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Estimating the Number of Organisms in Quantal Assays

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It has been demonstrated by virologists that, when procedural difficulties can be overcome and host variation can be eliminated, dosage-response data from virus assays conform with the one-particle theory of infection for both plaque counts and tissue cultures. Based on this theory, the number of virus particles from quantal virus assays can be estimated. Here a set of tables is presented from which the number of estimated particles can be obtained directly for several dilution factors and a number of dilution levels. Maximum likelihood estimation of particle counts is also illustrated using a computer program that we have prepared.

Quantitative methods as applied to quantal virus assays include two main methods of expressing the results of such assays. [The use of plaque counts is a related method which can be treated by the comparison of two observed Poisson variates (5).] One is the median lethal dose (LD_{50}) [median effective (ED_{50}) or median infective (ID_{50}) dose] method which estimates the dilution that would give 50% positive and 50% negative responses, and expresses the titers in multiples of the 50% lethal (effective or infective) dose. A method commonly used to determine LD_{50}, especially in the titration of antiviral assays, is the Reed-Muench method. The per cent of positive responses is calculated, not from the actual frequencies for various dilutions, but from the "accumulated sums" of positive and negative responses. The LD_{50} is then obtained by interpolation.

The second major method, the most probable number (MPN) method is based on the theory of Poisson distribution of small numbers. The dilutions of virus concentrate are considered samples of the original virus population present in the inoculum, and an estimate of the population is based on a sample.

Chang et al. (2) and Luria (10) discuss the advantages and disadvantages of LD_{50} and MPN methods and the model requirements justifying their applicability. In particular, Chang et al. (2) cite a number of controversies regarding the justification of the use of the MPN method in virus titration by host inoculation. They found in a statistical study of plaque counts of bacterial viruses that the experimental error was well within that expected when procedural difficulties were overcome and host variability was eliminated (3). The same was shown to hold for numerous animal viruses as well. Since not all animal viruses exhibit plaque formation on monolayer tissue cultures and reliance must be placed on the observation of cytopathogenic changes in tissue culture tubes, Chang et al. (2) carried out a study to confirm the belief that the dilution-response relationship observed in monolayer cultures would hold for conventional tissue culture tubes by employing the MPN technique. They found that the dose-response data were quantitatively reproducible and in conformity with the one-particle theory. Thus, it has been shown to be possible to express the results of virus determinations by methods which have the quantitative sense to indicate "how many organisms" are involved in the infective process (15), rather than to depend on conventional or commonly used LD_{50} methods such as Reed-Muench which are less satisfying and have the disadvantage that an adequate estimate of sampling error is virtually unobtainable (1).

Methods such as the MPN which provide a count of the number of particles involved in the infective process have the following advantages: (i) they extract more information from the data in that an estimate of the sampling error can be obtained; (ii) the number of virus particles is a more familiar measure of virus concentration for a nonvirologist than the indirect and nonuniformly expressed LD_{50} or titer;

(iii) the nomenclature for virus assays by plaque-counting methods and by tissue culture tubes would be more uniform if expressed in terms of number of particles. Chang et al. (2) mention a number of additional advantages of the MPN method.

Since it is often necessary to compare results which may have been obtained by two different methods such as the MPN and the ED_{50} methods, it is important to know that the MPN method of expressing results of virus assays may be easily converted to ED_{50} titers. We assume that the distribution of numbers of organisms in liquid is Poissonian and that from it we can make quantal determinations, i.e., response or no response types. The dose at which one-half of the subjects respond is the ED_{50} by definition. At this dose the probability that X = 0, that is, $P(X = 0)$, must equal 0.5. Because the model postulates a Poisson distribution, $P(X = 0)$ equals e^{-m}, the first term in the expansion of the Poisson. From the equation $e^{-m} = 0.5$, the solution m = 0.69 follows. Therefore, at ED_{50}, the number of organisms must always equal 0.69. For example, assume we have a fivefold dilution series from which we determine m to be 1,000 at log dose 4.1. It follows that m = 200 at log dose 4.8, m = 40 at log dose 5.5, and so on, as may be seen from Table 1. To find ED_{50}, we must look for the log dose which corresponds to m = 0.69. From Table 1, we see that m = 0.69 lies between m = 1.6 and m = 0.32; on the corresponding log dose scale we have the values 6.9 and 7.6, respectively. Therefore, by interpolation we find that ED_{50}, which must lie between these values, is about 7.2.

