In Vitro Replication of Cowpea Mosaic Virus RNA

I. Isolation and Properties of the Membrane-Bound Replicase¹

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Received for publication 9 July 1974

A fraction which contained the membrane-bound cowpea mosaic virus RNA replicase was isolated from cowpea mosaic virus-infected cowpea leaves. The replicase activity appeared on day 1 after inoculation, then increased to reach a maximal on day 4. The increase in enzyme activity preceded the most-rapid virus multiplication. The membrane-bound replicase activity was almost completely insensitive to actinomycin D and DNase. The corresponding fraction from healthy leaves had no RNA-dependent RNA polymerase activity. The viral RNA synthesis in vitro proceeded linearly for 20 min and required all four ribonucleoside triphosphates and Mg^{2+} ions. Mn^{2+} was a poor substitute for Mg^{2+} . The reaction was optimal at pH 8.2. During the whole period of RNA synthesis the in vitro synthesized RNA was at least 70% resistant against RNase in $2\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate), but completely digestible by RNase in $0.1\times$ SSC. Analysis of the products by sucrose gradient centrifugation followed by treatment of separate fractions with RNase demonstrated that both single- and double-stranded RNA were present. Double-stranded RNA sedimented at about 20S, with a shoulder at 16S to 17S. A minor part of the double-stranded RNA sedimented below 10S. Single-stranded RNA sedimented with the same rate as the two viral RNAs, 26S and 34S.

The genome of cowpea mosaic virus (CPMV) consists of two single-stranded RNA molecules which are separately encapsidated. Purified preparations of the virus contain two nucleoprotein particles (M and B) of similar size and with the same capsid protein composition but with different contents of RNA. Empty protein capsids (T) that lack RNA are also found. Both nucleoprotein particles, or both RNAs, are necessary for virus multiplication (6, 21).

The molecular weights of the RNAs sedimenting at 26S and 34S are 1.37×10^6 for M RNA and 2.02×10^6 for B RNA (L. Reijnders, A. M. J. Aalbers, A. van Kammen, and R. W. J. Thuring, 1974, *Virology*, in press). Hybridization studies and genetic analysis of some mutants have shown that the RNAs have no base sequences in common and that each RNA represents a unique piece of the CPMV genome (6, 21). Double-stranded RNA, which is specific for CPMV, has been isolated from CPMV-infected cowpea plants. Two double-stranded RNAs have been found, one corresponds to double-stranded B RNA and the other to double-stranded m RNA, suggesting that each RNA is synthesized separately (18, 19). Recently, the replication of CPMV RNA has been shown to be

associated with vesicular membranes of a cytopathic structure in CPMV-infected cells (1; G. A. De Zoeten, J. M. H. Assink, and A. van Kammen, *Virology*, in press). We attempted to isolate the CPMV-specific RNA replicase from infected leaves to gain more insight into the replication mechanism and the regulation of the synthesis of the two viral RNAs. We describe the isolation and properties of a crude CPMV RNA replicase which is bound to membranes. The enzyme complex catalyzed in vitro the incorporation of [³H]UMP into both double-stranded as well as single-stranded viral RNAs.

MATERIALS AND METHODS

Materials. Unlabeled ribonucleoside triphosphates, phosphoenol pyruvate (trisodium salt), RNase A (from bovine pancreas, 5 $\times$ crystallized type 1A), and RNase T1 (from *Aspergillus oryzae* grade III) were purchased from Sigma Chemical Co., St. Louis, Mo. Pancreatic DNase I (electrophoretically pure) was obtained from Worthington Biochemical Corp. Freehold, N.J.; [5-³H]UTP (ammonium salt) was obtained from The Radiochemical Center, Amersham; pyruvate kinase was obtained from Boehringer Mannheim GmbH (Mannheim, Germany), and Extran was obtained from Merck & Co. Actinomycin D was a generous gift from Merck, Sharp and Dohme. Solvane-100 sample solubilizer was obtained from Packard Instrument Co.

¹ Dedicated to J. Retèl.

Buffers. Buffer A contained 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), 0.01 M KCl, 0.01 M $MgCl_2$, 0.001 M EDTA, 5% (vol/vol) glycerol, and 0.003 M β -mercaptoethanol. Buffer C contained 0.01 M Tris-hydrochloride (pH 7.2), 0.1 M NaCl, 0.01 M EDTA, and 0.5% sodium dodecyl sulfate (SDS).

