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Myron K. Brakke
United States Department of Agriculture

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SYSTEMIC INFECTIONS FOR THE ASSAY OF PLANT VIRUSES[1]

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Myron K. Brakke

Crops Research Division, Agricultural Research Service, United States Department of Agriculture, Nebraska Agricultural Experiment Station, Lincoln

HISTORY

The first infectivity assays of plant viruses relied on the percentage of inoculated plants becoming systemically infected (22, 31). The use of systemic infections for assays became unfashionable, however, after the discovery of local lesions and the demonstration that their numbers depended on virus concentration (23). Continued emphasis on local lesions over the years led to the widely held belief that systemic infections could not be used for a reliable and accurate assay of plant viruses. There was never any good statistical evidence for this viewpoint. In fact, no thorough investigation has been published of the accuracy of "systemic assays" of plant viruses, as I shall term those assays based on the development of systemic symptoms in inoculated plants.

McKinney (31) successfully used systemic assays in an early investigation of the properties of tobacco mosaic virus. He inoculated plants by pin pricks through a wad of cotton soaked in inoculum and wedged in a leaf axil. This was apparently an efficient inoculation procedure because it always gave 100% infection with an undiluted extract from infected tobacco, and usually gave 100% with a 1:1000 dilution. McKinney made no attempt to analyze his results statistically to obtain an estimate of the virus concentration and of the error, but he did use dilution curves and recognized the principle of obtaining relative virus concentrations from dilutions giving identical results. Recalculation of his results shows that the percentage of infection decreased with dilution, as expected for a Poisson distribution. McKinney also stated the following seven necessary conditions for an infectivity assay, conditions which still hold for both systemic and local lesion assays. 1. The virus must lend itself to study in expressed fluids without losing its potency in a short time or upon dilution. 2. The plant must be easily grown throughout the year. 3. The plant must be uniformly susceptible and the symptoms definite. 4. The plants for a single experiment must be

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grown under the same conditions. 5. Growing conditions for all experiments must be standardized. 6. Accidental infection must be guarded against. 7. The inoculation technique must be uniform and efficient. Interestingly, this paper also contains the first report of the partial purification of a plant virus by centrifugation.

The accuracy of systemic assays for plant viruses was first investigated by Holmes (22), who concluded that 1350 plants would be needed to detect a twofold difference in concentration between two preparations. The theory and practice of bioassays in general were poorly developed at the time. Many of the techniques for working with plant viruses were also unsatisfactory. For example, Holmes inoculated tobacco plants with tobacco mosaic virus with an apparently inefficient pin-pricking method that never resulted in 100% infection. The percentage of infected plants changed more slowly with virus concentration than expected from a Poisson distribution. This flat dilution curve, coupled with the fact that Holmes calculated the number of plants needed for statistical significance at the 1% level, led to the high figure of 1350 plants. Recent results with cereal viruses indicate that 120–500 plants are needed to detect a two-fold difference between two preparations with statistical significance at the 5% level. The number of plants required depends on the dilutions inoculated, and percentage infection. Holmes soon improved the efficiency of his inoculation procedure by using the leaf-rubbing method, but at the same time realized the value of local lesions for assay. His early estimate of the accuracy of systemic assays was the only one in the literature for many years.

Many reports have appeared on statistical analyses of local lesions to assess the accuracy of their use for assays, and to test the goodness of fit of the number of lesions to that expected on the basis of various models, such as the Poisson distribution (4, 25, 28, 46). Bald (4) also showed that the percentage of plants systemically infected after inoculation with different dilutions followed the Poisson distribution in some cases.

Systemic assays have been used relatively frequently in recent years, but usually raw data have been reported without statistical analysis, or even interpretation as relative virus concentration. Brakke et al (10) reported dilution curves for wound tumor virus and plotted the logarithms of the percentage infection against the logarithm of the dilution. Brakke (6–8) used the maximum likelihood calculation of Finney (14) and the loglog transformation to interpret systemic assays of cereal viruses, while Pring & Timian (34) used the loglog transformation and a graphical method. Tu & Ford (41) reported systemic assays of maize dwarf mosaic virus interpreted with the arcsin transformation. Raymer & Diener (35) used systemic assays with potato spindle tuber virus, which is apparently a free nucleic acid, and Semancik & Weathers (39) used a similar assay for an apparently similar virus that causes exocortis of citrus. Lastra & Munz (27) calculated ID_{50} for assays of squash mosaic virus by the Reed-Muench method. Systemic assays have probably been used most frequently for cereal viruses and for insect-transmitted viruses. Whitcomb (42) has recently reviewed the bioassay of the latter class of viruses.

INTERPRETATION OF INFECTIVITY ASSAYS

The number of lesions or percentage of infected plants depends on the susceptibility of the host, efficiency of the inoculation procedure, concentration of inhibitors, specific infectivity of the virus, concentration of virus, and perhaps other factors. With proper experimental design, the number of lesions or infections could be used to measure any of these variables. However, I shall consider only the measurement of the concentration, or relative concentrations, of infectious virus particles. It will be assumed that the other variables are kept constant.

If an infectivity assay is to measure virus concentration, then the plant must respond differently to inoculation with different concentrations of virus. The response may be numbers of lesions, percentage of infected plants, or time for symptoms to appear. The relative concentration of two preparations is determined by finding the dilutions of the two preparations that give the same response, and not by comparing the response at equal dilutions. The relative concentration can be determined from the response at equal dilutions only if the change in response with dilution is known for both preparations.

In practice, it is only by luck that one would inoculate dilutions of two preparations giving the same response. It is almost always necessary to interpolate (or extrapolate) from the experimental results to determine the dilutions that would have given the same response. The interpolation is easier if a mathematical function, or transformation, of the response can be found that is a linear function of the dilution, or of a transformation of the dilution. The interpolation can be done by either a graphical or algebraic method. A graphical plot is easier to do and understand. It usually gives a satisfactory estimate of the concentration of virus and of systematic deviations from the model. However, it does not give a good estimate of the error nor a statistical estimate of goodness of fit.

An algebraic or statistical method is basically the same as a graphical method, but with the positions of the lines being calculated by formulae. An algebraic method should give estimates of the concentration of virus, the error, and the goodness of fit of the data to the model.

Several transformations may be used with systemic assays. If the data follow the Poisson distribution, the logarithm of the fraction or percentage of noninfected plants should be a linear function of the dilution with a slope that depends on the virus concentration. The loglog transformation also depends on the Poisson distribution. The loglog of the reciprocal of the fraction of healthy plants should be a linear function of the log of the dilution with a slope of one. The probit, angle, and logit tranformations should be linear functions of the log of the dilution. Finney (14) gives procedures for use of all these with bioassays with various organisms. However, the procedures can be used with plant viruses.