TABLE 1. *Log dose scale*

Log dose	m
4.1	1,000
4.8	200
5.5	40
6.2	8
6.9	1.6
(7.2, ED_{50})	(.69)
7.6	.32

MODEL FOR ESTIMATING NUMBER OF PARTICLES

The model for a dilution assay postulates that the variability in the dose-response relationship is caused by random variation in the number of virus particles and that there is constant host susceptibility. This reduces the problem to a physical one, that is, the dilutions of the virus concentration are viewed as samples of the original virus population present in the inoculum, and the objective of the assay becomes that of population estimation by sampling. The dilution method of estimating bacterial densities in water and milk without direct count, a method in use since 1915, is based on this principle. The MPN method was devised for estimation of numbers of viable bacteria by inoculation of broth cultures. This method is done by taking samples from the liquid, incubating each sample in a suitable culture medium, and observing whether growth of the organism has taken place. More generally, as discussed above, this method can be employed in any dilution type of bioassay where the responses are quantal, that is, where only the presence or absence of growth due to the organism is tested, provided that the experimental conditions are appropriate and host variation is eliminated.

The estimation of density is based on the application of probability theory under the following assumptions: (i) the organisms must be distributed entirely at random in bulk suspension from which small samples are to be removed so that the distribution of numbers of organisms in replicate samples will be Poissonian; (ii) visible growth must occur in every sample containing one or more infectious units (the one-particle theory of infection).

Under these assumptions the probability of a sterile sample will be e^{-m}, where m is the expected number of effective particles per unit volume of the undiluted suspension, and $1 - e^{-m}$ will be the probability that the sample is fertile, that is, contains one or more effective particles.

The problem of estimating m, "most probable number," from dilution series has interested a number of biologists and statisticians. McCrady (11) found approximate solutions for the estimatation of m from dilution series and attached probability values to certain patterns of response, for example, ++−−, +−+−, where + denotes growth and − no growth, the successive results corresponding to increasing dilutions. Chang et al. (2) provide a table for estimating the MPN for a five-tube (replicate) fourfold dilution series at three levels based on McCrady's multiple dilution probability equation. This equation was also developed by Greenwood and Yule in 1917. Chang et al. (2) give an approximate solution when the number of replicates is greater than five. When there are more than five replicate tubes, too many possible combinations of positive and negative tubes are involved, and the computation of the MPN by the multiple dilution probability

equation is time consuming. The equation was developed by H. A. Thomas for estimating coliform MPN in water analyses that do not fall under the conditions of the standard five-tube, 10-fold dilution MPN table of the American Public Health Association, 1936. For estimating the MPNCU of viruses by the tissue culture tube technique the equation is:

$$\text{MPNCU/ml} = \frac{\text{Total no. of positive tubes}}{[(\text{total ml of inocula in dilutions selected}) \times (\text{total ml of inocula in negative tubes})]^{1/2}}$$

It is said that the MPN estimates computed with this equation agree closely with those computed by the multiple dilution maximum likelihood equation when dilutions containing only positive tubes are excluded from the computation. No estimate of error is given for the MPN.

Haldane (9) worked out the standard error of m, mean number of particles, and was the first to emphasize that maximum information is provided by samples with an average density of 1.59 per unit. Cochran (4) reviewed the subject of "most probable number" and added his finding about the effect of the dilution factor on the standard error of m. Finney (6) developed a maximum likelihood method of estimation for m and the variance of m by using an equivalent deviate transformation. Peto (13) provided tables to aid in the computation of the maximum likelihood solution.

