubation. Experimental details are provided in the section on Materials and Methods. Incubation mixtures in both experiments contained 346 pmol (as nucleotide) of [^{3}H]poly(dU). In the experiment shown in A, between 2.0 and 100 units of enzyme was added, whilst 80 units of enzyme was present in the experiment shown in B.

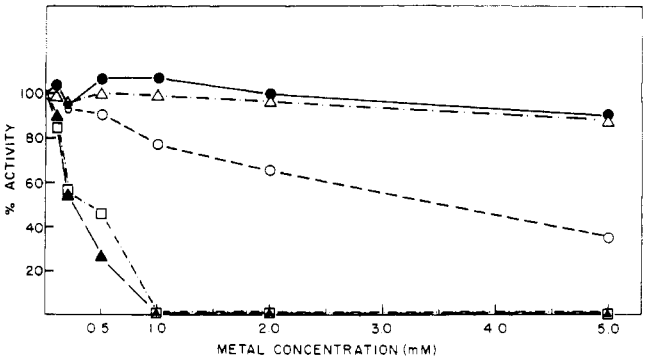

FIGURE 3: Effect of divalent cations on enzyme activity. Incubation mixtures were as described under Materials and Methods. [^{3}H]Poly(dU) [346 pmol (as nucleotide)] and 40 units of enzyme (fraction VI) were present. Activity of each metal is shown relative to that in the presence of 10 mM EDTA. (Closed circles) $MnCl_2$; (open triangles) $MgCl_2$; (open circles) $CoCl_2$; (closed triangles) $(Fe)_2Cl_3$; (open squares) $ZnCl_2$. The data obtained with $CaCl_2$ are omitted for the sake of clarity but were not significantly different from that observed in the presence of $MgCl_2$ or $MnCl_2$.

enzyme has no requirement for divalent cation. It retains full activity in the presence of EDTA and is not stimulated by either Ca^{2+}, Mg^{2+}, or Mn^{2+} (Figure 3). The heavy metal ions Fe^{2+}, Zn^{2+}, and Co^{2+} are inhibitory to enzyme activity (Figure 3). In the presence of between 30 and 50 mM NaCl, enzyme activity is stimulated twofold. This effect is not specific for NaCl; similar results were obtained in the presence of other anions and cations. The enzyme is insensitive to inhibition by 1.0 mM *N*-ethylmaleimide. In the presence of 1.0 mM *p*-chloromercuriphenylsulfonic acid, approximately 80% activity is retained.

Stability of Enzyme. The purified enzyme is stable to freezing for at least 3 months but loses activity on repeated freezing and thawing. When incubated at 50 °C for 10 min, fraction VI retains 57% of its activity at 37 °C. Heating at 65 °C for the same time destroys 97% of the activity.

Molecular Weight. In a 5–20% sucrose gradient containing 50 mM Tris-HCl, the enzyme sediments as a major peak at 2.65 S relative to a hemoglobin marker (Figure 4a). The minor peaks of activity at higher *S* values may reflect a tendency for

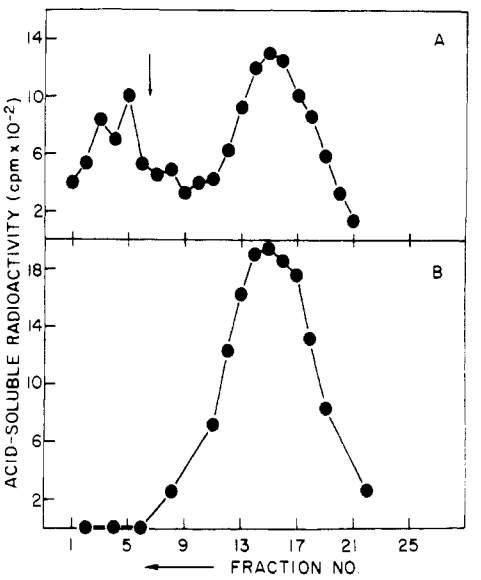

FIGURE 4: Sedimentation of uracil DNA *N*-glycosidase in 5–20% sucrose gradients. Experimental details are provided under Materials and Methods. (A) No sodium chloride present; (B) 0.3 N sodium chloride present. The arrow indicates the peak position of the hemoglobin marker used (4.2 S).

