COMPETITION STUDIES BETWEEN THE COMMON AND VM STRAINS OF TOBACCO MOSAIC VIRUS

by

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A Thesis Submitted to the Faculty of the

COMMITTEE ON AGRICULTURAL BIOCHEMISTRY AND NUTRITION

In Partial Fulfillment of the Requirement
For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1967

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ACKNOWLEDGEMENTS

The author expresses sincere appreciation to Dr. Albert Siegel for suggesting and guiding the work represented in this thesis and for his encouragement during the course of research. Helpful suggestions of experimental approaches were made by Drs. K. Matsuda, M. Zaitlin, and V. Hariharasubramanian and were also gratefully received. Support for the research and advice about thesis preparation by Dr. A. Kemmerer have been appreciated. Computerized statistical analyses of the data were made possible by the considerate work of Dr. R. Kuehl.

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ABSTRACT

Reports in the literature describe competitive inhibition of TMV-U1 infection on tobacco plants when this virus is inoculated in the presence of the strain TMV-VM. Reductions have been observed in the number of necrotic lesions induced by U1 when nonviral inhibitors of infection were applied to leaves at different times after U1 inoculation.

It was postulated that the progress of virus infection in the infective centers of inoculated leaves develops from an initial state of susceptibility to competitive inhibition to a state of resistance to such competition. Experiments were conducted on the tobacco species (Xanthi) which was selected because the ports of entry for the virus (Infectible Sites) on abraded leaves remain available to virus entry for a relatively long time. The relative number of distinctive U1 necrotic lesions produced was recorded as a function of the time between U1 inoculation and competitor (VM) application. The data appeared to demonstrate that U1 infective centers are not affected by viral competition at two minutes after U1 inoculation.

On the basis of these and other previously reported data it was hypothesized that one of the initial processes in plant virus infection, which begins at the moment of inoculation, is a growing association of

the infecting particle with the infectible sites. Determination of a concentration-infectivity graph for VM and verification of previously reported plots of inhibition of U1 infectibility as a function of different VM concentrations in the U1 inoculum are also reported.

INTRODUCTION

The mechanism of plant virus infection has become better understood through extensive studies of tobacco mosaic virus (TMV) infection of tobacco (Siegel and Zaitlin, 1964). This thesis attempts to elucidate the kinetics involved in establishment of irreversibility in TMV infection. A prerequisite for the establishment of TMV infection of susceptible leaves is that the leaf be abraded. Mechanical abrasion results in the formation of infectible sites (IS). The application of virus inoculum to IS generally results in infections. Although many virus particles may enter an infectible site, apparently only one particle may initiate infection at an IS (Siegel, 1959). After establishment of an infection, the process of virus replication occurs in the locus which is now called the infective center (IC). This process usually results in pathogenic symptoms on the host plant.

The experiments reported in this thesis were conducted on the host plant Nicotiana tabacum L. var Xanthi nc. (called Xanthi) which, when infected with TMV, produces necrotic lesions. Xanthi was used because IS produced on it remain available for infection for a relatively long period after abrasion (Siegel and Zaitlin, 1964). Host plants such as Xanthi are important in studies of infection kinetics because the numbers of lesions produced on them are a function of the concentration of infectious virus in the inoculum (Holmes, 1920).

A reduction in the relative number of lesions induced has been observed when a local lesion host is simultaneously inoculated with TMV and a virus which is not infectious to the plant (Kassanis, 1963). In a similar way infection by the common TMV-U1 strain was inhibited by TMV-VM infection as could be determined by the formation of fewer characteristic U1 lesions (Wu, Hudson, and Wildman, 1962). Other data have demonstrated that simultaneous inoculation with two different viruses almost never results in a mixed infection at single sites on the leaf (Kunkel, 1934). Results such as these have been interpreted to mean that only one virus particle may initiate the infection at any single IS, even though many particles may be present.

Infection of _N. sylvestris_ by TMV was unperturbed if exposed to a second infectious strain an hour after inoculation (Siegel, 1959). It was apparent that at some time after inoculation, the course of infection was irreversibly established. Thus questions arise concerning the association of TMV with infectible sites. Does there exist in the association of TMV with IS a period during which other virus particles may affect the association? Is the lability of U1 infected centers to competition with added virus followed by a period of resistance to competition? This thesis studies the kinetics of the development of such resistance to competition. The test of resistance involved the application of a competing, yet distinguishable virus strain at different

times after TMV-U1 inoculation. When in the presence of competitor, TMV-U1 produced relatively fewer lesions, it was assumed that the competitor had an effect on the infection process of TMV-U1. Data indicated that as the time lengthened between initial TMV inoculation and subsequent competitor application, the competing strain became less effective in reducing TMV lesion numbers. The apparent irreversibility of TMV attachment to IC was rapidly approached within the first two minutes of infection.

