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Characterization of an Infectivity Assay for the Ribonucleic Acid of Bacteriophage MS2[1]

JAMES H. STRAUSS, JR.,[2] AND ROBERT L. SINSHEIMER
Division of Biology, California Institute of Technology, Pasadena, California 91109

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An infectivity assay for MS2 ribonucleic acid (RNA), which uses bacterial spheroplasts of an F^- strain of *Escherichia coli*, has an efficiency of 10^{-5} infected spheroplasts per RNA molecule. The characteristics of this assay and the influence of several parameters are presented. Important variables include the duration of exposure of the cells to lysozyme-ethylenediaminetetraacetic acid, the duration of exposure of the spheroplast stock to the RNA solutions before dilution, the concentration of RNA, and the presence of competing RNA. The growth kinetics of the virus in the infected spheroplasts and the extent of lysis have also been studied.

This laboratory has used an infectivity assay for the ribonucleic acid (RNA) of the small bacteriophage MS2 in a variety of experiments, either as a test for the complete genome of the virus or to quantitate the number of RNA molecules present. Initially, the assay developed by Guthrie and Sinsheimer (9, 10) for the deoxyribonucleic acid (DNA) of phage ϕX174 was used, with the sole modification that the RNA was in 0.05 M tris-(hydroxymethyl)aminomethane (Tris) *p*H 7.0 instead of *p*H 8.1 (1, 3). The efficiency of this assay varies from about 2×10^{-8} to 5×10^{-7} infections per RNA molecule and is difficult to reproduce. This efficiency is the same as or slightly better than the results first reported by other investigators working with phage RNA (4, 11, 12, 14). In making this comparison, our unit is the RNA molecule, determined from absorbancy, rather than the phage plaque former; our viral stocks usually contain about 20% active plaque formers.

The report of Ginoza and Vessey (Abstr. FC5, Annual Meeting of the American Biophysical Society, 8th, 1964) led us to re-examine the RNA infectivity assay, and a more efficient and reproducible procedure was developed. A brief summary of the modified assay has been published (15). Recurring questions, however, lead us to publish the characteristics of this assay in more detail.

[1] The results presented here are part of a thesis by James H. Strauss, Jr., submitted to the California Institute of Technology in partial fulfillment of the requirements for the Ph.D. degree.

[2] Present address: Department of Biochemistry, Albert Einstein College of Medicine, Bronx, N.Y. 10461.

MATERIALS AND METHODS

PA, PAM, and 3XD are as described by Guthrie and Sinsheimer (10), except for an error in the composition of PA and PAM media. These should contain 1 g of glucose per liter. Bovine serum albumin (BSA) is a 30% sterile solution containing 0.1% NaN_3, as a preservative obtained from Armour Pharmaceutical Co., Kankakee, Ill. Tris chloride buffer (*p*H 7.0) is made by neutralization of a 1 M solution of Sigma Trizma Base with concentrated HCl to *p*H 7.0, at which time the temperature of the solution is 35 to 37 C.

The assay for bacteriophage MS2 is given in Davis and Sinsheimer (2), except that bacterial strain, *Escherichia coli* A19, was often used for seed bacteria. This strain, which lacks ribonuclease I, and strain Q13, which lacks both ribonuclease I and polynucleotide phosphorylase, were obtained from R. F. Gesteland (7).

The bacterial strain routinely used for the preparation of spheroplast stocks has been *Escherichia coli* K-12 strain W6. These F^- cells and the spheroplast stocks prepared from them are resistant to the mature virus, thus eliminating reinfection during the assay. All results refer to W6 spheroplast stocks unless noted otherwise.

Spheroplast stocks have also been prepared from the Hfr strains C3000, A19, and Q13. As these cells and the spheroplast stocks prepared from them are susceptible to the mature virus, reinfection by progeny phage will occur; therefore, such infected spheroplasts must either be assayed before reinfection occurs, or, alternatively, the time of incubation after infection must be carefully controlled. In the latter case, the assay is not strictly proportional to the concentration of RNA.