Many of these methods give similar results (e.g. 19). Table 1 gives results of several procedures applied to three systemic assays of barley stripe mosaic virus. In this experiment, A and B were two dilution series of the

same preparation, and C was a dilution series based on a twofold dilution of A and B. The probit calculation was done according to Finney (14) and gives the relative concentrations of two preparations. The relative concentration in this case is expressed as the difference between the logarithms of the virus concentration of two preparations, which is the logarithm of the ratio of virus concentration in one preparation to that in another. The Reed-Muench method (14) gives an estimate of the concentration infecting 50% of the plants, and the other methods, of the concentration infecting 63% of the plants. Except for the graphical analysis of A, all methods gave similar estimates of the virus concentration. Because of the one plant infected at $10^{-4.5}$ in series A, the graphical method gave a high figure.

The inadvisability of using the dilution endpoint is well illustrated by the data of Table 1. In series A, one plant was infected at a dilution of $10^{-4.5}$. Thus, according to the dilution endpoint, A had more than 10 times as much virus as B. There is a large sampling error associated with the small number of plants infected at the dilution endpoint, and the dilution endpoint has a correspondingly large error.

The convenient nonparametric methods such as the Reed-Muench and Spearman-Karber methods can give reliable results in some cases, but can be used only with assays spanning the full range from zero to 100% infection. Finney (14) gives qualified support to the Spearman-Karber, but not to the Reed-Muench method.

SAMPLING ERROR

A systemic assay would be highly accurate if an infinite number of plants could be inoculated, but in practice the number is always limited. The error due to the limited numbers is a sampling error that can be calculated from the binomial distribution. Suppose that virus concentration and other conditions were such that, if an infinite number of plants could be inoculated, exactly 10% would become infected. If one selected 30 plants from the infinite number, the probability of obtaining exactly three infected ones would be the same as the probability of obtaining exactly three infected plants if only 30 plants were inoculated.

The sampling error must particularly be kept in mind when dealing with small numbers of plants. For example, the difference from 1 infected plant out of 4 inoculated to 4 infected out of 4 is not highly significant, even though it is a difference of 25 to 100%. Regardless of the number of plants inoculated, a difference of 3 infected plants is not highly significant statistically. Faith should be placed in such small differences only if they can be repeated often enough to assume statistical significance.

If n plants are inoculated, and r plants become infected, the standard deviation of r/n is $P(1\text{-}P)/n$, where P is the probability of a plant becoming infected. If n were very large, then $P=r/n$. The variance of r does not depend on the manner in which r/n varies with virus concentration, nor on the degree of heterogeneity of the susceptibility of the host population. However,

TABLE 1. Concentration of virus calculated by different methods for three assays of barley stripe mosaic virus

Experimental results[a]					
A		B		C	
Dilution	r/n[b]	Dilution	r/n	Dilution	r/n
$10^{-1.5}$	31/31	$10^{-1.8}$	33/37	$10^{-1.6}$	30/34
$10^{-2.0}$	29/37	$10^{-2.3}$	25/32	$10^{-2.1}$	20/30
$10^{-2.5}$	15/34	$10^{-2.8}$	6/28	$10^{-2.6}$	6/32
$10^{-3.0}$	10/35	$10^{-3.3}$	3/26	$10^{-3.1}$	2/33
$10^{-3.5}$	2/31	$10^{-3.8}$	0/30	$10^{-3.6}$	2/30
$10^{-4.0}$	0/32			$10^{-4.1}$	0/26
$10^{-4.5}$	1/34				

Method	Estimated virus concentration A	B	C
$\overline{\text{Log } u}$ by loglog transformation and maximum likelihood.	2.26 ± 0.06[c]	2.28 ± 0.06	2.00 ± 0.06
$\overline{\text{Log } u}$ by weighted average	2.26 ± 0.06	2.30 ± 0.06	2.00 ± 0.06
$\overline{\text{Log } u}$ by graph of loglog	2.35 ± 0.18[d]	2.30 ± 0.19[d]	2.00 ± 0.19[d]
$\bar{u}$, weighted average	188 ± 16		
ID_{50}, dilution infecting 50% of plants, by Reed Muench	$10^{-2.50}$	$10^{-2.55}$	$10^{-2.22}$
Concentration relative to solution C, by probits	0.29 ± 0.20[e]	0.27 ± 0.20[e]	

[a] A and B were two different dilution series of the same preparation of virus. C was a series based on a 1:2 dilution of the preparation used for A and B. centration of virus in C was half that in A and B. The difference in the logarithms of the concentrations of A or B and C should be 0.30.

[b] The denominator, n, is the number of inoculated plants, and the numerator, r, is the number of infected plants.

[c] The figures after the $\pm$ sign are standard deviations based on sampling error.

[d] The figures after the $\pm$ sign are the smallest 95% confidence intervals of the points plotted.

[e] The 95% confidence interval of the virus concentration relative to that in C.

TABLE 2. Symbols and definitions

log	Logarithm to the base 10
ln	Naperian logarithms, to the base e
n_i	Number of plants inoculated with dilution i
r_i	Number of plants infected after inoculation with dilution i
p_i	r_i/n_i, fraction of inoculated plants becoming infected
q_i	$1-p_i=n_i-r_i/n_i$, fraction of inoculated plants remaining healthy
s	Experimental estimate of standard deviation
s^2	Experimental estimate of variance
u_i	Experimental estimate of number of virus particles in the undiluted preparation, obtained from results of inoculating dilution i
$\bar{u}$	Average value of u
w_i	Weighting coefficient for dilution i
z_i	Amount by which a preparation has been diluted to give dilution i. Dilution i has uz_i particles
P_i	Probability of an inoculated plant becoming infected. If an infinite number of plants were inoculated, $P_i=r_i/n_i$
Q_i	$I-P_i$
U	Actual number of virus particles. u is an experimental estimate of U
σ	Standard deviation based on sampling error and binomial distribution
σ^2	Variance based on sampling error and binomial distribution
$\sigma^2_{r_i}$	$n_iP_iQ_i$, variance of r_i
$\sigma^2_{p_i}=\sigma^2_{q_i}=P_iQ_i/n_i$,	variance of p_i and q_i
$\sigma^2_{\ln q_i}=P_i/n_iQ_i$,	variance of ln q_i
$\sigma^2_{u_iz_i}=P_i/n_iQ_i$,	variance of u_iz_i
$\sigma^2_{u_i}=P_i/n_iQ_iZ_i^2$,	variance of u_i
$\sigma^2_{\log \ln 1/q_i}=\sigma^2_{\log u_iz_i}=\sigma^2_{\log u_i}=P_i/(2.3)^2\ n_iQ_i(\ln Q_i)^2$	
$f(x)=e^{-u}u^x/x!$	Poisson distribution, where $f(x)$ is probability of finding x particles when the mean is u.

the plants inoculated with each dilution must be a random sample of the population. In statistical interpretation of systemic assays, it is customary to weight the value of r/n for each dilution with the reciprocal of the sampling variance. If a transform of r/n is used, the variance of the transform must be used. Some of these are given in Table 2.

