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In Vitro Replication of Cowpea Mosaic Virus RNA

II. Solubilization of Membrane-Bound Replicase and the Partial Purification of the Solubilized Enzyme

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A method for the solubilization of membrane-bound Cowpea mosaic virus RNA replicase has been developed bypassing the use of detergents. Solubilization has been achieved by washing the 31,000 × *g*-pellet containing the bound replicase with a Mg^{2+}-deficient buffer. This procedure had several advantages as compared to treatments with nonionic or ionic detergents: (i) the solubilized enzyme was stable at 4 C, (ii) more than 80% of the replicase could be solubilized without loss of total enzyme activity, (iii) the replicase was rather selectively released resulting in a two- to threefold increase in specific activity per se, and (iv) most of the green color from chloroplast fragments present in the crude replicase fraction remained membrane bound resulting in only slightly colored preparations of solubilized enzyme. The solubilized replicase has been further purified by DEAE-Bio Gel column chromatography. RNA synthesis directed by the DEAE-purified enzyme was template dependent and proceeded at a linear rate for at least 9 h.

The purification of the RNA replicases (RNA-dependent RNA polymerases) of the bacteriophages Qβ and f2 has proven to be of great value in the elucidation of the mechanism of phage RNA replication and its mode of regulation (3, 15, 16, 26, 31, 32, 38). In addition, by analysis of the subunit structure of the Qβ replicase the involvement of specific host proteins in bacteriophage replication has been revealed (see reference 5).

The study of eukaryotic virus RNA replication is greatly hampered by the lack of a pure and stable RNA replicase. Many attempts have been undertaken to purify the RNA replicases from cells infected with animal and plant RNA viruses. The first step in the purification of the eukaryotic replicases, comprising the detection and characterization of RNA-dependent RNA polymerase activity in virus-infected cells, has been reported for several animal and plant viruses (4–6, 8, 9, 21, 23, 27, 30, 36, 39). Most of the eukaryotic RNA replicases appeared to be bound to cytoplasmic membranes in tight association with endogenous RNA template (2, 9, 11, 14, 17, 18, 22, 29, 30). The further purification and characterization, however, depends on the availability of a soluble and template-dependent enzyme and thus demands that the replicase is released from the membranes and the template. From here the purification of eukaryotic replicases has met with many difficulties. The replicases are usually released from the membranes with the aid of nonionic and/or ionic detergents (2, 11, 14, 18, 19, 22, 24, 29, 34, 40). After solubilization the animal virus replicases still contain template RNA (2, 11, 14, 18, 22, 29, 34) which can only be removed laboriously, resulting in unstable enzyme preparations (29, 34).

In the case of plant virus RNA replicases, solubilization and removal of endogenous RNA could be attained for several viruses resulting in the partial purification and characterization of the replicase (10, 13, 24, 25, 40). Extensive purification of plant virus replicases, however, is also hampered by the lability of the enzymes.

Recently we reported the isolation and characterization of the membrane-bound cowpea mosaic virus (CPMV)-RNA replicase (39). In this paper we describe a procedure to release the CPMV replicase from the membranes that is very mild, does not involve the use of detergents, and yields a stable enzyme. After DEAE-Bio Gel column chromatography of the solubilized enzyme, a stable and template-dependent replicase is obtained.

MATERIALS AND METHODS

Materials. DEAE-Bio Gel A (control no. 13270 and 13758) was obtained from BioRad Laboratories, Rich-

mond, Calif., dithioerythritol (DTE) from Sigma Chemical Co., St. Louis, and phenylmethylsulphonylfluoride (PMSF) from Merck and Co. The source of all other chemicals has been previously mentioned (39). Actinomycin D was a generous gift from Merck, Sharp, and Dohme.

Buffers. Buffer A consisted of 0.05 M Tris-hydrochloride (pH 7.4), 0.01 M KCl, 0.001 M EDTA, and 0.003 M β-mercaptoethanol. Buffer B contained 0.05 M Tris-hydrochloride (pH 8.2), 25% (vol/vol) glycerol, 0.05 M KCl, 0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF. Buffer C was identical to buffer B except for the addition of 0.01 M $MgCl_2$. Buffer D contained 0.05 M Tris-hydrochloride (pH 8.2), 50% (vol/vol) glycerol, 0.05 M KCl, 0.001 M EDTA, 0.01 M DTE, and 0.5 mM PMSF. Buffer E contained 0.01 M Tris-hydrochloride (pH 7.2), 0.1 M NaCl, 0.01 M EDTA, and 0.5% sodium dodecyl sulfate.

