APPLICATION OF THE MOST PROBABLE NUMBER METHOD TO DETERMINE HEAT SENSITIVITY OF PROTOZOA

JOHN B. LOEFER AND ROY B. MEFFERD, JR.[1]

Southwest Foundation for Research and Education, and Trinity University, San Antonio, Texas

In order to determine the deleterious effect of specific agents or treatments on populations of micro-organisms, it is desirable to employ an end-point dilution method to determine number of viable survivors. Plating procedures have not been suitable for use with protozoa, except in the case of certain non-motile and pigmented forms. Lack of a suitable method has hampered studies on the sensitivity of protozoa to specific chemical substances or physical treatments. The most probable number method, frequently used by bacteriologists, was adapted for use in a study on heat sensitivity of Tetrahymena, and the results are described below.

Material and Methods

The ciliated protozoan, *T. pyriformis,* strain WB, as designated previously (Loefer, 1952) and used in an earlier investigation (Loefer and Matney, 1952), was employed in this study. Ingredients of the medium were: Bacto-Casitone, 2%; Bacto Yeast Extract, 0.25%; and salts in mg. % as follows: NaCl, 20; $CaSO_4$, 5; $MgSO_4$, 2.5; KNO_3, 20; KH_2PO_4, 10; K_2HPO_4, 10; $FeCl_3$, 0.5; pH, 7.1–7.3. Cultures in their log phase were used for all determinations. Exposure to specific temperatures was effected in 22 × 175 mm. Pyrex tubes in a constant temperature water bath (± 0.01° C.). A concentrated suspension of ciliates (0.1 ml.) was introduced into pre-heated media (20.0 ml.) and aliquots were removed aseptically at specific time intervals as indicated and transferred to cool (25° C.) dilution bottles containing 0.1% Bacto-Casitone plus inorganic salt solution, so as to obtain series of ten-fold dilutions. Suitable buffering and poising of the dilution fluid by the addition of a low concentration of regular medium is desirable. Additional advantages in the use of 0.1% medium for dilution are that the dilution bottles may be pretested for sterility prior to use, and also, since the bottles foam slightly when shaken, errors during the dilution process are avoided.

Most probable number technique

Five tubes containing approximately 10 ml. of regular medium (penicillin or streptomycin may be added to inhibit bacterial growth, if technique is not rigidly aseptic) were then inoculated with one ml. from each of the above-mentioned dilutions. After a suitable incubation period (96 hours), the tubes were checked for positive growth.

The most probable number of organisms in the original culture was found by reference to the data shown in Table I. The number of positive tubes (from each

[1] Post-doctoral Fellow in Cancer Research of the Damon Runyon Memorial Fund.

TABLE I

Most probable number per milliliter of sample, planting 5 portions in each of 3 dilutions in geometric series. (Modified from Hoskins, 1934)