METHODS

I. Conditions of Plant Growth

Seeds of _Nicotiana tabacum_ L. var Turkish Samsun and of _N. tabacum_ L. var Xanthi nc. (Xanthi) were germinated on a soil mixture of 1:2 sand:vermiculite. The plants were transplanted into two inch peat pots, which, after a period of seedling growth, were placed into four inch plastic pots containing the 1:1 sand:peat moss soil recommended by the University of California (Calif. Agric. Expt. Stn., 1956). The plants were periodically supplemented with Hoagland's nutrient solution (Went, 1957) and Vigaro (Swift and Co.). Natural light and a temperature range of 75°F to 90°F in the day and 50°F to 75°F at night were employed. Plants showing diseases and plants which had mature leaves exposed to insecticides were considered as possible sources of experimental error and were discarded.

II. Virus Preparation

Tobacco mosaic virus (TMV) strain U1 (Siegel and Wildman, 1954) has been maintained in this laboratory for several years. The TMV strain VM (Wu, Hildebrandt, and Riker, 1960) was obtained from Mr. W. Hudson at the Dept. of Botanical Sciences and Molecular Biology Institute, University of California, Los Angeles. In order to

make a pure VM preparation an individual, characteristically minute lesion of VM was transferred from a Xanthi leaf to the leaves of a plant of _N. tabacum_. Xanthi leaves develop typical necrotic local lesions when inoculated with strains of TMV whereas _N. tabacum_ develops a systemic infection. Following two weeks of development of the infection, shoots and inoculated leaves were assayed on Xanthi to reveal the presence of any contaminant viruses. Leaves of _N. tabacum_ plants containing only VM were harvested.

In order to purify the virus, frozen leaves in plastic bags were pulverized by pounding with the hand. The resulting leaf particles were further mascerated in Sorensen's M 1/15 phosphate buffer, pH 7, (Wildman and Ford, 1960) in an Omni-mixer (Ivan Servall, Inc.). A cheesecloth filtrate of this material was centrifuged at 12,000 x gravity for 30 minutes. The clear, brown supernatent yielded a white, opalescent virus pellet on further sedimentation at 105,000 x gravity for 60 minutes. The virus pellet was dissolved in Sorensen's buffer, 0.001 M in Versene, and recycled through low and high speed centrifugations until free of colored, particulate matter. Finally the pellet was dissolved and stored in pure Sorensen's buffer, pH 7. The best yields of virus were obtained following cold extraction procedures. A typical VM yield was 700 mg of virus per kilogram of leaves. The concentration of virus solutions was determined from the optical density at

260 mμ using a molecular absorption coefficient of 10^7 (Fraenkel-Conrat, 1966). The purity of VM preparations was determined by assay on Xanthi plants. U1 was found as a contaminant in about half of the VM preparations and these were discarded. TMV-U1 was prepared by the same procedures but with less concern for cold and sterile techniques.

III. Inoculation Procedures

Virus inocula were diluted with boiled M 1/15 phosphate buffer, pH 7, and supplemented with 50 mg of the abrasive Celite per ml of inoculum. Virus was applied to the leaves with plumbers' dope brushes which had a cheesecloth covering over the bristles. This cloth addition was a modification of the brush techniques of Wildman and Ford (1960) based on F. O. Holmes' (1929) idea to get maximum, very shallow leaf abrasion in conjunction with an even flow of inoculum. Inoculation was followed within ten minutes by a tap water rinse which was observed to alleviate post inoculation leaf wilt. When mixed inocula were used they contained U1 at 1 μg/ml (micro gram per milliliter) and VM at from 0.01 μg/ml to 160 μg/ml. In spray experiments individual leaves were abraded in the presence of 1 ug U1 per ml inoculum and then after different spans of time were sprayed with about 2 mls of VM inoculum (40 μg/ml) from a de Vilbiss atomizer. Re-rub experiments were similar but involved VM application by a second brushing. Nearly

200 brushes were used for each re-rub experiment making it impractical to cover the brushes with cheesecloth. In dip experiments U1-inoculated, excised leaves were exposed to VM by dipping into VM inoculum for 30 seconds. The leaf petioles were then inserted into holes in thin styrofoam sheets. These sheets were floated on water in glass covered turkey roasting pans (Wildman and Ford, 1960). During leaf incubation the pans were kept in a chamber which maintained a temperature of 18°C to 21°C and 500 foot candles of light at the leaf surface.

IV. Lesion Development and Counting

Intact plants were maintained on a 16 hour day of 400 foot candles of mixed fluorescent and incandescent light at a temperature of 80°F. Four days prior to inoculation Xanthi plants of the same age were selected and trimmed. After inoculation the most reliable lesion counts were made on the fourth day, since this was when the U1 lesions were clearly larger and colored with a grey ring and a tan center as contrasted with the minute brown lesions of VM. Counts of the U1 lesions were made by placing the leaf over a substage-lit glass plate and using a mechanical counter. Older leaves, deviations in environmental conditions, and harsh inoculation procedures tended to cause difficulty in distinguishing U1 and VM lesions and resulted in data not reported.

V. Experimental Design and Analysis of Data

Latin square assay schemes were used in order to minimize effects of leaf level and plant variation on the treatment averages. In the majority of experiments, where half leaves were analyzed, a 7 by 7 Graeco-Latin Square design was used. This design allowed statistical removal of experimental error due to differences in plants, leaf levels, and left and right sides of leaves. The Graeco-Latin Square had the added advantage that any particular pair of treatments could be found twice in direct comparison on the same leaf. Use of a prepared computer deck, ANOVA 44 by R. J. Weldon (professor of Systems Engineering, University of Arizona) allowed rapid determination of treatment means and of experimental error attributable to the above mentioned sources. The standard error of the means was calculated for several experiments.