Virus and plants. *Vigna unguiculata* (L.) Walp. var. "Blackeye Early Ramshorn" were grown in the greenhouse. Eight to ten days after sowing, the primary leaves were inoculated with crude sap from plants infected with a yellow strain isolate of CPMV. After inoculation, the plants were transferred to a growth chamber and further grown at 30 C with 75% relative humidity and continuous light. The leaves were harvested 4 days after inoculation (Fig. 1).

Virus was purified as described by Van Kammen (20), and the RNA was extracted with SDS and a mixture (1:1, vol/vol) of chloroform and phenol-cresol (12). The virus growth curve was determined as described by Van Griensven (Ph.D. thesis, Agricultural University, Wageningen, The Netherlands 1970).

Isolation of membrane-bound CPMV replicase. All operations were carried out at 4 C; the glassware used in the preparation of extracts and the assays of enzyme activity was heated at 150 C overnight. Centrifuge tubes were stored in 2% Extran and washed extensively with double-distilled water before use.

Portions (3 g) of freshly harvested, infected leaves from which the midribs were removed, were rinsed with double-distilled water, wiped with absorbent tissue, and homogenized with 35 ml of buffer A in a chilled mortar. The homogenate was filtered through two layers of "Miracloth," and the filtrate was centrifuged at $1,000 \times g$ for 10 min in a Sorvall SS34 rotor. The supernatant was adjusted to 20% (vol/vol) gly-

cerol and centrifuged at $31,000 \times g$ for 30 min; the resultant supernatant was discarded. The green pellet was washed twice by suspending it in buffer B with the aid of a Thomas homogenizer and sedimenting it at $31,000 \times g$ for 30 min; it was finally suspended in 0.5 ml of buffer B for each gram of tissue used. This suspension was used as the crude bound replicase.

Replicase assay. The standard assay mixture (total volume of 0.28 ml) contained 0.05 M Tris-hydrochloride (pH 8.2), 5% glycerol, 0.01 M $MgCl_2$, 0.01 M KCl, 0.01 M $(NH_4)_2SO_4$, 0.1 μ mol each of ATP, GTP, and CTP, 0.01 μ mol of UTP, 5 μ Ci of [3H]UTP (final specific activity of 0.4 Ci/mmol), 0.001 M EDTA, 1 μ mol of phosphoenolpyruvate, 10 μ g of pyruvate kinase, actinomycin D (20 μ g/ml), 3 mM β -mercaptoethanol, and enzyme preparation. The enzyme preparation was preincubated with actinomycin D for 5 min at 30 C, and then the reaction was started by the addition of the other ingredients. Incubation was at 30 C for 30 min or for the designated period of time in a shaking waterbath. The reaction was stopped by the addition of two 50- μ l samples to 5 ml of ice-cold 5% trichloroacetic acid which contained 2% $Na_2P_2O_7$, 2% NaH_2PO_4 , and 0.05% uridine. Bovine serum albumine (350 μ g per sample) was added as a carrier, and after 15 min at 0 C the acid-insoluble precipitates were collected on Whatman GF/A filters, washed five times with 5-ml portions of the trichloroacetic acid-phosphate-uridine solution, washed twice with 5 ml of 80% ethanol, and finally washed with 5 ml of diethyl ether (4). The filters were treated with 0.75 ml of a solubene-water mixture (9:1) for 2 h at 50 C to solubilize the precipitates and were subsequently counted with 10 ml of toluene-permablend scintillation cocktail containing acetic acid (2 ml/liter) (2). Under these conditions the counting efficiency for tritium was 40% in a Packard Tricarb scintillation counter.

Except where noted otherwise, the results are expressed as picomoles of [3H]UMP incorporated per milligram of protein per 30 min of incubation time. The values given are corrected for zero time values. Protein was measured by the procedure of Lowry et al. (9) with crystalline bovine serum albumin as a standard.