The true value of P is never known in an assay. In its absence, the best experimental estimate should be used. The best estimate is not obtained from the results of one dilution, but from considering the results of all dilutions. In effect, one makes a first estimate of the virus concentration, and on the basis of this estimate calculates an expected value, p, of P for each dilu-

tion and takes the weight appropriate to the expected value of P. The first estimate need not be highly accurate, but can usually be a simple average of the points known to have the most weight, or it can be obtained by a graphical procedure. If the second estimate differs considerably from the first, a third estimate of the concentration can be made by using weights from the second estimate.

The variance of the estimated mean value of the concentration is usually taken as the reciprocal of the sum of the weights of the individual points. This value of the variance is derived from the sampling error. It is a true estimate only if all the plants belong to the same population, if plants inoculated with each dilution are a random sample of the population, if the inoculation procedure was uniform throughout, and if the percentage of infected plants changes with dilution as assumed in the statistical model. If these conditions do not hold, the variance could be larger. For example, if the barley plants to be inoculated with one of a series of dilutions of barley stripe mosaic virus are wilted when inoculated, or vary in size, or are diseased with a root rot, they are not a random sample of the population and the resulting variance may be high. Or, if the statistical model assumes that the percentage of infection follows the Poisson distribution, whereas in fact the results deviate at a high percentage of infection because of host heterogeneity, then the actual variance of the mean value could be higher than that due to sampling error.

An experimental estimate of the variance can be obtained by considering the results from each dilution to constitute a separate estimate of the virus concentration. The deviation of these estimates from the mean estimate, obtained by considering all the dilutions, can be used in the usual way to calculate an experimental variance. The ratio of this variance to that derived from sampling error should have a chi square distribution. Unfortunately, both the estimates of the experimental variance and of chi square are usually based on very few points and can not be highly accurate. Nevertheless, if such an estimate of chi square is higher than expected at the 5% level, the estimate of the virus concentration from the assay is questionable.

THE POISSON DISTRIBUTION

The Poisson probability distribution has been used in the interpretation of counts of bacterial colonies, animal virus plaques, percentage of inoculated broth tubes showing bacterial growth, and percentage of inoculated animals infected with virus (2, 15, 20, 21, 32, 36). The Poisson distribution may also be applied to the interpretation of the systemic assays of plant viruses, which are similar statistically to these other assays. Many computational procedures developed for other assays can be used for systemic assays of plant viruses, and theoretical considerations, such as those for the effect of host heterogeneity, developed for assays with animals, should apply to those with plants.

According to the Poisson distribution, the fraction of plants remaining healthy, q, is e^{-uz} after inoculation with a solution containing uz particles per unit volume (Table 2). If $q = e^{-uz}$, then

$$\ln q = -uz \quad \text{or} \quad 2.3 \log q = -uz \qquad 1.$$

and

$$\log \ln 1/q = \log uz \qquad 2.$$

Suppose a 1:100 dilution of a virus preparation infects 14 out of 30 inoculated plants. Then the experimental estimate of UZ is $uz = 2.3 \log 30/16$ or 0.40 virus particles per unit volume. The size of this volume is not known, but it may be termed the "infection-initiating volume" (6). By definition, if the plant is to become infected, this volume must contain at least one virus particle that can develop into an infection under the conditions of the experiment. The size of this volume depends on host susceptibility and the inoculation procedure. The virus concentration determined by systemic assay is relative because the size of the "infection-initiating volume" is unknown. As with other infectivity assays, concentrations to be compared should be determined at the same time under the same conditions.

Several experimental estimates of q are made by inoculating plants with several dilutions, z, of virus. The problem is to obtain an estimate of U from the experimental values of q. Several methods are available, some of which have been reviewed (11, 14).

Let us consider first some methods based on equation 1. If $-\log q$ (or $\log 1/q$) is plotted against the dilution, z, a straight line through the origin should result with slope u. One difficulty with this plot is that z will be plotted on an arithmetic scale, whereas one usually inoculates a geometric series. The points will therefore be clustered near the origin. Suppose one inoculates dilutions of 1:10, 1:100, 1:1000 and 1:10,000. These points will be plotted at distances of 0.1, 0.01, 0.001, and 0.0001 units. Three of the points, 0.0001, 0.001 and 0.01, will be in the first 10% of the line and the point for 0.1 will be alone at the end of the line. If a line is drawn by eye, the slope will be determined almost exclusively by the point at 0.1, and the information in the other points will tend to be wasted.

The position of this line could be calculated algebraically with proper weighting of the points. One can also consider the value from each dilution as a separate assay and can then obtain an average of the results. For example, consider assay A of Table 1. From Table 3, we find that there was an estimated 1.51, 0.58, 0.34, 0.06, and 0.03 particles at dilutions of $10^{-2.0}$, $10^{-2.5}$, $10^{-3.0}$, $10^{-3.5}$, and $10^{-4.5}$, respectively. Multiplying these figures by the dilution factors ($1/z = 100$, 316, 1000, 3160, and 31,600, respectively) we obtain estimates of 151, 183, 340, 190, and 950 particles, respectively, from the five dilutions for the concentration of virus, u, in the original preparation. A simple average gives an estimate of 363 particles per infection-

TABLE 3. Number of particles (uz) per "infection-initiating volume," log uz, and weighting coefficients corresponding to percentage of inoculated plants becoming infected[a]

$100p$	uz	log uz	w	$1.96/\sigma\sqrt{n}$	$100p$	uz	log uz	w	$1.96/\sigma\sqrt{n}$
0.1		−3.004	0.005		31	0.371	−0.431	1.625	1.56
0.2		−2.698	0.011		32	0.386	−0.414	1.676	1.54
0.5		−2.300	0.027		33	0.401	−0.397	1.727	1.52
1	0.01	−1.998	0.053	8.72	34	0.416	−0.381	1.777	1.50
2	0.02	−1.695	0.106	6.20	35	0.431	−0.366	1.827	1.48
3	0.03	−1.516	0.159	5.08	36	0.446	−0.351	1.875	1.46
4	0.041	−1.389	0.212	4.33	37	0.462	−0.335	1.927	1.44
5	0.051	−1.290	0.265	3.90	38	0.478	−0.321	1.977	1.42
6	0.062	−1.208	0.318	3.53	39	0.494	−0.306	2.026	1.40
7	0.073	−1.139	0.373	3.27	40	0.511	−0.292	2.075	1.39
8	0.083	−1.079	0.424	3.08	41	0.528	−0.278	2.124	1.37
9	0.094	−1.025	0.477	2.91	42	0.545	−0.264	2.173	1.35
10	0.105	−0.977	0.530	2.75	43	0.562	−0.250	2.221	1.34
11	0.116	−0.934	0.583	2.61	44	0.580	−0.237	2.269	1.33
12	0.128	−0.893	0.635	2.50	45	0.598	−0.223	2.316	1.31
13	0.139	−0.856	0.688	2.41	46	0.616	−0.210	2.363	1.30
14	0.151	−0.822	0.741	2.32	47	0.635	−0.197	2.410	1.28
15	0.162	−0.789	0.794	2.24	48	0.654	−0.184	2.456	1.27
16	0.174	−0.758	0.846	2.17	49	0.673	−0.172	2.502	1.26
17	0.186	−0.730	0.899	2.11	50	0.693	−0.159	2.547	1.25
18	0.199	−0.702	0.951	2.05	51	0.713	−0.147	2.592	1.24
19	0.211	−0.676	1.004	1.99	52	0.734	−0.134	2.637	1.23
20	0.223	−0.651	1.056	1.94	53	0.755	−0.122	2.680	1.22
21	0.236	−0.628	1.109	1.89	54	0.777	−0.110	2.724	1.21
22	0.248	−0.605	1.161	1.86	55	0.799	−0.098	2.766	1.20
23	0.261	−0.582	1.213	1.82	56	0.821	−0.086	2.808	1.19
24	0.274	−0.562	1.265	1.78	57	0.844	−0.074	2.849	1.18
25	0.288	−0.541	1.316	1.74	58	0.868	−0.062	2.889	1.17
26	0.301	−0.521	1.368	1.71	59	0.892	−0.050	2.929	1.17
27	0.315	−0.502	1.420	1.67	60	0.916	−0.038	2.968	1.16
28	0.328	−0.483	1.471	1.64	61	0.942	−0.026	3.006	1.15
29	0.342	−0.465	1.523	1.62	62	0.968	−0.014	3.043	1.15
30	0.357	−0.447	1.574	1.59	63	0.993	−0.003	3.078	1.14

