Chem. 250, 3951-3959.
Howard, F. B., Frazier, J., & Miles, H. T. (1976) *Biochemistry 15,* 3783-3795.
Ikeda, K., Frazier, J., & Miles, H. T. (1970) *J. Mol. Biol. 54,* 59-84.
Lord, R. C., & Thomas, G. J., Jr. (1967) *Spectrochim. Acta 23A,* 2551-2591.
Lowe, M. J., & Schellman, J. A. (1972) *J. Mol. Biol. 65,* 91-109.
Miles, H. T. (1958) *Biochim. Biophys. Acta 30,* 324-328.
Miles, H. T. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51,* 1104-1109.
Miles, H. T. (1971) *Proced. Nucleic Acid Res. 2,* 205-232.
Miles, H. T., & Frazier, J. (1964) *Biochem. Biophys. Res. Commun. 14,* 21-28.
Miles, H. T., Lewis, T. P., Becker, E. D., & Frazier, J. (1973) *J. Biol. Chem. 248,* 1115-1117.
Morikawa, K., Tsuboi, M., Takabashi, S., Kyogoku, Y., Mitsui, Y., Iitaka, Y., & Thomas, G. J., Jr. (1973) *Biopolymers 12,* 799-816.
Ross, P. D., & Scruggs, R. L. (1965) *Biopolymers 3,* 491-496.
Shimanouchi, T., Tsuboi, M., & Kyogoku, Y. (1964) *Adv. Chem. Phys. 7,* 435-498.
Sober, H. A., Ed. (1966) *Handbook of Chemistry and Physics,* p D157, Chemical Rubber Publishing Co., Cleveland, Ohio.
Sutherland, G. B. B. M., & Tsuboi, M. (1957) *Proc. R. Soc. London, Ser. A 239,* 446-463.
Thomas, G. J., Jr. (1969) *Biopolymers 7,* 325-334.
Thrierr, J. C., Deubel, V., & Leng, M. (1972) *Biochimie 58,* 1115-1119.
Tsubor, M. (1969) *Appl. Spectrosc. Rev. 3,* 45-90.
Zimmerman, S. B. (1976) *J. Mol. Biol. 101,* 563-565.

Coat Protein Binds to the 3′-Terminal Part of RNA 4 of Alfalfa Mosaic Virus†

Corrie J. Houwing and E. M. J. Jaspars*

ABSTRACT: All four RNAs of alfalfa mosaic virus contain a limited number of sites with a high affinity for coat protein [Van Boxsel, J. A. M. (1976), Ph.D. Thesis, University of Leiden]. In order to localize these sites in the viral RNAs, RNA 4 (the subgenomic messenger for coat protein) was subjected to a very mild digestion with ribonuclease T1. The ten major fragments, apparently resulting from five preferential hits, were separated and tested for messenger activity in a wheat germ cell-free system, as well as for the capacity to withdraw coat protein from intact particles. Fragments which stimulated amino acid incorporation were assumed to contain the 5′ terminus. Strong evidence was obtained for the location of sites with a high affinity for coat protein near the 3′ terminus. The smallest fragment which has the 3′-terminal cytosine comprises only 10% of the length of intact RNA 4 but still possesses these sites. Evidence is presented that the complete coat protein cistron is in the complementing 90% fragment. Possibly, the high-affinity sites are entirely located in the 3′-terminal extracistronic part of RNA 4. They will have the same position in RNA 3 and, possibly, also in the other parts of the genome of alfalfa mosaic virus. The need of this genome for coat protein in order to become infectious may therefore find its explanation in the fact that a conformational change at the 3′ ends of the genome parts brought about by the coat protein is required for recognition by the viral replicase.

Alfalfa mosaic virus belongs to the group of plant viruses with a tripartite RNA genome. These viruses either have their genome parts (referred to as RNAs 1, 2, and 3) in separate virions of identical size (isocapsidic viruses) or of different size (heterocapsidic viruses). The heterocapsidic viruses share a unique biological property: they need a small amount of coat protein to start an infection cycle. Either coat protein itself or coat protein messenger from the same virus or from another heterocapsidic virus must be present to make the genome RNAs infectious. The coat protein messenger, referred to as RNA 4, is a small subgenomic RNA homologous to a part of RNA 3 (see Van Vloten-Doting and Jaspars, 1977, for a review). With the heterocapsidic AMV,[1] RNA 4 is mainly found in two copies in a special class of particles, the so called top component *a*. RNA 4 of AMV strain 425 has a length of about 800 nucleotides (Heijtink et al., 1977), of which 663 represent the cistron for the coat protein (Van Beynum et al., 1977; Koper-Zwarthoff et al., 1977).

The early function of the coat protein of the heterocapsidic viruses, though discovered in 1971 (Bol et al., 1971), is still a puzzling phenomenon. Apparently, the protein is needed in vivo, directly or indirectly, for an adequate expression of the coat protein cistron on RNA 3. If not, an infection with the three genome RNAs would start autonomously. However, no significant effect of the coat protein on the translation of AMV RNAs has been found in the cell-free systems from wheat germ (Van Vloten-Doting et al., 1975; Thang et al., 1975; Gerlinger et al., 1977; Rutgers, 1977) and Krebs ascites cells (Mohier et al., 1976), nor in oocytes of *Xenopus* (Rutgers, 1977).

Recent work from our group has shown that there are a few sites on the RNA molecules of AMV where coat protein sub-

† From the Department of Biochemistry, State University, Leiden, The Netherlands. *Received January 10, 1978.* This work was sponsored in part 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.).

[1] Abbreviations used: AMV, alfalfa mosaic virus; EDTA, ethylenediaminetetraacetate; TYMV, turnip yellow mosaic virus; tRNA, transfer RNA.

