PATHOLOGICAL AND ELECTRON MICROSCOPE STUDIES OF VARIOUS PLANT VIRUSES

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By

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INTRODUCTION

The first part of this report is concerned with the determination of the host ranges and the identity of three virus diseases of leguminous crops grown in Oklahoma.

The virus diseases studied were those of crotalaria, sesbania, and cowpeas. All of these legumes are of some economic importance in Oklahoma. Both crotalaria and sesbania are grown in Oklahoma as soil building green manure crops and though at present they are not grown extensively, they are becoming more widely used for this purpose. Cowpeas have been grown in Oklahoma for many years for soil improvement. They are also harvested for hay and for their seeds as well as being used for pasture.

Little research has been done on the viruses attacking either crotalaria or sesbania, but several virus diseases of cowpeas have been investigated and reported. In this study an attempt has been made to identify the three viruses and to determine whether they are among those already known or whether they are new to science.

The second part of the report is concerned with the use of the electron microscope in studying the morphology and structure of various plant viruses. In this section the necessary steps and stages in the purification and preparation of the viruses are discussed, and there is a general discussion of the techniques involved in this type of research.

The development of the electron microscope in the past few years has made it possible actually to see the form and structure of viruses and has opened up a whole new field of virus research.

CROTALARIA MOSAIC

Economic Importance of Host.

Crotalaria spp. generally known as rattlebox, has assumed economic and commercial importance in the United States in the last twenty years. The plant has been grown in India and other tropical areas for much longer periods. Three species are grown commercially in the United States, Crotalaria spectabilis Roth, C. striata D.C. and C. intermedia Kotschy (McKee and Pieters, 1937).

In Oklahoma, Crotalaria spectabilis is used as a green manure crop, but cannot be used for hay as most of the species are poisonous to livestock (Ligon, 1947). The fact that most species are poisonous to livestock makes the plant especially useful for the purpose of building up the soil, as farmers growing the crop must plow it under and cannot successfully use it as a hay crop. It is also useful in ridding the soil of nematodes as they are unable to live on Crotalaria. In addition to this the Crotalaria plant supports the growth of certain organisms which are the natural enemies of nematodes.

A virus disease of this plant would therefore cut down on the amount of foliage produced and thus limit the value of the plant as a green manure crop, as well as limiting its value for nematode control work. Further, depending on the host range of Crotalaria mosaic, the yields of other plants and crops of economic importance might be reduced by infection with the Crotalaria mosaic virus.

Source of Virus.

The Crotalaria virus under consideration was discovered by K. Starr Chester. It had been present for several years in the experimental plots

at Stillwater, Oklahoma. Five field plants were found to be infected with a bright yellow mosaic, accompanied by severe stunting and some distortion. These plants were potted and placed in the greenhouse and preliminary infection tests were made by Chester. Tests on Turkish tobacco gave no infection, but inoculations into five plants of C. spectabilis Roth in a period of approximately three weeks yielded one plant with symptoms of a bright yellow mosaic and one with symptoms of a green mosaic. Investigations were carried out with the bright yellow mosaic exclusively.

Review of the Literature.

Little has been reported on mosaic diseases of Crotalaria. Freeman Weiss of the United States Department of Agriculture has informed me that he has received reports of mosaic disease on Crotalaria mundyi, C. spectabilis Roth, C. striata D.C. (= C. mucronata Desv.), and C. usaramoensis Baker in Oklahoma. According to Weiss this virus has not been identified. He also reports a mosaic disease of C. spectabilis in Texas which is attributed to a pea virus. A mosaic of the following Crotalaria spp. characterized by mottling, leaf distortion, and blistering, stunting and shoot proliferation occurred in test plots in Virginia: C. incana L., C. intermedia Kotschy, C. lanceolata E. Mey., C. maxillasis Klotsch, C. spectabilis Roth., C. striata D.C. (= C. mucronata Desv.), and C. usaramoensis Baker. This mosaic is communicable to Vicia faba by juice inoculation (Johnson and Lefebvre, 1938). A mosaic disease of C. mucronata occurs in Puerto Rico which is not seed borne (Cook, 1931). A mosaic disease of C. juncea has been reported in Japan and one of C. saltiana Andr. in China. Zaumeyer (1940) induced a mosaic in C. retusa L., C. spectabilis and C. mucronata by artificial inoculation with one or another of the following viruses: pea mosaic 4 and 5, alsike clover mosaic 1 and 2.

Materials and Methods.

In attempting to determine the host range of Crotalaria mosaic the following plants were used: Crotalaria incana, Jimson weed, Datura stramonium L., tobacco, Nicotiana tabacum L. var. Turkish, Chinese and Groit cowpeas, Vigna sinensis (Tomer) Hassk. tomato, Lycopersicon esculentum Mill. and Vicia faba L.

In all cases inoculations were made in the following manner: The juice from a diseased crotalaria plant was expressed by grinding some of the leaves with a mortar and pestle. To this juice, in order to increase the volume to some extent and also to hold the virus in a neutral medium, was added a few c.c. of a potassium phosphate buffer solution of pH 7, made according to the directions of Clark and Lubs. A bent glass spatula bound at the bent and flattened end with cheese cloth was then dipped in the solution. The leaves of the plant to be inoculated were then dusted with Celite 535 in order to facilitate the entrance of the virus into the host plant, and the spatula, wet with the virus solution, was gently rubbed across two or three of the leaves. After a period of a minute or two the leaves were sprinkled with water to wash away the plant materials contained in the inoculum and further facilitate the entrance of the virus into the plant cells. The plants were then observed periodically for as long as two months in the case of Jimson weed.

Results of Inoculations.

Crotalaria incana L.

On March 17, 1947 ten plants of Crotalaria incana were inoculated with Crotalaria mosaic of the yellow mosaic type. On the 10th of April four of these plants showed symptoms of the disease which soon became pronounced.

The mosaic consisted of a bright yellow mottle, producing some distortion of the leaves and stunting. The yellow areas in general consisted of bands or streaks running approximately perpendicular to the long axis of the leaf.

Attempts were made to transmit the disease from the four infected plants by means of a dodder bridge to other healthy plants. Dodder, Cuscuta campestris Yuncker, was allowed to grow on the diseased plants until it had made a strong connection. It was then allowed to make a similar connection with healthy Crotalaria plants and after a period of from three days to a week the connection was broken. In no case was the virus transmitted through the dodder.

Datura stramonium L.

On March 17, 1947 one half of a flat containing more than 60 Jimson weed plants was inoculated with Crotalaria mosaic using the method of inoculation already described. The half of the flat which was not inoculated served as check plants.

These plants were observed periodically until May 21, 1947, or over a two months period. At this time the plants showed no symptoms of infection and were discarded.

Nicotiana tabacum L.

Seven plants of Turkish tobacco were inoculated with Crotalaria mosaic on March 1, 1947. After observation for a period of six weeks no symptoms of infection appeared and the plants were discarded.

Vigna sinensis (Torner) Hassk.

One quarter of a flat of Groit cowpeas and one quarter of a flat of Chinese cowpeas were inoculated with Crotalaria mosaic on March 1, 1947, and observed for a six weeks period. These were discarded at the end of the period as no symptoms were in evidence.

Lycopersicon esculentum Mill.

On March 1, 1947, one half a flat of tomatoes were inoculated with Crotalaria mosaic and observed for a period of five weeks. The plants were totally symptomless at the end of this time and were discarded.

Vicia faba L.

Ten broadbean plants were inoculated with Crotalaria mosaic and observed for a period of three weeks. No infection had occurred at the end of this time, and the plants were discarded.

Discussion.

As already pointed out, little pathological work has been done with any of the viruses of Crotalaria. It is impossible to determine whether or not this is the same virus of Crotalaria as that reported in the "Plant Disease Reporter" from Texas and Oklahoma, as no description of this mosaic is given in the literature. It is apparently not the same virus as that reported from Virginia in the "Plant Disease Reporter", for it does not produce blistering or shoot proliferation, and does not infect Vicia faba. From all that can be determined this Crotalaria mosaic is heretofore undescribed in any of the literature. It is regrettable that inoculum of this virus could not be preserved, but the virus was lost due to root disease in the host plants.

SESBANIA MOSAIC

Economic Importance of Host.

Sesbania spp. is grown as a soil building green manure crop (Ligon, 1947). At present it is grown only on limited areas throughout the State of Oklahoma and when closely drilled, produces 14 tons of green matter per acre or about 4 tons of dry matter. Here again, as with Crotalaria, a virus disease would in all probability reduce the amount of green manure produced and would be of further possible economic importance depending on whether or not it attacked other crop plants.

Review of the Literature on Sesbania Mosaic.

Very little pathological work has been done on the virus diseases of Sesbania. Weiss in "The Plant Disease Reporter" does not list any virus diseases of this plant, and Sesbania has received little notice in the general literature. At present Sesbania is cultivated on a comparatively small scale and for this reason, if no other, has received very little attention. Johnson (1942), however, in his work in the viroplasm hypothesis found a virus disease of Sesbania macrocarpa Muhl. which was symptomless on the host plant but which caused virus infection in soybeans. Johnson has not included any description of the symptoms caused by this virus on the soybean.

Review of the Literature on Soybean Mosaic.

As shown by the host reactions which follow, Sesbania mosaic was found to infect Ogden soybeans. The possibility exists, therefore, that the mosaic originally found in Sesbania may be an already recognized virus disease of soybeans and a review of the literature on soybean mosaic is therefore presented.

Soybean mosaic was first found in this country about 1915. This virus has been described by Gardner and Kendrick (1921) as producing stunting of the plants and petioles and shortening of the internodes to some extent. Leaflets are stunted, misshapen, and puckered with dark green puffy areas along the veins. The leaf is etiolated between these puffy areas and the leaflets tend to be asymmetrical, twisted, and curled downward about the margins. In addition to these typical mosaic symptoms, they further found a bronzing of the young leaves produced by a brown discoloration of short segments of the veins, and large splotches on the older leaves produced by a lace-like yellowing or browning of the veins (Kendrick and Gardner, 1924).

Reports of virus diseases of soybeans and the damage caused by the virus come from all over the world. Heinze and Köhler (1940) report a mosaic of soybeans in Germany which is closely related to or identical with that described for North America. A serious mosaic of soybeans is reported from Uganda, Africa, causing severe stunting of the plants with thickening, wrinkling and curling of the leaves (Hausford, C.G., 1934). In Shantung Province, China, infection of soybeans ranges as high as 100%. This soybean virus is undescribed but reported as being important in all soybean-growing regions of the country (Yu, 1939). Soybean mosaic has also been found in Quebec, Canada (Dickson, 1924), and in Rumania by Savulescu, Sandu-Ville, Aronescu, and Alexandri (1936). These Rumanian workers found that soybeans suffered losses in many districts from three forms of virus diseases: leaf curl, brown mosaic, and yellow mosaic.

Pierce (1935) working with a soybean virus found that the following hosts were not susceptible to the virus: Pisum sativum, Phaseolus vulgaris, Vicia faba, Melilotus officinalis, Trifolium pratense, Trifolium repens, Medicago sativa, Nicotiana tabacum, and Petunia hybrida. He describes the virus as being characterized by mottling of a more or less mild type under greenhouse conditions. Leaves were curled downward and malformed with dark-green areas interspersed over a light green (chlorotic) background. Johnson and Kohler (1943) state that soybean mosaic now appears to be common in all major soybean growing areas. They found symptoms to consist of a distortion of the leaves with dark green pouches along the veins or over the entire leaflets. Affected plants are stunted in growth, have flattened and stunted pods, and the yield of seed is materially reduced. The disease is seed borne and mechanically transmissible from plant to plant. They found that in addition to these typical mosaic symptoms, the leaves may remain smooth, the surface becoming "sharply mottled in angular designs". In other cases the leaf is wrinkled or crinkled quite differently from the puckering found in the first case. As pointed out by the authors, the variety of symptoms suggests that there are several soybean viruses rather than one. Different varieties may also produce somewhat different symptoms.

Source of the Virus.

In the fall of 1946 a single naturally infected Sesbania plant was found by K. Starr Chester in Experiment Station plantings at Oklahoma Agricultural and Mechanical College. The plant was potted and placed in the greenhouse by Chester.

Symptoms accompanying the disease were a moderate green mottling, distortion, and stunting.

Preliminary Tests.

Preliminary infection tests were made by Chester. Three plants of "Tobacco W", one of several species originally obtained from L. O. Kunkel, were inoculated with the juice from the diseased Sesbania plant. After a period of two weeks no infection was observed. Three plants of Turkish tobacco were also inoculated, but gave no symptoms of infection. Sixty Sesbania plants were inoculated with fresh virus juice using a cloth-covered spatula and water. Many of the plants inoculated in this manner showed infection.

Materials and Methods.

The same methods were used in attempting transmission of this disease to various hosts as were used in the transmission of Crotalaria mosaic. Attempts were made to transmit the virus to the following hosts: Datura stramonium L., Cucumis sativus L., Nicotiana tabacum L., Vigna sinensis (Torner) Hassk., Lycopersicon esculentum, Mill., Sesbania grandiflora Poir., Nicotiana glutinosa L., Pisum sativum L., Soja max (L.) Piper, and Phaseolus lunatus L.

Results of Inoculations.

Datura stramonium L.

One half a flat of Jimson weed was inoculated with Sesbania mosaic using the usual methods of inoculation and after observation for a period of about nine weeks was discarded as no symptoms had appeared at this time.

Cucumis sativus L.

One half a flat of cucumber plants were inoculated with Sesbania mosaic and observed over a six weeks period. The plants were discarded at the end of this time as no symptoms were in evidence.

Nicotiana tabacum L.

Following inoculation of seven plants of Turkish tobacco with Sesbania mosaic virus and observation for five weeks, no symptoms developed and the plants were discarded.

Vigna sinensis (Torner) Hassk.

One half a flat of Chinese cowpeas, one quarter of a flat of Groit cowpeas, and one half a flat of Black cowpeas were all inoculated with Sesbania mosaic. After observation for a period of from four to five weeks all were discarded except for a single Groit cowpea plant. This plant was thought to show possible symptoms of a virus disease. It was therefore potted and observed for approximately two months at the end of which time all previous symptoms had disappeared and the plant appeared to be perfectly normal.

Lycopersicon esculentum Mill.

Observation over a five weeks period of one half a flat of young tomato plants inoculated with sesbania mosaic showed no symptoms of disease. The plants were discarded at this time.

Nicotiana glutinosa L.

Ten pots containing one plant each of N. glutinosa were inoculated with sesbania mosaic and inspected frequently over a period of one month. No symptoms of disease having appeared at this time, the plants were discarded.

Pisum sativum L.