the enzyme to form aggregates in sucrose at low ionic strength. When sedimented in 5–20% sucrose containing 0.3 N NaCl (Figure 4b), the enzyme activity is detected as a single peak at 2.65 S. The Stokes radius was calculated at 19.3 Å and the molecular weight calculated from the Stokes radius and the sedimentation coefficient is 24 100 daltons. Gel filtration of the enzyme on Sephadex G-100 yields a molecular weight value of 23 600 relative to ovalbumin, ribonuclease, chymotrypsinogen, and bovine serum albumin markers. Finally, electrophoresis of fraction VI in polyacrylamide gel under reducing and denaturing conditions shows a single major band at a molecular weight of 24 000 daltons. We did not confirm that the major band contains enzyme activity, however. We conclude that the *N*-glycosidase has a molecular weight of approximately 24 000 and has no subunit structure.

Substrate Specificity. As indicated above, when crude extracts of *B. subtilis* are incubated with either [^{3}H]uracil-labeled phage PBS2 DNA or [^{3}H]poly(dU), all acid-soluble radioactivity is present as free uracil. Identical results were obtained with the purified enzyme. The K_m of the purified enzyme for [^{3}H]poly(dU) was determined at 1.1×10^{-9} mol/L and the V_{max} is 2 pmol of uracil released/min. It was previously shown that, when PBS2 DNA containing both [^{3}H]uracil and ^{32}P radioactive markers was incubated with crude extract in the presence of EDTA, only ^{3}H and not ^{32}P was present in the acid-soluble fraction, suggesting that the release of free uracil does not require and is not accompanied by breakage of phosphodiester bonds (Duncan et al., 1976a). In the present studies, the *N*-glycosidic function of the purified enzyme was demonstrated more directly. [^{3}H]Poly(dU) was incubated with enzyme to release 33% of the uracil. Aliquots of the polymer were then sedimented in neutral or alkaline sucrose gradients. As shown in Figure 5, in the neutral gradient the labeled polymer sediments as unit length molecules, indicating no breakage of phosphodiester bonds. Remaining radioactivity is present at the top of the gradient in the expected position of free uracil. In alkali, the labile phosphodiester bonds associated with apyrimidinic sites hydrolyze and all label sediments at the top of the gradient.

TABLE II: Effect of Natural and Synthetic Nucleic Acid Polymers on Uracil DNA N-Glycosidase Activity.[a]

Addition	% residual act. after addition of 1 nmol	8 nmol	20 nmol
Native DNA	92	67	58
Denatured DNA	82	46	42
Poly(dA)	100	94	85
Yeast tRNA	97	84	63
Poly(rC)	100	100	95
Poly(rA)	100	99	93
Poly(rA):poly(rU)	95	79	55
Poly(rG,rI)	90	64	51

[a] Incubations were performed as described in Materials and Methods. Incubation mixtures contained 75 pmol (as nucleotide) of [^{3}H]poly(dU) and 200 units of enzyme (fraction VI) as well as the amounts of natural or synthetic nucleic acid indicated. Incubations were at 37 °C for 20 min.

TABLE III: Degradation of Oligomers of [^{3}H]dU by Uracil DNA N-Glycosidase.[a]

Peak No. (from Figure 1)	Uracil as substrate (pmol)	Uracil as product (%)
1	34.5	0.8
2	7.2	2.1
3	4.7	0.6
4	21.7	38.6
5	13.6	36.9
6	15.2	59.4
7	33.9	71.1
8	23.8	74.6
9	25.3	69.9

[a] An ordered series of oligomers of [^{3}H]dU was prepared as described in the text. Incubation mixtures (0.4-0.5 mL) contained the amounts of dU (as nucleotide) indicated in the table. In addition, 10 mM EDTA-0.1 M Tris-HCl buffer (pH 7.5) and 7000 units of fraction VI were added. For each oligomer a control incubation without added enzyme was performed. Incubations were at 37 °C for 1 h and were terminated by placing tubes on ice. While on ice, the tubes were evaporated to dryness and the residue was resuspended in 5 μL of 10 mM Tris-HCl buffer (pH 8.6). This was spotted on silica gel thin-layer plates (Brinkmann) and developed with the top phase of a mixture of ethyl acetate-1-propanol-water as described by Friedberg et al. (1975). The thin-layer plates were fractionated and counted for radioactivity as described by Friedberg et al. (1975) and the percentage of free uracil liberated during incubation with enzyme was calculated.