0006-2960/78/0417-2927$01.00/0

units bind preferentially (Verhagen et al., 1976; Van Boxsel, 1976; Srinivasan et al., 1977). All four major RNA species of AMV as well as RNAs from other heterocapsidic viruses are able to withdraw protein subunits from intact AMV particles, whereas RNAs from less related viruses are, if at all, much less active in this respect. The presence of sites with a high affinity for coat protein on the RNAs of viruses which need the coat protein for infectivity suggests that some regulatory protein-RNA interaction takes place at an early stage of the life cycle. At a later stage these sites could function as nucleation sites in virion assembly (Driedonks et al., 1978). Therefore, it is of primary importance to know where these sites are located on the RNA molecules. To enter upon this problem, the monocistronic RNA 4 of AMV strain 425 was subjected to a partial digestion by ribonuclease T1. Large fragments resulting from single hits in the molecule were obtained. Since the AMV RNAs are capped (Pinck, 1975, 1976), and since the integrity of the cap structure is important for ribosome binding and translation of those eucaryotic messengers which have such a cap (Shatkin, 1976), we used amino acid incorporation in the wheat germ cell-free system as an easy test to identify fragments which contained the 5′ terminus of RNA 4. The reaction in which coat protein subunits are given off by virus particles to RNA made it possible to design three different assays for coat-protein affinity. The data obtained with these tests lead to the conclusion that the high-affinity sites are located near the 3′ terminus of RNA 4.

Materials and Methods

Viral nucleoproteins and RNAs were prepared, handled, and stored essentially as reported previously (Van Vloten-Doting and Jaspars, 1967, 1972; Bol et al., 1971; Rutgers, 1977). Strain 425 of AMV was used throughout. ^{35}S-labeled bottom component (specific activity 400 cpm/μg) was obtained as described by Verhagen et al. (1976). RNA 4 was extracted from top *a* fraction nucleoproteins and purified by preparative gel electrophoresis, so that no X- and Z-RNAs (Bol and Lak-Kaashoek, 1974; Bol et al., 1975; Heijtink and Jaspars, 1976) could be detected any longer when 6 μg of the preparation was subjected to analytical electrophoresis in a 4% polyacrylamide gel.

Transfer RNA. Unfractionated and uncharged tRNA from *Escherichia coli* B was purchased from Grand Island Biological Co. and made to 10 mg/mL in distilled water. Yeast $tRNA^{Phe}$ was obtained from Boehringer Mannheim.

Partial Digestion of RNA 4. A frozen sample of the RNA stock solution in 0.01 M sodium phosphate, pH 7.0, was thawed, diluted with the same buffer to 0.8 mg/mL, and heated for 5 min at 60 °C. Subsequently, the solution was dialyzed for 24 h at 0 °C against 0.01 M sodium acetate buffer, pH 5.0, 0.1 M NaCl. Digestion was for 15 min at 0 °C with 16 units of ribonuclease T1 (Merck) per mL. The enzyme was stored at 0 °C at a concentration of 5500 units/mL in 10^{-2} M sodium phosphate, 10^{-3} M NaN_3, pH 7.5. Samples to be added were diluted in distilled water to a concentration of 176 units/mL. The digestion was stopped by vigorously shaking with an equal volume of water-saturated phenol for 2 min in the cold. The water layer was extracted four times with ether and traces of ether were finally removed with a stream of nitrogen gas.

Terminal Labeling of RNA Molecules and End-Group Analyses. RNA 4, RNA 4 fragments, and yeast $tRNA^{Phe}$ were terminally ^3H-labeled by oxidation with $NaIO_4$ and subsequent reduction with NaB^3H_4 (10 Ci/mmol, The Radiochemical Centre, Amersham, England) essentially according to Ro-Choi et al. (1970). Instead of alcohol precipitation, gel filtration through Sephadex G-25 (Pharmacia) was used to remove excess reagents. Finally, the labeled RNAs were purified by preparative electrophoresis in acrylamide gels of appropriate concentrations. Specific radioactivities were approximately 4, 6, 13, and 18 × 10^4 cpm/μg in the case of RNA 4, fragment 62, fragments 29, and $tRNA^{Phe}$, respectively.

Alkaline hydrolysis and identification of the ^3H-labeled termini of the RNA molecules were performed essentially as described by Ohno et al. (1977). Unlabeled nucleoside trialcohol markers were prepared according to Randerath and Randerath (1973).

Polyacrylamide gel electrophoresis whether used for analytical or preparative purposes was performed at room temperature essentially according to Loening (1967). Samples were heated for 5 min at 60 °C. Preparative electrophoresis was carried out at 12 mA (80–100 V) in cylindrical gels of 8 × 1 cm in an apparatus as described by Popescu et al. (1971) and improved by Van der Marel (1976). Elution was performed with a peristaltic pump at a rate of 4.2 mL/h, unless otherwise indicated. Fractions of 0.71 mL were collected.

Messenger activity was measured in a wheat germ cell-free system as described by Pleij et al. (1976). Radioactive amino acids (The Radiochemical Centre, Amersham, England) were either [^{14}C]serine (per incubation mixture, 0.1 μCi; specific activity, 160 mCi/mmol) or [^{14}C]leucine (per incubation mixture, 0.1 or 0.145 μCi; specific activity, 324 mCi/mmol).

Protein-Binding and Virus-Labilization Assays. RNA solutions were dialyzed for 1 or 2 days at 0 °C against 10^{-2} M sodium phosphate, 10^{-3} M EDTA, pH 7.0. Samples of 0.1 mL of a suitable concentration were incubated for 40 h at 0 °C with either 20 μg of ^{35}S-labeled AMV bottom component or 2.5 μg of a gradient-purified unfractionated AMV preparation. The former incubation mixtures were centrifuged in 10–40% sucrose gradients in the same buffer for 9.5 h at 6 °C and 25 000 rpm in a Beckman SW 27.1 rotor. The total of the slow-sedimenting counts is a measure of the protein-binding activity of the RNA (Verhagen et al., 1976; Van Boxsel, 1976). To the latter incubation mixtures was added 0.1 mL of diluted leaf sap, and the incubation was continued for 15 min at 20 °C. The mixtures were then chilled in ice, diluted 26-fold with the above buffer, and inoculated onto eight half-leaves of French beans, side by side with standard mixtures made up and treated in the same way but having no RNA. The decrease in lesion number with respect to the standard reflects the extent to which the RNA is able to labilize the virus by removing protein from it. The diluted leaf sap was obtained by grinding primary leaves of French beans in a mortar, squeezing the slurry through cheesecloth, and diluting the sap ten times with the above buffer. The infectivity of free RNA is lost upon incubation with diluted sap, whereas the infectivity of virus is virtually unaffected. However, virus which has lost protein subunits to added RNA also becomes sensitive (Van Vloten-Doting and Jaspars, 1972). Protein-binding and virus-labilizing activities of RNA 4 fragments were read from calibration curves made with amounts of intact RNA 4 ranging from 1.4 to 5.8 pmol and from 0.1 to 1.0 pmol per incubation mixture, respectively. The activities were expressed as a/b × 100%, in which a/b is the ratio of the molar amounts of RNA 4 and of a given fragment that have the same effect in these assays. TYMV RNA tested as a control in an amount of 2.9 pmol in the protein-binding assay had an activity of 13%. When this RNA was tested in an amount of 0.13 pmol in the virus-labilizing assay, no significant decrease of infectivity was found.