Fisher (7) proposed an approximate method of estimating m that is particularly suitable for data from quantal virus assays. The estimation is based on counting the total number of sterile plates (T), of the dilution series with n replicates at each dilution level. If m is the number of organisms per tube at the highest concentration, the value of m for which the expected average number of sterile plates is equal to the observed number is given by the equation:

$$T = n(e^{-m} + e^{-m/a} + e^{-m/a^2} + \cdots + e^{-m/a^{s-1}}$$

where a = the dilution factor, s = the number of levels, n = the number of tubes or replicates, and T = the total number of sterile plates.

Although the most accurate estimate of the number of organisms per tube at any given level is obtained by solving the equation of maximum likelihood, Fisher has shown that 87.7% of the information is contained in the total number of fertile or sterile plates or tubes, counted without regard to level. The average value of the variance of the mean fertile level is $1/n \times (\log 2)/(\log a)$.

Fisher's equation, which yields an approximate estimate, has no closed-form solution. However, Table $VIII_2$ of Fisher and Yates (8) enables the solution of this equation to be obtained for twofold, fourfold, and tenfold dilution series, although some calculations are necessary.

TABLES FOR ESTIMATING PARTICLE DENSITY

We have prepared a set of tables (Tables 2–5; an abbreviated set of tables appears in reference 16), from which the number of organisms can be estimated by Fisher's equation by reading directly from the tables when T/n, the number of sterile plates divided by the number of replicates; a, the dilution factor; and s, the number of levels, are known.

Included are tables for twofold dilutions at 6, 8, 10, 12, 14, and 20 levels, for fourfold dilutions at 6, 8, 10, and 12 levels; and for tenfold dilutions at 4, 6, 8, and 10 levels, for values of T/n ranging from 0.4 to 5.0. Tables for fivefold dilutions, which were not among Fisher and Yates' tables, for 6, 8, 10, and 12 levels, are also included. Our tables were prepared by an iterative procedure using an IBM computer. Estimates of m are rounded to the nearest tenth in Tables 2 to 5 (except for tenfold dilution, ten levels, where estimates are rounded to the nearest integer), although computations were carried out with double precision to eight decimal places.

We have also written a computer program to give the maximum likelihood estimate of the number of organisms and the variance of this estimate directly from the maximum likelihood equations (*see Appendix*).

USE OF TABLES AND COMPARISON WITH OTHER METHODS

We shall illustrate the use of our tables by considering the data given by Fisher and Yates (8) for demonstrating their method.

Tests with potato flour containing rope spores (*B. mesentericus*) gave the following observations using five tubes each of 1 ml of dilutions 4, 2, 1 ... 1/128 g/100 ml (*E. C. Barton-Wright's data*) (dilution in grams per 100 ml followed by number fertile): 4, 5; 2, 5; 7, 5; 1/2, 5; 1/4, 4; 1/8, 3; 1/16, 2; 1/32, 2; 1/64, 0; 1/128.

There were twofold dilutions at ten levels; the total number of sterile plates is 19; total

TABLE 2. *Estimated particle density by number of dilution levels: twofold dilution*

