KINETICS OF CELL INFECTION AND PENETRATION BY THE VIRUS OF FOOT-AND-MOUTH DISEASE

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ABSTRACT

THORNE, H. V. (Research Institute, Animal Virus Diseases, Pirbright, England). Kinetics of cell infection and penetration by the virus of foot-and-mouth disease. J. Bacteriol. **84**:929-942. 1962.—The kinetics of cell infection by the virus of foot-and-mouth disease were determined, using acidification to pH 5.7 to eliminate virus from the system and to stop the reaction at required times. The infection curve was sigmoidal in form with an initial lag stage followed by a linear region of slope, at moderate concentrations, proportional to the virus and cell concentrations. The infection rate was very small below 15 C but increased rapidly with increasing temperature. Cell infection was 90% complete in about 3 min at 37 C and about 20 min at 25 C. By comparison with kinetic equations derived for a simple model of the infection process, adsorption and penetration rate constants were calculated. The adsorption rate constants were of the same order as those obtained from direct measurements of virus adsorption. The half-time for the penetration reaction was about 30 sec at 37 C and 6 min at 20 C; the rate decreased to near zero values below 15 C. The energy of activation for penetration was 24,000 cal/mole.

A study of the kinetics of infection of cells by animal viruses provides a means for exploring the kinetics and mechanism of virus penetration as well as being important for timing the following events of virus multiplication. Penetration is considered here to be the reaction between cell and virus particle, following adsorption, during which the virus particle loses normal infectivity and initiates the multiplication process. The number of cells infected at any time represents the number of cells into which virus has penetrated, the rate of infection of the cell population being determined by the over-all rate of the adsorption and penetration reactions. If allowance can be made for the contribution of the adsorption time, it should be possible to derive a rate constant, characteristic of the penetration reaction alone, from the rate of cell infection.

To determine the number of infected cells in a cell-virus mixture, it is necessary to eliminate virus particles in the liquid medium and attached to the cells; the speed of this elimination determines the accuracy with which the kinetics of a penetration reaction can be followed. Specific neutralizing antiserum of high titer eliminates free virus rapidly, but neutralization of adsorbed virus is often incomplete, particularly at high virus concentrations, and the procedures for removal of antiserum are disadvantageous.

The rapid inactivation of the virus of foot-and-mouth disease (FMD) at acid pH values permits the elimination of extracellular and cell-associated virus by momentary acidification of the medium to pH values in the range 5.5 to 6.0 (Cartwright and Thorne, 1958), and use of this method indicated that penetration of this virus was very rapid at 37 C. A more extensive investigation of the kinetics of infection of pig-kidney cells in suspension at several temperatures is recorded in this paper, and an attempt is made to characterize separately the kinetics of the process of penetration by comparing the results with the predictions of a theoretical model of the infection process. A summary of the results was presented previously (Thorne, 1961).

MATERIALS AND METHODS

Media. The growth medium for cells, and the medium normally used in studies of cell suspensions, was Earle's saline containing 0.01% yeast extract, 0.5% lactalbumin hydrolysate, and 10% ox or sheep serum (medium A). Monolayers in petri dishes were grown in an atmosphere of 5% CO_2 and air. The medium used for production of virus was medium B, i.e., medium A without

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serum. Agar overlay consisted of medium B to which were added 1.5% Difco agar and 5% ox or sheep serum. Phosphate-buffered saline (PBS) was the diluent for virus titrations (Dulbecco and Vogt, 1954). Penicillin (100 units/ml), streptomycin (100 units/ml), polymyxin (100 units/ml), nystatin (40 units/ml), and phenol red (1:100,000) were incorporated in all media.

Cell cultures. Pig-kidney monolayers were prepared essentially by the methods described by Sellers and Stewart (1959); these cultures were considerably variable both in growth and in sensitivity to virus. Secondary monolayers in 6-cm or 10-cm petri dishes were used for assay. Cell suspensions were prepared from primary monolayers grown (5 to 7 days) in Roux bottles. The monolayers were washed with Ca- and Mg-free PBS, treated with an ethylenediaminetetraacetic acid (EDTA)-trypsin mixture (0.01% EDTA plus 0.01% trypsin in Ca- and Mg-free PBS) for 15 to 30 min at 37 C, and the cells collected by centrifugation. The cells were washed and suspended in medium A and counted in a Fuchs-Rosenthal counting chamber to at least 400 cells. Counts in the presence of 0.1% trypan blue showed that 95% or more of the cells were unstained. Suspensions of the Stice pig kidney cell line were obtained from monolayers in the same manner. Suspensions of bovine tongue epithelial cells were prepared by the method described by Cartwright, Pay, and Henderson (1957).

