QUANTITATION OF VIRUSES BY THE PLAQUE TECHNIQUE

GERALD BERG, EUGENE K. HARRIS, SHIH L. CHANG, AND KENNETH A. BUSCH

Robert A. Taft Sanitary Engineering Center, U.S. Public Health Service, Cincinnati, Ohio

Received for publication 2 November 1962

ABSTRACT

BERG, GERALD (Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio), EUGENE K. HARRIS, SHIH L. CHANG, AND KENNETH A. BUSCH. Quantitation of viruses by the plaque technique. J. Bacteriol. **85**:691-700. 1963.—This paper presents the results of a study on overcrowding as it occurred with several strains of enteroviruses on monkey kidney cell layers. Each cell sheet area was 4,500 mm². Dispersion analysis of all plaque counts in the study revealed that the percentage error, in the absence of overcrowding, fluctuated within a range of 7 to 36% about a mean value of about 17%, which is close to what is expected from purely statistical variation in sampling. Overcrowding with Mahoney virus, a mixture of particles producing either rapidly or slowly expanding plaques, resulted primarily from the obscuring effect produced as rapidly expanding plaques obliterated infected foci produced by virus particles responsible for slow-forming plaques. Overcrowding occurred at plaque levels in excess of 35 to 40 per cell sheet. Overcrowding with Mahoney LP virus, a strain derived from a rapidly expanding Mahoney plaque which produced uniformly expanding plaques, did not occur until counts in excess of 60 to 70 per cell sheet were reached. Plaque obscuring was again responsible. Apparent overcrowding with Coxsackie A9 virus, when plaques were not permanently marked from the first day of counting, resulted from coalescence at levels above about 40 to 50 plaques per cell sheet. When plaques were permanently marked, overcrowding resulted from plaque obscuring which did not occur until levels in excess of about 120 per cell sheet were reached.

The development of the plaque technique (Dulbecco, 1952; Dulbecco and Vogt, 1954) made possible quantitative determinations in viral studies with a precision and accuracy previously unobtainable under practical conditions. This technique has now been used widely in quantitative studies for almost a decade, but few data are evident that demonstrate the precision and accuracy of the technique, and no data are available that define the mechanisms of plaque crowding or its effect on the precision and accuracy of the count.

This paper defines the precision and accuracy of the plaque technique and describes both the mechanism and the effect of plaque crowding.

MATERIALS AND METHODS

All virus stocks were prepared in rhesus (*Macaca mulatta*) monkey kidney cell cultures.

Poliovirus type I (Mahoney). This stock was a mixture of two strains. One of these, predominating in numbers, produced rapidly expanding plaques; the other produced slowly expanding plaques. This mixture of strains were used in these studies after 37 passages in cynomolgus (*Macaca cynomolgus*) and rhesus kidney cell cultures.

Poliovirus type I (Mahoney LP). This strain, obtained from the mixture just described, was isolated by passage of virus scraped from a rapidly growing plaque. On subsequent passage, this strain proved to be homogeneously fast-growing. It was used in this study after a total of 40 passages in cynomolgus and rhesus monkey kidney cell cultures.

Coxsackie A9 virus (CME456). This strain was isolated from a rectal swab taken from a healthy child and was used in this study after one passage in a rhesus monkey kidney cell culture.

Cell cultures. Cultures of trypsinized rhesus or African Green (*Cercopithecus aethiops sabaeus*) monkey kidney cells (Rappaport, 1956) were grown in rubber-stoppered 6-oz prescription bottles (Hsiung and Melnick, 1955) in a growth medium containing 96.28% Hanks' balanced salt solution (BSS), 0.5% lactalbumin hydrolysate, 3% calf serum, 0.22% $NaHCO_3$, and 50 units each of penicillin, streptomycin, and nystatin per ml. After the formation of a continuous cell sheet, the growth medium was re-

placed with a maintenance medium similar to the growth medium except that the serum and Hanks' BSS were replaced with Earle's BSS. The pH of the growth medium was 7.4; that of the maintenance medium was 7.6 or 8.0. Both were adjusted with NaOH. Maintenance medium was always changed shortly before inoculation.

The area of each cell sheet was 4,500 mm².

Test procedure. Dilutions were made in either Earle's or Hanks' BSS containing the same antibiotic concentrations present in the growth and maintenance mediums. The pH was adjusted with $NaHCO_3$ to 7.8 to 8.0.

In each experiment, a dilute virus stock was prepared from which all further dilutions were made. The dilution ratio was constant in all but one experiment. Each dilution was prepared by adding a sample of the dilute stock suspension to an appropriate quantity of diluent.

