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THE OCCURRENCE OF INFECTIOUS VIRUS RIBONUCLEIC ACID IN THE RIBOSOMAL FRACTION FROM TOBACCO MOSAIC VIRUS INFECTED TOBACCO LEAVES

(Met een samenvatting in het Nederlands)

by/*door*

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CHAPTER 1

INTRODUCTION

Little is known about the process of plant virus multiplication although much work has been done on the chemical and biological characterization of those viruses. The purpose of this paper is to support some evidence on the course of virus synthesis.

There are several reasons for studying the multiplication of plant viruses. First, infection of a plant with a virus usually causes typical symptoms of disease. These symptoms may often be very different in nature, e.g. necrosis of the tissue, mosaic on the leaves, deformation of leaves or other organs, stunt-

ing, morbid growth of the plant. The symptoms reflect the interaction of the process of virus multiplication with the physiological processes of the plant. The interaction seems to be specific since the same virus may cause different symptoms in different plants. On the other hand infection of the same plant with different viruses may result in various types of symptoms. This area of study is concerned with the specific problems of the biochemistry of plants.

Second, the multiplication of plant viruses has more general biochemical significance. Plant viruses, as far as is known are ribonucleoproteins. These particular ribonucleoproteins have the peculiar property of inducing the synthesis of specific virus ribonucleic acid and virus protein in the cell. In virus multiplication one can study the synthesis of a specific ribonucleic acid and protein and the relation between the two. This relationship is important in the study of nucleic acid and protein synthesis.

1.1. Choice of virus

Our study was performed with tobacco mosaic virus (TMV) because we thought this virus was a suitable model for our research.

Tobacco mosaic was the first plant disease recognized and identified as a virus disease (Iwanowsky, 1892; Beijerinck, 1898), when the filterability of the infectious agent was demonstrated. As such TMV played an important historical role in distinguishing viruses as pathogens from micro-organisms. Later TMV was the first virus isolated in paracrystalline form (Stanley, 1935) and characterized as a ribonucleoprotein (Bawden *et al.*, 1936). This leading role of TMV in the history of virus research is undoubtedly connected to the facts that TMV can be found in rather large amounts in diseased plants and that the TMV particle is rather stable. For that reason it is also relatively simple to prepare considerable quantities in purified form making TMV useful for chemical and physical research on viruses. The result has been that the chemical composition of TMV, its structure and its properties, are well known. Making use of this information one can easily demonstrate small amounts of the virus in specific tests and for this reason it is attractive as a model in the study of plant virus multiplication.

1.2. The components of TMV

Before giving the hypothesis which underlies this study we will describe briefly the composition and structure of TMV and the mutual relationship between its components. It does not seem necessary to discuss here extensively all data with regard to the structure of TMV. (For a review see e.g. Franklin *et al.*, 1959 and Fraenkel-Conrat, 1959). Only information relevant to our further discussion will be given.

TMV is a ribonucleoprotein consisting of 5–6% ribonucleic acid (RNA) and 94–95% protein. It is a rod shaped particle with a length of 300 mμ and a diameter of nearly 18 mμ. The molecular weight of TMV is about 4×10^7. The RNA is present as a long single chain of about 6,000 nucleotides. The protein consists of identical peptide chains of a molecular weight of about 17,300. They are called subunits and their total number is about 2,130. The subunits are held together by hydrogen bonds and ionic linkages only. The amino acid composition and sequence of the protein subunits is known. They

consist of a single chain of 158 amino acids. The rod shape of TMV was discovered from physical and electron microscope studies but the structure has been elucidated in more detail by X-ray studies of paracrystalline TMV preparations. The protein, in the form of subunits, is packed helix-like around the axis of the particle. The RNA, also wound in a helix, with a radius of 40 Å, is deeply embedded in the piling of protein subunits. In the middle of the rod is a hole with a diameter of 20 Å.

Using various treatments it is possible to separate the RNA and protein. By shaking a TMV preparation with water saturated phenol (GIERER and SCHRAMM, 1956), the protein can be extracted and it is possible to obtain the intact virus RNA without protein. The RNA alone has been found to be infectious. By treating TMV with acid or alkaline one can prepare the virus protein in its native form which has been found to be non-infectious. This demonstrates that the RNA carries the infectivity of the virus and probably induces the virus specific processes in the infected cell. The free virus RNA has only 0.1–0.5% of the infectivity of the virus. The minor infectivity of the RNA is not due to the destruction of the infectious component but it appears to be due to the greater lability of the RNA and the greater sensitivity to destructive agents to which it is exposed in the infectivity test. For it is possible to reconstitute TMV particles by mixing infectious RNA and virus protein under proper conditions and the resulting particles have nearly the same infectivity as the TMV from which the RNA and the protein were prepared.

Noteworthy experiments have confirmed these findings: when RNA of TMV, strain 1, is reconstituted with protein of TMV, strain 2 – both strains differing in composition of the protein – and this reconstituted virus inoculated onto tobacco plants, virus is synthesized in the plants with the protein going with the RNA of TMV strain 1 (FRAENKEL-CONRAT and SINGER, 1959). The susceptibility of the infection process to ribonuclease (CASTERMAN and JEENER, 1955) and to ultraviolet (UV) light (SIEGEL and WILDMAN, 1956; KLECZKOWSKI, 1960) further shows that the RNA is the essential factor for the infectivity of the virus.

Finally it is possible to prepare mutants of TMV *in vitro* by treating the TMV-RNA with nitrous acid (GIERER and MUNDRY, 1958; TSUGITA and FRAENKEL-CONRAT, 1962a, 1962b). These mutants differ in biological properties from the starting TMV and in some cases there is a change in the amino acid composition of the protein. Therefore it seems, without doubt, that the RNA determines *in vivo* the properties of the virus.

The protein of the virus protects the RNA against destroying agents. When the RNA is surrounded by protein it is much more infectious than equivalent amounts of free RNA which usually have only 0.1–0.5% of the infectivity of the virus. The protein surrounded RNA is much more resistant against heat, UV irradiation and chemicals than the RNA. When the virus protein is prepared in its native form by degradation of TMV, it retains the ability of reaggregation to the rod shape of the original virus under appropriate conditions of pH and ionic strength (SCHRAMM and ZILLIG, 1955). The shape of TMV appears to be primarily a function of the protein structure. TMV protein without RNA aggregates to rods of varying lengths. The aggregation is readily reversible by changes of pH or ionic strength. When the RNA is also present and becomes incorporated during aggregation, the protein becomes stabilized and forms rods of constant length of 300 mμ.

It seems wholly justified to regard the RNA of the virus as the genetic

component, in which the properties of a virus particle are transmitted to newly synthesized particles. It is obvious to assume then that the first step of virus multiplication will be multiplication of the RNA, followed by protein synthesis directed by the RNA. This hypothesis underlies our present study.

1.3. Precursors of TMV and the localization of plant virus synthesis

The experimental work already done on the biosynthesis of plant viruses will be reviewed here briefly. It bears mainly upon the demonstration of precursors of the complete TMV particle.

Engler and Schramm (1959) concluded that free infectious RNA occurs in TMV infected leaf cells. They compared the infectivity of RNA preparations obtained by homogenizing TMV infected leaves in the presence of phenol and phosphate buffer with the infectivity of RNA from leaf homogenates extracted with phenol after incubating them at 37°C for one hour. The latter treatment was to destroy all free infectious RNA. They attributed the differences in infectivity to the occurrence of free infectious RNA, the production of which had started before the synthesis of the virus protein. No conclusion could, however, be drawn about the real state of this RNA in the cell. Cochran (1957) deduced the presence of free infectious RNA in TMV infected tobacco plants from chromatographical experiments. Cochran's results could not be reproduced by Whitfeld *et al.* (1960) nor by the author. Cornuft (1960) described an electrophoretic method by which it should have been possible to isolate directly free infectious RNA from tobacco leaf tissue infected with TMV, but this method could not be reproduced in our laboratory. So at the beginning of this research there was only one unequivocal indication that besides TMV particles, infectious RNA which could be a precursor of TMV, occurs in leaves where virus multiplication takes place.

The occurrence of non-infectious virus-specific proteins in TMV infected tobacco plants is well established (Takahashi and Ishii, 1952; Jeener *et al.*, 1954; Commoner *et al.*, 1953). This protein now is generally called X-protein. It resembles the viral protein in its tendency to aggregate in rods under the proper conditions of pH and ionic strength. Van Rysselberghe and Jeener (1957) concluded from incorporation experiments with $^{14}CO_2$ that there is a precursor-product relationship between the X-protein and the protein in the virus. The X-protein is synthesized first and is then assembled to the rod shaped nucleoprotein particle.

The question of localization of the plant virus synthesis in the cell is not yet agreed upon. Zech and Vogt-Köhne (1955), using a microspectrophotometric technique, found indications that the virus RNA synthesis starts in the nucleus. They measured an increase of nucleic acid in the first hours after infection first in the nucleus of infected cells and later in the cytoplasm. This method, however, does not differentiate between virus RNA and normal RNA and no conclusion can be drawn about the site where biologically active virus RNA is formed.

Several reports have recently been published which claim that the nucleus is involved in TMV-RNA synthesis (Cornuet, 1962; Cochran *et al.*, 1962; Kim and Wildman, 1962). Evidence in this area is, however, not yet conclusive.

Boardman and Zaitlin (1958) reported that plastids are involved in virus synthesis, but these findings have not been supported by other research. From experiments with fluorescent antibodies Schramm and Röttger (1959) conclud-

ed that the nucleus and the chloroplasts do not take part in virus protein synthesis but that the virus protein is probably synthesized in the cytoplasm. The experiments of RYSSELBERGHE (1957) on the X-protein appear to point in the same direction. One can conclude that the final assemblage of protein and RNA is in the cytoplasm, but until there is more experimental evidence it is not safe to form definite conclusions.

1.4. SCOPE OF THE PRESENT INVESTIGATIONS

From studies on protein biosynthesis during the last decade it is known that protein synthesis, in which amino acids are united in a specific order, occurs at the ribosomes in the cytoplasm. We have assumed that virus protein synthesis does not follow a mechanism different from protein synthesis in healthy organisms. Therefore, we postulate that ribosomes will also be involved in virus protein synthesis. It may be expected, however, that a virus specific component is present during virus protein synthesis because such a specific product is delivered. This might be the virus RNA.

With that picture in mind we have studied the ribosomal fraction from healthy and TMV infected tobacco leaves and we think we have obtained good evidence that TMV-RNA occurs in the ribosomal fraction of TMV infected leaves without being incorporated in TMV protein. Since reports about ribosomes from tobacco leaves are not found in the literature, the isolation and properties of this fraction is also described.

CHAPTER 2

MATERIAL AND METHODS

2.1. PLANT MATERIAL

The 'White Burley' variety of *Nicotiana tabacum* was used in all experiments in which the ribosomal fraction from leaves was isolated. Leaves of *Nicotiana glutinosa* were used in the local lesion test for testing the infectivity.

Plants were grown in soil consisting of a mixture of sand, compost, leaf mold and farmyard manure. The soil was sterilized by steaming for 2 hours at 100 °C and sieved afterwards. The seeds were planted in sterile baskets. After about 3 weeks the seedlings were transplanted in boxes. After another 2 weeks the 'White Burley' plants were set in pots and one week later, about 6 weeks after sowing, they were put in a growing chamber under controlled conditions of light, temperature and humidity: 18–20 °C, 65–80 % relative humidity and 16 hours light of about 28,000 erg/cm^2 and 8 hours darkness each day. Usually the plants were used after two weeks under these conditions. For infecting the plants the leaves were dusted with Carborundum (600 mesh) and inoculated by rubbing a suspension of 50 μg TMV/ml on the leaves with the forefinger. The leaves were thoroughly washed after inoculation and kept under the same conditions of light, temperature and humidity as before. Leaves or whole plants were harvested whenever needed. Usually the fourth and fifth leaves were used.

Nicotiana glutinosa plants were grown in the greenhouse in boxes and leaves were picked whenever they were at the proper stage. The temperature in the

greenhouse was fairly constant during the whole year, but the light varied with the seasons and the weather. The susceptibility of the leaves to TMV and TMV-RNA in the local lesion test depends strongly on the lighting. It was necessary to shade the plants for the greater part of the year. This was done by means of cheesecloth and sheets of filter paper. The susceptibility of the leaves was frequently tested for control.

2.2. Tobacco mosaic virus

The TMV in all experiments was the strain commonly used in our laboratory. The virus was purified from leaves of *Nicotiana tabacum* var. 'White Burley' by the method of differential centrifugation which has become a standard procedure for the purification of TMV (Knight, 1962). The yield was usually between 1 to 2 g of virus per kg leaf material. The final preparation in water had one peak in the analytical ultracentrifuge with a sedimentation coefficient $S_0^{20} =$ 194 *S* and a hardly visible peak with a S_0^{20} of roughly 220 *S*. The latter should probably be attributed to aggregation of TMV in concentrated solutions to dimeric or trimeric particles. Under the electron microscope the vast majority of the particles were of uniform size of 300 × 18 mμ. The concentration of the purified TMV suspensions was determined by nitrogen determinations (Kjeldahl method) taking for the ratio TMV/N = 6.06 or spectrophotometrically. It was determined that a 0.02% suspension of TMV in water had an extinction at 260 mμ, using 1 cm cells, of $\alpha\,{}^{1\,cm}_{260} = 0.568$ and then TMV concentrations were based on this value.

2.3. Preparation of TMV-RNA

TMV-RNA was prepared by a slight modification of the method of phenol-extraction (Gierer and Schramm, 1956). Sodium lauryl sulphate was added to 8 ml of a TMV suspension containing 10 mg TMV/ml to a final concentration of 0.1%. The suspension was shaken with an equal volume of water saturated phenol for 8 minutes at 0–3 °C (cold room). The emulsion was broken by centrifugation at 600 × *g* for 7 minutes. The water layer was carefully taken off from the phenol layer with a pipette and once more shaken with 6 ml water saturated phenol for 6 minutes. The water and phenol layers were separated in the same way as before and the water layer was extracted 4 times with peroxide-free ether to eliminate the phenol. The TMV-RNA was precipitated with 2.5 volumes of cold ethanol. After flocculation, the precipitate was spun down and redissolved in water or phosphate buffer to the desired concentration. The sedimentation diagram in the analytical ultracentrifuge showed one homogeneous peak. When determined in 0.01 M sodium phosphate buffer, pH 7.0, the sedimentation coefficient S_0^{20} was 28 *S*.

2.4. Determination of sedimentation coefficients

Sedimentation coefficients S were determined in the analytical ultracentrifuge Spinco Model E, using Schlieren optics.

A series of concentrations was spun and from these the sedimentation coefficient at endless dilution S_0 was found by extrapolation to concentration zero. The S_0 values were corrected for temperature to 20 °C and recorded as S_0^{20}. The solvent used will be mentioned for individual cases.

At first the sedimentation coefficients were calculated from measurements of the photographs. Later the graphical method of MARKHAM (1960) was used for determining the S values.

2.5. LOCAL LESION TEST

The local lesion test was used for measuring the infectivity of preparations of TMV and TMV-RNA. Inoculations were made on detached leaves of *N. glutinosa*. The leaves were cut along the midrib into two halves, dusted with Carborundum (600 mesh), inoculated by rubbing the forefinger over every part once, and then rinsed with distilled water. After this the halves of each leaf – the one half inoculated with the test solution and the other with a standard virus solution for control – were put on wet filter paper in a petri dish and kept at 20 °C under continuous illumination by four parallel fluorescent tubes (providing about 38,000 erg/sec/cm^2). Six or more leaves were used in every test. The local lesions were counted 3 days after inoculation.

2.6. DETERMINATION OF PROTEIN AND RNA

Protein was estimated with Folin-Ciocalteu reagent according to the method of LOWRY *et al.* (1951). A standard curve was prepared from ovalbumin. The blue color was read at 750 mμ in an Unicam SP. 600 spectrophotometer. In contrast to LOWRY *et al.*, who reported the color being constant half an hour after addition of the reagent, it was found that 1½ hour was needed for the color to become constant.

RNA was estimated either by the orcinol reaction (SCHNEIDER, 1957), reading the green color at 550 mμ, or by hydrolysis of the RNA with 1 M perchloric acid and reading the extinction at 260 mμ. Standard curves were prepared from purified yeast RNA. Extinction measurements in the UV region were done in a Beckman DU spectrophotometer using 1 cm cells.

The concentration of TMV-RNA was determined by measuring directly the extinction at 260 mμ taking an extinction of 28.4 for a 0.1 % solution at a layer thickness of 1 cm.

2.7. DETERMINATION OF RNA IN WHOLE LEAVES AND IN VARIOUS FRACTIONS OF A LEAF HOMOGENATE

RNA in whole leaves and in fractions of leaf homogenates was determined according to the method of OGUR and ROSEN (1950) with some modifications. The procedure was the following. Samples of 5 g leaf tissue cut in small pieces were frozen at –13 °C and then ground into a fine powder using dry ice. The powder was put into 12–15 ml ethanol. While performing a fractionation procedure portions corresponding to 5 g leaf tissue were taken from each fraction and ethanol was added to a final concentration of 70 %. The alcoholic suspension was boiled during 2 minutes and then filtered with suction through a glass filter (G 4). Five ml acetone was layered on top of the precipitate and sucked through very slowly. The acetone treatment was repeated once and the filter cake dried in the air. When dry the white cake was thoroughly mixed on the filter with 8–10 ml of a 70 % ethanol, 1 % $HClO_4$ solution. After 10 minutes at room temperature the precipitate was filtered off again. The residue was then extracted twice with a mixture of ethanol and ether (3:1) by boiling for a few

minutes and then filtered on the same glass filter as before and dried. It was then quickly washed with 5 ml cold 0.2 M perchloric acid and finally extracted with 1.0 M $HClO_4$ at 4 °C for 16 hours. The precipitate was filtered off through the glass filter and thoroughly washed with 1.0 M $HClO_4$. The 1.0 M perchloric acid filtrate contained the hydrolysis products of RNA. The concentration was measured by determining the extinction at 260 mμ and estimating the concentration from a standard curve of purified yeast-RNA in 1.0 M perchloric acid.

2.8. Electron microscopic techniques

Preparation of ultra-thin sections

Small pieces of tobacco leaf were fixed with 1% OsO_4 in 0.028 M sodium veronal acetate buffer, pH 7.4, during 4 hours. The fixed tissue was washed with the veronal acetate buffer and dehydrated by putting it in respectively 50%, 70% and 96% ethanol for 5 minutes. Finally it was rinsed with absolute alcohol, 20 times during 10 minutes and once for 20 minutes.

The tissue was embedded by putting it in a mixture of butyl and methyl methacrylate (4:1) during 16 hours at room temperature, then in a similar mixture with 2% benzoyl peroxide for 2 hours and after that this mixture was placed at 60 °C for 24 hours. Sections were cut from the embedded tissue using a Porter-Blum ultramicrotome. The thickness of the sections was checked by means of the interference color of reflected light. Sections with a thickness of 500–600 Å were loaded onto grids with a formvar film. They were stained with 1.2% $KMnO_4$ in distilled water for 1 hour and then washed with 0.25% citric acid in distilled water for 1 minute and were finally washed with distilled water.

In case of fractions from a leaf homogenate, the pellets of centrifugations at different speeds were fixed with 1% OsO_4 in veronal acetate buffer pH 7.4 during 4 hours. The fixed pellets were further treated as described for the leaf pieces.

Sections of leaf or pellets were examined under the Siemens Elmiskop I electron microscope.

Positive staining of ribosomes

Ribosomes were precipitated by centrifugation at 105,000 × *g* and resuspended in buffer pH 7.0 containing 0.01 M ammonium acetate and 0.001 M magnesium acetate. A 0.1% suspension was sprayed onto grids with a 100 Å thick carbon film by means of a glass nebulizer. The grids were then immersed into a 2% solution of uranyl acetate in distilled water which was brought to pH 5.0 with 1 M sodium hydroxide. The preparations were left in this solution for two hours at room temperature. They were then washed by putting a drop of distilled water on the grid and sucking the water away with filter paper. This was repeated three times. The stained ribosomes were examined under the Siemens Elmiskop I electron microscope.

Measurement and counting of TMV particles

To 0.9 ml of a TMV suspension of the desired concentration 0.1 ml of a 0.3% serum albumin (bovine) solution in distilled water was added. The mixture was sprayed onto grids with formvar films by means of a glass nebulizer. The serum albumin served to improve the spreading of the droplets on the film. The preparations were shadow casted with palladium and examined in the Philips M 100 electron microscope.

CHAPTER 3

FRACTIONATION OF TOBACCO LEAVES

This chapter will describe the fractionation procedure selected for the isolation of the ribosomes from tobacco leaves. The distribution of the leaf-RNA over the fractions was examined. The greater part of the RNA occurred in a particulate fraction of nucleoproteins which sedimented at 105,000 × *g* for 1 hour. The origin in the cell of the nucleoprotein particles, the ribosomes, was traced using the electron microscope. The properties of this fraction will be discussed in more detail in the next chapter.

All results to be presented in Chapter 3 and 4 are from experiments with healthy tobacco plants.

3.1. SURVEY OF THE FRACTIONATION

The following experiment was carried out to get a general view of what happens in fractionation by differential centrifugation. Leaves of 'White Burley' tobacco without the midribs, were cut in pieces and ground along with buffer containing 0.5 M sucrose, 0.005 M tris-HCl (tris(hydroxymethyl)-aminomethane-hydrochloride), 0.01 M magnesium acetate, pH 7.0, in a mortar with a pestle and sand abrasive. The homogenate was filtered through glass wool and the filtrate centrifuged at 600 × *g* for 10 min. From this stage on, further fractionation was followed spectrophotometrically and by means of the analytical ultracentrifuge. The supernatant after 25,000 × *g* was spun at 105,000 × *g* (40,000 rpm) for 1 hour. The pellet was resuspended in buffer, (0.005 M tris-HCl, 0.001 M Mg acetate, pH 7.0) and then the suspension was clarified by centrifuging at 12,500 × *g* for 15 min. The last two centrifugations were repeated twice at high and low speed respectively. All treatments were done at 0–3 °C.

TABLE 1. Spectral data of fractions from tobacco leaf homogenates obtained by differential centrifugation.

Fraction	λ_{max} (mμ)	λ_{min} (mμ)	$\frac{E_{max}}{E_{min}}$	$\frac{E_{260}}{E_{280}}$
Supernatant after 25,000 × g for 15 min.	260	251	1.03	1.36
After 105,000 × g for 1 hour: supernatant	260	250	1.03	1.34
Pellet resuspended in tris-HCl buffer and clarified = M_1	260	242	1.20	1.52
M_1 1 hour at 105,000 × g; pellet resuspended in buffer and clarified = M_2	260	240	1.39	1.67
M_2 1 hour at 105,000 × g; pellet resuspended in buffer and clarified = M_3	260	239	1.46	1.89

Some spectral data of various fractions are recorded in Table 1. The successive fractions after each cycle of high and low speed centrifugation are referred to as M_1, M_2 and M_3 respectively. The fractions were diluted to about the same extinction at 260 mμ and the spectra were taken. The wavelengths of the maximum and the minimum in the spectra are given and also the ratios of the extinctions at a maximum and a minimum (E_{max}/E_{min}) and the ratios of the

extinctions at 260 mμ and 280 mμ (E_{260}/E_{280}). It can be seen from Table 1 that the differential centrifugation resulted in purification of a nucleoprotein. This is evident from the shift of the position of the minimum of M_1, M_2 and M_3 respectively to a lower wavelength whereas at the same time E_{260}/E_{280} and E_{max}/E_{min} increase.

