BIOCHEMICAL STUDIES OF WESTERN RING SPOT VIRUS

by

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A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1961

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Date thesis is presented August 8, 1960

Typed by Mary Adams

ACKNOWLEDGMENT

The author wishes to express her sincere gratitude to Dr. Tsoo E. King for his guidance, encouragement and advice in carrying out this project.

The author also wishes to acknowledge the invaluable help and advice of Dr. Vernon H. Cheldelin and Dr. Frank P. McWhorter.

The author is also under obligation to all the other people of the biochemistry group in the Science Research Institute for their frequent assistance at various times and also for the stimulating atmosphere which they helped to provide.

TO MY PARENTS

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

BIOCHEMICAL STUDIES OF WESTERN RING SPOT VIRUS

I. INTRODUCTION

The first significant distinction between viruses and micro-organisms came from the demonstration by Iwanowsky in 1892. He proved that the sap of mosaic diseased tobacco plants after passing a bacteria-proof filter candle was capable of inducing the disease in healthy tobacco plants. Nevertheless he remained firm in the belief that tobacco mosaic was a bacterial disease (66). Six years later Beijerinck confirmed Iwanowsky's results but drew different conclusions. Beijerinck considered that the cause of tobacco mosaic is fundamentally different from bacteria and proposed his theory of "contagium vivium fluidum" (14). Then in 1906 Baur showed that the so called variegation of Abutilon spp. could be transmitted by grafting and called this phenomenon "infectious variegation" (4). In 1912 Allard transferred tobacco mosaic virus to potato (1), and in 1916 Quanjer, Lek and Oortwijn Botjes discovered the infectious nature of potato leaf roll (98). The latter is especially significant because it disproves the theory of "senile decay". For some years after this discovery much attention was directed to the study of symptoms and new virus diseases. Some interesting viruses were brought to light during this period, among which may be mentioned those of tomato bushy stunt (115) and tobacco necrosis (117, p.231-245). In 1928

Holmes (64, p.67-73) first demonstrated the relationship between the concentration of tobacco mosaic virus and the number of local lesions which developed on the inoculated leaves of Nicotiana glutinosa. Holmes' observation has made possible the quantitative study of plant viruses and allows for comparative estimates of virus concentration. This technique has been extended to a number of other viruses; it is frequently used and is of great significance in plant virus research. In the meantime, Purdy Beal discovered that tobacco mosaic virus was a powerful antigen. She found that the virus, when inoculated into rabbits, gave rise to antibodies specific for that virus (97). This led to the discovery of the relationship between tobacco mosaic virus and cucumber virus 3 and 4 (8).

In 1930 Salman and LePelly (103, p.140-172) demonstrated the first latent plant virus, pancrinkle, in potatoes of King Edward variety. In fact no one has yet seen a King Edward potato plant free of this virus; it is possible that its presence accounts for some characteristics in potatoes. Several latent plant viruses were discovered, among which is the latent virus of sugar beet (116). The study of latent viruses is an important one, since they occur not only in plants but also in many different organisms. In 1929 Smith (114, p.215-224) discovered that the aphid Myzus persicae was the chief vector of potato leaf roll virus. The relationship between plant viruses and insect vectors has been under investigation for a number of years and is still incompletely

understood. Watson and Robert (131, p.543-547) have defined plant viruses in relationship to their insect vectors as "persistent and nonpersistent" according to the time the insects retained infectivity without reaccess to a source of virus. Most of the persistent viruses have leaf-hoppers for vectors. Kunkel (74), Black (16) and Maramorosch (83) in independent studies over a number of years have demonstrated that some of the plant viruses which can be transmitted by leaf-hoppers do multiply inside their insect vectors. This important discovery makes a link between the plant and animal viruses and may even suggest that some of these viruses started as insect viruses and mutated or became attuned to multiplication in plants.

Up to 1935 plant virus research was mainly concentrated on diseases and insect vectors. However since then the focus has been much on the virus itself. This is largely due to the significant discovery of Stanley (118). He isolated a large molecular weight crystalline proteinaceous material possessing the properties of typical mosaic disease from the diseased plants. This made the first correlation of biological properties with a physiochemical entity. Subsequent work showed by Bawden, Pirie, Bernal and Fankuchen (13) that this material is a nucleoprotein. In 1938 Bawden and Pirie purified and crystallized the virus of tomato bushy stunt (9, p.252-255). This was the first virus to be crystallized in the form of true three dimensional crystals, dodechedra, as compared with the paracrystals or liquid crystals

of tobacco mosaic virus (118). The purification of plant viruses together with the development of the electron microscope has enabled us to see the viruses, which were for so long invisible.

All these findings have provided a powerful stimulus to lead in a new era of virus research. However some of the difficulties which slowed the progress of modern virus research remained to plague current efforts. It is still a major problem to purify most viruses and it is an exacting task to correlate virus activity to a specific physiochemical entity. The development of new instruments and the refinement of techniques have aided in these efforts. Nevertheless, it is still significant that after 24 years of extensive studies only 16 plant viruses have been obtained in a highly purified form (121, p.38-70) and only 8 of them have been crystallized, as presented in Table 1.

The problems of isolating plant viruses from diseased plants are numerous and far from completely solved. Plant cells in general consist mainly of a rigid cell wall and a large vacuole filled with cell sap and surrounded by a thin layer of protoplasm in which the cell nucleus and the various plastids are embedded. It is not known with certainty how the virus is distributed in such cells, although it is probable that it develops in the cytoplasmic layer, and it is certain in the case of tobacco mosaic virus that a large proportion of virus particles is eventually deposited inside the vacuole sap in the form of crystalline masses (84, p.36-39). Recently Boardman and Zaitlin (19, 137, p.743-757) showed that tobacco mosaic virus

could be synthesized in chloroplasts.

It is not possible to form any general method for the isolation and purification of plant viruses. However the first requirement for the purification of plant viruses is that there should be a source of material containing sufficient virus to make its isolation possible. Thus the most suitable host plants have to be used, which should be very young, well nourished, growing vigorously, with the absence of inhibitors and freedom from contamination with unwanted viruses. In order to extract virus the cells have to be disrupted. Thus all the various components of the cells are mixed and an unphysiological suspension which is usually called sap is formed. From this mixture the virus is to be isolated.

A preliminary clarification of sap is essential. The general procedures are the coagulation or precipitation of noninfectious components and the removal from the suspension by gravity or centrifugal sedimentation, or filtration. From the clarified sap the virus can be further purified and concentrated by techniques which have been employed in general protein chemistry, such as salting out or chemical precipitation, acid or isoelectric precipitation, differential centrifugation, electrophoresis, chromatography and density gradient centrifugation. Among these the density gradient centrifugation (20, 21) has been proved very useful in the purification of viruses. The principle of the method is very simple. The formation of a density gradient is essential, sucrose and glycerol being commonly used as the solute.

Later Meselson et al. (88) described a modified technique using cesium chloride to form a concentration gradient. Two different techniques, namely equilibrium zonal centrifugation and rate zonal centrifugation have been satisfactorily employed (22).

When a concentrated and purified virus suspension is obtained, there are two questions related to the significance of the work: (1) Is the suspension truly homogeneous? and (2) are the particles really the infectious units? The problem of relating infectivity with the characteristic particles in a plant virus suspension is far more difficult than it is with animal viruses and bacteriophages. This is mainly due to the inadequacy of our assay procedure (119). Recently Schramm and Edger (106) developed a new infectivity assay technique, which consists both of systemic infection and local lesions. This method can give a fairly reasonable relationship between the characteristic particles and the infective units of tobacco mosaic virus. This is a very important finding, but it is still in its early stage and has not been applied for general use yet. Nevertheless because there is such a great difference between the number of characteristic particles present in a plant virus suspension and the number of infections which it can initiate, failure to obtain heterogeniety does not constitute proof that the characteristic particles are the virus. However evidence of heterogeniety in a virus suspension suggests but does not prove, that impurities are present. The virus may be pleomorphic, among which may be mentioned tobacco mosaic

virus (18) and potato X virus (5, p.177, 127); or purified virus may aggregate and/or dissociate during the purification processes and appear to be present in two or more forms.

In spite of the inadequacy of our assay procedure, useful methods are available for associating infectivity with the characteristic particles in the purified virus suspensions. The techniques used for the determination of homogeneity are essentially those physical methods employed in the general protein chemistry, such as ultracentrifugation analysis (121, p.35), electrophoretic mobility (22, 121, p.36), density gradient centrifugation (20, 21, p.279-289, 24, 88) and chromatography (109, 110, p.421-432). In addition, electron microscopy (134, p.203-213) has been considered the most convincing method to detect impurities, since we can examine the specimen directly under the electron microscope. Impurities in an amount of a few percent of the total solid fraction can be detected with ease unless they are morphologically indistinguishable from the virus particles.

Some of the plant viruses have now been crystallized (Table 1) or at least been shown to take up regular three dimensional arrays. This enables us to give a deeper understanding of virus structure, since it allows application of the method of X-ray crystal analysis. As the method of determining radial density distribution became refined, and protein labeled with heavy metal (Hg, Pb) became available, X-ray studies led to a rather specific and well supported model for the tobacco mosaic virus particles. This

important advance resulted from the work of Watson (130), Caspar (26) and others, but it is most prominently due to Franklin (46, 47, 48, 49) and her colleagues (50, p.447-461, 51, 52, 54, 65). A three dimensional model for the rod of tobacco mosaic virus was constructed, in which the rod is believed to be hollow, i.e. filled with solvent along its axis (40 Å diameter), and surrounded by protein building blocks arranged in a gently pitched helical array (49 units in 3 turns, 69 Å). The nucleic acid is believed to be threaded through in the form of a single helix, of the same pitch with a diameter of 80 Å and externally covered about 40 Å of protein. The latter however is not regarded as solid wall but a deeply grooved surface (47, 50, p.449-450, 58, 108). The helical structure of tobacco mosaic virus had also been confirmed by electron microscopic studies (94, 108, 135, p.212-213, 136, p.27-32). Not very much work has been done on the other plant viruses, Crick and Watson (33) have discussed the theoretical aspects of symmetry in the spherical plant viruses such as tomato bushy stunt and turnip yellow mosaic virus. Furthermore Caspar's X-ray diffraction studies have indicated 2-fold and 3-fold symmetry axes in bushy stunt virus (25). Recently by X-ray studies Franklin and Klug demonstrated that an icosahedral symmetry occurred in turnip yellow mosaic virus (53).

The physical study of purified virus has necessarily been associated with a chemical approach at whatever level was available at the time. Up to the present time the plant viruses that have

been isolated and analyzed have been proved to be ribonucleoproteins. These viruses, however, comprise only a few of the most stable plant viruses. The remarkable and important finding of Fraenkel-Conrat (37) and Gierer and Schramm (56, 57) of infectious nucleic acid isolated from tobacco mosaic virus nucleoprotein, followed by the extensive studies of the infectious nucleic acid (29, 39, 60, 107) and the reconstitution of tobacco mosaic virus indicated that nucleic acids are the essential elements in the sense of being the carrier of the genetic codes by which the new virus is constructed (30, 38, 42, 43, p.365-367, 45, 77). Several methods for the isolation of nucleic acid from the intact virus have been developed (28, 41, 56, 71, 80, 132). The methods by which the proximate constituents--purines, pyrimidines (15, 32, 113) and sugar (82, 89, 105)--can be identified and quantitated also have been well established.

The substantial part of the amino acids comprising the protein subunit of tobacco mosaic virus has already been worked out (17, 42, 69, 99) and comparisons with the amino acid composition of some tobacco mosaic strains have revealed significant difference (17, 69). Eight plant viruses have been analyzed for their nucleic acid composition as shown in Table 2. The base pattern is generally different, i.e. species-specific. Different strains of the same species have apparently the same base composition. This is in contrast with the amino acid composition. These discoveries stimulate another avenue of modern virus research, to establish the sequence of bases in the polynucleotide. However in the first

place we have to determine the form of the nucleic acid in the virus, i.e., whether the nucleic acid is in a single molecular structure of essentially unique character or whether it has a series of two or more types of molecular structures which are presented in certain constant or variable numbers. In other words, it is necessary to determine whether the nucleic acid isolated from the intact virus is essentially the same as it is present in the infective particle. Once we obtain the natural molecular species it will be certainly possible to establish the general pattern of structure. In the case of tobacco mosaic virus, the preliminary evidence favors a single polynucleotide chain (41, 50, p.453-454, 55, 58, 62) following the helical pattern of the protein subunits. Recently Reddi demonstrated that there is a significant difference in the structure of the nucleic acids of tobacco mosaic virus strains (100, 101, 102).