Samples (0.5 ml) of each dilution were inoculated onto cell sheets and incubated at room temperature (23 to 28 C) long enough for maximal adsorption of virus to cells (Berg, *unpublished data*). The infected cells were then overlaid with approximately 20 ml of an agar-base medium essentially the same as previously described (Hsiung and Melnick, 1955). Overlaid cultures were incubated at 36 C except during periods of plaque counting. Bottles were distributed randomly in the incubator between readings and identified only after readings to avoid bias.

Plaque counts. Plaques were counted and marked with a red wax pencil on the day they first became visible, and counts were continued daily thereafter until so much of the cell sheet was lysed counting became impractical. In some experiments, markings were permanent. Daily counts of new plaques were additive, so that each day's total was equal to that of the previous day or higher. In experiments where markings were temporary, the wax marks were removed from each bottle after the count was made and recorded, and all plaques were counted daily.

Measurement of plaque area. A celluloid sheet,

TABLE 1. *Relation of plaque counts to changing concentrations of Mahoney virus*

Plaque identification	Virus dilution	No. of bottles per dilution	Days of incubation									
			3		4		5		6		7	
			Mean count	SD	Mean count	SD	Mean count	SD	Mean count	SD	Mean count	SD
Temporary	1:1.00	7	87	±16	94	±11	TDTC*					
	1:1.26	8	76	±13	84	±9	TDTC					
	1:1.57	8	66	±6	76	±7	TDTC					
	1:1.98	8	50	±6	62	±7	56	±5	TDTC			
	1:2.50	8	38	±11	49	±7	47	±5	TDTC			
	1:3.13	8	33	±8	40	±7	39	±8	TDTC			
	1:4.00	8	27	±7	34	±5	34	±5	TDTC			
	1:5.00	8	24	±6	32	±5	32	±5	29	±4		
	1:6.25	8	20	±5	25	±5	26	±5	24	±3		
	1:8.00	5	12	±2	16	±4	16	±4	17	±4		
Permanent	1:1.00	8	45	±8	67	±10	71	±11	71	±11	71	±11
	1:1.26	8	30	±9	50	±9	56	±10	57	±9	57	±9
	1:1.57	8	26	±9	43	±8	50	±9	50	±9	50	±9
	1:1.98	8	26	±6	39	±10	45	±9	45	±9	45	±9
	1:2.50	8	28	±7	38	±7	41	±7	42	±7	42	±7
	1:3.13	8	22	±5	29	±4	31	±4	32	±5	32	±5
	1:4.00	8	15	±3	22	±5	24	±5	25	±5	25	±5
	1:5.00	8	16	±7	22	±7	24	±7	24	±7	24	±7
	1:6.25	8	10	±3	16	±4	18	±4	18	±3	19	±3
	1:8.00	7	8	±3	10	±4	11	±4	11	±4	11	±4

* Too difficult to count.

TABLE 2. *Relation of plaque counts to changing concentrations of Mahoney LP virus**

Virus dilution	No. of bottles per dilution	Days of incubation							
		2		3		4		5	
		Mean count	SD	Mean count	SD	Mean count	SD	Mean count	SD
1:1.00	6	152	±8	166	±7	167	±7	TDTC†	
1:1.26	5	136	±23	149	±19	151	±18	TDTC	
1:1.57	6	104	±12	119	±14	120	±14	TDTC	
1:1.98	6	87	±17	98	±17	99	±17	99	±17
1:2.50	6	64	±5	77	±6	78	±6	79	±7
1:3.13	6	57	±8	68	±8	69	±8	70	±8
1:4.00	6	48	±12	59	±13	60	±12	61	±12
1:5.00	6	37	±6	46	±6	48	±6	49	±6
1:6.25	6	30	±4	37	±6	39	±5	40	±6
1:8.00	6	25	±4	31	±5	33	±5	33	±5

* All plaques were identified permanently.
† Too difficult to count.

which covered the exact area of the cell sheet, was fitted with eight holes punched in such a fashion that they represented a random distribution of plaques when the celluloid sheet was placed over the cell sheet. The eight plaques closest to the punched holes were measured to the nearest millimeter with a millimeter rule.

Plaque diameters were determined on all counting days. Measurements were made in half the bottles containing each dilution inoculated. Since, each day, bottles were chosen for counting in random order, measurements of plaque diameters were made in randomly chosen bottles.

The average area of the plaques was determined for each day's count by squaring the diameter of each measured plaque and multiplying the average of the squared diameters by $\pi/4$.

