

ENDOGENOUS RNase-SENSITIVE
DNA POLYMERASE COMPLEX
FROM RAT TISSUES:
CHARACTERIZATION OF THE
REACTION AND THE PRODUCTS,
AND OF THE DNA
POLYMERASES RELEASED BY
RNase-TREATMENT

CENTRE FOR NEWFOUNDLAND STUDIES

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ENDOGENOUS RNase-SENSITIVE DNA POLYMERASE COMPLEX FROM RAT TISSUES:
CHARACTERIZATION OF THE REACTION AND THE PRODUCTS, AND OF THE DNA
POLYMERASES RELEASED BY RNase-TREATMENT

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By

Francesco Moranelli



May, 1977

To Suzanne

TABLE OF CONTENTS

	PAGE
ABSTRACT	(i)
ACKNOWLEDGEMENTS	(iii)
LIST OF FIGURES	(iv)
LIST OF TABLES	(viii)
LIST OF SCHEMES	(x)
LIST OF ABBREVIATIONS	(xi)
GLOSSARY	(xiii)
LITERATURE REVIEW	
I. Introduction	1
II. (A) Classification of Replicative DNA Polymerases	3
(B) Reaction Mechanism of Replicative DNA Polymerases	7
III. Characteristics of Various Replicative DNA Polymerases	11
(A) DNA Polymerase α	11
(a) Basic Distinguishing Features	11
(b) Purification	11
(c) Properties	13
(i) Template Specificity	13
(ii) Divalent Cation Requirement	14
(iii) Effect of Monovalent Cations	15
(iv) pH Optima	16
(v) Isoelectric Points	17
(vi) Size	18
(d) Subcellular Localization	21
(e) Inhibition Studies	22

	PAGE
(f) Biological Function	24
(B) DNA Polymerase β	27
(a) Basic Distinguishing Features	27
(b) Purification	27
(c) Properties	29
(i) Template Specificity	29
(ii) Divalent Cation Requirement	29
(iii) Effect of Monovalent Cations	30
(iv) pH Optima	30
(v) Isoelectric Point	30
(vi) Size	31
(d) Subcellular Localization	31
(e) Inhibition Studies	32
(f) Biological Function	32
(C) DNA Polymerase γ	35
(a) Basic Distinguishing Features	35
(b) Purification	36
(c) Properties	36
(i) Template Specificity	36
(ii) Divalent Cation Requirement	39
(iii) Effect of Monovalent Cations	39
(iv) pH Optima	40
(v) Isoelectric Point	40
(vi) Size	40
(d) Subcellular Localization	40

	PAGE
(e) Inhibition Studies	40
(f) Biological Function	40
(D) Mitochondrial DNA Polymerase	41
(a) Basic Distinguishing Features	41
(b) Purification	42
(c) Properties	42
(i) Template Specificity	42
(ii) Divalent Cation Requirement	44
(iii) Effect of Monovalent Cations	44
(iv) pH Optima	45
(v) Isoelectric Point	45
(vi) Size	45
(d) Submitochondrial Localization	45
(e) Inhibition Studies	46
(f) Biological Function	46
(E) RNA-Directed and RNase-Sensitive Endogenously-Templated DNA Polymerases	47
(a) Introduction	47
(b) Reverse Transcription with Bacterial Systems . .	51
(i) Exogenously-Templated Reaction Catalyzed by <i>E. coli</i>	51
(ii) Endogenous RNase-Sensitive DNA Polymerase	53
(c) Involvement of RNA in DNA Synthesis in Eukaryotic Cells	54
(i) RNA as a Primer in DNA Synthesis	54
(ii) RNA as a Template in DNA Synthesis	54
(d) Properties of the RNA-Directed DNA Polymerase .	55

	PAGE
(e) Nature of the Reaction Products of the RNA-Directed DNA Polymerases	58
(f) Biological Function of RNA-Directed DNA Polymerase	59
IV. Terminal Deoxynucleotidyl Transferase	62
V. Serological Analysis of DNA Polymerases	64
PURPOSE AND APPROACH TO THIS STUDY	67
MATERIALS AND METHODS	
I. Materials	
(A) Animals	68
(B) Biochemicals, Chemicals and Enzymes	68
(C) Buffers and Solutions	71
II. Methods	
(A) Preparation of Tissue Homogenates	73
(B) Preparation of the Endogenously-Templates RS-DP Activity from Rat Thymus	73
(C) Preparation of Exogenously-Templated Activities Derived from the RS-DP Complex from Rat Thymus	74
(D) Isoelectric Focusing	74
(E) Electrophoresis in Glycerol Gradients	76
(F) Siliconization of Glassware	78
(G) Isolation of the Reaction Products of the Endogenously-Templated DNA Polymerase	78
(H) Buoyant Density Analysis	80
(a) CsSO ₄ Density Gradient Centrifugation	80
(b) Guanidinium.Cl-CsCl Density Gradient Centrifugation	81

	PAGE
(c) Sucrose Density Gradient Centrifugation	81
(I) Sedimentation Velocity Analysis	81
(J) DNA Polymerase Assay	82
(K) Protein Determination	84
RESULTS	
I. Detection and Partial Purification of the Endogenously Templated Ribonuclease-Sensitive DNA Polymerase of Rat Thymus	85
(A) DNA Polymerase Activities in a Crude Extract of Rat Thymus	85
(B) Partial Purification of Rat Thymus DNA Polymerase . .	87
II. Properties of the Endogenous RNase-Sensitive DNA Polymerase	
(A) Requirements	91
(B) Heteropolymeric Nature of the Nucleic Acid Region Transcribed by the Endogenous RNase-Sensitive DNA Polymerase	91
(C) Preliminary Evidence in Favor of a Template Function for the RNA in the Endogenously-Templated RNase- Sensitive DNA Polymerase	93
(D) Properties of the Endogenous RNase-Sensitive DNA Polymerase	95
(a) Catalytic Properties	95
(i) Time-Course of the Reaction	95
(ii) Activity Versus Enzyme Concentration Curves	98
(iii) pH Optimum	101
(iv) Divalent Cation Requirements	104
(v) Effect of Potassium Chloride	104
(b) Inhibition Studies	104

	PAGE
(i) Effect of Actinomycin D and Distamycin A on the Endogenously-Templated Activity . .	104
(ii) Effect of N-ethylmaleimide on the RS-DP . .	110
(iii) Effect of Heparin	110
(iv) Effect of Polyamines on the RS-DP and DNA Polymerase α	110
(c) Physical Properties of the Endogenous Activity .	113
(i) Effect of Triton X-100 on the Endogenous RS-DP Activity	113
(ii) Buoyant Density of the Endogenous RS-DP .	115
(iii) Molecular Weight of the Endogenous RS-DP	118
(iv) Isoelectric Point of the Endogenous RS-DP	118
III. Purification of the Endogenous RNase-Sensitive DNA Polymerase	121
(A) Reasons for Further Purification	121
(B) General Observations	121
(C) Purification of DNA Polymerases from Rat Thymus . .	123
(D) Purification of the RS-DP from Rat Liver	132
IV. Analysis of the RS-DP Product of Rat Liver	140
(A) Effect of Nucleases and Alkali on the Product of the Endogenously-Templated DNA Polymerase	140
(B) Buoyant Density Analysis of the Products	142
(C) Size of the RS-DP Product after Various Treatments .	147
V. Isolation of the DNA Polymerase Activities Associated with the RS-DP Complex	154
(A) Effect of Nuclease Treatment on the Release of DNA- Directed DNA Polymerases from the Endogenously- Templated DNA Polymerase Complex from Rat Thymus . .	155

	PAGE
(B) Effect of Basic Proteins on the Endogenously-Templated DNA Polymerase Complex	160
(C) Effect of RNase Digestion Products on the Endogenously-Templated DNA Polymerase Complex	162
(D) Effect of RNase-Treatment on the Endogenous Activity in the Presence of Protease Inhibitors . . .	163
(E) Effect of RNase on More Extensively Purified Endogenously-Templated RS-DP Preparations	165
VI. Properties of the DNA Polymerases Released by RNase-Treatment of the Endogenous Activity	169
(A) Requirements	169
(B) Catalytic Properties	171
(a) Time-Course for the Peaks II and III Activities	171
(b) Activity Versus Enzyme Concentration Curve for Peak II	171
(c) pH Optimum	174
(d) Divalent Cation Optima	174
(e) Effect of Potassium Chloride	178
(f) Template Specificity	182
(i) Effect of Actinomycin D on the Exogenously RNA-Templated Activity with the Peak III Enzyme	184
(g) "Km's" for Native and "Activated" DNA Templates for Peaks II and III	188
(C) Inhibition Studies	188
(a) Effect of N-ethylmaleimide	188
(b) Effect of Heparin	192
(c) Effect of Ethidium Bromide	192
(d) Effect of Organic Solvents	192
(e) Effect of Polyamines	196

	PAGE
(D) Physical Properties of the DNA Polymerases Derived from the RS-DP Complex	200
(a) Molecular Weight	200
(b) Isoelectric Points of the Peak III Activity	203
DISCUSSION	207
I Evidence Suggesting RNA-Directed DNA Synthesis in Mammalian Cells.	207
(A) Indirect Evidence	208
(B) Direct Evidence	210
(C) Comparison of the Endogenously-Templated RS-DP Activity from Rat Thymus to that from Oncogenic RNA Viruses.. . . .	214
(a) Physical Properties	214
(b) Biochemical Properties	216
II Evidence Suggestive of Two Unique DNA Polymerases Associated with the Endogenously-Templated RS-DP Complex.	218
III Relationship of Peak II and III Activities to other Eukaryotic DNA Polymerases.	221
IV Speculations on the Possible Functions of the RNA-Directed DNA Polymerase in Normal Mammalian Cells.	223
APPENDIX I	
Relationship between Density and Refractive Index of Guanidinium.Cl-CsCl Solutions	227
REFERENCES	229
PUBLICATIONS ARISING FROM THIS WORK	251

ABSTRACT

An endogenously-templated DNA polymerase activity from rat thymus and liver has been partially purified and characterized, and the product of the reaction analyzed. The enzyme from both sources was shown to be sensitive to pretreatment with RNases [hence it is referred to as a RNase-sensitive DNA polymerase (RS-DP)]. The molecular weight of the RS-DP complex, estimated from Sepharose 6B gel filtration, is 280,000 daltons.

The RNA associated with the RS-DP is probably single-stranded (and therefore functions as a template) since the activity remained sensitive to RNase-treatment under conditions in which only single-stranded RNA is digested. The putative RNA template is heteropolymeric in nature, since all four nucleotides were incorporated into the DNA product to a similar extent (also indicating that the enzyme is not simply a terminal transferase). The enzyme is probably not of viral origin, as the activity was not stimulated by non-ionic detergents and also had a buoyant density (1.05 g/cm^3) in a sucrose gradient which differed greatly from that reported for the type-C viral activity ($1.22\text{-}1.24 \text{ g/cm}^3$). Inhibition studies with actinomycin D and distamycin A, however, revealed a similarity to the viral RNA-directed DNA polymerase (RD-DP), supporting the notion that the RNA in the RS-DP complex functions as a template. Additional supporting evidence for a template function for the RNA derives from buoyant density analysis of the product of the rat liver RS-DP activity.

The RS-DP activity differs from DNA-directed DNA polymerases in its preference for Mn^{2+} to Mg^{2+} as the divalent cofactor. Furthermore, the enzyme is not inhibited by N-ethylmaleimide and also responds dif-

ferently to heparin and polyamines than does the rat thymus DNA polymerase α .

Two lower molecular weight DNA polymerases (70,000 and 30-40,000 daltons, respectively denoted as Peaks II and III), were derived from the RS-DP complex by extensive RNase-treatment. These activities are probably not proteolytic fragments of a higher molecular weight DNA polymerase, since RNase-treatment in the presence of the protease inhibitors Trasylol and phenylmethylsulfonyl fluoride also resulted in the release of these activities.

The Peak II and III activities differ from each other in a number of their properties, and furthermore, differ from other eukaryotic DNA polymerases described in the literature, indicating that the enzymes are distinct and probably unique DNA polymerases.

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LIST OF FIGURES

<u>FIGURE</u>	<u>TITLE</u>	<u>PAGE</u>
1	Rat Thymus DNA Polymerase Activities in the 39,000 x g Supernatant	86
2	Profile on Rat Thymus DNA Polymerases on a Sepharose 6B Column	90
3	Time-Course of the Endogenous RS-DP Activity	97
4	Sigmoidal Relationship Between Activity and Concentration of RS-DP Complex	99
5	Inhibition of RS-DP Activity at High Concentrations of RS-DP Complex	100
6	Activity Versus RS-DP Concentration Curves in Siliconized and Non-Siliconized Tubes	102
7	pH Optimum of Endogenous RS-DP Activity	103
8	The Effect of Divalent Cations on the Endogenous RS-DP Activity	105
9	Effect of Potassium Chloride on the Endogenous RS-DP Activity	106
10	Effect of Actinomycin D on the Endogenous RS-DP Activity	108
11	Effect of Distamycin A on the Endogenous RS-DP and DNA Polymerase α of Rat Thymus	109
12	Effect of N-ethylmaleimide on the Endogenous RS-DP and DNA Polymerase α of Rat Thymus	111
13	Effect of Heparin on the Endogenous RS-DP and DNA Polymerase α of Rat Thymus	112
14	Effect of Polyamines on the Endogenous RS-DP and DNA Polymerase α of Rat Thymus	114
15	Effect of Triton X-100 on the Endogenous RS-DP Of Rat Thymus	116
16	Buoyant Density Analysis of the Endogenous RS-DP Activity	117
17	Molecular Weight Determination of the Endogenous RS-DP on a Sepharose 6B Column	119

<u>FIGURE</u>	<u>TITLE</u>	<u>PAGE</u>
18	Isoelectric Focusing of the Endogenous RS-DP Activity	120
19	Glycerol Gradient Electrophoresis Profile of Rat Thymus DNA Polymerases from the 39,000 x g Supernatant	130
20	Glycerol Gradient Electrophoresis of the Endogenous RS-DP of Fraction IV of the Rat Thymus Preparation	131
21	Activity Versus Enzyme Concentration Curve for the Endogenous RS-DP Activity from Fraction IV a of Rat Liver	137
22	Activity Versus Enzyme Concentration Curve of Fraction V of the Endogenous RS-DP Activity from Rat Liver	138
23	Effect of Actinomycin D and Distamycin A on the Endogenous RS-DP Activity from Rat Liver	139
24	Effect of S ₁ Nuclease Treatment on the Product Synthesized in the Presence of Actinomycin D, Before and After Heat Denaturation	143
25	Buoyant Density Analysis of the Product Synthesized in the Presence of Actinomycin D, on (A) Cs ₂ SO ₄ and (B) CsCl-Guanidinium-Cl Gradients	144
26	Buoyant Density Analysis of the Product Synthesized in the Presence of Actinomycin D, After (A) Heat Denaturation, (B) Alkali Treatment, (C) RNase A and T ₁ and (D) Nuclease S ₁ Treatments	146
27	Buoyant Density Analysis of the Product Synthesized in the Absence of Actinomycin D, Before (A) and After (B) Alkali-Treatment	148
28	Sedimentation Velocity Analysis of the Product Synthesized in the Presence of Actinomycin D; (A) Control, (B) After Heat-Treatment and (C) After Alkali-Treatment	149
29	Sedimentation Velocity Analysis of the Product Synthesized in the Absence of Actinomycin D, (A) Before and (B) After Alkali-Treatment	151
30	Elution Profile of Rat Thymus DNA Polymerases on a Sephadex G200 Column After Treatment with Various Nucleases	156

<u>FIGURE</u>	<u>TITLE</u>	<u>PAGE</u>
31	Profile of the DNA-Directed DNA Polymerases on a Sephadex G200 Column After RNase A and T ₁ Treatment	157
32	Profile of RD-DP and DD-DP Activities on a Sephadex G200 Column After RNase A and T ₁ Treatment of the Endogenous Activity	159
33	Effect of Oxidized RNase A, Histones and Lactoperoxidase on the Profile of the DNA Polymerases from Rat Thymus	161
34	DNA Polymerase Profile on Sephadex G200 of Native DD-DP After RNase-Treatment of the Endogenously-Templated Activity in the Presence of (A) Trasylol and (B) Phenylmethylsulfonyl Fluoride	164
35	Effect of Nucleases on Endogenously-Templated Activities of Rat Liver Exposed to 400 mM KCl	167
36	Time Course of the Peak II (A) and Peak III (B) Activities Derived from the RS-DP Complex	172
37	Activity Versus Enzyme Concentration Curves for Peaks II and III	173
38	Inhibition of the Peak III Activity at High Levels of Enzyme	175
39	The Effect of pH on the Peak II Enzyme	176
40	The Effect of pH on the Peak III Enzyme	177
41	The Effect of Divalent Cations on the Peak II Enzyme	179
42	The Effect of Divalent Cations on the Peak III Enzyme	180
43	The Effect of KCl on Peak II and III Activities	181
44	Dependence of Relative Efficiency of "Activated" to Native DNA for Peak II on Enzyme Concentration	185
45	Apparent Km's for Native and "Activated" DNA as Templates for the Peak II Enzyme	189
46	Apparent Km's for Native and "Activated" DNA as Templates for the Peak III Enzyme	190

<u>FIGURE</u>	<u>TITLE</u>	<u>PAGE</u>
47	Effect of N-ethylmaleimide on (A) Peak II and (B) Peak III Enzymes	191
48	Effect of Heparin on (A) Peak II and (B) Peak III Enzymes	193
49	Effect of Ethidium Bromide on the Peak II and III Enzymes	194
50	Effect of Polyamines on the Peak II and Peak III Enzymes	197
51	Effect of 1 mM Putrescine on the Activity Versus Enzyme Concentration Curve for Peaks II and III Activities	199
52	Molecular Weight Estimation of Peak II and III Activities on a Sephadex G200 Column	201
53	Molecular Weight Estimation of the Peak II and III Activities on a Sepharose 6B Column	202
54	Molecular Weight Estimation of the Peak II Activity (A) Before and (B) After RNase-Treatment on a Sepharose 6B Column	204
55	Molecular Weight Estimation of the Peak III Enzyme on a Sephadex G75 Column in the Presence of 1 M Salt	205
56	Isoelectric Focusing Analysis of the Peak III Enzyme	206
57	Relationship Between Density and Refractive Index of Guanidinium.Cl-CsCl Solutions	228

LIST OF TABLES

<u>NUMBER</u>	<u>TITLE</u>	<u>PAGE</u>
I	Comparison of Properties of DNA Polymerase I, II and III of <i>E. coli</i>	4
II	Classification of Replicative DNA Polymerases . . .	6
III	Properties of DNA Polymerase α	12
IV	Conversion of High Molecular Weight DNA Polymerases to Lower Molecular Weight Species . . .	19
V	Inhibitors of DNA Polymerase α	23
VI	Properties of DNA Polymerase β	28
VII	Inhibitors of DNA Polymerase β	33
VIII	Properties of DNA Polymerase γ	37
IX	Template Specificity of DNA Polymerase γ	38
X	Properties of Mitochondrial DNA Polymerase	43
XI	Criteria Used in Assigning a Template Function to RNA in DNA Synthesis	56
XII	Reaction Mixture for the DNA Polymerase Assays . . .	83
XIII	Partial Purification of Rat Thymus DNA Polymerases	88
XIV	Requirements of the RNase-Sensitive DNA Polymerase	92
XV	Incorporation of ^3H -Labelled Deoxynucleoside Monophosphates into DNA by the Endogenously- Templated RNase-Sensitive DNA Polymerase	94
XVI	Sensitivity of Endogenously-Templated DNA Polymerase Activity to RNase A and T_1 in the Presence of 200 mM NaCl	96
XVII	Purification of Rat Thymus DNA Polymerases	126
XVIII	RNase-Sensitivity of the Rat Thymus Endogenously- Templated Activity During the Course of Purification	128

<u>NUMBER</u>	<u>TITLE</u>	<u>PAGE</u>
XIX	Relative Specific Activity of Rat Thymus DNA Polymerases at Various Stages of Purification	129
XX	Purification of the Endogenous RNase- Sensitive DNA Polymerase from Rat Liver	135
XXI	RNase-Sensitivity of the Endogenously-Templated DNA Polymerase Activity from Rat Liver at Various Stages of Purification	136
XXII	Effect of Nucleases and Alkali on the RS-DP Product	141
XXIII	Analysis of the Sizes of Products after Various Treatments	152
XXIV	Requirements of the DNA Polymerase α and of the Peak II and III Activities Derived from the RS-DP Activity of Rat Thymus	170
XXV	Template Specificity of Rat Thymus DNA Polymerase α and the Peak III Activity Derived from the RS-DP Complex	183
XXVI	Template Specificity of Peaks II and III in the Presence of Mg^{2+} or Mn^{2+} as the Divalent Cation . . .	186
XXVII	Effect of Actinomycin D on the Peak III Activity with DNA and RNA Templates	187
XXVIII	Effect of Organic Solvents on the Peak II and III Enzymes	195

LIST OF SCHEMES

<u>NUMBER</u>	<u>TITLE</u>	<u>PAGE</u>
I	Template-Primer Specificity of Replicative DNA Polymerases	2
II	Requirements of Terminal Deoxynucleotidyl Transferase	3
III	Reaction Mechanism Proposed for DNA Polymerases . . .	9
IV	Provirus Hypothesis	49
V	Modes of Ribosomal Gene Amplifications (A) Semi-conservative Replication Model (B) Model of Tocchini-Valentini and Crippa	60
VI	Apparatus Used for Electrofocusing	75
VII	Apparatus for Electrophoresis in Glycerol Gradients	77
VIII	Flow-Chart for the Procedure used in Isolating the Product of the RS-DP Reaction	79
IX	Flow-Chart for the Purification of Rat Thymus DNA Polymerases	124
X	Flow-Chart for the Purification of the RNase Sensitive DNA Polymerase from Rat Liver	133
XI	Possible Modes of Association of the Endogenously Templated DNA Polymerase with Nucleic Acids	209
XII	Hypothetical Structures for the RS-DP Product	211-213
XIII	Central Dogma of Molecular Biology	224

LIST OF ABBREVIATIONS

ADD-DP	"Activated" DNA-Directed DNA Polymerase
AMV	Avian myeloblastosis virus
araCTP	1- β -D-arabinofuranosylcytosine 5'-triphosphate
BSA	Bovine serum albumin
CHT-GEN	Chymotrypsinogen
pCMB	p-chloromercuribenzoate
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide
dATP	2'-deoxyribosyladenine-5'-triphosphate
dCTP	2'-deoxyribosylcytidine-5'-triphosphate
DD-DP	DNA-Directed DNA polymerase
DEAE-cellulose	Diethylaminoethyl-cellulose
dGTP	2'-deoxyribosylguanine-5'-triphosphate
DMSO	dimethylsulfoxide
DNA (rDNA)	deoxyribonucleic acids (ribosomal)
DNase	deoxyribonuclease
dsDNA	double-stranded DNA
DTT	dithiothreitol
dTTP	2'-deoxyribosylthymidine-5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
EtOH	ethanol
LP	lactoperoxidase
NaPPi	Sodium pyrophosphate

NDD-DP	Native DNA-directed DNA Polymerase
NEM	N-ethylmaleimide
OVA	ovalbumin
PEG	polyethyleneglycol
PHA	phytohaemagglutinin
pI	isoelectric point
pmoles	picomoles (10^{-12} moles)
pOHgBZ	p-hydroxymercuribenzoate
PMSF	phenylmethylsulfonyl fluoride
RD-DP	RNA-directed DNA polymerase (reverse transcriptase)
RNA (rRNA, yRNA, TMV-RNA, Q β RNA Hb-mRNA)	ribonucleic acid (ribosomal, yeast, tobacco mosaic virus, phage Q β , haemoglobin message)
RNase	ribonuclease
rpm	revolutions per minute
RS-DP	RNase-sensitive DNA polymerase
RSV	Rous sarcoma virus
2-SH-EtOH	2-mercaptoethanol
SDS	sodium dodecyl sulfate
Sp. Act.	specific activity
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
Tris	tris(hydroxymethyl)amino-methane

GLOSSARY

In order to prevent any confusion that may arise from some of the terminology used in this dissertation, the following terms will be used as defined below:

Template: The nucleic acid strand dictating to the polymerases the sequence in which the deoxynucleotides will be incorporated.

Primer: The strand providing the 3'OH group essential for the initiation of DNA synthesis by DNA polymerases.

"Activated" DNA: DNA partially digested with pancreatic DNase I for the purpose of increasing the number of 3'OH initiation sites.

"Capped" DNA: Exonuclease III-treated "activated" DNA resulting in a stretch of single-stranded DNA.

RNA-directed DNA Polymerase: This term will be used when the function of the RNA is to act as a template.

RNA-dependent DNA Polymerase: This term will be used to indicate that the role of RNA in the reaction is to act as a primer in the initiation of the reaction.

RNase-Sensitive DNA Polymerase (RS-DP): Refers to the endogenously-templated DNA polymerase that is sensitive to RNase-treatment.

LITERATURE REVIEW

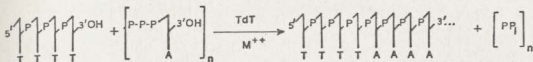
I. Introduction

DNA polymerases (DNA nucleotidyltransferase, EC 2.7.7.7), ubiquitously distributed in living organisms, are enzymes catalyzing the synthesis of DNA when supplied with the appropriate substrates. In order for an enzymatic reaction to occur, a DNA polymerase must be provided with a template-primer nucleic acid (DNA or RNA or combinations of), unless it is already associated with an endogenous template-primer. In addition it must be supplied with the four deoxynucleoside triphosphates and a divalent cation such as magnesium or manganese. The stoichiometry, as well as some of the template-primer combinations used in the catalysis of DNA synthesis by such enzymes, are depicted in Schemes I and II.

On the basis of template-primer or primer requirement by DNA polymerases (compare Schemes I and II), one can classify such enzymes into two basic categories (i) those that catalyze a *replicative-type* reaction (Scheme I) and (ii) those that catalyze a *terminal addition* reaction (Scheme II). The two classes are distinguishable on the basis of whether they do or do not require the direction of a DNA or RNA template with regard to which deoxynucleoside triphosphate will next be condensed in the polymerization step. In the *replicative-type* reaction, the sequence in which the nucleotides are incorporated in the newly synthesized DNA is determined by the DNA or RNA template, whereas in the *terminal addition* reaction no template is required, and the synthesized sequence is determined by the nucleoside triphosphate made available to the enzymes.

The *replicative-type* DNA polymerases may be further subdivided ac-

REQUIREMENTS OF TERMINAL
DEOXYNUCLEOTIDYL TRANSFERASE



SCHEME II

cording to their template-primer specificities as well as on the basis of other distinguishing features to be discussed in the next section.

II. (A) Classification of Replicative DNA Polymerases.

The DNA polymerase from *E. coli* initially discovered by Kornberg (1) was thought to function in the semi-conservative replication of DNA. The possible existence of multiple DNA polymerases in prokaryotes was not suspected however until the isolation by De Lucia and Cairns (2) of a mutant of *E. coli*, lacking DNA polymerase I but still capable of normal DNA replication. Shortly after the discovery of Cairn's mutant reports concerning DNA polymerase II (3), DNA polymerase III (4), and a complex form of DNA polymerase III (designated as DNA polymerase III*) (5) appeared in the literature. For the purpose of comparison, Table I outlines some of the distinguishing features of DNA polymerases I, II and III of *E. coli*.

The existence of multiple DNA polymerases (7-9) in eukaryotes was evident at an earlier stage of investigation than in the prokaryotic system and was attributed to the higher complexity of eukaryotic organisms. Within the past half decade, the compilation of data concerning various eukaryotic DNA polymerases has increased to such an extent, and the nomenclature system has varied so much that a tremendous amount of confusion

TABLE I¹. Comparison of Properties of DNA Polymerase I, II and III of *E. coli*

<u>Properties</u>	<u>DNA Polymerase</u>		
	<u>I</u>	<u>II</u>	<u>III</u>
Molecular Weight	109,000	120,000	160,000
Preferred Template	"Activated" DNA ²	"Gapped" DNA ³	"Gapped" DNA
No. of Molecules/Cell	400	100	10
Associated Exonucleases	5' → 3' 3' → 5'	3' → 5'	3' → 5'
Sensitivity to Sulfhydryl Reagents	No	Yes	Yes
<i>De Novo</i> DNA Synthesis	Yes	No	No
Inhibition by Pol. I Antiserum	Yes	No	No
Inhibition by araCTP ⁴	No	Yes	No
Effect of 10% EtOH	inhibition	inhibition	stimulation
Structural Genes	<i>pol A</i>	<i>pol B</i>	<i>dna E</i>

1 Abridged from Kornberg and Kornberg (6).

2 "Activated" DNA - DNA partially digested with pancreatic deoxyribonuclease I, for the purpose of increasing the number of 3'OH initiation sites.

3 "Gapped" DNA - exonuclease III-treated "activated" DNA resulting in stretches (i.e., gaps) of single-stranded regions in the DNA.

4 araCTP - 1-β-D-Arabinofuranosylcytosine 5'-triphosphate

has evolved as to which "replicative" DNA polymerase is actually being referred to by different investigators. In an attempt to eliminate this confusion, a number of scientists have proposed a uniform nomenclature for identifying eukaryotic DNA polymerases (10-12). The nomenclature system is based on the use of Greek letters in designating the DNA polymerases in the historical order in which they were discovered, analogous to the approach taken in the naming of bacterial DNA polymerases (10).

Table II outlines five main types of replicative DNA polymerases to be discussed in this review as well as some of their distinguishing features. In addition to these five types, there are also viral-induced DNA polymerases which will not be considered here [see Weissbach (10) for a brief review]. The table indicates that the classification is based on a combination of properties such as size, template specificity, intracellular localization, and sensitivity to sulfhydryl blocking reagents.

Previous attempts to classify DNA polymerases were based either on template specificity (14), subcellular localization (15), or size (16). Categorization according to template specificity alone had to be abandoned however, since enzyme preparation from various laboratories differ in the degree of contamination by nucleases, which would alter the templates and thus the enzymes' apparent specificity. Similarly, classification according to subcellular localization alone is not satisfactory, since the recovery of activity in various subcellular fractions is influenced by the isolation procedure used in preparing the subcellular components (17). The mitochondrial DNA polymerase is the only eukaryotic DNA polymerase classified with certainty on the basis of its subcellular localization, since drastic conditions are normally required to extract this polymerase

TABLE II¹. Classification of Replicative DNA Polymerases

<u>Properties</u>	<u>DNA Polymerase</u>				
	<u>α</u>	<u>β</u>	<u>γ</u>	<u>Mit.</u>	<u>RD-DP²</u>
Sedimentation Coefficient or Molecular Weight	6-8S	3.3S	6.1-6.3S	8-9S	Type I ³ 70,000 Type II 170,000 Type III 110,000
Subcellular Localization ⁴	C	N	C,N	Mit.	Oncogenic RNA viruses
Inhibition by thiol blocking reagents	+	-	+	-	+
Nuclease Activity	-	-	-	-	+
Template Specificity:					
"act" DNA	+	+	+	+	+
rA _n ·dT ₁₅	-	+	+	-	+
dT _n ·rA ₁₀	+	-	+	-	+
heteropolymeric RNA	-	-	-	-	+

1 Modified from Weissbach (10) and Holmes and Johnston (12).

2 Abbreviations used: RD-DP -- RNA-directed DNA polymerase, C -- cytoplasmic, N -- nuclear, Mit. -- mitochondrial.

3 According to Wu and Gallo (13).

4 Subcellular localization of the enzymes, when subcellular components are prepared in buffered aqueous media.

from that organelle (18). Classification on size alone also has its drawbacks, since the enzymes are well known for their ability to aggregate as well as to possibly associate with other proteins under certain conditions [e.g., (19) also see sections on Size below].

The RNA-directed DNA polymerases (RD-DP), the true "reverse transcriptases" from RNA oncogenic viruses (20, 21), can be distinguished both on the basis of their intraviral localization as well as on their unique ability to make DNA copies from natural RNA templates. RD-DPs from normal cells [i.e., avian (22) and mammalian (23-25)] can however be distinguished only on the basis of their ability to transcribe DNA copies from heteropolymeric RNA templates.

More detailed information concerning these various classes of replicative DNA polymerases will be discussed in subsequent sections. In accordance with the newly adopted nomenclature and for the sake of uniformity, all DNA polymerases that have been described in the literature, regardless of the terminology used by the authors, will be referred to as DNA polymerases α , β , and γ , on the basis of the distinguishing features outlined in Table II. Also, terminal deoxynucleotidyl transferase (TdT) will be briefly discussed for the purpose of comparison.

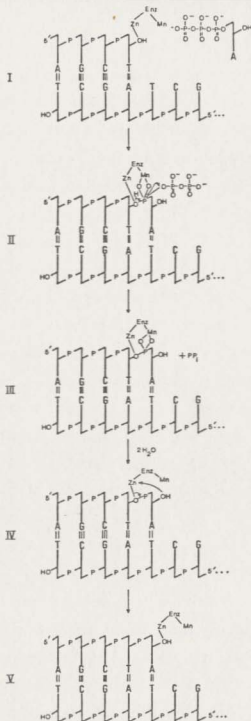
(B) Reaction Mechanism of Replicative DNA Polymerases

All replicative DNA polymerases reported to date, whether of eukaryotic or prokaryotic origin have the same basic requirements (16). Namely, all members of this class of enzymes require a template-primer, the four deoxynucleoside triphosphates, and a divalent cation (either Mg^{2+} or Mn^{2+}). Furthermore a 3'OH group on the primer strand is essential, since digestion of the template-primer with *micrococcal* nuclease, which pro-

duces 3'-phosphoryl termini, results in the abolition of template activity (16, 26, 27, 28, 29). The importance of the divalent cations has been shown to reside in their ability to promote the binding of the deoxynucleoside triphosphates to the enzyme (30), and not in promoting the formation of the template-primer-DNA polymerase complex. The latter function has been attributed to the presence of Zn^{2+} , which is found in all polymerases, regardless of their origin (16, 31-33).

Careful kinetic analysis of the role of each of the above mentioned components in the polymerization reaction, as well as magnetic resonance relaxation studies, have resulted in the elucidation of the reaction mechanism of DNA polymerases. Although much of this work has been carried out with *E. coli* DNA polymerase I, there is reason to believe that the same mechanism is operative in eukaryotic DNA polymerases. The proposed mechanism [Scheme III, modified from Slater *et al.* (30)] consists essentially of a two step process, a primer *elongation* step (Scheme III, steps I-III), and a primer *translocation* step (Scheme III, steps III-V).

The *elongation* step consists of the following events; (1) An initial binding of the polymerase to the primer-template DNA through coordination between the 3'OH group of the primer and the enzyme-bound Zn^{2+} (Scheme III, step I). (2) Binding of the deoxynucleoside triphosphates by coordination of the α -phosphoryl group to the enzyme-bound divalent cation (Mg^{2+} or Mn^{2+}). This ternary complex is in turn further stabilized by base pairing of the nucleotide to the template strand (Scheme III, step II). (3) A nucleophilic attack on the α -phosphoryl group by the 3'OH group subsequently occurs with a concerted displacement of pyrophosphate (Scheme III, steps II and III). At this point the nucleotidyl transfer is complete and the



REACTION MECHANISM PROPOSED FOR DNA POLYMERASES

SCHEME III

For an explanation, see text [modified from Slater *et al.* (30)].

translocation step, consisting of the following, begins: (1) Water ligands substitute for the phosphodiester ligand on the enzyme-bound Mn^{2+} (Scheme III, step IV). (2) Ligand substitution on the Zn^{2+} atom results in the dissociation of the 3'-oxygen atom of the previous nucleotide (now in a phosphodiester linkage) and coordination of the new 3'OH group at the end of the growing strand with the enzyme, thus bringing about *translocation* (Scheme III, step V).

As to whether this latter complex remains stable, and the same enzyme molecule repeats the same cycle over and over again (i.e., a processive process), or whether the enzyme is released after a single addition and binds to a different template-primer (i.e., a distributive process), is at present controversial. For example, Chang (34) and McClure and Jovin (35) claim that *E. coli* DNA polymerase I (34, 35) and calf thymus DNA polymerase α and β (34) dissociate from the template-primer complex after a single nucleotide addition, whereas Uyemura *et al.* (36), using *E. coli* DNA polymerase I claim that the mechanism is processive. Nevertheless, due to the high inefficiency of a distributive mechanism, it is probably safe to assume that a processive mechanism occurs *in vivo*, and that the mechanism is much more complex than is actually depicted in Scheme III. For example, the polymerizing complex may have other factors (i.e., DNA binding proteins) associated with it that would stabilize the template-primer-polymerase complex and render the mechanism processive. The *in vitro* observation of a distributive mechanism is certainly in support of the somewhat relaxed requirement for all four deoxynucleoside triphosphates which has often been observed for eukaryotic DNA polymerases (37-39).

III. Characteristics of Various Replicative DNA Polymerases

(A) DNA Polymerase α

(a) Basic Distinguishing Features

DNA polymerase α was the first DNA polymerase to be described in a mammalian system (40). The enzyme constitutes the major DNA polymerase in most eukaryotic cells, and may represent as much as 80-90 percent of the total cellular DNA polymerase activity in actively proliferating tissues (11). It can be distinguished from other cellular DNA polymerases on the basis of its high molecular weight ($> 100,000$ daltons), template specificity, and sensitivity to thiol blocking reagents. These distinguishing features are outlined in Table II. Table III summarizes some specific properties of α -polymerases isolated from various eukaryotic systems. The nomenclature systems used by various workers are also listed in Table III (column 2).

(b) Purification

The low levels of enzyme (approximately 1 mg/kg tissue) (12) even in highly proliferating tissues, as well as the apparent heterogeneity in size [(62), see also section on Size], have greatly hindered the purification of the enzyme. Nevertheless, substantial purification through conventional purification procedures has been achieved with preparations from a variety of sources. For example, the cytoplasmic enzyme from rat ascites hepatoma cells has been purified 490- and 560-fold (26, 42a), that from chick embryo to 50-60 percent homogeneity (63) and that from human KB cells to a specific activity of 7,300 units*/mg protein (28, 44a). In addition, the enzyme from normal human blood lymphocytes has been

* A unit is 1 nmole of labelled deoxynucleoside triphosphate incorporated per hour.

TABLE III. Properties of RNA Polymerase α

Source	Nucleotide or Subcellular Location	Template $\frac{A+G}{U+C}$	Reduction Coefficient (50%)	Cation Optima (mM) $\frac{Mg^{++}}{Mn^{++}}$	pH Optima	Reference
Rat Liver	Cytoplasmic (Smooth microsomal membrane) PI (soluble)	NR *** + + Poor NR	98 7.18 (250,000) 318 (250,000)	NR 10 + Required NR	6.8 - 7.4 8 NR	41 42, 43 42a
Rat Brain	Soluble	D>H	NR	3-5	8.6	44, 45, 46
Rat Brain Nucleus	(Nucleoplasm, cyto- plasm, ribosomes)	A > D > H	(100,000)	NR NR	7.4 (Triton-BCI)	47, 48
Cardiac Muscle	(Cytoplasm)	NR + NR	6-48	NR NR	7-5	49
Reticulocytes	Soluble	+ NR NR	80 and 118	4	NR	50
Boyal Brain Lymphocytes	Polymerase I Synthetic Template	Synthetic Template	78 and 108 (160,000)	5-10 NR	7.3 (complete dependent)	51
Bone Marrow	(Cytoplasmic)	+ NR NR	NR	varies with μ	Dependent on cation concentrations	52
Differentiating embryonic nuclei	(Soluble)	+ + NR	(130-150,000)	NR NR	NR	53a
Chick Embryo	Polymerase I (Cytoplasmic)	+ NR NR NR + NR	7.56 (140,000) 6-48	5-10 2-4	5.40 (dG) ₂ (dT) ₂ NR	53a, 53 53a
Hepoma LP Cells	RNA Polymerase	+ + NR	6.35	Template dependent	Template dependent	53
Aniline Hepatoma Cells	Polymerase C (Cytoplasmic) Polymerase P1 (Cytoplasmic and stromal fraction) Polymerase H (Nuclear)	+ - - A > H > D + NR NR "suppressed" RNA	7.28 (142,000) 6.48 (117,000) 8.78 7.48	3-8 3-5 1-3	7.0 - 7.5 (phosphate) 6.3 (phosphate) 7.3 (Triton-BCI) 6.5 (cytoplasm) 7.0 (phosphate) 6.5 (Triton-BCI) 6.5 (Triton-BCI)	38, 42a, 54 42a, 54, 55 55
Earlick Adipose Tumor Cells	(Nuclear and cytoplasmic)	+ NR NR	NR	7 +	NR	56
Chinese Hamster Cells	(Cytoplasmic and nuclear)	A > D > H (140-200,000)	6.1-6.23 6.1-200,000	10 NR	NR	57
BMK Cells	(Cytoplasmic)	+ NR NR	6.935	NR NR	7.94 (Triton-BCI)	58
BM-21/CL3 Cells	Polymerase I	+ Poor Poor	6-85	6-12 +	7.65	59
HeLa S3 Cells	Polymerase II (Nuclear)	A > D > H	10.58 (150,000)	5-10 NR	8	60
Human KB Cells	Polymerase C (Cytoplasmic) Polymerase B2 (Nuclear)	+ NR Poor + NR Poor	110,000-140,000 NR	10 NR 10 NR	9.2	28, 44a
					9.2	28, 44a

* Abbreviations used: A -- "activated" RNA; D -- heat-denatured RNA; N -- native RNA; NR -- not required.

** Symbols used: "+" indicates used, "-" not used, "Q" indicates inhibition and "P" activation.

purified 220-fold (51), and that from BHK-21/C13 cells (59) 154-fold through a simple extraction procedure followed by ion exchange chromatography on phosphocellulose and gel filtration on Sephadex G 100.

(c) Properties

(i) Template Specificity

Early work with DNA polymerase α revealed that relative to native DNA, denatured DNA was the preferred template for DNA synthesis (64). The ability of denatured DNA to act as an efficient template was later shown to be due to the presence of "hairpin" structures providing the 3'OH primer groups imperative for the initiation of DNA synthesis (65, 66). In general the most efficient heteropolymeric DNA templates are native DNA partially digested with pancreatic DNase I (also referred to as "activated" DNA) (26, 28, 37, 67-70), and "gapped" DNA (exonuclease III treated "activated" DNA) (28, 55). Surprisingly however, Sedwick *et al.* (28) have observed that when "activated" DNA is converted to "gapped" DNA (with very little increase in the number of 3'OH groups), there was no increase in its efficiency as a template. This is probably related to the inability of DNA polymerase α to traverse long gaps, consistent with an *in vitro* distributive mechanism [see section on Mechanism, II (B)].