Virus and plants. The growth of *Vigna unguiculata* (L.) Walp var. "Blackeye Early Ramshorn" plants and their infection with a yellow strain isolate of CPMV has been described (39). The primary leaves were harvested on day 4 after inoculation (39). Virus was purified as described by Van Kammen (35).

Isolation of CPMV-RNAs. CPMV nucleoprotein components (B and M) were separated by sucrose density gradient centrifugation in a Spinco Ti15 zonal rotor. RNA was isolated from the separate components by phenol-cresol-chloroform-sodium dodecyl sulfate extraction as described previously (39). B- and M-RNA were further purified by fractionation through a 34-ml linear 15 to 30% (wt/vol) sucrose gradient in buffer E at 20 C for 18 h at 22,500 rpm in an SW27 rotor.

Before centrifugation the RNA sample was heated at 65 C for 3 min and then quickly cooled at 0 C to prevent aggregation.

Isolation of membrane-bound CPMV-replicase. The details have been described elsewhere (39). In brief, 48 g of freshly harvested infected leaves were homogenized in eight portions of 6 g each with 35 ml of buffer A in a mortar. The filtered homogenate was centrifuged at 1,000 $\times$ *g* for 15 min. The supernatant was adjusted to 20% (vol/vol) glycerol and centrifuged at 31,000 $\times$ *g* for 30 min. The resulting pellet containing the bound replicase was directly used for the solubilization procedure.

Solubilization of membrane-bound replicase. An outline of the procedure is depicted in Fig. 1. The 31,000 $\times$ *g* pellet was resuspended in buffer B (1 ml for each gram of leaf tissue used) with the aid of a Thomas homogenizer and incubated for 60 min under continuous stirring on a magnetic stirrer at 4 C. The suspension was centrifuged at 4 C for 60 min at 31,000 $\times$ *g* in a Sorvall SS34 rotor. The clear, slightly green colored supernatant was carefully removed with a Pasteur pipette. To avoid contamination with the upper fluffy layer of the pellet, the last few milliliters were left in the centrifuge tube. The supernatant was saved and the residue was resuspended in buffer B (0.25 ml for each gram of leaf tissue used) and incubated for 30 min as described above. After centrifugation for 60 min at 31,000 $\times$ *g* the supernatant 2 was carefully removed with a Pasteur pipette.

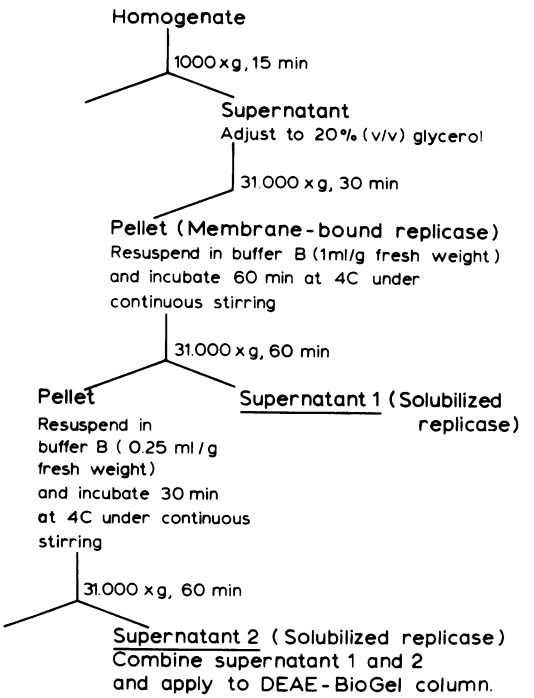

FIG. 1. *Scheme for the solubilization of membrane-bound CPMV replicase.*

Again great care was taken to avoid contamination with the material floating on top of the dark green sediment which was rather loosely packed now. The 31,000 $\times$ *g* pellet, washed with the Mg^{2+}-containing buffer C remained solid and firmly bound to the centrifuge tube. The two supernatants were combined and used directly for the ion-exchange chromatography. The pellet was resuspended in buffer B (0.25 ml for each gram of leaf tissue used) for testing of residual activity.

