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A Mathematical Analysis of Concomitant Virus Replication and Heat Inactivation

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A mathematical analysis of virus production with accompanying heat inactivation, from which the rate of virus release and total virus production are readilv calculated, is presented. Applications of this analysis for Sindbis and Chikungunya viruses are discussed.

Many viruses are heat labile, even at those temperatures which are optimal for their multiplication. In general, heat inactivation of infectivity follows first-order kinetics and the rate constant is temperature dependent. Significant loss of infectivity at 37 C occurs with influenza (11, 14), herpes (1, 20), Theiler's strain FA (12), Chikungunya (8), cytomegalic inclusion (22), mumps (9), rubeola (15, 19), rubella (16), respiratory syncytial (2), and avian tumor viruses (21). The half-life at 37 C varies widely, from 36 min to 9 hr, among these viruses (2, 3, 8, 9, 16, 21). Heat sensitivity is strongly influenced by the composition and *p*H of the suspending medium (4, 10, 13, 21). The addition of serum or other protein often decreases lability. Even so, rapid inactivation can occur while virus is being liberated from cells and is accumulating in the media. This makes a quantitative study of virus production invalid unless that virus which is produced and subsequently inactivated is taken into account. Rubin assumed a constant low rate of virus production in his analysis of virus yield and decay for Rous no. 1 chicken sarcoma (18). In contrast, this paper presents a mathematical analysis of virus production with concomitant virus inactivation. It provides a method for calculating the total virus in which no prior assumptions are made about the rate of release.

MATERIALS AND METHODS

Medium. Eagle's medium (6) modified with Hank's basal salts (7) was used throughout. In addition, the amino acid concentration was twice that originally recommended. This medium was supplemented with 3 to 5% newborn calf serum for fluid cultures.

Cells. Chick embryo (CE) monolayers were prepared from 10-day-old embryos (5). With 100-mm petri plates, we used 2×10^7 cells in 10 ml of Eagle's medium plus 5% calf serum, and, with 60-mm petri plates, we used 7×10^6 cells in 3.5 ml of medium. Plates were used within 24 hr.

Virus. Sindbis virus strain AR-339 was obtained from the American Type Culture Collection, and plaque-purified stocks were prepared in CE cell cultures. Stock virus was distributed in 1-ml amounts and was stored at −70 C.

Virus assay. Virus dilutions were made in cold Hanks' balanced salts solution (BSS; 7) containing 1% calf serum and were kept in an ice bath. Samples (0.5 ml) of each dilution were added to CE plates in triplicate and were allowed to adsorb for 1 hr at 37 C. During the adsorption period, the plates were tilted every 15 min. After adsorption, the monolayers were overlaid with Eagle's medium enriched with 3% calf serum and solidified with 1% Ionagar (Oxoid). Plates were then inverted and incubated at 37 C for 48 hr in an atmosphere of 10% CO_2. After incubation, 8 ml of neutral red (0.1 mg/ml) in BSS was added per plate and removed after 30 min of incubation. The plates were inverted and incubated at 37 C for an additional 2 hr. Virus titers were expressed as plaque-forming units (PFU) per ml.

Heat inactivation. The survival of infectious virus was determined by suspending virus in Eagle's medium supplemented with 5% calf serum, incubating the suspension at 37 C, and removing samples for assay at appropriate intervals.

Growth curves. In one series, CE monolayers on 60-mm petri plates were inoculated with 0.2 ml of virus, and, after 1 hr of adsorption, 5 ml of Eagle's medium supplemented with 3% calf serum was added per plate. At intervals thereafter, fluids from duplicate plates were collected, pooled, and stored at −70 C until assayed.

In the second series, five CE monolayers on 100-mm petri plates were inoculated with 0.5 ml of virus, held for attachment for 1 hr at 37 C, washed three times with BSS plus 1% calf serum, and overlaid with Eagle's medium containing 5% calf serum. At intervals thereafter, 0.5-ml samples of fluid were removed, pooled, and stored at −70 C. Samples of fresh medium (0.5 ml) were returned to each plate, and compensations were made for this dilution when titers were determined.

