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# Isolation and Subunit Structure of Polycytidylate-Dependent RNA Polymerase of Encephalomyocarditis Virus

**(BHK 21 cells/smooth membranes/electron microscopy)**

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**ABSTRACT** **A polycytidylate-dependent RNA polymerase of encephalomyocarditis virus was isolated from infected BHK 21 cells. The enzyme was associated with a smooth-membrane fraction, from which it was extracted by a mixture of sodium dodecyl sulfate, Triton X-100, and dithiothreitol, and further purified by chromatography on a Dowex-1 column and by glycerol gradient sedimentation. Analysis of a 6S glycerol gradient peak of RNA polymerase activity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed the presence of five polypeptides, of molecular weights 72,000, 65,000, 57,000, 45,000, and 35,000. The molecular weights of four of the polypeptides (72,000, 65,000, 45,000, and 35,000) are almost identical to the reported molecular weights of the four subunits of Qβ replicase.**

Great progress has been made in the isolation and elucidation of the structure and mode of action of RNA-dependent RNA polymerases (replicases) induced by some of the RNA bacteriophages in *Escherichia coli* (1–8). Qβ replicase can direct the synthesis of poly(G) with a poly(C) template, a reaction that is independent of host protein factors. (Synthesis of a strand complementary to the infecting RNA strand with a viral RNA template requires two host protein factors.) This result allowed the use of poly(C) as template in the assay for the virus-induced replicase, and facilitated development of procedures for purification of the enzyme (8).

Replicases of the simple RNA animal viruses, like the picornaviruses (9, 10), have not been obtained in a pure form, dependent for activity on an exogenous RNA template. This failure is most probably due to the relatively low amount of enzyme activity found in virus-infected cells, and to the fact, made apparent in studies with poliovirus (11, 12), that the replicase is firmly bound to smooth cytoplasmic membranes. Lack of an enzyme preparation capable of using a synthetic polyribonucleotide template has also hindered work on purification of the enzyme.

A study in our laboratory on replication of encephalomyocarditis (EMC) virus in BHK 21 cells showed that EMC replicase is also associated with a smooth cytoplasmic membrane fraction. Furthermore, after digestion of the viral RNA template with micrococcal nuclease the preparation manifested a poly(C)-dependent poly(G) polymerase activity, thus suggesting that poly(C) may be used for the assay of EMC replicase.

This report describes a purification procedure for EMC replicase based on its poly(C)-dependent poly(G) polymerase activity, and a preliminary analysis of its subunit structure.

Abbreviations; Replicase, RNA-dependent RNA polymerase: EMC, encephalomyocarditis: SDS, sodium dodecyl sulfate.

## MATERIALS AND METHODS

*Preparation and Storage of BHK 21 Cells Infected with EMC Virus.* Cells of a baby hamster kidney cell line (BHK 21), infected for 6.5 hr with EMC virus, contained a maximal amount of EMC replicase and, therefore, were a source of enzyme. They were grown in suspension culture in Eagle's minimal essential spinner medium (Medium F-14; Grand Island Biological Co) with 5% calf serum in 5% $CO_2$ in air. The cells were harvested by centrifugation (5 min at 500 × *g*), suspended in cold phosphate-buffered saline (pH 7.0) at a concentration of $10^8$ cells per ml, infected with a stock of EMC virus at a ratio of 10 plaque-forming units (PFU) per cell, and kept for 1 hr in crushed ice. The thick cell suspension was then diluted with warm (37°) Eagle's spinner medium to $1.5 \times 10^7$ cells per ml, and 250-ml aliquots were dispensed in 1-liter Bellco spinner flasks (Bellco Co., Vineland, N.J.) and placed on magnetic stirrers at 37° under 5% $CO_2$ in air. After 6.5 hr the infected cells were collected by centrifugation (10 min at 2000 × *g*), suspended in DTG buffer (see below) ($1.5 \times 10^8$ cells per ml), and stored at −70°. Replicase activity was stable for at least 4 months.