Sucrose-SDS density gradient centrifugation. The reaction mixture was made 2% in SDS, 1% in Brij 58, 1% in deoxycholate (DOC), and 10 mM in EDTA (SDS-Brij-DOC-EDTA detergent mixture) and incubated at 30 C for 20 min. A sample of the extract was directly layered onto a 34-ml linear 15 to 30% (wt/vol) sucrose density gradient in buffer C and centrifuged in an SW27 rotor at 22,500 rpm for 18 h at 20 C. Fractions were collected from the bottom. Two equal samples were taken from each fraction. One was treated with RNase (4 μ g of RNase A per ml + 4 U of T1 RNase per ml) in 2.5 ml of $2 \times$ SSC for 30 min at 30 C; the other was incubated for an equivalent period without RNase. Both samples were then made 5% in trichloroacetic acid, and, after the addition of carrier protein, they were processed as described above. CPMV RNAs with sedimentation coefficients of 26S and 34S were used as external marker.

FIG. 1. Time course of appearance of bound CPMV replicase activity in *Vigna* leaves infected with CPMV. Eight-day-old *Vigna* plants were inoculated and incubated with noninoculated control plants in a growth chamber. At daily intervals primary leaves were harvested and the bound replicase was isolated and assayed.

RESULTS

When an homogenate of CPMV-infected leaves was centrifuged at $31,000 \times g$, after a preliminary low-speed centrifugation to remove nuclei and chloroplasts, the sedimented material contained an RNA polymerase activity which was not present in the corresponding fraction from healthy leaves. The activity depended on the period between inoculation and harvesting of the leaves (Fig. 1). The virus-specific RNA polymerase activity, which will be referred to as bound CPMV RNA replicase activity, was detectable on day 1 after inoculation and increased rapidly to reach a maximum after 4 days. The activity then declined. The increase of CPMV RNA replicase preceded the rapid multiplication of CPMV in the leaves (Fig. 1). The replicase activity which developed depended on the age of the primary leaves at infection. Thus the activity which developed in 16-day-old primary leaves was only 60% of the activity developed in 8- to 10-day-old leaves, although the rates of increase were the same. In all subsequent experiments, primary leaves were inoculated 8 to 10 days after sowing and were harvested 4 days later.

Characteristics of the replicase reaction in vitro. (i) Requirements of the bound CPMV replicase. The omission of each of the three unlabeled ribonucleoside triphosphates (ATP, GTP, or CTP) from the reaction mixture caused a strong decrease in [^3H]UMP incorporation activity (Table 1). If all three unlabeled ribonucleoside triphosphates were left out, the replicase activity was almost completely suppressed. It was necessary to wash the constituents of the $31,000 \times g$ pellet twice before incubation to obtain this high degree of dependency. When the washing procedure was omitted, the incor-

poration of [^3H]UMP in the absence of the three added unlabeled ribonucleoside triphosphates was about 20% of that in the complete reaction mixture. These results suggest that there is a considerable endogenous nucleotide pool in the unwashed $31,000 \times g$ pellet.

The replicase was routinely assayed in the presence of actinomycin D. The RNA synthesis was only slightly higher in the absence of actinomycin D (Table 1). Moreover, when actinomycin D was replaced by DNase (30 $\mu\text{g}/\text{ml}$) a very similar incorporation of [^3H]UMP occurred. These results demonstrate that the $31,000 \times g$ fraction of the leaves had very little DNA-dependent RNA synthesizing activity. In contrast, the strong RNA polymerase activity in a fraction of the homogenate which sedimented in 10 min at $1,000 \times g$ was almost completely inhibited by actinomycin D. Clearly, the fractionation procedure achieved a good separation between the virus-specific and the host-specific RNA synthesis.

The bound CPMV RNA replicase required Mg^{2+} ions for its activity (Table 1), and the optimal concentration was in the broad range of 8 to 20 mM (Fig. 2). Higher concentrations of Mg^{2+} (up to 40 mM) caused only a small decrease in enzyme activity. Mn^{2+} could only partially replace Mg^{2+} (Table 1 and Fig. 2). At optimal concentrations the replicase activity was five times higher with Mg^{2+} than with Mn^{2+} ions. RNA synthesis in the presence of 10 mM Mg^{2+} was inhibited 40% by 2 mM Mn^{2+} (Table 1).