RESULTS

Precision of the plaque technique. The arithmetic means and standard deviations of the replicate counts at all dilutions of Mahoney virus are presented in Table 1. Similar determinations with the Mahoney LP strain and Coxsackie A9 virus are presented in Tables 2 and 3. At higher dilutions, where counts were not affected by overcrowding (see below), the percentage error (standard deviation divided by the mean and multiplied by 100) generally fluctuated about a mean value of 17% within a range of 7 to 36% (curve skewed to lower range).

Effect of incubation period on plaque counts. When overcrowding was absent, as judged by the proportional response of counts to dilution, 64 to 78% of the potential Mahoney plaques were sufficiently discrete to be countable on the first counting day (Table 1). With the Mahoney LP strain, 77% of the potential plaques formed under similar conditions (Table 2). With both viruses, 89 to 95% of all potential plaques had formed by the second counting day.

About 85% of the potential Coxsackie A9 plaques developed by the first day of counting (Table 3). An increase of only about 2% in plaque numbers appeared after the second counting day. In the experiment where marking was temporary, counting began 1 day later than in the experiment where marking was permanent. Here, too, few new plaques formed on subsequent days. This difference in time when plaques were discrete enough to be counted was a matter of hours and may reflect a difference in the time of day when the experiments were initiated and read or some variation in the conditions of the cell sheets.

Effect of coalescence on plaque counts. When plaques are not counted and permanently marked from the day of first appearance, contiguous groups of two or more plaques may coalesce and appear as a single plaque. On cell sheets containing numerous plaques that are not permanently marked when still small, large areas of coalescence make even approximations impossible by the day when all potential plaques have formed.

TABLE 3. *Relation of plaque counts to changing concentrations of Coxsackie A9 virus*

Plaque identification	Virus dilution	No. of bottles per dilution	Days of incubation							
			3		4		5		6	
			Mean count	SD	Mean count	SD	Mean count	SD	Mean count	SD
Temporary	1:1.67	3			70	±5	68	±7		
	1:2.00	5			60	±7	59	±5		
	1:2.22	4			52	±8	52	±6		
	1:2.50	5			52	±7	49	±6		
	1:2.87	5			37	±6	39	±8		
	1:3.33	5			46	±8	44	±7		
	1:4.00	5			35	±4	33	±5		
	1:5.00	5			27	±8	32	±6		
	1:6.67	5			21	±6	22	±5		
	1:10.0	5			18	±4	18	±5		
	1:20.0	4			6	±2	6	±2		
Permanent	1:1.26	6	136	±7	144	±8	145	±8	TDTC*	
	1:1.57	6	123	±9	131	±10	132	±11	TDTC	
	1:1.98	6	112	±13	120	±15	120	±15	TDTC	
	1:2.50	5	86	±13	98	±11	99	±11	TDTC	
	1:3.13	6	65	±6	72	±9	73	±9	TDTC	
	1:4.00	6	60	±5	66	±5	67	±6	TDTC	
	1:5.00	6	39	±6	45	±3	46	±3	46	±4
	1:6.25	6	27	±7	32	±7	34	±6	34	±7
	1:8.00	5	27	±5	33	±5	33	±8	33	±8

* Too difficult to count.

With Mahoney virus, when plaques were not permanently marked, counts in excess of about 56 per cell sheet could not be made on the third counting day (Table 1). By the fourth counting day, counts could not be made which exceeded approximately 29 plaques per cell sheet. When plaques were marked permanently, counting could be continued as long as some part of the cell sheet remained intact, thus avoiding the crowding effect of coalescence even at very high counts.

Because of their less discrete morphology, Coxsackie A9 plaques, when present in numbers exceeding about 46 per cell sheet, could no longer be enumerated by the fourth counting day, even when the plaques were marked permanently.

Plaque-obscuring phenomenon. The plaque-obscuring phenomenon results from the delay in formation of some plaques while others are expanding. Thus, the formation of independent plaques by viral particles located within or near the boundaries of expanding plaques is prevented.

Quantitative analysis of the overcrowding effect. The magnitude of overcrowding was determined by quantitative analysis.

1. Mahoney and Mahoney LP virus. In the absence of overcrowding, plaque counts are inversely proportional to dilution. Since the three determinations with these viruses were made with dilutions of a constant 1:1.26 ratio (i.e., 1:1, 1:1.26, ... 1:8.00), counts from this series should be consistent with the equation

$$X_k = C_o(1.26)^{-k} \quad (1)$$

where X_k represents a mean count at dilution k, C_o denotes the plaque-forming units (PFU) of the virus in the stock suspension, and k assumes values of 0, 1, 2, ... 9 of the dilution code.