Other Measurements. Extinction coefficients of nucleo-

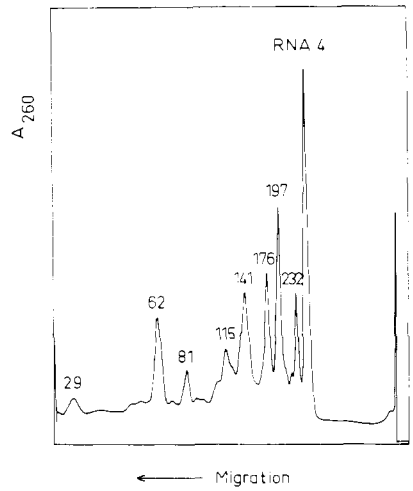

FIGURE 1: Densitometric scan of a 4% polyacrylamide gel in which 16 μg of a partial ribonuclease T1 digest was run. Conditions of partial digestion were as described in the text. Peaks were designated after their apparent molecular weights in thousands of daltons.

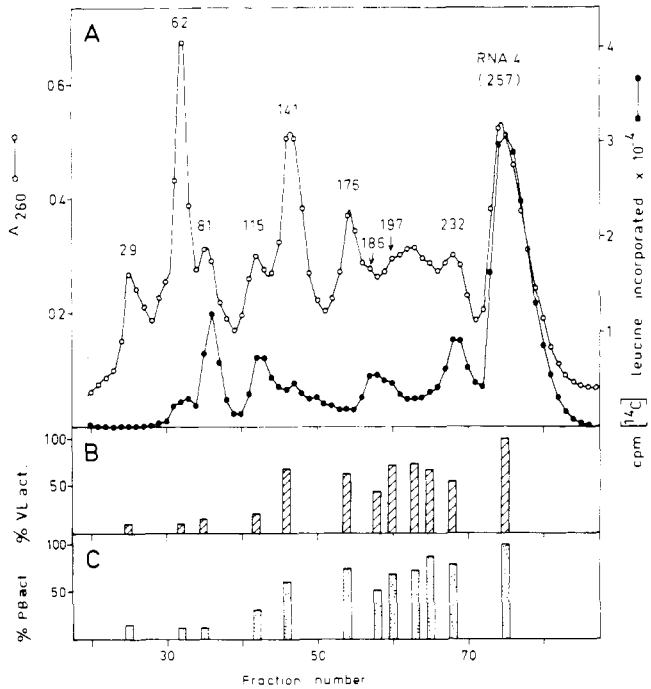

FIGURE 2: Preparative electrophoresis in 4% polyacrylamide gel of 435 μg of a partial ribonuclease T1 digest of RNA 4. (A) Profiles of absorbance at 260 nm (-O-O-) and messenger activity of 30-μL samples in the wheat germ system with [^{14}C]leucine as a precursor (-●-●-). Peaks were identified by analytical gel electrophoresis and numbered as in Figure 1, with the exception of 186. (B,C) Histograms of virus-labilizing (VL) and protein-binding (PB) activities, respectively, as defined in the text, of selected fractions on a molar basis with activities of RNA 4 set at 100%.

proteins and RNAs of AMV were taken from Heijtink et al. (1977), and $A_{260nm}^{0.1\%,1cm}$ values of tRNA (2.5×10^4 daltons) and TYMV RNA (2.0×10^6 daltons) of 24.0 (RajBhandary, 1968) and 23.3 (Haselkorn, 1962) were used, respectively. Radioactivity was measured as described elsewhere (Pleij et al., 1976; Heijtink et al., 1977). Infectivity assays were done according to Van Vloten-Doting and Jaspars (1967).

Results

Large Ribonuclease T1 Fragments of RNA 4. After partial digestion of RNA 4 by ribonuclease T1, analytical gel electrophoresis revealed a pattern of eight main peaks besides a peak of undegraded material (Figure 1). Apparent molecular weights of the fragments were estimated from their relative electrophoretic mobilities with RNA 4 and *E. coli* tRNA as markers of 257×10^3 (Heijtink et al., 1977) and 25×10^3 daltons, respectively. The RNA fragments were designated by their apparent molecular weights in thousands of daltons, as given in Figure 1. Though the electrophoresis was performed under nondenaturing conditions, so that conformation differences might have influenced the mobilities (Reijnders et al., 1973), it is remarkable that the eight fragments can be grouped in four pairs, each pair having a total molecular weight equaling the molecular weight of undegraded RNA 4. This suggests that fragmentation takes place preferentially at four sites. Proceeding degradation of part of the molecules could explain the nonstoichiometry of complementing fragments and the presence of many minor components.

