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# Reverse Transcriptase from Avian Myeloblastosis Virus

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From lots of 20 to 30 g of avian myeloblastosis virus RNA-dependent DNA polymerase was obtained in preparations of purity greater than 95% by using a two-step column chromatographic procedure employing DEAE (DE 52) and carboxymethylcellulose (CM 52). Yields of RNA-dependent DNA polymerase varied from approximately 20,000 to 35,000 U/g of virus. Specific activity of the enzyme was about 35,000 to 60,000 U/mg of protein. Free of detectable RNase activity, the product exhibited a molecular weight of about 160,000, an isoelectric point of 6.5, and approximately 2 mol of fatty acid per mol of enzyme.

Ready access to RNA-dependent DNA polymerase (RDDP) in relatively large amounts has greatly broadened the spectrum of biological and biochemical investigations feasible with the enzyme. Although avian myeloblastosis virus (AMV) is a convenient and, thus far, plentiful source of RDDP, continual efforts for improvement in the yield and specific activity of the enzyme are obviously desirable. Of serious concern, also, has been the hindrance to realization of the full enzyme potential for preparation of long complementary DNA chains due to traces of RNase, which is difficult to eliminate in large-scale RDDP purification.

Isolation of RDDP from various oncornaviruses has been reported (9, 11, 14, 15, 17). In earlier work with RDDP, there was much variation in the yield, relative purity, and specific activity of the enzyme preparations. Continued studies on the preparation of RDDP from AMV in this laboratory, however, have revealed a comparatively simple, two-step chromatographic procedure affording high yields of enzyme from large amounts of AMV. This report describes the methods of isolation and characterization of RDDP in preparations which were more than 95% pure and essentially free of RNase activity.

## MATERIALS AND METHODS

**Virus and chickens.** AMV was the BAI strain A derived initially (5) from a single bird. Tests by the interference technique (Victor Bergs, Life Sciences, Inc., St. Petersburg, Fla.) indicate that the agent in current use is a mixture of types A and B, with a predominance, usually, of type A virions (as much as 3:1). Leukemia was induced by intravenous inoculation into 1- or 2-day-old chicks of $10^{10}$ virus particles in 0.1-ml volumes of 1.5% bovine serum albumin-10% (wt/vol) LS 50 (a powder mixture of 2 parts of lincomycin hydrochloride and 1 part of spectinomycin sulfate tetrahydrate [Tuco, Div. of The Upjohn Co., Kalamazoo, Mich.]) in Simms solution (22). Birds used regularly since 1974 have been Hy-Line Brand variety W36, developed by Hy-Line International, Div. of pioneer Hy-Bred International, Inc., Johnson, Iowa, and obtained locally from Wallace Hatcheries, Dade City, Fla. With few exceptions, the chicks were males.

**Purification of virus.** All AMV used here or sent to other investigators was derived from the plasma of birds in the final stages of myeloblastosis. For 5 days after inoculation, the chicks were held in five-tiered brooders (model B735; Kuhl Poultry Equipment Co., Flemington, N.J.) and then transferred to single cages (model B73) on low racks. By inspection through hardware cloth cage tops, chicks at an appropriate stage of the disease (7 to 10 days post-inoculation) could be selected for bleeding. Frequent inspection was critical to avoid heavy losses, because affected birds succumbed quickly after onset of visible signs of illness.

Blood was drawn by heart puncture (5 to 10 ml per bird) into separate 12-ml centrifuge tubes containing 500 U of heparin (Scientific Protein Laboratories, Inc., Waunakee, Wis.) in 0.1 ml of calcium-free Ringer solution. Cells were sedimented by spinning the individual tubes at 3,000 × *g* for 10 min, and plasmas were pooled according to virus content as judged visually by cloudiness after passage through Schleicher and Schuell no. 588 fluted filter paper. The pooled plasma was clarified further by centrifugation for 10 min at 12,000 × *g* and passage through a double-layer Gelman glass filter (Gelman Laboratory Products, Ann Arbor, Mich.) of 1- (filter no. 66078) and 0.8-μm (filter no. 61630) porosities. Virus concentration in terms of numbers of particles per milliliter was estimated by adenosine triphosphatase activity (2). Virus was then sedimented in 50-ml plastic tubes by centrifugation at 59,000 × *g* for 40 min. The walls of the tubes were wiped free of lipid, and the pellets were stored at −80°C in 0.5-g (wet weight) virus amounts. In some instances, the plasma was frozen as such in a dry ice–alcohol bath in approximately 100-ml volumes in 250-ml plastic bottles. It should be emphasized that

the virus obtained here was derived from plasma which contained very little particulate material extraneous to the virus, such as cell debris or other constituents of fluids from tissue culture. In consequence, in this simple purification procedure, it was necessary only to wash away the soluble plasma components and there was no need to expose virus particles to damage by the hypertonicity of suspending media in gradient centrifugation (3).