REVIEW OF LITERATURE

I. Bioassay

F. O. Holmes (1929) reported that the TMV concentration in sap of infected plants could be determined when the sap was rubbed over leaves of *Nicotiana glutinosa*. The number of necrotic lesions produced on the leaves of this local lesion host were found to be a function of TMV concentration in the inoculum. Bioassay is much the same now but in place of plant sap, purified virus in M 1/15 phosphate buffer, pH 7 is commonly used as inoculum. Phosphate buffer has been used because it partially compensates for the deleterious effect of water on the production of lesions by TMV (Yarwood, 1952). Takahashi (1956) showed that the use of phosphate buffer in the presence of an abrasive such as 600 mesh carborundum increased assay sensitivity on *N. tabacum* L. var Xanthi nc. (Xanthi) by a factor of 4,600. Virus assays which follow the Latin Square scheme and use half leaves as the basic unit, have been shown to be efficient schemes for demonstrating treatment differences (Fry and Taylor, 1954).

II. The Infection Process

An average of at least 75,000 virus particles has been calculated to remain on inoculated leaves for each lesion that appears (Frankel-Conrat, Veldee, and Woo, 1964). Furumoto and Wildman

(1963b) showed that at least 1 in 10 TMV particles was infectious. This brings up a question of how many virus particles participate to induce one lesion at an infectible site (IS). Siegel (1966) has discussed evidence in support of the theory that there need be no more than one infectious particle in an infectible site when infection commences.

Virus entry into IS apparently requires that the IS have not sealed up, which in turn depends on how recently the IS were formed. Once an IS has been produced on N. glutinosa, the resistance of the IS against virus infection increases until resistance is almost complete at 30 minutes (Sheffield, 1936). On the host plant Xanthi the resistance of IS to infection does not appear until after a much longer period (Siegel and Zaitlin, 1964). When an IS has been infected the resultant infective center (IC) appears also to become resistant to applied materials. The lack of a deleterious effect of either water or detergent washes on the infections of freshly inoculated leaves has indicated a form of protection of the infecting particles within the IC. (Furumoto and Wildman, 1963a).

The studies of Siegel and Wildman (1956) and of Siegel, Ginoza, and Wildman (1957) provided information about the development of virus infection at infective centers. Rates of inactivation of IC as a function of UV (ultraviolet light) dose were determined at each

hour after virus inoculation. Within the first hour after virus inoculation UV inhibited IC from producing lesions at the same rate that it prevented virus *in vitro* from being infectious. The interpretation was that one hit of UV radiation on the infected leaf inactivated one virus particle and prevented one lesion. This evidence suggested that only one virus particle was needed to start a lesion and that at least a one hour lag period occurred before replicative processes began. A more complete review of the process of plant virus infection will be found in Siegel and Zaitlin (1964).

III. Location of the Virus

Trichomes have been demonstrated to be potential infectible sites (Hildebrand, 1958) as were the ectodesmata which surround them (Brants, 1965). Following the initiation of infections, Bald (1964 a,b) showed, through the alternate use of an RNA stain and RNase, that TMV-RNA was rapidly produced in nucleoli. What may have been TMV-RNA synthesis, independent of transcription from DNA, was observed in nucleoli of infected leaves (Smith and Schlegel, 1965). Indications of the presence of proteins in the IS led Stahmann and Gothoskar (1958) to study the effects of some model proteins on TMV inocula. Their results suggested that TMV was negatively charged and that the infectible site was positively charged. Such a charge

relationship was postulated to explain migration and specific binding of virus particles to the IS.

IV. Virus-Virus Interactions

When an equal quantity of the VM or U2 strain of TMV was mixed with U1 inocula, the VM was found to be a stronger competitive inhibitor of U1 infection (Wu, Hudson, and Wildman, 1962). The same researchers found that inhibition of U1 infection by inactivated VM or U2 was only one thousandth as pronounced as when infectious virus was used. They proposed that noninfectious viruses inhibited U1 by aggregating about U1 to delay infection and that infectious viruses acted by competing with U1 at the IS. Competition between the RNAs of different TMV strains has also been observed (Wu and Hudson, 1963).

U1 systemic infection, on the host plant *N. sylvestris*, inhibited U2 necrotic lesion formation when the two viruses were inoculated simultaneously (Siegel, 1959). However, if U2 necrotic infection was first established for one hour, subsequent inoculation with U1 had no effect on the development of U2 lesions. Observations such as these lent credence to assumptions made by Siegel for the purpose of developing an infection model. He assumed that only one virus particle may initiate an infection, that all IS are equally infectible, and that the IS are equally receptive to each kind of virus.