The spectrum of the fraction, referred to as M_3 in the table, is typical for a nucleoprotein with a rather high content of nucleic acid. By simultaneously examining the fractions in the analytical ultracentrifuge, it was found that this fractionation produced a purified component with a sedimentation coefficient S_0^{20} of 80 *S*. After that we determined the amount of RNA which could be extracted from leaf tissue and the part of the RNA going into the M fraction.

3.2. Amount of RNA extracted from the leaves

For determining the amount of RNA which could be extracted, portions of tobacco leaves without midribs were cut in pieces and two samples of 5 g each were taken. In both samples the amount of RNA was estimated according to the method of Ogur and Rosen described in 2.7. The rest of the leaf material was ground with 1.5 ml sucrose buffer (0.5 M sucrose, 0.005 M tris-HCl, 0.01 M Mg acetate) pH 7.0 per gram tissue and sand. The homogenate was filtered through glass wool. The filter cake was again ground together with the glass wool in 1 ml/gram tissue of the same sucrose buffer and filtered through glass wool a second time. The combined filtrates will be referred to from now on as the extract. From the extract two samples were taken corresponding to 5 g leaf tissue with the amount of RNA being determined as described in 2.7. Results are given in Table 2. It can be seen from this table that 70 to 80% of the RNA in the leaves could be extracted. Less effort in grinding resulted in a smaller amount of RNA in the extract. When the residue left after extraction was examined under the microscope, whole cells and cell fragments could always be seen. The fact that only 70–80% of the RNA could be extracted from the leaf material appears primarily due to incomplete disruption of the leaf cells in the extraction procedure.

Scheme 1. Fractionation of the extract of leaf homogenate.

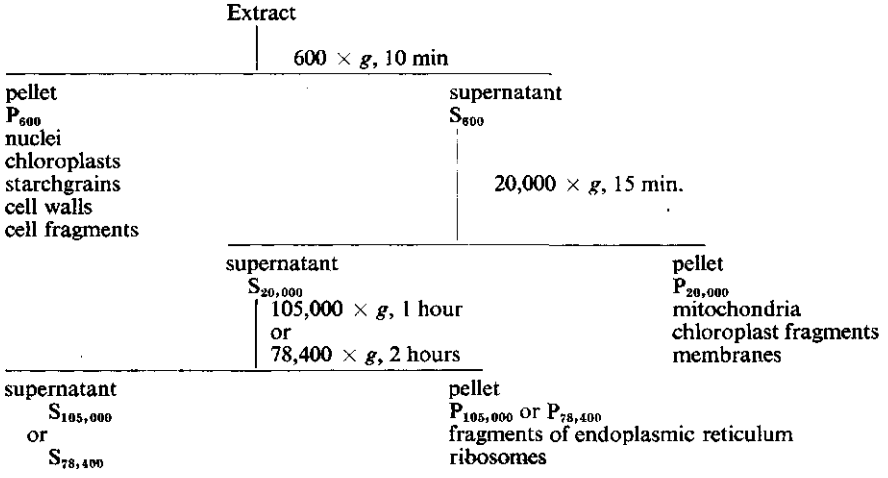


TABLE 2. RNA content of leaves and leaf cell fractions prepared by centrifugation of a leaf extract at increasing g-values. The numbers give the amount of RNA in milligrams per 5 g leaf tissue, except column 11 and 12 where just milligram amounts are given. For explanation of treatments and fractions see Scheme 1 and text.

1	2	3	4	5	6	7	8	9	10	11	12	13	14
Leaves	Extract	% extracted	P_{600}	S_{600}	% lost compared to extract	$P_{20,000}$	$S_{20,000}$	% lost compared to S_{600}	% in $S_{20,000}$ compared to extract	$S_{105,000}$ or $S_{78,400}$	$P_{40,000}$ or $P_{78,400}$	% in $P_{105,000}$ or $P_{78,400}$	Centrifugation
3.50	2.55	73%											
3.95	3.28	83%		2.9	12 %		2.8	3 %	85%	1.1	1.6	59%	1 hr 105,000 × g
2.37	1.78	75%		1.73	3 %		1.65	4 %	93%	0.16	1.0	86%	,,
3.86	3.12	80%	0.10	3.00	4 %	0.33	2.39	19 %	77%	0.23	2.16	90%	,,
3.27	1.64	50%		1.60	2.5%		1.59	0.5%	97%	0.65	1.00	61%	,,
3.50	1.85	53%	0.11	1.66	10 %	0.27	1.40	14 %	76%	0.5	0.76	60%	,,
	1.15			1.08	6 %		1.09	–	95%	0.13	0.80	86%	2 hr 78,400 × g
					av. 6 %			av. 7 %	av. 87%	0.61	1.16	65%	,,
										0.12	0.26	72%	,,
										0.52	2.2	81%	1.5 hr 105,000 × g
										0.27	1.06	80%	,,
												av. 74%	

3.3. Distribution of RNA over fractions of a leaf homogenate

The extract was fractionated by centrifugation according to Scheme 1, being a slight modification of the procedure described in 3.1. The cellular components found in each fraction are indicated.

The pellets were resuspended in buffer (0.005 M tris-HCl, 0.001 M Mg acetate) pH 7.0. Samples were taken from the fractions corresponding to 5 g leaf tissue and the RNA content was determined according to the method of Ogur and Rosen (2.7). The results of the RNA determinations are summarized in Table 2. It is seen that on the average more than 85% of the RNA from the extract was found in the $S_{20,000}$ fraction. The losses in the pellets from centrifugation at lower *g*-values were only small. It should be noted that the pellets P_{600} and $P_{20,000}$ were not washed so they may still have been contaminated by components from the supernatant liquid $S_{20,000}$. On an average, 6% of the RNA from the extract was lost in P_{600}, which contains nuclei, whole chloroplasts and small cell fragments, with 8% of the RNA being lost in $P_{20,000}$ which consisted mainly of mitochondria and chloroplast fragments.

The $S_{20,000}$ fraction was centrifuged either at 78,400 $\times$ *g*, in the no. 30 rotor of Spinco for 2 hours, or at 105,000 $\times$ *g* in the no. 40 rotor of Spinco for 1 or 1.5 hour. At this centrifugation the RNA was distributed in such a way that on the average 74% went into the pellet. However, there was a rather large variation in the percentages found in individual cases. We think this was due to non-ideal sedimentation – that is, sedimentation which was not always complete or situations where the pellet was not sufficiently packed. When this was overcome by taking a longer sedimentation period, e.g. 1.5 hour at 105,000 $\times$ *g*, more than 80% of the RNA was sedimented. This percentage was in our view a more reliable indicator of the amount of sedimentable RNA. Summarizing, we may conclude that more than 65% of the RNA in the original extract can be found in the ribosomal fraction. About 20% of the RNA is non-sedimentable at 105,000 $\times$ *g* and is left in the "soluble" fraction $S_{105,000}$.

Our data for tobacco leaf tissue may be compared with the results of Tissières *et al.* (1959) who found for *Escherichia coli* that 80–90% of the RNA occurred in the ribosomal fraction and 10–20% of the RNA in a fraction not sedimentable at 105,000 $\times$ *g*. Schneider *et al.* (1950) found further 65% of the RNA phosphate of mouse liver in the microsomes with 17% soluble RNA. Lyttleton (1960) determined for white clover leaves that about 20% of the RNA was not sedimentable even under conditions for which sedimentation of the nucleoproteins was complete.

3.4. Electron microscope observations

This section will describe the electron microscope observations on the occurrence of ribosomes in leaf cells and in the fractions isolated from leaf homogenates. Tobacco leaves consist mainly of mesophyll cells which can be divided morphologically into palisade and spongy parenchyma. Besides these cells there is a certain amount of transport tissue (the veins) and an epiderm with stomata and hairs.

Although a leaf is not composed of only one cell type, most of the cells do contain the structures responsible for virus multiplication. Thus in trying to isolate subcellular structures from leaves which are involved in virus synthesis,

the dilution by material from cells not participating should not be great. A fractionation at the cellular level did not appear necessary.

Photo 1 shows pictures of parts of ultrathin sections of a healthy tobacco leaf fixed with osmiumtetroxide. Photo 1a shows part of the cytoplasm of two neighbouring cells connected to each other by a plasmodesm. The cytoplasm is rich in darkly colored granules (the ribosomes) which have a diameter of about 150–180 Å. Furthermore, membrane structures which form part of the endoplasmic reticulum are visible. It can be seen that the membranes go through the plasmodesm from one cell to the other. At some places ribosomes appear to be attached to the membranes but this is probably only a chance occurrence. On the whole the membranes look rather smooth and are not set with particles so that they could be called rough surfaced. Most of the ribosomes do not seem to be attached to membranes. However, the ribosomes are not spread at random in the cytoplasm. In most cases they appear to be in clusters with some mutual connection among ribosomes close to each other. As far as this connecting structure could be viewed, it appeared to be different from the endoplasmic reticulum as the reticulum membrane appears to be much thicker. The reticulum membrane was found to be as thick as the nuclear membrane (photo 1b).

Ribosomes were found in all leaf cells and we have not observed any differences in the way they occurred.

Next we studied the fractions of the cell constituents gotten by differential centrifugation to see if it would be possible to localize the ribosomes in a particular fraction. The following pellets obtained by stepwise centrifugation of the extract were examined: 1) after 10 min at 600 $\times$ g; 2) after 7 min at 10,000 $\times$ g; 3) after 15 min at 20,000 $\times$ g; 4) after 10 min at 80,000 $\times$ g and 5) after 60 min at 105,000 $\times$ g. The pellets were fixed with 1% osmiumtetroxide in veronal-acetate buffer pH 7.4 during 4 hours and then embedded in methacrylate resin as described in 2.8. Ultra-thin sections of the pellets were examined (Photo 2).

It was not easy to establish the origin of the structures seen in the pellets with the electron microscope. Complete subcellular particles may be easily identified. The numerous fragments of those particles – often more or less swollen – were not always so quickly placed. Visible in the 600 $\times$ g pellet (P_{600}) were cell fragments and complete or somewhat damaged chloroplasts. No recognizable nuclei were detected with the electron microscope in this fraction. Nuclei, however, were found in this fraction with the light microscope. In the 10,000 $\times$ g and 20,000 $\times$ g pellets, mitochondria and perhaps proplastids, and a large amount of chloroplast fragments were found. No ribosomal particles were detected in the 10,000 $\times$ g pellet whereas in the 20,000 $\times$ g pellet a small group of granules with a ribosomal diameter was found once in a while enclosed between the vescicles of chloroplast fragments. Many membrane structures in the form of closed vescicles, could be seen together with ribosomes in the pellet after 10 minutes centrifugation at 80,000 $\times$ g. The vescicles in this fraction most probably did not originate from chloroplasts, but were fragments of endoplasmic reticulum. The 105,000 $\times$ g pellet ($P_{105,000}$) consisted chiefly of ribosomes contaminated with membranes (see Photo 2). These membranes could be eliminated further by resuspending the pellet and centrifuging at 20,000 $\times$ g and, if desired, repeating the high and low speed centrifugations.

It appears that the ribosomes constitute a well-defined fraction in the centrifuging scheme, notably the fraction which sediments at g values higher than

20,000 and in 1 hour at 105,000 × *g*. The particles in the 105,000 × *g* pellet have a diameter of 150 – 180 Å. They may be stained with uranyl acetate (Photo 3 and 4) as might be expected from particles rich in RNA (HUXLEY and ZUBAY, 1959). Besides many detached particles, the granules are in lines or clusters. Although the possibility of an artifact should be borne in mind, it seems more probable that the observed structure in the isolated ribosomal preparation corresponds to the cluster-like occurrence of the ribosomes in the cell.

3.5. DISCUSSION

A further discussion of the ribosomes and the endoplasmic reticulum in leaves is given here. The views concerning the endoplasmic reticulum have arisen from the work of PORTER (cf. PORTER, 1953) and PALLADE (cf. PALLADE, 1958) supported by others. The endoplasmic reticulum is described as a three dimensional network of thin membranes (about 7 mμ thick) which build an extensive system of spaces: vescicles, tubules, cisternae. The so-called ground substance of the cytoplasm is divided into two distinct phases by the existence of this internal membrane system: one represented by the content of the interconnected vesicles, the other by the surrounding cytoplasmic matrix. PALLADE and PORTER describe local differentiations within the endoplasmic reticulum and they distinguish rough-surfaced membranes to which ribosomes are attached and smooth membranes without these particles. The difference is illustrated, among others, by beautiful micrographs from rat pancreas (PALLADE, 1958) and liver. For plant tissue this difference appears to be less clear. PORTER and MACHADO (1960) report the occurrence of rough-surfaced and smooth-surfaced endoplasmic reticulum for meristematic cells of onion roots but the micrographs are less convincing in this case. The number of ribosomes attached to membranes is so small that the union of both may be by chance, meaning the evidence is not compelling. In the sections of tobacco leaf we did not distinctly observe rough-surfaced membranes comparable with those of liver and pancreatic cells.

Further proof of the occurrence of ribosomes attached to membranes in liver and pancreas can be shown by a fraction which can be isolated from homogenates of these organs consisting of fragments of the rough-surfaced endoplasmic reticulum. These fragments are called microsomes by PALLADE (1958). When the microsomal fraction from liver is fixed with osmiumtetroxide the microsomes appear as closed vesicles with ribosomes attached to the outer surface of the membranes when viewed in ultra-thin sections under the electron microscope. It may be noted that microsomes, being fragments, are found only *in vitro*, viz. homogenates. In the cell one can, at most, only point out the structures from which the microsomes can arise. The ribosomes may be loosened from the microsomes by treating them with a 0.1 % solution of sodium deoxycholate at pH 7.2. All these observations seem to prove the union of ribosomes and membranes.

Besides the ribosomes found attached to endoplasmic reticulum, one also finds unbound ribosomes dispersed in the cytoplasm between the endoplasmic canals of liver and pancreatic cells. They are sedimented after the microsomes and are referred to as the postmicrosomal fractions. PALLADE (1958) points out that "free" ribosomes, in the cell as well as in the pellet of the postmicrosomal fraction, frequently are in clusters or chains.

As far as is known to us, no clear differences in endoplasmic reticulum from plant tissue have been demonstrated justifying a distinction between rough-surfaced and smooth-surfaced reticulum for plants. Yet some authors (DUCET, 1961; BUVAT, 1958) claim such a distinction, while others (WHALEY *et al.*, 1960) accept it only with reservations.

One may have further doubts about the occurrence of rough-surfaced endoplasmic reticulum in plant tissue, since until now, no microsomes comparable in some degree to those from liver and pancreas, could be isolated from plant tissue homogenates. Reports of such isolations were not found in the literature, neither were we able to isolate such a fraction from tobacco leaves. We deliberately collected a fraction which sedimented in between the 20,000 $\times$ *g* and the 105,000 $\times$ *g* fraction, i.e. after 10 minutes at 80,000 $\times$ *g*, in the hope of detecting fragments of possible rough-surfaced reticulum; however, it was without success. The term microsomes, we concluded, should therefore not be used for leaf tissue and probably not for plant tissue at all. If, in fact, no rough endoplasmic reticulum exists in plants, then this difference from tissues of other organisms should be stressed as it may give proof of an important difference in organization at the subcellular level.

It seems conceivable that the structure in which ribosomes are attached to endoplasmic reticulum is limited to cells with a special function e.g. cells which produce proteins for secretion.

The fraction from tobacco leaves containing the ribosomes shows resemblance with the postmicrosomal fraction from pancreas obtained by PALLADE (1958) with the ribosomes dispersed in the cytoplasm. The ribosomes in the pellet appear to have some mutual coherence. This should not be confused with the presence of endoplasmic reticulum since the connecting structure is much thinner than the reticulum membranes. A possible functional explanation of this mutual coherence of ribosomes should be borne in mind, but as yet, this is only a question.

CHAPTER 4

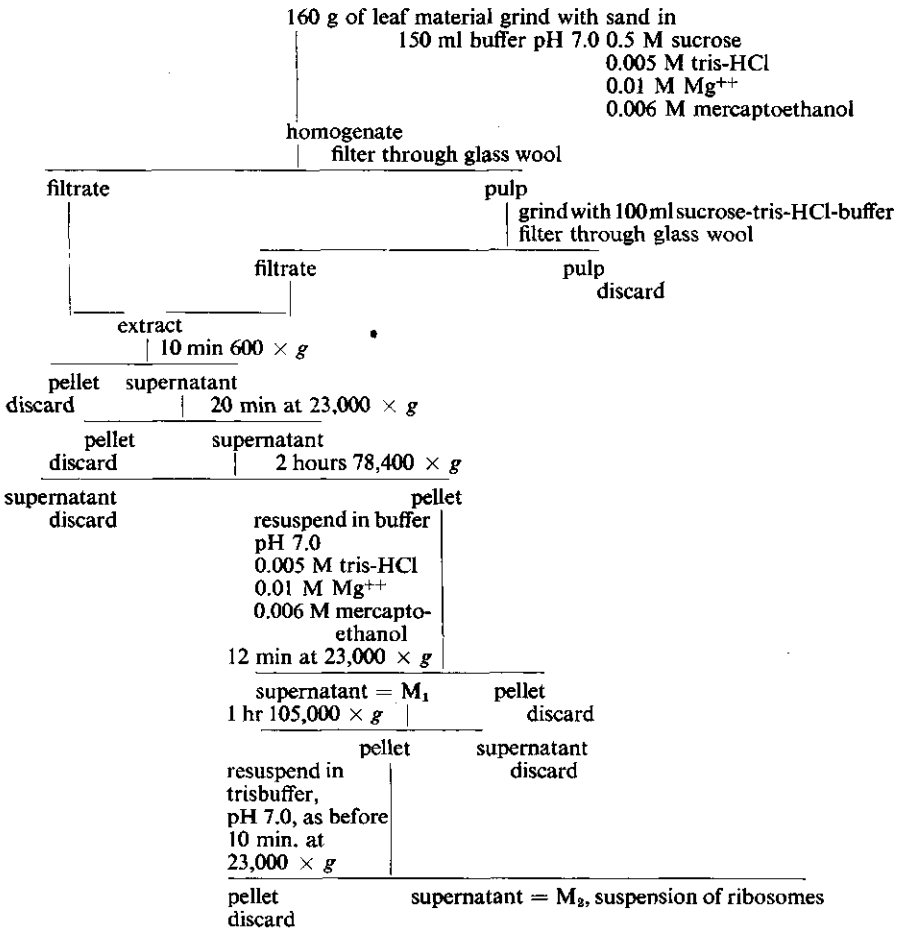
PROPERTIES OF RIBOSOMES FROM TOBACCO LEAVES

As described in the previous chapter, ribosomes are ribonucleoprotein particles with a diameter of 150–180 Å and occur in the cytoplasm. Ribosomes are one of the topics of biochemical study due to the fact that they play an important role in protein biosynthesis. The amino acids are linked to peptide chains at the ribosomes whereby the specific sequence of the amino acids in the protein is established. Recently T'SO (1962) gave a general review of the extensive literature on ribosomes to which we will refer. In his paper T'SO suggests all naturally occurring ribonucleoproteins be designated ribosomes. This implies that plant viruses belong to the ribosomes which appears to assume too much. We shall, therefore, not support his definition as given. The properties of these particles as isolated from healthy tobacco leaves will be described further in this chapter.

4.1. PREPARATION OF THE RIBOSOMAL FRACTION

The standard procedure used for isolating ribosomes from tobacco leaves is given in Scheme 2.

SCHEME 2. Standard procedure used for isolating ribosomes from tobacco leaves.



Midribs were cut from leaves of 'White Burley' tobacco and discarded. The rest of the leaf tissue was cut into pieces and 160 grams of the material was ground with 150 ml buffer pH 7.0 (0.5 M sucrose, 0.005 M tris-HCl (= tris (hydroxymethyl)aminomethane-hydrochloride),0.01 M Mg acetate, 0.006 M mercapto-ethanol (= MCE)) in a mortar with a pestle and about 100 grams of sand as an abrasive. All operations were performed in the cold room at 0–3 °C. The homogenate was filtered with suction through a thin layer of glass wool. The filter cake was ground once more with 100 ml of the same sucrose buffer used previously and again the extract was filtered through a glass wool layer.

The combined filtrates were centrifuged for 10 minutes at 600 × g. The pellet was discarded and the supernatant spun at 23,000 × g for 20 minutes in a Servall SS-1 centrifuge. The pellet was discarded and the supernatant centrifuged in the no. 30 rotor of the Spinco model L centrifuge at 78,400 × g for 2 hours. This time the supernatant was discarded and the pellet resuspended in buffer pH 7.0 (0.005 M tris-HCl, 0.01 M Mg^{++}, 0.006 M MCE) by gentle

homogenisation in a Potter-Elvehjem tube or by forcing the liquid through the needle of a syringe. The suspension was clarified by centrifugation at 23,000 × *g* for 15 min. The ribosomes were sedimented again by spinning at 105,000 × *g* for 1 hour in the Spinco no. 40 rotor. The supernatant was discarded and the ribosomes were resuspended in 0.005 M tris-HCl, 0.01 M Mg^{++}, 0.006 M MCE pH 7.0 buffer and again the suspension was clarified by centrifugation at 23,000 × *g* for 15 minutes. The final suspension of ribosomes was carefully decanted or taken off with a pipette. The yield was usually 35–40 mg RNA from 160 grams of leaf material. The suspension in 5–8 ml was colored light green or yellowish. In most of the experiments, the ribosomal preparation was used in this condition. Sometimes the ribosomes were further purified by another cycle of high and low speed centrifugation.

4.2. RNA AND PROTEIN CONTENT

The RNA and protein content of the ribosomal preparation was determined by the methods described in Chapter 2. Results are shown in Table 3. These determinations were made on ribosomal preparations which had three cycles of high and low speed centrifugation. Ribosomes had a somewhat higher protein content after two cycles of high and low speed centrifugation. Assuming that the preparations contained only protein and RNA, then the RNA to protein ratio corresponds to a RNA content of 42–47%.

TABLE 3. RNA and protein content of ribosomal preparations.

No.	Protein μg/ml	RNA $HClO_4$ method) μg/ml	RNA (orcinol method) μg/ml	RNA protein
1	1060	956	930	0.89
2	500	366	375	0.74
3	650		466	0.72

In testing for deoxyribonucleic acid (DNA) the diphenylamine reaction was always negative. No thymine could be detected by paper chromatography. So, it may be assumed that the preparations were free from DNA. No determinations of phospholipids were performed.

Ribosomes from white clover leaves have an RNA content of 52–56% (LYTTLETON, 1960), those from pea shoots contain 35–40% RNA (T'SO *et al.*, 1956), values of 60–65% have been reported for ribosomes from *E. coli* (TISSIÈRES *et al.*, 1959) and 40–45% for ribosomes from yeast (CHAO and SCHACHMAN, 1956).

4.3. UV ABSORPTION SPECTRUM

The ultra violet absorption spectrum of the ribosomal preparations had a maximum at 258–260 mμ and a minimum at 238–240 mμ. The ratio of the extinctions at the maximum and minimum was $E_{max}/E_{min} = 1.50–1.55$ and of the extinctions at 260 mμ and 280 mμ was $E_{260}/E_{280} = 1.80–1.86$. For less pure preparations these ratios were lower. A linear relationship was established between the extinction at 260 mμ and the concentration of the preparation up to an extinction of 2.0. From a number of simultaneous determinations of the

extinction at 260 mμ and the RNA content, it was concluded that a suspension containing 55 μg ribosomal RNA/ml had an extinction $\propto {}^{1\,cm}_{260} = 1.316$. We used this for estimating the concentration of the ribosomes. The concentration of ribosomes in all further experiments is expressed in milligrams ribosomal RNA, based on this value.

