SOME BIOLOGICAL AND PHYSICAL PROPERTIES OF CITRUS VIRUSES, WITH PARTICULAR EMPHASIS ON STUBBORN DISEASE

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INTRODUCTION

Of the many known diseases of citrus, those caused by viruses are least understood and most difficult to control. Control of these diseases has been approached through the development of virus free budwood. This method can be improved, and other methods developed through an understanding of the biological and physical properties of these viruses. Some of the more important of these properties are the serological relationships, host range, morphology of the virus, nucleic acid content, the temperature and pH of inactivation, and the chemical and physical properties which permit purification. The natural host range of the important citrus viruses as recognized by citrus virologists has been increasing steadily. Accurate serological diagnostic tests have only recently been developed for some of these virus pathogens. These serological tests have enabled citrus virologists to positively determine the presence of these viruses in citrus and herbaceous plants much more rapidly. Grant and Corbett (51, 52), in Florida, and Desjardins and Wallace (31) at the University of California at Riverside developed a method for the transmission of infectious variegation

(a virus disease of minor importance) which has enabled workers at the University of Arizona to transmit two of the more important viruses to herbaceous plant hosts. The advantage of transmitting a virus to an herbaceous plant is that the incubation period of the virus is much shorter than in the original woody host. This short period enables research workers to study such physical properties as the pH and temperature range of inactivation. Studies concerning virus morphology and nucleic acid content necessitate having a constant available source of the virus at hand. This is best accomplished by the use of herbaceous hosts. Therefore, as a result of research now in progress on the biological and chemical properties of citrus viruses, it should be possible to gain at least a limited understanding of some aspects of the physiology of parasitism in the near future. It is possible that with information of this nature new and more efficient control measures may be developed.

This paper will discuss and present experimental evidence of the serological relationships and morphological properties, attempts at purification, and studies on the pH and temperature of inactivation of psorosis and stubborn viruses, and some of the possible serological relationships of xyloporosis and cachexia viruses. The history and symptoms of psorosis, stubborn, xyloporosis, and cachexia, as they appear in the literature will also be summarized.

After the development of a serological test for stubborn disease was reported by Storm (90) and Storm and Streets (91) attempts were begun to develop a more rapid test applicable to use by workers with a minimum of equipment. The need for an extremely accurate test was apparent, and studies on the possible use of the agar diffusion technique were started. Serological tests provide an accurate diagnosis more quickly than symptom expression, which in citrus often takes years. The method of serum production for stubborn disease reported by Storm and Streets (91) was adapted to psorosis (92). An extensive check of all antisera produced was made in order to determine whether dual antisera had been produced. Details of these serological studies are reported herein.

Transmission of psorosis "A" and stubborn viruses to various herbaceous host plants was attempted, with a degree of success. Studies on the pH and temperature of inactivation were made. Several attempts at purification were made with little success. Studies on the morphology of these viruses yielded some useful information. The results of these studies have made it possible to speculate on some possible control measures.

Terminology

Whenever the term "healthy tree" is used in this paper

it means that this tree does not have the disease in question. Since there is no way of demonstrating that any given tree is free from disease, the criterion used in all these studies was that all plants used as controls were completely free from any evidence of the disease or diseases under study in that particular experiment. The terms "stubborn" and "stubborn disease" are used interchangeably in the literature when a worker is referring to the disease in which the causal agent is the stubborn virus. The term "stubborn" will be used in the above sense in this paper. When terms such as stubborn leaves, fruits, or trees are used, the implication will be that these leaves, fruits, or trees carry the stubborn virus. When "psorosis" is used without qualification as to the strain, the reference will be to psorosis "A." Xloporosis and cachexia will be considered as having the same causal virus or as being caused by strains of the same virus throughout this dissertation, except in Chapter I where the early history is discussed.

CHAPTER I

Survey of the Literature

Psorosis, stubborn, xyloporosis, and cachexia are among the most important virus diseases of citrus known (65, 66). These diseases all have a deleterious effect on yield, vigor, and appearance of citrus trees (65, 66). None of these diseases, so far as is known, has any insect vectors (65, 66).

Psorosis

Psorosis was the first citrus virus to be named and described. It was first reported by Swingle and Webber (94) in 1896. In 1932 Fawcett (33) gave the name psorosis "A" to the common form to distinguish it from the more destructive but less common form psorosis "B" (102). In addition to psorosis "A" and "B," Fawcett and his colleagues, according to Wallace (102), described four other types of psorosis: blind pocket, concave gum, crinkly leaf, and infectious variegation. All types of psorosis produce in common a chlorotic flecking of citrus leaves which occurs in early spring. In some years this flecking is not discernible.

Since this paper is only concerned with psorosis "A," the description of symptoms will cover only this disease.

In addition to the leaf symptoms described previously, bark symptoms are also produced. Small pustules usually appear on the bark of the main trunk in patches with or without gum formation. The pustules rupture and scales about 1/2 to 1/8 inches thick are formed. These lesions are first found in localized areas on the older bark of the trunks (Fig. 1) and limbs of the trees which are six to twelve or more years of age. As the scaling advances, deeper layers of bark and the wood are visibly affected, though still alive and functional. The rate of scaling varies from tree to tree. After the lesions have been present for some years, gum deposits often occur between layers of wood corresponding to the annual growth rings (Fig. 2). Large amounts of gum often form, depending on the environmental conditions. The xylem vessels become partly plugged with gum in the vicinity of the psorosis lesion (7). Later, the wood becomes light brown to reddish brown. The discoloration proceeds in an irregular fashion. The tree deteriorates; leaves are small, yellow, and few in number (66, 102).

Psorosis is found in all citrus species. This disease is one of the most destructive citrus diseases known, and is found wherever citrus is grown (66, 102). The only known means of spread is through the use of infected budwood (35, 102). In 1923, Lee (67) reported that psorosis was found in the Philippines, China, Japan, and many of the Pacific Islands. Fawcett (34) found this disease in Brazil,

Fig. 1. Trunk of a Washington navel orange tree severely infected with psorosis "A."

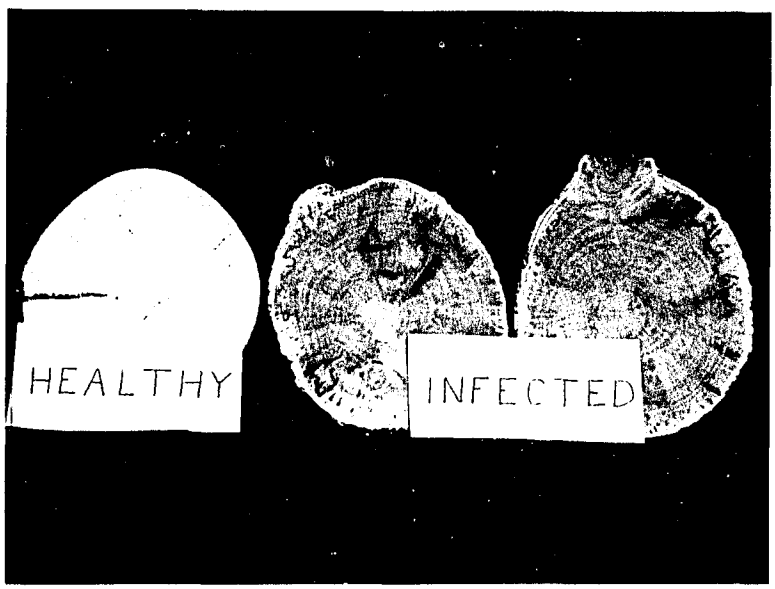

Fig. 2. Wood from healthy and psorosis "A" infected citrus branches. The wood of the infected branch is deeply stained with gum.

Argentina, and Paraguay. Rossetti (88) and Valiela (97) confirmed Fawcett's report. Malaguti et al. (69, 70) reported psorosis in Venezuela. Psorosis has been reported in all of the citrus producing areas of the United States (66, 102).

Three possible methods of control of psorosis have been suggested but each has its disadvantages. Budwood certification has the most promise of furnishing effective control (36, 103). However, in the past the difficulty of identifying virus-free budwood has limited the effectiveness of this method of control. Symptom expression in indicator plants is relatively slow and requires large greenhouse and screenhouse facilities (1, 103). Two methods of prolonging the productive life of trees infected with psorosis "A" have been used with some degree of success. The first method involves the scraping of the bark lesions with a scraper. This treatment seems to slow down the advance of the disease (33, 87). The second method involves the painting of a 1% dinitro compound (Dinitro-O-cyclohexyl-phenol, Dow DN-75) in kerosene over the lesion to 6 in. above and 3 in. below it (40, 43). This treatment also removes the scales, as did the first method. Both treatments serve only as a means of prolonging the economic life of the tree a few years. The disease will eventually render the plant commercially worthless in spite of these treatments (40, 43).

Wallace (101) reported that cross protection exists between strains of psorosis. If this is the case, it should be possible to inoculate rootstocks with a mild strain of the virus. This might protect the trees against a severe strain if such a strain were carried in the scion.

The only method of complete control suggested is the use of clean budwood. Production of such budwood might be simplified with the improved methods of diagnosis suggested in Chapters III and IV.

Fawcett, in a series of papers (37, 38, 39), proposed a system for naming citrus viruses according to the binomial system applied to the plant kingdom. Using this system of classification, he named the psorosis "A" virus Citrivir psorosis var. vulgare.

In 1960, Grant and Corbett (51) reported that they had been able to mechanically transmit infectious variegation from Eureka lemon to Crotalaria spectabilis Roth and Vigna sinensis Endl. (cowpea) by grinding infected leaves in a sucrose solution with activated charcoal. In 1962 Desjardins and Wallace (31) reported that they were able to mechanically transmit infectious variegation to the Chicago Pickling variety of cucumber, Cucumis sativa L. Desjardins and Wallace (30) have also produced a successful antiserum to the infectious variegation strain of psorosis. The research by these two groups of workers has furnished information which

transmission and the production of an antiserum to the psorosis "A" virus (92) as reported in Chapters II and III.

As a result of the research on the various strains of psorosis, new and more efficient diagnostic tools are now available.

Stubborn Disease

In the course of a three year performance study on navel oranges, Fawcett et al. (48) observed that some trees were stunted in growth and poor in production. As a result of some budding experiments with these trees in which the buds were slow in developing, he gave the name "stubborn" to this condition (44).

In the twenties and thirties, various symptoms were described by a number of workers and the diseases attributed to these symptoms were called crazy top (71), pink nose (71), acorn fruit (48), and acorn disease (54). All of these diseases were later shown to be part of the stubborn disease syndrome (44). Through a series of bud transmission experiments, Fawcett (41) established that stubborn disease was caused by a virus which he named Citrivir pertinaciae. However, Dimock (32) cautioned that bud transmission is not necessarily final proof of virus presence, since other pathogens, such as Verticillium and other fungi can also be transmitted this way.

Carpenter (13) reported that he had found stubborn in Arizona, California, Florida, and Texas. The first reported occurrence of stubborn outside the United States was made in 1953 (4), when this disease was found in Morocco. The report that stubborn was present in North Africa was soon confirmed by a number of other workers (17, 26). By 1961, stubborn had been reported in Corsica (100), Israel (23), Turkey (19), Greece (19), Egypt (28), and is considered to be wide-spread throughout the Mediterranean area (18). Klotz (66) states that this disease is probably world-wide in distribution. Stubborn is one of the most important virus diseases in Arizona (56), and the estimate of its national and international importance is increasing rapidly. (9, 13).

Stubborn affects many citrus species (13, 66). This disease has been reported on sweet orange (Citrus sinensis L.), Mandarin orange (Citrus reticulata Blanco), Tangelo (Citrus reticulata x C. paradisi Macf.), Shaddock (Citrus grandis (L.) Osbeck), and grapefruit (Citrus paradisi Macf.) (13, 66).