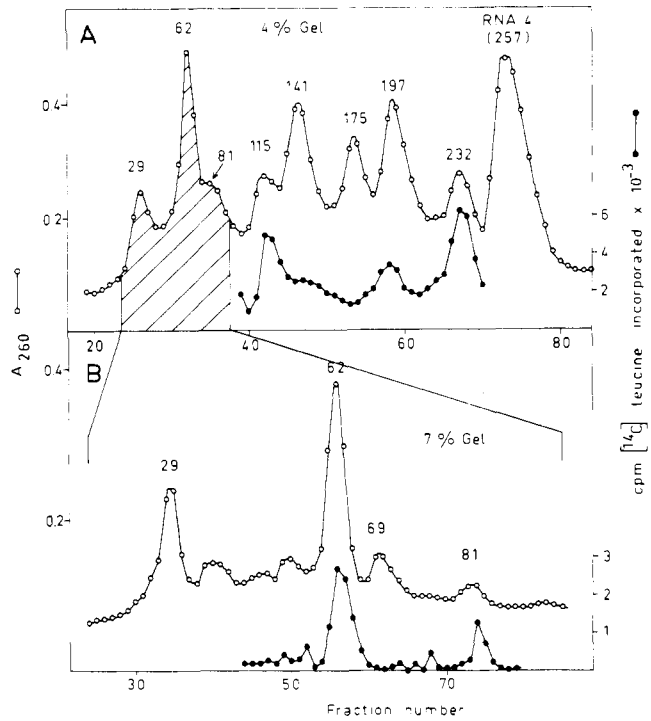

FIGURE 3: Preparative electrophoresis in polyacrylamide gels. Profiles of absorbance at 260 nm (-O-O-) and messenger activity in the wheat germ system with [^{14}C]leucine as a precursor (-●-●-). (A) 4% gel loaded with 360 μg of a partial ribonuclease T1 digest of RNA 4. (B) 7% gel loaded with the material from the hatched region (minus fractions 35 and 36) of two 4% gels as shown in A.

Messenger Activity of RNA 4 Fragments. A partial digest of RNA 4 was run in a preparative 4% polyacrylamide electrophoresis column (Figure 2A). The resolution of absorbance peaks as compared to that of an analytical electrophoresis run is less on the side of the smaller fragments and better on the side of the larger fragments. In the region between fragments 176 and 232 not one but several components could be distinguished. The molecular weights of two of these were estimated by interpolation to be 186 and 197×10^3 daltons. It is evident that fragments 62, 81, 115, and 232 have messenger activity, whereas fragments 29, 141, and 176 have not. Therefore, the former four fragments may be considered to have the 5′ terminus. It is uncertain whether fragment 197 is active in protein synthesis. The activity peak in this region seems to correspond with fragment 186 rather than with fragment 197. Fragment 29 could be inactive in spite of having the 5′ terminus, since leucine was used as the radioactive precursor and this amino

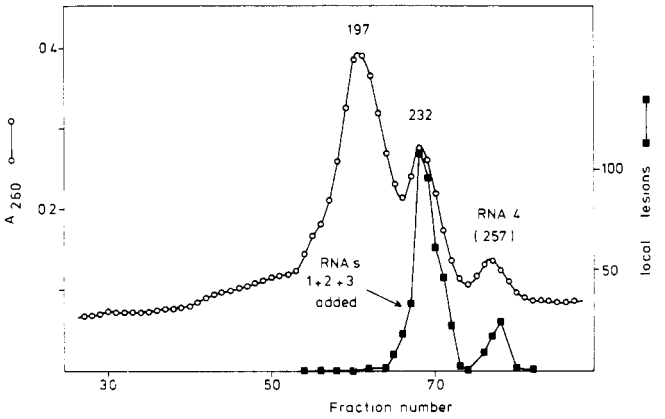

FIGURE 4: Preparative electrophoresis in 4% polyacrylamide gel of the largest fragments from a partial ribonuclease T1 digest of RNA 4 from two preceding preparative gel electrophoresis runs identical to that of Figure 3A. Fractions corresponding to the numbers 56 to 70 in that figure were combined and rerun. Profiles of absorbance at 260 nm (-O-O-) and total lesion number on 12 half-leaves after inoculation of 0.27 mL of each fraction to which 0.03 mL of a solution of 0.35 mg/mL of the three genome RNAs was added (-■-■-).

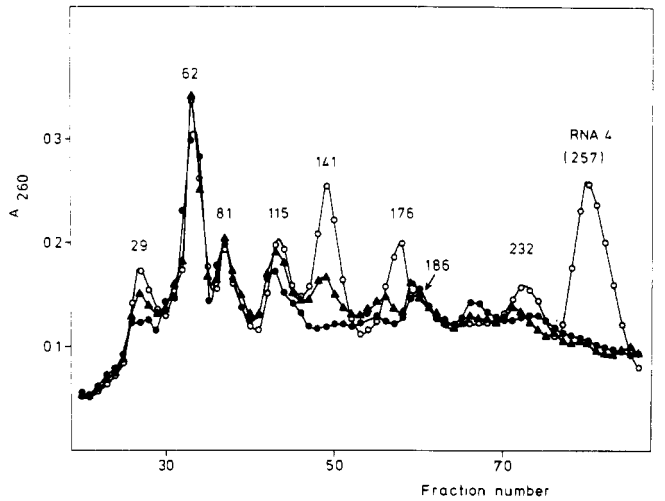

FIGURE 5: Preparative electrophoresis in 4% polyacrylamide gel of 234 μg of a partial ribonuclease T1 digest of RNA 4, before (-O-O-) and after incubation for 8 min at 20 °C (-▲-▲-) and 25 h at 0 °C (-●-●-) with 141 μg of gradient-purified unfractionated virus in 0.5 mL of 10^{-2} M sodium phosphate, 10^{-3} M EDTA, pH 7.0.

acid comes for the first time at position 24 of the coat protein. However, in another experiment, in which [^{14}C]serine (the amino acid at the first three positions of the coat protein) was used, fragment 29 was also inactive.

Because of the poor resolution in the region of the small RNA fragments in the eluate of a 4% preparative gel, the material in this region was precipitated with ethanol and rerun on a 7% preparative gel (Figure 3). In the eluate of the 7% gel, fragments 62 and 81 had messenger activity. A new inactive fragment was noticed, which by interpolation was found to have an apparent molecular weight of 69 × 10^3 daltons and therefore was designated 69. The latter fragment can be thought to be the complement of fragment 186, that in the pattern of this digest on the 4% gel was only seen as an activity peak close to the prominent absorbance peak of fragment 197. Thus, fragments 186 and 69 might reveal a fifth site for preferential ribonuclease attack.

