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Deoxyribonucleic Acid Polymerase with Rat Liver Ribosomes and Smooth Membranes. Purification and Properties of the Enzymes*

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ABSTRACT: Two enzymes that incorporate deoxyribonucleotides into DNA have been found in association with ribosomes and with a smooth membrane fraction isolated from rat liver. These enzymes have been partially purified by fractionation with ammonium sulfate and chromatography on DEAE-cellulose and phosphocellulose. The membrane and ribosome associated polymerases behave differently during the course of purification. Purified membrane polymerase is free of exo- and endonuclease activities but the purified ribosome polymerase contains a small amount of endonuclease activity. The purified enzymes have different pH optima but similar Mg^{2+} requirements. The ribosome-associated polymerase and the membrane-associated enzyme exhibit maximal activity at 9.0 and 8.0 pH units and at 15 and 10 mM Mg^{2+}, respectively. After purification both enzymes prefer "activated" DNA as primer, although the ribosome-associated enzyme retains some activity with native DNA primer. All four deoxyribonucleoside triphosphates are required by both enzymes for maximal activity, but the purified DNA polymerase from ribosomes has about 25% of maximal activity in the presence of only a single deoxyribonucleoside triphosphate. This activity does not appear to be a terminal addition enzyme since experiments with poly(dA) and poly[d(A-T)] demonstrate a requirement for base pairing of the deoxyribonucleoside triphosphate with the template. The properties of the ribosome- and smooth membrane-associated DNA polymerases suggest that they are not mitochondrial in origin. However, the DNA-synthesizing activity associated with ribosomes is similar to the activity extracted from highly purified rat liver nuclei. The DNA-synthesizing activity associated with the membrane fraction is not detectable in the KCl-extractable or nonextractable portion of rat liver nuclei.

Deoxyribonucleic acid polymerase activity has invariably been found in the postmicrosomal supernatant solution when mammalian tissue homogenates are prepared in aqueous media (Bollum and Potter, 1958; Davidson *et al.*, 1958). In fact, with the exception of a few specific studies of nuclear (Howk and Wang, 1969; Gold and Helleiner, 1964) and mtDNA polymerases (Kalf and Ch'ih, 1968; Meyer and Simpson, 1968), most of the investigations of the enzyme in mammalian tissues have used the postmicrosomal supernatant solutions as the starting material for isolation of the enzyme. Bollum and Potter (1958) and Keir and Smellie (1962) originally suggested that a portion of the DNA polymerase in mammalian cells may reside in the cytoplasm, as well as in the nucleus. However, these investigators acknowledged the possibility of artifact production during tissue homogenization. The recent demonstration of a unique mtDNA polymerase (Kalf and Ch'ih, 1968; Meyer and Simpson, 1968) clearly indicates that some of the DNA polymerase found in the cytoplasm is not representative of the nuclear enzyme. The question of whether the remaining DNA polymerase activity found in the cytoplasm is of nuclear origin must be examined critically and with an open mind. In particular, the demonstration of DNA associated with microsomes and polysomes (Bond *et al.*, 1969; Schneider and Kuff, 1969; Bell, 1969) raises the question of whether other unique ctDNA[1] polymerases might not exist. The demonstration of distinct membrane-associated and soluble DNA-synthesizing activities in bacteria (Okazaki *et al.*, 1970) clearly indicates the need for a critical examination of the distribution of DNA-synthesizing activity in higher organisms.

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[1] The abbreviations used are: ctDNA, cytoplasmic deoxyribonucleic acid; poly(dA), the homopolymer of deoxyriboadenylate; poly[d(A-T)], the alternating copolymer of deoxyriboadenylate and deoxyribothymidylate; poly(U), the homopolymer of uridylic acid; PMB, *p*-hydroxymercuribenzoate.

We have recently reported the occurrence of a deoxyribonucleotide-polymerizing activity with the free ribosomes and with a smooth membrane fraction from normal rat liver and hepatomas (Baril *et al.*, 1970). The activity associated with ribosomes preferred native DNA as primer, while the enzyme associated with the smooth membrane fraction showed a marked preference for denatured DNA as primer.

This paper reports the purification and properties of the deoxyribonucleotide-polymerizing activities associated with the ribosomes and the smooth membrane fraction of normal rat liver. The properties of the enzymes are compared to those of the extractable DNA-synthesizing activities from purified rat liver mitochondria and nuclei.

Experimental Section

Materials

Nonradioactive and radioactive deoxyribonucleotides were purchased from Schwarz BioResearch, Inc. Crystallized pancreatic ribonuclease and deoxyribonuclease and snake venom phosphodiesterase were obtained from Worthington. Special enzyme grade ammonium sulfate and sucrose were obtained from Mann Research Laboratories. The poly-[d(A-T)] copolymer and poly(dA) homopolymer were purchased from Biopolymers Laboratory. Calf thymus DNA, poly(U), and RNA were obtained from Sigma. Rat liver nDNA was extracted and purified according to Kidson (1967) from nuclei isolated by the method of Whittle *et al.* (1968). ^{3}H-labeled DNA was prepared from purified nuclei of 24-hr-regenerating rat liver as described by Ove *et al.* (1969a). Rat liver mtDNA was obtained by the procedure of Kalf and Grece (1967). DNA in 0.01 M Tris-HCl (pH 7.5) was denatured by heating for 10 min in a boiling-water bath followed by quick cooling in an ice-water bath. "Activated" calf thymus DNA was prepared by digestion with pancreatic DNase according to the procedure of Aposhian and Kornberg (1962). DEAE-cellulose was obtained from H. Reeve Angel and Co. (DE-52) and phosphocellulose was purchased from Sigma. The ion exchangers were precycled according to the manufacturer's directions. All other reagents were of the highest purity obtainable from commercial sources.

Animals and Tissues. Male rats (Holtzman, Madison, Wis.) weighing 150–200 g were used throughout this investigation. The animals were allowed to feed *ad libitum*, but were fasted overnight before sacrifice to deplete the level of liver glycogen. Rats were killed by cervical dislocation and the livers were removed, perfused with ice-cold 1% saline, and used for the fractionation of subcellular components as described under methods.

Methods

Tissue Fractionation. Perfused livers were washed in ice-cold 0.25 M sucrose–buffer A (50 mM Tris-HCl, pH 7.6 at 25°, 25 mM KCl, 5 mM magnesium acetate, and 1 mM dithiothreitol), blotted, weighed, minced, and then homogenized in four volumes of 0.25 M sucrose–buffer A using ten strokes with a loose-fitting Dounce homogenizer. Subcellular fractions were isolated from the homogenate as outlined in Figure 1. A crude nuclear fraction was isolated by centrifugation of the homogenate at 600*g* for 10 min. The sediment was resuspended in nine volumes of 0.25 M sucrose–buffer A and filtered through four layers of cheesecloth. Nuclei were purified from the filtered suspension by the procedure of Blobel and Potter (1967) as modified by Whittle *et al.* (1968). The pellets of purified nuclei were stored at −20°.

The supernatant solution from sedimentation of the crude nuclear fraction (S-1) was centrifuged at 10,000*g* for 15 min to sediment mitochondria. The mitochondrial pellet was washed three times in 0.25 M sucrose–buffer A plus 1 mM EDTA. This washed mitochondrial pellet was resuspended in 0.25 M sucrose–buffer A plus 1 mM EDTA and mitochondria were isolated on a discontinuous gradient of 0.5–2.0 M sucrose in buffer A plus 1 mM EDTA according to Kalf and Grece (1967).

Microsomes were isolated by centrifugation of the post-mitochondrial supernatant solution (S-2) at 105,000*g* for 1 hr. The postmicrosomal supernatant solution (S-3) was centrifuged at 78,000*g* for 15 hr in order to completely sediment ribosomes, membranes, and other sedimentable materials. The supernatant solution (S-4) was decanted and saved. The pellet (P-4) was resuspended in 1.3 M sucrose–buffer A by ten strokes with a loose-fitting Dounce homogenizer. Free ribosomes, membranes, and soluble materials were separated on a discontinuous sucrose gradient (0.8–2.0 M sucrose in buffer A) by centrifugation at 30,000 rpm (Spinco 30 rotor) for 24 hr as described previously (Baril *et al.*, 1970).