Native DNA has also been reported to be used by DNA polymerase α , however, this ability has been found to decrease with an increase in the degree of purification of the enzyme under investigation (37, 71, 72). Presumably the use of native DNA is due at least in part to the presence in the early purification steps of contaminating endonuclease activity capable of "activating" native DNA and thus rendering it an efficient template.

The synthetic deoxyhomopolymers, poly (dA) and poly (dT), investigated

as templates for calf thymus DNA polymerase α , are used only in the presence of complementary oligonucleotide primers (73), while in the case of poly (dC), no primer is required since at neutral pH this polymer is capable of forming "hairpin" structures to provide the initiation sites (73). The synthetic copolymer poly d(A-T) is used just as efficiently as "activated" DNA by polymerase α from bone marrow (52). The enzyme has also been reported to use polydeoxynucleotide templates initiated with oligoribonucleotides (62, 74, 75) as well as RNA-primed DNA as template (76). DNA polymerase α will not however use polyribonucleotides as templates when initiated with oligonucleotides (28, 37, 62, 70, 74, 75, 77).

Certain parameters to be kept in mind when testing poly- and oligonucleotides as template-primers are: the ionic strength of the medium, the divalent cation concentration and the temperature under which the assays are carried out since all of these factors affect the stability of the oligomer-polymer complexes (78). In addition the template preference of DNA polymerase α changes with the assay conditions (72), and also with the degree of purification of the enzyme - presumably because of the elimination, to various degrees, of contaminating nucleases at various stages of purification (37).

(14) Divalent Cation Requirement

All DNA polymerases reported to date, including DNA polymerase α , display an absolute requirement for a divalent cation for activity. This requirement is usually met by magnesium, when "activated" DNA is used as the template. Although manganese can to some extent replace magnesium (43, 45), it is much less effective. Optimal concentrations of Mg^{2+} lie in the range of 1 to 12 mM depending on assay conditions and the

source of the polymerase (26-28, 45, see also Table III). On the other hand, optimal Mn^{2+} concentrations are much lower, in the range of 0.05-0.40 mM, also depending on the assay conditions and the source of the polymerase (43, 45).

Divalent cation optima are difficult, if not impossible, to assess since a number of parameters of the DNA polymerase assay influence these values. Among such factors are the nature and concentration of the template, the concentrations of the deoxynucleoside triphosphates (53, 79, 80), and the pH (52). Thus the wide range of divalent cation optima reported may be due to the variations in assay conditions used in different laboratories. Craig and Keir (80) have pointed out that the higher the concentration of template and/or deoxynucleoside triphosphates, the higher is the optimum concentration of the divalent cation. This is at least partially due to the chelating action of the triphosphates, as well as the binding of the divalent cations to the nucleic acid template, especially at higher pH values. Contrary to this expected pH effect, DNA polymerase α from bone marrow exhibited an increase in divalent cation optimum upon decreasing the pH of the assay (52). Enzymes incapable of using Mn^{++} have also been reported. For example, DNA polymerase α from the nuclear membrane-chromatin fraction of rat ascites hepatoma cells was found not to catalyze DNA synthesis in the presence of Mn^{++} (27, 42a, 54). This is probably not a unique feature of the enzyme but may merely reflect the assay conditions used.

(iii) Effect of Monovalent Cations

Monovalent cations have been reported to both inhibit (27, 41, 42a, 47, 48, 51, 54, 59, 63, 81, 82), as well as stimulate DNA polymerase activity (50, 52, 58, 83, 84). The nature of the mechanism for this

stimulation is complex, and probably involves both the enzyme and the template. Some workers have found that the stimulation is specific for only certain cations (50, 58, 83). Moreover, the effect has been shown to be influenced by the type of template used (51a, 63). Chick embryo DNA polymerase α was inhibited in the presence of salt when "activated" DNA was used as the template, whereas with poly (dA)·poly (dT) the optimum concentration of KCl was 40 mM (51a, 63). With regard to specific ion effects, Lazarus and Kitron (58, 85) have shown that the cytoplasmic DNA polymerase α activity from baby hamster kidney (BHK) cells was enhanced by monovalent cations in the order: $\text{NH}_4^+ > \text{K}^+ > \text{Na}^+ > \text{Cs}^+ > \text{Li}^+$, with the latter cation being inhibitory. The effect seemed to be related to the crystalline radii of the cations, and it was concluded that a crystalline radius of 1.42 was optimal for stimulation (58). They also made the interesting observation that the sulfate salt of ammonium ions was substantially less stimulatory than the chloride salt. However, this may be due to the inhibitory side effects of sulfate ions on enzymes involved in phosphoryl transfer (58).

(iv) pH Optima

In general the pH optimum of DNA polymerase α lies near neutrality. For example, polymerases α from rat thymus (83), rat intestinal mucosa (47, 48), rat ascites hepatoma cells (26, 42a, 54), chick embryo (51a, 63), cardiac muscle (49), normal human lymphocytes (51), and BHK-21/Cl3 cells (59), displayed pH optima between 6.8 and 7.65. It is important to note however, that the optimum pH depends on a variety of parameters, such as the nature of the template (51, 53) and buffer (27, 42a, 54, 55, 58), as well as on the nature and concentration of the divalent and monovalent cation used (52). In particular, DNA polymerase α from hepatoma

LF cells exhibited a pH optimum in the range of 7 to 7.4 when assayed with the template poly [d(A-T)·d(T-A)], pH 7.6 to 8.4 with poly (dC), and 6.8-7.8 with heat-denatured DNA as template (53). With regard to the nature of the buffer, the enzyme from rat ascites hepatoma cells had a pH optimum of 7.0 in potassium phosphate, 7.8 in Tris-HCl, and 9.5 in glycine-NaOH buffer (55). An increase in pH optimum has been observed in the case of DNA polymerase α from bone marrow (52) when the divalent cation concentration in the reaction mixture was decreased. This was also found to be the case when the monovalent cation concentration was altered (52). Thus it appears that the determination of an absolute pH optimum for the enzyme is a difficult task due to the influence exerted upon this parameter by other components normally present in the assay systems. This would explain the wide range of pH optima reported from the various laboratories engaged in the characterization of DNA polymerases.

(v) Isoelectric Points

DNA polymerase α from a variety of sources has been shown to be an acidic protein. This conclusion is based on the relative affinity of the enzyme for ion exchange resins such as DEAE-cellulose and phosphocellulose (44a). In particular, the isoelectric point (pI) of DNA polymerases α from normal human lymphocytes (51) has been directly shown, by isoelectric-focusing, to have a value of 4.5. This value is probably too low, however, since any nucleic acid that may be associated with the polymerase must be eliminated prior to determining pI's by isoelectric-focusing; otherwise the pI's obtained will be those of a protein-nucleic acid complex, and hence too acidic (86).

(vi) Size

DNA polymerase α from various sources (62, 70, 84, 87-93) appears to be heterogeneous in size [see Tables III (column 4) and IV]. This heterogeneity may be due to aggregation (44a, 52, 78, 94), proteolysis (12, 92, 95), association with other proteins of the replicative complex (37, 62, 70, 78, 87, 88, 91, 96), or merely association with nucleic acids (91). In general, the size of the enzyme is in excess of 100,000 daltons although a polymerase α of 87,000 daltons has been reported in *Drosophila melanogaster* (79). The variety of sizes observed for the "large" DNA polymerase from a number of sources, and the molecular species detected under various dissociation conditions are shown in Table IV. The size of some of the species detected under dissociating conditions indicates the possibility of confusion between DNA polymerases α and β when identification is made on the basis of molecular weights alone. Apparently, some of the large polymerases reported are merely an aggregate form of DNA polymerase β or a mixture of both DNA polymerases α and β (see comments in Table IV).

A lack of correlation between the molecular weights as estimated by gel filtration and by sedimentation velocity analysis has been observed (62, 70, 109, 110). However, this may be explained by the apparent asymmetric shape of the DNA polymerase molecule as suggested by Holmes and Johnston (109). These authors determined that an axial ratio of 10 to 1 for DNA polymerase α of rat liver would explain the discrepancy in apparent molecular weights. The same explanation was offered by Caruso *et al.* (110) for DNA polymerase α of *Xenopus laevis* which behaved as a 300,000 dalton species by gel filtration, and as a 145,000 dalton species by sedimentation velocity analysis in glycerol gradients.

TABLE IV. Conversion of High Molecular Weight DNA Polymerases to Lower MW Species

Source	Initial Size	Treatment	Species Detected	References	Comments
BHK cells	10.7 - 138	(a) Heparin (b) 0.45 M NaCl	3.38 6.38, 3.58	97 97, 98	Original species consisted of both α and β , change in size accompanied by change in template preference.
Rat ascites hepatoma	400,000 daltons	(a) storage for 1 week at 0° C (b) freezing at -80° C (c) replacement of 2- β -ETOH with 2- β -ETOH (d) conversion treatment with DNase, RNase, and bromelain	70-80,000 daltons	99	Change in size accompanied by change in template preference.
				100	
Human KB cells	85 and 108 (frozen prep'n)	0.45 M NaCl	6.48	44	
I - Cytoplasmic (frozen prep'n)		0.45 M NaCl	6.48	44	
II - Nuclear >150,000 daltons		0.15 M K-phosphate	45,000 daltons	19	Aggregate of β .
HeLa cells	10.38 and 11.78	0.125 M $(NH_4)_2SO_4$	6.58	94	
Calf thymus (Cytoplasmic)	6.7 - 8.58 8.58 (130-100,000) 85	2 M urea Freezing and thawing urea	5.48 (90,000) 10,000-40,000 8.58 and 48 7.38, 3.38	91 91 93	Mixture of both α and β . 3.28 species probably a proteolytic fragment of 7.38 enzyme.
Rat liver	6-88 6-88	High salt and alkaline pH Cesium chromatography on phosphocellulose	7.18 3.58	101 102	
Marine normal and regenerating liver	6-88	0.25 M KCl	3.58	90, 103-105	Aggregate of DNA polymerase β .
Marine liver mitochondria	10.58	0.25 M KCl 0.50 M KCl	8.88	106	Mitochondrial DNA polymerase.
Marine testis	6-88	0.125 M KCl	6-88, 4.78, and 3.58	90, 103-105, 107	Mixture of both α and β .
JLS-V10 cells	110,000	SDS, guanidinium-HCl or Triton X 100	70,000 and 35,000 daltons	108	Probably aggregate of DNA polymerase β .

The ionic strength at which the sedimentation coefficients and the molecular weights are estimated also influences the estimated size. For example, at an ionic strength below 0.07, DNA polymerase α aggregates to 10.7S (52, 94, 111); at physiological or higher ionic strengths, however, the enzyme has a sedimentation value of 6-8S (14, 52, 78). It has also been claimed that the size of the enzyme from calf thymus can range from 130,000 to 450,000 daltons depending on the conditions to which it is exposed (78).

The heterogeneity of calf thymus DNA polymerase α has been studied in detail by Holmes *et al.* (62, 92, 93). These workers have resolved a partially purified enzyme preparation into three species (denoted as enzymes A, B, and C) on a DEAE-cellulose column (62). Enzyme A has been shown to have a sedimentation coefficient of 8S, enzyme B 5.2S, and enzyme C 7.2S, corresponding to molecular weights of 200-230,000, 100-110,000, and 155-170,000 respectively (12, 62). The relative amounts of these species varied from one preparation to another, and furthermore, in some preparations two A species (denoted as A_1 and A_2) were obtained (12, 93). Enzyme A could be converted to the C form by 2.8 M urea treatment (93) and enzyme B has been suggested to probably represent a proteolytic degradation product of enzyme C (12, 62, 92, 95). If poly (dA)·(dT)₁₀ was used as the template instead of "activated" DNA, an additional species (enzyme D, 6.6-7S or 140-150,000 daltons) was detected on the DEAE-cellulose column (62).

With regard to the subunit structure of DNA polymerase α , Holmes *et al.* (62) have concluded that the 8S (enzyme A) species is made up of a 55,000 dalton subunit associated with an active subunit of 155,000 daltons (enzyme C). This finding is consistent with the observation by

Sedwick *et al.* (44a) for polymerase α from human KB cells, except that these authors reported a subunit size of 77,000 daltons. More recently, Matsukage *et al.* (112) have shown that polymerase α of mouse myeloma consists of two subunits, one of 150,000 and the other of 60,000 daltons. Discrepancies in subunit size between enzymes from human KB cells, and calf thymus or mouse myeloma are probably due to the use of non-homogeneous enzyme preparations; before arriving at any final conclusions it is essential that homogeneous enzyme preparations be obtained.

(d) Subcellular Localization

Both early (113), as well as recent studies (37, 43, 69, 95) directed at determining the subcellular localization of DNA polymerase α are in conflict with the presumed site of action of the enzyme - that is, the major portion of the DNA polymerase activity in mammalian cells is usually recovered in the cytoplasm. Explanations have been offered for this, namely, it has been suggested that the finding of this activity in the cytoplasm may be due to leakage from nuclei during their isolation, or that it may represent *de novo* cytoplasmic synthesis of enzyme to be translocated to the nucleus when needed for DNA synthesis (114). Although a number of investigators have failed to detect any DNA polymerase α activity in the nucleus (37, 43, 69, 95, 127, 128), other workers were successful in doing so (28, 41, 68, 125, 126). Chang and Bollum (15, 37) have shown however that removal of the nuclear outer membrane yields nuclei devoid of DNA polymerase α , and that failure to remove the outer membrane yields nuclei displaying polymerase α activity.

Non-aqueous isolation methods for nuclei have revealed that DNA polymerase α could indeed be found in nuclei (7, 115-117). It has been shown, either through the use of non-aqueous isolation media (120), or the use

of 30% glycerol supplemented with 4 mM CaCl_2 with no buffer (118-119), or through enucleation of cells by treatment with cytochalasin B (121), that as much as 90% of the total DNA polymerase α activity can be found in the nucleus (118-121). Thus it appears that the finding of DNA polymerase α in the cytoplasm is most likely due to its leakage from the nucleus, and thus the paradox with regard to subcellular localization and function has been resolved.

The possibility that DNA synthesis may be initiated at the nuclear membrane aroused interest in trying to locate the polymerase α at such a site. Thus, the enzyme has been reported to be associated with the nuclear membrane-chromatin fraction (54, 122, 123), but it is not clear whether the enzyme is bound only to the DNA or nucleoprotein complexes (123), or whether it is an integral part of the membrane itself. The enzyme has also been reported to be associated with smooth microsomal membranes (42, 43, 54, 124).

(e) Inhibition Studies

The possibility of using inhibitors as tools for distinguishing various DNA polymerases and for determining the biological function of DNA polymerases, as well as their potential use for cancer chemotherapy has stimulated much of the work directed at exploring the effect of various inhibitors on DNA polymerases. A list of some of the inhibitors investigated for their effect on DNA polymerase α is given in Table V. With regard to their possible use in distinguishing DNA polymerase α from other DNA polymerases, the sulfhydryl blocking reagents, N-ethylmaleimide (NEM) (26, 27, 42, 42a, 48, 51, 51a, 52, 55, 57-60, 84, 88, 97-99), p-chloromercuribenzoate (p-CMB) (26, 27, 41, 42, 43, 44, 48, 51a, 55, 84, 99, 122), and p-hydroxymercuribenzoate (p-OHHgBz) (53),

TABLE V. Inhibitors of DNA Polymerase α

INHIBITOR	SOURCE OF POLYMERASE	REFERENCE
NEM*	BHK cells (+)**	58, 59, 97, 98
	Rat ascites hepatoma (+)	26, 27, 55, 99, 42a
	Rat intestinal mucosa (+)	48
	Bone marrow (+)	52
	Chick embryo (+)	51
	HeLa cell S ₃ nuclei (+)	60
	Developing chick heart (+)	27
	Chinese hamster cells (+)	57
	Cultured mouse L929 cells (+)	84
	Normal human blood lymphocytes (+)	51
	Mouse myeloma (+)	88
	Morris hypatoma (+)	42
pCMB	Rat ascites hepatoma (+)	26, 27, 55, 42a, 99
	Adult rat liver (+)	43, 41
	Rat intestinal mucosa (+)	48
	Chick embryo (+)	51
	Human KB cells (+)	28, 44a
	Cultured mouse L929 cells (+)	84
	Morris hepatoma (+)	42
pOHHgBz	Calf thymus nuclear membrane (+)	122
	LF hepatoma (+)	53
Heparin	BHK cells (+)	58, 59, 97, 98
Ethidium Bromide	Reticulocytes (+)	50
	Bone marrow (+)	52
	Human KB cells (+)	28, 44a
Actinomycin D	Bone marrow (+)	52
Acridine Orange	Human KB cells (+)	28, 44a
Nalidixic Acid	Rat intestinal mucosa (+)	48
	Bone marrow (-)	52
araCTP	Developing chick heart (+)	27
	Rat liver (+)	129
Haem	Reticulocytes (+)	50
	Bone marrow (+)	52
AF/013	Reticulocytes (+)	50
	Bone marrow (+)	52
NaPPi	Rat ascites hepatoma (+)	26, 27, 55, 42a, 99
	Human KB cells (+)	28, 44a
Methanol, Ethanol and Isopropanol	Mouse myeloma (stimulated)	88
	Rat ascites hepatoma (+)	26, 27, 55, 42a, 99

* For list of abbreviations refer to page (xi)-(xii).

** "+" indicates inhibition and "-" indicates no effect.

enable us to distinguish DNA polymerase α from β . As noted in Table V, polymerase α is extremely sensitive to these compounds whereas DNA polymerase β , as we shall see later, is relatively resistant.

Heparin has also been reported to inhibit DNA polymerase α from BHK cells (58, 59, 97, 98). Furthermore, haem and the rifamycin derivative 3-formyl rifampicin SV:O-n-octyloxime (AF/013) have been reported to inhibit the polymerase α from reticulocytes (50) and bone marrow (52). Other compounds reported to have an inhibitory effect on DNA polymerase α are: sodium pyrophosphate (26-28, 42a, 44, 55, 99) 1- β -D-arabinofuranosyl cytosine 5'-triphosphate (araCTP) (27), and isopropanol, methanol, and ethanol (26, 27, 42a, 55, 99). However, in the case of the latter compounds, their effect on the polymerase α from mouse myeloma was of a stimulatory rather than of an inhibitory nature (88).

Less specific inhibitors, exerting their effect by binding to the template, consist of compounds such as ethidium bromide (28, 44a, 50, 52), actinomycin D (52), nalidixic acid (48, 52), and acridine orange (28, 44a).

(f) Biological Function

The most unambiguous approach in determining a function for a particular enzyme (e.g., DNA polymerase α), is to isolate a mutant lacking or having a defective enzyme, and correlating this deficiency with the loss of a specific biological function (e.g., DNA replication). Such an approach is relatively simple when dealing with prokaryotes, but with eukaryotes it is extremely difficult to obtain such mutants. Due to this difficulty, alternate approaches have been taken in the attempt to assign a biological function to DNA polymerases. Such approaches have consisted of comparisons between the relative levels of different DNA polymerase activities and the *in vivo* rates of DNA synthesis in tissues

and cell cultures challenged to proliferate, comparisons of the relative levels of different DNA polymerases in tissues from quiescent to actively proliferating states, and the disappearance of the enzyme in terminally differentiating systems, as well as a comparison in properties of the various DNA polymerases and that of an actively proliferating complex (i.e., effect of unwinding proteins on polymerases as well as their ability to use RNA-primed DNA templates). All such evidence is circumstantial and does not lead to the assignment of a function with certainty.

A series of studies making use of such indirect approaches have implicated DNA polymerase α with DNA replication. A positive correlation between the level of activity of this enzyme and the *in vivo* rate of DNA synthesis has been reported in the following systems: regenerating rat liver (42, 95, 127, 130), mouse L cells (95), HeLa cells (60, 131, 132), BHK cells (133), chemically induced tumors (78, 95), PHA-stimulated normal human lymphocytes (51, 134), tissue culture cells at various growth rates (8), erythropoietine-induced mouse spleens (135), and phenylhydrazine-induced reticulocytosis in mouse spleens (78, 95). In addition, it has been shown that terminally differentiating chick myoblasts lose their DNA polymerase α activity while they retain polymerase β (53a). Although incapable of undergoing further mitosis, the myotubes retain the ability to repair DNA (53a). Furthermore, it has been observed that a decrease in DNA polymerase α activity occurs during development of mouse embryo brain (136). All the results obtained with the above systems clearly indicate that the level of DNA polymerase α varies according to the rate of cell proliferation and thus parallels DNA replication.

A temperature-sensitive mutant of the smut fungus *Ustilago maydis* having a heat labile DNA polymerase activity has been isolated (137).

This organism apparently has no DNA polymerase β (138, 153) so all activity is due to polymerase α . Consistent with a replicative role for DNA polymerase α , the temperature-sensitive mutant loses the ability to replicate its nuclear DNA at the restrictive temperature while retaining the ability to replicate its mitochondrial DNA [P. Unrau, personal communication to Banks *et al.* (138)].

Generally speaking, replication is a complex process involving the participation of not only DNA polymerase, but also of several other accessory proteins (139). As in the case of prokaryotic systems, a number of reports dealing with protein factors having a stimulatory effect on DNA synthesis in eukaryotic systems have appeared in the literature (140-146). It is interesting to note that when such stimulatory factors were investigated with regard to their effects on *in vitro* DNA synthesis, in the presence of either polymerases α and β , a substantial stimulation of activity was observed with the polymerase α only and no such enhancement was evident with β (140, 141, 143, 146) or mitochondrial DNA polymerase (140).

The observation in both bacterial (147-149) and mammalian (74, 150-152) systems that RNA serves as a primer for *in vivo* DNA synthesis has led to a comparative study on the ability of both polymerases α and β to make use of RNA-primed DNA templates for DNA synthesis (62, 74-76). The overall conclusion from such investigations is that only DNA polymerase α possesses the ability to use RNA-primed DNA templates for DNA synthesis, consistent with a possible replicative function for this enzyme.

Thus there exists a great deal of evidence that DNA polymerase α is the replicating enzyme, even though much of this evidence is circumstantial.

(B) DNA Polymerase β (a) Basic Distinguishing Features

Although DNA polymerase β was unambiguously identified in 1971, it was probably discovered much earlier (7-9). The enzyme represents approximately 5-15% of the total DNA polymerase activity in actively proliferating tissues and about 50% in non-proliferating tissues (10). It can be distinguished from other cellular DNA polymerases on the basis of its low molecular weight as well as its resistance to sulphydryl blocking reagents (10, 12, see Table II). Chang (153) has recently analyzed various organisms for DNA polymerase β activity and has concluded that the enzyme is widely distributed in cells from multicellular animals but is absent in bacteria, plants, and protozoa.

Some reported properties of DNA polymerase β are summarized in Table VI.

(b) Purification

Unlike DNA polymerase α , DNA polymerase β has been purified to homogeneity (44a, 89, 145, 159-161, 163). This success can undoubtedly be attributed to the tight association of the polymerase with chromatin, making it much easier to eliminate the bulk of other cellular proteins (159-161). The enzymes from human KB cells (44a, 159), calf thymus chromatin (160, 161) and Novikoff hepatoma cells (163) have been purified to homogeneity as indicated by SDS-gel electrophoresis (44a, 159) and analytical ultracentrifugation (160, 161, 163). In addition partially purified enzymes have been prepared from other systems: 154-fold purification from BHK-21/C13 cells (59, 96), 100 to 600-fold from rabbit bone marrow (37) and 290-, 2,200-, and 9,000-fold purification from normal human lymphocytes (51), rat ascites hepatoma cells (27, 42a, 54,

TABLE VI. Induction of RNA Polymerase E

Source	Nucleoside or (nucleotide) added	Template			Induction Coefficient (C/D)		Station (hr., C/D)		pH	Station
		A	B	E	++	+	hr.	hr.		
Adult rat liver	(nuclear)	++	++	++	3-4 x (29,000)		15-25	HR	7.4-8	41, 125
	(nuclear)	++	HR	+	HR		10	HR	50 (hr.23)	49
	(nuclear)	++	-	-	3.58 (44,000)		10	HR	7.7 (Trie-4023)	47
	(ribosomal, nuclear)	++	HR	+	HR		10-15	+	9 (glycine)	43
Regenerating rat liver	RNA polymerase I (nuclear & ribosomal)	+	HR	HR	(25,000)		15	HR	HR	42
Rat brain	(nuclear)	HR	+	+	3-48		5-5	HR	7.5	45, 46
Rat intestinal muscle	(nuclear)	++	-	+	HR (39,000)		HR	HR	50	48
Differentiating embryonic muscle	RNA polymerase	++	+++	HR	(18-22,000)		HR	HR	HR	53a
Rat anterior thymic cells	RNA polymerase P ₂ (nuclear, anterior thymic)	++	+	++	(44,000)		16	0.5	↓	9.8 (glycine) 27, 42a, 54, 137
Chick embryo	RNA polymerase Cyttoplasmic RNA poly- merase II (nuclear & cytoplasmic)	HR	HR	+	(27-31,000)		-	+	no effect	HR
		HR	HR	+	35 (59,000 and 27,000)		HR	HR	120	HR
Rabbit bone marrow & spleen	(nuclear and cyto- plasmic)	+	HR	HR	3.35 (30-40,000)		8-14	0.15-0.50	HR	8.4
Rabbit bone marrow	(nuclear and cyto- plasmic)	+	HR	HR	3.35 (29-30,000)		5-20 (data)		Template dependent	37
Human KB cells	RNA polymerase K1	++	Poor	Poor	(35-38,000)		20	HR	120	9.2
KB cells	(nuclear)	HR	+	+	HR 2.4-2.35		4	2	HR	4.6 (Trie-maleate)
Cardiac muscle		HR	+	++	HR 3.58		HR	HR	HR	9.0
HeLa S3 cells	RNA polymerase	+++	+	++	HR 3.58 (45,000)		15-25	HR	HR	9.0
Calif thymus	(cytoplasmic)	+	HR	HR	+(44-45,520)		6-12 (data)	HR	40-100	9.2 (formal- dehyde)
HEC cells	(nuclear)	+	HR	HR	HR 2.475		HR	HR	↑	6.15 (Trie-4023)
HEC-21/213 cells	RNA polymerase II (nuclear)	+	Poor	Poor	3.35		4-12	HR	60	8.5 - 8.9
Normal human blood lymphocytes	RNA polymerase II	+	HR	HR	3.35 (20,000)		5-10 (data)	HR	HR	7.9
Chinese hamster ovary cells	(nuclear)	++	HR	HR	45 (49,000)		HR	HR	HR	8.10
Neuro L329 cells	(nuclear)	++	HR	+	HR (25,000)		HR	HR	↓	HR
Neuro spleen		+	HR	HR	2.58		HR	HR	HR	HR

1. Symbols used: A, B, R and S refer to "activator", heat denatured, native and synthetic templates respectively. HR indicates not reported. An arrow pointing downwards ("↓") indicates inhibition and one pointing upwards ("↑") indicates stimulation. A "no" sign means that the designated template or divalent cation is used, whereas a "+" sign means that it is not used.

157) and chick embryo (158), respectively. DNA polymerase β from the cytoplasmic fraction of chick embryo has also been purified to 90% homogeneity (63).

(c) Properties

(i) Template Specificity

Of all natural templates examined, "activated" DNA is the most efficient template used by DNA polymerase β (43, 69, 163, see also Table VI). DNA templates are preferred over RNA templates; for example, the enzyme will copy poly (dA)·(dT)₁₅ more efficiently than poly (rA)·(dT)₁₅ (51, 69, 159, 161, 164). It has also been reported that "activated" DNA and poly (rA)·oligo (dT)₁₅ are equally good templates (51, 69, 165).

DNA polymerase β will not use DNA templates primed by oligo RNA primers. For example, poly (dT)·oligo (rA) will not be copied to any great extent (76). In addition, the enzyme will not initiate synthesis on an heteropolymeric RNA primer (79). The enzyme does utilize the following synthetic templates: poly d(A-T) (26, 27, 42a, 52, 89, 156, 157), poly (rA)·poly (dT) (51, 51a, 156), poly (dA)·(dT)₁₀ (51a, 52, 89), poly (rA)·poly (rU) (51), and poly (dC)·(dG)₁₅ (15).

(ii) Divalent Cation Requirement

The optimal Mg^{2+} concentration for DNA polymerase β from various sources lies in the range of 3-25 mM depending on the source of the enzyme as well as the assay conditions used (see Table VI). As in the case of polymerase α , the optimal concentration is affected by both the nature of the template (69) as well as pH (26, 27, 55, 157). When synthetic templates are used, the preferred divalent cation is Mn^{2+} . Optimum Mn^{2+} concentrations have been reported in the range of 0.25-2 mM (see Table VI). In the case of the chick embryo DNA polymerase β , the divalent

cation requirement could be satisfied only by Mn^{2+} and not Mg^{2+} (158).

(iii) Effect of Monovalent Cations

As noted previously (Section III A(c)iii) the effect of monovalent cations on DNA polymerase α was primarily inhibitory. On the other hand, the effect of such cations on DNA polymerase β is usually stimulatory (see Table VI). The optimum monovalent cation concentrations for enzymes from various sources has ranged from low [e.g., 30-70 mM (42, 154, 155), 50 mM (156), and 60 mM (59, 96)] to high [e.g., 100 mM (44a, 28, 159), 120 mM (63), and 40-200 mM (160, 161, 166)]. DNA polymerase β from chick embryo (158) however, was not affected by salt, and that from mouse L929 cells (84), was actually inhibited.

(iv) pH Optima

In contrast to DNA polymerase α , the pH optimum for DNA polymerase β lies in the alkaline range (see Table VI). However, in the case of the enzyme from KB cells (39), a pH optimum near neutrality (pH 6.6) in Tris-maleate buffer has been reported. As with DNA polymerase α , the optimum pH is influenced both by the nature of the template (69, 160, 161) as well as the nature of the buffering species used (see Table VI).

(v) Isoelectric Point

Most investigators have reported that DNA polymerase β is a basic protein. Isoelectric points of 9.15, 9.20 and 9.40 have been reported for the enzymes from chick embryo (158), human KB cells (44a, 28, 159) and normal human lymphocytes (51) respectively. DNA polymerase β from calf thymus chromatin is also a basic protein with an pI of 9.5. Amino acid analysis of the enzyme, however, revealed no predominance of basic amino acids in the polypeptide, thus the basic pI can only be explained by assuming that a significant percentage of the acidic amino acids are in

the amide form (L.M.S. Chang in ref. 78).

(vi) Size

In comparison to DNA polymerase α , which has a sedimentation value of 6-8S, the sedimentation coefficient of DNA polymerase β is low, usually in the range of 3-4S. In chick embryo, two forms of DNA polymerase β have been found with molecular weights of 27,000 and 54,000 daltons and it has been suggested that the 27,000 dalton species represents a subunit of the larger enzyme form (51a, 63). However, in the case of DNA polymerase β from calf thymus (160) and human KB cells (167), it has been shown by SDS-gel electrophoresis that the enzyme consists of a single polypeptide chain of 42,000-45,000 daltons, while polymerase β from Novikoff hepatoma cells (163) has a molecular weight of 32,000 daltons. Except in the case of SDS-gels, it must be kept in mind that caution must be exercised when determining the size of the enzyme since it has the ability to aggregate to larger size species (53a, 159, 168, see also Table IV).

(d) Subcellular Localization

DNA polymerase β is found mainly in the nucleus (15, 28, 37, 41, 49, 57, 58, 60, 63, 69, 84, 96, 101, 125, 154, 155, 170, 171), although its presence in the cytoplasm (15, 37, 171), and association with the ribosome fraction in both hepatoma cells (42, 71) and in normal and regenerating rat liver (42, 43, 71, 124) has also been reported. Recent reports indicate, however, that its presence in the cytoplasm as well as in the ribosome fraction is probably a fractionation artefact due to leaching of the enzyme from the nucleus (118-121, see Section III A(d)). Its association with ribosomes is certainly not surprising since the enzyme is a basic protein, and the non-specific adsorption of such proteins to ribosomes is a well known phenomenon (172). This suggestion is supported

by the observation that DNA polymerase β can be dissociated from the ribosome fraction through a 0.2 M salt wash (71). An alternate explanation previously offered, is that the cytoplasmic presence of the enzyme may merely reflect the site at which the *de novo* synthesis occurs prior to translocation to the nucleus (114), its site of action.

(e) Inhibition Studies

Unlike DNA polymerase α , polymerase β is relatively insensitive to various inhibitors (compare Tables V and VII). For example, DNA polymerases β from BHK cells (58, 59, 96), rat ascites hepatoma cells (27, 42a, 99) and HeLa S_3 cells (60) (see Table VII), are insensitive to N-ethylmaleimide whereas the polymerases α from the same sources are sensitive (see Table V). In general, polymerase β is resistant to both NEM and pCMB although polymerase β from some sources [e.g., rat intestinal mucosa nuclei (48)] has been shown to be sensitive to such sulfhydryl blocking reagents. Polymerase β from BHK cells also was not inhibited by heparin (58, 59, 96), a compound which has a marked inhibitory effect on polymerase α (58, 59, 97, 98). Of special interest also is the nucleotide analogue araCTP which has no effect on DNA polymerase β from developing chick heart (51a) and rat liver (129) whereas it inhibited polymerase α from both sources. Acridine orange also had an inhibitory effect on polymerase α from human KB cells and no effect on polymerase β from the same source (28, 44a, 176). The effects of a series of other inhibitors on both activities are listed in Tables V and VII. These studies indicate that some inhibitors could in fact be used to distinguish the two polymerases.

(f) Biological Function

Approaches identical to those used for DNA polymerase α have also

TABLE VII. Inhibitors of DNA Polymerase β

INHIBITOR	SOURCE OF POLYMERASE	REFERENCE
NEM*	BHK cells (-)**	58, 59, 96
	Rat ascites hepatoma cells (-, +)	42a, 27, 99, 157
	Rat liver nuclei (+)	41, 43, 166
	Rat intestinal mucosa nuclei (+)	48
	HeLa S ₃ cells (-)	60
	Developing chick heart (-)	51a
	Chinese hamster cells (-)	57
	Normal human lymphocytes (-)	51, 169
	Regenerating rat liver and Morris hepatoma (-)	42
	Rat ascites hepatoma cells (-)	42a, 27, 99, 157
	Rat liver nuclei (+, -)	41, 43, 155
pCMB (pOHgBz)	Rat intestinal mucosa nuclei (+)	48
	Human KB cells (-)	28, 176
	Chinese hamster cells (-)	57
	Regeneration rat liver and Morris hepatoma (-)	42
Heparin	BHK cells (-)	58, 59, 96
Ethidium Bromide	Rat liver nuclei (-)	41, 43, 155
	Human KB cells (-)	28, 176
Acridine Orange	Human KB cells (-)	28, 176
Nalidixic Acid	Rat intestinal mucosa nuclei (- stimulates)	48
araCTP	Developing chick heart (-)	51a
	Rat liver (-)	129
NaPPi	Human KB cells (+)	28, 176
	Chinese hamster cells (+)	57
Methanol, Ethanol and Isopropanol	Rat ascites hepatoma (stimulated)	42a, 27, 99, 157
	Calf thymus chromatin (-)	160
Acetone	Calf thymus chromatin (-)	160
o-phenanthroline	Chick embryo (+)	63, 158

* For list of abbreviations refer to page (xi)-(xii).

** A minus (-) indicates no effect, whereas a plus (+) indicates inhibition.

been tried in attempting to determine the biological role of DNA polymerase β . The activity of polymerase β , however, unlike DNA polymerase α , shows little correlation with *in vivo* rates of DNA synthesis in a large variety of systems, among which are: regenerating rat liver (42, 95, 127, 130), tissue culture cells grown under various conditions (8, 95, 173), HeLa cells (60, 131, 132), BHK cells (133), chemically induced tumors (78, 95), PHA-stimulated normal human lymphocytes (51, 134), erythropoietine-induced mouse spleens (135) and phenylhydrazine-induced reticulocytosis in mouse spleens (78, 95). Reports contrary to this have, however, appeared in the literature. For example, Mayer *et al.* (174) found that when normal human lymphoid cells are stimulated with the mitogen PHA, DNA polymerase β is the only polymerase that manifests itself prior to DNA replication, whereas α and γ increase after DNA synthesis has occurred. Similar results were obtained by Ooka and Daillie (175) upon analysis of the cell cycle of synchronized KB cells. These authors observed little change in the level of DNA polymerase α throughout the cell cycle, whereas the level of β decreased in G_2 , M and early G_1 phases, and then increased to a maximum in the middle of the S phase. Thus, there is circumstantial evidence that polymerase β , as well as α , may participate in DNA replication.

A repair function for β has also been suggested. Wicha and Stockdale (53a) have shown that although terminally differentiating chick myoblasts lose their DNA polymerase α activity (as well as their ability to divide), they still retain DNA polymerase β and the ability to carry out DNA repair. Furthermore, Bertazzoni *et al.* (134) have shown that polymerase α in PHA-stimulated normal human lymphocytes has a temporal relationship to replication whereas β increased in level after replication had occurred,

coincident with a stage in which the cells were capable of performing DNA repair synthesis after UV damage. Thus according to their results DNA polymerase β is involved in DNA repair rather than replication.

Other features of DNA polymerase β which argue against a possible replicative function include: (a) its inability to be stimulated by DNA binding proteins (132, 140, 141, 143, 146) and (b) its inability to use RNA primers for DNA synthesis (62, 74-76, 79). However, the latter point only suggests that polymerase β may not be involved in initiation and does not preclude the possibility of its being involved in a later stage of replication. Indeed Weissbach *et al.* (132) have shown that polymerase α needs to participate only immediately after the RNA primer has been synthesized, and that after an initial addition of deoxynucleotides, chain elongation can be carried out by either α , β or γ .

(C) DNA Polymerase γ

(a) Basic Distinguishing Features

This is the most recent of the eukaryotic DNA polymerases to be described (77, 177). Its discovery was undoubtedly stimulated by the finding of the RNA-directed DNA polymerase (reverse transcriptase) activity in oncogenic RNA viruses. The enzyme, like the mitochondrial enzyme, is usually responsible for a minor fraction of the total cellular DNA polymerase activity, representing only approximately 1% of the total activity (131), although a level as high as 15% has been reported in some systems (178).

The enzyme has been shown to be ubiquitously distributed in rat tissues (178), in HeLa cells (77), human lymphoblastoid cells (179), calf thymus (180), mouse myeloma (87), human leukemic cells (14), murine

cells (170, 181, 182), rat and mouse cells (177, 183), and regenerating calf lens (184).

The enzyme can be distinguished from DNA polymerase α on the basis of its ability to use poly (rA)·(dT)₁₂ as template-primer, and from DNA polymerase β on the basis of its size, sensitivity to thiol blocking reagents, and ability to use poly (dT)·(rA)₁₀ as template-primer (see Table II). The enzyme can also be distinguished from viral reverse transcriptase on the basis of its inability to transcribe heteropolymeric RNAs into complementary DNA products.

(b) Purification

The extremely low levels of DNA polymerase γ have to date made it difficult to obtain a homogeneous preparation, although the activity from *Drosophila melanogaster* (79) has been purified to apparent homogeneity (2,300-fold with a sp. act. of 46,750 units/mg protein). The activities from other sources have also been purified quite extensively (see Table VIII). DNA polymerase γ from HeLa S₃ cells has also been extensively purified (60,000-fold, giving a sp. act. of 25,000 units/mg protein) by Knopf *et al.* (188).

(c) Properties

(i) Template Specificity

Table IX outlines the template specificity of DNA polymerase γ from various sources. The most specific and most efficient template-primer used by the enzyme is poly (rA)·oligo (dT). Poly (rA)·poly (dT) is also efficiently used for the synthesis of polydeoxythymidylate by most of the reported polymerases γ . Other synthetic templates may be either used or not used depending on the source of the enzyme and/or the reaction conditions employed (see Table IX). Whether such reported differences

TABLE VIII. Properties of DNA Polymerase Y

Source	pH	$\frac{2+}{Mn}$	Cation Opt. $\frac{2+}{Mg}$	K^+	pH Opt.	Purification	References
HeLa cells WI 38 cells	NR	++	+	.12 M (I)* .075 M (II)	NR	500-fold	77
HeLa S3 cells	160-330,000	.5 mM [(A) _n ·(dT) ₁₂₋₁₈]	5 mM ("A" DNA)	.13 M	8.5 [(A) _n ·(dT) ₁₂₋₁₈]	60,000-fold (25,000 units/mg protein)	188
Normal rat liver and hepatomas	NR	0.5 mM	5-20 mM	<50 mM	8-9.5	NR	177
Chick embryo	NR	.25-.5 mM (rA _n -dT _n) 1 mM	7.5 mM ("A" DNA) Not used	.075-.1 M .12 M	NR NR	NR NR	185 189
<i>Drosophila</i> <i>melanogaster</i>	87,000	.4 mM (synthetic templates)	1 mM "A" DNA	NR	8.5	2,300-fold	79
Cultured murine cells	NR	.5 mM	NR	.125 M	NR	NR	178
Calf thymus	68,000 63,000	.5 mM	partially 2+ replaces Mn	NR	NR	450-fold	180
House myeloma	230,000	NR	NR	NR	NR	18,000-fold	190
Chick embryo heart	50,000	Used	Not used	NR	NR	1,000-fold	186
Mammalian cells	NR	Most efficient	partially 2+ replaces Mn	NR	NR	NR	191

* "I" refers to DNA polymerase YI and "II" to polymerase YII. "A" DNA refers to "activated" DNA, and "NR" to not reported.

TABLE IX. Template Specificity of DNA Polymerase γ

Source	Act. DNA		Natural RNA	Templates					$r(A-U)_n$	References
	primed	RNA		$rA_n \cdot dT_{12}$	$dA_n \cdot \text{oligo } dT$	$dT_n \cdot rA_n$	$dA_n \cdot dT_n$	$rA_n \cdot rU_n$	$d(A-T)_n$	
HeLa cells	I	+	-	+	NR	NR	NR	-	NR	77
	II	+	-	+	NR	NR	NR	-	NR	77
Normal rat liver and hepatomas	-	-	-	NR	NR	+	NR	+	-	177
Chick embryo	+	+	NR	+	NR	+	+	-	NR	189, 186, 185
<i>Xenopus laevis</i> oocyte	+	+	NR	+	+	+	NR	+	NR	79
Murine spleen	NR	NR	NR	+	NR	NR	NR	NR	+	187a
Mouse embryo	NR	NR	NR	+	NR	+	NR	NR	NR	170
Mouse myeloma	-	NR	NR	+	+	NR	NR	NR	NR	87, 190
Cultured murine cells	+	NR	-	+	NR	+	NR	NR	NR	178
Calf lens	NR	NR	NR	+	NR	NR	NR	NR	NR	184
Calf thymus	NR	NR	NR	+	NR	NR	NR	NR	NR	180
Mammalian cells	NR	NR	NR	+	NR	+	NR	NR	NR	191
Rat mammary tumors	NR	NR	NR	+	-	NR	NR	NR	NR	187

* Symbols used: A plus ("+") indicates that the designated template has been reported to be used by the enzyme, whereas a minus ("-") indicates that it is not used. "NR" refers to not reported, "I" to DNA polymerase γ I and "II" to polymerase γ II.

reflect real differences in the enzymes, or changes in the assay conditions, or in the degree of contamination by various other DNA polymerases, is not clear at present. "Activated" DNA has been reported to be both used (77, 79, 138, 178, 185) as well as not used (87, 177), by this class of enzymes. Natural RNA templates are either not used as template (77, 177, 178) or partly used (185) - that is, only the poly (rA) tract of the mRNAs examined were transcribed. This inability to use natural RNAs as templates for heteropolymeric DNA synthesis distinguishes DNA polymerase γ from viral reverse transcriptases.