(1) x			(2)	(1) x			(2)	(1) x			(2)	(1) x			(2)	(1) x			(2)	(1) x			(2)
1	.1	.01		1	.1	.01		1	.1	.01		1	.1	.01		1	.1	.01		1	.1	.01	
0	0	0		1	0	0	2	2	0	0	4.5	3	0	0	7.8	4	0	0	13	5	0	0	23
0	0	1	1.8	1	0	1	4	2	0	1	6.8	3	0	1	11	4	0	1	17	5	0	1	31
0	0	2	3.6	1	0	2	6	2	0	2	9.1	3	0	2	13	4	0	2	21	5	0	2	43
0	0	3	5.4	1	0	3	8	2	0	3	12	3	0	3	16	4	0	3	25	5	0	3	58
0	0	4	7.2	1	0	4	10	2	0	4	14	3	0	4	20	4	0	4	30	5	0	4	76
0	0	5	9	1	0	5	12	2	0	5	16	3	0	5	23	4	0	5	36	5	0	5	95
0	1	0	1.8	1	1	0	4	2	1	0	6.8	3	1	0	11	4	1	0	17	5	1	0	33
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2	3	1	1	14	4	1	1	21	5	1	1	46
0	1	2	5.5	1	1	2	8.1	2	1	2	12	3	1	2	17	4	1	2	26	5	1	2	64
0	1	3	7.3	1	1	3	10	2	1	3	14	3	1	3	20	4	1	3	31	5	1	3	84
0	1	4	9.1	1	1	4	12	2	1	4	17	3	1	4	23	4	1	4	36	5	1	4	110
0	1	5	11	1	1	5	14	2	1	5	19	3	1	5	27	4	1	5	42	5	1	5	130
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3	3	2	0	14	4	2	0	22	5	2	0	49
0	2	1	5.5	1	2	1	8.2	2	2	1	12	3	2	1	17	4	2	1	26	5	2	1	70
0	2	2	7.4	1	2	2	10	2	2	2	14	3	2	2	20	4	2	2	32	5	2	2	95
0	2	3	9.2	1	2	3	12	2	2	3	17	3	2	3	24	4	2	3	38	5	2	3	120
0	2	4	11	1	2	4	15	2	2	4	19	3	2	4	27	4	2	4	44	5	2	4	150
0	2	5	13	1	2	5	17	2	2	5	22	3	2	5	31	4	2	5	50	5	2	5	180
0	3	0	5.6	1	3	0	8.3	2	3	0	12	3	3	0	17	4	3	0	27	5	3	0	79
0	3	1	7.4	1	3	1	10	2	3	1	14	3	3	1	21	4	3	1	33	5	3	1	110
0	3	2	9.3	1	3	2	13	2	3	2	17	3	3	2	24	4	3	2	39	5	3	2	140
0	3	3	11	1	3	3	15	2	3	3	20	3	3	3	28	4	3	3	45	5	3	3	180
0	3	4	13	1	3	4	17	2	3	4	22	3	3	4	31	4	3	4	52	5	3	4	210
0	3	5	15	1	3	5	19	2	3	5	25	3	3	5	35	4	3	5	59	5	3	5	250
0	4	0	7.5	1	4	0	11	2	4	0	15	3	4	0	21	4	4	0	34	5	4	0	130
0	4	1	9.4	1	4	1	13	2	4	1	17	3	4	1	24	4	4	1	40	5	4	1	170
0	4	2	11	1	4	2	15	2	4	2	20	3	4	2	28	4	4	2	47	5	4	2	220
0	4	3	13	1	4	3	17	2	4	3	23	3	4	3	32	4	4	3	54	5	4	3	280
0	4	4	15	1	4	4	19	2	4	4	25	3	4	4	36	4	4	4	62	5	4	4	350
0	4	5	17	1	4	5	22	2	4	5	28	3	4	5	40	4	4	5	69	5	4	5	430
0	5	0	9.4	1	5	0	13	2	5	0	17	3	5	0	25	4	5	0	41	5	5	0	240
0	5	1	11	1	5	1	15	2	5	1	20	3	5	1	29	4	5	1	48	5	5	1	350
0	5	2	13	1	5	2	17	2	5	2	23	3	5	2	32	4	5	2	56	5	5	2	540
0	5	3	15	1	5	3	19	2	5	3	26	3	5	3	37	4	5	3	64	5	5	3	920
0	5	4	17	1	5	4	22	2	5	4	29	3	5	4	41	4	5	4	72	5	5	4	1600
0	5	5	19	1	5	5	24	2	5	5	32	3	5	5	45	4	5	5	81	–	–	–	——

(1) Significant number: positive tubes* with dilutions 1x, 0.1x and 0.01x.

(2) Most probable number per milliliter.

* The dilution "x," used in the tubes, representing the first digit in the significant number, is corrected as follows:

Where x is a dilution of	*Multiply MPN value by*
1:10	1
1:100	10
1:1000	100
etc.	

group of five) in the entire series of geometric dilutions may be represented as a multi-digit number, *e.g.*, 5 5 5 4 3 1, in which the first digit represents the number of positive tubes from the 1 : 10 dilution, and the last number from the 1 : 1,000,000 solution. The number to be used for reference to the table, *i.e.*, the significant number, is 5 4 3 1. The first digit of the significant number represents the number of tubes of the highest dilution from which all were positive, or the lowest dilution used, in the event less than 5 are positive. Since the significant number must consist of three digits, any fourth digit is added to the third, and the example given above now becomes 5 4 4. Reference to column 1 of the table indicates a most probable number of 350. Since the dilution for the first digit of the significant number was 1 : 1000, the most probable number is multiplied by the correction factor indicated in the table. In the illustration given, we obtain 35,000 organisms per milliliter as the most probable number.