TABLE 3. *(Continued)*

100p	uz	log uz	w	$\frac{1.96}{\sigma\sqrt{n}}$	100p	uz	log uz	w	$\frac{1.96}{\sigma\sqrt{n}}$
64	1.02	0.009	3.113	1.13	82	1.71	0.234	3.422	1.00
65	1.05	0.021	3.147	1.12	83	1.77	0.248	3.410	1.08
66	1.08	0.032	3.179	1.12	84	1.83	0.263	3.392	1.09
67	1.11	0.045	3.210	1.11	85	1.90	0.278	3.368	1.09
68	1.14	0.057	3.240	1.11	86	1.97	0.294	3.336	1.10
69	1.17	0.069	3.267	1.11	87	2.04	0.310	3.298	1.10
70	1.20	0.081	3.294	1.10	88	2.12	0.326	3.250	1.11
71	1.24	0.093	3.318	1.10	89	2.21	0.344	3.193	1.12
72	1.27	0.105	3.341	1.09	90	2.30	0.362	3.124	1.13
73	1.31	0.117	3.362	1.09	91	2.41	0.382	3.041	1.14
74	1.35	0.129	3.380	1.09	92	2.53	0.402	2.941	1.16
75	1.39	0.142	3.397	1.09	93	2.66	0.425	2.822	1.19
76	1.43	0.154	3.409	1.08	94	2.81	0.449	2.679	1.21
77	1.47	0.167	3.421	1.08	95	3.00	0.476	2.504	1.27
78	1.51	0.180	3.428	1.08	96	3.22	0.508	2.289	1.32
79	1.56	0.193	3.433	1.08	97	3.51	0.545	2.016	1.41
80	1.61	0.207	3.434	1.08	98	3.91	0.592	1.656	1.55
81	1.66	0.220	3.430	1.08	99	4.61	0.663	1.136	1.88

[a] The symbols have the following meanings:

p = the fraction of inoculated plants that become infected. 100p is the percentage infection.

uz = the number of infectious particles per infection-initiating volume; since log uz = log 2.3 log $1/q$, where $q = 1 - p$, the figures in the log uz column may be used for the loglog function to plot against the log of the dilution.

w = the weighting coefficient for log uz, $w = (2.3)^4 Q(\log Q)^2/P$. w should be multiplied by n, the number of plants inoculated, to give nw, which is the reciprocal of the sampling variance, and is the weight for the results obtained at that dilution.

1.96σ = The 95% confidence interval of the corresponding log uz, or of log ln $1/q$. The figures given must be divided by $n^{1/2}$, where n is the number of plants inoculated, to give 1.96σ.

initiating volume in the original preparation. The high figure of 950 was obtained from the infection of a single plant, and a simple average gives much more weight to this estimate than is justified.

According to Cornell & Speckman (11) an unweighted least squares estimate of the concentration is given by

$$\bar{u}_{ls} = \frac{\sum_i z_i(-\ln q_i)}{\sum_i z_i^2} = \frac{\sum_i u_i z_i^2}{\sum_i z_i^2} \qquad 3.$$

A weighted average may be obtained by multiplying each estimate, u_i, by the reciprocal of the corresponding sampling variance. From Table 2, the sampling variance for u_i is $P_i/nQ_iZ_i^2$ and the weight would be $nQ_iZ_i^2/P_i$. The weighted average would be

$$\bar{u}_{wls} = \frac{\sum_i n_i Q_i z_i^2 u_i/P_i}{\sum_i n_i Q_i z_i^2/P_i} \qquad 4.$$

which, according to Cornell & Speckman (11), is a weighted least squares estimate of $\bar{u}$. The symbols Q and P are used to signify that the weight should be based on the probability of a plant being healthy or diseased. Since Q and P are not known, an experimental estimate has to be used, but that estimate should be the best available, which would be one based on a preliminary estimate of the average, $\bar{u}$. The variance of the weighted average estimate of $\bar{u}$ would be the reciprocal of the sum of the weights,

$$1/\sum_i n_i Q_i z_i^2/P_i$$

This would be a variance based on the sampling error; an experimental estimate of the variance based on the differences between the average estimate of $\bar{u}$, and the individual estimates, u_i, could also be obtained.

The weighted average estimate of the virus concentration of preparation A, Table 1, obtained by application of equation 4, is 188 ± 26, considerably less than the simple average of 363. The least squares estimate of $\bar{u}$ (by equation 3) is 155. In this case, the estimate of 188 is closer to the other estimates reported in Table 1 for preparation A than either 155 or 363. However, Cornell & Speckman (11) found that the least squares estimate (equation 3) gave a less biased result than the weighted least squares (equation 4) estimate for some assays they investigated.

Estimations of $\overline{\log u}$ based on equation 2 have some advantages over those of $\bar{u}$ based on equation 1. Log u is more apt to be normally distributed than is $\bar{u}$. A plot of log ln $1/q$ against log z gives a straight line with a slope of one. Points for a geometric progression series are evenly spaced along the line (Figure 1). If log z is read at log ln $1/q = 0$, then $\overline{\log u} = -\log z$. This is the dilution that should infect 63% of the plants or ID_{63}. An ID_{50} could be obtained from the point where the line crosses log ln $1/q = 0.16$, which corresponds to 50% infection.