Assay of DNA Polymerase. Unless otherwise indicated the standard assay contained the following in a total volume of 0.5 ml; 0.02 μmole each of TTP, dGTP, dCTP, dATP plus [^{3}H]TTP (final specific activity of 0.5 mCi/μmole in the assay for mtDNA polymerase and 0.025 mCi/μmole in all other assays), 8 μmoles of $MgCl_2$, 20 μmoles of glycine (pH 8.0 for membrane and mitochondria polymerase and pH 9.0 for the nuclear and ribosomal enzyme), 0.5 μmole of dithiothreitol, 100 μg of calf thymus DNA (native, heat denatured, or "activated" by pancreatic DNase), and 0.01–0.1 mg of protein of each fraction assayed. The reaction is linear for at least 2 hr using quantities up to 200 μg of crude protein or up to 100 μg of purified fractions. The incubation was at 37° for 1 hr and the reaction was terminated by addition of: 100 μg of bovine serum albumin as carrier, 0.1 ml of 0.1 M sodium pyrophosphate, and 5 ml of cold 10% trichloroacetic acid. After 30 min at 4°, the tubes were centrifuged at 2000 rpm for 10 min and the supernatant solution discarded. The precipitate was dissolved in 0.5 ml of normal NaOH and 5 ml of cold 10% trichloroacetic acid was added to each tube. After 30 min at 4°, the precipitates were collected on glass fiber filters (Whatman GF/C) that had been prewashed with 0.1 M sodium pyrophosphate. After sample application the filters were washed with 5% trichloroacetic acid, followed by 95% ethanol, and dried under a heating lamp. Radioactivity was measured in 10 ml of Spectrofluor scintillator (4 g of 2,5-diphenyloxazole and 50 mg of 1,4-[bis(5-phenyloxazol-1-)]benzene per l. of toluene). The counts were not corrected for self-absorption. Terminal deoxyribonucleotidyl transferase activity was measured under identical conditions but the unlabeled deoxyribonucleoside triphosphates were omitted from the incubation mixture. One unit of activity is equal to 1 mμmole of labeled deoxyribonucleoside triphosphate incorporated into acid-insoluble product per hr at 37°. Specific activity is expressed as units per milligram of protein.

Assay of Other Enzymes. Deoxyribonuclease activity was assayed by the method of Geiduschek and Daniels (1965) using ^{3}H-labeled DNA (50 cpm/μg DNA) from regenerating rat liver. Incubation conditions for the assay of native DNA preferring endonuclease were according to O'Connor (1969) and for denatured DNA preferring endonuclease were as described by Curtis and Smellie (1966). The incubation was from 1 to 24 hr at 37°. Nonspecific phosphodiesterase was assayed by the method of Bernardi and Griffe (1964) with cal-

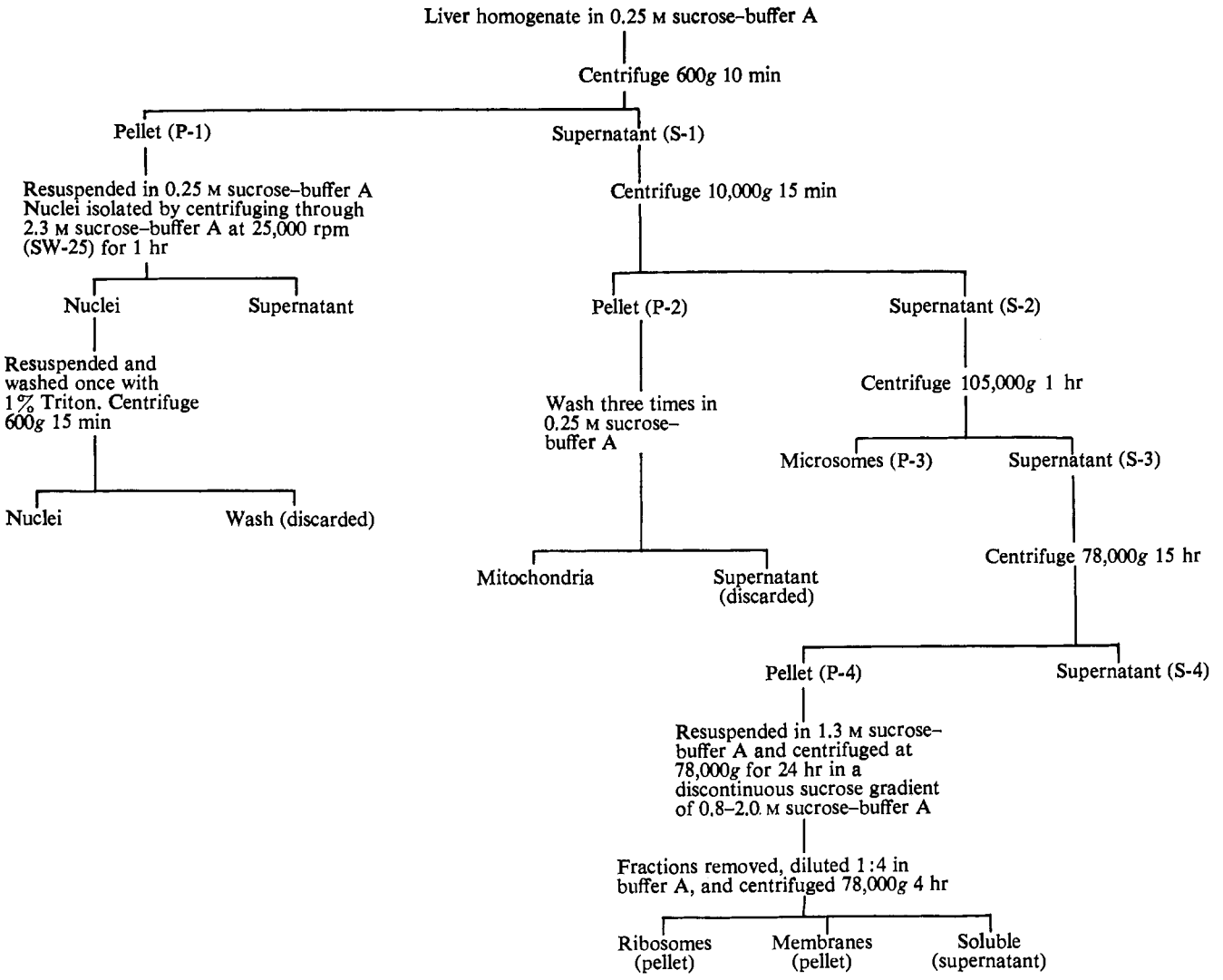

FIGURE 1: Schematic representation of procedures used for subcellular fractionation of rat liver. All manipulations were carried out at 4°. See text for detailed description.

cium bis(*p*-nitrophenyl)phosphate (Sigma) and specific phosphodiesterases were measured according to Razzell and Khorana (1959). Succinic dehydrogenase (EC 1.3.99.1) was assayed spectrophotometrically by the method of Slater and Bonner (1952) or Ziegler and Reiske (1967). Monoamine oxidase (EC 1.4.3.4) was assayed as described by Tabor *et al.* (1954) and glucose 6-phosphatase by the method of Hers *et al.* (1951).

Chemical Measurements. DNA was determined by the Burton (1962) modification of the diphenylamine reaction, or for purified DNA samples by measurement of absorption at 260 mμ. RNA was measured by the procedure of Fleck and Munro (1962) using $E^{1\%}_{1\,cm} = 312$. Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin (Sigma) as standard.

Results

Distribution of DNA Polymerase Activity in Subcellular Fractions. Rat liver homogenates were fractionated by differential centrifugation as shown in Figure 1. Purified nuclei were treated according to Howk and Wang (1969) for solubilization of the extractable nDNA polymerase, and the mitochondrial pellet (P-2) was extracted for mtDNA polymerase by the method of Meyer and Simpson (1968). Other particulate fractions were resuspended by brief homogenization in buffer A. The specific activities of DNA polymerase measured in the various subfractions are shown in Table I. A valid estimation of the per cent of total DNA polymerase with each of these fractions cannot be made because of differences in extraction conditions, recovery, and DNase distribution. The nuclear fraction exhibited a high specific activity of a DNA polymerase that preferred native DNA primer, confirming previous observations (Howk and Wang, 1969; Meyer and Simpson, 1968). The specific activity in the mitochondrial fraction was extremely low, but significant polymerase activity was found in the microsome fraction. Considerable activity was also observed in the postmicrosomal supernatant solution, as noted by others (Bollum and Potter, 1958; Davidson *et al.*, 1958; Iwamura *et al.*, 1969; Ove *et al.*, 1969b, 1970).