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Results

Heat inactivation data for Sindbis virus at 37 C in Eagle's medium enriched with 5% calf serum are shown in Fig. 1. At any given time, the inactivation rate of infectious units was proportional to the number of survivors. This is similar to the death rate of bacteria (17). Since the half-life of infectious Sindbis virus under these conditions was approximately 4 hr, inactivation must be considered when interpreting growth data for this virus.

Sindbis virus growth in CE monolayers is shown in Fig. 2 and 3. Initially, the virus present is that inoculum not removed by washing. After a period of little or no virus increase, there was an exponential rise, followed by a plateau. Such a plateau is common in virus growth: when the virus is stable, the plateau indicates the cessation of virus production; with a heat-sensitive virus, the plateau reflects an equilibrium between virus release and virus inactivation. Thus, the plateau seen in Fig. 2 after 10 hr indicates a continued liberation of virus balanced against virus inactivation. A decrease in virus became apparent only after 24 hr. On the other hand, the titers in Fig. 3 leveled off and then dropped. This decrease in infectious virus during the final 8 hr resembled the inactivation data presented in Fig. 1, suggesting that virus production had halted and that the virus which had been produced was being inactivated at the expected rate.

If virus particles are being inactivated throughout the 24 hr period, then infectivity assays do not represent the total virus produced in this interval. Instead, each assay represents the plaque-forming virus which has been released and has escaped heat inactivation. When compared to the maximal virus titer, the initial virus concentration is negligible and, therefore, its inactivation does not contribute significantly to the difference between total virus and detectable virus. In addition, early in the period of exponential growth there has been little time for inactivation, and the experimental growth curve would be expected to approximate closely total virus produced. However, with increasing time, inactivation of released virus becomes more and more significant, so that a mathematical analysis of the data is necessary to determine the total virus produced.

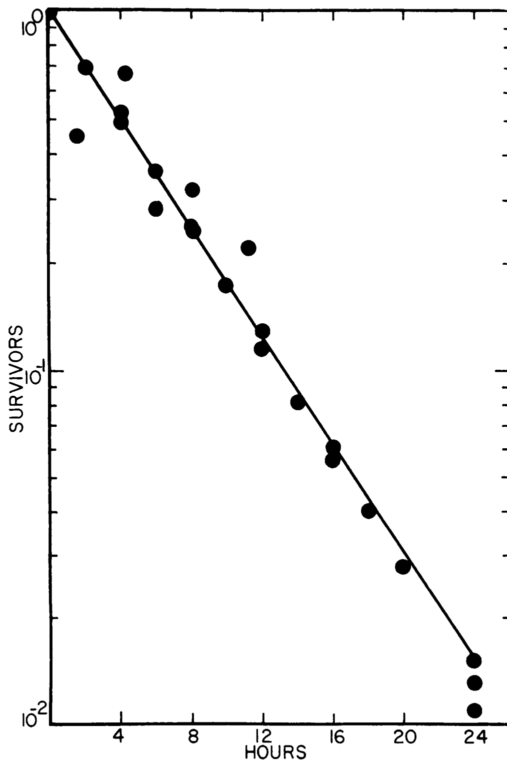

FIG. 1. *Inactivation of Sindbis virus at 37 C. Virus was suspended in Eagle's medium plus 3% calf serum. Samples were removed at intervals and were assayed for infectious virus on CE monolayers.*