(ii) Effect of monovalent ions and pH. The enzyme activity was not affected by up to 60 mM $(\text{NH}_4)_2\text{SO}_4$, but higher concentrations caused inhibition (Fig. 3). The activity was not

TABLE I. Requirements of the bound CPMV replicase

Reaction conditions	Percent of control
Complete*	100
- ATP	22
- GTP	20
- CTP	24
- (ATP, GTP, CTP)	6
- Mg^{2+}	5
- Mg^{2+} + Mn^{2+} (2 mM)	20
+ Mg^{2+} (10 mM) + Mn^{2+} (2 mM)	59
- Actinomycin D	108
- Actinomycin D + DNase (30 $\mu\text{g}/\text{ml}$)	98

* The complete reaction mixture was as described in Materials and Methods.

FIG. 2. Effect of Mg^{2+} and Mn^{2+} ions on the bound CPMV replicase activity. Assay conditions were as described except for the concentration of divalent cation.

affected by KCl at concentrations of up to 0.4 M. The enzyme activity was maximal at pH 8.2. Between pH 7.9 and 8.5 the enzyme displayed at least 90% of its activity (Fig. 4).

(iii) **The time course of the bound CPMV replicase reaction.** RNA synthesis by the CPMV RNA replicase was linear for 20 min, but declined to zero 30 to 40 min after incubation (Fig. 5). The host cell activity in the $31,000 \times g$ fraction of healthy leaves was negligible (Fig. 5).

The products of the bound-replicase reaction. (i) **Synthesis of RNase-resistant RNA.** The product of the RNA replicase reaction was characterized by determining the degree of RNase resistance of the synthesized RNA at various reaction times. Equal samples of the reaction mixture were incubated in 2 ml of $2 \times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate) (high salt) and $0.1 \times$ SSC (low salt) with 25 μ g of RNase A and 25 U of RNase T1 per ml for 30 min at 30 C. At least 70% of the RNA synthesized throughout the reaction consisted of mate-

FIG. 3. Effect of $(\text{NH}_4)_2\text{SO}_4$ on the bound CPMV replicase activity. Assay conditions were as described except for the concentration of $(\text{NH}_4)_2\text{SO}_4$.

FIG. 4. Effect of pH on the bound CPMV replicase activity. Assay conditions were as described except for the pH of the assay mixture.

FIG. 5. Time course of RNA synthesis by the bound CPMV replicase. A batch enzyme reaction mixture was incubated under standard assay conditions. At the indicated times, two 50- μ l samples were removed, immediately added to 5 ml of 5% trichloroacetic acid containing 2% $\text{Na}_2\text{P}_2\text{O}_7$, 2% NaH_2PO_4 , and 0.05% uridine, and further processed as described. O, Activity of a corresponding fraction from healthy leaves.

rial resistant to RNase in high salt (Fig. 6). In contrast, all of the RNA was made soluble by RNase in low salt. In some experiments the portion of RNase-resistant material was somewhat higher during the first 5 min of incubation than later. As a control, samples of the reaction mixture were incubated in $2 \times$ SSC without RNase. No loss of acid-precipitable counts was observed in these incubations. These results suggest that newly made RNA is, for the most part, present in partially double-stranded replicative structures.

(ii) **Analysis by sucrose-SDS-density gradient centrifugation.** The products of the replicase reaction were also analysed by sucrose gradient centrifugation. A complete reaction mixture was incubated for 30 min and then treated with the SDS-Brij-DOC-EDTA detergent mixture for 20 min at 30 C. A sample was layered onto a linear 15 to 30% sucrose gradient containing 0.5% SDS. A typical sedimentation profile of the labeled products is shown in Fig. 7. The bulk of the synthesized RNA sedimented at about 20S, with a shoulder at 17S. There was some material sedimenting slower than 10S and two minor peaks with sedimentation coefficients of 26S and 34S. The S values of the two fastest sedimenting peaks corresponded with those of CPMV middle-component and bottom-component RNA, respectively, used as external markers on a separate gradient. The RNA in the fractions of the sucrose gradient was tested for RNase resistance. The RNA sedimenting at 20S, 17S, and slower was found to be partially resistant to RNase; the 26S and 34S RNAs were completely hydrolyzed (Fig. 7). As the S values