**Purification of RDDP.** In routine isolation of RDDP, lots of about 20 to 30 g of AMV were purified through two or three cycles of alternate low- and high-speed centrifugation (3,000 and 59,000 × *g*, respectively) and suspended (13) in 10 mM Tris-hydrochloride (pH 8.1)–0.15 M NaCl–1 mM EDTA in concentrations of 20 to 50 mg/ml. Virions were disrupted by Triton X-100, deoxycholate, and KCl (13). The crude lysate was stirred for 30 min at 4°C, centrifuged for 10 min at 25,000 × *g*, and diluted 10-fold with 10 mM potassium phosphate buffer (pH 7.3) containing 0.2% Triton X-100, 2 mM dithiothreitol (DTT), and 10% glycerol. All phosphate buffers used in this procedure contained these additional reagents. RDDP in the diluted lysate was adsorbed to column (8 by 8 cm) of DEAE (DE 52) at a flow rate of 2.5 to 3 liters/h. The column was washed exhaustively with 10 mM potassium phosphate buffer (pH 7.3), and the enzyme was eluted from the column with 0.3 M potassium phosphate (pH 7.3).

The active fractions from the DE 52 column, which contained 5 to 10% of the protein from the lysate, were diluted 10-fold with 10 mM potassium phosphate and loaded onto a column (4.6 by 8 cm) of carboxymethylcellulose (CM 52) with a flow rate of 200 ml/h. After the column was developed with a linear gradient of 10 to 300 mM potassium phosphate (500 ml), alternate fractions (4.5 ml) were assayed for protein and RDDP. Active fractions from CM 52 chromatography were pooled, concentrated 2.5-fold by dialysis against 0.2 M potassium phosphate containing 2 mM DTT, 0.2% Triton X-100, and 50% glycerol, and stored at −20°C.

**Assay of RDDP.** Reaction mixtures for the assay of RDDP consisted of 50 mM Tris-hydrochloride, pH 8.3, 6 mM $MgCl_2$, 40 mM KCl, 100 μg of bovine serum albumin per ml, 0.5 mM [$^3$H]TTP (10 to 20 cpm/pmol), 0.2 mM polyriboadenylate:deoxythymidylate$_{12-18}$, and RDDP diluted sufficiently with 10 mM potassium phosphate (pH 7.3) to produce linear incorporation kinetics for at least 30 min. The acid-insoluble product of this reaction was collected on 1.5-cm GF/C filter disks and processed for detection of radioactivity by liquid scintillation spectrometry (2). One unit of enzyme is defined as that amount which incorporates one nanomole of TMP into an acid-insoluble product in 10 min at 37°C under these conditions.

**RNase assay.** Raji cells were grown for 24 h at 37°C in RPMI 1640 culture medium supplemented with 10% fetal bovine serum and 10 to 20 μCi of [$^3$H]uridine per ml. Cells were collected by centrifugation at 12,000 × *g* for 10 min and disrupted by suspension in 0.1 M sodium acetate (pH 5.0)–0.5 M NaCl–0.5% sodium lauryl sarkosinate. After phenol extraction and ethanol precipitation, 28S [$^3$H]RNA was isolated by rate zonal sedimentation. Gradient fractions containing 28S RNA were diluted threefold with acetate buffer, reextracted with phenol, precipitated with ethanol, and stored at −20°C.

For the RNase assay, 0.1-ml reaction mixtures containing 20 mM Tris-hydrochloride, pH 7.4, 0.15 mM NaCl, 1 to 5 μg of tritium-labeled 28S RNA (specific activity, about $2 \times 10^5$ cpm/μg), and 20 to 40 U of purified RDDP were incubated for 60 min at 37°C. This mixture was layered on top of a preformed, linear, 5.2-ml, 15 to 30% (wt/vol) sucrose gradient containing 0.1% sodium lauryl sarkosinate, 0.1 M NaCl, and 0.05 M sodium acetate, pH 5.0. Gradients were centrifuged at 40,000 rpm in an SW50.1 rotor for 5 h at 4°C and fractionated by collection of drops through a 23-gauge needle inserted into the bottom of the gradient. A 50-μl portion of each 30-drop sample was transferred to a 1.5-cm GF/C disk and processed for determination of acid-insoluble radioactivity (2).