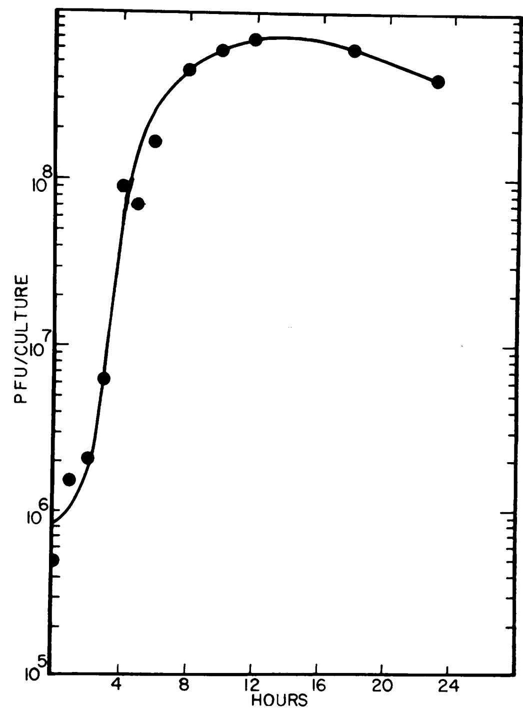

FIG. 2. *Growth curve of Sindbis virus in CE monolayers. Input multiplicity was 5 PFU/cell. Each point represents virus in pooled samples from five plates.*

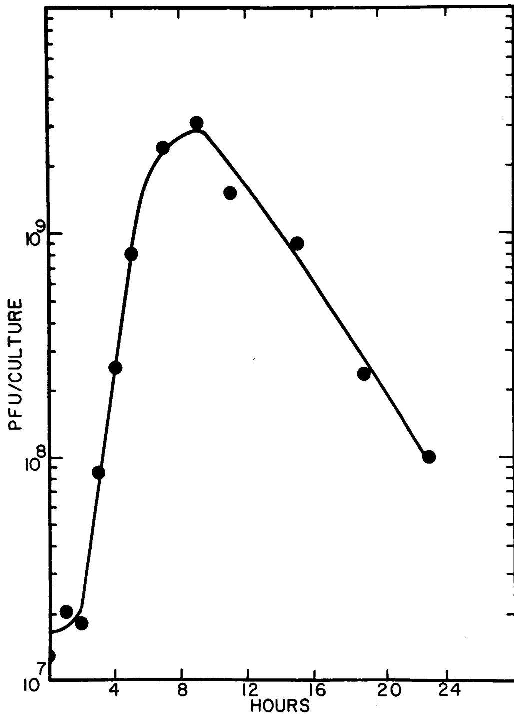

FIG. 3. *Growth curve of Sindbis virus in CE monolayers. Input multiplicity was 10 PFU/cell. Each point represents an assay of the fluids from duplicate plates.*

ANALYSIS

Definition of terms. (i) $F(t)$: let $F(t)$ denote the fraction of virus escaping inactivation after t hours at 37 C. $F(t)$ = PFU at time t/PFU at time 0. Thus, $0 \leq F(t) \leq 1$. $F(0) = 1$, i.e., at the beginning of the inactivation period, no virus has been inactivated. The inactivation data with Sindbis virus, which are a plot of log $F(t)$ versus time, are approximated by a straight line. Therefore, $F(t) = 10^{at+b}$ and, since the line slopes down, $a < 0$. The y-intercept $b = 0$. (ii) $G(t)$: let $G(t)$ denote the number of PFU which are detectable at time t. Thus, a plot of $G(t)$ versus time is the experimentally determined growth curve. (iii) $H(t)$: let $H(t)$ denote the total PFU released from t_0 (onset of virus release) to time t, including those PFU which have been inactivated and are not experimentally detectable at time t. Thus, $H(t_2) - H(t_1)$ = the number released from time t_1 to time t_2, and $H'(t)$ is the rate of virus release per hour at time t. Notice that here and in the following derivation, prime notation is used. $H'(t)$ is the same as $dH(t)/dt$. (iv) Q: let Q denote the number of PFU present initially (residual virus).