*Fractionation of Infected BHK 21 cells by Isopycnic Sucrose Gradient Centrifugation.* When BHK 21 cells, infected for 6.5 hr with EMC virus, are homogenized in cold distilled water, most of the EMC replicase activity is in the postmitochondrial fraction. Caliguiri and Tamm (12) have shown that in HeLa cells infected with poliovirus, the virus-induced replicase is associated with a smooth membrane fraction that can be separated by isopycnic sucrose gradient centrifugation. This method was slightly modified and was used for isolation of a smooth membrane fraction from the postmitochondrial fraction of cells infected with EMC virus.

All operations were done at 0–4°. $3 \times 10^9$ frozen infected cells were thawed and collected by centrifugation (5 min at 15,000 × *g*). The cell pellet was suspended in 14 ml of distilled water and homogenized in a Dounce homogenizer (Kontes Glass Co.) by 20 strokes with pestle A, followed by 20 strokes with pestle B. The homogenate was centrifuged (20 min at 15,000 × *g*), and the supernatant fluid (about 10 ml) was collected. TKMD (see below) × 100 buffer (0.01 volume) was added, and the supernatant was made 30% in sucrose by addition of 60% sucrose (w/v) solution in TKMD buffer. The volume was brought to 16 ml with 30% sucrose in TKMD

TABLE 1. *Distribution of EMC replicase, protein, and lipid-P in the isopycnic sucrose gradient fractions**

| Fraction (% sucrose) | Replicase (units)‡ | Protein (mg) | Specific activity (units/mg protein) | Lipid P (μmol) |
|---|---|---|---|---|
| Cytoplasmic extract (10)† | 5200 | 81.6 | 63.7 | 13.86 |
| *1.* 10.2 (3.3) | 120 | 0.8 | 160.0 | 0.69 |
| *2.* 18.0 (8.2) | 2640 | 1.2 | 2203.0 | 5.10 |
| *3.* 26.0 (7.8) | 450 | 6.5 | 69.0 | 4.14 |
| *4.* 28.0 (7.4) | 165 | 20.0 | 8.2 | 0.83 |
| *5.* 32.0 (7.6) | 172 | 23.1 | 7.4 | 0.96 |
| *6.* 40.0 (12.5) | 185 | 13.5 | 13.6 | 0.82 |
| *7.* 46.5 (7.0) | 160 | 7.8 | 20.0 | 0.86 |
| *8.* 51.0 (5.6) | 158 | 6.5 | 26.8 | 0.04 |
| Pellet — | 86 | 0.6 | 14.3 | — |

* Prepared from $3 \times 10^9$ infected cells.

† Volume of fraction, ml.

‡ 10-μl Aliquots were assayed for replicase activity (see *Methods*).

buffer and divided into two 8-ml aliquots. Two discontinuous sucrose density gradients were prepared by layering solutions of sucrose prepared in TKMD buffer in the following order: 2.5 ml of 60%, 5.6 ml of 45%, 5.6 ml of 40%, 8 ml of the infected cell supernatant in 30% sucrose, 5.6 ml of 25%, and 2.5 ml of TKMD buffer on top. The gradients were centrifuged for 20 hr at 25,000 rpm in a Spinco SW25.1 rotor. After centrifugation, the tubes contained eight unequal layers, delineated either by membrane bands or by a difference in opalescence. The unequal fractions were collected, starting from the top, by a syringe. The greater part of the replicase activity was found in the second fraction from the top (fraction 2). Each gradient tube contained about 4 ml of fraction 2.