T/n[a]	No. of Levels					
	6	8	10	12	14	20
0.4	38.4	153.7	614.6	2,458.4	9,833.7	629,359.2
0.5	33.0	132.0	528.1	2,112.4	8,449.5	540,766.0
0.6	28.8	115.2	460.8	1,843.1	7,372.2	471,821.9
0.7	25.4	101.6	406.5	1,625.8	6,503.4	416,217.0
0.8	22.6	90.4	361.5	1,446.2	5,784.7	370,221.0
0.9	20.2	80.9	323.7	1,295.7	5,178.9	331,449.9
1.0	18.2	72.8	291.3	1,165.2	4,660.8	298,289.7
1.1	16.5	65.8	263.3	1,053.1	4,212.5	269,598.5
1.2	14.9	59.7	238.8	955.2	3,820.9	244,539.4
1.3	13.6	54.3	217.3	869.1	3,476.3	222,481.4
1.4	12.4	49.5	198.2	792.7	3,170.9	202,937.8
1.5	11.3	45.3	181.2	724.7	2,898.8	185,525.8
1.6	10.4	41.5	166.0	663.8	2,655.3	169,939.0
1.7	9.5	38.1	152.3	609.1	2,436.4	155,928.6
1.8	8.7	35.0	139.3	559.7	2,238.9	143,290.0
1.9	8.0	32.2	128.8	515.0	2,060.2	131,852.8
2.0	7.4	29.7	118.6	474.5	1,898.0	121,474.0
2.1	6.8	27.4	109.4	437.6	1,750.5	112,032.3
2.2	6.3	25.3	101.0	404.0	1,616.0	103,424.2
2.3	5.8	23.3	93.3	373.3	1,493.1	95,560.5
2.4	5.4	21.6	86.3	345.2	1,380.7	88,364.1
2.5	5.0	20.0	79.9	319.4	1,277.6	81,768.0
2.6	4.6	18.5	73.9	295.8	1,183.0	75,713.3
2.7	4.3	17.1	68.5	274.1	1,096.1	70,148.4
2.8	4.0	15.9	63.5	254.0	1,016.0	65,028.6
2.9	3.7	14.7	58.9	235.9	942.3	60,310.3
3.0	3.4	13.7	54.6	218.6	874.4	55,960.5
3.1	3.2	12.7	50.7	202.9	811.6	51,945.8
3.2	2.9	11.8	47.1	188.4	753.7	48,237.5
3.3	2.7	10.9	43.8	175.0	700.1	44,809.6
3.4	2.5	10.2	40.7	162.6	650.6	41,638.6
3.5	2.3	9.4	37.8	151.2	604.7	38,703.4
3.6	2.2	8.8	35.1	140.6	562.3	35,985.1
3.7	2.0	8.2	32.7	130.7	522.9	33,466.1
3.8	1.9	7.6	30.4	121.6	486.4	31,130.6
3.9	1.7	7.1	28.3	113.1	452.6	28,964.4
4.0	1.6	6.6	26.3	105.3	421.2	26,954.3
4.5	1.0	4.6	18.4	73.7	294.7	18,859.6
5.0	0.6	3.2	12.9	51.7	206.8	13,238.0

[a] T/n is the total number of sterile tubes of the dilution series divided by the number of replicates for each level.

number of fertile plates is 31. In our notation T/n is 3.8; reading from Table 2, m equals 30.4, and the number of organisms per gram is estimated to be 760. Fisher and Yate's more cumbersome procedure yielded 760, the same result. Based on the maximum likelihood equations, m is estimated as 766.25. Finney's estimate using "an equivalent deviate transformation" for the maximum likelihood equation is 766. Using Thomas's equation as given by Chang et al. (2), the estimate is 740, where we have

$$11/\sqrt{5}(1/4 + 1/8 + 1/16 + 1/32 + 1/64 + 1/128) \times (1.1/4 + 2.1/8 + 3.1/16 + 3.1/32 + 5.1/64 + 5.1/128) = 11/1.487 = 7.398$$

$$7.4 \times 100 = 740$$

The expected variance of the mean fertile level is 0.2, and the fiducial limits of the number of tubes at levels 0.025 and 0.975 for P are obtained from $3.8 \pm 1.96 \times 0.45 = 2.918$ and 4.682. Reading from our tables, the values are $57.8 \times 25 = 1{,}442.50$ and $16.2 \times 25 = 405.0$, again in close agreement with Fisher

TABLE 3. *Estimated particle density by number of dilution levels: fourfold dilution*