Rate of virus production. Consider a growth curve. Until t_0, no virus is released, i.e., $H'(t) = 0$ for $0 \leq t < t_0$. The amount of virus detectable at any time $t > t_0$; i.e., $G(t)$ depends on the fraction of virus present initially which has not been inactivated, $QF(t)$, plus the total virus released from t_0 to t, minus those PFU which have been inactivated from t_0 to t. The portion of the growth curve after the onset of virus release t_0 can be subdivided into very small intervals, and the virus contributed to $G(t)$ by each of these intervals is $F(t-x)\Delta H(x)$, where $t > t_0$. Therefore

$$G(t) \simeq QF(t) + \sum_{x=t_0}^{x=t} F(t-x)\ \Delta H(x)$$

$$G(t) = QF(t) + \int_{t_0}^{t} F(t-x)\ dH(t)$$

But
$$F(t-x) = 10^{a(t-x)} = 10^{at}\,10^{-ax} = F(t)/F(x)$$

and
$$dH(x) = H'(x)dx$$

$$G(t) = QF(t) + \int_{t_0}^{t} F(t)\frac{H'(x)}{F(x)}\,dx$$

Since $F(t)$ is constant with respect to the integral, the equation simplifies to:

$$\frac{G(t)}{F(t)} = Q + \int_{t_0}^{t} \frac{H'(x)}{F(x)}\,dx$$

$$\left(\frac{G(t)}{F(t)}\right)' = \frac{H'(t)}{F(t)}$$

$$\frac{F(t)\,G'(t) - G(t)\,F'(t)}{[F(t)]^2} = \frac{H'(t)}{F(t)}$$

$$\frac{F(t)G'(t) - G(t)F'(t)}{F(t)} = H'(t)$$

$$G'(t) - G(t)\frac{F'(t)}{F(t)} = H'(t)$$

But $F'(t) = a\ ln\ 10\ F(t)$, so

$$G'(t) - aG(t)ln\ 10 = H'(t)$$

The only method available to determine $G'(t)$ is graphical. Since $G(t)$ has a large range, it is impossible to determine $G'(t)$ with any accuracy unless $G(t)$ is plotted as a logarithmic function. The following alters the equation so that $G'(t)$ can be determined from a graph of $G(t)$ on semilog paper or from a plot of log $G(t)$ versus time. Let $g(t)$ denote the function of t so that $G(t) = 10^{g(t)}$. By substituting $10^{g(t)}$ for $G(t)$, an equation is obtained for $H'(t)$, the rate of virus production at time t. The slope $g'(t)$ can be determined directly from a graph of log $G(t)$ versus time plotted on a Cartesian coordinate system.

$$\text{Since } G(t) = 10g(t),\ G'(t) = G(t)g'(t)1n10$$

$$H'(t) = G(t)g'(t)1n\ 10 - aG(t)1n10$$

$$H'(t) = G(t)1n10\ (g'(t) - a)$$

$$H'(t) = 2.3G(t)\ (g'(t) - a)$$

The rate of virus production at any time t where $t > t_0$ can be calculated from this equation. Let $R(t)$ denote

$H'(t)$; i.e., $R(t)$ is the rate of virus production at time t.

$$R(t) = 2.3\, G(t)\, [g'(t) - a]$$

Total virus. The total virus $H(t)$ can be calculated graphically. If $R(t)$ is plotted against time in hours, by definition the area bounded by (i) the positive x-axis, (ii) the graph $R(t)$, (iii) a vertical line through t, and (iv) a vertical line through t_0 (a time just after the onset of virus release) is the total virus produced up to time t, i.e., $H(t)$. This area can be determined graphically with a planimeter. It should be noted that the choice of t_0 is made arbitrarily and must be as close to the onset of virus release as is possible. Total virus can be calculated for appropriate time intervals, and a growth curve can be plotted from these results. Note that total virus refers to virus which was released from cells as infectious virus and includes heat-inactivated virus as well as the remaining infectious virus. It does not refer to incomplete or initially defective viruses.

