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BIOASSAY OF CLOVER WOUND TUMOR VIRUS AND THE MYCOPLASMALIKE ORGANISMS

WHITCOMB, R F

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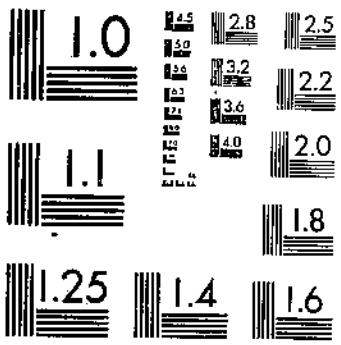

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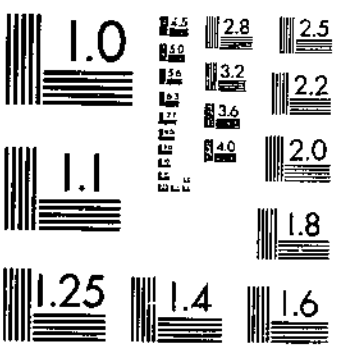

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Bioassay of Clover Wound Tumor Virus and The Mycoplasmalike Organisms of Peach Western X and Aster Yellows

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Bioassay of Clover Wound Tumor Virus and the Mycoplasmalike Organisms of Peach Western X and Aster Yellows

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INTRODUCTION

Chiu and others (*12, 13*)[1] recently developed a method of assaying phytarboviruses (*53*) on monolayer cultures of leafhopper cells (*5*). This method, the development of which culminated a decade of efforts and progressive advances (*23, 24, 26, 32, 33, 44*) may, for some disease agents at least, replace insect injection, a technique which is by contrast one of the most cumbersome and laborious of assay methods.

In 1967 it was suggested (*15, 25*) that the agents of "yellows diseases" may be organisms such as mycoplasmas or chlamydiae. Recently, preliminary reports on cultivation (*11, 30*) have appeared. If the currently held theory of etiology proves to be true, many leafhopper-borne disease agents that are currently assayed by injection may eventually be assayed by counting colonies on agar plates. It is unlikely, however, that injection of insects will ever be abandoned as a research tool. Some plant disease agents that propagate in their vectors may not prove amenable to tissue-culture assays. Certain agents, such as that of peach Western X-disease, are acquired by their vectors so poorly from known plant hosts that injection may be a preferred method of infecting insects (*26, 56*). Even for agents that are acquired easily by their insect vectors, injection provides a means of standardizing dosage and the approximate time at which infection begins. New plant disease agents will need to be studied by this means. Thus, there is a continuing need for an understanding of the events occurring after injection, especially aspects relating to assay (*50*).

[1] Italic numbers in parentheses refer to Literature Cited, p. 28.

THE PROBLEM

Various solutions containing particles of a plant disease agent, or dilutions of the solutions, are injected into a number of insects. Although the number of test insects is theoretically unlimited, the patience of an operator is usually exhausted by the time 400 to 600 insects have been injected. Furthermore, if subsequent infection of the insects is to be verified by means of test plants, there will prove to be a limiting number of plants that can be maintained in the available facilities. Given these limitations, the problem is to derive from the admittedly laborious procedure not only a maximum efficiency of estimation, but any other information that may be incidentally available.

METHODS

Test Insects

For assays of clover wound tumor virus (WTV), the vector *Agallia constricta* Van Duzee was injected. The vector *Colladonus montanus* Van Duzee was used for assays of the agent of peach Western X-disease (WX), and *Macrosteles fascifrons* (Stål) was injected with the aster yellows (AY) disease agent.

With all three of these leafhopper species, last-instar nymphs, collected shortly before molting to the adult stage, are most desirable as test insects. Such nymphs are of known age, are usually highly susceptible to infection, are easily pierced by sharp needles, and are more likely to survive the injection operation than adults. However, if the "total-latent-period" assay method is used, in which insects are maintained for long periods on test plants, it may be necessary to select young male insects. This prevents the deposition of eggs and the destruction of the test plant by the progeny.

After collection, the insects should be stored at temperatures slightly above freezing in a moist chamber to prevent desiccation. They are injected under a continuous flow of moist CO_2 at room temperature under a dissecting microscope, after which they should be returned as soon as possible to a healthy, preferably immune, plant, upon which they survive well.

Needles

Whereas much classical work has been done with needles pulled in gas flames (*47*), those who have been introduced to machine-pulled needles will certainly insist on them. Not only do machines produce a uniform product, but many, including myself, are unable to produce any needle of their quality in a flame.

The Injection Procedure

If inoculum is freshly prepared and is kept cold in an ice bath, it will not coagulate, even if it is a crude extract from plants or insects. Small amounts of liquid are drawn into the needle with a low vacuum; small amounts are expelled by momentarily closing off a T-joint inserted into the compressed-air line leading to the needle. The alternative method of drawing up liquid and expelling it by mouth is made undesirable by saliva produced by the operator. If large, turgid nymphs have been selected, the operation should proceed rapidly. Groups of 10 anesthetized insects can be injected in 1 to 2 minutes; in this way exposure to lethal doses of CO_2 can be avoided.

Mortality of the Injected Insects

Jensen and others (*26*) summarized the factors known to produce mortality of injected leafhoppers. These included the WX disease agent, bacterial contaminants (*58*), old age, and a variety of effects ascribed to "wounding." Premature death occasionally occurred in adults that had not been injected. These deaths could possibly be ascribed either to microbial infection (*36*, *58*), or to poor nutrition during the immature stages, induced by the toxicity of the nymphs to the celery plants on which they were reared. A single last-instar nymph produced enough toxin to kill a 2- or 3-leafed celery seedling.

It is likely that, if suitable insects are used, if sufficient care is taken to preclude microbial contamination, if adequate nutrition is provided throughout nymphal instars, and if insects are rejected if they have been pierced more deeply than necessary, that 95- to 100-percent survival can be obtained.

Scoring of Diseased Insects

Much effort has been spent on the serological scoring of insects by the precipitin ring test (*52*), fluorescent antibody test (*35*, *45*), ring time test (*49*, *51*), and hemagglutination (*20*, *42*). For agents that have high antigen titers, the Ouchterlony test (*37*) should also prove useful.

Another method of scoring diseased insects—lethality of the agent to the vector—is available in the case of the WX agent (*26*). Although percentages of surviving insects were clearly correlated with dosage when high concentrations of inocula were injected, incidental deaths hopelessly obscured the results if lower concentrations were injected.

Despite the introduction of other scoring methods, most assays still require test plants. Furthermore, until the transmission of disease agents from insect to plant is well understood, such phenom-

ena as the fate of insects on test plants, the transmission of disease agents to plants, and the course of the infection in the insects and plants will remain a reasonable area of interest to vector specialists.