Physical treatment of the infective centers of the U1, U2, U8, and VM strains of TMV with heat revealed that only the IC of VM were resistant enough to the treatment to continue the process of infection (Wu, 1963). Rappaport and Wu (1962) challenged the stability of U1- and of VM-IC against UV irradiation and found U1-IC to be more resistant. This was interpreted to mean that infective centers of VM were inactivated faster by UV than were those of U1. They then irradiated mixedly inoculated N. glutinosa leaves with the result that greater numbers of U1 lesions were produced than in the absence of UV. Their explanation was that many IS were blocked from developing into lesions by the mutual inhibition between the two TMV strains. Since VM was more sensitive to UV than was U1, then there apparently came a time when some blocked IS were left with only infectious U1 particles and therefore resulted in U1 lesions. Since UV acts by breaking RNA, they suggested that competitive inhibition occurred at an RNA level.

Later, Wu (1964) observed that VM inhibition of U1 on leaves of N. glutinosa was not affected by UV 10 hours after inoculation. This meant that in the IS where mutual inhibition had occurred the relationship between the two virus particles had changed in 10 hours. On the basis of UV inactivation rates he interpreted that both U1 and VM virus particles had passed through a two hour period during which time unsheathing and binding of the viruses may have taken place. From the

second hour on, decreasing rates of U1 rescue from VM competition indicated to him a growing association of VM particles with the IC.

RESULTS

I. The Infectivity of VM as a Function of Concentration

A commonly used test of infection characteristics of a virus preparation is the relationship displayed between virus concentration and the number of lesions produced. The results of such a series of experimental determinations for VM are shown in Figure 1. It can be seen that VM concentrations less than 0.5 µg/ml induced lesions on Xanthi in proportion to the virus concentration. That is to say that the graph is approximately linear in the low concentration range. It can also be seen that increases in VM concentrations above 0.5 µg/ml were not met with as great an increase in relative lesion numbers as was observed at lower VM concentrations. The concentration-infectivity plot for U1, also shown in Figure 1, is from Wildman (1959). The plot of VM infectivity on Xanthi approximates that of U1 which was obtained from experiments performed by Wildman on the plant *N. glutinosa*.

II. The Competition of VM with U1 when both Viruses are Inoculated Simultaneously

Distinction was clearly made between the necrotic lesions of U1 and of VM on Xanthi leaves infected with both viruses. Occasionally it was found that several VM lesions had coalesced to form a necrotic area which appeared similar to the lesions induced by U1. In cases