(ii) Divalent Cation Requirement

Divalent cation optima as well as other properties of polymerase γ are listed in Table VIII. With synthetic templates, the divalent cation requirement is met by Mn^{2+} , whereas with natural DNA templates, the requirement is best satisfied by Mg^{2+} (see Table IX). The optimum Mn^{2+} concentration is usually about 0.5 M (177, 178, 180, 185), although an optimum of 1 mM (189), has also been reported. In general, with synthetic templates, Mg^{2+} is incapable of replacing Mn^{2+} (see Table VIII).

(iii) Effect of Monovalent Cations

Monovalent cations have in general been reported to stimulate DNA polymerase γ from various sources. The optimum KCl concentration usually lies in the vicinity of 125 mM (77, 138, 178, 189), although lower optimum concentrations have also been reported [i.e., 75 mM for polymerase γ II from HeLa cells (77), and 75-100 mM for chick embryo DNA polymerase γ (185)]. In contrast to this, it has been reported that polymerase γ from normal liver (177) is inhibited by concentrations of KCl greater than 50 mM.

(iv) pH Optima

In general polymerase γ displays maximum activity at alkaline pH, namely in the region of 8-9.5 (79, 177, see also Table VIII).

(v) Isoelectric Point

An isoelectric point of 5.8 has been reported for the enzyme from mouse myeloma (190).

(vi) Size

The enzymes from various sources appear to differ in size, ranging from 50,000 daltons in chick embryo (186) to 230,000 daltons (6-8S) in mouse myeloma (190) (see Table VIII).

(d) Subcellular Localization

In the case of HeLa cells, two distinct DNA polymerases γ have been reported, one in the cytoplasm (γ I) and one in the nucleus (γ II) (77). Ninety percent of DNA polymerase γ from mouse myeloma was found to be cytoplasmic in origin (178).

(e) Inhibition Studies

DNA polymerase γ from HeLa cells (188), developing chick embryo (189) and L-cells (178), has been shown to be sensitive to the thiol blocking reagents pCMB (178, 188, 189) and NEM (188), and in this respect differs from polymerase β . In addition the HeLa cell enzyme has also been shown to be sensitive to sodium pyrophosphate and phosphonoacetic acid (188).

(f) Biological Function

Like other DNA polymerases, the biological function of DNA polymerase γ is still an enigma. However, some interesting observations that may link the enzyme with a replicative function have been made. First, both forms of polymerase γ from HeLa cells have very low K_m 's for deoxy-

nucleoside triphosphates (approx. $0.5 \mu\text{M}$) (192), which are approximately 10-fold lower than those for polymerase α and β . There is reason to suspect that a DNA polymerase with a low K_m for the deoxynucleoside triphosphates plays an important role in replication, since the concentration of the deoxynucleoside triphosphates is relatively low at the beginning of the S phase (192a, 192b). Thus it would be expected that DNA polymerase γ would function more efficiently at the beginning of this phase. Secondly, it has been observed that polymerase γ like polymerase α , increases in activity during the S phase of the cell cycle (131). However, unlike DNA polymerase α , and like polymerase β , DNA polymerase γ is not affected by DNA binding proteins (132), nor can the enzyme use RNA primers for DNA synthesis.

(D) Mitochondrial DNA Polymerase

(a) Basic Distinguishing Features

As the name implies this DNA polymerase is restricted to the mitochondria. The enzyme constitutes only a minor fraction ($< 1\%$) of the total cellular DNA polymerase activity (78, 193). On the basis of differences reported in the size and properties (i.e., stimulation by salt) of mitochondrial DNA polymerase, Tibbetts and Vinograd (194) and Hecht (106) have speculated on the possible existence of multiple DNA polymerases. Experimental evidence in support of this has recently been provided by Radsak and Seidel (193).

Mitochondrial DNA polymerase is distinguishable from other eukaryotic DNA polymerases not only on the basis of subcellular localization, but also on its biochemical characteristics. The enzyme has a relatively high sedimentation coefficient (8-9S) (154), thus easily distinguishable

from DNA polymerase β (see Table II), and is fairly resistant to inhibition by thiol blocking reagents (195, 196), hence differing from polymerase α . It also has the unique feature of being unable to transcribe poly (dC) \cdot (dG)₁₂ which is efficiently utilized by other eukaryotic DNA polymerases (10). The enzyme in addition is extremely heat labile compared to DNA polymerases α and β , having a half-life of less than five minutes at 45^o C (195, 196). The enzyme was initially thought to have an endonuclease activity associated with it, however this activity has recently been shown to merely represent a contaminant (197). Some specific properties of the mitochondrial DNA polymerase are summarized in Table X.

(b) Purification

The extreme lability of mitochondrial DNA polymerase made the purification of the enzyme difficult; even so, reasonable degrees of purification have been achieved (see Table X). The enzyme is easily separable from other cellular DNA polymerases since it is tightly associated with mitochondria, and also because of its binding characteristics to ion exchange absorbents relative to other cellular DNA polymerases (195, 201, 202).

(c) Properties

(i) Template Specificity

Mitochondrial DNA polymerases from different sources exhibit differences in template preference (109-111). However such differences may be attributed to various degrees of purity of the enzyme [e.g., contaminating nucleases (110, 111, 194)], and also to the existence of more than one DNA polymerase in the mitochondria (193). Like DNA polymerase α , mitochondrial DNA polymerase is unable to copy oligodeoxy-

TABLE X. Properties of Mitochondrial DNA Polymerase*

Source of Mitochondria	MR or Sedimentation Coefficient	Cation Optima		pH Opt.	Purification	Reference
		Mg ²⁺	Mn ²⁺			
Rat intestinal mucosa	100,000 daltons	5 mM	2 mM (30% as efficient as Mg ²⁺)	40 mM (50% [†])	400-fold	198
Rat liver	NR	12 mM	NR	NR	22-fold	181, 201
	150,000 (9.2S)	12 mM	NR	NR	NR	43, 154
	NR	7.5 (changes with purification)	NR	150 mM	60-fold	18
	NR	3-10 mM	1.5 mM (20-30% as effective as Mg ²⁺)	150 mM	3,550-fold	196
	NR	NR	NR	6-7-fold stimulation	NR	
Marine liver	150-170,000 (8.8S)	NR	NR	150 mM (7-fold stimulation)	NR	106
Mouse testis	(7-10S)	4-8 mM	NR	NR	NR	104
Bovine spermatozoa mitochondria (1)	150,000 (8.8S)	8-14 mM	NR	150-170 mM	NR	199
HeLa cells	106,000	10 mM (+50 mM KCl)	NR	NR	234-fold	195
	100,000 (45,000 & 60,000 subunits)	20 mM (- KCl)	NR	NR	ap. act. 1,000 units per mg protein	200
	NR	NR	NR	NR	NR	197
Yeast	150,000	50 mM	NR	50 mM (no significant activation)	NR	202

* For abbreviations and symbols used in this Table, see Table III.

nucleotide initiated homopolymers such as poly (A)·(dT)₁₂₋₁₈, poly (U)·(dA)₁₂₋₁₈, poly (C)·(dG)₁₂₋₁₈, or poly (I)·(dC)₁₂₋₁₈ (197), and in this regard is distinguishable from polymerase γ (192, 195). Although poly (dC)·(dG)₁₅ is efficiently utilized by other DNA polymerases, the mitochondrial DNA polymerase is incapable of using it as a template to any appreciable extent (10). Early studies on template specificities showed a high preference for circular mitochondrial DNA as a template (201). This, however, was presumably due to the generation of 3'OH initiation sites by a contaminating endonuclease in the enzyme preparation (201).

(ii) Divalent Cation Requirement

Magnesium is the preferred divalent cation for the mitochondrial DNA polymerase (see Table X). The Mg^{2+} optima lie in the range of 5-20 mM. One must keep in mind however that the optimum may change with the degree of purification of the enzyme (18) and also with monovalent cation concentration (195). For example, the HeLa cell mitochondrial DNA polymerase exhibited a Mg^{2+} optimum of 10 mM in the presence of 50 mM KCl and an optimum of 20 mM in the absence of the salt (195). Manganese can also be used by the enzyme, but it can only partially replace magnesium (196, 198).

(iii) Effect of Monovalent Cations

One of the most unique features of mitochondrial DNA polymerase is the large stimulation in activity observed in the presence of high concentrations of monovalent cations (18, 106, 196, 199). A 5- to 7-fold stimulation in activity of the enzyme from murine (106) and rat liver (18) mitochondria has been observed with 150 mM KCl. The activity from rat intestinal mucosa was stimulated 50% in the presence of 40 mM salt

(198) and that from HeLa cell mitochondria 60% in the presence of 50 mM salt (195). In the view of work by Radsak and Seidel (193), such large differences in salt concentration optima may be due to the researchers examining different mitochondrial DNA polymerases.

(iv) pH Optima

Various pH optima have been reported for the mitochondrial DNA polymerase (see Table X). The range lies between 7.5 and 9.2 and is dependent on the nature of the buffer (198, 199, 201), and probably on the degree of purification of the enzyme.

(v) Isoelectric Point

On the basis of its high affinity for phosphocellulose, the mitochondrial enzyme is a basic protein (195, 201, 202).

(vi) Size

The mitochondrial DNA polymerase, like DNA polymerase α , is of relatively large size and its molecular weight appears to be dependent on the degree of purification of the enzyme preparation used for the determination. The partially purified enzymes from rat intestinal mucosa (198) and HeLa cell mitochondria (195) behave as species of 100,000 and 106,000 daltons, respectively. The most highly purified enzyme reported, from HeLa cell mitochondria (197), also has a molecular weight of 100,000-110,000 and by SDS-gel electrophoresis appears to be made up of two subunits with molecular weights of 45,000 and 60,000. This mitochondrial DNA polymerase represent mitochondrial DNA polymerase I according to Radsak and Seidel (193), and the 150,000 dalton species reported by others (see Table X) may represent mitochondrial DNA polymerase II.

(d) Submitochondrial Localization

The observation by Schultz and Nass (61), that the specific activity

of endogenously-primed DNA synthesis is higher in membrane components than in the whole mitochondria, in addition to the possible initiation of replication at a membrane site in other systems (54, 122, 123), has stimulated the investigation of the submitochondrial localization of mitochondrial DNA polymerase (203). Through selective disintegration of the outer and inner membranes of the mitochondria, it was established that the DNA polymerase is fixed to the inner side of the inner membrane (203). This is consistent with the recent observation that DNA synthesis occurs in conjunction with a DNA inner membrane complex of rat liver mitochondria (204).

(e) Inhibition Studies

Mitochondrial DNA polymerase from HeLa cells (195), like polymerase β , is relatively insensitive to NEM when compared to DNA polymerase α . The activities from both murine liver mitochondria (106) and rat intestinal mucosa mitochondria (198), however, are sensitive to this thiol blocking reagent. Among some of the most potent inhibitors of mitochondrial DNA polymerase are the mutagenic dyes ethidium bromide (106, 196) and acriflavin (196, 202). Other inhibitors include potassium phosphate buffer (47), pyrophosphate (196, 198, 201), inorganic phosphate (198), nalidixic acid (198), and actinomycin D (196).

(f) Biological Function

Substantial evidence indicates that mitochondrial DNA replication is independent of the mitotic cycle of the cell (206). For example, it has been shown that the incorporation of precursors into mitochondrial DNA proceeds continuously and at a greater rate than the synthesis of nuclear DNA. Nass (205) has reported that mitochondrial DNA synthesis proceeds at 18 times the rate of nuclear DNA synthesis in the 12 hr

regenerating rat liver. Further evidence for this independence derives from the higher turnover rate of mitochondrial DNA relative to nuclear DNA (202, 205). Such observations suggest a considerable degree of autonomy in the biogenesis of mitochondria. However considerable evidence exists that the synthesis of the polymerase, or of a factor affecting the polymerase activity, may be under both nuclear (202, 207, 208) as well as mitochondrial control (193).

The most obvious function for mitochondrial DNA polymerase is the replication of mitochondrial DNA. Circumstantial evidence in support of such a function has been provided by Schultz and Nass (61) who have observed that the rate of synthesis of mitochondrial DNA is reflected in the specific activity of mitochondrial DNA polymerase. A repair function for the enzyme has also been suggested (207, 208). Namely, it has been observed that when the protozoan *Tetrahymena pyriformis* is subjected to irradiation with UV light or X-rays, or exposed to ethidium bromide (207, 208) a 40-fold increase in the level of mitochondrial DNA polymerase activity occurs. As to whether one or both of the mitochondrial DNA polymerases recently reported (193) is or are involved in each of the above-mentioned functions is at present not known.

(E) RNA-Directed and RNase-Sensitive Endogenously Templated DNA Polymerases

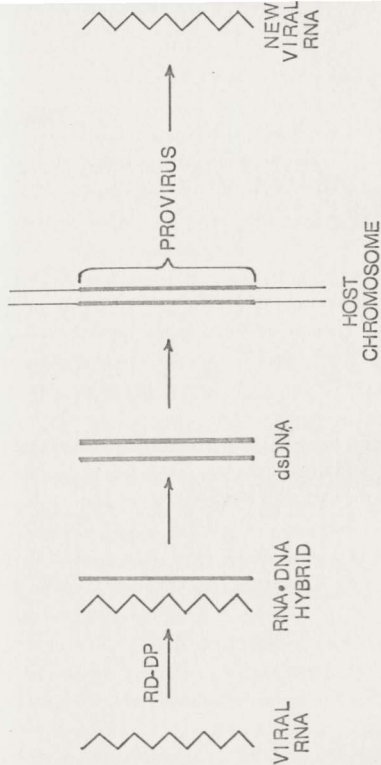
(a) Introduction

Endogenously templated DNA polymerases sensitive to RNase may be either RNA-directed or RNA-primed subject to whether the RNA moiety of the template-primer functions as a template or as a primer, respectively. RNase-sensitive activities falling in both categories have been described.

The RNA-directed DNA polymerases (RD-DP), the true "reverse transcriptases" were originally discovered in oncogenic RNA viruses independently by Mizutani and Temin (20) and Baltimore (21). Their discovery was the outcome of an attempt to explain how oncogenic RNA viruses could stably transform cells. Although the answer to this query was suggested by Temin (209) as early as 1964 in his *provirus hypothesis* (Scheme IV), formulated on the basis of indirect evidence, it was not widely accepted until the discovery of RD-DP (20, 21) which provided unambiguous evidence for it. Since its initial discovery in 1970 (20, 21), work on this enzymatic system in oncogenic RNA viruses has generated a voluminous literature and has been the subject of a number of recent reviews (13, 210, 211); thus it will not be dealt with in this particular review.

The nature of the reaction catalyzed by RD-DP suggested its possible involvement in a number of processes inherent to normal cells - that is, the enzyme might participate in functions other than the neoplastic transformation of cells by oncogenic RNA viruses. Namely, RD-DP could possibly function in the amplification of genes (212-217), cell differentiation (22), antibody formation (218) and memory (219, 220). Consequently, a number of workers have attempted to detect such an activity in various biological systems. In addition, the potential use of such an activity as a marker for oncogenicity made it necessary to determine whether its presence was limited to oncogenic RNA viruses or whether it was more widely distributed (221). Initial studies having these objectives in mind made use of the synthetic polynucleotide poly (rA)·oligo (dT) (177, 178, 221) as template-primer; however, it is now clear that such studies dealt with DNA polymerase γ (see Section III, C).

Other investigators have taken the approach of looking for endogenous-



PROVIRUS HYPOTHESIS

templated DNA polymerases that were sensitive to RNases. However, even this approach resulted in ambiguities, since it is now well known that RNA may serve as a primer for replicative DNA synthesis (62, 74-76). Thus, some investigators were probably detecting a replicating complex consisting of DNA polymerase α , a DNA template and an RNA primer, as suggested by Reitz *et al.* (222) in their study of the endogenous RNase-sensitive DNA polymerase complex from PHA-stimulated normal human lymphocytes.

The possibility that RNA may serve as either primer or template makes it imperative that certain criteria be met prior to declaring that a true "reverse transcriptase" exists in normal cells. Such criteria have been outlined by Gallo (223):

(1) If the activity is endogenously templated, it must be shown to be RNase-sensitive. Preliminary evidence for the function of RNA as a template may be obtained by showing this sensitivity with low concentrations of RNases in the presence of 0.2 M salt (223a).

(2) The enzyme must require all four deoxynucleoside triphosphates for maximum activity, in order to eliminate the possibility of terminal addition, and also to show that poly (rA) tracts of the RNA are not the only sequences transcribed.

(3) The reaction should be partially resistant to actinomycin D, or distamycin A.

(4) An RNA-DNA hybrid intermediate must be detected in the early part of the reaction.

(5) In the case of exogenously templated activities it must be shown that the product is DNA, and that it is complementary to the RNA template.

In addition, it can be determined whether the polymerase is of viral

origin by examining whether the activity is activated or enhanced by treatment with non-ionic detergents. This follows from the observation that viral reverse transcriptases are encapsulated in the virion and are inaccessible to the substrates unless the virion is first disrupted (13, 210, 211). Such evidence cannot however be conclusive since it could still be argued that viral components which have not yet been assembled into intact virions, may be present in the tissue.

(b) Reverse Transcription with Bacterial Systems

(i) Exogenously Templated Reactions Catalyzed by *E. coli* DNA Polymerase I

Numerous reports in the literature (224-231) have indicated that *E. coli* DNA polymerase I is capable of carrying out the reverse transcription reaction when supplied with heteropolymeric RNA templates. Cavalieri and Carroll (224) have provided evidence that single-stranded RNA and ribosomal RNA from rat liver are capable of directing the Kornberg enzyme to synthesize complementary DNA. Evidence in support of the actual use of RNA as the template was provided by the observation that the reaction was sensitive to pretreatment of the RNA with RNase and not DNase. Further support was provided by the isolation of the RNA:DNA hybrid intermediate. Furthermore Karkas (225) has shown with the same enzymic system that the longer strand of the template-primer (phage ϕ_1 RNA:DNA), be it either RNA or DNA will serve as the template whereas the shorter one will function as the primer. Gulati *et al.* (226) through the use of various heteropolyribonucleotides such as AMV-RNA, Q β RNA, globin mRNA and *Drosophila* RNA, in attempting to work out conditions under which the *E. coli* enzyme will use RNA templates, have concluded that the relative ratio of enzyme to template-primer is critical in observing reverse transcription. That is, a ratio of enzyme to template exceeding

four is essential for the reaction to occur. Travaglini and Loeb (229) in carrying out a similar study have also observed this dependence on the ratio of enzyme to template, and have determined that a ratio of enzyme to template of ten was critical in obtaining large size products. Loeb *et al.* (227) have also made the observation that the Kornberg enzyme is capable of transcribing both DNA as well as 28S rRNA and TMV-RNA under identical conditions. The reaction was shown to meet the usual criteria of sensitivity to RNase-treatment, resistance to actinomycin D, and complementarity between the RNA template and the DNA product. TMV-RNA was minimally effective by itself, however upon addition of oligo (dT), its effectiveness as a template was greatly enhanced. The RD-DP reaction was also found to be abolished by antiserum against *E. coli* polymerase I. Cavalieri *et al.* (228) have carried out a kinetic analysis of the RNA-directed and DNA-directed reactions catalyzed by the Kornberg enzyme and have observed that unlike the DD-DP reaction, the RNA-dependent reaction occurred allosterically. This cooperative phenomenon was observed with both hemoglobin mRNA as well as poly (rA)·(dT)₁₀ as templates.

Travaglini and Loeb (229) have also provided evidence that the Kornberg enzyme has a double-stranded RNA endonuclease associated with it, which enables it to create additional 3'OH initiation sites on the RNA template. Both the RD-DP and the DD-DP activities of DNA polymerase I are probably catalyzed by the same enzyme since they co-fractionated on a gel filtration column as well as on an isoelectric-focusing column. Furthermore, both activities were equally susceptible to inhibition by the zinc chelator o-phenanthroline.

(ii) Endogenous RNase-Sensitive DNA Polymerase

Experimental data showing that hereditary information could be transferred to different bacterial species by a specific RNA from *E. coli* K 12 Hfr showdomycin-resistant mutant, led to the search for an enzyme capable of carrying out reverse transcription (232). This led to the discovery of an RNA-bound DNA polymerase in this *E. coli* strain (233, 234), as well as a DNA polymerase that specifically transcribed the transforming RNA into a complementary DNA copy (232-234).

The endogenous RNase-sensitive DNA polymerase was partially purified through a combination of $(\text{NH}_4)_2\text{SO}_4$ fractionation, DEAE-cellulose chromatography and gel filtration on a Sephadex G 200 column. This endogenously-templated activity has been shown to meet all of the criteria for establishing that RNA is the actual template. The reaction has been shown to be sensitive to RNase, the DNA-RNA hybrid intermediate has been isolated, and the DNA product was hybridizable to the transforming RNA template. In addition, base composition analysis of the product further confirmed complementarity to the RNA template, and the product was also shown to have a sedimentation coefficient (6S) identical to that of the template. In contrast to results obtained by others (224-231), this activity does not seem to reside in the Kornberg enzyme, and it specifically transcribes transforming RNA into a complementary DNA copy (232-234). No other RNAs, either homopolymeric or heteropolymeric were used by the enzyme, nor was native or "activated" calf thymus DNA used as template. Low levels of activity were observed with an *E. coli* DNA template; however, upon pretreatment with RNase A and T_1 , the activity was abolished (232-234).

The endogenously templated activity had a strict requirement for a

divalent cation, preferring Mg^{2+} to Mn^{2+} , and displayed a pH optimum of 7.65 (232-234). In addition the enzyme was found to be totally resistant to NEM and 50% inhibited by 0.2 M salt (233). These latter two properties distinguish the enzyme from *E. coli* polymerases II and III. Its response to NEM is identical to that of *E. coli* polymerase I, whereas the effect of salt differs in that the Kornberg enzyme is totally resistant to 0.2 M salt (6). In addition the activity was inhibited 40-50% by rifampicin and rifamycin derivatives (233).

(c) Involvement of RNA in DNA Synthesis in Eukaryotic Cells

(i) RNA as a Primer in DNA Synthesis

An endogenously RNA-primed DNA templated DNA polymerase activity has been reported in PHA-stimulated normal human lymphocytes (237). The activity was initially found to possess some of the properties of the RNA-directed reaction, except that the RNA·DNA hybrid intermediate could not be detected. This inability to detect the hybrid was initially attributed to the presence of RNase H (an RNase specific for the RNA moiety of RNA·DNA hybrids) in the enzyme preparation. Further analysis of the system by the same investigators (222) led them to conclude however, that the role of RNA in their system was merely to participate in the priming of a DNA templated reaction (222). The role of RNA as a primer in *in vitro* DNA synthesis by DNA polymerase α has already been documented above (section III, A (c) (2)).

(ii) RNA as a Template in DNA Synthesis

DNA polymerases directed by RNA templates have been detected either in association with the RNA template (endogenous RNase-sensitive) or as exogenously RNA-directed DNA polymerases. Endogenously templated RNase-sensitive enzymes have been reported in chicken embryos (22, 235, 236),

uninfected rat cells (23), human sperm heads and seminal fluid (24, 238), sea urchin (239) and normal human cell lines (240). Activity, dependent upon exogenously added RNA templates, has been detected in allantoic fluid of uninfected chicken eggs, using globin mRNA as template (241), chick embryo brain using purified RNA from chick embryonic skin (242), and mitochondria from rat liver and cerebral cortex using rat liver RNA (243).

The criteria used by the authors in assigning a template function to the RNA have been tabulated in Table XI. The evidence in favor of such a function for the RNA is by no means conclusive for every system listed in the table. Further experimental data regarding the nature of the product(s) need to be provided in order to assess the true function of the RNA in some of the endogenously and exogenously templated reactions listed.

(d) Properties of the RNA-Directed DNA Polymerases

Unlike α -, β -, γ -, and mitochondrial-DNA polymerases, little work has been done with regard to determining optimal conditions for the putative RD-DPs. Indeed much of the work has been directed at establishing whether the RNA serves as a template or as a primer. Even so, much of the experimental work is not conclusive. Some of these studies have merely used RNase-sensitivity (239, 245, 243, 247) as the criterion for a template function for the RNA. Others, in addition, have made use of the partial resistance to actinomycin D that such enzymes display (24, 238, 246). More dependable studies have, on the other hand, in addition to these properties, made use of more stringent criteria such as the isolation of an RNA-DNA hybrid (22, 23, 235, 236, 241, 244), as well as hybridization of the product back to the RNA template (22, 23,

TABLE XI. Criteria Used in Assigning a Template Function to RNA in DNA Synthesis

I. Endogenously Templated DNA Polymerases:

Source of Activity	RNAse Sensitivity	Resistance to Act. D	Detection of Hybrid	Hybridization	References
Chicken embryos	++	+	+	+	22, 235, 236, 244
Uninfected rat cells	+	+	+	+	23
Normal human cell line	+	NR	+	NR	240
Human sperm heads	+	+	NR	NR	24, 238
Sea urchin	+	NR	NR	NR	239
Human lymphoid cell line	+	NR	NR	NR	245

II. Exogenously Templated DNA Polymerases:

Allantoic fluid of uninfected chicken eggs (globin mRNA)	+	+	+	+	241
Popliteal lymph nodes (iRNA)	+	+	NR	NR	243
Chicken embryos (AMV -RNA)	+	NR	NR	NR	246
Rat liver and cerebral cortex mitochondria (rat liver RNA)	+	NR	NR	NR	247

* A "+" means that the criteria indicated has been met, whereas NR indicates that it has not been reported. "iRNA" refers to immune RNA, "mRNA" to messenger RNA and "AMV-RNA" to avian myeloblastosis viral RNA.

235, 236, 241, 244).

As in the case of the replicative DNA polymerases, all RD-DP activities have a strict requirement for all four deoxynucleoside triphosphates for maximum activity, as well as an absolute requirement for a divalent cation (22, 23, 24, 236, 240, 243, 245, 248). It is imperative that the incorporation of all four deoxynucleoside triphosphates be shown to occur, otherwise one risks the danger of detecting a polymerase γ instead of a true reverse transcriptase. This occurred in the chick embryo system in which Rougeon *et al.* (185) observed that the poly (rA) regions of globin mRNA and 60-70S AMV RNA were preferentially transcribed, and not the heteropolymeric regions.

Few of the activities detected have been characterized with respect to the optimal assay conditions. In the case of the endogenous activity from chicken embryo, it has been reported, however, that the divalent cation optima is 15 mM in the case of Mg^{2+} , and 1 mM for Mn^{2+} . Furthermore, Mn^{2+} at its optimal concentration was only 30% as efficient as Mg^{2+} (22, 248). Also a broad pH optimum centering around 8 has been reported (248). In addition the enzyme has been shown to require the presence of a thiol-reducing reagent for maximum activity (248).

The available evidence does not support a viral origin for the endogenous RNase-sensitive DNA polymerases from animal tissues. With the exception of the endogenous RNase-sensitive DNA polymerase from the cell-free seminal fluid (24, 238), none of the putative reverse transcriptases reported are stimulated by nonionic detergents (22, 23, 24, 235, 236, 238, 240, 241, 243, 244, 246, 247). This is consistent with a non-viral origin for the enzymes. Buoyant density analysis of some of

the activities also differentiates these enzymes from those of oncogenic RNA viruses. For example, the endogenous activity from chicken embryos which was found to be present in a particulate fraction, has been reported to have a buoyant density of 1.05 g/cc (22), and that from human lymphoid cell lines (245) 1.15 g/cc. The activity from sperm heads which was not enhanced by non-ionic detergents had a buoyant density of 1.21-1.25 g/cc (237, 238) and the one from cell-free seminal fluid (24, 238) which was activated by detergent, 1.22 g/cc.

(e) Nature of the Reaction Products of the RNA-Directed DNA Polymerases

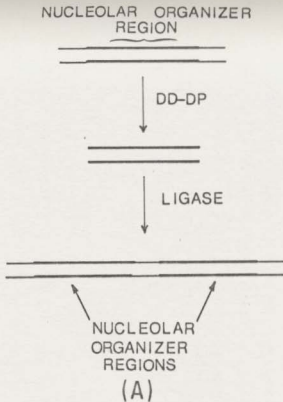
The product of the chick embryo endogenous activity (22, 235) and that from uninfected rat cells (23), consists of a mixture of single and double-stranded DNA. Kang and Temin (22, 235) have also analyzed in detail the product of a reaction that was carried out in the presence of actinomycin D. Sucrose gradient analysis revealed sedimentation coefficients of 6S and 30S. The fast sedimenting product, however, was shown to disappear when treated with RNase, alkali, or heat. In addition part of the product (35%) which banded in the RNA region on a CsCl gradient, disappeared upon treatment with alkali, and subsequently banded in the density range of 1.4-1.55 g/cc (i.e., the density range for DNA). When it was heated for 10 minutes at 100° C, much of the peak in the RNA region disappeared; however, some remained, and if this was subsequently treated with RNase A and T_1 , it disappeared. This suggested both hydrogen bonding as well as phosphodiester linkages between the template and the product (235). When the product was treated with S_1 nuclease, an enzyme which degrades single-stranded regions of either RNA or DNA, the density profile had two peaks, one in the RNA region, and one

in the DNA region, suggesting that the product contained both DNA·RNA hybrids, as well as double-stranded DNA. The density of the RNA·DNA hybrid after treatment with S_1 nuclease may be the result of double-stranded RNA remaining attached to the hybrid (235). Furthermore, Kang and Temin (22) have found that the product of the endogenous chicken embryo activity hybridized 40% with the RNA from the chicken cell fraction, but did not hybridize to RNA from avian leukosis and Rous sarcoma viruses.

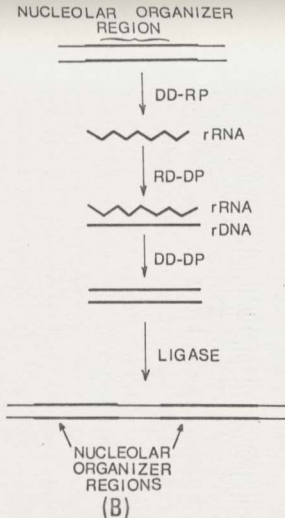
(f) Biological Function of RNA-Directed DNA Polymerase

By analogy to the function of viral RD-DP in the production of a provirus for incorporation into the host-cell DNA (Scheme IV), the animal cell RD-DP may be involved in producing DNA copies of RNA transcripts for the purpose of amplifying selected regions of the chromosome. Evidence for this hypothetical function has not been generally accepted, however, and the biological role of eukaryotic reverse transcriptase remains a mystery.

Of all the possible functions that an RNA-directed DNA polymerase may take part in, its possible role in ribosomal gene amplification is the one most thoroughly studied. There are basically two possible routes through which the process of ribosomal gene amplification may occur (Scheme V). The first postulates that the entire ribosomal DNA complement of the chromosomal nucleolar organizer is used as a template for DNA replication, in the classical semi-conservative manner (Scheme VA). An alternate route (Scheme VB) has been proposed by Tocchini-Valentini and Crippa (212, 213, 217), who have suggested that the first step in the amplification process is the formation of an RNA transcript of the entire ribosomal DNA unit which then serves as a template for the RNA-



SEMI-CONSERVATIVE
REPLICATION
MODEL



MODEL OF TOCCHINI-VALENTINI
& CRIPPA

SCHEME V

Modes of Ribosomal Gene Amplification

directed DNA polymerase, yielding an RNA·DNA hybrid intermediate which would subsequently be converted to a double-stranded DNA. Finally, polycistronic ribosomal DNA would be linked together by a ligase (Scheme VB). Their hypothesis is based on observations made with *Xenopus* oocytes at the pachytene stage, a stage coincident with the amplification process. They observed that (i) When oocytes are incubated in the presence of ^3H -uridine and ^{14}C -thymidine, a peak of ^3H -uridine incorporation bands at the density of ribosomal DNA [which has a higher G-C content and thus a greater density in CsCl gradients than non-ribosomal DNA (249)]. This material was resistant to RNase unless first denatured, suggesting that the product is an RNA·DNA hybrid (212-214, 217). (ii) The rifampicin derivative 2'5'-dimethyl-N (4')-benzyl N [desmethyl] rifampicin, which is known to inhibit viral RD-DP, inhibited formation of the ribosomal DNA peak but not the non-ribosomal DNA (212, 213, 217). This was confirmed autoradiographically by Ficq and Brachet (215) who have shown that the amplification of rDNA which takes place in the extrachromosomal "caps" was inhibited by the same drug. (iii) The isolation of an RNA-DNA hybrid intermediate (214, 216, 217) as well as the discovery of an enzyme capable of using the rRNA from the complex, as a template for DNA synthesis (214, 217), further substantiated this hypothesis.

Although this series of papers provide substantial evidence that RD-DP may be involved in gene amplification, Bird *et al.* (250) have been unable to confirm these results. They have not been able to detect either the RNA template (47S), nor the hybrid intermediate. In addition, these workers have suggested that the radioactivity after uridine labelling is attributable to the conversion of uridine to deoxycytidine and subsequent incorporation into DNA. This explanation is not satis-

factory however, since it was shown that 95% of the uridine counts were sensitive to alkali (216, 217) and also sensitive to RNase A after heat denaturation (214, 216, 217), suggesting that uridine was not converted to deoxycytidine. It is conceivable that these contradictory observations are a result of the two groups looking at different stages of the amplification process. Indeed, Tocchini-Valentini *et al.* (217) have pointed out that growth conditions must be carefully standardized to study the sequence of events which occur during the amplification process. For example, when ovaries of tadpoles of apparently the same age as the controls, but grown in the presence of the hormone β -estradiol are labelled in organ culture, the *de novo* synthesis of rDNA is no longer sensitive to RNase. The use of β -estradiol by Bird *et al.* (250) to obtain sex reversion of the animals could influence the normal growth of the tadpoles and therefore affect the timing of the development of the ovaries relative to the external morphology of the animals.

IV. Terminal Deoxynucleotidyl Transferase

This subject has recently been reviewed in detail by Bollum (251) and will thus be only briefly considered here. As pointed out earlier, TdT catalyzes the consecutive addition of nucleoside triphosphates onto the 3'OH terminus of single stranded DNA (see Scheme II). Although it has been claimed that the enzyme is present only in the thymus (252), or in thymus derived cells (253), more recent experimental data suggests a wider distribution for this activity. For example, TdT has been reported to be present in chromatin from plant tissues (254), human bone marrow (255), human leukemic cells (256-258) and human brain (259). The high stability of the enzyme has aided in obtaining a homogeneous

preparation (260).

Briefly, the enzyme is a low molecular weight protein of approximately 35,000 daltons, consisting of two subunits of 8,000 and 26,500 daltons (260). Unlike the replicative DNA polymerases, TdT requires only a single deoxynucleoside triphosphate for activity, with dATP being the best substrate. The presence of all four deoxynucleoside triphosphates actually inhibits the activity, and thus TdT is easily distinguishable from the replicative DNA polymerases. One of the prerequisites for the primer polynucleotide is the absence of any secondary structure involving the 3'OH group (251). Oligonucleotides as short as trinucleotides are among the most efficient primers for the enzyme.

Like the replicative DNA polymerases, TdT has an absolute requirement for a divalent cation, presumably for the binding of the deoxynucleoside triphosphates. The divalent cation preference changes, however, depending on the nature of the nucleotide base. For example, with pyrimidine triphosphates, Co^{2+} is preferred, whereas with purines Mg^{2+} works best (251). The enzyme displays a pH optimum of 7.2 when cacodylate or HEPES are used as buffers, while other buffer anions inhibit the activity.

In addition to a general ionic strength inhibition effect, anions such as PO_4^{3-} , SO_4^{2-} , Cl^- and others (251) have also been shown to inhibit. Furthermore, pyrophosphate, one of the by-products of the reaction, is inhibitory. Although TdT, like DNA polymerase β , has a low molecular weight, unlike this enzyme, TdT is susceptible to the sulphydryl blocking reagent pCMB (261). Metal ligands such as EDTA and o-phenanthroline (and not m-phenanthroline) are also effective inhibitors.

The function of TdT remains enigmatic, although it has been suggested

that it may play a role in the immune system by diversifying the section of the genome coding for the variable region of the immunoglobulin chains (262). More recently, however, Johnson and Morgan (263) have questioned the actual existence of TdT. These workers have shown that the successful finding of TdT depends on the purification method used, and that it can be obtained from a replicative DNA polymerase upon prolonged storage of the enzyme. Thus, the possibility exists that TdT may be a proteolytic fragment of a DNA polymerase, although this does not necessarily preclude a possible function for the enzyme, since *in vivo* processing of a DNA polymerase to a TdT may very well occur.

V. Serological Analysis of DNA Polymerases

Serological analysis of the relationship between calf thymus DNA polymerases α and β by Chang and Bollum (264), and rat liver polymerases α and β by Mizutani and Temin (265), revealed that the two enzymes were immunologically related. This was consistent with the model postulated by Hecht (104) and Lazarus and Kitron (97) that polymerase β represents a subunit of polymerase α . Later experimental data, however, revealed that polymerase β could aggregate to a size similar to that of polymerase α (105) and thus the apparent conversion of α to β (97, 104) merely represented a disaggregation of polymerase β , and not a dissociation of polymerase α as originally postulated. It now appears likely that the polymerase α used to raise antibodies was probably contaminated with an aggregate of β , and therefore the two activities were erroneously found to be related. Consistent with this explanation are the recent observations (94, 266, 267) that the two activities are immunologically distinct. For example, it has been shown that the anti-

body against partially purified polymerase α from HeLa cells did not cross-react with polymerase β from the same source (94), although the same antiserum did inhibit the α activity from Chinese Hamster cells. This observation did suggest common amino acid sequences among polymerases α from different species (94). The lack of relationship between polymerases α and β has also been confirmed with enzymes from human lymphoid cells (267) and chick embryo (266). In addition, these enzymes (α and β) were not inhibited by antibody against primate type C viral reverse transcriptases (268, 269), or against AMV reverse transcriptase (266). Furthermore, antibody against human polymerase α did not cross-react with reverse transcriptase from five RNA tumor viruses (267).

Antiserum against calf thymus polymerase α was found not to inhibit TdT or *E. coli* polymerases I and II; however, it did cross-react with mitochondrial DNA polymerase (264). This same antiserum was also found to cross-react with both α and β from a number of mammalian tissues such as mouse L cells, PHA-stimulated normal human lymphocytes, rat liver, and rabbit bone marrow (264). In the light of the possibility that there were antibodies against both α and β in the serum preparation (see above) these observations suggest that polymerase α from a variety of mammalian cells share common polypeptide sequences, and also that polymerases β are serologically related.

Brun *et al.* (266) have also observed that of the antibodies directed against α and β from chick embryo, only the β polymerase antibody was found to cross-react with β from avian and mammalian cells, whereas antiserum against polymerase α did not cross-react with the enzyme from mammalian cells. Spadari *et al.* (94), on the other hand, have shown

that antiserum against HeLa cell polymerase α had the same inhibition pattern on polymerase α from Chinese Hamster cells. Thus it appears that polymerases β from both avian and mammalian cells are serologically related whereas polymerases α are not.

Polymerase γ from HeLa cells is not inhibited by antibodies against reverse transcriptase from Mason-Pfizer monkey virus, Woolly monkey virus, or Rauscher murine leukemia virus (192). It has also been observed that polymerase γ from human lymphocytes is not inhibited by antibody against Simian Sarcoma virus reverse transcriptase (179, 267, 268).

Antibody against calf thymus TdT has been prepared by Bollum (270) who has shown that it is able to cross-react with TdT from human, chicken, mouse and rat tissues. The serum was, however, unable to neutralize polymerases α and β . This is in agreement with similar work carried out by Kung *et al.* (271), who in addition have shown that anti-TdT serum does not cross-react with *E. coli* polymerase I, or with reverse transcriptase of Maloney mouse leukemia virus. Similar studies by Chan and Srivastava (272) have recently shown that antibody against calf thymus TdT also inhibited TdT from acute leukemic lymphoblasts and Molt-4 cells but not DNA polymerases α , β and γ from these cells.

The chick embryo endogenous RNase-sensitive DNA polymerase is not serologically related to DNA polymerase α or to viral reverse transcriptases, although it was found to be partially or completely neutralized by antibody against polymerase β from the same source (273).

PURPOSE AND APPROACH TO THIS STUDY

The purpose of the work to be described in this dissertation is two-fold. First to investigate whether RNA-directed DNA synthesis occurs in mammalian cells, and secondly to determine whether the DNA polymerases associated with the RNase-sensitive DNA polymerase complex, previously reported by the author (281), are novel eukaryotic DNA polymerases, or whether they are related to the known enzymes.

The approach taken for the first objective, was to rigorously analyze the endogenously-templated RNase-sensitive DNA polymerase previously described (281), and in this regard some of the work described is an extension of the work previously reported. The experimental approaches taken were aimed at determining the nature of the RNA associated with the RS-DP complex, as well as the nature of the product synthesized. In addition, the response of the activity to inhibitors such as actinomycin D and distamycin A, which would reveal the nature of the nucleic acid template was investigated.

With regard to the second objective, a method previously reported by the author (281) was used to isolate the DNA polymerases associated with the RS-DP complex, and the properties of the enzymes derived from it were determined and compared to the reported properties of other eukaryotic DNA polymerases.

MATERIALS AND METHODS

I. Materials

(A) Animals

Female rats (100-150 g) of the Sprague-Dawley strain were used for all experiments, and were obtained from either the Animal Unit, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, or from Canadian Breeding Farm and Laboratories Ltd., St. Constant, Quebec.

(B) Biochemicals, Chemicals, and Enzymes(a) Deoxynucleoside Triphosphates

Unlabelled deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) were obtained as their sodium salts, from Sigma Chemical Co., St. Louis, Mo. Stock solutions of 50 mM and 5 mM were prepared in distilled water and stored at -20°C until required.