FIG. 6. Time course of total RNA synthesis and of the fraction resistant to RNase. A 2-ml reaction mixture was incubated under standard assay conditions. At the indicated times two 50- μ l portions were removed. One sample was immediately assayed for total acid-insoluble radioactivity (●); the second portion was incubated in 2 ml of $2\times$ SSC containing 25 μ g of RNase A per ml and 25 U of RNase T1 per ml for 30 min at 30 C (○); the third portion was heated with RNase A (25 μ g/ml) and RNase T1 (25 U/ml) in 2 ml of $0.1\times$ SSC (■); and the last sample was incubated in 2 ml of $2\times$ SSC without RNase (Δ). The reactions were stopped by adding trichloroacetic acid to a final concentration of 5%. Carrier protein was added, and the acid-insoluble precipitates were processed.

FIG. 7. Sucrose-SDS density gradient centrifugation of replicase reaction product. The reaction mixture contained (in 0.84 ml) 0.75 ml of enzyme preparation, 0.75 μ mol each of ATP, GTP, and CTP, 0.075 μ mol of [3 H]UTP (final specific activity of 0.8 Ci/mmol), Macaloid (0.5 mg/ml, previously incubated at 40 C for 90 min), actinomycin D (20 μ g/ml), and the other ingredients as described. The reaction mixture was incubated for 30 min at 30 C and subsequently deproteinized by adjustment to 2% SDS, 1% Brij 58, 1% DOC, and 10 mM EDTA. A portion of the extract (0.75 ml) was layered onto a 34-ml 15 to 30% linear sucrose gradient in buffer C and centrifuged at 20 C and 22,500 rpm for 18 h in an SW 27 rotor. Fractions

of 20S and 17S agree reasonably with those expected for double-stranded bottom-component and middle-component RNA, respectively, it was concluded that the membrane-bound CPMV replicase produced, *in vitro*, mainly double-stranded RNA but also an appreciable amount of single-stranded virus RNA. The sedimentation profile shown in Fig. 7 was very characteristic and reproducible for the RNA products obtained by treatment of the reaction mixture with the SDS-Brij-DOC-EDTA detergent mixture and direct layering onto the sucrose-SDS-gradients. A similar profile was obtained when the RNA was extracted from the reaction mixture with SDS and a mixture of chloroform and phenol-cresol (1:1) (12). Extraction of the RNA by SDS and phenol-cresol without chloroform resulted, however, in low recoveries, and very little of the material extracted had a sedimentation coefficient greater than 15S.

DISCUSSION

Our results show that CPMV-infected cowpea leaves contain an RNA-dependent RNA polymerase activity which appears to be closely bound with cytoplasmic membranes, and which is able to synthesize *in vitro* both double-stranded and single-stranded viral RNA species. The fraction of cell constituents sedimenting at $31,000 \times g$ for 30 min from a leaf homogenate contains most of the cytoplasmic membranes, and with CPMV-infected leaves it also contains the specific RNA polymerase activity. Recently it was demonstrated that the cytoplasm of CPMV-infected leaf cells contains a characteristic cytopathic structure consisting of vesicular membranes embedded in amorphous electron-dense material (De Zoeten et al., *Virology*, in press; J. M. H. Assink, Ph.D. thesis, Agricultural University, Wageningen, The Netherlands, 1974). It was further shown that the replicative form of CPMV RNA is associated with the vesicles of the cytopathic structure in which a rapid *in vivo* incorporation of [3 H]uridine took place. The replication of CPMV RNA therefore appeared to be associated with the vesicles of the cytopathic structure. The occurrence of *in vitro* RNA polymerase activity in the membrane fraction of CPMV-infected leaves confirmed this assumption. The RNA polymerase activity was virus specific as shown by its appearance and increase after inoculation of cowpea leaves

were collected from below and two equal portions were assayed for total RNA (●) and RNase-resistant RNA (○).

with CPMV. Furthermore, the RNA polymerase incorporated [^3H]UMP into RNase-resistant double-stranded RNA and also produced labeled single-stranded middle- and bottom-component CPMV RNA. By chopping the infected leaves, it has been possible to isolate the cytopathic structures more or less intact (De Zoeten et al., *Virology*, in press). The structures sedimented at $1,000 \times g$ for 15 min together with the nuclei and chloroplasts. Homogenization by mortar and pestle resulted in a replicase-containing fraction which was only slightly contaminated by nuclear or chloroplast RNA polymerase activity. More than 90% of the RNA polymerase activity in the $31,000 \times g$ pellet was resistant to actinomycin D and insensitive to DNase, indicating that it contained only a minor contamination from the DNA-dependent RNA polymerase of the cell. In contrast, the $1,000 \times g$ pellet from a homogenate of both infected and healthy leaves contained a high [^3H]UMP incorporation activity, which was almost completely inhibited by actinomycin D.