Virus strains. Strain M11 (type O) virus grown at 37 C in pig-kidney monolayers and harvested after 8 hr was used in the main investigation, but a few experiments were done with Dutch 57 and Ven. 1 (type O) strains grown in surviving fragments of cattle tongue epithelium in tissue culture. Virus suspensions were stored at 4 C and used within 10 days.

Assay of virus and infected-cell suspensions. M11 virus suspensions were titrated as described by Thorne and Cartwright (1961). After acidification to remove virus, the number of cells infected with the M11 strain was determined in the same manner except that 10% serum was incorporated in the PBS diluent. This gave a 50% higher plating efficiency for infected cells than PBS alone. Final titers were based on a total count of at least 100 plaques.

Dutch 57 and Ven. 1 virus and infected-cell suspensions were titrated by intraperitoneal inoculation of unweaned mice (Skinner, 1951), using a threefold dilution interval and ten mice per dilution. End points were calculated by the method of Spearman-Kärber (Kärber, 1931).

Acid treatment. To remove virus from infected-cell suspensions, 1 N CH_3COOH or 1 N HCl was added. Acidification to pH values in the range of 5.5 to 6.0 was found to be suitable, and pH 5.7 was chosen for routine use. In kinetic experiments, samples were taken rapidly from cell-virus suspensions by pipetting volumes of 2 ml into small bottles containing the quantity of acid, in a volume of 0.1 ml, required to bring the pH to 5.7. The pH was restored to neutrality after 1 min by the addition of 1 N NaOH.

RESULTS

Applicability of the pH method. The rapid rate of inactivation of the virus of FMD at acid pH is well established (Pyl and Klenk, 1933; Bachrach et al., 1957*a*; Cartwright and Thorne, 1958). For the strains used in this work, the rate constants for inactivation at pH 5.7 and 25 C were greater than 10 min^{-1}, corresponding to at least 99.999% inactivation of infectivity in 1 min. In the presence of cells, the rate of inactivation of all virus, free and adsorbed, was determined in the following manner. After varying periods of adsorption, the pH was changed instantaneously to 5.7; at intervals, the pH of a sample was rapidly restored to pH 7.6 with 1 N NaOH, the cells immediately disrupted with sodium dodecyl sulfate (Cartwright and Thorne, 1958, 1959), and the residual virus assayed. By this means, the infected cells, which would otherwise register as infective units, were destroyed and the residual cell-associated virus dispersed. In a typical experiment with bovine tongue epithelial cells, for a virus input of 10^7 ID_{50}/ml the residual virus was less than $10^{1.5}$ ID_{50}/ml, i.e., less than 0.1% of the number of cells infected ($10^{4.5}$/ml), within 30 sec of adding acid. (A sample of the original suspension, treated with antiserum for 15 min and washed five times by centrifugation with PBS before disruption, still contained 10^4 ID_{50}/ml. Washing six times without antiserum treatment gave a final titer of 10^5 ID_{50}/ml.) The majority of adsorbed virus, as well as unadsorbed virus, therefore was inactivated at pH 5.7 within 30 sec.

Similar results were obtained with pig-kidney cells. As a result of the loss of infectivity of most

of the M11 virus adsorbed by pig-kidney cells (Thorne and Cartwright, 1961), cell-associated virus was much less than in the other virus-cell systems. After 30 min adsorption time and one washing, cell-associated virus was only of the same order numerically as the number of infected cells and, after six washings, was reduced 100-fold. Acidification alone reduced the amount of cell-associated virus another 100-fold. As in the previous experiments with a 50% end-point assay, the number of infected cells was unchanged after times of at least 30 min at 25 C and pH 5.7 (Table 1), and the yields of virus at 37 C, 200 to 300 plaque-forming units (pfu)/cell, were equal to the maximal yields obtained without pH inactivation (Cartwright and Thorne, 1958).

Infection of bovine tongue epithelial and pig-kidney cells by Dutch 57 and Ven. 1 strains. Figure 1 shows the log titers of the numbers of tongue epithelial cells infected, determined by 50% end-point assay, after increasing times of contact of cells and virus at several temperatures. The cells were kept in suspension in all experiments by intermittent shaking. At high virus concentrations ($\sim 10^8$ ID_{50}/ml), the number of cells infected was near maximum after a time as short as 1 min at 37 C, within the accuracy of the assay, and it was concluded that the two events of adsorption and penetration took place very rapidly; the longest time for penetration was 1 min plus the negligible time for pH inactivation. At 30 and 20 C, the initial rate of the reaction was decreased. There was no significant cell infection at 4 C. Results with pig-kidney cells were similar in all cases.