We have previously reported (Baril *et al.*, 1970) that most of the DNA polymerase activity in the postmicrosomal supernatant solution can be sedimented by centrifugation at 78,000 *g* for 15 hr. Centrifugation of the resulting pellet (P-4) on a discontinuous sucrose gradient showed that most of the DNA polymerase of P-4 is associated with the free ribosome and the membrane fractions. Electron microscopic analysis indicated

TABLE I: Comparison of DNA Polymerase Activity in Subcellular Fractions.

Fraction	Total Units[b]		Sp Act. (Units/mg)	
	N[c]	D[c]	N[c]	D[c]
Nuclear extract (P-1)	91 (12)	69 (8)	1.44 (0.3)	1.1 (0.2)
Mitochondrial extract (P-2)	22	4	0.05	0.01
Microsomal extract (P-3)	133 (19)	55 (10)	1.70 (0.2)	0.7 (0.1)
Postmicrosomal supernatant (S-3)	492 (97)	350 (58)	0.83 (0.2)	0.58 (0.1)
Centrifugation of S-3				
Pellet (P-4)	366 (52)	438 (67)	1.59 (0.3)	1.90 (0.3)
Supernatant (S-4)	56 (15)	0	0.07 (0.2)	0
Sucrose-Gradient Fractions of Pellet (P-4)				
Nonsedimentable	0	0	0	0
Membranes	198 (33)	460 (86)	1.22 (0.2)	2.84 (0.4)
Free ribosomes	102 (21)	22 (4)	1.23 (0.2)	0.26 (0.06)

[a] Fractionations based on 50 g of liver. The standard assay containing 0.1 mg of native or heat-denatured calf thymus DNA as primer was used to assay all fractions. [b] 1 unit = 1 mμmole of [^{3}H]TMP incorporated per hr. [c] N = native and D = heat-denatured calf thymus DNA. Values = mean plus and/or minus standard error for from 5 to 10 determinations except for the mitochondria which represents the average of 3 determinations with 10–15% variation.

that the free ribosome and membrane fractions were free from contamination by whole cells, nuclei, or mitochondria and that the membrane fraction was composed of smooth elements (Baril *et al.*, 1970). The DNA polymerase activity associated with the membranes preferred heat-denatured DNA as primer, whereas the enzyme in the free ribosome fraction markedly preferred native DNA as primer.

Elimination of Mg^{2+} from the isolation medium had no effect on the specific or total activity of DNA polymerase with either the free ribosome or membrane fraction. However, addition of 5 mM EDTA to S-1 (see Figure 1) increased the specific and total activity of DNA polymerase in the free ribosome fraction, as well as the amount of free ribosome fraction obtained after sucrose gradient centrifugation of pellet P-4, but had little effect on the yield of membrane-associated DNA polymerase activity. Variations in the method and extent of homogenization did not significantly alter the specific activity of membrane- or ribosome-associated enzymes, although they did alter the total yield of these fractions (E. F. Baril *et al.*, 1970, unpublished data). These and other observations lead us to believe we are not dealing with an artifact, although we have no concrete evidence to substantiate that belief.

TABLE II: Purification of DNA Polymerase from Nuclei.[a]

Fraction	Total Protein (mg)	Total Units	Sp Act. (Units/mg)	Yield (%)
Nuclear extract (30,000*g* supernatant)	49	58.0	1.19	100
Ammonium sulfate	31	43.7	1.42	75
DEAE-cellulose	11	25.2	2.32	43
Phosphocellulose	1	10.2	10.20	17.8

[a] The standard assay containing 0.1 mg of "activated" calf thymus DNA as primer was used to assay all fractions.

After sucrose gradient centrifugation low but significant activity was observed in the nonsedimentable fraction using native DNA as primer. Activity was absent from this fraction when KCl was eliminated from the buffer. It is believed to appear in the soluble fraction after dissociation from microsomes by KCl. The fact that the nonsedimentable activity prefers native DNA primer may indicate that it is dissociated by KCl from ribosomes and not from the membrane fraction.

Properties of P-4 Subfractions. The postmicrosomal pellet (P-4) had no detectable activity of the mitochondrial enzymes, succinic dehydrogenase and monoamine oxidase. It did contain glucose 6-phosphatase activity, 112 mμmoles of P_i/min per mg, indicating the presence of endoplasmic reticulum in this fraction.

The free ribosomes obtained by sucrose gradient centrifugation of P-4 had an RNA/protein of 0.7 and the smooth membrane fraction a ratio of 0.06. There was no detectable DNA in either the free ribosome or membrane fraction derived from P-4.

Purification of Nuclear Polymerase. The purification procedure is summarized in Table II.

PREPARATION OF EXTRACT. Rat liver nuclei were isolated and purified by the procedure of Whittle *et al.* (1968) as described in Methods. The pellets of purified nuceli were resuspended in about five volumes of buffer A plus 1.0 M KCl and the mixture was stirred at 0–4° for 1 hr. The material was then dialyzed overnight against 500 volumes of buffer A, 20% ethylene glycol. The dialyzed extract was centrifuged at 30,000*g* for 30 min and the supernatant decanted and saved.

AMMONIUM SULFATE FRACTIONATION. Solid ammonium sulfate was slowly added to the 30,000*g* supernatant to a concentration of 25% saturation with constant stirring at 0–4° and subsequent stirring for 30 min. The mixture was centrifuged at 10,000*g* for 10 min and the supernatant solution decanted. Additional ammonium sulfate was added to the supernatant solution to give 40% saturation and after stirring for 30 min the mixture was centrifuged at 10,000*g* for 10 min. The supernatant solution was decanted. The 25–40% $(NH_4)_2SO_4$ precipitate usually contained negligible DNA polymerase

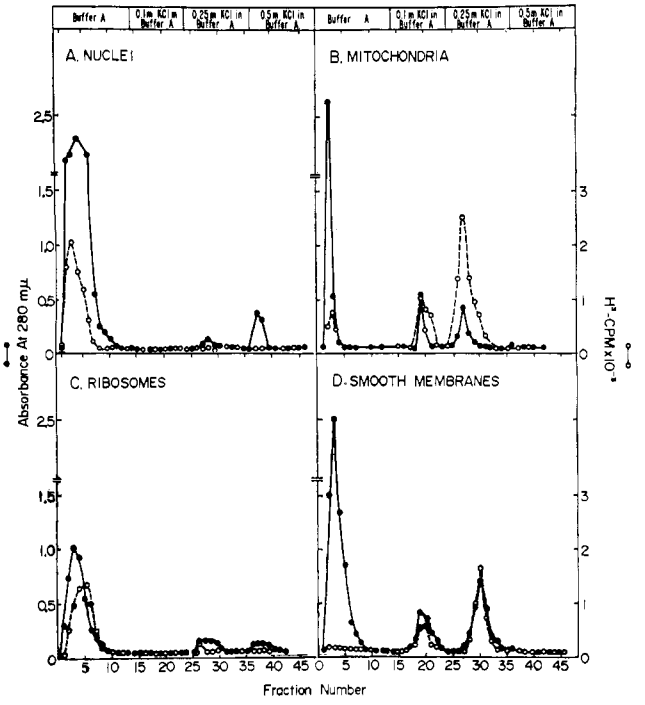

FIGURE 2: Elution profiles from DEAE-cellulose chromatography of $(NH_4)_2SO_4$ fractions of rat liver nuclear, mitochondrial, ribosomal, and smooth membrane fractions. (A) 40–65% $(NH_4)_2SO_4$ fraction of the KCl extract of purified nuclei. (B) 40–65% $(NH_4)_2SO_4$ fraction of three-times-washed mitochondrial pellet. (C) 40–65% $(NH_4)_2SO_4$ fraction from 0.2 M KCl extract of ribosomes. (D) 25–40% $(NH_4)_2SO_4$ fraction from smooth membrane of cytoplasm. A 1.2 × 18 cm column was used in each case and 3-ml fractions collected. The absorbance at 280 mμ (●) was measured and 0.1-ml aliquots of the dialyzed fractions were used to assay DNA polymerase activity (○) with [^{3}H]TTP and "activated" calf thymus DNA primer as described in the text.