*Assay of Endogenous Replicase Activity* measures conversion of [$^3$H]GTP into an acid-insoluble product. The standard reaction mixture (0.3 ml) contained: 50 mM Tris·HCl (pH 8.0) at 25°, 4 μM $MgCl_2$, 0.1 M KCl, 3 μg of actinomycin D, 20 μg of creatine phosphokinase, 5 mM creatine phosphate, 50 nmol each of ATP, CTP, and UTP, and 0.66 nmol of [$^3$H]GTP (Radiochemical Center, 500–1000 cpm/pmol). Poly(C)-dependent poly(G) polymerase was assayed in the same reaction mixture modified by the omission of ATP, CTP, and UTP, and addition of 100 μg of poly(C) (Miles Laboratories). Because of its extreme lability, the poly(G) polymerase was assayed immediately after collection of each fraction. The tubes were incubated for 20 min at 37°. Reactions were terminated by addition of 100 μg of bovine-serum albumin and 3 ml of 10% cold trichloroacetic acid containing 10 mM sodium pyrophosphate. The precipitate was collected on a glass-fiber filter (Whatman GF/A), washed with 5% trichloroacetic acid containing 1 mM sodium pyrophosphate, then with 95% ethanol, dried, and counted in 5 ml of toluene-based scintillation solution in a Packard Tri-Carb counter. One unit of enzyme activity is the amount catalyzing the incorporation of 1 pmol of GMP in 20 min at 37° into an acid-insoluble product.

TABLE 2. *Purification of EMC replicase*

| Step of purification | Protein (mg) | Lipid-P (μmol) | Activity (units) | Specific activity (units/mg protein) |
|---|---|---|---|---|
| 1. Cytoplasm | 81.60 | 13.26 | 5200 (100)* | 63.7 (1)† |
| 2. Fraction 2 | 1.20 | 5.10 | 2640 (51) | 2200 (34) |
| 3. Pellet of Fraction 2 | 0.53 | 4.70 | 1870 (36) | 3527 (55) |
| 4. 0.6 M NaCl Dowex eluate | ~0.008 | — | 105 (2) | 13200 (206) |
| 5. Glycerol gradient peak I | ~0.004 | — | 215 (4) | 53000 (850) |

The enzyme was prepared from $3 \times 10^9$ infected cells. 5-μl Aliquots from steps *1–3* were assayed for replicase activity, and 25-μl aliquots from steps *4* and *5* were assayed for activity of poly(C)-dependent poly(G) polymerase.

* Yield (%).

† Purification (fold).

*Sodium Dodecyl Sulfate(SDS)–Polyacrylamide Gel Electrophoresis.* Proteins were dissociated by heating at 100° for 1.5 min with 1% SDS and 1% 2-mercaptoethanol, and separated by SDS–polyacrylamide gel electrophoresis (13) with 10% acrylamide and 0.27% bis-acrylamide. The gels were stained by Coomassie Brilliant Blue and destained electrophoretically. The stained protein bands were scanned by an Elphor densitometer (Munchen, W. Germany).

*Analytical Methods.* Protein was determined by the method of Lowry *et al.* (14), with crystalline bovine albumin (Sigma) as standard. Samples with interfering materials were first precipitated with 5% trichloroacetic acid. Lipid phosphorus was determined (15).

*Buffers.* DTG buffer: 10 mM Tris·HCl (pH 8.0) at 25°–1 mM dithiothreitol–20% glycerol. TKM buffer: 10 mM Tris·HCl (pH 7.8)–10 mM KCl–1.5–mM $MgCl_2$. TKMD buffer: TKM buffer plus 1 mM dithiothreitol. TMG buffer: 10 mM Tris·HCl (pH 7.0)–10 mM $MgCl_2$–5% glycerol.

## RESULTS

*Separation of Membrane-Bound EMC Replicase by Isopycnic Sucrose Gradient Centrifugation.* Infected cells were fractionated by isopycnic sucrose gradient centrifugation, and the separated layers were assayed for replicase activity

TABLE 3. *Appearance of poly(C)-dependent poly(G) polymerase activity after digestion of membrane-free pellet with micrococcal nuclease*

| Exp. no. | Additions | [$^3$H]GMP incorporation (cpm) Before nuclease | After nuclease |
|---|---|---|---|
| 1 | None | 3980 | 96 |
| 2 | 100-μg poly(C) | 3965 | 780 |
| 3 | 100-μg ribosomal RNA (BHK 21) | 3943 | 102 |