This graphical estimation of $\overline{\log u}$ agrees well with those made by more elaborate methods if the line is drawn carefully and with some attention to

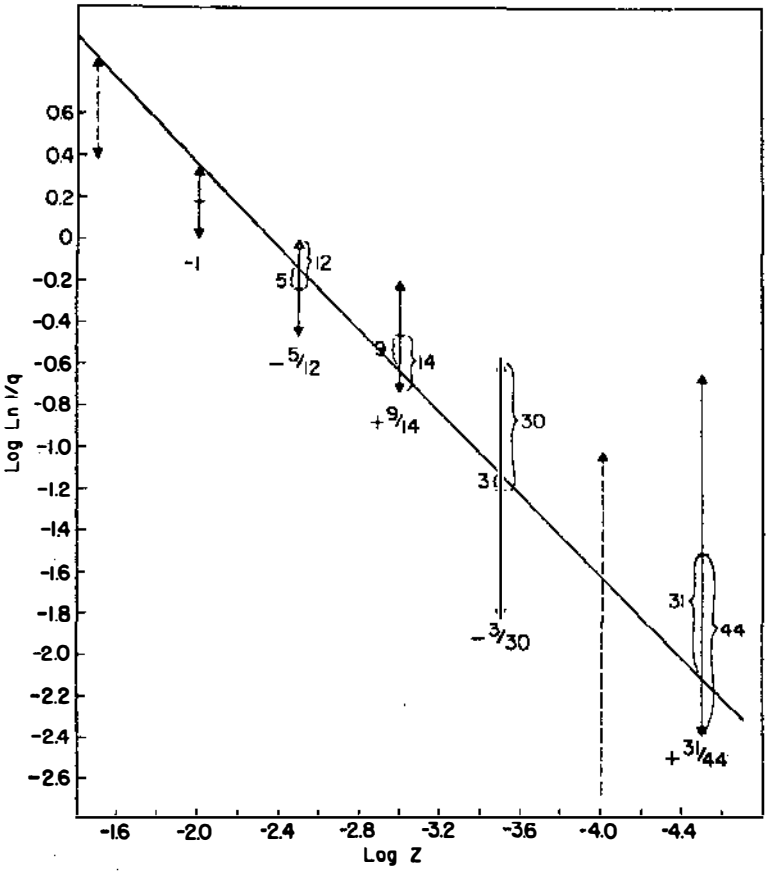

FIGURE 1. Graphical analysis of an assay of barley stripe mosaic virus in barley. The data for the assay are given in Table 4. Log ln $1/q$ (column 5) is plotted against the log of the dilution (column 2), the points being indicated by a short horizontal line. A vertical line equal in length to the 95% confidence interval (column 7) is drawn through each point. A line of slope -1 is drawn through the points in such a way as to intersect all the confidence intervals. If possible, the same number of points should be above and below the line. As a further help in selecting the position of the line, one can add the distances to the line of the points above and below the line, with distances being measured in confidence interval units. The point at log $z=-2$ is 1 confidence interval below the line (-1). The point at log $z=-2.5$ is 5/12 of confidence interval below the line (-0.41) and the point at log $z=-3.5$ is 3/30 below (-0.10). The point at log $z=-3.0$ is 9/14 of a confidence interval above ($+0.64$) and that at log $z=-4.5$ is 31/43 ($+0.72$) above. The sum of the distances below the line is -1.51, and above is 1.36. The line should be moved down a slight amount. It now crosses the line at log $uz=0$ at 2.36. Since the line is slightly too high, we'll make the estimate 2.35. That is, at a dilution of $10^{-2.35}$, 63% of the plants should have been infected, or there was one particle of virus per infection-initiating volume. Therefore, there must have been $10^{2.35}$ particles per infection-initiating volume in the undiluted preparation, or log $u=2.35$.

The dilutions with 100% or 0% infected plants are indicated by dashed lines showing upper and lower limits calculated as described in the text.

TABLE 4. Data for plotting the loglog function for graphical estimation of virus concentration

1	2	3	4	5	6	7
dilution, z	Log z	r/n[a]	p[b]	log ln $1/q$[c]	$2\sqrt{n\sigma}$[f]	2σ
$10^{-1.5}=1/31.6$	−1.5	31/31	1	> 0.38[d]		
$10^{-2}=1/100$	−2.0	29/37	0.78	+0.18	1.08	0.18
$10^{-2.5}=1/316$	−2.5	15/34	0.44	−0.24	1.33	0.23
$10^{-3.0}=1/1000$	−3.0	10/35	0.29	−0.46	1.62	0.27
$10^{-3.5}=1/3162$	−3.5	2/31	0.06	−1.21	3.53	0.62
$10^{-4}=1/10{,}000$	−4.0	0/32	0.0	< −1.03[e]		
$10^{-4.5}=1/31{,}620$	−4.5	1/34	0.03	−1.52	5.08	0.87

[a] n is the number of plants inoculated and r is the number infected.
[b] p is the fraction of plants infected, equals r/n.
[c] log ln $1/q$ equals log uz and is obtained from Table 3.
[d] There is a 95% probability that log ln $1/q$ is greater than log 2.38 = 0.38.
[e] There is a 95% probability that log ln $1/q$ is less than 0.477-log 32 = −1.03.
[f] σ is the standard deviation of log ln $1/q$ and 2σ is approximately the 95% confidence interval. Values of $n^{1/2}2\sigma$ are from Table 3.

weighting the points. Values of the log ln function and of the corresponding standard deviations are given in Table 4. The line in Figure 1 is a plot of assay A of Table 1 and is drawn to minimize the distance of the points from the line with distances measured in standard deviation units. If the line can be drawn to pass through all the 95% confidence intervals, then the goodness of fit is probably satisfactory. The smallest confidence interval of the individual points can be used for the confidence interval of the average—it should be an overestimate. Small systematic deviations of the results from the Poisson distribution can be judged by inspection of the plots of several assays. For example, if the points at high percentages of infection consistently fall below the line, one might suspect host heterogeneity. None of these graphical procedures gives the best estimate from a statistical viewpoint, but they are simple and quite reliable.

Another simple procedure would be to calculate an unweighted average of log u. This would be equivalent to drawing a separate line for each point and obtaining an estimate of log u from each line. However, since the line has a slope of one, the estimate of log u can be obtained simply by subtracting log z from log ln $1/q$ = log uz. The first five columns of Table 5 would suffice. A simple average for assay A in this case would be 2.45, which is higher than most of the estimates in Table 2, but not as high as the simple arithmetic average of u was.

The calculation of the weighted average of log u for assay A of Table 1 is shown in Table 5. Values of log uz = log ln $1/q$ and the weighting coefficient were obtained from Table 3. The weight is the reciprocal of the sampling

TABLE 5. Calculation of weighted average of log u for an assay of barley stripe mosaic virus on barley

1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9	10	11
log z	r/n	p	log uz	log u	expected log uz	w	nw	nw log u	log u-$\overline{\log u}$	$nw(\log u - \overline{\log u})^2$
−1.5	31/31	1								
−2.0	29/37	0.78	+0.18	2.18	0.25	3.41	126.2	275.0	−0.08	0.81
−2.5	15/34	0.44	−0.24	2.26	−0.25	2.22	75.5	170.6	00	00
−3.0	10/35	0.29	−0.46	2.54	−0.75	0.84	29.4	74.7	+0.28	2.30
−3.5	2/31	0.06	−1.21	2.29	−1.25	0.29	9.0	20.6	0.01	0.00
−4.0	0/32	00								
−4.5	1/34	0.03	−1.52	2.98	−2.25	0.03	1.0	3.0	+ .76	.59
			SUMS				241.1	543.9		3.70

[a] The dilution is z, and log z is the log of the dilution. If the dilution is $10^{-1.5}$ or 1:31.6, $z = 1/31.6$, and $\log z = \log 1/31.1 = \log 1 - \log 31.6 = -\log 31.6 = -1.5$.