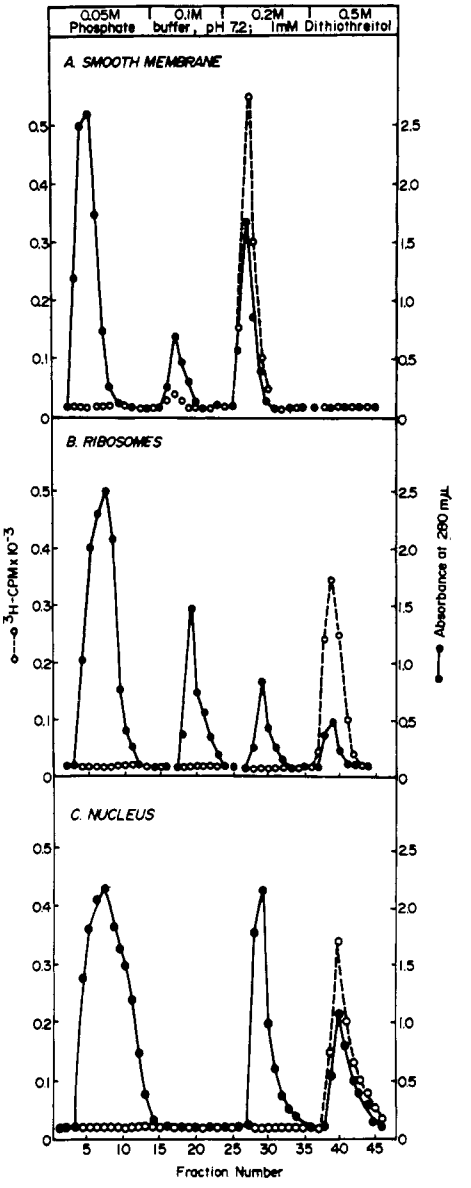

FIGURE 3: Phosphocellulose chromatography of the pooled DEAE-cellulose fractions with highest DNA polymerase activity. (A) Smooth membrane: tubes 29–32 from DEAE-cellulose were pooled, dialyzed, and the dialyzed fraction chromatographed on a 0.9 × 10 cm column of phosphocellulose, as described in the text. (B) Ribosomes: tubes 2–7 from DEAE-cellulose were pooled, dialyzed, and chromatographed on phosphocellulose as in part A. (C) Nucleus: tubes 2–6 from DEAE-cellulose were pooled, dialyzed, and chromatographed on phosphocellulose as in parts A and B. Fractions of 3 ml were collected and the absorbance at 280 mμ was measured (●). After dialysis against buffer A, 20% ethylene glycol, 0.2-ml aliquots were assayed for DNA polymerase activity (○) using "activated" calf thymus DNA primer, as described in text.

activity and was discarded. Ammonium sulfate was added to the supernatant solution to 65% saturation after removal of the 25–40% $(NH_4)_2SO_4$ precipitate. After stirring, the mixture was centrifuged and the supernatant solution decanted and discarded. The precipitate was suspended in a minimal volume of buffer A, 20% ethylene glycol and dialyzed overnight against 4 l. of the same buffer.

DEAE-CELLULOSE CHROMATOGRAPHY. After dialysis of the 40–65% $(NH_4)_2SO_4$ fraction, 2–4 ml (9 mg/ml) was applied to a DEAE-cellulose column (1.2 × 18 cm) previously equilibrated with buffer A. The column was washed with buffer A, 20% ethylene glycol and then eluted with stepwise increases in potassium chloride concentration from 0.1 to 0.4 M in buffer A, 20% ethylene glycol. The absorbance at 280 mμ was monitored and 3-ml fractions were collected at a flow rate of about 0.5 ml/min. DNA polymerase activity was not bound to DEAE-cellulose under these conditions and all of the activity appeared in the column wash (Figure 2A). The contents of tubes with the highest polymerase activity were pooled and dialyzed overnight against several volumes of 0.05 M potassium phosphate buffer (pH 6.8), 1 mM dithiothreitol, and 20% ethylene glycol.

PHOSPHOCELLULOSE CHROMATOGRAPHY. The dialyzed fraction of DNA polymerase from DEAE-cellulose, usually 9–10 ml (2 mg/ml), was applied to a phosphocellulose column (0.9 × 10 cm) that was equilibrated with 0.05 M potassium phosphate buffer (pH 6.8), 1 mM dithiothreitol, and 20% ethylene glycol. The column was washed with the same buffer and then eluted by stepwise increases in potassium phosphate concentration from 0.1 to 0.5 M potassium phosphate buffer (pH 7.2), containing 1 mM dithiothreitol and 20% ethylene glycol. The collected fractions were dialyzed overnight against 500 volumes of buffer A containing 20% ethylene glycol. DNA polymerase activity was firmly bound to phosphocellulose and eluted at 0.5 M potassium phosphate concentration (Figure 3). The fractions were dialyzed overnight against buffer A, 20% ethylene glycol. Fractions having the highest specific activity were pooled and kept at 4°.

TABLE III: Purification of DNA Polymerase from Ribosomes.[a]

Fraction	Total Protein (mg)	Total Units	Sp Act. (Units/mg)	Yield (%)
Free ribosomes	463	324	0.7	100
0.2 M KCl extract	43	292	6.8	90
Ammonium sulfate	28	301	10.7	93
DEAE-cellulose	19	306	16.1	94
Phosphocellulose	0.4	143	358.0	44

[a] The assay was the same as for Table II.

TABLE IV: KCl Solubilization of Microsomal DNA Polymerase.[a]

KCl Concn (M)	DNA Polymerase Activity: Pellet Total Units[b]	Pellet % [c]	Supernatant Total Units[b]	Sp Act.[d]	Supernatant % [c]
Control	5.9	96	0.3	0.24	4
0.05	4.7	77	1.4	0.66	23
0.15	2.8	46	3.3	1.52	54
0.20	1.9	30	4.3	6.61	70
0.60	1.9	31	4.2	3.87	69

[a] Potassium chloride was added at the indicated concentrations to aliquots of microsomes suspended in buffer A; the control was an aliquot in buffer A only. The material was kept at 4° for 1 hr and centrifuged at 105,000*g* for 4.5 hr. The supernatant solution was decanted, pellets were resuspended in equal volumes of buffer A, and both were dialyzed overnight against buffer A. The standard assay system was used with native calf thymus DNA primer. [b] one unit = 1 nmole of [^{3}H]TMP incorporated per hr. [c] Per cent of activity in resuspended microsome pellet. [d] One unit per mg of protein.

Purification of Mitochondrial DNA Polymerase. Washed mitochondrial pellets (see Figure 1) or mitochondria isolated by sucrose gradient centrifugation according to Kalf and Grece (1967) were resuspended in 50 mM Tris (pH 8.0 at 25°), 1.0 M KCl, 1 mM EDTA, and 1 mM dithiothreitol. The suspension was subjected to three cycles of freezing and subsequent thawing at 25°, and was then extracted with buffer A, 1.0 M KCl, and 1 mM EDTA with stirring for 4 hr at 4°. The extract was centrifuged at 30,000*g* for 30 min, the supernatant solution decanted and recentrifuged at 105,000*g* for 4 hr. The final supernatant solution was decanted and dialyzed overnight against 4 l. of 50 mM Tris (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol.

Solid ammonium sulfate was added to the dialyzed extract to 25% saturation with constant stirring at 4°, and the solution was stirred for an additional 30 min. After sedimentation of the precipitate the supernatant solution was decanted and the procedures were repeated for steps of 25–40 and 40–65% saturation of ammonium sulfate. The precipitates, 0–25, 25–40, and 40–65% $(NH_4)_2SO_4$ fractions were resuspended in minimal volumes of buffer A, 20% ethylene glycol. The ammonium sulfate fractions and the fraction soluble in 65% saturation of ammonium sulfate were dialyzed overnight against 4 l. of the same buffer. Most of the polymerase activity was present in the 40–65% $(NH_4)_2SO_4$ fraction. This fraction was then chromatographed on DEAE-cellulose under the conditions described for chromatography of the extract from nuclei.

Purification of Ribosome-Associated Polymerase. Fractions of free ribosomes or ribosome-containing material were used as starting material for purification of the ribosome-associated DNA polymerase with qualitatively similar results. The fractions used include: free ribosomes obtained by sucrose gradient fractionation of P-4, or by EDTA or Triton X-100 treatment of microsomes; isolated rough endoplasmic reticulum; the total microsome fraction (P-3). The highest yield of the enzyme was obtained when the whole microsome fraction was used as starting material for the purification (E. F. Baril *et al.*, 1970, unpublished data).