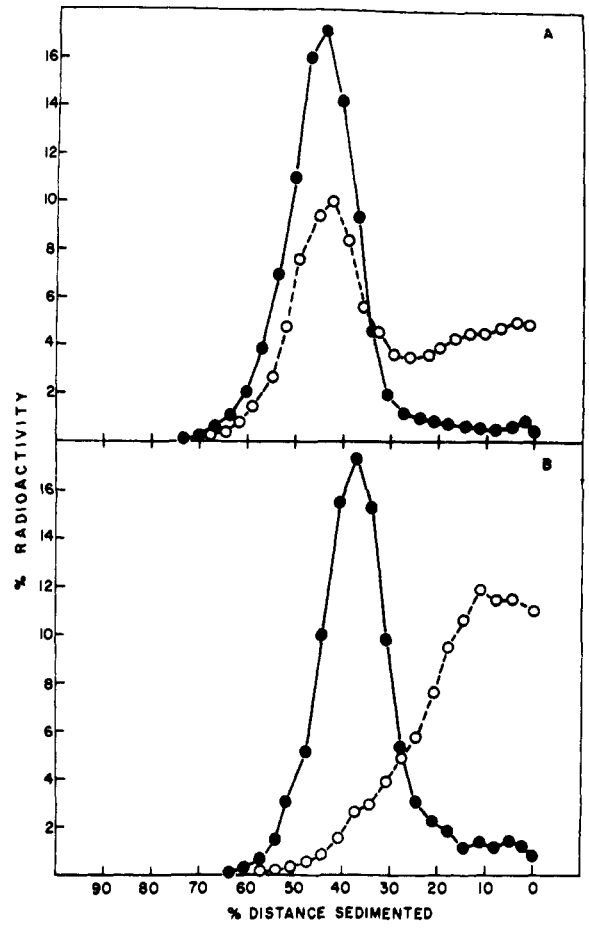

FIGURE 5: Demonstration of N-glycosidic mode of action of enzyme on [^{3}H]poly(dU). Experimental details are provided under Materials and Methods. The direction of sedimentation is from right to left. (A) Sedimentation in neutral gradients; (B) sedimentation in alkaline gradients. (Closed circles) No enzyme added; (open circles) enzyme present.

It was demonstrated in earlier studies that the enzyme does not release acid-soluble radioactivity from [^{3}H]thymine-labeled T7 DNA, or [^{3}H]adenine-labeled *E. coli* DNA (Friedberg et al., 1975). These results were confirmed in the present studies using [^{3}H]thymine-labeled *E. coli* DNA and [^{3}H]adenine-labeled PBS2 DNA. In addition, no free cytosine or guanine was detected when poly(dC) or poly(dG) was incubated with the enzyme. The enzyme did not promote the release of acid-soluble radioactivity from either [^{3}H]uracil-labeled RNA or [^{3}H]poly(rU):poly(dA). Free uracil was not released from either [^{3}H]deoxyuridine or dUTP. Furthermore, when present in concentrations up to tenfold greater than that of poly(dU) (as nucleotide equivalents), deoxyuridine or dUTP did not inhibit enzyme activity. Both native and particularly heat-denatured *E. coli* DNA inhibit the action of the enzyme on [^{3}H]poly(dU) in a concentration-dependent fashion (Table II). Inhibition was also observed in the presence of poly(rA):poly(rU), yeast tRNA, and poly(rG,rI). Poly(rC), poly(rA), and poly(dA) produced insignificant inhibition (Table II).

When enzyme was incubated with the sized oligomers obtained by digestion of [^{3}H]poly(dU) with pancreatic DNase, the smallest oligomer sensitive to attack was the material present in peak 4 (Figure 1), presumed to be the tetranucleotide $(pU)_4$ (Table III).

