PURIFICATION OF THE DNA POLYMERASE OF AVIAN MYELOBLASTOSIS VIRUS

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SUMMARY

DNA polymerase from avian myeloblastosis virus has been purified by a combination of column chromatography and gel filtration methods. The isolated enzyme sediments at approximately 6 S and consists of two subunits of molecular weights 110 000 and 69 000. It is free of RNA and DNA endonuclease activity. The enzyme possesses the RNA-, DNA-, and hybrid-directed polymerase activities found in the virion.

INTRODUCTION

The discovery[1,2] of a ribonuclease-sensitive DNA polymerase activity in oncogenic RNA viruses was quickly extended[3-7] to a wide variety of oncornaviruses[8]. It was further shown that the product DNA was complementary to the RNA of the virion used as the source of the enzyme preparation[3,9-11]. These findings were promptly followed by experiments that established the existence in these viruses of polymerase activities that respond to double-stranded DNA[9,12-15] and, with a very high efficiency, to synthetic homopolymeric duplexes[13,16] composed of polyribonucleotides polydeoxyribonucleotides, and hybrid structures of the two. The size of the DNA product synthesized was generally much less than that of the template employed[3,4,9,11].

In addition to the DNA polymerase activities, evidence was also found for DNA endo- and exonucleases[13,17], ligase[17], and a nucleosidetriphosphate phosphotransferase[18] in the virion.

Purification of the RNA dependent DNA polymerase is a necessary prerequisite to an unambiguous analysis of the reaction mechanism. Purity is also required to delineate the relation of this polymerase activity to the others observed with DNA and synthetic homopolymeric duplexes as templates. Finally, pure enzyme should permit a decision on whether the nuclease, ligase and phosphotransferase activities are collectively or individually inherent and necessary components of the DNA polymerase function.

We report here the purification and characterization of the DNA polymerase activity from avian myeloblastosis virus.

Abbreviations: AMV, avian myeloblastosis virus; BBOT, 2,5-bis-2-(5-*tert.*-butylbenzoxazolyl)thiophene.

MATERIALS

Whatman microgranular DEAE-cellulose, DE52, 1.0 mequiv/g dry weight, and phosphocellulose, P11, 7.4 mequiv/g dry weight were obtained from Reeve Angel. Sephadex G-200 and CM-Sephadex C-50 were purchased from Pharmacia Fine Chemicals. Clarkson Chemical Company provided hydroxylapatite. Unlabeled nucleoside triphosphates and dithiothreitol came from P–L Biochemicals. Miles Laboratories supplied polynucleotides and *Micrococcus lysodeikticus* DNA. Tritiated nucleoside triphosphates were obtained from New England Nuclear, Schwarz BioResearch, and Amersham-Searle. Nucleic acid polymers were the generous gifts of Drs. F. Bollum (University of Kentucky), A. N. Nussbaum (Hoffman-LaRoche), and L. A. Underkofler (Miles Laboratories). Acrylamide and methylene bisacrylamide came from Bio-Rad Laboratories. Nonidet P-40 was a product of Shell Chemical Co.

METHODS

(1) Purification of avian myeloblastosis virus

Avian myeloblastosis virus (AMV), BAI strain A, was obtained by methods previously described[19] from the blood of chicks in the terminal stage of myeloblastic leukemia[20] and from infected myeloblasts suspended in tissue culture. Virus from blood plasma was purified essentially as described by CARNEGIE et al.[21]. As a final step, the virus suspension was sedimented at 27 000 rev./min in the Spinco SW27 rotor through 12 ml of 20 % glycerol in 0.01 M Tris–HCl (pH 8.5), 0.15 M NaCl, 1 mM EDTA (Tris–NaCl–EDTA buffer) onto a 6-ml pad of glycerol. The virus was removed from the pad, suspended in the same buffer without glycerol, and stored at −70°.

Myeloblastosis virus produced in tissue culture was supplied by Dr. J. W. Beard, Duke University. The culture fluid had been concentrated 50-fold by centrifugation and contained from $2.5 \cdot 10^{12}$ to $5 \cdot 10^{12}$ virus particles per ml[22]. After centrifugation at $3000 \times g$ for 10 min, the virus was concentrated against a 6-ml pad of glycerol at 27 000 rev./min for 1 h in the Spinco SW 27 rotor. Further purification was as previously described[3].

(2) Isolation of AMV RNA

Purified virus from blood plasma was lysed by adding sodium dodecyl sulfate to 0.5 %. The suspension was extracted twice with phenol–cresol solution (prepared according to KIRBY[23] and equilibrated with Tris–NaCl–EDTA buffer) and the RNA was precipitated by addition of 0.1 vol. of 3 M NaCl and 2 vol. of 95 % ethanol. After a second alcohol precipitation, the RNA was layered onto a 10 to 30 % glycerol gradient containing 0.01 M Tris–HCl (pH 7.4), 0.1 M NaCl, and 1 mM EDTA in the Spinco SW 41 rotor. After centrifugation at 41 000 rev./min and 5° for 3 h, fractions were collected dropwise from the bottom of the tube and those containing the 70-S RNA component were pooled and alcohol precipitated.

(3) Preparation of polynucleotide duplexes

Polynucleotide duplexes were formed by annealing equimolar amounts of two complementary homopolymers at concentrations of approximately 100 µg/ml each in 0.01 M Tris–HCl (pH 7.4), 0.2 M NaCl at room temperature for 15 min.

(4) Polyacrylamide gel electrophoresis of proteins

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed by a modification of the method of SHAPIRO et al.[24].

Protein samples were precipitated with an equal volume of 10 % trichloroacetic acid, allowed to stand at 0° for 15 min, and centrifuged at 16 000 × g for 30 min. Recovery was greater than 95 %. The pellet was thoroughly drained, and the precipitated protein was dissolved in 25–50 µl of 0.01 M sodium phosphate (pH 7.8), 1 % sodium dodecyl sulfate, 1 % 2-mercaptoethanol. After 30 min at 60°, glycerol was added to 10 % and the sample was layered onto the gel. Gels contained 5 % acrylamide, 0.25 % methylene bisacrylamide, 0.1 % sodium dodecyl sulfate, and 0.1 M sodium phosphate (pH 7.8). Electrophoresis was performed at 10 mA per gel for 15 min and then at 15 mA per gel for 75 min.

Gels were stained for 2 h in a 0.25 % solution of Coomassie brilliant blue in 10 % acetic acid, 50 % methanol. They were destained by diffusion in 7 % acetic acid, 5 % methanol and stored in the same solvent.

