AN ABSTRACT OF THE THESIS OF

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Title STUDIES ON VIRUSES ISOLATED FROM ROSES WITH ROSE MOSAIC SYMPTOMS

Abstract approved

(Major professor)

Previous work on the rose mosaic virus has produced uncertainty concerning the identity and properties of this virus. This work was undertaken to help establish the true identity of the virus and to determine some of its properties.

A mechanically transmissible virus associated with rose mosaic virus disease of *Rosa dillecta*, var. Red Delight was purified by homogenizing infected tissue in pH 9.5 borate buffer containing thioglycollic acid and activated charcoal, clarifying the filtrate with ether-CCl₄ and concentrating the suspension by alternate high and low speed and density gradient centrifugation. The ultraviolet absorption of the purified virus preparation was measured and the percent RNA and protein were calculated as 24.8 and 75.2 percent respectively. The virus suspension consisted of spherical particles, as determined by electron microscopy, with two size ranges at 20-30 μ and 35-45 μ. An antisera to the virus was prepared. Attempts were made to determine...
the serological relationship of the virus to other known viruses, but the results were not conclusive.
STUDIES ON VIRUSES ISOLATED FROM ROSES WITH ROSE MOSAIC SYMPTOMS

by

THOMAS RAY WATSON

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Dean of Graduate School

Date thesis is presented June 4, 1965

Typed by Lucinda M. Nyberg
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STUDIES ON VIRUSES ISOLATED FROM ROSES
WITH ROSE MOSAIC SYMPTOMS

INTRODUCTION

It generally is recognized that basic information concerning the identity and nature of the viruses in roses is essential for effective control of the diseases. One of the viruses found in roses, designated as the rose mosaic virus, was isolated by White in 1932 (33). Only a few studies have been made on the properties of this virus. Fulton (9) found that the virus was very unstable, losing all its activity in buffer within 50 minutes. He reported that cysteine hydrochloride and sodium sulfite preserved the infectivity of the virus for at least three hours. Willison, et al. (36) found that a rose virus could be partially purified in a potassium cyanide-sodium hydrogen sulfite solution. They found that their preparation contained particles with a mean diameter of 28 mµ and 40 mµ. Fulton (10) reported that plant juice containing the rose mosaic virus could be clarified by absorbing the host tissue on hydrated calcium phosphate.

Halliwell (14) claimed to have partially purified the rose mosaic virus by using Fulton's clarification method, followed by alternate low and high speed, and density gradient centrifugation. On the basis of Halliwell's work, Halliwell and Milbrath (15) claimed that rose mosaic virus was a strain of tomato ring spot virus. This conclusion
of Halliwell and Milbrath was not accepted by Fulton (12).

Because the previous studies have produced uncertainty concerning the identity and properties of rose mosaic virus, the present investigation was undertaken with the purpose of verifying or disproving previous conclusions. The goals of this investigation were: 1. to isolate from roses a rose mosaic virus free from contamination by other viruses, 2. to find a reliable method of purifying the virus, 3. to determine the properties and morphology of the virus, and 4. to develop an antiserum to the virus.
REVIEW OF LITERATURE

Methods of Virus Purification

A survey of the methods used in purifying viruses was made. Methods must be found to 1. preserve the virus activity in vitro, 2. remove the host proteins without inactivating the virus, and 3. concentrate the virus. Since methods which are successful with one virus may not be successful with another, it is a matter of trial-and-error to determine which method will be successful with a particular virus.

Many viruses which are stable and active within the plant are inactivated when the plant cells are macerated. It is believed that this inactivation is caused by substances produced when the cell is broken down and the contents exposed to the atmosphere (19, p. 38). One group of these inactivating substances is believed to be produced by air oxidation, and hence mild reducing agents have been used successfully by many workers to preserve the virus activity in vitro. Sodium sulfite and bisulfite were among the first such reducing agents to be used for this purpose (28; 6). Other agents, such as cysteine hydrochloride, sodium glutathione, and sodium ascorbate also were found to prevent oxidative inactivation (6). Recently (21) diethyldithiocarbamate and thioglycollic acid have been found to be good stabilizing agents. Complexing agents, such as potassium cyanide (19), and chelating agents, such as ethylenediamine tetraacetic acid (EDTA)
(19), which tie up the oxidative catalysts also have been found useful in preserving activity.

A buffer solution, which keeps the pH sufficiently removed from the isoelectric point of the virus to prevent precipitation, also is necessary to preserve the activity in vitro. Dilute phosphate buffers (28) have been used most extensively, especially since it was found that phosphate ions had a special effect in increasing the activity of several viruses (38). Recently it has been reported that magnesium ions added to the phosphate buffer increase further the activity of several plant viruses (17). Dilute borate buffers also were used to preserve activity (29). Some viruses which are unstable in phosphate buffers have been found to be stable in these borate buffers (30; 23).