^3H -Labelled deoxynucleoside triphosphates [thymidine-(methyl- ^3H)5'-triphosphate, (10-20 curies/mmol), deoxyadenosine- $^3\text{H}(\text{G})$ * 5'-triphosphate, (5.04 curies/mmol), deoxyguanosine- $^3\text{H}(\text{G})$ 5'-triphosphate, (1.03 curies/mmol), and deoxycytidine-(5- ^3H) 5'-triphosphate, (25.6 curies/mmol)], were purchased as their tetrasodium salts from New England Nuclear, Boston, Mass. The solvent (50% ethanol in water) was removed from each nucleotide solution by directing a gentle stream of nitrogen gas over the surface of the solution. The concentration of each solution was adjusted to 0.1 mM by the addition of an appropriate quantity

* G refers to a general labelling of the base in the nucleotide.

of "cold" deoxynucleoside triphosphate. The solutions were stored at -20°C until required.

(b) Templates

Native calf thymus DNA (Type I) was purchased from Sigma and stock solutions of 0.70 mg/ml in distilled water were prepared and stored at -20°C until required. Heat-denatured DNA was prepared from native DNA solutions by heating at 95°C for 10 minutes followed by rapid cooling in ice-water. "Activated" calf thymus DNA was either a gift from Dr. L.A. Loeb (Fox Chase Center for Cancer Research, Philadelphia, Pennsylvania) or prepared as described by Loeb (274) by subjecting the DNA to a limited digestion with pancreatic DNase I until maximum priming ability with sea urchin DNA polymerase (also a gift from Dr. L.A. Loeb) or rat thymus DNA polymerase α was obtained.

Yeast tRNA was obtained from Worthington, Freehold, N.J. The other RNA templates: Q β RNA, and 23S rRNA and 16S + 23S rRNA from *E. coli* were obtained from Miles Laboratories Inc., Elkhart, Indiana. Solutions of various concentrations, as indicated in the legends, were prepared in distilled water and stored at -20°C until needed.

(c) "Carrier" DNA

Calf thymus DNA (Type V) purchased from Sigma was stored at a concentration of 2 mg/ml in distilled water at either -20°C or $0-4^{\circ}\text{C}$.

(d) Cleland's Reagent

Dithiothreitol (DTT) was purchased from Sigma and 0.2 M (for use in the DNA polymerase reaction mixtures) and 1 M (for the buffer solutions) solutions were prepared in distilled water and stored at -20°C until

required.

(e) Inhibitors

Ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide) was purchased from Calbiochem, Los Angeles, California. Actinomycin D, heparin, and N-ethylmaleimide (NEM) were from Sigma. Distamycin A was a gift from Dr. Mario Ghione, Farmitalia, Milano, Italy.

(f) Other Chemicals

Trizma base (tris-[hydroxymethyl]aminomethane), EDTA (ethylene-diamine-tetracetic acid), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), and Triton X-100 (octyl phenoxy polyethoxyethanol) were obtained from Sigma. Phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), guanidine-HCl, and Cs_2SO_4 (grade 1) were also from Sigma. Trasylol was obtained from Boehringer Ingelheim (Canada) Ltd., Montreal, Quebec.

Calf thymus histones and oxidized RNase A were from Sigma, and "RNase-free" lactoperoxidase (LP) further purified by the method of Rombouts *et al.* (275) was a gift from Dr. C. Michalski (Faculty of Medicine, Memorial University of Newfoundland). Azocoll and the polyamines: putrescine-dihydrochloride, spermidine-trihydrochloride, and spermine-tetrahydrochloride were from Calbiochem. CsCl, sodium pyrophosphate, polyethylene glycol 6000 (PEG) ($\text{MW}_{\text{av.}}$ 6,000-7,500) and glycine were from J.T. Baker, and perchloric acid and glycerol from Fisher Scientific, Montreal, Quebec. Cetyltrimethylammonium bromide (CTAB) was purchased from the British Drug House, Toronto, Canada. Enzyme grade sucrose was

obtained from Schwarz/Mann, Orangeburg, N.Y. and Siliclad from Clay Adams, Parsippany, N.J. Other chemicals were of the highest purity available.

(g) Enzymes

Electrophoretically purified bovine pancreatic DNase I, obtained from Sigma, was prepared at a concentration of 1 mg/ml in water and stored at -20° C until required. Both bovine pancreatic RNase A (crystalline powder) and RNase T₁ from *Aspergillus oryzae* (as an ammonium sulfate suspension at 0.54 mg/ml) were obtained from Sigma. A stock solution of 2 mg/ml RNase A was prepared in distilled water, heated for 10 minutes at 95° C to destroy any contaminating DNase, and stored at -20° C until needed. Under assay conditions capable of detecting as little as 0.025 μ g of DNase per ml (using ^3H -DNA as substrate), no DNase activity could be detected in the RNase solutions at a concentration as high as 400 μ g per ml (highest level assayed). S₁ nuclease from *Aspergillus oryzae* was obtained from Miles Laboratories Inc. and was used at a concentration found to degrade only single-stranded nucleic acids (data not shown).

(C) Buffers and Solutions

(a) Buffer A

20 mM Tris-HCl, pH 8.0 (at 25° C), 40 mM KCl, 1 mM EDTA and 0.5 mM DTT.

(b) Buffer B

20 mM glycine-NaOH, pH 8.5, 40 mM KCl, 1 mM EDTA and 0.5 mM DTT.

(c) Stopping Solution

1 M perchloric acid, plus 0.05 M sodium pyrophosphate.

(d) Washing Solution

0.5 M perchloric acid, plus 0.025 sodium pyrophosphate.

(e) Electrofocusing Solutions

(i) Gradient Solutions.

The dense gradient solution consisted of 30 ml glycerol and 1.8 ml Ampholines, pH 3-10 (LKB Produkter, Sweden) (40% solution) made up to 50 ml with water. The light gradient solution consisted of 0.6 ml Ampholines, pH 3-10 (40% solution) and 49.4 ml water.

(ii) Electrode Solutions.

The lower electrode solution consisted of 120 g sucrose, 140 ml of distilled water, and 2 ml of concentrated phosphoric acid. The upper electrode solution consisted of 1 percent NaOH in distilled water.

(f) Electrophoresis Solutions

(i) Gradient Solutions.

The dense gradient solution consisted of 45% glycerol and 5 mM Tris-HCl, pH 8.0. The light gradient solution consisted of 20% glycerol in 5 mM Tris-HCl, pH 8.0.

(ii) Electrode Solutions.

The upper electrode solution consisted of 20 mM Tris-HCl, pH 8.0, and the lower electrode solution of 50% glycerol in 20 mM Tris-HCl, pH 8.0.

II. Methods

(A) Preparation of Tissue Homogenates

Female Sprague-Dawley rats (100-150 g) were first stunned and then sacrificed by cervical dislocation. The appropriate tissue was removed and quickly immersed in several volumes of ice-cold isotonic (0.14 M) KCl solution. The tissue was then blotted, weighed, minced with scissors and homogenized in 3 volumes of Buffer B unless otherwise indicated. The homogenization in the case of rat thymus was carried out in a Dounce homogenizer (Kontes Glass Company, Vineland, N.J.) using three strokes of the loose-fitting pestle (pestle A) followed by three strokes with pestle B. To enhance the extraction of DNA polymerase, the homogenate was further frozen and thawed twice, after which it was centrifuged at $39,000 \times g$ for 30 minutes in an International or Sorvall RC 2B centrifuge. The $39,000 \times g$ supernatant after further purification as indicated served as the source of the enzyme.

In the case of rat liver, the tissue was homogenized in a Waring Blender (Waring Products Service Center, New Hartford, Conn.), using three fifteen second bursts at low speed, and another three at high speed, at $0-4^{\circ} \text{C}$. The homogenate was then treated as indicated in the legends.

(B) Preparation of the Endogenously-Templated RS-DP Activity from Rat Thymus

The $39,000 \times g$ supernatant was fractionated by gel filtration on a Sepharose 6B or Sephadex G 150 column as indicated, the active fractions were pooled, and concentrated by dialysis against Buffer B (unless

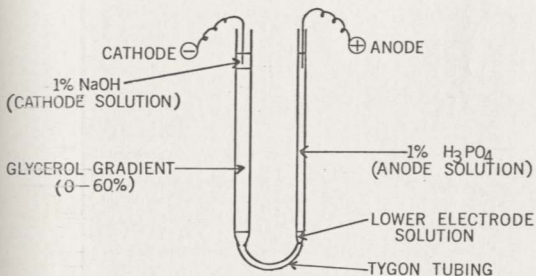
otherwise indicated) containing 30% PEG. For all experiments described, activity versus enzyme concentration curves were carried out for every preparation to assure that assays were carried out in a linear range of enzyme concentration.

(C) Preparation of Exogenously-Templated Activities Derived from the RS-DP Complex from Rat Thymus

One ml of endogenously-templated activity partially purified on a Sepharose 6B column was treated with 200 μ g RNase A and 10 μ l of RNase T₁ for three hours at 25° C, and subsequently fractionated on a Sephadex G 200 column (1.5x30 cm) equilibrated with Buffer B. Approximately 1.2 ml fractions were collected and assayed (as described below) for exogenously-templated DNA polymerase activity with native DNA as the template. The active fractions for the second and third peaks of activity eluting from the column were pooled separately and when necessary, concentrated as described above. Activity versus enzyme concentration curves were carried out with every preparation, in order to assure that assays were conducted in a linear range.

(D) Isoelectric Focusing

The procedure for isoelectric focusing was modified from that described by Vesterberg and Svensson (276) to include preequilibration of the column prior to the application of the sample. The apparatus used was similar to that described by Poiesz *et al.* (32) and is shown schematically in Scheme VI. Basically, it consists of two Falcon serological pipettes (of 10 ml and 5 ml capacities) connected at the tips by Tygon tubing. Sufficient lower electrode solution (see Materials) was added to the lower electrode chamber (5 ml pipette) to fill the



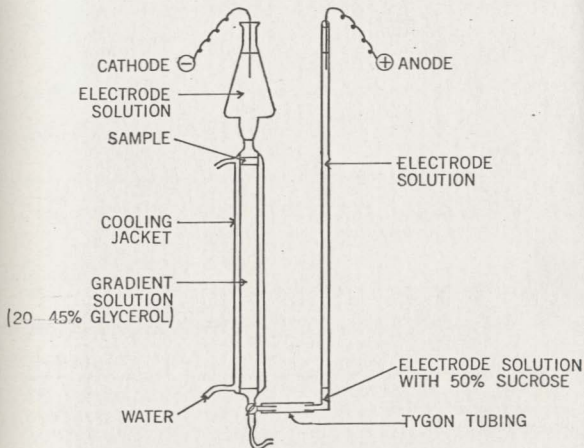
APPARATUS USED FOR
ELECTROFOCUSING

SCHEME VI

gradient chamber (10 ml pipette) up to the bottom graduation mark. The Tygon tubing was then clamped, and a 10 ml linear density gradient [(0-60% glycerol, see Materials, Section C(e)(i)] was prepared with a gradient mixer. Several milliliters of upper electrode solution was then carefully layered on top of the gradient, and sufficient 1% phosphoric acid was added to the lower electrode chamber to approximately balance the hydrostatic pressure of the gradient. The clamp was removed from the Tygon tubing, platinum electrodes were then positioned in each electrode solution and connected to a power supply. The column was pre-equilibrated at 400 volts for 4 hours (0-4° C) before inserting the sample (in 20% glycerol) into the gradient through a thin Tygon tubing. Equilibration was continued for a further 12 hours at 500 volts. At the end of the focusing, 0.5 ml fractions were collected from the bottom of the 10 ml pipette by pumping distilled water onto the top of the gradient.

(E) Electrophoresis in Glycerol Gradients

The apparatus used for glycerol gradient electrophoresis is depicted in Scheme VII and consists essentially of a condenser connected through a three-way stopcock to a glass tube by means of a piece of Tygon tubing. The condenser was connected through a ground-glass joint at the top to the bottom of an Erlenmeyer flask fitted with a complementary ground-glass joint. In preparing the column, the two chambers were connected by means of the three-way stopcock, and lower electrode solution was added to a level adjacent to the water inlet of the cooling jacket. The stopcock was then closed and a 75 ml linear glycerol gradient [20-45%, see Materials, Section C(f)(i)] was prepared with a gradient mixer. The



APPARATUS FOR ELECTROPHORESIS
IN GLYCEROL GRADIENTS

sample, in 10% glycerol, was carefully layered on top of the gradient followed by a layer of approximately 5 ml of 5% glycerol in 20 mM Tris-HCl, pH 8.0. Upper electrode solution was then carefully added to within 1 cm of the ground glass joint. The Erlenmeyer flask was joined to the top of the column and carefully filled with upper electrode buffer. The lower electrode chamber was also filled with the same solution to a level which just balanced the pressure when the two chambers were connected. Platinum electrodes were positioned at the top of each chamber and electrophoresis was carried out at 0-4° C for the lengths of time and at the voltage indicated in the appropriate legends. At the end of the run, the three-way valve was positioned to allow the gradient solution to be collected from the bottom of the condenser, by pumping distilled water into the top of the chamber.

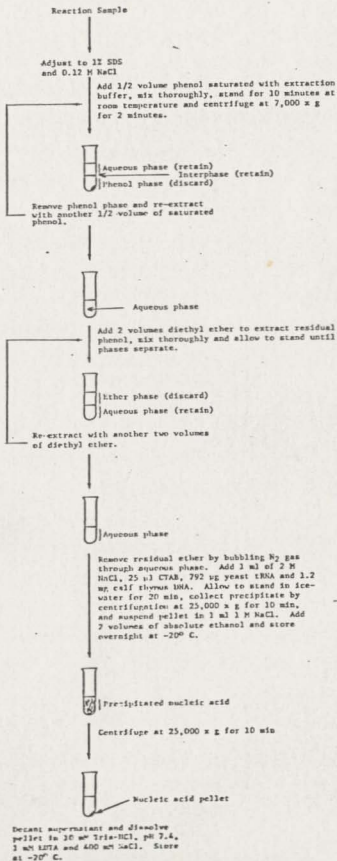
(F) Siliconization of Glassware

Unless otherwise indicated, the test tubes used in carrying out the assays were siliconized with Siliclad [see Materials, Section I(B)(f)] according to the directions given by the manufacturer.

(G) Isolation of the Reaction Products of the Endogenously-Templated DNA Polymerase

The product of the RS-DP reaction was isolated by a modification of the method of Reitz *et al.* (277). A flow chart for the procedure is shown in Scheme VIII. A 100-fold increased reaction mixture was used for the isolation of the product. The reaction was terminated by the addition of SDS (to a final concentration of 1%) and 2 M NaCl was added to give a final concentration of 0.12 M NaCl. The product was then twice extracted with one-half volumes of phenol saturated with RNA ex-

Extraction Procedure for the Endogenously-Templated RN-DP Product



traction buffer (10 mM Tris-acetate, pH 5.2, 10 mM Mg-acetate, and 60 mM NH_4Cl). Residual phenol in the aqueous phase was extracted twice with two volumes of diethyl ether, and the residual ether removed by bubbling nitrogen gas through the aqueous phase. One ml of 2 M NaCl and 25 μl of 0.1 M CTAB were added, followed by the addition of 792 μg yeast tRNA and 1.2 mg of calf thymus DNA as "carriers" for the radioactive product. After standing in ice-water for 20 minutes, the precipitate was collected by centrifugation at 25,000 x g for 20 minutes. The pellet was then dissolved in 1 ml of 1 M NaCl and reprecipitated with 2 volumes of absolute ethanol (stored overnight at -20°C). The precipitate was then collected by centrifugation at 25,000 x g for 10 minutes, dissolved in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 400 mM NaCl, and stored frozen at -20°C until needed.

(H) Buoyant Density Analysis

(a) Cs_2SO_4 Density Gradient Centrifugation

Appropriate volumes of samples (containing sufficient ^3H -DNA for subsequent analysis) were diluted to 1 ml with TNE buffer (10 mM Tris-HCl, pH 7.2, 1 mM EDTA and 0.1 M NaCl) (277) and added to 4 ml of a solution of Cs_2SO_4 (1 g/ml) in TNE buffer. The mixtures were then centrifuged in the Beckman SW 50.1 rotor at 20°C for 64 hours at 35,000 rpm. Eight drop fractions were collected from the bottom of each tube and the acid precipitable radioactivity in 100 μl aliquots of each fraction was determined by adding first, 1 ml of 10% cold trichloroacetic acid (TCA), followed by 200 μg of "carrier" DNA. After thorough mixing, the precipitate was transferred onto Whatman GF/C glass fiber discs and washed successively with two aliquots of cold water, one of washing

solution, twice again with water and finally with 95% EtOH. The sample was then dried and counted as described below for the DNA polymerase assays.

(b) Guanidinium·Cl-CsCl Density Gradient Centrifugation

The guanidinium·Cl-CsCl solution was prepared as described by Enea and Zinder (278) and consisted of 12.88 g CsCl and 2.4 g guanidinium chloride in 10 ml of 10 mM Tris-HCl, pH 7.4. A 4.8 ml volume of the guanidinium·Cl-CsCl solution was mixed with 277 μ l of sample and centrifuged at 38,000 rpm at 15° C in the Beckman SW 50.1 rotor for 72 hours. After centrifugation eight drop fractions were collected and the radioactivity in 100 μ l aliquots of each fraction determined as described above.

(c) Sucrose Density Gradient Centrifugation

Buoyant density analysis of the DNA polymerases was carried out on a 10-45% sucrose gradient (in Buffer B), centrifuged at 45,000 rpm for 2 hrs in the Beckman SW 50.1 rotor. At the end of the centrifugation, eight drop fractions were collected and the DNA polymerase activities determined as described below.

(I) Sedimentation Velocity Analysis

Sedimentation velocity analysis of the product was carried out in 5-20% sucrose gradients (in Buffer B) in a Beckman SW 50.1 rotor, centrifuged for 4 hours at 50,000 rpm. At the end of the run, eight drop fractions were collected and the radioactivity in 150 μ l aliquots of each fraction was determined as described above. Sedimentation values and

molecular weights were calculated by the procedure of Martin and Ames (278a), using 4.0S (25,000 daltons) tRNA in a separate tube as a standard.

To determine the size of the rat liver enzymes similar gradients were run using the Beckman SW 36 rotor, centrifuged for 16 hours at 35,000 rpm. At the end of the run, 19 drop fractions were collected, and enzymatic activities were determined as described below. Sizes were again estimated as described by Martin and Ames (278a) by comparison to pig heart fumarase (8.5S*) standards included in each gradient.

(J) DNA Polymerase Assay

The reaction components for the DNA polymerase assays are summarized in Table XII. Reactions were carried out for 5 minutes in the case of the endogenously-templated activity and for 30 minutes in the case of the exogenously-templated activities at 37° C. At the end of the incubation period, the reaction was stopped by first cooling the tubes in ice-water, followed by the addition of 1 ml Stopping Solution at 0° C (which precipitates the DNA product) and 0.2 mg "carrier" DNA. The mixture was then thoroughly mixed with a vortex mixer, followed by centrifugation at 3,500 rpm for 20 minutes in a Sorval RC 3 centrifuge with a swinging bucket rotor. The supernatant, containing the bulk of the unincorporated label, was then removed by aspiration. The pellet was dissolved in 1 ml 0.2 M NaOH at room temperature, and reprecipitated by the addition of 2 ml ice-cold Stopping Solution. The acid insoluble product was collected by vacuum filtration on a Whatman GF/C glass fiber disc, washed with 2-3 ml volumes of ice-cold distilled water (3 times), washing solution (two times), and again with two volumes of water. Finally, the

* Massey, V. (1952) *Biochem. J.* 51, 490-494.

TABLE XII. Reaction Mixture for the DNA Polymerase Assays

<u>Reaction Component</u>	<u>Endog.</u> <u>(μl/assay)</u>	<u>Native</u> <u>(μl/assay)</u>	<u>Activated</u> <u>(μl/assay)</u>
Glycine NaOH pH 8.5 (1 M)	2	2	2
MgCl ₂ (0.1 M)	2	2	2
BSA (2 mg/ml)	2	2	2
DTT (0.2 M)	1	1	1
KCl (1 M)	1	1	1
dATP (5 mM)	1	1	1
dCTP (5 mM)	1	1	1
dGTP (5 mM)	1	1	1
dTTP (0.1 mM)	-	2	2
³ H-dTTP (0.1 mM)	4	2	2
"Activated" DNA (1.2 mg/ml)	-	-	10
Native DNA (0.7 mg/ml)	-	10	-
H ₂ O	10	-	-
Enzyme Preparation	<u>25</u>	<u>25</u>	<u>25</u>
Total Volume	50	50	50

filter disc was washed with 95% ethanol, dried under an infra-red heat lamp, and the radioactivity incorporated determined with a Beckman LS 233 liquid scintillation counter. The scintillation solution unless otherwise indicated consisted of 6 g of scintillation grade PPO (2,5-diphenyloxazole) (Kent Laboratories, Vancouver, B.C., Canada) per liter of toluene. Disposable plastic counting vials from New England Nuclear were used.

(K) Protein Determination

Protein concentration was estimated by the method of Lowry *et al.* (279), using crystalline bovine serum albumin as standard or from a nomograph (Calbiochem) based on the method of Warburg and Christian (280).

RESULTS

I. Detection and Partial Purification of the Endogenously-Templated Ribonuclease-Sensitive DNA Polymerase of Rat Thymus

(A) DNA Polymerase Activities in a Crude Extract of Rat Thymus.

Work with rat thymus DNA polymerases was carried out initially with the 39,000 x g supernatant of a rat thymus homogenate. Upon assessing the DNA polymerase activities at various concentrations of the extract, both in the absence and presence of native and "activated" DNA templates, it was observed that in addition to exogenously-templated activities, an endogenously-templated DNA polymerase activity also was present (Fig. 1). This latter activity was found to be partially sensitive to RNase A treatment, and was totally abolished upon pretreatment of the extract with both RNase A and T_1 . As can be seen in Fig. 1, at an extract concentration of 1.6% (w/v), RNase A treatment abolished 80% of the endogenous activity, whereas with a combined treatment of RNase A and T_1 , essentially all of the endogenously-templated DNA polymerase activity was eliminated.

When assessing DNA polymerase activities using crude extracts, many problems might be encountered, and thus uncertainties with regard to levels of activity observed might arise. For example, factors such as nucleases which would destroy the template and product, phosphatases, which would destroy the nucleoside triphosphate substrates, and dilution of the labelled deoxynucleoside triphosphate, would be among some of the factors that would interfere with DNA polymerase assays and cast doubt on experimental observations. For these reasons, it was necessary to develop a purification scheme prior to investigating the nature of

FIGURE 1. Rat Thymus DNA Polymerase Activities in the 39,000 x g Supernatant.

The 39,000 x g supernatant of a 25% (w/v) rat thymus homogenate in Buffer A was prepared as described in *Materials and Methods*. Final concentrations of extract in the reaction mixtures (expressed as "% Homogenate") were obtained through appropriate dilutions with Buffer A. Assays were as described in *Materials and Methods* except that Tris-HCl, pH 8.0 replaced glycine-NaOH, and the reactions were carried out for 15 min at 37° C. The sp. act. of the dTTP was 3,500 cpm/pmole.

Native DNA-dependent DNA polymerase (●—●), "activated" DNA-dependent DNA polymerase (■—■), and endogenously-templated DNA polymerase (preincubated for 30 min at room temperature) in the absence of RNases (○—○), in the presence of 200 µg RNase A/ml (□—□), or 200 µg RNase A/ml plus 8.75 µg RNase T₁/ml (310,000 units/mg protein) (▲—▲) were determined. All assays were carried out in duplicate, and the average value plotted.

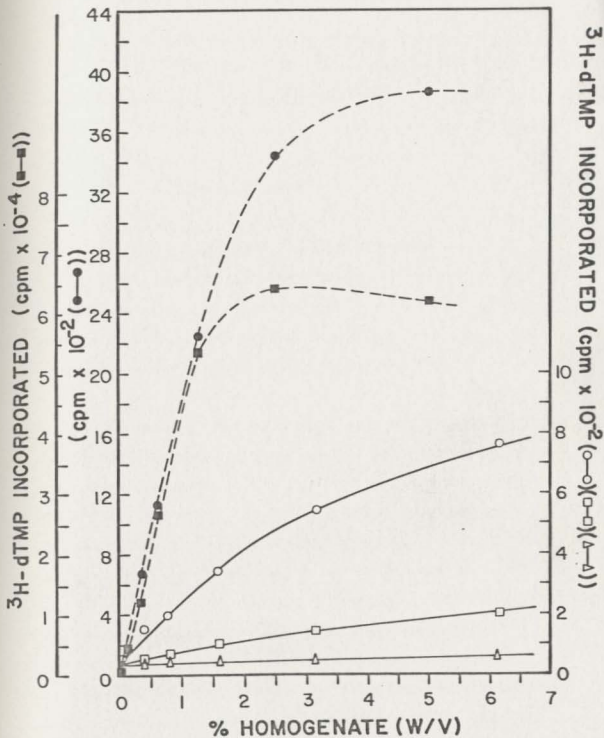


FIGURE 1. Rat Thymus DNA Polymerase Activities in the 39,000 x g Supernatant.

the endogenously-templated RNase-sensitive DNA polymerase as well as some of its properties. Furthermore it was of interest to determine whether the endogenously- and exogenously-templated activities were separable. Thus, purification procedures were undertaken with the two objectives of removing interfering factors and determining if one protein was responsible for the polymerase activities.

(B) Partial Purification of Rat Thymus DNA Polymerases

The purification method in the initial experiments involved gel filtration chromatography on a Sephadex G150 column. Analysis of the fractions eluting from such a column revealed that both the endogenously- and exogenously-templated activities eluted in the void volume (281). The overall purification (relative to the crude extract) achieved on the Sephadex G150 column is shown in Table XIII A. The endogenously-templated activity was purified approximately 1.5-fold with a recovery of 90%, whereas the exogenously-templated activities were purified 2.1- and 1.3-fold with recoveries of 124% and 75% respectively, when native and "activated" DNA were used as templates (Table XIII A). The less than 100% recovery of endogenously-templated activity and the increased recovery of the native DNA-templated DNA polymerase activity might suggest a possible connection between the two activities. Namely, some of the DNA polymerase complexed to the endogenous template might be dissociated, thus increasing the amount of "free" enzyme to make use of the native DNA template. Alternatively, one might postulate instability on the part of the endogenously-templated activity, as well as the removal of factor(s) originally present in the crude extract that interfered with the native DNA-directed DNA polymerase assay.

TABLE XIII. Partial Purification of Rat Thymus DNA Polymerases

PROCEDURE A

Enzyme Fraction	Template	Total Protein (mg)	Total Activity (pmol/hr)	Sp. Act. (pmole/hr/mg protein)	Purification	Yield (%)
I. 39,000 x g Supernatant	Endogenous	145	2,700	19	1	100
	Native DNA	145	17,700	122	1	100
	"Activated" DNA	145	451,100	3,110	1	100
II. Sephadex G 150	Endogenous	84	2,440	29	1.52	90
	Native	84	22,000	262	2.14	124
	"Activated" DNA	84	340,300	4,060	1.30	75

PROCEDURE B

I. 39,000 x g Supernatant	Endogenous	408	5,056	12.39	1	100
II. Sepharose 6B	Endogenous	133	5,349	40.21	3.57	105

(A) The 39,000 x g supernatant of a thymus homogenate was prepared as described in *Methods*. A 10 ml aliquot was then fractionated on a Sephadex G150 column (3 x 40 cm) and the void volume fractions were pooled. Enzyme activities were assessed as described in *Methods*, except that glycine-NaOH was replaced with a similar concentration of Tris-HCl, pH 8.0, and the sp. act. of the ³H-dTTP was 2,820 cpm/pmole.

(B) The 39,000 x g supernatant was prepared as described in *Methods*, and a 20 ml aliquot was fractionated on a Sepharose 6B column (5 x 50 cm) equilibrated with Buffer B. The endogenously-templated activity was determined as described in *Methods*. The specific activity of the ³H-dTTP was 6,000 cpm/pmole.

Protein concentrations were determined by the method of Lowry *et al.* (279) for both preparations.

It was apparent from Sephadex G150 chromatography that these activities were associated with species of molecular weight greater than the exclusion limit of the gel; thus, in an attempt to achieve a better purification of the enzymes, as well as possibly resolving them from each other, gel filtration on a Sepharose 6B column was carried out. As can be seen in Fig. 2, little resolution of the activities was achieved; however, a substantial amount of extraneous material (as observed from the high turbidity of the fractions) eluted ahead of the DNA polymerases. This is reflected in the higher purity of the endogenously-templated activity relative to the crude extract, as shown in Table XIII B. On this column, the endogenously-templated activity was purified approximately 3.6-fold relative to the specific activity observed in the crude extract.

FIGURE 2. Profile of Rat Thymus DNA Polymerases on a Sepharose 6B Column.

A 25% (w/v) rat thymus homogenate was prepared as described in the legend to Fig. 1, and a 1 ml aliquot of the 39,000 x g supernatant was fractionated on a Sepharose 6B column (1.5 x 25 cm) equilibrated with Buffer A, and eluted with the same Buffer. DNA polymerase activities were assessed under the conditions described in the legend to Fig. 1 except that the time of incubation was 30 min.

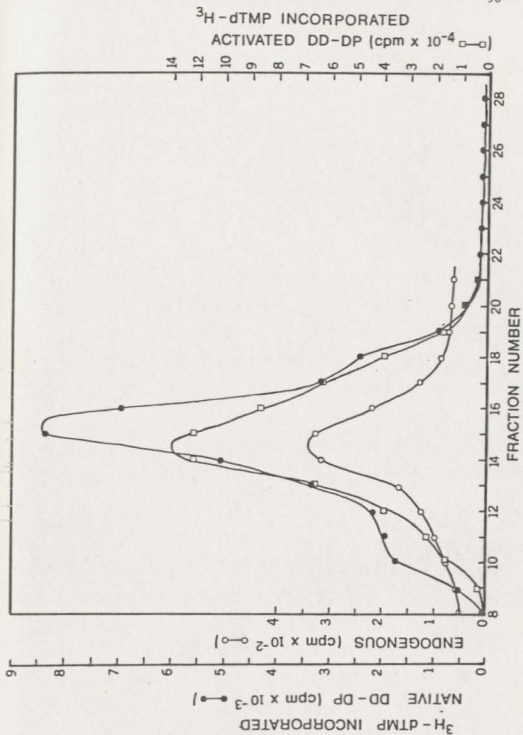


FIGURE 2. Profile of Rat Thymus DNA Polymerases on a Sepharose 6B Column.

II. Properties of the Endogenous-RNase-Sensitive DNA Polymerase

(A) Requirements

The reaction requirements for the endogenously-templated DNA polymerase partially purified on a Sephadex G150 column are shown in Table XIV. Consistent with the expected properties of a replicative DNA polymerase rather than those of a terminal deoxynucleotidyl transferase, the endogenously-templated DNA polymerase requires the presence of all four deoxynucleoside triphosphates for maximum activity. Deletion of the three unlabelled nucleotides resulted in approximately one-eighth of the activity, while the deletion of each individually was less effective. An absolute requirement for a divalent cation was evident. Both Mg^{2+} and Mn^{2+} were used by the enzyme although with different efficiencies. Manganese at a concentration one-tenth that of Mg^{2+} , proved to be twice as efficient as Mg^{2+} . Pretreatment with pancreatic RNase A was found to abolish 96% of the activity, suggesting that RNA is required either as a template or as a primer.

(B) Heteropolymeric Nature of the Nucleic Acid Region Transcribed by the Endogenous RNase-Sensitive DNA Polymerase

In the course of attempting to determine whether an RNA-directed DNA polymerase existed in eukaryotes, Rougeon *et al.* (185) have observed in an enzyme preparation from chick embryo that only the poly(A) tracts of the mRNAs examined as templates, were transcribed. This was not consistent with the presence of a "true" reverse transcriptase since such an enzyme does not discriminate between homopolymeric and heteropolymeric regions of the RNA template (13). Although the data presented in Table

TABLE XIV. Requirements of the RNase-Sensitive DNA Polymerase*

Reaction Conditions	Incorporation of [³ H]dTMP (%)
EXPERIMENT A	
Complete	100
-dATP, -dCTP, -dGTP	12
-dATP	29
-dCTP	46
-dGTP	48
Complete ¹ (enzyme preincubated 30 min at 24° C)	81
+RNase A (enzyme preincubated 30 min at 24° C in presence of 200 µg RNase A/ml)	4
EXPERIMENT B - Divalent Cation Requirement	
Complete	100
-Mg ⁺⁺	0
-Mg ⁺⁺ , +Mn ⁺⁺ (0.5 mM)	213

1 ml of the 39,000 x g supernatant prepared as described in the legend to Fig. 1 was fractionated on a Sephadex G150 column (1.5 x 30 cm) equilibrated with Buffer A. The eluted fractions were assayed for endogenous activity in a total volume of 50 µl containing: 67 µM of each of dATP, dCTP, and dGTP, 8 µM of [³H] dTTP (sp. act. 3,500 cpm/pmole), 4 mM dithiothreitol, 3.34 mM MgCl₂, 26.6 mM Tris-HCl pH 8.0, and 25 µl of enzyme preparation in Buffer A. 15 minute incubations at 37° C were carried out, and the samples subsequently treated as described in the legend to Fig. 1. The most active fractions, which eluted in the void volume, were pooled and used as the source of activity. Assay conditions were altered from those just described, as noted in the table, and all assays were carried out in duplicate. In experiments A and B, 100% activity corresponds to 1,300 cpm and 890 cpm/assay, respectively.

1 Two 100 µl aliquots, one of which contained 20 µg RNase A, were incubated for 30 minutes at 24° C.

* Reproduced from Moranelli (281).

XIV with regard to the requirement for all four deoxynucleoside triphosphates for maximum activity does suggest the synthesis of heteropolymeric DNA product, the evidence is not conclusive. In fact, Sarin *et al.* (281a) have observed that when reverse transcription was carried out with *E. coli* DNA polymerase I with AMV-RNA as template, the DNA polymer synthesized was mostly homopolymeric, only 2-20% of the total DNA product synthesized was heteropolymeric. For these reasons, an experiment was designed to determine the quantity of each deoxynucleoside monophosphates incorporated into DNA product by the endogenously-templated DNA polymerase (Table XV). The results indicated that all four nucleotides were incorporated into the DNA product in comparable quantities. There were 1.30, 1.15, 1.67 and 0.85 pmoles per hour of each of ^3H -dAMP, ^3H -dTMP, ^3H -dCMP and ^3H -dGMP respectively, incorporated into the product (see Table XV). Thus the template regions transcribed by the endogenously-templated DNA polymerase were heteropolymeric in nature.

(C) Preliminary Evidence in Favor of a Template Function for the RNA in the Endogenously-Templated RNase-Sensitive DNA Polymerase

As pointed out earlier [see Literature Review, Section IIIE(a)], preliminary evidence in showing whether RNA serves as a template in the endogenously-templated RNase-sensitive DNA polymerase (RS-DP) reaction may be obtained by demonstrating the RNase-sensitivity at low concentrations of RNases in the presence of 0.2 M salt (223a). This follows since RNase A and T_1 specifically degrade single-stranded RNA, and the presence of 0.2 M salt will stabilize RNA-DNA hybrids, therefore protecting the RNA moiety of the hybrid from the RNases. Thus, if the RNA

TABLE XV. Incorporation of Tritium-Labelled Deoxy-nucleoside Monophosphates into DNA by the Endogenously-Templated RNase-Sensitive DNA Polymerase.

<u>Labelled Precursor</u>	<u>Amount of Incorporation</u>	
	<u>cpm/hr</u>	<u>pmoles/hr</u>
^3H -dATP	7,870	1.30
^3H -dTTP	7,920	1.15
^3H -dCTP	5,930	1.67
^3H -dGTP	810	0.85
^3H -dATP, ^3H -dTTP, ^3H -dGTP and ^3H -dCTP	22,160	-

The enzyme sample was partially purified on a Sepharose 6B column, and assays were carried out as described in *Methods* except that the concentrations of the deoxynucleotides were at 8 μM each. Either one or all four of the nucleotides in the assay were ^3H -labelled. The assays were carried out in triplicate using 5 minute incubations and the average values, corrected for background and normalized to 1 hr incubations are shown. The specific activities of ^3H -dATP, ^3H -dTTP, ^3H -dCTP and ^3H -dGTP were 6,040, 6,870, 3,540 and 950 cpm/pmole respectively.

serves as a template in the RS-DP reaction, then the endogenously-templated activity should be sensitive to low concentrations of RNases in the presence of 0.2 M salt, whereas if its function is to act as a primer, the activity should be resistant to the RNase-treatment. The experimental results shown in Table XVI indicate that even under the high salt conditions a substantial proportion (46-58%) of the activity is abolished by relatively low concentrations of RNases, compared to the levels normally used to eliminate most of the activity (see Table XIV). Although the results obtained (Table XVI) are not quantitative since the salt concentration must be reduced in the reaction mixture when assessing the endogenous activity, thus destabilizing DNA-RNA hybrids and making the putative RNA primer susceptible to nuclease digestion, the data do provide qualitative evidence in favor of a template function for the RNA.

(D) Properties of the Endogenous-RNase-Sensitive DNA Polymerase

(a) Catalytic Properties

(i) Time-Course of the Reaction.

Figure 3 shows the time-course of the endogenous RNase-sensitive DNA polymerase (RS-DP) reaction. As can be seen, the reaction was linear for only approximately five minutes, after which the rate decreased and eventually plateaued. The reason for this short period of linearity is not clear, and attempts to extend it were unsuccessful. The depletion of deoxynucleoside triphosphates is only partially responsible for this short period of linearity since the addition of fresh substrates to the reaction mixture at 15 minutes after initiation of the reaction increased the total amount of DNA synthesized by only 50%.

TABLE XVI. Sensitivity of the Endogenously-Templated DNA Polymerase Activity to RNase A

and T_1 in the presence of 200 mM NaCl.

<u>Treatment</u>		<u>Activity</u> <u>(cpm)</u>	<u>Activity Relative</u> <u>to Control Sample I</u> <u>(%)</u>	<u>Activity Relative</u> <u>to Control Sample II</u> <u>(%)</u>
(A)	Control sample I	1,190	100	-
(B)	Control sample plus addition of RNases prior to assay (Control II)			
	(1) 20 μ g RNase A + 1.05 μ g T_1	310	26	100
	(2) 1.2 μ g RNase A + 1.05 μ g T_1	590	49	100
(C)	Sample pretreated with			
	(1) 20 μ g RNase A + 1.05 μ g T_1	170	14	54
	(2) 1.2 μ g RNase A + 1.05 μ g T_1	250	21	42

- (A) 100 μ l of the enzyme preparation was adjusted to 200 mM NaCl by the addition of 6 μ l of 4 M NaCl. This was followed by incubating for 1 hr at 24° C and 10 μ l aliquots were subsequently assayed in duplicate for endogenous activity for 15 minutes at 37° C.
- (B) Two 100 μ l aliquots of sample were preincubated as in (A) and the denoted quantities of RNases added just prior to the assessment of activity as described in (A).
- (C) Two 100 μ l aliquots of sample were adjusted to 200 mM NaCl as in (A) and treated with the denoted quantities of RNases for 1 hr at 24° C. 10 μ l aliquots were then assayed for endogenously-templated activity as in (A). The sp. act. for RNase T_1 was 1.6×10^6 units/mg protein and for ^3H -dTTP, 3,500 cpm/pmol.

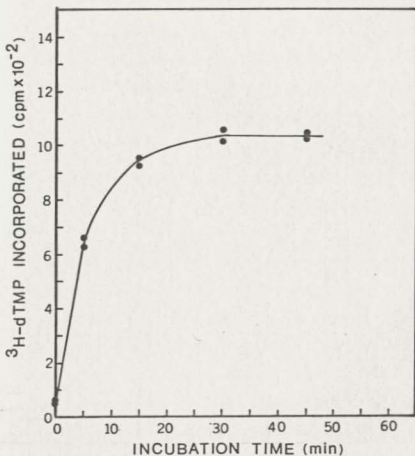


FIGURE 3. Time-Course of the Endogenous RS-DP Activity.

The enzyme was prepared as described in the legend to Table XIV. Assays for the endogenous activity were carried out as described in Table XIV, except that the incubation time was varied as indicated.

Further evidence that the substrates are not entirely depleted after 15 minutes was provided by the observation that the addition of "activated" DNA to the reaction at 15 minutes elicited the synthesis of twenty times more product than observed for the endogenous RS-DP reaction. However, the rate with "activated" DNA as template was only approximately 30% of the initial rate of polymerase α in the preparation (data not shown). The further addition of enzyme at 15 minutes resulted in approximately a 20% increase in activity above the plateau level (data not shown).

The reduced rate of polymerase activity for the RS-DP after the first 10 minutes is not due to inactivation of the enzyme nor hydrolysis of the template at 37° C, since preincubation of the enzyme preparation at 37° C for periods as long as one hour prior to addition of substrates did not reduce the level of endogenously-templated activity (data not shown). Thus it appears that the reason for the short period of initial rate is probably due to the depletion of active template (not by hydrolysis of the template), the depletion of substrates, or to an efficient inhibition of the activity by the by-product(s) of the reaction (e.g., pyrophosphate).

(ii) Activity Versus Enzyme Concentration Curves.

The RS-DP activity versus concentration curve was found to be non-linear (Fig. 4). That is the activity observed at low concentrations of the enzyme was less than proportional to the activity observed at higher concentrations. In addition, at sufficiently high concentrations of the enzyme, a very pronounced inhibition occurred (Fig. 5). The reason for these observations is not clear, although they may suggest the possible presence of both a positive effector as well as an inhibitor. The possibility that the template-DNA polymerase complex dis-

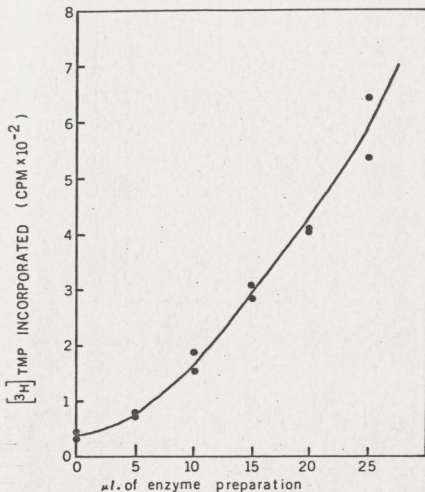


FIGURE 4. Sigmoidal Relationship Between Activity and Concentration of the RS-DP Complex.