Example. Total virus production for a heat-sensitive virus can be determined from its growth curve and inactivation rate. With Fig. 1 providing the latter, the rates of virus release in PFU per cell per hour were determined for each assay period in the growth curves in Fig. 2 and 3 from the formula:

$$R(t) = 2.3\, G(t)\, [g'(t) - a].$$

In addition, total virus yield was calculated as described previously for each assay period. The relationship among total virus, observed virus, and the rates of virus release is shown in Fig. 4 and 5 for the growth curves in Fig. 2 and 3, respectively. As would be expected, virus production halted before 12 hr in the growth curve in Fig. 3, whereas it was maintained throughout the 24-hr period in Fig. 2.

With prolonged virus production, virus measured at 24 hr represented 20% of the total produced. Where virus production ceased early, virus detected at 24 hr constituted only 2% of the total produced.

Example 2. This analysis permits quantitative interpretation of data obtained with other viruses in similar situations where inactivation accompanies production. Data amenable to such analysis frequently appear in the literature. For example, Heller (8) described the effect of actinomycin D on the growth of Chikungunya virus in CE monolayers. Figure 6 is a reproduction of these data (reprinted here with permission of the publishers, Academic Press, Inc., New York). In the control culture, the maximal virus titer was reached at 6 hr, fell slightly, and then declined rapidly. Heller ascribed this loss of infectivity to heat inactivation alone. Figure 6 provides sufficient data for analysis. Total virus was calculated for the control as well as for the actinomycin D-treated culture. Figure 7 depicts the relationship between total and observed virus titers. Significantly greater amounts of virus were produced in the actinomycin D-treated cells. This difference is not as dramatic as is suggested in Fig. 6. At 20 hr, because of heat decay, the 10-fold difference appeared as greater than 1 million-fold. The determination of total virus may not affect the validity of Heller's interpretation; it does present a more realistic appraisal of virus production.

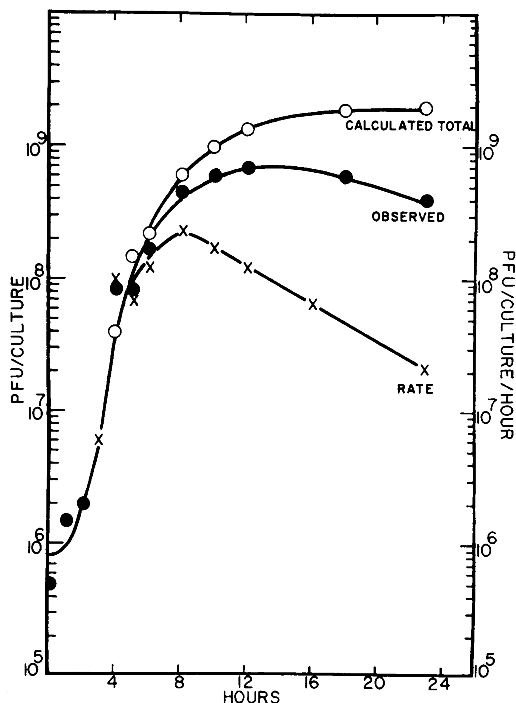

FIG. 4. *Relationship among calculated rate of virus release, calculated total virus, and observed virus for growth curve of Sindbis virus in Fig. 2.*