Symptoms of stubborn on navel orange according to Fawcett (41) are production of multiple buds and abnormal branching resulting in a brush-like growth. The leaves are broader and shorter than normal. There is usually an unseasonable, somewhat chlorotic flush of growth in the fall. Often some of the fruits have a rind normal at the stem end,

becoming abruptly thinner and smoother over the rest of the surface, producing an acorn appearance. These acorn-shaped fruits tend to be sour and bitter. Other reports (13, 45, 48) include in the symptomatology of orange and grapefruit the common occurrence of off-season or fall bloom in the infected trees. Abnormal leaf drop in the fall and twig dieback are also common. Fawcett and Klotz (46, 47) reported that stubborn was one cause of non-bearing in oranges and grapefruit.

The most complete description of stubborn symptoms was published by Carpenter (13) in 1959 and summarized by Storm (90) in 1960. Carpenter defined three categories on Marsh grapefruit trees. Type I trees show the most serious symptoms, Type III the mildest. According to Carpenter (13), the number of stubborn fruits on an infected tree varies from year to year. However, the acorn-shaped fruit remains the most reliable symptom of the disease. Chapot (19) claims that any fruit with a curved columella is diagnostic of stubborn.

Carpenter (13) also states that symptoms in navel and other sweet oranges except Valencia have a range of symptom expression similar to grapefruit. He states that the Valencia orange is little affected by stubborn.

In 1958, Carpenter and Hield (16) cast doubt on the reliability of the long-accepted blue albedo symptom of this disease. In the course of fruit set experiments with

they observed that this chemical apparently induces blue albedo in grapefruit. They concluded that blue albedo may not be a specific indicator of stubborn, but merely a characteristic though non-specific symptom.

A number of reports have recently appeared on the internal changes in citrus thought to be associated with stubborn. Bové et al. (9) showed that stubborn fruits contained less soluble sugar than normal fruits, and that there was a good deal of variation in the amino acid content between healthy and diseased fruits. They concluded that stubborn induces clear-cut chemical changes in the juice from grapefruit and orange fruits.

After treating stubborn diseased trees with iron chelates, Hilgeman (55) concluded that although there was a slight increase in yield and an improvement in appearance of treated trees, the value of these compounds in the control of this disease has not yet been established. However, he did suggest three possible ways in which the stubborn virus might be acting: (a) The virus might interfere with auxin action; this would tend to explain the stunting, off-season blooming, and the fall defoliation of diseased trees. (b) The virus might be inducing bud mutations, because frequently the disease is expressed in one or a few branches on a tree. (c) The stubborn virus might be a latent virus, whose expression in the form of visible symptoms is brought about by adverse environmental conditions, such as weather.

The possibility that a virus might be able to induce genetic abnormalities in fruit trees was also made by Nyland (74) as a result of his work with stone fruit viruses.

Storm (90) and Storm and Streets (91) reported that studies of cleared leaves from several varieties of healthy and diseased citrus trees indicated that there were fewer calcium oxalate crystals in the stubborn infected leaves. This report tends to confirm earlier observations by McGeorge (71) that there is less total calcium in stubborn diseased grapefruit leaves. Johnson and Storm (64) found that McGeorge's conclusion also held for Washington navel orange trees.

Calavan and Christiansen (11) state that most of the symptoms attributed to stubborn by Fawcett et al. (48) are not sufficiently specific to be diagnostic. They claim that many of the trees diagnosed as stubborn on the basis of gross symptomatology have proven to be adversely affected by such factors as root rot, cachexia, exocortis, inferior rootstocks, scionic incompatibility, and/or heredity. However, Carpenter (14), basing his statement on his indexing program, states that stubborn is not caused by tristeza, psorosis, vein enation, or cachexia viruses either singly or in combination. Calavan and Christiansen (11), however, are correct in stating that most of the

stubborn symptoms are vague, and without stubborn fruit on the tree it is a simple matter to mistake stubborn for any number of other diseases. With the recent production of an antiserum produced from antigen sources obtained from Carpenter which is specific for the stubborn virus, a positive diagnosis is possible any time of the year (91, 92).

In the past, most of the research on stubborn has been conducted in the field, but in the last few years laboratory investigations have been started (9, 19, 64, 91). As a result of these studies, methods for the positive diagnosis of stubborn have been developed. Since there has not been an insect vector reported for this disease, it is probably spread exclusively by infected propagating stock. Thus, by determining whether trees which are to be used for a bud source are free of the virus, and using only virus-free buds in propagation, the spread of this disease should be stopped in the near future.

Xyloporosis

Xyloporosis was first reported by Reichert and Perlberger (86) in 1934 on Palestine sweet lime (C. auranti-folia (Christm.) Swingle) in what is now Israel. Since 1934, xyloporosis has been found to be widespread throughout the Mediterranean area (83, 84), in North America (66), and in South America (42, 88). Reichert (83) claims that this disease is as important to world citrus production as

tristeza (quick decline).

In addition to Palestine sweet lime, the xyloporosis virus attacks sweet orange, sour orange, tangelo, mandarin orange, grapefruit, lime, and shaddock (12, 66, 77, 80). This disease is being recognized in more and more areas and its reported host range is being increased rapidly (12, 83).

The symptoms as described on Palestine sweet lime develop in three stages: (a) Small round to ovoid depressions occur in the wood of the stock below the bud union. These depressions or pits result from deformation of the wood (Fig. 3). Corresponding protuberances or pegs, which fit into these pits, are formed on the bark. These effects usually occur just below the bud union and do not extend into the scion. (b) The bark is further depressed and small depressions develop into larger patches and bands. An appreciable overgrowth of the scion usually occurs. Pits become more numerous, giving the wood a sieve-like appearance. The stock becomes elastic and is easily bent over by the weight of the top. (c) Parts of the bark become brown; browning occurs from the inside through to the surface, the bark splits, turns black, and peels away in pieces. The adjacent wood darkens. All of the leaves produced are small and branches wither slowly until the entire top dies.

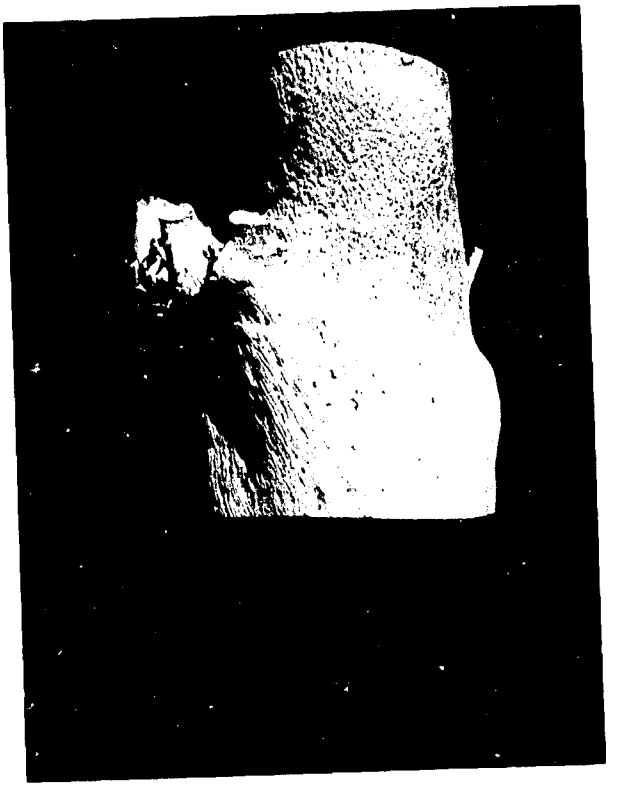

Fig. 3. Wood from trunk of a xyloporosis infected tree. This photograph shows the heavy wood pitting in the scion.

Since xyloporosis is spread through infected propagating stock, control has been approached through bud certification programs (1, 49, 103). It takes at least two to three years to test a bud source for the presence of xyloporosis (103), thus making control an expensive and time-consuming process. A more rapid means of diagnosis is needed.

Cachexia

Childs first reported cachexia on Orlando tangelo in 1950 (20). Since then it has been found on Rough lemon (C. jambhiri (Lushington) Rusk), Citrange (C. sinensis x Poncitrus trifoliata (L.) Raf.), and Cleopatra mandarin. Transmission experiments (22) demonstrated the virus nature of cachexia and showed that grapefruit could be a symptomless carrier. This was confirmed by Olson (75) in 1952. The host range of the cachexia virus has been extended to include sweet orange, kumquat (C. aurantifolia x Fortunella japonica (Thumb.) Swingle), and a large number of mandarin hybrids (24, 27, 77). Cachexia is probably more important than first thought, since new hosts are being found regularly. Cachexia has been reported in many of the citrus producing areas in the Mediterranean (2, 72), and the Western hemisphere (10, 26, 49).

The symptoms of cachexia are similar to those of xyloporosis, for the first suggestion that the disease may

be present is the development of numerous growth cracks in the outer bark. The wood has elongated pits and the bark develops growth ridges which fit into these pits. Cachexia differs from xyloporosis in that brown gum deposits form in the pits and the bark phloem. As in xyloporosis, the bark becomes brown and bark scaling follows. With the entrance of secondary organisms and the onset of various nutrient deficiencies, complete degeneration of the tree occurs. Control again is a matter of prevention (66), that is, the use of virus-free budwood and non-susceptible root stocks (22, 66). As the case with xyloporosis, the determination that a bud source is free of the cachexia virus requires several years after buds from suspected trees are grafted into citrus indicator plants (1, 22, 103).

Childs is of the opinion that cachexia and xyloporosis are caused by the same virus or strains of the same virus (21). He bases this opinion on the fact that the symptoms of both diseases are similar, both have similar host ranges, and that in transmission experiments he has obtained symptoms of both xyloporosis and cachexia on different indicator plants when indexing a single plant (22, 24, 25, 27). This opinion has been confirmed by Calavan and his colleagues (10, 12), and Olsen and his colleagues (76, 77), and it is generally accepted by most citrus virologists that xyloporosis and cachexia are

caused by the same virus, or different strains of the same virus. Grant et al. (53) concluded that xyloporosis and cachexia are different viruses, or at least different strains of the same virus on the basis of observations on cachexia in sweet lime and Orlando tangelo. Reichert and Bintal (85) also claim that xyloporosis and cachexia are different viruses. They base their claim on observations of cachexia in Clementine sweet lime and on Grant's report. However, Calavan, Christiansen, and Weathers (12), as a result of some very precise work involving comparative reactions of Orlando tangelo and Palestine sweet lime to cachexia and xyloporosis, concluded that these diseases are caused by one virus or different strains of one virus. Carpenter and Furr (15) cautioned that wood pitting can occur in undiseased seedlings. It is therefore advisable to use this symptom with care when making a diagnosis.

Reichert (81, 82, 83) has claimed for years that stubborn disease is the same as xyloporosis. Since he has no experimental data and bases his conclusion entirely on field observations, most citrus virologists do not accept this hypothesis.

It is apparent from the information presented in this chapter that none of these diseases have had adequate laboratory investigation. Yet psorosis, stubborn, and xyloporosis-cachexia are of great importance to world citrus

production. Laboratory research on these viruses is in the early stages (30, 31, 52, 92), and more rapid methods of positive diagnosis are being found (30, 31, 52, 64, 92). From this research effort new and more precise control measures should be derived.

CHAPTER II

Serological Experiments

Serological techniques have found wide application in the diagnosis of plant virus diseases (5, 99). This has been particularly true in the potato virus work in Europe (98, 99). The advantage of using serological techniques in the diagnosis of virus diseases is that they are rapid, accurate, and easily applicable once an antiserum has been prepared (5, 99).

Since most of the citrus virus diseases are known to be transmitted by infected budwood, the need for accurate diagnosis of bud source trees is apparent. Serology fills this need in that it provides a rapid, accurate, and inexpensive method of diagnosis.