DEAE-Bio Gel column chromatography. Thirty milliliters of a packed DEAE-Bio Gel slurry was washed with 200 ml of buffer B, poured into a column, and equilibrated with buffer B. The combined supernatants containing the solubilized enzyme were applied to the column (1.6 by 15 cm) and the unadsorbed material was washed out with buffer B. Then the bound material was eluted with a linear 0.05 to 0.4 M KCl gradient in the same buffer and 2.7-ml fractions were collected at a flow rate of about 16 ml/h. The fractions containing template-dependent replicase activity were pooled and dialyzed against 2 liters of buffer B saturated with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 31,000 $\times$ *g* for 20 min, dissolved in 2 ml of buffer B, dialyzed for 2 h against 1 liter of buffer B to remove residual $(NH_4)_2SO_4$, and finally dialyzed overnight against buffer D. The enzyme solution was divided in aliquots (200 μl) and stored frozen in liquid nitrogen.

The DEAE-Bio Gel was regenerated and reequilibrated by washing the slurry on a Büchner funnel with buffer B containing 0.5% Sarkosyl and 1.0 M KCl until the green color had disappeared, and finally washed with buffer B.

Replicase assay. The standard assay mixture (total volume of 0.240 ml) contained 0.05 M Tris-hydrochloride (pH 8.2), 5 to 10% glycerol, 0.01 M $MgCl_2$, 0.025 M KCl, 0.013 M $(NH_4)_2SO_4$, 0.001 M EDTA, 0.25 μmol each of ATP, GTP, and CTP, 0.01 μmol of UTP, 5 μCi of [^{3}H]UTP (specific activity 12 to 14 Ci/mmol), 1 μmol of phosphoenol pyruvate, 10 μg of pyruvate kinase, 5 μg of actinomycin D, 0.004 M DTE, 25 μg of CPMV-RNA, and enzyme. Assay mixtures were incubated for 30 min at 22 C and the reactions were terminated by the addition of 3 ml of ice-cold 10% of trichloroacetic acid containing 4% $Na_4P_2O_7$ and 4% NaH_2PO_4. After the addition of bovine serum albumin (350 μg per sample) the mixture was left on ice and then the acid-insoluble precipitates were collected on Whatman GF/A filters, washed five times with 5-ml aliquots of 5% trichloroacetic acid containing 2% $Na_4P_2O_7$ and 2% NaH_2PO_4, five times with 1 N HCl containing 0.1 M $Na_4P_2O_7$, twice with 80% ethanol, and finally with ether. The filters were then processed as described previously (39). In the case of a time course experiment a batch enzyme reaction mixture was incubated in the dark, because of the presence of actinomycin D, and at the times indicated two 50-μl samples were taken and immediately spotted on numbered GF/C filters. The filters were collected in 5% trichloroacetic acid containing 2% $Na_4P_2O_7$ and 2% NaH_2PO_4, and washed batchwise with two changes each, respectively, of the trichloroacetic acid-phosphate solution, hydrochloric acid-phosphate solution, ethanol, and ether as described above.

RESULTS

Solubilization of the membrane-bound replicase. Solubilization of membrane-bound proteins can be attained by several methods (28, 33). To investigate the most suitable method to release CPMV replicase from the membranes, the effect of different detergents and high-ionic strength was examined first.

In these experiments the 31,000 × *g* pellet containing the membrane-bound replicase was isolated, resuspended in 1 ml of buffer C for each gram of leaf tissue used, and treated for 30 min at 4 C with (i) detergents such as Nonidet P40, Triton X-100, Brij 58, Lubrol W, Tween 80, or deoxycholate in concentrations ranging from 0.1 to 2% or with mixtures of the different detergents; (ii) detergents in combination with high salt (0.25 to 2.0 M KCl), and (iii) high salt (0.25 to 2.0 M KCl). The suspensions were then centrifuged for 60 min at 31,000 × *g*. Several treatments appeared to be very effective in dissolving proteins from the 31,000 × *g* pellet as shown by the reduced size and protein content of the residual pellet. However, there was a considerable loss of replicase activity, recoverable in pellet plus supernatant, sometimes up to 50%. Moreover the results were disappointing with regard to subsequent purification because of the lability of the solubilized replicase. Therefore, we examined another method known to release proteins from membranes (28, 33), namely, subjection of membranes to divalent cation depletion.