Infection tests with one half a flat of American Wonder Peas showed no symptoms after two months.

Phaseolus lunatus L.

Infection tests with half a flat of Bush Lima Beans yielded no symptoms after two months.

Sesbania grandiflora Poir.

Ten young Sesbania plants were inoculated with Sesbania mosaic. At the end of approximately one month's time all 10 plants showed typical symptoms of the mosaic. Mosaic symptoms in Sesbania are characterized by a mottle of the leaves and a pronounced puckering and distortion, the latter very pronounced on some leaves accompanied by stunting and dwarfing of the plant. The diseased plant may often recover, at least for a period, from the effects of stunting and attain normal or nearly normal size. Dwarfing and stunting seem to take place primarily in the young infected plant. As the plant matures it appears to overcome this effect and assume normal size. The other symptoms apparently persist throughout the life of the plant, i.e. mottle, puckering of the leaf surfaces and leaf distortion, although these symptoms may appear more pronounced at one time than at another. Particularly does this variation in symptoms appear in the case of plants kept in the greenhouse during the summer when temperatures range from 90° to 110° F or even higher. Under these conditions the symptoms become masked and do not become pronounced again until cooler weather has set in.

As is the case with many viruses, the symptoms are generally noticeable first on the young leaves. While the older leaves may appear normal, three weeks to one month's time after inoculation with Sesbania mosaic, the newly developing young leaves will begin to show a characteristic mottle, distortion, and puckering, and as the leaves develop, the puckered

condition becomes more pronounced and at least some of the leaves become so distorted as to be hardly recognizable.

Soja max (L.) Piper

Twenty-two young soybean plants of the Ogden variety were inoculated with Sesbania mosaic. After a period of from three weeks to a month, seventeen of the plants showed infection with the mosaic. Subinoculations were then made from the diseased to healthy soybean plants and these in turn showed definite symptoms in a period ranging from three weeks to a month's time. Following this, cross-inoculations were made in which healthy soybeans were inoculated with the juice from diseased Sesbania plants while healthy Sesbania was inoculated with the juice from diseased soybeans. Ten healthy plants of soybeans and Sesbania were used. All of the plants inoculated showed symptoms of the disease.

Symptoms on Soybeans.

Ogden soybeans infected with Sesbania mosaic are stunted in growth and dwarfed in appearance. The leaves are characterized by an intense and striking mottle showing very sharply contrasted areas of yellow and dark green. These alternate dark and green areas are generally angular in appearance and give the leaf a flecked aspect. On some leaves the yellow chlorotic areas predominate while on others the green areas are predominant.

Transmission attempts with dodder.

Attempts were made to transmit Sesbania mosaic to both Sesbania and cowpeas by means of a dodder bridge using Cuscuta campestris Yuncker. The attempts were a complete failure as it was practically impossible to cause the dodder to attach itself to the Sesbania plant. This may be

accounted for by the woody nature of Sesbania, or by the host specificity of the strain of dodder which was used.

Conclusions.

As shown by the preceding experiments, the mosaic originally found on Sesbania is readily transferable into Ogden soybeans and just as easily transferred back into healthy Sesbania plants.

Symptoms of the disease on soybeans strongly suggest that it is one of the less common soybean mosaics described by Johnson and Kohler (1943) and described here in the review of literature. Particularly does the angular design of the mosaic infection on the leaf as described and photographed by them suggest that the virus originally found on Sesbania is the same as, or very closely related to, their soybean mosaic.

It is certain that Sesbania mosaic is not the same as the ordinary type of cowpea mosaic as described by several authors.

COWPEA MOSAIC

Economic Importance of Host.

Cowpeas have been grown in Oklahoma for many years. About half the cowpeas grown in the State in the last few years have been used for purposes of soil improvment. Part of the crop is harvested for hay, part is used for pasture and the remainder is harvested either for the edible seed or for purposes of seed. Due to the increase in the use of cowpeas for purposes of soil improvement, any cowpea mosaic may become an important factor in cowpea production. In the case of the use of the seed for seed certifi-

cation a mosaic would be of the greatest importance, as some mosaics of the cowpea have been shown to be seed borne and may infect as much as 30 to 40 percent of the seed. In one known instance a 40-acre field of cowpeas in Oklahoma was disqualified for certification because it was 100 percent infected with mosaic.

Source of the Virus.

In the fall of 1946 a strain of cowpea mosaic was found by K. Starr Chester in a field plant in Experiment Station plantings at Stillwater, Oklahoma. This plant was potted and placed in the greenhouse by Chester who made preliminary infection tests with the virus.

Preliminary tests.

On October 17, 1946 three plants of tobacco N, already described under Sesbania Mosaic, were inoculated by Chester with Cowpea Mosaic. These plants were observed at various periods until November 9 at which time no infection had occurred. Three plants of Turkish tobacco were then inoculated and in a period of about three weeks a light green mottle was noted on one of these plants. This plant later developed necrotic systemic infection of tobacco ringspot type. Subinoculations from the diseased Turkish tobacco plant to three healthy plants of Turkish tobacco caused the appearance of symptoms in nine days' time. At this time two of the plants showed large necrotic local lesions, thin and transparent. Two weeks later these showed typical systemic necrotic ringspot. As there was no virus of this type in culture in the greenhouse at the time, there was little chance that the infection was accidental.

Review of Literature on Tobacco Ringspot.

Ringspot infection on some of the agronomic varieties of N. tabacum in Virginia was reported in 1922 by Fromme and Wingard and in 1924 by

Wingard and Godkin. Wingard (1928) states that the symptoms of the disease are restricted to the leaves of most of the host plants, but may appear on the stems and fruits in some cases. Infection usually results in the formation of rings and lines that spread out in a zigzag manner. Wingard has divided the hosts infected with tobacco ringspot into four groups depending on the type of symptoms found on the various hosts and has grouped those which show strikingly similar symptoms together. Valleau (1932) has shown that there are at least two types of tobacco ringspot, the common type of symptoms being recognized by the presence of large chlorotic and necrotic ring- and-line patterns. With this type of tobacco ringspot the leaves retain nearly normal color and appearance. The chlorotic pattern is various shades of green with little or no yellowing. The other strain of the virus, however, causes the formation of distinctly yellow patterns. The entire leaf of the plant becomes bleached slightly or turns a light yellowish green to nearly white. In the case of infection with the green type of ringspot, the lower leaves of the plant are generally infected with a chlorosis, giving a blotched appearance, which seems to be a symptom of ringspot. Valleau further states that ringspot virus, either of the green or yellow type, retards the development of the pollen grains and he suggests that ringspot virus may cause pollen sterility. As shown by Price, (1932), several species of Nicotiana infected with tobacco ringspot show "recovery" as a normal behavior of plants infected with the disease and they do not develop further symptoms on reinoculation with the disease. When inoculations are made from tobacco plants infected with tobacco ringspot to Vigna sinensis, typical red lesions develop on the surfaces of the inoculated leaves, and the number of these can be used to determine the concentration of the virus (Price, 1936).

Review of Literature on Cowpea Mosaic.

There has been comparatively little published in the literature on cowpea mosaic. Elliott (1921) first reported a cowpea mosaic in Arkansas. This disease was later found in other states, but as far as is known, no pathological work has been done on the Arkansas virus.

McLean (1941) reported a cowpea mosaic at Oklahoma Agricultural and Mechanical College which caused a severe dwarfing of the cowpea varieties Progressive White and Arlington. Other symptoms of this disease were vein-clearing in the developing leaves. Following this or associated with it, was a typical mottling of light and dark green. Accompanying the mottling there was frequently a slight arching or convex cupping of the leaflets.

Harding (1941) working at Oklahoma Agricultural and Mechanical College has reported a cowpea mosaic which he found to be sap-transmissible to cowpeas and certain members of the Solanaceae. In Nicotiana glutinosa the symptoms of the mosaic began as a distinct vein-clearing in the leaves. This graduated into a diffuse blistery mottle accompanied at times by scattered areas of necrosis, especially at the edges of the leaves. He found that the leaves curled downward and were usually reduced in size. In Nicotiana tabacum the symptoms began as pronounced vein-clearing in the leaves of young plants. This changed to a mild mosaic as the plants grew, which either persisted or eventually disappeared. In petunia a mild mosaic was produced with no leaf distortion or necrosis. In cowpeas the virus causes a very severe disease. It began as a brilliant yellow mosaic in the young leaves. The succeeding leaves had a striking veinal mosaic which was accompanied by a severe downward crinkling. Often the plants were short-lived.

Snyder (1942) reports a virus disease of the asparagus bean, Vigna sesquipedalis Wight, which takes the form of a mosaic both on this host and on cowpeas. The close relationship of the asparagus bean to the cowpea, both belonging to the same genus, warrants an examination of the mosaic and an attempt to determine its relationship to the cowpea mosaic under investigation.

Diseased asparagus beans show a light and dark mosaic, often accompanied by a downward rolling of the leaflets which is frequently pronounced. The dark green areas tendto become puffy and result in mild rugose symptoms or leaf distortion. The dark green areas frequently form broad bands along the main veins of the leaflet, leaving the remainder of the leaf light green in color. The infected plants are often dwarfed, especially if infection has taken place through the seed.

The virus is transmitted both mechanically and by the aphid Macrosiphum pisi (Kalt.) and Snyder believes that other aphids which feed naturally on the asparagus bean may also be vectors of the virus.

The seed-borne nature of the virus emphasizes its similarity to the viruses of the cowpea. The fact that the asparagus bean mosaic does not infect catjang, Vigna catjang Walp., differentiates it from McLean's cowpea mosaic which does; however, McLean did not test his cowpea virus on the asparagus bean.

Snyder states that on the basis of symptoms, seed transmission, aphid vectors, and properties, the asparagus bean mosaic clearly belongs to the group of viruses that cause, respectively, the seed-borne mosaics of common bean, soybean, and cowpea. He considers that biologically these viruses may be considered to be physiologic forms of the same virus species.

Yu (1946) has reported a mosaic disease of cowpeas from China which was prevalent along the Yangtze Valley and, according to him, was not related to other common bean or soybean viruses. He states that the symptoms vary with varieties. The first symptom is a clearing-of-veins which starts at the base of the leaf and spreads over the entire leaf. This is very pronounced in some varieties. After 10-12 days, conspicuous mottling appears with chlorotic areas well demarcated from the green tissue. Irregular patches of light green develop which often contain islets of normal green frequently parallel to the veins. Accompanying the mosaic there is customarily a slight convex cupping, arching, or inward rolling of the leaves. The leaves are often puckered and show deep marginal indentations. In some varieties lesions develop which turn dark brown toward the end of the growing season. Dark reddish brown discolorations may be produced on the veins and the top leaves become stunted, misshapen and puckered with dark green areas along the veins. There is also a general stunting of the plants, shortening of internodes, and a tendency toward excessive branching.

As well as being transmitted mechanically, the virus was also found to be transmitted by three types of aphids, and was found to infect both lima and adzuki beans.

Yu states that this cowpea mosaic resembles asparagus bean mosaic in symptoms and properties, while its host range resembles that of McLean's cowpea mosaic. He considers these three viruses to be very closely related.

An experimental strain of cucumber mosaic has been found by Price (1934) which produces a mosaic in cowpeas and which is therefore called cowpea mottling strain. Except for the mosaic reaction on cowpea, all

other host reactions are like typical cucumber mosaic. Price has shown that all other strains of cucumber mosaic produce only localized infection in the form of necrotic red lesions on cowpeas.

Further Infection Studies with Cowpea Mosaic.

Following the preliminary infection tests made by Chester in the fall of 1946 further systematic studies were started early in 1947 to work out at least a partial host range of the virus and to determine whether or not this virus, originally found in cowpeas, could be classed as a strain of tobacco ringspot.

Materials and Methods.

Studies on host range were carried out on the following: Nicotiana tabacum L., Nicotiana glutinosa L., Vigna sinensis (Torner) Hassk., Lycopersicon esculentum Mill., Pisum sativum L., Phaseolus lunatus L., Soja max (L.) Piper, Cucumis sativus L., Datura stramonium L., Petunia hybrida Vilm., and Capsicum frutescens L.

The method of inoculation of the above hosts was the same as that described under Crotalaria Mosaic, Celite being used as an abrasive. The plants were observed over varying periods and the results of the inoculations were recorded.

Results of Inoculations.

Wth the exception of those hosts indicated, all of the following hosts were inoculated with virus from Nicotiana tabacum which had been originally infected from cowpea containing the mosaic virus.

Nicotiana tabacum L.

Seven young plants of N. tabacum were inoculated with cowpea mosaic.

After a period of 10-14 days, two of the seven plants showed symptoms of the disease. The symptoms originally consisted of a light green mottle, developing after a period of three weeks into a systemic necrotic infection of the ringspot type. The plants were preserved and after a period of from two to three months lost all ringspot symptoms, the light green mottled effect remaining on some of the leaves of the diseased tobacco plants. Occasionally faint ringspot patterns again became manifest on the lower leaves of the tobacco plants, but these again disappeared after a comparatively short period of time.

In order to check these results ten additional plants of Turkish tobacco were inoculated with cowpea mosaic. Again, no infection was found after observation for a three to four weeks period.

With the high temperatures in the greenhouse during the summer all symptoms of the disease became masked.

One of the Turkish tobacco plants inoculated in April 1947 showed, in addition to the ringspot symptoms, several yellow spots indicating the possible presence of mutants. From these yellow spots inoculations were made, using a #2 insect needle, into thirty young plants of Turkish tobacco in an attempt to isolate possible mutants. After two months' observation no symptoms of disease had developed and the plants were discarded.

Seven plants of Turkish tobacco were inoculated with cowpea mosaic in the spring of 1948 from diseased Nicotiana glutinosa. After a period of five weeks no symptoms of any sort had developed on the plants. This was in marked contrast to the symptoms found on the inoculation of Turkish tobacco one year previous to this and suggested possible attenuation of the virus.

Nicotiana glutinosa L.

Five young Nicotiana glutinosa plants were inoculated with the cowpea virus and in a period of three to five days, all plants showed symptoms of a disease. Symptoms consisted of definite lesions and a crinkling and curling of the leaves. As the disease became systemic, these symptoms disappeared, and only a light yellow mottle of the leaves remained, accompanied by a slight distortion in some of the leaves. Inoculations from these diseased plants into healthy Nicotiana glutinosa plants five to six months later produced only a light yellow mottle with slight distortion of some of the leaves, and since this time further inoculations into healthy Nicotiana glutinosa plants have yielded only these symptoms, no lesions or conditions of crinkling and curling of the leaves being found.

Vigna sinensis (Torner) Hassk.

Attempts were made to transmit the cowpea mosaic into four types of cowpeas: Chinese, Groit, Black, and Blackeye.