Infection of pig-kidney cells by the M11 strain. To compare theory and experiment, a study of the kinetics of cell infection under various conditions, using a plaque assay, was made. The sensitivity of cell suspensions and assay monolayers

TABLE 1. *Survival of infected pig-kidney cells at pH 5.7*

Time	No. of infected cells (pfu/ml × 10^{-3})	
	Expt 1	Expt 2
min		
1	80	100
5	72	91
10	78	114
15	75	96
40	93	96

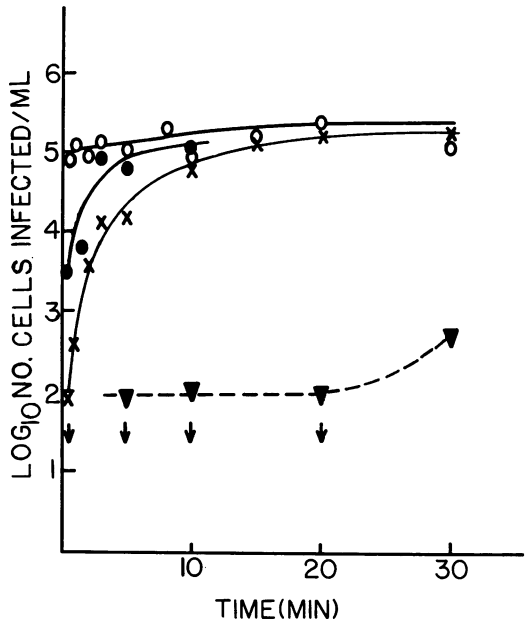

FIG. 1. *Kinetics of infection of tongue epithelial cells by the Dutch 57 strain at several temperatures* (○, *37 C;* ●, *30 C;* ×, *21 C;* ▼, *4 C;* $10^{7.8}$ ID_{50}*/ml; 4.0* × 10^6 *cells/ml;* ↓ *signifies no positive reactions*).

to virus varied and influenced the values of some of the experimental parameters, but experiments of the same type were repeated several times to show that the observed relationships were generally valid. When variables other than temperature were studied, the experiments were performed at 25 or 37 C. The form of the cell-infection curve was better revealed at 25 C than at 37 C, and experiments of longer duration were possible without complication by virus multiplication. Experiments with medium A and medium B gave similar results; results quoted are for medium A only.

Influence of time and temperature. The general form of the curve for temperatures from 20 to 37 C giving the number of cells infected at different times after mixing cells and virus is shown by a typical curve for 25 C (Fig. 2A). An initial transient portion of increasing slope was followed by a linear region, where the greatest rate of increase occurred, and finally by a region from 15 to 20 min onwards (for 25 C), where the rate decreased to zero. At high virus concentrations, incubation for 4 hr after this time (at 25 C) produced no further increase in the number of infected cells (Fig. 2B) despite the continued

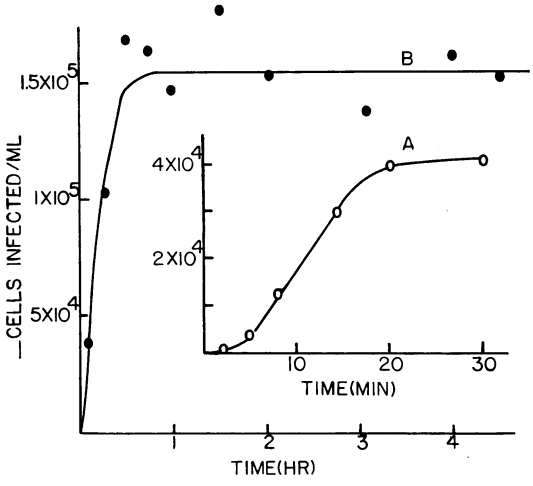

FIG. 2. *Kinetics of infection of pig-kidney cells by the M11 strain at 25 C;* (A) *1.5 × 10⁵ pfu/ml, 5 × 10⁵ cells/ml;* (B) *prolonged adsorption, 1.3 × 10⁷ pfu/ml, 5.4 × 10⁵ cells/ml (different cell preparations).*