Independently TISSIÈRES *et al.* (1962) reported an extinction of 130 at 260 mμ representing a concentration of 5.2 mg ribosomal RNA per ml for *E. coli* ribosomes.

4.4. INFLUENCE OF MAGNESIUM IONS

We examined the sedimentation diagram of the ribosomal preparation and looked at the influence of the concentration of the magnesium ions on the ribosomes. It is known (TISSIÈRES *et al.*, 1959) that ribosomes from *E. coli* having a sedimentation coefficient of 70 *S* can be split reversibly into two subunits of 50 *S* and 30 *S* respectively dependent on the Mg^{++} concentration. Ribosomes from pea seedlings having a sedimentation coefficient of 80 *S* have been reported to split reversibly into a 60 *S* and a 40 *S* component dependent on the concentration of Mg^{++} ions (T'SO, 1958).

When the ribosomes were in 0.005 M tris-HCl buffer 0.01 M Mg^{++}, pH 7.0, the preparation had mainly two peaks with sedimentation coefficients $S_0^{20} = 80$ *S* and $S_0^{20} = 112$ *S* respectively (see Fig. 1a and 1b). Usually they also had small peaks of a 40 *S* and a 60 *S* component (We shall refer to these peaks as the 40 *S* and 60 *S* peaks, although the real measured values of the sedimentation coefficient fluctuated somewhat between 37–45 *S* and 58–66 *S*). When the Mg^{++} concentration was reduced to 10^{-3} M and further to 10^{-4} M the 112 *S* component gradually disappeared but there was no increase of 60 *S* and 40 *S* components or a decrease of the 80 *S* peak. Even dialysing the preparation against 10^{-4} M Mg^{++} for 20 hours did not cause splitting of the 80 *S* component. When

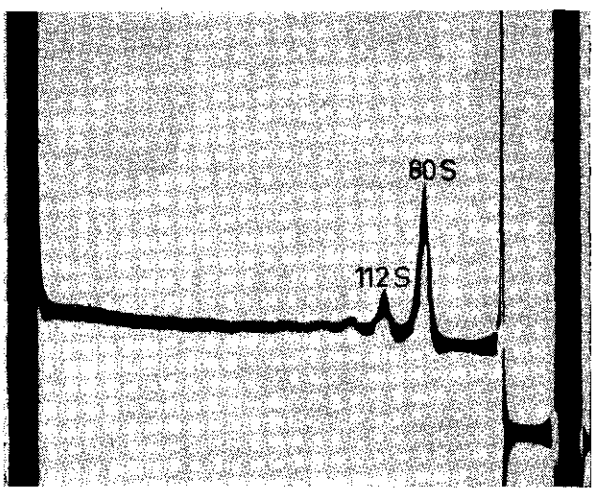


FIG. 1a. Sedimentation diagram of a ribosomal preparation from healthy leaves in 0.005 M tris-HCl, 0.01 M Mg acetate pH 7.0. Sedimentation from right to left. Speed 44,770 rpm. Temperature 8.7 °C. Photo taken about 10 minutes after starting the rotor.

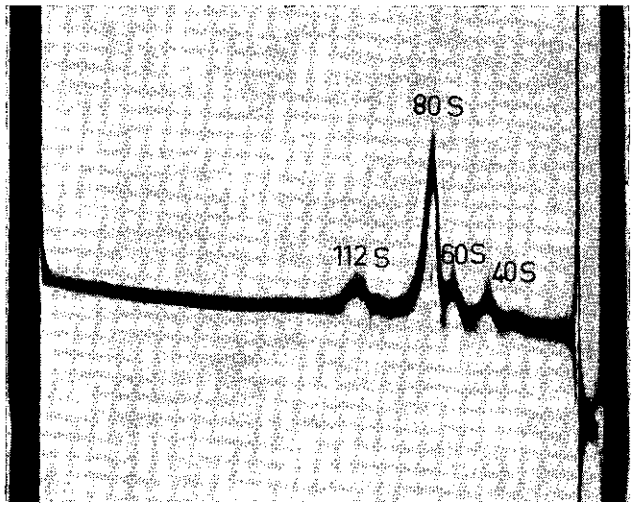


FIG. 1b. Sedimentation diagram of a ribosomal preparation from healthy leaves in 0.005 M tris-HCl, 0.001 M Mg acetate, pH 7.0. Sedimentation from right to left. Speed 44,770 rpm. Temperature 9.2 °C. Photo taken about 10 minutes after starting the rotor.

the Mg^{++} concentration was then increased to 10^{-2} M, a small 112 *S* peak appeared again but the 40 *S* and 60 *S* components did not disappear. It seemed that the ribosomal preparation contained mainly particles with a sedimentation coefficient of 80 *S*, which could not be split dependent on the Mg^{++} concentrations. In this respect the behavior of leaf ribosomes is not the same as that of the 70 *S* ribosomes from bacteria (TISSIÈRES *et al.*, 1959) or of the 80 *S* pea ribosomes (T'SO *et al.*, 1958). Neither the 40 *S* and 60 *S* component present in the preparation aggregated at higher Mg^{++} concentrations. Since it was difficult to raise 60 *S* and 40 *S* particles from 80 *S* particles, and both components are persistent, it seems probable that 60 *S* and 40 *S* are naturally occurring particles in the leaf. At a concentration of 10^{-2} Mg^{++}, some aggregation of 80 *S* particles to 112 *S* particles occurs and this aggregation is reversible. Depending on the Mg^{++} concentration, the 112 *S* peak may vanish or reappear.

When the concentration of the Mg ions was increased beyond 10^{-2} M the particles aggregated still further and became precipitable at low centrifugal forces. Between 0.03 and 0.05 M Mg^{++} there was complete precipitation of the ribosomes. This precipitation was not reversible, for the precipitate did not dissolve and it was impossible to resuspend the ribosomes. This is in contrast to the findings of TAKANAMI (1959) for ribosomes of rat liver. TAKANAMI purified rat liver ribosomes by precipitating them with Mg^{++} ions and redissolving after that.

Addition of a chelating substance such as ethylenediaminetetra-acetic acid (EDTA) to a concentration of 0.01–0.02 M caused splitting of the 80 *S* particles with components with a sedimentation coefficient of about 20 *S* and 30 *S* arising. When these were sedimented by high speed centrifugation for a few hours and the pellet was resuspended in 0.005 M tris-HCl buffer pH 7.0, containing 10^{-2} Mg^{++}, no 80 *S* or any other particles were reconstituted.

The sedimentation diagram was the same when the ribosomes were suspend-

ed in 0.02 M potassium phosphate buffer pH 7.0, instead of tris-HCl buffer, or in lower phosphate concentrations of the same pH. The influence of the Mg^{++} concentration was the same as in tris-HCl buffer. When the phosphate concentration was increased to 0.05 M, the ribosomes dissociated, the 80 *S* peak decreased and lower *S* components appeared. For example in a certain preparation of ribosomes in 0.05 M phosphate pH 7.0, a 75 *S* peak (corresponding to the 80 *S* component), 18 *S*, 34 *S* and 44 *S* peaks occurred side by side (see Fig. 2). This dissociation could not be reversed by lowering the phosphate concen-

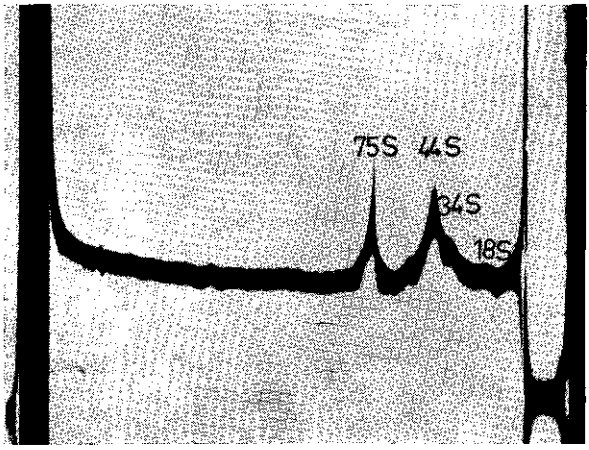


FIG. 2. Sedimentation diagram of a ribosomal preparation from healthy leaves in 0.05 M potassium phosphate pH 7.0 after 18 hours at 0–3 °C. Sedimentation from right to left Speed 44,770 rpm. Temperature 8.4 °C. Photo taken about 12 minutes after starting the rotor.

tration and adding magnesium acetate. The dissociation in 0.05 M phosphate increased with pH in the sequence 6.5, 7.0, 7.5, and was complete at pH 8.0. The dissociation at higher pH's could not be reversed by lowering the pH as reported by T'so (1958) for ribosomes from pea seedlings.

4.5. RNA FROM THE RIBOSOMAL FRACTION

The RNA from the ribosomes was prepared by extraction with water-saturated phenol according to the method of GIERER and SCHRAMM (1956) as modified by HALL and DOTY (1959). In this modification sodium lauryl sulphate is added to the ribosomes to a final concentration of 0.1 % and the suspension is stirred for 5 min at room temperature before extracting the preparation with water-saturated phenol as described in 2.3. The final preparation of RNA in 0.02 M sodium phosphate at pH 7.0 had a sedimentation diagram (see Fig. 3) with two distinct peaks as revealed in the analytical ultracentrifuge. These two components had sedimentation coefficients in 0.02 M sodium phosphate pH 7.0 of $S_0^{20} = 25.3$ *S* and $S_0^{20} = 15.4$ *S* respectively. Both components occurred in a proportion which varied between 25.3 *S*:15.4 *S* = 1:1 — 1:1.25. Besides the two main components, some slower sedimenting material was always present in a broad band in varying amounts up to 15 % as determined by measuring the surfaces of the peaks.

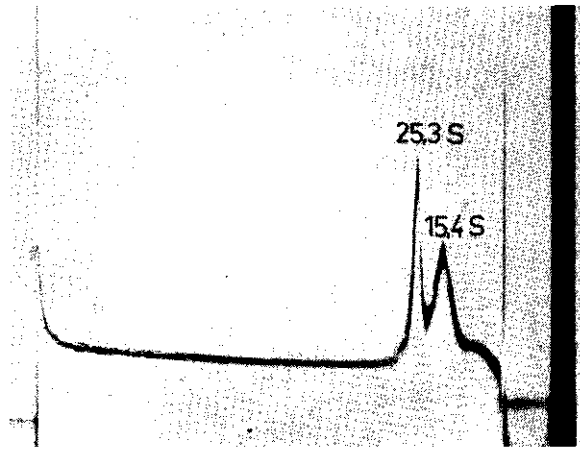


FIG. 3. Sedimentation diagram of RNA prepared from healthy leaf ribosomes by phenol extraction. Solvent 0.02 M sodiumphosphate pH 7.0. Sedimentation from right to left. Speed 50,740 rpm. Temperature 7.1 °C. Photo taken about 22 minutes after starting the rotor.

Lauryl sulphate is said to inhibit the ribonuclease present in latent form in ribosomes (ELSON and TAL, 1959) and set free when the ribosomes are broken down. Furthermore it aids in giving a quicker separation of protein and RNA. We were able to confirm that sodium lauryl sulphate has a favorable effect on the preparation of RNA. Omitting the pretreatment with lauryl sulphate resulted in the production of low weight material, broad peaks of about 3–4 *S* and 6–10 *S* arose, while none of the heavier component was found.

The RNA preparations were found to be stable to the extent that the sedimentation diagram did not show a marked difference after overnight storage of the RNA in the refrigerator.

The occurrence of low weight material in the preparation of ribosomal RNA might be explained by assuming internal breakdown of RNA in the ribosomes or breakdown during preparation by phenol extraction. The first possibility can not be accepted as no increase of low weight material was observed when the ribosomes were stored during the night at 0–3 °C before preparing the RNA. Even though the breakdown during preparation of the RNA cannot be excluded one should bear in mind that this low weight RNA may also have another source. It might be a completely other kind of RNA not originating from the ribosomal particle. For example, it could be messenger RNA which is known to attach to the ribosomes as has been shown by NOMURA, HALL and SPIEGELMAN (1960), BRENNER *et al.* (1961) and GROS *et al.* (1961) especially in the presence of a rather high magnesium ion concentration as in our case. Some of the RNA might also be transfer-RNA which transports the amino acids to the ribosomes for protein synthesis.

4.6. AMINO ACID INCORPORATION

As mentioned before, ribosomes are involved in protein biosynthesis. It was, of course, of great interest to see if the ribosomes from tobacco leaves had preserved some of their biosynthetical activity. Many cell-free systems from

micro-organisms and animal organs capable of protein synthesis *in vitro* have been described (for a review see HOAGLAND, 1960). For plants, however, only WEBSTER (1959) and RAACKE (1959) have reported cell-free systems capable of *in vitro* amino acid incorporation into protein. Both used pea seedlings as starting material.

The course of protein synthesis from amino acids follows briefly. First amino acids are activated at the carboxyl end group by adenosine triphosphate (ATP) and amino acid activating enzymes. The activated amino acids are subsequently linked to low-molecular, soluble, non ribosomal RNA, so-called transfer-RNA. This reaction is catalysed by enzymatic preparations which contain amino acid activating enzymes and require ATP as a source of energy. The next step is the association of the intermediate soluble RNA-amino acid compound with the ribosomes where formation of the peptide bond takes place and the specific sequence of amino acids is established. This last step is dependent on guanosine triphosphate (GTP) and ATP, while a specific, so-called messenger-RNA, is involved in specifying the sequence of amino acids (HOAGLAND, 1960; MATTHAEI and NIRENBERG, 1961; NIRENBERG and MATTHAEI, 1961).

Therefore, the requirements for a system which incorporates amino acids in protein *in vitro* are: amino acids, ATP, soluble enzymes (amino acid activating enzymes and other enzymes essential to the process), transfer-RNA, GTP and ribosomes with messenger RNA. Usually an ATP-generating system is also added to the system e.g. phospho(enol)pyruvate and pyruvate phosphokinase. The incorporation is measured by using one or more amino acids labeled with ^{14}C and demonstrating labeling of protein. The incorporation should be dependent on the energy source. The protein is measured as the acid insoluble material.

First we have tried to isolate the soluble enzymes from tobacco leaves. We did not succeed, however, in demonstrating amino acid activating enzymes in any fraction from tobacco leaves. Therefore, we had to exclude tobacco leaves as a source of soluble enzymes for the present. It is known that yeast is rich in amino acid activating enzymes (cf. VAN DER VEN, 1957). Indeed we had no difficulties in isolating an amino acid activating enzyme fraction from yeast. This fraction was prepared according to the method of CHAO and SCHACHMAN (1956). One hundred grams of fresh baker's yeast (eventually after storage in the deep freezer) were ground in a mortar with 250 grams of Carborundum (100 mesh) which was added gradually as more cell sap was set free. All operations were performed in the cold room (0–4 °C). At the beginning a small amount of buffer, 0.01 M tris-HCl, 0.01 M Mg acetate, 0.06 M KCl, 0.006 M MCE, pH 7.8 was added to facilitate grinding. The mixture of broken yeast cells and Carborundum was extracted four times with 25 ml buffer. The extract was centrifuged for 5 min at 800 $\times$ *g*. The precipitate was added to the Carborundum in the mortar and once again ground and extracted four times with 25 ml buffer. This extract was also spun at 800 $\times$ *g* for 5 min. The two extracts were then combined and centrifuged at 23,000 $\times$ *g* for 20 min. The pellet was discarded and the supernatant centrifuged for 60 min at 23,000 $\times$ *g*. Again the pellet was discarded and the supernatant was spun for 2 hours at 105,000 $\times$ *g*. The supernatant was carefully taken off from the pellet. It contained the soluble enzymes including amino acid activating enzymes and the transfer-RNA. The amino acid activating activity of the fraction was checked by the hydroxamate method (HOAGLAND *et al.*, 1956). This fraction was used in the amino acid incorpora-

tion experiments. For these experiments, the reaction mixture had the following composition per ml:

Ribosomes from tobacco leaves	3	mg RNA
Soluble enzymes from yeast	3.2	mg protein
ATP	10	μM
GTP	0.1	μM
Phospho(enol)pyruvate	5	μM
Phospho(enol)pyruvate kinase	20	μg
l-amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, *iso*-leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine) each	0.05	μM
l-leucine (1-^{14}C)	0.028	μM ($\pm$ 0.2 μC)
Tris buffer-HCl	50	μM
Mercapto-ethanol	10	μM
KCl	50	μM
Magnesium acetate	10	μM
pH = 7.8		

TABLE 4. Incorporation of amino acids with ribosomes from tobacco leaves.

	Counts per min/mg protein
I Complete reaction mixture	55 167 150
II Complete mixture, without ribosomes	8 8 5
III Complete mixture without ATP	7 4 10

This is essentially the incubation medium of MATTHAEI and NIRENBERG (1961). Incubation was for 30 min at 37°C. The incorporation of amino acids was measured with ATP and without ATP in the medium, and without ribosomes. After one-half hour the reaction was stopped by adding an equal volume of 10% perchloric acid.

The precipitate was spun down and washed respectively with 5% hot perchloric acid, hot 96% alcohol, a mixture of alcohol-ether-chloroform (2:2:1) and ether. Finally the protein was suspended in acetone and transferred to a counting plate and counted in an infinitely thin layer with a gas flow counter (30% efficiency) (SIEKEVITZ, 1952).

Results are shown in Table 4. It is seen that a distinct energy dependent incorporation was found.

Although it has not yet been shown that the incorporation is really in a true

alpha peptide linkage in protein, it seems probable in view of the dependences that such an incorporation is involved here.

The incorporation of amino acids with ribosomes from tobacco leaves is being studied further and will be the subject of another paper. We only mention the first findings as a proof of the quality of the ribosomes.

4.7. Stability of the ribosomal preparations

The stability of the ribosomal preparations is of great importance. We have tried, therefore, to form an idea about the stability of the tobacco leaf ribosomes. The level of ribonuclease in the preparations is of special interest. Ribonuclease in known to occur in leaves of higher plants (Holden and Pirie, 1955a) and the ribosomes are susceptible to the action of this enzyme. When 25 μg/ml pancreatic ribonuclease was added to the ribosomes in a concentration of 1–2 mg RNA/ml the suspension became turbid within half an hour and the ribosomes flocculated. When heated to 60 °C the ribosomal preparations flocculated within a few minutes whereas a considerable amount of acid soluble material absorbing at 260 mμ was set free. The ribosomes could not stand freezing and thawing. After such a treatment the preparations were turbid and sedimented at low speed. (20,000 × *g*).

When a suspension of ribosomes was stored at 0–4 °C or at 25 °C it remained water clear. The release of acid-soluble material which absorbs at 260 mμ was less than 1% over a period of 20 hours at 0–4°C and 1–4% at 25°C. For precipitating the acid insoluble substance we used the McFadyen's reagent (0.25% uranyl acetate, 2.5% trichloro-acetic acid in water (McDonald, 1955). However, it might be objected that the release of acid soluble material is not a sufficient criterion for the absence of breakdown of ribosomes. The RNA might be inactivated by forming pieces which are still so big that they are precipitated by acid. As mentioned in 4.4, no change in the sedimentation diagram of ribosomes was observed after storage at 0–4 °C overnight or at room temperature for 8 hours. There appeared not to be autolysis of the ribosomes to an appreciable degree. There also was no increase of low weight material in RNA preparations made from ribosomes which had been stored at 0–4°C overnight (see 4.5). This seems to exclude appreciable internal breakdown. To test further the level of ribonuclease which might be present at the outside of the ribosomes, we added purified yeast nucleic acid (0.5 mg/ml) to the ribosomes (1 mg RNA/ml) but could not detect acid soluble material within 4 hours. A further proof of the low level of ribonuclease in the ribosomal preparation was shown by the fact that the ribosomes were capable of amino acid incorporation (4.6). Ribonuclease should strongly inhibit amino acid incorporation.

4.8. Medium used for the ribosomal preparations

Most of our experiments were done in a buffered medium containing 0.005 M tris-HCl, 0.006 M MCE, 0.01 M Mg^{++}, pH 7.0. The ribosomes were found to be rather stable in this solvent. Phosphate buffer of pH 7.0 and a molarity of 0.02 M or less also appeared to be a useful medium. Yet we preferred the tris buffer because Grunberg – Manago *et al.* (1955) reported that the presence of phosphate could bring about breakdown of RNA by the enzyme polyribonu-

cleotide phosphorylase. We did not demonstrate this as a real danger but preferred to avoid taking the chance.

The presence of a reducing substance seems to be very necessary. Sap of tobacco leaves contains active oxidizing agents as revealed by the rapid brown coloring of extracted sap. Oxidizing agents inactivate ribonucleic acid (PIRIE, 1959) and should be inhibited. The addition of mercapto-ethanol to the grinding medium prevented the coloring of the sap. MCE was maintained in the medium at all further stages since its stabilizing effect on the protein synthesizing activity of ribosomes has been reported (MATTHAEI and NIRENBERG, 1961) and the loss of this activity might be due to the breakdown of some RNA.

4.9. DISCUSSION

The only other isolation of ribosomes from leaves has been described by LYTTLETON (1960) who worked with white clover. Ribosomes from pea seedlings have been extensively studied (T'SO *et al.*, 1956; cf. T'SO, 1958; T'SO, 1962; WEBSTER, 1959; LETT and TAKAHASHI, 1962). LYTTLETON (1960) isolated ribosomes from wheat germ. Whereas the ribosomes from pea seedlings were found to dissociate reversibly in smaller units dependent on the concentrations of magnesium ions, the ribosomes from white clover leaves could not be split reversibly. But their stability was dependent on the presence of magnesium ions. Still more independent on the Mg^{++} concentration, are the ribosomes from tobacco leaves as has been shown in 4.4. There has been no explanation for this heterogeneity of ribosomes until now. Heterogeneity of ribosomes has been pointed out by TISSIÈRES *et al.* (1962) who described two kinds of 70 *S* ribosomes in preparations from *E. coli*, one with dissociation dependent on Mg^{++} concentration and another kind which does not dissociate in this way. According to these authors the latter kind should be particularly involved in protein synthesis. They refer to them as "active" ribosomes. T'SO *et al.* (1961) reported heterogeneity for the dissociation of ribosomes from rabbit reticulocytes without correlating this with a difference in synthesing ability of the two classes. Whatever may be the reason for this heterogeneity, whether it be a difference in ability to synthesize protein or a difference caused by the type of cell or the kind of tissue the ribosomes are originating from, it is an intriguing problem. Tobacco leaves might be a good source of the non-dissociable kind of ribosomes.

CHAPTER 5

THE PROCESS OF VIRUS MULTIPLICATION IN LEAVES OF 'WHITE BURLEY' TOBACCO INFECTED WITH TMV

The further aim of our research was to analyse the ribosomal fraction from leaves infected with TMV for the occurrence of virus ribonucleic acid. We hypothesized that the occurrence of virus ribonucleic acid in this fraction as it might play a role in the virus protein synthesis (see 1.4).