[b] r is the number of infected plants and n is the number inoculated.

[c] p is the fraction infected and equals r/n.

[d] uz is the number of infectious particles per infection-initiating volume at the dilution z. $\log u = \log(2.3 \log 1/1-p)$ and is obtained from Table 3.

[e] $\log u = \log uz - \log z$. For example at a dilution of $10^{-2.5}$, $\log u = -0.24 - (-2.5) = 2.5 - 0.24 = 2.26$.

[f] *An inspection of the data suggests the average of log u should be about 2.25, if little weight is given to the* one plant infected at $10^{-4.5}$. If 2.25 is taken as an estimate of $\overline{\log u}$, then the expected $\log uz = \log z + 2.25$.

[g] Weighting coefficient, w, from Table 3, corresponding to expected values of log uz.

[h] The weighting coefficient, w, from column 7 multiplied by the number of inoculated plants from column 2.

Average of log u is $\overline{\log u} = 543.9/241.1 \pm 1/(241.1)^{\frac{1}{2}} = 2.26 \pm 0.064$. Chi square is 3.70 with 4 degrees of freedom and is not significant. If the 10^{-2} dilution is omitted, $\overline{\log u} = 2.35 \pm 0.085$, and $\chi^2 = 2.49$ with 3 d.f.

variance. Since this depends on the number of plants inoculated, it can not be tabulated conveniently. The weighting coefficient given in Table 3 must be multiplied by the number of plants inoculated for that dilution. As discussed earlier, the weighting coefficient should be calculated using the probability of infection P. Since this is unknown, an experimental estimate must be made. In the example of Table 5, weights were based on an estimated value of $\overline{\log u}$ of 2.25. The results would have been almost the same if weights had been based on $\overline{\log u} = 2.30$ or even 2.35.

The variance of the weighted average is the reciprocal of the sum of the weights, $1/\sum_i n_i w_i$. This estimate of the variance is based on sampling error. Another estimate of the variance, s^2, may be calculated from the deviations of the individual points from the average. These deviations should be squared and weighted before being added.

$$s^2 = \frac{\sum_i n_i w_i (\log u_i - \overline{\log u})^2}{\sum_i n_i w_i} \qquad 5.$$

If s^2 is divided by the first estimate of the variance, $1/\sum_i n_i w_i$, an estimate of χ^2 is obtained.

$$\chi^2 = \sum_i n_i w_i (\log u_i - \overline{\log u})^2 \qquad 6.$$

An alternate form of equation 6 is

$$\chi^2 = \sum_i n_i w_i (\log u_i)^2 - \frac{\left(\sum_i n_i w_i \log u_i\right)^2}{\sum_i n_i w_i} \qquad 7.$$

A slide rule was used for the multiplications in Table 5, and equation 6 was used for the calculation of χ^2. Use of equation 7 involves subtraction of large numbers and a slide rule is not sufficiently accurate.

Finney (14) has given a maximum likelihood method for calculating concentrations of particles from quantal assays such as systemic assays of plant viruses. A few comparisons have been made between results with this calculation and a weighted average as demonstrated in Table 5. The results of the two methods were similar (Table 1). The maximum likelihood method of Finney gives some weight to dilutions infecting none or all of the plants, while the weighted average does not. The maximum likelihood method is probably preferable on a theoretical basis. In practice, the maximum likelihood calculation of Finney (14) is only slightly more work than the weighted average of Table 5, but is more difficult to understand.

NO INFECTED PLANTS OR NO UNINFECTED ONES

If no plants are infected among those inoculated with a dilution, z, one cannot estimate the virus concentration, but one can estimate the upper limit of virus concentration if it is assumed that the assay follows the Poisson distribution. Let Y be the probability of a plant remaining healthy. If an infinite number of plants were inoculated, Y would be the fraction healthy. If n plants were inoculated, the probability of all n being healthy is Y^n. There will be at least a 0.95 probability of at least one infected plant if Y^n is 0.05 or less. When $Y^n = 0.05$, then $Y = 0.05^{1/n}$ is the fraction remaining healthy if a large number were inoculated. Since $Y = e^{-Uz}$

$$e^{-Uz} = 0.05^{1/n}$$

Taking natural logarithms of both sides gives

$$-Uz = 1/n \ln 0.05$$

$$-Uz = \frac{2.3}{n} \log 0.05 = \frac{2.3\ (-1.301)}{n} = \frac{-3.0}{n} \qquad 8.$$

Therefore, if the average number of particles per infection-initiating volume were greater than $3/n$, where n is the number of plants inoculated, there would be a 0.95 probability of at least one plant becoming infected,

If no infected plants are obtained, and if it is assumed that the sample parameters are the same as the population parameters, then there is a 0.95 probability that the average number of virus particles is equal to or less than $3/n$. On a logarithmic scale, there would be a 0.95 probability that the log uz was less than 0.477 minus log n.

The probability of a plant being infected is $1-Y$ if Y is the probability of its being healthy. If there is a probability of 0.95 that at least one plant will be healthy, then there is a probability of 0.05 that all will be diseased. Then $(1-Y)=0.05^{1/n}$, where n is the number of plants inoculated. Proceeding as above

$$Y = e^{-Uz} = 1 - 0.05^{1/n}$$

and

$$Uz = -2.3 \log (1-0.05^{1/n}) \qquad 9.$$

If Uz were less than the value given by this equation, there would have been a 0.95 probability of at least one healthy plant. If no healthy plants were obtained, and if it is assumed that sample parameters are the same as the population parameters, then there is a probability of 0.95 that the amount of virus equals or exceeds uz given by the equation. If n is 5, 8, 10, 15, 20, 30, 40, or 50, then uz is 0.80, 1.17, 1.35, 1.71, 1.97, 2.35, 2.63, and 2.85, respectively.

EFFECT OF HOST HETEROGENEITY

Host heterogeneity has more effect on the results at high concentrations of virus than at low concentrations (3). The number of infected plants will be less than expected when high percentages of plants are infected. If the data are plotted as a loglog function, the data will deviate from a straight line at high percentages of infection, but not at low (Figure 2) (40). If one wishes to use the results for a bioassay, it may be necessary to discard the data from those dilutions infecting more than a certain percentage of plants. Alternatively, the data may be used to assess host heterogeneity (40). A continuous distribution of host susceptibility should result in a curved line with the loglog plot (Figure 2). If two populations of different, but uniform, susceptibilities are present in the host, a curve with a shelf should result (Figure 3).