**Polyacrylamide gel electrophoresis.** Isoelectric focusing was carried out in 9% polyacrylamide gels (6 by 70 mm) containing 1.7% carrier ampholytes (13). Dissociating gel electrophoresis was carried out in Tris-acetate-buffered sodium dodecyl sulfate (pH 6.4) as recommended by Bio-Rad Laboratories.

**Adsorbents.** DE 52 and CM 52 were obtained from Whatman, Inc., Clifton, N.J., and washed immediately before use. DE 52 (200 g) was batch washed in 2 liters of 0.2 NaOH in 50% ethanol. This was followed by washes with 4 liters of water to neutrality, then with 0.2 N HCl (2 liters) and, finally, with water to neutrality. CM 52 (100 g) was washed with 0.2 N HCl in ethanol, followed successively by washes with water 0.2 N NaOH, and water.

All of each adsorbent was used to pack the respective columns. It should be emphasized that each adsorbent was washed immediately before preparation of RDDP to avoid RNase contamination resulting from even slight microbial growth. Each adsorbent was discarded after use in a single preparation.

**Chemicals.** Nucleotides were obtained from either Miles Laboratories, Inc., Elkhart, Ind., or P-L Biochemicals, Milwaukee, Wis. New England Nuclear Corp., Boston, Mass., was the source of [$^3$H]TTP (47 Ci/mmol). GF/C paper for filter disks was from Whatman, Inc., Clifton, N.J. Reagents for disk gel electrophoresis were from E-C Apparatus Corp., St. Petersburg, Fla; carrier ampholytes were purchased from Bio-Rad Laboratories, Richmond, Calif.; and molecular-weight markers were from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. A Bio-Rad gel Pro-pHiler was used to measure the pH gradient in acrylamide gels.

## RESULTS

**Production of AMV.** Host response to AMV is dependent particularly on the genetic constitution (7) and age of the bird and on the dose and route of inoculation of the virus (6). Another factor observed in the use of large numbers of chicks in numerous serial passages was adaptation or variation in infectivity of the virus. Before access to Hy-Line Brand W36, Hy-Line 934 exhibited high response to AMV and produced virus in high concentrations in the plasma. In

contrast, in the beginning, W36 birds were less susceptible to infection with AMV, and the yield of virus for 2 years was relatively low. At present, however, the susceptibility to and yield of virus in these birds are very high after serial passages for more than 2 years at intervals of approximately 2 weeks. As an example, 287 chicks, chosen at random, yielded a total of 610 ml of plasma (2.13 ml/bird) with a virus concentration of $1.6 \times 10^{12}$ virions per ml, or a total of $976 \times 10^{12}$ virions.

The hydrated density of AMV measured in bovine serum albumin in physiological saline solution (21) is 1.059, and the degree of hydration in this medium is 80% by volume. From these data and a mean radius at 70 nm for the hydrated particles (mean sedimentation coefficient corrected to water at 20°C, 692) 1 g of virus (wet weight) comprises $2.66 \times 10^{14}$ virions. Thus, the yield of virus from 287 chicks was $976 \times 10^{12}$ virions or 3,660 mg (wet weight) of the agent, equivalent to 12.75 mg or $3.4 \times 10^{12}$ virus particles per bird. Inasmuch as $10^{10}$ particles were contained in the initiation inoculum, the yield per bird, $3.4 \times 10^{12}$ virions, represented an average increase of 340-fold.

An unexplained aspect of response was the distinct differences in birds reared on different local farms, despite a common genetic source. Incidence of myeloblastosis response, about 85% positive, was great in the Hy-Line W36 chicks, but, as noted, a large dose ($10^{10}$ virions) was necessary to produce this result.

**Yield and purity of RDDP.** Table 1 shows the results of fractionation of the virus lysate from 26 g (wet weight) of purified virus. Loss of activity was about 40%, in contrast to the elimination of more than 99% of the protein. The enzyme purification factor was about 246-fold, and the yield of transcriptase was 26,300 U/g of virus. Specific activity of the enzyme in this preparation was 56,400 U/mg of protein. Results observed with seven lots of 19 to 26 g of virus (Table 2) over a period of almost 1 year showed a variation of 23,100 to 35,100 enzyme units per g of virus and a specific activity of 35,400 to 56,700 U/mg of protein. All RDDP activity that eluted from ED 52 was adsorbed to CM 52, a weak cation exchanger, but there was minimal adsorption of nonspecific proteins. This efficiency of CM 52 is illustrated in Fig. 1A, where the major protein peak is coincident with RDDP activity. After densitometric tracing of a stained sodium dodecyl sulfate gel of concentrated material from this peak of activity (Fig. 1B), the enzyme was estimated to be more than 95% pure.