Overcrowding may be discerned when the corrected counts are computed by multiplying the mean counts by their dilution factors, i.e., $(1.26)^0$,

$(1.26)^1$, $(1.26)^2$, ... $(1.26)^9$. When overcrowding is not significant, corrected counts vary randomly about a mean value which estimates C_o. These calculations with the data in Tables 1, 2, and 3 show that the corrected counts tended to increase with dilution until a critical dilution was reached. At dilutions above this point, the corrected counts leveled off and fluctuated generally within the limits of sampling variations.

C_o was estimated by averaging the corrected mean counts of the four highest dilutions where overcrowding, as indicated by the random variations of the mean values of the counts about their theoretical means, was absent. With C_o estimated, expected counts at the test dilutions were computed from equation 1. These expected counts are themselves estimates and, therefore, subject to statistical sampling error. This is taken into account in the significance test described below.

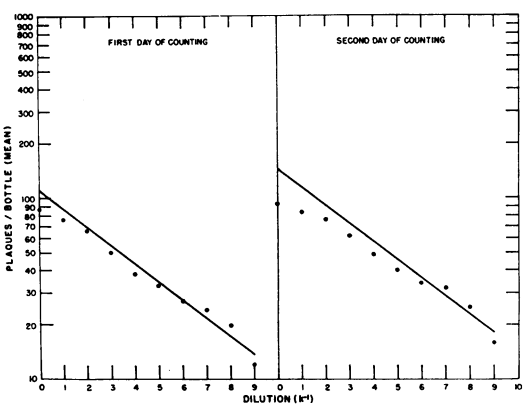

FIG. 1. *Overcrowding with Mahoney virus. Plaque marking temporary.*

To demonstrate overcrowding and the dilution pattern of the counts before and after the appearance of all potential plaques in the three tests, C_o and the expected counts were computed for each significant counting day. In Fig. 1, 2, and 3, logarithmic plots of expected counts against the dilution code are presented with actual mean counts represented by individual points.

Examination of the curves for the days of maximal counts in Fig. 1, 2, and 3 shows clear evidence of overcrowding above certain count levels. In each case, statistical significance tests were performed on the data. Since, under the "null" hypothesis of noncrowding (equation 1), we may reasonably assume approximately normal distribution of observed mean counts about expected values, the tests consist simply of computing for each dilution the ratio

$$Z = \frac{\text{observed mean count} - X_k \text{ (equation 1)}}{\sqrt{\frac{s}{n} + \text{variance of } X_k}}$$

where n is the number of bottles at that dilution.