Serological experiments with stubborn, psorosis, and xyloporosis (cachexia) will be discussed in this chapter. Two approaches were taken: (a) a study using various antisera to develop methods suitable for field identification of viruses in suspected hosts, and (b) studies of the virus titer in stored whole leaves and plant sap. These latter studies were of importance because most of the plants were located either at Yuma or in the Salt River Valley, and

most of the laboratory work was done in Tucson, Arizona, a distance of 250 and 125 miles respectively, this easy access to the plants being tested was not possible.

The first successful serological work involving a citrus virus disease was done in 1960 with stubborn (90). This research was reported in the following year (91). In 1962 Desjardins and Wallace (30) reported that they had produced an antiserum for infectious variegation, a mild strain of psorosis, and Storm and Streets (92) reported the production of an antiserum to psorosis "A." These recent reports indicated the possibility of using serological techniques in the various citrus budwood programs throughout the world.

A. Field Test of Antisera

MATERIALS AND METHODS: The antisera used in these investigations were prepared by using fruits as the antigen sources. Antisera II-1 through II-6 (Table 1) were prepared from obvious stubborn fruits furnished by Dr. J. B. Carpenter. Antigen sources for sera 1 through 4 came from the University of Arizona Citrus Experiment Station at Tempe from trees which were diagnosed as stubborn by Drs. R. H. Hilgeman and J. B. Carpenter. Antiserum S4-12 was prepared from the obviously psorosis infected tree shown in Fig. 1.

Fruits were peeled, liquefied in a blender, and the homogenate was strained through four layers of cheesecloth. The juice was then centrifuged in an International Centrifuge (model number PR-2) for 15 minutes at 5° C at 10,000 times gravity. The supernatant was again centrifuged as above. The resulting supernatant was centrifuged for 90 minutes at 5° C at 24,500 times gravity. Final pellets were resuspended in 5 ml. water, mixed with an adjuvant, and injected into rabbits. Injections were made at weekly intervals for five weeks. Two weeks after the final injection, the rabbits were bled and the blood serum separated and stored in a deep freezer at -16° C until used. This method was described in detail by Storm (90) and Storm and Streets (91) in 1961. Three types of antisera were produced; one to stubborn, one to psorosis, and one to both. Antisera prepared from eleven sources were used (Table 1). All were tested by three methods; (a) the microprecipitin test (98), Fig 4, (b) the Ouchterlony agar double diffusion test (5, 29), Fig. 5, and (c) the chloroplast agglutination test (5, 99), Fig. 6.

When fruits were used in the microprecipitin test or the agar double diffusion test, the initial preparations of the fruits were made in the same manner as that used in preparation of materials for injection into rabbits. When leaf material was used in these tests 5 g. of leaves were ground in 50 ml. of pH 7 phosphate buffer. The homogenate was strained through four layers of cheesecloth and centrifuged in the same manner as the fruit preparations.

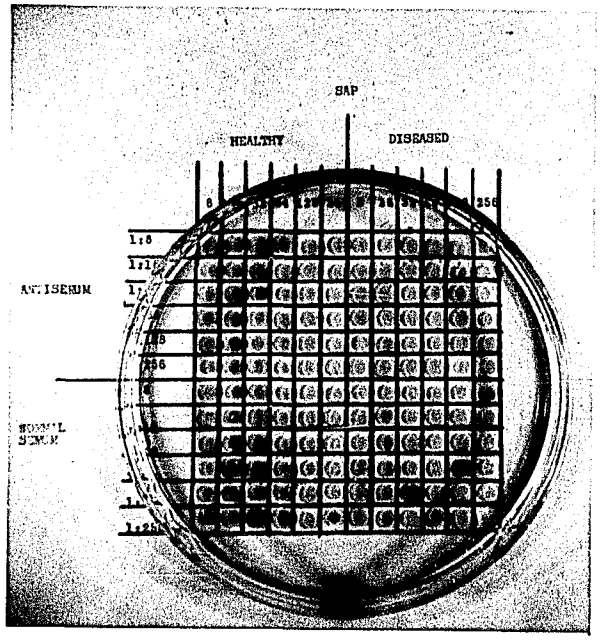

Fig. 4. Photograph of microprecipitin test. The drops are covered with paraffin oil to protect them from evaporation.

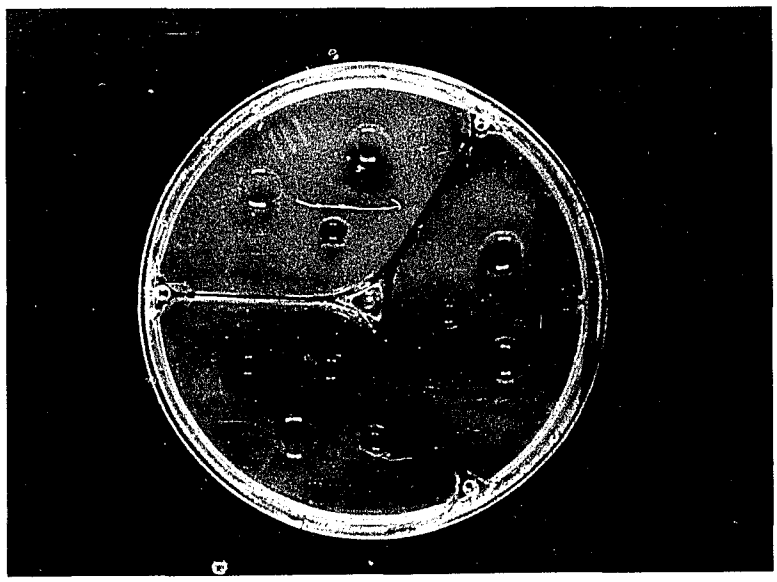

Fig. 5. Photograph of agar double diffusion test. Normal sera and antisera are placed in small wells, and plant sap in the larger ones.

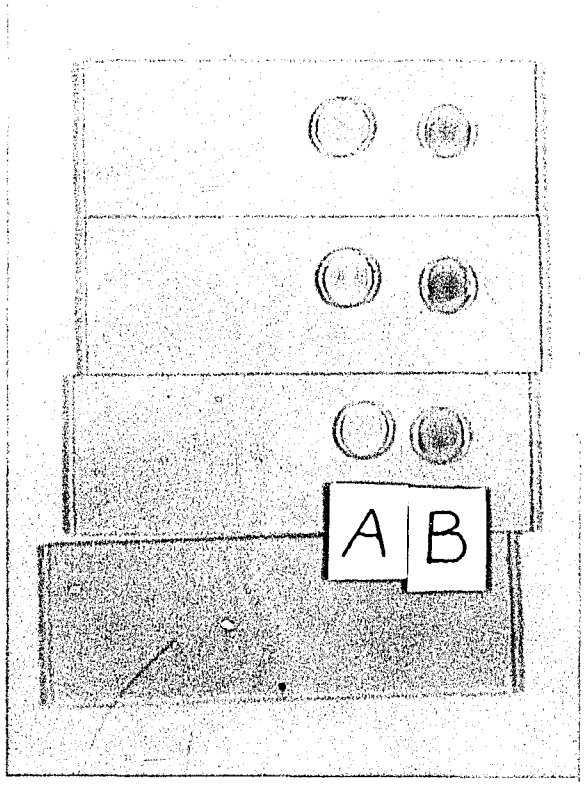

Fig. 6. This photograph of the chloroplast agglutination test shows a positive test for stubborn disease in Row B, which is a mixture of antiserum and sap from an infected tree. Row A is a mixture of sap from a healthy tree and antiserum.

Table 1. Source, titer, type of antiserum

Serum Number	Antigen Source Variety	Titer Stubborn	Psorosis
1	Valencia	1:32	-
2	Valencia	1:256	1:256
3	White Grapefruit	1:32	-
4	Redblush	1:32	1:128
II-1	Marsh I*	1:256	1:64
II-2	Marsh II*	1:128	-
II-3	Marsh III*	1:64	-
II-4	Koethen	1:64	-
II-5	Robertson navel	1:128	-
II-6	Trovita	1:256	-
S4-12	Washington navel	-	1:128

* Refers to Carpenter's symptom types.

The microprecipitin tests were made as described by van Slogteren (98). Plant sap from infected and healthy trees was clarified as previously described, and normal sap and antiserum were diluted with distilled water in a series from 1:8 to 1:256. One drop each of all possible combinations of sap and serum dilutions were mixed as individual droplets on a six inch petri plate which had been previously coated with 0.5% Formvar (Fig. 4). The drops of mixed sap and serum were covered with paraffin oil and the petri plates were stored in the refrigerator overnight. The following morning they were removed and after standing at room temperature for one hour, were examined microscopically.

The Ouchterlony agar double diffusion test was performed in petri dishes three inches in diameter, the bottoms of which were coated with 0.1% agar in distilled water and dried. One gram of agar or 0.5 gram of Ion two agar and 0.85 g of Na Cl were added to 100 cc of 0.01 M phosphate

buffer, pH 7, and the mixture was autoclaved at 15 psi for 20 minutes. Merthiolate was added to produce a final concentration of 1:10,000 of merthiolate. Sixteen ml of warm agar were added to each petri dish and allowed to solidify. The reservoir holes in which the sera were placed were cut with a number 2 cork borer; those used for the antigen preparation were made with a number 4 cork borer. All reservoirs were equally spaced from one another. The agar plugs were removed and the plates were stored in a refrigerator at 5° C until used. After the antigens and antisera had been placed in the reservoirs, plates were kept in a moist chamber at room temperature and examined daily.

Leaf material was prepared for the chloroplast agglutination test in the following manner: (a) A few leaves were placed in a blender with 50 ml of phosphate buffer at pH 7. (b) The homogenate was filtered through four layers of cheesecloth. (c) One drop each of healthy and diseased plant sap was placed on a glass slide which had been previously treated with formvar (Fig. 6). (d) One drop of antiserum was mixed with each drop on the slide. (e) Slides were stored in a moist chamber at room temperature for two hours, then results were read with the aid of a microscope and tabulated. Each sample was checked in triplicate.

All antisera used in these tests were first absorbed with juice from healthy fruits. After an antiserum was shown to be serologically active by the agar diffusion test, and

the titer established by the microprecipitin test, large numbers of leaf samples were checked by the chloroplast agglutination test. Three trees were used as controls for all of these experiments. Two were seedling trees on their own rootstocks. Samples from these trees were received from Dr. R. M. Allen at Yuma, and were as free from known citrus viruses as can be determined by present techniques. The other tree was an exceptionally healthy white grapefruit about twenty years old which in four years of observation showed no symptoms of stubborn, psorosis, or xyloporosis.

All leaf and flower bud samples to be tested were collected in a random manner around the tree from no higher than seven feet above the ground. Samples were collected from some of the trees several times during the year. None of the leaf or bud samples were stored frozen, although some leaf samples were stored in the refrigerator at 5° C for two weeks before they were used.

RESULTS AND CONCLUSIONS: A positive diagnosis of stubborn, psorosis, and possibly xyloporosis-cachexia can be accomplished by the chloroplast agglutination test if young or immature leaves are used in conjunction with the appropriate antiserum. The chloroplasts break up when the leaves are tested as outlined, but a positive test is obtained by the agglutination of chloroplast fragments and other cell debris.

When young (immature) leaves collected from trees known to have stubborn disease were checked by the

chloroplast agglutination test, only 2% of the samples gave an inconclusive test, while none checked free from stubborn. When mature leaf samples from some of the same trees were tested, 48% of the samples were inconclusive and 24% tested stubborn free. The data collected in this stubborn disease series of tests are shown in Table 2 and summarized in Fig. 7.

Table 2. Results of the chloroplast agglutination test on mature and immature stubborn and psorosis infected leaves.*

Disease	Number of trees	Number of samples	Kind of Leaves	Number Positive	Number Negative	Number Inconclusive
Stubborn	162	553	Immature	542	0	11
Stubborn	54	108	Mature	30	26	52
Psorosis	141	219	Immature	210	0	9
Psorosis	10	50	Mature	15	12	23

* Normal serum checks of healthy and diseased sap gave negative results in all cases.