TABLE 1. *Summary of RDDP purification*[a]

| Protein fraction | RDDP activity (U) | Protein (mg) | Sp act of RDDP (U/mg of protein) | Purification (-fold) |
|---|---|---|---|---|
| Crude lysate | 1,064,923 | 2,951 | 361 | |
| DE 52 | 929,678 | 239 | 3,890 | 12 |
| CM 52 | 683,380 | 12 | 56,356 | 246 |

[a] These data were derived during purification of RDDP from 26 g of AMV (preparation G-977, Table 2).

TABLE 2. *Yield and specific activity of various preparations of RDDP*

| Preparation | Amt of AMV (g) | Amt of RDDP (U/g of AMV) | Sp act of RDDP (U/mg of protein) |
|---|---|---|---|
| G-777 | 22 | 23,119 | 41,176 |
| G-877 | 20 | 30,960 | 52,965 |
| G-977 | 26 | 26,283 | 56,356 |
| G-1077 | 25 | 35,137 | 51,673 |
| G-1877 | 23 | 31,541 | 56,663 |
| G-678 | 19 | 32,201 | 41,220 |
| G-778 | 24 | 31,065 | 35,351 |

**RNase activity.** In addition to other advantages of the two-step chromatographic isolation of RDDP was the essentially complete elimination of RNase. After incubation of Raji cell 28S ribosomal RNA with RDDP for 60 min at 37°C, no degradation of RNA was detectable by sedimentation on a sucrose gradient (Fig. 2).

**Stability.** Detailed studies of RDDP stability have not been made. At −20°C, however, most preparations lost no more than 10% activity after storage for 1 year in phosphate-buffered glycerol (data not shown). Repeated freezing and thawing are detrimental to enzyme activity.

**Isoelectric point.** Results of measurement of the isoelectric point of RDDP made after migration of a 75-μl sample into the focusing gel are shown in Fig. 3. The focused gels were sliced, polypeptides were extracted, and RDDP activity of each fraction was determined. As is evident in Fig. 3, the peak of activity focused as a sharply defined band at pH 6.5, thus defining the enzyme as a slightly acidic protein.

**Glycoprotein and fatty acid assays.** During these studies, it was noted that RDDP adsorbed to an affinity column of concanavalin A coupled to Sepharose 4B and, subsequently, could be eluted with 0.2 M KCl. Because RDDP could not be eluted from concanavalin A-Sepharose with alpha-methyl-D-mannoside, the possible presence of carbohydrate and fatty acid residues in RDDP was investigated. Purified RDDP (lot G-1477) was precipitated by dialysis against 50 mM Tris-hydrochloride (pH 8.0). The precipitate was collected in new, un-