INCUBATION PERIODS IN PLANTS

Wound Tumor Virus

The incubation period of WTV in crimson clover (length of time from the date insects were placed on the plant to the first date of symptomatic expression) varied from 16 to 60 days. The distribution of incubation periods had a range of about 30 days. The frequency distribution curves (fig. 1) were skewed to the left, with a tail extending

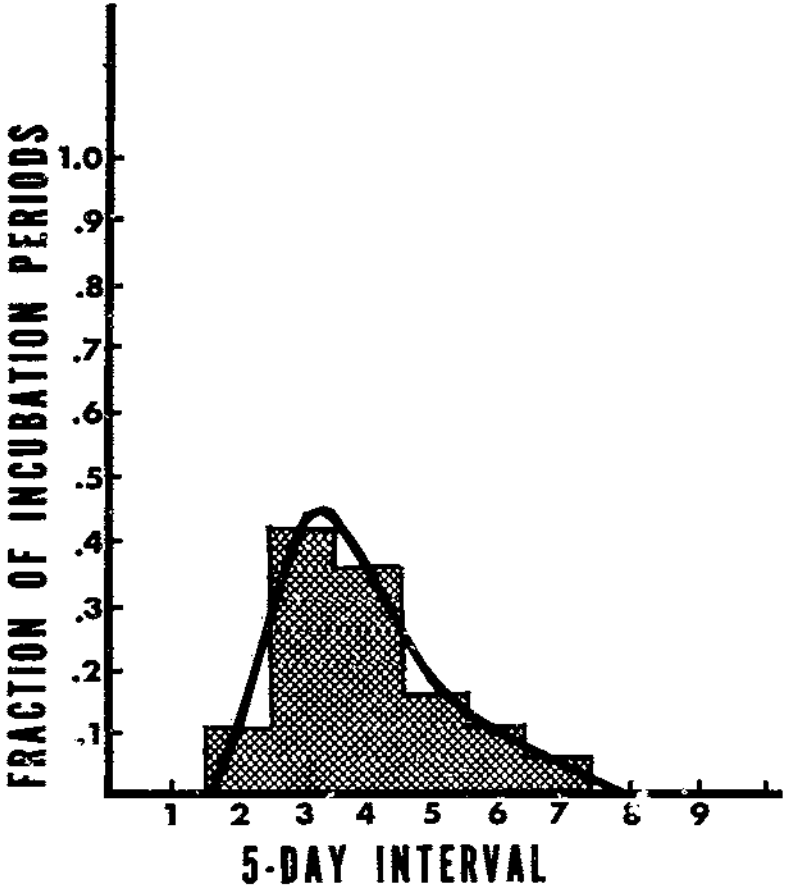

FIGURE 1.—Distribution of incubation periods of WTV in crimson clover. Modal time for incubation period was usually 19 to 21 days after the start of a 7-day inoculation feeding interval on the plants.

to the right. Although such curves seemed at first to give an equally good fit to the incubation periods from either first or subsequent transmissions by individual insects, a chi-square analysis (table 1) demonstrated that first transmissions were somewhat more likely to result in lengthy incubation periods than subsequent ones.

TABLE 1.—*Chi-square test of significance between distributions of WTV incubation periods in first and subsequent transmissions*

Experiment and position of transmission	Incubation periods		
	First four 5-day intervals	Last two 5-day intervals	Total for each position
	Number	*Number*	*Number*
Experiment No. 1:			
First	[1] 15	1	16
Other	13	0	13
Experiment No. 2:			
First	17	3	20
Other	27	0	27
Experiment No. 3:			
First	18	8	26
Other	22	3	25
Experiment No. 4:			
First	13	2	15
Other	44	3	47
Experiment No. 5:			
First	8	2	10
Other	49	6	55
Totals:			
First	71	16	87
Other	155	12	167
Total for each set of intervals	226	28	254

χ^2 observed $=7.3$
χ^2 $(\alpha=0.05)=3.84$
χ^2 $(\alpha=0.01)=6.63$

[1] Numbers of incubation periods of WTV in crimson clover.

Western X-Disease Agent

The first celery plants infected in a series of transmissions by a given vector frequently develop WX symptoms only after an unusually long incubation period. Kunkel (*28*) noted a similar phenomenon with the aster yellows disease agent.

These classical observations have now been extended with WX to the entire period of inoculativity of the vector. As shown in figure 2, inoculativity of WX by its vector, after an initial steep rise, decreases to levels as low as those in early stages of infection. If this decrease in transmission results from lower concentration of agent being inoculated into the plant, then incubation periods in celery should show a corresponding increase. For example, the last transmission in a series should result in a longer incubation period. The data of figure 3 support this expectation.

We have also noted that incubation periods preceding and following "skips" in transmission by single insects also tend to be longer than other transmissions in the series. Duffus (*16*) has made similar observations with aphid vectors. Although the titer of WX in *C. montanus* probably reaches higher levels than many other disease agents in their vectors, transmission of the WX agent to celery is very poor.

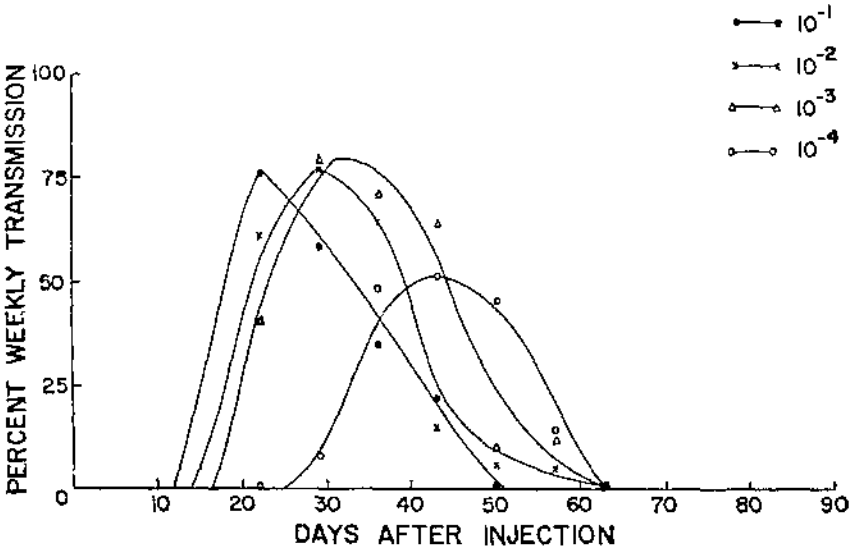

FIGURE 2.—Transmission of the Western X-disease agent in weekly inoculation intervals on healthy celery following injection of serial tenfold dilutions of extract from infected vectors. Only insects that at some time transmitted WX were used in the calculations. Interpretation of dosage relationships has been discussed in a previous publication (*54*).