The enzyme was prepared and the activity assessed as described in Table XIV, except that various quantities of enzyme preparation were used and incubations were for five minutes.

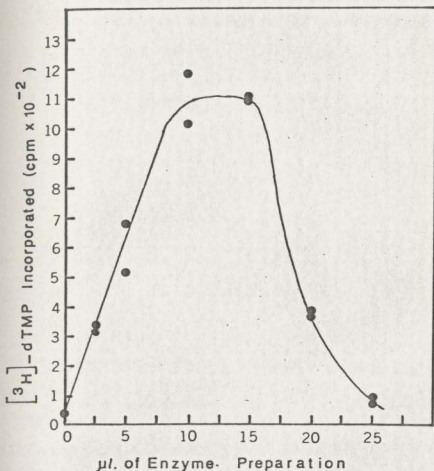


FIGURE 5. Inhibition of the RS-DP Activity at High Concentrations of the RS-DP Complex.

The sample used for the experiment described in Fig. 4 was 6-fold concentrated, and the activity assessed as described in the same figure. The enzyme sample was concentrated by dialysis against 30% PEG in Buffer A.

sociates at low concentrations with a concomitant loss of activity, and aggregates to an inactive form at high concentrations also exists.

Sigmoidal activity versus enzyme concentration curves for the exogenously-templated RD-DP activity derived from the endogenously-templated activity have been previously observed by Moranelli (281). Such curves were also obtained by Penner (personal communication) with DNA polymerases α and β of rat thymus. However the sigmoidicity could be abolished by carrying out the assays in siliconized test tubes (Penner, personal communication). It therefore appeared that the sigmoidicity was due to the adsorption of the enzyme to the glassware which reduced the effective concentration of the enzyme, thus accounting for the less than proportional level of activity at lower enzyme concentrations. Thus activity versus enzyme concentration curves for the RS-DP complex were determined in both siliconized and non-siliconized tubes (Fig. 6). As can be seen, the curve was still sigmoidal regardless of whether the glassware was or was not treated with Siliclad. Thus, it would appear, at least in the case of RS-DP, that the sigmoidicity is not due to adsorption of the enzyme to the glassware.

(iii) pH Optimum.

The pH optimum of the endogenous RS-DP activity is shown in Fig.

7. In Tris-HCl buffer, the pH optimum was quite broad and seemed to occur at about pH 7 to 7.5 although a lower limit was not obtained. Even at pH 8, the enzyme was still approximately 90% as active as at pH 7.5.

In glycine-NaOH buffer, on the other hand, the pH optimum was more alkaline, namely in the range of 8 to 8.5. Furthermore the enzyme was more active in glycine-NaOH buffer than in Tris-HCl. This buffer effect is not uncommon and has been observed with other DNA polymerases (27, 42a,

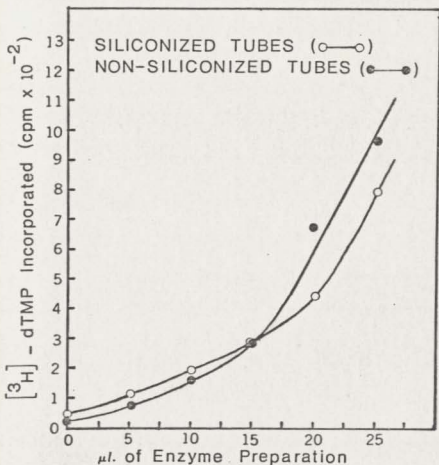


FIGURE 6. Activity Versus RS-DP Concentration Curves in Siliconized and Non-Siliconized Tubes.

The endogenously-templated activity was prepared in Buffer B and partially purified on a Sepharose 6B column. Assay conditions for the activity were identical to those described in *Materials and Methods* except that various quantities of enzyme preparation were used. Duplicate assays were carried out in both siliconized and non-siliconized tubes as indicated in the figure, and the average plotted. The sp. act. of the [³H] dTTP was 6,800 cpm/pmole.

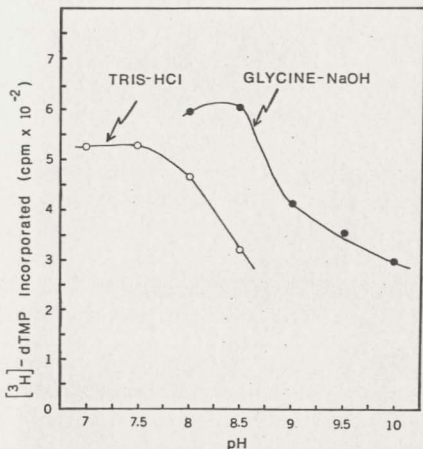


FIGURE 7. pH Optimum of the Endogenous RS-DP Activity.

The enzyme sample was prepared as described in Fig. 6, and a 1 ml aliquot was dialyzed for several hours against 1 liter of Buffer B solution lacking the glycine-NaOH. Reaction conditions were as described in *Materials and Methods*, except that the 40 mM glycine-NaOH at pH 8.5 in the reaction mixture was replaced by 40 mM of the buffers at the appropriate pH as indicated in the Figure. Assays were carried out in duplicate, and the average plotted. The sp. act. of the [³H]dTTP was approximately 6,500 cpm/pmol.

54, 55, 58, 67, 69, 198, 199, 201).

(iv) Divalent Cation Requirements.

Figure 8 shows the divalent cation effect on the RS-DP activity. As with other DNA polymerases, an absolute requirement for a divalent cation was evident. It is noteworthy that Mn^{2+} at its optimum concentration (1-2 mM) was twice as effective as Mg^{2+} at its optimum (3-5 mM). This is consistent with the preference for Mn^{2+} previously reported from this same system (281) for the exogenously-templated RD-DP activity, as well as the divalent cation preference of RD-DP activities from oncogenic RNA viruses when RNA is the template (13, 210, 211). This observation distinguishes the RS-DP from the DNA-directed DNA polymerases where Mg^{2+} is the preferred cation [see Literature Review, sections III(A)(c)(ii), III(B)(c)(ii), III(D)(c)(ii)].

(v) Effect of Potassium Chloride.

Concentrations of KCl greater than 40 mM inhibited the endogenously-templated RS-DP activity (Fig. 9). This inhibition however was small, and at a concentration of 100 mM KCl, about 90% of the activity observed at 40 mM salt still remained. The effect of salt was identical to its effect on the exogenously-templated RD-DP activity previously reported (281), and differed from the effect on DNA polymerase α cofractionating with the RS-DP activity. Polymerase α was previously shown to be inhibited to a far greater extent in the presence of high salt (281).

(b) Inhibition Studies

(i) Effect of Actinomycin D and Distamycin A on the Endogenously-

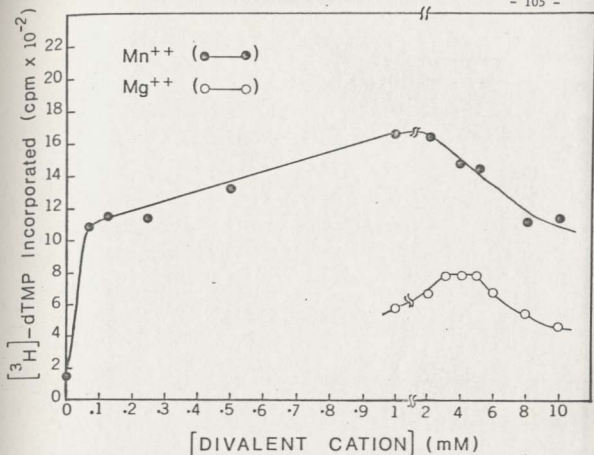


FIGURE 8. The Effect of Divalent Cations on the Endogenous RS-DP Activity.

The enzyme sample was prepared as described in Fig. 6, and an aliquot was dialyzed for several hours against 1 liter of Buffer B lacking EDTA. Reaction conditions were as described in *Materials and Methods*, except that the concentration of Mg^{2+} was varied as indicated in the figure, or was replaced by various concentrations of Mn^{2+} . All assays were carried out in duplicate and the averages plotted.

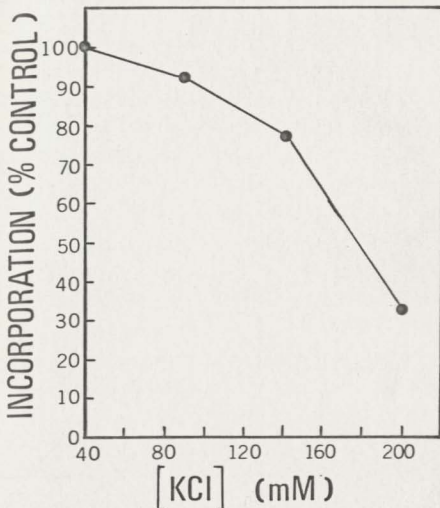


FIGURE 9. Effect of Potassium Chloride on the Endogenous RS-DP Activity.

The enzyme sample was prepared as described in Fig. 6, and the reaction conditions were as described in *Materials and Methods*, except that the concentration of KCl was varied as indicated. Assays were carried out in duplicate, and the averages plotted. 100% activity is equal to 1,275 cpm/assay. The sp. act. of [^3H]TTP was 6,500 cpm/pmole.

Templated Activity.

Interest in the effects of actinomycin D and distamycin A on the endogenous activity lies in their specificities. Both actinomycin D and distamycin A inhibit DNA-directed nucleic acid polymerases, and their inhibition mechanism seems to reside in their ability to intercalate between G-C (actinomycin D, 282) and A-T (distamycin A, 283) base pairs in double-stranded DNA. These inhibitors on the other hand, will not interact with single-stranded RNA. This property has allowed them to be used as tools in the delineation of the RNA-directed reaction (284) and has provided the necessary conditions for the isolation of RNA-DNA hybrid intermediates characteristic of the RD-DP reactions (285). Thus, it would be expected that if the RS-DP is a "true" reverse transcriptase, the activity should be partially resistant* to these inhibitors.

The effects of actinomycin D and distamycin A on the endogenous RS-DP are depicted in Figs. 10 and 11 respectively. At concentrations of 50 and 100 μg per ml of actinomycin D, the RS-DP was inhibited approximately 55% (Fig. 10). Polymerase α from the same source on the other hand was inhibited 88-92% at the same concentrations of actinomycin D (281).

Analogous results were obtained with distamycin A (Fig. 11). At concentrations of 25 to 200 μg of inhibitor per ml, 60-70% of the RS-DP activity was abolished, whereas essentially all of the DNA polymerase α

* This follows since "reverse transcriptases" catalyze a two step reaction; an initial step in which the RNA-DNA hybrid is formed which is resistant to these inhibitors and a second step in which the hybrid is converted to double-stranded DNA, which is sensitive.

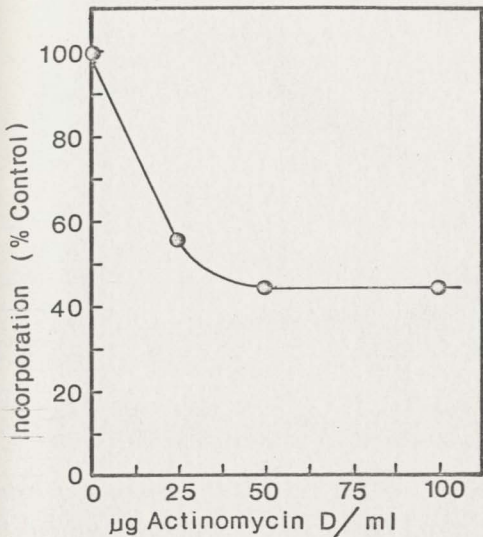


FIGURE 10. Effect of Actinomycin D on the Endogenous RS-DP Activity.

The enzyme preparation was obtained as described in Fig. 6, and assay conditions were identical to those described in *Materials and Methods*, except that the activity was assessed in the presence of the inhibitor concentrations indicated. The averages of duplicate assays have been plotted. 100% activity is equal to 2,040 cpm of [^3H]dTMP incorporated (sp. act. = 6,500 cpm/pmole).

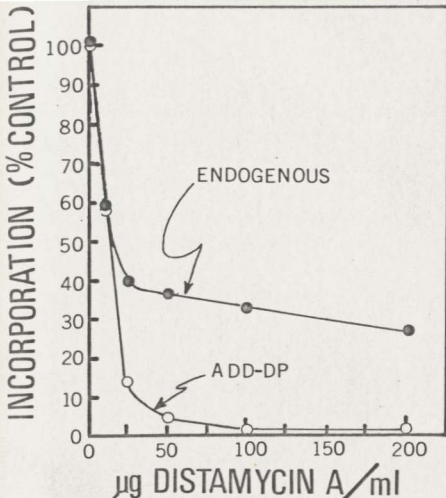


FIGURE 11. Effect of Distamycin A on the Endogenous RS-DP and DNA Polymerase α of Rat Thymus.

The enzyme from the 39,000 x g supernatant was partially purified by chromatography on a Sephadex G150 column. The active fractions eluting from the column were pooled and concentrated as described in *Materials and Methods*. Assay conditions for the endogenous activity and DNA polymerase α (ADD-DP assayed with an "activated" DNA template) were as described in *Materials and Methods*, except that 40 mM Tris-HCl, pH 8.0 was used in the case of DNA polymerase α . Both activities were assayed in duplicate for 5 min at 37° C in the absence or presence of the concentrations of distamycin A indicated. 100% activity for the endogenously-templated activity, and for DNA polymerase α , was 540 cpm (sp. act. 3,500 cpm/pmole) and 56,300 cpm (sp. act. 3,500 cpm/pmole) respectively.

activity was eliminated (Fig. 11).

The results obtained with these inhibitors provide strong evidence in favor of a template function for the RNA in the endogenously-templated DNA polymerase complex.

(ii) Effect of N-Ethylmaleimide on the RS-DP.

The effect of the sulfhydryl blocking reagent NEM on the RS-DP and on DNA polymerase α from rat thymus is shown in Fig. 12. As can be seen, the RS-DP activity was hardly affected by the inhibitor even at concentrations as high as 200 μ g per ml, whereas polymerase α was inhibited more than 95% at the same inhibitor concentration. These results indicate that the RS-DP activity is not related to DNA polymerase α and γ which are sensitive to sulfhydryl blocking reagents (see Table II). The response of RS-DP to NEM is, however, similar to that of DNA polymerase β and mitochondrial DNA polymerase.

(iii) Effect of Heparin.

Fig. 13 shows the effect of heparin on the RS-DP and on rat thymus DNA polymerase α . At a concentration of 50 μ g of inhibitor per ml, the RS-DP activity (Fig. 13, endogenous) was stimulated approximately 25%, whereas polymerase α (Fig. 13, DD-DP) was inhibited by 25%. Higher concentrations of inhibitor were inhibitory towards both activities. At a concentration of 200 μ g heparin per ml the RS-DP was reduced approximately 75%, and DNA polymerase α more than 90% relative to the control levels.

(iv) Effect of Polyamines on the RS-DP and DNA Polymerase α .

The effects of polyamines (putrescine, spermidine, and spermine) on

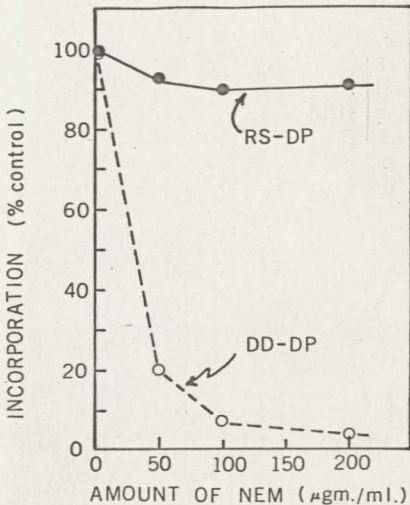


FIGURE 12. Effect of N-Ethylmaleimide on the Endogenous

RS-DP and DNA Polymerase α of Rat Thymus.

The enzyme preparation was partially purified as described in Table XIV. Assays for the endogenous (RS-DP) activity were also as described in Table XIV, and for DNA polymerase α as described in Fig. 11, except that the reaction was carried out for 15 minutes. The DNA polymerase α preparation was the Peak I activity described later (Fig. 30C). 100% activity was taken as the activity observed in the absence of inhibitor. All assays were carried out in duplicate, and the averages plotted.

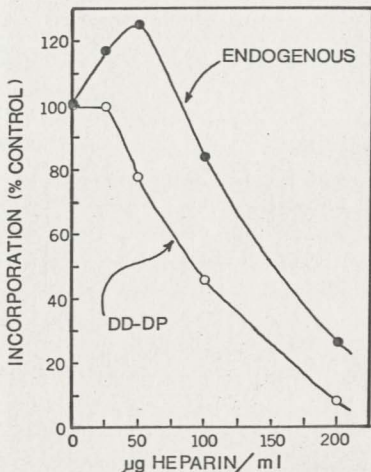


FIGURE 13. Effect of Heparin on the Endogenous RS-DP and DNA Polymerase α of Rat Thymus.

The enzyme was partially purified by gel filtration on a Sepharose 6B column and assay conditions were as described in *Materials and Methods*, using "activated" DNA as the template for DNA polymerase α (DD-DP) and 40 mM Tris-HCl, pH 8 as a buffer in place of glycine-NaOH. Duplicate assays were carried out for 5 minutes and the averages plotted. 100% activities for the endogenously-templated enzyme, and for DNA polymerase α were 1,590 cpm (sp. act. 6,500 cpm/pmole) and 74,280 cpm (sp. act. 3,500 cpm/pmole), respectively.

the RS-DP activity as well as on DNA polymerase α are shown in Fig. 14. It was observed that at a concentration of 4 mM putrescine, the RS-DP was not affected whereas DNA polymerase α was inhibited by more than 60% (Fig. 14A). Spermidine on the other hand was inhibitory to both activities, although the effect on DNA polymerase α was more pronounced than that on the RS-DP (Fig. 14B). Low levels of spermidine (5 to 80 μ M) had essentially no effect on both activities (data not shown) while spermine in the concentration range of 2.5 to 40 μ M produced a slight stimulation of DNA polymerase α and an inhibition of the RS-DP activity (10-30%, Fig. 14C). Higher concentrations (0.5 to 4 mM) were found to inhibit both activities, although the inhibition effect on DNA polymerase α was greater than that on the RS-DP (Fig. 14D). On the basis of these findings it is thus possible to distinguish the RS-DP from DNA polymerase α .

(c) Physical Properties of the Endogenous Activity

(i) Effect of Triton X-100 on the Endogenous RS-DP Activity.

Viral reverse transcriptases are normally sequestered in the virion, and cannot be assessed for activity unless access to the substrates is first promoted by disrupting the viral envelope (13, 210, 211). This is normally achieved by treating the virus particles with a non-ionic detergent such as Triton X-100 which results in a large stimulation of reverse transcription (13, 210, 211). The stimulatory effect however is observed only within a narrow range of detergent concentrations, and at high concentrations, inhibition is observed (13, 210, 211).

The possibility that the endogenous RNA-directed DNA polymerase of

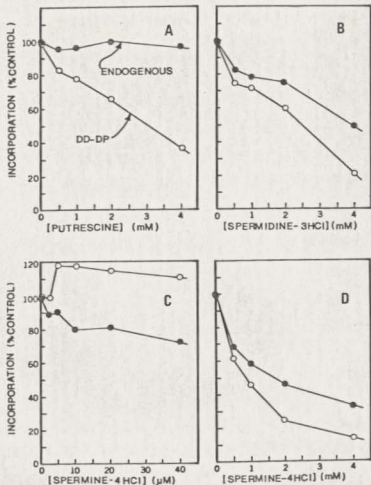


FIGURE 14. Effect of Polyamines on the Endogenous RS-DP and DNA Polymerase α of Rat Thymus.

The enzyme preparation and reaction conditions were as described in the legend to Fig. 13, except that in the case of DNA polymerase α (DD-DP), 5 μ l of 1.2 mg/ml "activated" DNA per assay was used instead of 10 μ l. In addition, assays for both enzymes were carried out in the absence or presence of various concentrations of polyamines as indicated. Assays were for 5 minutes in duplicate for both activities. For the endogenously-templated enzyme and DNA polymerase α 100% activity was 1,160 and 86,200 cpm/assay, respectively.

rat thymus is of viral origin was tested by examining the effect of detergent treatment (Fig. 15). It was previously pointed out by Wu and Gallo (13) that freezing and thawing of certain virus preparations may be sufficient to disrupt the virions, making the detergent treatment unnecessary. Thus, in the experiment shown in Fig. 15, the thymus homogenate was not frozen and thawed as normally done, hence if the enzyme is of viral origin a large activation of the activity should be observed upon treatment with detergent. Within the concentration range of 0.01-1% (v/v) of Triton X-100, no stimulation of activity was observed (Fig. 15). This is consistent with a non-viral origin for the enzyme, although the possible presence of viral components not assembled into intact virions cannot be excluded by this data.

(ii) Buoyant Density of the Endogenous RS-DP.

The endogenous RS-DP from chicken embryos (22) was reported to be particulate in nature. In the present study part of the RS-DP activity from rat thymus was also found to be particulate, with 40 to 60% of the total activity in the cell extract pelleting in a one hour centrifugation at 164,900 x g (data not shown).

Buoyant density analysis in sucrose gradients of the enzyme partially purified on a Sepharose 6B column revealed a density of 1.06 g/cm^3 for the main component of the RS-DP activity (Fig. 16), almost identical to that reported by Kang and Temin (22) for the endogenously-templated activity from chicken embryos. In addition, a minor component at a density of approximately 1.13 g/cm^3 was also observed (Fig. 16). The proportion of enzyme activity observed at these two densities was found to depend on the degree of purification of the enzyme sample. For example, when

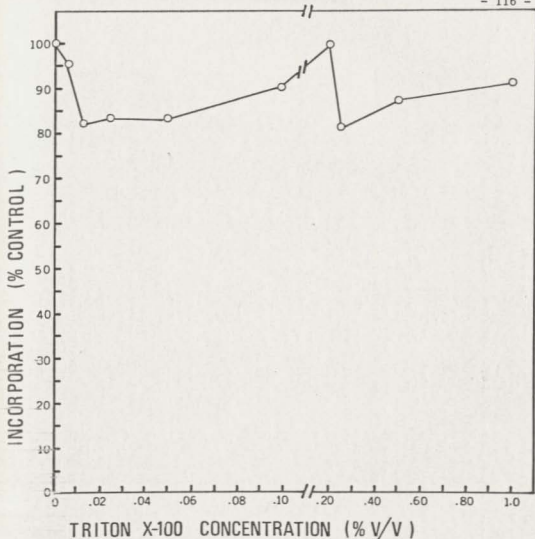


FIGURE 15. The Effect of Triton X-100 on the Endogenous RS-DP of Rat Thymus.

A rat thymus homogenate was prepared as described in *Materials and Methods*, except that it was not frozen and thawed. The activity from the 39,000 x g supernatant was partially purified on a Sepharose 6B column and the pooled active fractions were tested for their response to Triton X-100. Assays (in duplicate) were carried out as described in *Materials and Methods*, except that the indicated concentrations of detergent were included. 100% activity prerepresents 1,345 cpm of [^3H]dTMP incorporated (sp. act. = 6,500 cpm/pmole).

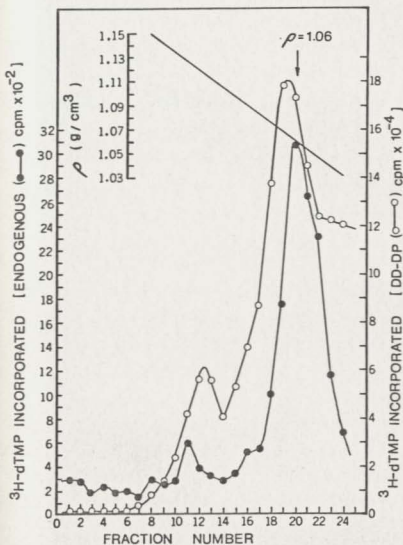


FIGURE 16. Buoyant Density Analysis of the Endogenous RS-DP Activity.

The buoyant density of an enzyme sample (400 μl) partially purified on a Sepharose 6B column was determined as described in *Materials and Methods*. Assays for the endogenous activity were as described in *Materials and Methods* except that the incubation time was 30 minutes as for DNA polymerase α . Assay conditions for DNA polymerase α were as described in the legend to Fig. 14.

the 39,000 x g supernatant was used, the amounts of enzyme at the two densities was equal (data not shown). This variation may be due to the adsorption of other components present in the extract, to the RS-DP complex thus producing heterogeneity in the buoyant density of the complex. The buoyant densities observed for the RS-DP activity further argue against a viral origin for the enzyme. This follows since viral particles have buoyant densities of 1.14 to 1.17 g/cm³, and disrupted virions densities of 1.22-1.24 g/cm³ (13).

(iii) Molecular Weight of the Endogenous RS-DP.

The size of the endogenous RS-DP activity, as determined by its elution volume on a Sepharose 6B column is shown in Fig. 17. Calibration of the column with the marker proteins - fumarase, aldolase and bovine serum albumin gave an estimated molecular weight of approximately 280,000 daltons.

(iv) Isoelectric Point of the Endogenous RS-DP.

As expected for a nucleic acid protein complex, the pI of the RS-DP complex was acidic (Fig. 18). That is, the activity focused at a pH of approximately 4.5. The polymerase α activity also had an acidic pI, namely 4.8. This low pI for polymerase α , as in the case of the RS-DP activity, was probably due to the association of nucleic acid with it (86).

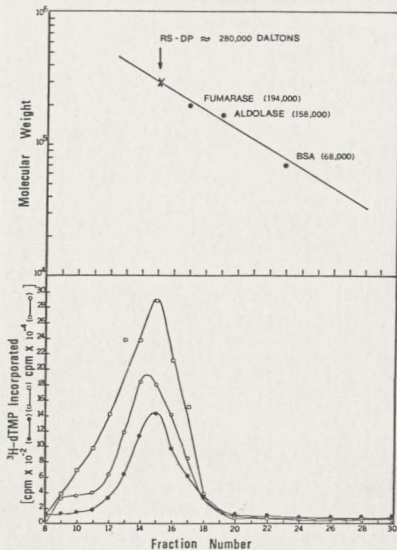


FIGURE 17. Molecular Weight Determination of the Endogenous RS-DP on a Sepharose 6B Column.

A 1 ml aliquot of an enzyme preparation (in Buffer A) initially eluted from a Sepharose 6B column with Buffer A and subsequently concentrated by dialysis against 30% (w/v) PEG in Buffer A, was rechromatographed on a Sepharose 6B column (approximately 1.5 x 24 cm) equilibrated with the same buffer, and 1.2 ml fractions were collected. Activities with native DNA ($\square \rightarrow \square$), "activated" DNA ($\circ \rightarrow \circ$), and no template ($\bullet \rightarrow \bullet$) were determined as described in *Materials and Methods* except that glycine-NaOH was replaced by Tris-HCl, pH 8.0 in all cases, and all assays were for 30 minutes. The specific activity of [^3H]dTTP was in all cases approximately 3,500 cpm/pmole.

The same column was calibrated with the marker proteins BSA, Aldolase and Fumarase.

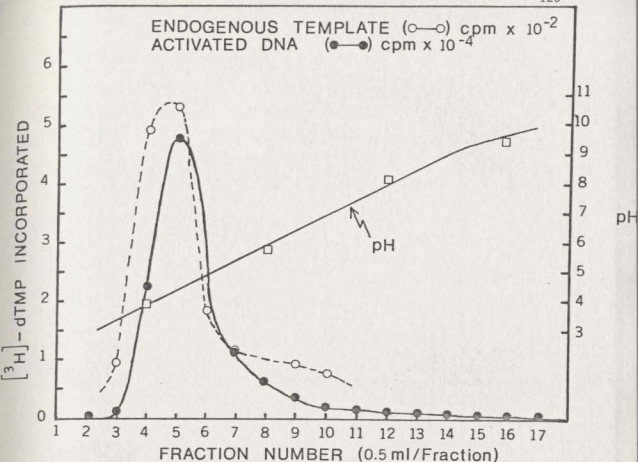


FIGURE 18. Isoelectric Focusing of the Endogenous RS-DP Activity.

Isoelectric focusing was carried out as described in *Materials and Methods*, using 400 μl of the 39,000 \times g supernatant of a rat thymus homogenate prepared in Buffer A. Assay conditions were as described in *Materials and Methods*, except that glycine-NaOH was replaced with 40 mM Tris-HCl, pH 8.0, and 10 minute assays were carried out.

III. Purification of the Endogenous RNase-Sensitive DNA Polymerase

(A) Reasons for Further Purification

The purpose of the work reported in this section was two-fold:

(1) to attempt to resolve the various DNA polymerases detected in rat thymus, and (2) to obtain a sufficiently pure preparation of the endogenously-templated RS-DP complex free of interfering factors (i.e., nucleases such as RNase H) to allow the isolation of a product that reflected the state in which it was originally synthesized. Two types of enzyme preparation were used: rat thymus, for the first objective, and later on, rat liver for pursuing the second objective.

(B) General Observations

When attempting to purify an enzyme from a cell extract, a useful initial step would be the use of a method that would enable a reduction in the large volume of extract that one would normally start out with. Among such methods are: ammonium sulfate precipitation, precipitation at acid pH, or precipitation with polyethylene glycol (PEG) (283a). The first of these methods was not found to be very useful in the purification of the RS-DP activity, since only low recoveries of the enzyme complex were obtained, and in addition the enzyme was found in every $(\text{NH}_4)_2\text{SO}_4$ cut taken [0-60% saturation of $(\text{NH}_4)_2\text{SO}_4$ at 10% saturation intervals, data not shown]. Acid precipitation of the enzyme at pH 5.5 (adjusted with Na-acetate) was more successful, and it was found that 40 to 110% of the initial activity could be precipitated at this pH (data not shown). However, large variations in recovery from preparation to preparation were obtained when using this method (low recoveries were

most often observed, data not shown) making it unsuitable. PEG precipitation on the other hand, gave consistently higher yields of activity with recoveries of 80% to greater than 100%, and furthermore, the recovery (if less than 100%) could be improved by additional PEG treatment. A level of 3.2% (w/v) PEG was found to be quite effective in precipitating most of the RS-DP activity.

Subsequent gel filtration of the PEG precipitates on Sepharose 6B columns was normally found to result in low recoveries of enzyme (< 60%) as well as a decrease in the specific activity of the RS-DP. Therefore other methods, such as ion exchange chromatography on DEAE-cellulose and on phosphocellulose, as well as further precipitation with 20 mM $MgCl_2$ were investigated. Unlike the endogenous RS-DP activity of *E. coli* K 12 (233) which survived passage through a DEAE-cellulose column, the RS-DP from rat thymus and liver could not be recovered from such columns. This is not surprising when one considers the high efficiency with which DEAE-cellulose removes nucleic acids from cell extracts. Essentially all of the endogenous activity loaded onto phosphocellulose columns could be recovered, although very little purification was normally achieved with this cation exchanger. Precipitation of the endogenous activity with 20 mM $MgCl_2$ was found to be very useful when relatively concentrated enzyme samples were used. Little precipitation of the activity occurred when the enzyme samples were dilute.

Good yields as well as substantial further purification of the RS-DP activity could be achieved by centrifugation at 164,900 x g for two hours. The only disadvantage with this latter method, however, was that a substantial amount of activity was found in both the pellet and the

supernatant. The specific activity in the 164,900 x g supernatant was usually high, however, so the pellet fraction was discarded. Further observations regarding this particular step of purification will be dealt with below (see Results, Section VE).

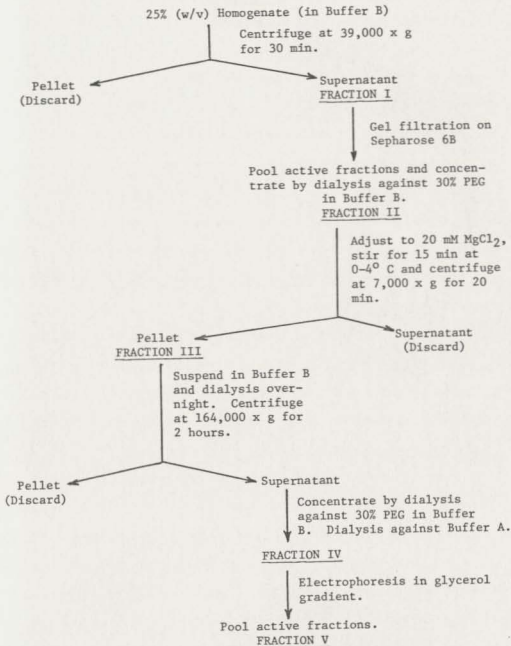
One further observation that deserves mention is that one can concentrate the enzyme sample quite easily by dialysis against 30% (w/v) PEG. Apparent recoveries of activity using this concentrating method were usually greater than 100% of the activity observed in dilute samples. The reason for this is related to the non-linear shape of the activity versus enzyme concentration curve for the RS-DP (see Figs. 4 and 6). However, as shown in Fig. 5 when the enzyme sample is too concentrated, very little activity might be detected. If this is the case, by carrying out the appropriate dilution, one may recover the original activity.

The non-linearity of the activity versus enzyme concentration curve has made it imperative that enzyme concentration curves be obtained at every stage of purification so as not to underestimate the activity. Thus, for every value reported in the subsequent tables (Tables XVII and XX), activity versus enzyme concentration curves were carried out, and the activity observed in the linear range of the curve was taken as the true value.

(C) Purification of DNA Polymerases from Rat Thymus

A flow chart for the purification of rat thymus DNA polymerases is shown in Scheme IX. The 39,000 x g supernatant was subjected to chromatography on Sepharose 6B, precipitation with 20 mM $MgCl_2$,

Flow Chart for the Purification of Rat Thymus DNA
Polymerases



centrifugation at 164,900 x g (50,000 rpm in Beckman 50 Ti rotor) for two hours, and subsequent electrophoresis in a 20-45% glycerol gradient. The overall purification achieved for the RS-DP and for native and "activated" DNA-directed DNA polymerases is shown in Table XVII. Although over 99% of the total protein was eliminated by the procedures used, the purification achieved for the three activities was relatively low. The endogenously-templated activity was purified approximately 30-fold whereas the exogenously-templated activities with native and "activated" DNA templates were purified 5- and 15-fold respectively.

The much higher recovery (185%) of endogenously-templated activity relative to the level observed in the 39,000 x g supernatant is probably due to the removal of an inhibitor (also supported by the observed inhibition at high concentrations of enzyme sample, Fig. 5). Conversely a substantial reduction in the recoveries of the exogenously-templated activities was observed (approximately 29% and 30% recovery for the native and "activated" DNA-directed activities respectively) due to the fact that only the fractions containing substantial levels of endogenously-templated activity were pooled at this stage of purification, and in addition the exogenously templated activities were relatively unstable at this stage of purification. Subsequent purification steps (see Table XVII) were accompanied by reduced yields of the RS-DP and native DNA-templated DNA polymerase activity, whereas the activity with "activated" DNA increased at the stage of $MgCl_2$ precipitation (Table XVII, Fraction III) relative to gel filtration (Table XVII, Fraction II), and subsequently decreased with further fractionation (see Table XVII).

While working out conditions under which electrophoresis could be

TABLE XVII. Purification of Rat Thymus DNA Polymerases

	Enzyme Fraction	Template	Total Protein (mg)	Total Activity (pmole/hr)	Sp. Act. (pmol/hr/mg protein)	Purif.	Yield (%)
I.	39,000 x g Supernatant	Endogenous	674	6,730	10	1	100
		Native DNA	674	103,000	153	1	100
		"Activated" DNA	674	3,233,000	4,800	1	100
II.	Sepharese 6B	Endogenous	132	12,500	95	9.5	185.7
		Native DNA	132	29,500	223	1.5	28.6
		"Activated" DNA	132	955,900	7,240	1.5	29.6
III.	20 mM MgCl ₂	Endogenous	57	4,600	81	8.1	68.8
		Native DNA	57	19,500	340	2.2	18.9
		"Activated" DNA	57	1,490,000	26,200	5.5	46.1
IV.	164,900 x g Supernatant	Endogenous	35	3,270	94	9.4	48.7
		Native DNA	35	9,420	270	1.8	9.1
		"Activated" DNA	35	863,500	24,700	5.0	26.7
V.	Electrophoresis	Endogenous	4.2	1,300	300	30.0	18.7
		Native DNA	4.2	3,400	810	5.3	3.3
		"Activated" DNA	4.2	312,000	73,600	15.3	9.6

A 49 ml volume of the 39,000 x g rat thymus supernatant in Buffer B was subjected to the fractionation procedures shown in Scheme IX. DNA polymerase assays were as described in *Methods*, except that the time of incubation was for 5 min. The specific activity of ³H-dTTP was 5,770 cpm/pmole for the endogenously-templated activity, and 2,900 cpm/pmole for the exogenously-templated activities. Protein concentrations were determined by the method of Lowry *et. al.* (279) for fractions I to IV and spectrophotometrically (280) for fraction V.

carried out in glycerol gradients, it was observed, that the DNA polymerase activities could be resolved into at least two peaks, one of which remained at the origin (Fig. 19). However, upon using Fraction IV (164,900 x g supernatant, Table XVII), a single broad peak of RS-DP activity was observed (Fig. 20). The reason for double peaks of activity with less pure preparation may be due to protein-protein or protein-nucleic acid interactions. Figure 19 also shows that the position of the DNA polymerases in the glycerol gradients was dependent on the length of time the voltage was applied.

Table XVIII shows the effect of ribonuclease treatment on the endogenously-templated activity at various stages of purification. Initially (Fraction I) the activity was totally sensitive to this treatment, whereas with later fractions, substantial levels of endogenous activity still remained after RNase-treatment, - Fraction II displaying the highest level of resistance (~ 39%). Approximately 75% of the endogenous activity in Fraction V was sensitive to RNase-treatment. It is not clear whether the activity resistant to RNases is DNA-directed, or whether the residual activity was due to incomplete hydrolysis of the RNA. Evidence in support of the latter was the observation that the more concentrated the enzyme preparation, the more difficult it was to totally abolish the endogenous activity.

The relative specific activities of the endogenously- and exogenously-templated activities are shown in Table XIX. The variation in the relative specific activity ratios of the RS-DP relative to the exogenously-templated DNA polymerases is in support of the notion that the activities are catalyzed by different enzymes. The ratio of the specific activities

TABLE XVIII. RNase-Sensitivity of the Rat Thymus Endogenously-Templated Activity During the Course of Purification

	Enzyme Fraction	Control (cpm)	Activity after RNase-treatment (cpm)	Activity Remaining (%)
I.	39,000 x g Supernatant	170	0	0
II.	Sepharose 6B	1780	700	39.0
III.	20 mM MgCl ₂	1580	120	7.5
IV.	164,900 x g Supernatant	1150	400	35.0
V.	Electrophoresis	1770	470	26.0

Aliquots (200 μ l) of each fraction described in Table XVII were incubated either in the absence or in the presence of 40 μ g RNase A and 2 μ l RNase T₁ for 3 hrs at room temperature. Assays for the endogenous activity were as described in *Methods*. The sp. act. of ³H-dTTP was 5,770 cpm/pmole.

TABLE XIX. Relative Specific Activity of Rat Thymus DNA Polymerases at Various Stages of Purification

Enzyme Fraction	Sp. Act. (pmoles/hr/mg protein)		Relative Sp. Act.	
	Endog.	Native	Endog.	Native
I. 39,000 x g Supernatant	10	153	1	15.3
II. Sepharose 6B	95	223	1	2.4
III. 20 mM MgCl ₂	81	340	1	4.2
IV. 164,900 x g Supernatant	94	270	1	2.9
V. Electrophoresis	300	810	1	2.7
				481
				76.5
				322
				264
				250

The specific activity data from Table XVII is expressed as the activity (for each fraction) with native and "activated" DNA templates relative to the endogenously-templated activity.

FIGURE 19. Glycerol Gradient Electrophoresis Profile of Rat Thymus DNA Polymerases from the 39,000 x g Supernatant.

Glycerol gradient electrophoresis was carried out as described in *Materials and Methods*, using 4 ml (A) and 3 ml (B) aliquots of a 39,000 x g supernatant further purified by precipitation at pH 5.5 (adjusted with 0.2 M sodium acetate), and gel filtration on a Sepharose 6B column equilibrated with Buffer A. The voltage (800 V) was applied for 6.5 (A) and 12.5 (B) hrs, after which 2.5 ml fractions were collected and assayed for DNA polymerases as described in *Materials and Methods*, except that glycine-NaOH was replaced by Tris-HCl, pH 8.0, and assays were for 30 minutes.

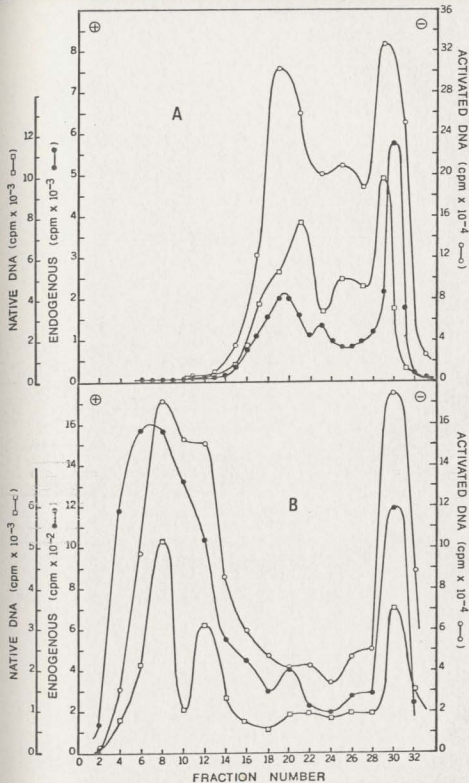


FIGURE 19. Glycerol Gradient Electrophoresis Profile
of Rat Thymus DNA Polymerases from the 39,000
x g Supernatant.

FIGURE 20. Glycerol Gradient Electrophoresis of the Endogenous RS-DP of Fraction IV of the Rat Thymus Preparation.

A 2.5 ml aliquot of Fraction IV (see Table XVII) of the rat thymus preparation was electrophoresed as described in *Materials and Methods* for 13.25 hours. At the end of the run, 2 ml fractions were collected and assayed for endogenous activity as described in *Materials and Methods* except that assays were for 30 minutes. The A_{280} profile and the A_{260}/A_{280} ratios were determined with a Beckman DB-G Spectrophotometer.