25-μl Aliquots were assayed for enzyme activity with a reaction mixture containing all four ribonucleoside triphosphates. BHK 21 ribosomal RNA was isolated from purified ribosomes of uninfected BHK 21 cells.

and for protein and lipid phosphorus content. Fraction 2 contains about 50% of the replicase activity and only 1.5% of the protein (Table 1). In agreement with the results in poliovirus-infected HeLa cells (12), fraction 2 showed a high phospholipid P to protein ratio, typical for the composition of membrane material. Most of the replicase found in fraction 2 could be pelleted by centrifugation. 8 ml of fraction 2 was diluted with 16 ml of TKMD buffer and centrifuged for 2 hr at 50,000 rpm (Spinco rotor 50). The pellet was suspended in 1 ml of TKMD buffer, and replicase activity and protein and lipid phosphorus content were determined in both pellet and supernatant. The pellet contained about 70% of the replicase, more than 90% of the lipid phosphorus, and about 40% of the protein (Table 2).

The material in the pellet was examined in a JEM 100B electron microscope, by negative staining with 2% phosphotungstic acid. Electronmicrographs reveal the presence of unique structures that seem to be composed of multimembranous layers arranged in close concentric circles. Fractions 1 and 3 contained only a few of these structures. They were not detected in the other gradient fractions, nor in isopycnic gradient fractions obtained by a similar separation of a cytoplasmic preparation from unifected cells.

*Poly(C)-Dependent Poly(G) Polymerization by EMC Replicase.* Attempts to remove the endogenous RNA template from the membrane-bound EMC replicase by digestion with micrococcal nuclease were not successful. Under these conditions the activity of the membrane-bound enzyme was unaffected. However, the enzyme did become sensitive to nuclease action if it was first separated from the bulk of the membrane material as follows: fraction 2, obtained from $3 \times 10^9$ infected cells, was diluted with 2 volumes of TKMD buffer and centrifuged for 2 hr at 50,000 rpm (Spinco rotor 50). The pellet was suspended in 1 ml of TKM buffer containing 20 mM dithiothreitol and 0.05% Triton X-100, and kept for 18 hr at 2°. The material was divided into three parts, and each was loaded on a 4.5-ml 5–20% sucrose gradient in TKM buffer containing 20 mM dithiothreitol and 0.05% Triton X-100, and centrifuged for 16 hr at 50,000 rpm in Spinco rotor SW50.1. Fractions of 0.5 ml were collected, and the small pellets were suspended, each in 0.15 ml of TKMD buffer containing 0.5 M KCl and 30% (v/v) glycerol. The pellet and fractions were examined for replicase activity and lipid phosphorus. The pellet contained about 50% of the replicase and only 2% of the lipid phosphorus, thus indicating that the replicase was separated from the bulk of the membrane material. At this stage the endogenous template RNA of the enzyme became susceptible to the action of micrococcal nuclease. The three pellet suspensions were combined; to the 0.45 ml were added 1 μmol of $CaCl_2$ and 25 μg of micrococcal nuclease (Worthington), and the mixture was incubated for 30 min at 25°. Nuclease activity was terminated by the addition of 5 μmol of ethyleneglycol-bis-(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA). Aliquots were tested for ability to synthesize an RNA dependent on endogenous template RNA and for activity of poly(C)-dependent poly(G) polymerase. Nuclease treatment abolished all of the endogenous template RNA-dependent replicase activity, but now, in the presence of poly(C) the preparation manifested poly(G) polymerase activity (Table 3).