Molecular weights of polypeptide chains were determined as described by WOLF et al.[25] using as molecular weight markers polymers of ribonuclease A prepared ith diethyl pyrocarbonate. Electrophoresis of proteins at pH 8.9 in Tris–glycine buffer was performed as described by DAVIS[26].

(5) Protein determination

Protein was measured by the method of LOWRY et al.[27] using crystalline bovine albumin (Fraction V) as standard.

(6) Polymerase assay

The assay mixture for homopolymer templated reactions (total volume 0.1 ml) contained the following in µmoles: Tris–HCl (pH 8.3), 5.0; MgCl$_2$, 0.6; 0.02 each of the required labeled and unlabeled deoxynucleoside triphosphates; and double-stranded homopolymer template, $1.2 \cdot 10^3$ pmoles polymer phosphate in each strand. Reactions were incubated at 37° for 10 min and terminated by the addition of cold 5 % trichloroacetic acid.

After 10 min, the acid-precipitable radioactivity was collected on nitrocellulose filters and counted in 0.4 % 2,5-bis-2-(5-*tert*.-butylbenzoxazolyl)thiophene(BBOT) in toluene.

Assays using natural RNA and DNA templates were prepared identically except that they contained 0.02 µmoles each of three unlabeled nucleoside triphosphates and 4 nmoles of the fourth labeled triphosphate. Templates were used at levels from 1 to 2 µg per 0.1 ml assay.

Specific activities of the ³H-labeled triphosphates were 35–50 counts/min per pmole for homopolymer-templated reactions and 350–500 counts/min per pmole for those using natural RNA or DNA templates.

(7) Preparation of AMV DNA polymerase

The procedure is described for 60 mg of purified viral protein. Larger amounts have been handled successfully by scaling up the various steps proportionately.

12 ml of AMV (5 mg/ml in 0.01 M Tris–HCl (pH 8.5), 0.15 M NaCl, 1 mM EDTA) were mixed in order with 1.2 ml Nonidet P-40, 1.2 ml 10 % sodium deoxy-

cholate, and 3.6 ml 4 M KCl until homogeneous. The mixture was kept at 0° for 15 min and then was centrifuged at 16 000 × *g* for 10 min. The pellet was discarded and the supernatant diluted to 10 times its volume with 0.01 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, 10 % glycerol.

The solution was applied to a 1.2 cm × 11.0 cm column of DEAE-cellulose carefully equilibrated with the same buffer. The column was washed with 80 ml of 0.05 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, 10 % glycerol, and eluted with 40 ml 0.3 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, 10 % glycerol. The flow rate was about 36 ml/h.

The peak activity fractions from the DEAE-cellulose column were pooled and diluted to 3 times their volume with 0.01 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10 % glycerol. The material was loaded onto a 0.9 cm × 8.0 cm column of CM-Sephadex C-50 previously equilibrated with the same buffer. The column was washed with 8 ml of 0.1 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10 % glycerol and eluted with 12 ml of 0.3 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10 % glycerol. A flow rate of 15 ml/h was maintained. The peak fractions were pooled, glycerol was added to 50 %, and the enzyme stored at −20°.

(8) Phosphocellulose column chromatography of AMV DNA polymerase

The peak fractions from a DEAE-cellulose column (about 5 mg protein) were pooled and diluted 6-fold with 0.01 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10 % glycerol and applied to a 0.9 cm × 9.0 cm column of phosphocellulose equilibrated with the same buffer.

The column was eluted with a 150-ml gradient from 0.05 M potassium phosphate (pH 8.0) to 0.5 M potassium phosphate (pH 8.0) containing 2 mM dithiothreitol and 10 % glycerol. The flow rate was maintained at about 0.4 ml/min and about 1.5-ml fractions were collected.

(9) Hydroxylapatite column chromatography of AMV DNA polymerase

The peak fractions from a phosphocellulose column were pooled and diluted 5-fold with 0.01 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, 10 % glycerol and loaded onto a 0.9 cm × 9.0 cm column of hydroxylapatite equilibrated with the same buffer.

The column was eluted with a 150-ml gradient from 0.05 to 0.5 M potassium phosphate (pH 7.2) containing 2 mM dithiothreitol and 10 % glycerol. The flow rate was maintained at about 0.2 ml/min and about 2.0-ml fractions were collected.

(10) DNA cellulose chromatography of AMV DNA polymerase

DNA cellulose was prepared essentially as described by ALBERTS AND HERRICK[28]. Clean cellulose (Munktell 410) was washed several times with boiling ethanol and distilled water to remove remaining pyridine. It was then pre-cycled with base and acid (0.1 M NaOH, water, 0.01 M HCl) and washed to neutrality with water. The cellulose was then thoroughly dried, first in air and then by lyophilization.

Calf thymus DNA was dissolved in 0.01 M Tris-HCl (pH 7.4), 1 mM EDTA at a concentration of 1 mg/ml. The DNA solution was poured into petri dishes and mixed with the cellulose to form a slurry (approximately 1 g cellulose to 3 ml DNA solution). The material was extensively air dried, ground to a powder, and lyophi-

lized. Slow, complete drying seems to be essential for good adsorption of the DNA. The powder was resuspended in 0.01 M Tris–HCl (pH 7.4), 1 mM EDTA, 0.15 M NaCl, washed twice with the same buffer, and checked for DNA adsorption by measuring optically the amount of DNA released by boiling. About 30–40 % of the input DNA was taken up by the cellulose.

For chromatography of AMV DNA polymerase, a 0.5 cm × 10 cm column of DNA cellulose was exhaustively equilibrated with 0.01 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10 % glycerol. Approximately 60 µg of AMV DNA polymerase (phosphocellulose fraction) was applied to the column in about 0.03 M potassium phosphate buffer. The column was eluted with a 32-ml linear gradient from 0.01 to 0.5 M potassium phosphate (pH 8.0) containing 2 mM dithiothreitol, 10 % glycerol. The flow rate was maintained at 8 ml/h and 0.5 ml fractions were collected.

(11) Glycerol gradient centrifugation of AMV DNA polymerase

AMV DNA polymerase (phosphocellulose fraction, approximately 0.7 mg) was layered over a 10 to 30 % (v/v) glycerol gradient in 0.2 M potassium phosphate (pH 8.0), 2 mM dithiothreitol in the Spinco SW 50.1 rotor. Bovine serum albumin was run on a parallel gradient to serve as marker. The proteins were sedimented at 50 000 rev./min and 1° for 9.5 h and 10-drop fractions were collected dropwise from the bottoms of the tubes through a 20-G needle.