Discussion

Activities that catalyze the removal of free bases from polymeric deoxyribonucleotides including DNA are recognized as a distinct class of enzymes referred to as DNA N-glycosidases (Lindahl, 1976). The first report of such an activity was by Kirtikar and Goldthwait (1974), who showed that an enzyme activity previously designated as endonuclease II of *E. coli* (Friedberg and Goldthwait, 1969; Friedberg et al., 1969) can catalyze the removal of both O^6-methylguanine and 3-methyladenine from DNA alkylated with N-methyl-N-nitrosourea. This enzyme has also been shown to promote the release of N^6-(12-methylbenz[a]anthracenyl-7-methyl)adenine and N^2-(12-methylbenz[a]anthracenyl-7-methyl)guanine from DNA treated with 7-bromomethyl-12-methylbenz[a]anthracene (Kirtikar et al., 1975). Laval (J. Laval, personal communication) has reported the isolation from *M. luteus* of a 3-methyladenine DNA N-glycosidase activity that is free

of associated phosphodiester hydrolase activity. Lindahl (1976) has also reported the isolation of a 3-methyladenine DNA *N*-glycosidase from *E. coli*, but it is not yet established whether or not this activity has the same properties as endonuclease II.

DNA *N*-glycosidase activity specific for uracil has been reported in both *E. coli* (Lindahl, 1974, 1976) and *B. subtilis* (Friedberg et al., 1975; Tomita and Takahashi, 1975; Duncan et al., 1976a; Katz et al., 1976). The enzyme from *E. coli* has recently been extensively purified and characterized and shown to resemble that from *B. subtilis* in most respects (T. Lindahl, personal communication). Our interest in the enzyme in *B. subtilis* stems largely from the recognition that this organism is the host for a group of bacteriophages that naturally contain uracil (and in some examples 5-hydroxymethyluracil) in their DNA instead of thymine (Takahashi and Marmur, 1963). We have previously shown that following infection of *B. subtilis*, phage PBS2 (which contains uracil in its DNA) codes for the expression of an inhibitor of the uracil DNA *N*-glycosidase activity (Friedberg et al., 1975; Duncan et al., 1976a). Work in our laboratory has been directed toward the purification and characterization of both the host enzyme and the phage induced inhibitor. The purification and characterization of the latter and its interaction with purified uracil DNA *N*-glycosidase will be reported in a subsequent publication.

The enzyme from *B. subtilis* is a relatively small protein with no subunit structure. It has no requirement for divalent cation and is not stimulated by metals such as magnesium, manganese, or calcium. The heavy metals, iron, zinc, and cobalt are inhibitory. The present studies confirm more directly our previous contention that the enzyme is a *N*-glycosidase (Friedberg et al., 1975), by showing that the enzyme has no associated phosphodiester hydrolase activity on uracil-containing DNA.

The substrate specificity of the enzyme is very stringent. Our studies indicate that at least three parameters determine its catalytic activity.

(a) Uracil is the only base identified as being recognized by the enzyme. Based on previous studies from this laboratory (Friedberg et al., 1975) together with the results of the present studies, neither thymine, cytosine, adenine, guanine, bromodeoxyuracil, nor 5-hydroxymethyluracil are recognized as substrate.

(b) The uracil must bear a certain proximity to a deoxyribose moiety since uracil in ribonucleic acid is not recognized as a substrate. In addition, the deoxyribose must be on the same strand as the uracil as evidenced by the observation that the copolymer poly(rU):poly(dA) is not attacked. However, it is not certain that the uracil and the deoxyribose moieties have to be in the same nucleotide; e.g., the enzyme may conceivably recognize a single uridine moiety present in covalent linkage in a deoxypolymer.

(c) A polymeric structure is required since neither deoxyuridine, deoxyuridine monophosphate, nor deoxyuridine triphosphate is recognized as substrate. Our studies indicate that the oligomer $(pU)_4$ is the smallest sized single-stranded oligomer degraded by the enzyme.