T/n[a]	No. of Levels			
	6	8	10	12
0.4	992.7	15,883.0	254,128.7	4,066,059.9
0.5	802.9	12,846.0	205,535.8	3,288,573.1
0.6	661.1	10,577.0	169,231.6	2,707,705.1
0.7	551.3	8,820.0	141,120.5	2,257,927.7
0.8	463.7	7,418.9	118,702.2	1,899,235.8
0.9	392.3	6,276.1	100,417.2	1,606,675.4
1.0	333.1	5,329.8	85,276.2	1,364,419.8
1.1	283.7	4,539.6	72,632.8	1,162,125.3
1.2	242.3	3,877.3	62,036.7	992,586.7
1.3	207.6	3,321.4	53,142.0	850,272.2
1.4	178.4	2,853.7	45,659.6	730,552.9
1.5	153.7	2,458.7	39,339.3	629,429.5
1.6	132.7	2,123.1	33,969.0	543,503.7
1.7	114.7	1,835.9	29,374.9	469,997.8
1.8	99.3	1,588.8	25,420.8	406,733.2
1.9	86.0	1,375.2	22,003.6	352,057.7
2.0	74.4	1,190.4	19,045.9	304,734.3
2.1	64.4	1,030.5	16,488.3	263,813.5
2.2	55.8	892.6	14,282.0	228,511.4
2.3	48.4	773.9	12,382.6	198,121.2
2.4	42.0	671.8	10,748.4	171,974.7
2.5	36.5	583.8	9,340.3	149,444.8
2.6	31.7	507.7	8,123.0	129,967.7
2.7	27.6	441.6	7,066.4	113,062.2
2.8	24.0	384.1	6,146.1	98,337.5
2.9	20.9	333.9	5,343.0	85,488.0
3.0	18.1	290.1	4,642.4	74,277.9
3.1	15.8	252.0	4,032.5	64,519.8
3.2	13.7	219.0	3,503.3	56,052.3
3.3	11.9	190.3	3,045.3	48,724.2
3.4	10.3	165.6	2,649.3	42,388.9
3.5	9.0	144.2	2,306.6	36,906.0
3.6	7.8	125.6	2,009.2	32,147.9
3.7	6.8	109.4	1,750.3	28,004.6
3.8	6.0	95.2	1,524.1	24,386.0
3.9	5.2	82.9	1,326.3	21,221.2
4.0	4.5	72.1	1,153.4	18,455.1
4.5	2.2	35.9	574.9	9,198.6
5.0	1.1	18.0	287.9	4,606.7

[a] T/n is the total number of sterile tubes of the dilution series divided by the number of replicates for each level.

TABLE 4. *Estimated particle density by number of dilution levels: fivefold dilution*

T/n[a]	Number of Levels			
	6	8	10	12
0.4	2,935.5	73,387.9	1,834,698.0	45,867,450.6
0.5	2,322.1	58,052.1	1,451,303.2	36,282,580.5
0.6	1,869.5	46,738.1	1,168,451.8	29,211,294.5
0.7	1,528.0	38,200.9	955,022.9	23,875,571.6
0.8	1,263.3	31,582.5	789,562.2	19,739,056.1
0.9	1,052.3	26,307.9	657,696.4	16,442,409.6
1.0	880.1	22,003.3	550,081.7	13,752,042.8
1.1	737.2	18,430.9	460,773.5	11,519,337.1
1.2	617.7	15,442.2	386,055.8	9,685,395.0
1.3	517.7	12,942.7	323,568.6	8,089,215.7
1.4	434.6	10,864.4	271,609.1	6,790,228.2
1.5	365.8	9,146.2	228,654.3	5,716,358.6
1.6	309.1	7,727.7	193,192.1	4,829,802.2
1.7	262.0	6,551.7	163,783.9	4,094,597.3
1.8	222.7	5,567.2	139,180.4	3,479,509.2
1.9	189.4	4,735.3	118,382.4	2,959,559.6
2.0	161.0	4,025.7	100,642.6	2,516,064.9
2.1	136.7	3,417.4	85,433.8	2,135,844.7
2.2	115.8	2,896.0	72,399.8	1,809,993.9
2.3	98.1	2,451.7	61,292.8	1,532,318.9
2.4	83.0	2,076.3	51,906.8	1,297,671.4
2.5	70.4	1,761.2	44,030.6	1,100,765.8
2.6	59.9	1,497.5	37,426.9	935,923.0
2.7	51.0	1,276.0	31,900.3	797,506.7
2.8	43.6	1,088.8	27,218.9	680,473.1
2.9	37.2	929.1	23,227.6	580,689.3
3.0	31.7	792.0	19,800.2	495,004.4
3.1	27.0	673.9	16,846.6	421,165.4
3.2	22.9	572.2	14,305.3	357,633.1
3.3	19.4	485.3	12,132.7	303,318.0
3.4	16.5	411.6	10,291.4	257,286.4
3.5	14.0	349.7	8,742.1	218,552.6
3.6	11.9	297.7	7,441.7	186,042.0
3.7	10.2	253.9	6,347.2	158,679.8
3.8	8.7	216.8	5,419.9	135,498.5
3.9	7.4	185.1	4,628.1	115,701.8
4.0	6.0	157.9	3,947.2	98,680.5
4.5	2.8	69.8	1,745.9	43,647.2
5.0	1.2	31.5	788.9	19,723.4