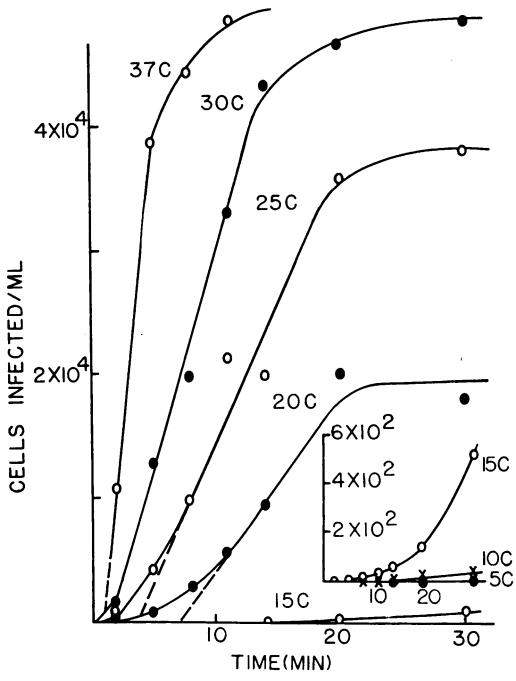

FIG. 3. *Kinetics of infection of pig-kidney cells by the M11 strain at several temperatures (8.0 × 10⁴ pfu/ml; 5.2 × 10⁵ cells/ml).*

presence of 50% of the original virus in the liquid medium.

Two parameters will be used to characterize the experimental curves: the slope of the linear portion of the curve in infected cells/min and the intercept which the linear portion makes on the time axis. The general features of the curve were the same for all temperatures examined above 15 C (Fig. 3). Increasing the temperature from 15 C at constant cell and virus concentration increased the rate of the reaction and resulted in an increase in slope and a decrease in the intercept. The rate of cell infection was very small at 15 C and near zero at 10 and 5 C (Fig. 3, inset). The proportion of cells infected at 30 min at different temperatures for constant cell and virus concentrations is shown for two experiments in Table 2.

TABLE 2. *Influence of temperature on the number of pig-kidney cells infected by the M11 strain after adsorption for 30 min*[a]

Expt	Temp (C)						
	5	10	15	20	25	30	37
1	0.02	0.2	2	40	80	100	100
2	0.01	—	1.7	37	90	90	100

[a] Results are expressed as cells infected as percentages of the number at 37 C.

Influence of virus concentration. Increasing the virus concentration at constant cell concentration and temperature increased the final number of cells infected (Thorne and Cartwright, 1961), the number of cells infected at all prior times, and the slope (Fig. 4 and 5). At moderate virus concentration (below ca. 10^6 pfu/ml), the slope increased proportionally to the virus concentration (Fig. 6). The intercept remained constant within limits of error up to ca. 5×10^5 to 10^6 pfu/ml; this was most apparent at 25 C where the intercept was sufficiently large for significant variations to be observed. Above ca. 10^6 pfu/ml, the intercept decreased; e.g., an increase in virus concentration from 10^6 to 10^7 pfu/ml reduced the intercept from about 5 min to about 2 min.

Influence of cell concentration. Increasing the cell concentration increased the rate of cell infection (Fig. 7). At a fixed virus concentration, the slope was proportional to the cell concentration up to about 5×10^5 cells/ml. At higher cell concentrations, the slope increased more slowly (Fig. 8). The intercept (Fig. 7) was independent of cell concentration up to 5×10^5 to 10^6 cells/ml

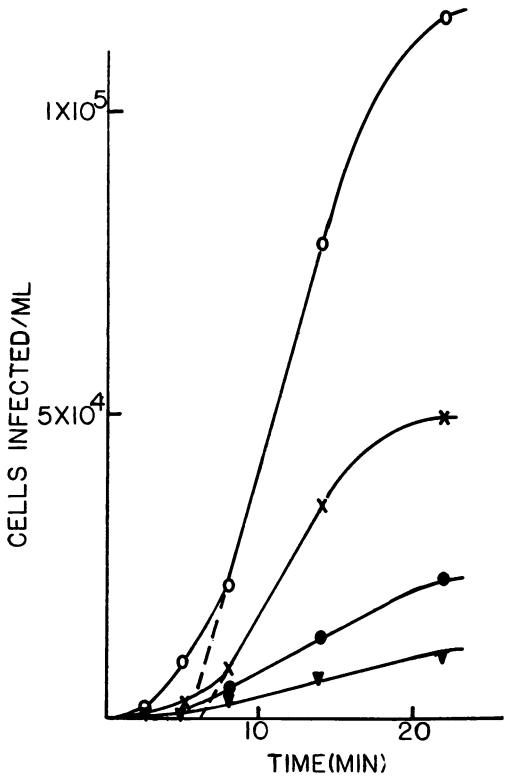

FIG. 4. *Influence of virus concentration on the kinetics of infection of pig-kidney cells by the M11 strain at 25 C* (○, *1.8* × *10⁵*; ×, *9.0* × *10⁴*; ●, *4.5* × *10⁴*; ▼, *2.3* × *10⁴ pfu/ml; 1.0* × *10⁶ cells/ml*).