Chinese cowpeas

One quarter of a flat of Chinese cowpeas was inoculated with the mosaic in March 1947. These were observed during a period of more than five weeks. There being no satisfactory evidence of disease in any of the plants, they were finally discarded.

Groit cowpeas

A similar procedure with Groit cowpeas using one quarter of a flat of the plants showed no infection of any of the plants inoculated.

Black cowpeas

Approximately one year after the infection tests on Chinese and Groit cowpeas six young Black cowpeas were inoculated from Nicotiana glutinosa

containing the cowpea mosaic. After a period of from three to four weeks two of the plants inoculated showed symptoms consisting only of a very mild vein clearing on two or three of the upper leaves. These symptoms persisted for a period of from two to three weeks and then disappeared. Following the disappearance of the vein clearing symptoms a moderate mottle was produced on several of the leaves of the plants infected. The mottle consisted of a rather blotchy pattern containing alternate dark and light green areas but was far from being as marked as the mosaic found on the original cowpea host.

Blackeye Cowpeas

Shortly after the middle of June of 1948 thirty plants of Blackeye cowpeas in the field were inoculated with juice from a diseased Black cowpea plant. By the first of July symptoms began to appear in the form of vein clearing on two of the plants inoculated. A few days later the vein clearing symptoms on one of the plants had disappeared an a mild mosaic developed with only slight leaf distortion or none.

By the middle of July a total of three plants of the 30 originally inoculated showed the mild mosaic symptoms. At this time all other cowpeas in the same field planting, none of which had been inoculated, were checked for any virus symptoms which might be present. Of these uninoculated plants, eight were found to have symptoms of apparently the same type of mild mosaic as that found in the inoculated plants. This fact strongly suggested the possibility that the diseased cowpeas found in both inoculated and uninoculated plants was due to a seed-borne virus present in the seed at the time of planting. The possibility also exists that the mild mosaic inoculated into the 30 cowpeas was transmitted by insects to some of the other plants present.

No definite conclusions can be drawn at this time from the above observations as to whether or not the cowpea mosaic considered in this report was transmitted to Blackeye cowpeas.

Capsicum frutescens L.

Ten Ruby King Pepper plants were inoculated with cowpea mosaic and observed over a period of five weeks. These failed to show any symptoms of infection.

Lycopersicon esculentum Mill.

Approximately 50 tomato plants were inoculated with cowpea mosaic. After observation for a period of five weeks the plants were symptomless.

Pisum sativum L.

Inoculation of one half a flat of ordinary garden peas gave no symptoms on any of the plants.

Phaseolus lunatus L.

No disease symptoms were observed upon inoculation of one half a flat of beans and subsequent observation for a two months period.

Soja max (L.) Piper

One quarter of a flat of Ogden soybeans was inoculated with cowpea mosaic. These failed to show any infection after seven weeks.

Cucumis sativus L.

Infection tests with cucumber failed to yield any symptoms of the disease.

Datura stramonium L.

Inoculation of one half a flat of Jimson weed and subsequent

Observation for a period of seven weeks showed the plants to be non-susceptible to cowpea mosaic.

Petunia hybrida L.

Seven petunia plants were inoculated with cowpea mosaic from Nicotiana glutinosa. After observation of the plants for a four weeks period, no symptoms of the disease had developed, and the plants were discarded.

Discussion.

A comparison of the host reactions of this cowpea mosaic with cucumber mosaic and with the cowpea mosaics isolated and studied by McLean (1941), by Harding (1941), and by Yu (1946) is shown in the following table.

Table 1. REACTIONS OF CERTAIN VIRUSES AFFECTING COWPEAS

Infection on	McLean's Cowpea Mosaic	Harding's Cowpea Mosaic	Newton's Cowpea Mosaic	Yu's Cowpea Mosaic	Cucumber Mosaic Cowpea Mottling Strain	Snyder's Asparagus Bean Mosaic
Nicotiana tabacum	--	Vein clearing, then mild mosaic	Very mild ring spot or none	--	Mosaic, distortion	--
N. glutinosa*	--	Very severe distortion	Mosaic, slight distortion	--	Mosaic, distortion	--
Tomato	--		--	--	+	
Potato	--			--	+	
Petunia	--	Mild mosaic	--	--	+	
Jimson weed	--		--	--	+	
Pepper	--	Severe mosaic	--	--	+	
Eggplant					+	
Cowpea var.	Mild mosaic	Very severe mosaic	Moderate mosaic	Mosaic	Mosaic	
Groit			--			
Chinese			--			
Black			Mild mosaic			
Blackeye		--	Mild mosaic			
Pea	--		--	--		--
Field Pea	--					
Sweetpea	--			--		--

Table 1 Continued

Infection on	McLean's Cowpea Mosaic	Harding's Cowpea Mosaic	Newton's Cowpea Mosaic	Yu's Cowpea Mosaic	Cucumber Mosaic Cowpea Mottling Strain	Snyder's Asparagus Bean Mosaic
Bean	--	--	--	--	+	Occasional infection
Mungbean	--					
Soybean			--			--
Lima bean	+		--	+		
Vicia	--			--	+	
Cucumber	--	--		--	+	
Squash		--			+	
Cabbage		--	--			
Zinnia	--			--	+	
Catjang	+					--
Asparagus bean						
Seed transmission	+	?	?	+	?	+

*N. glutinosa is the best differential host of these viruses.

Viruses which cause only local lesions on cowpea: tobacco ringspot (large blotchy lesions); ordinary cucumber mosaic (small, punctate lesions). Alfalfa mosaic, tomato bushy stunt, peawilt causes wilting disease of cowpea.

Comparison with Cucumber Mosaic

As is indicated by its name, cucumber mosaic produces a definite mosaic pattern on cucumber. Inoculations of the cowpea mosaic studied produced no infection when inoculated into cucumber plants. Cucumber mosaic also infects tomato, Jimson weed, and bean, while the cowpea mosaic infects none of these.

Comparison with Harding's Cowpea Mosaic

In Turkish tobacco Harding's cowpea mosaic produced vein clearing followed by a mild or marked mosaic. The cowpea mosaic under consideration produced only a very mild ringspot or none at all, accompanied by a green mosaic and only slight distortion of the leaves or none. In Nicotiana glutinosa Harding's cowpea mosaic produced very severe distortion of the leaves while this cowpea mosaic produced a mosaic pattern with only occasional slight distortion or none.

Comparison with McLean's Cowpea Mosaic

As shown by the table, McLean's cowpea mosaic produced only a mild mosaic in cowpeas and no reaction in either Nicotiana tabacum or Nicotiana glutinosa.

Comparison with Snyder's Asparagus Bean Mosaic

As indicated in the preceding table the asparagus bean mosaic does not attack either Nicotiana tabacum or N. glutinosa, while it has been shown that the cowpea mosaic reported here originally attacked both of these hosts. The difference in the reactions of the two types of tobacco to these two viruses serves to differentiate them from one another. The possibility does exist, however, that this cowpea virus may be related to the asparagus bean virus as well as to other cowpea viruses, for all of them produce mosaic symptoms on the cowpea which seem to vary chiefly in intensity of expression.

Comparison with Yu's Cowpea Mosaic

As indicated earlier, the cowpea mosaic studied by Yu attacked the lima bean. This was not the case with the cowpea mosaic considered in this report. Furthermore, while this cowpea mosaic was found capable of infecting Turkish tobacco originally, Yu's cowpea mosaic did not attack this host.

The symptoms of Yu's virus on cowpeas were also quite different from the symptoms found in the case of the cowpea mosaic reported here. Apparently the only similarities are found in the fact that both produce vein clearing in the early stages and both are mosaics. Beyond this, however, not only does Yu's mosaic produce quite different symptoms in many ways, but the symptoms are much more marked in their effects.

Comparison with Tobacco Ringspot

As already stated tobacco ringspot causes the formation of rings and lines which spread out in zigzag manner in Nicotiana tabacum. This is in contrast to the very mild ringspot or none at all produced by the cowpea virus in the same host. It has been further shown that ordinary tobacco ringspot produces typical red lesions on the leaves of cowpea while the cowpea virus produced a moderate mosaic in cowpeas originally.

Conclusions.

From the above reactions I am led to believe that the virus studied is an undescribed virus of the cowpea, but possibly related to those already described. There is a possibility, however, that it may be a very mild strain of tobacco ringspot incapable of producing red lesions on the cowpea, but producing instead a moderate mosaic in some instances. There is also evidence which would lead to the conclusion that attenuation of the virus has taken place during the period of a year and one-half in which it has

been in culture. The original symptoms of the virus in the cowpea frm which it was isolated consisted of a moderate mosaic. Inoculations were made from the original plant into Nicotiana tabacum where a mild ringspot was produced together with a light green mottle. The virus was cultured in this host for a period of approximately six months before being inoculated back into cowpeas. Inoculations into both Groit and Chinese cowpeas did not produce any symptoms of the disease. As the variety of the original cowpea plant containing the virus is unknown, either varietal resistance or possible attenuation of the virus caused by passage through a host other than the original one is indicated. Other evidence to support this latter supposition is the fact that original inoculations into Nicotiana glutinosa produced crinkling and curling of the leaves followed by a mosaic pattern. Inoculations after a period of from four to six months into the same host produced only the mosaic with none of the symptoms of crinkling or curling, thus indicating a possible modification in the nature of the original virus. Still further evidence of possible attenuation of the virus is seen in the fact that inoculations of the cowpea virus which had been maintained in Nicotiana glutinosa for a year's time failed to produce any symptoms in Nicotiana tabacum. This latter reaction may be due to the fact that the inoculations were made in late May when temperatures in the greenhouse were comparatively high.

There are a number of examples found in the literature showing that the properties of the virus of a given plant may be definitely changed by its transfer to other host species. Walker (1926) reported this type of phenomenon in working with the mosaic diseases of cucumber. Cross inoculations by him seemed to indicate that the properties of the virus were determined by the character of the juices of the host plant in which the disease was found. Garsner and Lackey (1928) passed curly-top of sugar beets through resistant varieties of beets and found that attenuation had taken place.

Attenuation can also be caused by high temperatures as shown by Holmes (1934) in his work with tobacco mosaic and by other workers as well. High temperatures may have been a definite factor in attenuation of the cowpea mosaic under consideration, as this virus went through an entire summer in the greenhouse where temperatures at times were as high as 110° F or even higher.

SUMMARY

A strain of cowpea mosaic was found in Experiment Station plantings at Stillwater, Oklahoma in 1946.

The virus was found to be sap-transmissible to Nicotiana glutinosa L., Nicotiana tabacum L., and to Black Cowpeas. Infection was not demonstrated in Chinese or Groit cowpeas, tomato, peas, beans, Ogden soybeans, cucumber, or Jimson weed.

Comparison of this virus with cucumber mosaic virus and the cowpea mosaic viruses of McLean, Harding, and Yu as well as with Snyder's asparagus bean mosaic virus, showed it to be distinct from these, but possibly related to them. Comparison with tobacco ringspot indicated the possibility that the virus might be a mild strain of tobacco ringspot, although differing from tobacco ringspot in its inability to produce the characteristic red lesions on cowpeas.

Reactions over varying periods of time on Nicotiana tabacum, Nicotiana glutinosa, and cowpeas indicate either varietal resistance or attenuation of the virus in the case of cowpeas and attenuation in the two species of tobacco used.

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ELECTRON MICROSCOPE STUDIES OF PLANT VIRUSES

INTRODUCTION

In the Spring of 1947 a grant was made to the Research Foundation of Oklahoma A. and M. College by the United States Public Health Service for the purpose of conducting basic research on plant viruses with the electron microscope. It was felt that such studies of plant viruses would aid in an understanding of the nature of animal and human viruses, and, as plants are easier to work with in studying virus diseases, it was also felt that a further understanding of the general nature of viruses might be gained more readily by studies on those affecting plants. Much of our present knowledge of viruses has been gained through investigation of plant viruses, in particular tobacco mosaic virus which was the first virus to be weighed, measured, counted, and crystallized.

Since the development of the electron microscope further investigations of viruses have been made possible. Here again the preponderance of the research has been with tobacco mosaic which apparently lends itself very readily to all types of investigation. At present not more than a dozen different plant viruses have been investigated with the electron microscope, and yet several entirely distinct types of virus morphology have been detected. It appears, therefore, that further electron microscope research on viruses in general should aid in extending our knowledge of these entities.

Purpose.

The primary object of the following electron microscope studies was to investigate various plant viruses with the purpose in mind of determining their morphological characteristics--primarily size and shape. Studies of morphology and structure, particularly over a wide range of different viruses,

should aid considerably in establishing a natural classification of viruses and perhaps show a correlation between physical structure and the type of symptoms produced on the plant host. Electron microscope studies to date have led to a confirmation of size, shape, and molecular weight of viruses as determined by other means. Studies on the morphology of tobacco mosaic have confirmed the findings of Lauffer (1942) and other workers regarding the particle size of the virus.

In general it may be said that the use of the electron microscope in conjunction with physical and chemical studies of viruses may offer a means of determining the effects of either chemical or physical experimentation with the virus.

With all these possibilities in mind and the intent of pursuing the problem as far as time would permit, research with the electron microscope was begun in September of 1947.

ELECTRON OPTICS

History.

The development of the electron microscope is the result of the observations of numerous workers. It has been known ever since the principles of light microscopy were developdd that short wave emanations could be substituted for ordinary light and much greater magnifications obtained. In general it was found that the laws governing ordinary light optics were applicable to electron optics. Deep-seated qualitative differences, however characterize the behavior of electrons as contrasted with the behavior of light. (Zworykin et al. 1945)

A number of discoveries and observations, however, were necessary before the invention of the electron microscope was possible.

Electrons were discovered in 1897 by J. J. Thompson. These newly found particles were at first treated as being subject to the laws of Newtonian mechanics. A treatment of this type, however, did not explain the behavior of electrons in atoms which is manifested in atomic line spectra. This difficulty was overcome by the development of quantum mechanics using Einstein's special theory of relativity and Planck's quantum relation (Zworykin et al., 1945). The characteristics of wave motion associated with moving electrons was demonstrated by de Broglie (1924). He showed in his work with the hydrogen atom that electrons have a definite wave length and by so doing laid the groundwork for the development of the electron microscope.

Another important step in the development of the electron microscope was the discovery by Busch (1926) that calculations in terms of light optics can be applied to the behavior of an electron beam in an electrical field.

By this time much of the necessary theory had been developed and practical applications began to follow. About 1931 and 1932 workers in both the United States and Germany demonstrated electron images independently of one another, and soon after this Marton (1934) reported the first application of the electron microscope to biological objects. Following these initial steps, development of the instrument was quite rapid, although it was several years before very practical instruments were built. Hillier and Prebus in 1939 built the well-known Toronto model, the design of which was used by the Radio Corporation of America in manufacturing the model produced by them (Eisenstark, 1948).