The 31,000 × *g* pellet containing the membrane-bound replicase was isolated, resuspended in the Mg^{2+}-deficient buffer B (Fig. 1), and incubated for 60 min at 4 C under continuous stirring. After centrifugation it was found that the distribution of the replicase activity had changed drastically. Table 1 compares the distribution of enzyme activity between pellet and supernatant after washing with Mg^{2+}-deficient or Mg^{2+}-containing buffer. More than 90% of the replicase activity remained bound to membranes in the case of the Mg^{2+}-containing buffer wash. However, after washing the 31,000 × *g* pellet with the Mg^{2+}-deficient buffer B, 70 to 80% of the replicase was released to supernatant 1.

The pellet obtained after the Mg^{2+}-deficient buffer wash showed about the same size as the control and contained almost all of the green material; in contrast, detergents dissolved the membranes almost completely.

When the washing procedure was repeated, a further 40 to 50% of the remaining replicase activity was released. So, by washing twice with the Mg^{2+}-deficient buffer B, more than 80% of the replicase activity was solubilized (Table 1). One of the main features of this solubilization procedure was the rather selective release of replicase. The specific activity of the solubilized enzyme had increased two- to threefold with respect to the membrane-bound enzyme. Moreover, no loss in total enzyme activity occurred as was the case with detergents. Another

TABLE 1. *Distribution of CPMV replicase activity after washing the 31,000 × g pellet containing the membrane-bound replicase with Mg^{2+}-deficient or Mg^{2+}-containing buffer*[a]

Replicase activity after	Washing with Mg^{2+}-deficient buffer		Washing with Mg^{2+}-containing buffer	
	Pellet	Supernatant	Pellet	Supernatant
1st wash	20–30%	70–80%[b]	92%	8%
2nd wash	10–20%	80–90%[c]		

[a] The 31,000 × *g* pellet containing the membrane bound replicase was isolated from 24 g of CPMV-infected Vigna leaves and washed with either 24 ml of buffer B or 24 ml of buffer C as described in Fig 1. The second wash with the Mg^{2+}-deficient buffer was performed with 6 ml of buffer B.

[b] Supernatant 1.

[c] Supernatant 1 plus 2.

striking feature of the solubilized enzyme proved to be its stability. The enzyme could be stored in buffer B at 0 to 4 C for several days without significant loss in activity. RNA synthesis by the solubilized enzyme was slightly stimulated by the addition of template RNA and continued for at least 60 min (Fig. 2). The membrane-bound enzyme did not respond to the addition of template and catalyzed UMP incorporation for about 30 to 40 min as was shown previously (39).

DEAE-Bio Gel chromatography. The combined 31,000 × *g* supernatants containing the released enzyme were used for chromatography on a DEAE-Bio Gel column (Fig. 3). Most of the green color still present in the soluble enzyme preparation moved through the column and characterized the flow-through material. The bulk of the replicase activity eluted at about 0.10 to 0.14 M KCl and comprised the first absorbancy at 280 nm (A_{280}) peak resolved by the KCl gradient. The second A_{280} peak contained mostly nucleic acids as judged by the A_{260}-A_{280} ratio which was greater than 2.0. In most experiments the replicase peak was not symmetrical and skewed to the right yielding a shoulder or sometimes a minor peak (Fig. 3, fractions 52 to 56). This minor peak appeared to contain replicase molecules slightly contaminated with template RNA as indicated by some residual enzyme activity in the absence of added template. However, RNA synthesis directed by the bulk of the replicase was template dependent (Fig. 4). Thus, chromatography of the solubilized replicase on a DEAE-Bio Gel column affords an almost complete separation of replicase from nucleic acids concomitant with the removal of a considerable amount of con-

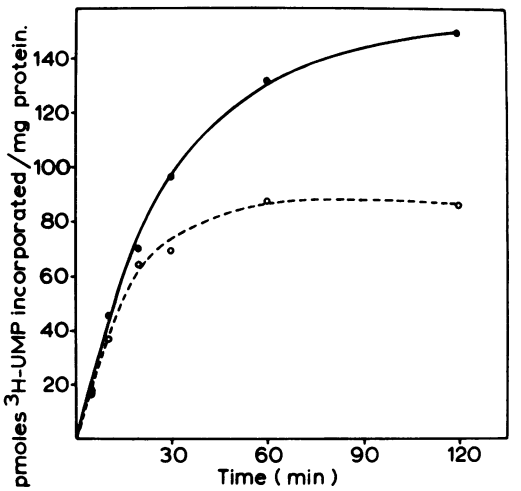

FIG. 2. *Time course of RNA synthesis by the solubilized CPMV replicase before DEAE-Bio Gel chromatography. Membrane-bound replicase was isolated and solubilized as described in Fig. 1. An enzyme reaction mixture (0.72 ml) was incubated under standard assay conditions in the dark in the presence (●) or absence (○) of unfractionated CPMV RNA (75 μg). At the times indicated, two 50-μl aliquots were removed and assayed for acid-insoluble radioactivity.*