For the analysis it was necessary to know how virus multiplication progressed after inoculating the leaves. On the basis of this knowledge one could then choose the suitable moment for searching for virus RNA. In this chapter we will describe the process of TMV multiplication in leaves of 'White Burley'

tobacco grown under our conditions (see 2.1). Also discussed will be the problems which emerged from those experiments and their influence on further experiments which were planned.

5.1. Determination of virus increase in inoculated tobacco leaves

Leaves of 'White Burley' tobacco plants were inoculated with a suspension of 50 μg TMV/ml and then thouroughly rinsed with tap water. At various times after inoculation the inoculated leaves of 6 plants were taken as a sample. The midribs were cut from the leaves and the remaining leaf tissue was ground with an equal amount in weight of 0.02 M sodium phosphate buffer pH 7.0. The homogenate was filtered through a glass filter and the filtrate used for the infectivity test on *Nicotiana glutinosa* by the local lesion assay-method (see 2.5). The filtrate was inoculated undiluted and in dilutions 1:10 and 1:100. The infectivities of the extracts at different times after inoculation are recorded in Table 5. The increase of the infectivity is shown graphically in Fig. 4. It can

Table 5. Infectivity of the extract from leaves of 'White Burley' tobacco at different times after inoculation with TMV tested by local lesion assay on *N. glutinosa*. Numbers given are numbers of local lesions on test halves over the numbers of lesions on control halves inoculated with 0.5 μg TMV/ml. Six leaves were used in each assay.

Time after inoculation:	0 hr	24 hr	30 hr	47 hr	71 hr	95 hr	119 hr
Dilution of extract							
undiluted	10/ 78	2/ 70	4/124	346/132	692/ 62	1696/ 60	
1: 10	17/584	4/144	41/848	1288/440	2489/251	2450/216	2506/100
1:100	2/418	1/166	6/880	131/353	1211/278	1484/154	947/106

be seen that no virus multiplication was observed during the first 30 hours after inoculation. It does not follow as a matter of course that virus multiplication did not occur earlier but at least it was below the limit of detection by the

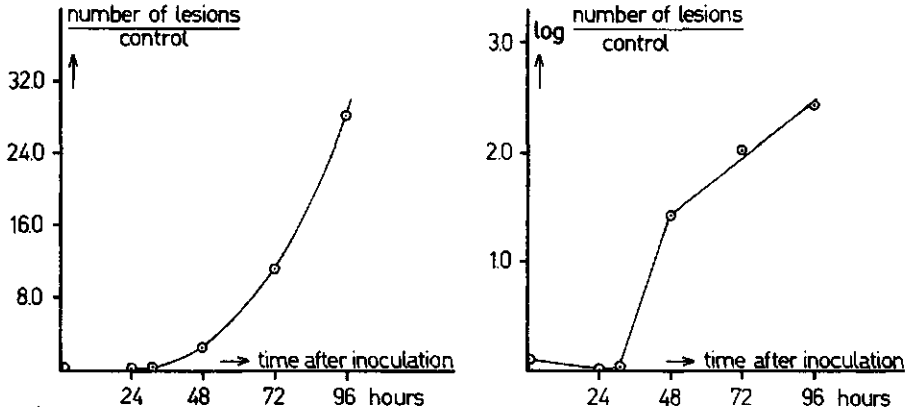


Fig. 4. The increase of tobacco mosaic virus in leaves of 'White Burley' tobacco inoculated with 50 μg TMV/ml at time zero. The increase of TMV was determined by measuring the infectivity of sap extracted from the leaves at various times after inoculation.

method of local lesion assay. At time zero, a small amount of virus was found attributable to virus inoculated onto the leaf. This amount decreased during the first 24 hours due to the fact that some of the virus had penetrated into the leaf cells while some virus may also have been inactivated. The infectivity still found after 24 hours was mainly due to virus particles which did not penetrate the cells but stuck to the surface of the leaf and thus were not washed away.

The period of time during which no virus multiplication was observed is generally referred to as the latent period. For our conditions, this period appeared to be nearly 30 hours. After 30 hours the first increase of virus could be detected, followed by an outburst of virus during the next 18 hours. Then the multiplication went on rapidly and it continued steadily until 96 hours after infection. After this period the curve flattened and the amount of virus reached a maximum.

When looking at the curve, one should take into account the following. At inoculation only a very small part of the total number of leaf cells are infected. The virus can penetrate into the leaf cells through wounds made by the rubbing the Carborundum dusted leaf surface with the inoculum. This treatment results inevitably in only a limited number of cells being wounded and infected.

What exactly happens during the latent period is not known. The fact that less virus can be demonstrated after 24 hours than at time zero might indicate that some of the original virus has been converted into a less stable form which is destroyed during extraction. This is also suggested by experiments which demonstrate the sensitivity of the infection process to ultraviolet light and ribonuclease during the first few hours (SIEGEL *et al.*, 1957; CASTERMAN *et al.*, 1955).

As soon as the synthesis of virus particles has started, the virus may spread to other cells and the number of cells in which virus multiplication occurs increases. Therefore, the curve indicating the synthesis of TMV in the leaves probably reflects only a small increase in the amount of virus per infected cell with the main increase coming from the number of cells becoming infected. This is especially true in the beginning. When after 48 hours the virus increase is highest, but still regular, most of the cells have probably become infected and the curve reflects mainly the increase of virus particles per cell.

5.2. DISCUSSION

The process of virus infection in the leaf involves the synthesis of particles at all possible stages. The ideal situation would be when all cells were infected at the same time with the synthesis of virus particles proceeding synchronously in all cells. That would present the easiest situation for studying the stages which are passed during the multiplication process. The actual situation is, however, far from this ideal. Only a small number of cells are infected upon inoculating the leaf. In these cells virus synthesis will start before spreading the infection to other cells. When finally the vast majority of the cells are infected there may be a great difference in the stage of the process for various cells. Some may be nearly saturated with virus with others just starting virus synthesis. It makes little sense to start an analysis for precursors of complete virus particles or for intermediate stages in virus synthesis shortly after inoculation. The dilution of any virus specific compound by healthy cell material would be so large, making the chance of detection very small. Just as one has to wait until

many virus particles have been synthesized so that they come within the reach of the detection method, one also has to wait for sufficient precusor material to be present. For that reason it appears necessary to wait for the infection to spread over the majority of the cells. This seems to us a minimum requirement but it creates other difficulties.

The intermediate stage in virus multiplication which we wanted to investigate was the stage in which virus protein was being synthesized. We assumed that virus ribonucleic acid is involved in this protein synthesis and, therefore, we wanted to examine the ribosomal fraction of infected leaf cells to see if virus ribonucleic acid occurs in a form not yet coated with virus protein.

The most characteristic property of virus ribonucleic acid is its infectivity, so one should probably use this property for demonstrating the presence of virus ribonucleic acid. When isolating a ribosomal fraction from infected leaves, the fraction contains a considerable amount of virus particles newly synthesized at an earlier time. It is impossible to use infectivity to demonstrate small quantities of infectious ribonucleic acid with the fair amount of virus present. Infectious virus nucleic acid has only 0.1–0.5% of the infectivity of equivalent amounts of virus (FRAENKEL-CONRAT, 1959). Hence the infectivity of small amounts of ribonucleic acid will be easily shaded by that of the virus.

Since we had to choose the lesser of two evils, we accepted the disadvantage of a considerable contamination of the ribosomal fraction with virus. When the rate of virus synthesis is maximal there will be the largest number of virus protein synthesizing centres suitable for analysis. For that reason we made most of our analysis at 60 hours after inoculating the leaves. The separation of the TMV particles from the ribosomes was attempted by the use of TMV antiserum. The antibodies are known to react specifically with TMV protein forming complexes with the virus which can be precipitated by low speed centrifugation. "Free" nucleic acid not surrounded by TMV protein, but perhaps attached in some way to the ribosomes, will not react with antiserum.

Eliminating the TMV particles from ribosomal preparations will be described in the next chapter.

CHAPTER 6

THE USE OF TMV ANTISERUM FOR SEPARATING TMV FROM RIBOSOMES

The antigenic properties of viruses are well known. When viruses are injected into animals the production of antibodies is stimulated. Antibodies are modified serum globulins which react specifically with the corresponding antigen. The reaction is a combination of the antigen with the antibody which can be detected by secondary effects such as: complex formation which gives rise to the separation of a precipitate, complement fixation and production of anaphylaxis or neutralization of activity (in case of a virus: neutralization of infectivity) (cf. MATTHEWS, 1957).

The reaction is very specific because the combination is due to the presence on the surface of the antigens of so-called determinant groups which may combine with corresponding groups on the surface of the provoked antibody.

Because of the specificity, serological methods have found wide applications

in virus research. In the field of plant viruses, antisera are used for diagnostic purposes, for characterization of viruses and for determination of virus concentrations. They also have been of value for demonstrating the presence of non-infectious, virus-related substances in diseased plants. For example, the presence of X-protein in TMV infected tobacco plants was demonstrated serologically (JEENER *et al.*, 1953). The precipitation reaction has been most used with plant viruses. In this reaction the binding of antigen and antibody gives rise to the formation of a precipitate. In our case where the issue is the separation of TMV from ribosomal suspensions the precipitation reaction was also suitable.

6.1. THE PRECIPITATION REACTION

The amount of antigen precipitated by antibodies in the precipitation reaction depends on the proportion of the concentration of antigen and antibody. When the ratio of antibody to antigen is gradually increased by adding increasing quantities of antibody to a constant amount of antigen, one may observe a number of phenomena which follow.

When only a very small amount of antibodies is added to antigen, no precipitation occurs. This is referred to as the zone of complete inhibition. With increasing amounts of antibodies there follows a zone of partial inhibition in which some precipitate is formed but antigen and antibody remain also in the supernatant liquid. Then there is a zone of antigen excess in which the added antibodies are completely precipitated but still some antigen remains in the supernatant. Next is the equivalence zone where both antigen and antibody are completely precipitated and can not be demonstrated in the supernatant. Finally, there is a zone of antibody excess and a zone of extreme antibody excess where the antigen is completely precipitated in antigen-antibody aggregates and increasing amounts of antibodies are found in the remaining liquid (cf. BOYD, 1956). This description applies to rabbit antisera. In the case of antisera from horses there is also inhibition of precipitation at the antibody excess zone but this does not hold for rabbit antisera.

For TMV, the ratio of antibody to antigen (R) is 0.25 in the zone of equivalence (KLECZKOWSKI, 1961). In the zone of antibody excess, R is 2.0 according to this author, but also higher values up to 3.4 and 4.1 have been reported (KLECZKOWSKI, 1941; SCHRAMM *et al.*, 1941). According to KLECZKOWSKI (1961) the maximum value of R seems to vary from one antiserum to another.

The precipitate of antibody and antigen is built up by a three dimensional lattice of virus and antibody particles. The criterion for the precipitation reaction is, in most cases, the visibility of the precipitate. However, when very high dilutions of antigen and antibody are used there is no visible flocculation though formation of microfloccules occurs (BLACK *et al.*, 1946) which can be observed with the electron microscope. Thus formation of floccules goes further than the visible endpoint of the precipitin reaction.

It can be calculated from the R values mentioned that in the equivalence zone, such as $R = 0.25$, about 60 antibody molecules are available for each TMV particle in the precipitate (the particle weight of TMV is 4×10^7 and of γ-globulin 160,000–170,000). At antibody excess, $R = 2.0$ or more, thus there may be 450 or more antibody particles for each TMV particle. This suggests already that the TMV particles are rather tightly bound together. Indeed they

are not easily soluble and can be separated from the solvent without difficulty by centrifugation at 10,000 *g*.

Antisera are mostly used for the purpose of identification. They have been seldom applied, however, for separating a special component from a mixture which we are planning. DOUNIN and POPOVA (1938) used precipitation with antiserum for preparing pure antigen suspensions. These authors prepared an antiserum against healthy plant sap. Infective sap was allowed to react fully with this antiserum so that all constituents of normal plants were precipitated. In this way a pure antigen was obtained. More recently GOLD (1961) used anti-host serum in the purification of tobacco necrosis virus to remove host substances inhibiting virus infectivity and having chemical and physical properties similar to those of the virus. On the other hand, the quantitative and selective separation of antibodies from an antiserum by precipitation with antigens has been performed many times. This is done, for example, when various strains of a virus are differentiated in cross absorption tests (see BAWDEN, 1954).

We have found TMV antibodies efficient for separating TMV from ribosomal preparations of TMV infected tobacco leaves. From the description of the precipitin reaction given in the previous sections it will be clear that it is necessary to add so much antiserum that the zone of equivalence, or still better the zone of antibody excess, is reached. The TMV antibody aggregates can then be easily separated from the ribosomal suspension by centrifugation at low speed.

6.2. PURIFICATION OF ANTISERUM

TMV antiserum was made available by courtesy of Ir. D.H.M. VAN SLOGTEREN from the Laboratorium voor Bloembollenonderzoek at Lisse and as a gift from Mr. D. MAAT from the Instituut voor Plantenziektenkundig Onderzoek at Wageningen. The antiserum was obtained from rabbits injected with concentrated purified virus suspensions. The antisera from Lisse were absorbed with healthy plant sap for eliminating antibodies against normal plant proteins which could still have been present in the virus preparations used for injection. To do this 2 ml of sap from healthy tobacco plants were added to 0.5 ml serum. The mixture was centrifuged, the pellet discarded and the supernatant lyophilized. The freeze-dried material was supplied to us.

The antiserum was purified before using for the following reasons. First, antiserum may give rise to aspecific flocculation, which is not due to the interaction of an antigen and its antibodies. This can be avoided by isolating the γ-globulin fraction from the serum (cf. VAN DER VEKEN *et al.*, 1962). Another reason is the fact that the sera have been absorbed with healthy plant sap. By using the absorption procedure, only specific antibodies are left which we need but at the same time, substances from normal plant sap are added in excess. Thus we may add enzymes having a harmful effect on the ribosomes or the RNA in the ribosomal preparations, e.g. ribonuclease which is known to occur practically always in plant sap. Actually we found that the crude antiserum contained a considerable amount of ribonuclease activity. To purify the antiserum we used the method of fractionation with $(NH_4)_2SO_4$ following the directions of JAGER and NICKERSON (1948). The γ-globulin fraction was precipitated from the absorbed antiserum by adding $(NH_4)_2SO_4$ to 33% saturation. This method appeared advantageous as we learned from work on the purification of plant

ribonuclease (FRISCH-NIGGEMEYER and REDDI, 1957) that hardly any ribonuclease was precipitated at this ammonium sulphate concentration.

Procedure:

Freeze-dried antiserum was suspended in buffer, 0.005 M tris-HCl, 0.006 M MCE, 0.01 M Mg^{++}, pH 7.0, and centrifuged at 1000 × *g* to eliminate non-soluble material. To the clear suspension solid $(NH_4)_2SO_4$ was added in portions with constant stirring, to 33% saturation. The suspension was stored at 0–4 °C overnight. The precipitate was spun down at 1000 × *g*. The supernatant was taken off carefully with a pipette and discarded. The pellet was resuspended in the starting volume of 33% saturated ammonium sulphate solution and left at room temperature for one hour. The precipitate was again spun down by centrifugation at 1000 × *g* for 30 minutes. The supernatant was taken off and discarded and the precipitate was dissolved in the same tris buffer pH 7.0 as before. One more centrifugation at 10,000 × *g* was given to eliminate possible non-dissolved material. The γ-globulins were reprecipitated with $(NH_4)_2SO_4$ and the whole procedure repeated. Finally the γ-globulin suspension was dialyzed overnight against tris buffer pH 7.0.

6.3. COMPONENTS OF THE ANTISERUM

The degree of purity was checked by paper electrophoresis as described by VAN KAMPEN and ZONDAG (1955). Drops of antiserum were applied to 2 cm wide strips of Whatman no. 1 filter paper, soaked before in 0.055 M veronal buffer pH 8.8. The serum on the strips was subjected to electrophoresis during 11 to 12 hours at 0.25 mA per cm paper width, using the same veronal buffer. Next the paper strips were dried at 105 °C for 5 to 10 min and then colored by immersing the paper strips in a solution of 0.75 gram azocarmine B in 50% methyl alcohol and 10% acetic acid in water for 10 min. The coloring was followed by a series of successive washings in 10% acetic acid until the blank parts of the paper were decolorized and finally the strips were dried at 105 °C. For measuring the color intensities of the different bands on the paper, the strips were made transparent by soaking them in a 1:1 mixture of paraffin oil and α-bromonaphtalene. The strips were then laid between two plane parallel glass slides and the color intensities were measured determining the extinction in an EEL scanner.

The result is shown in Figure 5. The dotted line gives the electropherogram of the original antiserum absorbed with healthy sap. The solid line is the electropherogram after fractionation with $(NH_4)_2SO_4$. Three peaks are found in the crude serum presumably coming from mainly albumin and β and γ-globulin respectively. The three components were not well separated in the crude antiserum although normal serum of a rabbit could easily be resolved in three separate components. Probably the bad separation of albumin and β and γ-globulin in the antiserum is due to the large amount of contaminating protein from the plant sap added in the absorption procedure.

The serum contained mainly γ-globulin after fractionation with $(NH_4)_2SO_4$. Measuring the area under the peaks indicated 93% γ-globulin and presumably 2.2% albumin and 4.7% β-globulin. A further precipitation with $(NH_4)_2SO_4$ according to the procedure described, diminished the amount of impurity but generally we used the antiserum after two precipitations.

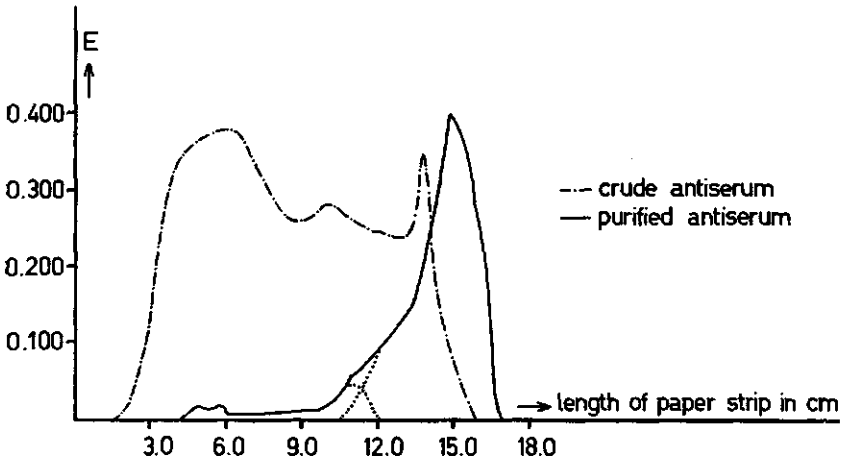


FIG. 5. Electropherogram of TMV antiserum from rabbit, absorbed with healthy plant sap, and of the γ-globulin fraction isolated from this antiserum by fractionation with ammonium sulphate. For further explanation see text.

6.4. RIBONUCLEASE ACTIVITY OF THE ANTISERUM

For testing further the quality of the purified antiserum, we examined the ribonuclease activity of the preparation. It is known that ribonuclease will rapidly destroy RNA even when present only in a very low concentration. Ribonuclease (RNase) activity was measured by the method described by MCDONALD (1955), measuring the release of RNA fragments soluble in acid uranyl acetate. A standard curve relating the RNase activity with the enzyme concentration was made using commercial crystalline RNase from pancreas

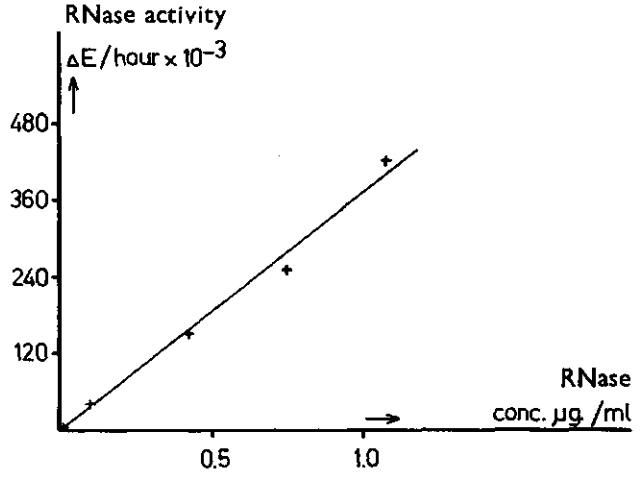


FIG. 6. The relation between the activity and the concentration of pancreatic ribonuclease using purified yeast RNA 0.5 mg/ml as a substrate. The activity was measured as the release of material soluble in acid uranyl acetate (McFadyens reagent). The soluble material was measured by determining the extinction E at 260 mμ.

(see Fig. 6). Mixtures containing 0.5 mg purified yeast RNA per ml and ribonuclease varying from 0.01 to 1 μg per ml in tris buffer (0.005 M tris-HCl, 0.006 M MCE, 0.01M Mg acetate) pH 7.0 were incubated at 25 °C. At different times, samples of 1 ml were taken from the reaction mixtures and added to 1 ml McFadyens reagent (= 0.25% uranyl acetate, 2.5% trichloroacetic acid in water). After 30 minutes at room temperature the precipitate was filtered off through Schleicher and Schüll no. 589[3] filter paper and the extinction at 260 mμ of the 1:5 diluted filtrate was measured against 1:10 diluted McFadyens reagent. The increase of the extinction over time served as a measure for the enzyme activity. Since this increase was not linear at higher enzyme concentrations, the initial velocity was used.

Hereafter the ribonuclease activity of the purified antiserum was estimated by incubating antiserum (titer 1/128) in the concentration used in the experiments with ribosomes, with 0.5 mg purified yeast RNA per ml. No proper ribonuclease activity could be measured. The only fact here to mention is that the RNase activity was less than 0.01 μg pancreatic ribonuclease per ml. At the same time the RNase activity of non-purified antiserum was found to be equivalent to 1 μg per ml.

A more sensitive test of the RNA destroying effect of the antiserum appeared to be the effect on the infectivity of TMV-RNA. For this, the infectivity of TMV-RNA with and without addition of crude and purified antiserum was determined by incubating these mixtures at 25 °C and inoculating samples taken at different times onto *N. glutinosa* leaves. The results are shown in Table 6. One ml antiserum (titer 1/120) was added to 1 ml TMV-RNA.

TABLE 6. The infectivity of TMV-RNA in the concentration of 20 μg/ml incubated at 25 °C with and without addition of crude and purified antiserum.

Time in min	TMV-RNA 20 μg/ml	TMV-RNA + purified antiserum 20 μg TMV-RNA/ml	TMV-RNA + crude antiserum 20 μg TMV-RNA/ml
0	216	190	0
30	305	238	0
60	401	81	0
120	216	22	0

The numbers given are the number of local lesions on 6 half leaves of *N. glutinosa*.

The crude antiserum destroyed the infectivity of TMV-RNA immediately. The infectivity of TMV-RNA, when nothing was added started to decrease after 2 hours. When TMV-RNA was incubated with purified antiserum, the infectivity decreased more rapidly than without antiserum but remained at a high level for at least half an hour. The effect, though not desirable, did not appear disastrous for our experiments. It should be kept in mind that besides the destroying effect, the antiserum acts as an inhibitor of infection (GIERER and SCHRAMM, 1956).