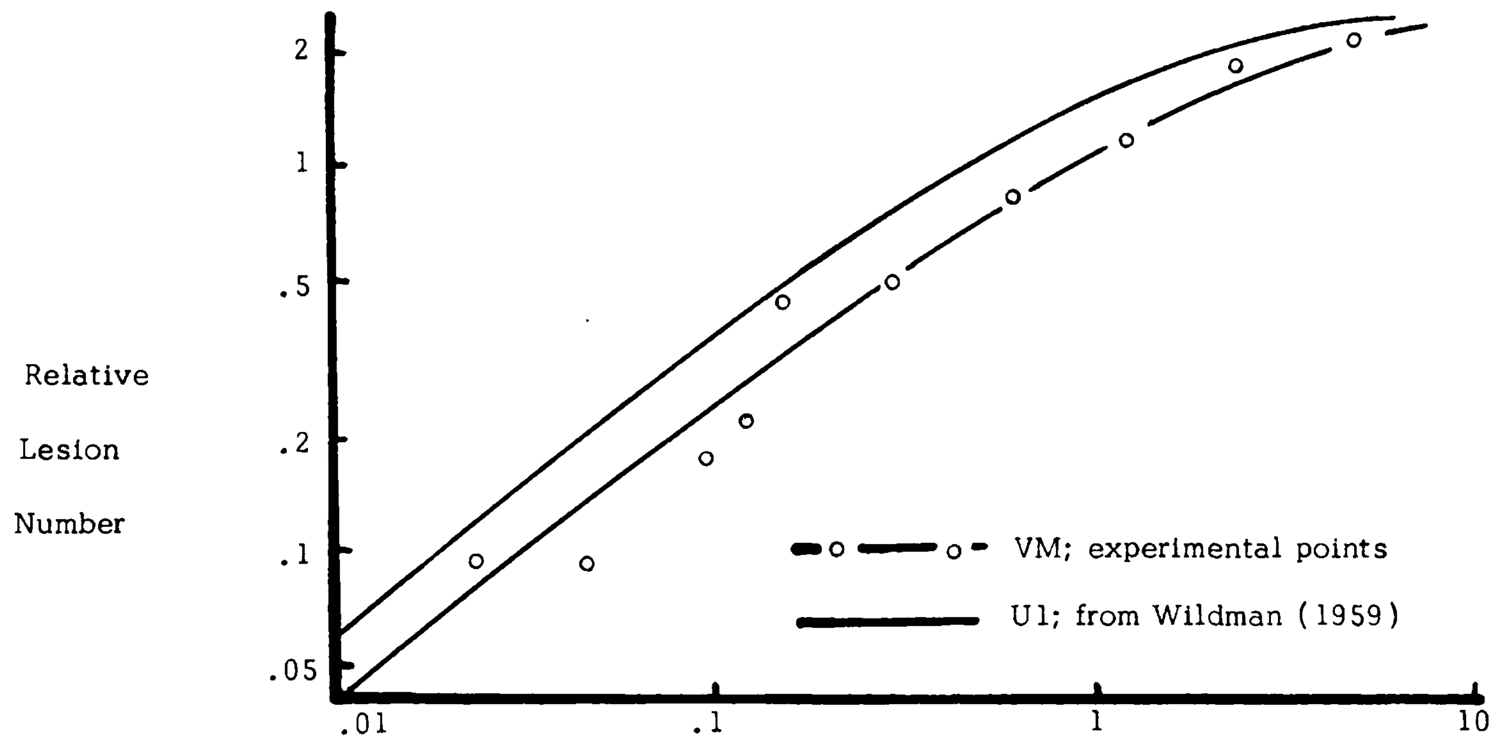

Figure 1 Relative Lesion Producing Capacities of VM and U1

where lesions of VM and U1 could not be differentiated, the data were not taken.

Characteristics of competition between U1 and VM were first reported by Rappaport and Wu (1962) who used the local lesion host N. glutinosa. They reported a reduction in the number of U1-induced lesions when VM was added to U1 inocula and the degree of this competitive inhibition was shown to be a direct function of the VM concentration. Their experiments were repeated for this thesis on the local lesion host Xanthi and are reported in Figure 2. The inhibitory effect of VM on the number of lesions induced by a standard (1 µg/ml) U1 inoculum becomes quite evident at VM concetrations greater than 1 µg/ml. Data from similar experiments performed by other workers on N. glutinosa are shown for comparison (Rappaport and Wu, 1962). In the region where U1 was inoculated in the presence of VM at concentrations below 0.5 µg/ml the infectivity of U1 appears to be unperturbed. In this same region all but a few of the points fall close to a straight line.

III. Kinetics During the Establishment of Irreversible Association of U1 with Infectible Sites

The method used to test for the presence of an irreversible association of U1 with infectible sites was to expose the U1 inoculated leaf to a competing virus, VM. U1 was brushed on Xanthi leaves and

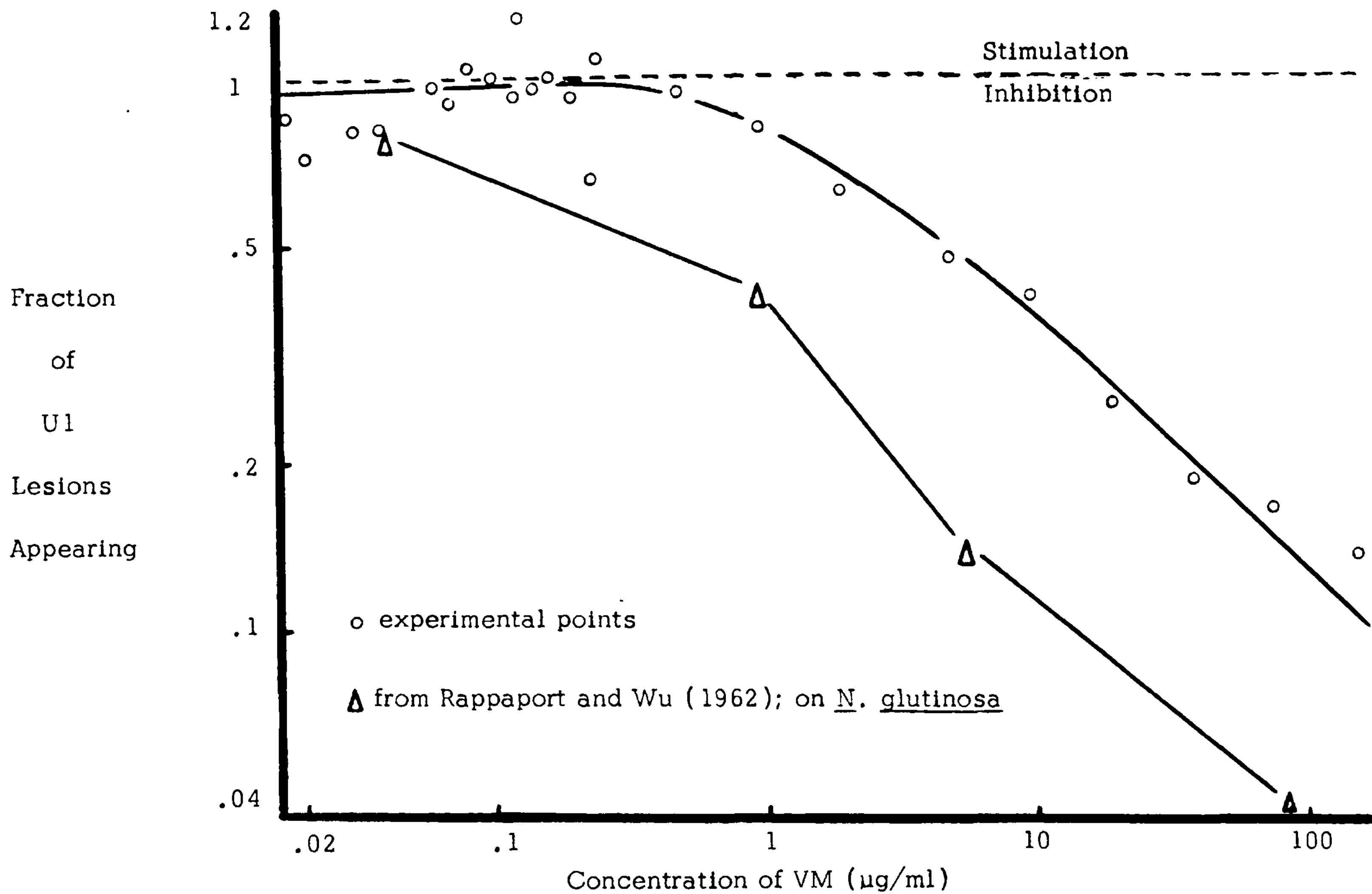

Figure 2 The Inhibition of U1 (1 µg/ml) When Inoculated in the Presence of VM on Xanthi