Today R.C.A. and other companies are producing and marketing various types of electron microscopes. R.C.A. manufactures both a "Universal" type and a console type instrument. The former is operated at 60 kilovolts while the latter operates at 30 kilovolts.

Basic Principles of Electron Optics.

As distinguished from the light microscope, the electron microscope uses electrons rather than light, and in the place of glass lenses, electrical magnetic fields. Furthermore the chamber through which the electrons pass in the electron microscope must be evacuated to a comparatively high degree, which is unnecessary in the light microscope. A comparison of the two instruments is given in figure 1.

Vacuum is necessary in the electron microscope in order to increase the mean free path of the electrons. As shown by Zworykin et al., (1945) the mean free path of an electron at a pressure of 10^{3} mm Hg is about $10^{-4.5}$ cm while at a pressure of 10^{-6} mm Hg the mean free path becomes 10^{5} cm. Creation of a vacuum therefore is necessary to "clear a path" for the electrons.

In specimen-viewing the image as seen in the electron microscope is created by the scattering of electrons while in the case of the light microscope, vision depends on the absorption and reflection of light from the object under consideration.

As shown in figure 1, the electron microscope consists of a filament from which the electrons are emitted, and several magnetic coils through which the electrons pass and are first concentrated to produce a strong bombardment of the specimen and then "fanned out" by successive coils to

enlarge the image of the specimen. The image is projected on a fluorescent screen and either viewed indirectly in the case of the R.C.A. "U" type or directly in the case of the R.C.A. console model. The filament as well as the magnetic coils are all incased in a vacuum chamber which is evacuated by means of an oil diffusion pump contained in the instrument. This equipment, together with the electrical mechanisms necessary to step up the voltage, constitutes the instrument. Electrons are emitted from the filament due to the high voltage which is induced.

Uses and Limitations of the Electron Microscope.

As is the case with many new mechanical inventions, they are at first considered "wonder machines" and their powers and applications are generally over-emphasized. No doubt the electron microscope is in many ways a "wonder machine", but it is far from being the solution to all physical, chemical, and biological problems. It should be regarded as a very useful tool in connection with other tools and processes of modern science. To no extent should it be regarded as making the light microscope obsolete, but as a means of examining objects too small to be seen with the light microscope and oftentimes to be used in conjunction with the latter.

The primary advantage of the electron microscope lies in the fact that objects heretofore too small to be seen with the light microscope may now be viewed and studied with it. Coupled with this advantage are the advantages of depth of focus and sharpness of image (Eisenstark, 1948). The extreme magnification of the instrument has made possible the observation of the fine details of physical surfaces such as metals, crystals, teeth, and numerous other types of surfaces as well as viruses, bacteriophage, protein molecules, and many other structures.

The electron microscope has the limitation and disadvantage that colors can not be distinguished. All images appear either dark or light, the shades of darkness or lightness depending on the density and thickness of the material as well as on the strength of the electron beam. Another disadvantage of the instrument lies in the fact that many types of materials must be prepared very carefully before they can be viewed successfully. This is particularly true in the study of viruses and bacteriophage where hours are often consumed in preparing the material. This difficulty is partially due to the inability of the electron microscope to record colors which might assist in differentiating these small entities from foreign or contaminating material present.

With the light microscope it is generally possible to study live material while one of the limitations of the electron microscope is the fact that only dead material may be observed. This, of course, is a distinct disadvantage in that conclusions based on the appearance of dead material may not be applicable to living material and that living processes such as motion cannot be studied. All living specimens to be viewed with the electron microscope are killed in the instrument by desiccation due to the high vacuum necessary. The possibility of any living material surviving this desiccation process and being seen in the living state is further precluded by the presence of heat produced by electron bombardment and by the electrons themselves.

These are the general disadvantages in the use of the electron microscope. Other disadvantages exist depending on the field of research under consideration, i.e. materials must be so thin for satisfactory observation that in some fields of research this constitutes a major problem. At present a high speed microtome is in the process of devel-

opment which may remedy this particular problem (Merrill and Fullam, 1947).

REVIEW OF THE ELECTRON MICROSCOPE IN PLANT VIRUS RESEARCH

A number of plant viruses have been investigated with the electron microscope. As in the case of other types of virus investigations, tobacco mosaic has received the greatest amount of attention and there are numerous publications on various types of investigations of tobacco mosaic. Anderson and Stanley (1941) studied the reactions between tobacco mosaic and its antiserum. Studies on particle length of tobacco mosaic were made by Takahashi and Rawlins (1948). These same workers (1947b) also carried out electron microscope investigations on mutation in tobacco mosaic. Their investigations showed no differences in morphological structure between ordinary tobacco mosaic and a yellow mutant. Rawlins, Roberts and Utech (1946) also investigated the lenth of tobacco mosaic particles and indicated some of the probable causes of end-to-end aggregation of the particles. Electron micrographs of very highly concentrated solutions of tobacco mosaic were made by Wyckoff (1947) which show the extreme concentration possible in the case of tobacco mosaic. Oster and Stanley (1946) studied, by means of the electron microscope, tobacco mosaic taken from the hair cells of diseased tobacco plants and concluded from their findings and those of other workers that "particles 15 by 280$m\mu$ in size, represent tobacco mosaic virus." Sigurgeirsson and Stanley (1947) carriedout further experiments on the size of the basic entities of tobacco mosaic and found evidence of end-to-end aggregation of the basic units on standing at 4° C. for various periods of time.

Otherplant viruses have been studied with the instrument although none as extensively as tobacco mosaic.

Two strains of potato X-virus have been examined and found to be indistinguishable morphologically (Takahashi and Rawlins, 1946). These same authors made studies of squash mosaic virus (1947a) and concluded that the virus had a particle size of 30 $m\mu$. Takahashi (1948) made further examinations of squash mosaic which he had crystallized. Electron micrographs of two other plant viruses, southern bean mosaic and tomato bushy stunt, showed that they were spherical bodies of approximately the same size (Price, Williams and Wyckoff, 1946). Cucumber mosaic viruses 3 and 4, as well as tobacco necrosis virus were studied by Stanley and Anderson (1941).

The various viruses mentioned above constitute practically all the plant viruses which have been studied with the electron microscope. In addition to these, one plant virus which is transmitted by insects has recently been studied with the electron microscope. This is the yellow-dwarf virus of potatoes (Black, Mosley, and Wyckoff, 1948). To the best of my knowledge this is the only case in which a virus requiring insect vectors for its transmission has been viewed and photographed with the electron microscope.

Those acquainted with the multitude of different viruses which attack plants will see from the above account that only a comparatively small proportion of plant viruses have been studied with the electron microscope. It may be said, therefore, that this phase of research with all its numerous ramifications has barely begun. A similar condition exists in both the fields of animal and human virus diseases and investigations in any of these fields of virus research may very probably aid research in the other closely related fields.

MATERIALS AND METHODS

The electron microscope is of comparatively recent development, and for this reason, techniques of preparation of material for examination, photographing of the material, and the various types of quantitative as well as qualitative studies are as yet in the pioneer stage, although rapid progress has been made in these lines. A number of problems, then, present themselves to the beginner in the study of viruses with the electron microscope. These are: (1) Becoming acquainted with the electron microscope to such an extent that the best results can be obtained with it; (2) mastery and development of the technique of preparation of the material for examination including both the technique of mounting the material for observation and also that of purification of the material so that when mounted it will be as free as possible of foreign material; (3) interpretation and identification of the material viewed with the electron microscope; and (4) photography of the viruses to give the best definition and contrast.

All studies were made with a type EMC-2 electron microscope. With an instrument of this type it is possible to enlarge the material viewed 2000 or 5000 diameters directly, with further useful photographic enlargement to 30,000 diameters. In all these studies the 5000 diameter magnification was used exclusively. The image of the object being studied can be viewed directly on a fluorescent screen with this instrument. The screen holding the material to be studied is bombarded with electrons from a biased electron gun. The electrons travel at about one-third the speed of light. Electrons are absorbed by solid materials in the field of the electron gun leaving the electrons which are not blocked by these objects to pass on into a magnetic field where they are "fanned out" and arrive on the viewing screen. The

dark areas seen on the viewing screen then represent the images of the particles which blocked the passage of the electrons.

A number of viruses were obtained for examination. These were kept in culture in growing plants in the greenhouse. Prior to the initiation of the actual electron microscope work, a rather large collections of viruses was built up so that work could proceed without interruptions due to a lack of materials. This collection was added to from time to time as different viruses were obtained, and at present includes the following:

1. Alfalfa Mosaic Virus
2. Barley Mosaic Virus
3. Bean Mosaic Virus
4. Cabbage A Virus
5. Cabbage B Virus
6. Cowpea Mosaic Virus
7. Cucumber Mosaic Virus
8. Cucumber Mosaic Virus (bindweed strain)
9. Geranium Mosaic Virus
10. Hippeastrum Mosaic Virus
11. Potato Leafroll Virus
12. Potato Spindle Tuber Virus
13. Potato Virus A
14. Potato X-Virus
15. Sesbania Mosaic Virus
16. Severe Etch Virus
17. Squash Mosaic Virus
18. Tobacco Mosaic Virus

19. Tobacco Ringspot Virus

20. Violet Mosaic Virus

21. Watermelon Mosaic Virus

22. Wheat Mosaic Virus

The collection appears to be the largest in the United States apart from collections in Wisconsin, Kentucky, and on the East and West coasts.

Purification and Preparation of Virus Material.

The process of purification of the viruses to be examined consisted of extracting the juice either by pressing or grinding of the leaves of the diseased plant and subsequent centrifugation and filtration. Entities as small as viruses must be in a relatively pure condition before satisfactory examination with the electron microscope is possible. Comparatively small amounts of plant material which occur when plant juices are pressed out, and large amounts which occur when the plant material is ground to express the juice, must be removed before good results, or even any useful results, can be obtained.

In general diseased leaves containing the virus to be studied were harvested and either frozen overnight, as in the case of tobacco mosaic, or used directly without freezing. Freezing of plant material makes extraction of the juices much easier and gives a greater yield of juice. The juices were extracted with a mortar and pestle, an attempt usually having been made to press the juice out with the pestle rather than grinding up the leaves. This procedure yielded a fairly clear juice. The raw juice was then partially cleared in an ordinary centrifuge at from 3000-4000 r.p.m. for a period of from 20 to 30 minutes. This threw down the coarser and heavier material and generally left a turbid looking solution.

The juice was then passed through a sintered glass filter covered with a layer of Celite 535 about 2-3 mm. thick. At times additional Celite was added to the juice prior to filtration through Celite. The juice was filtered under suction pressure using either a water tap or a small evacuating pump. Filtration through Celite is a modification of the technique used by Takahashi and Rawlins (1946) in examining potato X-virus. Passage through this type of a filter yielded an opalescent filtrate characteristic of pure virus suspensions, due to their colloidal properties, ranging in color from brownish-yellow to a light greenish-yellow. This process sufficiently removed the plant material so that the virus could be viewed in the electron microscope. Some of the prepared juice was then diluted with distilled water at dilutions ranging generally from 1-10 to 1-20 and was then ready to mount for observation. Dilution was used with a dual purpose in mind: dilution of the remaining colloidal plant material and dilution of the virus itself. Unless diluted in this manner, many viruses have such a high concentration that observation of the separate virus particles is practically impossible.

Any filtration methods that are adequate for the separation of the virus from the plant material have a decided disadvantage. While it has been shown numerous times that the virus passes through certain filters and is therefore partially purified by the removal of much of the larger parts of the colloidal material originally present, nevertheless it is also a known fact that these plant products which are filtered out may in themselves act as very fine filters and may retain the virus by mechanical means and by electrical charge phenomena. McKinney (1927) has shown that in the filtration of the extract of plant leaves the filtrate becomes decidedly clearer as the filtration process proceeds. In order to test this

point with viruses he passed mosaic-diseased plant extract through Büchner funnels and found that a considerable portion of the virus was held back by the slime composed of the plant materials. The amount of virus present, therefore, in a filtrate is directly proportional to the amount of plant material present at the time of filtration. Where it is possible to obtain a relatively pure juice prior to the filtration process, the amount of virus retained on the filter is small. In the case of tobacco infected with virus diseases, much of the juice can be extracted in a comparatively pure state by firmly pressing the leaves rather than grinding them, provided the leaves have been frozen and then allowed to thaw out before the pressing process. In the case of viruses attacking other plants such as cabbage, it is practically impossible to press more than a few drops of the juice out even after the leaves have been frozen and thawed, consequently grinding must be resorted to.

At the time this work was initiated filtration, combined in some cases with attempts at chemical precipitation, was the only means at hand feasible for purification of the viruses. It was recognized at the time, however, that only partial and limited success could be expected using these methods of purification. In the case of tobacco mosaic which exists in high concentration, is relatively very stable, comparatively large in size, and readily recognized in the electron microscope, these means of purification are adequate and can be expected to yield good results. However, in the cases of other viruses which are far less concentrated, less stable, and of comparatively small size, these methods are inadequate for good results. The primary step in all electron microscope plant virus research is to "clean up" the material to be examined without losing all or the greater portion of the virus. For this purpose other methods than those involving

filtration have been found necessary and far superior.

Particularly in the earlier preparation of various viruses by filtration methods for electron microscope observation, contamination with colloidal plant material proved the limiting factor. This was due to the fact that, not only did the contamination of these plant materials make observation and identification of the virus under consideration practically impossible, but also these contaminating plant particles often became large enough to absorb ions in an amount which was sufficient to release heat to the extent that the supporting membranes were shattered and broken.

In an attempt to reduce the amount of plant material present to a minimum, several procedures were used.

Following filtration through sintered glass filters covered with a layer of Celite 535, the juice containing the virus was passed through a Seitz filter. In several cases this gave an almost colorless solution showing that much of the plant material had been removed. Examination with the electron microscope of mounts prepared in this manner gave very clear fields, but only in the case of tobacco mosaic was it possible to locate virus particles, and even in this case the amount of virus found was very small. This technique therefore had to be abandoned as it apparently filtered out the major portion of the viruses as well as the plant material.

Attempts were also made to "clean up" the virus suspensions by means of sintered glass filters layered with Celite and placed one above the other. The diseased juice was then passed into the upper filter under suction pressure from a small evacuating pump. From the upper funnel the juice dripped into the lower funnel and the process was repeated. Suction was maintained from one filter to the other by a tight rubber jacket which pre-

vented the outside air from rushing in at the points of contact of the two funnels. This method of purification, while aiding materially in removing plant materials, appeared to have the same drawbacks as the Seitz filtration procedure. Filtration of this type was not investigated extensively, and was completely abandoned following the purchase of an ultracentrifuge for virus and other types of research by the Research Foundation of Oklahoma Agricultural and Mechanical College.