*Isolation of a Soluble Poly(C)-dependent EMC Replicase.* Thus, EMC replicase, when freed of its endogenous RNA template, manifests a poly(C)-dependent poly(G) polymerase activity. The fact that under identical assay conditions extracts of uninfected cells did not show any poly(G) polymerizing activity suggested that poly(C) may be used as template for the routine assay of EMC replicase, provided the enzyme has been separated as an RNA-free protein. Attempts to extract the replicase from material treated with micrococcal nuclease by 1 M KCl, 1 M $NH_4Cl$, nonionic detergents, and other means were not successful. Therefore sodium dodecyl sulfate (SDS), which acts as an efficient membrane solubilizer and separates proteins from nucleic acids, was used, along with Triton X-100 and dithiothreitol, for extraction of a soluble and RNA-free replicase from membranes of fraction 2. In order to avoid extensive protein denaturation and reformation of protein–RNA complexes, the SDS and RNA were rapidly removed from the extract by adsorption on a column of Dowex 1-×8 by a method similar to that described (16). The poly(C)-dependent EMC replicase eluted from the Dowex column was further purified by sedimentation through a 5–20% glycerol gradient as follows: fraction 2 was treated as described in *Results*. After the 18-hr incubation at 2°, the suspension was brought to about 8° and a 10% solution of SDS was added to give a final concentration of 0.3% SDS. The clarified solution was immediately applied to a Dowex 1-×8 column (0.5 × 4 cm), equilibrated with 50 mM sodium phosphate (pH 7.0) and operated at room temperature. The column was then eluted with 3 ml of cold TMG buffer, followed by 3 ml of cold TMG buffer containing 0.6 M NaCl. Fractions of 0.25 ml were collected into ice-cooled tubes and assayed for activity of endogenous RNA-dependent EMC replicase and poly(C)-dependent poly(G) polymerase. Almost all of the protein was eluted by TMG buffer alone (Fig. 1). The first portion of the effluent contained some activity of endogenous RNA-dependent replicase, while the remainder contained only poly(C)-dependent poly(G) polymerase. Further elution with 0.6 M NaCl released a barely detectable amount of protein, but a significant amount of poly(C)-dependent poly(G) polymerase activity. The active fractions of the 0.6 M NaCl eluate were pooled, loaded on a 26-ml gradient of 5-20% glycerol, in

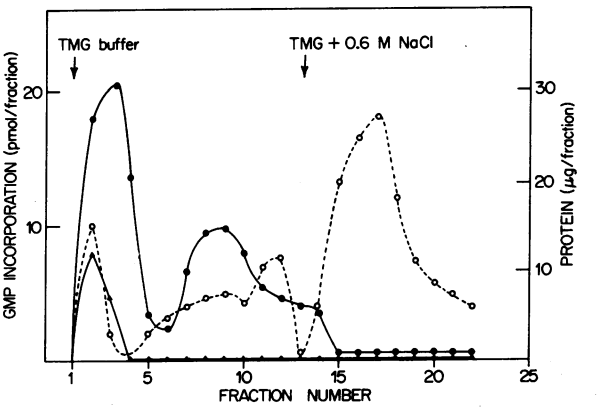


FIG. 1. Dowex-1 chromatography of poly(C)-dependent replicase. The enzyme was extracted from a fraction-2 pellet by a mixture of dithiothreitol, Triton X-100, and SDS, and chromatographed on a 0.5 × 4 cm Dowex-1 column. (●——●) protein: (○——○) poly(C)-dependent activity: (▲——▲) endogenous template RNA-dependent activity.

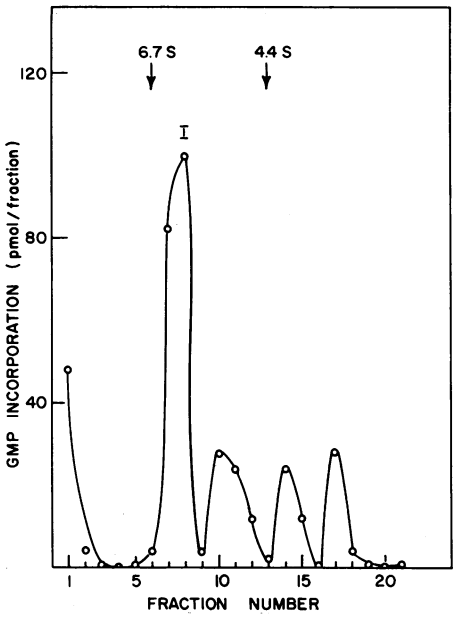


FIG. 2. Glycerol gradient centrifugation of poly(C)-dependent replicase. Pooled active fractions of the 0.6 M NaCl eluate from the Dowex-1 column were loaded on a 5–20% glycerol gradient, centrifuged, and assayed for activity. Alcohol dehydrogenase (6.7 S) and bovine serum albumin (4.4 S) were used as sedimentation markers. (O——O) poly(C)-dependent replicase activity.