[a] T/n is the total number of sterile tubes of the dilution series divided by the number of replicates for each level.

and Yates's results of 1,440 and 407. While Thomas' equation for the MPN method is easy to apply and gives reasonable results, its disadvantage lies in the fact that it provides no estimate of variability.

The maximum likelihood estimate of the mean number of organisms is in close agreement with the estimate using the results of Fisher and of Finney. However, the confidence interval obtained by direct solution of the maximum likelihood equation differs from that obtained by Fisher's method and by Finney because both Fisher and Finney obtained the standard error for the logarithm of the number of particles.

By the direct maximum likelihood solution, we have m = 30.65, V(m) = 81.639, SE(m) = 9.0354. The 95% confidence interval is $30.65 \times 25 \pm 1.96 \times 9.035 \times 25$. Using the approximation $V(\ln x) \doteq V(x)/x^2$, as Finney and Fisher did, $V(\ln m) \doteq 81.639/30.65^2 = 0.086903$; $SE(\ln m) \doteq 0.2948$. The confidence interval,

TABLE 5. *Estimated particle density by number of dilution levels: tenfold dilution*

T/n[a]	No. of Levels			
	4	6	8	10
0.4	916.6	91,655.2	9,165,522.3	916,552,231
0.5	695.1	69,506.5	6,950,650.6	695,065,054
0.6	520.0	52,005.7	5,200,571.4	520,057,142
0.7	386.9	38,694.3	3,869,430.8	386,943,083
0.8	292.5	29,254.7	2,925,467.3	292,546,730
0.9	227.1	22,712.7	2,271,273.5	227,127,353
1.0	180.2	18,022.9	1,802,289.0	180,228,905
1.1	144.9	14,487.3	1,448,733.3	144,873,330
1.2	117.0	11,699.0	1,169,901.0	116,990,097
1.3	94.2	9,420.6	942,060.6	94,206,057
1.4	75.1	7,511.6	751,162.7	75,116,265
1.5	59.0	5,896.6	589,665.0	58,966,502
1.6	45.6	4,556.4	455,640.4	45,564,037
1.7	35.0	3,504.4	350,445.3	35,044,531
1.8	27.2	2,725.9	272,590.9	27,259,088
1.9	21.6	2,158.5	215,850.3	21,585,035
2.0	17.3	1,734.7	173,469.3	17,346,930
2.1	14.0	1,406.0	140,599.3	14,059,934
2.2	11.4	1,141.8	114,179.8	11,417,980
2.3	9.2	923.1	92,313.5	9,231,349
2.4	7.4	738.3	73,833.6	7,383,362
2.5	5.8	581.1	58,114.2	5,811,422
2.6	4.5	450.3	45,028.7	4,502,866
2.7	3.5	347.3	34,731.4	3,473,146
2.8	2.7	270.8	27,081.2	2,708,118
2.9	2.1	214.8	21,481.1	2,148,113
3.0	1.7	172.8	17,283.2	1,728,320
3.1	1.4	140.2	14,019.0	1,401,902
3.2	1.1	113.9	11,390.8	1,139,080
3.3	0.9	92.1	9,212.9	921,292
3.4	0.7	73.7	7,370.8	737,082
3.5	0.6	58.0	5,803.1	580,306
3.6	0.4	45.0	4,497.6	449,760
3.7	0.3	34.7	3,470.0	347,006
3.8	0.2	27.1	2,706.4	270,636
3.9	0.1	21.5	2,147.1	214,708
4.0	0.0	17.3	1,727.7	172,769
4.5	0.0	5.8	580.2	58,022
5.0	0.0	1.7	172.8	17,276