We wondered whether one or more of the largest fragments could yield the complete coat protein as their translation product. In that case, the fragment(s) would function in vivo

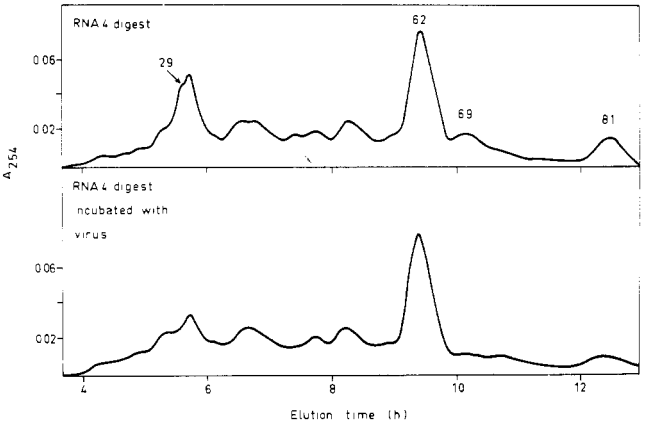

FIGURE 6: Profiles of absorbance at 254 nm recorded by an LKB Uvicord III of the eluates of preparative electrophoresis runs in 7% polyacrylamide gels. Elution rate was 3.2 mL/h. The gels were loaded with 86 μg of the smallest fragments from a partial ribonuclease T1 digest of RNA 4, obtained from several preceding electrophoresis runs such as those of Figures 2A and 3A, before (upper panel) and after incubation for 24 h at 0 °C with 52 μg of virus in 0.5 mL of 10^{-2} M sodium phosphate, 10^{-3} M EDTA, pH 7.0 (lower panel).

like RNA 4 in activating the genome. From 720 μg of partial digest separated in two preparative electrophoresis runs in 4% polyacrylamide columns, the material in the region of the fragments 186 to 232 was concentrated by ethanol precipitation and reelectrophoresed in the same way. When a mixture of the three genome RNAs was added to the fractions of the latter run, infectivity was clearly associated only with fragment 232 and with the remainder of RNA 4 (Figure 4). As reported elsewhere (Rutgers, 1977), the product of fragment 232 in the wheat germ system was indistinguishable in electrophoretic mobility from authentic coat protein, whereas the product of fragment 186 was clearly smaller.

On the basis of the messenger activities it is reasonable to suppose that from each of the following pairs of fragments, 232 + 29, 186 + 69, 115 + 141, 81 + 176, and 62 + 197, the first member represents a left (5′ terminal) part and the second member the complementing right (3′ terminal) part of RNA 4.

Virus-Labilizing and Protein-Binding Activities of RNA 4 Fragments. Selected fractions from the eluate of Figure 2A were tested for their capacity to withdraw coat protein from intact virus particles by two different assays. Both the virus-labilization assay and the protein-binding assay gave low values in fractions with a maximum of messenger activity and vice versa, as can be seen in Figure 2B,C, respectively. Fragment 29 is an exception, since it has no messenger activity and still is low in virus-labilizing and protein-binding activities. Fragment 232, though active as a messenger, is remarkably active as a protein-binding molecule.

Shift in the Electrophoretic Mobility of RNA 4 Fragments Resulting from Protein Binding. When coat-protein molecules bind either to RNA 4 or to its fragments there will be a considerable increase in particle weight. Furthermore, a neutralization of negatively charged phosphate groups is likely to occur. Both changes may be expected to reduce the electrophoretic mobility. In fact, when RNA 4 was incubated with virus for 40 h at 0 °C and then electrophoresed in a preparative gel of 4% polyacrylamide, its peak was converted into slower migrating polydisperse material (result not shown). Partial digests of RNA 4 were also incubated with virus particles and it turned out that the various fragments behaved very differently (Figure 5). The peaks of fragments 62, 81, 115, and 186 remained virtually unchanged or decreased only slightly,

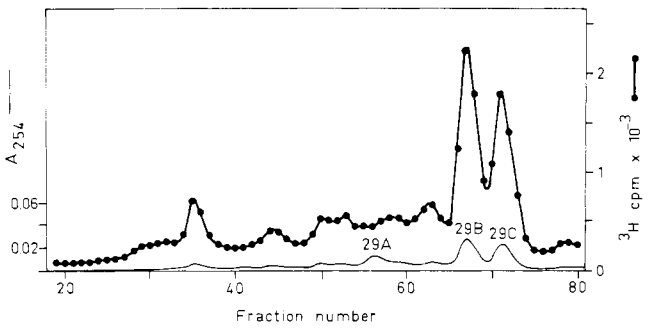

FIGURE 7: Preparative electrophoresis in 13% polyacrylamide gel of about 40 μg of a preparation of fragments 29 obtained from three preceding electrophoresis runs in 4% gels similar to that of Figure 2A and terminally ^{3}H-labeled as described in the text. Elution rate was 3.2 mL/h. The profile of absorbance at 254 nm recorded by an LKB Uvicord III reveals the presence of three main peaks which are designated as 29A, 29B, and 29C. Radioactivity was determined in samples of 5 μL and was not corrected for the background.

whereas the peaks of fragments 141, 176, and 232 disappeared. The peak of fragment 29, though clearly decreasing, did not disappear completely. To investigate more in detail what happened to the smaller fragments, a similar digest was prefractionated on a preparative electrophoresis column of 4% polyacrylamide, and the material eluting first was precipitated with ethanol and rerun on a 7% preparative gel, before and after incubation with virions. Figure 6 shows the recorder tracks of the eluates which are more detailed than the absorbance patterns obtained from measurements of the fractions. Now it is evident that the material called fragment 29 is, in reality, a mixture of different fragments of which at least one binds coat protein. It can also be seen that the peak of fragment 69 disappeared, whereas that of fragment 62 remained unchanged. Thus, from these mobility-shift observations it is even more clear than from the foregoing assays that right fragments are capable of withdrawing coat protein from particles whereas left fragments are not, with the exception of fragment 232 which represents about 90% of the original molecule.