The current hypothesis indicates that RNA[1] must carry the

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[1] The following abbreviations will be used:

RNA	ribonucleic acid
WRSV	western ring spot virus
TRV	tobacco ringspot virus
BYMV	bean yellow mosaic virus
Tris	Tris(hydroxymethyl)-aminomethane
EDTA	ethylenediaminetetracetic acid tetrasodium salt
M	molar; moles per liter; or one molar
N	normal; equivalents per liter; or one normal
cm.	centimeter
mm.	millimeter
μ	micron
mμ	millimicron
Å	Angstrom = 10^{-8} cm.
g.	gravity, i.e., in: 8,000 x g., it refers to 8,000 times force of gravity
lbs.	pounds

genetic information, or in other words, the RNA can induce the formation of its own kind and can in addition induce the formation of a highly specific protein with which the RNA eventually combines. As the problem of polynucleotide sequence is solved, perhaps the interrelationship between the polynucleotide and the polypeptide could be easily attacked. Hart and Smith (63) have tried to combine semisynthetic polynucleotides with tobacco mosaic virus-protein. However the product is far less stable than the structure formed with the homologus protein and nucleic acid. This finding implied that the biological action of a nucleic acid is dependent upon the exact arrangement of its nucleotide residues. This is in line with Reddi's findings that the base structures are different in different strains of tobacco mosaic virus (100, 101, 102). In view of the great diveristy in the length and composition of the polymers tested in Hart and Smith's experiment, they concluded that the arrangement of the nucleotide residues has little to do with the ability of a nucleic acid to form stable organized complexes with protein; or in other words that any ribonucleic acid (not mononucleotide) would interact with tobacco mosaic virus protein in the same manner.

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[1]

mg.	milligram
% w/v	i.e., 30%(w/v) of ammonium sulfate, means that 30 grams of ammonium sulfate were added to 100 ml. of solution
% v/v	i.e., 25%(v/v) of 90% ethanol, means that 25 ml. of 90% ethanol was added to 100 ml. of solution
cfp	refers to the purity of the reagent; it is the best grade manufactured by California Corporation for Biochemical Research

The character of the infectious particles represented only a part of the significant information to be gained from the laboratory aspects of virology. The interaction of the virus particles and the host cell is the most important aspect as far as the true expression of the viral activity is concerned. Information in this field is more difficult to observe and interpret than the physical and chemical properties of the virus particles. The knowledge about the mechanism of virus self-replication and the interaction with plant cells is still insufficient, since it has not been possible to separate plant cells in a way that would permit investigation of virus activity within single cells as in the case of bacterophages.

The appearance of abnormal proteins in the infected plants has been demonstrated by several workers (12, 31, 67, 123, 124, 125, 126). Furthermore it has been shown that the tobacco mosaic virus associated protein has a structure similar to tobacco mosaic virus (50, p.453), the same fourteen amino acids are in approximately the same percentage as in tobacco mosaic virus (92). Several kinds of experimental techniques which have been conducted by different groups of workers to search for the correlation between the abnormal proteins and the tobacco mosaic virus have been reviewed by Porter (95, p.76-79). Conflicting results have been reported, however Van Rysselberge and Jeener's tracer work (128, 129) does suggest strongly that the soluble antigen, and/or the abnormal proteins are the direct precusors of the virus. Recently Takahashi (123, p.497-499) found that X-protein reconstitutes fully infectious

virus if permitted to aggregate in the presence of native virus RNA. Thus Takahashi concluded that X-protein is the surplus viral protein rather than a precusor, a degradation product, or a synthesized material rejected during assembly of the virus.

There is good evidence to support the idea that the intact tobacco mosaic virus particle is disrupted just before or early in the process of initial infection. The probability that the particles are degraded to their protein and nucleic acid moieties came from two different types of experimentations. Hamers-Casteman and Jeener (61) found that there is an initial phase in infection, of about two hours duration, during which tobacco mosaic virus multiplication can be inhibited in tobacco leaves by infiltrated ribonuclease. This sensitivity seems to necessitate the separation of virus protein and nucleic acid, since it has been shown that RNA is not susceptible to the action of this enzyme when it is combined with the protein moiety. A similar conclusion regarding disruption of virus particle was reached by Siegel (111, 112) using the technique of irradiating infective centers with ultraviolet at various times after initiation of infection. The intact virus showed a lag period for resistance to ultraviolet light, but little or no lag period for the isolated nucleic acid. This was confirmed by Fraenkel-Conrat et al. (44) and Schramm and Enger (106). Thus it was concluded that when infection is initiated with an intact virus particle, the nucleic acid is freed from the protein soon after the establishment of infection and prior to virus replication.

The biochemical study of plant viruses is still very largely in the early stage. Most of the results have emerged from the researches of a single plant virus, tobacco mosaic virus. It is unlikely that all the plant viruses will behave in the same manner as tobacco mosaic virus. As a matter of fact it has been shown that the base pattern of nucleic acids is significantly different. This might reflect some of the biological properties of the individual virus. Thus biochemical studies of more plant viruses are highly desirable.

The present thesis will report the biochemical behavior of a new plant virus, western ring spot virus (WRSV).

Western ring spot virus was first discovered by McWhorter from wilted pea plants found near Weston, Oregon in 1950. The name, western ring spot virus has been proposed by McWhorter (81). WRSV has been found by extensive test plant studies to occupy a position intermediate between the classical bean yellow mosaic virus and the classical tobacco ringspot virus. It seems somewhat more closely related to the bean yellow mosaic than to the ringspot virus.

Western ring spot virus occurs naturally in potatoes and legume crops in many areas of the Pacific Northwest. It has always been isolated from plants where it was present in combination with other viruses, such as with enation mosaic in peas, with bean yellow mosaic in beans, with alfalfa mosaic in potatoes. In legume crops WRSV behaves somewhat like an activator, its

presence being evidenced by far greater injury to the plants than the injury usually created by other viruses which may be present. In the field in combination with other viruses, WRSV may be transmitted by pea aphids, however in a pure form it is rarely aphid transmittable (2, p.27-29).

In 1955 Kuehl (72, p.1-30) made the first biochemical investigations on this virus. A method for the partial purification of the virus by use of alcohol precipitation, ammonium sulfate fractionation, and differential centrifugation was developed. However the final preparation was purified only about two fold with less than 10% recovery and was unstable. It lost its infectivity completely within 30 hours. Thus further studies along this line are highly desirable.

The work presented in this thesis consists of two main parts: (1) a purification procedure in a pattern similar to Kuehl's method but with many more precautions in operations, and (2) some physical and chemical properties. The modified method can give about 100 to 200 fold of purification with 10 to 20% recovery. Crystallization of the virus from the purified preparations was successful. The viral nucleic acid was isolated. However the protein moiety of WRSV has not been isolated in a satisfactory state by any of the known and modified techniques.

II. MATERIALS AND METHODS

1. Cultivation of western ring spot virus (WRSV)

A pure culture of western ring spot virus (WRSV) was supplied by Dr. Frank P. McWhorter, Professor of Plant Pathology in this college. Plants of Bountiful bush bean (Phaseolus Vulgaris L.) were used as hosts for multiplication of WRSV. All plants were grown under greenhouse conditions with natural illumination. Plants were inoculated twelve to sixteen days after planting. At that time primary leaves were about two inches long but the first trifoliate leaves had not yet appeared. Prior to inoculation leaves were lightly dusted with 400 grit carborundum. The virus in buffer solution was applied by rubbing across the primary leaves with fingers. The inoculated leaves were then rinsed well with tap water for removal of the carborundum and buffer salts.

2. The assay for infectivity

Up to the present time, no host has been found which may produce consistently local lesions from WRSV suitable for quantitative titration. The method used throughout this study was the systemic infection method (119, p.198-200). The assay depends on the fraction of plants becoming systemically infected after manual inoculation with serial dilutions of the solution to be tested. The initial visible symptom was curling of the first trifoliate leaflets. These leaves became subsequently distorted and discolored. About a month after inoculation the primary leaves began to show discoloration and necrosis. The specific

infectivity is arbitrarily defined as follows:

Specific infectivity =

$$\frac{\text{number of plants becoming infected}}{\text{number of plants inoculated}} \text{ (per mg. of protein)}$$

The maximum specific infectivity as described in the tables refers to the above ratio by using the maximum number of plants infected divided by the total number of plants inoculated.

3. Protein determination

Two methods were employed in the present studies.

A. Weichselbaum's method (133): Samples containing 1 to 20 mg. of protein were precipitated with an equal volume of 9% trichloroacetic acid. The precipitate collected from centrifugation was then dissolved in a suitable amount of 0.1 N NaOH solution. Subsequent steps were the same as described by Weichselbaum (133). Blanks were used for colored samples.

B. Turbidimetric method: Samples containing less than 1 mg. of protein were determined by the turbidimetric method of Kunitz (73, p.434). To 2 ml. of sample solution was added 3 ml. of 10% trichloroacetic acid. The reaction mixture was shaken constantly for 20 seconds and then read immediately at 550 mμ. It should be understood that nucleic acid was also precipitated by trichloroacetic acid.

In both cases crystalline bovine serum albumin served as the standard. Crystalline bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, Missouri.

4. Phosphorus determination

Total organic phosphorus was determined by the modified method (3) of Fiske and SubbaRow (36, p.397-398).

5. Ribose determination

Ribose was determined by the modified orcinol method (105), and desoxyribose was determined by the diphenylamine method (105). Both orcinol and diphenylamine were recrystallized twice from benzene and n-hexane respectively (105). D-ribose (A grade) and desoxyribose (A grade) were used as standard. They were purchased from California Corporation for Biochemical Research, Los Angeles 63, California.

6. Electron microscopy

A. Preparation of collodion film on stainless steel grids: After numerous trials the following procedure was developed and found to be very satisfactory for making films in the electron microscopy. The grids were first supported on an aluminum supporter by the aid of scotch tape. The supporter was then placed in a glass jar (diameter: 10 inches) filled with sufficient water to make the water level about 1 to 2 inches above the grids. Two drops of 25% collodion solution were then added on the water surface. It was allowed to stand for exactly 60 seconds; during this period a strong thin collodion film was formed. The supporter was then removed very gently from the glass jar and a smooth film was subsequently covered on the grids. It was left at room temperature for about 15 minutes and then stored in a

vacuum desiccator for at least 6 hours before use.

B. Preparation of specimens: Purified virus preparations were sprayed on collodion film supported by electron microscope grids and dried in air or by freezing. After being shadowed with chromium or palladium, they were viewed in an RCA-EMU-2D electron microscope fitted with 10 mil condenser aperture, externally centrable 1 mil objective aperture and electrostatic compensator.

The apparatus for making the specimens was originally designed in the Bacteriology department, Oregon State College, and a slight modification was made as shown in Figure 2. It consists of 3 main parts: (I) a bottle with 2 side arms, (II) a specimen holder, and (III) a modified atomizer. The side arm B was specially designed for adjusting the level of the cooling mixture in the bottle as used in the freezing drying technique. Side arm B can also be used for adjusting the pressure inside of the bottle.

The grids with collodion film were put into the blocks of the specimen holder. The specimen holder was then inserted into side arm A of the bottle. 0.1 ml. of sample was pipetted through tube C of the atomizer. Tube C was then connected with other parts of the atomizer by a rubber tubing as shown in Figure 2. The atomizer with the bottle was then assembled and the side arm B was closed with a rubber stopper or cork. The rubber bulb was squeezed once or twice. Then the system was allowed to equilibrate for one minute. The specimen holder with specimens was taken out and kept under vacuum overnight. The specimens were then shadowed

with palladium or chromium at an angle of 25 degrees and viewed under the electron microscope.

Electron microscope screen desks (or grids) were purchased from Ernest F. Fullam, Inc., P.O. Box 444, Schenectady 1, New York. Twenty-five percent collodion solution was made from commercial collodion and reagent grade amyl acetate.

7. Paper chromatography of WRSV-nucleic acid

A. Method of hydrolysis: Several methods of hydrolysis have been reported (87, 113). N HCl at 100°C for 1 hour was used in the present experiment. Sixteen mg. of lyophilyzed virus was placed in a conic centrifuge tube and 0.3 ml. of N HCl was added. The tube was then stoppered with a serum bottle cap and placed in a boiling water bath for 1 hour. Most of the protein became insoluble after this treatment and was separated by centrifugation. The supernatant fraction was used for paper chromatography immediately. Under these conditions only adenine and guanine were liberated into the form of the free base. Cytidylic acid and uridylic acid remained combined as nucleotides and only a small amount of uridine and cytidine were formed (87).

B. Paper chromatography and spectrophotometry: Nucleosides and nucleotides were determined by paper chromatographic and spectrophotometric techniques (15). The hydrolysates obtained were applied to filter paper strips (20 x 46 cm.). A synthetic mixture of authentic compounds which consisted of adenine, guanine, cytidine, uridine, cytidylic acid and uridylic acid and a N HCl

blank were also applied on the same paper strip. The paper strips were then developed in an isopropyl alcohol-HCl solvent system by the descending technique at 20°C. After 26 hours the paper strips were hung upside down to dry overnight in the hood.

The separated bases were located on the paper chromatogram by their absorption of ultraviolet light. Guanine gave a blue fluorescent spot. A mineralight lamp served as a light source. The spots on the chromatogram and blanks from the corresponding place were cut out and each was extracted with constant shaking for two hours at 25°C with 5.0 ml. of N HCl in a stoppered flask. The extracts were decanted from the paper and centrifuged to remove shreds. The optical density of the eluates were recorded in a Cary Recording spectrophotometer with the appropriate extract of a paper blank to adjust the instrument to zero.

Uracil (A grade), cytidine(hemi)-sulfate(cfp), uridine(cfp), adenine(cfp), uridylic acid(cfp) and cytidylic acid (Lot No. 4802) were used as standard for comparison. All of them except cytidylic acid were purchased from California Corporation for Biochemical Research, Los Angeles 63, California. Cytidylic acid was obtained from Schwarz Laboratories, Inc., New York 17, New York.