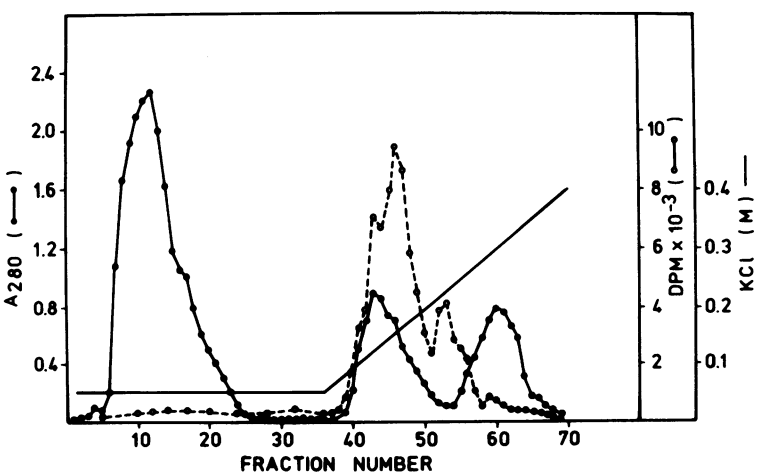

FIG. 3. *DEAE-Bio Gel column chromatography of solubilized CPMV replicase. Solubilized replicase (57 ml, 43 mg of protein) was prepared from 48 g of infected Vigna leaves as described in Fig. 1 and applied to a DEAE-Bio Gel column (1.6 by 15 cm) equilibrated with buffer B. After the flow-through material had emerged, the column was developed with a 108-ml linear 0.05 to 0.4 M KCl gradient in buffer B. Fractions of 2.7 ml were collected at a flow rate of 16 ml/h; 100-μl aliquots of each fraction were assayed for replicase activity for 60 min (○); (●) A_{280} nm; (——) KCl molarity.*

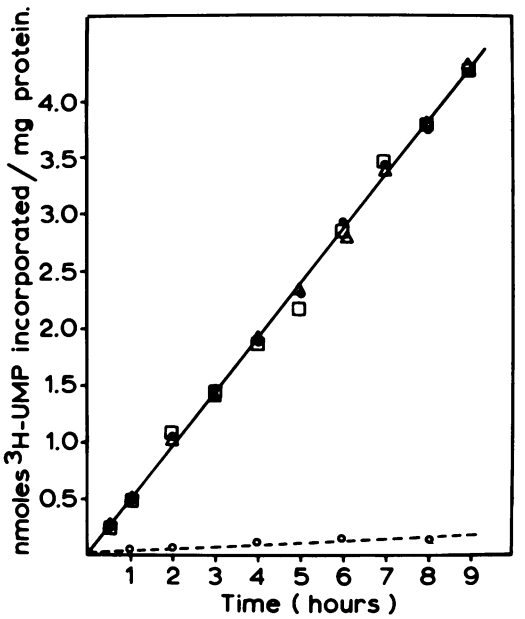

FIG. 4. *Time course of RNA synthesis by the solubilized CPMV replicase after DEAE-Bio Gel chromatography. Membrane-bound replicase from 48 g of infected Vigna leaves was solubilized and purified by DEAE-Bio Gel column chromatography. The appropriate fractions were pooled, precipitated by dialysis against buffer B saturated with $(NH_4)_2SO_4$, and further processed as described in Materials and Methods. A 1.2-ml reaction mixture containing 0.125 ml of enzyme solution (230 μg of protein) was incubated in the dark under standard conditions in the presence of respectively 100 μg of CPMV-B-RNA (●), 100 μg of CPMV-M-RNA (△), 50 μg of CPMV-B-RNA plus 50 μg of CPMV-M-RNA (□), or in the absence of RNA (○). At the times indicated, two 50-μl aliquots were removed and assayed for acid-insoluble radioactivity.*

tamination eluting in the flow through. The profile (Fig. 3) was quite reproducible for more than 10 different experiments and was obtained with two different batches of DEAE-Bio Gel (no. 13270 and 13758). One batch (no. 13639) was inferior because all the nucleic acids coeluted with the proteins in one peak at low ionic strength.