(12) Assay of contaminating nuclease activities

Ribonuclease activity in the CM-Sephadex enzyme was measured by following the breakdown of ^{3}H-labeled *Escherichia coli* 4-S and 5-S RNA on polyacrylamide gels. The RNA (15 µg) and enzyme (0.35 µg) were incubated in 0.025 ml of the standard assay mixture lacking deoxyriboside triphosphates and template. After 0 min and 60 min of incubation at 37°, sodium dodecyl sulfate was added to 1 %, and the samples subjected to electrophoresis on polyacrylamide gels as described by BISHOP *et al.*[29]. The gels were frozen, cut into 1-mm slices, dried on filter paper strips, and counted in 0.4 % BBOT in toluene.

Deoxyribonuclease activity was measured by following the breakdown of ^{3}H-labeled *E. coli* DNA by alkaline sucrose gradient centrifugation.

Two standard reaction mixtures were prepared omitting the deoxyribonucleotides and including the labeled DNA (approximately 0.30 µg). Purified AMV DNA polymerase (1.4 µg) was added to one, and both were incubated at 37°. After 30 min, EDTA was added to 5 mM and sodium dodecyl sulfate to 0.5 %, and the samples were layered onto 5 to 20 % sucrose gradients containing 0.1 M NaOH, 0.9 M NaCl, and 1 mM EDTA in the Spinco SW 50.1 rotor. After centrifugation at 50 000 rev./min for 3 h at 10°, fractions were collected dropwise from the bottom of the tube and precipitated with trichloroacetic acid. Insoluble material was collected on nitrocellulose filters and counted in BBOT–toluene scintillation fluid.

RESULTS

Extraction of the DNA polymerase from virions

Solubilization of the AMV DNA polymerase was effected by treatment of the

virus particles with detergent (0.7 % deoxycholate; 7 % Nonidet P-40) and salt (0.8 M KCl) at 0°. Glycerol gradient analysis in 0.1 M potassium phosphate showed that over 95 % of the enzyme activity sedimented at 8.4 S or less after treatment. Assays were performed using poly (rA) · poly (rU), AMV 70-S RNA, and *M. lyso-deikticus* DNA as templates. Specific activities and recoveries were determined with poly (rA) · poly (rU). Lower concentrations of detergent or salt, while capable of releasing the enzyme from the virion, left much of the activity attached to material that sedimented at higher *s* values. After release the enzyme showed an absolute requirement for added template.

The extract was centrifuged at low speed to remove a small amount of material that reduced the flow rate of the DEAE-cellulose column. The pellet contained a negligible amount (< 3 %) of the activity.

Chromatography of AMV DNA polymerase on DEAE-cellulose

The solubilized enzyme was diluted 10-fold to reduce the concentrations of salt and detergent and loaded onto a column of DEAE-cellulose. The column was then exhaustively washed with 0.05 M potassium phosphate buffer, which removes the detergents and much of the protein. Virtually all the activity was retained throughout loading and washing. The enzyme was then eluted with 0.3 M potassium phosphate buffer. About 5 % of the protein is recovered from the column, together with most of the activity.

Fig. 1 shows a DEAE-cellulose column assayed, respectively, with three different templates: the homopolymer duplex poly (rA) · poly (rU), 70-S AMV RNA, and *M. lysodeikticus* DNA. With each template, the activity is found generally to coincide with the protein peak. The slight displacement of activity observed with various templates appears to be due to their differential sensitivity to contaminating proteins and salts.

Generally, greater than 90 % of the starting activity is eluted with the 0.3 M potassium phosphate. The DEAE-cellulose column step yields about a 20-fold enrichment of the enzyme.

CM-Sephadex chromatography of AMV DNA polymerase

The peak activity fractions from the DEAE-cellulose column were pooled, diluted 3-fold with low salt buffer, and loaded onto a column of CM-Sephadex. The column was washed with 0.1 M potassium phosphate and eluted with 0.3 M potassium phosphate. All of the enzyme activity is retained by the column during loading and washing. Fig. 2 shows the profiles obtained by assaying the column with three different templates. About 0.5–1 % of the total protein is eluted with the 0.3 M buffer.

The degree of purification after CM-Sephadex chromatography and the amount of activity recovered have varied with different batches of virus, but highly reproducible results are obtained when the same starting material is used. In every case, the column effectively removes all the acidic protein contaminants as measured by polyacrylamide gel electrophoresis at pH 8.9. Preparations using different batches of virus produced in tissue culture have yielded enzyme of 30–60-fold higher specific activity than the crude extract. Variability in yield is probably due to the instability of the enzyme. The history of the virus preparation may be of paramount importance

in obtaining high yields and specific activities. It appears that certain treatments (*e.g.* multiple freezing and thawing) adversely affect the stability of the enzyme.

An actual enzyme preparation carried through the CM-Sephadex step is summarized in Table I.

The CM-Sephadex enzyme was assayed as described in METHODS for RNA and DNA endonuclease activities. As can be seen in Figs. 3 and 4, no detectable break-
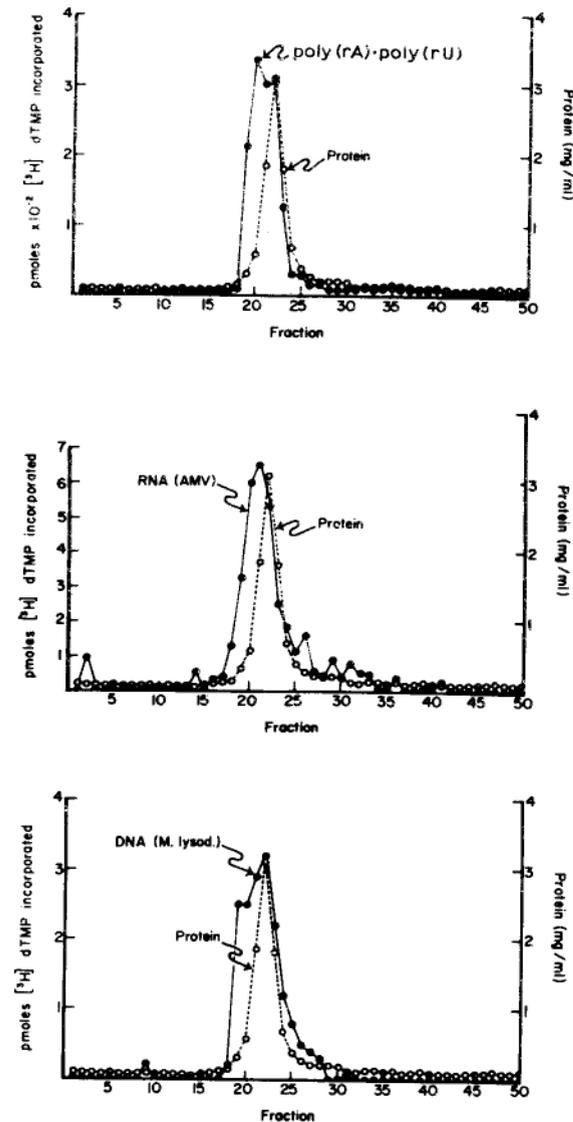
Fig. 1. DEAE-cellulose chromatography of AMV DNA polymerase. Avian myeloblastosis virus (600 mg) was solubilized as described in METHODS and chromatographed on a 2.5 cm × 25 cm column. Fractions of 4.4 ml were collected from the 0.3 M phosphate eluent. Assays using poly (rA) · poly (rU), AMV 70-S RNA, and *M. lysodeikticus* DNA were performed using 2 μl, 5 μl and 5 μl, respectively, from each fraction.

down of the nucleic acids occurred when incubated with purified enzyme under standard conditions, minus the deoxyriboside triphosphates.