The purification procedure involves successive steps of KCl extraction, ammonium sulfate fractionation and chromatography on DEAE-cellulose and on phosphocellulose, as shown in Table III.

POTASSIUM CHLORIDE EXTRACTION. The free ribosomal or microsomal pellet was resuspended in ten volumes of 50 mM Tris-HCl (pH 7.6 at 20°), 200 mM KCl, 5 mM magnesium acetate, and 1 mM dithiothreitol by ten strokes with a loose-fitting Dounce homogenizer. After 1 hr at 4°, the suspension was centrifuged at 105,000*g* for 4.5 hr. The supernatant solution, the microsomal KCl extract, contains the DNA polymerase activity originally associated with the ribosomal or microsomal fraction. As shown in Table IV, 70% of the activity is present in the supernatant solution when resuspension and centrifugation is conducted in the presence of KCl concentrations of 0.2 M or greater. Replacement of KCl by NH_4Cl gives similar results.

The microsomal KCl extract was dialyzed overnight against 500 volumes of buffer A containing 20% (v/v) ethylene glycol. Ethylene glycol was found to stabilize the activity of the crude and purified enzyme fractions for at least 3 weeks at 4° and at least 6 months at −20°.

AMMONIUM SULFATE FRACTIONATION. To the dialyzed KCl extract was added solid ammonium sulfate to a concentration of 40% saturation with constant stirring at 4°. After stirring for 30 min, the mixture was centrifuged at 10,000*g* for 10 min. The precipitate was discarded and ammonium sulfate was added to the supernatant solution to 65% saturation. After stirring for 30 min and centrifugation, the precipitate was dissolved in 2–5 ml of buffer A. Ammonium sulfate was removed by overnight dialysis against 4 l. of buffer A, 20% ethylene glycol.

DEAE-CELLULOSE CHROMATOGRAPHY. The dialyzed 40–65% saturated ammonium sulfate fraction was chromatographed on a DEAE-cellulose column under the conditions previously described for purification of DNA polymerase from nuclei. As in the case of the nuclear preparation, all of the activity appeared in the column wash (Figure 2C). The column wash was pooled and dialyzed overnight against 500 volumes of 0.05 M potassium phosphate buffer (pH 6.8), 1 mM dithiothreitol, and 20% ethylene glycol.

PHOSPHOCELLULOSE CHROMATOGRAPHY. The dialyzed fraction from DEAE-cellulose was chromatographed on phospho-

cellulose under the conditions described for purification of DNA polymerase from nuclei. As shown in Figure 3, DNA polymerase activity from ribosomes eluted at the same potassium phosphate concentration, 0.5 M, as that from nuclei.

Purification of DNA Polymerase Associated with Smooth Membranes. The purification procedure is summarized in Table V.

AMMONIUM SULFATE FRACTIONATION. The smooth membrane fraction from sucrose gradient centrifugation of the P-4 pellet was resuspended in buffer A by brief homogenization. The protein concentration was adjusted to about 20 mg/ml by addition of buffer A. Solid ammonium sulfate was slowly added to a concentration of 25% saturation with continual stirring at 4° and with additional stirring for 30 min. The material was centrifuged at 10,000*g* for 10 min. The precipitate was discarded and ammonium sulfate was added to the supernatant solution to a concentration of 40% saturation. After stirring for 30 min the mixture was centrifuged and the supernatant solution was discarded. The precipitate was suspended in a minimal volume of buffer A containing 20% ethylene glycol and dialyzed overnight against 4 l. of the same buffer.

DEAE-CELLULOSE CHROMATOGRAPHY. The dialyzed ammonium sulfate fraction was chromatographed on DEAE-cellulose under the conditions described for purification of the nuclear polymerase. DNA polymerase activity was present in fractions eluting with 0.1 and 0.25 M KCl, although most of the activity eluted with 0.25 M KCl (Figure 2). The tubes containing DNA polymerase activity that had eluted with 0.25 M KCl were pooled and dialyzed overnight against 500 volumes of 0.05 M potassium phosphate buffer (pH 6.8), 1 mM dithiothreitol, and 20% ethylene glycol.

PHOSPHOCELLULOSE CHROMATOGRAPHY. The dialyzed fraction from DEAE-cellulose was chromatographed on phosphocellulose under the conditions described for the purification of the nuclear enzyme. The membrane-associated polymerase activity all eluted with 0.2 M potassium phosphate (Figure 3A). The fractions were dialyzed overnight against buffer A,20% ethylene glycol. Fractions having the highest activity were pooled and were kept at 4°.

COMPARISON OF POLYMERASES ELUTED FROM DEAE-CELLULOSE. A comparison of the elution profiles of DNA polymerase from the ammonium sulfate fractions of nuclei, mitochondria, ribosomes, and smooth membranes is shown in Figure 2A–D.

The enzymes from purified nuclei and ribosomes appear quite similar at this stage of the purification. Neither enzyme was bound to DEAE-cellulose and all of the activity appeared in the column wash (Figure 2A,C). The column washes also contained considerable nuclease activity. The DNA polymerases from both nuclei and ribosomes showed a marked preference for native DNA as primer. In the presence of only a single deoxyribonucleoside triphosphate the activities of both n- and rDNA polymerases were about one-half of those observed in the presence of all four deoxyribonucleoside triphosphates.

Chromatography of extracts of washed mitochondrial pellets (P-2) produced multiple peaks of polymerase activity (Figure 2B). Most of the activity eluted with 0.1 and 0.25 M KCl. The DNA polymerase activity from mitochondrial extracts remained low throughout purification and a 20-fold increase in the specific activity of labeled deoxyribonucleoside triphosphate was required in the assay for detection. The addition of ATP and an ATP-generating system or higher Mg^{2+} (Wintersberger and Wintersberger, 1970) and KCl (Meyer and Simpson, 1968) concentrations to the assay system failed to raise the activity from mitochondrial extracts to the level observed with extracts from nuclei, ribosomes, or membranes.

TABLE V: Purification of DNA Polymerase from Smooth Membranes.[a]

Fraction	Total Protein (mg)	Total Units	Sp Act. (Units/mg)	Yield (%)
Membranes from sucrose gradient	163	89.6	0.55	100
Ammonium sulfate	62	72.0	1.16	80
DEAE-cellulose	15	67.1	4.55	75
Phosphocellulose	1.2	46.2	40.00	52

[a] The assay procedure is given in Table II.

As shown in Figure 2D, the elution pattern of DNA polymerase from extracts of the smooth membrane fraction is similar to that observed for extracts of P-2, the mitochondrial pellet (Figure 2B). However, the polymerase activity in chromatographic fractions from smooth membranes was 15–20 times higher than that in the chromatographic fractions from mitochondria. Although the crude membrane-associated enzyme was active with denatured, and to a lesser extent with native DNA as primer, the fractions from DEAE-cellulose required activated DNA as primer for maximal activity. The change in primer preference is probably related, in part, to removal of nucleases. After chromatography on DEAE-cellulose nuclease activity appeared in the column wash while most of the polymerase activity eluted at 0.25 M KCl. Also, the addition of pancreatic DNase or small amounts of the column wash to the incubation mixture containing the purified enzyme markedly increased the activity with native, and to a lesser extent with denatured, DNA primers (E. F. Baril *et al.*, 1970, unpublished data).