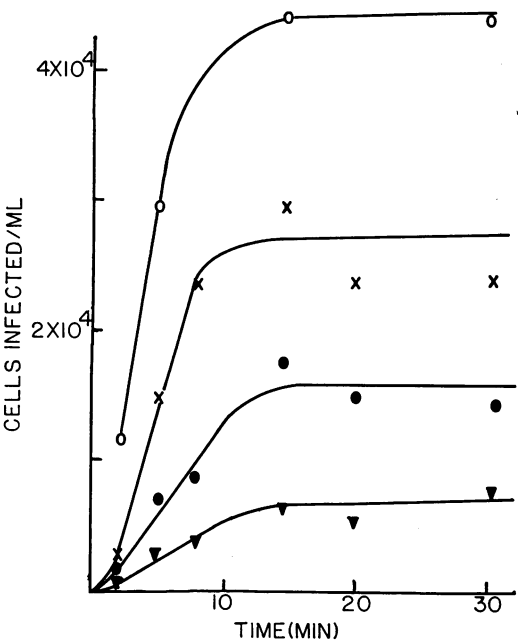

FIG. 5. *Influence of virus concentration on the kinetics of infection of pig-kidney cells by the M11 strain at 37 C* (○, *2.0* × *10⁵*; ×, *1.0* × *10⁵*; ●, *5.0* × *10⁴*; ▼, *2.5* × *10⁴ pfu/ml; 4.0* × *10⁵ cells/ml*).

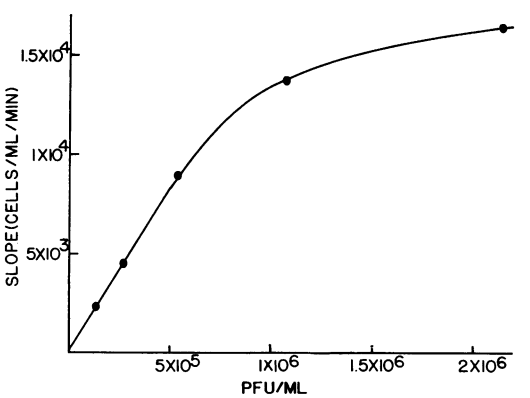

FIG. 6. *Influence of virus concentration on the slope of the M11-pig kidney cell infection curve at 25 C* (*4.0* × *10⁵ cells/ml*).

at a fixed virus concentration, and decreased at higher concentrations.

The combination of high cell and high virus concentrations produced very rapid rates of cell infection; e.g., 4 × 10^6 cells/ml and 2 × 10^7 pfu/ml gave a rate of 2.2 × 10^5 infected cells per ml per min at 25 C.

Early stages of the reaction. It follows from mathematical treatment of the infection process (see below) that, for the earliest stages of the reaction, the number of infected cells should be proportional to the square of the time (t). When cell-infection curves were replotted as a function of t^2, the existence of an initial linear region was apparent at all temperatures, and, when the final number of cells infected was small, e.g., at 20 C, the curve was linear up to at least 15 min. Linearity at 37 C was demonstrated by sampling at times up to 2 min (Fig. 9). The slopes of all t^2 plots were found to be proportional to initial cell and virus concentrations. Figure 10 shows the influence of virus concentration.

Variations between cell preparations. The varying rates of adsorption and proportions of cells infected for different preparations were described previously (Thorne and Cartwright, 1961). Similar variations were apparent in the present experiments. The rates of infection at 37 C of

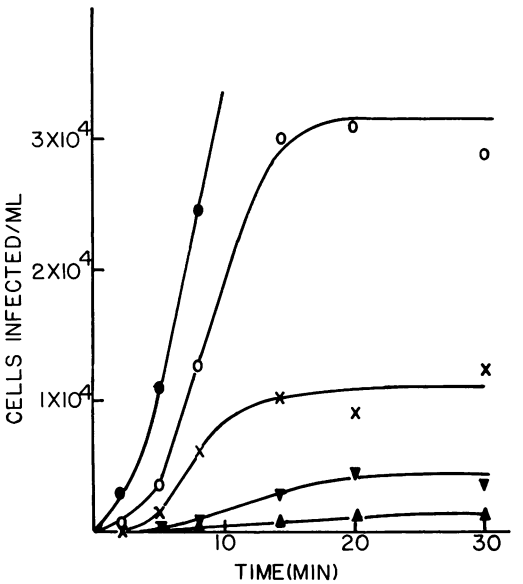

FIG. 7. *Influence of cell concentration on the kinetics of infection of pig-kidney cells by the M11 strain at 25 C (●, 2.0 × 10⁶; ○, 5.0 × 10⁵; ×, 2.0 × 10⁵; ▼, 5.0 × 10⁴; ▲, 1 × 10⁴ cells/ml; 1.7 × 10⁵ pfu/ml).*