Whatman No. 52 filter paper was used without any treatment for chromatography. The isopropyl alcohol-HCl solvent system (8.75 M isopropyl alcohol, 2 N HCl) was used for development of chromatograms. Isopropyl alcohol was fractionally distilled and the fraction boiling at 82°C/760 mm. was collected and stored in

the dark.

All glassware used for the assay of these purine and pyrimidine bases was cleaned with ethanol, nitric acid, and then tap water, distilled water and finally redistilled water.

8. Amino acid composition of WRSV-protein

A. Method of hydrolysis: Samples containing 2 to 6 mg. of protein were hydrolyzed at 105°C under 15 lbs. pressure with 20% of HCl for 10 to 14 hours in sealed pyrex test tubes. Excess HCl was removed by repeated evaporation in vacuo and subsequent dilution with water. The solution was then filtered through a sintered glass funnel. The samples were finally made up to volume.

B. Column chromatography: The amino acid composition of the acid hydrolysate of WRSV-protein was determined by Moore and Stein's ion exchanger technique (90). Two sizes of columns (0.9 x 150 cm., and 0.9 x 15 cm.) filled with amberlite IR-120 (particle size: 56 μ. in diameter) were used.

(a) Neutral and acidic amino acid: The column used for the analysis of these amino acids was 0.9 x 150 cm. long. 0.2 N sodium citrate buffer, pH 3.27, and subsequently 0.2 N sodium citrate buffer, pH 4.25, were used as the eluting agent. The change of buffer from pH 3.27 to pH 4.25 for the elution was made when the eluent volume was 2.15 times that at which the aspartic acid peak had emerged. In the present experiment, the aspartic acid peak always appeared between fractions number 57 and 59.

Each fraction was 2 ml.

The column was first equilibrated with 0.2 N sodium citrate buffer, pH 3.27 at 50°C for about 5 hours under 6 lbs. pressure. 0.5 ml. of acid hydrolysate equivalent to about 1.5 mg. of protein was fed onto the column under gravity; then washed with 0.2 N sodium citrate buffer, pH 2.20. The column was then eluted with 0.2 N citrate buffer, pH 3.27 under 6.0 lbs. pressure with a flow rate of about 10 minutes per fraction. The 0.2 N sodium citrate buffer, pH 4.25 was introduced at the position as described in the preceding paragraph. The elution was completed in about 40 hours.

(b) Basic amino acids: The 0.9 x 15 cm. column was used and the amino acids were eluted with 0.35 N sodium citrate buffer, pH 5.27. The same technique was used as that for the neutral and acidic amino acids, except that the flow rate was adjusted to 5 minutes per fraction of 2 ml. with 5.0 lbs. pressure. The complete elution took about 6 hours.

Both columns were first standardized by a known mixture of 18 amino acids and ammonium chloride. The resolution of peaks was satisfactory. Fractions were analyzed by the modified photometric ninhydrin method of Moore and Stein in a 4 N acetate buffer, pH 5.5 (91). The color was read at 570 mμ. in general and 440 mμ. for proline, in a Beckman spectrophotometer model-B. The Integration of each amino acid peak was performed by simple addition of color yields after subtraction of the base line color. A leucine

solution containing 0.12 μmole/ml. was used as the standard. The color yields reported by Moore and Stein (91) were used.

Ninhydrin (reagent grade) and hydrindantin (anhydrous) were obtained from Pierce Chemical Co., Rockford, Illinois.

Methyl cellosolve used for the ninhydrin color reagent was fractionally distilled and the fraction boiling at 122°C/760 mm. was collected.

A Cambridge pH meter was used for adjusting the pH of buffers used.

9. Isolation of WRSV nucleic acid

The WRSV nucleic acid was isolated by the use of the modified detergent method as described by Knight (71). To 10 ml. of virus preparation (about 2 to 3 mg. of protein per ml.) were added 4.0 ml. of 5% duponol-C aqueous solution. The reaction mixture was adjusted to pH 8.6 with 0.1 N HCl and 0.1 N NaOH respectively, and then incubated at 40°C for 15 to 18 hours. Following this treatment, 4.2 g. of ammonium sulfate (30% w/v) was added and the precipitate was centrifuged out. The liquid layer was pipetted out with a long needle syringe and stored in the refrigerator overnight. During that period, most of the nucleic acid precipitated out and was collected by centrifugation at 12,000 x g. for 30 minutes. The nucleic acid thus obtained was further purified by dissolving in 5 ml. of ice cold water and precipitated with 10 ml. of cold ethanol. This process was repeated once more. A few drops of 3 M acetate buffer, pH 4.7 was added to complete the

precipitation. The intact virus was further separated by centrifugation at 80,000 x g. for 1 hour. The nucleic acid remained in the supernatant layer.

Commercial Duponol-C was recrystallized twice from n-hexane. Trypsin (2 x crystalline) was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio. Ribonuclease (5 x cryst.) was purchased from Sigma Chemical Co., St. Louis, Missouri.

All other reagents used throughout the present studies were reagent grade. Redistilled water was used except where otherwise indicated.

III. RESULTS

1. Properties of the sap from infected plants

Sap of infected plants was used as the starting material for all experiments. Infected plants were ground in a commercial electric meat grinder. The sap was first strained through cheese cloth and then centrifuged at 7,000 x g. for 10 minutes to remove starch granules and other debris. Both the yield of the sap and the protein content were dependent upon the season. About 5 ml. of sap were obtained per plant with 10 to 20 mg. protein per ml. in summer and 1 to 2.5 ml. of sap per plant with 20 to 28 mg. protein during the rest of the year.

Table 3 indicates that the virus occurred in the leaves and stems as well as in the petioles of the plants. It can be seen that the specific infectivity is higher in trifoliate leaves, stems and petioles than in the primary leaves. The roots were not studied. Thus all experimental work described was done by using sap from the whole infected plant except the roots.

A. Dilution end point: Positive infections were obtained fairly regularly at dilution of 10^{-2} or in the order of one-tenth mg. of protein and not infrequently at dilution of 10^{-3}. There seems little doubt that the virus content of the sap is variable and depends upon cultural conditions of the plants.

B. Acidity: The acidity of the sap was found rather constant, namely pH 5.7 to 5.8. It did not vary with its protein content or virus concentration.

C. Thermal inactivation point: Ten ml. of the crude sap was incubated at the proper temperature for 10 minutes, then cooled in an ice bath and its infectivity was measured immediately. The virus in crude saps was inactivated completely after heating for 10 minutes at 55°C as shown in Table 4. Under such conditions 50% of the protein precipitated out.

D. Effect of storage: WRSV in crude saps was almost completely inactivated on standing for three days at 5°C as shown in Table 5. The inactivation might be caused by some proteolytic enzymes or other agents present in the sap. On the other hand, the whole plant exclusive of the roots could be stored at the same temperature without significant loss in infectivity as shown in Table 6. Likewise purified preparations were relatively stable. The reason for the unmistakable increase of virus infectivity after storage of 45 to 56 hours was not explained.

2. Purification

During the present study, a method for the partial purification of western ring spot virus was developed. It consisted of precipitation of the virus with ethanol, extraction with acetate buffer, fractionation with ammonium sulfate, and differential centrifugation.

Crude sap from infected plants which had been kept at 5°C for 45 to 50 hours was used as the starting material. All the subsequent operations were carried out at 0 to 5°C except as otherwise indicated. Two hundred and fifty ml. of 90% ethanol

(by weight) were added to 1,000 ml. of sap over a period of 30 minutes with rapid stirring. After standing for 10 minutes the suspension was centrifuged for 10 minutes at 10,000 x g.. The supernatant liquid (S-1) was discarded. The precipitate (P-1) was extracted with 300 ml. of 0.1 M acetate buffer, pH 5.5. The mixture was centrifuged 20 minutes at 12,000 x g.. The extraction was repeated once more with 200 ml. of buffer. The supernatant fractions (S-2) from the extraction were combined and the residue (P-2) was discarded.

One hundred and fifty grams of ammonium sulfate were added to 500 ml. of supernatant liquid (S-2) in a 20 minute period. The mixture was left in the ice bath for an additional 20 minutes. The precipitate (P-3) thus formed was collected by centrifugation at 6,000 x g. for 5 minutes and resuspended in 150 ml. of 0.1 M phosphate buffer, pH 6.0. The supernatant liquid (S-3) was discarded. The suspension was first centrifuged for 20 minutes at 10,000 x g. and the precipitate (P-4) was discarded. The supernatant liquid (S-4) was further centrifuged for 60 minutes at 80,000 x g. in a Spino model L centrifuge equipped with rotor No. 30. The supernatant liquid (S-5) was discarded, and the glassy, green-brown pellet (P-5) was suspended in 15 ml. of 0.1 M phosphate buffer, pH 6.0. The suspension was again centrifuged for 10 minutes at 9,000 x g.. The residue was extracted twice with 10 ml. each of the phosphate buffer. The supernatant fractions (S-6) were combined. The fraction (S-6) was further

centrifuged for 60 minutes at 80,000 x g.. The supernatant liquid (S-7) was discarded and the light brown glassy pellet (P-7) was suspended in 10 ml. of 0.1 M Tris buffer, pH 8.0. The suspension was finally centrifuged for 10 minutes at 7,000 x g. to remove any heavy particles. The final extract was dialyzed against water for 40 hours at 4°C. The scheme for the purification is summarized in Figure 1.

The virus infectivity and protein content of each fraction from a typical experiment are presented in Table 7.

Solutions of virus prepared by the method described were opalescent and pale yellow-green in color. The yields of virus were somewhat variable. About 10 to 20% recovery in the final preparation was obtained as determined by the infectivity measurement (Table 8).

3. Properties of the purified western ring spot virus

A. Crystallization: When the purified virus preparations were stored at 5°C in aqueous solution small crystals gradually deposited (Figure 3). These crystals usually were needle shaped but some appeared to be derived from dodecahedra. The crystals had the same type of ultraviolet absorption spectrum as that of the fresh preparation both being typical of nucleoprotein (Figure 4). These crystals collected by centrifugation were suspended in 0.1 M Tris buffer, pH 8.0 and tested for infectivity by the local lesion technique, horse bean plants serving as the local lesion host. It was found that the crystals could initiate local

lesions as did the fresh preparation. However, in the crystalline state the virus is more labile. It could be inactivated by washing with glass redistilled water.

B. Ultraviolet absorption spectrum and nucleic acid content: The ultraviolet absorption spectrum of the purified WRSV was observed in a Cary Recording Spectrophotometer, Model 11. It was typical of nucleoprotein with a minimum at 245 mμ. and a maximum at 259 mμ., as shown in Figure 4.

The purified virus preparation was a ribonucleoprotein, since the preparation showed a positive reaction with orcinol but a negative reaction with diphenylamine. Thus the virus most probably does not contain desoxyribose.

The western ring spot virus isolated contains about 37% of nucleic acid by weight calculated from the phosphorus content and 33 to 40% of nucleic acid from ribose analysis. Under the experimental conditions only the purine bonded ribose was analyzed (82), thus a conversion factor was used which will be discussed later.

C. Dilution end point: Positive infections were obtained fairly regularily at dilution of 10^{-3} or in the order of 1 microgram of protein per ml. for the purified virus preparations. No inactivation was observed at such dilutions even in the absence of a protective colloid.

D. Thermal inactivation point: Western ring spot virus in the purified preparations (0.2 mg./ml.) was completely inactivated

after heating for 10 minutes at 55°C with or without the presence of 1 mg./ml. bovine serum albumin as shown in Table 9.

E. pH effect on stability: It was found that WRSV in the purified preparations was stable between pH 6.0 to 8.0 for 2 hours at 5°C. However, the infectivity was decreased by increasing the acidity, as shown in Table 10.

F. Trypsin digestion: One ml. of purified virus (about 1.5 mg./ml.), either in aqueous solution or in 0.1 M Tris buffer, was incubated with 2 mg. of trypsin at pH 7.0 for 24 hours at 4°C and 37°C respectively. The incubation mixtures were then separated by centrifugation. The precipitates were suspended into 10 ml. of 0.1 M Tris buffer, pH 8.0. Corresponding dilutions were also made for the supernatant fractions. The results of the infectivity assay are presented in Table 11. It appeared that WRSV was completely inactivated by trypsin digestion and that this inactivation was irreversible.

G. Electron microscopy: Western ring spot virus appeared to consist of both rods and spherical particles under the electron microscope as shown in Figures 5 and 6. However the relative distribution of rods and spherical particles was apparently dependent upon the pH and/or the nature of the buffer medium used for the last step of purification. If pH 8.0, 0.1 M Tris buffer was used, particles in the rod form predominated whereas in acidic solutions such as pH 6.0, 0.1 M phosphate buffer practically only those in the spherical form were evident (Figure 7). However

at a given pH value (such as 8.0) but in Tris buffer, the ratio of rod to spherical particle is much higher than in phosphate buffer. From electron micrographs (Figures 5 and 6), it can be seen that the rods are actually in the form of a necklace instead of a smooth cylindrical rod. The diameter of the rods and the spherical particles is about the same, about 25 mμ..