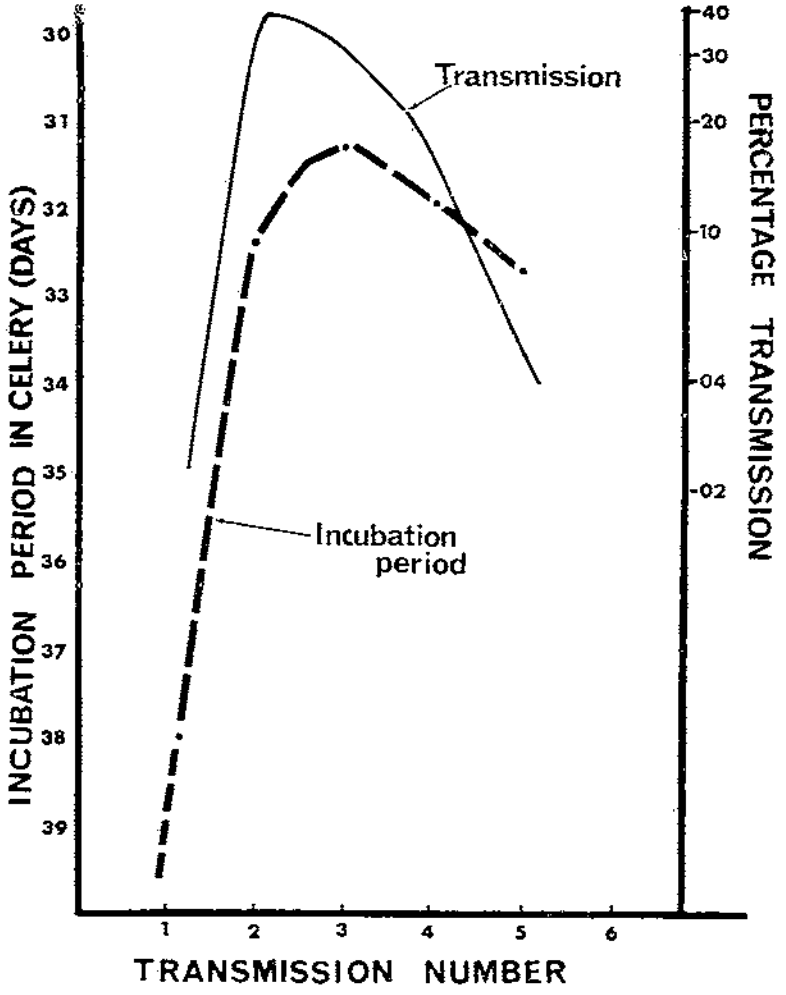

FIGURE 8.—Two methods of measuring amount of western X-disease agent transmitted to celery by *Colladonus montanus*. Solid line represents percentage of infected test plants over the 6-week course. Dotted line represents the incubation period of the infections in celery initiated during the same 6-week course.

Therefore, a plant such as celery, which happens to be very poorly susceptible to a particular disease agent, is a good tool for studying variations in the inoculative potential of vectors. Thus, the system has provided the best evidence to date that leafhopper vectors do, in fact, have different inoculative potentials from time to time, rather than a simple ability or disability to transmit.

Insects transmitting the WX agent tend to undergo a definite

"transmission-free" period (TFD) before death. The duration of this period depends (fig. 4) on the age of the insect at the time of death. The presence of such a period before death, when insects failed to transmit WX, seems at first to suggest a discontinuous change in the vector's status from transmitter to nontransmitter. There is some histological evidence for such a change (*56*). Serous cells of *C. montanus* are eventually destroyed by WX infection, but the other nine cell types of the principal salivary gland, despite certain cytological changes (*56*), apparently remain more or less functional.

We have speculated (*56*) that multiplication of WX agent in serous cells may be required for transmission. The dependence of the length of the TFD on longevity of the vector (fig. 4), however, suggests that histopathological changes induced by WX may not greatly affect the transmission mechanism. The longevity of vectors varies considerably within groups of insects treated identically (insects living

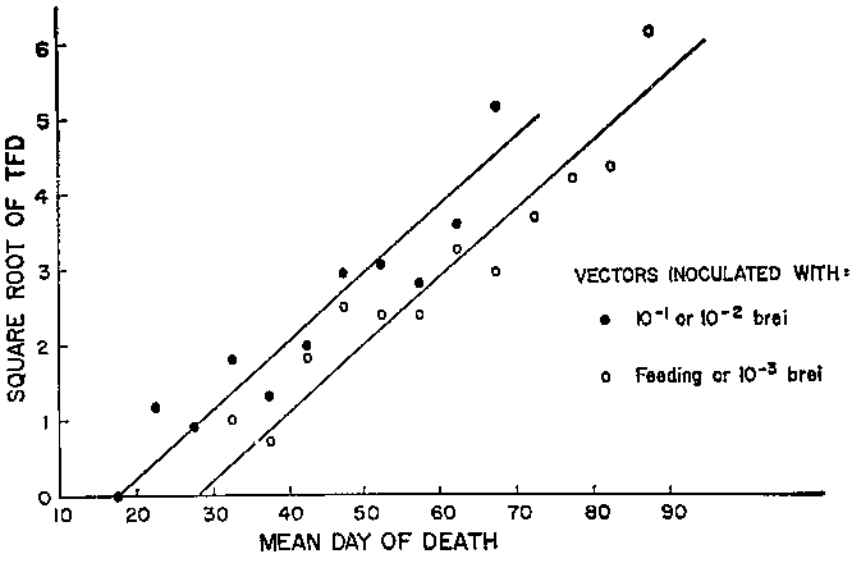

FIGURE 4.—Dependence of the number of transmission-free days (TFD) before the death of *Colladonus montanus* adults upon the longevity of the vector. (Mean day of death=days after injection of WX agent). Because longevity of the vectors depended on the injected concentration or route of entry of WX, two lines were drawn: One for the more concentrated inocula (10^{-1} or 10^{-2} dilutions of extract) and another for more dilute inoculum (10^{-3} dilution), or for infections initiated when insects fed on diseased plants.

as long as 83 days after injection were found to carry advanced histopathological symptoms (*57*)). It is doubtful that infected insects failing to transmit WX for 10 to 36 days before death suffered all the while from total loss of a vital function such as salivation. It is significant also that insects that happened to die during early stages of infection transmitted well in their last inoculation interval.

In summary, WX transmission probably decreases to a certain extent for reasons related to aging and decreased feeding rate of the insect, as in the transmission of pea enation mosaic virus (*48*). However, the major cause of decrease in inoculativity is probably a steep decay of WX agent after the initial growth phase. Decreases due to failure of individual serous cells to secrete watery saliva, or total impairment of transmission as a result of the failure of all serous cells, may occur to a limited extent, but probably are relatively unimportant factors in the overall transmission curve.

QUANTAL RESPONSE ASSAYS

Dilution Curve Theory

Brakke and others (*10*) interpreted WTV dilution curves by a graphical method. Logarithms of the percentages of leafhoppers that infected crimson clover were plotted against the logarithms of the concentrations of virus extract originally injected into the insects. It was found that the points fell approximately upon a straight line, in the range of 0 to 50 percent infected insects. Dilution curves of extracts to be compared were, therefore, fitted to straight lines of the same slope, and differences in concentration were estimated graphically.

The above method is in contrast to the classical Poisson dilution theory, as applied to bacteriophage (*17*), animal viruses (*38*), sigma virus of *Drosophila* (*39*), and wheat streak mosaic virus on individual wheat plants (*8*, *9*).

According to the Poisson interpretation, the chance presence or absence of a virus particle at the site of initiation of infection is responsible for the characteristic "dosage mortality curve" obtained. The proportion, Q, of insects that receive no particle at the site of infection is e^{-x} where e is the base of natural logarithms, and x is the average number of infectious particles reaching the infection site; i.e., the concentration of particles at the infection site. If the initial concentration of particles in the extract is c, the concentration of a dilution of that extract is x/f^n, where f is the dilution factor, and n the number of times the extract has been diluted. Then $Q=e^{-cf^n}$, and

$\log_e \frac{1}{Q} = cf^n$. A plot, therefore, of $\log_{10} \log_e \frac{1}{Q}$ against $\log_{10} c$ should give a straight line with unit slope.

Dilution Curve of WTV

Data from two published sources (*6*, *10*) were analyzed, as well as data not previously published in their entirety obtained in the study of vectorless WTV (*7*). None of the three sets of data gave a good fit to Poisson theory. When $\log_{10} \log_e \frac{1}{Q}$ was plotted against the logarithm of virus concentration, the best-fitting straight lines, determined graphically, gave slopes of 0.67, 0.50, and 0.48. In only one of the 26 dilution curves examined was a slope as great as 1.00 obtained.