One of the most striking properties of the DEAE-purified enzyme was its capacity to perform RNA synthesis at a linear rate for at least 9 h (Fig. 4), demonstrating a remarkable stability of the DEAE-purified enzyme. The same kinetics of RNA synthesis were obtained with enzyme stored in liquid nitrogen for at least 1 month.

No significant differences occurred when either B-RNA, M-RNA or both RNAs from CPMV were used as template.

DISCUSSION

The solubilization procedure with the Mg^{2+}-deficient buffer B has several features which makes it superior to methods using detergents. (i) More than 80% of the membrane-bound replicase is solubilized without loss of total enzyme activity. (ii) The released enzyme is stable in buffer B for several days at 0 to 4 C without special precautions. (iii) The supernatant containing the released replicase is only slightly colored. Almost all of the green material is retained in the dark green pellet. In contrast, detergents dissolved the membrane pellet almost completely. (iv) The release of the replicase is rather selective, resulting in a two- to threefold increase in specific activity per se. Furthermore, this method is not limited in use to only CPMV-infected leaves. The same procedure has been applied successfully for the solubilization of tobacco mosaic virus replicase and alfalfa mosaic virus replicase from the membranes of infected tobacco leaves (P. M. Romaine and M. Zaitlin; C. M. Clerx-van Haaster and J. F. Bol, personal communication).

Divalent cation depletion of membranes is a well-established method to solubilize ATPases (1, 12, 28, 33). These proteins, which can be easily and selectively detached from the membranes, are thought to be bound to the surface of the lipid bilayer or to surface proteins (28). Most of these proteins contain a large excess of acidic side chains and a low content of hydrophobic residues (28, 33). The divalent cations are thought to be required for binding because they neutralize the repulsive electrostatic charges of the membrane and form salt bridges between carboxyl groups of the proteins and phosphate groups of the phospholipids (28). Mg^{2+} ions were also found to be essential in binding DNA-dependent RNA polymerase to chloroplasts from maize (7). Bottomley et al. (7) found that very low Mg^{2+} concentrations were critical for solubilization of the enzyme. From studies concerning the in vivo replication of the bacteriophage MS2, Haywood (20) concluded that replicase components or factors required for complementary-strand synthesis are bound to membranes even in the absence of divalent cations and that the polymerase is no longer bound to these factors during the synthesis of the bulk of the progeny single-stranded RNA.

Our results demonstrate that CPMV repli-

case can be released from the membranes with a Mg^{2+}-deficient buffer and therefore may resemble the ATPases with respect to the kind of binding. Whether the replicase is bound directly to the membranes or to RNA chains which, in turn, are attached to the membranes is not clear. We have to emphasize that the leaves were homogenized in a Mg^{2+}-deficient buffer. In spite of this the replicase was found to be associated with the membrane. Just after the isolation of the 31,000 $\times$ g pellet the addition of Mg^{2+} ions appeared to be essential for the prolonged association with the membranes. So the absence of Mg^{2+} ions during the isolation of the 31,000 $\times$ g pellet did not essentially alter the attachment to the membranes probably because of a high Mg^{2+} pool in the plants. It even facilitated the solubilization procedure because the replicase could now be released directly in contrast to the ATPase-containing membranes which need to be washed five times or more before they release the enzyme (28).

Purification and removal of endogenous template RNA by DEAE-Bio Gel chromatography did improve the ability of the replicase to perform RNA synthesis considerably. Before the DEAE step, RNA synthesis directed by the soluble enzyme was only partially stimulated by RNA and leveled off after about 60 min, whereas RNA synthesis directed by the DEAE enzyme continued for at least 9 h. Whether the DEAE enzyme is capable of reinitiating and producing full-length single-stranded minus and plus strands has still to be determined. Experiments relating to these questions are presently in progress.