The clarification of plant sap; i.e., the removal of the host components, appears to be the most critical step in the purification of a virus. The clarifying treatment must remove the host components without damaging or entraining the virus. Host proteins are removed from the virus solution essentially by three processes: coagulation or precipitation, adsorption or absorption, and ultracentrifugation (32, p. 300-357). Agents which have been used to cause coagulation or precipitation are: heat (5), freezing (29), inorganic salts (27), ethanol (19), organic solvents such as butanol, chloroform, ether, carbon tetrachloride (24), and acids (19). Diatomaceous earth (Celite) and activated charcoal (8) are common adsorption agents. High speed
Centrifugation will remove most host proteins, but, unless there has been a previous clarification, the virus often is removed also (25).

After plant sap has been clarified, it usually is necessary to concentrate the virus before its properties can be studied. For the more stable viruses, concentration can be performed by precipitating the virus with a high concentration of a salt such as ammonium sulfate (26), with 95 percent ethanol (5), or with acids (27). Another common method of concentrating the virus is by alternate high and low speed centrifugation (36). The clarified sap is first centrifuged at high speed to remove the virus from suspension. Then the pellet is resuspended in the buffer and centrifuged at low speed to remove host material. By repeating the cycles, most of the host material can be removed and the virus concentrated in a pellet. Loss of virus can be minimized by recycling the discarded fractions.

Another method of reducing the volume of clarified sap is by dialysis against saturated sucrose solutions at low temperature (25). The final concentrate then is dialyzed against buffer to remove the sucrose. Another efficient concentration method, density gradient centrifugation, was introduced by Brakke (7). Density gradients, usually of sucrose, are set up in plastic centrifuge tubes and a small volume of the virus suspension is layered on top of each tube. The tubes then are placed in a swinging bucket rotor and centrifuged at high speed until separation is accomplished. The various layers are
assayed to determine which contains the virus. This infective layer can be centrifuged, resuspended, and then layered on another density gradient for further concentration and separation.

During purification it is necessary to determine if the virus is still present and active, and if the impurities have been removed. There are two principal ways of assaying for infectivity of a plant virus (4, p. 148-170). In one, the systemic method, the plant is inoculated with the virus and later the entire plant shows virus disease symptoms. Many plants are inoculated with dilutions of the test solution and other plants with dilutions of a control solution. From the numbers of plants that become infected, it is possible to estimate the concentration of active virus. The main drawbacks to this method are that excessive numbers of plants are required, and a lengthy time is required for symptoms to appear. The other method, discovered by Holmes (16), is based on the fact that the virus induces localized necrotic lesions at the site of the inoculation in leaves of certain hosts. The number of lesions, compared with the number from a control on other leaves (or other half leaves) is a quantitative measure of the active virus concentration. Since it has been found that the numbers of lesions produced by a given inoculum varies between leaves of the same plant, statistical methods are used to reduce variation. There are several other variables which affect the accuracy of the method; hence results of local lesion assays must be interpreted cautiously.
In addition to their biological properties, viruses are characterized by their physical and chemical properties, as measured with an analytical ultracentrifuge (20), a spectrophotometer (38) and an electron microscope (35); and by their serological properties (2).

The analytical ultracentrifuge is used to measure the sedimentation rates of the particles in a virus suspension. If the rates are uniform, the suspension is considered homogeneous.

A quantitative determination of the components of a mixture can be made with a spectrophotometer (3) if each component absorbs most strongly at a sufficiently different wavelength. It is necessary to obtain the absorption spectra of each component in the pure state, and then the spectra of the mixture. It is fairly simple to calculate the concentrations of the components of a two-component mixture; but it is much more difficult when there are three components.

Warburg and Christian (38) measured the absorption of a pure enolase sample and a pure nucleic acid sample at 280 and 260 m\(\mu\). They then measured the absorption of known mixtures of the two pure substances. After calculating the extinction coefficients they were able to devise a table from which the relative concentrations of enolase and nucleic acid could be obtained from the measured absorbance at 280 and 260 m\(\mu\) of any mixture of the two substances. This table can be used for determining the protein and nucleic acid concentration of a virus by assuming that the virus solution contains only the
pure virus, and that the virus protein has identical ultraviolet absorption characteristics to that of enolase. The electron microscope can be used to study the morphology of the virus particles. Particles are characterized by their size, shape and ability to withstand distortion by specific preparation procedures. In serology, a virus suspension is used to induce the production of antiserum in the blood of warm-blooded animals. The antiserum will react specifically against the type of proteins present in the original suspension. By this means it is possible to determine if the virus solution contains host protein impurities, and also, if the virus is contaminated by, or related to, other known viruses.
MATERIALS AND METHODS