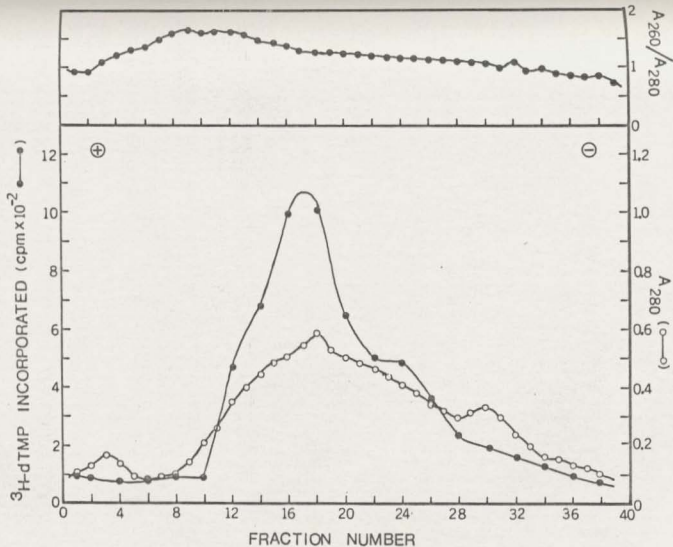


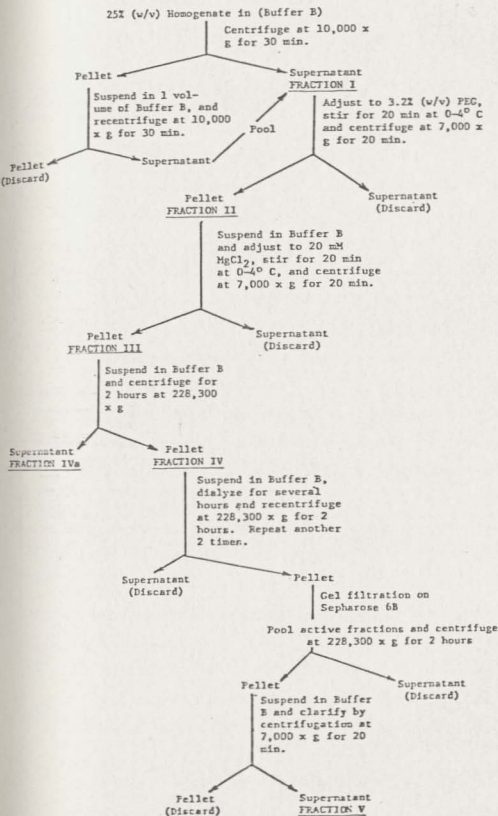
FIGURE 20. Glycerol Gradient Electrophoresis of the Endogenous RS-DP of Fraction IV of the Rat Thymus Preparation.

observed with native and "activated" DNA templates changes from 1:31 in Fraction I and II to 1:77 in Fraction III and 1:92 in Fractions IV and V. Therefore, the activity with native DNA as template relative to that with "activated" DNA as template decreases to a constant relative specific activity. This is probably due to the loss of nucleases capable of "activating" native DNA through partial hydrolysis in the early steps of the purification procedure, rendering it an efficient template. Thus the two exogenously-templated activities may be due to the same enzyme with a constant ratio of approximately 1:90 respectively with native and "activated" DNA templates.

(D) Purification of the RS-DP from Rat Liver

Having examined the effect of various purification procedures on the RS-DP complex, it was decided to apply these methods to rat liver, for the purpose of obtaining enough RS-DP complex to attempt both the isolation of sufficient quantities of product as well as the putative RNA template. This would hopefully permit both the analysis of the endogenous reaction product as well as hybridization studies of the product to the RNA template. The first of these objectives was realized, the latter however could not be carried out, as it proved difficult to isolate an active RNA fraction from the complex.

The procedure used for the purification of the enzyme from rat liver is depicted in Scheme X. In the case of rat liver, a 10,000 x g supernatant was obtained, the pellet was washed by centrifugation with one volume of buffer, and the two supernatants pooled (Fraction I). This extract was then subjected to PEG (3.2% w/v) precipitation and sub-



sequently to MgCl_2 precipitation (see Scheme X). This was followed by centrifugation at 228,300 x g for two hours and retention of the pellet fraction, which was washed three times (by centrifugation at 228,300 x g) and subsequently chromatographed on a Sepharose 6B column. Active fractions were pooled and again pelleted at 228,300 x g. The pellet was then suspended and much of the insoluble material removed by centrifugation at 7,000 x g for 20 minutes. The overall purification achieved with this procedure is summarized in Table XX. The activity was purified approximately 21-fold relative to the specific activity in the crude extract, with a yield of 12.5%. Over 99% of the total protein was eliminated.

Table XXI shows the effect of RNase-treatment on the endogenous activity. Fractions I and II were completely sensitive to RNase-treatment, whereas subsequent fractions contained substantial levels of RNase-resistant activity. The higher resistance of the 228,300 x g supernatant fraction (Fraction IVa) may be partially due to the presence of a natural RNase inhibitor normally found in rat liver (286). The endogenous RNase-sensitive activity in Fraction V was 87% abolished by the RNase treatment.

Since inhibition occurred at high concentrations of enzyme from the 228,300 x g supernatant (Fig. 21), it was decided to discard this fraction and carry out any further purification with the 228,300 x g pellet which did not show any inhibition at high levels of extract (data not shown). The fraction obtained after gel filtration of the 228,300 x g pellet on Sepharose 6B and recentrifugation of the active fractions at 228,300 x g for two hours was also linear with enzyme concentration

TABLE XX. Purification of the Endogenous RNase-Sensitive DNA Polymerase From Rat Liver

	<u>Enzyme Fraction</u>	<u>Total Protein (mg)</u>	<u>Total Activity (pmoles/hr)</u>	<u>Sp. Act. (pmoles/hr/mg protein)</u>	<u>Purif.</u>	<u>Yield (%)</u>
I.	10,000 x g Supernatant	3,942	31,700	8.0	1	100
II.	PEG precipitate	730	25,000	34.3	4.3	79
III.	20 mM MgCl ₂	625	31,700	50.7	6.3	100
IV.	(a) 228,300 x g Supernatant	120	6,860	57.1	7.1	22
	(b) 228,300 x g Pellet	570	42,900	75.2	9.4	135
V.	*Sephacrose 6B of fraction IV (b)	23.9	3,980	166.3	20.7	12.5

A 156 ml volume of the 10,000 x g supernatant from rat liver was subjected to the fractionation procedure outlined in Scheme X. Assays for the endogenously-templated DNA polymerase activity were as described in *Methods* (sp. act. for ³H-dTTP = 6,500 cpm/pmole), and the protein concentrations were determined by the method of Lowry *et. al.* (279).

TABLE XXI. RNase-Sensitivity of the Endogenously-Templated DNA Polymerase Activity from Rat Liver at Various Stages of Purification

Enzyme Fraction	Endogenous Activity		Activity Remaining after RNase-treatment (%)
	Control (cpm/5 min)	After RNase-treatment (cpm/5 min)	
I. 10,000 x g Supernatant	300	0	0
II. PEG precipitate	710	0	0
III. 20 mM MgCl ₂	640	20	3
IV. (a) 228,300 x g Supernatant	510	240	47
(b) 228,300 x g Pellet	710	190	27
V. Sepharose 6B of fraction IV (b)	2,140	280	13

Aliquots (200 μ l) of each fraction described in Table XX were incubated for 3 hrs at 25°C either in the absence or presence of 40 μ g RNase A and 1.08 μ g of RNase T₁. The endogenous activity was assessed as described in *Methods*.

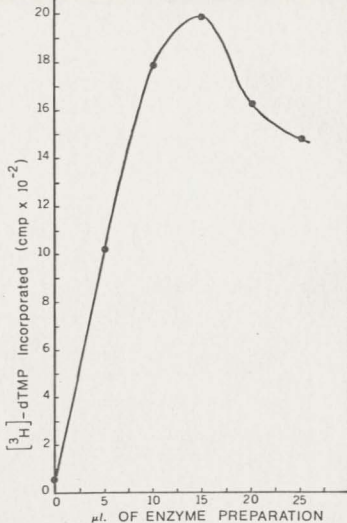


FIGURE 21. Activity Versus Enzyme Concentration Curve
for the Endogenous RS-DP Activity from
Fraction IV (a) of Rat Liver.

Fraction IV (a) obtained as described in Table XX was assayed as described in *Methods* except that the quantity of enzyme was varied. Duplicate assays were carried out, and the averages plotted.

(Fig. 22). Figure 23 shows the effect of actinomycin D and distamycin A on the rat liver endogenous activity. At least 75% of the activity was found to be sensitive to these inhibitors.

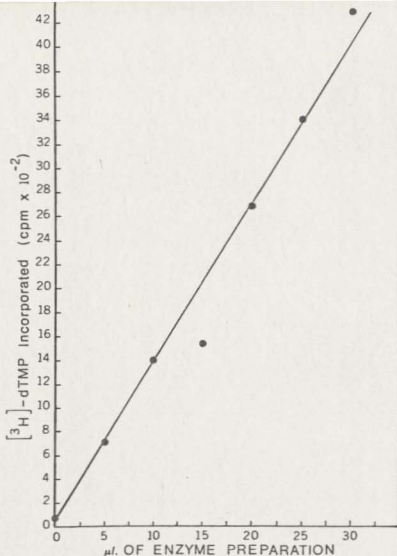


FIGURE 22. Activity Versus Enzyme Concentration Curve
of Fraction V of the Endogenous RS-DP
Activity from Rat Liver.

Experimental conditions were identical to those described for Fig. 21, except that the enzyme preparation was Fraction V, obtained as described in Table XX.

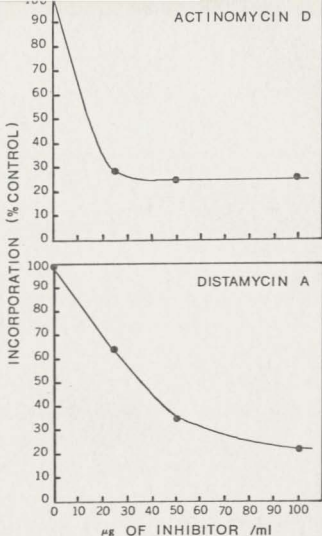


FIGURE 23. Effect of Actinomycin D and Distamycin A on the Endogenous RS-DP Activity from Rat Liver.

Fraction V from Table XX was assayed as described in *Methods* except that various concentrations of the inhibitors as indicated in the figure were included. 100% activity is equal to 3,450 cpm of ^3H -dTMP incorporated.

IV. Analysis of the RS-DP Product of Rat Liver

The necessity of carrying out a product analysis of the endogenous RS-DP activity has already been pointed out [see Literature Review, section IIIE(a)]. The possible functions for RNA as either a template or a primer has made it difficult to unambiguously assign a particular function to the RNA in the RS-DP complex solely on the basis of RNase sensitivity. For this reason the nature of the product was analyzed both on the basis of its response to various nucleases as well as on its buoyant density after various treatments. The results of this study are presented in this section.

(A) Effect of Nucleases and Alkali on the Product of the Endogenously-Templated DNA Polymerase

Fraction V of the rat liver preparation described in the previous section was used for the analysis of the product of the RS-DP reaction. Thus a five minute reaction product from a 100-fold scaled up assay mixture was prepared in the absence and the presence of actinomycin D, the product isolated by phenol extraction as described in Materials and Methods, and subjected to analysis as described in the following experiments.

Table XXII shows the effect of DNase I and RNase A and T_1 as well as alkali treatment on the DNA polymerase product. As expected the RNase and alkali treatment destroyed little or none of the product. However, treatment with DNase I (which destroys both single and double-stranded DNA, ref. 287) was found to abolish 57% of the product synthesized in the presence of actinomycin D, and 47% of the product synthesized in its absence (Table XXII). The acid precipitable counts remaining after DNase I

TABLE XXII. Effect of Nucleases and Alkali on the RS-DP Products

<u>Treatment</u>	Product (+ Act. D)		Product (- Act. D)	
	<u>cpm</u>	<u>%</u>	<u>cpm</u>	<u>%</u>
Control	2,250	100	4,220	100
+ DNase I	960	43	2,220	53
+ RNase A + T ₁	1,960	87	4,320	102
+ Alkali	2,120	94	3,700	88

An aliquot of ³H-labelled product (2,250 cpm, synthesized in the presence of actinomycin D, and 4,220 cpm, synthesized in the absence of inhibitor) was treated with either 0.1 µg DNase I in the presence of 1 mM MgCl₂, or 4 µg RNase A and 1.08 µg RNase T₁ for 1 hr at 37°C, or 0.3 M NaOH heated for 10 min at 95°C. The radioactivity remaining after these treatments was determined as described in *Methods*.

treatment may represent DNA product in the form of an RNA-DNA hybrid.

Nuclease S_1 is an endonuclease acting on single-stranded DNA or RNA (288, 289). Its effect on the DNA product synthesized in the presence of actinomycin D, before and after heat denaturation is shown in Fig. 24. Approximately 25% of the native product is susceptible to nuclease S_1 treatment, whereas after heat denaturation, over 85% is converted to acid soluble material. The first result suggests that at least 75% of the product is in a double-stranded form (either DNA-DNA or RNA-DNA). The second result is consistent with about 15% of the product being covalently linked to its complementary template (or alternatively, capable of rapid renaturation).

(B) Buoyant Density Analysis of the Products

As pointed out earlier, proof of a template function for the RNA in the endogenous RS-DP, requires that the RNA-DNA hybrid intermediate be isolated. The aim of the next series of experiments was to determine whether an RNA-DNA hybrid is produced by the RS-DP. Its isolation would enable the unambiguous assignment of a template function to the RNA.

With the above objective in mind, the product (synthesized in the presence of actinomycin D), was analyzed by isopycnic gradient centrifugation in both Cs_2SO_4 as well as $CsCl$ -guanidinium- Cl gradients. The results obtained in these experiments are shown in Fig. 25 (A, Cs_2SO_4 , and B, $CsCl$ -guanidinium- Cl). In the Cs_2SO_4 gradients, calf-thymus DNA was found to band at a density of 1.40 g/cm^3 (as determined in a separate experiment), whereas the product of the RS-DP reaction, banded at a density of 1.44 g/cm^3 . *E. coli* 23S rRNA was found to band at a density

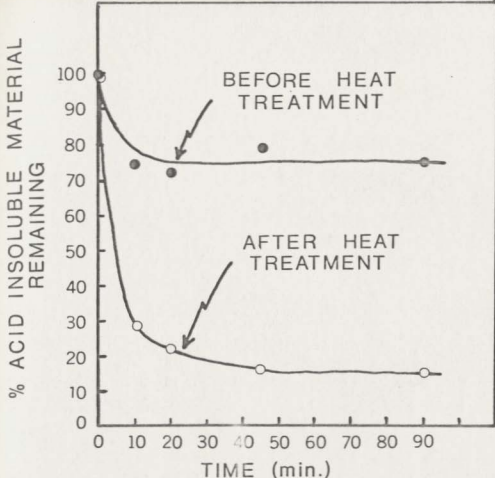


FIGURE 24. Effect of S_1 Nuclease Treatment on the Product Synthesized in the Presence of Actinomycin D, Before and After Heat Denaturation.

Samples of the product synthesized in the presence of actinomycin D were used either in their native state or after heat-denaturation for 10 minutes at 95°C followed by rapid cooling in ice-water. Aliquots of 50 μl of the native or heat denatured product were mixed with 45 μl of S_1 buffer (25 mM sodium acetate pH 4.5, 0.3 M NaCl and 6 mM ZnSO_4) and 5 μl of S_1 nuclease (4.69 units) and incubated at 37°C for the times indicated. The reactions were stopped by the addition of 1 ml 10% TCA, 200 μg of "carrier" DNA, and the samples were processed as described in *Methods*. Duplicate assays were carried out, and the averages plotted. 100% for the native product (before heat treatment) was 875 cpm, and for the heat-treated product, 930 cpm.

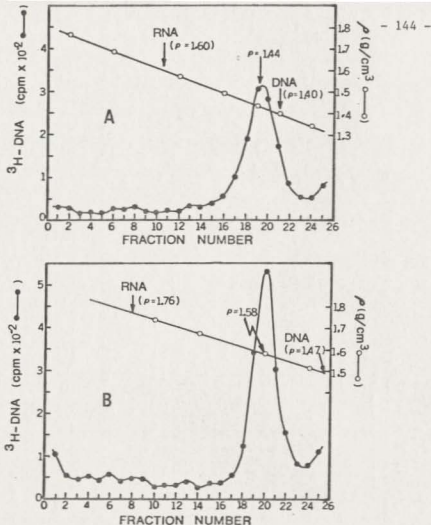


FIGURE 25. Buoyant Density Analysis of the Product Synthesized in the Presence of Actinomycin D on (A) Cs_2SO_4 and (B) $\text{CsCl-Guanidinium}\cdot\text{Cl}$ Gradients.

About 3,500 cpm of product synthesized in the presence of actinomycin D was analyzed on a Cs_2SO_4 gradient (A) as described in *Methods*. In (B), approximately 5,200 cpm of a similar product was analyzed as described in *Methods* on a guanidinium·Cl- CsCl gradient. The positions of the marker nucleic acids (*E. coli* 23S rRNA and calf thymus DNA) were determined in parallel runs and they were detected by reading the absorbance at 260 nm. Densities were determined using an Abbe Refractometer and converting the refractive indices to density using "Isotables" (Instrumentation Specialties Company, Lincoln, Nebraska) in the case of Cs_2SO_4 , and for guanidinium·Cl- CsCl as described in Appendix I.

of 1.60 g/cm^3 (Fig. 25A). This suggests that the product of the RS-DP contains substantially more DNA than RNA, resulting in a density much closer to that of DNA than RNA. In order to achieve a better resolution between RNA, RNA-DNA hybrids, and DNA, equilibrium centrifugation was carried out in guanidinium-CsCl gradients as described by Enea and Zinder (278). Under the conditions described (278), RNA bands essentially at the bottom of the gradient, whereas the DNA bands at the top. Thus a better separation between the RNA-DNA hybrid and the DNA should be possible. In addition, any artefacts produced by the possible presence of protein associated with the nucleic acid (which would make it less dense) should be minimized by the denaturation due to the guanidinium-chloride (290). The product synthesized in the presence of actinomycin D was found to have a buoyant density of 1.58 g/cm^3 on these gradients (Fig. 25B), whereas DNA and RNA banded at buoyant densities of 1.47 and 1.76 g/cm^3 , respectively. As expected, the guanidinium-CsCl gradients did indeed provide a better resolution of the hybrid product from the DNA region. As to whether this was merely due to the greater resolving ability, to the ability of guanidinium to remove any proteins that may be complexed to the nucleic acid (290) or a combination of these effects is not known. Further analysis of the product was carried out with guanidinium-CsCl gradients.

Figure 26 shows the effect of (A) heat denaturation, (B) alkali treatment, (C) RNase A and T_1 treatment and (D) S_1 nuclease treatment on the buoyant density of the product. Upon heat denaturation, the product still remained in the hybrid region ($\rho = 1.52 \text{ g/cm}^3$) (Fig. 26A) indicating a phosphodiester linkage between the template and the product,

FIGURE 26. Buoyant Density Analysis of the Product Synthesized in the Presence of Actinomycin D, After (A) Heat Denaturation, (B) Alkali Treatment, (C) RNase A and T₁ and (D) Nuclease S₁ Treatments.

Aliquots of ³H-labelled product (200 μ l containing approximately 5,200 cpm) in total volumes of 277 μ l were treated as follows:

- (A) Heated for 10 min at 95°C, followed by rapid cooling in ice-water.
- (B) Adjusted to 0.3 M NaOH and heated for 10 min at 95°C.
- (C) Treated with 20 μ g RNase A and 1 μ l RNase T₁ and incubated for 1 hr at 37°C.
- (D) The product was preincubated for 1 hr at 37°C in the presence of 75 μ l of Nuclease S₁ buffer (see legend to Fig 24) and 2 μ l of S₁ nuclease (5 x 10⁵ units/ml.).

After these treatments, the buoyant density analysis was carried out on guanidinium.Cl-CsCl gradients as described in Methods.

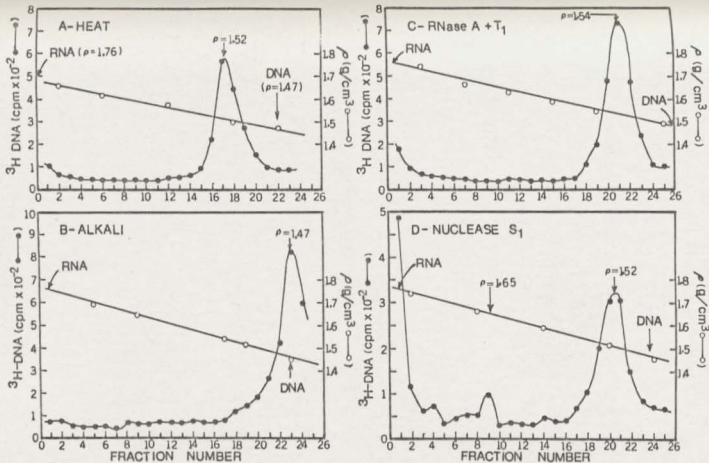


FIGURE 26. Buoyant Density Analysis of the Product Synthesized in the Presence of Actinomycin D, After (A) Heat Denaturation, (B) Alkali Treatment, (C) RNase A and T₁ and (D) Nuclease S₁ Treatments.

whereas alkali treatment (Fig. 26B) shifted the product to the density of DNA (1.47 g/cm^3). Treatment with RNases yielded a product that had a somewhat lower density (1.54 g/cm^3), but still banded in the hybrid region (Fig. 26C), suggesting that most of the RNA associated with the complex is not single-stranded. However, the small decrease in density compared to that of the untreated control sample (1.58 g/cm^3 , Fig. 25B) suggests that some single-stranded RNA was initially associated with the product. The effect of S_1 nuclease on the product is shown in Fig. 26D. As can be seen some product remained at intermediate densities (1.52 and 1.65 g/cm^3) while a substantial amount was recovered at the density of RNA ($\rho = 1.75 \text{ g/cm}^3$) in the bottom fraction. For further comments concerning these results see Discussion.

The product synthesized in the absence of actinomycin D was similar to that synthesized in its presence. The main peak had a density ($\rho = 1.53 \text{ g/cm}^3$) closer to that of DNA than RNA. In addition, a small amount of product banded in a hybrid region closer to the density of RNA (Fig. 27A). Alkaline hydrolysis of the product was accompanied by a shift to the DNA region on the gradient (Fig. 27B).

(C) Size of the RS-DP Product after Various Treatments

Sedimentation velocity analysis of the product was carried out for two purposes: to estimate the size of the product, as well as to further substantiate whether the RNA associated with the product was covalently linked to it. The product synthesized in the presence of actinomycin D was found to sediment as a 10.4 S species (Fig. 28A) relative to the sedimentation rate of yeast tRNA (4.0 S). Heat de-

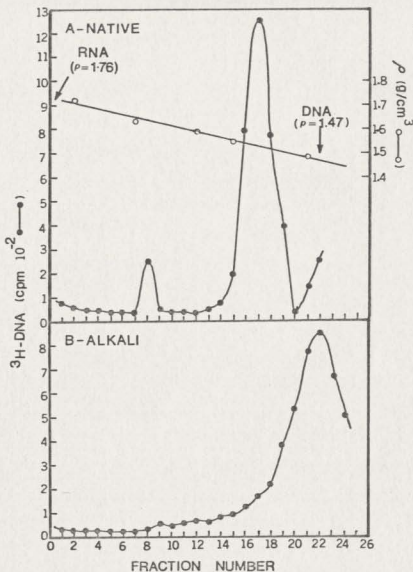


FIGURE 27. Buoyant Density Analysis of the Product Synthesized in the Absence of Actinomycin D, Before (A) and After (B) Alkali-Treatment.

Aliquots of ^3H -labelled product (200 μl containing approximately 6,500 cpm) in total volumes of 266 μl were analyzed by equilibrium centrifugation in guanidinium-Cl-CsCl gradients as described in *Methods*.

(A) No pretreatment.

(B) Prior to buoyant density analysis the product was treated as described in the legend to Fig. 26 (B).

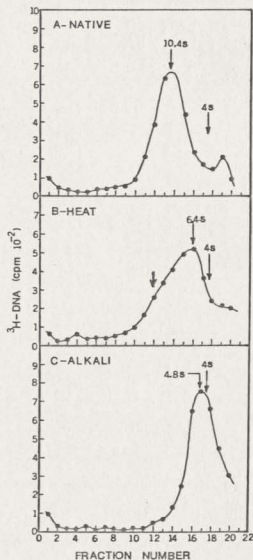


FIGURE 28. Sedimentation Velocity Analysis of the Product Synthesized in the Presence of Actinomycin D; (A) Control, (B) After Heat-Treatment and (C) After Alkali-Treatment.

Sedimentation velocity analysis was carried out as described in *Methods* using a 5-20% sucrose gradient in the Beckman SW 50.1 rotor.

(A) 200 μl of product (approximately 5,200 cpm) + 35 μl of distilled water.

(B) As in (A) except that the sample was heated for 10 minutes at 95°C followed by rapid cooling in ice-water.

(C) 200 μl of product plus 35 μl of 2 M NaOH, heated for 10 minutes at 95°C .

naturation of the product prior to sedimentation velocity analysis, resulted in a more heterogeneous size distribution, with the peak fraction having a sedimentation coefficient of 6.4 S (Fig. 28B). Higher molecular weight components were clearly also present as shoulder in the 12.8 S region. Alkali treatment of the product prior to sedimentation velocity analysis, resulted in a relatively homogeneous product with a sedimentation value of 4.8 S (Fig. 28C). These results suggest that RNA is covalently linked to the DNA product.

The product synthesized in the absence of actinomycin D is again similar to the one synthesized in its presence. The native product has a sedimentation value of 9.6 S (Fig. 29A), and upon alkali treatment it decreases to a size (4.8 S) (Fig. 29B) identical to that of the product synthesized in the presence of actinomycin D (Fig. 28C).

Table XXIII summarizes the different sizes of product observed with and without various treatments. Furthermore in the same table, the sedimentation values have been converted to apparent molecular weights (based on a value of 25,000 daltons for 4.0 S tRNA) in order to compare the product sizes more easily, and to obtain an idea of how long the RNA template may be. If one considers the size of the product after alkali treatment (33,000 daltons, see Table XXIII), which represents single stranded DNA product, and compares this to the size distribution observed after heat denaturation (50,600 - 143,000 daltons) (which gives a measure of the size of single-stranded covalently linked RNA-DNA), one can obtain a rough estimate of the size of the RNA associated with the hybrid. In carrying out such calculations, one finds that the size of the RNA associated with the product ranges from 18,000 to 110,000

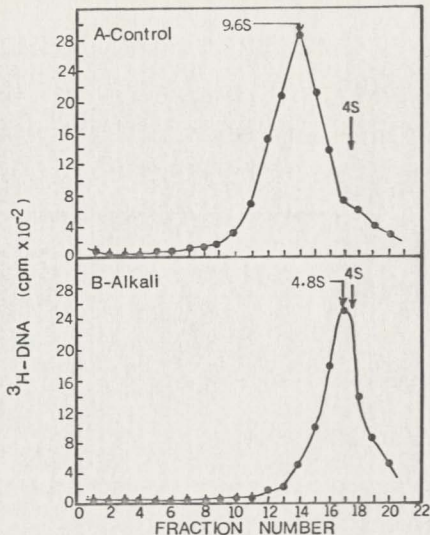


FIGURE 29. Sedimentation Velocity Analysis of the Product
Synthesized in the Absence of Actinomycin D₁
(A) Before and (B) After Alkali-Treatment.

The product synthesized in the absence of actinomycin D
 (6,500 cpm/experiment) was analyzed as described in Fig. 28.

(A) No pretreatment.

(B) Treated with 0.3 M NaOH at 95° for 10 minutes.

TABLE XXIII. Analysis of the Sizes of the Products After
Various Treatments

I. Product Synthesized in the Presence of Actinomycin D:

<u>Treatment</u>	<u>Sedimentation Coefficient</u>	<u>Molecular Weight</u>	<u>Number of Nucleotides</u>
A. Control	10.4S	105,000	320
B. Heat Denaturation			
(1) main peak	6.4S	51,000	150
(2) shoulder	12.8S	143,000	430
C. Alkali	4.8S	33,000	100

II. Product Synthesized in the Absence of Actinomycin D:

A. Control	9.6S	93,000	280
B. Alkali	4.8S	33,000	100

The sedimentation values were calculated from the sedimentation rates or distances of the ³H-labelled products relative to the sedimentation of the 4S tRNA marker (see Figs. 28 and 29). The method of Martin and Ames (278a) was used to calculate the sedimentation coefficients and the apparent molecular weights, taking the 4S marker to be 25,000 daltons. The approximate number of nucleotides was estimated by dividing the molecular weight by 330 (the average molecular weight of a single nucleotide residue).

daltons. This corresponds to RNAs of 50 to 330 nucleotides long. These large sizes for the RNA are inconsistent with a primer function, where RNAs of 10 to 20 nucleotides long (corresponding to molecular weights of 3,300 to 6,600 daltons) usually serve as primers (152, 291). The observed large size for the RNA, is however consistent with a template rather than a primer function. The lower ranges of size observed for the RNA are probably due to the hydrolysis of the RNA by RNase H (which was confirmed to be present in the enzyme preparation, data not shown).

Attempts in trying to abolish the RNase H activity, in order to obtain better results, were unsuccessful. Some workers had previously reported that RNase H is sensitive to the sulfhydryl blocking reagent NEM (291a). Since it had been observed (Fig. 12) that the RS-DP activity was resistant to NEM, it would seem to be advantageous to synthesize product in the presence of this inhibitor to prevent the nuclease activity. However, it was found that the RNase H activity in the enzyme preparation from rat liver could not be abolished by NEM (data not shown), making the experiment unfeasible.

V. Isolation of the DNA Polymerase Activities Associated with the RS-DP Complex

The observation by others (22) that the DNA polymerase from the endogenous RS-DP complex, when dissociated from the endogenous template primer, acquires the property of a DNA-directed DNA polymerase, has made the identification of the enzyme uncertain. For this reason a method was developed in previous work (281) which permitted the identification of the enzyme originally associated with the complex. The procedure used takes advantage of the fact that the complex is of high molecular weight and is sensitive to RNase-treatment. It was observed that treatment of the complex with RNase A for three hours at 25° C, followed by passage through a gel filtration column abolished the endogenously-templated DNA polymerase activity. However, if DNA polymerase activity was assayed in the presence of 16S + 23S rRNA as template, a DNA polymerase species could be detected in the 110,000 dalton region of the column. This peak of activity was specifically produced only if the endogenously-templated DNA polymerase activity was abolished by the RNase-treatment (281). This relationship between the endogenous RS-DP activity, and the DNA polymerase activity produced after RNase-treatment was used as a criterion in establishing that the two activities were identical in origin (281). Further evidence in support of this is provided by the properties of the RS-DP activity reported in this dissertation, which were similar to the properties previously reported for the exogenously-templated RD-DP activity produced by the RNase-treatment of the endogenous RS-DP complex (281, see also Discussion).

The uncertainty which had previously arisen regarding the purity

of the RNA used as the template (281), as well as the enhanced release of activity now obtained through a combined treatment with RNase A and T_1 has resulted in the need for further characterization of these polymerases.

(A) Effect of Nuclease Treatment on the Release of DNA-Directed DNA Polymerases from the Endogenously-Templated DNA Polymerase Complex from Rat Thymus

The effect of treatment with various nucleases on the profile of the rat thymus DNA polymerases eluting from a Sephadex G 200 column are shown in Fig. 30. The enzyme in the control sample (Fig. 30A) was incubated at 25° C for three hours prior to gel filtration; all of the DNA polymerase activities whether endogenously- or exogenously-templated eluted from the column in the void volume. If the preparation was pretreated with RNase A alone (Fig. 30B), most of the endogenously-templated RS-DP activity was abolished, and some polymerase activity capable of using native DNA as template became apparent at the lower molecular weight position. Figure 30C shows the profile obtained when the pretreatment included both RNase A and T_1 ; at least two new peaks of DNA polymerase activity were detectable with native DNA as the template. In other experiments, these peaks of activity were more distinct than shown in Fig. 30C, i.e., the level of activity observed between the first and second peak was substantially lower (Fig. 31).

Figure 30D shows the effect of DNase I on the profile of the activities on the same column. Essentially all of the endogenous activity was recovered in the same position as in the control experiment,

- 195a -

FIGURE 30. Elution Profile of Rat Thymus DNA Polymerase on a Sephadex G200 Column After Treatment with Various Nucleases.

A rat thymus homogenate was prepared in Buffer A, and a 5 ml aliquot of the 39,000 x g supernatant was first fractionated on a Sephadex G150 column (3 x 36 cm), the void volume fractions containing all DNA polymerizing activities (total volume = 32 ml) were pooled and concentrated 5-fold by dialysis against Buffer A containing 30% PEG. The concentrate was divided into 1 ml aliquots, each of which was further fractionated, after various treatments, on a Sephadex G200 column (1.5 x 30 cm) equilibrated with the same buffer. (A) Sample fractionated without any treatment, (B) preincubated for 3 hr at 24° C in the presence of 200 µg RNase A, (C) in the presence of 200 µg RNase A and 8.75 µg RNase T₁ (sp. act. 565,000 units/mg), and (D) 1 µg DNase I. Activities were assessed in the absence of template (○—○), presence of native DNA (●—●), and in the presence of "activated" DNA (◊—◊), as described in the legend to Fig. 1.

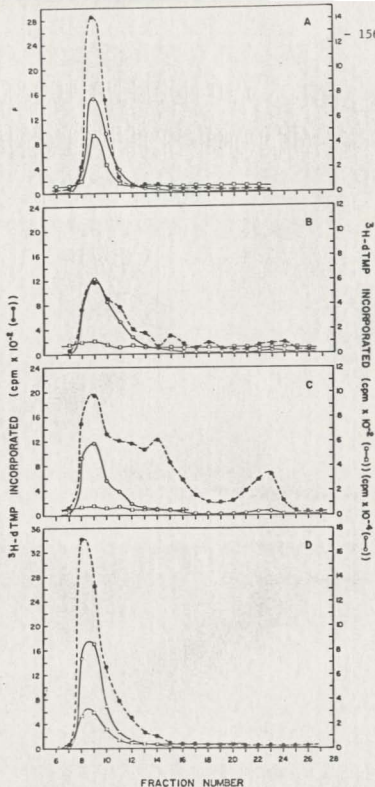


FIGURE 30. Elution Profile of Rat Thymus DNA Polymerases on a Sephadex G200 Column after Treatment with Various Nucleases.

FIGURE 31. Profile of the DNA-Directed DNA Polymerase on a Sephadex G200 Column After RNase A and T_1 Treatment of an Homogenate Prepared in Buffer A.

A 1 ml aliquot of the 39,000 x g supernatant of an homogenate prepared in Buffer A was treated for 3 hr at 24° C in the presence of 200 μ g RNase A and 30.5 μ g RNase T_1 (sp. act. 310,000 units/mg), after which it was fractionated on a Sephadex G200 column (1.5 x 30 cm) equilibrated with Buffer A. 25 drop fractions (\sim 1.5 ml) were collected and assayed for native DNA-dependent DNA polymerase (●—●), "activated" DNA-dependent DNA polymerase (○—○), and endogenous activity (□—□), as described in the legend to Fig. 1.

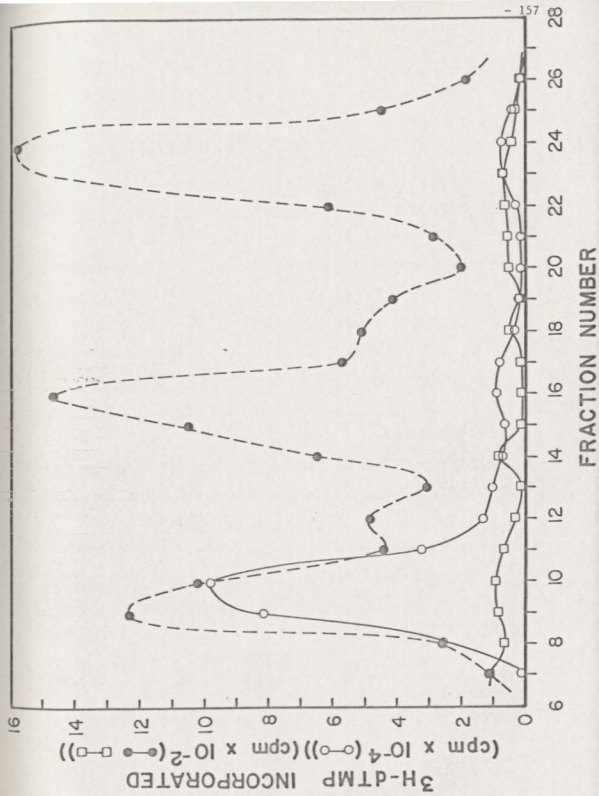


FIGURE 31. Profile of the DNA-directed DNA Polymerase on a Sephadex G200 Column after RNase A and T_1 Treatment of an Homogenate Prepared in Buffer A.

and no apparent shift of DNA polymerase activities to the lower molecular weight forms was observed. The concentration of DNase I (1 $\mu\text{g/ml}$) in this experiment was sufficiently low not to interfere with the assay of DNA polymerase activity in the column fractions. Higher concentrations of DNase (up to 200 $\mu\text{g/ml}$) have also been used with similar results (data not shown), although with somewhat lower recoveries of DNA polymerase activities (especially with exogenous DNA templates) in the void volume.

These experiments demonstrate that the endogenous activity is sensitive to RNase and not to DNase, and that furthermore, the reciprocal relationship between the endogenously-templated activity and the lower molecular weight DNA polymerase activities (i.e., a decrease in level of endogenously-templated activity after RNase-treatment is accompanied by the appearance of the lower molecular weight DNA polymerases) suggests that the lower molecular weight enzymes are derived from the RS-DP complex.

Figure 32 shows the elution profile of another sample that was treated with both RNase A and T_1 on a Sephadex G 200 column. In this case the fractions were assayed with both native DNA as well as 16S + 23S rRNA as templates. As can be seen, the profile of the RD-DP activity was found to closely parallel that of the DD-DP activity. These results differ from those previously reported in that the second peak of activity eluting from the column is of a molecular weight smaller than that previously observed for the RD-DP activity (281). The reason for this may be due to either the more efficient digestion of RNA by the combined RNases-treatment, or more likely due to less aggregation

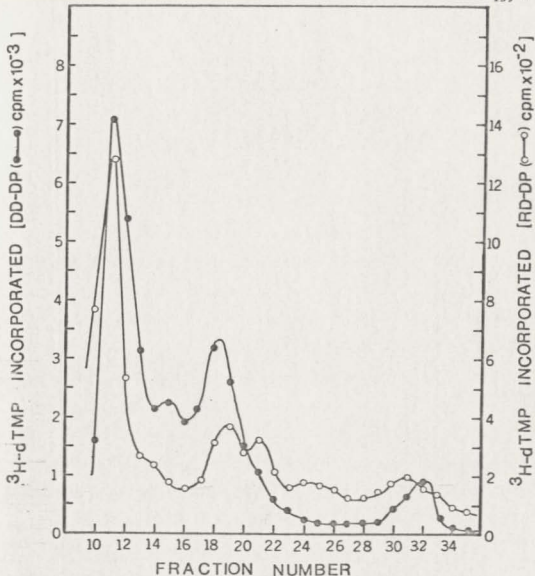


FIGURE 32. Profile of RD-DP and DD-DP Activities on a Sephadex G200 Column After RNase A and T_1 Treatment of the Endogenous Activity.

The exogenously-templated activities were prepared as described in *Methods*. DNA polymerase assays with native DNA template (DD-DP) were also as described in *Methods*. In the case of the RD-DP activity, assay conditions were similar to those for the endogenous activity except that 10 μ l water was replaced with 10 μ l of 1.32 mg/ml 16S + 23S rRNA.

occurring with the lower molecular weight species. Support for this latter statement evolves from the observation that further RNase-treatment of the higher molecular weight RD-DP activity previously reported (281) did not further reduce its size, and also on the basis that the lowest molecular weight species has been often observed to aggregate (data not shown) when rechromatographed. These results indirectly suggest that the enzyme may aggregate, depending on pH and buffer ions, since the previous work was carried out at pH 8.0 in Tris-HCl, and the present work at pH 8.5 in glycine-NaOH buffer.

(B) Effect of Basic Proteins on the Endogenously-Templated DNA Polymerase Complex

The interaction of RNase A with nucleic acids (including DNA) is a complex phenomenon (292, 293), and RNase A has actually been shown to have a strong unwinding effect on DNA (292, 294). Thus the possibility exists that RNase may be displacing the DNA polymerases competitively from an endogenous nucleic acid rather than through its ability to hydrolyze RNA. This problem was approached in the following experiments by testing the effect of other highly basic proteins on the endogenous activity.

When the enzyme preparation was pretreated with either oxidized RNase A (which lacks nuclease activity) (Fig. 33, top panel), histones (middle panel) or lactoperoxidase (LP) (lower panel), at a concentration identical to that used in carrying out RNase A-treatment (200 µg/ml), the lower molecular weight DD-DP activities derived from the RS-DP, were not obtained, nor was the level of endogenous activity reduced by the

Figure 33: Effect of Oxidized RNase A, Histones and Lactoperoxidase on the Profile of the DNA Polymerases from Rat Thymus.

The endogenously-templated activity was prepared in buffer A as described in Methods. Aliquots of 1 ml were treated with (A) 200 μ g oxidized RNase A, (B) 200 μ g calf thymus histones, or (C) 200 μ g lactoperoxidase for 3 hrs at 25°C prior to fractionation on a Sephadex G150 column. DNA polymerase activities [endogenously-templated (●—●), native DNA templated (□—□), and "activated" DNA (■—■)] were assessed as described in Methods, except that glycine-NaOH was replaced by a similar concentration of Tris-HCl, pH 8.0.

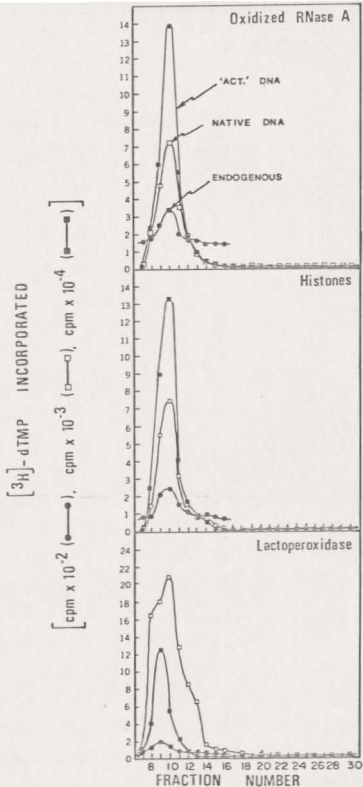


FIGURE 33: Effect of Oxidized RNase A, Histones and Lactoperoxidase on the Profile of the DNA Polymerases from Rat Thymus.

oxidized RNase A and the histones. In the case of the LP, the commercially available enzyme initially used did decrease the level of endogenous activity, and this was accompanied by the shift of DNA polymerase activity to a lower molecular weight position (data not shown). This LP was found to be contaminated with RNase activity, and the effect on the endogenous activity could thus be attributed to this contaminant. The LP used in this experiment (Fig. 33, lower panel), had been further purified (275) to eliminate the RNase activity. Even so, some endogenous activity was abolished, and the level of activity with native DNA as template was both increased as well as partially shifted to a lower molecular weight position (Fig. 33, lower panel), suggesting that the LP was not completely free of RNase. The increased level of exogenously-templated DD-DP may be due to more enzyme being made available to use the native DNA template.