Purification and Characterization of Fragment 29. Since the material from 4% preparative gels with an apparent molecular weight of about 29 × 10^3 daltons contained fragments with protein-binding activity, which probably represented the extreme right part of RNA 4, it was decided to purify and investigate this material. However, from 4 mg of crude RNA 4 the yield of fragments 29 was only about 60 μg. For further work the material had to be labeled radioactively. This was done by 3′-terminal periodate oxidation and subsequent reduction with sodium [^{3}H]borohydride. When analyzed electrophoretically on a preparative column of 13% polyacrylamide, three main absorbance peaks, called fragments 29A, 29B, and 29C in order of decreasing mobility, were seen, the last two of which were clearly labeled (Figure 7). Central fractions of the labeled peaks 29B and 29C were examined for their protein-binding capacity with the aid of the mobility-shift test and for the nature of their labeled end groups. To make the reaction conditions more comparable to those of the experiments of Figures 5 and 6, undegraded RNA 4 was added to the incubation mixture with virus particles. To the mixture were also added purified fragment 62 and yeast tRNA^{Phe} which were labeled in the same way. Analysis of the mixture on a 9% preparative gel revealed that the peak of fragments 29 was reduced, whereas those of the control RNAs were not (Figure 8A,B). On this gel, fragments 29B and 29C were not separated. To find out if both the fragments reacted similarly,

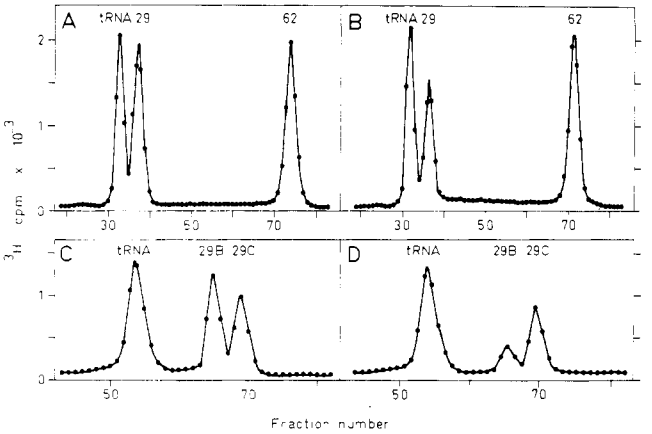

FIGURE 8: Preparative electrophoresis in 9% (A,B) and 13% polyacrylamide gels (C,D) of mixtures of terminally ^{3}H-labeled RNAs before (A,C) and after incubation (B,D) with 600 ng of gradient-purified unfractionated virus in 0.1 mL of 10^{-2} M sodium phosphate, 10^{-3} M EDTA, pH 7.0, for 24 h at 0 °C. Elution rate was 3.2 mL/h. The gels were loaded with the following RNA mixtures: (A,B) 30 ng of yeast tRNA^{Phe}, 32 ng of fragment 29B, 25 ng of fragment 29C, 129 ng of fragment 62, and 784 ng of RNA 4; (C,D) 30 ng of yeast tRNA^{Phe}, 32 ng of fragment 29B, 25 ng of fragment 29C, and 913 ng of RNA 4. Radioactivity was determined in samples of 0.65 mL and was not corrected for the background.

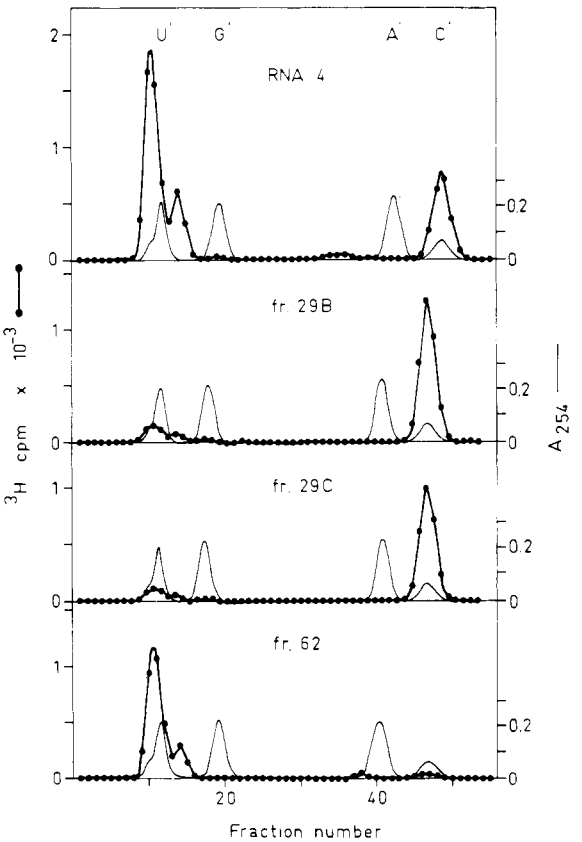

FIGURE 9: Analyses of ^{3}H-labeled end groups of RNA 4 and its fragments 29B, 29C, and 62. Alkaline hydrolysates of the RNAs were chromatographed on a phosphocellulose column together with the four nucleoside trialcohols.

a reaction mixture without fragment 62 was analyzed on a 13% preparative gel. The result is shown in Figure 8C,D. It appears that the peak of fragment 29B is more reduced than that of 29C, which presumably means that it has a higher affinity for the coat protein.

After alkaline hydrolysis it was found that fragments 29B and 29C both had a cytosine at their 3′-termini. Cytosine is also

found with intact RNA 4 besides the labeled 5′-terminal cap structure (Pinck, 1975, 1976) (Figure 9). From the work of others (Takanami and Imaizumi, 1977; Ohno et al., 1977), the oxidized and reduced cap is known to elute from a phosphocellulose column somewhat faster than the trialcohol of uridine. This confirms the 3′-terminal position of fragments 29B and 29C. The cap structure is also found after alkaline hydrolysis of the labeled fragment 62, which confirms its 5′-terminal position. Some cap derivatives seem to be present also in the hydrolysates of fragments 29B and 29C. However, we found the same in the hydrolysate of terminally ^{3}H-labeled yeast tRNA^{Phe}, so that this material is more likely to be a contamination from the NaB^3H_4 preparation (Randerath and Randerath, 1973; Ohno et al., 1977).