Virus Isolation and Culture

The virus used in the early work was obtained from Dr. J. A. Milbrath of Oregon State University, and was the culture used by Halliwell (14) in his work. Since it appeared that this virus source may have been contaminated with tobacco and/or tomato ringspot virus, another source of the rose mosaic virus was sought. The virus designated as RDRMV used in most of this work was obtained directly from rose. A rose plant, Rosa delecta var. Red delight, with severe symptoms of the rose mosaic virus disease, was obtained from the Chase Gardens in Eugene, Oregon. The virus was transmitted mechanically from the rose to Bansie soybean in the following manner: One gram of young rose leaves was rapidly frozen in liquid nitrogen and then crushed to a fine powder with a sterilized mortar and pestle (22). The powder was wiped onto carborundum-dusted Bansie soybean leaves with a sterilized pestle and the leaves then were rinsed with water. The virus could also be transmitted, but not as successfully, by grinding the rose leaves in a 0.5 percent (w/v) Na₂HPO₄ and quickly wiping the suspension onto the carborundum-dusted soybean leaves with a pestle.

The virus was increased in the soybean plants in a greenhouse kept at a daytime temperature of about 24°C and a night time
temperature of about 15°C. The soybeans were planted in a sandy loam soil, in No. 10 cans. Fifteen to twenty-five seeds were tamped into the soil in each can and then covered with a one-half inch layer of sand. In the winter the soybeans were ready for inoculation 14-15 days after planting. The time was somewhat shorter in the summer.

**Inoculum Preparation**

Six to seven days after the soybean plants were inoculated, 100 g of leaves were mixed 1:1 or 1:2 (w/v) with 0.05 M borate buffer, pH 9.5. The mixture was ground in a blender and the juice was expressed through two layers of cheesecloth to remove the large pieces of plant tissue. This inoculum was used immediately after preparation.

**Infectivity Assay**

Virus infectivity was determined by assay on two host plant species. *Momordica balsamina* developed necrotic local lesions on the primary leaves 5-6 days after inoculation. The number of lesions was counted and the average number per leaf was reported. However, the *M. balsamina* was not a dependable local lesion host. Sometimes inoculated *M. balsamina* had no lesions at all, even with a known high concentration of virus. Therefore it was necessary to use the Bansie soybean as a systemic assay host for many of the experiments. A positive assay was the death of the terminal tips of the plants about
8-10 days after inoculation with the virus.

Purification Experiments

The inoculum described above was used in all purification experiments unless otherwise noted. A Servall angle centrifuge was used for low speed centrifugation, 15 minutes at 5000 rpm (3500 g) for small samples, and a Servall Superspeed centrifuge, model SS-3, 15 minutes at 4000 rpm (1980 g) was used for large samples. A Spinco Model L centrifuge was used for all high speed centrifugations. A No. 40 rotor, one hour at 38000 rpm (95300 g) was used if the sample did not exceed 144 ml, and a No. 30 rotor, one hour at 2900 rpm (73500 g) was used for larger samples.

Density gradient columns were prepared about one hour before centrifugation in the following manner: A 40 percent (w/v) and a 20 percent (w/v) solution of sucrose in distilled water was used to prepare a continuous gradient column in 3 x 1 inch plastic centrifuge tubes with a density gradient former. Then five ml of the virus suspension were floated on top of each gradient and centrifuged at 24500 rpm (51,700 g) for four hours in the SW 25.1 rotor of the Spinco Model L Ultracentrifuge. Afterward, opalescent bands were detected by shining a light vertically through the tubes. These bands were removed from the tubes with a syringe and a hypodermic needle which was bent at a right angle.
Absorption Spectrophotometry

The ultraviolet absorption spectra of the partially purified virus solution was measured with a Beckman DB spectrophotometer. The absorption was measured relative to the solvent in which the virus was suspended. The height of the absorption peaks at 260 and 280 mµ was measured and, with the aid of a nomograph devised by E. Adams (1), based on the work of Warburg and Christian (37), the approximate nucleic acid and protein content of the sample was calculated.

Electron Microscopy

Specimen grids were prepared in the following manner. A single drop of the purified virus in borate buffer was placed onto a Formvar- or collodion-covered grid. A single drop of a 1:2000 polystyrene latex suspension was added as an internal size standard. The latex particles had a mean diameter of 264 mµ. After standing several minutes, drops were pulled down to a thin film, then allowed to air dry. They were shadowed with a platinum-palladium alloy and were examined with a RCA type EMU2-D electron microscope.