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LITERATURE CITED

1. **Abrams, A.** 1965. The release of bound adenosine triphosphatase from isolated bacterial membranes and the properties of the solubilized enzyme. J. Biol. Chem. **240**:3675-3681.
2. **Arlinghaus, R. B., and J. Polatnick.** 1969. The isolation of two enzyme-ribonucleic acid complexes involved in the synthesis of foot-and-mouth disease virus ribonucleic acid. Proc. Natl. Acad. Sci. U.S.A. **62**:821-828.
3. **August, J. T., A. K. Banerjee, L. Eoyang, M. T. Franze de Fernandez, K. Hori, C. H. Kuo, U. Rensing, and L. Shapiro.** 1968. Synthesis of bacteriophage Qβ RNA. Cold Spring Harbor Symp. Quant. Biol. **28**:73-81.
4. **Baltimore, D.** 1964. In vitro synthesis of viral RNA by the polio virus RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **51**:450-456.
5. **Baltimore, D., H. J. Eggers, R. M. Franklin and I. Tamm.** 1963. Polio virus-induced RNA polymerase and the effects of virus specific inhibitors on its production. Proc. Natl. Acad. Sci. U.S.A. **49**:843-849.
6. **Baltimore, D., and R. M. Franklin.** 1963. A new ribonucleic acid polymerase appearing after Mengo virus infection of L-cells. J. Biol. Chem. **238**:3395-3400.
7. **Bottomley, W., H. J. Smith, and L. Bogorad.** 1971. RNA polymerase of maize: partial purification and properties of the chloroplast enzyme. Proc. Natl. Acad. Sci. U.S.A. **68**:2412- -2416.
8. **Bové, J. M., C. Bové, M. J. Rondot, and G. Morel.** 1965. Chloroplasts and virus-RNA synthesis. Biochemistry of chloroplasts, p. 329-339. *In* T. W. Goodwin (ed.), Proc. NATO Adv. Stud. Inst. Aberyst-wyth, vol. 2, Academic Press Inc., London.
9. **Bradley, D. W., and M. Zaitlin.** 1971. Replication of tobacco mosaic virus. II. The in vitro synthesis of high molecular weight virus-specific RNAs. Virology **45**:192-199.
10. **Brishammer, S., and N. Juntti.** 1974. Partial purification and characterization of soluble TMV replicase. Virology **59**:245-253.
11. **Caliguiri, L. A., and A. G. Mosser.** 1971. Proteins associated with the polio virus RNA replication complex. Virology **46**:375-386.
12. **Carreira, J., J. A. Leal, M. Rojas, and E. Muñoz.** 1973. Membrane ATPase of Escherichia coli K12. Selective solubilization of the enzyme and its stimulation by trypsin in the soluble and membrane-bound states. Biochim. Biophys. Acta **307**:541-556.
13. **Clark, G. L., K. W. C. Peden, and R. H. Symons.** 1974. Cucumber mosaic virus-induced RNA polymerase: partial purification and properties of the template-free enzyme. Virology **62**:434-443.
14. **Ehrenfeld, E., J. V. Maizel, and D. F. Summers.** 1970. Soluble RNA polymerase complex from polio virus-infected HeLa cells. Virology **40**:840-846.
15. **Federoff, N. V., and N. Zinder.** 1972. Properties of the phage f2 replicase. I. Optimal conditions for replicase activity and analysis of the polynucleotide product synthesized in vitro. J. Biol. Chem. **247**:4577-4585.
16. **Federoff, N. V., and N. Zinder.** 1972. Properties of the phage f2 replicase. II. Comparative studies on the ribonucleic acid-dependent and poly(C)-dependent activities of the replicase. J. Biol. Chem. **247**:4586-4592.
17. **Friedman, R. M., J. G. Levin, P. M. Grimley, and I. K. Berezesky.** 1972. Membrane-associated replication complex in arbo virus infection. J. Virol. **10**:504-515.
18. **Girard, M., D. Baltimore, and J. E. Darnell.** 1967. The polio virus replication complex: site for synthesis of polio virus RNA. J. Mol. Biol. **24**:59-74.
19. **Hadidi, A., and H. Fraenkel-Conrat.** 1973. Characterization and specificity of soluble RNA polymerase of brome mosaic virus. Virology **52**:363-372.
20. **Haywood, A. M.,** 1973. Two classes of membrane binding of replicative RNA of bacteriophage MS2. Proc. Natl. Acad. Sci. U.S.A. **70**:2381-2385.
21. **Horton, E., S. L. Liu, L. Dalgarno, E. M. Martin, and T. S. Work.** 1964. Development of ribonucleic acid polymerase in cells infected with Encephalomyocarditis virus single- and double-stranded RNA by the isolated polymerase. Nature (London) **204**:247-250.
22. **Lazarus, L. H., and R. Barzilae.** 1974. Association of foot-and-mouth disease virus replicase with RNA template and cytoplasmic membranes. J. Gen. Virol. **23**:213-218.
23. **Martin, E. M., and J. A. Sonnebend.** 1967. Ribonucleic acid polymerase catalyzing synthesis of double-stranded arbovirus ribonucleic acid. J. Virol. **1**:97-109.
24. **Mouches, C., C. Bové, and J. M. Bové.** 1974. Turnip