Chemical precipitation in combination with filtration methods was also attempted, largely with tobacco mosaic virus, in order to purify the material. The method consisted in adjusting the pH of the virus suspension and salting out the virus protein, using the methods of Stanley (1935). This method was also abandoned at an early stage in favor of ultracentrifugal methods of purification.

Ultracentrifugation

Development of the Ultracentrifuge

The ultracentrifuge offers an ideal means for the purification and concentration of viruses and eliminates the difficulties encountered when filtration methods are used. The highly purified produce obtained is admirably suited for examination with the electron microscope.

Svedberg *et al.* (1924, 1926) is responsible for the development of the ultracentrifuge with which he produced forces greater than 500,000 times the force of gravity. As designed by him, the ultracentrifuge was used for two types of work: (1) the determination of molecular weight of colloidal materials through study of sedimentation equilibria, and (2) the separation of different types of colloidal material, made possible by differences in their sedimentation rates (Alexander, 1926).

Colloidal particles, including viruses, do not settle out of suspension when acted upon by the gravitational force of the earth or even the centrifugal force of the low speed centrifuge. In order to bring about the sedimentation of colloidal particles it is necessary to subject them to centrifugal forces many times stronger than the force of gravity. With the ultracentrifuge very powerful centrifugal fields can be developed, and Svedberg (1938) has operated the ultracentrifuge at speeds which give centrifugal forces as high as 900,000 times that of gravity.

Modifications of the quantitative type of ultracentrifuge of Svedberg have been developed which are comparatively simple in construction and design. A centrifuge of this type has been developed by Beams which uses air under pressure to drive a rotating head. The rotating head rides upon the cushion of air which is also used to propel it. The ordinary friction of moving parts is thus eliminated, the only friction involved being that created by the air in driving the head. Ultracentrifuges of this type have the advantage of simplicity of construction and eliminate the necessity of replacing worn out parts. Holes are bored in the head of the centrifuge, generally at an angle, and tubes containing the material to be centrifuged are inserted in the holes. The principle of the anglehead centrifuge is well known and need not be discussed here. It may be stated briefly that the velocity necessary to settle material in the angle head centrifuge is much less than that required for the same material in centrifuges with tubes in the horizontal plane. A centrifuge of the type described above was obtained by the Research Foundation of Oklahoma A. and M. College for the purification of viruses and other biological work. The rotating head was constructed of aluminum and contained four holes bored at right angles to one another. Each of these holes held approximately 0.5 cc. of liquid.

The head was rotated by air under pressure which entered through equidistant holes in the base of the centrifuge. On passing through these holes the entering air struck small flanges etched in the base of the aluminum head and rotated the head. It was possible to develop high speeds with this centrifuge at very low air pressures. As shown by graph No. 1 speeds greater than 36,000 r.p.m. can be obtained at a pressure of 10 pounds, while at a pressure of 90 pounds a speed of 120,000 r.p.m. was possible, this latter speed being equal to 300,000 times the force of gravity. While high speeds are necessary to obtain high centrifugal fields of force, another consideration of prime importance is the radius through which the suspended colloidal material will rotate, for the centrifugal force is directly proportional to the radius as is shown by the equation:

$$f_c = w^2 mr \qquad (1)$$

where:

f_c = centrifugal force
w = angular velocity
m = mass
r = radius

Forces developed with the ultracentrifuge are generally expressed in gravitational units which are also directly proportional to the radius about which the material is rotated. The equation for gravitation force is $f_g = mg$, m being the mass and g being the gravitational constant. Dividing equation (1) for centrifugal force, by the force due to gravity, the force in gravitational units becomes:

$$F = \frac{w^2 r}{g} \qquad (2)$$

Preliminary trials were made with the ultracentrifuge in order to become acquainted with the technique of operating it. Attempts were made to develop small tubes to act as suitable containers for the virus suspen-

sions which were to be placed in the aluminum head. Ordinary gelatin capsules proved ineffective as they dissolved quite readily in distilled water. Both collodion and parlodion also proved ineffective. Tubes made of these latter materials became wrinkled and distorted when revolved at high speeds. Custom-made pyrex glass tubes were finally ordered from a laboratory supply house and proved satisfactory.

It was found that when liquids were revolved at high speeds in the open head of the centrifuge, evaporation of the liquid proceeded rapidly. It was necessary, therefore, to have a closely fitting cover made for the head which was held in place by a small central bolt. This cover when fitted with a gasket prevented evaporation almost completely.

Extensive use of the ultracentrifuge in the virus studies included in this paper was limited by the time element. The centrifuge was not received until January 1948 and further delay was incurred by the fact that preliminary trials with it were necessary before it could be used, and also due to the fact that there was much delay in procurring the small pyrex glass tubes necessary for the work. The major portion of the virus preparations, therefore, had to be made using filtration and low speed centrifugation techniques. The groundwork, however, has been laid for the use, at this institution, of the ultracentrifuge in virus research as well as in other types of biological investigations.

Preparation with the Ultracentrifuge.

As already indicated, the purpose of the ultracentrifuge in virus research work is to highly purify the material and eliminate foreign and contaminating materials as much as possible. It cannot be repeated too often or stressed too strongly that in virus research the purification of the

material under investigation to the highest degree possible is particularly important and necessary. Only after high grade purification methods have been used can successful investigations and interpretations be made of virus entities. Viruses as seen under the electron microscope, without the use of shadowcasting techniques which are discussed later, are generally of a rather shadowy and indefinite nature and do not consist of well-resolved forms which are easily seen and recognized.

In general the technique of preparation of diseased plant materials consisted first of all in harvesting the diseased tissues, generally the infected leaves, and grinding or pressing them with a mortar and pestle to express the juices. The leaves were sometimes frozen over night before grinding or pressing, or they were ground or pressed directly after being harvested. In most cases a few c.c. of distilled water was added to the leaves while extracting the juice in order to facilitate extraction and to increase the volume. These extracted juices were then passed through cheesecloth to remove the larger amount of plant material which occurred particularly when the material was ground rather than pressed. As indicated earlier, pressing of juices is suitable for some types of plant material, while with other types grinding is necessary. Following these preliminary steps the juice may be further filtered through Celite as done by Stanley (1940) who also added dipotassium phosphate to the juice. In these studies, however, filtration other than through cheesecloth was not used. The juice was then purified by differential centrifugation which consisted of centrifuging the material at alternate high and low speeds. This method is commonly used by most workers in the field of virus research. Stanley (1940) used this method in the purification of tomato bushy stunt virus. Rawlins, Roberts, and Utech (1946) also used

this method, alternately centrifuging tobacco mosaic at 3000 r.p.m. and 35,000 r.p.m. In the crystallization and preparation of squash mosaic Takahashi (1948) used this method, and it may be said that it is the generally accepted procedure in virus purification from either plant or animal materials.

In these studies the juice was first run in the low speed centrifuge at 3000 r.p.m. to 5000 r.p.m. for a period of from one half to one hour. This served to throw down the heavier plant material and left, in the case of tobacco juice, a dull brown liquid. Small amounts of the brown liquid were then centrifuged for periods of 1 1/2 to 3 hours at 40,000-60,000 r.p.m. These high speed runs were generally begun at a speed of 60,000 r.p.m. for a period of about one half hour at which time the pressure on the line which furnished air for operation of the ultracentrifuge had dropped off until it reached a constant pressure of 12 pounds which corresponds to a speed of about 40,000 r.p.m. Centrifugation was then carried out at this speed during the remainder of the run. Upon completion of the ultracentrifugation process the small tubes containing the plant juice were removed from the ultracentrifuge and the supernatant liquid was carefully decanted and discarded, it having been assumed that the major portion of the virus particles now were in the material thrown down in the bottom of the tube. This precipitate which in some cases consisted of small pellets, and in other cases was in the form of a fine sediment, was then resuspended in a small amount of distilled water. In this manner the virus was again suspended in a liquid medium while the originally colloidal plant material, due to the high speed centrifugal process, was clumped together to form small lumps. The resuspended virus was then centrifuged in a low speed centrifuge at approximately 3000 r.p.m. for half an hour to an hour. Following this the lumps of col-

loidal plant material were found as a sediment in the bottom of the tube. The supernatant liquid bearing the virus was then carefully decanted and small drops of the purified suspension were used in the preparation of mounts for electron microscope examination.

In all cases the differential centrifugation was only repeated once, that is, the material was first run at a low speed, then at a high speed, and finally at a low speed. Some workers have repeated this process several times in order, it is assumed, to further purify the material. I have found, however, in my investigations, up to the present time that one complete cycle of high and low speed centrifugation has proved sufficient to remove the greatest part of the contaminating material, and unless exceptionally pure material is desired, the additional purification obtained is not worth the added time involved.

An important consideration that must not be overlooked in the use of the ultracentrifuge in purifying plant material is the fact that while ordinary contaminating plant substances may be practically eliminated with the instrument, and those remaining quite readily identified as ordinary contaminants, there still remains the possibility that plant proteins of high molecular weight and comparatively large size may be thrown down along with the viruses. These high molecular weight proteins may be readily confused with the virus particles as they are very similar in appearance. This is especially true if the virus under investigation is spherical in shape, as proteins themselves also seem to have a spherical structure in general. High molecular weight proteins were isolated by Loring, Osborne, and Wyckoff (1938) from the broad bean and pea plant, and Takahashi (1948) has isolated a purified high molecular weight substance

from squash plants which shows a striking resemblance to the virus of the squash plant as isolated by him.

Preparation for Examination with the Electron Microscope.

Mounting Technique

Following the purification of virus material either by centrifugal or filtration processes, it was necessary to mount this material for examination with the electron microscope. For mounting material of this type, small copper or steel screen discs are used. These discs are slightly more than 3 mm. in diameter and have numerous small perforations, approximately square in shape, through which the material being studied may be viewed in the electron microscope. Over these perforated metal discs a very fine, thin film of transparent material is placed to serve as a supporting membrane for the viruses. Various materials have been used for this purpose. Among these are Collodion, Parlodion, and Formvar. Viruses examined in these studies have been mounted on membranes of both Parlodion and Formvar. Parlodion, when used, is dissolved in amyl acetate in a 1% solution. In using Formvar, ethylene dichloride is used as a solvent and about 1/2% of Formvar by weight is dissolved in this. Tests with both Formvar and Parlodion showed the latter to be the better material for use as a supporting membrane, although many workers report highly successful results using Formvar.

The following method of preparation of specimen material was used throughout the work. Distilled water was placed in a small, chemically clean cylindrical glass dish approximately 5 inches by 2 1/2 inches. After the surface of the water had become comparatively still a drop of either Parlodion or Formvar was dropped on the surface of the water with

an ordinary dropper. These substances when dropped on a water surface spread out rapidly to form a very thin film which solidifies as the solvent material evaporates. As already mentioned, Parlodion dissolved in amyl acetate proved to be the better material to serve as a supporting membrane. Films made of Formvar broke much more readily when subject to electron bombardment in the electron microscope, and upon breaking almost invariably rolled and twisted in such a way as to become useless for the purposes intended. Parlodion, on the other hand, withstood electron bombardment much more effectively, and when breaking did occur, there was much less tendency to roll and twist. After the film had solidified and become "set" on the water surface, the small metallic screens were placed on the surface of the film. Generally from four to eight of these screens were placed on any one film in two closely spaced rows. An ordinary microscope slide which had been well washed and dried was then carefully pressed down on the screens and supporting membrane so that the membrane adhered to the edges of the slide. The metal screens were thus held between the slide and the membrane. Retaining hold of the microscope slide between the thumb and the forefinger, the microscope slide was pushed into the water slightly and tilted at a small angle along its longitudinal axis. An ordinary teasing needle was then used to cut the membrane parallel to its length. The slide was then pushed deeper into the water, rapidly turned through an angle of 180° and taken out of the water. The purpose in cutting the membrane was primarily to make certain that when the slide was turned through 180° only the original layer of the membrane covered the screens and a second layer was not deposited. At this point the screens were on the upper side of the microscope slide with a single layer of the membrane covering them. The excess water then was drained off of the microscope slide and film and slide were allowed to dry under a Petri dish cover, the latter serving to prevent the accumulation

of dust and foreign particles on the surface of the film. The drying process was generally hastened by placing the slide on a tripod ringstand and warming it from below with an ordinary 40-watt light bulb. This dried the membrane fairly rapidly without damaging it. When the membrane was completely dry, a small drop of the virus suspension under consideration was placed on each of the screens covered with the membrane. The virus suspension was allowed to remain on each screen for a period of from two to five minutes and sometimes longer. The suspension was generally deposited on the membrane surface by means of an ordinary dropper which had been drawn out to a fine point or by means of a micropipette. The major portion of the drop was removed from the screen with the same type of dropper or micropipette. After removal of the drops the remaining suspension was allowed to dry again and after drying was washed with distilled water to remove as much as possible of the salts and foreign material present (Cater and Stanley, 1946).

All the preceding steps having been carefully carried out, the virus under consideration was ready for examination with the electron microscope.

In the preparation of tobacco mosaic it was possible to use a further refinement in technique of preparation. Tobacco mosaic after having been prepared for electron microscope examination by filtration methods was then shadowgraphed, a technique which is discussed in the following pages.

Shadowgraphing.

A technique of comparatively recent origin is known as shadowgraphing. Essentially it consists in the formation of a dark area resembling a shadow on one side of the specimen under investigation. This procedure has the effect of giving the shadowgraphed material a raised or three-dimensional

appearance which has proved of considerable assistance in the study of biological as well as other types of material. In the case of virus studies this technique has been almost indispensable in the recognition, measurement, and identification of some of the viruses investigated so far.

Technique of shadow casting.

This technique was originally developed as a means of measuring the heights of objects under the electron microscope. The material under consideration having been purified and mounted prior to examination with the electron microscope, is then placed on a stage under a bell jar. The bell jar and contents are then evacuated and a metallic substance, chromium, gold, uranium, or some other metal, is then vaporized by means of a tungsten filament. (Other metals such as molybdenum may also be used for filaments.) The particles of vaporized metal travel in straight lines and bombard the surface supporting the specimen obliquely at a suitable angle. High points of the surface, such as virus particles, intercept the metal particles in the form of a fine film, particularly on the side from which the metals are "shot". Areas immediately behind the high points are blocked from the metallic spray, and in a negative appear as shadows and serve to make the particles in the field stand out from the underlying surface (Williams and Wyckoff, 1945a). The principle compares perfectly to the casting of a shadow by objects exposed to light rays.