Serology

Rose mosaic virus antiserum was obtained from domestic rabbits in the following manner. About 20 ml blood was taken from an
untreated rabbit, and the serum was separated and stored in a refrigerator to be used as a "normal serum" control. The rabbit was injected with two ml portions of partially purified virus in borate buffer solution every other day for two weeks. Then 10-20 ml blood was taken from the rabbit and the antiserum separated. This antiserum was exposed to various antigens, purified virus, host material, and other viruses, in the Ouchterlony Agar Double-Diffusion test (2). In this test the antibodies and antigen diffuse through the agar and after a suitable incubation period, precipitates are formed if the serological test is positive.
Attempts were made to find local lesion and systemic hosts for the rose mosaic virus, RDRMV. Of the plants tested, only black cowpea (*Vigna sinesis*), Bansie soybean (*Glycine max*), *Chenopodium amaranticolor*, and *Momordica balsamina* gave any definite symptoms. Black cowpea showed both local lesion and systemic reactions, but the reaction was too undependable for the plant to be used either for virus propagation or virus assay. *C. amaranticolor* showed a systemic reaction, but it required 3-4 weeks for the symptoms to appear. Soybean showed a systemic reaction within 6-8 days; hence it was selected as a propagation and systemic host for the virus. When inoculations were made on sunny days, fewer soybeans showed symptoms. *M. balsamina* showed only local lesions, which usually appeared within 5-6 days after inoculation. Sometimes *M. balsamina* had no symptoms at all, but it was the best available local lesion assay host. When the virus-inoculated controls showed no symptoms, the experiment had to be discarded. The unsusceptible species tested are listed below: APONCYNACEAE: *Vinca rosa*; CHENOPODIACEAE: *Chenopodium quinoa*, *Chenopodium alba*; COMPOSITAE: *Calendula officinales*, var. Pacific beauty, *Helianthus annus*; CRUCIFERAЕ: *Brassica campestris*, *Brassica rapa*, var. Purple top,
PHYSICAL CHEMICAL PROPERTIES

The RDRMV inoculum was prepared by the method described in the materials and methods section, and then the following properties were determined.

Dilution End Point

The dilution end point was about $10^{-2}$ since only 30 percent of the plants were infected after inoculation with virus at this dilution. No plants were infected with dilution $10^{-3}$ or greater.

Thermal Inactivation Point

The RDRMV survived ten minutes at $50^\circ$, but not ten minutes at $55^\circ$. Further attempts to obtain a more exact thermal end point failed because the indicator plants apparently were not susceptible on the day that the inoculations were made.

Longevity in Vitro

Two kinds of experiments were made for the resistance to inactivation in vitro. In the first, the samples of extracted juice were kept in distilled water at room temperature and the activity tested every half hour. In the second, the crude sap in borate buffer was centrifuged for 15 minutes at 7600 rpm and then stored in a refrigerator.
at 4.4⁰ C. The activity was tested at frequent intervals for 48 hours. The virus remained active in vitro for two hours at room temperature. Longevity was increased to 24 hours when it was stored in buffer at 4.4⁰ C.
VIRUS PURIFICATION

Buffer Selection

Attempts were made to determine the type, concentration and pH of buffer which would have the greatest preserving effect on the activity of the virus. Leaves from infected plants were homogenized in the desired buffer and kept at 4°C. Inoculations were made on soybean and M. balsamina after short intervals of time. Table I shows the effect of type and concentration of buffer on the infectivity of the rose mosaic virus. The virus was most stable in borate buffer containing 0.05 M borate ion. Table II shows the effect of pH of the 0.05 M borate buffer on infectivity of the virus. The virus was most stable in buffer solutions of pH 9.5.

TABLE I. EFFECT OF BUFFER KIND AND CONCENTRATION ON VIRUS INFECTION.

<table>
<thead>
<tr>
<th>Buffer, pH 7.0</th>
<th>Percent soybean plants showing symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 1/2 hour</td>
</tr>
<tr>
<td>Distilled water</td>
<td>56</td>
</tr>
<tr>
<td>0.10 M phosphate</td>
<td>71</td>
</tr>
<tr>
<td>0.02 M phosphate</td>
<td>75</td>
</tr>
<tr>
<td>0.50 M phosphate</td>
<td>0</td>
</tr>
<tr>
<td>0.05 M borate</td>
<td>82</td>
</tr>
<tr>
<td>0.10 M borate</td>
<td>90</td>
</tr>
<tr>
<td>0.50 M borate</td>
<td>78</td>
</tr>
</tbody>
</table>
TABLE II. EFFECT OF pH OF 0.05 M BORATE BUFFER ON VIRUS INFECTIVITY.

<table>
<thead>
<tr>
<th>Buffer pH</th>
<th>Soybean % plants showing symptoms</th>
<th>M. balsamina Average lesions per leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 1/2 hr</td>
<td>After 1 1/2 hr</td>
</tr>
<tr>
<td>7.5</td>
<td>89</td>
<td>41</td>
</tr>
<tr>
<td>8.5</td>
<td>86</td>
<td>21</td>
</tr>
<tr>
<td>9.5</td>
<td>82</td>
<td>3</td>
</tr>
</tbody>
</table>

Presence of Reducing Agent

The sap from the homogenized infected leaves turned dark upon standing. Since it was presumed that this darkening was due to oxidation, attempts were made to find a reducing agent which would prevent the darkening. Sodium sulfite, 0.05, 0.1 and 0.5 M; 0.01 M cysteine hydrochloride, 0.01 M sodium diethyldithiocarbamate, and 0.01 M thioglycollic acid were tested; only the thioglycollic acid prevented the darkening and preserved the virus activity.