Cells to be converted to spheroplasts are grown in 3XD to 3×10^8 to 5×10^8/ml (as observed with a Petroff-Hausser counter) and collected by centrifuga-

tion at room temperature. For each 20 ml of original cell suspension, the following (in milliliters) are added at room temperature: 1.5 M sucrose, 0.35; 30% BSA, 0.17; lysozyme solution (2 mg/ml in 0.25 M Tris, *p*H 8.1), 0.02; and 4% disodium ethylenediaminetetraacetic acid (EDTA), 0.04. Addition of EDTA initiates the "conversion period," and subsequent addition of Mg^{++} terminates it. For conversion periods of 1 min or less, the Mg^{++} is added as 9.6 ml of PAM medium. For conversion periods of 1 min or more, 9.6 ml of PA medium is added immediately after EDTA, followed by 0.2 ml of 10% $MgSO_4$ after the desired conversion period. All spheroplast stocks are routinely chilled in ice water before use.

Infection of the spheroplast stock with MS2 RNA is initiated by adding 0.4 ml of stock to 0.4 ml of the RNA suspension. Normally the RNA is in 0.05 M Tris (*p*H 7.0), in a 37 C water bath. The "infection period" is terminated and the "incubation period" is initiated by dilution of this infection mixture with 3.2 ml of PAM medium to give the "incubation mixture". B-D Cornwall Continuous Pipetting Outfits (Becton, Dickinson and Co., Rutherford, N.J.) are used for dispensing both the spheroplast stock and PAM medium.

The infectivity of the RNA preparation is measured either by direct assay of the infected spheroplasts, or by assay of the progeny phage produced. Infected spheroplasts are assayed after a 10- to 15-min incubation period, by use of the method of Guthrie and Sinsheimer (10). The assay based upon production of progeny phage uses an incubation period of 1.5 to 3 hr. The infected spheroplasts are then lysed either by freeze-thawing (once), by dilution into distilled water for 10 min, or by addition of a few drops of $CHCl_3$ and vigorous mixing; the released phage are then titered.

The results are expressed as infected spheroplasts per RNA molecule or as progeny plaque-forming units (PFU) per RNA molecule. The latter figure can be related to the former by the average burst size as measured in single-burst experiments.

Single-burst experiments were performed by use of RNA at a concentration such that progeny phage were present in only 10 to 25% of the tubes.

The preparation of MS2 RNA has been described (16). The concentration of RNA is expressed in molecules per milliliter, determined from the absorbancy of the RNA preparation. Tobacco mosaic virus (TMV) was prepared from infected tobacco leaves by the method of Simmons, as described in Friesen and Sinsheimer (6). RNA was isolated from TMV by the method of Fraenkel-Conrat et al. (5). Lysates of *E. coli* C3000, prepared by lysozyme-EDTA treatment of the cells and subsequent lysis with a neutral detergent (8), were kindly provided by G. N. Godson. Ribosomal RNA was prepared by phenol extraction of ribosomes isolated from these lysates.

RESULTS

Preparation of spheroplast stocks. Short periods of conversion of cells to spheroplasts consistently provided more efficient stocks, and the presence of BSA improved the measured infectivity of RNA preparations. The data in Table 1 illustrate these observations.

TABLE 1. *Effect of the duration of the conversion period on the efficiency of the spheroplast stock*

Expt[a]	Conversion period (min)	PFU/RNA molecule	
		+BSA	−BSA
1	1	1.7×10^{-4}	0.28×10^{-4}
	3	1.6×10^{-4}	0.24×10^{-4}
	6	1.4×10^{-4}	0.27×10^{-4}
	10	0.6×10^{-4}	0.17×10^{-4}
	20	0.6×10^{-4}	0.15×10^{-4}
2	0.5	1.4×10^{-4}	0.16×10^{-4}
	1	0.6×10^{-4}	0.11×10^{-4}

[a] Infection period was 10 min at 35 C, and the incubation period was 90 min at 35 C.