The similar elution profiles of DNA polymerase from extracts of washed mitochondrial pellets and smooth membranes suggested possible crosscontamination of these fractions. Mitochondrial pellets (P-2) were further purified by sucrose gradient centrifugation according to Kalf and Grece (1967). The isolated mitochondria were lysed, extracted, and subjected to ammonium sulfate fractionation as described in Methods. The 40–65% $(NH_4)_2SO_4$ fraction was chromatographed on DEAE-cellulose under the conditions indicated in Figure 2 and the results are shown in Figure 4. All of the polymerase activity was eluted by 0.1 M KCl, in agreement with results previously reported by Meyer and Simpson (1968) for purification of rat liver mtDNA polymerase. The absence of polymerase activity in the column wash and in the fractions eluting with 0.25 M KCl suggests that the activity observed in these fractions when unpurified mitochondria were used (Figure 2B) was due to polymerase(s) associated with smooth membrane and/or nuclei and ribosomes contaminating P-2. A considerable portion of the endoplasmic reticulum of rat liver sediments with the mitochondrial (P-2) and crude nuclear (P-1) pellets, as demonstrated by Blobel and Potter (1967) and Loeb *et al.* (1967). In addition, upon rechromatography on DEAE-cellulose, the 0.25 M KCl polymerase fractions from smooth membranes eluted at the same KCl concentration (0.25 M). The DNA polymerase activity from mitochondria that elutes with the 0.1 M KCl fraction (Figure 4) has

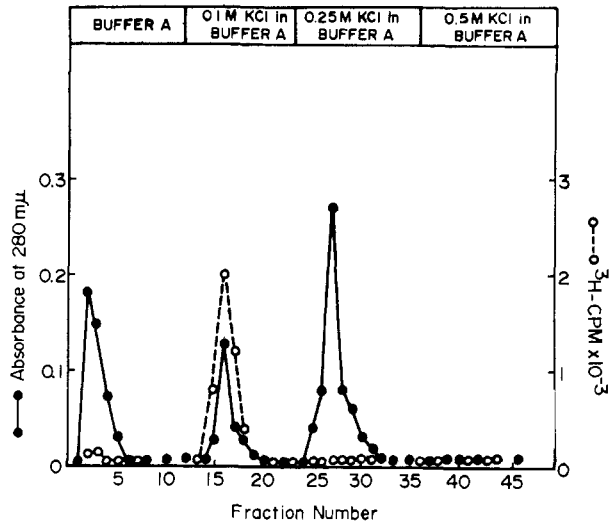

FIGURE 4: Chromatography on DEAE-cellulose of the 40–65% $(NH_4)_2SO_4$ fraction from rat liver mitochondria that had been further purified by sucrose gradient centrifugation according to Kalf and Grece (1967). A 1 × 12 cm column was used and 1 ml (5 mg/ml) of sample was applied to the top of the column. Fractions of 2 ml were collected and the absorbance at 280 mμ (●) was measured. After dialysis, 0.2-ml aliquots of fractions were used to assay for DNA polymerase activity (○) using "activated" calf thymus DNA primer as described in the text.

low activity relative to the activity from nuclei, ribosomes, or smooth membranes. Activated DNA was more efficient as primer than native or denatured calf thymus, or rat liver, n-, or mtDNA. The low activity with rat liver mtDNA contrasts with the results of other investigators (Kalf and Ch'ih, 1968; Meyer and Simpson, 1968) and the reason for it is unknown at this time. The polymerase activity had an optimum pH, 7.5, and optimum Mg^{2+} concentration, 12 mM, similar to reported values for rat liver mtDNA polymerase (Kalf and Ch'ih, 1968; Meyer and Simpson, 1968). The activity of the DEAE-cellulose fraction was not increased by raising salt concentrations to 0.15 M KCl, in contrast to the findings of Meyer and Simpson (1968) but in agreement with those of Wintersberger and Wintersberger (1970) for yeast mtDNA polymerase.

Comparison of Elution Profiles on Phosphocellulose. The column wash from DEAE-cellulose chromatography of the nuclear or ribosomal extract and the 0.25 M KCl fraction from DEAE-cellulose chromatography of the smooth membrane extract were chromatographed on phosphocellulose and the results are shown in Figure 3. The polymerase activity from smooth membranes elutes at a lower phosphate concentration (0.2 M) than does that from the nucleus or ribosomes (0.5 M).

The ribosome-associated polymerase at this stage is purified about 500-fold over the activity in free ribosomes (Table III) and about 1500-fold over the activity in S-2. The membrane-associated polymerase is purified about 80-fold over the activity in isolated membranes (Table V) and more than 300-fold over the activity in S-2. Attempts at further purification by chromatography on hydroxylapatite or gel filtration on Sephadex G-200 led to considerable losses in total activity with minimal increases in the specific activity of the polymerases.

Properties of Purified Polymerases. EFFECT OF pH. The ribosomal and nuclear polymerases behave similarly with respect to pH, but the membrane polymerase has different characteristics.

Ribosome-associated DNA polymerase has its maximal activity at pH 9.0 in glycine buffer using activated calf thymus DNA as primer. The activities at pH 8.0 and 10.0 are about 60 and 50%, respectively of the optimal value. Purified DNA polymerase from highly purified rat liver nuclei has its maximal activity at pH 9.0, confirming results of Howk and Wang (1969) with DNA polymerase from rat liver nuclei.

The membrane-associated DNA polymerase has maximal activity at pH 8.0 in Tris-HCl buffer using activated calf thymus DNA as primer. The activity declines rapidly to about 35% of the maximum at pH 7.5 and about 50% at pH 9.0. This differs from the rat liver mitochondrial polymerase reported by Kalf and Ch'ih (1968) to have a pH optimum of 7.5 in Tris-HCl and 33% of maximal activity at pH 8.0.

REQUIREMENT FOR DIVALENT METALS. The purified membrane, nuclear, and ribosome-associated polymerases have an absolute requirement for Mg^{2+} (Table VI). Membrane-associated polymerase requires 7–10 mM Mg^{2+} for maximal activity while the ribosomal, as well as nDNA polymerase, has maximal activity in the presence of 10–15 mM Mg^{2+}. Magnesium could not be replaced by Mn^{2+}. Less than 25% of the maximal activity obtained with Mg^{2+} was observed when Mn^{2+} was used in the assay.

PRIMER REQUIREMENTS. There is no incorporation with the purified nuclear, ribosome-, or membrane-associated DNA polymerases in the absence of added DNA primer (Table VI). The activities with native or denatured DNA as primer progressively declined during the purification while the activities with activated DNA increased. The purified polymerases require activated DNA for maximal activity although the

TABLE VI: Basic Requirements for Deoxyribonucleotide Incorporation into DNA.[a]

Reaction Mixture	[^{3}H]TMP Incorpd with (pmoles/hr)		
	Nuclear Enzyme	Ribosomal Enzyme	Membrane Enzyme
Complete: added primer			
Native calf thymus DNA	583	449	50
Native calf thymus DNA + DNase I	1467	1681	970
Denatured calf thymus DNA	194	100	38
"Activated" calf thymus DNA	2668	2800	1907
Rat liver nDNA	736	622	63
Rat liver mtDNA	31	38	12
None	0	0	0
RNA (rat liver)	0	0	0
Poly(U)	0	0	0
Complete: activated calf thymus DNA as primer	2668	2800	1907
$-Mg^{2+}$	0	0	0
−dCTP, dGTP	772	979	173
−dCTP, dATP	466	583	15
+PMB (3×10^{-4} M)	2541	2268	26

[a] The standard assay was used with indicated modifications. 10 μg of the respective phosphocellulose fractions was used for enzymes.

purified nuclear and ribosomal polymerase retains some activity with native DNA primer. Activity of the polymerases with native DNA primer is increased by addition of small amounts of pancreatic DNase (0.01 μg) to the incubation mixture, suggesting that the decline in activity with purification is due to removal of nucleases rather than to alterations of the enzymes. No incorporation was observed with the nuclear, ribosome-, or membrane-associated polymerases using poly(U) or RNA as primer.

REQUIREMENTS FOR DEOXYRIBONUCLEOSIDE TRIPHOSPHATES. All four deoxyribonucleoside triphosphates are required for maximal activity of the nuclear, ribosome-, and membrane-associated DNA polymerases (Table VI). In the absence of two deoxyribonucleoside triphosphates the activity of the membrane polymerase is only 10% of that with the complete system, and there is essentially no activity in the presence of only a single deoxyribonucleoside triphosphate ([^{3}H]TTP). This indicates that the purified membrane-associated DNA polymerase is essentially free of terminal transferase activity (Bollum *et al.*, 1964; Krakow *et al.*, 1961).

However, the nuclear and ribosome-associated DNA polymerase has significant activity (35% of maximum) in the presence of two deoxyribonucleoside triphosphates. The activity with a single deoxyribonucleoside triphosphate is even higher with less purified fractions of the enzyme (over 50% of maximum). These data may indicate that the ribosome-associated enzyme is composed mainly of terminal transferase. However, the increased activity in the presence of all four deoxyribonucleoside triphosphates and the lack of incorporation of ribonucleoside triphosphates (Table VI) are not compatible with the properties of terminal transferase that have been reported (Bollum *et al.*, 1964; Krakow *et al.*, 1961; Gottesman and Canellakis, 1966; Wang, 1968). A high rate of incorporation with fewer than four deoxyribonucleoside triphosphates has previously been observed with mammalian DNA polymerase preparations (Howk and Wang, 1969; Greene and Korn, 1970). Studies with DNA polymerase from KB cells have shown that the incorporation does not occur through the provision of the missing deoxyribonucleoside triphosphates by a pyrophosphorolysis reaction or by the combined action of exonuclease and nucleoside diphosphokinase activity in the enzyme preparation (Greene and Korn, 1970).