Clarification of Plant Sap

Various methods for clarifying the sap from the infected leaves were tried. Since this was a very critical step in the purification procedure, the clarification methods and results are described in some detail.
Hydrated Calcium Phosphate

Because of previous reports (10; 14) that the rose mosaic virus could be clarified by adsorption on hydrated calcium phosphate (HCP), this method was tried to provide a reference for other methods. Fulton's (10) procedure was followed. HCP was prepared by mixing equal volumes of 0.1 M Na₂HPO₄ and 0.1 M CaCl₂. This produced a milky suspension, which, after ten minutes flocculated into large enough particles to be filtered on a Büchner funnel. The precipitate was washed with distilled water, and the resulting paste had the consistency of cold cream. Systemically infected leaves were ground in phosphate buffer (1:1 1/2 w/v) which contained 0.01 M sodium di-ethyldithiocarbamate and 0.02 M sodium thioglycolate. The mixture was expressed through cheese cloth and the juice was centrifuged for 15 minutes at 1980 g. The HCP paste was added to the plant sap in a ratio of 6 ml to 10 g of original tissue and stirred for five minutes and then centrifuged for 15 minutes at 1980 g. The supernatant was a light green color. This was inoculated onto both soybean and M. balsamina indicator plants. Table III gives the results of the infectivity assay. The infectivity of the virus was completely lost during the treatment with the HCP.
### TABLE III. EFFECT OF CLARIFICATION TREATMENTS ON VIRUS INFECTIVITY.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Date</th>
<th>Soybean % plants infected Before treatment</th>
<th>Soybean % plants infected After treatment</th>
<th>M. balsamina Ave. lesions per leaf Before treatment</th>
<th>M. balsamina Ave. lesions per leaf After treatment</th>
</tr>
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<tbody>
<tr>
<td>HCP</td>
<td>13 Apr.</td>
<td>90.0</td>
<td>0</td>
<td>3.3</td>
<td>0</td>
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<tr>
<td></td>
<td>16 Apr.</td>
<td>86.6</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>Ether-CCl₄</td>
<td>7 Apr.</td>
<td>100.0</td>
<td>100.0</td>
<td>7.0</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>13 Apr.</td>
<td>81.5</td>
<td>37.6</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>22 Apr.</td>
<td>96.0</td>
<td>87.5</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>26 Apr.</td>
<td>89.5</td>
<td>95.0</td>
<td>---</td>
<td>---</td>
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<tr>
<td>Celite-Charcoal</td>
<td>19 Feb.</td>
<td>---</td>
<td>---</td>
<td>5.8</td>
<td>12.0</td>
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<tr>
<td>Charcoal</td>
<td>25 Dec.</td>
<td>---</td>
<td>---</td>
<td>6.2</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>26 Jan.</td>
<td>70.0</td>
<td>100.0</td>
<td>---</td>
<td>---</td>
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<tr>
<td></td>
<td>10 Feb.</td>
<td>87.5</td>
<td>77.8</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>15 Feb.</td>
<td>100.0</td>
<td>89.0</td>
<td>---</td>
<td>---</td>
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<tr>
<td></td>
<td>17 Feb.</td>
<td>69.0</td>
<td>91.0</td>
<td>---</td>
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<tr>
<td></td>
<td>25 Feb.</td>
<td>77.9</td>
<td>77.8</td>
<td>---</td>
<td>---</td>
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<tr>
<td></td>
<td>5 Mar.</td>
<td>11.1</td>
<td>75.0</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Ether-Carbon tetrachloride

Following the procedure of Wetter (32) for tobacco rattle virus, 25 percent ether and 25 percent CC1₄ were shaken for ten minutes with the infected plant sap in borate buffer. The light tan aqueous layer was centrifuged and the supernatant was assayed. Table III gives the results of the assay of the supernatant of several experiments. The ether-CCl₄ treatment clarified the plant sap and also preserved the rose mosaic virus infectivity.
Butanol

Using the method described by Gooding (13), a 7 percent (v/v) butanol-plant sap solution was stirred for one-half hour in a 40°C water bath. The suspension was centrifuged at 3500 g for 15 minutes to remove the host material. The light tan supernatant was stirred for another one-half hour on the water bath, centrifuged again and, the clarified supernatant was assayed. The butanol treatment completely inactivated the virus.