6.5. SEPARATION OF TMV FROM RIBOSOMES

For separating TMV from ribosomes an equal volume of purified antiserum was added to ribosomal preparations from TMV infected leaves containing

varying amounts of TMV. The dilution end point of the antiserum, tested to 10 μg TMV/ml was usually 1/120 as determined in the tube test. The mixture was put in an incubator at 37 °C for 10 minutes. The temperature of the suspension starting at 4 °C, however, never went above 25 °C. Depending on the virus concentration visible flocculation or only a little turbidity could be seen. The suspension was centrifuged in the cold room at 12,500 — 15,000 × *g* for 10 min. The pellet contained the TMV with the ribosomes in the supernatant. When the virus concentration was high, the TMV was not removed completely by one addition of antiserum. Then another portion of antiserum was added and the treatment repeated.

The effect of the antiserum treatment on the ribosomal preparations was tested in various ways. To a preparation of ribosomes from healthy leaves which had as spectral characteristics $E_{max}/E_{min} = 1.54$ and $E_{260}/E_{280} = 1.86$ purified TMV was added to a concentration of 125 μg/ml. This virus concentration caused a countless number of lesions on *N. glutinosa*. The suspension was treated with antiserum as described. The precipitate was spun down. The remaining supernatant had $E_{max}/E_{min} = 1.55$ and $E_{260}/E_{280} = 1.88$. The ribosomal RNA was recovered for 95% and the suspension had an infectivity of only 1 lesion per half leaf tested on *N. glutinosa*.

In Fig. 7 the sedimentation diagram is shown of a ribosomal preparation from leaves 60 hours after infection with TMV from which TMV was separated by two treatments with antiserum. The peak near the meniscus demonstrates the excess of γ-globulin present. When compared with the diagram of ribosomes from healthy leaves, no indication of breakdown of the ribosomes by the antiserum can be found.

No release of UV absorbing substances could be detected in preparations of ribosomes incubated with antiserum at 25 °C for 2 hours which might point to slow breakdown of the ribosomes.

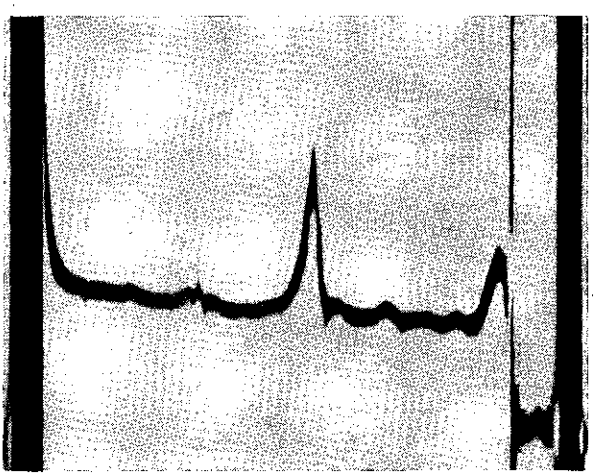


FIG. 7. Sedimentation diagram of a ribosomal fraction from diseased leaves after TMV was precipitated by two treatments of purified antiserum. Sedimentation is from right to left. Speed 44,770 rpm. Temperature 9.7 °C. Photograph taken about 15 minutes after rotor reached full speed. The medium was 0.005 M tris-HCl, 0.01 M Mg acetate, with excess of antiserum. Beside the 80 *S* peak of the ribosomes, there is a big peak near the meniscus due to the excess of γ-globulin.

The ribosomal fraction isolated from secondarily infected leaves a few days after symptoms appeared on these leaves consisted to a large extent of TMV. The UV. spectral characteristics $E_{max/min} = 1.05$ and $E_{260/280} = 1.39$ were not even those of ribosomes and indicated a large amount of contaminating material, presumably TMV. Three additions of antiserum were necessary to lower the TMV concentration to the point where the ribosomes predominated over TMV and $E_{max/min}$ and $E_{260/280}$ became 1.42 and 1.83 respectively. This further demonstrated that TMV may be selectively separated from the suspensions.

6.6. Conclusion

It was concluded that purified antiserum was a suitable means for separating TMV from ribosomes and thus for preparing ribosomal preparations free from contaminating TMV from TMV infected leaves. The application of antiserum for this purpose will be further described in Chapter 8.

As discussed before, an opinion about the presence of TMV-RNA not yet surrounded with TMV protein in ribosomal preparations can be demonstrated best on basis of the infectivity. For this, we had to be certain that no TMV was left after the antiserum treatments. It is now known that antiserum has a neutralizing effect on the infectivity of the virus. This may be a specific effect of the antibodies or a non-specific effect of the serum, also produced by heterologous sera and normal sera (Rappaport and Siegel, 1955; Fraenkel-Conrat and Singer, 1957).

Whatever the reason, infectivity is not a sufficient criterion for checking if TMV is still left after treatment with antiserum. Some may be left but does not demonstrate infectivity because of the inhibiting action of antiserum.

It appeared necessary, therefore, to look for a method of determining the TMV concentrations independent of infectivity. This will be described in the next chapter. It also bears upon the problem that when two infectious components – TMV and TMV-RNA – are present at the same time, it is necessary to determine one of the components independent of the other to learn the contribution of each.

Chapter 7

DETERMINATION OF TMV CONCENTRATIONS BY PARTICLE COUNTING

After treating the ribosomal preparations from TMV infected leaves with antiserum for eliminating the contaminating TMV, a small but definite infectivity was left in the remaining suspension of ribosomes. For example, in three experiments at a concentration of ribosomes of 1 mg RNA/ml, 2, 17 and 34 local lesions were produced on 6 half leaves while the control suspension of 0.5 μg TMV/ml gave 251, 601 and 327 lesions respectively. The question arose if those infectivities were due to TMV left after the antiserum precipitations or caused by another infectious component in the ribosomal preparations.

It appeared impossible to decide upon two infectious components on the basis of the infectivity test only. It was necessary to determine the concentration of one of the components independent of the other. Obviously it is more

attractive then to look for a method for determining TMV particles as these particles are so well characterized in their physical and chemical properties.

STEERE (1955) has given a survey of the methods available for determining TMV concentrations. The various methods fall into two groups: those that measure biological activity and those that depend on chemical, physical or morphological properties of the virus particles. The biological assay methods, in which the infectivity of a virus suspension is measured, are the most sensitive. Virus concentrations of 10^{-6} mg TMV/ml can be measured by these methods.

Since we needed to distinguish the physical nature of the infectious agent we had to use one of the non-biological methods. Among these the method of concentration determination by particle counting with the electron microscope is the most sensitive. Furthermore this method does not make high demands on the purity of the preparation in which the TMV has to be determined. This made it the most suitable for our purpose.

7.1. THE METHOD OF PARTICLE COUNTING WITH THE ELECTRON MICROSCOPE

The method of virus concentration determination by particle counting with the electron microscope was developed by BACKUS and WILLIAMS (1950). The procedure requires a suspension of particles of uniform size and known concentration as a standard. As reference particles, polystyrene spheres (Dow Chemical Co.) were used. The virus suspension and the polystyrene particles were mixed in known proportions and sprayed onto the grids of the electron microscope by a nebulizer. By this method the suspension was distributed as small droplets over the film of the grid. The number of polystyrene spheres and the number of characteristic virus particles were counted in a number of droplets. From the ratio of these numbers, the number of virus particles in one milliliter may be calculated and from that in turn the concentration. The method is very simple; the only requirement is that the virus particles be easily identified with the electron microscope. It is not necessary to have pure virus preparations when the virus may be easily distinguished from the other components present. The rigid 300 $\times$ 18 mμ rods of TMV, may be singled out without difficulty from electron microscopic pictures of droplets of ribosomal suspensions (see Photo 5). Disadvantages of the method are that the use of an electron microscope is rather expensive and the counting is rather tiresome.

The method has been used for measuring the increase of the amount of TMV in tobacco leaves after inoculation (STEERE, 1952), for determining the particle weight of among others TMV (WILLIAMS, BACKUS and STEERE, 1951) and for estimating the number of TMV particles necessary to get one local lesion (STEERE, 1955) and other applications. BACKUS and WILLIAMS (1950) have determined the amount of 10^{-4} mg TMV/ml by this method and found it to be reproducible within a 5% variability.

7.2. USEFULNESS OF PARTICLE COUNTING FOR DETERMINING TMV CONCENTRATIONS

To check the usefulness of the method, a number of dilutions were made from a TMV suspension with a known concentration. To 0.8 ml of each dilution was added 0.1 ml of polystyrene suspension containing 4 mg/ml and 0.1 ml of 0.3% serum albumin solution. The serum albumin was to give a better spreading of the droplets when they were sprayed onto the formvar film of the grids for the

electron microscope. The preparations were shadow casted with palladium and viewed in the Philips M 100 electron microscope. From a large number of droplets, varying from 50 to 100, the number of TMV particles and the number of polystyrene spheres were counted. The number of spheres gave the volume of suspension viewed. For comparison with the counted number of TMV particles, the number of TMV particles was calculated which might be expected to be

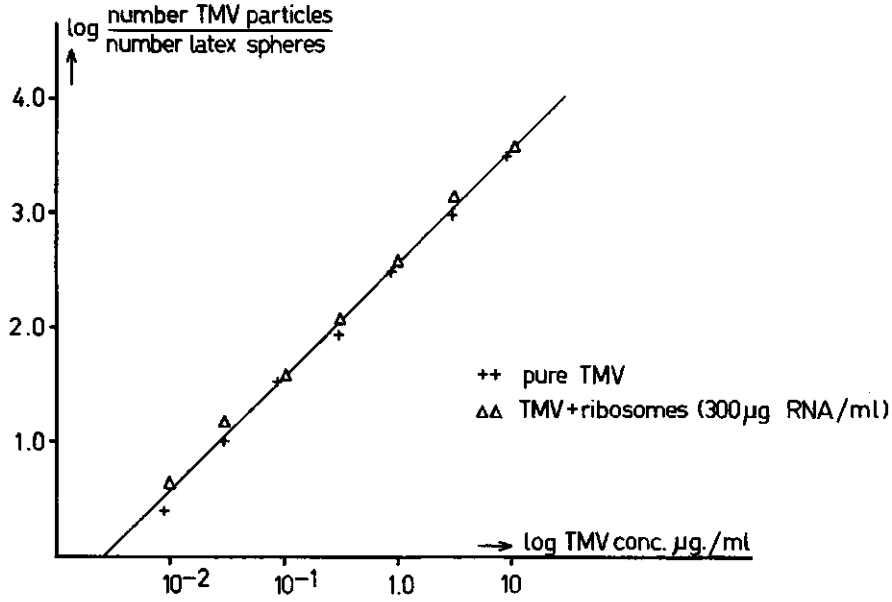


FIG. 8. The relation between the concentration of TMV and the number of particles counted with the electron microscope in a given volume. To 0.8 ml TMV suspension was added 0.1 ml 0.3% serum albumin solution and 0.1 ml of a polystyrene suspension corresponding with 4.5 mg polystyrene. The suspension was sprayed onto the grids with a nebulizer. The ratio of the number of TMV particles to the number of polystyrene spheres is plotted against the virus concentration.

present in this volume at the known concentration. The results are shown in Table 7 and again graphically in Fig. 8. The same countings were made in the presence of ribosomes in a concentration of 300 μg RNA/ml in the TMV suspensions.

In the calculations made in Table 7 the diameter of the polystyrene spheres was assumed to be 260 mμ. This is the generally accepted value (GEROULD, 1950). Using this dimension we found the length of the TMV to be 304 mμ taking a rather small sample of 27 particles. The density of polystyrene is 1.052 gram/cm^3 (WILLIAMS *et al.*, 1951). The starting polystyrene suspension was purified from contaminating spheres of larger and smaller dimensions by centrifugation. The added 0.1 ml polystyrene corresponded to 4×10^{11} spheres. For the calculation, we used in addition Avogadro's number $N = 6 \times 10^{23}$ and the molecular weight of TMV 4×10^7.

The countings were done directly on the electron microscope screen at a magnification of 10,000 × and a reading glass with a magnification of 2 ×.

TABLE 7.

I. Particle counts of a series of TMV concentrations with a constant concentration of 4×10^{11} latex spheres per ml.

Concentration of TMV µg/ml	Number of TMV particles counted	Number of spheres counted	Volume counted $\times 10^8 \mu^3$	Number of TMV particles/ml calculated from the concentration	Number of TMV particles in counted volume (calculated)
0.009	1	3935	9.8	1.4×10^8	1.3
0.03	4	3885	9.7	4.5×10^8	4.4
0.09	19	5543	13.8	13.5×10^8	18.6
0.3	49	5609	14.0	45×10^8	63
0.9	133	4045	10.1	135×10^8	136
3.0	217	2219	5.55	450×10^8	250
9.0	744	2296	5.7	1350×10^8	770

II. Particle counts of a series of TMV concentrations with 4×10^{11} latex spheres per ml and a contamination of ribosomes of 300 µg RNA/ml.

Concentration of TMV µg/ml	Number of TMV particles counted	Number of spheres counted	Volume counted $\times 10^8 \mu^8$	Number of TMV particles/ml calculated from the concentration	Number of TMV particles in counted volume (calculated)
0.01	1	2228	5.57	1.6×10^8	0.9
0.03	4	2519	6.30	4.6×10^8	2.0
0.1	12	3146	7.87	16×10^8	12.4
0.31	32	2691	6.73	46×10^8	31
1.0	48	1241	3.11	157×10^8	49
3.1	171	1214	3.04	465×10^8	140
10.5	511	1375	3.44	1574×10^8	540

Droplets were counted which either fitted on the screen in their entirety or were not so large that confusion might arise about parts already counted when moving the droplet over the screen. There is no variation in the ratio of standard to unknown particles with the droplet size as has been established by Nixon (1958). It is necessary to examine only whole droplets for the particles present to exclude the possibility of an unequal distribution of particles over the droplet surface. Hence, counting on the screen has an advantage in comparison with counting from photographs (BACKUS and WILLIAMS, 1950) because one is not restricted to only the small droplets which fit the screen. It means droplets of larger size may also be used.

Table 7 shows a good agreement between the concentration as determined by particle counting and the concentration in the region of 10 μg TMV/ml to 0.01 μg TMV/ml. The presence of a considerable amount of contaminating material i.e. 300 μg RNA/ml as ribosomes had no influence on the count.

At low concentrations of virus the counted volume had to be at least large enough so that one particle could be expected to be present in the volume with certainty. Table 7 indicates that the number of spheres used in the experiment was much too large. Such a large number of spheres had to be counted before a reasonable volume was examined, that it was unnecessarily tiresome. Furthermore, the surface taken by the spheres was so large that the possibility of missing particles lying under the edges or in the shadow of the spheres must be kept in mind.

For those reasons, the number of spheres was drastically decreased to 10^{10} per ml in further experiments; 0.1 ml of a suspension of 1 mg/ml was added each time. The average number of spheres per 500 μ^3 droplet was then 5 (500 μ^3 being about the volume of a droplet fitting the screen at the magnification used). Droplets were inspected until a total of 1,500 polystyrene spheres had been counted. The counted volume was then $15 \times 10^4\mu^3 \pm 2.6\%$. At a concentration of 0.01 μg TMV/ml 22–23 particles may be expected in $15 \times 10^4\mu^3$.

As shown in Table 7 and Figure 8 there is a good agreement between the dilution of the virus suspension and the decrease in the number of particles counted. The fitting of the points to the line indicates that the sample counted was large enough.

When a large number of particles is counted, e.g. at 0.1 μg TMV/ml with on the average 225 particles in $15 \times 10^4\mu^3$ the counting can be used for concentration determination. The standard deviation being the square root of the number of counted particles will then be small. At lower concentrations the standard deviation of the countings will be so large that no accurate concentration determination can be done. However, it remains possible to make a statement with sufficient certainty about the concentration of TMV being below a distinct level at a certain counting. This was sufficient for our purpose since our main problem was that the TMV concentration should not be underestimated from the counting.

It may be assumed that the virus particles in the suspension were distributed according to a Poisson distribution. Taking the counted number of particles as the mean, it is possible to determine the upper confidence limit for the mean. We read these limit values from a table by VAN KLINKEN and PRINS (1954) (see Table 8). For example, with 14 particles counted in a volume of $15 \times 10^4\mu^3$, there is 5% probability that a value of 22 or more will be found. So there is 95% certainty that the actual number of particles is below 22. With 13

TABLE 8. Upper confidence limit for the mean of the Poisson distribution $p_n = \frac{m^n \cdot e^{-m}}{n!}$ (Copied from Van Klinken & Prins, 1954).

m	Confidence coefficient 1 — p					
	0.9	0.95	0.98	0.99	0.995	0.999
0	2.3	3.0	3.9	4.6	5.3	6.9
1	3.9	4.7	5.8	6.6	7.4	9.2
2	5.3	6.3	7.5	8.4	9.3	11.2
3	6.7	7.8	9.1	10.0	11.0	13.1
4	8.0	9.2	10.6	11.6	12.6	14.8
5	9.3	10.5	12.0	13.1	14.2	16.5
6	10.5	11.8	13.4	14.6	15.7	18.1
7	11.8	13.1	14.8	16.0	17.1	19.6
8	13.0	14.4	16.2	17.4	18.6	21.2
9	14.2	15.7	17.5	18.8	20.0	22.7
10	15.4	17.0	18.8	20.2	21.4	24.1
11	16.6	18.2	20.1	21.5	22.8	25.6
12	17.8	19.4	21.4	22.8	24.1	27.0
13	19.0	20.7	22.7	24.1	25.5	28.4
14	20.1	21.9	24.0	25.5	26.8	29.9
15	21.3	23.1	25.2	26.7	28.2	31.2
16	22.5	24.3	26.5	28.0	29.5	32.6
17	23.6	25.5	27.8	29.3	30.8	34.0
18	24.8	26.7	29.0	30.6	32.1	35.4
19	25.9	27.7	30.2	31.9	33.4	36.7
20	27.0	29.1	31.5	33.1	34.7	38.0

particles this confidence is 98 % and with 16 particles 90 %. In this way the upper limit of the TMV concentration could be estimated. In the cases where we have estimated the virus concentration less than 0.01 μg TMV/ml, we have used at least the 95 % upper confidence limit.

7.3. CONCLUSION

The particle counting method can not be used to determine accurately concentrations of TMV below 0.1 μg/ml. Below this concentration the method can be used, however, for estimating the upper limit of the TMV concentration and it is possible to ascertain the concentration of TMV being below a distinct value, e.g. less than 0.01 μg/ml.

The consequence of this information for our problem was that it was impossible to say that the low infectivities in the ribosomal preparations after treatment with antiserum mentioned at the beginning of this chapter, were not due to TMV. The sensitivity of the local lesion test for TMV is such that numbers of 10–50 lesions on half leaves might arise from the presence of 0.01 μg TMV/ml or less in the inoculum.

There is of course the problem that by particle counting one does not determine the number of infectious particles. It is possible that characteristic particles occur which are non-infectious. Since it was impossible to solve this problem, it appeared to be safest to assume all characteristic particles to be infectious.

The counted particles were not all of uniform length. On Photo 5 it can be seen that particles smaller than 300 mμ occur in the preparation. These might arise from fragmentation of particles resulting from preparation for the electron

microscope. Therefore, we preferred to consider the fragments also as "characteristic" particles, not making any discrimination for infectivity on basis of the length of the particle, except where two fragments clearly belonged together.

The presence of infectious RNA in the ribosomal preparations was investigated by extracting the RNA with phenol which will be described in the next chapter. It has been of great importance to know the possible TMV concentration in the starting suspension of ribosomes for interpreting the infectivity of these RNA preparations. To do this job the method of particle counting has been found to be very useful.

CHAPTER 8

INFECTIOUS RNA IN RIBOSOMAL PREPARATIONS FROM TMV INFECTED TOBACCO LEAVES

In Chapter 6 a method was described for separating TMV particles from ribosomal preparations coming from TMV infected leaves. For this separation TMV antibodies were used. The efficiency of the separating procedure was checked by particle counting with the electron microscope. Chapter 7 discussed how this procedure was inadequate to demonstrate the presence of another virus specific infectious component besides TMV. This implied, however, that the virus RNA, if any occurred in the ribosomal fraction, would be infectious. There is still the possibility that virus RNA does not occur in free form known to be infectious but might be found, for example, as a part of a structure in close connection with the ribosomes. As such, the infectivity might not have been demonstrated because the virus RNA would not have been active in the infectivity test.

Keeping in mind this possibility of latent occurrence of virus RNA in the ribosomes, we looked for a method of demonstrating infectious RNA by removing it from latency. Therefore, we prepared the RNA from the ribosomal preparations by phenol extraction. Any structure which might have inhibited the infectivity of any potential virus RNA, should have been destroyed by this procedure. An extra advantage of phenol extraction is that the contribution to the infectivity from the remaining contaminating TMV will be greatly reduced. The RNA of this TMV set free by phenol extraction will have only 0.5% or less of the infectivity of the equivalent amount of TMV. Whereas 0.01 μg TMV/ml may still represent a considerable infectivity, the equivalent amount of free RNA ($0.05 \times 0.01 = 5 \times 10^{-4}$ μg TMV-RNA/ml assuming 5% RNA in TMV) is well below the limit of what can be detected in the local lesion test.

8.1. Procedure in demonstrating the presence of infectious RNA

We used the following procedure. Ribosomes were prepared from TMV infected leaves at different times after inoculation by the method described in Chapter 4. After two cycles of high and low speed centrifugation, the ribosomal preparation (= M_2), usually in the concentration of 4–6 mg RNA/ml, was treated in the following way. A small sample was set apart for testing the infectivity. To the larger part, an equal volume of purified antiserum was added. The γ-globulin fraction had a titer of 1:120 as tested against 10 μg TMV/ml.

The mixture was placed in an incubator at 37 °C during 10 minutes. The temperature of the mixture, starting cold, never raised beyond 25 °C during this period. The suspension was then centrifuged at 15,000 × *g* for 15 minutes in the cold room whether or not a visible precipitate had formed. The supernatant (= M_2A_1, i.e. M_2 once treated with antiserum) was carefully decanted from the pellet (= P_1). M_2A_1 was divided in two equal portions after taking a sample for the infectivity test. An equal volume of antibody suspension was added to one portion and the mixture put again in an incubator at 37 °C for 10 minutes. The suspension was centrifuged at 15,000 × *g* for 15 minutes, after which the supernatant (= M_2A_2, i.e. M_2 after 2 treatments with antiserum) was carefully taken off from the pellet with a pipette. The procedure is also given in Scheme 3. We have already described in Chapter 6 that the treatments with antiserum had no harmful effect on the ribosomes.

From M_2A_2 and the remaining half of M_2A_1, the RNA was prepared according to HALL *et al.* (1959) as described in Chapter 4.5. The infectivities of the different fractions, M_2, M_2A_1 and M_2A_2 and of the RNA from M_2A_1 (= RNA_1) and from M_2A_2 (+ RNA_2) were compared at the concentration of 1 mg RNA/ml. An example is given in table 9 of the results of two preliminary experiments with a ribosomal preparation from leaves 63 hours after inoculation with TMV.