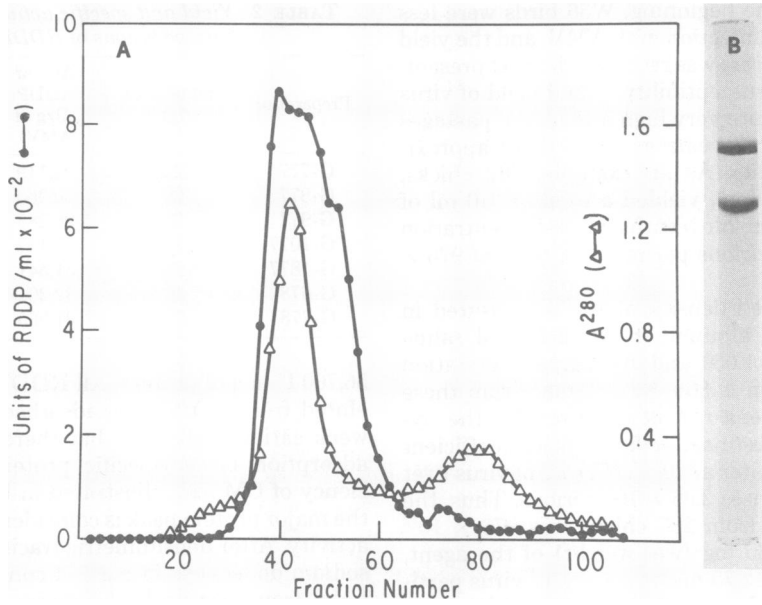


FIG. 1. *(A) Chromatography of AMV RDDP on CM 52. After adsorption of RDDP onto a column (4.6 by 8 cm) of CM 52, the column was developed with a linear gradient (500 ml) of 10 to 300 mM potassium phosphate containing 0.2% Triton X-100, 2 mM DTT, and 10% glycerol. Alternate fractions (4.5 ml) were assayed for RDDP activity. A different preparation was eluted without Triton X-100 to determine the elution positions of proteins (absorbance at 280 nm) present in the chromatogram. Fractions 36 to 62 were pooled and concentrated 2.5-fold by dialysis against 200 mM potassium phosphate (pH 7.3) containing 0.2% Triton X-100, 2 mM DTT, and 50% glycerol for further analysis. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of RDDP in CM-52 pool concentrated as described for (A). The top band is the beta polypeptide (molecular weight, 92,000), and the bottom band is the alpha polypeptide (molecular weight, 68,000). Molecular weights were determined by comparison with positions of standard proteins run in separate gels (data not shown).*

used polycarbonate tubes by centrifugation at 12,000 × *g* for 10 min and suspended in freshly distilled water. This cycle of centrifugation and resuspension of RDDP was repeated six times, with a final yield of 7.05 mg of enzyme. The material then was prepared for analysis of carbohydrate and fatty acid content by gas-liquid chromatography and mass spectrometry. Results of these analyses, which were done by R. A. Laine and R. L. Lester, Department of Biochemistry, University of Kentucky Medical Center, Lexington, indicated measurable amounts of fatty acid (Table 3) but no significant amount of carbohydrate. The purified preparation of RDDP contained 1.0 mol of fatty acid per 101,600 g of protein, or approximately 2 mol of fatty acid per mol of reverse transcriptase. This amount of fatty acid could be the result of contamination, or it might well be an integral component of the reverse transcriptase.

## DISCUSSION

Because of the current extensive use of reverse transcriptase, as evident both from the literature and distribution to large numbers of investigators, it is of much practical importance to devise procedures for increasing the yield and specific activity of the enzyme. Yields of virus appear to have been increased, due, in part at least, to adaptation of the agent to the birds now in use. Use of young chicks and adjustment of the infecting dose are critical. Incidence of infection decreases rapidly with age (6), and increase in dose may result in rapid death of the host without occurrence of significant concentrations of virus in the plasma (unpublished data). It is notable that the dose of $10^{10}$ virions is much greater than the dose of $2.6 \times 10^7$ virions required to yield an incidence of 50% in Line 15 chicks, which were used previously for quantitative studies on AMV (5).

Factors primarily responsible for the high yields and specific activities of RDDP, which are large in comparison with analogous findings in early work with the enzyme, are not yet fully understood and are still under study. The oversized chromatography columns permit rapid processing of large volumes and thus minimize exposure of the enzyme to degradative activities possibly present in the crude lysate. Further-

more, use of the weak cation exchanger CM 52 results in minimal adsorption of nonspecific proteins eluted from DE 52. Although high levels of purity of RDDP or components of RDDP are attainable in a single step by adsorption to polycytidylate-agarose (17) or polyuridylate-Sepharose (11), this approach is not economically feasible for large-scale production of RDDP.

Usefulness of RDDP in a very broad range of biological problems is indicated by the increasing use of the enzyme in current studies. The lack of species template specificity and consequent ability of AMV reverse transcriptase to synthesize complementary DNA from mRNA templates from diverse sources provides an important and sensitive tool for a variety of experiments in the study of gene frequency, mRNA metabolism, and transcription of chromatin in vitro. It is now possible to obtain complementary DNA preparations that are complete and faithful copies of their respective mRNA templates