The enzyme has a high binding affinity for poly(dU) ($K_m = 1.1 \times 10^{-9}$ M). However, the observation that other nucleotide polymers can inhibit enzyme activity to varying degrees indicates a nonspecific binding affinity for these.

It is of interest to speculate on the biological role of the uracil DNA *N*-glycosidase. The enzyme is apparently quite ubiquitous in nature and has been demonstrated in mammalian cells and tissues in addition to the prokaryotes *B. subtilis* and *E. coli* (Lindahl, 1976; Sekiguchi et al., 1976). Since dUTP is present in cells as a normal precursor for DNA synthesis, it is distinctly possible that uracil is incorporated into DNA at a low frequency and the function of the *N*-glycosidase is to ensure its repair by excision. We refer to such a DNA repair mode as base excision repair to distinguish it from nucleotide excision repair such as that involving excision of pyrimidine dimers. The biological consequences of uracil incorporation into DNA are not clear, however, since, at least in terms of the fidelity of the genetic code, a U → T transition is not inappropriate. Conceivably enzymatic functions required for normal replication and/or transcription of DNA are critically affected by this transition. Recently Duncan et al. (1976b) have reported the isolation of uracil DNA *N*-glycosidase defective mutants of *E. coli* (*ung*$^-$) which demonstrate no loss of viability under normal growth conditions.

An alternative mode of generating uracil in DNA is by deamination of cytosine. Studies in this laboratory (R. Da Roza, E. C. Friedberg, B. Duncan and H. Warner, manuscript in preparation) have shown that *ung*$^-$ cells are more sensitive to treatment with nitrous acid than *ung*$^+$ cells, suggesting that the uracil DNA *N*-glycosidase is involved in the excision repair of deaminated cytosine.

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References

Ackers, G. K. (1964), *Biochemistry 3,* 723.
Bollum, F. J. (1968), *Methods Enzymol. 12B,* 591.
Carrier, W. L., and Setlow, R. B. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1599.
Duncan, B. K., Rockstroh, P. A., and Warner, H. R. (1976b), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1493.
Duncan, J., Hamilton, L., and Friedberg, E. C. (1976a), *J. Virol. 19,* 338.
Forger, J. M., III, Choie, D. D., and Friedberg, E. C. (1976), *Cancer Res. 36,* 258.
Friedberg, E. C., Ganesan, A. K., and Minton, K. (1975), *J. Virol. 16,* 315.
Friedberg, E. C., and Goldthwait, D. A. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 934.
Friedberg, E. C., Hadi, S. M., and Goldthwait, D. A. (1969), *J. Biol. Chem. 224,* 5879.
Gates, F. T., and Linn, S. (1977), *J. Biol. Chem. 252,* 2802.
Helling, R. B., Goodman, H. M., and Boyer, H. W. (1974), *J. Virol. 14,* 1235.
Katz, G. E., Price, A. R., and Pomerantz, M. J. (1976), *J. Virol. 20,* 535.
Kirtikar, D. M., Dipple, A., and Goldthwait, D. A. (1975), *Biochemistry 14,* 5548.
Kirtikar, D. M., and Goldthwait, D. A. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2022.
Lindahl, T. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3649.
Lindahl, T. (1976), *Nature (London) 259,* 64.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56,* 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.

Rushizky, G. W., and Sober, H. A. (1962), *Biochim. Biophys. Acta 55*, 217.
Sedgwick, W. D., Wang, T. S. F., and Korn, D. (1972), *J. Biol. Chem. 247*, 5026.
Sekiguchi, M., Hayakawa, H., Makimo, F., Tanaka, K., and Okada, Y. (1976), *Biochem. Biophys. Res. Commun. 73*, 293.
Takahashi, I., and Marmur, J. (1963), *Nature (London) 197*, 794.
Thomas, C. A., and Abelson, J. (1966), *Proced. Nucleic Acid Res.*, 553.
Tomita, F., and Takahashi, I. (1975), *J. Virol. 15*, 1081.
Weimann, G., and Randerath, K. (1963), *Experientia 19*, 49.