When the infection period was shorter than in Table 1 (see below), the conversion period was still optimal at about 30 sec. In this case, omission of the BSA led to a more drastic reduction in efficiency.

Spheroplast stocks prepared with the short conversion period contained less than 50% completely rounded forms by microscopic examination. Most of the cells were affected by the lysozyme treatment, however, as evidenced by partial rounding. The efficiency of these stocks was stable for at least 24 hr in the cold.

The best results were obtained with the Armour 30% solution of BSA; 30% solutions of BSA made from both Armour and Sigma Fraction V powder were also tried with much poorer success.

When the cell concentration of W6 is allowed to go beyond 5×10^8/ml, the resulting spheroplast stocks are definitely less efficient.

Spheroplast stocks prepared from the Hfr strains C3000, A19, and Q13 were about 10% as efficient as those prepared from the F^- strain W6.

Infection and incubation periods. The length of the infection period is critical. The result of varying the length of the infection period with several spheroplast stocks is shown in Fig. 1. The sharpness of the optimum depended in part upon the concentration of the stock, and less concentrated stocks provided a broader plateau. The decline in progeny phage titer as the infection period was lengthened was a consequence of a loss of infected spheroplasts, for single-burst experiments indicate that the average burst size was the same for infection periods of 30 sec and 10 min.

The best results were obtained by mixing equal volumes of RNA suspension and spheroplast

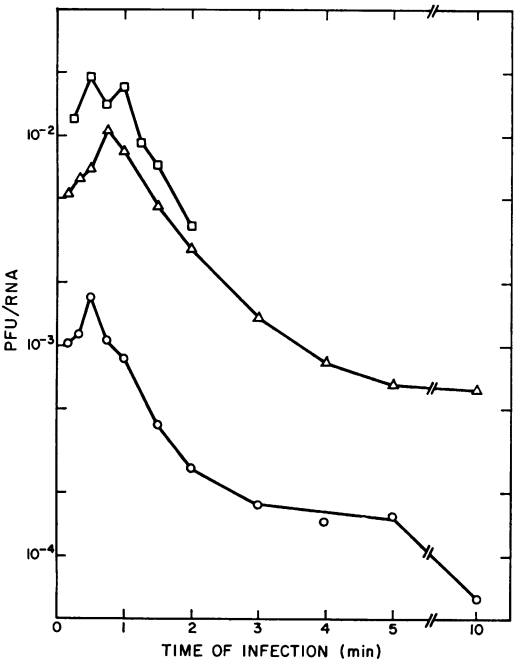

FIG. 1. *Effect of the duration of the infection period on the progeny phage titer. Infection and incubation were at 37 C. The concentrations of the spheroplast stocks and the lengths of the incubation periods in the three experiments were: 1.2 × 10^9/ml, 90 min (○); 8 × 10^8/ml, 90 min (△); and 5.4 × 10^8/ml, 3 hr (□).*

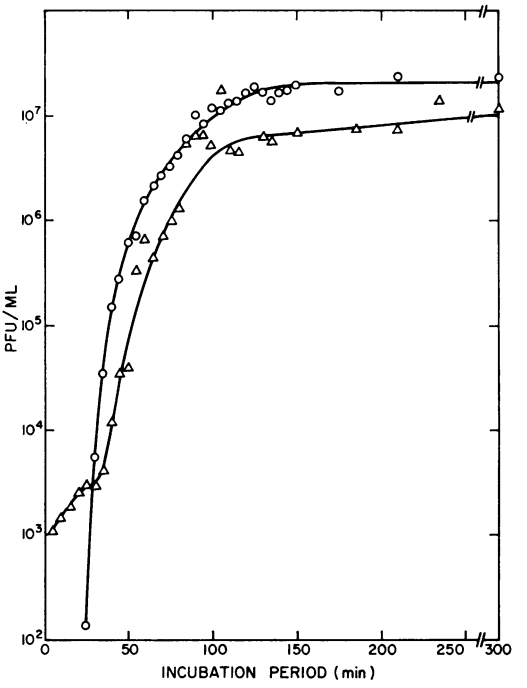

FIG. 2. *Growth curve of MS2 in RNA-infected spheroplasts. MS2 RNA was used at 5 × 10^{10} molecules/ml. Infection was for 30 sec at 37 C. Incubation was also at 37 C. At intervals, samples were removed and infective centers were assayed without artificial lysis of the infected spheroplasts (△), or after lysis with $CHCl_3$(○).*