Enzyme stored in 50 % glycerol at −20° has retained greater than 90 % of its activity for more than 5 weeks.
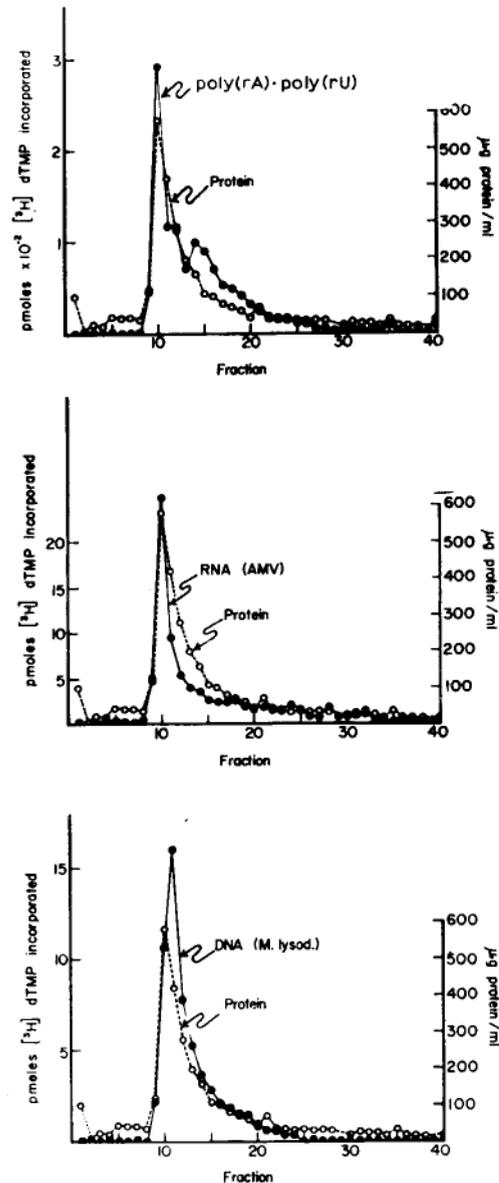
Fig. 2. CM-Sephadex chromatography of AMV DNA polymerase. The peak fractions (19–24) of the DEAE–cellulose column shown in Fig. 1 were pooled and chromatographed on a 1.5 cm × 17.5 cm column. Fractions of 1.5 ml were collected from the 0.3 M phosphate eluent. 1 μl, 5 μl, and 5 μl were used, respectively, to assay activity with poly (rA) · poly (rU), AMV RNA, and DNA.

TABLE I

PURIFICATION OF DNA POLYMERASE FROM AVIAN MYELOBLASTOSIS VIRUS

Assayed in the standard assay mixture using poly (rA) · poly (rU) as template. Specific activity expressed as pmoles dTMP incorporated per 10 min per μg of protein.

Fraction	Total protein (mg)	Specific activity (units/μg)	Total activity (units)	Yield (%)
(1) Solubilized virus	155.4	26.4	$4.1 \cdot 10^6$	100
(2) DEAE-cellulose column pool	8.0	487.5	$3.9 \cdot 10^6$	95
(3) CM-Sephadex pool	1.5	909.0	$1.4 \cdot 10^6$	34
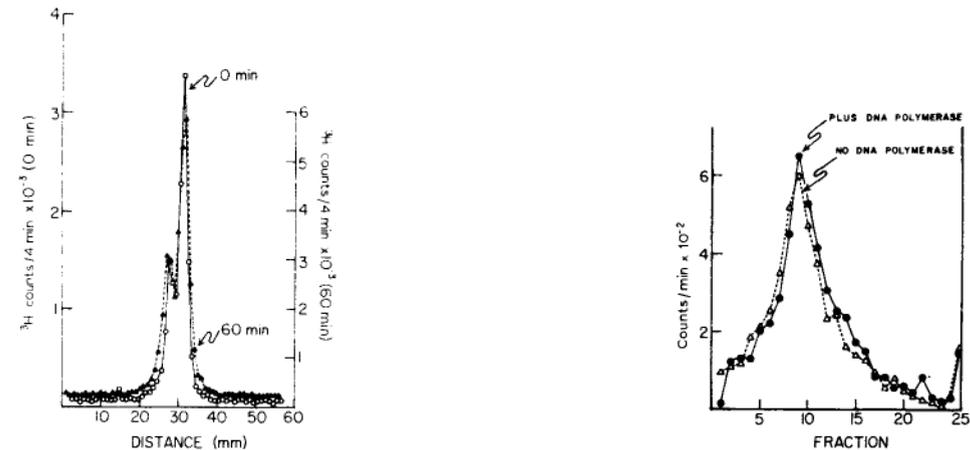
Fig. 3. Assay of ribonuclease activity in CM-Sephadex enzyme. ^{3}H-labeled *E. coli* 4-S and 5-S RNA (30 000 disint./min per μg) were incubated with CM-Sephadex enzyme as described in METHODS and resolved on 4.8 % pre-swollen polyacrylamide gels at 10 mA per gel for 60 min.

Fig. 4. Assay of deoxyribonuclease activity in CM-Sephadex enzyme. ^{3}H-labeled *E. coli* DNA (69 000 disint./min per μg) was incubated with CM-Sephadex enzyme as described in METHODS and examined by alkaline sucrose density gradient centrifugation.

Evidence for two components in the AMV DNA polymerase

Peak fractions from several columns were pooled, denatured with sodium dodecyl sulfate and mercaptoethanol and analyzed on 5 % sodium dodecyl sulfate-polyacrylamide gels. Fig. 5 shows the band patterns at various stages of purification. After DEAE cellulose chromatography there are still over 25 different polypeptide chains present. The second gel reveals that after CM-Sephadex two bands become very prominent suggesting that they may be responsible for enzyme activity. This is further supported by the third gel of material passed through a Sephadex G-200 column. Fig. 6 shows the activity profile from the Sephadex G-200 column and shows that the enzyme is separated from smaller ultraviolet-absorbing material.