then VM was applied by spray, dip, or a second abrasive brushing (re-rub). VM was used at 40 μg/ml because this concentration was not a wasteful excess but still inhibited U1 (1 μg/ml) infection by 75 percent (Figure 2). Data were collected in terms of the relative number of U1 lesions appearing as a function of the time between U1 inoculation and VM application. The host plant Xanthi was used because, according to Siegel and Zaitlin (1964), the rate of IS disappearance is so slow on this host that the number of infectible sites should be essentially constant throughout the inoculation periods (up to four minutes). Ambiguities in distinguishing U1 lesions from coalesced groups of VM lesions arose most often in dip and re-rub experiments and least often in spray experiments.

The results of experiments in which VM was applied by spraying leaves previously inoculated with U1 are presented in Figure 3. The points plotted are the means of several determinations together with the standard error of the means. A control treatment, "mix", is also shown in order to indicate the degree of U1 inhibition when U1 and VM were inoculated simultaneously. Although considerable variation was encountered in performing the experiments, it can be seen that inhibition of U1 infection was achieved when VM was presented to infectible sites which would otherwise have developed into U1 lesions. The

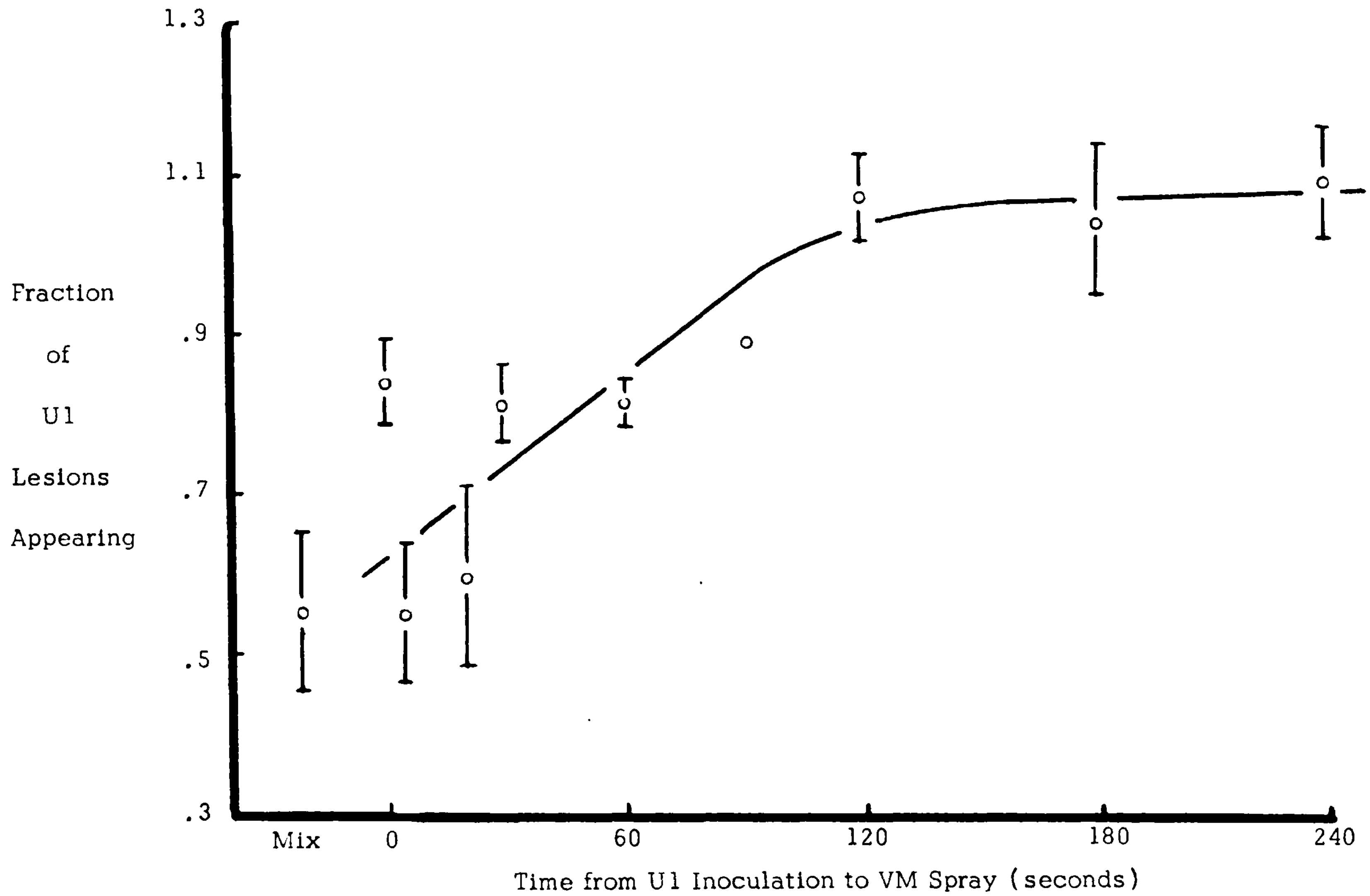

Figure 3 Development of Resistance of U1 Infection Against VM Competition --Spray Experiments