H. Relationship between the characteristics of the particles and infectivity: Purified preparations appeared to consist of both rods and spherical particles as described in the preceding section. Experimental results showed that both rods and spherical particles are infectious or correlated with infection. When healthy bountiful bush bean plants were processed in the same way as infected tissue, a barely detectable pellet was obtained after high speed centrifugation. The preparation was examined under the electron microscope in the same manner as the virus preparation. No rods or particles similar to those in the virus preparation were found in the healthy plant preparations.

It was found that the virus preparations containing more rods possessed higher stability than that with spheres in aqueous solution. Preparations containing mainly spherical particles are practically completely inactivated after 5 days storage at 5°C; but on the other hand, preparations with rod particles as the main component can stand for 14 days at 5°C, retaining about 50% infectivity.

Magnesium ion appeared to enhance the virus infectivity.

8.0 ml. of virus preparation (0.1 M, pH 8.0 Tris buffer) was dialyzed against 0.1 M Tris buffer, pH 8.0 containing 0.005 M EDTA for 36 hours. The dialyzed solution was centrifuged at 7,000 x g. for 10 minutes to remove the small amount of insoluble material. The supernatant liquid was then divided into 2 equal portions. One was dialyzed against water overnight (E-H), and the other was dialyzed against 0.008 M magnesium sulfate solution overnight (E-Mg.).

Both samples were examined under the electron microscope; it was found that (E-Mg.) contains more rods than (E-H). This is in line with the fact that the infectivity of (E-Mg.) was about double that of (E-H) (Table 12).

4. Isolation of WRSV nucleic acid

The WRSV nucleic acid isolated by the modified detergent method (71) has the absorption spectrum shown in Figure 8 with a very symmetrical peak over 257 mμ. with E maximum/E minimum = 1.83, E 257/E 280 = 1.96, and $E^{1\%(P)}_{257m\mu.} = 2.3 \times 10^3$. Here E is the absorbance (log. I_o/I). $E(P)_{257m\mu.}$ was also calculated to be 7160; here E(P) = 30.98 E/cl. where E: extinction, c: concentration of phosphorus in the solution in g./liter, and l: thickness of the absorbing layer in cm. (27).

The nucleic acid preparation gave a negative biuret reaction. The phosphorus analysis indicated that 0.314 mg. of ribonucleic acid had been isolated from 20 mg. of WRSV. It corresponds to 4% yield based on the protein to nucleic acid ratio of 100:37.

5. Purine and pyrimidine composition of WRSV-nucleic acid

The composition of nucleic acid in WRSV was analyzed by methods of Bendick (15), and Markham and Smith (87, 113) with slight modification.

The results are presented in Table 13. It can be seen that WRSV-nucleic acid contains a large proportion of guanine and cytosine and has a purine/pyrimidine ratio of 0.933. The absolute amount of each individual nucleoside or nucleotide was calculated by comparing the absorbance and the appropriate peak region of each individual substance with a known concentration of standard sample. The resolution of these nucleotides and nucleosides under the experimental conditions was quite satisfactory.

6. Amino acid composition of WRSV-protein

The amino acid composition of WRSV-protein was analyzed by Moore and Stein's ion exchanger columns (90). The results are presented in Table 14. It was found that WRSV-protein contains 16 amino acids, which are aspartic acid, serine, threonine, glutamic acid, proline, glycine, alanine, cystine, valine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine; and a high ammonia content in the acid hydrolysate. It appeared that WRSV-protein is a basic protein. This is in line with the fact that WRSV is more stable in the neutral or basic solution.

The necessary separate determinations of tryptophan and cysteine have not been possible with the amount of material

available.

7. Isolation of WRSV-protein:

Attempts to isolate the protein moiety of WRSV failed. Several methods were tried. They were:

A. Fraenkel-Conrat's acetic acid method (40): To 5.0 ml. of virus solution (containing about 15 mg. of protein) was added 10 ml. of cold glacial acetic acid with constant stirring for about 1 hour, then centrifuged at 10,000 x g. for about 30 minutes. The supernatant fraction was diluted with an equal volume of water and dialyzed against 3 liters of water for 50 hours at 5°C with 8 transfers.

Some precipitate appeared about 20 hours after dialysis. A few drops of 5 N acetate buffer, pH 4.7, was added to the dialyzed solution to complete the precipitation. This suspension was then centrifuged at 80,000 x g. for 1 hour in a Spinco model L centrifuge equipped with rotor No. 30. The precipitate was collected and suspended in 5.0 ml. of water and adjusted to pH 8.0 with 0.1 N NaOH and 0.1 M acetic acid. The mixture was then centrifuged at 105,000 x g. for 1 hour to remove the undissociated virus particles. The supernatant liquid fraction showed a typical nucleoprotein spectrum in the ultraviolet region. This fraction was resistant to ribonuclease digestion. These results indicated that the protein moiety was not separated from the intact virus molecule. Several modifications were also performed including: (a) prolonging the incubation time of the virus solution with

acetic acid, such as 12 hours, 24 hours, or 30 hours at 5°C., (b) performing the acetic acid reaction at room temperature, (c) dialyzing the virus solution against 0.1 M acetate buffer, pH 4.6 for 30 hours at 5°C. None of these techniques seem to help the splitting of the virus molecule without denaturation. Several buffer solutions such as 0.1 M phosphate buffer, pH 6.0, pH 7.0, and 0.1 M Tris buffer, pH 8.0 have been used for the extraction of the precipitate collected from the centrifugation after dialysis. It did not seem to improve the extraction or in other words most of the precipitate was insoluble.

B. Ethanolamine method (42, 93): 10 ml. of virus solution was dialyzed against 3 liters of 0.1% ethanolamine solution, pH 10.5, for 30 hours at 5°C. The dialyzed solution was then centrifuged at 80,000 x g. for 1 hour in a Spinco model L centrifuge equipped with rotor No. 30. The supernatant fraction was then fractionated with ammonium sulfate. Four fractions were collected. They were 0.0 to 0.15, 0.15 to 0.35, 0.35 to 0.60, and 0.60 to 0.90 saturation of ammonium sulfate. All these fractions were extracted first with 0.1 M phosphate buffer, pH 7.0, and then with 0.1 M Tris buffer, pH 8.0. However most of the precipitate was insoluble. Only small portions were extracted. The ultraviolet absorption spectrum of each extract was examined by the Cary Recording spectrophotometer. A 260 mμ. peak appeared at the ultraviolet absorption spectrum of the extracts from the 0.60 to 0.90 ammonium sulfate saturation fraction. This indicated that some of the virus

molecules were indeed resolved. In other words the 0.60 to 0.90 fraction was a mixture of the viral nucleic acid, the viral protein and probably some undissociated virus molecules. There was no peak but a plateau from 255 mμ. to 280 mμ. of 0.15 to 0.35, and 0.35 to 0.60 fractions. Due to the insolubility of the product obtained from the treatment with ethanol amine, all fractions were very dilute. This fact hampered the progress of further fractionation.

DISCUSSION

That WRSV is distinct from other virus species has been proved by inoculation into different test plants and by cytological analysis of its effects on and in host cells (81). These studies indicate that this virus should be placed intermediary between tobacco ringspot virus (TRV) and bean yellow mosaic virus (BYMV). Some physical and chemical properties of TRV have been determined (34, 68, 84, p.96-98, 120), but these of BYMV have not been effectively studied. Successful isolating of WRSV should therefore permit exact comparison with TRV and suggest the approach to study of BYMV. The latter virus is widely distributed throughout North America and is commonly mixed with WRSV in bean plants. Characterization and identification cannot be made without the purified product being free from plant proteins and host nucleic acid.

Bean yellow mosaic virus is rather unstable. On the other hand WRSV is biologically stable. Thus it should be a suitable model for biochemical analysis at the molecular level.

1. Isolation of the virus

Since the discovery of WRSV by McWhorter (81) in 1950, practically no work in its biochemistry has been done until the preliminary study in isolation by Kuehl (72, p.1-30). However, the final preparation obtained by Kuehl's method possessed a specific infectivity of about only twice that of the crude sap.

The preparation is not stable and the recovery is lower than 10%. These facts suggest that inactivation during the isolation may occur. An improved method with respect to purity, yield and stability has been worked out in the present study. The following points need further elaboration.

A. Preliminary treatment of the starting material: It has been found that the preliminary treatment not only can increase the virus yield but also facilitates the removal of pigments. The treatment is simple; the whole infected plant, with exclusion of roots, is kept in a plastic bag and stored at 5°C for 45 to 50 hours before grinding. This increase in infectivity may be due to the inactivation of some proteolytic enzymes or natural antagonists in the plant. The process of freezing of tissues before grinding has been used for several plant viruses to improve subsequent isolation (121, p.17-19). However this technique has been found not suitable for WRSV. The virus is inactivated rapidly under freezing conditions.

B. Alcohol precipitation: One of the modifications is the employment of 25% (v/v) of 90% ethanol in the precipitation of the virus from the clarified sap. It has been found under such conditions that 30% more virus can be recovered from the supernatant liquid fraction as presented in Table 15. The ethanol precipitate thus obtained is directly extracted with 0.1 M acetate buffer, pH 5.5, instead of extraction with 0.1 M phosphate buffer, and subsequent operation as used by Kuehl (72, p.11-21). Two

different kinds of buffers, 0.1 M acetate and 0.1 M phosphate both at pH 5.5 have been used. As far as the final preparation is concerned, the preparation from the extraction with acetate buffer is far more infective and stable than from the original Kuehl's method (Table 16).

C. Differential centrifugation: The virus collected from the first ultracentrifugation has a very low solubility in 0.1 M phosphate buffer, pH 6.0. In an attempt to increase the virus concentration in the final preparation, two buffers, ammonium carbonate and Tris-HCl, both at pH 8.0, have been studied. It has been found that extracts thus derived have lower specific infectivities than those obtained from phosphate buffer (Table 17). Thus phosphate buffer is still employed but with one more cycle of differential centrifugation to further remove compounds of low molecular weights. The precipitate collected from this second ultracentrifugation is then extracted with 0.1 M Tris buffer, pH 8.0. By this method the virus in Tris buffer has been found to be more stable than that in the corresponding extract with phosphate buffer as shown in Table 18.

Virus preparations purified by the modified method always appeared a pale yellow-green color. The pigment cannot be removed by either ammonium sulfate fractionation or repeated differential centrifugation. Similar cases have been reported by Bawden and Pirie (7), and Ginoza et al. (59) for the purification of tobacco mosaic virus; and Dorner (35), and Loring (78) for the purified

potato X. Several methods which have been found successful to remove plant pigments in the purification of plant viruses have been tried also, such as: clarification with chloroform emulsion, developed by Schneider (104), with n-butanol and chloroform emulsion, reported by Steere (120); treatment with adsorbents, like Lloyd's reagent and active carbon (96), adjust to low pH (76, p.493), and trypsin digestion (7, p.282). None has been found successful. Either the virus is inactivated or it is firmly adsorbed. Preparative chromatography and electrophoresis have now been extensively used for the removal of the firmly bounded pigments from the partial purified plant viruses. However, in view of the low thermal inactivation point and also the rapid inactivation by freezing of the purified WRSV, these techniques may not be suitable.

D. Crystallization: Small crystals deposited from the virus preparation appeared to be in two forms, needle and dodecahedra. Separation of these crystals has not been performed because of the limited amount of material available. These crystals could initiate local lesions on horse bean plants as did the fresh preparations. However the virus in the crystal state is very unstable. It will lose its infectivity completely after being washed twice with water. This may be due to surface denaturation, since its ultraviolet absorption spectrum does not change.

2. Electron microscopy

When examined under the electron microscope, WRSV appeared to consist of both rods and spherical particles. Both forms of

particles are infectious as shown in Figures 5 and 6. The rods are actually in the form of a necklace instead of smooth cylinders. In other words, the spherical particles are the segments of rods or the rods are end to end aggregates of the spherical particles. Complete separation of these two types of particle has not been successful. It is suggested that both particles may exist in an equilibrium state.

Spheres $\rightleftharpoons$ rods

The morphology of the virus is dependent upon the position of the equilibrium. Aggregation of protein molecules is usually due to secondary bondings. Present data indicate that the morphology of WRSV is a function of the pH and the nature of the buffer salts. At pH 8.0, Tris buffer, particles in the rod form predominate (Figure 5) whereas in the acidic side, such as at pH 6.0, practically only those in the spherical form exist (Figure 7). It has also been found that preparations containing more rods possess higher stability than the others. The equilibrium state probably exists in vivo. Electron microscopic examination of unpurified plant juice prepared by pressing the infected plants through a squeezer has thus been made (6). Both rods and spherical particles as in the purified preparations have been observed (Figure 9). The indefinite length of the rods which occurs in both purified and unpurified preparations indicates that the tangled appearing rods from the purified preparations are the native form and are not an artifact formed during purification. This may reflect some of its

biological properties. In the case of tobacco mosaic virus Lauffer and Stanley (75, p.319) have claimed that as the pH approaches the isoelectric point from either side, one encounters aggregation of the rod shaped particles, at first end to end aggregation predominating and later side to side aggregation. The isoelectric point of WRSV has not been determined. However the amino acid analysis and the amide content of WRSV suggest that the viral protein is basic. The end to end aggregation of rods of potato X has likewise been shown by Takahashi and Rawlins (127).