It is of interest to note (Table II) that the Sephadex G-200 protein, which shows only two bands, retains the ability to respond to all the templates that were active with the crude detergent-disrupted virion preparations. Too much weight should not be given to the relative responses to the synthetic templates since they vary from one polymer preparation to another.
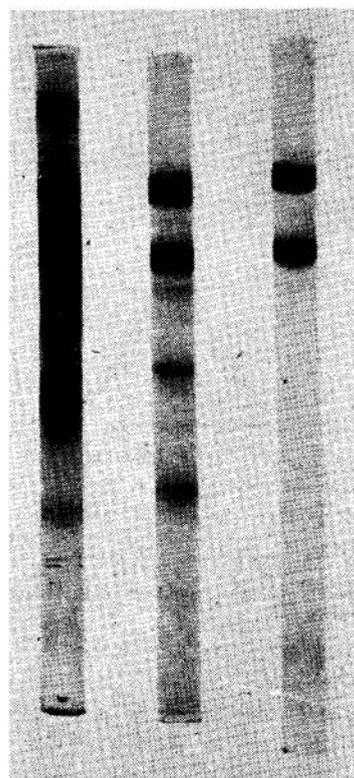
Fig. 5. Sodium dodecyl sulfate–polyacrylamide gels of DEAE-cellulose, CM-Sephadex and Sephadex G-200 enzyme fractions. Sodium dodecyl sulfate gels of AMV DNA polymerase were run after purification by DEAE-cellulose chromatography (left), CM-Sephadex chromatography (middle), and Sephadex G-200 chromatography. They contain, respectively, 32, 30, and 28 μg of protein.

Fig. 6. Sephadex G-200 chromatography of AMV DNA polymerase. The peak fractions (475 μg protein in 1 ml) from a CM-Sephadex column were pooled and loaded onto a 0.9 cm × 54 cm column of Sephadex G-200 equilibrated with 0.3 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 5 % glycerol. The column was eluted at a flow rate of 7 ml/h and 0.6 ml fractions were collected. 10-μl aliquots from each fraction were used to assay with each template.

To gain further information on the association of the two principal bands with the polymerase activity, the behavior of the protein on other columns was examined.
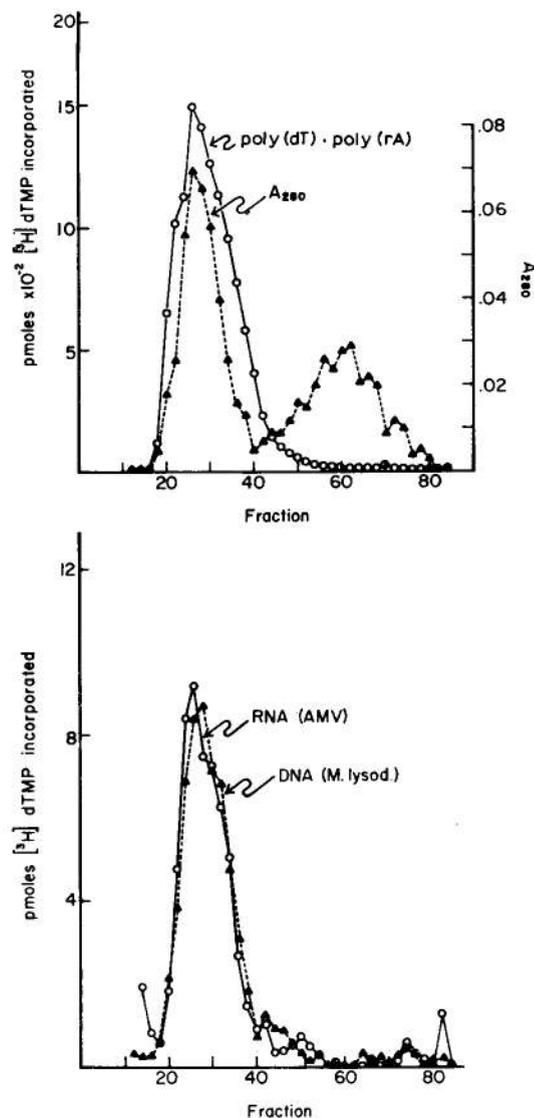
TABLE II

ACTIVITY OF PURIFIED AMV DNA POLYMERASE WITH VARIOUS TEMPLATES

Assays were performed as described in METHODS except that all reactions were incubated at 37° for 10 min. Each contained 0.6 μg of Sephadex G-200 enzyme. [^{3}H]dTTP was used in all reactions except those with poly (dC) · poly (dG) and poly (rI) · poly (rC) where the label was in dGTP. Homopolymer duplex templated reactions contained 1.2 · 10^3 pmoles polymer phosphate in each strand. AMV RNA and *M. lysodeikticus* DNA were present at 1.2 μg and 2 μg per reaction, respectively.

Template	Labeled base incorporated (pmoles)
Poly (dT) · poly (rA)	1294.6
AMV RNA	8.9
M. lysodeikticus DNA	7.6
Poly (dC) · poly (dG)	235.6
Poly (rA) · poly (rU)	440.5
Poly (rI) · poly (rC)	311.4