Results obtained in the psorosis series were much the same as those obtained for stubborn. Only 4% of the immature leaf samples known to have psorosis tested inconclusive; none checked psorosis-free. When mature leaf samples were used from some of the same trees, only 30% of the samples tested positive for psorosis; all of the rest were either inconclusive or negative (Fig. 8). The data for these tests are presented in Table 2.

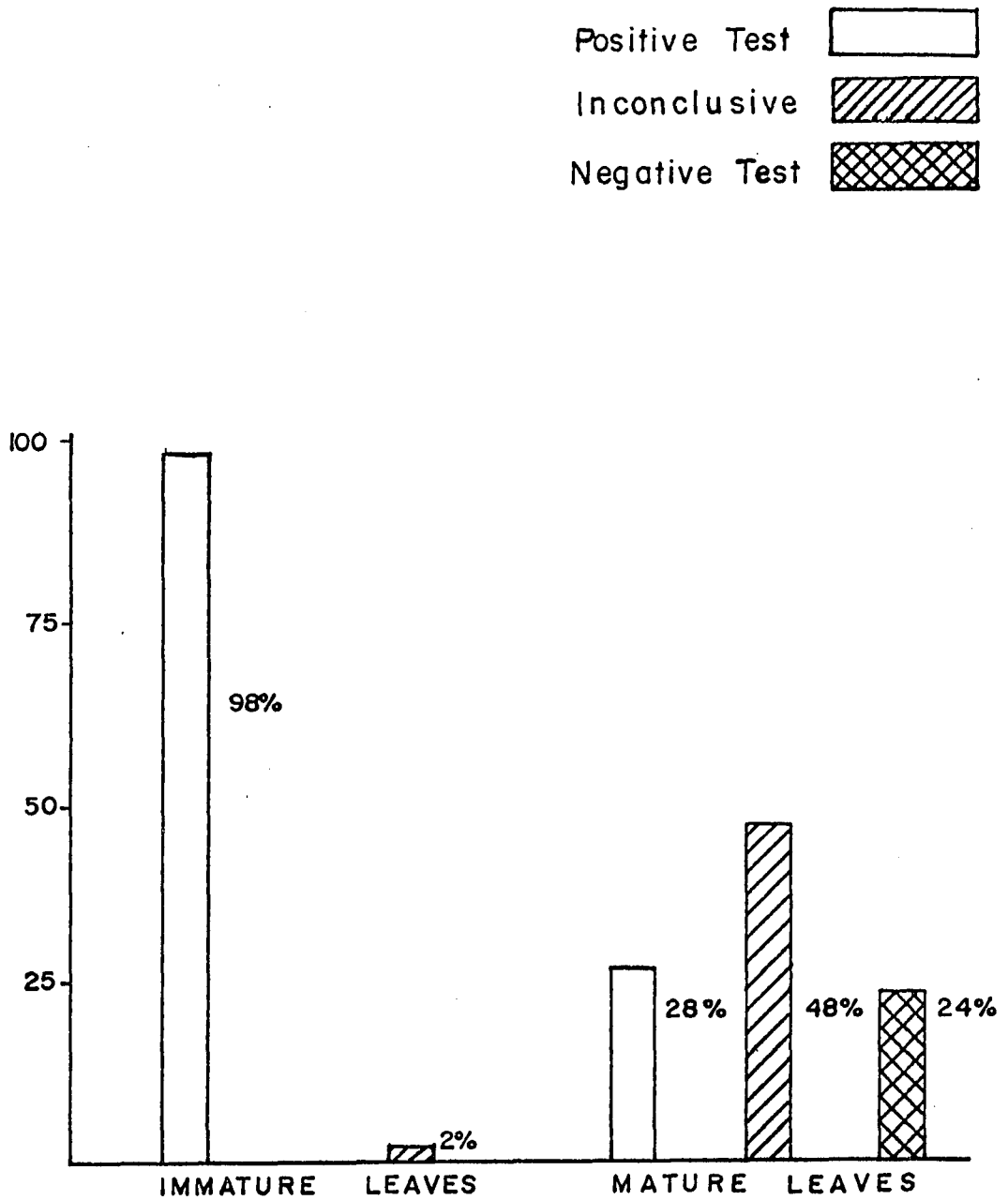

Fig. 7. Results of serological agglutination tests on mature and immature leaves taken from stubborn diseased trees

Leaves infected with xyloporosis (cachexia) were tested with stubborn and psorosis antisera by the chloroplast agglutination test (see Table 3 for data). All of the samples checked with psorosis antisera checked negative. When tested with the stubborn antisera, 85% of the samples gave a positive test, 9% tested negative, and 5% inconclusive (Fig. 9).

Table 3. Results of the chloroplast agglutination test with stubborn or psorosis antisera on leaves infected with either xyloporosis (cachexia), seedling yellows, tristeza, or exocortis.*

Type of Antisera	Disease In Tree	No. of Trees	No. of Samples	No. Pos.	No. Neg.	No. Incon.
Stubborn	Xylop.	137	1014	866	97	51
Psorosis	Xylop.	31	62	0	62	0
Stubborn	Seedling Yellows	2	12	0	12	0
Psorosis	Seedling Yellows	2	4	0	4	0
Stubborn	Tristeza	2	12	0	12	0
Psorosis	Tristeza	2	4	0	4	0
Stubborn	Exocortis	21	168	0	168	0
Psorosis	Exocortis	21	42	0	42	0

* Normal serum checks of healthy and diseased sap gave negative results.

Percentages given in Figures 7, 8, and 9 have been rounded to the nearest whole number.

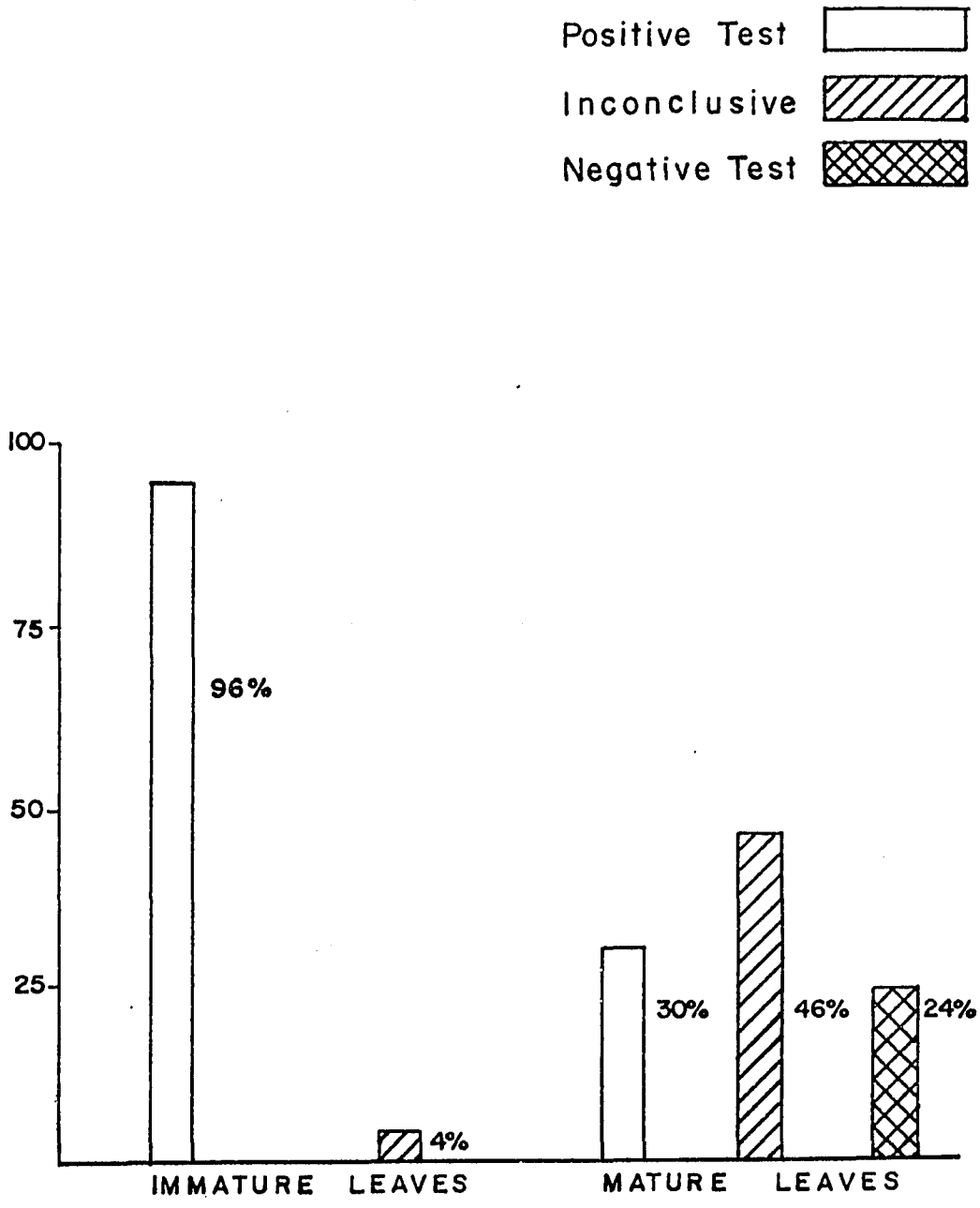

Fig. 8. Results of serological agglutination tests on mature and immature leaves taken from psorosis infected trees.

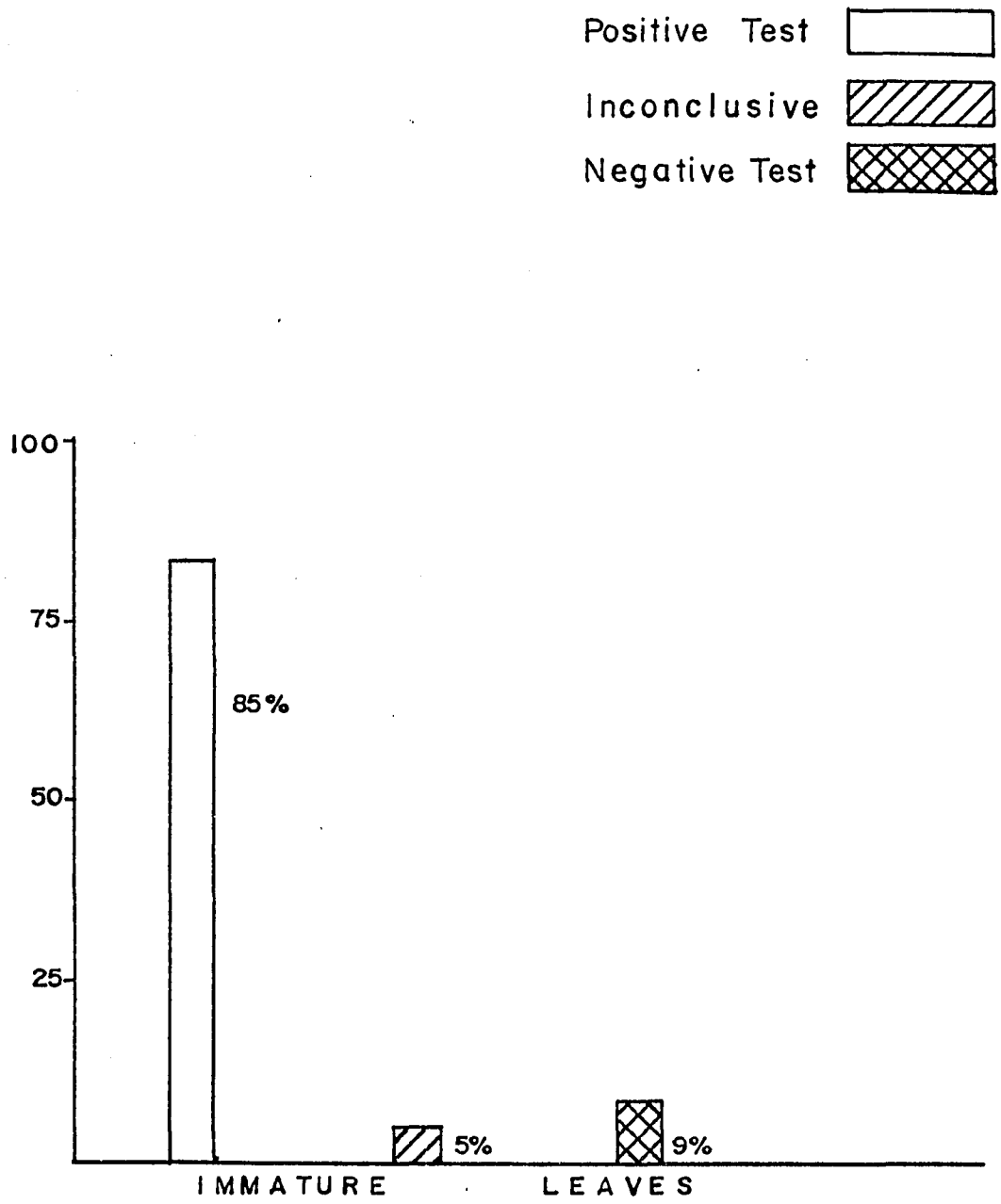

Fig. 9. Results of serological agglutination tests made with stubborn antiserum on leaves from xyloporosis (cachexia) infected trees.