8.2. INTERPRETATION AND CHECKING OF THE RESULTS

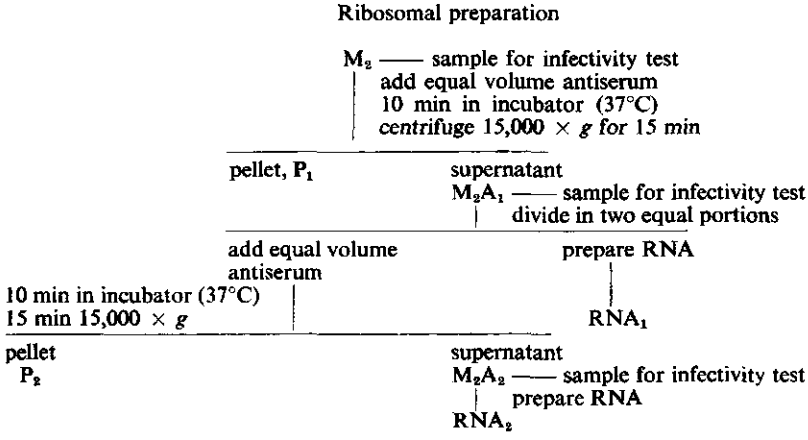
As is seen from Table 9, a considerable amount of infectivity occurred in the RNA_1 and RNA_2 fractions. These infectivities were about equal and much higher than those in the M_2A_2 fraction. The amount of TMV-RNA which would give approximately the same number of lesions as the RNA_1 and RNA_2 was calculated as follows. Assuming that TMV-RNA has 0.5 % of the infectivity of the equivalent amount of complete virus and that TMV contains 5 % RNA, a quantity of 0.5 μg TMV – which gives approximately 415 lesions – is equivalent to 0.05 × 0.5 = 0.025 μg TMV-RNA and 100/0.5 × 0.025 = 5 μg TMV-RNA would have the same infectivity. Thus 27 lesions by RNA_2 correspond roughly with 27/415 × 5 = 0.3 μg TMV-RNA.

TABLE 9. Infectivity of the fractions mentioned in Scheme 3.

Fraction	Concentration of inoculum μg RNA/ml	Infectivity*)	
		experiment I	experiment II
M_2	1000	4212/738	2770/937
M_2A_1	1000	43/483	94/1007
M_2A_2	1000	1/434	12/1197
RNA_1	1000	39/549 (= 0.07)	279/631 (= 0.44)
RNA_2	1000	27/415 (= 0.06)	284/987 (= 0.29)
P_1	1000		438/1321
P_2	resuspended in a few drops of water		186/1483

*) Infectivity is expressed as the number of lesions found in local lesion assay on *N. glutinosa*. Number of lesion on test halves over number of lesions on control halves are given. Six half leaves were used in each assay; control halves were inoculated with 0.5 μg TMV/ml.

SCHEME 3. Procedure for separating TMV from ribosomes.



After each antiserum treatment, the infectivity in the M fractions decreased considerably as is also shown by the infectivities found in the pellet. The RNAs extracted from M_2A_1 and M_2A_2 respectively, however, do not differ very much in infectivity. This might indicate that the amount of infectious RNA disappearing as TMV in the second antiserum precipitation is small in regard to the total amount of infectious RNA present. This might be explained by the presence of infectious RNA in the ribosomal preparation in another form than TMV which is not affected by antiserum. It seemed worthwhile to look into this possibility further. Various possibilities which might explain the infectivity of the RNA_1 and RNA_2 preparations were checked.

First, the TMV concentration in the M_2A_2 fraction might be greatly underestimated by the infectivity test so that in fact there was not such a big difference in virus content between M_2A_1 and M_2A_2. Such underestimation could be due to inactivation of virus by addition of serum proteins (RAPPAPORT and SIEGEL, 1955) or to the formation of small aggregates of virus and antibodies which were not spun down but would, nevertheless, reduce the number of infectious units. For that reason, the TMV concentration was checked in further experiments by particle counting with the electron microscope.

Secondly, the presence of ribosomal RNA might have a stimulating effect on the infectivity of TMV-RNA increasing the relative infectivity of TMV-RNA in the presence of ribosomal RNA from the usual 0.5% to a much higher value. A known amount of TMV was added to ribosomes from healthy leaves and the infectivity of this TMV was compared with the infectivity of the RNA obtained after phenol extraction of the mixture (see Table 10). It was found that the ribosomes did not inhibit the infectivity of TMV appreciably. The infectivity of the RNA prepared from the mixture was not abnormally high. From the data of Table 10 it may be estimated, for example, that 0.22 μg TMV/ml had the same infectivity as 1.6 μg TMV-RNA/ml. Hence a relative infectivity of $(0.05 \times 0.22)1.6 \times 100 = 0.7\%$ was found. Usually we found the relative infectivity of TMV-RNA prepared from TMV in the range of 0.07–0.7% and on an average of 0.5%. This is only slightly higher than the range of 0.05–0.5% as recorded by FRAENKEL-CONRAT (1959). It is clear that in no way the infectivities

TABLE 10. Infectivity of a mixture of ribosomes from healthy leaves (M_2) and TMV and of the RNA prepared from this mixture by phenol extraction*.

Inoculum	Concentration of TMV in inoculum µg/ml	Infectivity = number of lesions on test halves (t)** / number of lesions on control halves (c)	t/c
TMV	21.5	2997/127	23.3
	2.15	1518/127	12
	0.22	275/127	2.16
M_2 + TMV	21.5	2687/112	24
	2.15	1376/112	12.3
	0.22	197/112	1.76
	Concentration of TMV-RNA in µg/ml		
(M_2 + TMV)-RNA	10.3	854/140	5.97
	2.1	398/140	2.79
	0.21	35/140	0.245

* A mixture of ribosomes from healthy leaves and TMV in 0.005 M tris-HCl, 0.001 M Mg acetate buffer pH 7.0, containing 2.38 mg RNA/ml and 64.6 µg TMV/ml was diluted 1:3, 1:30, 1:300, giving TMV concentrations of 21.5, 2.15 and 0.22 µg/ml, and inoculated onto *N. glutinosa* leaves. The same concentrations of pure TMV in tris-HCl buffer were also inoculated. RNA was prepared from 15 ml of this mixture by phenol extraction as described in 2.3 page 7. After alcohol precipitation the RNA was resuspended in 3.75 ml 0.01 M sodium phosphate pH 7.0 and inoculated undiluted and in the dilutions 1:5 and 1:50. Normally the yield of TMV-RNA in the extraction procedure is 80%. Taking this into account, the concentration of TMV-RNA will be $(15 \times 64.6 \times 0.05 \times 0.80)/3.75 = 10.3$ µg/ml. Hence the inoculated concentrations contained respectively 10.3, 2.1 and 0.21 µg TMV-RNA/ml.

** Six half leaves were used in each assay; total numbers of lesions on 6 half leaves are given. The control suspension contained 0.1 µg TMV/ml.

of RNA_1 and RNA_2 could be explained by the special conditions in which they were prepared.

It is of interest to notice that the presence of ribosomes preserved the infectivity of TMV-RNA. RNA prepared from TMV suspensions with a concentration of 60 μg/ml had a very low infectivity (<0.01%) whereas the same TMV concentration produced a normal RNA infectivity in the presence of ribosomes (2.38 mg RNA/ml) as shown in Table 10.

In further testing the procedure, TMV was added to a ribosomal preparation from healthy leaves in the ratio of 3 μg TMV:1 mg RNA. This was again separated from the ribosomes by one precipitation with antibodies. The TMV concentration was < 0.01 μg TMV/mg RNA as was checked by particle counting under the electron microscope. One particle was counted to 1,500 polystyrene spheres in a mixture containing 10^{10} spheres/ml and 333 μg RNA/ml. No infectivity was found in the M_2A_1 fraction nor was the RNA prepared from M_2A_1 by phenol extraction found to be infectious.

The nature of the infectivity in RNA_1 and RNA_2 was established by the fact that it was destroyed by incubation with 5 μg pancreatic ribonuclease/ml for two hours or by standing overnight at room temperature. This showed the labile RNA nature of the infectivity. The infectivity could be preserved by storing the RNA fractions at –30° C.

8.3. Further results of experiments made 60–62 hours after inoculation

In Table 11, the results are given of a number of experiments starting from leaves 60–62 hours after inoculation with 50 μg TMV/ml. From the table it can be seen that in all experiments, RNA with a high infectivity could be prepared by phenol extraction of ribosomal preparations from which the TMV had been separated to various degrees. In most of the experiments it was found that the infectivity of RNA_1 and RNA_2 after respectively one and two antiserum treatments, differed only little although there was a considerable change in TMV concentration as it appeared from the infectivity tests as well as from the particle countings.

For determining the TMV concentration by particle counting, M_2, M_2A_1 and M_2A_2 fractions in a concentration of 333 μg RNA/ml and a constant concentration of polystyrene spheres were sprayed and counted under the electron microscope. Simultaneously the ratio of virus particles to polystyrene spheres was determined in a series of TMV concentrations (0.1–10 μg/ml) with the same concentration of spheres. The linear relation between the ratio of virus particles to spheres and to the virus concentration served as a reference line from which the virus content in the M fractions could be estimated.

The usefulness of the procedure became clear from the fact that in a few cases tested a good agreement was found between the TMV concentration in the M fractions and in the pellets after precipitation.

The infectivities of RNA_1 and RNA_2 as estimated by the calculation described in the previous section and from the infectivity of TMV-RNA preparations tested in the same period as the experiments were performed, corresponded to 0.1–0.8 μg TMV–RNA except in the experiment of column IV (Table 11), where exceptionally high values were found (1–2.5 μg TMV-RNA). The latter may be due to some mistake or it may be connected to the high rate of synthesis in the leaves used as was apparent from the TMV content of the M_2 fraction

TABLE 11. TMV concentration as determined by particle counting and infectivity of fractions obtained according to Scheme 3.

Concentration of TMV by particle counting in μg TMV/mg RNA/ml						Infectivity by local lesion assay* ***									
Exp. no.	I	II	III	IV	V	I		II		III		IV		V	
						conc. of inoculum μg RNA/ml	t/c	conc. of inoculum	t/c	conc. of inoculum**	t/c	conc. of inoculum	t/c	conc. of inoculum	t/c
M_2	46.5	40	125	206	120	–	–	100	2627/329	100	4196/374	100	3225/143	–	–
M_2A_1	1	1	0.15	5	7	–	–	100	28/293	100	128/521	100	59/178	–	–
M_2A_2	<0.01	<0.05	<0.04	0.04	1	–	–	200	26/365	200	50/671	200	8/279	–	–
P_1	45	–	110	–	120	–	–	100	628/377	c	1833/564	100	980/274	–	–
P_2	1.1	–	0.1	–	6	–	–	100	5/235	c	131/601	c	100/288	–	–
RNA I						1000	18/52	1000	32/97	1000	57/373	1000	140/54	1000	15/209
RNA II						1000	32/40	1000	20/87	1000	76/408	1000	40/40	1000	36/255

* The infectivity is given as the quotient $\frac{t}{c} = \frac{\text{number of local lesions on test halves}}{\text{number of local lesions on control halves}}$

6–12 leaves were used in each assay. The control inoculum had 0.1 μg TMV/ml in experiment I, and 0.1 μg TMV/ml in the other four.

** The pellets were resuspended in buffer and diluted to a volume corresponding to a concentration of 100 μg RNA/ml. Where the concentration is indicated with c this has not been done, instead concentrated pellet suspensions in a small volume were used.

*** For comparison: 0.5 μg TMV-RNA/ml had an infectivity ot 35/136 and 0.1 μg TMV-RNA/ml, 71/390 (0.1 μg TMV/ml as control).

(206 μg/mg RNA). Since the greatest possible TMV-RNA concentration originating from TMV could be 0.005 μg/ml this means there was 20–160 times as much infectious RNA as might be expected from contaminating TMV. On the other hand, 0.1–0.8 μg TMV-RNA might have originated from 2–16 μg TMV/mg RNA. This would have been easily detected with the electron microscope and would have been precipitated with the antiserum.

In the experiments reported in Table 11 the procedure was not yet completely standardized. The virus antibody aggregates were spun down at the rather low speed of 600 × *g* (VAN KAMMEN, 1961). This appeared not to be satisfactory in all experiments. Sometimes it was found that the virus content of M_2A_2 was not reduced to less than 0.05 μg/mg RNA as tested by particle counting. In those cases virus was found as small aggregates of TMV particles which were obviously not precipitated by the centrifugation or which whirled up because of insufficient packing of the pellet. For those reasons we took it a practice to spin down the TMV – antibody complexes at 12,500 — 15,000 × *g* for 12 min. This was found to be sufficient for precipitating all aggregates. When TMV suspensions with a concentration of 0.1–10 μg/ml were treated with antiserum all TMV particles were spun down at 12,500 × *g* for 12 min and no TMV particles could be detected in the remaining supernatant under the electron microscope.

Furthermore, it was found unsatisfactorily that the infectivity of the RNA was not estimated from simultaneous inoculations of TMV-RNA on leaves of the same group of test plants. In the experiments of Table 11, the TMV-RNA infectivities were determined in independent experiments. Since the susceptibility of the leaves could vary considerably the estimation of the amount of infectious RNA was rather crude. It seemed that this could be improved by inoculating a series of TMV-RNA concentrations with each experiment by way of a standard curve.

Also the particle counting had to be improved to admit more accurate statements about the virus concentration after antiserum treatments. This will be described in the next section when experiments are reported which combine all suggested improvements.

8.4. THE AMOUNT OF INFECTIOUS RNA AT DIFFERENT TIMES AFTER INOCULATION

Table 12 gives the results of a series of experiments performed at different times after inoculating the leaves. The concentrations of TMV are given as determined by particle counting as well as the infectivity of the successive fractions as determined by the local lesion test.

For the particle counting, M_2, M_2A_1 and M_2A_2 fractions were diluted to 420 μg RNA/ml and 0.8 ml of each of these dilutions was mixed with 0.1 ml 0.3% serum albumin solution and 0.1 ml of a polystyrene suspension containing 10^{10} spheres. When the upper limit of the TMV concentration had to be estimated in M_2A_1 or M_2A_2 fractions, the preparations were examined and counted under the electron microscope to 1,500 spheres. This corresponded with a volume of $15 \times 10^4 \mu^3$. At a concentration of 0.01 μg TMV/ml 22–23 particles may be expected in this volume (*cf.* page 40). When 1–3 virus particles were counted against 1,500 spheres (see Table 12) the upper limit of the virus concentration could be determined by means of Table 8 (page 41) given for the upper confidence limit for the mean of a Poisson distribution. For example, when 2 virus

particles were counted there is 98% certainty that these are not 7.5 particles. For the undiluted M_2A_2 or M_2A_1 fraction it is then 98% certain that there were not 22.5 particles/mg RNA/15 $\times$ $10^4\mu^3$ and this is recorded in the table as: TMV concentration $<$ 0.01 μg/mg RNA/ml.

The results of Table 12 confirm the findings presented in the previous section. From the Table it can be seen that the virus content of the M_2 fraction in μg TMV/mg RNA/ml increased with time after inoculation. After about 36 hours, the virus content increased rapidly but the amount of TMV was still so small that it was separated from the ribosomes by one antiserum treatment. After 60 hours the virus concentration had become so high that it could not be reduced to less than 0.01 μg/mg RNA/ml by one precipitation with antiserum. This may differ of course with the titer of the antiserum. A second antiserum

TABLE 12. TMV concentration, as determined by particle counting, and infectivity of portions obtained according to Scheme 3 at different times after inoculation.

Time after inoculation	Fraction	Number of virus particles/ Number of polystyrene spheres	TMV concentration* by particle counting μg/mg RNA/ml	Infectivity**
24 hours	M_2	49/250	0.4	179/352
	M_2A_1	1/1500	<0.01	1/205
	RNA_1			11/807 (= 0.014)
36 hours	M_2	36/117	0.6	217/301
	M_2A_1	1/1500	<0.01	2/251
	RNA_1			5/903 (= 0.006)
38 hours	M_2	68/122	1.1	385/324
	M_2A_1	2/1500	<0.01	17/601
	P_1	210/129	1.1	–
	RNA_1			27/1633 (= 0.017)
48 hours	M_2	479/112	8.5	629/135
	M_2A_1	3/1500	<0.01	2/190
	M_2A_2	1/1500	<0.01	1/227
	RNA_1			21/935
	RNA_2			23/1048 (= 0.022)
48 hours	M_2	617/200	6.2	1892/214
	M_2A_1	3/1500	<0.01	31/214
	M_2A_2	1/1500	<0.01	21/204
	RNA_1			8/599
	RNA_2			13/499 (= 0.026)
60 hours	M_2	1660/100	33.3	4703/201
	M_2A_1	72/1100	0.13	895/811
	M_2A_2	2/1500	<0.01	218/1269
	RNA_1			26/455
	RNA_2			23/506 (= 0.045)
	P_2	126/600	0.14	
71 hours	M_2	1565/50	63	2667/654
	M_2A_1	544/109	10	1437/586
	M_2A_2	3/1500	<0.01	34/327
	RNA_1			435/706
	RNA_2			15/641 (= 0.023)

* 10^{10} polystyrene spheres per ml; M_2, M_2A_1 and M_2A_2 in the concentration of 333 μg RNA/ml. P fractions were suspended in a volume of H_2O to a concentration corresponding to 1 mg RNA/ml.

** Concentration of inoculum was 1000 μg RNA/ml in all cases. Control suspension was 0.5 μg TMV/ml. The ratio is the number of lesions on test halves to number of lesions on control halves.

treatment was necessary 60 hours after inoculation for reducing the TMV concentration below 0.01 μg/mg RNA/ml. Whereas the virus content of M_2A_1 after 60 hours was low and thus the contribution from this virus to the infectious RNA of RNA_1 small, this was not true any more after 71 hours. The virus content of the M_2A_1 fraction was clearly reflected in the infectivity of RNA_1. After 48 hours and 60 hours there was no appreciable difference between the infectivities of the RNA_1 and the RNA_2 fractions. A distinct decrease in virus content could be detected after 60 hours when the M_2A_1 fraction was treated a second time with antiserum. Over the whole range of times, the amount of infectious RNA was 70–160 times as much as might be expected from TMV contaminations of at most 0.01 μg/ml.

In Table 13 the RNA infectivities are evaluated in terms of TMV-RNA infectivity. The amount of infectious RNA at different times after inoculation is estimated. This has been done in various ways.

TABLE 13. Infectivity of ribosomal RNA_2 at different times after inoculation.

Time after inoculation in hours	Number of lesions from RNA (1 mg/ml)/ Number of lesions from 0.5 μg TMV/ml	Equivalent amount of TMV-RNA μg/ml (calculated)	Equivalent amount of TMV-RNA μg/ml (standard-curves)
24	11/ 807 = 0.014	0.07	0.04
36	5/ 903 = 0.006	0.03	0.1
38	27/1633 = 0.017	0.08	0.16
48	23/1048 = 0.022	0.11	0.1
	13/ 499 = 0.026	0.13	0.1
60	23/ 506 = 0.045	0.22	0.12
71	15/ 641 = 0.023	0.12	0.14

The control suspension was the same for all experiments reported in Table 13. It contained 0.5 μg/TMV/ml. The infectivities of the RNA extracted from the ribosomes may be compared in reference to the infectivity of the control. From the ratio of the infectivity of the RNA to control TMV, it is possible to calculate roughly the amount of TMV-RNA with an infectivity equivalent to the infectivity of ribosomal RNA. For this it was assumed that TMV contains 5% RNA and that the relative infectivity of TMV-RNA with regard to TMV is 0.5%. The calculated values are given in the third column of Table 13. It might give the impression that the amount of infectious RNA in the ribosomes increased up to 60 hours after inoculation and then decreased again. This comparison does not take into account that the relative infectivity of TMV-RNA may vary considerably and is not always 0.5%. It may vary from 0.05–0.5%. This variation depends on the condition of the leaves used in the test. For that reason, it seems better to estimate the equivalent amounts of TMV-RNA from the infectivities of a series of TMV-RNA concentrations inoculated in each experiment. These values are given in the last column of Table 13. It can be seen that the amount of infectious RNA increases in the beginning, but soon becomes constant. It did not appear practicable to standardize the growing of the *N. glutinosa* test plants in such a way that the same relative infectivity for TMV-RNA was always found even when the same TMV-RNA preparation was used all the time, as was done in the experiments of Table 12. Portions of TMV-RNA

were kept frozen at –30°C and did not loose their infectivity under these conditions. It is known that the infectivity of TMV-RNA may vary dependent on the condition of growth and the age of test plants (BAWDEN and PIRIE, 1957). Since we could not control this variation, it seemed best to determine the amount of infectious RNA for each case individually. Therefore, we place the most trust in the amounts given in column 4 of Table 13.

Tentatively we should like to conclude that there is about 0.12 μg infectious RNA per mg ribosomal RNA during the period of maximum virus multiplication in the leaves. The most rapid increase of virus in the leaves starts at 36–38 hours after inoculation. It is just preceded by the increase of infectious RNA in the ribosomal fraction. It seems possible that the amount of virus RNA in the ribosomal fraction is also a determining factor for the speed of virus increase.

8.5. CONCLUSION

Some time after inoculation of the leaves, the virus content became so high that the TMV could not be separated from the ribosomes by two treatments of antiserum. Separation was no longer practicable. Perhaps if a γ- globulin fraction with a higher titer of antibodies were available, those difficulties may be overcome.

The concentration of TMV was so high in secondarily infected leaves with symptoms that our separating procedure did not work either. The concentration of ribosomes also appeared rather low. Generally we got the impression that in those cases the greater part of the M_2 fraction consisted of TMV particles. This, however, needs further investigation.

Our method of separating TMV from ribosomes seems limited to those cases where the concentration of TMV particles in the ribosomal preparations is not so high.

We tried to perform reconstitution of the infectious RNA with TMV-protein (FRAENKEL-CONRAT and SINGER, 1959). This would have been a good test for the nature of the RNA. All our trials were, however, negative. Although it was possible to produce rod-shaped, ribonucleoprotein-like particles, the reconstitution resulted in complete loss of infectivity. In view of the fact that the infectious RNA constituted only 0.1 ‰ of the total RNA in the preparations, this was not amazing in itself.

In the above description we have given evidence pointing to the presence of infectious RNA in the ribosomal fraction from TMV infected tobacco leaves. This RNA is probably not combined with virus protein since it was not separated from the ribosomal suspension by TMV antibodies. The RNA may, to some extent, be called free RNA. Whether it is really free or forms part of another structure is an unanswered question at the present. There appears to be some connection between the ribosomes and the infectious RNA we have demonstrated. For the RNA remains with the ribosomes during preparation by the procedure of high and low speed centrifugation whereas unbound RNA would have been lost. How specific this binding is has still to be established. When the RNA plays a role in virus protein synthesis a close structural connection with the ribosomes may be expected.

CHAPTER 9

DISCUSSION

In the previous chapters we presented experimental evidence showing the occurrence of infectious RNA in the ribosomal fraction from TMV infected tobacco leaves. This chapter will be used to try to evaluate the significance of our findings.

The approach to unravelling the mechanism of a biochemical process (e.g. the process of virus protein synthesis) is twofold. First, one may try to isolate the components from the cell which probably play a role in the cell process. If this isolation is done by careful fractionation of the cell constituents, it may also make it possible to localize the process in the cell. Second, it has to be decided in which way the components are involved in the process under study. Our analysis of the ribosomal fraction from TMV infected leaves bears upon the first approach.

We postulated that the ribosomes would play a role in virus protein synthesis and that a virus specific component should be present in the ribosomal fraction determining the nature of the protein to be synthesized. To prove this, the ribosomal fraction from TMV infected tobacco leaves was isolated. The TMV particles were separated from the fraction by means of TMV antibodies and then the occurrence of infectious RNA, presumably viral RNA, in the fraction was established. This RNA could not have originated from TMV particles. It has now to be determined if, and in which way, the virus RNA in the ribosomal fraction was involved in virus protein synthesis. Some suggestions can be offered for this based on recent reports from the literature.