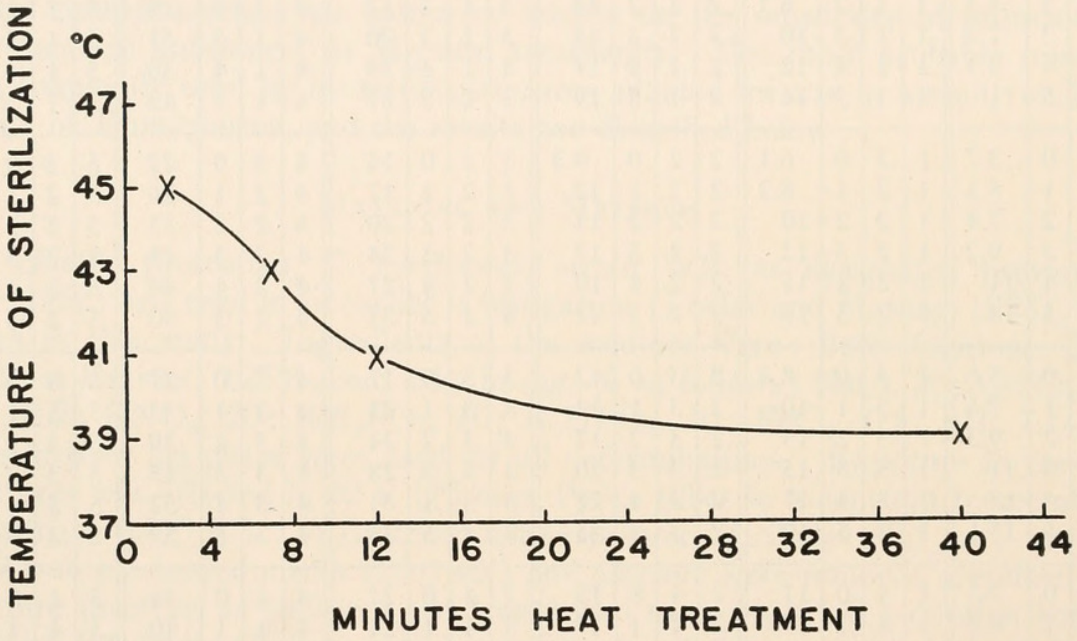

FIGURE 1. Thermal death time for *Tetrahymena pyriformis* WB.

Correlations were made between direct microscopic counts (Hall, Johnson and Loefer, 1935) and most probable number determinations. Average results of either did not vary by more than 5% when replicate determinations were made. The slightly higher count observed consistently in direct counts is readily explicable since any culture contains a certain number of non-viable organisms which will not be detected in the most probable number determination.

EXPERIMENTAL

Viable survivors at specified time intervals were determined following treatment of cultures at 39, 41, 43 and 45° C. In addition, thermal death time at each temperature was determined and these data are presented in Figure 1. The sharp increase in the thermal biological effect as the temperature is raised is due to the high Q_{10} of protein denaturation. At 37° C., several hours were required to obtain a sterile culture.

Although survivor curves were obtained at each of the temperatures tested, those following treatment at 39 and 45° C. are presented as being typical (Fig. 2). As opposed to the case following treatment with ultraviolet radiation (Mefferd and Loefer, 1952), there was a close correlation between loss of motility and loss of viability. In all cases there is a period of relatively slow death rate preceding the period of most rapid killing. A part of this lag undoubtedly is a result of the time required to raise individual cells to the temperature of the water bath, but this explains only a part of the effect. The most logical explanation remains that given by Rahn (1931) which assumes that there is more than one molecule which must be destroyed in order to cause death of the cell. A possibility which must be considered