To this end, the DEAE-cellulose enzyme was put on phosphocellulose instead of CM-Sephadex and eluted with a phosphate gradient. As shown in Fig. 7, the enzyme activity elutes as a single sharp peak. The inset on Fig. 7 shows the sodium dodecyl sulfate gel analysis of the peak region and reveals the same two principal bands along with some minor contaminants. Fractions 32–39 of the active region from Fig. 7 were pooled and chromatographed on hydroxylapatite. The elution profile of the activity with a phosphate gradient is shown in Fig. 8 along with the sodium dodecyl sulfate gel of the peak region. Here we see the two principal bands as virtually the only detectable components. Enzyme eluted from another phosphocellulose column was then separated by affinity chromatography on DNA cellulose as described in METHODS. Once again (Fig. 9) the enzyme was recovered in a single sharp peak which, when analyzed by gel electrophoresis, was seen to contain the same two polypeptide chains.
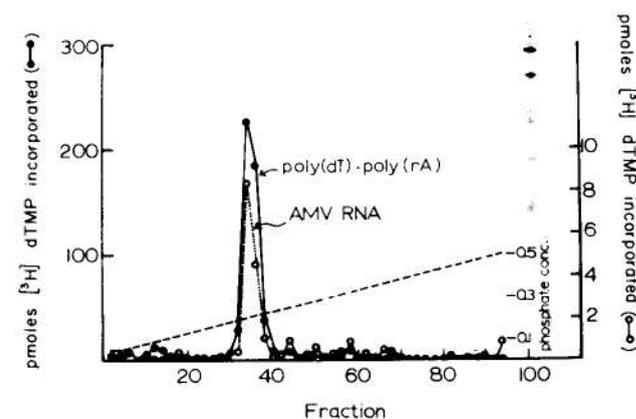
Fig. 7. Phosphocellulose column chromatography of AMV DNA polymerase. AMV DNA polymerase (DEAE-cellulose fraction) was chromatographed on phosphocellulose as described in METHODS. 2 μl from each fraction were used to assay poly (dT) · poly (rA) templated activity and 20 μl from each for AMV RNA activity. A sodium dodecyl sulfate–polyacrylamide gel of the peak fraction is shown.
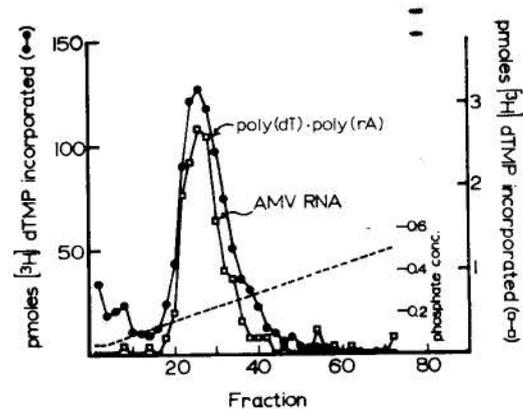
Fig. 8. Hydroxylapatite column chromatography of AMV DNA polymerase. AMV DNA polymerase (phosphocellulose fraction) was chromatographed on hydroxylapatite as described in METHODS. 10 μl from each fraction were used to assay poly (dT) · poly (rA) templated activity and 20 μl from each for for AMV RNA activity. A sodium dodecyl sulfate–polyacrylamide gel of Fractions 26–37 is shown. Fractions 18–25 gave an identical but considerably lighter pattern.
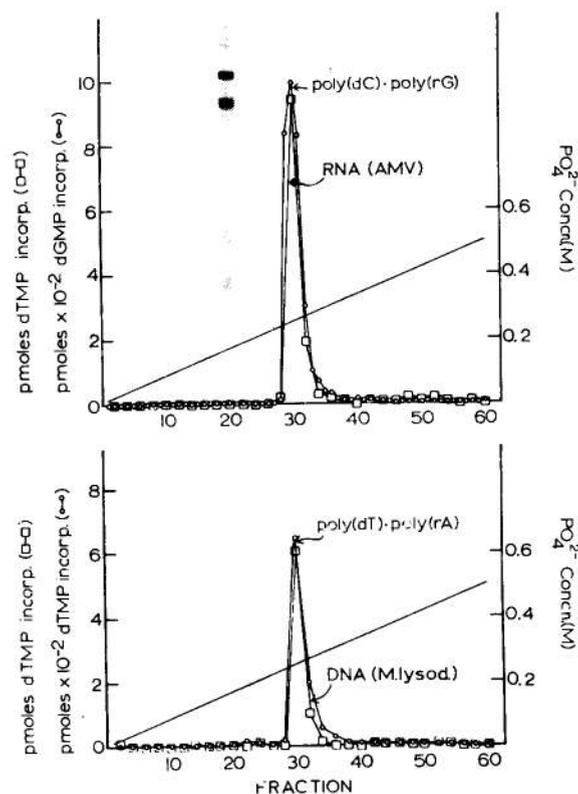
Fig. 9. DNA cellulose chromatography of AMV DNA polymerase. AMV DNA polymerase (phosphocellulose fraction) was chromatographed on DNA cellulose as described in METHODS. 10 μl from each fraction were used to assay for activity with each of the indicated templates. A sodium dodecyl sulfate–polyacrylamide gel of fractions 29–32 inclusive is shown.

Sedimentation of purified polymerase

The two components appear to behave as a single complex on a variety of columns. It was of obvious interest to see whether this held true in a sedimentation analysis. A phosphocellulose enzyme preparation was accordingly run on a glycerol gradient containing 0.2 M potassium phosphate as described in METHODS. Fig. 10 shows that the activity sediments as a sharp band with an $s_{20,w}$ of 6 S corresponding
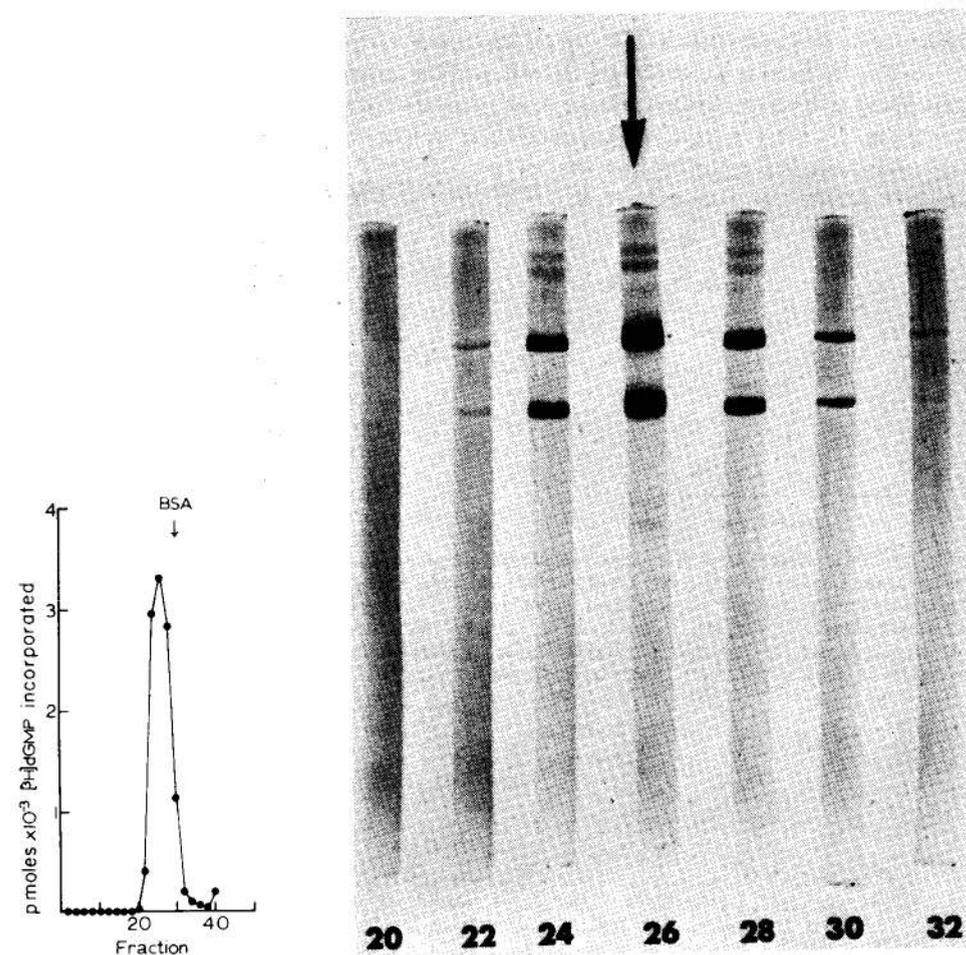
Fig. 10. Glycerol gradient centrifugation of AMV DNA polymerase. Centrifugation was performed as described in METHODS. Phosphocellulose enzyme was precipitated with an equal volume of saturated $(NH_4)_2SO_4$ (4°, pH adjusted to 7.4 with NH_4OH) suspended in 0.1 ml of buffer and layered onto the gradient. The activity was located using poly (dC) · poly (rG) as the assay template and 10 μl from each fraction. The polymerase activity sedimented at approximately 6 S calculated with reference to bovine serum albumin (BSA, arrow) run on a parallel gradient.