Figure 5 and table 2 show the result of two unsuccessful attempts to fit the data (*6*, *10*) graphically to the theoretical curve. In each case there was a wide discrepancy between theoretical and observed values. Such deviations are considered to be too serious to justify application of the theoretical dilution curve to data so far obtained. Under these circumstances, there seems to be no reason why the scheme of Brakke and others (*10*) should not be used as an empirical method (*49*).

Dilution Curve of the WX Agent

Whitcomb and others (*54*) in earlier experiments used tenfold dilution steps. In two later experiments, however, half-log dilution steps were used (tables 3 and 4).

The results of one experiment (table 2) indicate that percentages of infected vectors can, in rare instances, be meaningless as indicators of the concentration of agent that was injected. An error in labeling solutions or insects seems to be precluded by the latent periods in the insects, which were normal for each injected concentration. Furthermore, no conceivable reconstruction of the data, assuming mislabeling, would improve its conformity to expectation. Finally, whereas in this experiment there was a large fraction of "immune insects," it was not unusual to observe smaller percentages of seemingly immune insects in other injection experiments. Recent research suggests two possible mechanisms for immunity. We have observed in the course of our WX studies that adipose cells were altered by stress. Since adipose tissue might be the major site of infection after injection of the Western X-disease agent, physiological alteration of such cells might render the insects immune. Recent suggestions (*15*, *25*) that agents of yellows diseases may be Mycoplasmalike agents offer another possible explanation: infection with a Mycoplasma not

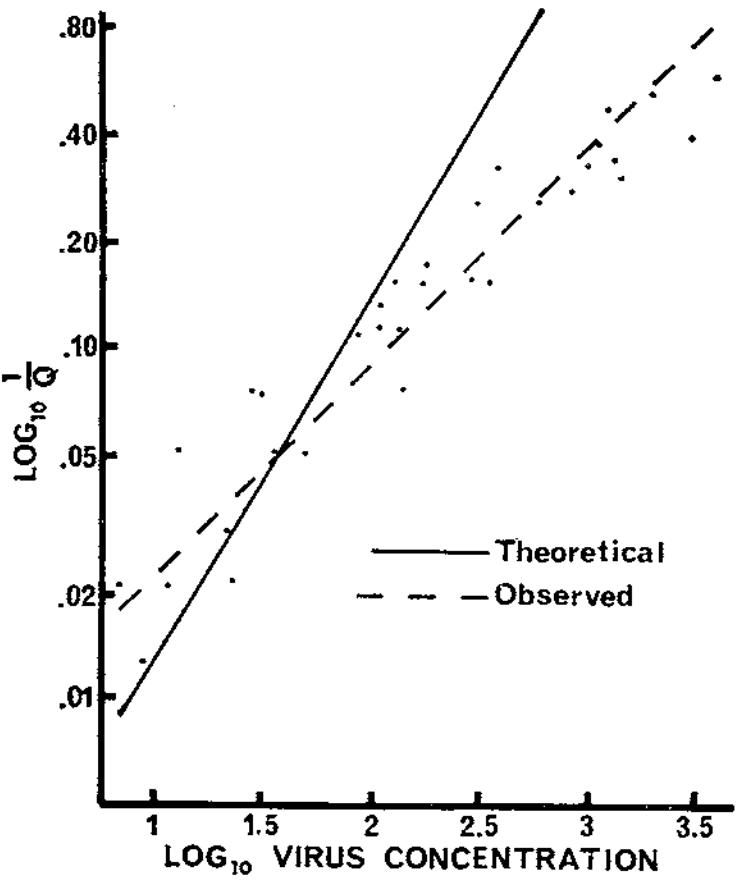

FIGURE 5.—Data of Brakke and others (*10*) eye-fitted to the best-fitting straight line. The slope of the observed line is 0.67, compared with the value of 1.00 predicted by Poisson theory. The logarithm of virus concentration at the highest dilution was arbitrarily taken to be 1.0; therefore, the abscissa is a measure of *relative* virus concentration.

pathogenic to plants might protect a leafhopper against an agent that does cause plant disease. Such an agent has recently been visualized by electron microscopy (*36*) and would be expected to be present in the injected extracts.

In a second experiment a number of special precautions were taken. *C. montanus* adults carrying the Western X-disease agent were extracted in 10 ml. 0.85 percent NaCl, emulsified with Genetron 113, and clarified very briefly at 1,000 g., after which the pH was adjusted to 7.3. This extract was centrifuged for 10 minutes at 5,000 r.p.m.

TABLE 2.—*Analysis of quantal response: Attempt to fit data of Black and Brakke* (6) *to a Poisson distribution*

[P=observed fraction of transmitting insects. Q=1—P, which, according to the Poisson interpretation, $=1-e^{-x}$, where x is the average number of successful particles per insect. According to the Poisson interpretation, the calculated c values (x/d where d is the dilution) should be homogeneous for the three dilutions of each passage]

Passage	Log_{10} dilution	d	P	Q= 1—P	Log_e Q=x	c
			Number			
1	−2	1	0.30	0.70	0.36	0.36
	−3	10	.05	.95	.05	.50
	−4	100	.05	.95	.05	5.00
2	−3	1	.19	.81	.21	.21
	−4	10	.04	.96	.04	.40
	−5	100	.00	1.00	.00	----
3	−3	1	.20	.80	.22	.22
	−4	10	.15	.85	.16	1.60
	−5	100	.06	.94	.06	6.00
4	−2	1	.41	.59	.52	.52
	−3	10	.11	.89	.12	1.20
	−4	100	.00	1.00	.00	----
5	−2	1	.37	.63	.46	.46
	−3	10	.17	.83	.19	1.90
	−4	100	.04	.96	.04	4.00
6	−2	1	.54	.47	.76	.76
	−3	10	.25	.75	.29	2.90
	−4	100	.00	1.00	.00	----
7	−2	1	.57	.43	.84	.84
	−3	10	.25	.75	.28	2.80
	−4	100	.07	.93	.07	7.00

TABLE 3.—*Analysis of quantal response. Abnormal dilution curve of WX agent*

Dilution [1]	Fraction transmitting [2]		$\overline{LP}$
Number	*Number*	*Percent*	*Days* [3]
$10^{-1.0}$	11/33	33	24
$10^{-1.5}$	4/24	17	23
$10^{-2.0}$	2/26	08	28
$10^{-2.5}$	7/28	25	26
$10^{-3.0}$	4/30	13	26
$10^{-3.5}$	7/28	25	26
$10^{-4.0}$	12/35	34	30
$10^{-4.5}$	12/25	48	34
$10^{-5.0}$	11/27	41	32

[1] Dilution calculated on basis of the original weight of extracted insects.

[2] Numbers of insects transmitting WX agent to celery test plants, divided by the number of insects surviving through the possible latent period.

[3] Latent periods of WX agent in individual vectors were assumed to be the midpoint of the interval during which their first WX transmission to celery occurred. The average latent period ($\overline{LP}$) for a group was calculated: $\frac{\Sigma LP_i}{N}$, where LP_i was the latent period of a single vector, and N was the number of latent periods observed.

in the SW 39 L rotor of the Spinco [2] Model L centrifuge. Half-log dilutions of the supernatant were made in 0.1 M glycine, 0.01 M $MgCl_2$, 0.01 M Na_2SO_3, after which 50 nymphs were injected with each dilution within 1½ hours of final centrifugation.