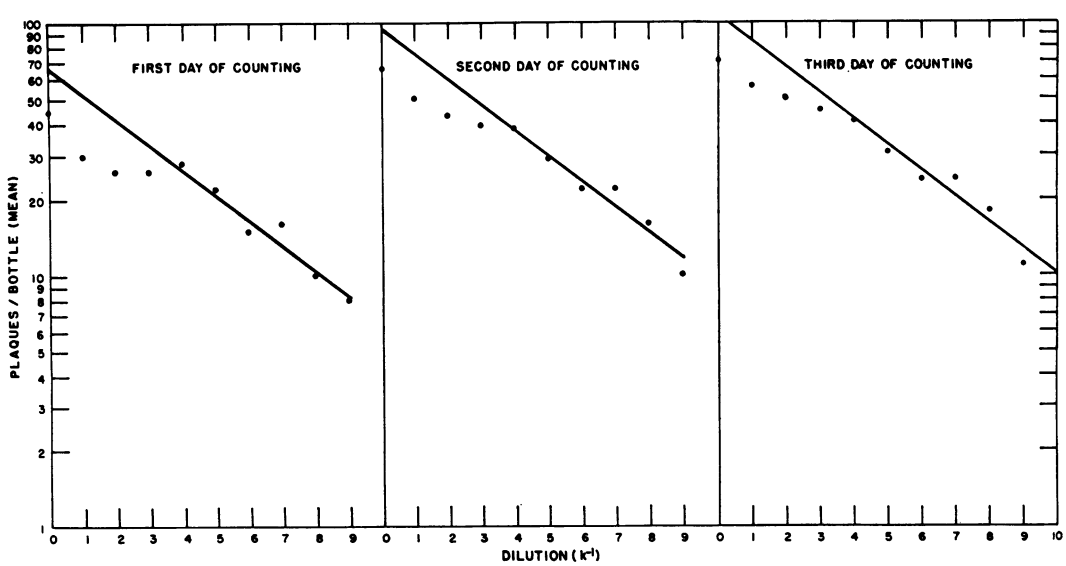

FIG. 2. *Overcrowding with Mahoney virus. Plaque marking permanent.*

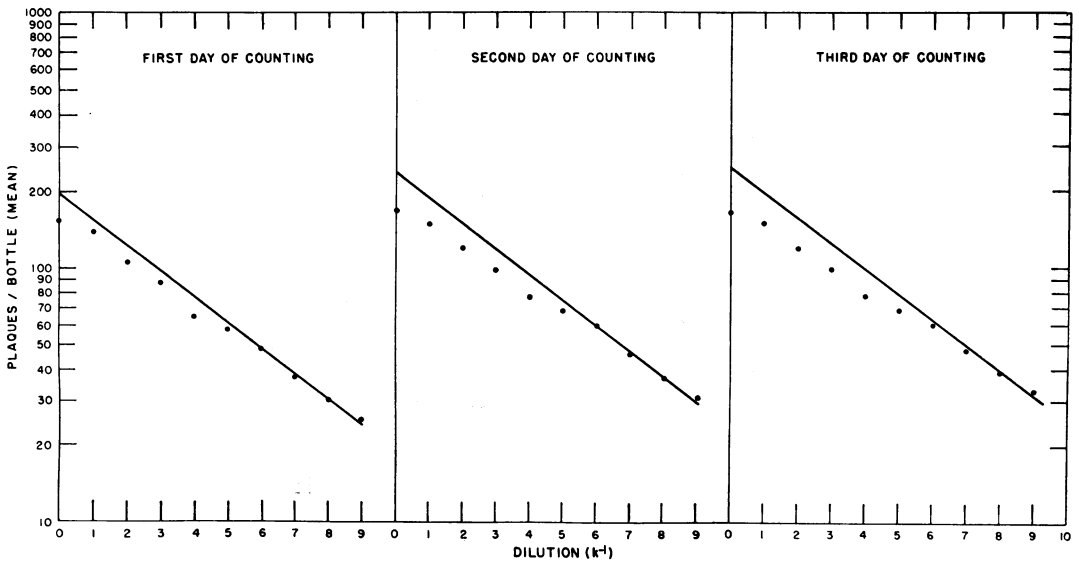

FIG. 3. *Overcrowding with Mahoney LP virus. Plaque marking permanent.*

TABLE 4. *Test for significant deviation of observed counts at points of apparent overcrowding*

Virus	Plaque identification	Observed plaque count*	Expected plaque count	*Z* value	Significance†
Mahoney	Temporary	39	46.1	−2.0	S
		47	58.0	−4.0	S
		56	73.2	−5.3	S
	Permanent	41	41.5	−0.2	NS
		45	52.2	−1.7	S
		50	66.0	−3.5	S
Mahoney LP	Permanent	60	62.5	−0.5	NS
		69	78.5	−2.3	S
		78	99.0	−5.3	S
Coxsackie A9	Temporary	44	45.0	−0.2	NS
		39	51.5	−2.6	S
		49	60.0	−2.4	S
	Permanent	120	124	−0.5	NS
		132	156	−3.4	S
		145	195	−6.6	S

* Values were determined at those points immediately preceding apparent overcrowding and then at the next two lower dilutions.

† NS = not significant at the 95% level; S = significant at the 95% level.

From equation 1, the variance of X_k equals $(1.26)^{-2k}$ times the variance of C_o. The latter variance is a function of the variances of the observed mean counts at high dilutions from which C_o was calculated. The statistics needed to calculate Z are available in Tables 1, 2, and 3. Using a 5% one-tailed criterion, since we anticipate overcrowding and not undercrowding, if Z is less than −1.65 we may reject the hypothesis of noncrowding at that dilution.

The results of these tests demonstrated that, although occasional significant deviations occurred at high dilutions, only at or below those dilutions at which apparent overcrowding occurred did the observed counts deviate consistently and with increasing significance from the expected counts. Z values for the highest dilutions at which significant crowding could not be demonstrated and Z values for the next two lower dilutions showing significant and increasing deviations from expected values are presented in Table 4. Inspection of Fig. 1, 2, and 3 indicates clearly, as counts increase above those for which Z values are presented, that the gaps between observed and expected values broaden increasingly. We can state with assurance, therefore, that the critical count levels apparent in Fig. 1, 2, and 3 represent real signals of overcrowding at these and higher count levels.

a. Mahoney virus. When plaques produced by Mahoney virus were temporarily marked, consistent underestimation of the PFU appeared at counts over about 35 per bottle (Fig. 1). When the plaques were permanently marked, the near-maximal noncrowded count of about 40 per bottle was not significantly different (Fig. 2). Since permanent marking of plaques did not prevent overcrowding, overcrowding on the day of maximal counts must be due primarily not to coalescence of plaques but to obscuring of slowly expanding infected foci. (Infected foci are defined as areas containing virus-infected cells but not yet visible as plaques.)