stock to initiate infection. Large deviations from equal volumes sharply decreased the infectivity of MS2 RNA. Maximal production of phage required dilution of the infection mixture at least fivefold with PAM medium before incubation; further dilution was without effect.

The optimal temperature for incubation was 36 to 38 C. Incubation at lower temperatures decreased the burst size. The infection period may be conducted at 30 C or 37 C with equal efficiency (with proper adjustment in the length of the infection period), as long as the incubation period is conducted at 37 C.

Maximal phage production required 2.5 to 3 hr of incubation at 37 C. The phage titer was then stable for at least another 2 hr (Fig. 2).

Tris and phosphate buffers of varying ionic strength were tested as the RNA solvent. Buffer concentrations above 0.05 M were sharply inhibitory to the assay. The results with buffer concentrations from 0.001 to 0.05 M were somewhat dependent upon the spheroplast stock. Most often an optimum at 0.005 to 0.01 M was observed with little difference between Tris and phosphate buffers. The results of two experiments showing this effect are given in Table 2.

TABLE 2. *Influence of the buffer concentration of the RNA solvent on progeny PFU produced*

Expt[a]	Buffer concn (M)	PFU/RNA molecule	
		Tris	Phosphate
1	0.01	4.7×10^{-3}	4.7×10^{-3}
	0.05	2.6×10^{-3}	1.2×10^{-3}
	0.1	0.7×10^{-3}	0.1×10^{-3}
	0.2	—	10^{-6}
2	0.001	6.2×10^{-3}	6.2×10^{-3}
	0.005	9.2×10^{-3}	8.5×10^{-3}
	0.01	10.4×10^{-3}	6.2×10^{-3}
	0.05	7.0×10^{-3}	2.4×10^{-3}

[a] Infection period was 30 sec at 37 C, and the incubation period was 3 hr at 37 C.

RNA solvents at three different values of *p*H were tested. No difference was found between 0.05 M Tris at *p*H 7.0 or 8.1; 0.05 M sodium acetate at *p*H 5.0 was an order of magnitude less efficient than 0.05 M Tris at *p*H 7.0.

The presence of up to 10% of the organic sol-

vents formamide, dimethylformamide, or dimethylsulfoxide in the RNA solvent had little effect on the assay (not more than 30% inhibition).

Growth of the virus and lysis of the spheroplasts. A growth curve of MS2 in RNA-infected spheroplasts is shown in Fig. 2. By analogy with growth curves of phage-infected bacteria, the "eclipse period" is 25 to 30 min. Lysis of some of the infected spheroplasts began 5 to 10 min later; phage production and lysis continued for about 2 hr. In this experiment, the infected centers apparently increased between 5 and 20 min, but more frequently the infected-center count was constant between 5 and 25 min after infection.

About one-third of the infected W6 spheroplasts eventually lysed spontaneously (Fig. 2). This lysis took place in the incubation tube and was not produced in the plating procedure. Experiments with A19 spheroplasts, which are infectible by the mature virus, indicate that about one-third of the infected A19 spheroplasts similarly lysed in the incubation tube. Growth curves of MS2 in RNA-infected A19 spheroplasts did not begin to level off until after 3 hr of incubation; there was then a slower rise in phage titer for at least another 2 hr. After 5 hr, apparent burst sizes of 10^8 were produced, because some of the infected spheroplasts lyse, leading to multiple infection cycles. A single-burst experiment was performed to quantitate the fraction of infected A19 spheroplasts which lyse. In this 100-tube experiment, 75 tubes contained no phage. Of the 25 tubes which did contain phage, 17 tubes had burst sizes from 35 to 3,800 and an average burst size of 1,400. The remaining tubes had apparent burst sizes from 1.6×10^6 to 6×10^9 and an average apparent burst size of 9×10^8. Thus, two-thirds of the bursts had reasonable values for a single burst; in the other one-third, lysis of the original infected spheroplast was clearly followed by subsequent reinfection cycles.