Fig. 11. Sodium dodecyl sulfate–polyacrylamide gels of glycerol gradient peak. The peak fractions 20, 22, 24, 26, 28, 30 and 32 from the glycerol gradient shown in Fig. 10 were each precipitated with trichloroacetic acid and analyzed by electrophoresis in sodium dodecyl sulfate gels as described in METHODS. The gels show that the polypeptide chains sediment together and that they coincide with the activity peak. The amount of protein recovered on each gel corresponds to the relative activity of the respective fractions. The arrow indicates the activity peak.

to a molecular weight of 110 000. It may be noted that a similar sedimentation value has been reported[30] for the DNA polymerase of the Rous sarcoma virus. The peak fractions (20–32 inclusive) were precipitated and analyzed electrophoretically in sodium dodecyl sulfate–acrylamide gels. As may be seen from Fig. 11, the same two bands are observed as the principal components in the peak region. It is evident that they are sufficiently tightly complexed to behave as a single physical entity under these conditions.

Mole ratios and molecular weights of the two components

The molecular weights were determined on sodium dodecyl sulfate–acrylamide gels using polymers of ribonuclease A as molecular weight markers. As may be seen from Fig. 12, the two chains have molecular weights of 110 000 and 69 000.

If these two polypeptides are in fact subunits of the same enzyme, then the relative number of each present in an enzyme unit should be proportional to their
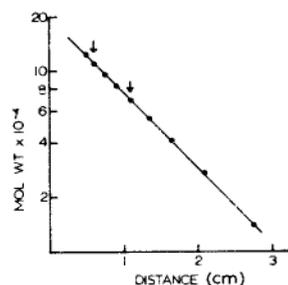
Fig. 12. Determination of molecular weights of subunits. The molecular weights of the polypeptide chains present in AMV DNA polymerase extracts were determined on sodium dodecyl sulfate–polyacrylamide gels as described in METHODS. The circles show the positions of polymers of ribonuclease A (mol. wt. 13 700) run as markers. The arrows mark the positions of the two major bands run on a parallel gel, indicating molecular weights of 110 000 and 69 000. Molecular weights of marker polymers range from 13 700 to 123 300.
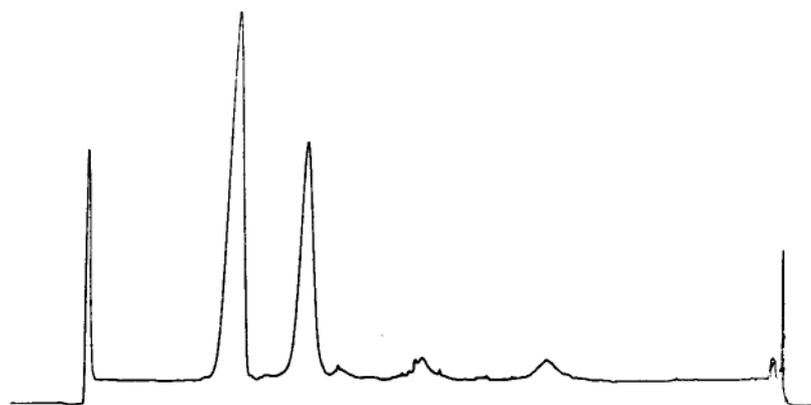
Fig. 13. Scan of sodium dodecyl sulfate–polyacrylamide gel of CM-Sephadex enzyme. Sodium dodecyl sulfate–polyacrylamide gels were scanned, using a 0.05 mm slit, in the Gilford Model 2400 spectrophotometer equipped with linear transport. The pattern shows that the enzyme is approximately 90 % pure assuming that all proteins are stained equally by Coomassie blue.

molecular weights. It has been shown[31] that the amount of Coomassie brilliant blue bound to various proteins differed by less than 10 %. By scanning stained sodium dodecyl sulfate gels, the amount of each protein present can be determined[32,33]. A typical scan is shown in Fig. 13 from which the ratio of the two components is readily obtained.

Gels containing various amounts of enzyme from different sources were scanned and measured with the results shown in Table III. It is evident that the weight ratio of the chains found in the various preparations is that expected if the two components are present in equal numbers.

TABLE III

WEIGHT RATIO OF CHAINS

Gels were scanned as described by BERG[32]. The area under each peak was estimated by multiplying the peak height by the peak width at half-height.

Gel No.	Enzyme preparation	110 000 mol. wt. polypeptide : 69 000 mol. wt. polypeptide
34	DEAE-cellulose enzyme	1.3
53	CM-Sephadex enzyme	1.9
63	CM-Sephadex enzyme	1.7
64	Sephadex G-200 enzyme	1.5
67	CM-Sephadex enzyme	1.5
69	Sephadex G-200 enzyme	1.8
75	Phosphocellulose enzyme	1.4
83	Hydroxylapatite enzyme	1.3
88	Glycerol gradient enzyme (Fraction 24, Fig. 11)	1.7
89	Glycerol gradient enzyme (Fraction 26, Fig. 11)	1.6
90	Glycerol gradient enzyme (Fraction 28, Fig. 11)	1.6

Average of 11 gels:	1.6
Expected value (assuming one of each polypeptide chain per enzyme molecule):	1.6

TABLE IV

REQUIREMENTS OF AMV DNA POLYMERASE REACTION

Complete system: 50 mM Tris–HCl, pH 8.3; 6 mM $MgCl_2$; 0.2 mM each dATP, dCTP, dGTP; 0.04 mM [^{3}H]dTTP, 665 counts/min per pmole; 0.7 μg AMV DNA polymerase, phosphocellulose fraction; 2 μg AMV RNA; 0.4 mM dithiothreitol; 100 mM KCl; incubated 20 min at 37°.