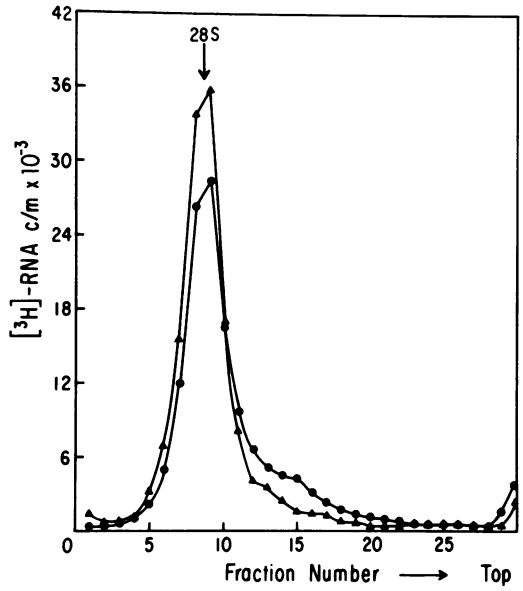


FIG. 2. *Determination of RNase in purified AMV reverse transcriptase. RDDP (41.2 U) was incubated with 5 μg of $^3$H-labeled 28S Raji cell ribosomal RNA (specific activity, 2 × $10^5$ cpm/μg) in 0.02 M Tris-hydrochloride (pH 7.4) and 0.15 M NaCl for 60 min at 37°C. The 0.1-ml reaction mixture was layered onto a linear 15 to 30% sucrose gradient (5.2 ml) containing 0.05 M sodium acetate, pH 5.0, 0.1 M NaCl, and 0.1% sodium dodecyl sulfate and centrifuged in an SW50.1 rotor at 40,000 rpm for 5 h at 0°C. The gradients were fractionated by collecting 30-drop samples from the bottom of the gradient. A 50-μl portion of each sample was processed for determination of acid-insoluble [$^3$H]RNA by liquid scintillation spectrometry.*

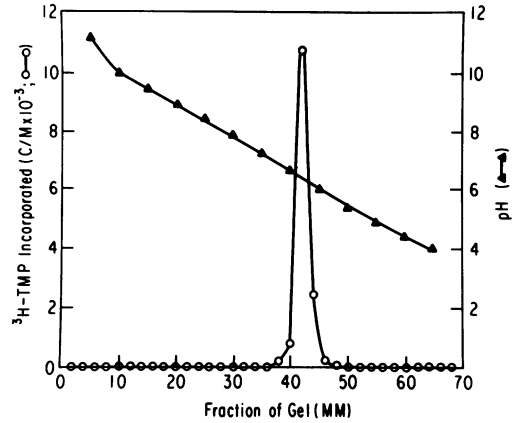


FIG. 3. *Isoelectric focusing of purified AMV RDDP. RDDP in the concentrated CM 52 pool, as described in the legend to Fig. 1A, was dialyzed against 50 mM Tris-hydrochloride (pH 8.0) containing 2 mM DTT and 50% glycerol to remove potassium phosphate. The material was focused in an 8% polyacrylamide gel containing 1.7% carrier ampholytes (1.4% of 3 to 10 ampholytes and 0.3% of 8 to 10 carrier ampholytes). After determination of the pH gradient with miniature electrodes (Bio-Rad Pro-pHiler), the gel was frozen and sliced into 1-mm sections. Polypeptides were extracted from the sections overnight at 4°C with 0.1 M potassium phosphate (pH 7.3) containing 2 mM DTT and 10% glycerol. A 10-μl portion of each extract was added to 50 μl of RDDP reaction mixture, incubated for 10 min at 37°C, and assayed for the presence of acid-insoluble [$^3$H]TMP on GF/C disks as previously described (1).*

TABLE 3. *Fatty acid content of AMV reverse transcriptase*

| Fatty acid[a] | % Of total fatty acids |
|---|---|
| 16:0[b] | 50.1 |
| 18:1 | 23 |
| 18:0 | 14.1 |
| 23:0 | 4.6 |
| 24:0 | 2.8 |
| 26:0 | 5.2 |

[a] Fatty acids were identified both by retention time during gas chromatography and by atomic mass as determined with a mass spectrometer. Approximately 2 mol of fatty acid was found per mol of RDDP after analysis of 7.05 mg of enzyme.

[b] The first number indicates the length of the carbon chain, and the second number indicates the number of unsaturated bonds per chain.

(4, 8, 10, 18). Full-length copies of 35S RNA have been synthesized in good yield after scrupulous exclusion of nuclease activity (19). Clearly, such complementary DNAs provide good candidates for the insertion of eucaryotic structural genes into bacterial plasmids (10, 12, 16, 20). It is evident that, without this source of the C-type RNA virus and the derivative tran-

scriptase available in large amounts, work in the area of tumor viruses, as well as many significant studies in other biological and biochemical fields, would probably be severely restricted.

## ACKNOWLEDGMENTS

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