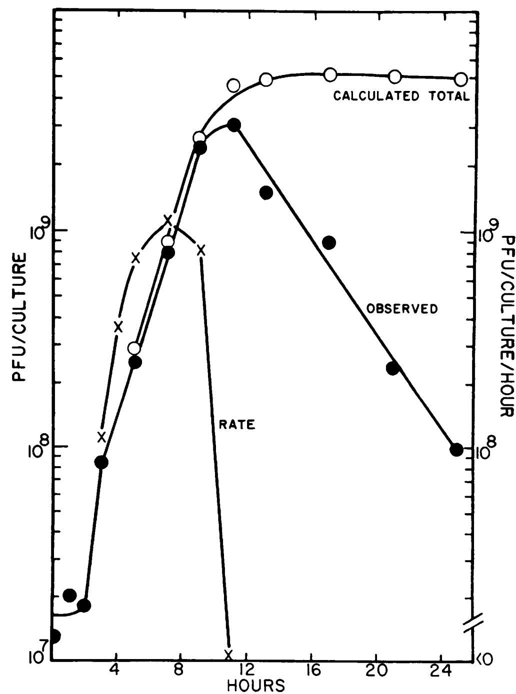

FIG. 5. *Relationship among calculated rate of virus release, calculated total virus, and observed virus for growth curve of Sindbis virus in Fig. 3.*

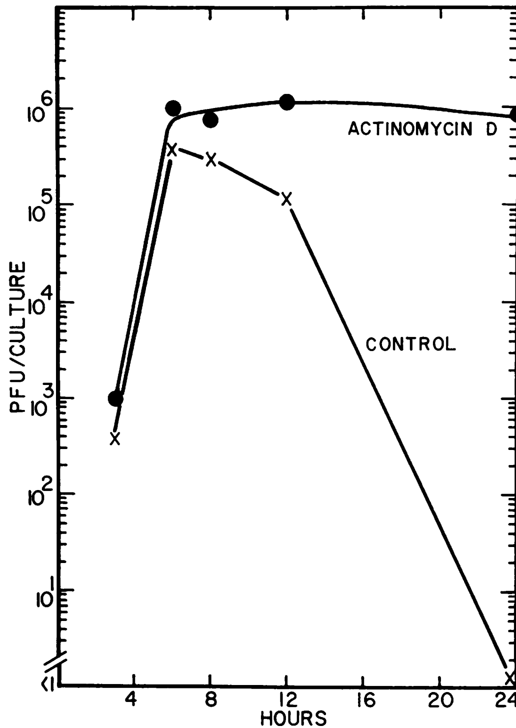

FIG. 6. *Growth curves of Chikungunya virus in CE monolayers. (After Fig. 1, E. Heller, Virology* **21**:*654, 1963.)*

Direct experimental evidence to support this analysis can be obtained by determining virus particle numbers as well as infectious units. Such studies are now in progress.

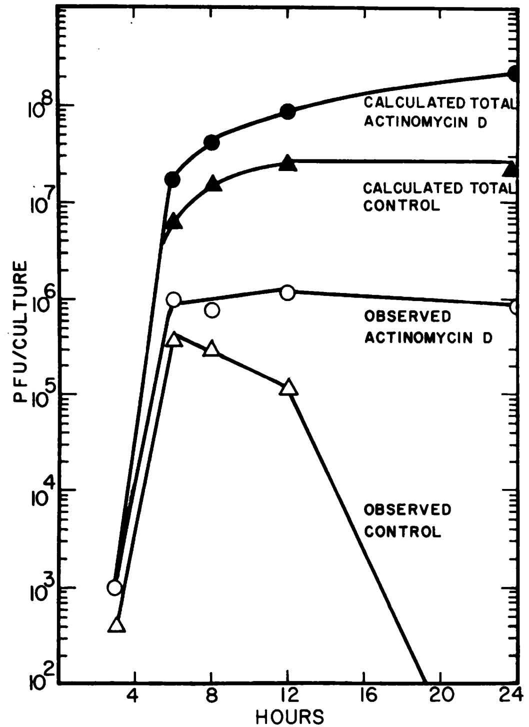

FIG. 7. *Relationship between the calculated total virus and observed virus in growth curves of Chikungunya virus. Calculations are based on the growth curves in Fig. 6. (After Fig. 1, E. Heller, Virology* **21**:*654, 1963.)*

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