In the course of evaluating the eleven antisera, a few trees infected with other common citrus viruses were checked by means of the chloroplast agglutination test. Trees infected with tristeza, seedling yellows, and exocortis all checked negative with both stubborn and psorosis antisera (Table 3).

Stubborn disease and psorosis can be diagnosed by the use of the chloroplast agglutination test if the appropriate antiserum is used. Old, mature leaves infected with stubborn and/or psorosis yield erratic results when tested by the serological agglutination test.

The results as summarized in Fig. 9 indicate that xyloporosis (cachexia) might be a related strain of the stubborn virus. These data tend to support the claim of Reichert (81, 82, 83, 84) that stubborn and xyloporosis are caused by the same virus. Although no experiments were designed to measure possible differences between xyloporosis and cachexia, in the few cases that these strains were noted, no difference was discernible in their serological activity.

B. Effects of Storage on the Serological Activity of Stored Leaves and Raw Plant Sap

Since most of the serological tests reported in this chapter were performed in Tucson, Arizona, a city some distance from the commercial citrus areas of the state, it became necessary to determine the effects of storage on the materials

collected and transported. For this reason studies on virus stability in stored leaf material and raw sap were begun.

MATERIALS AND METHODS: Leaf materials were collected as described in section A of this chapter from healthy, psorosis, and stubborn trees from a grove near Tucson. Stubborn Marsh grapefruit and psorosis-infected Washington navel orange trees were used in these studies. Since it was discovered early in these studies that freezing causes the agglutination of cellular fragments after grinding, and the purpose of this experiment was to determine whether storage would affect the results of the chloroplast agglutination test, no material was stored in the frozen state. The leaves were transported from the point of collection to the laboratory in Tucson in sealed plastic bags which contained a moist paper towel, and bags of samples were transported in an ice chest. Upon arrival at the laboratory, these samples were transferred to a refrigerator and remained there until used. Maximum time of transit was not more than 45 minutes.

Serum number 2, the highest titer dual antiserum for stubborn and psorosis, was used in all of these tests. The microprecipitin test was used in order that an estimate of titer reduction could be obtained. Stored whole leaf material was checked by the agglutination test. These leaf samples were tested on the day they were collected and 2, 4, 7, 14, 28, and 35 days after collection. Raw sap from homogenized leaves was tested the day of collection and each

day thereafter for nine days.

A leaf sample of 5 g was ground in 50 ml of phosphate buffer in a blender, then strained through four layers of cheesecloth. At this point a portion of the sample was set aside for the raw sap studies, and three drops of this aliquot were used in the agglutination test. The remainder was filtered through one-half inch of cellite on one sheet of Whatman filter paper in a Buchner funnel. This filtrate was then diluted 1:64 and checked by the microprecipitin test. Raw sap was stored in the refrigerator with one part merthiolate per 10,000 parts sap to prevent microorganism activity. These experiments were repeated three times.

RESULTS AND CONCLUSIONS: In all cases in which stored whole leaves were used, the serological activity remained constant for the duration of the experiment. This was true for both the stubborn and psorosis material.

Raw sap expressed from stubborn material kept its activity for two days. The activity then dropped off to half of its original activity (Fig. 10) and remained constant until the eighth day; then dropped to a point where the material had a titer of 1:16.

Raw sap from psorosis infected leaves had no serological activity the second day. This experiment was then repeated with readings at one hour intervals (Fig. 11). The activity remained constant for one hour, then dropped off until at the fifth hour there was no discernible activity.

Whole leaves infected with the psorosis and stubborn viruses can be stored for one month at 5° C without any measurable loss in serological activity. These experiments measured only the stability of the protein coat of the virus; thus it is possible that the virus nucleic acid is being destroyed, which would cause loss in infectivity. For this reason it is probably better to use only fresh preparations for experimental work with stubborn.

Serological activity of raw sap from psorosis infected trees drops off rapidly in storage, indicating that the protein coat is being affected. If this is so, the virus nucleic acid is being exposed to the destructive forces in the preparation, and is probably being destroyed. Raw sap from psorosis material cannot be stored any length of time. This indicated that fresh preparations must be used in all experiments.

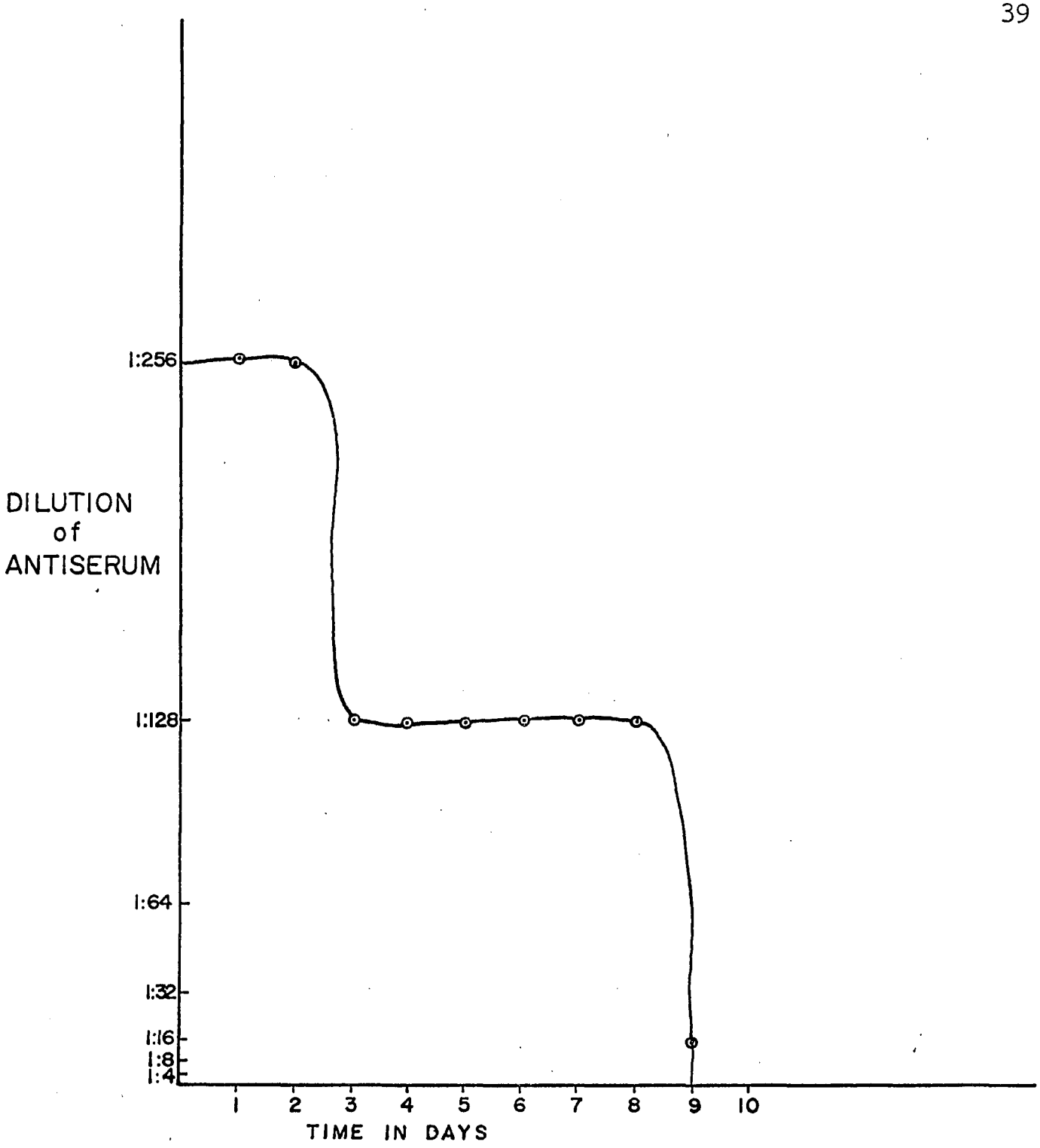

Fig. 10. Effects of storage on raw sap from stubborn infected leaves.

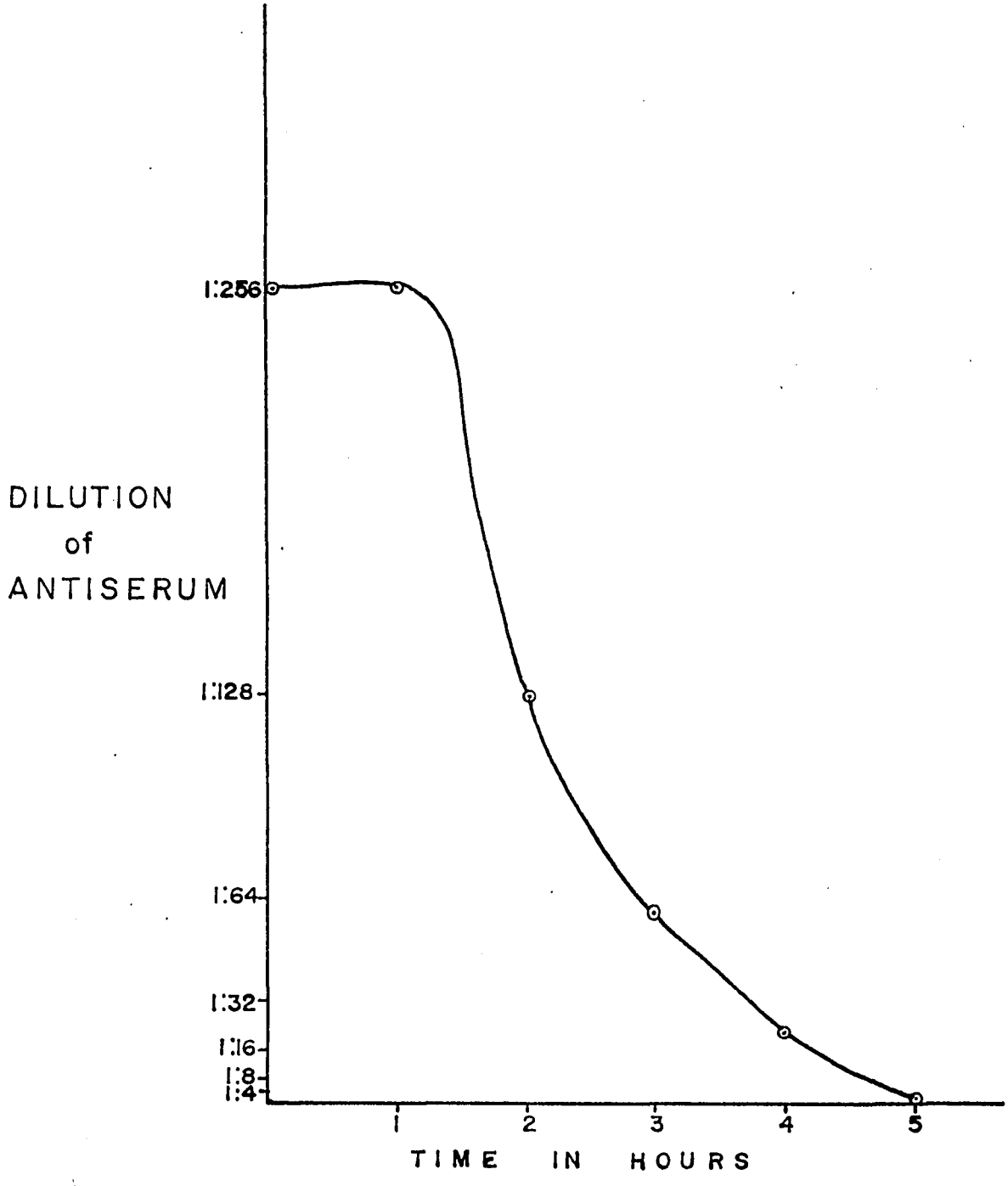

Fig. 11. Effects of storage on raw sap from psorosis infected leaves.