The results (table 4) do not conform to Poisson dilution curve theory in one important respect. Apparently only about 83 percent of test insects were susceptible to the WX agent. Therefore, observed percentages should be transformed by multiplying them by 1.2, in effect, ignoring the hypothetical immune insects. When this was done, the series of percentages obtained were compatible with Poisson statistics (table 4). Of course, if percentages of infection of susceptible insects do not follow the Poisson distribution, such a transformation will not improve the conformity to Poisson statistics.

[2] Trade names are used in this publication solely for the purpose of providing specific information. Mention of any specific equipment, trade product, or commercial company does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement by the Department over similar products or companies not mentioned.

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TABLE 4.—*Analysis of quantal response: WX dilution curve*

Log dilution [1]	Insects responding				[6] P(1.2)	[7] $\overline{LP}$	[8] $\overline{LP_T}$
	Positive [2]	Negative [3]	Unknown [4]	Fraction transmitting [5]			
	Number	*Number*	*Number*	*Percent*	*Percent*	*Days*	*Days*
1.0	33	7	5	82.5	99	22.0	62.4
1.5	35	6	7	85.4	100	26.3	63.2
2.0	21	5	9	80.8	97	30.1	69.3
2.5	22	12	12	64.7	78	30.8	66.1
3.0	10	23	12	30.3	36	32.1	66.4
3.5	2	24	12	7.7	9	[9] 31.9	[9] 66.5
4.0	0	37	5	0.0	0	----	----
4.5	1	32	12	3.0	4	[9] 35.0	[9] 59.0
5.0	0	35	9	0.0	0	----	----

[1] Dilution calculated on basis of the original weight of extracted insects.

[2] Positives=number of insects transmitting WX agent to celery.

[3] Negatives=number of insects failing to transmit WX agent to celery, despite survival through the possible latent period.

[4] Unknown=number of insects that died before end of a possible latent period and whose inoculative potential, therefore, was unknown.

[5] Fraction infected was computed from the positive-negative data.

[6] The transformation P(1.2) was derived from the assumption that on the average only 83.3 percent of insects were susceptible to WX infection. The effect of this transformation is to ignore apparently immune insects.

[7] Latent periods of WX agent in individual vectors were assumed to be the midpoint of the interval, in days, during which their first transmission of WX agent to celery occurred. The average latent period for a group was $\overline{LP}=\Sigma LP_i/N$, where LP_i was the latent period of a single vector, and N was the number of latent periods observed.

[8] The mean total latent period ($\overline{LP_T}$) was computed by averaging the total latent periods (number of days between injection of WX agent into the insect and first appearance of symptoms in celery plants inoculated by the insect) for each injected leafhopper that transmitted to celery.

[9] These numbers were calculated from very small sample sizes.

Factors Possibly Affecting Dilution Curves

Effects of preinoculation storage and injected volume.—Two effects were tested: (1) The effect of injected volume, (2) storage of vectors before injection. In each experiment, 200 large last-instar nymphs of *A. constricta* were collected and stored at 2° C. in a glass tube in the cold room, but not in a moist chamber. Groups of 50 were weighed, then injected with a 10^{-1} dilution of WTV-infected sweet clover root

tumors in 0.003 M KH_2PO_4, 0.0066 M K_2HPO_4, 0.225 M NaCl. Half of the vectors were injected with as little solution as could be introduced, half with as much as could be introduced. After injection the insects were reweighed to determine the total weight of inoculum that had been injected into all insects in a group. It was found that by the above technique the injected volume could be varied over a sevenfold range. In addition, nymphs stored in the cold room for 7 or 8 hours before injection accepted more injected fluid.

All injected vectors were stored at room temperature in a moist chamber, then transferred to Grimm alfalfa (*Medicago sativa* var. Grimm). After 3 weeks on alfalfa, the vectors were transferred in colonies of two to healthy crimson clover test plants for a 2-week inoculation access period. Survival of the insects did not vary significantly from group to group. The transmission for colonies of two was recorded and calculated (*52*) into single-insect transmission (P_1).

The P_1 values (table 5) varied widely from treatment to treatment. A satisfactory interpretation of the results can be constructed as follows: The principal storage effect is assumed to be upon the volume that can be injected. Therefore, differences in proportions of infected insects are ascribed to the differences in injected volume. It is also assumed that in the range of volumes used, the probability that a single infectious particle will initiate infection does not depend on the volume. This is equivalent to assuming that, for example, injection of seven times as much inoculum of one concentration is the same as injecting the same volume of an inoculum seven times as concentrated.

To assess the proposed relationship between volume and concentration, the relationship between concentration and percentage infected vectors first needed to be assessed. The test for conformity for the Poisson-binomial interpretation of dilution curves (plotting of $\log_{10} \log_e \frac{1}{q}$ vs. $\log_{10} c$) was performed.

Although the deviation of such lines from unit slope excludes the Poisson interpretation (see "Dilution Curve of WTV," p. 10), the method provides an empirical method for plotting transmission percentages. Data from the two experiments reported here could be best fitted to a straight line (fig. 6) with a slope, 0.57, which is very similar to dilution-curve slopes. This suggests that volume error in insect injection can be partly controlled by measuring the magnitude as a mean for a group of injected insects, and then correcting the concentrations as calculated by dilution.

Because variations in the volumes of injected inocula are almost certainly random, they would not be expected to result in systematic deviation of the dilution curve from a theoretical slope. Single points

TABLE 5.—*Effect of injected volume and pre-inoculation storage on fraction of WTV-infected insects after injection*

Experiment number[1] and time stored at 2° C.	Experimental treatment			Experimental results—Colonies of two infective		Calculated values	
	Initial weight	Gain in weight	Time in moist chamber at room temperature	Fraction	Percentage	Calculated percent single insect transmission[2]	Confidence intervals[3]
Hours	*Grams*	*Grams*	*Hours*	*Number*	*Percent*	*Percent*	
Experiment No. 1:							
1.5	0. 0833	0. 0036	7. 5	9/22	41	23	35–12
2.75	. 0850	. 0008	6. 5	3/21	14	7	16– 3
7.75	. 0799	. 0057	----------	13/23	57	34	48–23
8.75	. 0761	. 0015	----------	7/21	33	18	30–11
Experiment No. 2:							
2.00	. 0938	. 0051	7. 0	18/22	82	58	72–42
2.75	. 0847	. 0014	7. 5	14/21	67	42	55–29
8.00	. 0847	. 0057	----------	21/23	91	70	86–52
8.50	. 0803	. 0018	----------	14/22	64	40	52–28

[1] Fifty nymphs were injected for each storage-time group in each experiment.

[2] Fraction of single-insect transmission (P_1) was calculated: $P_1 = 1-(1-P_2)^{1/2}$.

[3] Ninety-five-percent confidence intervals for P_1, determined from confidence intervals for P_2 determined graphically from Clopper and Pearson, in Dixon and Massey (*14, p. 415*).