It therefore appears that the ability of RNase A and T_1 to release the polymerases originally associated with the endogenous nucleic acid is due to their nuclease activity, and not merely to a competitive displacement of the enzyme from a nucleic acid-protein complex.

(C) Effect of RNase Digestion Products on the Endogenously-Templated DNA Polymerase Complex

Another possible explanation for the release of the DNA polymerases from the endogenously-templated DNA polymerase complex is that the products of the RNase digestion (mono- and oligo-nucleotides) may be affecting the enzyme in such a way that disruption of the complex may occur. Thus the total RNA present in the enzyme preparation was

isolated by phenol extraction, and subsequently hydrolyzed with RNase A and T_1 and the total digestion products were used to treat the RS-DP preparation. No effect on the endogenous activity was observed with this treatment, nor were any exogenously-templated activities observed in the lower molecular weight regions of the column (data not shown).

(D) Effect of RNase-Treatment on the Endogenous Activity in the Presence of Protease Inhibitors

The possibility that some low molecular weight DNA polymerases are active proteolytic fragments of a higher molecular weight DNA polymerase was considered, particularly since proteolysis has been shown to occur with *E. coli* DNA polymerase I (295, 296) and possibly with DNA polymerase α from eukaryotes (12, 62, 92, 95). Thus it became of interest to examine whether proteolysis during the course of RNase treatment might be producing the lower molecular weight DNA polymerases. The approach taken in this study consisted of carrying out the RNase-treatment in the presence of the protease inhibitors - Trasylol (aprotinin) and phenylmethylsulfonyl fluoride (PMSF). The presence of these inhibitors should abolish the proteolytic activity of any serine proteases (in the case of PMSF, 297) and estero-proteases (in the case of Trasylol, 298). The results of this study are shown in Fig. 34, and were qualitatively similar to the results obtained when the RNase-treatment was conducted in the absence of protease inhibitors (see Fig. 31). The results however were not quantitative, in that more of the second peak was recovered when the RNase treatment was conducted in the presence of PMSF (Fig. 34B). The reason for this is probably related

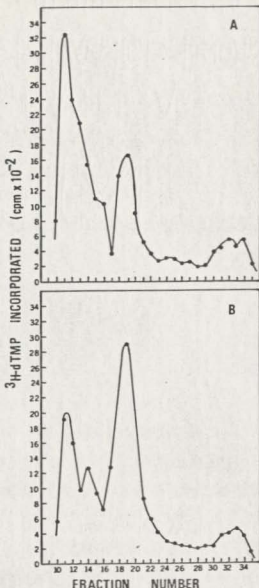


FIGURE 34: DNA Polymerase Profile on Sephadex G200 of
Native DD-DP After RNase - Treatment of the
Endogenously-Templated Activity in the Presence
of (A) Trasyolol and (B) Phenylmethylsulfonyl
Fluoride.

Two 1 ml aliquots of endogenously-templated DNA polymerase preparation were treated with 200 μg RNase A and 10 μl of RNase T, in the presence of (A) 5 μl of 0.5 M PMSF (in DMSO), and (B) 10 μl of Trasyolol, for 3 hrs at 25°C , followed by fractionation on a Sephadex G200 column. DNA polymerase activity was assessed with native DNA as the template, as described in Methods.

to the length of storage of the sample, since it was observed that the initial control (RNase-treatment in the absence of protease inhibitors) yielded less activity and the control run after the experiments with the protease inhibitors yielded even more activity in the regions where the second and third peaks of activity eluted.

The absence of proteases was further suggested by assessing the endogenously-templated DNA polymerase preparation used in this study for protease activity with the general protease substrate Azocoll (Calbiochem). No protease activity could be detected with this substrate (data not shown).

Thus it is unlikely that the released activities are active proteolytic fragments of a higher molecular weight DNA polymerase, although this possibility cannot be totally excluded since a protease insensitive to Trasylol and PMSF, and incapable of hydrolyzing collagen (Azocoll) may be present in the preparation.

(E) Effect of RNase on more Extensively Purified Endogenously-Templated RS-DP Preparations

Ribonuclease-treatment of more highly purified preparations of the RS-DP (such as Fraction V of the rat thymus preparation described above), gave results similar to those obtained with the less purified samples used in Sections VA, B, C and D.

Some interesting observations made during the course of working out procedures for purifying the RS-DP from rat liver merit some consideration. For example, in the attempt to separate the endogenous activity from the ribosome fraction, it was observed that the presence of 400 mM KCl in

the buffer, would allow the endogenous activity to remain in the 228,300 x g supernatant after centrifugation. However upon dialyzing out the salt, so as to enable assessment of the endogenous activity, it was observed that the complex was not sensitive to RNase-treatment, nor was it sensitive to DNase-treatment. Specifically, if the sample was treated with either RNases, or DNase I, and subsequently passed through a Sepharose 6B column, the endogenous activity was still recovered and no apparent release of DNA polymerase activities occurred (data not shown). However, if the sample was sequentially treated with DNase I and then with the RNases, or vice versa, it was observed that the lower molecular weight DNA polymerases could be detected on sucrose gradients (see Fig. 35). If the sample was not treated at all, two high molecular weight peaks of endogenous activity were obtained on these gradients [Fig. 35 (1)], however, if treated with a combination of RNase A and T_1 and DNase I [Fig. 35 (4)], a very low molecular weight species could be detected. For a discussion of the sizes observed for these species, see Discussion.

Similarly, it was observed that a thymus preparation of endogenous RS-DP stored for as long as one month at 0-4° C, was no longer sensitive to RNase-treatment, nor was it found to be partially resistant to actinomycin D (data not shown). In addition this endogenous activity was not as sensitive to KCl as the original RS-DP enzyme. These observations suggest that the DNA polymerases may switch from an RNA to a DNA template or vice versa (see Discussion).

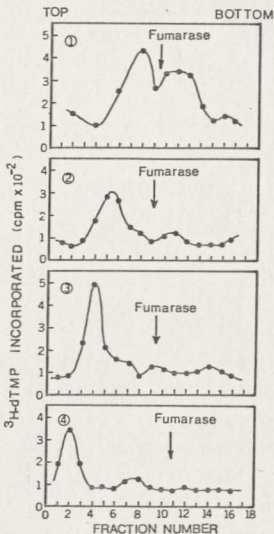


FIGURE 35: Effect of Nucleases on the Endogenously-Templated Activities of Rat Liver Exposed to 400 mM KCl.

The endogenously-templated DNA polymerase from a rat liver sample was prepared as described in Table XX. Fraction IV(b) was suspended in Buffer B containing 1% Triton X-100 and re-sedimented at $228,300 \times g$ for 2 hours (all of the original activity was found in the pellet). The pellet was then suspended in Buffer B containing 400 mM KCl and re-centrifuged. At this stage, all of the endogenously-templated activity was found in the supernatant fraction. The salt concentration was then reduced to 40 mM by overnight dialysis against Buffer B, and the enzyme sample concentrated as described in *Methods*.

- (1) An aliquot was analyzed on a 5-20% sucrose gradient (Beckman SW 36 rotor, see *Methods*).

Another 1 ml aliquot was treated with 200 μg RNase A + 5.4 μg RNase T_1 for 3 hrs at 25°C and fractionated on a Sepharose 6B

FIGURE 35 (Contd.)

column. At this stage it was observed that the DNA polymerase activity eluted as a complex with an endogenous template, regardless of the RNase-treatment. Another aliquot was then treated with 50 μ g DNase I and 1 mM $MgCl_2$, for 3 hrs at 25°C and fractionated by gel filtration as above. Even with this treatment, the activity eluted as a complex with an endogenous template.

(2) The sample treated with DNase I, was then treated with RNases as above and (3) that initially treated with RNases was then treated with DNase I as above, and analyzed by sedimentation velocity as for sample (1).

(4) Another 1 ml aliquot of the sample was analyzed after pre-treatment with a combination of RNase A + T_1 and DNase I as above.

In (1), the endogenous activity was assayed as described in *Methods*, and in (2), (3) and (4) DNA polymerase activity was assessed using native DNA as the template as described in *Methods*.

VI. Properties of the DNA Polymerases Released by RNase-Treatment of the Endogenous Activity

Having available a method that resulted in the isolation of the DNA polymerases associated with an endogenous RNA template, the properties of the released activities were examined to determine whether these enzymes were related to any of the known eukaryotic DNA polymerases. In addition it might be expected that such analysis of the properties would enable one to biochemically distinguish between RNA-directed and DNA-directed DNA synthesis.

(A) Requirements

The requirements of rat thymus DNA polymerase α , and the activities released from the endogenously-templated DNA polymerase complex (Peaks II and III) are shown in Table XXIV. All three DNA polymerases required a full complement of deoxynucleoside triphosphates for maximum activity. Deletion of the three unlabelled deoxynucleoside triphosphates from the reaction mixture resulted in only 17%, 35% and 34% of the activity for DNA polymerases α , Peak II and Peak III, respectively. With the deletion of a single deoxynucleoside triphosphate at a time, a substantial amount of activity still remained especially with the Peak II enzyme (see Table XXIV). This is probably related to a distributive mechanism of DNA synthesis, in which the enzyme dissociates from the template when a region of DNA is encountered for which complementary deoxynucleoside triphosphates are not available, and then binds again to another region for which the nucleotides are available.

It is further shown in Table XXIV that all three enzymes require a

TABLE XXIV. Requirements of the DNA Polymerase α and of the Peak II and III Activities Derived from the RS-DP Activity of Rat Thymus

Reaction Conditions	d[³ H]TMP Incorporated (%)		
	DNA Polymerase α	Peak II	Peak III
Complete	100	100	100
-dATP, -dCTP, -dGTP	17	35	34
-dATP	39	70	58
-dCTP	57	93	70
-dGTP	44	95	57
-MgCl ₂	0	0	0
-MgCl ₂ + MnCl ₂ (0.5 mM)	65	86	87
+ DNase (2 μ g added at 15 min)	2	0	3
+ RNase A (4 μ g added at 15 min)	99	92	91

The three peaks of activity were prepared as described in the legend to Fig. 30 C. Reaction conditions for the complete systems are identical to those described for Fig. 1, except that native DNA was used as the template, and deletions made accordingly as denoted in the Table. All assays were done in duplicate, and incubated for 30 min at 37° C. 100% activity for DNA polymerase α , and Peaks II and III are 2,500 cpm, 720 cpm, and 370 cpm/assay respectively.

divalent cation for activity. Furthermore, it was observed that Mn^{2+} can partially replace Mg^{2+} , and at a concentration of 0.5 mM was 65%, 86% and 87% as effective as Mg^{2+} for DNA polymerase α , Peak II and Peak III activities respectively.

The product of the reactions for all three polymerases was sensitive to DNase I and not RNase A, suggesting that the product is DNA (Table XXIV).

(B) Catalytic Properties

(a) Time-Course for the Peak II and III Activities

The reactions catalyzed by Peak II (Fig. 36A) and Peak III (Fig. 36B) DNA polymerases were linear for at least 45 minutes, the longest period of time investigated. A slight non-linearity between time and incorporation may exist at short periods of incubation, however, the deviation from linearity was not great (Fig. 36).

(b) Activity Versus Enzyme Concentration Curve for the Peak II and Peak III Activities

The relationship between activity and enzyme concentration for Peaks II and III are shown in Fig. 37. Both activities displayed a sigmoidal relationship between these two parameters, that is the activity observed at low enzyme concentrations was less than proportional to the amount of enzyme added. The sigmoidicity was not due to a contaminating nuclease in the enzyme preparation which could possibly "activate" the native DNA template by nicking it, since this non-linearity was also observed when "activated" DNA was used as the template (Fig. 37B, D). Nor was this phenomenon due to adsorption of the enzyme to the glassware,

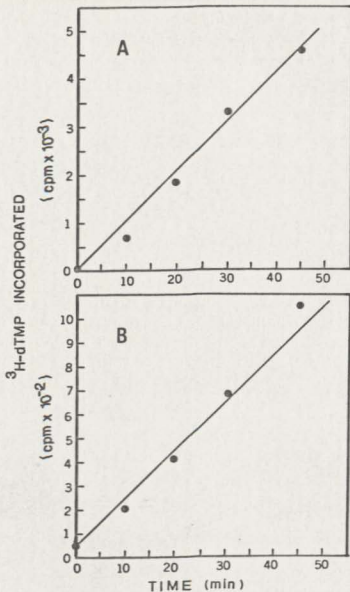


FIGURE 36. Time-Course of the Peak II (A) and Peak III (B) Activities Derived from the RS-DP Complex.

Peaks II and III were prepared as described in *Methods*, and assayed with native DNA as the template for the times of incubation indicated. Duplicate assays were carried out and the averages plotted.

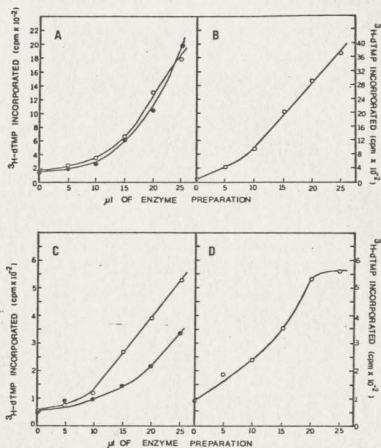


FIGURE 37. Activity Versus Enzyme Concentration Curves
for Peaks II and III.

The preparation of the enzymes and assay conditions were as described in *Methods* except that the final concentration of "activated" DNA was 0.12 mg/ml instead of 0.24 mg/ml, and the quantities of the enzymes used were varied as indicated. Peak II was assayed with both native DNA (Panel A), either in siliconized (○—○) or non-siliconized (●—●) tubes, and "activated" DNA (Panel B) in siliconized tubes. Peak III was similarly assayed with native (Panel C) and "activated" (Panel D) DNA as templates. All assays were carried out in duplicate for 20 minutes at 37° C, and the averages plotted. The sp. act. of ³H-dTTP was 3,500 cpm/pmole.

since the shape of the curve did not greatly change when the assays were carried out in siliconized tubes. Some adsorption of enzyme or of some factor affecting the activity may be occurring in the case of Peak III (Fig. 37C), since the curve obtained when assays were carried out in non-siliconized tubes, was more sigmoidal than when assays were carried out in siliconized tubes (Fig. 37C).

A very pronounced inhibition effect, similar to the one observed for the endogenous RS-DP (see Fig. 5), was obtained when high concentrations of Peak III were used (Fig. 38). In non-siliconized tubes, however, the inhibition was less pronounced than in siliconized tubes. This is probably due to the adsorption of an inhibitor by the untreated glassware.

(c) pH Optimum

The pH optima for Peaks II and III are shown in Figs. 39 and 40 respectively. In the case of Peak II, maximum activity was observed in Tris-HCl buffer, and the optimum was very broad, with almost equal levels of activity being observed at pHs from 7.5 to 8.5 (Fig. 39). Less activity was observed in glycine-NaOH buffer, and the optimum was quite sharp at pH 8.5, although substantial levels of activity were still apparent at pH 9.5. In the case of Peak III (Fig. 40), higher levels of activity were observed in glycine-NaOH buffer, with a maximum between pH 8 and 8.5. The activity in Tris-HCl at pH 8.0 was only about 50% as great as in glycine-NaOH at the same pH.

(d) Divalent Cation Optima

The divalent cation optima for Peaks II and III are shown in Figs.

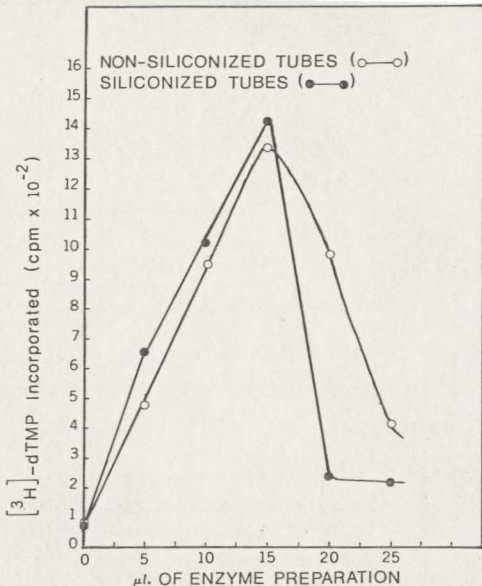


FIGURE 38. Inhibition of the Peak III Activity at High Levels of Enzyme.

The Peak III enzyme used for the experiment described in Fig. 37 was 5-fold concentrated by dialysis against Buffer B containing 30% (w/v) PEG and assayed, using native DNA as template, as described in the same figure except that the length of incubation was 30 minutes. Duplicate assays were carried out and the averages plotted.

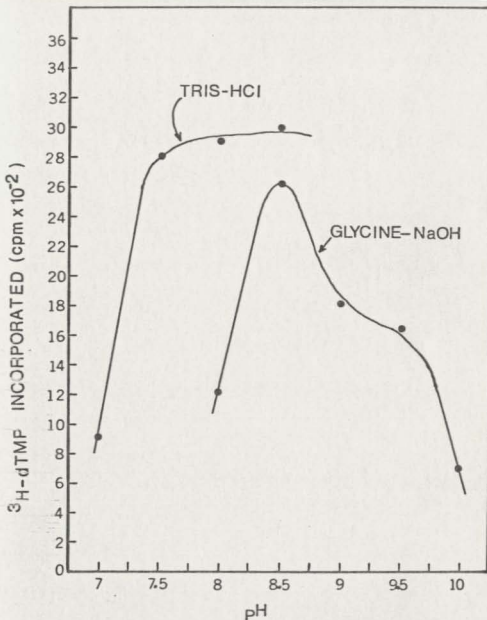


FIGURE 39. The Effect of pH on the Peak II Enzyme.

The Peak II activity was prepared as described in *Methods* and 1 ml aliquot was dialyzed for several hours against 1 liter of Buffer B lacking glycine-NaOH. Reaction conditions were as described in *Methods*, except that the 40 mM glycine-NaOH, pH 8.5 was replaced by an equal concentration of the indicated buffers, at the appropriate pH. Duplicate assays were carried out and the averages plotted.

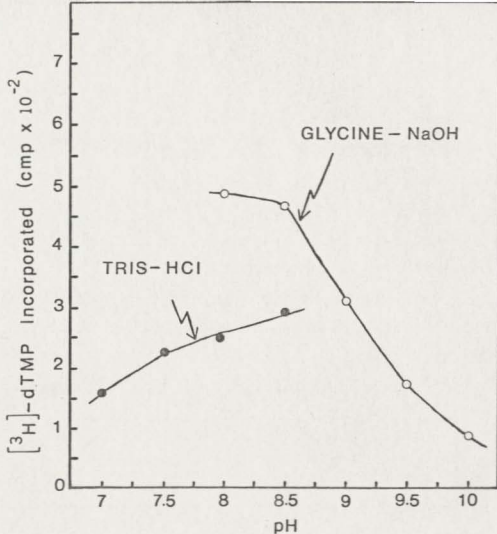


FIGURE 40. The Effect of pH on the Peak III Enzyme.

A Peak III sample was obtained and treated as described for the Peak II activity in the experiment depicted in Fig. 39.

41 and 42 respectively. Both peaks of activity showed a high preference for Mg^{2+} as the divalent cation. Although Mn^{2+} was used by both enzymes, it was much less effective than Mg^{2+} . The optimum Mg^{2+} concentration for both activities was very broad and was found to lie between 4 and 8 mM (Fig. 41) and 1 to 6 mM (Fig. 42) for Peaks II and III respectively. Mn^{2+} at 5.5 mM for Peak I and 4 mM for Peak II was only about 25% as effective as Mg^{2+} at the same concentrations for both enzymes (Figs. 41 and 42). This preference for Mg^{2+} when DNA templates are used is in sharp contrast to the divalent metal requirement of the RS-DP activity (see Fig. 8), and the putative RD-DP activity from this system reported previously (281), which prefer Mn^{2+} as the divalent cation activator.

(e) Effect of Potassium Chloride

The optimum KCl concentration for Peak II (Fig. 43A) was found to lie between 25 and 50 mM, with the activity at these salt concentrations being 3 to 4-fold greater than that observed in the absence of salt. At concentrations greater than 50 mM, KCl inhibited the enzyme until at about 180 mM salt, the level of activity had declined to the level observed in the absence of salt.

For Peak III, the optimal KCl concentration was higher than that for Peak II (Fig. 43B), namely between 100 and 150 mM. As in the case for Peak II the maximum stimulation relative to the activity in the absence of salt was 3 to 4-fold (Fig. 43B). Also at KCl concentrations above the optimum, an inhibitory effect was seen relative to that activity observed at the optimum salt concentration.

In contrast to these observations, the effect of KCl on the endogenously-templated activity (see Fig. 9) and the RD-DP activity previously re-

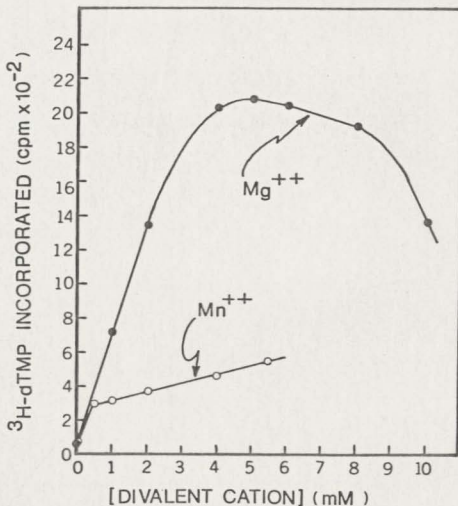


FIGURE 41. The Effect of Divalent Cations on the Peak II Enzyme.

A Peak II enzyme sample was prepared as described in *Methods*, and a 1 ml aliquot was dialyzed for several hours against Buffer B lacking EDTA. Reaction conditions were as described in *Methods*, with native DNA as the template, except that the concentration of Mg^{2+} was varied, or was replaced by the indicated concentrations of Mn^{2+} . All assays were carried out in duplicate, and the averages plotted.

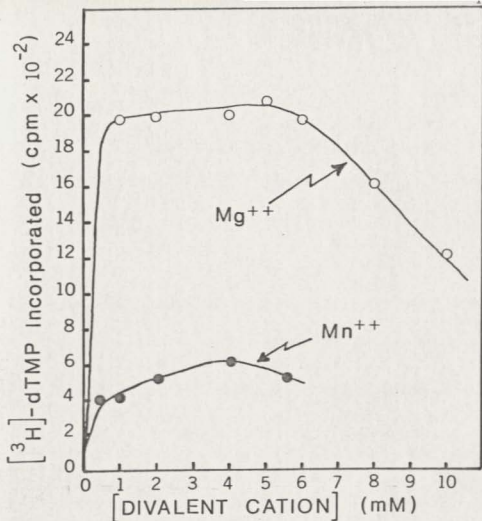


FIGURE 42. The Effect of Divalent Cations on the Peak III Enzyme.

A Peak III enzyme sample was treated in a manner identical to that described for the Peak II activity in Fig. 41.

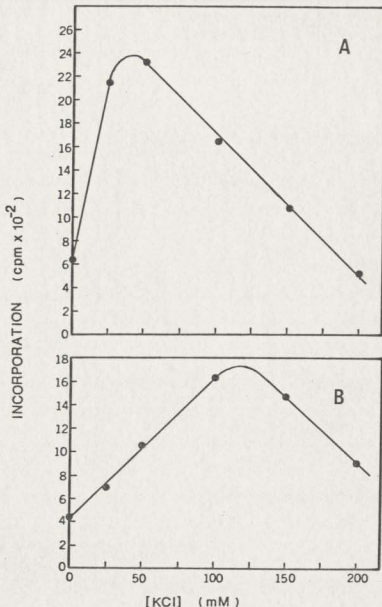


FIGURE 43. The Effect of KCl on Peak II and III Activities.

The two enzymes were obtained as described in *Methods*, and the KCl in the sample was removed by dialysis against Buffer B lacking KCl. The reactions were carried out with native DNA as the template, as described in *Methods*, except that the concentration of KCl was varied as indicated. Duplicate assays were carried out and the averages plotted. Panel A represents Peak II activity and Panel B, Peak III.

ported (281) was primarily inhibitory at concentrations exceeding 40 mM.

(f) Template Specificity

The efficiency with which DNA polymerase α , and the lowest molecular weight DNA polymerase (Peak III) derived from the RS-DP complex, make use of DNA and RNA templates is shown in Table XXV. Both enzymes showed a preference for "activated" DNA over native DNA: however, the relative efficiencies with which these two templates were used differed. The low molecular weight DNA polymerase (originally associated with the RS-DP complex), had only a 3.4-fold preference for "activated" DNA relative to native DNA, compared to a 29.5-fold preference for DNA polymerase α .

The amount of endogenously-templated DNA polymerase activity remaining in the DNA polymerase α preparation after RNase-treatment and gel filtration was dependent on the concentration of the enzyme. Samples assayed immediately after gel filtration rarely showed endogenously-templated activity, however upon concentrating the sample, as in the case of the DNA polymerase α preparation used in the study described in Table XXV, which was 6-fold concentrated after the gel filtration step, exhibited a level of 21% endogenous activity relative to the activity observed with native DNA. Upon further RNase-treatment however this level was reduced to 12% of the native DNA-directed DNA polymerase activity. The Peak III activity on the other hand displayed virtually no endogenous activity regardless of whether the sample was or was not concentrated.

The template specificities of DNA polymerase α and the Peak III enzyme unlike those of Peak II from the Sepharose 6B column, are fairly consistent. In contrast, with the Peak II enzyme, the relative

TABLE XXV. Template Specificity of Rat Thymus DNA Polymerase α and the Peak III Activity Derived from the RS-DP Complex

	<u>DNA Polymerase α</u>		<u>Peak III</u>	
	<u>(^3H-dTTP Incorporated)</u>		<u>(^3H-dTTP Incorporated)</u>	
	<u>c.p.m. per assay</u>	<u>%</u>	<u>c.p.m. per assay</u>	<u>%</u>
Native calf thymus DNA	5,340	100	8,060	100
"Activated" calf thymus DNA	157,570	2950	27,680	340
Denatured calf thymus DNA	93,130	1740	3,490	43
16S + 23S rRNA	2,500	47	620	8
RNase treated 16S + 23S rRNA ¹	2,030	38	161	2
Endogenous (-template)	1,140	21	80	1
+ RNase ²	640	12	-	-

The enzymes were prepared as described in *Methods*, except that they were fractionated on a Sepharose 6B (3 x 36 cm) column instead of Sephadex G200, and a 5 ml aliquot of the enzyme preparation was treated with 200 μg RNase A and 8.75 μg RNase T₁ for 3 hr at 25°C. DNA polymerase α represents the first peak of activity that eluted from the column. Reaction conditions were as described in *Methods*, except that glycine-NaOH was replaced by a similar concentration of Tris-HCl, pH 8.0. The concentrations of the templates were 6.9, 12, and 6.9 μg per assay, respectively, for native, "activated" and denatured DNA, and 13.9 μg per assay for 16S + 23S rRNA. All assays were carried out in duplicate for 30 minutes. The values shown for the RNase-treated samples (see footnotes 1 and 2) are based on control samples preincubated in the absence of RNases.

1. The rRNA (1.39 mg/ml) was treated with 177 μg RNase A + 10.3 μg RNase T₁ per ml for 1 hr at 24°C.
2. An aliquot of DNA polymerase α preparation was incubated for 1 hr at 24°C with 178 μg RNase A + 7.8 μg RNase T₁ per ml.

specificity towards "activated" and native DNA templates varies with enzyme concentration, from a ratio of about 48 to 1 with 5 μ l of enzyme to about 12 to 1 with 25 μ l of enzyme (Fig. 44). This variable ratio is primarily the result of a sigmoidal relationship between enzyme concentration and incorporation when native DNA is used as the template (see also Fig. 37).

When the enzymes derived from the RS-DP complex were resolved on a Sephadex G 200 column, the preference for native DNA relative to "activated" DNA was increased. For Peak II activity, the "activated": native DNA ratio of activities was 3.54, whereas for Peak III it was 1.65 when Mg^{2+} was the divalent cation (Table XXVI). With Mn^{2+} , the ratios were 7.66 and 4.62 for Peaks II and III respectively. With denatured DNA as template, the relative efficiency as compared to native DNA was increased when Mn^{2+} was the divalent cation. Thus it seems that single-stranded templates may be more efficiently used when Mn^{2+} is the divalent cation.

RNA templates are not efficiently used by the Peak II enzyme, although the efficiency with which they are used was increased when Mn^{2+} was the divalent cation as compared to Mg^{2+} (see Table XXVI). On the other hand the Peak III enzyme used RNA templates very efficiently; RNA was one-fifth as effective as native DNA when Mg^{2+} was the divalent cation, and almost equally effective or even preferred (e.g., 23S rRNA) when Mn^{2+} was the divalent cation (Table XXVI).

(i) Effect of Actinomycin D on the Exogenously RNA-Templated Activity with the Peak III Enzyme

The effect of actinomycin D on the DNA- and RNA-directed DNA polymerase activity with the Peak III enzyme is shown in Table XXVII. As can

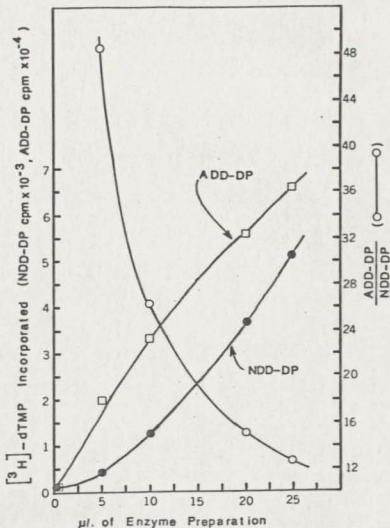


FIGURE 44. Dependence of Relative Efficiency of "Activated" to Native DNA for Peak II on Enzyme Concentration.

A Peak II sample was prepared as described in *Methods*, except that the sample was fractionated on a Sepharose 6B column instead of a Sephadex G200 column. Assays for the DNA polymerase activity with native (NDD-DP) and "activated" (ADD-DP) DNA templates were also as described in *Methods*, except that the quantity of enzyme was varied. Duplicate assays were carried out, and the averages plotted.

TABLE XXVI. Template Specificity of Peaks II and III in the Presence
of Mg^{2+} or Mn^{2+} as the Divalent Cation.

Template	Peak II					Peak III				
	Incorporation				Relative Activity Mg^{2+}/Mn^{2+}	Incorporation				Relative Activity Mg^{2+}/Mn^{2+}
	Mg^{2+}		Mn^{2+}			Mg^{2+}		Mn^{2+}		
	(cpm)	(%)	(cpm)	(%)		(cpm)	(%)	(cpm)	(%)	
Native DNA	4,400	(100)	940	(100)	4.70	36,300	(100)	5,900	(100)	6.10
"Activated" DNA	15,600	(354)	7,180	(766)	2.20	60,000	(165)	27,400	(462)	2.20
Denatured DNA	870	(20)	920	(98)	0.90	18,200	(50)	9,900	(166)	1.80
16S + 23S rRNA	220	(5)	430	(46)	0.50	7,100	(20)	6,800	(114)	1.05
yRNA	40	(0.90)	210	(22)	0.19	6,940	(19)	6,300	(107)	1.10
Q β RNA	3	(0.06)	55	(6)	0.05	6,130	(17)	3,900	(66)	1.56
23S rRNA	60	(1.40)	270	(29)	0.24	7,030	(19)	9,200	(154)	0.77

The enzyme samples were prepared as described in *Methods*. The reaction conditions were as described for the endogenously-templated activity, except that water was replaced with 10 μ l of the nucleic acid template indicated and the divalent cation concentration for Mg^{2+} was 4 mM, and for Mn^{2+} , 2 mM. The concentrations of the nucleic acids stock solutions were as follows: 0.7, 1.2, and 0.7 mg/ml for native, "activated", and denatured DNA respectively, and 1.64, 1.41, 1.25 and 1.35 for 16S + 23S rRNA, yRNA, Q β RNA and 23S rRNA respectively. Assays were carried out in duplicate and the average tabulated.

TABLE XXVII. Effect of Actinomycin D on the Peak

III Activity with DNA and RNA Templates.

<u>Template</u>	<u>Control</u> <u>(cpm)</u>	<u>+ Actinomycin D</u> <u>(cpm) (% Control)</u>	
Native DNA	840	190	22
16S + 23S rRNA	800	810	101
yRNA	910	660	72
Q β RNA	660	300	46
23S rRNA	650	180	36

Reaction conditions were as described in Table XXVI with Mn²⁺ as the divalent cation, except that in the experiments with actinomycin D, 50 μ g/ml of inhibitor was included in the reaction mixture.

be seen, the presence of 50 μ g actinomycin D per ml reduced the level of activity to 22% of the control level when native DNA was used as the template. With RNA templates, the presence of inhibitor was less effective. In fact, with 16S + 23S rRNA as template, no inhibition was observed, whereas with yRNA, Q β RNA and 23SS rRNA, the level of activity was reduced to 72%, 46% and 36% respectively relative to the control level. These observations are consistent with the RNAs acting as the templates for DNA synthesis, and that the activity observed with 16S + 23S rRNA as template probably represents the initial step of a typical reverse transcription reaction.

(g) "Km's" for Native and "Activated" DNA Templates for Peaks II and III

The apparent Km's for native and "activated" DNA as templates for Peaks II and III are given in Figs. 45 and 46 respectively. Template saturation curves in all cases were hyperbolic. Double reciprocal plots of these data yielded apparent Km's for native DNA of approximately 4.8 μ g per ml for both enzymes (Figs. 45B and 46B). The "Km's" for "activated" DNA for both enzymes were about twice as high as those for native DNA (Figs. 45D and 46D), suggesting that the activities derived from the RS-DP complex have a preference for native DNA relative to "activated" DNA.

(C) Inhibition Studies

(a) Effect of N-ethylmaleimide

The effect of the sulfhydryl blocking reagent-NEM on Peaks II and III is shown in Fig. 47. As in the case of the endogenous RS-DP activity, both of these enzymes were resistant to NEM. In this respect,

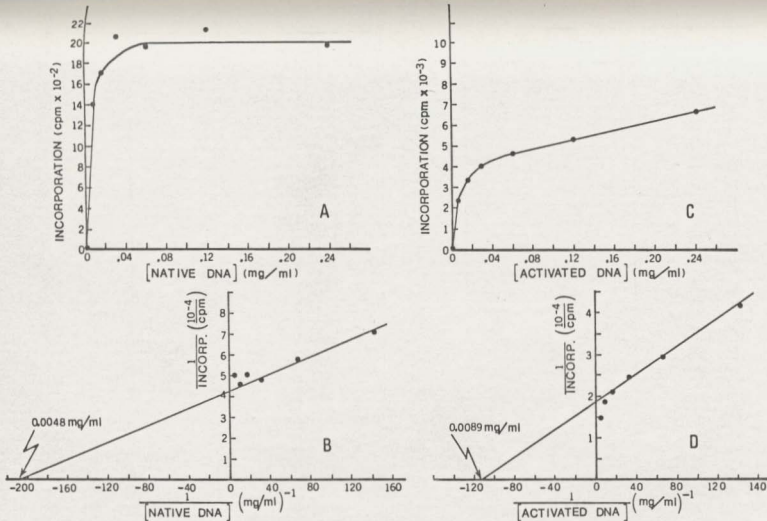


FIGURE 45. Apparent K_m 's for Native and "Activated" DNA as Templates for the Peak II Enzyme.

The Peak II enzyme was prepared as described in *Methods*. Reaction conditions were also as described in *Methods*, except that the concentrations of native and "activated" DNA templates were varied as indicated in the figure. All assays were carried out in duplicate, and the averages plotted.

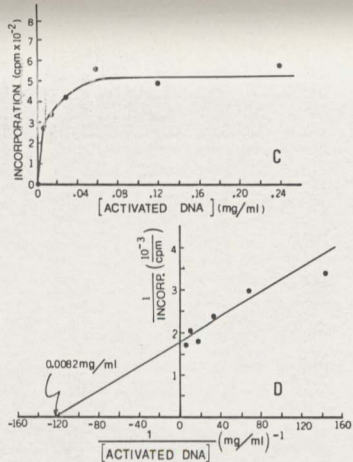
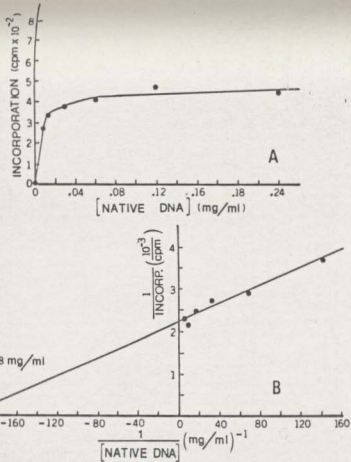


FIGURE 46. Apparent K_m 's for Native and "Activated" DNA as Templates for the Peak III Enzyme.

The Peak III enzyme was prepared as described in *Methods*, and the activity assayed as described for Fig. 45.

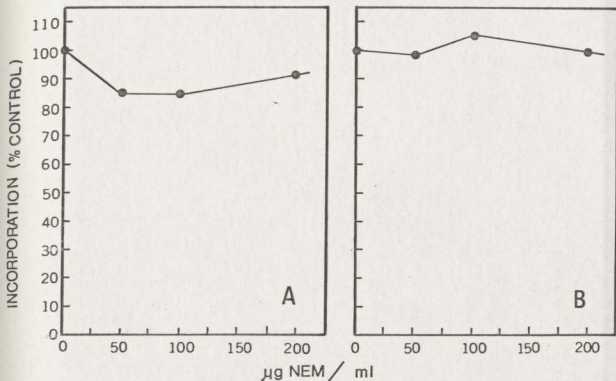


FIGURE 47. Effect of N-Ethylmaleimide on (A) Peak II and (B) Peak III

Enzymes.

The Peak II (Panel A) and Peak III (Panel B) activities were prepared as described in *Methods*, except that the fractionation was carried out on a Sepharose 6B column. The reactions were also carried out as described in *Methods*, with native DNA as the template, except that the reaction mixtures also contained the quantity of inhibitor indicated, and the incubation times were only 15 minutes. 100% activity for Peak II represents 1,870 cpm of ^3H -dTMP incorporated, and for Peak III, 700 cpm.

the activities are similar to DNA polymerase β (see Table VII) and HeLa cell mitochondrial DNA polymerase (195), and different from DNA polymerase α (see Table V) and γ (188) which are sensitive to this inhibitor.

(b) Effect of Heparin

Heparin is a potent inhibitor of the Peak II and III activities (Fig. 48A and B). Both activities were inhibited more than 90% at a concentration of 50 μg per ml. In contrast, the presence of 50 μg of heparin per ml stimulated the RS-DP activity approximately 25% and inhibited DNA polymerase α about 25% (Fig. 13). The response of Peaks II and III to this inhibitor distinguishes them from DNA polymerase β which is quite resistant to this inhibitor relative to DNA polymerase α (see Tables V and VII).

(c) Effect of Ethidium Bromide

Ethidium bromide at a concentration of 100 μg per ml was found to inhibit more than 90% of the Peak II and III activities (Fig. 49). At lower concentrations of the inhibitor (12.5 μg per ml), Peak II was stimulated slightly whereas Peak III was inhibited by about 15%. In contrast to this, the endogenous activity was not affected by the inhibitor at a concentration of 20 μg per ml (data not shown), while the released activities showed substantial inhibition ($\sim 25\%$) at this concentration.

(d) Effect of Organic Solvents

The effects of various organic solvents on the released activities are tabulated in Table XXVIII. In the case of Peak II, substantial stimulation was observed in the presence of 10% methanol, ethanol, acetone

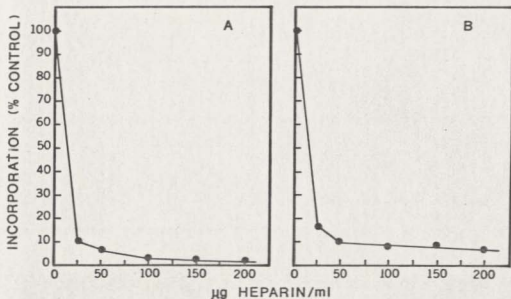


FIGURE 48. Effect of Heparin on (A) Peak II and (B) Peak III Enzymes.

Enzyme samples were prepared as described in *Methods*, and assayed in duplicate under the conditions described with native DNA as template except that the reaction mixtures also contained the quantities of heparin indicated. 100% activity for Peak II (Panel A) was equal to 1,940 cpm and for Peak III (Panel B), 1,280 cpm of ^3H -dTTP incorporated.

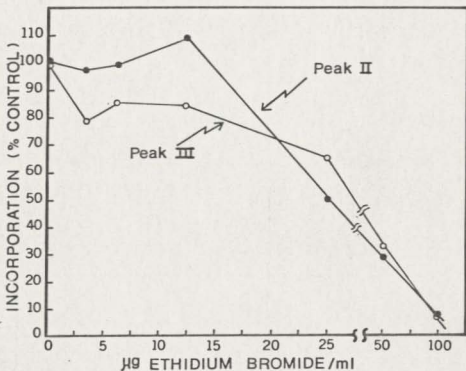


FIGURE 49. Effect of Ethidium Bromide on the Peak II and III

Enzymes.

Peak II and III enzymes were assayed in duplicate with native DNA as the template as described in *Methods*, except that the quantities of ethidium-bromide indicated were included in the reaction mixtures. 100% activity for Peak II represented 1,680 cpm and for Peak III, 1,570 cpm of ^3H -dTTP incorporated.

TABLE XXVIII. Effect of Organic Solvents on the Peak
II and III Enzymes.