In some of the earlier work using the shadowgraphing or shadowcasting technique, chromium was used as the shadowing metal. As pointed out by Williams and Wyckoff (1945b) this metal is suitable as long as one is dealing with bacteria and most viruses that are large compared with the thickness of the metallic layer deposited on them during the shadowcasting process. In the case of smaller particles, i.e. certain other viruses, a false im-

pression of size and shape is given by the metals of lower electronic scattering power, such as chromium. As shown by Williams and Wyckoff (1945b), gold, which has a higher electron scattering power than chromium, is admirably suited for the formation of very fine films and gives, therefore, a more accurate representation of the specimen. Gold is capable of giving a calculated thickness of 5 to 10Å as compared with chromium which gives a calculated thickness of ca. 80Å.

This report includes shadow-cast photographs of tobacco mosaic only. At the time this work was done, shadowcasting equipment was in the process of being built at Oklahoma Agricultural and Mechanical College, but was not completed until a later date. Through the courtesy of Mr. Hugh Barton of Phillips Petroleum Company at Bartlesville, Oklahoma, tobacco mosaic preparations were shadowgraphed with gold and pictures taken.

The advantages of shadowcasting of viruses can be listed as follows:

1. A three dimensional effect is produced, giving greater clarity of the specimen.
2. Details of morphological and physical structure are enhanced.
3. The shadow formed in the process may be used in quantitative measurements of height.
4. A means of assistance in identification and recognition of the virus is offered by this technique.
5. Greater definition and contrast is obtained in the photography of the viruses.

The process of shadowcasting is being used extensively today in many fields of research with the electron microscope and is by no means confined in its use to the field of biology. It serves admirably in the investigation

of the minute details of all types of surface structures and makes visible structural details which otherwise might be invisible.

Replica Technique.

Often closely associated with the shadowgraphing process is the replica technique. It is particularly useful in the examination of materials too thick to be viewed directly under the electron microscope, and has found wide and extensive applications in the field of biology. The technique consists of placing a film of plastic material, Formvar, collodion, or other plastic, over the surface of the specimen. The plastic, upon drying, gives a replica of the specimen and may then be stripped off and examined under the electron microscope.

The replica technique was developed by Schaeffer and Harker (1943) and was used by Williams and Wyckoff (1945a) in preparing gold-shadowed specimens of tobacco mosaic. They were interested in the preparation of a very smooth substrate in order not to confuse the ultimate structure of collodion films with tobacco mosaic virus. For this purpose they used polished glass microscope slides which had been chemically cleaned. On these surfaces they placed drops of the virus suspension which were allowed to dry and were gold-shadowed in a vacuum chamber. They then coated the microscope slides with a thin layer of collodion. When dry, the collodion layer was stripped off the slide together with its adhering gold. This eliminated the fine structure of the collodion because its structure was not brought out by the shadow-cast gold.

The replica technique was not used in any of the studies discussed in this report.

RESULTS OF ELECTRON MICROSCOPE EXAMINATIONS

Tobacco Mosaic Virus.

The virus first examined in these studied with the electron microscope was tobacco mosaic mirus. To the beginner in electron microscope virus research this virus has several distinct advantages which recommend its use. As mentioned previously, it is comparatively stable, exists in high concentrations, is of relatively large size, and is quite readily isolated from the host material. In addition to this it has been photographed so often that from photographs in the literature the beginner can recognize it quite readily under the electron microscope.

Two means of preparation of tobacco mosaic were used. Originally the material was prepared by the filtration methods already described. Using this method it was possible to view the virus with the electron microscope and to obtain photographs of it. With the assistance of Mr. Hugh Barton some of this material was gold-shadowed and photographed. The striking difference between the two types of material, i.e. gold-shadowed and non-gold-shadowed, can be seen from the photographs which follow. Virus entities that have not been shadowed appear only as dark rods, while the gold-shadowed material stands out from the field in bold relief and gives a much clearer conception of the morphology and structure of the virus. In addition the photographs of the gold-shadowed tobacco mosaic virus, in the case of end-to-end aggregation of the separate virus entities, show the points or boundary lines where the particles have aggregated themselves end to end.

At the beginning of the studies using the ultracentrifuge as a means of purifying virus material, tobacco mosaic was again used, largely to be-

come acquainted with the technique involved and the results which might be expected. As shown by the photographs which follow, it is possible to obtain, not only very high concentrations of the material, but also fields of virus entities which are free of contaminating plant material. As seen from the photographs, tobacco mosaic appears in such a concentrated form as to present the appearance of a web or net.

From measurements made of the particle size of the isolated material, and from comparisons with pictures of tobacco mosaic virus found in the literature, and from the examination of healthy tobacco preparations, it has been determined that the material isolated from diseased Turkish tobacco was the infectious entity causing the symptoms known as tobacco mosaic in the host. The basic units of the virus appear to be rod-like structures averaging 15 x 280 as has been shown by other workers. End-to-end aggregation of the basic virus units has also been observed in these studies. In some cases this phenomenon appeared in a very pronounced manner, while in other instances it was hardly noticeable. No attempt has been made in these studies to determine the probable causes of this phenomenon. Studies of this type have been undertaken by Rawlins, Roberts, and Utech (1946) and by other workers.

Severe Etch Virus.

Leaves of Turkish tobacco infected with severe etch virus were harvested, frozen overnight, and purified using filtration techniques.

Examination with the electron microscope yielded two types of particles not found in healthy tobacco. One of these was rod-shaped while the other consisted of much smaller spherically-shaped particles. The rod-shaped entities suggested the presence of tobacco mosaic virus which was later confirmed by examination and inoculation tests of the host plants from which the extract examined was obtained. It was found that the host plants ori-

ginally containing only severe etch had become accidentally contaminated with tobacco mosaic virus.

Photographs taken of the extracts containing both viruses reveal the presence of red-shaped and spherical particles. The small spherical particles may be seen scattered between the rod-shaped tobacco mosaic virus. These spherical particles appear to be all of a similar size and in a sufficient concentration to suggest that they may be the agents of severe etch.

Isolation of severe etch virus from tobacco mosaic was not completed in time to attempt preparation of severe etch by ultracentrifugal means.

Sesbania Mosaic Virus.

Following the initial success with tobacco mosaic virus using filtration techniques, an attempt was made to isolate and study the virus causing sesbania mosaic.

Leaves of sesbania plants, which had been placed in the dark for about 20 hours to cut down on the starch contents of the leaves, were harvested and prepared for examination in the usual manner. Both healthy and diseased leaves were prepared in order to compare the two in an endeavor to determine whether there were any entities present in the diseased preparations which were not found in the healthy material and which could possibly be identified as the virus producing the mosaic in sesbania. Several preparations of both healthy and diseased leaves were made using slight modifications in filtration methods.

In spite of the fact that all precautions were taken to prepare pure material for examination wihh the electron microscope, nothing approximating conclusive results could be arrived at. Foreign plant materials were

generally present to such an extent that the presence of the virus could have been easily masked, particularly if the virus were spherical in shape and therefore similar in appearance to the plant materials.

In the preparation of all viruses from plant materials by filtration methods, dilution of the juice following the completion of filtration procedures was necessary. It was necessary to dilute the final extract in order to decrease the heavy concentration of plant materials otherwise present. Dilution, in addition to decreasing the amount of plant particles present, also decreases the number of virus particles present in any given preparation. In the case of viruses such as sesbania mosaic virus, which, unlike tobacco mosaic virus, may be present in the plant extract in comparatively small concentrations, dilution of the material may also hinder recognition of the viruses as such, for in general it may be said that the more concentrated a virus preparation is, the easier it becomes to identify. Particularly is this true in the study of viruses heretofore not examined with the electron microscope.

Studies on this virus were finally abandoned due to the inability to obtain conclusive results using filtration and dilution methods.

Cucumber Mosaic Virus.

Diseased Turkish tobacco leaves infected with two strains of cucumber mosaic, one, ordinary cucumber mosaic, and the other a strain found on bindweed by K. Starr Chester in Stillwater, were prepared for examination with the electron microscope, originally using filtration and dilution procedures and later using ultracentirfugal methods.

Results using filtration and dilution methods were very unsatisfactory. No structures could be consistently recognized as characteristic of diseased material.

With the ultracentrifuge somewhat more conclusive results were obtained of the presence of what may be the virus producing cucumber mosaic. Time was the limiting factor in carrying these investigations further.

Cabbage A virus.

Using filtration and dilution methods, an attempt was made to investigate the virus designated as cabbage A virus. For this purpose leaves of diseased cabbage had to be thoroughly ground with a mortar and pestle as the cabbage leaf yields practically no juice by ordinary pressing methods and very little juice when ground. Even though rather complicated filtration methods were used to remove the plant materials present in large amounts due to the fine grinding of the leaves, mounts made of the juice when examined with the electron microscope were so heavily laden with plant substances that recognition of the presence of a virus was impossible.

Time did not permit ultracentrifugal preparation and subsequent examination of cabbage A virus with the electron microscope.

Cowpea Mosaic Virus.

Preparations were made of the cowpea mosaic, symptoms and host range of which were discussed in the first part of this report.

Using Nicotiana glutinosa as the host plant, specimens were prepared for examination under the electron microscope using both filtration and ultracentrifugal techniques.

As in the case of all other filtration preparations with the exceptions of tobacco mosaic virus, examination with the electron microscope failed to give results that were in any way conclusive. Electron microscope examination of cowpea mosaic virus prepared by ultracentrifugal techniques, however, showed the presence of apparently spherically shaped particles, some of these in a highly aggregated state, that may be the infective agent causing cowpea mosaic. It cannot be definitely stated that the particles shown in the photographs which follow are the cowpea virus. More extensive investigation than has at this time been possible would be necessary before conclusions of this nature could be drawn. It is significant, however, that bodies of the type shown were not found in any of the examinations of healthy Nicotiana glutinosa. Gold shadowing of diseased preparations would, no doubt, aid markedly in the determination of the nature of the particles observed.

Wheat Mosaic Virus.

Wheat grown in experimental plantings at Oklahoma Agricultural and Mechanical College, and showing symptoms of a mosaic, was prepared for electron microscopic examination. The ultracentrifuge was used exclusively in the preparation of the diseased material. Healthy wheat leaves were prepared in a like manner for purposes of comparison with the diseased extract.

As shown by the photographs which follow, a number of similarly shaped particles appeared in the field under the electron microscope. These seem to show a definite homogeneity in their size and appearance and further appear to be aggregated to some extent. Similar types of bodies have not been found in extracts of healthy wheat leaves. Again, it is impossible at this time to draw definite conclusions as to the nature of the particles observed. More thorough investigations are necessary before definite conclusions can be made.

As pointed out earlier, even after careful purification and the complete, or practically complete removal of foreign substances from electron microscope preparations, the possibility still exists that homogeneous particles found under the electron microscope may be high molecular weight proteins rather than viruses. This fact must be continually kept in mind in the investigation of viruses with the electron microscope.

CONCLUSIONS

Electron microscope studies of plant viruses have shown that it is possible to isolate and observe the agents causing the various virus infections. The results also indicate that careful preparation of the specimens using adequate techniques is necessary before the desired results can be obtained, and furthermore, that the accurate and proper interpretation of the material under consideration is only possible through extensive investigations of that material. In the identification of particles viewed with the electron microscope as the causal agent of any particular virus disease, the necessity of examining numerous fields of both healthy and diseased extracts is of prime importance.

Each virus investigated with the electron microscope constitutes a separate and distinct problem in some respects. While the general technique of preparation is the same for all, the physical and chemical properties of the virus under consideration appear to have a definite effect on the ease with which adequate preparations are made. This point may be illustrated by a comparison of the preparation and observation of tobacco mosaic as contrasted with cucumber mosaic. In the case of the former, specimens are easily prepared and the virus can be readily observed and

identified under the electron microscope. In the case of the latter, satisfactory preparation and identification is more difficult. This is probably accounted for by a difference in physical and chemical properties such as original degree of concentration in the host, size of virus proteins, and general stability, which involves optimum pH, longevity in vitro, effect of temperature and various other effects, all of which must be taken into account in the preparation of the material for electron microscopic examination. Another factor which must be considered in virus preparations using the ultracentrifuge, and one which varies depending on the molecular weight of the particular virus under consideration, is the force necessary to throw down the virus. As pointed out earlier, the force is directly proportional to the speed and the radius through which the virus suspension is revolved, all of which must also be taken into consideration.

SUMMARY

A brief outline of the history and development of the electron microscope, together with the basic principles of electron optics and the various techniques of preparation of virus specimens is presented, including filtration and ultracentrifugation methods.

Examination of tobacco mosaic virus definitely showed the presence of rod-shaped bodies 15mμ x 180mμ which were identified as the agent causing tobacco mosaic. Examination of cowpea mosaic virus, wheat mosaic virus, and severe etch virus revealed spherically-shaped bodies which are believed to be the entities producing each of the respective diseases, but will require further investigation before arriving at definite conclusions. The study of cucumber mosaic virus and sesbania mosaic virus revealed no agents which could be identified as the viruses producing these diseases, and an

investigation of Cabbage A virus proved entirely unsatisfactory due to the preponderance of plant materials present.

It is concluded that plant viruses may be investigated and identified with the electron microscope with the use of proper and adequate techniques. Each virus under investigation must be regarded, at least in part, as a separate and distinct problem, and the nature of each virus must be taken into account in preparing it for electron microscope examination.