System	[^3H] dTTP incorporated (pmoles)
Complete	12.4
—$MgCl_2$	0.0
—$MgCl_2$ +0.6 mM $MnCl_2$	3.5
—dATP	0.4
—dCTP	0.3
—dGTP	0.5
—AMV RNA	0.1
—dithiothreitol	8.6
—KCl	5.3

Properties and requirements of the reaction with purified AMV DNA polymerase

The available information on the optimal conditions and requirements of RNA-dependent DNA polymerases have thus far been derived from studies with detergent-disrupted virion preparations. Table IV summarizes a reexamination with a purified AMV polymerase. It will be noted that the reaction is dependent on RNA, the presence of all four deoxyriboside triphosphates, and a divalent cation, Mg^{2+} being superior to Mn^{2+} over a wide range of concentrations. The addition of dithiothreitol and KCl lead to marked improvement. Fig. 14 shows that the effect of the dithiothreitol is most readily apparent in the later stages of prolonged syntheses.
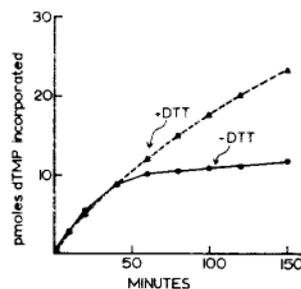
Fig. 14. Effect of dithiothreitol on AMV DNA polymerase activity. Reaction kinetics in the presence ($\triangle$) and absence ($\bullet$) of 0.4 mM dithiothreitol (DTT) are shown. The template is AMV 70-S RNA. The curves show that dithiothreitol is necessary for long-term synthesis. The exact time varies at which the reaction without dithiothreitol ceases.

DISCUSSION

The two-column procedure described for preparing purified DNA polymerase from avian myeloblastosis virus is a rapid and convenient procedure for obtaining reasonably pure enzyme in good yield. At the CM-Sephadex stage, enzyme from tissue culture virus is 90 % pure or better. Substitution of phosphocellulose for CM-Sephadex chromatography gives similar results. If one starts with virus isolated from the plasma of infected birds, enzyme of even higher purity is obtained at corresponding stages of purification. Removal of residual contamination can be achieved by $(NH_4)_2SO_4$ concentration followed by either Sephadex G-200 or hydroxylapatite chromatography, or centrifugation through glycerol gradients.

Two polypeptide chains are found to be associated with the activity in 1 : 1 ratio throughout purification by DEAE-cellulose, CM-Sephadex, Sephadex G-200, phosphocellulose, hydroxylapatite and DNA cellulose affinity chromatography as well as by glycerol gradient centrifugation. This suggests that they are subunits of the polymerase. However, proof that the two polypeptides are in fact subunits will require examination of the activities of the separated and reconstituted proteins. The possibility that the subunits may each have one or more of the activities associated with them individually cannot be ruled out at this time.

There is a 1.6-fold discrepancy between the molecular weight of the active form as determined by glycerol gradient centrifugation (110 000) and the sum of the molecular weights of the two polypeptide chains determined on sodium dodecyl sulfate–polyacrylamide gels (110 000 and 69 000). Assuming that the molecular

weight determinations are not in error, the fact that the ratio of the chains is constant across the activity peak (Table III) in the glycerol gradient suggests that the discrepancy is due to differences in molecular asymmetry of the subunits as compared with assembled proteins. A quantitatively similar situation obtains with $Q\beta$ replicase in which the sum of the molecular weights of the subunits determined on sodium dodecyl sulfate gels is 1.7 times that determined by sedimentation for the native enzyme[34,35].

The value obtained for the sedimentation coefficient of the enzyme would seem to exclude the possibility that the molecule contains more than one of each subunit.

It is worth emphasizing that association of the two major polypeptides, even through an extensive series of purification steps, does not constitute proof that they represent the desired enzyme. As with all such enzyme purifications, minor contaminants (*e.g.* 1 % or less) that might not be detectable could conceivably be responsible for the activity. We cannot prove rigorously at this time that the two principal polypeptide chains constantly observed in our active preparations compose the polymerase. However, in addition to their invariant presence and constant 1 : 1 ratio, several additional considerations argue for their being the enzyme components.

Throughout purification, there have been no minor bands that are consistently present in all preparations. The most frequently observed contaminant is a pair of bands representing approximately 4 % of the total protein. From their gel mobilities, they have molecular weights of 190 000 and 210 000 and therefore would be expected to sediment much more rapidly, either individually or as a complex, than the polymerase. They are not enriched relative to the two major bands during the course of purification of the activity. It seems likely that they represent aggregates of the major bands due to the relatively large amount of protein applied to the gels.

Were the activity due to any contaminant present as a few percent of the protein, the specific activity of the purified enzyme would be many-fold greater than that observed. The most active preparations of *E. coli* DNA polymerase[36] assayed with poly [d(A–T)], $Q\beta$ replicase[37] assayed with $Q\beta$ RNA, and *E. coli* transcriptase assayed with poly [d(A–T)][38] and calf thymus DNA[39] have specific activities*, respectively, of 1.2, 3.2, 4.5, and 1.2 moles nucleoside monophosphate incorporated per sec per mole enzyme. The most active preparations of AMV DNA polymerase incorporate 1.2 moles dXMP per sec per mole enzyme. This value is in excellent agreement with those for the other purified polymerases and argues that the principal protein components are responsible for activity. These calculations encompass both initiation and chain elongation. They are not based on any assumptions concerning the numbers of enzyme molecules participating, but rather on the reported enzyme specific activities.

If in fact the enzyme is essentially pure, the amount of protein recovered should correlate with the number of enzyme molecules expected per virion. It is reasonable to assume that there is at least one polymerase molecule per virus particle. Since the virion contains $3.0 \cdot 10^8$ daltons of protein[40] and the molecular weight of the AMV DNA polymerase is $1.8 \cdot 10^5$ daltons, one enzyme molecule per virion

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* The highest value obtained for enzyme fractions described as homogenous was used. The reported specific activities were converted from arbitrary units to moles of nucleoside monophosphate per sec per mole of enzyme.

would represent 0.06 % of the total protein. Normally, 0.3–1 % of the starting protein is recovered, which would correspond to 5–17 enzyme molecules per virus particle. Were the enzyme a 2 % contaminant of our preparation, each virion would contain only 0.1–0.3 polymerase molecule.

The availability of pure enzyme makes possible a re-examination of the nature of the intermediates and the final product uncomplicated by possibly irrelevant or interfering enzyme activities.

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