[a] T/n is the total number of sterile tubes of the dilution series divided by the number of replicates for each level.

using common logs, is $1.48643 \pm 1.96 \times 0.12803$. Taking antilogs we have (17.20, 54.62). Multiplying by 25, the 95% confidence interval is (430.00, 1,365.50). Finney obtained the interval (429, 1,370). We note that the interval based on the log of the density is not symmetric about the estimate of the density or number of particles. The results of these methods are summarized in Table 6.

Tables 2-5 which we have prepared can be readily extended to additional dilution levels and values of T/n by using the program we have developed. The tables are applicable to complete dilution series, that is, where the proportion of sterile sample ranges from 0 to 1 including both points if the data fit the Poissonian model, (12, 14).

TABLE 6. *Comparison of results obtained by different methods of estimation*

Method	m	95% Interval	Length of interval
Our maximum likelihood solution	766	323-1,209	886
Fisher and Yates	760	407-1,440	1,033
Our Table 2	760	405-1,442	1,007
Finney	766	429-1,370	941
Thomas' equation	740		

APPENDIX

Maximum Likelihood Solution for the Density of Particles

Suppose that a liquid contains V ml and a sample v ml and that there are actually b organisms in the liquid. Under the assumptions stated earlier, there will be no growth if and only if the sample contains no organisms.

Consider a single organism. The probability that it lies in the sample is the ratio of the volume of the sample to the volume of the liquid, v/V. The probability that it is not in the sample is $1 - v/V$. By the multiplication theorem, the probability that none of the b organisms is in the sample is $p = (1 - v/V)^b$. When v/V is small, p is approximated by $p = e^{-vb/V}$. Since b/V is the density, μ, of organisms per ml, we have $p = e^{-v\mu}$, where p is the probability that the sample is sterile. If n samples are taken each of volume v and if s of these samples are found sterile, the proportion of sterile samples is an estimate of p, and hence we obtain an estimate, m, of the density μ by $s/n = e^{-vm}$.

If p is the probability that a sample is sterile, the probability that s out of n samples are sterile is given as

$$\frac{n!}{s!(n-s)!} p^s (1-p)^{n-s}$$

Since $p = e^{-v\mu}$, this is

$$\frac{n!}{s!(n-s)!} e^{-sv\mu}(1 - e^{-v\mu})^{n-s}.$$

If we have samples at several dilution levels, the likelihood function is given by

$$L = \Pi_{i=1}^{k} \frac{n_i!}{n_i!(n_i - s_i)!} e^{-s_i v_i \mu}(1 - e^{-\mu v_i})^{n_i - s_i}$$

where k is the number of dilution levels.
The log likelihood is

$$\ln L = \text{constant} - \mu \Sigma v_i s_i + \Sigma(n_i - s_i) \ln (1 - e^{-\mu v_i})$$

$$\frac{d \ln L}{d\mu} = -\Sigma v_i s_i + \Sigma(n_i - s_i) \frac{v_i e^{-\mu v_i}}{1 - e^{-\mu v_i}}$$

$$\frac{d^2 \ln L}{d\mu^2} = -\Sigma(n_i - s_i) v_i^2 \left[\frac{e^{-\mu v_i}}{(1 - e^{-\mu v_i})^2}\right] \quad (1)$$

$$\text{Var}(\hat{\mu}) = -\frac{1}{d^2 \ln L / d\mu^2} = \frac{-1}{-\Sigma(n_i - s_i) v_i^2 \left[\frac{e^{-\mu v_i}}{(1 - e^{-\mu v_i})^2}\right]} \quad (2)$$

By the usual maximum likelihood procedures, letting $\hat{\mu} = m$, m is obtained by setting equation 1 equal to zero and solving for $\hat{\mu}$ and the variance by solving equation 2. A computer program for the IBM model 7094 computer using an iterative procedure was written to obtain direct solutions for equations 1 and 2.

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