effect of VM on infected IS which would have been the centers of U1 lesions is compared to the competitive inhibition observed when U1 and VM are inoculated simultaneously (mix). This evidence reveals that after exposure of infectible sites to one virus strain a reduced level of competition can still be observed on subsequent exposure of the IS to a second strain. The data further indicate that the period of lability of U1-infective centers to VM interference lasts for approximately two minutes after U1 inoculation on Xanthi. Greater than two minutes after inoculation of one viral strain, it appears that the infection develops a level of commitment to that viral strain which cannot be reversed by application of another strain.

Experiments where VM was applied by dipping an excised U1-inoculated leaf into VM inoculum (Figure 4) showed results similar to those of spray experiments. Although the data are again subject to considerable variation, they essentially reproduce the features of the spray experiments.

A series of experiments parallel to the spray and dip experiments above were attempted in which VM was applied to U1-inoculated leaves by a second abrasive rub. This re-rub technique, which was observed to be slightly more damaging to leaves than the spray and dip techniques, resulted in highly inconsistant data. One particular problem with re-rub experiments was a complete lack of U1 inhibition

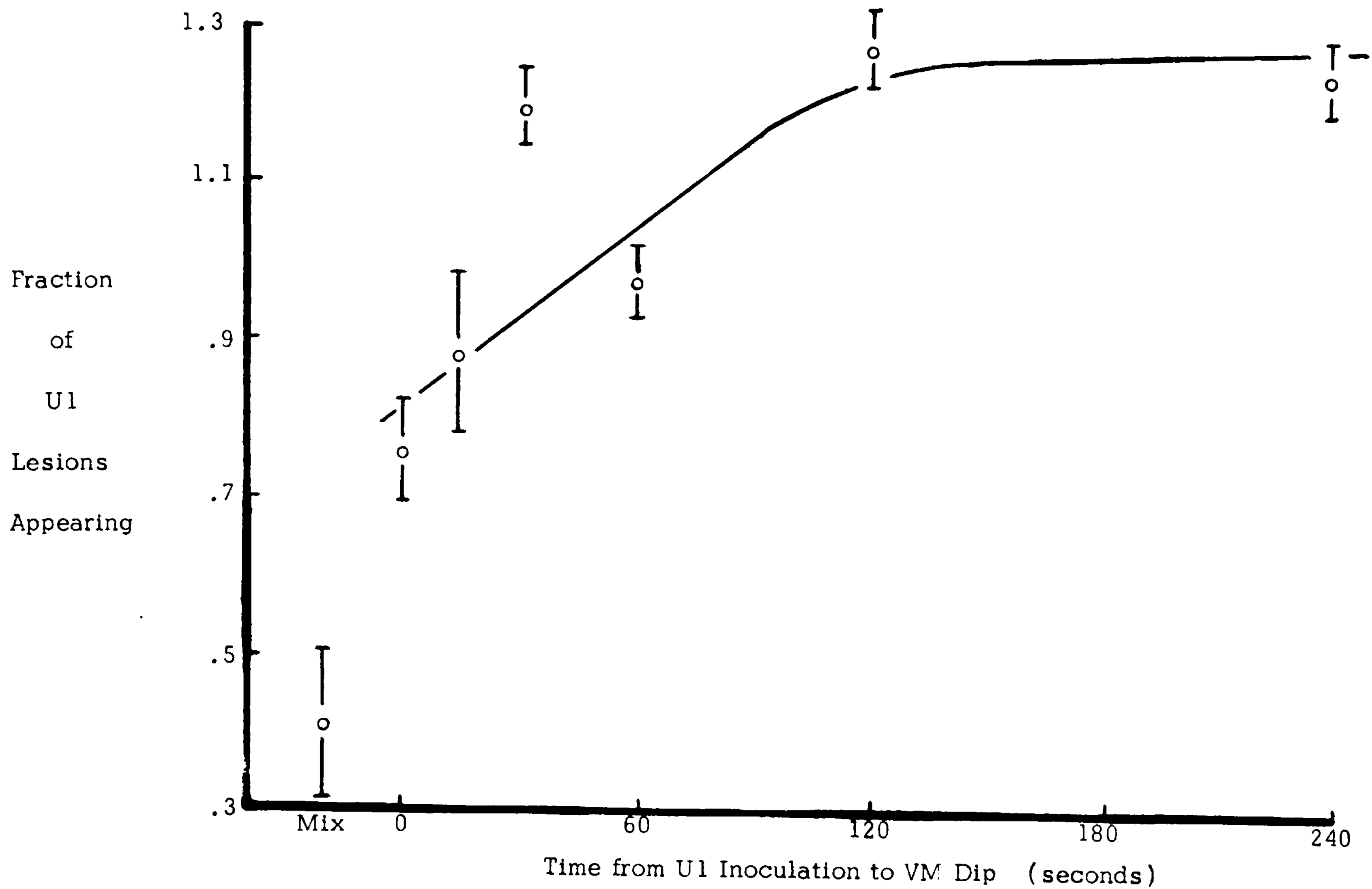

Figure 4 Development of Resistance of U1 Infection Against VM Competition --Dip Experiments