9.1. A POSSIBLE MODE OF ACTION OF THE INFECTIOUS RNA IN THE RIBOSOMAL FRACTION

A breakthrough in the study of protein biosynthesis is found in the work of NIRENBERG and MATTHAEI (MATTHAEI and NIRENBERG, 1961; NIRENBERG and MATTHAEI, 1961). They succeeded in isolating a stable cell-free system from *E. coli* in which the amount of amino acids incorporated into protein by means of the ribosomes was dependent upon the addition of RNA preparations which functioned as templates for the protein to be synthesized. They found that synthetic polyribonucleotides could stimulate the amino acid incorporation. When a specific polyribonucleotide was used it resulted in specific incorporation of one or more amino acids. For example, when polyuridylic acid was added, incorporation of *l*-phenylalanine was brought about into a polypeptide which could be identified as poly-*l*-phenylalanine. With polyribonucleotides of a different composition, other correspondingly different polypeptides were found (MATTHAEI *et al.*, 1962; SPEYER *et al.*, 1962; LENGYEL *et al.*, 1961, 1962). This established a direct relationship between the composition of the RNA added and protein to be synthesized. RNA was found to be the carrier of the information for the amino acid sequence in protein. This fitted very well with the finding of BRENNER *et al.* (1961), who demonstrated, using the system of Coli bacteria infected with T2 bacteriophage, that ribosomes are non-specialized structures which can synthesize protein depending upon the so-called messenger they happen to contain. A rapid metabolizing RNA which had the capacity to

attach itself to the ribosomes was considered this messenger. Especially since VOLKIN *et al.* (1958) had found such a rapid metabolizing RNA in T2 infected *E. coli* to have a base composition quantitatively similar to that of phage DNA. This suggested the possibility for this RNA to transfer the information coded in the DNA to the protein synthesizing centres. At the same time GROS *et al.* (1961) found an RNA with a high rate of turnover present in normal *E. coli* cells. Tentatively it was assumed that this RNA had the function of determining the amino acid sequence in the protein.

Further studies supported this opinion. Various virus RNA's, TMV-RNA (NIRENBERG and MATTHAEI, 1961; TSUGITA *et al.*, 1962), RNA from coliphage f2 (NATHANS *et al.*, 1962) and RNA from polio virus (WARNER *et al.*, 1963b) were found to stimulate the amino acid incorporation with cell free ribosomal preparations from *E. coli*. The protein synthesized in this way *in vitro* was compared with the authentic virus proteins. In the case of coliphage f2-RNA, good evidence was obtained that the protein produced under influence of phage RNA was, for the greater part, f2 coat protein. In both the other cases, the products of *in vitro* experiments had structural similarities to the viral proteins. These findings all point strongly to the virus RNA being the primary protein structure determining factor in virus protein synthesis. On these grounds it seems reasonable to assume that the virus RNA demonstrated in the ribosomal fraction from tobacco leaves also functions as a carrier of structural information in TMV protein synthesis.

9.2. THE CAPACITY OF VIRUS PROTEIN SYNTHESIS

There appear to be two ways to demonstrate the possible role of TMV-RNA in TMV protein synthesis in leaves. First, a cell free system from leaves analogous to the *E. coli* system, actively synthesizing protein, might be isolated. Thus, the influence of TMV-RNA and other plant virus RNA's on this system might be studied. This is attractive because it demands making accessible the protein synthesizing mechanism in leaves or other parts of the plants which has not been accomplished yet. The interaction of virus RNA with the ribosomes will be of special interest in that connection.

Second, it does not seem impossible to attempt the isolation from TMV infected leaves of a ribosomal fraction containing the infectious RNA, and to demonstrate that this fraction would be capable of virus protein synthesis. The following calculations derived from the results of Chapter 8 give an impression of the possible activity of this process and the ratio between virus RNA content and virus protein synthesis.

From the data represented in Table 12 on page 49 it can be seen that in the period of 48–71 hours after inoculation of the leaves there is an increase of roughly 30 μg TMV/mg RNA in 12 hours. This does not take into account the losses of TMV and RNA during the fractionation. This means that there is an increase of 0.04 μg TMV/mg RNA/min or 6×10^8 TMV particles/mg RNA/min. Assuming that each TMV particle contained 2.130 TMV protein units, (FRANKLIN *et al.*, 1959) this increase corresponds to a production of 1.3×10^{12} protein molecules/mg RNA/min. It is clear that in leaf tissue, TMV protein synthesis is a rapidly producing process which should be suitable for study also *in vitro*. At the other hand, we found (see Table 13, page 50) in the period of 48–71 hours after infection, approximately 0.12 μg TMV-RNA/mg RNA. This

corresponds to 3.6×10^{10} molecules TMV-RNA/mg RNA, assuming a molecular weight of 2×10^6 for TMV-RNA (GIERER, 1958a and b; BOEDTKER, 1959), and a rate of synthesis of $(1.3 \times 10^{12})/(3.6 \times 10^{10}) = 36$ TMV protein molecules/min/TMV-RNA molecule.

We calculated already from our data that each minute about 6×10^8 TMV particles/mg RNA/min were synthesized. Assuming the virus RNA in the ribosomal fraction was in a steady state, this means that 1.7% of the 3.6×10^{10} molecules TMV-RNA/mg RNA were incorporated into a TMV particle per minute and again supplied by new RNA synthesis.

On the average, a virus RNA molecule could then stay 60 minutes in the ribosomal fraction. In these 60 minutes there will be synthesized per TMV-RNA molecule $60 \times 36 = 2{,}160$ protein molecules. This should mean that one TMV-RNA molecule might effect the synthesis of about the number of protein molecules present in one TMV particle. This seems reasonable in itself and offers an attractive picture of TMV synthesis. Naturally these calculations contain a good deal of speculation, but they may serve to indicate how our findings of 0.12 μg TMV-RNA/mg RNA and a rate of TMV synthesis of 30 μg TMV/mg RNA are in proportion.

9.3. VIRUS RNA AND THE MESSENGER CONCEPTION

The idea of a messenger was deduced by JACOB and MONOD (1961a and b) from genetical experiments on enzyme induction and repression. According to their conception, the messenger is the component which carries the structural information from structural genes to the protein synthesizing center. As such, the messenger is a transcription of the information in the DNA of the genes which passes to the ribosomes where a second transcription into protein occurs. This messenger should furthermore be rather unstable, according to these authors, with a high rate of turnover.

The discovery that in phage infected cells (VOLKIN *et al.*, 1958) growing yeasts (YČAS and VINCENT, 1960) and *E. coli* (GROS *et al.*, 1961) there exists a rapidly renewed RNA which has the properties of having base ratios analogous to its homologous DNA, gave good support to the view that RNA could function as a messenger. The RNA, furthermore, had the capacity of attaching itself to the ribosomes and could hybridize with homologous DNA demonstrating complementarity in its sequence to its parental DNA (SPIEGELMAN, 1961). It has been demonstrated that the same population of ribosomes produces bacterial proteins and phage specific protein in a phage infected cell (BRENNER *et al.*, 1961). An enzyme fraction was isolated which brings about the synthesis of RNA from the nucleoside-5′-triphosphates using DNA as a primer. The produced RNA has a quantitatively similar base composition compared to the primer DNA (HURWITZ *et al.*, 1961; WEISS, 1960; STEVENS, 1960). The sequence of amino acids in the proteins synthesized *in vitro* with ribosomes has been demonstrated to be dependent on the addition of a specific RNA (NIRENBERG and MATTHAEI, 1961). Therefore, an abundance of evidence appears to point to RNA as the messenger as postulated by JACOB and MONOD. However, if RNA from the RNA viruses is called a messenger RNA, it is not in the sense of the messenger postulated by JACOB and MONOD. There is no indication that virus RNA is synthesized dependent of DNA or that it functions as an intermediate between DNA and the protein synthesizing center. On the contrary there are

some indications that virus RNA synthesis is not dependent on DNA. Actinomycin D which inhibits DNA-dependent RNA synthesis in mammalian *L* cells inhibits synthesis of DNA containing vaccinia virus but does not affect the yield of RNA containing Mengo virus (REICH *et al.*, 1961, 1962). No complementarity could be detected between the RNA of $MS\varnothing_2$ phage and the DNA of the infected host *E. coli* ('DOI and SPIEGELMAN, 1962). Although definite evidence is lacking, it seems dangerous to consider virus RNA a messenger as defined by JACOB and MONOD.

There seems to be good evidence that there is some interference between multiplication of RNA containing viruses and the genetically governed protein synthesis. The symptoms developed in virus infected plants point in this direction. This may be, however, a secondary effect. The important point is to find out if RNA viruses are multiplying independent of DNA. Then there could still be another mechanism for control of protein synthesis besides the one suggested by JACOB and MONOD.

Another difference between virus RNA and messenger RNA is that the viral RNA active in virus protein synthesis does not seem to be an unstable intermediate as is postulated for the messenger RNA of the gene regulated protein synthesis. The fact that the viral RNA is degraded during *in vitro* protein synthesis in some cases, may be a property of the *in vitro* system, but this breakdown needs not necessarily to occur *in vivo* too (BARONDES and NIRENBERG, 1962; WARNER *et al.*, 1963b).

9.4. THE POSSIBLE CONNECTION BETWEEN THE VIRUS RNA AND THE RIBOSOMES

The role of the ribosomes in transmitting information concerning the amino acid sequence to protein has now become a passive one. The way the messenger RNA is attached to the ribosomes has become of particular interest. The messenger RNA's found, vary considerably in size, having sedimentation coefficients from 3 *S* to 30 *S*. Considering the long molecules such as the virus RNA's mentioned before it becomes a puzzling question how the long chain of about 6000 nucleotides interacts with the small ribosomes of about 180 Å in diameter. Recently the suggestion was put forward (WARNER *et al.*, 1963a; WETTSTEIN *et al.*, 1963) that a multiple ribosomal structure should be the really active structure in protein synthesis. In this multiple ribosomal structure, ribosomal units of 80 *S* are held together by RNA which could be the messenger. The number of ribosomes involved might vary presumably with the length of the messenger and the size of the protein molecule to be synthesized. The way these units are working is not yet agreed upon. We should like to point out in this connection the clusters and chains of ribosomes visible in the electron micrographs of Photo's 3 and 4 (see 3.4). We have not fractionated our ribosomal preparations for isolating ribosomal structures of different sizes, although we were well aware of the fact that the preparations contained components of different sizes (Chapter 3). In light of the recent findings mentioned, the possibility exists that the infectious RNA we have isolated from the ribosomal preparations from tobacco leaves originates from the more complicated, multiple structures, shown in the electron micrographs.

We are inclined to believe that the virus RNA isolated from our ribosomal fractions from tobacco is rather firmly bound to the ribosomes. It seems improbable that the infectious RNA in the preparations was due to a non-specific

binding of free RNA to the ribosomes during homogenization. It appears doubtful that such a non-specific binding could give enough protection to the RNA to endure the whole procedure and still remaining infective. Further fractionation of the ribosomal preparations will clarify this point.

Finally it may be argued whether or not some virus protein is attached to the ribosomal structure with which the infectious virus RNA forms a complex, particularly when this RNA is active in protein synthesis. A question may be raised if this protein would not also react with the anti TMV γ-globulin. Indeed COWIE *et al.* (1961) and NOVELLI (1961) found β-galactosidase attached to the ribosomes of β-galactosidase synthesizing cells and they could detect it with serological methods. If some protein was present it did not disturb our separation of TMV and infectious ribosomal RNA. One should not conclude from this that no virus protein is bound to the ribosomes but there may be a distinct serological difference between the protein bound to the ribosomes and the unbound protein, similar as it appeared in the case of β-galactosidase studied by COWIE *et al.* (1961). If TMV protein was attached to the ribosomes it would be single TMV protein subunits. It seems very probable that there are important serological differences between this protein and the protein as it occurs in aggregated form in protein X and TMV. For that reason we do not consider this possibility as a serious objection against our procedure.

Further research trying to answer the questions raised in this chapter will contribute to the solution of the role played by the ribosomal fraction in virus protein synthesis and virus multiplication.

SUMMARY

1. The occurrence of infectious ribonucleic acid not yet surrounded with virus protein in tobacco mosais virus (TMV) infected tobacco leaves was studied. The possibility that it plays a role in virus protein synthesis has been considered. Special attention was paid to the ribosomal fraction from infected leaves as it was supposed that this fraction would be involved in virus protein synthesis and consequently the presence of a virus specific component could be expected in this fraction.

2. The ribosomes represent a well defined fraction of tobacco leaf homogenate containing roughly 66% of the ribonucleic acid extracted from the leaves.

3. In the cytoplasm of tobacco leaf cells the ribosomes were found to be scattered in groups or clusters, and were not attached to the endoplasmic reticulum, suggesting that there is possibly some connection between the particles.

4. No microsomes could be isolated from the leaf homogenate. The ribosomes were also found occurring in chains or clusters in the isolated ribosomal fractions similar to these in the cytoplasm. In addition many free particles were also present.

5. Tobacco leaf ribosomes were found to have a diameter of 150–180 Å and a RNA: protein ratio of 0.72–0.89. The analytical ultracentrifuge revealed that the ribosomal preparations contained mainly 80 *S* particles besides small quantities of 40 *S*, 60 *S* and 112 *S* components. The 80 *S* particles could not be reversibly dissociated dependent on the Mg-ion concentration.

6. The ribosomal preparation had the capacity of incorporating amino acids in vitro to some extent, depending on an energy source. The incorporation was achieved by means of a soluble enzyme fraction containing amino acid activating enzymes and transfer-RNA obtained from yeast. A similar enzyme fraction isolated from leaves did not serve the purpose.

7. The ribosomal preparations were quite stable. They did not show autolysis when stored at 4 °C over night. Such preparations did not prove any measurable ribonuclease activity.

8. After inoculating a leaf, virus increase is first determined by two factors: the increase in the number of infected cells and the increase in the quantity of virus per cell. Later, the number of infected cells does not increase anymore and the virus increase depends only on the increase of virus per cell. Hence the best time to undertake an analysis for intermediates of virus synthesis would be obviously when most of the cells are infected.

9. The ribosomal fraction of TMV infected leaves contained also a lot of TMV some time after infection. This TMV could be separated from the ribosomes by adding an excess of TMV antibodies and then centrifuging the virus -antibody aggregates at 12,500 $\times$ *g* for 15 min. Infectious RNA not yet surrounded by virus protein would not react with the TMV antibodies.

10. To prevent destruction of ribosomes it was necessary to purify the antiserum and to isolate the γ-globulin fraction from the antiserum containing the antibodies. This fraction could be precipitated from the antiserum by adding ammonium sulphate to 33% saturation. The isolated γ-globulin fraction did not indicate any measurable ribonuclease activity. Correspondingly it did not cause a rapid inactivation of TMV-RNA.

11. The method of particle counting under the electron microscope was found to be very useful in checking the degree to which the TMV particles could be separated from the ribosomes. This method can be used for quantitive determinations to a concentration of 0.1 μg TMV/ml. At further lower concentrations the number of particles becomes too small for an accurate concentration determination. However, the method can still be used for estimating the upper concentration limit starting from a Poisson distribution for the particles in suspension.

12. After the TMV particles had been eliminated to a concentration of less than 0.01 μg/mg ribosomal RNA a certain amount of infectious RNA could be isolated from the ribosomal preparations by phenol extraction corresponding to 0.1 μg TMV-RNA/mg ribosomal RNA. This was 70–160 times as much as could be expected to originate from any contaminating TMV. This demonstrated the occurrence of infectious RNA in the ribosomal fractions.

13. The amount of infectious RNA becomes measurable twenty four hours after inoculating the leaves. It showed an increase during the next 12 hours to an amount equivalent to 0.12 μg TMV-RNA/mg ribosomal RNA and remained at that level for the next 48 hours. The increase of infectious RNA occurred just before the rapid virus increase started. This suggests that the amount of infectious RNA present in the ribosomal fraction also determines the rate of virus synthesis.

SAMENVATTING

In dit proefschrift wordt onderzoek beschreven waaruit blijkt dat in bladeren van 'White Burley' tabak geïnfecteerd met tabaksmozaïekvirus (TMV), infectieus ribonucleïnezuur voorkomt dat niet omgeven is met viruseiwit. Dit ribonucleïnezuur zou een rol kunnen spelen bij de virussynthese.

In de inleiding wordt uit hetgeen bekend is over de chemische en biologische eigenschappen van TMV de hypothese afgeleid waarvan bij het onderzoek werd uitgegaan. TMV is een staafvormig ribonucleoproteïne – 300 mμ lang en met een diameter van bijna 18 mμ – dat voor 5 tot 6 % uit ribonucleïnezuur (RNA) bestaat en voor 94 tot 95 % uit eiwit. Het eiwit zit in de vorm van identieke deeltjes spiraalsgewijs rondom de as van het staafje gepakt. Het RNA is één lange streng die, gewonden in een spiraal, diep ligt ingebed in de stapeling van de eiwitdeeltjes. Het is mogelijk om het eiwit en het RNA te scheiden en in natieve vorm te bereiden. Daarbij blijkt het RNA, hoewel in veel geringere mate dan het complete virusdeeltje, infectieus te zijn terwijl het eiwit niet infectieus is. Dit wijst er op dat het RNA de drager is van de infectiositeit van het virus en waarschijnlijk de virusspecifieke processen in de geïnfecteerde cel induceert. Nog op een andere manier wordt de rol van het RNA bij de virusvermenigvuldiging duidelijk gedemonstreerd. Het is namelijk mogelijk door tevoren afzonderlijk bereid infectieus RNA en viruseiwit te mengen weer TMV-deeltjes te verkrijgen, die vrijwel niet verschillen van de oorspronkelijke virusdeeltjes. Gebruikt men nu voor een dergelijke reconstitutie respectievelijk RNA en eiwit van twee TMV-stammen, die verschillen in eiwitsamenstelling, dan wordt, wanneer het gereconstitueerde virus wordt geïnoculeerd op tabaksplanten, virus gesynthetiseerd met eiwit dat hoort bij het virus waar het RNA uit werd bereid. Hieruit blijkt dat het virus-RNA bepaalt welk eiwit er gevormd wordt. Men mag om deze redenen het RNA van het TMV beschouwen als de genetische component waarin de eigenschappen van een virusdeeltje worden overgedragen op nieuw te maken deeltjes.

Uit onderzoek over de biosynthese van eiwitten is komen vast te staan dat de stap in het syntheseproces in de cel, waarbij de aminozuren in een specifieke volgorde aaneengeschakeld worden, plaats vindt aan kleine nucleoproteïne deeltjes, de zgn. ribosomen. Het lijkt aannemelijk dat de synthese van viruseiwit niet volgens een essentieel ander mechanisme verloopt dan de normale eiwitsynthese. Daarom kan men er van uitgaan dat de ribosomen ook betrokken zullen zijn bij de viruseiwitsynthese. Het is dan wel te verwachten dat er bij de viruseiwitsynthese een virusspecifieke component aanwezig is, die de eiwitsynthese dirigeert en zorgt voor het specifieke produkt. Deze dirigerende component zou het virus-RNA kunnen zijn. Ons onderzoek was erop gericht aan te tonen of en, zo ja, hoeveel virus-RNA er voorkomt in de ribosomale fractie van met TMV geïnfecteerde bladeren.

In het tweede hoofdstuk wordt beschreven hoe de planten die bij het onderzoek gebruikt werden, respectievelijk *Nicotiana tabacum* var. 'White Burley' voor het isoleren der ribosomen, en *Nicotiana glutinosa* als toetsplant voor TMV, werden opgekweekt. Er worden verder een aantal algemene methoden vermeld waarvan gebruik werd gemaakt.

In het derde hoofdstuk wordt de fractionering van homogenaten van gezonde tabaksbladeren behandeld volgens een methode waarbij afwisselend bij hoog en laag toerental gecentrifugeerd wordt (Schema 1). Wanneer bladeren gemalen worden in een mortier, in buffer met zand als schuurmiddel, kan 70 tot 80 % van

het RNA uit het blad geëxtraheerd worden. Van het geëxtraheerde RNA komt ongeveer 66% terecht in de ribosomale fractie die sedimenteert na de kernen, chloroplasten en mitochondriën. Ongeveer 16% wordt teruggevonden in de zwaardere fractie die kernen, chloroplasten, mitochondriën en fragmenten daarvan bevat, terwijl 20% niet sedimenteerbaar is na anderhalf uur centrifugeren bij 105.000 × *g* (Tabel 2). De herkomst van de RNA-rijke fractie in de cel werd onderzocht door elektronenmicroscopisch de inhoud van bladcellen te vergelijken met die van de verschillende fracties. De osmiofiele deeltjes met een diameter van 150 tot 180 Å, de ribosomen, die in een grote hoeveelheid in het cytoplasma voorkomen werden teruggevonden in de RNA-rijke fractie. De ribosomen vormden dus een duidelijke fractie van het celhomogenaat.

Bij de bestudering van de coupes van bladcellen (Foto's 1a en 1b) viel het op dat de ribosomen niet of slechts incidenteel vastzitten aan het endoplasmatisch reticulum. Toch lijkt het of de ribosomen niet willekeurig verspreid liggen maar vooral voorkomen in groepjes, met onderling enig verband. Soortgelijk was de situatie in de geïsoleerde ribosomenfractie. Microsomen, fragmenten van endoplasmatisch reticulum met ribosomen eraan, kwamen hierin niet voor. Behalve afzonderlijk kwamen de ribosomen vaak voor in ketens of groepjes, hetgeen een onderling verband suggereert (Foto's 3 en 4). De mogelijke functionele betekenis van deze structuur is nog niet duidelijk.

In hoofdstuk 4 wordt beschreven hoe de eigenschappen van de ribosomen uit gezonde tabaksbladeren verder werden onderzocht en vergeleken met die van ribosomen uit ander organismen. De verhouding RNA : eiwit van een ribosomenpreparaat (Schema 2) varieerde van 0,72 tot 0,89, wat overeenkomt met een gehalte van 42 tot 47% RNA wanneer het preparaat alleen RNA en eiwit zou bevatten. In de analytische ultracentrifuge toonde het sedimentatiediagram (Fig. 1 en 2) dat de fractie bestond uit een hoofdcomponent met een sedimentatiecoëfficiënt van 80 *S* en kleine hoeveelheden van 40 *S*, 60 *S* en 112 *S* componenten. De verhouding waarin de componenten voorkomen is weinig afhankelijk van de magnesiumionen-concentratie in het medium, in tegenstelling tot met wat beschreven is voor ribosomen van andere organismen. Het was niet mogelijk de 80 *S* component reversibel te dissociëren in afhankelijkheid van de magnesiumionen-concentratie. Pogingen om de ribosomen te dissociëren met ethyleendiaminetetra-azijnzuur (EDTA), hoge fosfaatconcentraties of verhoging van de pH leidden alle tot irreversibele splitsing en beschadiging van de ribosomen. De ribosomenfractie bezat, zij het in geringe mate, het vermogen om *in vitro* aminozuren te incorporeren in eiwit, afhankelijk van een energiebron (Tabel 4). Daarbij werd een enzymfractie, met aminozuuractiverende enzymen en transfer-RNA uit gist gebruikt. De ribosomenpreparaten waren stabiel. Wanneer ze bewaard werden bij 0°C in buffer pH 7.0, 0.005 M tris, 0.01 M Mg^{++}, 0.006 M mercapto-ethanol vertoonden ze geen autolyse. De preparaten vertoonden geen belangrijke RNA splitsende activiteit.