INHIBITION BY PMB. The purified membrane-associated DNA polymerase activity is sensitive to inhibition by *p*-hydroxymercuribenzoate while the nuclear and ribosome-associated polymerase activity is only slightly lowered in the presence of PMB. As shown in Table VI, a concentration of PMB of 3×10^{-4} M, in the presence of dithiothreitol, resulted in over 85% inhibition of the membrane-associated polymerase activity. The purified nuclear and ribosome-associated polymerase activity is inhibited less than 20% at this concentration of PMB. Increasing the PMB concentration to 6×10^{-4} M completely inhibits the membrane-associated DNA polymerase activity and results in less than 30% inhibition of the purified nuclear and ribosome-associated DNA polymerase activity.

LOCATION OF [^{3}H]TMP INCORPORATED BY RIBOSOMAL POLYMERASE. The location at which incorporation into DNA occurs in the presence of only a single deoxyribonucleoside triphosphate was determined by studying the kinetics of release of acid-soluble radioactivity from the product by snake venom phosphodiesterase. Values are expressed as per cent of the acid-soluble deoxyribonucleotides released during the total hydrolysis of the product by snake venom phosphodiesterase according to Adler *et al.* (1958). The percentages of acid-soluble radioactivity and of optical density at 260 mμ that were released at different times of a 180-min incubation are given in Table VII. During the initial 15-min incubation, 67% of the incorporated [^{3}H]TMP was released, although less than 5% of the total deoxyribonucleotides were released during that time. After 3-hr incubation with snake venom phosphodiesterase 76% of the incorporated radioactivity had been released, but only 20% of the polymer had been hydrolyzed. These data indicate that in the presence of a single deoxyribonucleoside triphosphate incorporation into DNA by the ribosomal enzyme is limited to sites at or near the deoxyribonucleoside ends of the preexisting DNA chains.

TABLE VII: Action of Venom Phosphodiesterase on Product DNA.[a]

Incubn Time (min)	Radioactivity[b] Released (%)	OD Released (%)[c]
15	67	4
30	68	5.3
60	70	10.0
120	71	15
180	76	20

[a] Purified native calf thymus DNA was incubated for 1 hr with 10 μg of purified ribosomal polymerase in the presence of [^{3}H]TTP only, as described in Methods. The labeled DNA was isolated and 0.7 mg (1.12 OD_{260}; 2700 cpm) was incubated with 25 μg of snake venom phosphodiesterase for the indicated times. The radioactivity and OD_{260} rendered acid soluble were determined. [b] Per cent of total acid soluble counts after 24-hr incubation (2050 cpm). [c] Per cent of total acid soluble after 24-hr incubation (0.8 optical density unit).

Template Specificity of Nuclear, Ribosomal, and Membrane Polymerases. Base pairing with the template is required for incorporation of deoxyribonucleoside triphosphates into an acid-insoluble product by the nuclear, ribosomal, and membrane polymerases, as shown in Table VIII. Incorporation does not occur to any marked extent by the noncomplementary limited terminal addition reaction (Bollum *et al.*, 1964; Krakow *et al.*, 1961). As previously discussed, significant incorporation occurred with the nuclear and ribosomal polymerase with native calf thymus DNA as primer in the presence of a single deoxyribonucleoside triphosphate. The incorporation of [^{3}H]TTP was increased by the presence of a second deoxyribonucleoside triphosphate, and when the second deoxyribonucleoside triphosphate was dATP the incorporation of [^{3}H]TTP represented about 30% of that obtained when all four deoxyribonucleoside triphosphates were present. With the homodeoxyribopolymer, poly(dA), as primer the nuclear, ribosomal, and membrane polymerases promoted the incorporation of the complementary ([^{3}H]TTP) but not the homologous ([^{3}H]dATP) deoxyribonucleoside triphosphate into an acid-insoluble product. With the copolymer poly[d(A-T)] as primer, incorporation of [^{3}H]TTP occurred in the presence of dATP, but there was little incorporation in the absence of dATP or in the presence of dCTP. There was no incorporation of [^{3}H]dCTP. These results are not consistent with the presence of significant

TABLE VIII: Template Specificity of DNA Polymerases.[a]

Template	Deoxyribonucleotides Added	[^{3}H]Deoxyribonucleotides Incorpd with (pmoles/hr)		
		Nuclear Enzyme	Ribosomal Enzyme	Membrane Enzyme
Calf thymus DNA (native)	[^{3}H]TTP	179	128	0
	[^{3}H]TTP, dATP	248	316	<5
	[^{3}H]TTP, dCTP	203	186	0
	[^{3}H]TTP, dGTP	293	192	<5
	[^{3}H]TTP, dATP, dCTP, dGTP	1365	1035	42
Poly(dA)	[^{3}H]TTP	384	448	339
	[^{3}H]dATP	0	0	0
Poly[d(A-T)]	[^{3}H]TTP, dATP	165	174	76
	[^{3}H]TTP, dCTP	17	25	<5
	[^{3}H]dCTP	0	0	0

[a] The standard assay was used except for the changes in template and deoxyribonucleoside triphosphates as indicated and the incubation was for 2 hr at 37°. The amount of templates used was poly(dA), 11 μg; poly[d(A-T)], 18 μg; calf thymus DNA (native), 100 μg. The enzymes were 10 μg of the respective phosphocellulose fractions.

terminal transferase activity with the purified nuclear, ribosomal, and membrane polymerases.

NUCLEASE ACTIVITY. The purified membrane-associated polymerase is free from exonuclease or endonuclease activity (Table IX). Incubation of the enzyme with native or heat-denatured ^{3}H-labeled rat liver nuclear DNA (prepared as described in the Experimental Section) under conditions for assay of native (O'Connor, 1969) and denatured DNA (Curtis and Smellie, 1966) preferring DNase activities of rat liver did not result in loss of label even after 24-hr incubation at 37°. The purified membrane-associated polymerase is also free from specific and nonspecific phosphodiesterase activity.

TABLE IX: Deoxyribonuclease Activity in Purified DNA Polymerase Fractions.[a]

Enzyme Fraction	% Loss of ^{3}H Label after Incubn for			
	1 hr		24 hr	
	N[b]	D[b]	N[b]	D[b]
Membrane polymerase	0	0	0	0
Ribosomal polymerase	0	0	0	37
Nuclear polymerase	0	0	0	55

[a] DNase activity was assayed by the method of Geiduschek and Daniels (1965) using ^{3}H-labeled regenerating rat liver DNA (50 cpm/μg) as substrate. Incubation conditions for assaying native preferring DNase activity were according to O'Connor (1969) and for denatured preferring DNase activity as described by Curtis and Smellie (1966). The incubation mixtures were in a total volume of 0.5 ml and contained 100 μg (5000 cpm) of labeled DNA, native or heat denatured, and 10 μg of the phosphocellulose fraction of purified nuclear, membrane or ribosome-associated DNA polymerase. Incubation was at 37° for 1 or 24 hr. [b] N = native, D = heat-denatured ^{3}H-labeled DNA.

Incubation of purified nuclear and ribosomal polymerases under the same conditions shows that both enzyme preparations do contain nuclease activity that acts with denatured DNA. Although there is no detectable loss of label from denatured DNA after 1 hr of incubation at 37°, approximately 55 and 37% loss of radioactivity was observed after 24-hr incubation with the nuclear and ribosomal polymerase preparations, respectively. Gel filtration of the digest on Sephadex G-100 indicates that the loss of label is probably due to the presence of endonuclease since no detectable mononucleotides were released (E. F. Baril *et al.*, 1970, unpublished data). No loss of label was observed when native DNA was incubated with the purified nuclear or ribosomal enzymes (Table IX). However, preliminary experiments involving alkaline and neutral sucrose gradient centrifugation show that purified ribosomal polymerase produces some single-strand breaks in native DNA (E. F. Baril *et al.*, 1970, unpublished data). It is possible that the same endonuclease is responsible for the single-strand breaks observed with native DNA, as well as the more extensive degradation of denatured DNA. The purified nuclear and ribosomal polymerases are free of specific and nonspecific phosphodiesterase activity.