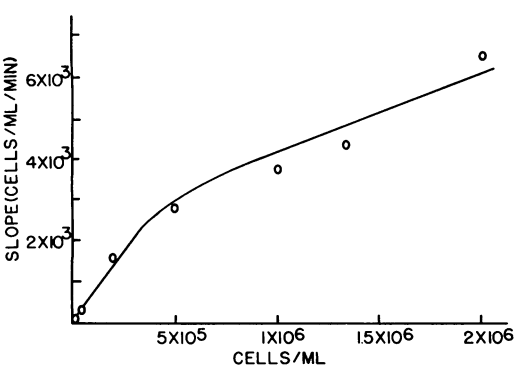

FIG. 8. *Influence of cell concentration on the slope of the M11-pig kidney cell infection curve at 25 C (1.7 × 10⁵ pfu/ml).*

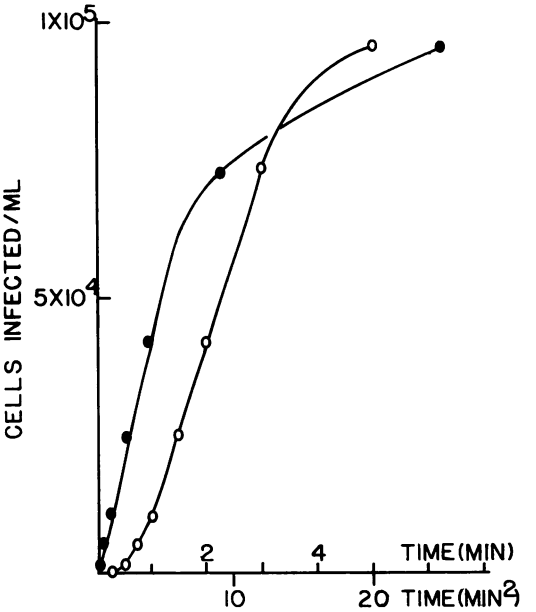

FIG. 9. *Initial stages of infection of pig-kidney cells by the M11 strain at 37 C plotted vs. (○) t and (●) t² (4.4 × 10⁶ pfu/ml; 5.2 × 10⁵ cells/ml).*

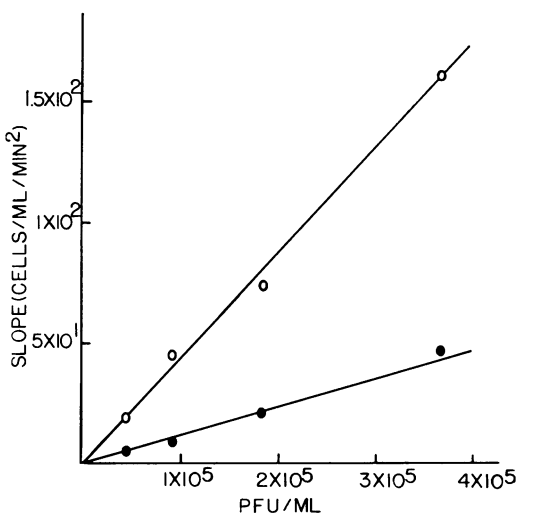

FIG. 10. *Influence of virus concentration on the slope of the M11-pig kidney t² plot at 25 C (○, 8.5 × 10⁵; ●, 1.7 × 10⁵ cells/ml).*

four cell suspensions, each derived from a separate kidney, were obtained during the early stages of the reaction (Fig. 11). The curves differ significantly in slope when the number of cells infected is plotted against t, as shown, or t^2. For clarity, only three curves are shown.

Suspensions of the Stice cell line gave curves of the same form as primary pig-kidney cells, with an intercept of 5 min at 25 C. However, the number of cells infected and the slope at a given virus concentration were much less than for primary cells. This was consistent with the very slight adsorption of virus by the Stice cell line (Thorne and Cartwright, 1961).