in the mix control in 13 of the 21 experiments performed. Graphs of the data from re-rub experiments showed little internal consistency and did not reflect to a useful degree any of the aspects common to Figures 3 and 4.

DISCUSSION

I. The Concentration-Infectivity Relationship for VM

The data reported in this thesis were obtained as numbers of necrotic lesions produced by TMV strains on leaves of *Nicotiana tabacum* L. var Xanthi nc. (Xanthi). The first experiments presented (Figure 1) defined a concentration-infectivity plot for the TMV strain VM. A plot of this type for VM has not previously been published. Comparison of the VM plot with the concentration-infectivity plot of U1 taken from Wildman (1959) and shown in the same figure, reveals a great similarity. Both plots showed approximately linear relationships at concentrations below 0.5 µg/ml and slopes of nearly 1 in the same region. A slope of 1 can be interpreted to signify that proportionate increases of virus applied to available IS result in proportionate increases in the conversions of IS to infective centers. Thus, in at least this quantitative aspect, the association of VM with IS appears to be by a process similar to that for U1.

II. The Effect of VM on U1 Infection after Simultaneous Inoculations

The rate of inhibition of U1 lesion production by VM as a function of VM concentration is clearly displayed in Figure 2. In general it can be seen that this observation duplicates that of Rappaport and Wu (1962) which is also shown in Figure 2. The portion of the new curve

involving greater than 1 μg/ml VM shows higher U1 relative infectivity than that in the previous report. Such a discrepancy might be expected if the VM preparation used in the current work produced fewer lesions per mg of virus than the VM preparation of Rappaport and Wu (1962). The difference between the two curves may also reflect different sensitivities of the two host species used. The new data expand on the work of Rappaport and Wu (1962) at VM concentrations between 0.1 and 2.5 μg/ml and make it clear that VM has little effect on U1 in this low range. The results can also be submitted as corroboration of the results of two other similar reports: Wu, Hudson, and Wildman (1962); Wu and Hudson (1963).

III. Increasing U1 Associations with Infectible Sites

The data for competition experiments which involved a time lag between U1 and VM applications were presented in Figures 3 and 4. In each experiment there was a treatment of U1 inoculation alone which was used to represent 100 percent relative U1 infectivity. Since each of the other treatments (except mix) involved an identical U1 inoculation followed later by VM application, then these other treatments can be considered to have started out with the capacity to induce full U1 infectivity comparable to 1 μg/ml U1. It can be assumed that at the time of VM application the U1 inoculated leaves had 100 percent of the U1-IS associations already initiated. Any reduction of U1

infectivity resulting from VM applications must then be attributable to VM either blocking or replacing U1 associations at IS.

The data represented in Figures 3 and 4 show that VM caused varying degrees of inhibition of U1 infection when applied up to two minutes after U1 inoculation on Xanthi leaves. Inspection of the graphs reveals that the degree to which VM interferes with U1 infection steadily decreases during the first two minutes. More than two minutes after U1 inoculation, application of VM no longer is capable of decreasing U1 infectivity. Interpretation of these results may be made in light of the above discussion. Of the established relative number of U1 associations with IS made at the time of U1 inoculation, the number which are labile to interference by VM decreases as a function of time after U1 inoculation. The suggestion is that within the first two minutes after U1 inoculation the association of U1 particles with IS is in a transition state. The transition appears to be toward secure U1 interactions with IS, which become inviolable to interference by competing viruses. The decreased availability of U1 infectible sites to interaction with VM is probably not a function of physical sealing up of abraded tissue since it was shown earlier that IS on Xanthi remain open for a long time (Siegel and Zaitlin, 1964).

It appears that the resistance of U1 infective centers to competition with VM represents specific U1 associations with the IS.

The experiments in this thesis have indicated that a virus can develop a relationship with an abraded leaf which can protect the virus's ability to induce an infection. Stahmann and Gothoskar (1958) proposed that TMV associations with IS may be due to an attraction between opposite charges on the virus particle and on the IS. Wu and Rappaport (1961) determined the apparent heat of inactivation of IS and suggested that a protein is an integral part of IS. Wu (1964) suggested that a virus which is prevented from inducing a lesion may still develop an intimate association with the IS which it entered. The associations he described were apparently strong enough to decrease UV sensitivity of the virus. Thus it appears that abraded host leaves provide particular loci with which a virus must associate in order to cause infection. Incorporation of the virus into the IS seems to develop right from the time of inoculation and may be the initial step in the progress of infection.

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