Comparison of the assay for infected spheroplasts and the assay for progeny phage. Plating for infected spheroplasts was one-tenth to one-third as efficient per infective center, as was the assay for progeny phage. The number of infected spheroplasts may be calculated from the final phage titer, by use of the burst size. For W6 spheroplasts, the burst size was 2,000 in a number of single-burst experiments. To determine a burst size for A19 spheroplasts, the concentration of infective centers was calculated from the frequency of bursts in a single-burst experiment (assuming a Poisson distribution). This concentration, together with a growth curve of MS2 in RNA-infected A19 spheroplasts, defines an apparent burst size as a function of the duration of the incubation period.

The number of infected spheroplasts calculated in either case from the progeny phage titer was usually threefold higher than that measured by direct plating.

The top agar used in the assay for infected spheroplasts was PAM medium containing BSA and agar. By analogy with the conditions in liquid medium, it is probable that only one-third of the infected spheroplasts lysed on the plate, leading to the observed lower efficiency.

Effect of RNA concentration on the assay. A typical dilution curve of MS2 RNA is shown in Fig. 3. Duplicate points have been included to illustrate that reproducibility was usually quite good. Phage production is linear with RNA concentration over at least four orders of magnitude. The dilution curve of MS2 RNA (Fig. 3) becomes nonlinear in the concentration range of 10^{11} to 10^{12} molecules/ml (or 10^{10} to 10^{11} molecules/ml upon dilution into the incubation mixture as presented in Fig. 3), and is completely saturated by 10^{12} molecules/ml. As 0.004 absorbancy units correspond to 10^{11} MS2 RNA molecules/ml, the infection is saturated when the RNA concentration is in the range 0.004 to 0.02 absorbancy units/ml.

With the better stocks, about 1% of the spheroplasts can be infected with saturating amounts of RNA.

Inhibition of the assay by TMV RNA and *E. coli* ribosomal RNA is shown in Fig. 4. Inhibition began when the competing RNA concentration was about 0.004 absorbancy units/ml. When the competing RNA concentration was greater than 0.004 absorbancy units/ml, the inhibition was

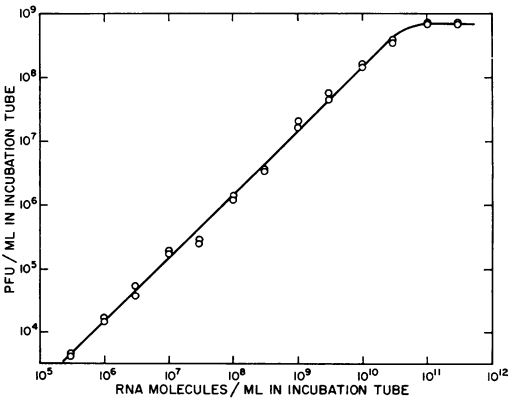

FIG. 3. *Dilution curve of MS2 RNA. Infection was for 30 sec at 37 C, and incubation was for 90 min at 37 C. The line shown has a slope of 1.00 and corresponds to* 1.5×10^{-2} *PFU per RNA molecule.*