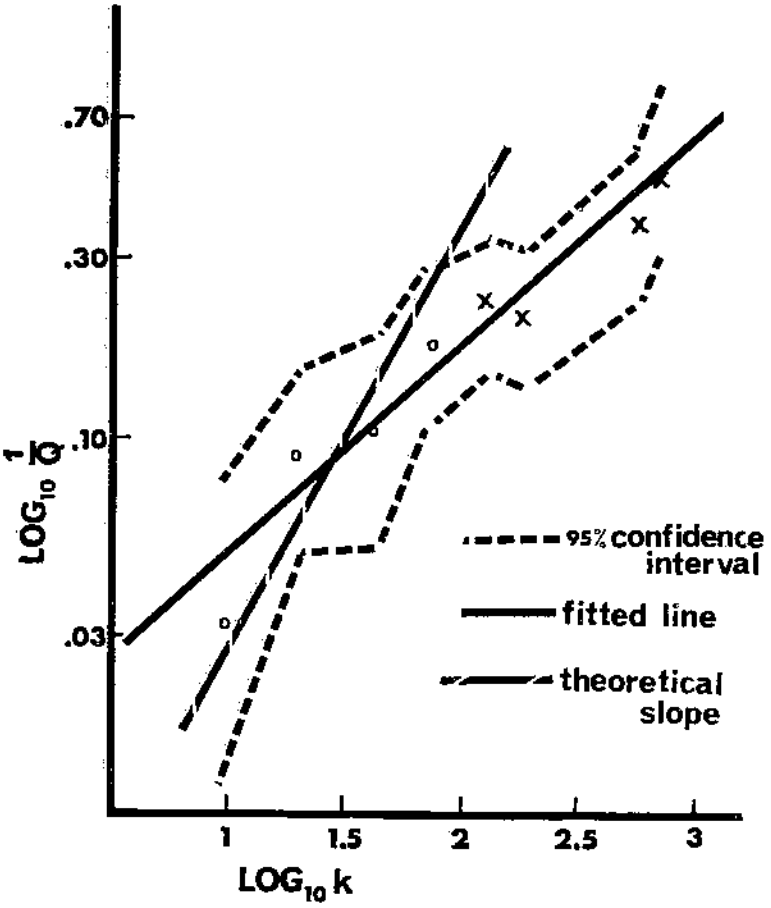

FIGURE 6.—Graphical analysis of data of table 5. The P_i values for table 5 were converted to the appropriate logarithm (ordinate). Abscissa is the logarithm of the ratio of volume injected to the smallest volume. (o=experiment 1, x=experiment 2.) The fitted line, with a slope of 0.57, is similar to dilution-curve slopes of WTV, which indicates that fluctuations of volume or concentration produce similar effects on the fraction of injected insects.

on the curve, however, might be too high or too low, as a result of high or low volumes being injected into some groups of insects. The size of the orifice of the injection needle is the most probable source of such variation in routine assays.

Specific infectivity.—The specific infectivity of disease agents varies widely (*41*). Errors resulting from this factor should, however, be random and should not affect the slope of the dilution curve.

Reversible aggregation.—One type of error that could systematically bias the dilution curve is reversible complex formation or aggregation (*2*). If the agent in an undiluted extract is bound in complexes that dissociate upon dilution, the slope of the dilution curve will be less than one.

Background materials.—When virus is diluted, the other constituents in the medium are also diluted. If substances inhibitory to infection are present, a slope of less than one will be observed. Since two of the sets of WTV data (p. 17) were taken from virus preparations partly purified by rate density gradient centrifugation, inhibitory substances in the extract should have sedimentation coefficients similar to that of the virus. Of course, among the sundry inhibitory substances that might be present (*41*), a prime suspect might well be inactive WTV particles.

Variation in susceptibility of test insects.—Perhaps the most likely cause for a slope less than unity is host heterogeneity (*1*, *43*). Nagaraj and Black (*34*) selected a nontransmitter race of insects from the *A. constricta* colonies, but it was not determined whether these insects failed to acquire virus, support multiplication, or transmit the virus. Other studies of nontransmitter races (*4*, *27*) also failed to elucidate the relationship between "incompetence" and viral multiplication. In addition to genetic variation, it is likely that there are irregular physiological variations (*29*) or perhaps intrastadial variation in the susceptibility of single insects to infection by plant-disease agents.

GRADED-RESPONSE ASSAYS

Despite its widespread use, quantal assay has inherent limitations. Suppose we wish to ask a question such as: "What is the virus content in each of 20 fractions from a purification procedure?" It is impractical or impossible to dilute each of 20 inocula several times and inject all dilutions into sufficiently large samples of test insects. At least 20 and preferably 30 observations are necessary for a reliable estimate of a percentage, and at least three dilutions of a fraction may be required to find the range of dosage responses where percentages are between 0 and 100. Thus, for 20 fractions, we would have to inject 1,200 insects, assuming 100-percent survival. Except in rare circumstances, such an effort would be impractical. Furthermore, we frequently wish to assay fluids, such as salivary material or hemolymph, which are available in only small quantities and would be difficult or impossible to dilute accurately. For these applications it has been necessary to employ a graded-response assay.

Dynamics of Infection Process

The basis for graded-response assay lies in the dynamics of the infection process. This was first studied by Black (*3*), who obtained variable results after permitting *M. fascifrons* to feed on plants infected with the aster yellows agent, but concluded that the agent increased during the incubation period. Whitcomb and Black (*52*) measured the concentration of viral soluble antigen after injection of WTV. Whitcomb and others (*55*) estimated the concentration of the WX agent in the hemolymph of *C. montanus* at various times after injection. Reddy and Black (*40*) estimated the concentration of WTV in solutions injected into *A. constricta* in order to follow growth of the virus after acquisition feeding. Sinha and Chiykowski (*46*) followed the increase of the aster yellows agent in various organs of its vector. Plus (*39*) studied the growth curves of sigma virus in *Drosophila*.

The general sense of these contributions is as follows: Infection initiated by injection of a disease agent is followed by an increase of the agent, which follows the course of a logistic growth curve. Transmission of the disease agent is closely correlated with the time when maximum titer is reached in the insect. The transmission curve rises steeply from below the threshhold of detection to a high level within a short period of time. Following the logistic growth phase of the agent, there occurs a decrease in the concentration of the agent, which is correlated with decreasing transmission frequency.

Graded-response methods using parameters of transmission curves are based on the concentration-dependence of the time placement of the transmission curves. Thus, a family of similar curves (fig. 2), whose logistic growth phases were found to have similar slopes, was generated by plotting transmission results from injected dilutions of a disease agent.

T_{50} Method

The dependence described above can be used in a number of ways. Any property of the transmission curve can be selected as an operative statistic. The first approach we used (*55*) was to calculate the T_{50}, at a time at which 50 percent of insects that would eventually transmit were transmitting at least once weekly. The T_{50} of an unknown could be related to the T_{50} of values obtained from a dilution series by graphic interpolation.

Latent-Period Method

In later work we used a latent-period method of analysis for the WX agent. Such assays had been used in a less quantitative way in earlier

studies (*22*, *31*). For purposes of this analysis, as in the T_{50} analysis, it was necessary to transfer the insects to new test plants five times. Thus, the inoculation feeding was divided into five intervals, not necessarily equal. To compute a latent period, it was assumed that successful transmission events in each interval occurred at the instant of the median time of that interval. All data from insects injected with a given inoculum were used to compute the average latent period resulting from injection of that inoculum. This average latent period was then referred to a standard curve, preferably obtained in the same experiment under the same experimental conditions.

In practice, seasonal differences in the greenhouses in Berkeley, Calif., were minimal, and good agreement was obtained in the absolute values of latent periods at different times of year. It would be much preferable and is necessary in Beltsville, Md., to perform such assays in controlled-growth facilities. If this is done, it is not absolutely necessary (although still highly desirable) to include a dilution-curve control in each experiment.