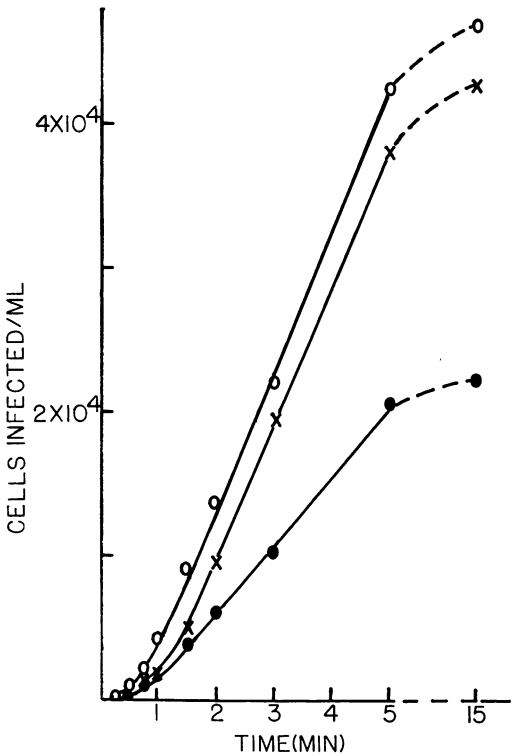

FIG. 11. *Kinetics of infection of different preparations of pig-kidney cells by the M11 strain at 37 C (2.1 × 10⁵ pfu/ml; 4.0 × 10⁵ cells/ml).*

Cell infection after adsorption at low temperature. Although infection is almost completely suppressed below 15 C, adsorption of virus is comparatively unaffected (Thorne and Cartwright, 1961), and at 4 C is still about a third of the rate at 37 C. To eliminate the influence of the adsorption time on the cell-infection curve, adsorption was completed at low temperature before increasing the temperature to allow penetration. The virus and cell suspensions were mixed at 4 or 10 C for 1 hr, diluted 50-fold into medium at the required temperature, and the number of cells infected at intervals after dilution determined.

In both medium A and B, a rapid increase from the origin with no lag was observed (Fig. 12).

Influence of medium constituents on infection. Cell-infection curves, using PBS, medium A, or medium B as suspending media, were indistinguishable, showing that the additional constituents in the latter two media were unimportant for infection. The omission of Ca and Mg ions reduced the number of cells infected after 30 min contact of virus and cells 50-fold; this is in accordance with the results of Bachrach et al. (1957*b*), who showed that Ca and Mg ions were essential for adsorption of the virus to bovine kidney cells. On addition of the ions at this point, a normal infection curve was obtained.

The infection process was not influenced by the presence of ribonuclease. This was evident both from the identity of the infection curves with and without serum, since ribonucleases are present in serum, and from experiments with cells in suspension in PBS, which showed no influence of added ribonuclease up to 100 μg/ml.

THEORY

For the purpose of deriving a rate constant to characterize penetration, only a simple model of cell infection will be considered. Cell infection is considered to take place by two reactions: (i) adsorption of the virus particle to the cell surface, and (ii) irreversible reaction of the virus with the

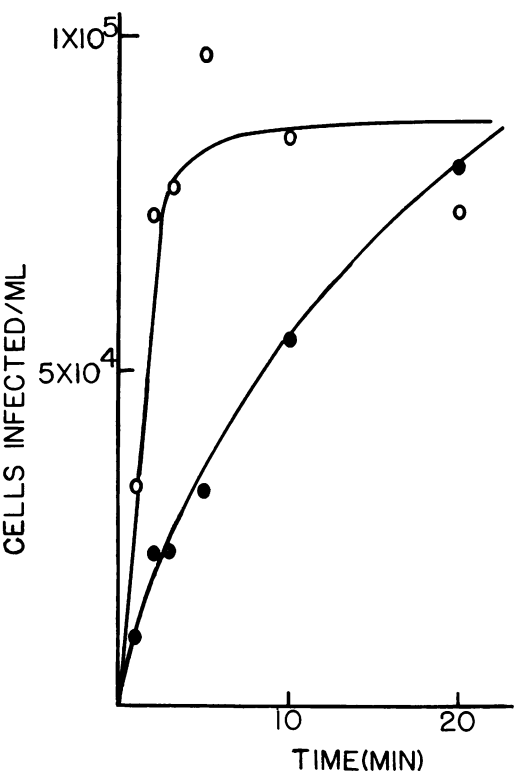

FIG. 12. *Kinetics of infection of pig-kidney cells by the M11 strain after prior adsorption at 10 C (○, 37 C; ●, 25 C; 4.5 × 10⁶ pfu/ml; 8.0 × 10⁵ cells/ml).*

cell (penetration), which causes infection and initiates the production of virus. This model resembles mathematically the reaction between an enzyme and its substrate (Laidler, 1958).