Chloroform-Butanol Emulsion

Following the method of Steere (24), two volumes of 1:1 chloroform-n butanol solution was added to one volume of plant sap and stirred for 15 minutes. The emulsion was broken by centrifugation at 3500 g and the greenish-brown aqueous layer was removed by decantation and assayed on indicator plants. The rose mosaic virus was completely inactivated by this method.

Charcoal and Celite-Charcoal

Following the suggestion by Corbett (8) that activated charcoal was a good clarification agent for several virus suspensions, activated charcoal (Norite A) was added to the expressed sap and the mixture was stored in the refrigerator for 30 minutes. The mixture
was centrifuged at 1980 g for 10-15 minutes and the supernatant was assayed for infectivity. Table III shows the results of seven experiments with charcoal. Not only did the charcoal preserve the infectivity of the rose mosaic virus, but the charcoal also appeared to increase the virus infectivity. Experiments were performed using celite and then charcoal to clarify the plant sap. The result, as noted in Table III, was about the same as with the charcoal alone.

**Modified Charcoal-Ether-CCl₄ Method**

A modified method for the clarification of the infected plant sap was devised. In this method 100 g of infected soybean leaves were homogenized in 100 ml of 0.05 M borate buffer, pH 9.5, containing 10 g activated charcoal and 0.002 moles thioglycollic acid. The homogenate was expressed through one layer of cheesecloth and the filtrate was centrifuged at 1980 g for 15 minutes and then at 7900 g for five minutes. The supernatant was shaken with 25 percent anhydrous ether and 25 percent CCl₄, and then centrifuged at 1980 g for 15 minutes.

**Concentration**

After the virus suspension was clarified, it was concentrated by alternate low and high speed centrifugations. The final pellet was resuspended in borate buffer, layered on a sucrose density gradient and centrifuged at 51700 g for four hours. Three distinct bands,
separated by transparent zones, were formed during the centrifugation. The lowest band (designated zone 1) contained the infective virus. This band was centrifuged at 95300 g for two hours and the resulting pellet was resuspended in borate buffer and layered on 30 percent sucrose. Two bands were formed, designated zone 1b and zone 2b.

**Purification Procedure**

A summary of the procedure by which RDRMV could be successfully purified is given in Figure 1. Table IV shows the results of the stepwise infectivity assays of four different purification experiments, which followed the procedure given in Figure 1. The assay on soybean indicated that the virus was concentrated without a loss of infectivity, but that 20-30 percent of the infectivity was lost in the density gradient centrifugation. The assay on M. balsamina indicated that the infectivity of the virus was increased about six times in the purification procedure, before the density centrifugation was made.
Purification of Rose Mosaic Virus

Leaves homogenized in 0.05 M Borate Buffer (pH 9.5) containing 0.02 M thioglycollic acid and 10 percent activated charcoal

Homogenate expressed through 1 layer cheesecloth

(1) filtrate

- centrifuged 4000 rpm 15 min — discard residue
  - centrifuged 8000 rpm 5 min — discard residue

(2) 25 percent anhydrous ether

- shake 5 min
  - 25 percent CCl₄
    - shake 5 min

- centrifuged 4000 rpm 15 min — discard residue
  - centrifuged 8000 rpm 5 min — discard residue

(3) supernatant

- centrifuged 38,000 rpm 60 min — discard supernatant

(4) resuspend in 0.05 M Borate Buffer (pH 9.5) 1 hr

- centrifuged 3500 rpm - 5 min — discard residue

(5) supernatant

- density gradient centrifugation - 24,500 rpm - 4 hrs

(6) zone 1

- centrifuged 38,000 rpm - 2 hrs — discard supernatant

(7) pellet

- resuspend in 0.05 M Borate Buffer (pH 9.5) 1 hr
  - centrifuged 38,000, sw 39 on 30 percent sucrose

(8) purified virus zones

Figure 1. Outline of procedure used for the purification of rose mosaic virus (RDRMV).
### TABLE IV. PURIFICATION OF ROSE MOSAIC VIRUS. INFECTIVITY ASSAY.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>% soybean plants infected or ave. les./leaf on M. balsamina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>Homogenate in buffer (control)</td>
<td>---</td>
</tr>
<tr>
<td>Homogenate in buffer with charcoal</td>
<td>100%</td>
</tr>
<tr>
<td>After ether-CCl₄ clarification</td>
<td>100%</td>
</tr>
<tr>
<td>After high-low speed cycles</td>
<td>100%</td>
</tr>
<tr>
<td>Zone 1</td>
<td>Used for Producing Antiserum</td>
</tr>
<tr>
<td>Zone 2</td>
<td>0</td>
</tr>
<tr>
<td>Zone 3</td>
<td>0</td>
</tr>
</tbody>
</table>
ULTRAVIOLET ABSORPTION

The ultraviolet absorption of the rose mosaic virus suspension was measured with a Beckman DB spectrophotometer. The absorption spectra of the purified virus from zone 1 is shown in Figure 2, curve B. This is compared with the spectra of the healthy host supernatant from high speed centrifugation in Figure 2, curve A. Both preparations showed curves typical of nucleo-proteins with maxima near 260 mւ and minima at 235-240 mւ.