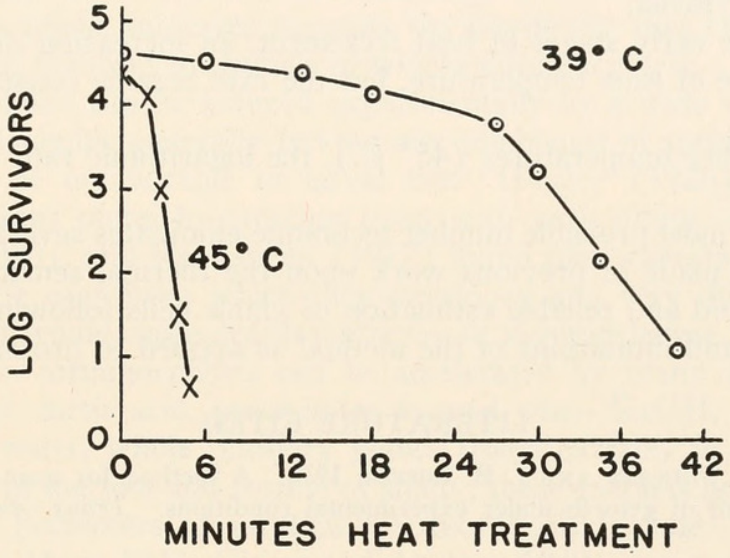

FIGURE 2. Survivor curves for cultures of *Tetrahymena pyriformis* WB subjected to 39 and 45° C.

is that the protozoan population varies widely in the thermal resistance of its members (Jahn, 1933). When large inocula are employed, it is not difficult to select a population of cells with markedly enhanced viability at high temperatures (Mefferd and Campbell, 1952). The authors have, by such techniques, obtained strains of *T. pyriformis* which are capable of growth at 37° C. and even higher. However, in the usual population of a few million cells, the portion which is heat resistant would only negligibly affect the survival curve.

Our data agree generally with those reported by Jahn (1933) for *Euglena gracilis*, and the slight difference may be explained by two major modifications in our technique which varied from his, *viz.*, the introduction of a very small volume of concentrated organisms into a large volume of pre-heated medium, and the quantitative determination of viable cells utilizing a technique (most probable number) which is dependent only upon whether or not an aliquot contains one or more viable cells. This eliminates the many factors which may influence methods based upon comparative growth rates, such as unequalized population densities and the difference in nutritional supplementation of the media between a culture in which no organisms are killed and another in which many have been killed and lysed. The importance of either of these factors is evident.

It is important for the successful application of the technique that incubation of tubes be continued for an extended period of as many as six days or so. In each series there are tubes which contain as few as one organism. Starting from this small inoculum, considerable time is required to achieve a detectable number of cells in the tube. A small quantity of a suitable antibiotic in the dilution and growth media is a distinct advantage in inhibiting chance bacterial contaminants.

Summary

1. Utilizing the most probable number technique, thermal death time and viable survivor curves over a series of temperatures (39, 41, 43 and 45° C.) were determined for Tetrahymena.

2. During the early stages of heat treatment, an increasing death rate was observed in the case of each temperature, but the rate became constant (logarithmic) after a time.

3. At the higher temperatures (45° C.), the logarithmic rate was achieved in a very short time.

4. Use of the most probable number technique eliminates several of the criticisms which have been made of previous work upon the thermal sensitivity of protozoa, and enables a rapid and reliable estimation of viable cells following heat treatment. The advantages and limitations of the method as applied to protozoa are discussed.

LITERATURE CITED

Hall, R. P., D. F. Johnson and J. B. Loefer, 1935. A method for counting protozoa in the measurement of growth under experimental conditions. *Trans. Amer. Micr. Soc.*, **54**: 298–300.

Hoskins, J. K., 1934. Most probable numbers for evaluation of coli-aerogenes. Tests by fermentation tube method. *Public Health Rep.*, **49**: 393–405.

Jahn, T. L., 1933. Studies on the physiology of the euglenoid flagellates. IV. The thermal death time of *Euglena gracilis* Klebs. *Arch. f. Protisten.*, **79**: 249–262.

Loefer, J. B., 1952. Some observations on the size of Tetrahymena. *J. Morph.*, **90**: 407–414.

Loefer, J. B., and T. S. Matney, 1952. Growth inhibition of free-living protozoa by actidione. *Physiol. Zool.*, **25**: 272–276.

Mefferd, R. B., Jr., and L. L. Campbell, Jr., 1952. Studies on a thermophilic variant of *Bacillus globigii*. *Tex. Rep. Biol. Med.*, **10**: 419–420.

Mefferd, R. B., Jr., and J. B. Loefer, 1952. Lethality of ultraviolet radiation for *Tetrahymena pyriformis*. *Proc. Soc. Protozool.*, **3**: 17.

Rahn, O., 1931. The order of death of organisms larger than bacteria. *J. Gen. Physiol.*, **14**: 315–337.