Organic Solvent	Peak II		Peak III	
	Incorporation (cpm)	(% Activity)	Incorporation (cpm)	(% Activity)
Control	390	100	860	100
+ 10% methanol	500	127	810	95
+ 10% ethanol	465	118	720	84
+ 10% butanol	24	6	0	0
+ 10% acetone	480	122	755	88
+ 10% DMSO	620	158	1,040	121

Peaks II and III were obtained as described in *Methods*. The average of duplicate assays is shown and were carried out for 30 minutes at 37° C using native DNA as the template under the conditions described in *Methods*, except that the indicated organic solvent was included.

and dimethylsulfoxide (DMSO), whereas a 94% inhibition was observed with 10% butanol. In the case of Peak III, 10% methanol, ethanol or acetone slightly inhibited the activity (6-16% inhibition), and 10% butanol totally abolished it. Ten percent DMSO stimulated Peak II by 57% and Peak III by 21%. The response of these enzymes to methanol, ethanol and acetone is similar to that reported for DNA polymerase β (see Table VII), and differs from that for polymerase α which is extremely sensitive (Table V).

(e) Effect of Polyamines

The influence that polyamines exert on the Peak II and III activities is shown in Fig. 50. Either stimulation or inhibition was observed depending both on the type as well as the concentration of the polyamine used. Furthermore, as described below, the polyamines effect Peaks II and III differently. For example, putrescine at a concentration of 0.5 mM was found to stimulate Peak II by about 20% (Fig. 50A), and Peak III by about 75% (Fig. 50B).

A greater stimulation of Peak III activity relative to that of Peak II was also observed with spermidine (Fig. 50C and D), and a smaller inhibition at high concentrations (e.g., 4 mM). Spermine strongly inhibited the Peak II activity (Fig 50E), whereas its effect on Peak III was stimulatory at low concentrations (up to 1 mM) and inhibitory at higher concentrations (above 1 mM). Concentrations of spermine as low as 40 μ M (Fig. 50G and H) did not effect either of the two activities.

In contrast to the stimulatory effect that putrescine had on Peak II and III activities, it had no effect on the RS-DP activity, and inhibited DNA polymerase α (Fig. 14A). Spermidine on the other hand at

FIGURE 50. Effect of Polyamines on the Peak II and Peak III Enzymes.

The Peak II and III enzymes were assayed with native DNA as the template as described in *Methods*, except that the reaction mixtures also contained the concentrations of polyamines indicated. All assays were carried out in duplicate, and the averages plotted. 100% activity for Peak II is equal to 2,400 cpm and 1,250 cpm for Peak III of ^3H -dTTP incorporated. The sp. act. of ^3H -dTTP was 3,600 cpm/pmole.

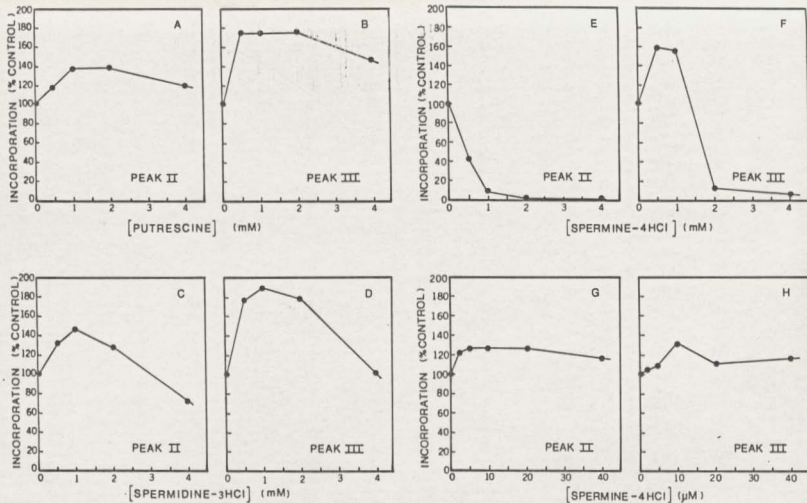


FIGURE 50. Effect of Polyamines on the Peak II and Peak III Enzymes.

concentrations which inhibited the RS-DP and DNA polymerase α (Fig. 14B), stimulated the DNA polymerases derived from the RS-DP complex (Fig. 50C and D). In the case of spermine, 20% stimulations of both the RS-DP (Fig. 14C), and the activities derived from it (Fig. 50G and H) were observed in the concentration range of 50 to 40 μ M, whereas the polymerase α was inhibited by about 20% (Fig. 14C). At much higher concentrations of spermine (0.5-1 mM) all of the activities except the Peak III activity (which was actually stimulated by 60%) were inhibited (see Figs. 14D and 50 E and F). It would thus appear on the basis of these data, that the mechanism whereby polyamines influence DNA polymerases is very complex, and their site of action may be both on the nucleic acid template as well as on the DNA polymerases.

Chiu and Sung (46) have suggested that polyamines may stimulate DNA polymerases by promoting linearity in the activity versus enzyme concentration curve at low concentrations of enzyme. The presence of 1 mM putrescine in the assay for the Peak II and III activities was found not to abolish the sigmoidicity observed in its absence. In the case of Peak II (Fig. 51, top) at low concentrations of the enzyme, no apparent stimulation by putrescine was observed, however, at higher concentrations of enzyme (15 and 20 μ l), a 20 to 25% stimulation resulted. With 25 μ l of enzyme, the polyamine inhibited the activity approximately 70%. Thus the effect of putrescine on the Peak II activity depends on the enzyme concentration used.

In the case of Peak III (Fig. 51, bottom) putrescine stimulated the activity at all concentrations of enzyme used, however the degree of stimulation was not identical at all concentrations. Theoretically, if

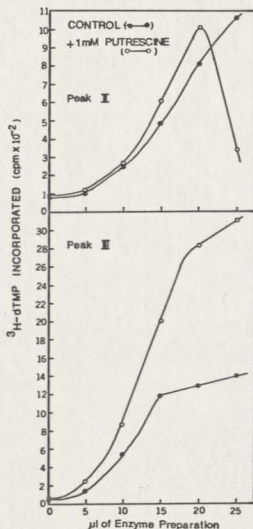


FIGURE 51. Effect of 1 mM Putrescine on the Activity
Versus Enzyme Concentration Curve for
Peaks II and III Activities.

The enzymes were prepared as described in *Methods* and assayed with native DNA as the template, using various quantities of the enzymes as indicated, either in the presence or absence of 1 mM putrescine. All assays were carried out in duplicate, and the averages plotted.

polyamines were to stimulate DNA polymerases by making the activity proportional to enzyme concentration at low levels of enzyme (i.e., make the activity versus enzyme concentration curve linear), the percentage stimulation should be greatest at low concentrations of enzyme and minimal at the highest concentrations. This was not found to be the case.

(D) Physical Properties of the DNA Polymerases Derived from the RS-DP Complex

(a) Molecular Weight

The determination of the molecular weight of the released activities proved to be difficult mainly because of their ability (especially Peak III) to both aggregate, and also to adsorb onto the Sephadex. For example, a calibration curve for a Sephadex G 200 column (Fig. 52) shows the Peak II activity (indicated by the first arrow) at a molecular weight position of 40,000 daltons. The Peak III activity (second arrow) eluted much later than the total column volume. The enzyme behaved similarly on Sephadex G 150 (data not shown). On Sepharose 6B, this anomolous behaviour was not apparent, the Peak II activity eluted at a position of 70,000 daltons, and the Peak III activity at approximately 30,000 daltons (Fig. 53)*.

Rechromatography of the RNase-released activities resulted in the

* With the sample used in this particular experiment, the 110,000 dalton species previously reported (281) to be derived from the endogenous activity is also apparent.

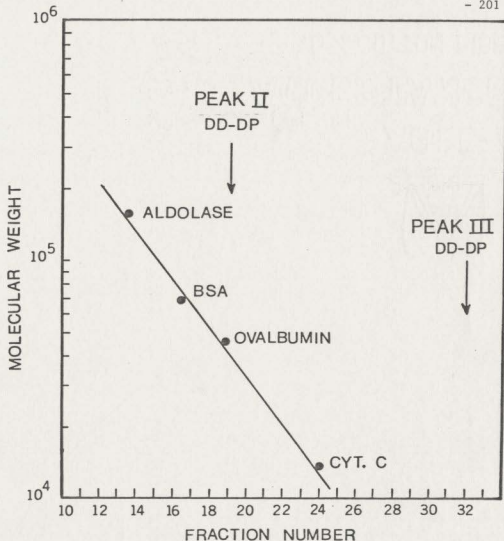


FIGURE 52. Molecular Weight Estimation of Peak II and III Activities on a Sephadex G200 Column.

The enzymes were prepared as described in *Methods* and the Sephadex G200 column used for fractionation was calibrated with the marker proteins: cytochrome C (13,700), ovalbumin (45,000), BSA (67,000) and aldolase (158,000).

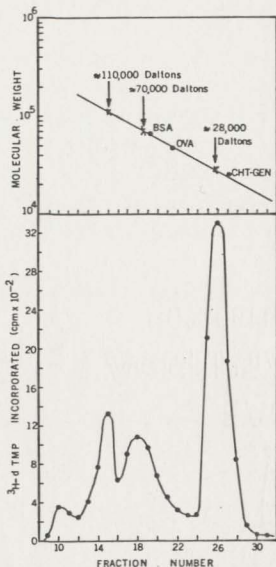


FIGURE 53. Molecular Weight Estimation of the Peak II and III Activities on a Sepharose 6B Column.

The enzymes were prepared as described in *Methods* using a Sepharose 6B column calibrated with the marker proteins, bovine serum albumin (BSA, 67,000), ovalbumin (OVA, 45,000) and chymotrypsinogen (CHT-GEN, 25,000). DNA polymerase assays were as described in *Methods* with native DNA as template.

following observations: The Peak II activity still eluted from a Sepharose 6B column as a 70,000 dalton species, either without further RNase-treatment (Fig. 54A), or following RNase-treatment (Fig. 54B). When the Peak III activity (after it was concentrated) was rechromatographed on the Sepharose 6B column, the activity no longer eluted at the same position. In fact it appeared to aggregate to such an extent that the activity was found throughout the column fractions (data not shown). When chromatographed on a Sephadex G 75 column, in the presence of 1 M KCl in the buffer, the activity eluted as a 40,000 dalton species (Fig. 55).

(b) Isoelectric Points of the Peak III Activity

Analysis of the Peak III activity on an isoelectric focusing column revealed a heterogeneous population of DNA polymerases (Fig. 56) with three main peaks of activity at pI's of 6.4, 8.0 and 8.4 with native DNA as template, and two enzyme peaks with pI's of 8.2 and 6.4 with "activated" DNA as template. This heterogeneity probably does not represent unique enzyme species since these results were not reproducible, and other isoelectric points were obtained in other experiments (data not shown). The inconsistencies in isoelectric points observed from one preparation to another may be due to aggregation, association of factors with the enzyme that give it different template specificities, or to association with the ampholytes used to establish the pH gradient. Attempts at abolishing these artefacts were unsuccessful.

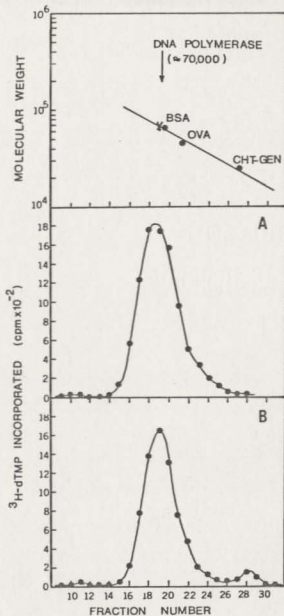


FIGURE 54. Molecular Weight Estimation of the Peak II
Activity (A) Before and (B) After RNase-
Treatment on a Sepharose 6B Column.

The Peak II enzyme was prepared as described in *Methods* and two 1 ml aliquots were refractionated on a Sepharose 6B column (approximately 1.5 x 25 cm) calibrated with the denoted marker proteins, either without any further treatment (A) or after further RNase-treatment as described in *Methods*. Assay conditions were as described in *Methods* using an "activated" DNA template.

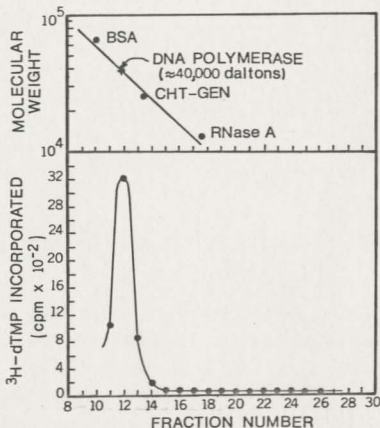


FIGURE 55. Molecular Weight Estimation of the Peak III Enzyme on a Sephadex G75 Column in the Presence of 1 M Salt.

A Peak III sample was prepared and concentrated as described in *Methods*. The KCl concentration was adjusted to 1 M by the addition of an appropriate volume of 2 M salt to the enzyme sample. A 1 ml aliquot was then fractionated on a Sephadex G75 column (approximately 1.5 x 25 cm) equilibrated with Buffer B containing 1 M KCl. A 10 μ l aliquot of each fraction diluted to 25 μ l with Buffer B was assayed for DNA polymerase activity using "activated" DNA as the template. The column was calibrated with the marker proteins indicated.

FIGURE 56. Isoelectric Focusing Analysis of the Peak III Enzyme.

A 0.4 ml aliquot of the Peak III enzyme obtained from a Sepharose 6B column was electrofocused in a 10 ml column and fractions were collected as described in *Methods*.

DNA polymerase activities were assessed using native and "activated" DNA templates as described in *Methods* except that glycine-NaOH pH 8.5 was replaced by Tris-HCl pH 8.0 at the same concentration.

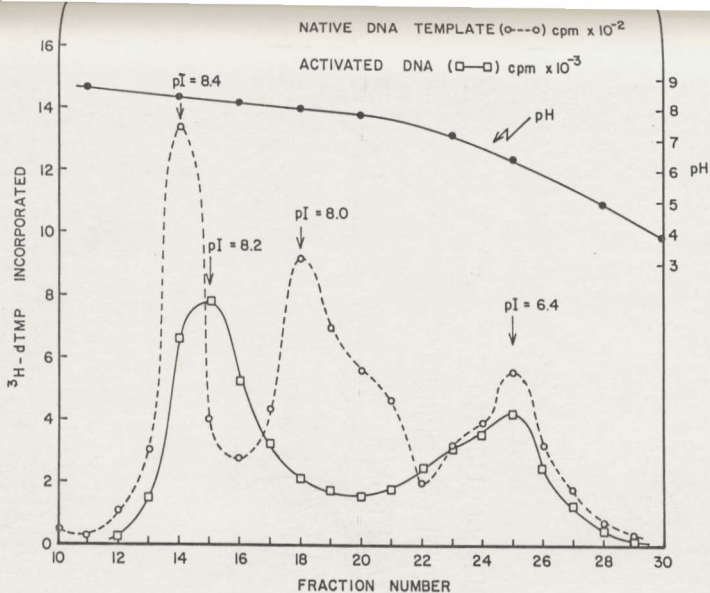


FIGURE 56. Isoelectric Focusing Analysis of the Peak III Enzyme.

DISCUSSION

I. Evidence Suggesting RNA-Directed DNA Synthesis in Mammalian Cells

It is now clear that the term "RNA-directed", when applied to a DNA polymerase, does not describe a unique feature of the enzyme, but rather a function that the enzyme may execute, given the appropriate reaction conditions. That is to say, RD-DP activities described to date, with the exception of the RD-DP activity from *E. coli* described by Beljanski's group (232-234), also function as DNA-directed DNA polymerases. Indeed, the "true" RD-DPs ("reverse transcriptases"), from oncogenic RNA viruses, show a greater efficiency in transcribing DNA templates than RNA templates (13, 210, 211). This dual function of DNA polymerases has made it necessary to closely assess the nature of any putative RNA-directed DNA synthesis to rigorously exclude the possibility of a DNA-directed function. This is especially important when one considers that the RNA-directed activity is normally quite low relative to the large amounts of DNA-directed activity. It is thus conceivable that minor levels of apparent RNA-directed activity may be elicited by traces of DNA contaminants in the RNA preparations used as exogenous templates, or, in the case of the endogenously-templated activities, in the enzyme preparation itself.

The recent finding that RNA may also serve as a primer, rather than as a template, in certain enzyme systems (62, 74-76, 147-152, 222, 299), has further complicated the matter of trying to establish whether RNA-directed DNA synthesis occurs in mammalian cells. This is particularly important to take into account when dealing with endogenously-templated RNase-sensitive activities (222, 299).

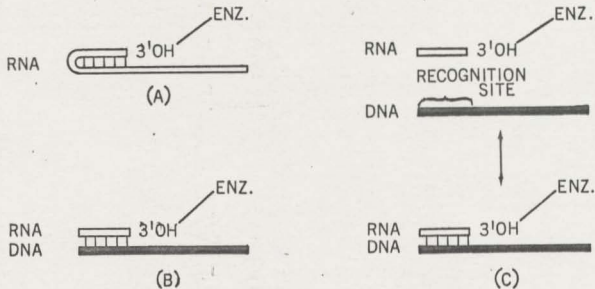
In the work described in this dissertation, the evidence in support of a template function for the RNA in the endogenously-templated RS-DP complex has been both indirect as well as direct.

The essential criteria for establishing that a DNA polymerase is RNA-directed were outlined earlier [see Literature Review, Section III E(a)].

(A) Indirect Evidence

The observation that the endogenously-templated DNA polymerase activity is sensitive to RNase-treatment clearly indicates that RNA has a function in the reaction. The possible functions of RNA in the complex are depicted in Scheme XI, which basically postulates either a template-primer [Scheme XI (A)] or a primer function [Scheme XI (B) and (C)] for the RNA. Under the conditions normally used in carrying out the RNase-treatment (i.e., with high levels of RNase A), the RNA in all three possible structures would be susceptible to the nuclease digestion. However, under the conditions described in Table XVI (i.e., low levels of RNase A in the presence of 200 mM NaCl) only the structures depicted in Scheme XI (A) and (C) would be susceptible. The sensitivity to RNase-treatment at high salt concentrations (Table XVI) therefore tends to rule out Structure (B); in addition, the observation that the size of the RS-DP complex was unaffected by DNase-treatment disqualifies the structures shown in Scheme XI (B) and (C), and lends further support to that depicted in Scheme XI (A).

Further support for a template function for the RNA emerges from the observation that the RS-DP activity is only partly sensitive to actinomycin D (Fig. 10) and distamycin A (Fig. 11). This follows from the fact that these inhibitors influence DNA-directed nucleic acid synthesis



SCHEME XI

Possible Modes of Association of the Endogenously-Templated
DNA Polymerase with Nucleic Acids

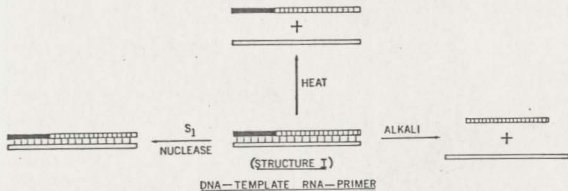
Under conditions of low concentrations of RNases and 200 mM KCl, the RNA template in (A) would be susceptible to RNase A and T_1 digestion, in (B) the RNA primer would not be susceptible to the RNases and in (C) the RNA would be susceptible to RNase hydrolysis only when dissociated from the DNA template.

and not RNA-directed synthesis (284, 285).

(B) Direct Evidence

More conclusive evidence with regard to a template function for the RNA was obtained from buoyant density analysis of the product. Three types of products may be produced by the RS-DP activity when the reaction is carried out in the presence of Actinomycin D (see Scheme XII) assuming either a template-primer [Structures II and III, Scheme XII (B) and (C) respectively] or a primer (Scheme XII (A), Structure I) function for the RNA. Structure III takes into account the possibility that the RNase-H activity (contaminating the enzyme sample, data not shown) may have hydrolyzed some of the RNA template. Included in each Scheme are also some predictions regarding the expected buoyant density and sedimentation velocity properties of the product after various treatments, as well as indications as to whether the data presented in Results, Section IV, are consistent with the predictions.

A number of experimental findings are inconsistent with Structures I and II [see Scheme XII (A) and (B)]. For example, in the case of Structure I, the predicted effects on the buoyant density of the product after heat- and S_1 nuclease-treatment, the sedimentation velocity analysis after heat-treatment, and the sensitivity to S_1 nuclease-treatment, are inconsistent with the experimental findings. For Structure II, the predicted buoyant density of the native structure, and that of the structure after heat-treatment or S_1 nuclease-treatment are not consistent with the experimental observations. In addition, the partial sensitivity to S_1 nuclease-treatment (25% of the product was sensitive, see Fig. 24), and the size of the product after heat-denaturation (Fig. 26A), are also

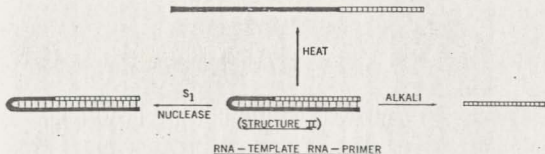


SCHEME XII (A)

<u>Predictions</u>	<u>Agreement with Experimental Observations</u>
1. The buoyant density of the native product should be close to that of DNA.	Yes*
2. After heat-treatment, the buoyant density of the product should be closer to that of RNA than it was before heating.	No
3. After alkali-treatment, the buoyant density of the product should be identical to that of DNA.	Yes
4. S_1 nuclease-treatment should not greatly alter the buoyant density of the product.	No
5. The product should be totally resistant to S_1 -nuclease unless first heat-denatured.	No
6. After heat-treatment the size of the product should be less than or equal to one-half the size of the native product.	No
7. After alkali-treatment the size of the product should be less than one-half the size of the native product.	Yes

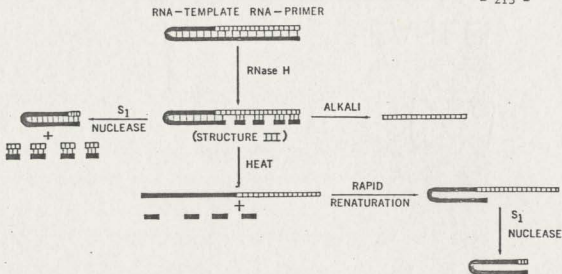
Symbols used: RNA -
 DNA -
 Newly synthesized DNA -

* A "Yes" denotes that the prediction is in agreement with the experimental observations, whereas a "No" indicates that it is not.



SCHEME XII (B)

<u>Predictions</u>	<u>Agreement with Experimental Observations</u>
1. The buoyant density of the product should be closer to that of RNA than DNA.	No
2. After heat-treatment, the buoyant density of the product should not change.	No
3. After alkali-treatment, the buoyant density of the product should be identical to that of DNA.	Yes
4. S_1 nuclease-treatment should not greatly alter the buoyant density of the product.	No
5. The product should be totally resistant to S_1 -nuclease, unless first heat-denatured.	No
6. After heat-treatment, the size of the product should be equal to the size of the native product.	No
7. After alkali-treatment, the size of the product should be less than one-half the size of the native product.	Yes



SCHEME XII (C)

Predictions

Agreement with
Experimental Observations

- | | |
|---|-----|
| 1. The buoyant density might be closer to that of DNA than RNA depending on how much RNA-template has been degraded by the RNase H. | Yes |
| 2. After heat-treatment, the buoyant density of the product should move closer to that of DNA. | Yes |
| 3. After alkali-treatment, the buoyant density of the product should be identical to that of DNA. | Yes |
| 4. After S_1 nuclease-treatment, the buoyant density of some of the product should be close to that of RNA, and the rest of hybrid density. | Yes |
| 5. The native product should be partially sensitive to S_1 nuclease, and after heat denaturation completely or almost completely sensitive. | Yes |
| 6. After heat-treatment, the size of the product should be heterogeneous, depending on the position at which RNase H has cleaved. | Yes |
| 7. After alkali-treatment, the size of the product should be less than or equal to one-half the size of the native product. | Yes |

inconsistent with the predictions for Structures I and II.

Structure III on the other hand, which differs from Structure II in that RNase H has partially degraded the RNA template, is consistent with all experimental findings [compare predictions in Scheme XII (C) to experimental findings shown in Figs. 24, 25, 26 and 28 and Table XXIII].

Thus, the experimental results obtained upon buoyant density and sedimentation velocity analysis of the product after various treatments, as well as the sensitivity of the product to S_1 nuclease-treatment, are consistent with a template function for the RNA. It is apparent however, from the same results, that the RNA template did not remain intact, possibly because of the RNase H activity contaminating the enzyme sample.

It is also apparent, from the analysis of the DNA product synthesized in the absence of actinomycin D (see Fig. 27), that the structure of the product is mainly RNA-DNA hybrid, although some double-stranded DNA was formed. This is most likely related to the short period of the reaction (5 min) in which mostly RNA-DNA hybrid is synthesized. The second step of the reaction, the formation of double-stranded DNA, has only occurred to a minor extent during the short period of reaction time used.

(C) Comparison of the Endogenously-Templated RS-DP Activity from Rat Thymus to that from Oncogenic RNA Viruses

(a) Physical Properties

An obligatory step, in assessing the RD-DP activity from oncogenic RNA viruses, is the disruption or "loosening up" of the virus capsids in

order to make the enzyme accessible to the substrates. This is usually achieved by treating the viral particles with a non-ionic detergent such as Triton X-100 [see Literature Review, Section III E(a)]. This treatment however, was not found to be essential for the assessment of the endogenously-templated RNase-sensitive DNA polymerase activity from rat thymus (see Fig. 15) and in this respect the activity does not appear to be of viral origin. In addition, the activities from both sources differ greatly in their buoyant densities, with the viral enzymes displaying buoyant densities of 1.22 to 1.24 g/cm³ (13), compared to 1.05 g/cm³ for the enzyme from rat thymus (Fig. 16), obtained in sucrose gradients.

In view of the observation that a significant amount (40-60%) of the rat thymus RS-DP complex can be pelleted at high centrifugal forces (164,900 x g for one hour), this enzyme-nucleic acid complex would seem to be particulate in nature, as also reported by others (22-24, 236, 238, 241, 248). The activity found in the particulate fraction may be a fractionation artifact however, produced by the adsorption of the complex to components present in the microsomal fraction (see legend to Fig. 35). This conclusion is supported by the observation that the rat liver enzyme can be washed from the pellet fraction with 400 mM salt present in the buffer. However the activity could not be solubilized by treatment of the pellet fraction with 1% Triton X-100 in the buffer. These observations are inconsistent with a microsomal origin for the RS-DP complex, but are consistent with a possible ionic interaction between the complex and the ribosome fraction. However the possibility that the salt is causing a disaggregation of a large aggregate of the complex, cannot be totally excluded.

Teitz (300, 301) has reported an RNA-directed DNA polymerase activity

in "normal" rat thymus tissue cultures, and has shown its source to be a type C particle on the basis of the density at which the particles banded in a sucrose gradient, and by the large increase in RD-DP activity upon treatment with non-ionic detergents. The cultured cells however, were not normal; histological studies revealed that the morphology of the cultured cells (within 24-48 hours from the beginning of the culture) differed greatly from that of freshly prepared rat thymus cells (302). Furthermore, no C-type particles were observed in the rat thymus tissue taken directly from the normal living rats, even though the presence of such particles in germ-free mice is common (301).

(b) Biochemical Properties

The properties of the cellular RS-DP activity are similar in some respects, to the properties reported for the viral enzyme. On the basis of the stringent requirement for a full complement of deoxynucleoside triphosphates for maximum activity, both activities fall in the category of *replicative* DNA polymerases. The responses of the enzymes from both sources to the inhibitors actinomycin D and distamycin A are also similar, although the site of action of these inhibitors is on the template, and not on the enzyme itself. In this regard both activities differ from the DNA-directed DNA polymerases, which are more sensitive to these inhibitors than are the RS-DPs. The rat thymus RS-DP activity is also similar to the activity from mammalian C-type RNA tumor viruses, in that both enzymes show a preference for Mn^{2+} over Mg^{2+} as the divalent metal cofactor (13, and Fig. 8). This feature, however, is not an absolute criterion for reverse transcription since the activities from the avian viruses, the type B virus from mice, and the Mason-Pfizer monkey virus

all prefer Mg^{2+} (13).

Significant differences between the properties of the rat thymus enzyme and those reported for oncogenic RNA viruses have also been observed. For example, the rat thymus enzyme was inhibited by concentrations of KCl greater than 40 mM (see Fig. 9), whereas the viral enzymes display optimal activity at KCl concentrations between 50 and 100 mM (13). Of greater significance, perhaps, is the observation that the activity from rat thymus was insensitive to NEM (see Fig. 12), whereas that from oncogenic RNA viruses is sensitive to this inhibitor (303, 304). A significant difference in the response of the enzymes from both sources to the inhibitor heparin has also been observed. Namely, the activity from Rauscher murine leukaemia virus was reported (305) to be inhibited by heparin (50% inhibition in the presence of 14 to 20 μ g of heparin/ml) whereas the activity from rat thymus was stimulated (25% stimulation in the presence of 50 μ g heparin/ml) (see Fig. 13).

II. Evidence Suggestive of Two Unique DNA Polymerases Associated with the Endogenously-Templated RS-DP Complex

The evidence that the two DNA polymerases arising after RNase-treatment of the endogenously-templated activity are derived from the RS-DP complex has already been dealt with in detail (see Results, Section V). The released activities seem to be distinct enzymes on the basis of the differences between them, observed in their physical and kinetic properties. For example, their sizes differ, with the Peak II enzyme having a molecular weight of 70,000 daltons, whereas the Peak III enzyme is approximately 30-40,000 daltons in size (see Figs. 53-55). In addition, the Peak III enzyme has the ability to aggregate under low salt conditions, whereas the Peak II enzyme does not. Also, at low salt concentrations, both enzymes will adsorb to Sephadex columns, although to different extents, with the Peak III activity being adsorbed more strongly* (see Fig. 52). The Peak II activity is not merely an intermediate produced during the RNase-treatment, since further treatment of this activity with RNases did not further reduce its size [see Fig. 54 (b)], nor is it an aggregate of the Peak III enzyme, since rechromatography in the presence of 1 M salt in the buffer did not reduce its size (data not shown).

Differences between Peaks II and III are also evident in their re-action properties. For example, significant differences between the two activities in their responses to pH and buffer anions are evident (compare Figs. 39 and 40). Furthermore the two activities differ with regard to

* This adsorption phenomenon is an advantage in the purification of these enzymes. In fact the protein concentration in the Peak III fractions from the Sephadex G 200 column is so low that it could not be estimated by the A_{260}/A_{280} ratio. Fluorometric determination of protein concentrations in a Peak III sample has revealed a concentration of 20 μ g protein per ml, when compared to a BSA standard (data not shown).

divalent (Figs. 41 and 42) and monovalent (Fig. 43 A and B) cation optima.

The efficiencies with which the Peak II and III activities transcribe various DNA and RNA templates also differ. For example, the activity with native DNA, relative to that observed with "activated" DNA, is better for the Peak III than for the Peak II enzyme (see Table XXVI). In addition the Peak III enzyme uses RNA templates much more efficiently in the presence of either Mg^{2+} or Mn^{2+} as the divalent cation, whereas the Peak II enzyme uses them very inefficiently if at all (Table XXVI).

Reports concerning the use of polyamines in distinguishing different DNA polymerases have been both positive (44, 45, 46) as well as negative (306). Chiu and Sung (44, 45, 46) have claimed that polyamines could be used to distinguish DNA polymerases α and β from rat brain, whereas Yoshida *et al.* (306) claim that it is impossible to distinguish the same polymerases derived from calf thymus, on the basis of their response to polyamines. These contradictory observations are probably related to the assay conditions used, since the effect of polyamines on DNA polymerases depends on a number of parameters inherent in the assay (306). With regard to the Peaks II and III activities described in this present work, it was observed that under identical assay conditions, the two activities responded differently. For example, at a concentration of 1 mM spermine (see Fig. 50 E and F), the Peak II enzyme was inhibited by more than 90% whereas the Peak III activity was stimulated approximately 60%. In the presence of putrescine and spermidine both enzymes were stimulated, but to different extents. Thus on the basis of these observations, it is possible to distinguish the Peak II and III activities on the basis of their response to polyamines.

The responses of the two activities to organic solvents also differ.

The Peak II activity was significantly stimulated by the presence of 10% methanol, ethanol or acetone, while the Peak III activity was slightly inhibited. In addition, the two activities were stimulated to different extents by 10% DMSO (see Table XXVIII).

The enzymes are similar however, in that they both display sigmoidal activity versus enzyme concentration curves (see Fig. 37), and in addition, respond similarly to NEM (Fig. 47), heparin (Fig. 48) and ethidium bromide (Fig. 49).

Thus on the basis of physical as well as biochemical properties, the Peak II and III activities derived from the RS-DP complex represent two DNA polymerase species.

The possibility that the two DNA polymerase species derived from the endogenously-templated RS-DP complex are proteolytic fragments of a higher molecular weight species has not been totally excluded (see pages 163-165). It is still possible that an undetected proteolytic activity, insensitive to PMSF and Trasylol, in the enzyme preparation or in the RNase solutions may be responsible for the production of the Peak II and III activities. However, if a contaminating protease (or proteases) was involved in the generation of the Peak II and Peak III activities, this would not make the released DNA polymerases any less unique. First of all, they must be derived from a unique fraction of the total DNA polymerases (i.e., the RNase-sensitive DNA polymerase) since the amount of released activity is always small (less than 1% of the total DNA polymerases). Secondly, the released activities, even if they were generated by proteolysis, have the interesting property of a relatively high affinity for native DNA which may be of biological significance, regardless of whether the enzymes were generated by proteolysis or RNase-treatment alone.

III. Relationship of Peak II and III Activities to Other Eukaryotic DNA Polymerases

The observation that the Peak II and III activities require a full complement of deoxynucleoside triphosphates for maximum activity, indicates that the enzymes are *replicative-type* DNA polymerases. However, a comparison of the properties of these two enzymes, with the reported properties of other eukaryotic DNA polymerases, suggests that they are unique enzymes.

Various features of the DNA polymerases derived from the RS-DP complex distinguish them from other eukaryotic DNA polymerases. For example, the two activities are different from DNA polymerase α in that they are highly resistant to inhibition by NEM, whereas DNA polymerase α is extremely sensitive (compare Figs. 12 and 47). In addition, the Peak II and III activities are 3- to 4-fold stimulated by relatively high concentrations of KCl (Fig. 43), whereas DNA polymerase α from rat thymus was inhibited by the presence of salt (281). Furthermore, DNA polymerase α from the same source was substantially less sensitive to heparin inhibition than were the Peaks II and III activities (compare Figs. 13 and 48). The insensitivity of the RNase-released activities to organic solvents such as methanol, ethanol and acetone (see Table XXVIII) also distinguishes them from DNA polymerase α , which has been reported to be extremely sensitive to inhibition by these compounds (26, 27, 42a, 55, 99). The observed differences between the responses of DNA polymerase α and those of the Peaks II and III activities to polyamines (see Figs. 14 and 50) further distinguishes these enzymes. In addition, the relatively small sizes of Peaks II and III (70,000 and 30-40,000 daltons, respectively) relative to the size of polymerase α (see Table III) further substantiates

that they are unique enzymes.

The Peak II and III activities may be distinguished from DNA polymerase γ on the basis of their response to NEM. As we have seen, the Peak II and III activities are resistant to NEM inhibition, whereas DNA polymerase γ has been reported to be sensitive to NEM inhibition (188). The Peak III activity is also different from DNA polymerase γ in that it is able to transcribe heteropolymeric RNAs into DNA, whereas DNA polymerase γ cannot (see Table IX).

The Peak II and III activities are not related to the mitochondrial DNA polymerase, both on the basis of their different sizes (mit.DNA polymerase having a sedimentation value of 8-9S, see Table II), as well as their different subcellular localizations.

Although the Peak II and III activities (Peak III in particular) do have some features in common with DNA polymerase β , (especially their insensitivity to NEM inhibition), they are distinguishable from this enzyme in other respects. For example, the RNase released activities use native DNA as template, whereas DNA polymerase β seems not to use it (37, 160), although contradictory observations have been reported (see Table VI). In addition, DNA polymerase β is relatively resistant to inhibition by heparin, compared to DNA polymerase α (58, 59, 96-98), while the Peak II and III activities are much more sensitive to this inhibitor than is polymerase α (compare Figs. 13 and 48). The Peak II activity also differs from DNA polymerase β on the basis of size.

Thus on the basis of a series of biochemical characteristics, Peaks II and III are distinguishable from other eukaryotic DNA polymerases, and are unique DNA polymerase species.

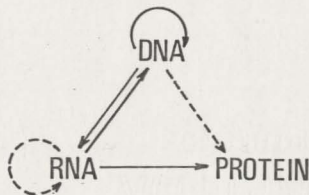
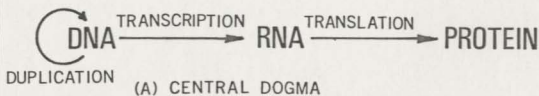
IV. Speculations on the Possible Functions of the RNA-Directed DNA Polymerase in Normal Mammalian Cells

Although the *Central Dogma* of molecular biology proposed by Crick (307) in 1958 stated that "once information has passed into protein it cannot get out again", many molecular biologists felt also that the transfer of information from RNA to DNA violated this dogma. On the basis of the gross chemical composition of *E. coli* (1% DNA, 6% RNA, and 15% protein) (308), they intuitively visualized the information transfer processes in cells as depicted in Scheme XIII (A) [modified from Watson (209)]. Hence, the initial indirect evidence (209) for a reversal of the transcription process was not widely accepted. However, the discovery of the RNA-directed DNA polymerase in oncogenic RNA viruses (20, 21) raised serious questions with regard to the concept of unidirectional information transfer. This led Crick (310) to clarify what the *Central Dogma* originally stated. In addition, he reclassified the types of information transfer that may or may not occur in biological systems, as follows (310):

<u>General Transfers</u>	<u>Special Transfers</u>	<u>Unknown Transfers</u>
DNA \longrightarrow DNA	RNA \longrightarrow RNA	Protein \rightarrow RNA
DNA \longrightarrow RNA	DNA \longrightarrow Protein	Protein \rightarrow DNA
RNA \longrightarrow Protein	RNA \longrightarrow DNA	Protein \rightarrow Protein

The *General Transfers* constitute modes of information transfer that occur in all cells. *Special Transfers* are those that do not occur in most cells, but may occur under special circumstances, and *Unknown Transfers* constitute those that have never been shown to occur.

The widespread occurrence of RD-DP [as supported by reports in the literature (22-24, 235, 236, 238-243, 281) and this present work], which



(B) REVISED CENTRAL DOGMA

SCHEME XIII

Central Dogma of Molecular Biology

In this scheme, the solid arrows indicate *General Transfers* and the broken arrows *Special Transfers*. The *Unknown Transfers* remain as suggested by Crick (310) [see text, from Moranelli, (281)].

has recently become apparent, suggests that the information transfer from RNA to DNA no longer constitutes a *Special Transfer* but rather a *General Transfer*. Hence, a further revision of the *Central Dogma* is in order since information transfer from RNA to DNA seems to have a much more general distribution than assumed by Crick (310). Thus, we might now represent the *Central Dogma* as depicted in Scheme XIII (B).

The revised version of the *Central Dogma* suggests that the function of the RD-DP is to transfer information from RNA to DNA. The number of biological processes in which this information transfer may occur are numerous, although any participation by RD-DP in such functions is at present largely speculative. The suggested processes include differentiation during embryonic development (22, 158, 186, 189, 311), the specialization of immunocompetent cells for the production of specific antibodies (218) and possibly the process of memory (219, 220).

The role of RD-DP in the amplification of rRNA genes in *Xenopus* oocytes has already been suggested by the observations of a number of workers (212-217) and has been discussed above [see Literature Review, Section III E(f)] although their evidence is at present, still questionable (250). A similar function in the amplification of ribosomal genes in human liver cells, after treatment with 3,3',5-L-triiodothyronine (312), and in the regenerating lens of *Triturus* (313) may also occur.

A function in the transfer of information from one cell to another, as in the case of the transfer of showdomycin-resistance in *E. coli* (232) is another possible process in which RD-DP may participate. Finally, such an activity may possibly function in DNA replication, as suggested by Loeb *et al.* (227), although at this time there is no evidence whatsoever

for RNA acting as a template in this process.

Arguments in favor of the appearance of RNA prior to the manifestation of DNA during the course of evolution have been presented (314). If this were the case, the importance of the RD-DP in the process of evolution would be evident.

Although any biological function assigned to the RD-DP is at this time purely speculative, this enzyme may yet prove to be very versatile in its biological function. The widespread distribution of the activity, and the apparently unique nature of the enzyme(s) involved in catalyzing the RD-DP reaction, support this conclusion.

APPENDIX I

Relationship Between Density and Refractive Index
of Guanidinium·Cl-CsCl Solutions

The relationship between density and refractive index can be expressed by the equation for a straight line:

$$\begin{aligned}
 y &= ax - b \\
 \text{or} \quad \rho &= a \eta_D^{25} - b
 \end{aligned}
 \tag{1}$$

where "a" is the slope of the line, "b" is a constant, η_D^{25} is the refractive index, and ρ the density. The slope (a) as determined from a plot of ρ vs η_D^{25} (Fig. 57) was found to be 24.4 and the constant "b" is equal to 33.0.

Using these values for "a" and "b", one can convert the refractive index measurements (η_D^{25}) determined with an Abbe Refractometer, to the corresponding density using equation (1).

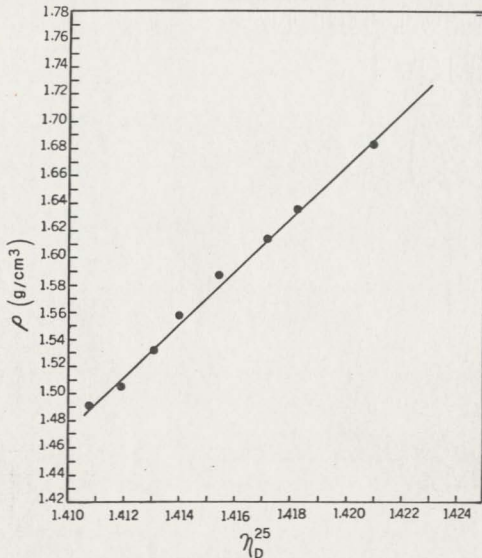


FIGURE 57. Relationship Between Density and Refractive Index of Guanidinium·Cl-CsCl Solutions.

A buoyant density gradient of guanidinium·Cl-CsCl solution was prepared as described in *Methods* except that the sample was substituted with 277 μl of distilled water. The refractive index of each fraction was read at 25° C using an Abbe Refractometer, and densities (g/cm^3) were determined by weighing 100 μl aliquots on an analytical balance.

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PUBLICATIONS ARISING FROM THIS WORK

1. Moranelli, F. and Penner, P.E. (1975) "Properties of a Low Molecular Weight Cytoplasmic DNA Polymerase", *Proc. Can. Fed. Biol. Soc.* 18, 639.
2. Moranelli, F. and Penner, P.E. (1975) "DNA Polymerase Activity Associated with Endogenous Template: Release by Ribonuclease Treatment", *Can. J. Biochem.* 53, 574-582.