CHAPTER III

Mechanical Transmission

Transmission of a woody plant virus to an herbaceous host is one of the most important steps to be taken before the disease involved can be studied extensively in the laboratory. Since a fruit tree is difficult to move, a constant source of the virus being studied is seldom available to the worker. However, if the virus can be transmitted to an herbaceous host, laboratory personnel can have available a constant source of the virus in plants of the same physiological age. The symptoms on an herbaceous host, because of the host's shorter life cycle, often can be used as a means of diagnosing a given virus disease. If, as is the case with many citrus virus diseases, there is a latent period of several years before the symptoms appear on the tree, the advantages of an herbaceous host are apparent. If by chance the symptoms produced on the herbaceous plant are of the local lesion type, a measure of the relative titer can be obtained by comparing the number of lesions produced per given treatment.

Grant and Corbett (51, 52) reported the first successful mechanical transmission of a citrus virus to an herbaceous host. They were able to transmit infectious variegation

(a strain of psorosis) to Crotalaria spectabilis and to cowpea, and back into Eureka lemon. Desjardins and Wallace (31) reported mechanical transmission of infectious variegation to cucumber in 1962. Holmes (57) reported the mechanical transmission of potato mottle virus to and from citrus. It is thus apparent that viruses can be transmitted to and from citrus. However, to date no one has reported success in the mechanical transmission of either the stubborn or psorosis "A" viruses.

The discussion of the experimental work on the transmission of woody plant viruses will be limited in this chapter to studies of stubborn and psorosis "A" diseases. A number of herbaceous plants were used in these experiments, but successful transmission was obtained only when the cucumber variety White Spine was used.

MATERIALS AND METHODS: Citrus leaves from healthy trees and infected trees known to have the disease in question were ground in a blender in a 2% sucrose solution in a 0.1 M pH 7 phosphate buffer at a rate of 5 mg/g of leaf material. Before grinding, activated charcoal (Norite AO) at a rate of 0.05 g/g of leaf material and cellite also at 0.05 g/g of leaf material were added to the sucrose buffer solution. Leaves and solution were pre-cooled in the refrigerator at 5° C until used. After grinding, the plant juice was filtered through four layers of cheesecloth. This juice was placed in a beaker in a tray of ice water and taken to the

greenhouse, where it was immediately used in the inoculations. Inoculations of the cotyledonary leaves were made with a stiff bristle brush. Attempts were made to transmit the stubborn virus and psorosis "A" virus to Papago pea, Bountiful bean, Chenopodium amaranticolor, Kentucky 57 tobacco, and White Spine cucumber, and from cucumber to Koethen sweet oranges. In all cases the transmission of the virus in question to cucumber was confirmed serologically by the chloroplast agglutination test. One Washington navel orange tree seriously infected by psorosis and a stubborn Marsh grapefruit tree which had been observed to show all the disease symptoms over a three year period were used. Both trees were tested serologically, and these tests confirmed the original diagnosis. The control material came from the healthy white grapefruit tree described in Chapter II.

RESULTS AND CONCLUSIONS: Both the stubborn virus and the psorosis virus were transmitted to cucumber. Time of day of the inoculation had an important effect on the per cent transmission. One-half hour before dawn proved to be best (Table 4). Probably it would be possible to obtain as high a percentage of transmission any time of day if one had facilities to place the plants in the dark for a few hours before inoculation. It is possible that a higher temperature at the surface during the day inhibited transmission. Inoculation experiments at dawn were repeated three times over a period of one year. The data for these experiments are reported in Table 5.

Table 4. Effect of time of inoculation per cent transmission of the stubborn and psorosis viruses.

Time of day	Number of cucumber plants inoculated	Virus used	Number of plants infected	% transmission*
1/2 hour before dawn	40	stubborn	36	90
	40	psorosis	31	78
11:30 A.M.	40	stubborn	11	27
	40	psorosis	16	40
4:00 P.M.	40	stubborn	4	10
	40	psorosis	2	5

* Rounded to nearest whole number.

Table 5. Results of mechanical transmission experiments.

Virus Used	Source of Inoculum	No. cucumber Plants Inoculated	No. Infected	Per Cent * Transmission
Stubborn	Marsh Grapefruit	40	36	90
Stubborn	Marsh Grapefruit	40	29	73
Stubborn	Marsh Grapefruit	132	113	86
Psorosis	Washington Navel	40	31	78
Psorosis	Washington Navel	40	32	80
Psorosis	Washington Navel	120	106	88
Stubborn	Cucumber	40	17	43
Psorosis	Cucumber	40	19	48

* Rounded to nearest whole number.

Attempts to transmit the psorosis virus from cucumber to Koethen sweet orange may have been successful, as a faint mottle appeared in the young leaves. Leaf symptoms take at least three months to appear, and the mottle was very faint. Stubborn disease appears in citrus trees when they are six to twelve years old, so no hope of short term diagnosis existed. A serological test was not performed on these trees after inoculation.

In cucumber the stubborn virus produced extreme dwarfing frequently accompanied by an almost complete lack of internode elongation at the top of the plant (Figs. 12 and 13). Plants usually become chlorotic and die within a month. Presence of the stubborn virus was confirmed serologically by the chloroplast agglutination test (Chapter II).

Psorosis "A" symptoms first appear in cucumber five to seven days after inoculation as a slight chlorotic mottling which is difficult to see and too faint to photograph. A diagnosis was obtained by collecting the cotyledonary leaves after dark, removing the chlorophyll with 95% ethyl alcohol, and staining with Iodine potassium iodide as described by Bawden (6). Chlorotic areas stand out as black or dark blue spots because of starch accumulation in these chlorotic areas (Fig. 14). There is a slight dwarfing effect produced by the psorosis "A" virus which is noticeable about 28 days after inoculation. This effect is not as pronounced as with the stubborn virus (Fig. 15). The psorosis

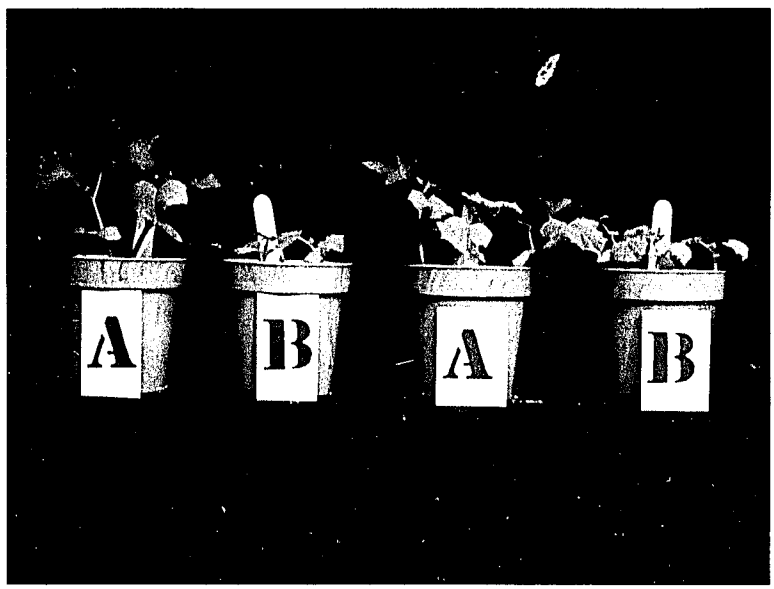

Fig. 12. Cucumber plants "B" inoculated with the stubborn virus. Plants "A" were inoculated with sap from a healthy tree. Photograph was taken 14 days after inoculation.

Fig. 13. Photograph of stubborn inoculated plants "B" with control inoculated with sap from healthy tree. Twenty-eight days after inoculation.

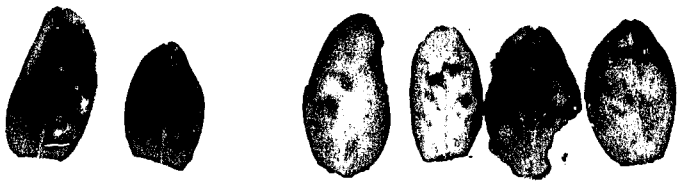

Fig. 14. Photograph of cotyledonary leaves stained with IKI from cucumber plants inoculated with sap from leaves taken from healthy and psorosis "A" infected citrus trees.

Fig. 15. Plant "A" is the control. Plant "B" has been inoculated with sap from a psorosis infected citrus tree. C has been inoculated with sap taken from a stubborn tree.

virus becomes systemic after two or three weeks in cucumber, producing a slight mosaic pattern in the leaves. This systemic infection was confirmed serologically by the chloroplast agglutination test.

It was noted in the course of these experiments that neither stubborn nor psorosis were transmitted when the temperature at the time of inoculation was above 28° C. This was a chance observation and no experiments were designed to determine the effects of temperature on mechanical transmission. Grant and Corbett (51, 52) found similar conditions to be true in their work with infectious variegation.

Since both stubborn and psorosis "A" can be mechanically transmitted to an herbaceous host, an additional diagnostic technique is available. It should now be more convenient to begin a laboratory study of both viruses, as virus sources can be easily brought into the laboratory.

CHAPTER IV

Preliminary Studies Concerning the Purification of the Stubborn Virus

Studies discussed in this chapter concern attempts to purify the stubborn virus using morphological purity as the criterion for purity. These studies were not completed. However, the work reported should furnish a good point of departure for future research of this type.

Two basic approaches were taken: (a) clarification of plant sap by ethanol dialysis and (b) filtration through Millipore filters. Combinations of these two approaches were also used.

To date, no citrus viruses have been purified. This is understandable, since laboratory research on these viruses has just recently begun. With proper equipment the purification of the stubborn virus should be accomplished with very little difficulty.