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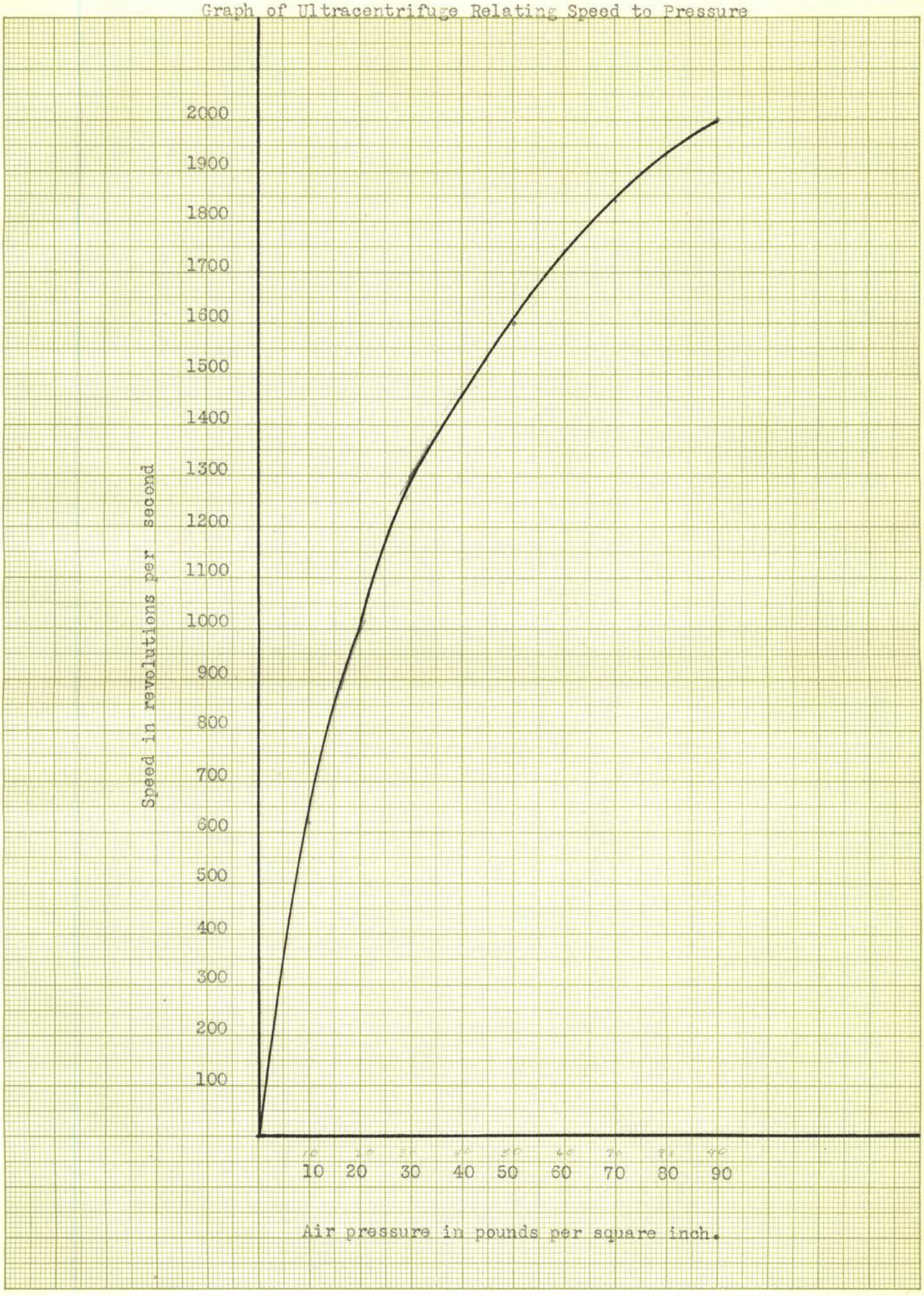
Graph of Ultracentrifuge Relating Speed to Pressure



Crotalaria Mosaic Virus

Explanation to Plate 1

A. Healthy and diseased crotalaria plant. Note reduction in leaf size, distortion and bright yellow mosaic pattern on diseased plant, left.

B. Two healthy, right, and two diseased, left, crotalaria plants, showing dwarfing and stunting due to crotalaria mosaic virus.

PLATE 1

A

B

Sesbania Mosaic Virus

Explanations to Plate 2

A. Healthy sesbania leaf

Explanations to Plate 3

A. Diseased sesbania leaf. Note the distortion, puckering, and mosaic pattern on the leaf.

Explanations to Plate 4

A. Healthy sesbania plant

B. Diseased sesbania plant. Note the distortion, puckering and reduction in size of the leaves.

Explanations to Plate 5

A. Top view of a healthy sesbania plant

B. Top view of a diseased sesbania plant.
Note extreme distortion, puckering, and crinkling of some of the infected leaves.

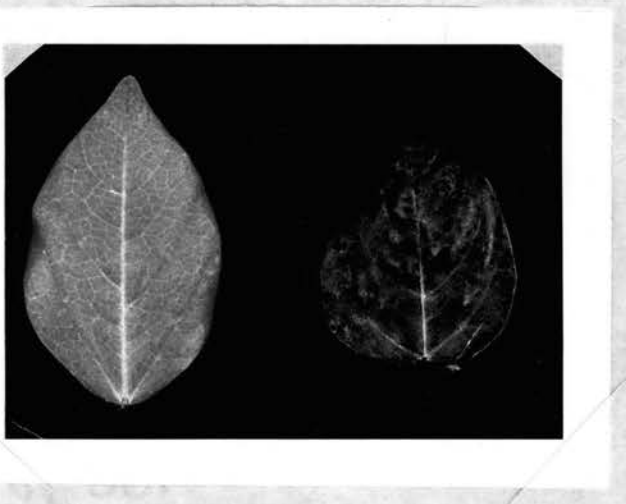
Explanations to Plate 6

A. Healthy, left, and diseased, right, leaflets of sesbania. Note reduction in size, distortion, and puckered effect of diseased leaflet.

B. Healthy, left, and diseased, right, leaflets of Ogden soybean infected with sesbania mosaic. Note the angular mosaic symptoms, vein clearing, and distortion of the edge of the diseased leaflet.

C. Healthy, right, and diseased, left, leaves of Ogden soybeans infected with sesbania mosaic.

PLATE 6



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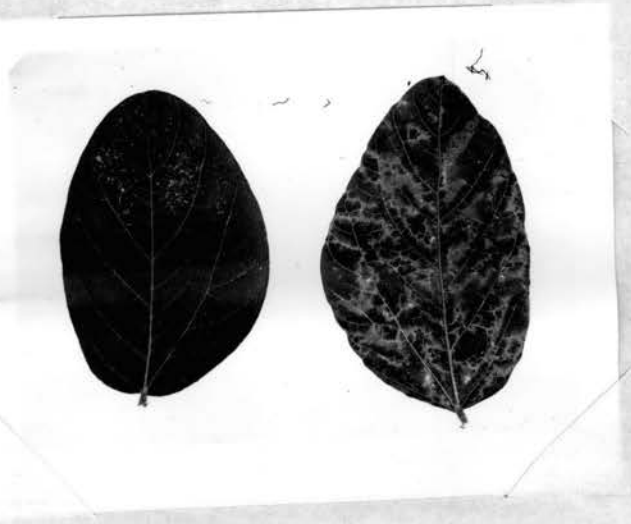

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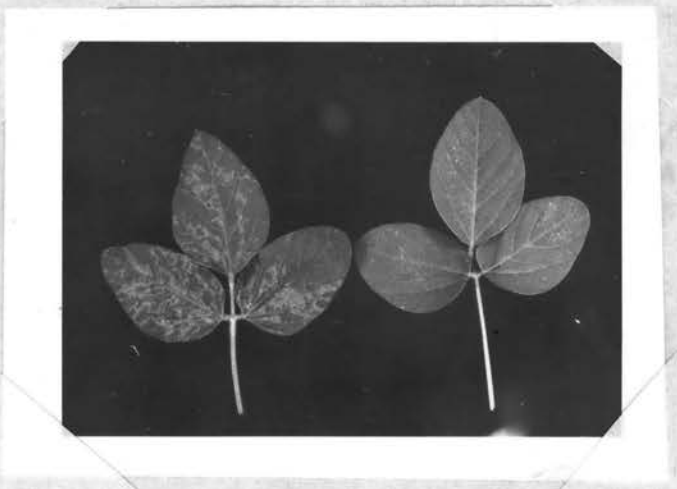


C

Cowpea Mosaic Virus

Explanations to Plate 7

A. Healthy cowpea leaf

B. Diseased cowpea leaf. Note the mild symptoms of vein clearing on the central leaflet due to infection with cowpea mosaic.

Explanations to Plate 8

A. Healthy *Nicotiana glutinosa*

B. Diseased *Nicotiana glutinosa* infected with cowpea mosaic virus. Note the mild mosaic and distortion of some of the upper leaves.

Explanations to Plate 9

A. Healthy *Nicotiana glutinosa* leaf

B. Leaf of *Nicotiana glutinosa* infected with cowpea mosaic. Note the leaf distortion and mild mosaic symptoms.

PLATE 7

A

B

PLATE 9

A

B

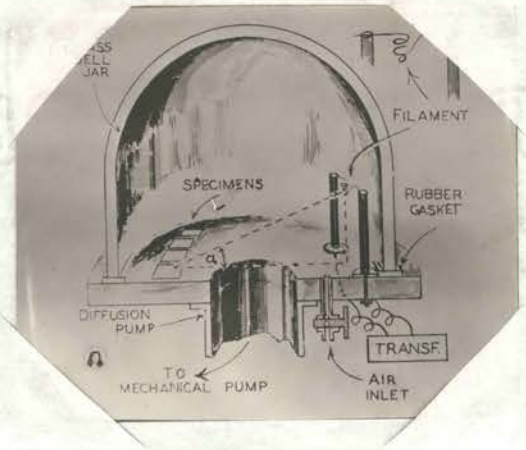
The Electron Microscope

Explanation to Plate 10

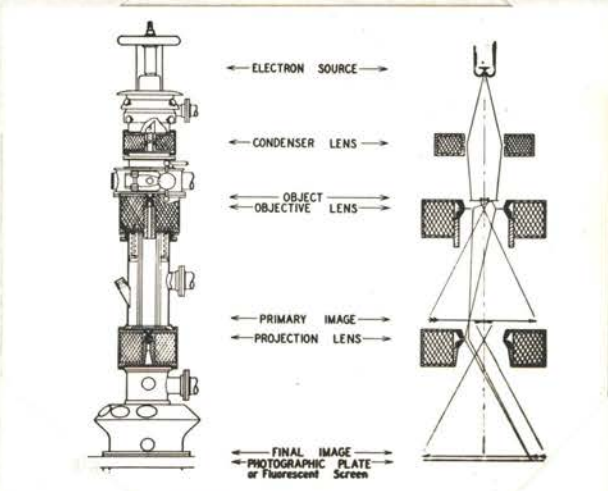
A. Shadowgraphing apparatus

B. Optics of the Electron Microscope

PLATE 10


FILAMENT
SPECIMENS
RUBBER GASKET
DIFFUSION PUMP
TRANSF.
TO MECHANICAL PUMP
AIR INLET

A


ELECTRON SOURCE
CONDENSER LENS
OBJECT
OBJECTIVE LENS
PRIMARY IMAGE
PROJECTION LENS
FINAL IMAGE
PHOTOGRAPHIC PLATE
or Fluorescent Screen

B

Tobacco Mosaic Virus

Explanations to Plate 11

A. Gold-shadowed specimen of tobacco mosaic virus. X 21,100 approx. Note the rod-like structures which are the virus and the segmentation of the long rods showing end-to-end aggregation of individual virus particles. The white objects in the field are plant contamination. The specimen was prepared by filtration methods.

B. Gold-shadowed specimen of tobacco mosaic virus. X 40,000 approx. Extreme enlargement has caused a fuzzy appearance of the specimen.

Explanations to Plate 12

A. Gold-shadowed specimen of tobacco mosaic virus showing the dark effect of heavy gold shadowing. X 40,000 approx.

B. Tobacco mosaic virus X 10,000 approx. The specimen has not been gold-shadowed. Note the long rods caused by end-to-end aggregation of the virus particles.

Explanations to Plate 13

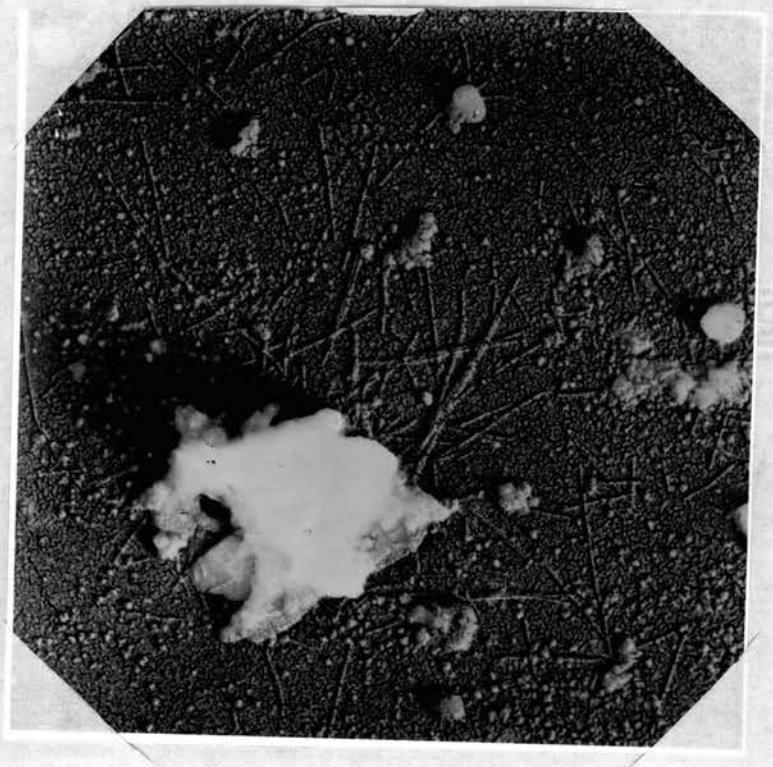
A. Tobacco mosaic virus showing extreme end-to-end aggregation of the virus particles. X 20,000 approx.

Explanations to Plate 14

A. Tobacco mosaic virus prepared with the ultracentrifuge. X 8,750 approx. Note high concentration of virus particles producing a webbed effect. Dark areas are due to the aggregation of the virus.

B. Tobacco mosaic virus prepared with the ultracentrifuge. X 8,750 approx. Note the absence of foreign material from the field. Dark areas are produced by aggregation of the virus.