Synthesis of Ribosomal 5S RNA by Isolated Nuclei from HeLa Cells in Vitro†

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ABSTRACT: The fidelity of 5S ribosomal RNA transcription in isolated HeLa cell nuclei has been studied by molecular hybridization using an *E. coli* hybrid plasmid which contained *Xenopus* 5S DNA as a probe. As a prerequisite, the incubation conditions were optimized for the synthesis of a specific gene product in nuclei. The synthesis of 5S RNA was dependent on the presence of Mg^{2+}, while increasing quantities of Mn^{2+} progressively inhibited its formation. The most dramatic effect on the amount of 5S RNA synthesized was exerted by the ionic strength of the medium. An optimum was observed at 50 mM NH_4Cl while a significant depression occurred at higher ionic strengths and only 20% or less of the maximal 5S RNA synthesis occurred at 150 to 200 mM for monovalent ions, respectively. At these latter concentrations, bulk RNA synthesis was still very active, indicating a clear dissociation of 5S and bulk RNA syntheses. The synthesis of hybridizable 5S RNA sequences is sensitive to high concentrations of amanitin, demonstrating that RNA polymerase C is responsible for their synthesis. It was shown, however, that conditions for maximal activity of enzyme C in isolated nuclei do not warrant an optimal production of ribosomal 5S RNA, reemphasizing the necessity of specific assay systems for the analysis of defined transcription products. Under optimized incubation conditions, 0.059 pmol of ribosomal 5S RNA was synthesized per 2×10^6 nuclei during 40 min of incubation. This corresponds to 18 000 molecules per nucleus, demonstrating an efficient reinitiation of 5S RNA synthesis. The fidelity of 5S rRNA transcription was assayed by subjecting the in vitro products to a combination of size fractionation and molecular hybridization. Two low-molecular-weight products were identified by gel electrophoresis representing 5S RNA and a compound which is probably the 4.5S precursor to tRNA. Hybridization of the individual gel fractions to 5 S containing plasmid DNA showed that only the component which comigrated with mature in vivo 5S RNA hybridized. Moreover, it has been observed by Sephadex G-100 gel filtration that there are no hybridizable 5S sequences in RNA of high molecular weight. Hybridization of in vitro 5S RNA is completed to essentially 0% by the addition of a great excess of cold in vivo 5S RNA. Chromatography of T1 ribonuclease digests of 5S RNA synthesized in vitro on diethylaminoethyl-Sephadex in the presence of 7 M urea revealed a similar oligonucleotide pattern obtained from in vivo 5S ribosomal RNA. Transcription of antisense RNA is therefore unlikely and, from these results, we conclude that isolated nuclei synthesize 5S rRNA accurately with respect to sequence and size.

The synthesis of low-molecular-weight RNA species in isolated cell nuclei has been studied by several investigators under different experimental conditions (Price and Penman, 1972; Reeder and Roeder, 1972; Marzluff et al., 1974; Weinmann and Roeder, 1974; Udvardy and Seifart, 1976; Weil and Blatti, 1976; Sarma et al., 1976). In view of the lack of a specific assay system, none of them have, however, optimized the conditions for the synthesis of a specific gene product. Thusfar the criteria of optimization were either maximal incorporation of labeled precursors into bulk RNA or the linearity of this process during a defined incubation period, and, since total RNA synthesis and the production of a specific class of RNA are not necessarily correlated, this question was investigated. In addition we attempted to resolve the question whether the 5S-sized product formed in nuclei was homologous in sequence to ribosomal 5S RNA and, alternatively, whether sequences detectable by molecular hybridization were necessarily of 5S size. It was, therefore, analyzed whether high-molecular-weight RNA products contained sequences for 5S RNA, possibly indicating a read-through of transcription or, alternatively, whether 5S rRNA sequences were detectable in low-molecular-weight fragments resulting from incorrect initiation or premature termination of RNA polymerase. It was hitherto impossible to obtain sufficient amounts of a complementary probe required for quantitative studies of this kind in isolated nuclei because of the presence of large amounts of endogenous RNA. Current advances in the construction of bacterial plasmids containing foreign DNA (Cohen et al., 1973) enabled us to employ this chimeric DNA as a probe for specific DNA–RNA hybridization analyses. We have chosen to study the synthesis of 5S RNA because of a number of significant

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