MATERIALS AND METHODS: All attempts to purify the stubborn virus were made with leaf material. Morphological purity was used as the criterion for purity, and serological tests were performed after each step to determine whether there had been a loss in the serological activity of the virus in that step. Leaf material plus 0.1 M phosphate buffer at pH 7

or pH 7 buffer and ethanol at the rate of 5 ml/g of leaf material was homogenized in a blender and pressed through four layers of cheesecloth. In the first set of tests, frozen and unfrozen leaves from healthy and diseased trees were homogenized in either 20, 25, 30, 35, 40, 45, or 50% buffer and ethanol (Steere, 89, page 21). The strained homogenate was placed in dialysis tubing for 24 hours in a refrigerator. All solutions used in these tests were precooled in a refrigerator for at least 24 hours. Ice was added to the water in which the dialysis tubing was placed. Water was changed and ice added every 6 hours. Material from the dialysis tubing was filtered through one-half inch of cellite and one piece of Number 1 Whatman filter paper. The resulting solution was centrifuged for 15 minutes at 2,450 times gravity at 5° C, and the supernatant was then centrifuged at 100,000 times gravity at 0° C for one hour in a Spinco high speed centrifuge (Model L). The resulting pellet was resuspended in 5 ml of phosphate buffer at pH 7 containing standard particles and sprayed on formvar coated electron microscope grids with a Nebulizer atomizer. The grids were shadow cast with paladium and examined in an electron microscope. Electron microscope preparation techniques are standard and can be found in any text on electron microscopy (Anderson, 3).

In the second set of experiments, healthy and diseased fresh leaves were homogenized in phosphate buffer and filtered through a Millipore filter apparatus (catalogue

number XX10 025 00) and cellite. One inch of cellite was placed on top of each 25 mm filter to prevent clogging of the filter. The plant sap was first passed through cellite and a Millipore prefilter, then cellite and a 5.0 micron filter, and finally through cellite and an 0.45 micron filter. Fresh ground material, ground material which was stored in the refrigerator for 24 hours, or for 48 hours were used in these experiments. After filtration, serological activity was checked and the material was centrifuged, prepared, and examined in the electron microscope as described in the previous paragraph. Healthy material was collected from the same trees used in serological experiments described in Chapter II.

All leaf material used in these studies was collected in a random manner around the trees and from no higher than seven feet above the ground. This material was stored in a refrigerator for 24 hours before it was homogenized in the blender. Only young or immature leaves were used.

RESULTS AND CONCLUSIONS: The best ethanol treatment resulted from the grinding of fresh leaves in 30% buffered ethanol buffered to pH 7 (Table 6). If there was any loss in serological activity in this treatment, it was not detectable with the microprecipitin test. When leaves were frozen before treatment the titer dropped from 1:256 to 1:64 in the best of the series, i.e., the 30% ethanol series.

Table 6. Effects of ethanol dialysis on virus titer and amount of cell debris.

Per Cent Ethanol	Virus titer of fresh leaves	Virus titer after dialysis	Relative amount of cell debris
20	1:256	1:128	4**
25	1:256	1:128	3
30	1:256	1:128	2
35	1:256	1:64	2
40	1:256	1:32	--
45	1:256	1:32	--
50	1:256	1:16	--
30*	1:256	1:64	2

* Leaves frozen prior to treatment.
** 1 = no cell debris, 4 = much cell debris.

In all cases using ethanol at 35% and above, the titer dropped. Titer of fresh leaves treated with 20, 25, and 30% ethanol remained constant. Upon examination after centrifugation, the electron microscope showed that the 20 and 25% ethanol treatment had much more cell debris than the 30% treatment. Samples of diseased sap had a large number of rod-shaped particles which did not appear in the check samples. These samples also had large amounts of cell debris not removed by the treatment and were not pure.

In the filtration studies, no difference in serological activity could be determined between samples of fresh sap, sap held at room temperature, and sap stored in the refrigerator. When this material was examined in the electron microscope, considerable debris was found, and virus-like particles were present in the diseased material.

The 30% ethanol experiments were repeated with fresh leaf material from healthy and diseased trees. After centrifugation, the resuspended pellets of each type were combined and run through the Millipore filter series without cellite. The filtrate was again centrifuged as previously described, tested serologically, and examined in the electron microscope. Rod-shaped particles were still evident in the diseased material. Although the preparations had less debris than any previously described, they were not pure.

The rod-shaped particles disappeared when the material was absorbed with stubborn antiserum at pH 7 and centrifuged at 24,500 times gravity at 5° C for fifteen minutes before being sprayed on the microscope grids, indicating that the particles were probably the particles of the stubborn virus, or the virus protein.

DISCUSSION: The stubborn virus, as was determined in Chapter III, is relatively stable in stored sap. The virus protein is capable of withstanding harsh treatment as determined by the ethanol experiments. Thus, with proper equipment, purification should not be too difficult a problem. Steere (89), in a summary paper on virus purification, suggests a number of techniques which should be helpful in accomplishing this purification. Preliminary studies reported in this chapter indicate that the stubborn virus is probably a rod-shaped particle.

In a series of papers, Horne and his co-workers (58, 59, 60, 61, 62), discussing human and other animal viruses, suggest a method of determining virus fine structure. This involves the use of potassium phosphotungstate as a virus stain. The virus, after staining, is sprayed on carbon coated microscope grids and examined. The methods described in these papers, if accompanied by the purification methods discussed in this chapter, should enable one to determine whether the rod-shaped particles observed conform to known virus morphology.

These studies were completed before it was known that the stubborn virus could be mechanically transmitted. No attempt has been made to transmit this semi-purified preparation.

These rod-shaped particles could probably be obtained in much purer form by a combination of density gradient centrifugation followed by electron microscope examination.

Now that an indicator plant is available, these experiments should be repeated, and both serological activity and infectivity should be checked in every step. It is possible that the rod-shaped particles seen in the electron microscope were non-infectious virus protein, since it is possible to destroy the nucleic acid of a virus without appreciably changing the serological properties of its protein sheath.

CHAPTER V

Some Physical Properties of the Psorosis and Stubborn Viruses

One possible approach to control of citrus virus diseases by the production of "clean" (virus free) budwood lies in the heat treatment of whole trees or propagating stock. Nyland (73) has been successful in freeing some stone fruit lines of the ringspot virus by means of heat treatment. Before such treatments are undertaken it is necessary to determine what the temperature of inactivation is *in vitro* for the viruses involved. If this temperature is too high *in vitro*, then this approach to control will not be practical.

In order to proceed intelligently with virus purification it is helpful to know the pH at which the virus in question is inactivated. This information enables one to avoid spending much time on experiments in which the activity of the virus has been destroyed in one of the initial steps.

Takahashi and Rawlins (95) developed a method of predicting the shape of a virus by stream double refraction. Since some evidence has been collected which indicated that the stubborn virus might be rod-shaped (Chapter IV), attempts were made to verify this by the stream double refraction method.

All studies reported in this chapter were made with both the stubborn and psorosis "A" viruses. These studies grew out of work reported in the previous chapter and should aid in the development of methods of producing clean budwood, help in purification studies, and have already resulted in the prediction of the shape of both viruses in question. There have been no reports of these types of studies on any of the citrus viruses.

Thermal Inactivation

MATERIALS AND METHODS: Healthy and stubborn and psorosis infected citrus leaves were collected in a random manner around the trees from no higher than seven feet above the ground. Leaves from stubborn trees were homogenized in a blender in 0.1 M phosphate buffer at the rate of 5ml/g leaf material. The homogenate was filtered through four layers of cheesecloth and then heat treated. Treatment F was prepared from frozen leaves. The buffer solution was also frozen before grinding. The homogenate was then inoculated into cucumber. In all cases except the room temperature check, the material was heated for ten minutes in a water bath at a given temperature as stated by Bawden (6). This material was then checked for serological activity by the microprecipitin test which was described in Chapter II.

Leaves from healthy and psorosis infected trees were ground in 20% sucrose solution of 0.1 M phosphate buffer to which activated charcoal and cellite had been added at the same rate as described in Chapter III. In all cases, except treatment F and the room temperature check, the material was heated for ten minutes in a hot water bath at a given temperature. In treatment F the leaf material and sucrose, buffer, charcoal, and cellite solution were frozen before grinding. All psorosis materials were inoculated into White Spine cucumbers as in Chapter III.

Two sets of controls were used with both the stubborn and psorosis viruses. One of the controls was prepared from diseased material at room temperature. The other control was prepared from healthy leaves in the same manner as the diseased leaves.

Temperatures used in heat treating were: room temperature (about 19° C), 30, 40, 50, 60, 70, 80, 90, 95, and 100° C. Twenty-four plants were used in each treatment.

RESULTS AND CONCLUSIONS: The serological activity of the stubborn virus was completely destroyed in the 50° C treatment and had begun to decline in the 40° C treatment (Table 7). It might be possible to obtain stubborn free budwood by placing the scion wood in a moist chamber at 45° C for varying lengths of time, then budding it on a

seedling rootstock and checking the leaves from the bud for serological activity.

The results of treatment F indicate that it is not advisable to freeze stubborn leaves before using them in virus studies, as the titer is reduced by freezing.

Table 7. Results of heat treatment of sap from stubborn trees.

Treatment	Temp. used	Serum Dilution 1:8	1:16	1:32	1:64	1:128	1:256
ck	Rm. temp	4**	4	4	4	4	4
F	Frozen	3	3	2	2	2	2
G	30° C	4	4	4	4	4	4
H	40° C	4	4	4	4	3	2
I	50° C	1*	1	1	1	1	1
Healthy check	Rm. Temp.	1	1	1	1	1	1

1* = no precipitation, 4** = maximum precipitation.

The psorosis virus was transmitted to cucumber in a few cases after the 70° C treatment (Table 8) There was no apparent reduction of psorosis virus titer when leaves were frozen before grinding. It would appear that heat treatment of psorosis budwood is not feasible, since the virus appears to resist a temperature of 70° C. This temperature would probably kill the budwood.

Table 8. Results of heat treatment of sap from psorosis trees.

Treatment	Temp. used	No. Plants in treatment	No. Plants Infected	Per Cent Infected
ck	Rm. Temp.	24	23	96
F	Frozen	24	22	92
G	30° C	24	22	92
H	40° C	24	11	46
I	50° C	24	12	50
J	60° C	24	9	37
K	70° C	24	2	8
L	80° C	24	0	0
M	90° C	24	0	0
N	95° C	24	0	0
O	100° C	24	0	0
Healthy Check	Rm. Temp.	22	0	0

pH of Inactivation

MATERIALS AND METHODS: Stubborn and healthy leaf materials were collected, prepared, and checked in the same manner as described in the previous experiment. Instead of heating the materials, aliquots of the leaf sample were ground in a series of buffered solutions of pH 2.2 to 8.0. McIlvaine's standard buffer solutions as given by Jacobs and Gerstein (63) were used in all cases. This series of citric acid-sodium phosphate buffer solutions consisted of the following pH: 2.2, 2.6, 3.0, 3.6, 4.0, 4.6, 5.0, 5.6, 6.0, 7.0, and 8.0.

Psorosis and healthy leaf materials were also collected and prepared in the same manner as described in the previous experiment, and placed in a series of sucrose solutions with buffers as described in the previous experiment.

RESULTS AND CONCLUSIONS: The serological activity of the stubborn virus began dropping off at pH 6 and was not measurable at pH 5 (Table 9). The virus protein must have been completely denatured at pH 5, or was so tightly adhering to the chloroplast fragments that it was filtered out with the cell fragments.

Table 9. Results of grinding stubborn leaves in different pH solutions.

pH	Serum Dilutions					
	1:8	1:16	1:32	1:64	1:128	1:256
5.0	1*	1	1	1	1	1
5.6	4**	3	3	2	1	1
6.0	4	4	3	3	2	2
7.0	4	4	4	4	4	4
8.0	4	4	4	4	4	4
7.0***	1	1	1	1	1	1

1* No precipitation, 4** Maximum precipitation
7.0*** Healthy check.

Table 10. Results of inoculation studies in which psorosis infected leaves were ground in different pH solutions.

pH	No. of Plants Treated	No. of Plants Infected	Per Cent Infected
2.2	20	0	0
2.6	20	0	0
3.0	19	0	0
3.6	20	0	0
4.0	20	0	0
4.6	20	0	0
5.0	17	2	12
5.6	17	4	24
6.0	17	12	71
7.0	16	15	94
8.0	17	15	89
7.0*	15	0	0

* Healthy check.