If the results are interpreted by other methods, the results of host heterogeneity will show up in different ways. The probit transformation is based on the assumption that host susceptibility has a normal distribution. If the host plants are heterogenous and the distribution of susceptibility is close to a normal distribution, a probit plot should give a straight line whose slope is a function of the variance.

ASSAYS BASED ON INCUBATION PERIOD

Most systemic assays depend on the percentage of inoculated plants that become infected. However, the time from inoculation to appearance of

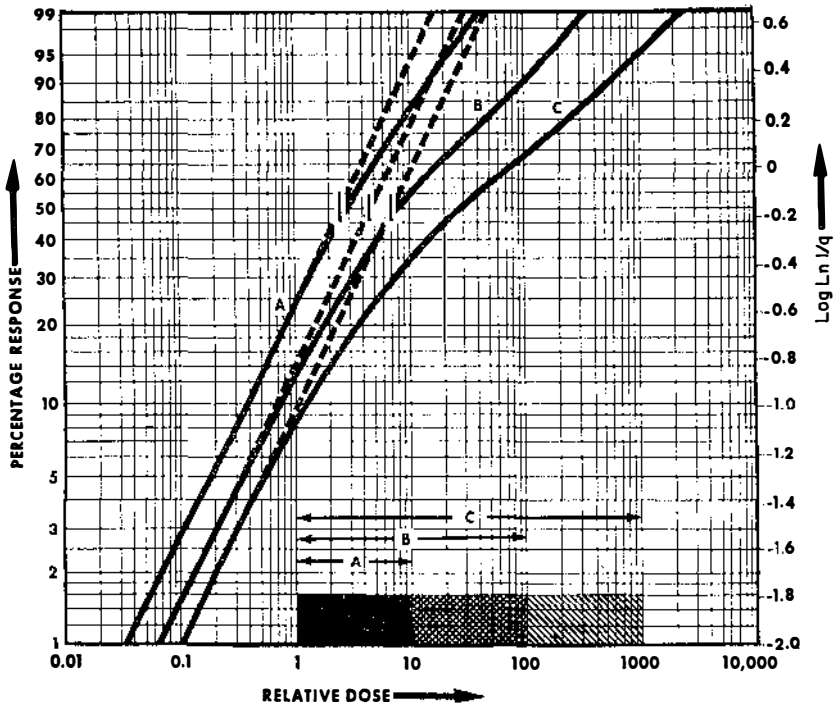

FIGURE 2. Dose response probabilities for continuous distribution of host susceptibility over a 10-fold range (A), a 100-fold range (B), or a 1000-fold range (C). Shortley & Wilkins, 1965.

symptoms may also be used. Empirically, with animal infections, the average incubation period often is a linear function of the logarithm of the concentration of an inoculated pathogen (32, 40). Theoretical models have led to a similar relation for high concentrations of virus (13, 18, 40). When less than 50% of the individuals are infected, most infections should develop from a single particle and the incubation period should not be a function of dose. When all inoculated individuals become infected, most infections should develop from more than one virus particle and the incubation period should be dose-dependent.

An added complication in plants is the fact that development of systemic symptoms depends on movement of virus. The theory of virus movement is not completely understood, but it appears that virus movement can depend on the concentration of virus inoculated (41). Systemic symptoms never develop in some plants under conditions where plant growth is rapid compared to virus movement. With some virus-host combinations, therefore, the incubation period could be dose-dependent even though less than 50% of the inoculated plants become infected.

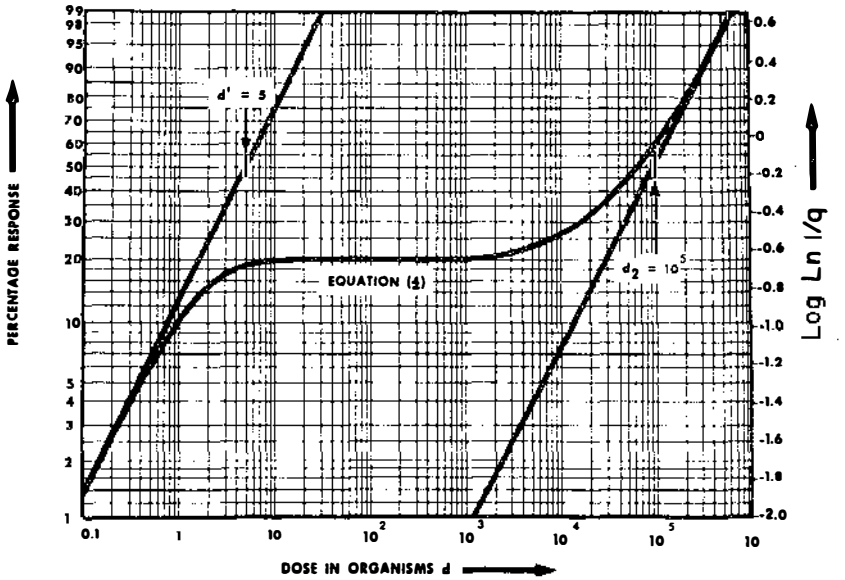

FIGURE 3. Hypothetical response from a mixed population of two groups of uniform hosts. It was assumed that 20% of the hosts have an ID_{50} (d^1) of 5 organisms, and 80% an ID_{50} (d_2) of 10^5 organisms. Shortley & Wilkins, 1965.

The incubation period often changes relatively slowly with animal virus concentration. A plot of incubation time against the logarithm of the virus concentration may be linear over several log units. If plant viruses follow a pattern similar to that of animal viruses, an assay based on incubation period should give an estimate of virus concentration with a relatively small number of plants. It is difficult to predict how many plants would be needed for a given accuracy, since the variance in this type of assay is usually an experimental one calculated from the data for each assay (14). The variance of the mean incubation time often increases with the mean, and transformations may be necessary before analysis of variance.

The incubation period has seldom been used for systemic assay of plant viruses. Hooker & Benson (24) reported that the average incubation period of potato virus X in *Datura tatula* L. decreased as the concentration of virus in the inoculum increased. Raymer & Diener (35) took readings on tomato plants inoculated with potato spindle tuber virus every other day. They added all the positive readings. The sooner a plant showed symptoms, the more it contributed to the total for each dilution, since each infected plant was counted at each reading. If readings are made regularly until all plants become infected, the total number of positives should be a linear function of the logarithm of the virus concentration, if the mean incubation period is such a linear function. If the plants do not all become infected, the data can

be split with data from dilutions resulting in 100% infection used for an incubation period assay, and data from higher dilutions used for a quantal assay (14). Alternatively, the data may be combined in an empirical manner. Thus, Raymer & Diener (35) multiplied the total number of positives for each dilution by the logarithm of the dilution factor, and added these products to give an "infectivity index."