Computation of latent-period data from a large experiment can be readily accomplished on a single data sheet (table 6) by employing certain shortcuts.

Latent periods in the Vector

The Poisson interpretation of dilution curves has certain implications in the expected distribution of latent periods in the insect. If a large majority of infections are initiated by single particles as predicted, theoretically when less than about 40 percent of insects become infected, the latent periods resulting from those infections should have a maximum value (or more accurately, a maximum *mean* value, with a variance depending on the variability in the insect population injected). Examination of the distributions obtained experimentally (fig. 7) after the injection of WX agent into *C. montanus* empirically supports this expectation.

Half-$\log^{10}$ dilutions (in terms of weight of tissue from infected vectors) were injected. It is suggested that most latent periods resulting from injections of suspensions diluted a hundredfold (−2.0) or more resulted from single-particle, or at most double- or triple-particle infection; such latent periods should be maximal. This interpretation is consistent with Poisson statistics, which predict for example that when 65 (1.2) = 78 percent of vectors are infected after injection of a hundredfold dilution, 42.3 percent of the infected vectors would receive 1 particle, 32 percent would receive 2 particles, 15.4 percent would receive 3 particles, and 6.4 percent would receive 4 particles.

If my interpretation is correct, latent periods in insects can reflect the difference between infections caused by one and by about five or more particles.

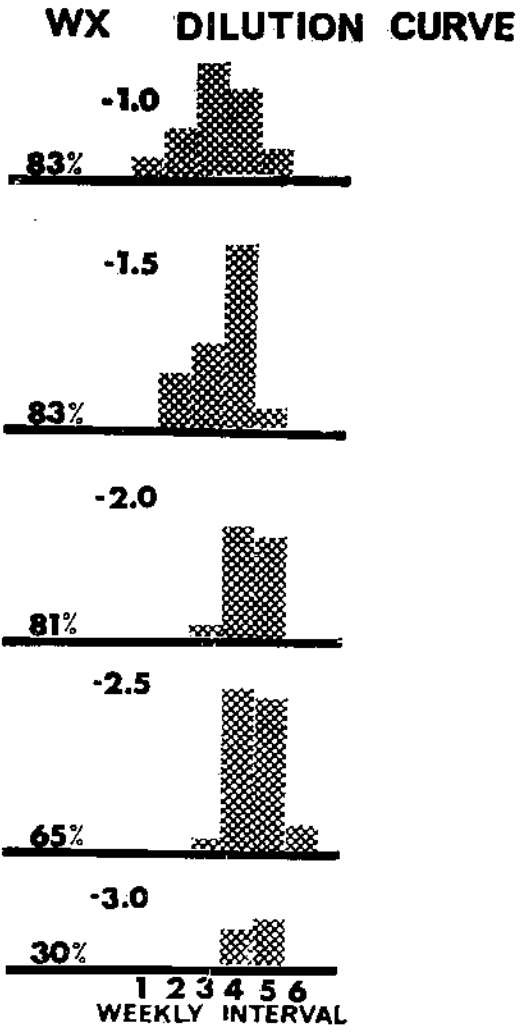

FIGURE 7.—Percentages of infection and frequency distributions of latent periods of the Western X-disease agent in *Colladonus montanus*.

TABLE 6.—*Example of calculation of average latent period of WX agent in leafhoppers*

[The experimental treatments of WX inocula are followed by injection of the extract into leafhoppers. Surviving leafhoppers are transferred to new test plants at intervals 10 to 50 days after injection, the range in which all latent periods fall. Three- or four-day intervals are preferable, but intervals, not necessarily equal, of any length may be chosen. A preliminary calculation is made of differences between intervals in days]

Symbol and definition	Interval (i)			
	1	2	3	4
	Days	*Days*	*Days*	*Days*
D=Range of interval in days after injection	20–24	24–31	31–38	38–52
H_i=Midpoint of interval in days after injection	22	27.5	34.5	45
d_i=Difference between midpoint of interval and midpoint of first interval (H_i-H_1)	0	5.5	12.5	23

CALCULATION OF LATENT PERIOD USING ABOVE H_i AND d_i VALUES

Inoculum tested	Vectors transmitting during interval (n_i)					Surviving vectors including non-transmitters	Percentage transmission [1]	Weighted average period between H_1 and transmission $\frac{\Sigma n_i d_i}{N}$	Average latent period $\overline{LP}=H_1+\frac{\Sigma n_i d_i}{N}$
	1	2	3	4	Total time				
	Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Percent*	*Days*	*Days*
A	0	0	0	0	0	28	0		
B	1	1	13	4	19	24	79. 17	13. 68	35. 68
C	6	7	9	0	22	24	91. 67	6. 86	28. 86
D	6	8	8	1	23	24	95. 83	7. 26	29. 26
E	4	9	5	4	22	28	78. 57	9. 27	31. 27
F	12	10	1	0	23	23	100. 00	2. 93	24. 93
G	7	10	5	0	22	28	78. 57	5. 34	27. 34
H	1	1	7	0	9	18	50. 00	10. 33	32. 33

[1] This value is not used to calculate the latent period, but will be needed elsewhere.

Total-Latent-Period Method

Although the use of latent periods in the insect has enabled workers to perform assays which otherwise would not have been possible, there are various objections to the method. It is laborious to make even five transfers, as was necessary for the WX agent. Also, the latent-period method necessarily entails making discontinuous observations. This can easily lead to quirks and contradictions when an unfortunate interval is chosen.

With the aster yellows agent, the shorter time scale for incubation periods in plant and insect makes the selection of intervals a critical matter. It has turned out that the total latent period—the latent period in the insect, plus the latent period in the plant—is a function of virus concentration. Actually, this observation was first made with the WX agent (table 4) despite the fact that the lengthy incubation period in the plant, 28 days minimum to 70 days maximum, obscured the results somewhat and augured against routine use. It was predicted that the method should work best with an agent with a short latent period in the plant, especially if that latent period was not greatly affected by the age of the plant. The transmission of the aster yellows disease agent to summer daisy (*Chrysanthemum carinatum* Schousb.), china aster (*Callistephus chinensis* (L.) Nees.), or plantain (*Plantago major* L.) fulfills these requirements.

In two respects, the standard biological operations are modified with this assay. It is necessary to use young males or nymphs sterilized by gamma radiation for injection. If this is done, the small test plants can be held in the controlled environment chamber, and AY symptoms read without removal of the cage, with the insect still present.

The principal steps of the technique are the same as for any graded-response assay: A dilution-curve control is performed, and the responses (in this case, the total latent periods) are plotted graphically (fig. 8). Unknown samples are injected under the same conditions as the control, and subsequent biological operations performed under the same environmental conditions. The standard assumption of bioassay, that unknown aliquots produce responses that bear a similar relationship to concentration as aliquots of the control dilutions, must be made. For example, the dispersity of the agent in the various inocula must be comparable. If there is a steep time-decay curve for infective units, this can alter the "effective concentration" determined. In the case of agents that propagate in leafhoppers, these factors may frequently be operative. Results of two experiments with the AY agent are presented (tables 7, 8; fig. 8) as examples of this method.