On the third day of counting, when plaques were temporarily marked, some overcrowding from coalescence became apparent, for at the lower dilutions counts dropped below those of the previous day (Table 1).

b. Mahoney LP virus. With plaques permanently marked, little or no overcrowding occurred at levels below 60 to 70 plaques per bottle by the time near-maximal counts appeared (Fig. 3).

Mahoney LP virus had been obtained by passage of virus from a rapidly expanding plaque of Mahoney virus, a mixture of virus particles producing both rapidly and slowly expanding plaques (see Materials and Methods). The Mahoney LP virus produced plaques that expanded more uniformly, which probably accounts for the relatively high counts obtainable before plaques were obscured by overcrowding.

With the Mahoney virus, plaque obscuration probably resulted from obliteration of slowly expanding infected foci by rapidly expanding plaques. This situation is more readily visualized when one considers that on the same cell sheet plaques of the slowly expanding strain are only about 1 to 2 mm in diameter when those of the rapidly expanding strain are about 4 to 6 mm in diameter.

2. Coxsackie A9 virus. A 1:1.26 constant dilution ratio was used throughout the determination with permanently marked Coxsackie A9 plaques (Table 3). Expected counts were computed from equation 1. A changing dilution ratio was used with the temporarily marked plaques, and expected counts were computed from equation 2, a modification of equation 1:

$$X_k = \frac{C_o}{d_k} \qquad (2)$$

X_k and C_o are the same as in equation 1; d_k is the dilution factor for the k dilution. For example, d_k is 2 for a 1:2 dilution, 3 for a 1:3 dilution ... 20 for a 1:20 dilution.

In the absence of overcrowding, mean counts in the permanently marked test plot linearly against dilution on a semilog scale, and mean counts in the temporarily marked test plot linearly against d_k on a log-log scale.

Values of C_o were estimated as described. Expected counts for the permanently marked plaque data in Table 3 were computed from equation 1, and expected counts for the temporarily marked plaque data in Table 3 were computed from equation 2. Appropriate plots of the expected counts against the dilution code are