From the effect of magnesium ion on the viral infectivity it appeared that Mg^{++} might act as an stabilizing agent for the viral infectivity. In other words, Mg^{++} acts as cement to link small particles into more stable rods. It seems more likely that bonds weaker than a covalent bond, probably coordinate bonds, are involved between the Mg^{++} ion and virus particles. However no evidence is available to demonstrate whether the metallic component is associated with the virus protein, with the nucleic acid, or with both. Stabilization of potato yellow dwarf virus by magnesium ion has likewise been demonstrated by Brakke (23, p.469-474). Loring and Waritz (79) have demonstrated the occurrence of small amounts of relatively firmly bounded iron, copper, calcium, and magnesium in tobacco mosaic virus. They have suggested that these metals may be responsible for binding the small nucleotide into the large, more organized structure necessary for infection.

3. Nucleic acid moiety of western ring spot virus

WRSV has been identified as ribonucleoprotein by the orcinol method. However this method does not differentiate ribose from other pentoses. The nature of the sugar may be determined by isolation and conversion into derivatives, or by paper chromatography in various solvents. Using methods by which the nucleic acids were isolated from tobacco mosaic virus (82), cucumber mosaic virus (86) has been unequivocally identified as D-ribose. At the present time, pentose nucleic acid isolated from those sources may be called ribonucleic acid. The nucleic acid content calculated on the ribose content is based on the fact that only purine bonded pentose reacts with orcinol under the experimental conditions. The total nucleic acid was determined by first assaying for purine nucleotide by selectively measuring the ribose bonded to purine and then, knowing the purine pyrimidine ratio (48.6:51.4) from chromatographic studies, the total content of nucleic acid was calculated. In view of the varied ribose content of the final purified virus preparations, the virus preparations may be contaminated with a small amount of carbohydrate such as potato X as reported by Dorner et al. (35) and Loring (78, p.463-465).

Since the yield of the nucleic acid prepared from WRSV was very low, intact virus particles were used for the analysis of purine and pyrimidine composition. The aqueous solution of the purified virus preparation was first lyophilyzed before hydrolysis.

Thus a more concentrated hydrolysate could be obtained. Isopropyl alcohol-HCl solvent system was chosen as the solvent for developing the chromatogram, since it has not only a high resolving power, but also it permits the analysis of large quantities of guanine.

One fact that emerges from the present analysis of nucleic acid composition of WRSV (this was also observed by Knight[82], Markham and Smith [86, 87, 113]) is that the general base pattern of plant viruses is very different. It has been suggested that nucleic acid composition might be useful for characteristion of virus "genera" and thus be useful in the classification of plant viruses. WRSV-nucleic acid has a rare guanine-cytosine type composition. This might reflect some of its biological properties. This is in line with the current hypothesis that RNA carries the genetic information of the intact virus.

4. Protein moiety of western ring spot virus and its amino acid composition

The amino acid analysis of WRSV is not completely satisfactory, as presented in Table 14. However it does give qualitative information, such as lack of methionine in WRSV-protein. The virus contains 16 amino acids in the acid hydrolysate with very high amide content. Possible causes of deviation of the amino acid analysis may be due to: (1) the conditions of hydrolysis were not ideal, some amino acid might be destroyed, especially when the intact virus was used (99). (2) Furthermore, the virus preparation might be contaminated with host protein(s). Since WRSV

appears to be pleomorphic, the purity of the virus preparations could not be determined by conventional methods. The electron micrographs of the purified preparations appear very clean, however this would not eliminate the possibility of the presence of inert protein molecules with morphology similar to the virus particles. (3) In experiment 1 (Table 14), an unusually high base line was obtained all the way through the analysis of the eluates from the column for the acidic and neutral amino acids. This might be due to contamination by ammonia from the atmosphere.

The necessary separate determinations of cysteine and tryptophan have not been possible, since they were limited by the amount of material available.

Attempts have been made to isolate the native protein from WRSV but without any success. In view of the amino acid composition of WRSV protein, it appears to be a protein with a high content of amide. The current hypothesis suggests that the protein moiety of the virus serves as an "overcoat" of the viral nucleic acid. This view has been developed from the extensive studies of tobacco mosaic virus. In order to isolate the native protein from WRSV several conditions had been tried. It was found that under acidic conditions, such as 67% acetic acid, the protein was practically completely denatured. This might be due to the hydrolysis of the amide groups of the protein moiety with the formation of some free carboxylic groups, such that the bond distribution, especially some secondary bondings, such as salt

linkages and hydrogen bonding would be changed. Or in other words, some bondings were destroyed and/or created and a random distribution might be formed, so that the protein is denatured. Under basic conditions, such as pH 10.5 (0.1% ethanolamine buffer), some of the viruses were dissociated as indicated in the ultraviolet absorption spectrum, but the yield was very poor and highly contaminated with the viral nucleic acid and/or the undissociated virus.

All these findings indicate that WRSV is more stable at basic than at acidic pH values. This is in line with the results obtained from the pH effect on the infectivity of WRSV (Table 10). Alkaline treatment for the isolation of native protein seems to offer some promise for further fractionations. However this prospect has not as yet been able to be performed as limited by the amount of material available.

Table 1. Crystalline Plant Viruses.

Name	Culture	Crystal Form	Reference
Southern Bean Mosaic	bountiful bean	rhombic prism & rhombic bipyramids	96
Squash mosaic	Zucchini squash	rod shaped microcrystal	122
Tobacco necrosis	potato	lozenge shaped plate	10
	tobacco	variable dodecahedron, bipyrimids, irregular laminae	10
	Princeton	no true crystal	10
	Rothamsted	flat plate, triclinic prism, hexagonal plate	12
Tobacco mosaic	tobacco	needle or tactoids	118
Tobacco ring spot	Caserta squash	plate-like crystal octahedron	120
Tomato bushy stunt	tomato	rhombic dodecahedron	11
Turnip yellow mosaic	Chinese cabbage or turnip	octahedron, small birefringent needle	85
Western ring spot	Bountiful bush bean	needle and dodecahedra	present thesis

Table 2. Nucleotide composition of ribo-nucleic acid of some plant viruses.

No.	Virus	Strain	Adenine	Guanine	Cytosine	Uracil	Purine/pyrimidine	Reference
1	cucumber	CV3	1.03	1.02	0.73	1.23	1.05	70
2	cucumber	CV4	1.03	1.03	0.77	1.18	1.05	70
3	potato X	PX	1.37	0.87	0.91	0.85	1.27	35
4	southern bean mosaic	SBM	1.03	1.04	0.92	1.01	1.07	35
5	tobacco mosaic	TMV	1.19	1.01	0.74	1.05	1.24	70
6	" "	M	1.18	1.05	0.77	1.03	1.23	70
7	" "	J 14D1	1.20	1.01	0.74	1.07	1.22	70
8	" "	GA	1.17	1.04	0.77	1.04	1.22	70
9	" "	YA	1.19	1.02	0.74	1.06	1.23	70
10	" "	HR	1.17	1.03	0.72	1.08	1.22	70
11	tobacco ringspot	TRS	0.96	0.99	0.93	1.13	0.95	68
12	tomato bushy stunt	BS	1.10	1.11	0.82	0.98	1.22	35
			0.995	1.12	0.875	1.01	1.12	87
13	turnip yellow mosaic	TY	0.91	0.69	1.53	0.89	0.65	87
14	western ring spot	906	0.630	1.30	1.46	0.62	0.933	present thesis

The figures are expressed in terms of molar proportions of the constituent nucleotide calculated to an arbitrary total of 4.

Table 3. Infectivity of sap from different parts of infected plants.

Part of plant	Infectivity at dilution of: 1:10	1:100	Protein (mg./ml.)
Primary leaves	18/20	15/20	14.0
Trifoliate	20/20	20/20	28.3
Stem and petioles	20/20	17/20	5.8

Dilutions were made with 0.1 M phosphate buffer, pH 6.0. The figures given for the infectivity refer to the number of plants becoming infected over the number of plants inoculated. This notation is used throughout the paper except where otherwise stated.

Table 4. The effect of temperature on the infectivity of crude sap.

Temperature (°C)	Protein (mg./ml.)	Infectivity at dilution of: 1:1	1:10	1:100
0	20.0	31/31	20/20	2/20
26	20.2	18/18	19/20	12/17
35	20.0	16/17	20/20	10/22
55	9.9	1/19	0/17	1/15
70	4.5	0/22	0/22	0/29
100	3.5	0/26	0/14	0/24

10 ml. of crude sap was incubated at the temperature given for 10 minutes, then cooled in an ice bath and its infectivity was measured. Dilutions were made with 0.1 M Tris buffer, pH 8.0 after cooling.

Table 5. The effect of storage on the infectivity of crude sap.

Sample	Infectivity at dilution of:		
	1:1	1:10	1:100
0 hour	20/20	20/20	9/20
5 hours	18/20	20/20	20/20
9 "	18/20	11/20	9/20
22 "	10/20	7/20	0/20
32 "	8/20	4/20	0/20
49 "	4/20	5/20	0/20

Crude sap from infected plants were stored at 5°C at different lengths of time before the infectivity was measured. Dilutions were made with 0.1 M phosphate buffer, pH 6.0.

Table 6. The effect of storage of the whole infected plants on the infectivity of crude sap.

Sample	Infectivity at dilution of: 1:1	1:10	1:100
0 hour	15/20	15/20	10/20
5 hours	20/20	16/20	8/20
10 "	19/20	17/20	11/20
24 "	19/20	14/20	14/20
33 "	19/20	20/20	13/20
37 "	20/20	13/20	6/20
45 "	20/20	16/20	18/20
16 "	13/20	16/20	6/20
21 "	17/20	17/20	7/20
25 "	16/20	20/20	10/20
35 "	17/20	11/20	8/20
44 "	14/20	16/20	7/20
48 "	20/20	17/20	11/20
56 "	17/20	17/20	17/20

The whole infected plants with the exclusion of roots were stored at 5°C for different lengths of time before the infectivity was measured. Dilutions were made with 0.1 M phosphate buffer, pH 6.0.

Table 7. Infectivity and protein content of different fractions in purification.

Fraction No.	Total Protein (mg.)	Total volume (ml.)	Infectivity at dilution of: 1:1	1:10	1:100	1:1000	Maximum specific infectivity
Sap	15,600	850	13/14	10/14	8/17	0/16	2.56
S-2	440	400	20/20	9/14	3/17	0/19	16.1
S-4	196	70	11/11	16/16	10/12	7/13	192
S-6	31.5	35	---	20/20	9/13	4/17	262
S-8	14.0	8	---	20/20	13/14	13/18	418

Specific infectivity:

$$\frac{\text{Number of plants becoming infected}}{\text{Number of plants inoculated}} \text{ per mg. of protein}$$

Maximum specific infectivity refers to specific infectivity calculated from the highest dilution. Dilutions were made with the same buffer as used in the preparation of the respective fraction.

Table 8. Infectivity and protein content of final preparations from three representative experiments.

Experiment No.	Sample	Total protein (mg.)	Maximum specific infectivity	Recovery
1	control	15,600	2.56	
	virus prep.	14.0	418	14.6%
2	control	22,800	2.16	
	virus prep.	15.0	254	10.5%
3	control	44,200	1.36	
	virus prep.	54.5	254	19.5%

The controls refer to crude sap used for purification.

Table 9. The effect of temperature on the infectivity of purified virus preparations.

Temperature (°C)	Infectivity at dilution of: 1:10		1:100	
60	0/17	0/20	0/20	0/20
55	0/17	0/20	0/20	0/20
50	0/23	6/23	0/24	2/20
45	9/23	20/20	8/23	18/20
40	23/23	---	9/23	---
38	12/17	13/13	---	20/20
22	23/26	20/20	18/18	20/20
4	18/18	26/26	46/70	20/20

Virus solutions at 0.2 mg. of protein per ml. were incubated at the temperature indicated for 10 minutes and cooled in an ice bath. Dilutions were made with 0.1 M Tris buffer, pH 8.0 after cooling.

Table 10. The effect of pH on the stability of a purified virus preparation.

pH	Infectivity at dilution of: 1:100	1:500
8.6 (Tris buffer, 0.1 M)	20/20	9/16
8.0 " " "	22/22	24/24
7.0 (phosphate buffer, 0.1 M)	22/22	25/25
6.0 " " "	20/21	12/15
5.5 (acetate buffer, 0.1 M)	11/21	10/24

Virus solutions were incubated in the respective buffer for 2 hours at 5°C before infectivity was measured.

Table 11. Inactivation of a purified virus preparation by trypsin.

Sample	Infectivity at dilution of: 1:10	1:100
Supernatant fraction from incubation mixture at 4°C	0/14	--
Precipitate fraction from incubation mixture of 4°C	0/17	--
Supernatant fraction from incubation mixture of 37°C	0/17	--
Precipitate fraction from incubation mixture of 37°C	0/11	--
Trypsin solution (2 mg./ml.)	0/12	--
WRSV without trypsin treatment (4°C)	--	12/18

One ml. of virus solution (1.5 mg./ml.) was incubated with 2 mg. of trypsin at pH 7.0 and at the temperature indicated for 24 hours. The incubation mixture was then separated by centrifugation. Dilutions were made with 0.1 M Tris buffer, pH 8.0.

Table 12. Effect of magnesium ion on the infectivity of a virus preparation.