One has the option of using the latent period in insect vectors, or the total time for the latent period in insects and the incubation period in plants, with viruses that have to be assayed by feeding or injecting insects (42). Reports have appeared on the change of incubation periods or latent periods with pathogen concentration for leaf-hoppers injected with aster yellows "virus" and western X disease "virus" (30, 43, 44), but subsequent reports have indicated that these agents are not virus (12, 45).

AGREEMENT OF PLANT VIRUS SYSTEMIC ASSAYS WITH POISSON THEORY

There is no reason to believe that there is a basic difference in the initial steps of infections leading to the observable local lesions and to systemic symptoms. If the laws governing initiation of virus infection are such that numbers of local lesions follow the Poisson distribution, then the numbers of systemically infected plants should also, providing that every infection leads to a lesion or to a systemically infected plant. Development of systemic symptoms depends on movement of virus. The numbers of plants showing systemic symptoms might follow different patterns in plants where virus movement is slow and uncertain than in those plants where movement is rapid.

Local lesions of plant viruses were likened to bacterial colonies when the use of local lesions for infectivity assays was proposed. The numbers of bacterial colonies was first shown by Fisher et al (15) to follow a Poisson distribution. This conclusion was reached because the mean number of colonies was proportional to the concentration; and because the observed variance in the colony counts was equal to the mean, as it should be for the Poisson distribution. These same two criteria have been used in subsequent investigations of counts of bacterial colonies and animal virus plaques (2, 33, 36). Several researchers have investigated the relation between counts of plant virus local lesions and the Poisson distributions using, however, different criteria than were used for bacterial colonies (4, 28, 46). The maximum number of countable lesions has been equated with the number of susceptible sites, a questionable procedure, and the increase in numbers of lesions from zero to the maximum with increasing concentration of virus in the inoculum has then been compared with numbers predicted by the Poisson distribution. Based on this criterion, the numbers of local lesions have followed the Poisson distribution in some cases, but not in others (17, 26). As to application of the criteria used for bacterial colonies, the numbers of local lesions appear to be proportional to virus concentration when

numbers of lesions are small. However, the variance of lesion counts appears to be higher than the variance of the Poisson distribution (e.g. see 25).

Early investigations of the fit of local-lesion numbers to the Poisson distribution were based on the assumption that all sites had the same susceptibility. The effect of host heterogeneity on the Poisson distribution as applied to assays of animal pathogens has been reported (3, 40). Furomoto & Mickey (16) developed a mathematical model for plant virus local lesions based on two probability functions, the Poisson distribution to describe the probability of a virus particle being placed near a cell, and a further probability function to describe the chances of the particle entering the cell. The same model should hold if the second probability function describes host-site heterogeneity. Furomoto & Mickey (17) showed that this model could satisfactorily explain the number of tobacco mosaic virus lesions, but it has not been tested with other viruses. Finally, the overlap problem and the possibility of suppression of late lesions by early ones because of immunity effects have been neglected in theoretical investigations of lesion numbers. Theories developed for other counting problems suggest that overlap of lesions should reduce the countable number even at moderate numbers (1, 29).

In short, the evidence is not yet conclusive that the Poisson distribution, suitably modified for host and site heterogeneity, satisfactorily explains the numbers of plant-virus local lesions and their observed variance. It can not be concluded that systemic assays should follow a Poisson distribution because of basic similarities to local lesions.

Many systemic assays do satisfactorily fit the Poisson distribution (4, 6–9). However, the sampling error is usually large enough that the results of any one assay will satisfactorily fit any of several models. Results of many tests would have to be combined to obtain a statistically significant test of goodness to fit to a particular model to the exclusion of other models.

Cereals are well suited to systemic assays because large numbers of plants can be grown in a small space. I have analyzed 332 assays of wheat streak mosaic, barley stripe mosaic, and brome mosaic viruses by the maximum likelihood method with the loglog transformation (14). The value of Chi square was significant at the 5% level in 59 of these assays. However, Chi square would have been significant at the 5% level in only 18 of the assays, if data from dilutions that infected more than 63% of the plants had been omitted. It is probable that the host plants were not uniformly susceptible or the inoculation procedure was not uniform, or both.

More recently we have calculated the weighted average of the logarithm of virus concentrations for 14 assays of barley stripe mosaic virus on barley as illustrated in Table 5 (Pring & Brakke, unpublished). The average value of Chi square was 4.78 for 3 degrees of freedom. Each assay had 4 dilutions at quarter log unit steps. Chi square in this case is the ratio of the experimental variance, s^2, for a single dilution to the variance of the mean calculated from sampling error. If these estimates of variance were the same, Chi

square would equal the degrees of freedom. Since the average value of Chi square was 1.6 times the number of degrees of freedom, it appears that the actual variance in the results is higher than that calculated from sampling error. The high Chi square could result from host heterogeneity, variation in inoculation technique, deviations from the Poisson model, or other sources.

Comparisons of means of the logarithms of virus concentrations with the standard deviations based on sampling error should be done with equations based on the normal distribution, not on Student's t distribution (14). If comparisons of means are made in this way for assays where Chi square is consistently high, it may be wise to multiply the sampling variance by the average Chi square divided by the number of degrees of freedom. Comparison of means simply by finding if confidence intervals overlap has the effect of increasing the variance.

Several of the insect-transmitted viruses that are not manually transmissible to plants have been assayed by feeding or injecting insect vectors, which then transmit the viruses to plants (42). Results from injecting wound tumor virus into leafhoppers (5, 10) do not fit a Poisson distribution (42). The number of infected plants increased more slowly with virus concentration than expected for a Poisson distribution.

A dilution curve from infections initiated by aphids which had fed through membranes on different concentrations of barley yellow dwarf virus deviated from a Poisson distribution at high dilutions (37). The deviation does not appear to be statistically significant since it was the result of the infection of only a few plants. Rochow & Brakke (38) presented results from feeding aphids several fivefold dilutions of barley yellow dwarf virus. The data are limited, but in most cases the number of infected plants decreased approximately as expected from the Poisson distribution.

Whitcomb (42) has suggested that most plant virus assays that depend on insect transmission are more suitable for an interpretation based on incubation periods than for one based on presence or absence of infection. However, the published data are rather limited for any definite conclusion.

CONCLUSIONS

Advantages of systemic assays of plant viruses are simplicity of experimental design and interpretation. Numerous computational schemes and statistical models developed for assay of animal pathogens are available. The assay may be based on the percentage of infected plants, on the incubation period, or both.

The evidence suggests that the percentages of infected plants obtained approaches that expected with the Poisson distribution. Deviations from the Poisson distribution can indicate host heterogeneity, virus instability, or other phenomena. Since host heterogeneity causes deviations at high percentages of infection, half-log unit or even twofold dilutions should be inoculated to obtain sufficient plants in the linear region. The loglog transformation appears to be the best of those based on the Poisson distribution.

Tenfold dilutions are probably adequate for assays based on incubation periods, since a linear relation between incubation period and log dilution may hold over a wide range.

The disadvantage of a systemic assay is the number of plants required. This may be overcome in part by using very young plants, or by use of plants such as cereals that require little space.

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