Discussion

The results of the present investigation show clearly that the high-affinity sites are confined to the right half of RNA 4 and, presumably, to the extreme right quarter of the molecule. The latter assumption is based on the finding that in the patterns of Figure 2 fragment 186, which encompasses three quarters of the intact RNA 4, coincides with a peak of messenger activity and with local minima of protein-binding and virus-labilizing activities. The precise activities of fragment 186 are difficult to estimate because of its being sandwiched between the clearly active fragments 176 and 197. Furthermore, the electrophoretic mobility of fragment 186 seems not to be affected by incubation with particles (Figure 5), which is also in favor of its not binding coat protein. Moving further toward the 3′ end of RNA 4, we arrive at the enzymatic hit which gives rise to the left fragment 232 and the right fragment 29. Fragment 232 contains the complete coat-protein cistron. This can be inferred from its molecular weight, the length of the coat protein, and the recently elucidated nucleotide sequence at the 5′ terminus of RNA 4, which shows that the triplet for the first amino acid of the coat protein has the positions 40 to 42 from the 5′ end (Koper-Zwarthoff et al., 1977). Moreover, infectivity studies (Figure 4) indicate that fragment 232 gives rise to a functional product in vivo. Between the end of the cistron and the 3′ terminus of fragment 232 there must be another 20 nucleotides, which could well be responsible for the unmistakable affinity of this fragment for coat protein (Figure 5). Apparently, the small fragment 29 which comprises about 90 nucleotides is not able to withdraw coat protein from virus particles, for it has no significant protein-binding or virus-labilizing activity (Figure 2). However, it is evident that a considerable amount of fragment 29 disappears from the electrophoretic pattern after incubation with virus particles (Figures 5, 6, and 8). Probably, because of the small size and charge of fragment 29, a complex with a virus particle once formed will not dissociate again (Verhagen et al., 1976; Van Boxsel, 1976). The heterogeneity of fragment 29 seen upon electrophoresis in 13% gels could arise from alternative hits a few nucleotides apart in a region about 90 nucleotides from the 3′ terminus which is susceptible to low concentrations of ribonuclease T1. This holds only for fragment 29B and 29C, since fragment 29A is not terminally ^{3}H-labeled and therefore must be an internal fragment. It is remarkable that the apparently smaller fragment 29B has a higher affinity for coat protein (Figure 8). Therefore, it is likely that fragments 29B and 29C differ in conformation, which apart from any difference in size would be sufficient to explain the mobility difference under nondenaturing conditions.

The positioning of the sites with a high affinity for coat protein near the 3′ terminus of RNA 4 opens an interesting way for speculation about the biological function of the coat protein. It is very likely that RNA 4 is homologous with the 3′-terminal part of RNA 3, since this has been found to be the situation in the isocapsidic brome mosaic virus (Bastin et al., 1976) and since the initiation site of the coat-protein cistron seems to occupy an internal position as judged from the low frequency of translation both in vivo (Bol et al., 1971) and in vitro (Thang et al., 1975, 1976; Mohier et al., 1975a,b, 1976; Van Vloten-Doting et al., 1975, 1977; Gerlinger et al., 1977; Mohier, 1977; Rutgers, 1977). Recently, a sequence of 161 nucleotides at the 3′ terminus of RNA 4 of brome mosaic virus has been elucidated, and it has been shown that all four RNAs of this virus have the same or nearly the same sequence at their 3′ termini (Dasgupta and Kaesberg, 1977). If the analogy between brome and alfalfa mosaic viruses goes so far that also the four RNAs of AMV have a long track of nucleotides at their 3′ termini in common, this would imply that in all four RNA species high affinity sites for the coat protein would be located 3′ terminally. Such a tendency of the coat protein to attach to the 3′ end of the genomic RNAs would suggest a function in replication rather than in translation. No significant influence of the coat protein has been found in any of the in vitro translation studies mentioned above. These data taken together lead us to the following hypothesis: When the three genome RNAs of AMV enter a cell in the absence of the coat protein of the virus, translation will start normally and all information of the genome will be translated with the exception of the coat protein cistron on RNA 3, which has an internal initiation site. Among the translation products there might be one or more proteins which have, alone or in combination with certain cellular proteins, replicase activity. However, this replicase would not be able to recognize its natural templates, the three genome RNAs, unless these have special structural features. In contrast to the situation with the isocapsidic viruses, such structural features would be absent in the naked RNAs of the heterocapsidic viruses. With the isocapsidic viruses, the 3′-terminal parts of all RNA species have a tRNA-like structure, which upon incubation with plant enzymes, ATP, and amino acids leads to a specific charging with tyrosine (Hall et al., 1972; Kohl and Hall, 1974). This has been related to a possible role of a plant protein-synthesis elongation factor in the replicase complex (Hall and Wepprich, 1976; Bastin and Hall, 1976). It is known that the bacterial elongation factors Tu and Ts form part of the replicase of bacteriophage Qβ (Blumenthal et al., 1972; Landers et al., 1974). With the heterocapsidic viruses, association of the viral coat protein with the 3′-terminal regions of the RNAs would be required for the formation of recognition sites for the replicase. Given the presence of a few coat protein molecules, replication could start and would indirectly give rise to the synthesis of RNA 4, which is an efficient messenger for coat protein, so that replication could go on.

In the above hypothesis, the structure at the 3′ terminus of the AMV RNAs plays a crucial role. What do we know about it? Unlike the RNAs of the isocapsidic brome and cucumber mosaic viruses which have a 3′-terminal adenosine (Dasgupta and Kaesberg, 1977; Takanami and Imaizumi, 1977), RNA 4 and possibly also the other RNAs of AMV end in a cytosine residue. The RNAs of AMV could not be charged with any amino acid under conditions used for the charging of brome mosaic virus RNAs (T. C. Hall, personal communication; A. J. Van Agthoven, L. Bosch, and E. M. J. Jaspars, unpublished results). Addition of a few coat-protein molecules per molecule of RNA 4 affects certain details of the thermal melting profile of the RNAs, which demonstrates that changes in the base-paired structure take place (Srinivasan and Jaspars, to be published). Nevertheless, attempts to charge AMV RNAs to

which coat protein was added with an amino acid have been unsuccessful (J.-P. Tijssen and E. M. J. Jaspars, unpublished results). However, a 3′-terminal structural change brought about by the coat protein and leading to the formation of a replicase recognition site will not necessarily entail the recognition by both a tRNA nucleotidyltransferase and an aminoacyl-tRNA synthetase. RNA-synthesizing enzymes isolated from healthy and AMV-infected tobacco plants have been shown to use AMV and other viral RNAs as templates without much preference (Bol et al., 1976; Le Roy et al., 1977; Clerx-Van Haaster and Bol, to be published). However, it will be interesting to investigate the copying process in more detail and to see whether AMV RNAs with coat protein on their high-affinity sites in some respect are better templates. Furthermore, the above hypothesis predicts that an infection cycle can be started with a mixture of genome RNAs in which only the high-affinity sites of RNA 3 are occupied by coat protein. Work along these lines is in progress.