None of the plants inoculated with psorosis material ground at pH 4.6 or below became infected by the virus (Table 10). The virus was apparently completely inactivated by exposure to pH 4.6 and the stability of the virus was adversely affected at pH 6.0.

Stream Double Refraction Studies

MATERIALS AND METHODS: A monocular microscope was modified according to the directions given by Takahashi and Rawlins (95). The condenser was removed and a polarizer substituted. An analyzer was substituted for the ocular. The objective was removed and a rubber stopper with a blackened piece of glass tubing replaced it. A glass cylinder which had been painted black and had a pipette with a rubber bulb cemented to it was placed on the stage. The polarizer was turned on its vertical axis until its vibration direction made an angle of 45° with the direction of the liquid expelled from the pipette. The analyzer was then turned until the field was dark as a result of the nicol prisms being crossed.

Healthy sap or sap from stubborn or psorosis infected leaves homogenized in a blender and filtered through Number one Whatman filter paper and one-half inch of cellite in a Buchner funnel was placed in the cylinder. A small amount of liquid was drawn into the pipette. When the rubber bulb was squeezed, the reaction was read in the manner described

by Takahashi and Rawlins (95). Stubborn material was collected from some of the same trees used in Chapter IV.

RESULTS AND CONCLUSIONS: When the sap from stubborn leaves was expressed from the pipette, the liquid was seen to be doubly refractive, and appeared as a bright streak across the dark field, indicating that the juice from stubborn leaves contained rod-shaped particles. Juice from psorosis infected leaves was double refractive only on the edges of the stream expressed from the pipette. This indicated that the sap contained disk-shaped particles or very short rods. Sap from healthy leaves examined in the same way showed no double refraction.

These results support the electron microscope observations in Chapter IV that sap from stubborn leaves contains rod-shaped particles.

CHAPTER VI

Additional Studies on the Diagnosis of Stubborn Disease of Citrus

The chloroplast agglutination test on stubborn disease is often difficult to read. This is because the chloroplasts burst when citrus leaves are ground. In cases where low titer antisera are being used, the agglutination is not complete and appears as small agglutinated clumps of cell debris thoughout the droplet. If the chloroplasts could be removed intact from citrus leaves the agglutination reaction could probably be read without the use of a microscope.

Attempts were made to determine whether the paper chromatography of amino acids might furnish an additional means of diagnosing stubborn. If leaves could be used in these tests one might be able to determine whether a tree was carrying the stubborn virus long before the tree was old enough to bear fruits.

Chloroplast Studies

MATERIALS AND METHODS: Citrus leaves were ground with either a mortar and pestle or a blender in gradients of buffered and unbuffered sucrose solutions. The resultant

sap was examined under a microscope to determine whether the chloroplasts remained intact. Sucrose solutions used were 5, 4, 3, 2, 1, 0.5, 0.25, 0.01, and 0.005 molar.

RESULTS AND CONCLUSIONS: In all cases checked the chloroplasts burst upon grinding. Since the object of these tests was to develop a method of obtaining whole citrus chloroplasts, the results indicated that any further efforts in this line would entail a more complex technique. The study was abandoned.

Chromatography of Healthy and Diseased Leaves

MATERIALS AND METHODS: Leaves were collected from stubborn and healthy navel orange trees. These leaves were ground in a blender with sufficient absolute alcohol so that the final concentration of alcohol was about 80% by volume. The insoluble material was removed by filtration and washed with 80% ethanol. Then three times the volume of chloroform was added to each volume of ethanol extract. After thorough shaking the resulting aqueous layer (upper) was removed and concentrated to one tenth its original volume by evaporation. This method of extracting free amino acids from plant materials is given by Block and Weise (8) on page 16 of their book.

These extracts were spotted on Number one Whatman Chromatograph paper at full strength, one half, one fourth, and one eighth dilutions. These dilutions were made with

distilled water. Ascending chromatographs were made. The solvent used was n-butyl alcohol:acetic acid:distilled water in the proportion of 250:60:250. Each treatment had three replications and the experiment was repeated three times. After the solvent front had traveled to about an inch from the edge of the paper, the papers were dried, sprayed with 0.25% ninhydrin, and placed in an oven at 60° C for an hour. Then they were examined and spots were marked. Fluorescent spots were determined with ultra-violet light with a wave length of 2537 A.

RESULTS AND CONCLUSIONS: No significant differences could be found on comparing the healthy and diseased materials. Perhaps bidirectional chromatographs might be more helpful in showing possible differences in free amino acid content of healthy and stubborn citrus leaves, since Bové et al. (9) claim such differences exist in the fruits. Possibly another solvent system would give better results.

CHAPTER VII

Discussion

Psorosis, stubborn and xyloporosis-cachexia are among the most important citrus diseases which at present are not well controlled. These diseases are most insidious, since all remain latent in a tree from two to many years. Thus a young citrus tree may appear vigorous and healthy, and yet produce little marketable fruit when it matures. The life of this tree may be considerably shortened. Although it is carrying a virus which will destroy its economic utility, it appears to be normal and healthy. Methods must be developed to prevent trees carrying these viruses from being planted. This will entail the use of accurate diagnostic methods such as those described in Chapters II and III.

The older citrus groves which have trees infected with the psorosis, stubborn, and xyloporosis-cachexia viruses, either singly or in combination, are at present left to the ravages of these diseases, for there are no adequate methods of controlling these viruses once they are in the tree. In order to find methods of curing virus diseases or controlling viruses in trees, a more complete understanding of the behaviour of these viruses must be attained.

Before an understanding of the physiology of parasitism can be achieved, some of the basic biological and physical properties of these viruses must be determined. This dissertation presents introductory laboratory work in this field.

Antisera can be produced to the stubborn (90, 91) and psorosis "A" (92) viruses. Through the use of the agglutination reaction, one can determine in a matter of hours whether any given tree (without consideration of its age) is carrying these viruses. There could be instituted a serological check of all trees in nurseries before they are sold and planted in groves, with the subsequent destruction of all trees carrying one of these diseases. Although this would be expensive, the cost would be of little consequence when compared with the amount of money saved because of the reduction of losses in the years following. Thereafter nurseries could be supplied with virus free propagating stock.

The importance of certified virus free budwood has been emphasized by many workers (1, 25, 49, 72, 78, 80, 84, 93, 103). As pointed out earlier many of these citrus viruses do not lend themselves to an indexing program since some take two or more years to express themselves in the most susceptible citrus test variety. Now, however, bud sources can be checked for both stubborn and psorosis "A" by either serological tests or by mechanical transmission to cucumber. Weathers and Calavan (104) suggested the use of nucellar seedlings as a method of freeing citrus clones

from virus diseases, since these viruses are not usually passed through the seed. Serology and mechanical transmission could also be used to index such nucellar seedlings, since in the past identification of the viruses was the limiting factor in the use of nucellars.

Probably xyloporosis-cachexia would lend itself to the production of an antiserum, as there is now some evidence that it is related to stubborn and reacts with a stubborn antiserum. With a bank of antisera for stubborn, xyloporosis-cachexia, and psorosis, the citrus industry would have an excellent start toward relief of its virus problems. By producing antisera from obvious xyloporosis-cachexia antigen sources, one could make some conclusive statements as to their relationship to each other and to the stubborn virus.

The antisera used in these tests could be much improved, as the titer is relatively low. The best method of serum improvement lies in the production of more pure and highly concentrated antigens. The stubborn virus would probably be simplest to purify as there is now some preliminary data on purification, and the virus appears to be highly stable. Since it can be transmitted to cucumber, this might serve as a method of separating the stubborn virus from some of the other viruses found in citrus. Gold (50) has suggested the use of an antihost serum, made from healthy plants of the same variety as the infected plant

which is to be used in virus purification. This method should remove much of the normal plant protein from infected plant sap.

In 1961, Thornberry (96) made a plea for a standardized set of test plants for indexing citrus viruses. In doing this he was referring to the citrus varieties used in indexing. However, since the time involved for symptom expression is so great, it is the author's opinion that methods such as serology and transmission to herbaceous hosts will have to be developed if any practical indexing program is to be maintained on a scale sufficient to bring about the needed results.

Pfaeltzer (79) used 2.5% nicotine to enhance the mechanical transmission of stony pit virus of pear to a number of herbaceous hosts. This method might be applied to the stubborn and psorosis viruses to determine whether nicotine might affect the transmission at different times of day, as dawn is an inconvenient time to perform transmission experiments.

Since xyloporosis-cachexia react with a stubborn antiserum, they are probably related to the stubborn virus and could quite possibly be transmitted in a manner similar to the methods used in the transmission of stubborn.

Transmission of the xyloporosis and cachexia strains of the xyloporosis virus to an herbaceous host should be attempted. Cucumber would probably be the most likely host

with which to begin these transmission experiments. Time of inoculation has a great effect on the per cent transmission of stubborn, thus indicating that inoculations should be tried at different times of day.

By combining serological and transmission diagnostic techniques with a heat treatment of stubborn budwood, it might be possible to obtain some stubborn-free citrus lines. Since insect transmission is not known to be a problem, such lines would be easy to maintain and could serve as bud sources for the industry.

Psorosis probably would not lend itself to the heat treatment process, as its in vitro thermal inactivation point is too high. However, it might be possible to use nucellar seedlings as a source of psorosis free budwood as suggested by Weathers and Calavan (104), even though some citrus viruses will be passed through nucellar seedlings.

Experiments to determine stability in storage should be repeated with the stubborn and psorosis viruses, and begun with the xyloporosis-cachexia virus. At the time these experiments were conducted, no herbaceous host was known. All information on stability concerns the virus protein and does not reflect infectivity.

Bové et al. (9) indicated that these are some gross differences in the amino acid content of stubborn and healthy fruits. They did not identify the amino acids. Thus it

might be profitable to conduct more chromatography experiments using leaves, fruits, and standard amino acids as this might indicate in what manner the virus is altering the physiology of the host.

As a result of the recent trend toward laboratory research with citrus viruses, the future of the industry appears brighter. Methods of detecting latent viruses occurring in immature trees have been developed for a few diseases and should eliminate the prospect of new groves being planted which carry these viruses.

CHAPTER VIII

Summary

As a result of serological experiments which were begun in 1958, antisera have been produced to the stubborn virus and the psorosis "A" virus. The chloroplast agglutination test has been applied to both of these viruses, thus making possible field testing for these viruses. A survey of citrus viruses made with these antisera indicates that the xyloporosis-cachexia virus might be related to the stubborn virus.

Studies on the stability of the stubborn and psorosis "A" viruses indicate that both are highly stable in whole leaves stored under refrigeration. The psorosis virus is unstable when stored as raw sap, while the stubborn virus is quite stable stored in this manner.

Both stubborn and psorosis "A" can be mechanically transmitted from citrus to cucumber and from cucumber to cucumber. Transmission in both cases is greatly influenced by the time of inoculation. The early morning hours (i.e., just before dawn) are the best time to inoculate host plants.

Attempts to purify the stubborn virus have met with little success. However, electron microscope examination

of partially purified sap indicated that there were virus-like rods present in the preparation from stubborn leaves. The presence of these rods was confirmed by stream double refraction.

The thermal inactivation point of the stubborn virus appears to be low enough to indicate that virus inactivation studies on budwood might yield virus free buds. The thermal inactivation point of the psorosis "A" virus is sufficiently high to rule out success in this case.

Although no success was obtained in amino acid chromatographic studies of stubborn material, other workers have recently reported that such differences do exist. The chromatographic studies were not pursued after the initial experiments yielded negative results. In the light of this new information, it might be advisable to repeat this work with other solvent systems.

In view of the large amount of successful laboratory research reported in the last few years which make available more information on some of the most important citrus viruses, it may be possible to develop new and less expensive control measures than now exist.

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