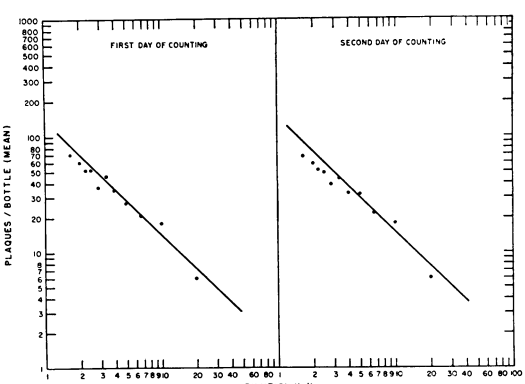

FIG. 4. *Overcrowding with Coxsackie A9 virus. Plaque marking temporary.*

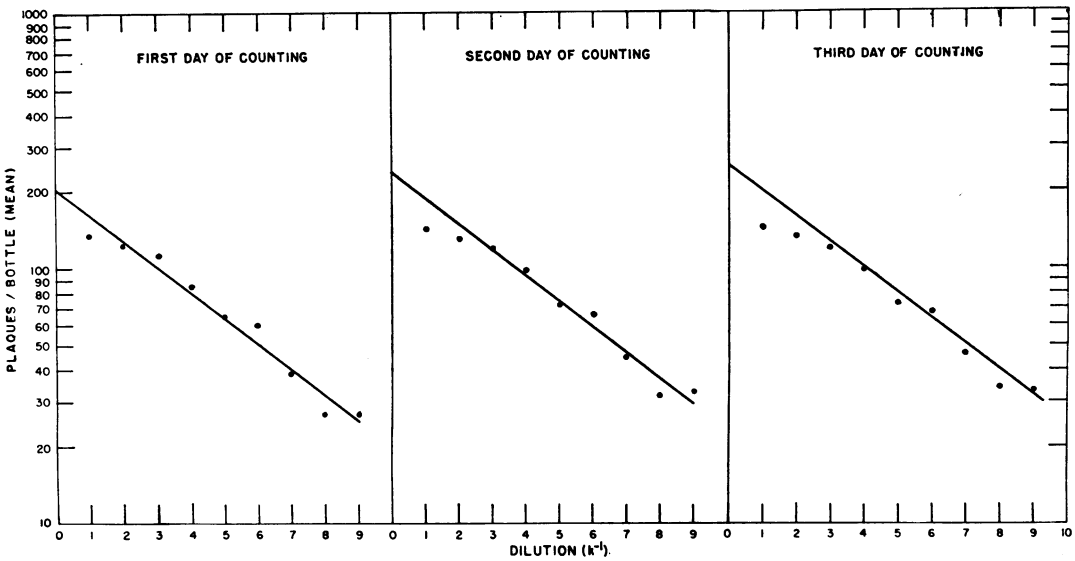

FIG. 5. *Overcrowding with Coxsackie A9 virus. Plaque marking permanent.*

TABLE 5. *Average proportion of cell sheet area covered by plaques at near-maximal noncrowded counts**

Virus	Plaque identification	Total no. of plaques measured	Mean plaque area	SE	Proportion of cell sheet area covered by plaques†
			mm^2		
Mahoney	Temporary	320	19.0	±0.5	0.14
	Permanent	248	42.2	±1.0	0.38
Mahoney LP	Permanent	319	39.3	±0.9	0.57
Coxsackie A9	Temporary	115	17.4	±0.9	0.18
	Permanent	216	14.2	±0.4	0.38

* At least 96% of total plaques formed.
† Area of overlapping was not taken into consideration.

presented in Fig. 4 and 5 with actual mean counts represented by individual points.

Figure 4 demonstrates that with plaques temporarily marked there was underestimation at counts of 40 to 50 per cell sheet on the day of near-maximal counts. Underestimation was indicated by the consistent location of the mean counts below the theoretical curve. When the plaques were permanently marked, the mean counts fluctuated randomly about the theoretical curve on all counting days at all dilutions, with the exception of the two lowest (Fig. 5). Underestimation of about 23% is suggested at expected counts of approximately 190 per bottle.

Significant Z values were obtained consistently at mean counts of 39 and 120 plaques per bottle and higher in the tests with temporary and permanent markings, respectively (Table 4). The points of consistent departure of the observed from the expected counts (Fig. 4 and 5) seem to give a reliable estimate of the critical count levels.

The suggestion of overcrowding at counts above 40 to 50 per cell sheet with temporarily marked plaques contrasts with the absence of overcrowding at counts below 120 per bottle when plaque marking was permanent. This implies that coalescence is primarily responsible for overcrowding at levels between about 40 to 50 and 120 plaques per bottle. At counts above 120

per bottle, plaque obscuring may result in additional overcrowding.

Relationship of total plaque area and cell sheet area in overcrowding. With the method described in the previous section, plaque diameters were measured on each counting day, and average plaque areas were calculated together with their standard errors (standard deviations of the means). The proportion of each cell sheet area covered by plaques was determined by multiplying the average plaque area by the count and dividing the result by the cell sheet area (4,500 mm^2). This method of computing total plaque areas does not take into consideration overlapping, which should occur randomly, and thus exaggerates the actual cell sheet area covered when overlapping occurs.

Table 5 demonstrates that on the day when nearly all (over 96%) potential plaques had formed in the temporarily marked Mahoney test, only 14% of the total cell sheet area was covered by plaques at counting levels just below those at which overcrowding became manifest. Coalescence reduced plaque counts after the second counting day.

When Mahoney plaques were permanently marked, counts exceeding 96% of the final total were not evident until the third counting day, since there were no losses from coalescence. Here, 38% of the cell sheet was covered by plaques before overcrowding occurred.

Mahoney LP plaques expanded at a more uniform rate. On the third counting day, when nearly all potential plaques had appeared, 57% of the cell sheets containing near-maximal noncrowded counts were covered by plaques. Since permanent plaque marking eliminated coalescence, and a uniform plaque expansion rate reduced plaque obscuring, a greater area of the cell sheet was consumed before overcrowding became evident. With permanently marked bottles, the 50% increase in cell sheet area consumed by Mahoney LP plaques before overcrowding occurred is consistent with the 50% increase in the number of Mahoney LP plaques that could be counted before maximal noncrowded counts were reached.

With temporarily marked Coxsackie A9 plaques on sheets containing near-maximal noncrowded counts, 18% of the cell area was covered when almost all potential plaques were manifest. When plaque marking was permanent, 38% of the cell sheet area was covered by plaques under similar conditions. With both temporarily and permanently marked Coxsackie A9 tests, over 98% of the potential plaques appeared by the second counting day. The 111% increase in cell area covered by permanently marked Coxsackie A9 plaques reflects overcrowding due to coalescence and is reasonably consistent with the 131% increase in near-maximal noncrowded counts.