Sample	Infectivity at dilution of:			Maximum specific infectivity
	1:10	1:100	1:1000	
Control	22/22	19/19	5/25	135
E-H	18/18	11/19	3/21	179
E-Mg	24/24	14/20	6/16	416

Control refers to the purified virus preparation without any treatment.

E-H: The virus preparation dialyzed against 0.005 M EDTA solution and followed by water.

E-Mg: The virus preparation dialyzed against 0.005 M EDTA solution and followed by 0.008 M of magnesium sulfate solution.

Table 13. Purine and pyrimidine composition of WRSV-nucleic acid.

Compound	Experiment 1	Experiment 2
guanine	0.324	0.338
adenine	0.156	0.155
cytosine	0.365	0.363
uracil	0.154	0.145

Purines/pyrimidines: 0.933

Precentages of the bases of WRSV nucleic acid, calculated to an arbitrary total of 1.

Table 14. Amino acids composition of WRSV-protein.

	Moles residues/10^5 grams WRSV		
	Experiment 1	Experiment 2	Average
Aspartic acid	48.2	41.2	44.7
Threonine	25.6	25.0	25.3
serine	22.2	16.8	19.5
glutamic acid	47.7	38.4	43.1
proline	21.9	19.2	20.6
glycine	40.8	39.0	39.9
alanine	35.2	38.7	37.0
cystine	37.5	44.8	41.2
valine	17.1	13.9	15.5
isoleucine	20.9	32.3	26.6
leucine	34.7	43.5	39.1
tyrosine	12.9	12.8	12.9
phenylalanine	22.4	23.7	23.1
lysine	39.9	32.0	36.0
histidine	20.7	13.7	17.2
ammonia	116.0	124.0	120.0
arginine	31.9	17.5	24.7
TOTAL	595.6	576.5	586.4

Table 15. Infectivity and protein content of the supernatant fraction from different concentrations of alcohol fractionation.

Final concentration of ethanol (%)**	Infectivity at dilution of: 1:1	1:10	1:100	1:1000	Protein (mg./ml.)
18	--	9/10	18/20	9/20	6.0
18	--	6/17	3/18	--	6.1
18	--	8/18	3/18	--	5.8
22.5	--	10/14	0/14	--	5.5
22.5	1/12	--	--	--	4.7

** Experimentally 90% ethanol (by weight) was used to bring the final concentration as indicated.

Table 16. Infectivity and protein content in the extract resulting from the extraction of the ethanol precipitate by different buffers.

Sample	Infectivity at dilution of: 1:1	1:10	1:100	Protein (mg./ml.)
Acetate buffer (0.1 M, pH 5.5) extract	10/11	11/13	0/13	3.0
Phosphate buffer (0.1 M, pH 5.5) extract	8/12	11/12	0/16	4.8
Phosphate buffer (0.1 M, pH 6.0) extract first, then follow Kuehl's operation (72)	9/11	10/11	8/10	6.3

The volume of the extract is the same in all three cases.

Dilutions were made with the same buffer as used for extraction.

Table 17. Infectivity and protein content in the extract resulting from the extraction of the precipitate obtained from the first ultracentrifugation with different buffers.

Sample	Infectivity at dilution of:						Protein (mg./ml.)
	1:10		1:100		1:1000		
	I*	II**	I	II	I	II	
Bicarbonate buffer extract	1/18	1/14	1/18	0/20	0/20	--	4.0
Tris buffer extract	7/17	5/12	1/16	0/20	1/18	--	4.8
Phosphate buffer extract	7/15	1/13	2/14	0/20	2/18	--	0.5

* The infectivity was measured immediately.

** The infectivity was measured after storing at 5°C for 2 days.

Bicarbonate buffer used was 0.1 M, pH 8.0

Phosphate buffer used was 0.1 M, pH 6.0.

Tris buffer used was 0.1 M, pH 8.0.

Table 18. Comparison of the infectivity and protein content of virus preparations made from Kuehl's original method (72) and the modified method.

Method	Infectivity at dilution of: 1:10	1:100	1:1000	Protein (mg./ml.)
Kuehl's	2/6	0/11	--	1.4
"	2/20	0/20	--	0.6
"	2/32	--	--	1.85 (72)
Modified	18/20	4/19	0/20	1.2
"	16/17	13/17	3/19	3.5

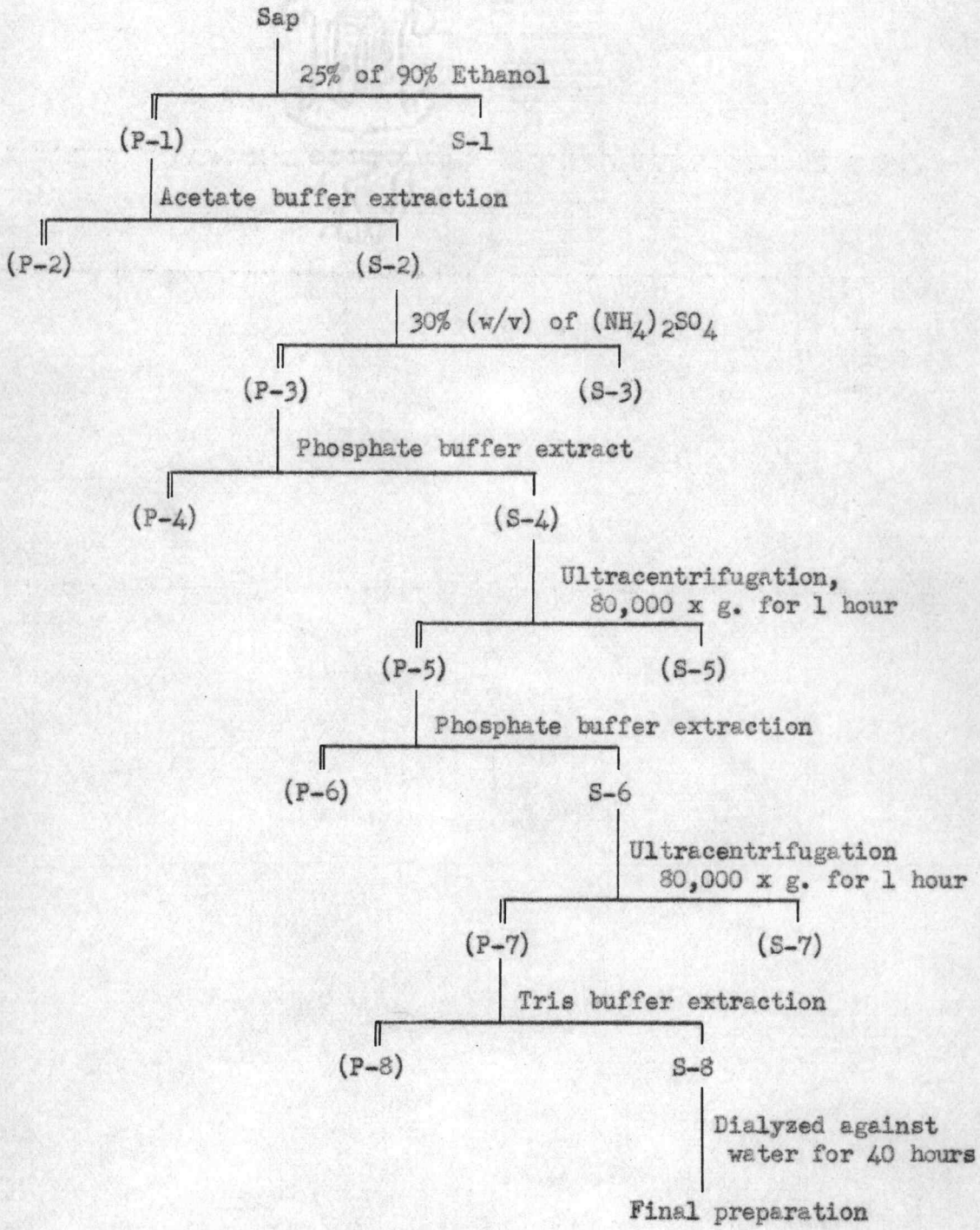

Figure 1. Purification scheme.

Figure 2. Apparatus for making specimens for electron microscopic examination.

I. A bottle with side arms:

Side arm "A" used to connect the specimen holder.

Side arm "B" used to adjust the level of cooling mixture and the pressure inside of the bottle.

II. A specimen holder with 6 blocks for holding the grids.

III. A modified atomizer with the modified tube "C" for receiving the sample.

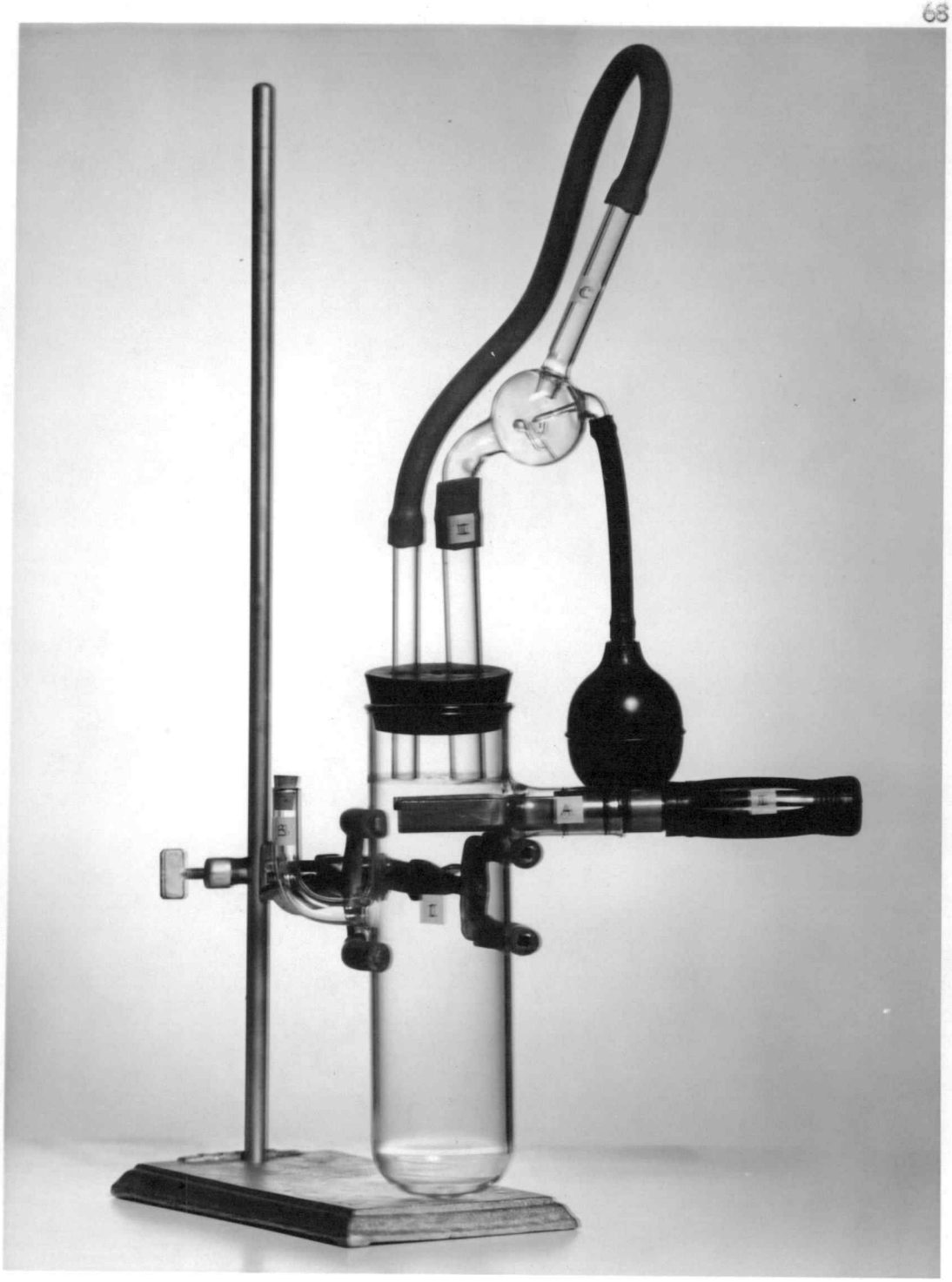

Figure 2.

Figure 3. Crystal picture of purified WRSV.

Crystals of western ring spot virus obtained from the aqueous solution of a purified virus preparation after storage at 5°C for about a week. Magnification about 600x.