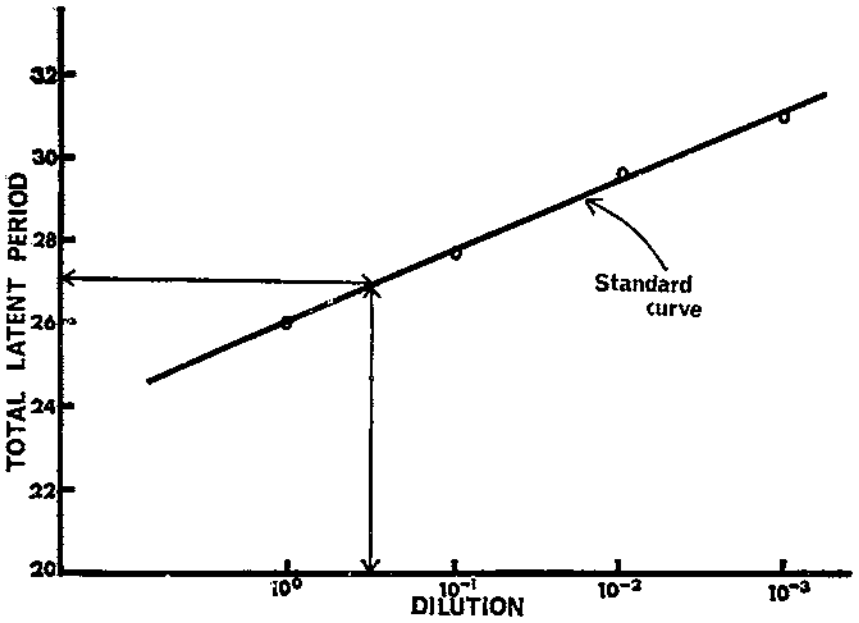

FIGURE 8.—Relationship of total latent period to AY concentration. The line drawn is a standard curve fitted to the points of the dilution curve (table 8). Total latent periods observed after injection of treated extracts can be converted to AY concentration by means of this standard curve.

SUMMARY AND DISCUSSION

Concepts and problems of the bioassay of a leafhopper-borne plant virus—clover wound tumor virus (WTV), and of two "yellows disease" agents—the peach Western X-disease agent (WX) and the agent of aster yellows disease (AY), are discussed. The principal details of assay were found to be similar for both types of agent. Machine production of needles and improved methods for the transfer and maintenance of test insects make such bioassays less laborious and cumbersome than they once were. Interpretative methods now need to be developed to realize the fullest potential of existing bioassay techniques.

Dosage-response curves obtained by injecting disease agents into leafhoppers had problematical interpretations. Although the fraction of infected insects decreased in groups injected with lower concentrations of disease agents, the observed slope of the dosage-response curve was always less than the theoretical unit slope if crude inocula were

TABLE 7.—*Total-latent-period method: Aster yellows transmission to summer daisy*

[Four buffers were tested with the aster yellows agent. Final yield of agent was determined by injecting single concentrations of each, and comparing the total latent period (time in insect and plant before symptoms could be detected) from each with a standard curve whose slope was derived from control dilutions. Only two dilutions were used; it would have been preferable to have had three or four. Graphic interpolation of concentration as in figure 8. Clear differences are asserted between alkaline and acid pH and between buffers C and D]

Material tested	Fraction transmitting	Total latent period	Log_{10} units AY
Dilution of extract:	*Number*	*Days*	
10^{-1}	24/28	29. 4	2
10^{-2}	22/25	33. 9	1
Buffer and pH:			
A(6.1)	22/26	30. 9	1. 5
A(5.5)	37/39	30. 9	1. 5
B(6.1)	25/25	32. 3	1. 25
C(8.0)	38/38	29. 4	2
D(8.0)	35/36	27. 9	3

injected. Reversible complexing and aggregation of the particles of disease agents, as well as variations in susceptibility among the test insects, are likely factors in reduction of the slope of the dosage-response curves in both WX and WTV assays. Other factors, such as interference in the establishment of infection by "background" materials, or by inactive virus particles, may also reduce the slope.

Storage of test insects before injection apparently increases their capacity for acceptance of fluid. The volume of inoculum injected could be deliberately varied by a sevenfold factor. The dosage response was correspondingly affected by such an increase. Variations in the volume of inoculum injected into test insects and failures to detect insects infected by injection were additional sources of error. Such errors, however, should be randomly distributed and should not affect the slope of the dosage-response curve. Further experiments with known dilutions of purified virus are highly desirable, since dosage-response curves provide important clues regarding the nature of the infection process.

Incubation-period data, long overlooked as sources of information about virus concentration, were found to be most adaptable to analysis. Estimation of incubation-period statistics, such as the average

TABLE 8.—*Total-latent-period method: Aster yellows transmission to aster* [1]

Material tested	Time [2]	Fraction transmitting	Total latent period [3]	Log_{10} units AY [4]	Log units AY corrected for time [5]
Dilution of extract:	*Minutes*	*Number*	*Days*		
10^{-1}	0	9/10	12.8	3.0	----------
10^{-2}	20	23/25	15.8	2.0	----------
10^{-3}	40	15/17	18.5	1.0	----------
10^{-4}	150	13/15	14.0	2.5	----------
Treatment:					
1	60	24/30	15.8	2.0	2.2
2	80	13/28	18.5	1.0	1.3
3	95	6/24	18.8	.9	1.3
4	120	0/25	----------	.0	.0

[1] Four treatments of increasing intensity were applied to an AY extract. Analysis as in table 7.

[2] Time between preparation of inoculum and injection.

[3] Time between injection of an insect and first appearance of symptoms on asters.

[4] The value of 1 was arbitrarily assigned to the extract at 10^{-3}.

[5] The AY concentrations were corrected for time by constructing a time-decay curve with the two replicate injections of 10^{-1} inoculum, and assuming that all inocula decayed at the observed rate.

latent period or T_{50} (time at which 50 percent of transmitters were transmitting at least once weekly) for groups of insects, allowed the estimation of concentration of the disease agent in many samples within a single experiment and permitted construction of infectivity-scanning curves for fractions from purification procedures. The best statistical treatment of incubation-period data has not been determined, but several empirical methods are presented as interim procedures until more sophisticated analyses (*18*, *19*, *21*) are available.

Incubation periods in plants, as well as insects, were dosage-dependent, although the dependence in plants was more difficult to demonstrate. This difficulty was attributed to the steep growth curves of disease agents in their vectors, which seemingly assure that most successful transmission events are induced by concentrations of agent greatly in excess of the minimum concentration required to produce the minimum incubation period in the plant. The earliest, or the latest, transmissions by vectors transmitting the WX agent to celery tended

to have longer incubation periods in the plant than other transmissions. Celery, which is poorly susceptible to WX, was an ideal plant for demonstrating fluctuation of the inoculative potential of WX vectors. With more susceptible plants, the threshhold concentration of disease agent for minimum incubation period is quickly reached.

With any given set of environmental conditions, the length of the incubation period of AY and WX infection in plants was independent of the original concentration of virus originally injected into the vector. This made it possible to discern between incubation periods in insects receiving different virus concentrations as follows:

$$^{l}\text{total} = {}^{l}\text{insect} + {}^{l}\text{plant}$$

where ^{l}plant was a constant mean value with constant variance. In the case of WX, this total-latent-period method was difficult to employ because the incubation period in celery, 26 to 50 days, was accompanied by high variability. However, development of AY symptoms in aster, plantain, or summer daisy required only 6 to 18 days, and was less variable. Thus, in total-latent-period assay, transfers were usually not required to determine the incubation period, only one dilution needed to be injected with each inoculum, and fewer insects were required for the single injection, whereas a minimum of five transfers were required to determine the latent periods in *C. montanus* before transmission of the WX agent.

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