STABILITY OF ENZYMES. The crude enzyme preparations (prior to DEAE-cellulose chromatography) from nuclei, ribosomes, and smooth membranes are stable in the presence of 20% ethylene glycol for at least 3 weeks at 4°, and for at least 6 months when frozen at −20°. Purified enzyme fractions are very labile even in the presence of ethylene glycol, DNA, or albumin, with a 50% loss of activity in 6 days at 4° and a greater loss when frozen. Incubation at 37° for 1 hr in the absence of DNA results in a 40% loss of activity. Thus, the labile state of the purified enzymes impedes studies of the extent of DNA synthesis.

Discussion

Systematic fractionation of a rat liver homogenate revealed considerable DNA polymerase activity associated with free and bound ribosomes and with a smooth membrane fraction, as well as with nuclei and a lesser amount with mitochondria.

There was very little DNA polymerase activity in the nonsedimentable (soluble) portion of the homogenate. This distribution of DNA polymerase activity led us to question whether the activity in each of these subfractions is due to one of the known deoxyribonucleotide-incorporating enzymes in rat liver, namely, n- and mtDNA polymerases and terminal transferase.

It seems unlikely that either the ribosome- or membrane-associated polymerase is an artifact derived from mitochondria (*i.e.*, mtDNA polymerase). There was no detectable activity of characteristic mitochondrial enzymes, such as succinic dehydrogenase and monoamine oxidase, in the postmicrosomal pellet (P-4) from which both ribosome- and smooth membrane-associated polymerases could be isolated. The specific activities of even the crude DNA polymerase preparations from ribosomal and smooth membrane fractions were markedly higher than that of partially purified DNA polymerase from mitochondria. DNA polymerase activities from both the ribosomal and smooth membrane fractions eluted from DEAE-cellulose at ionic strengths different from that required to elute the DNA polymerase activity from purified mitochondria. Finally, the pH optima, Mg^{2+} requirements, primer preferences and responses to salt concentrations of both the ribosome- and membrane-associated polymerases differ from the reported properties of rat liver mtDNA polymerase (Kalf and Ch'ih, 1968; Meyer and Simpson, 1968).

It is probable that the ribosome-associated polymerase is identical to the nuclear DNA polymerase. The ribosome-associated DNA polymerase fractionates in the same manner as that from highly purified rat liver nuclei. Moreover, the enzymatic properties of the ribosomal polymerase are similar to those of crude or partially purified nDNA polymerase. The properties of our partially purified nuclear and ribosome-associated DNA polymerases are comparable to those of the rat liver nDNA polymerase studied by Howk and Wang (1969).

The ribosome-associated and nDNA polymerase is active in the presence of only a single deoxyribonucleoside triphosphate. A high activity of mammalian DNA polymerase in the presence of fewer than four deoxyribonucleoside triphosphates has previously been observed by others (Howk and Wang, 1969; Greene and Korn, 1970; Chang and Hodes, 1968). The high activity in the presence of only one deoxyribonucleoside triphosphate might suggest that the activity is due to terminal transferase. However, the properties of the nuclear and ribosome-associated polymerase differ from those previously reported for terminal transferase (Wang, 1968; Bollum *et al.*, 1964; Krakow *et al.*, 1961). Wang (1968) has reported that terminal transferase from rat liver nuclei is inhibited by the presence of all four deoxyribonucleoside triphosphates, has maximal activity at pH 7.1 in the presence of 5 mM Mg^{2+}, shows a marked preference for denatured and activated DNA, and incorporates ribonucleoside triphosphates, as well as deoxyribonucleoside triphosphates into DNA. In contrast, we find that the nuclear and ribosome-associated polymerase requires all four deoxyribonucleoside triphosphates for maximal activity, has a pH optimum of 9.0 in the presence of 15 mM Mg^{2+}, shows a preference for native and activated DNA and does not incorporate ribonucleoside triphosphates into DNA. Moreover, the nuclear and ribosome-associated polymerase shows a strict requirement for base pairing of the incorporated deoxyribonucleoside triphosphate with the template. We are currently analyzing, by alkaline cesium chloride gradient centrifugation, the nature of the product formed by the polymerases when polydeoxyribohomo- and copolymers are used as templates.

The membrane-associated polymerase does not appear to be identical with the nuclear and ribosome-associated polymerase. The membrane-associated polymerase has a different net charge than the ribosomal or nuclear polymerase, as shown by their different affinities for DEAE-cellulose and phosphocellulose. The purified polymerases show similar requirements for Mg^{2+}, but have different pH optima and markedly different sensitivities to inhibition by *p*-hydroxymercuribenzoate. The most striking difference between the partially purified polymerases is that the membrane-associated enzyme is virtually inactive unless all four deoxyribonucleoside triphosphates are present, while the nuclear and ribosome-associated polymerase has considerable activity in the presence of fewer than four deoxyribonucleoside triphosphates. Although the crude enzyme fractions have different primer preferences, these are progressively lost during the purification and the purified enzymes all require activated DNA for maximal activity. The purified membrane-associated polymerase is free from both endo- and exonuclease activity. However, the purified nuclear and ribosome-associated polymerase has some associated endonuclease activity which produces single-strand breaks in DNA (E. F. Baril *et al.*, 1970, unpublished data).

Based partially on the evidence presented here, we conclude that rat liver contains a membrane-associated deoxyribonucleotide-polymerizing activity that has properties different from both mitochondrial DNA polymerase and the extractable DNA polymerase from purified rat liver nuclei. Rat liver also contains a ribosome-associated deoxyribonucleotide polymerizing activity that has properties identical with those of the extractable DNA polymerase of purified nuclei but different from those described for terminal transferase from rat liver nuclei (Wang, 1968).

Further investigation is required to define the properties and particularly the function of the enzyme present in the nucleus and ribosomes of rat liver and of that associated with the smooth membrane fraction. We have observed that the activity of the membrane-associated enzyme is markedly increased in proliferating tissues such as tumors (Baril *et al.*, 1970), regenerating and fetal rat liver (E. F. Baril *et al.*, 1970, unpublished data). In addition, distributional studies of ribonucleotide reductase and thymidine kinase activities in these same tissues have shown that the highest specific activities of these enzymes are found in the smooth membrane fraction (Dr. H. Elford, Duke University, personal communication). There have been recent reports suggesting that the enzymes for DNA replication in bacteria are membrane associated (Smith *et al.*, 1970; Knippers and Stratling, 1970; Okazaki *et al.*, 1970). It is possible that we are observing a similar situation in rat liver. It is of utmost importance to determine the degree of involvement of either or both of the polymerases in DNA replication, as opposed to DNA repair.

The extranuclear distribution of the DNA polymerases reported here is somewhat enigmatic. We cannot exclude the usual consideration that their presence in cytoplasmic fractions is an artifact due to damage and leakage of nuclei during the fractionation. We have, however, been unable to demonstrate the presence of DNA in the postmicrosomal pellet (P-4) from normal or 24-hr regenerating rat liver following a 2-hr pulse of [^{3}H]thymidine. Moreover, the association of the different polymerases with specific organelles creates some doubt as to an artifactual cytoplasmic association of these enzymes. The membrane-associated polymerase is

not found in highly purified normal rat liver nuclei, although it is found in nuclei from hepatomas and regenerating rat liver. Further experimentation is required to ascertain whether or not the smooth membrane fraction contains fragments of the nuclear membrane with which the membrane polymerase may actually be associated.

It is feasible that these enzymes originate in the cytoplasm and are transported to the nucleus, a possibility originally suggested by Bollum and Potter (1958) and Keir and Smellie (1962). There has been a recent report of the translocation of DNA polymerase from the cytoplasm to the nucleus during sea urchin development (Fansler and Loeb, 1969). Also, Mueller (1969) has shown a dependence of DNA synthesis in isolated HeLa cell nuclei on cytoplasmic protein factors present in the postmicrosomal supernatant solution. Moreover, the synthesis of histones has been shown to occur in the cytoplasm and not the nucleus (Robbins and Borun, 1967; Gallwitz and Mueller, 1969). Thus, there is increasing evidence in eukaryotic cells for the transport of essential nuclear components from the cytoplasm to the nucleus. Numerous alternative explanations can account for the results we have reported here. These basic observations, however, do suggest a possible nucleocytoplasmic interaction in DNA synthesis in rat liver.

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