PLATE 11



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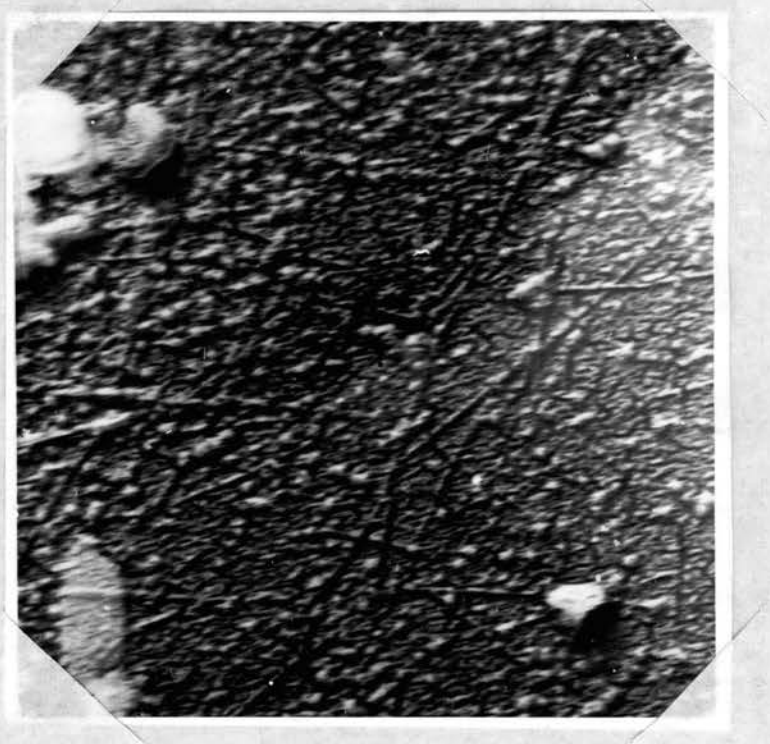

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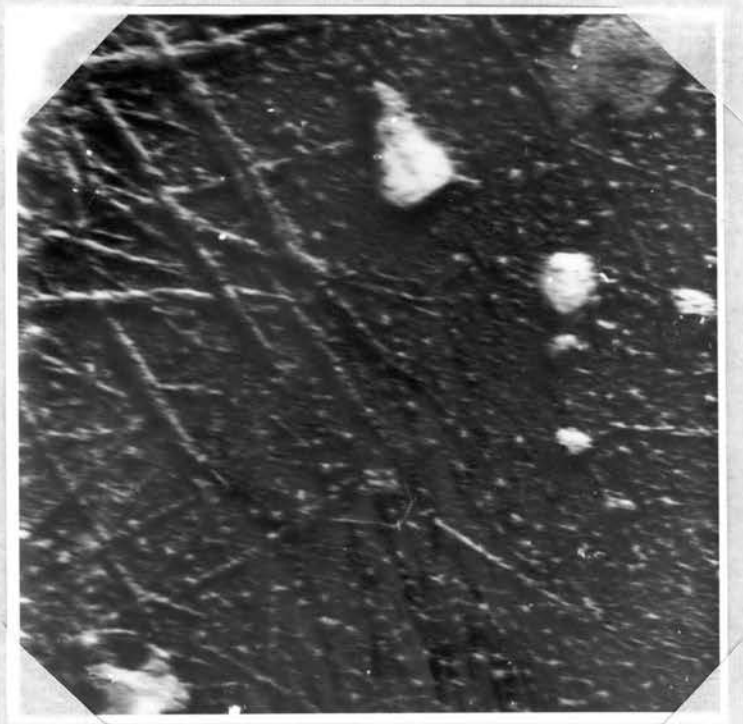
PLATE 12



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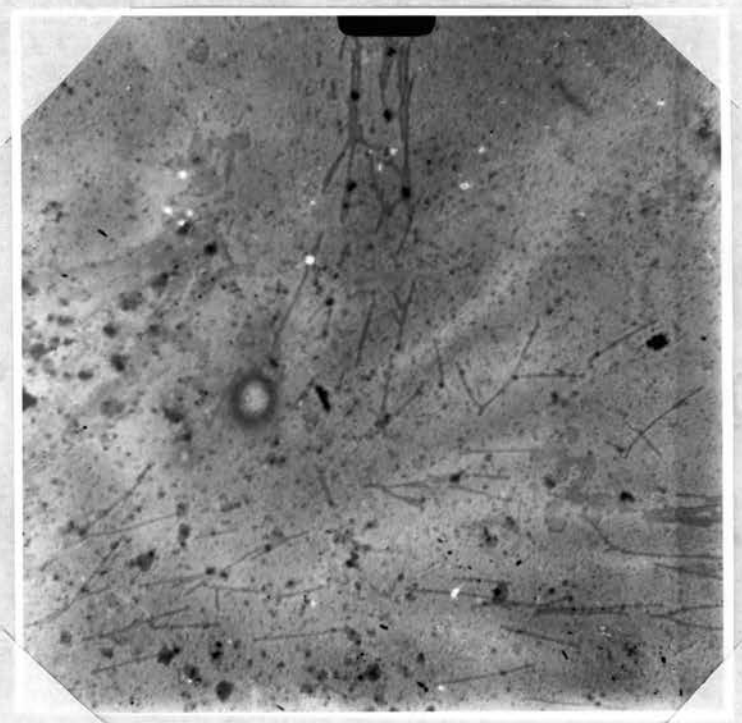

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PLATE 13

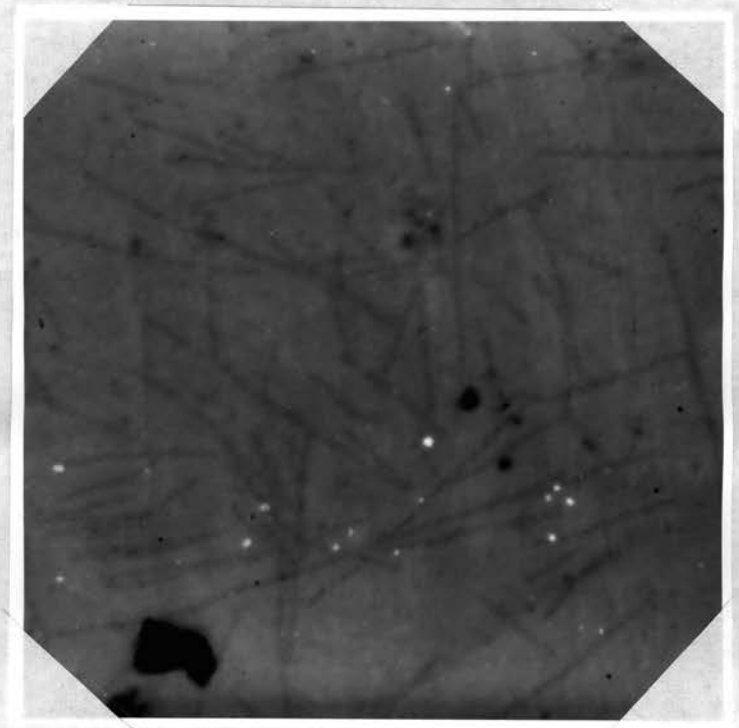

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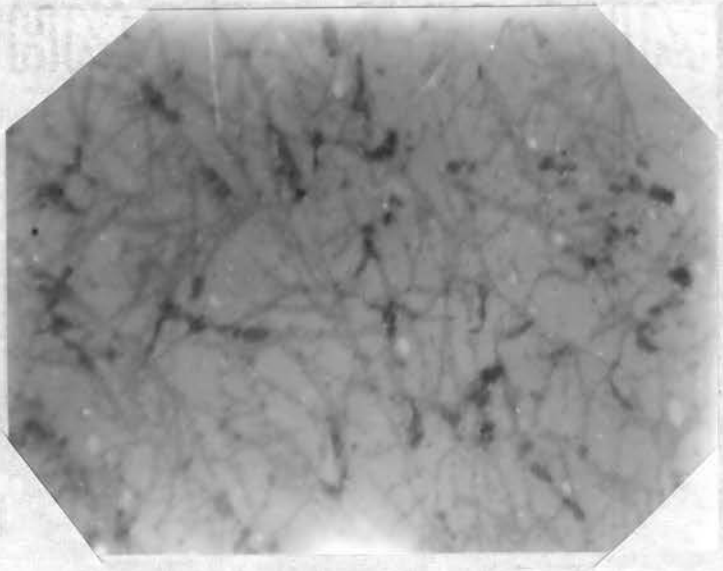
PLATE 14



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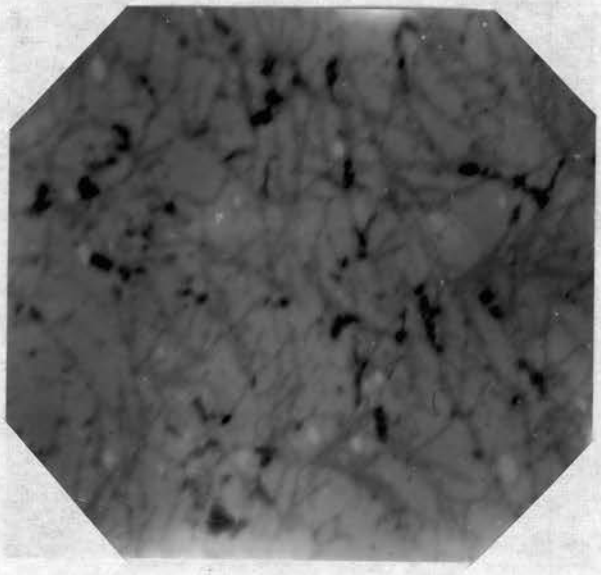


B

Sesbania Mosaic Virus

Explanations to Plate 15

A. Tobacco mosaic virus, rod-shaped particles, and spherical particles believed to be sesbania mosaic virus. X 10,000 approx.

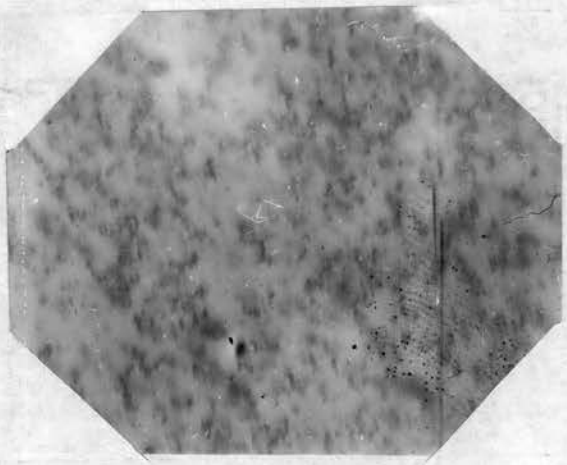
Cowpea Mosaic Virus

Explanation to Plate 16

A. Extreme aggregation of apparently spherical particles believed to be cowpea mosaic virus. X 7,500 approx. Specimen prepared with the ultracentrifuge.

B. Specimen from cowpea infected with cowpea mosaic virus and prepared by filtration methods. Note the masking of what may be the cowpea mosaic virus by contaminating colloidal plant material. X 7,500 approx.

PLATE 16



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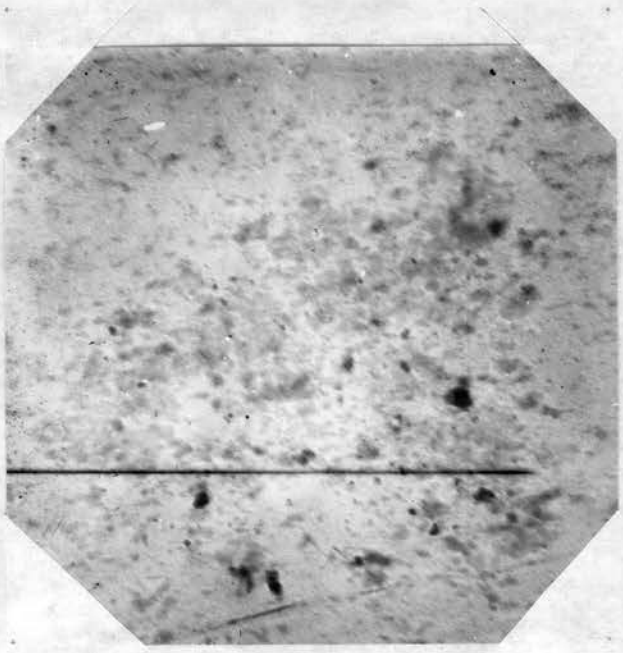


B

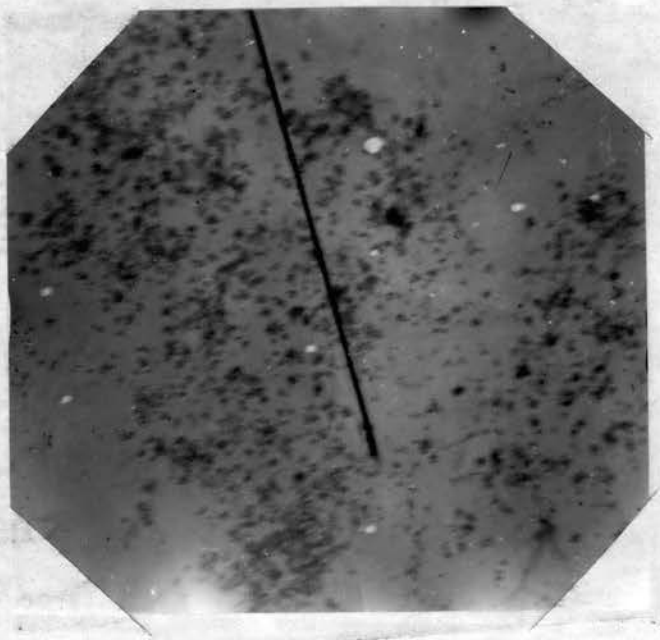
Wheat Mosaic Virus

Explanation of Plate 17

A. Aggregation of apparently spherical particles believed to be wheat mosaic virus. X 8,750 approx. Specimen prepared with the ultracentrifuge.

B. Particles believed to be wheat mosaic virus. The double or paired appearance of each particle is due to vibration in the electron microscope at the time the photograph was made. Specimen prepared with the ultracentrifuge. Note the lack of contamination or foreign substances. X 8,750 approx.

PLATE 17



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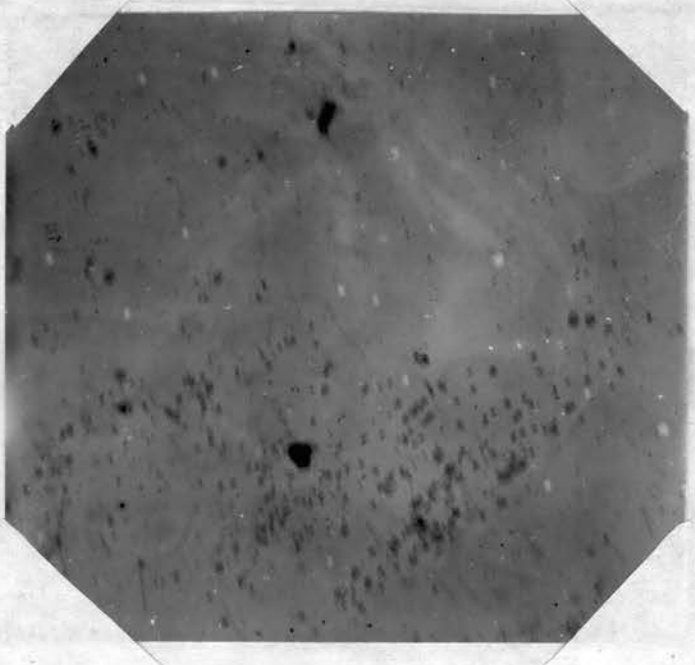

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Typist:
Evelyn M. Preston