The percent ribonucleic acid (RNA) and protein were calculated from the absorption of 280 mւ and 260 mւ, using the nomograph devised by E. Adams (1). The healthy host preparation contained 37.1 percent RNA and 62.9 percent protein; the purified virus preparation contained 24.8 percent RNA and 75.2 percent protein.

To check the validity of this method of determining the percent RNA, the absorption spectra of tobacco mosaic virus, purified by alternate centrifugation and density gradient centrifugation, was obtained, and the percent RNA calculated. The following values of percent RNA were obtained when the TMV was suspended in the given solvents: in phosphate buffer, 5.2, 6.0, 6.35; borate buffer, 5.5; sucrose, 4.5, 5.6 percent. This was compared with the value given by Knight (18) of 5.0 percent RNA.
Figure 2. A healthy host preparation (A) and an ultraviolet absorption spectra of a purified preparation of Rose Mosaic Virus (B).
Portions of the purified virus preparations from steps 7 (zone 1) and 8 (zones 1b, 2b) of the purification procedure (Figure 1) were examined with the electron microscope. The resulting photomicrographs showed spherical particles of various sizes. Figure 3 shows an enlargement of one of the photomicrographs of the preparation taken from zone 1 in the purification procedure.

The size of each particle was measured when projected on a 5 x 5 foot screen. The numbers of particles measured were: zone 1, 67; zone 1b, 54; zone 2b, 14. The particle sizes were sorted into 5 mµ categories and are shown in histograms in Figure 4, A-C. In zone 1 there were two size distribution maxima, one at 20-25 mµ, the second at 35-40 mµ. In zone 1b there were also two size distribution maxima; one at 25-30 mµ, the second at 35-40 mµ. In zone 2b there was 1 maximum, between 35-45 mµ.
Figure 3. Electron micrograph of the partially purified preparation of rose mosaic virus (RDRMV).
Figure 4. Particle size distribution of RDRMV. (A) zone 1, (B) zone 1b, (C) zone 2B.
SEROLOGY

Antisera were produced to the purified virus preparation from step 4 of the purification procedure (Figure 1). The antisera reacted against the soybean host material, indicating that the original virus preparation contain host proteins. Most of the host antibodies were removed from the antisera by absorbing them with a healthy pellet obtained by carrying healthy host material through the purification procedure to step 4. The host material and the absorbed antibodies were removed by centrifuging at 3500 g for 15 minutes. The resulting antisera reacted with purified and crude extracts of the red delight rose mosaic virus (RDRMV).

Many attempts were made to determine if the RDRMV virus was serologically related to any of the viruses available in this laboratory. The viruses and antisera used were obtained from Dr. John A. Milbrath, except the Ford Tom RSV and antiserum that were obtained from Dr. Richard Ford, Oregon State University. The results are shown in Table V. From these results it is not possible to tell whether there is any definite serological relationship between the RDRMV virus and any other known virus.
TABLE V. NUMBERS OF POSITIVE AND NEGATIVE SEROLOGICAL REACTIONS BETWEEN TRS, Tom RSV, NRS, AND RM VIRUSES + ANTISERA (+ INDICATES A POSITIVE REACTION AND - INDICATES A NEGATIVE REACTION).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Antiserium Tom RS</th>
<th>NRSV</th>
<th>RMV</th>
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<tbody>
<tr>
<td></td>
<td>Ford's TRSV</td>
<td>TRSV</td>
<td>TRSV 17ao</td>
</tr>
<tr>
<td>TRSV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grogan's TRSV</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>TRSV 17ao</td>
<td>1</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>Tom RSV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McWhorter's Tom RSV</td>
<td>3</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>Milbrath's Tom RSV</td>
<td>1</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>Grogan's Tom RSV</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Ford's Tom RSV</td>
<td>0</td>
<td>1</td>
<td>*</td>
</tr>
<tr>
<td>PRSV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prunus RSV</td>
<td>0</td>
<td>1</td>
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</tr>
<tr>
<td>RMV</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Halliwell's RMV</td>
<td>1</td>
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<td>1</td>
</tr>
<tr>
<td>S1N-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milbrath's RMV</td>
<td>1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Red Delight RMV</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* indicates no trial
DISCUSSION

The primary objectives of this work have been partially fulfilled. A virus has been isolated from roses, but it has not been determined whether or not this virus definitely is free from other contaminants because the serological tests are inconclusive. A method has been found for purifying the virus. The percent RNA and protein in the virus preparation have been determined, and it has been shown that the virus particles have a size distribution with two maxima. An antiserum to the virus has been developed, but it has not been shown conclusively whether or not the virus is related to other known viruses.