The CPMV-replicase had some features in common with other plant viral RNA replicases. (i) The enzyme was found in a membrane fraction (3, 8, 15). (ii) The rapid increase of the replicase activity preceded the maximal virus multiplication (13, 16). (iii) The enzyme required Mg^{2+} ions for its activity, and Mn^{2+} ions are a poor substitute (5, 11, 22). (iv) The RNA synthesis proceeded linearly for about 20 min and then leveled off (3, 8, 17). (v) Most of the in vitro synthesized RNA was resistant to RNase in high salt ($2 \times \text{SSC}$) (8, 14, 22). It is notable that the membrane-bound CPMV replicase also produced some single-stranded middle- and bottom-component RNA. It seems premature to conclude that these single-stranded viral RNAs were the result of complete de novo synthesis in vitro. The possibility should be kept in mind that the replicase only completed by elongation the synthesis of viral RNA strands which were then released from the replicase-template complex. Such single-stranded viral RNAs would have radioactivity over a variable length at one end, depending on the site where the elongation of the pre-existing strand was started. This has not been demonstrated nor excluded in our experiments. Neither is there any indication for reinitiation of RNA chain synthesis. Laflèche et al. (8) demonstrated with turnip yellow mosaic virus that, in vitro, the RNA replicase only elongated pre-existing RNA strands. No release of single-stranded RNA occurred in that system. The same probably holds for the in vitro tobacco mosaic virus RNA replicase reaction, as

suggested by Bradley and Zaitlin (3). The synthesis of single-stranded plant viral RNAs in vitro has been reported for the membrane-bound brome mosaic virus RNA replicase (7).

However, the labeled product of the CPMV replicase consisted predominantly of partially RNase-resistant RNA sedimenting at 20S and 17S. Previous studies (18, 19) have demonstrated that there are two size classes of double-stranded RNA, corresponding with double-stranded middle- and bottom-component RNA of CPMV. The in vitro labeled 20S and 17S RNase-resistant RNA therefore represented replicative structures corresponding to each of the two viral RNAs.

The significance of the partly RNase-resistant RNA sedimenting below 10S is not clear. Such RNA has not been noted in preparations of double-stranded RNA from CPMV-infected leaves (18, 19). The RNA sedimenting below 10S might have arisen as a by-product of the in vitro synthesis. For example, the RNA synthesized in vitro by the RNA polymerase induced by cucumber mosaic virus in cucumber cotyledons consisted of low-molecular-weight double-stranded RNA; however, no high-molecular-weight RNA was found, making the relationship of these RNA species to the replication mechanism obscure (10). The slowly sedimenting RNase-resistant RNA might also be due to some limited degradation of the 20S material during the extraction procedure. The nature of the products was found to depend to some extent on the extraction procedure. We preferred to extract the RNA from the reaction mixture before sucrose gradient centrifugation with the SDS-Brij-DOC-EDTA detergent mixture because it gave highly reproducible results and the recovery of labeled RNA was very high (more than 90%). In preliminary experiments, the RNA was extracted from the reaction mixture by means of phenol-cresol and SDS. However, this method gave low recoveries and, in addition, no RNA sedimented faster than 15S. This might indicate that degradation can occur to a variable extent during extraction. The true nature of the partially RNase-resistant RNA sedimenting slower than 10S and also the other RNA components found in the gradient require further investigation. However, our results show that CPMV-infected *Vigna* leaves can provide an available in vitro system to analyze the replication of a multicomponent plant RNA virus.

ACKNOWLEDGMENTS

We thank Ine Jongen-Neven for her skilful and patient technical assistance and S. G. Maynew for his help in correcting the English.

This investigation was supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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