Because plaques in the temporarily marked test were slightly larger than those in the permanently marked test on the day when near-maximal counts appeared (Table 5), the cell sheet area covered at this time in the temporarily marked test was disproportionately large when compared with the area covered in the permanently marked test. Had the average plaque areas been the same in the two experiments, 17.4 mm^2 for example, then the cell sheet area covered in the permanently marked test would have been about 130% greater than in the temporarily marked test. The discrepancy in average plaque size was probably due to a difference in the times of day when the tests were done and the plaques counted, or to some qualitative differences in the cell cultures used.

Discussion

Overcrowding with Mahoney virus, at the level of 35 to 40 plaques per cell sheet, when almost all potential plaques had formed, resulted primarily from the obscuring effect. Rapidly expanding plaques obliterated infected foci produced by virus particles responsible for slowly expanding plaques. Coalescence was not primarily responsible for overcrowding at this level. This is indicated by the inability of permanent plaque marking to reduce overcrowding and the lack of overcrowding at this plaque level with the Mahoney LP virus which produced more uniformly expanding plaques.

When plaques were marked temporarily and 98% of the potential plaques had formed, apparent overcrowding with Coxsackie A9 virus resulted from coalescence at counts above approximately 40 to 50 per cell sheet. Overcrowding was not suggested on cell sheets with permanently marked plaques until counts above 120 per cell layer were reached. At this level, plaque obscuring may be responsible for additional overcrowding.

Although no plaque obscuring was evident with Coxsackie A9 virus at counts below 120 plaques per bottle, obscuring of Mahoney LP plaques began at a much lower level. Both viruses produced uniformly expanding plaques. However, Mahoney LP plaques expanded at a more rapid rate and did not reach near-maximal numbers until the third counting day, whereas near-maximal counts of Coxsackie A9 plaques appeared by the second counting day. Envelopment of late-forming infected foci by expanding Mahoney LP plaques may account, in large part, for the plaque obscuring at counting levels of 60 to 70 plaques per bottle with this virus, while no plaque obscuring occurred with Coxsackie A9 virus at counting levels below 120 per bottle.

The sizes of plaques at near-maximal noncrowded levels, their number, and the area of the cell sheets on which they form are the major factors which define overcrowding. Nevertheless, it seems impossible to determine a precise relationship among these three factors that would allow the composition of a mathematical formula permitting the prediction of maximal noncrowded counts; there seems to be no way to include such factors as rate of plaque appearance and expansion, and plaque morphology in such an equation.

The problem of obtaining maximal noncrowded counts without extensive preliminary work is difficult. The scope of the complexity is suggested by the 35 to 40 plaque maximum per cell sheet (4,500 mm^2) which we obtained with Mahoney virus when contrasted to the 400+ plaque maximum per cell sheet (1,964 mm^2) obtained by Postlethwaite (1960) with vaccinia virus. Vaccinia plaques were less than 1.3 mm^2 in area when maximal counts were reached.

Certainly, the arbitrary limit of 40 to 50 plaques per cell sheet (2,828 mm^2) set by others (Dulbecco and Vogt, 1954) is an over-simplification. This limit is based on the assumption that significant overcrowding occurs only as a result of coalescence when neighboring plaques are separated by less than 3 mm at their centers.

In view of the present work, however, the following recommendations seem to be in order. (i) Ascertain that virus stocks to be used for quantitative work contain only particles which produce uniformly appearing and expanding plaques. (ii) Count plaques beginning on the first day of appearance and mark them permanently. Plaques appearing on subsequent days should be added to those counted earlier. (iii) Assume that maximal noncrowded counts occur when about 40% of the cell sheet is covered by plaques (with no adjustment for overlapping).

ACKNOWLEDGMENT

We are indebted to Robert T. Hyde for careful editorial review of the manuscript.

LITERATURE CITED

DULBECCO, R. 1952. Production of plaques in monolayer tissue cultures by single particles of an animal virus. Proc. Natl. Acad. Sci. U.S. **38**:747–752.

DULBECCO, R., AND M. VOGT. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exptl. Med. **99**:167–182.

HSIUNG, G. D., AND J. L. MELNICK. 1955. Plaque formation with poliomyelitis, Coxsackie, and orphan (Echo) viruses in bottle cultures of monkey epithelial cells. Virology **1**:533–535.

POSTLETHWAITE, R. 1960. A plaque technique for the titration of vaccinia virus in chick embryo cells and some features of vaccinial infection in this system. Virology **10**:466–482.

RAPPAPORT, C. 1956. Trypsinization of monkey-kidney tissue: an automatic method for the preparation of cell suspensions. Bull. World Health Organ. **14**:147–166.