TKMD buffer, and centrifuged for 20 hr at 25,000 rpm in a Spinco rotor SW25.1. Alcohol dehydrogenase ($s_{20,w}$ = 6.7) and bovine serum albumin ($s_{20,w}$ = 4.4) were run in a parallel gradient as protein markers. Each gradient was fractionated into 20 fractions, and 25-$\mu$l aliquots were assayed for activity of poly(C)-dependent replicase. A distinct "heavy" peak (I) of replicase activity sedimented behind alcohol dehydrogenase, but there was also some tailing of "light" peaks of low activity at the upper part of the gradient (Fig. 2). From the relative positions of peak I and the marker proteins in the gradient we may estimate that the replicase of peak I has a sedimentation coefficient of about 6 S. The presence of some replicase at upper regions of the gradient may be due to a change in sedimentation properties or loss of subunits as result of the SDS treatment.

*Subunit Analysis of EMC Replicase.* The pooled fractions of peak I (Fig. 2) contained a minute amount of protein, about 4–6 $\mu$g. This was lyophilized and analyzed by SDS–polyacrylamide gel electrophoresis. The densitometer tracing and photograph (*insert*) in Fig. 3 show three major proteins and two minor ones (labeled I, II, III, IV, and V). The approximate molecular weights of the proteins based on electrophoretic mobility are 72,000, 65,000, 57,000, 45,000, and 35,000 for proteins I–V, respectively. At this stage of purification the enzyme was very unstable and it could not be further purified. Thus, we do not know whether all five proteins are true subunits of the replicase, or whether one or two of the minor components are contaminants.

## DISCUSSION

Previous attempts to isolate EMC replicase by conventional methods used in protein purification were not successful. The enzymatic activity, found in the postmitochondrial fraction, did not adsorb to various adsorbents used in protein chromatography. Studies (12) that showed that poliovirus replicase was bound to smooth cytoplasmic membranes, suggested that a similar situation may exist in BHK 21 cells infected with EMC virus. This was found to be so, and it probably explains the failure to purify the enzyme by conventional means. At this point we realized that further purification of the enzyme would be possible only after its release from the cytoplasmic membranes. However, since the assay of the enzyme was dependent on the presence of an RNA template, it was necessary either to release a complex composed of enzyme and RNA template or a free enzyme protein; in the latter case, an alternative polyribonucleotide template would be required for assay of its activity.

Treatment of the cytoplasmic membranes with 20 mM dithiothreitol and 0.05% Triton X-100, followed by centrifugation through a sucrose gradient of a similar composition, separated the greater part of the membrane material from the complex of replicase–RNA template. The enzymatic activity, which sedimented to the bottom of the tube, was associated with an insoluble aggregate. However, at this stage the template RNA in the aggregate became sensitive to the action of micrococcal nuclease. It thus became possible to free at least part of the enzyme from its viral RNA template and to prove, upon addition of poly(C), that EMC replicase can also manifest an activity of a poly(G) polymerase. The results suggested the possible use of poly(C) in further purification studies. Attempts to extract the replicase by high salt concentrations or nonionic detergents were not successful. Therefore, another method, extraction of the