In hoofdstuk 5 wordt het verloop van de virusvermenigvuldiging in bladeren van 'White Burley' tabak beschreven na inoculatie met TMV (Fig. 4). Na een latente periode van 24 tot 30 uur neemt de hoeveelheid virus snel toe. In het begin wordt de toename van het virus bepaald door twee factoren: toename van het aantal cellen waarin virus vermenigvuldigd wordt en toename van de hoeveelheid virus per cel. Maar op een gegeven moment zal het grootste aantal cellen geïnfecteerd zijn en de eerste factor geen rol meer spelen. Wanneer men tussenprodukten van de virussynthese wil opsporen is het gewenst te werken in een

stadium waarin zoveel mogelijk cellen deelnemen aan de virusvermenigvuldiging. De verdunning met gezonde celbestanddelen is dan het geringst, de concentratie van mogelijke tussenprodukten het grootst. Men moet daarom aanzienlijke tijd (48–60 uur) na inoculatie wachten voordat men een analyse kan beginnen. Het feit dat de infectie in alle cellen niet synchroon verloopt brengt met zich mee dat alle stadia van de vermenigvuldiging naast elkaar voorkomen. Voordat de infectie zich verbreid heeft over de grote meerderheid der cellen zal in een deel van de cellen al een aanzienlijke hoeveelheid virus gevormd zijn. In ons geval, waar we het voorkomen van infectieus RNA willen vaststellen, geeft dit bijzondere problemen. De infectiositeit is de meest karakteristieke eigenschap van het virus-RNA en hiervan zouden we dus bij voorkeur gebruik willen maken om het RNA aan te tonen. Daarvoor was het dus nodig eerst andere infectieuze deeltjes, met name de virusdeeltjes zo volledig mogelijk te verwijderen.

De wijze waarop TMV kan worden verwijderd uit een ribosomenpreparaat zonder de ribosomen te beschadigen wordt in hoofdstuk 6 beschreven. Antilichamen tegen TMV reageren specifiek met het TMV-eiwit en zullen niet reageren met RNA dat niet omgeven is met viruseiwit. De aggregaten van virus en antilichamen kunnen door centrifugeren bij laag toerental uit de ribosomensuspensie worden geprecipiteerd. Om a-specifieke reacties te voorkomen werd de γ-globuline fractie uit het antiserum geïsoleerd door gefractioneerde precipitatie met ammoniumsulfaat (Fig. 5). Het zo gezuiverde antiserum bleek goed in staat TMV en ribosomen te scheiden, zonder de ribosomen te beschadigen. Nadat het TMV met behulp van antilichamen gescheiden kon worden van de ribosomen bleef de vraag bestaan hoe volledig deze scheiding was. De infectiositeitstoets kon hierop geen antwoord geven. Daarom werd (hoofdstuk 7) een bepaling gebruikt voor de TMV-concentratie die niet gebaseerd is op de infectiositeit. Door de zeer karakteristieke structuur van TMV zijn de virusdeeltjes gemakkelijk te herkennen onder de elektronenmicroscoop (Foto 5). Door het aantal deeltjes in een bepaald volume te tellen is het mogelijk de virusconcentratie te bepalen. Hoewel in het gebied van de zeer lage concentraties ($< 0{,}1$ μg TMV/ml) geen nauwkeurige bepaling van de concentratie meer kon worden gedaan was het wel mogelijk de bovenste grens van het mogelijke concentratiegebied nauwkeurig vast te stellen. Het bleek dat met het antiserum de virusconcentratie tot $< 0{,}01$ μg TMV/mg ribosomen-RNA teruggebracht kon worden. In hoofdstuk 8 wordt beschreven hoe uit ribosomenfracties waaruit in verschillende mate het TMV verwijderd was, door extractie met fenol, RNA kon worden geëxtraheerd met een infectiositeit overeenkomende met 0,1 μg TMV-RNA/mg ribosomen-RNA of meer. Dit RNA kon niet van TMV afkomstig zijn zoals ook door verschillende controle-experimenten werd aangetoond (Tabel 11). De hoeveelheid infectieus RNA was 70 tot 160 maal zo groot als afkomstig zou kunnen zijn uit de onmeetbare TMV-verontreinigingen, die achtergebleven kunnen zijn in het ribosomenpreparaat. Kleine meetbare TMV-verontreinigingen bleken bovendien weinig invloed te hebben op de hoeveelheid infectieus RNA die geëxtraheerd kon worden (Tabel 10). Een serie proeven uitgevoerd op verschillende tijdstippen na inoculatie (Tabel 12 en 13) toonde aan dat 24 uur na de inoculatie het vrije infectieuze RNA meetbaar wordt om dan toe te nemen tot 0,12 μg TMV-RNA/mg ribosomen-RNA (0.01 %) en ongeveer constant te blijven tot 72 uur na inoculatie. De toename van infectieus RNA vindt plaats vlak voor het virus snel begint toe te nemen. Dit suggereert dat de viruseiwitsynthese bepaald wordt door de hoeveelheid vrij virus-RNA aanwezig in de ribosomenfractie.

In hoofdstuk 9 wordt een algemene discussie gegeven en geprobeerd aanknopingspunten te vinden tussen onze resultaten en die van recent onderzoek over de eiwitbiosynthese.

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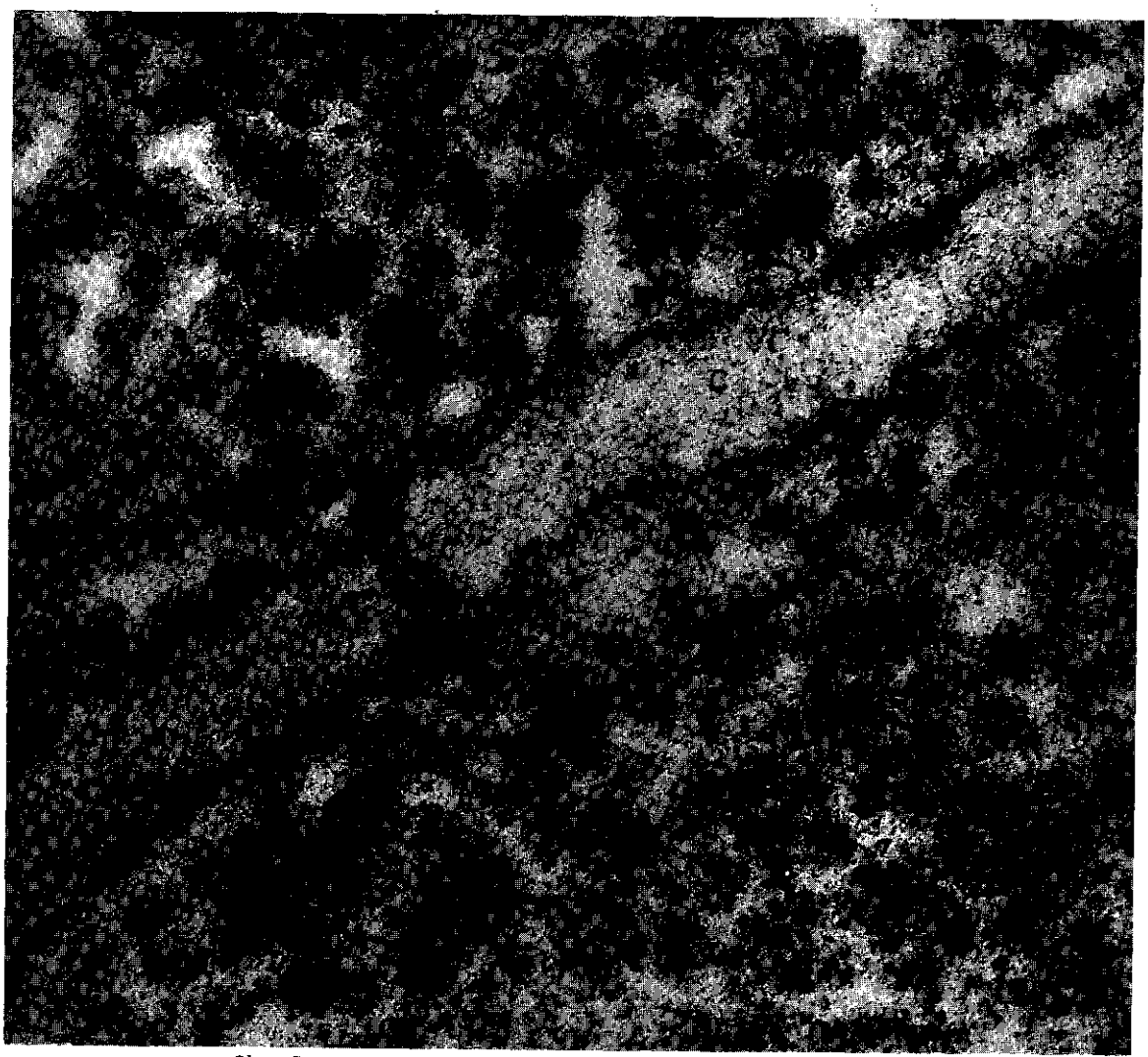

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PHOTO 1a. Ultra thin section of tobacco leaf.
Young leaf, fixed with OsO_4 and stained with $KMnO_4$ as described in 2.8 page 9. Part of the cytoplasm of two neighbouring cells is shown connected by a plasmodesm. The young age of the cells is apparent from the cell wall which is not yet completely stretched. In the cytoplasm are many ribosomes appearing as darkly colored granules, Membrane structures which form part of the endoplasmic reticulum are also seen. Magnification 120,000.

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PHOTO 1b. Ultra thin section of tobacco leaf
Older leaf cell already containing a large vacuole. The photo represents part of the nucleus and part of a vacuole with a strip of cytoplasm with a mitochondrion between the two. Groups of ribosomes are scattered through the cytoplasm. The membranes in the cytoplasm, presumably parts of the endoplasmic reticulum, have a thickness similar to the nuclear membrane. There is no evident relationship between the ribosomes and the membranes. Magnification 60,000.

n = nucleus
r = ribosomes
p = plasmodesm
nm = nuclear membrane
e = endoplasmic reticulum
v = vacuole
m = mitochondrion
c = cell wall

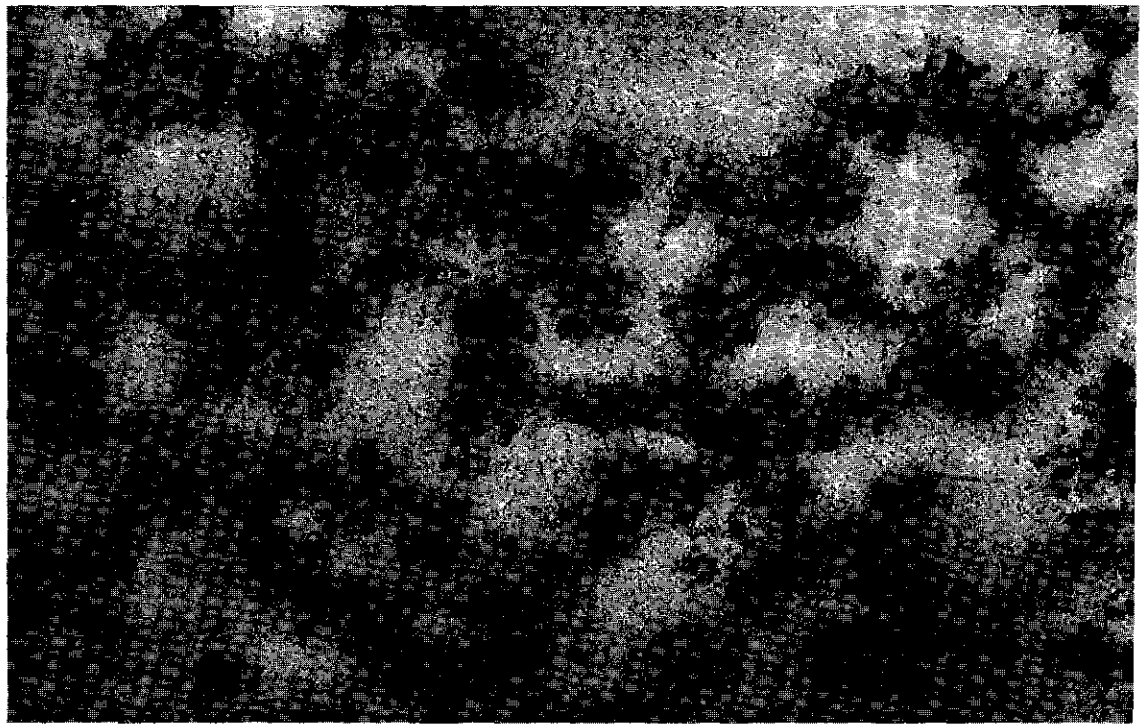

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PHOTO 2. Ultrathin section of a 105,000 × *g* pellet ($P_{105,000}$), fixed with OsO_4 and stained with $KMnO_4$ as described in 2.8. The pellet consists of ribosomes with a contamination of membranes. Magnification 120,000.
r = ribosomes m = membranes.

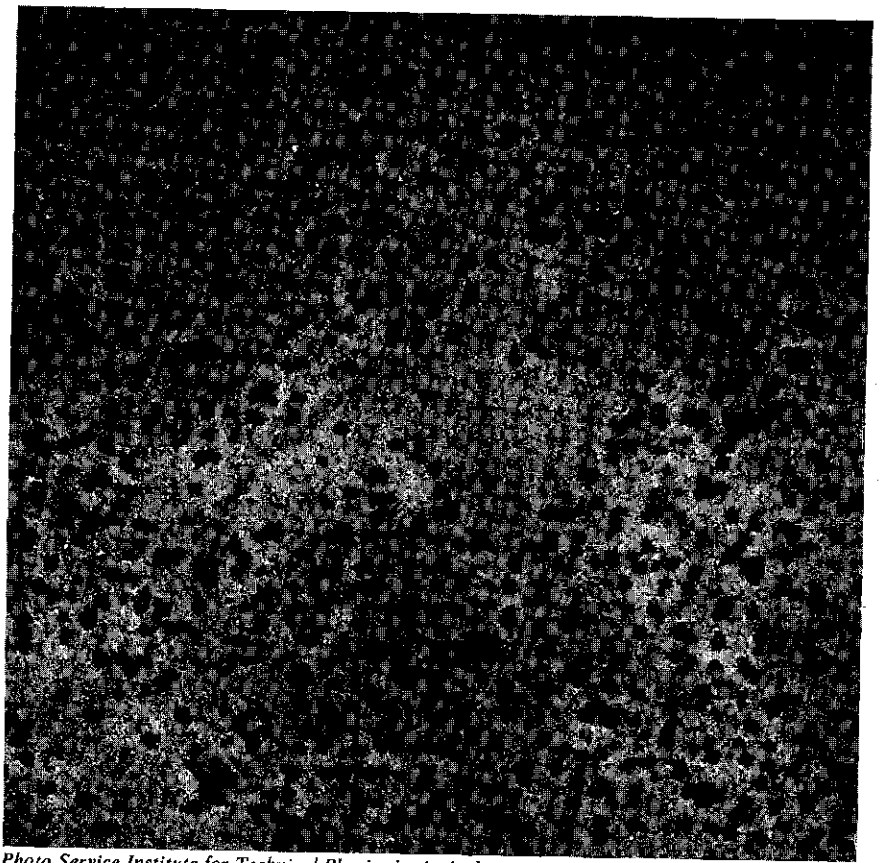

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PHOTO 3. Ribosomes from healthy leaves after two precipitations at 105,000 × *g* and positively stained with uranyl acetate as described in 2.8. Besides free particles, the ribosomes often appear in chains or clusters. Magnification 100,000.

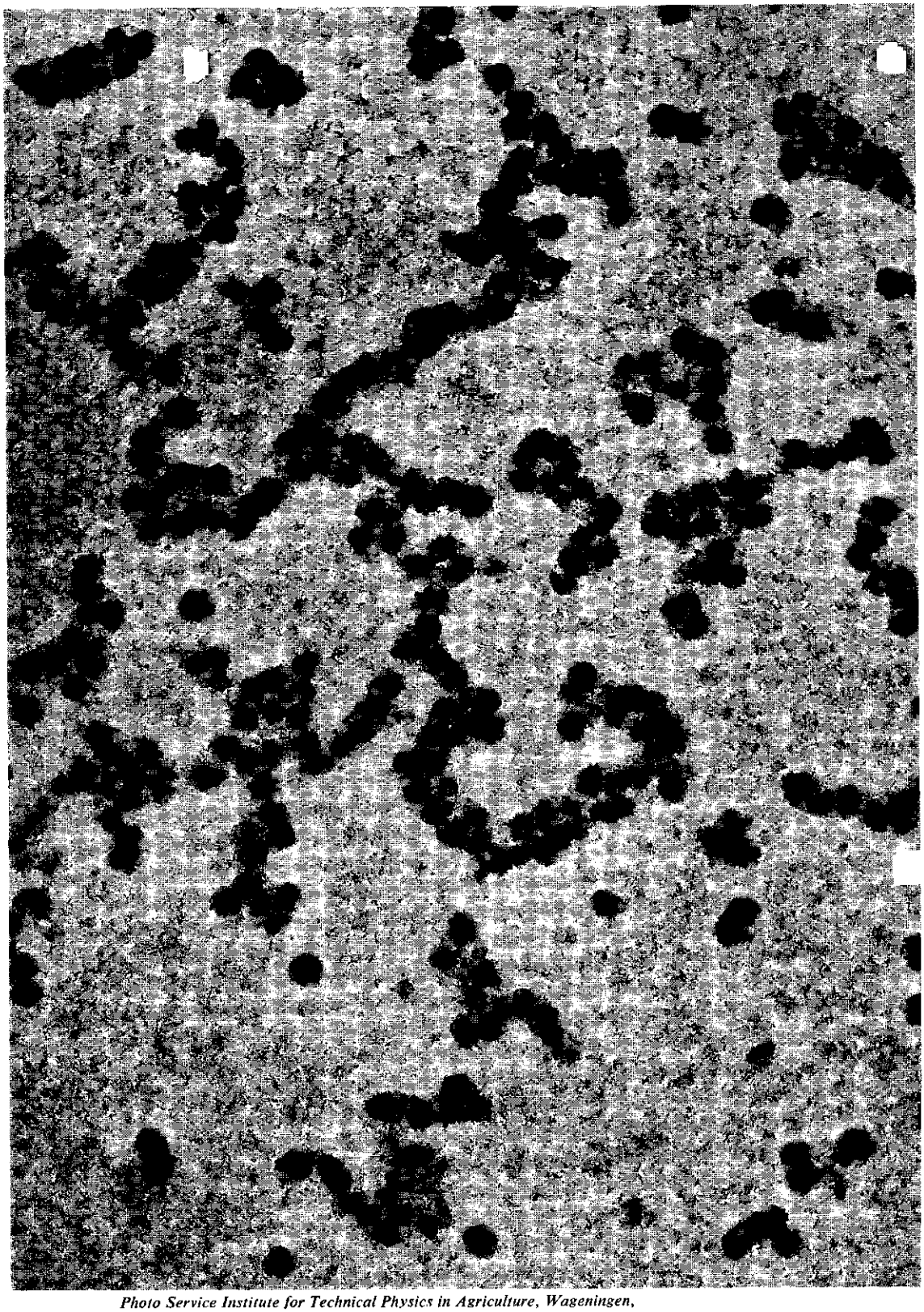

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PHOTO 4. Positively stained ribosomes from healthy leaves, purified by two cycles of high and low speed centrifugation. Magnification 200,000.

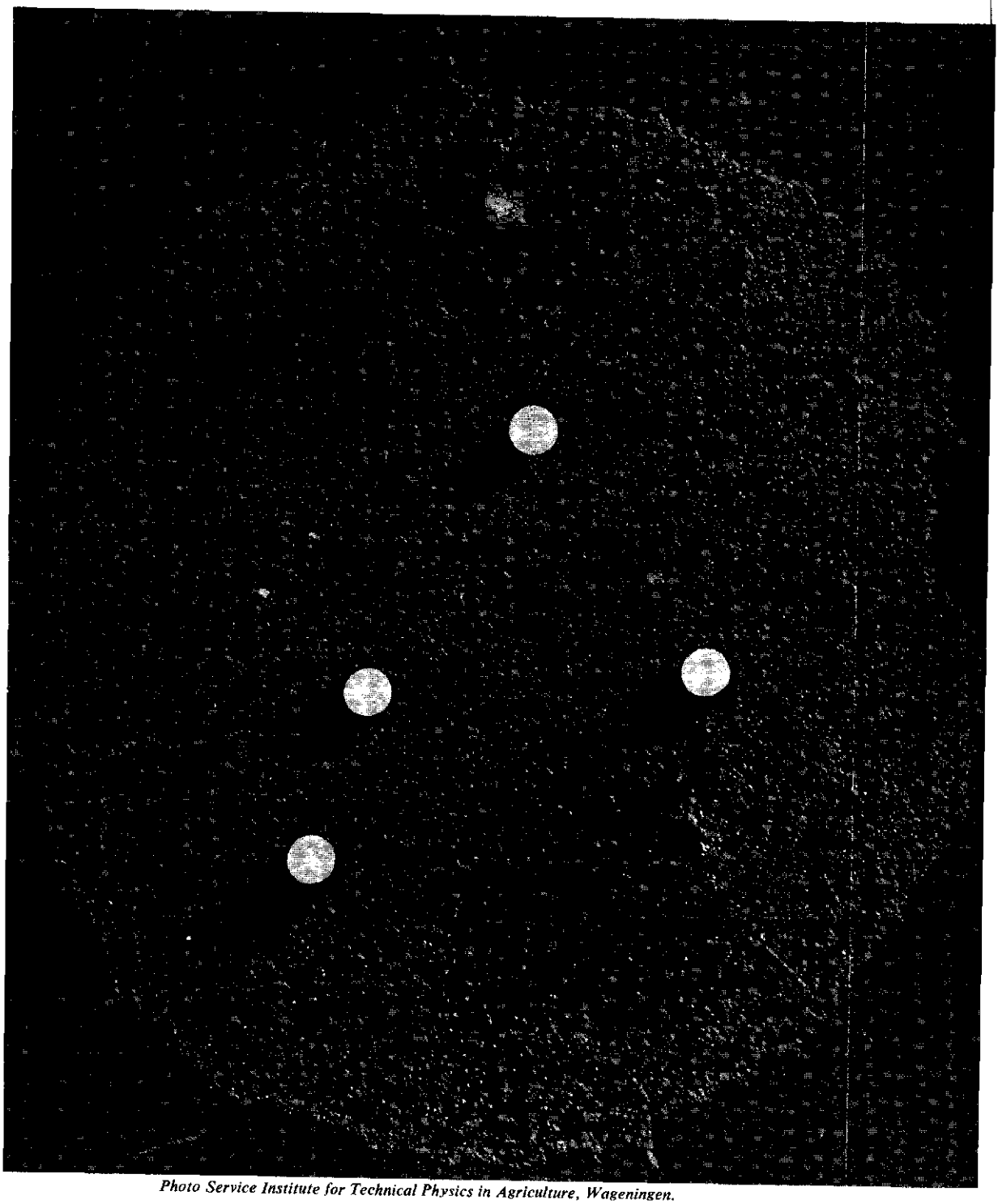

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PHOTO 5. Droplets of an M_2 fraction (page 42) containing about 4–5 μg TMV/mg RNA. A suspension of 333 μg RNA/ml, 0.1 mg polystyrene/ml and 0.03% serum albumin was sprayed onto the formvar film of the grids of the electron microscope. Shadow casting is with palladium. Magnification 24,000.