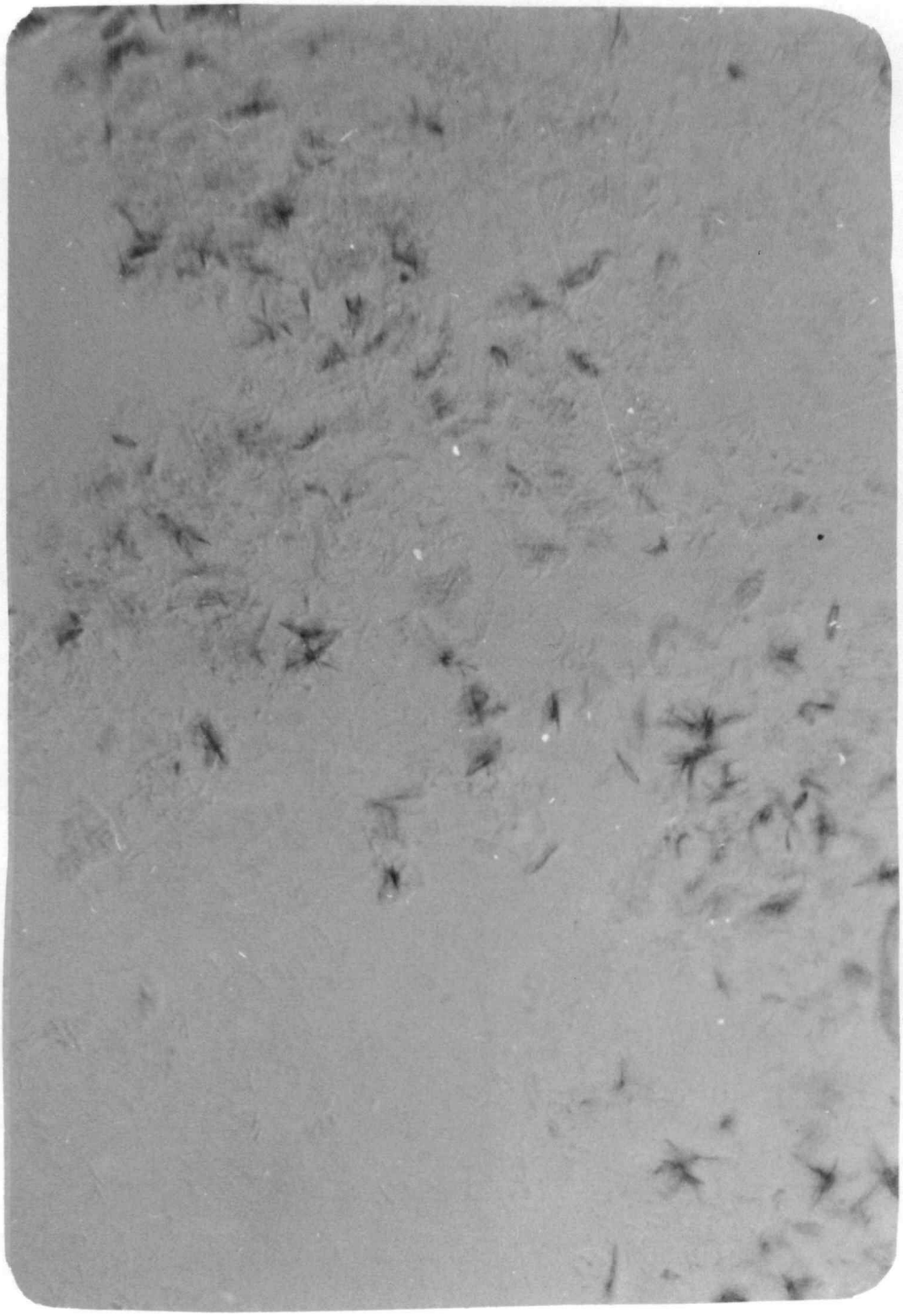

Figure 3.

Figure 4. Ultraviolet absorption spectrum of purified WRSV

Absorption spectrum of WRSV (0.123 mg./ml.) in a 1 cm. layer recorded on a Cary model 11 Spectrophotometer. 0.1 M Tris buffer pH 8.0 was used as the medium.

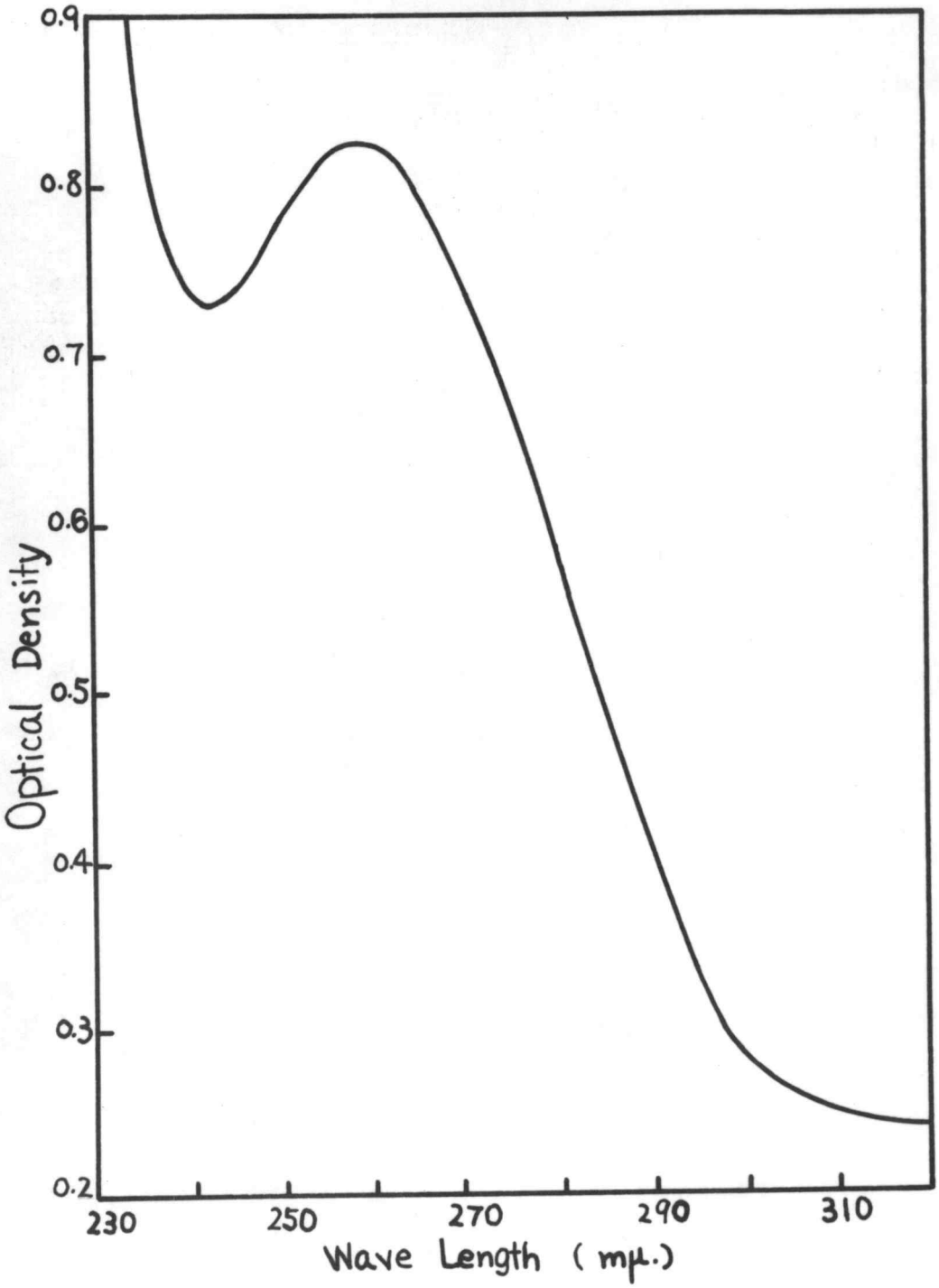
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Optical Density
230
250
270
290
310
Wave Length (mμ.)

Figure 4.

Figure 5. Electron micrograph of purified WRSV, No. 1.

The virus sample was prepared by the purification scheme as shown in Figure 1. The specimens were made with a smaple which was diluted 10 times with distilled water, air dried technique was used, shadowcast with palladium at a 25° angle; magnification 80,000 x.

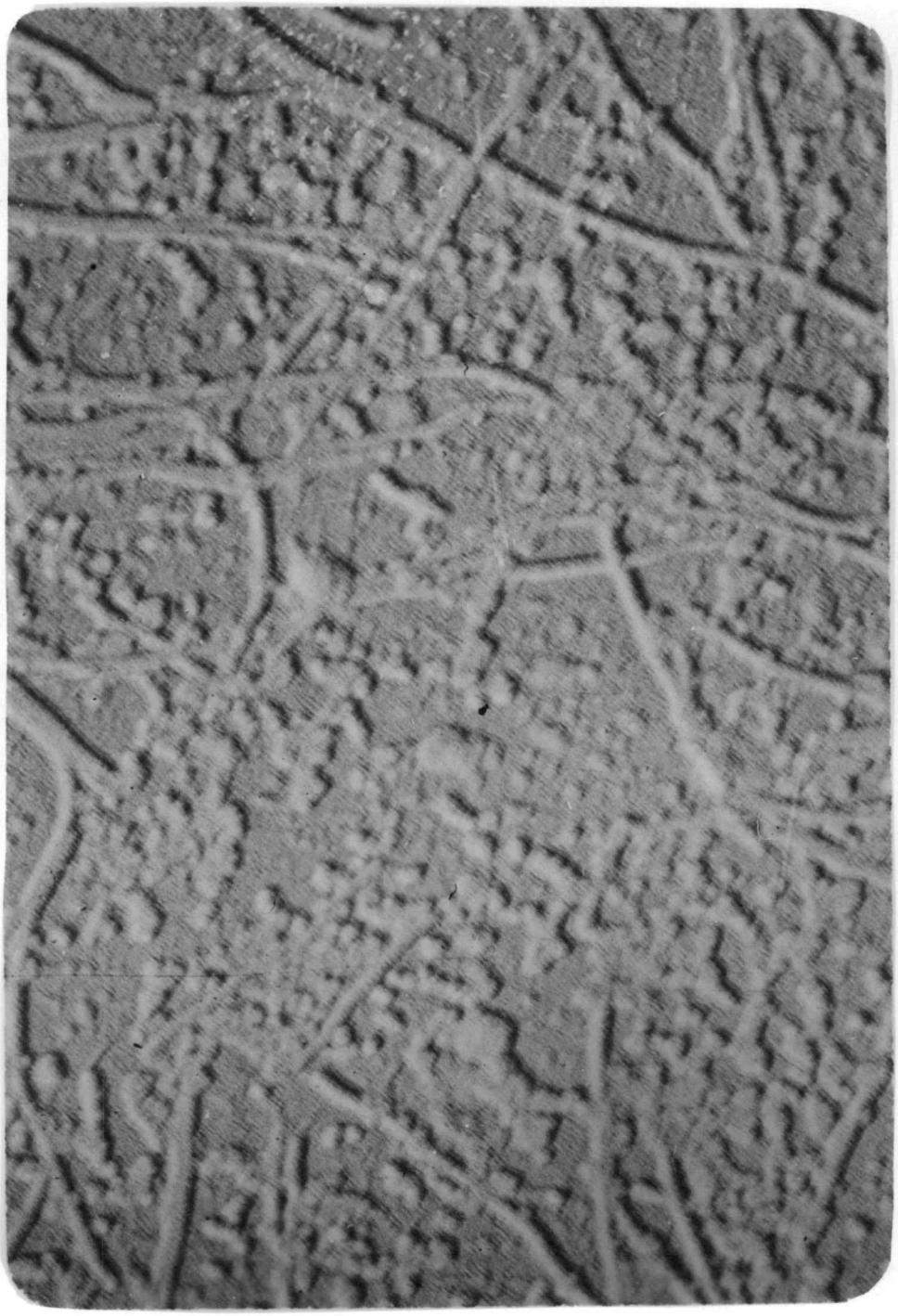

Figure 5.

Figure 6. Electron micrograph of purified WRSV, No. 2.

The virus sample was prepared by the purification scheme as shown in Figure 1. The specimens were made with a sample which was diluted 20 times with distilled water, air dried technique was used, shadowcast with palladium at a 25° angle; magnification 60,000 x.

Note the segments of the rods.