The RDRMV could not be purified by the method used by Halliwell (14) and Fulton (10) because the HCP either absorbed the virus particles, or the virus particles were entrained by the absorbed host material. In either case, the virus was removed from suspension by the HCP.

It is believed that the enhancing effect of charcoal on the infectivity of the virus was caused by the charcoal absorbing inhibitors from the virus suspension. The ether-\text{CCl}_4 may also have been effective in removing virus inhibitors.

The percent RNA calculated for the RDRMV of 24.8 percent is of the same order of magnitude as that given by Knight (18) for other spherical mosaic viruses. On the other hand, the value obtained for
the RDRMV was not similar to the 13.0 percent calculated from the absorption spectra given by Halliwell (14, p. 28).

The particle size distribution of three preparations of the RDRMV gave a maximum at 35-40 m\(\mu\). The distribution of two of the preparations gave also a maximum at 20-30 m\(\mu\). These sizes are similar to the two particle sizes found by Williams, et al. (37). It is possible that the RDRMV contains two different viruses, or that the rose mosaic virus occurs in two sizes. Weintraub (31) found that the particle size distribution of Tom RSV gave two maxima (25-30 m\(\mu\) and 45-50 m\(\mu\)) when the virus was obtained from Gomphrena and tobacco; but that just one maximum (25-39 m\(\mu\)) occurred when the Tom RSV was obtained from petunia and cucumber.

The size distribution of all of the particles in the photomicrograph of Halliwell's thesis (14, p. 29) showed a maximum at 25-35 m\(\mu\). The size distribution of the particles obtained by Halliwell is not the same as the size distribution in the RDRMV.

The serological relationships between the RDRMV and other known viruses presents a confusing picture. The RDRMV antiserum reacts with Grogan's Tom RSV, but this virus also reacts with Milbrath's TRSV antiserum. RDRMV reacts with Milbrath's TRSV and Tom RSV antisera, but not with Ford's TRSV or Grogan's Tom RSV antisera. Although time was not available, it would be well to use additional samples of known viruses and virus antisera.
Suggestions for Further Work

After the course of this investigation was reviewed, several additional experiments could be suggested:

1. The greatest difficulty encountered in this work was in obtaining the assay for virus infectivity. This is the greatest need for further work -- to determine the conditions under which the indicator plants produced symptoms when inoculated with the virus. There appeared to be many factors, other than concentration of virus, which affected the appearance of virus symptoms on indicator plants. These factors should be studied systematically, and the conditions found under which the indicator plants are most susceptible to producing the symptoms of virus infection. Some of these factors are the following: temperature, amount of light received by the plants, time of day, position on the leaf inoculated, maturity of the leaf, position of the leaf on the stem, and position of the leaf in its daily movement cycle.

2. Work should continue in finding a better local lesion host and then a study should be made relating the number of virus particles in a given preparation to the number of lesions produced.

3. Since it appeared that the virus preparation contained two
different size particles, it would be profitable to attempt to
separate the particles (perhaps by repeated density gradient
centrifugation), and then study the properties of each. It is
conjectured that the small particles are a strain of TRSV and
the large particles are a strain of rose mosaic virus. If this
were correct, it would answer some of the questions that have
been raised by the serology studies of this and earlier work.
It would also be interesting to determine if one or both of the
particles is necessary to produce the symptoms of the rose
mosaic disease in roses.

4. More work could be done on the mechanical transmission of
the rose mosaic virus from roses to a herbaceous host. This
could include a study of the factors which affect transmission,
such as time of year and maturity of the roses and herbaceous
plant.

5. Further attempts should be made to produce antisera with a
high concentration of virus antibodies. If this cannot be done,
perhaps the antisera could be concentrated by precipitating
the antibodies with ammonium sulfate. If a concentrated anti-
sera could be obtained, field testing of roses for latent
virus may be initiated.
SUMMARY

1. A rose mosaic virus (RDRMV) was isolated from a rose plant, *Rosa dillecta*, var. Red Delight by mechanical transmission to Bansie soybeans.

2. The virus was purified by homogenizing infected soybean tissue in pH 9.5 borate buffer containing thioglycollic acid and activated charcoal, clarifying the filtrate with ether-CCl₄, and concentrating the suspension by alternate high and low speed and density gradient centrifugation.

3. The ultraviolet absorption of the purified virus preparation was measured and the percent RNA and protein were calculated as 24.8 and 75.2 percent respectively.

4. The virus suspension consisted of spherical particles, with two size ranges, 20-30 mµ and 35-40 mµ, as determined by electron microscopy.

5. An antiserum to the virus was prepared.

6. Attempts were made to determine the serological relationship of the virus to other known viruses, but the results were not conclusive.
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