Acknowledgments

We are especially indebted to Dr. Tineke Rutgers for performing the amino acid incorporation assays. We are also grateful to Mr. C. Geerlof for a gift of ^{35}S-labeled bottom component of AMV. Drs. Lous Van Vloten-Doting, J. F. Bol, and S. Srinivasan made useful comments on the manuscript.

References

Bastin, M., and Hall, T. C. (1976), *J. Virol. 20,* 117.
Bastin, M., Dasgupta, R., Hall, T. C., and Kaesberg, P. (1976), *J. Mol. Biol. 103,* 737.
Blumenthal, T., Landers, T. A., and Weber, K. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1313.
Bol, J. F., and Lak-Kaashoek, M. (1974), *Virology 60,* 476.
Bol, J. F., Van Vloten-Doting, L., and Jaspars, E. M. J. (1971), *Virology 46,* 73.
Bol, J. F., Brederode, F. T., Janze, G. C., and Rauh, D. K. (1975), *Virology 65,* 1.
Bol, J. F., Clerx-Van Haaster, C. M., and Weening, C. J. (1976), *Ann. Microbiol. (Paris) 127A,* 183.
Dasgupta, R., and Kaesberg, P. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 4900.
Driedonks, R. A., Krijgsman, P. C. J., and Mellema, J. E. (1978), *Eur. J. Biochem. 82,* 405.
Gerlinger, P., Mohier, E., Le Meur, M. A., and Hirth, L. (1977), *Nucleic Acids Res. 4,* 813.
Hall, T. C., and Wepprich, R. K. (1976), *Ann. Microbiol. (Paris) 127A,* 143.
Hall, T. C., Shih, D. S., and Kaesberg, P. (1972), *Biochem. J. 129,* 969.
Haselkorn, R. (1962), *J. Mol. Biol. 4,* 357.
Heijtink, R. A., and Jaspars, E. M. J. (1976), *Virology 69,* 75.
Heijtink, R. A., Houwing, C. J., and Jaspars, E. M. J. (1977), *Biochemistry 16,* 4684.
Kohl, R. J., and Hall, T. C. (1974), *J. Gen. Virol. 25,* 257.
Koper-Zwarthoff, E. C., Lockard, R. E., Alzner-deWeerd, B., RajBhandary, U. L., and Bol, J. F. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 5504.
Landers, T. A., Blumenthal, T., and Weber, K. (1974), *J. Biol. Chem. 249,* 5801.
Le Roy, C., Stussi-Garaud, C., and Hirth, L. (1977), *Virology 82,* 48.
Loening, U. E. (1967), *Biochem. J. 102,* 251.
Mohier, E. (1977), Ph. D. Thesis, University of Strasbourg.
Mohier, E., Hirth, L., Le Meur, M.-A., and Gerlinger, P. (1975a), *Virology 68,* 349.
Mohier, E., Hirth, L., Le Meur, M. A., and Gerlinger, P. (1975b), *Fed. Eur. Biochem. Soc. Meet., Proc. 39,* 171.
Mohier, E., Hirth, L., Le Meur, M.-A., and Gerlinger, P. (1976), *Virology 71,* 615.
Ohno, T., Sumita, M., and Okada, Y. (1977), *Virology 78,* 407.
Pinck, L. (1975), *FEBS Lett. 59,* 24.
Pinck, L. (1976), *Ann. Microbiol. (Paris) 127A,* 175.
Pleij, C. W. A., Neeleman, L., Van Vloten-Doting, L., and Bosch, L. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 4437.
Popescu, M., Lazarus, L. H., and Goldblum, N. (1971), *Anal. Biochem. 40,* 247.
RajBhandary, U. L. (1968), *J. Biol. Chem. 243,* 556.
Randerath, K., and Randerath, E. (1973), *Methods Cancer Res. 9,* 3.
Reijnders, L., Sloof, P., Sival, J., and Borst, P. (1973), *Biochim. Biophys. Acta 324,* 320.
Ro-Choi, T. S., Moriyama, Y., Choi, Y. C., and Busch, H. (1970), *J. Biol. Chem. 245,* 1970.
Rutgers, A. S. (1977), Ph.D. Thesis, University of Leiden.
Shatkin, A. J. (1976), *Cell 9,* 645.
Srinivasan, S., Jaspars, E. M. J., and Hinz, H.-J. (1977), *FEBS Lett. 80,* 288.
Takanami, Y., and Imaizumi, S. (1977), *Virology 77,* 853.
Thang, M. N., Dondon, L., Thang, D. C., Mohier, E., Hirth, L., Le Meur, M. A., and Gerlinger, P. (1975), *Colloq. Inst. Natl. Sante Rech. Med. 47,* 225.
Thang, M. N., Dondon, L., and Mohier, E. (1976), *FEBS Lett. 61,* 85.
Van Beynum, G. M. A., De Graaf, J. M., Castel, A., Kraal, B., and Bosch, L. (1977), *Eur. J. Biochem. 72,* 63.
Van Boxsel, J. A. M. (1976), Ph.D. Thesis, University of Leiden.
Van der Marel, P. (1976), Ph.D. Thesis, University of Leiden.
Van Vloten-Doting, L., and Jaspars, E. M. J. (1967), *Virology 33,* 684.
Van Vloten-Doting, L., and Jaspars, E. M. J. (1972), *Virology 48,* 699.
Van Vloten-Doting, L., and Jaspars, E. M. J. (1977), *Comp. Virol. 11,* 1.
Van Vloten-Doting, L., Rutgers, T., Neeleman, L., and Bosch, L. (1975), *Colloq. Inst. Natl. Sante Rech. Med. 47,* 233.
Van Vloten-Doting, L., Bol, J., Neeleman, L., Rutgers, T., Van Dalen, D., Castel, A., Bosch, L., Marbaix, G., Huez, G., Hubert, E., and Cleuter, Y. (1977), in Nucleic Acids and Protein Synthesis in Plants, Bogorad, L., and Weil, J. H., Ed., New York, N.Y., Plenum Press, p 387.
Verhagen, W., Van Boxsel, J. A. M., Bol, J. F., Van Vloten-Doting, L., and Jaspars, E. M. J. (1976), *Ann. Microbiol. (Paris) 127A,* 165.