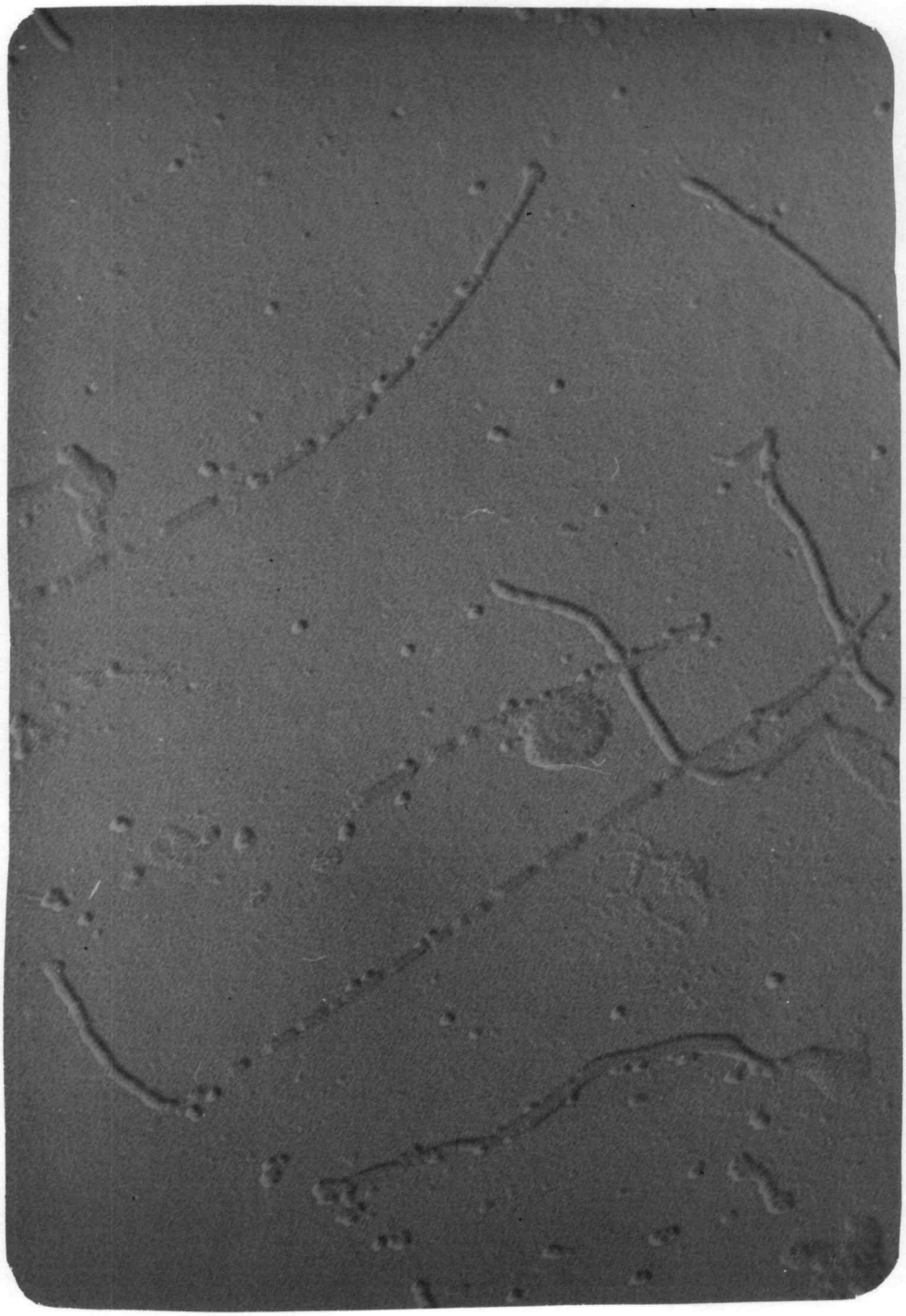

Figure 6.

Figure 7. Electron micrograph of purified WRSV, No. 3.

The virus sample was prepared by the purification procedure as shown in Figure 1 except 0.1 M phosphate buffer, pH 6.0, instead of Tris buffer, pH 8.0, was used for the extraction in the last step. The specimens were made with a sample which was diluted 10 times with distilled water, air dried technique was used, shadowcast with palladium at a 25° angle; magnification 75,000 x.

Figure 7.

Figure 8. Ultraviolet absorption spectrum of a nucleic acid preparation isolated from WRSV.

Absorption spectrum of WRSV-nucleic acid (4.2 micrograms of organic phosphorus per ml.) in a 1 cm. layer recorded on a Cary model 11 spectrophotometer. Water was used as the solvent.

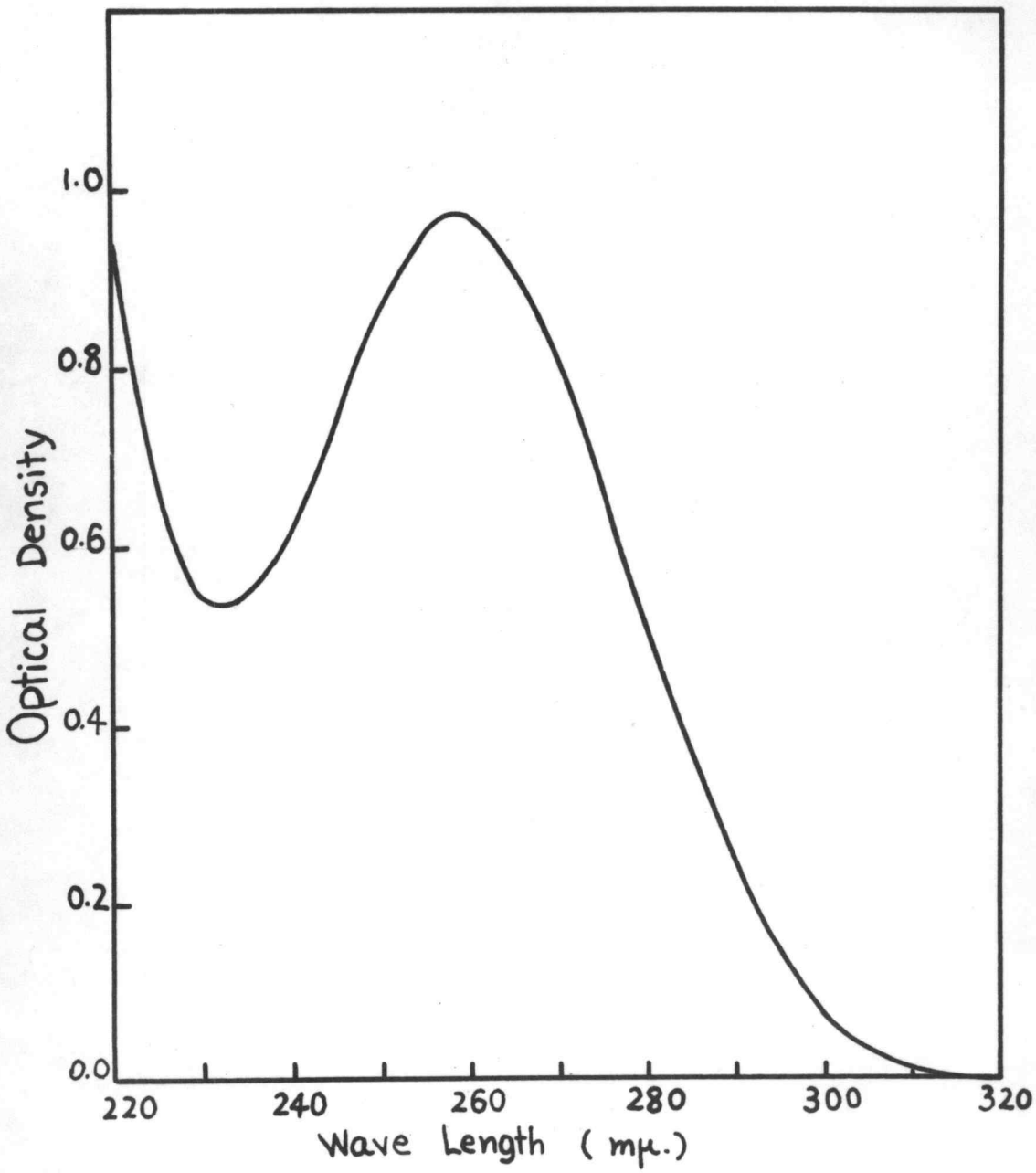
Optical Density
1.0
0.8
0.6
0.4
0.2
0.0
220
240
260
280
300
320
Wave Length (mμ.)

Figure 8.

Figure 9. Electron micrograph of unpurified WRSV from infected bean plants.

The virus sample was prepared by pressing the infected plants through a squeezer and then diluted 10 times with distilled water, air dried technique was used, shadowcast with palladium at a 25° angle; magnification 70,000 x.

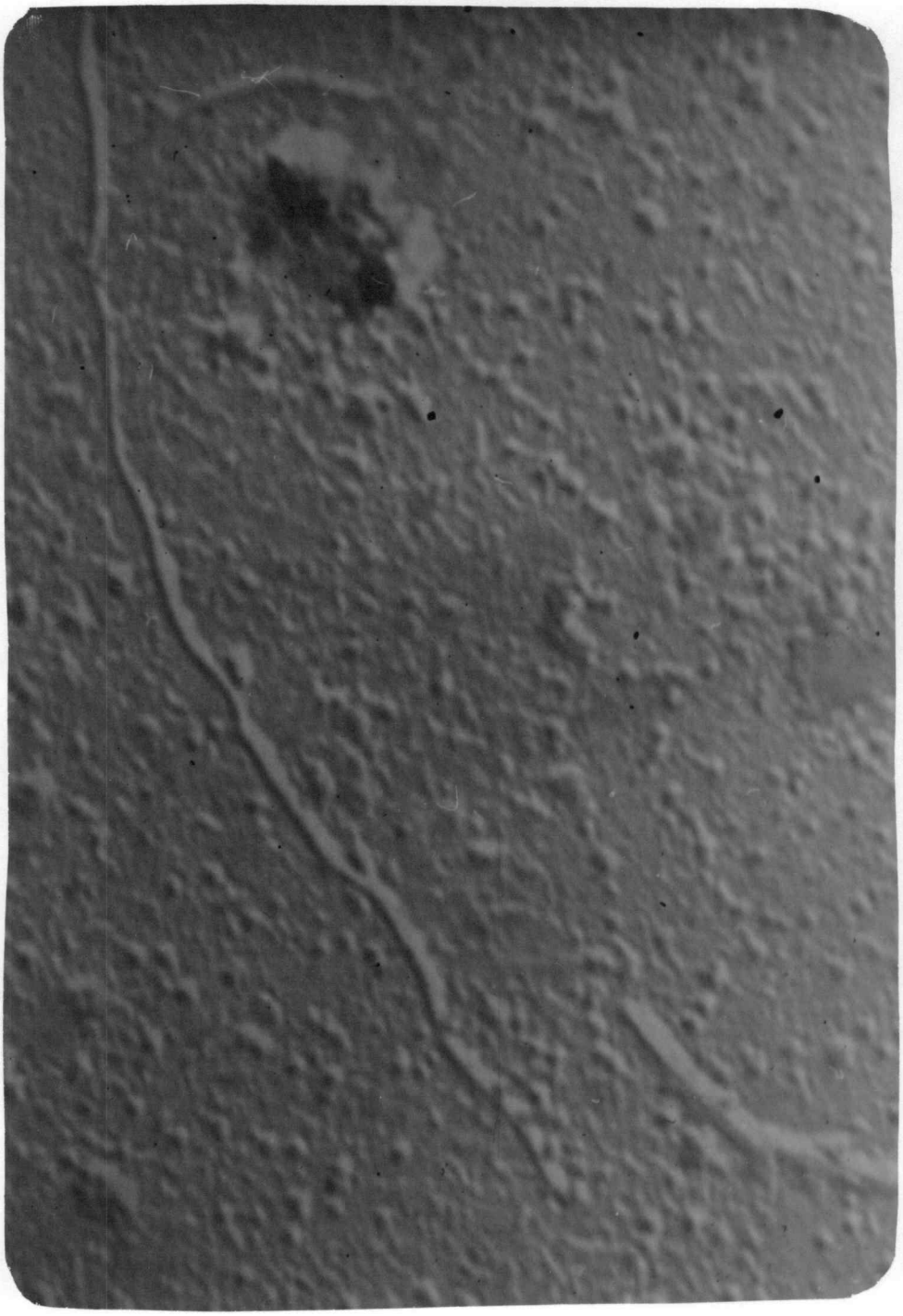

Figure 9.

SUMMARY

A method for the isolation of western ring spot virus (WRSV) from infected bean plants has been developed. It consisted of the precipitation of the virus with ethanol, extraction with acetate buffer, and fractionation with ammonium sulfate. This preparation was then subjected to repeated differential centrifugation. The purified sample was about 100 to 200 times more potent than the crude sap in specific infectivity. Recovery usually ranged from 10 to 20%.

Crystallization of the virus occurred in aqueous solution at 5°C within a week. Both these crystals and fresh preparations initiated local lesions on horse bean plants.

The virus in the crude sap as well as in the purified preparations exhibited both rod and spherical patterns under the electron microscope. The relative distribution of rods and spheres was dependent upon the pH and the nature of buffer salt used for the extraction of the virus in the last step of purification. An equilibrium between these two forms is suggested. The relative distribution of these particles *in vivo* would be, thus, controlled by pH which might be slightly varied with physiological conditions.

Western ring spot virus was labile to trypsin at pH 7.0, even at 4°C. It showed a thermal inactivation point at 55° C. The dilution end point of the purified virus was 10^{-3}, i.e., in

the order of micrograms of protein of the purified preparation per ml.. WRSV in the purified preparations was rapidly denatured by freezing.

Both the crystalline and the fresh virus preparations showed the typical ultraviolet absorption spectrum of a nucleoprotein. Nucleic acid content of WRSV was found to be about 37% by weight. The viral nucleic acid was isolated. Its absorption spectrum was symmetrical with the peak at 257 mu.. The $E^{1\%(p)}_{257m\mu}$ was 2.3×10^3. $E_{maximum}/E_{minimum}$ was 1.83, and E_{257}/E_{280} was 1.96, where E is the absorbance ($\log I_0/I$). The purine and pyrimidine composition of WRSV-nucleic acid was determined by chromatographic and spectrophotometric techniques after appropriate hydrolysis. The nucleic acid appeared to be a guanine-cytosine type and showed a purine/pyrimidine ratio of 0.933.

The amino acid composition of WRSV-protein was analyzed by Moore and Stein's technique on ion exchanger columns. Sixteen amino acids (aspartic acid, serine, threonine, glutamic acid, proline, glycine, alanine, valine, cystine, leucine, isoleucine, tyrosine, phenylalanine, lysine, histidine, and arginine) and a high ammonia content were found in the acid hydrolysate of the intact virus.

Attempts were made to isolate the native protein from WRSV but without success. Possibilities of the failure were discussed.

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