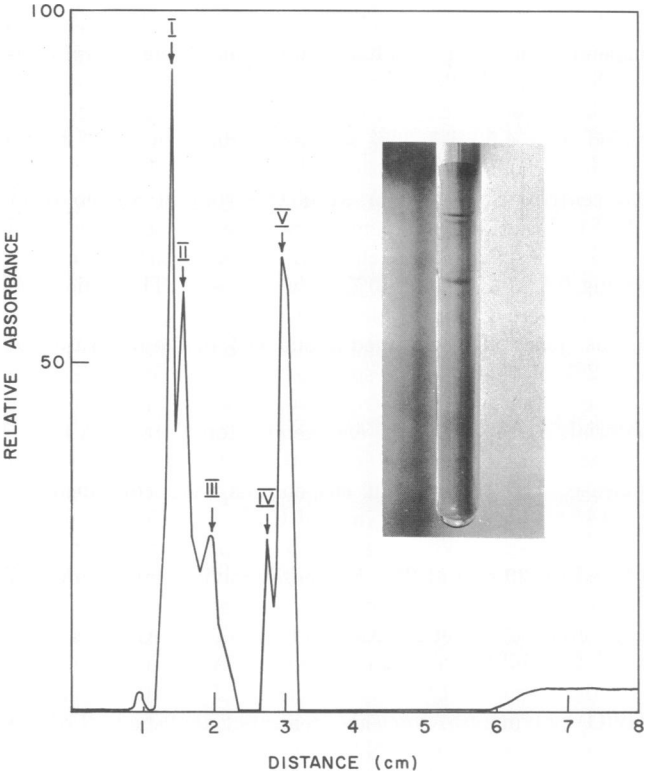


FIG. 3. SDS–polyacrylamide gel electrophoresis of poly(C)-dependent replicase in peak I of glycerol gradient. The SDS–polyacrylamide gels (10%) were prepared and run as described by Weber and Osborn (13).

enzyme from membranes of fraction 2 by a mixture of SDS, Triton X-100, and dithiothreitol, was tested. Although SDS causes extensive protein denaturation and dissociation into polypeptide subunits, a partial amount of enzymatically active replicase was extracted in a soluble, poly(C)-dependent form, and was further purified by chromatography on a Dowex-1 column and by glycerol gradient fractionation. A highly purified poly(C)-dependent poly(G) polymerase was eluted from the Dowex column by TMG buffer containing 0.6 M NaCl (Fig. 1). The active fractions contained about 2% of the protein loaded on the column and did not manifest any endogenous template RNA-dependent RNA synthesis, suggesting that complete separation between the enzyme and its template RNA has occurred. It should be noted that the total activity of poly(C)-dependent replicase found after sedimentation through the glycerol gradient was about twice as much as that eluted from the Dowex column by 0.6 M NaCl (Table 2). This result may be due to removal of nucleases or to renaturation of some of the SDS-denatured enzyme. Although enzyme activity doubled after the 20-hr sedimentation at 4°, the resulting fraction (peak I) lost activity rapidly (half-life from 2–4 hr) when kept at the same temperature. This final fraction contained only about 4% of the original activity, but appears to be extensively purified, as judged by SDS–polyacrylamide gel electrophoresis. Probably as a result of the SDS treatment, the greater part of the replicase dissociated completely into subunits.

SDS–polyacrylamide gel electrophoresis of the material in peak I revealed five polypeptides (I–V) of approximate molecular weights 72,000, 65,000, 57,000, 45,000, and 35,000, respectively (Fig. 3). At the present state of enzyme purification it is not clear whether all five polypeptides constitute true subunits of EMC replicase. However, there seems to be a remarkable similarity between the molecular weights of polypeptides I, II, IV, and V and the molecular weights of the four subunits of Qβ replicase: 70,000, 65,000, 45,000, and 35,000 for subunits I–IV, respectively (6, 7). This rather surprising observation, if not fortuitous, may be understood if we take into account the fact that Qβ replicase contains three polypeptides (I, III, and IV) of host *E. coli* origin. It may be that certain proteins that are similar in structure and function exist both in *E. coli* and BHK 21 cells, and that during infection with Qβ bacteriophage or EMC virus they are removed from the cellular pool and incorporated into the structure of the newly formed replicase. The recent progress in characterization of the *E. coli* proteins that form part of the poly(C)-dependent Qβ replicase (17, 18), and current work in our laboratory aimed at determination of the origin and functions of the proteins that compose the poly(C)-dependent replicase of EMC virus may help to clarify this problem.

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