yellow mosaic virus-RNA replicase: partial purification of the enzyme from the solubilized enzyme-template complex. Virology **58**:409-423.

25. **Mouches, C., C. Bové, C. Rarreau, and J. M. Bové.** 1975. TYMV-RNA replicase, p. 109-120. *In* Proceedings of the INSERM-EMBO workshop on in vitro transcription and translation of viral genomes, vol. 47. Editions INSERM, Paris.
26. **Palmenberg, A., and P. Kaesberg.** 1974. Synthesis of complementary strands of heterologous RNAs with Qβ replicase. Proc. Natl. Acad. Sci. U.S.A. **71**:1371-1375.
27. **Ralph, R. K., and S. J. Wojcik.** 1969. Double-stranded tobacco mosaic virus RNA. Virology **37**:276-282.
28. **Razin, S.** 1972. Reconstitution of biological membranes. Biochim. Biophys. Acta **265**:241-296.
29. **Rosenberg, H., B. Diskin, L. Oron, and A. Traub.** 1972. Isolation and subunit structure of polycytidylate-dependent RNA polymerase of encephalomyocarditis virus. Proc. Natl. Acad. Sci. U.S.A. **69**:3815-3819.
30. **Semal, J., and R. I. Hamilton.** 1968. RNA synthesis in cell-free extracts of barley leaves infected with brome-grass mosaic virus. J. Gen. Virol. **10**:79-89.
31. **Spiegelman, S., N. R. Pace, D. R. Mills, R. Levisohn, T. S. Eikhom, M. M. Taylor, R. L. Peterson, and D. H. L. Bishop.** 1968. The mechanism of RNA replication. Cold Spring Harbor Symp. Quant. Biol. **28**: 101-124.
32. **Stavis, R. L., and J. T. August.** 1970. The biochemistry of RNA bacteriophage replication. Annu. Rev. Biochem. **39**:527-560.
33. **Steck, T. L., and C. F. Fox.** 1972. Membrane proteins, p. 27-76. *In* C. F. Fox and A. Keith (ed.), Membrane molecular biology. Sinauer Associates Inc. Publishers, Stamford, Conn.
34. **Traub, A., H. Rosenberg, B. Diskin, and E. Kalmar.** 1975. Isolation and properties of the replicase of encephalomyocarditis virus, p. 135-142. *In* Proceedings of the INSERM-EMBO workshop on in vitro transcription and translation of viral genomes, vol. 47. Editions INSERM, Paris.
35. **Van Kammen, A.** 1967. Purification and properties of the components of cowpea mosaic virus. Virology **31**:633-642.
36. **Weening, C. J., and J. F. Bol.** 1975. Viral RNA replication in extracts of Alfalfa mosaic virus infected Vicia Faba. Virology **63**:77-83.
37. **Weissmann, C., M. A. Billeter, H. M. Goodman, J. Hindley, and H. Weber.** 1973. Structure and function of phage RNA. Annu. Rev. Biochem. **42**:303-328.
38. **Weissmann, C., G. Feix, and H. Slor.** 1968. In vitro synthesis of phage RNA: the nature of the intermediates. Cold Spring Harbor Symp. Quant. Biol. **28**:83-100.
39. **Zabel, P., H. Weenen-Swaans, and A. van Kammen.** 1974. In vitro replication of cowpea mosaic virus RNA. I. Isolation and properties of the membrane-bound replicase. J. Virol. **14**:1049-1055.
40. **Zaitlin, M., C. T. Duda, and M. A. Petti.** 1973. Replication of tobacco mosaic virus. Virology **53**:300-311.