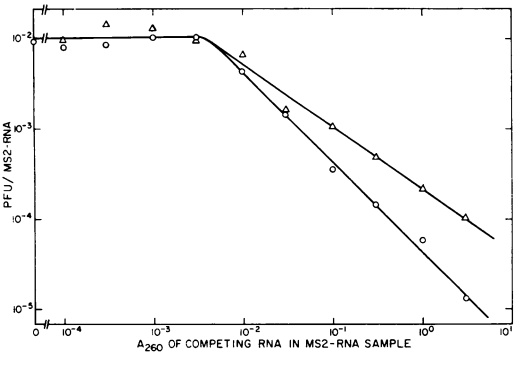

FIG. 4. *Inhibition of the RNA infectivity assay by TMV RNA and ribosomal RNA. MS2 RNA at a concentration of 10^{10} molecules/ml was assayed in the presence of increasing amounts of TMV RNA (○) or Escherichia coli ribosomal RNA (△). Infection was for 30 sec at 37 C, and incubation was for 3 hr at 37 C.*

linear with increasing concentration in the case of TMV RNA. This implies a strict competition between the various RNA for uptake or any other limiting factor. For *E. coli* ribosomal RNA, however, the inhibition goes as the 0.7 power of the concentration, indicating that MS2 RNA has a competitive advantage.

The influence of external ribonuclease on the assay. The release of ribonuclease I into the medium during conversion of *E. coli* cells to spheroplasts has been reported by Neu and Heppel (13). Semiquantitative measurements of the amount of ribonuclease in these spheroplast stocks indicate the presence of activity equivalent to that of about 15 mμg/ml of pancreatic ribonuclease. Of this amount, roughly one-third is contributed by ribonuclease contamination of the commercial BSA.

However, the optima in the lengths of the conversion period and the infection period are not a result of this ribonuclease activity. Spheroplast stocks formed from the strains A19 and Q13, which lack ribonuclease I, show the same optima. Furthermore, removal of ribonuclease in the BSA by treatment with bentonite has little effect on these optima for spheroplasts of W6, A19, or Q13 (a slight protection was noted in a 10-min infection period).

DISCUSSION

The efficiency of the modified assay, by use of the F^- strain W6, has varied from 2×10^{-2} to 2×10^{-3} progeny PFU per RNA molecule. This can be restated as 10^{-5} to 10^{-6} infected spheroplasts per RNA molecule. With care, the efficiency is normally the higher value. Glowacki (*personal communication*) has obtained an efficiency of 2×10^{-2} PFU/RNA molecule with this method for both MS2 RNA and Qβ RNA.

We have found that the presence of BSA stimulates the RNA assay. Knolle and Kaudewitz (12) and Ginoza and Vessey (Abstr. FC5, Annual Meeting of the American Biophysical Society, 8th, 1964) found that BSA had no effect. Our finding that Fraction V BSA will not substitute for the Armour 30% solution may have some bearing on this. Also, the more efficient the assay, the greater the stimulation by BSA.

The optima in the duration of the conversion period and the infection period for RNA infection, which are in contrast to observations concerning infection with the DNA of bacteriophage φX174 (9, 10; *unpublished observations*), appear to be related to the metabolic integrity of the cell and the dynamics of RNA-host interaction. In the case of φX DNA, longer periods of lysozyme-EDTA treatment increase the efficiency of nucleic acid uptake by the spheroplasts. The spheroplasts become less efficient for RNA infection with increased conversion period, presumably because of increasing structural damage to the cell which is of greater consequence than the possibility of increased uptake.

The optimum in the duration of the infection period means that RNA molecules which have been adsorbed and are capable of producing progeny have at least a 90% probability of becoming noninfective, unless the mixture is diluted quickly with PAM medium. φX DNA, however, is apparently stable under these conditions, and more infected spheroplasts accrue as the infection period is lengthened.

These empirical optima, however, provide only a partial explanation for the 1,000-fold difference in efficiency of infection with MS2 RNA as compared with φX DNA. In experiments with both W6 and A19 spheroplasts, the efficiency of DNA infection could be improved about 10- to 20-fold through the use of longer conversion and infection periods than those optimal for RNA infection.

Also, the ultimate capacity of the spheroplast stocks is the same in either case; about 1% of the cells can be infected with saturating concentrations of φX DNA (10) or MS2 RNA. Thus, at present, the difference in efficiency of the two nucleic acids cannot be explained.

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