To simplify the treatment, cells are assumed to have one and the same site for adsorption and penetration. Virus and cells are assumed to react according to the equation

$$V + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} VC \xrightarrow{k_3} C^* \qquad (1)$$

where k_1 and k_2 represent the rate constants governing adsorption of virus to the cells and dissociation of virus, if this occurs, from the virus-cell complex (VC), respectively, and k_3 the rate constant governing penetration of the virus and rate of production of infected cells (C^*). V and C represent concentrations of free virus and uninfected cells, respectively. The differential equation describing the process is then

$$\frac{d^2C^*}{dt^2} + (k_2 + k_3)\frac{dC^*}{dt} = k_1 k_3[V][C] = k_1 k_3 \cdot \left[Vo - C^* - \frac{1}{k_3}\frac{dC^*}{dt}\right]\left[Co - C^* - \frac{1}{k_3}\frac{dC^*}{dt}\right] \qquad (2)$$

where Vo and Co are initial concentrations of V and C, respectively.

It is not possible to obtain analytical solutions for equations of this type, but, approximate solutions can be obtained. Some of these are presented in the following.

(i) If only the earliest stages of the reaction are considered, when t is small and $C \sim Co$ and $V \sim Vo$, then equation 2 reduces to

$$\frac{d^2C^*}{dt^2} + (k_2 + k_3)\frac{dC^*}{dt} = k_1 k_3[Vo][Co] \qquad (3)$$

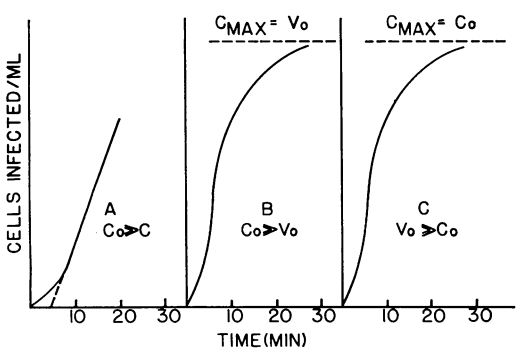

FIG. 13. *Theoretical forms (schematic) of the cell infection curve for* (A) $Co \gg C^*$, (B) $Co \gg Vo$, *and* (C) $Vo \gg Co$.

With boundary conditions $t = 0$, $C^* = 0$, $dC^*/dt = 0$, the solution to this equation is

$$C^* = \frac{k_1 k_3 VoCot}{k_2 + k_3} + \frac{k_1 k_3 VoCo}{(k_2 + k_3)^2}e^{-(k_2+k_3)t} - \frac{k_1 k_3 VoCo}{(k_2 + k_3)^2} \qquad (4)$$

which is of the form shown in Fig. 13A. The exponential term decreases in importance with time, and the plot becomes linear after the initial lag. The intercept of the extrapolated linear portion of the curve on to the horizontal axis, τ, is given by

$$\tau = \frac{1}{k_2 + k_3} \qquad (5)$$

and is independent of the cell and virus concentrations. The slope of the straight line is

$$\frac{dC^*}{dt} = \frac{k_1 k_3 VoCo}{k_2 + k_3} \qquad (6)$$

and is proportional to both Co and Vo. From these two equations, the value of the product k_1k_3 can be derived from the experimental slope and intercept and the values of Co and Vo.

When the following approximations are applicable, the individual values of k_1, k_2, and k_3 can be derived from equations 5 and 6. (a) For $k_3 \gg k_2$

$$\tau \cong \frac{1}{k_3} \qquad (7)$$

and the slope becomes

$$\frac{dC^*}{dt} \cong k_1 VoCo \qquad (8)$$

(b) For $k_2 \gg k_3$

$$\tau \cong \frac{1}{k_2} \qquad (9)$$

and

$$\frac{dC^*}{dt} \cong \frac{k_1 k_3 VoCo}{k_2} \qquad (10)$$

For times very soon after mixing cells and virus, when t is small, the exponential in equation 4 can be expanded and, neglecting the third and higher powers of t, C^* becomes

$$C^* \cong \frac{k_1 k_3 VoCo}{2} t^2 \qquad (11)$$

C^* is therefore initially a linear function of t^2, and from this equation another estimate of k_1k_3 can be obtained. For particular relationships between the values of the parameters k_1, k_2, and k_3, therefore, estimates can be made of the values of k_1, k_2, k_3, and k_1k_3 from the preceding equations.

(ii) A second solution can be derived when $Co \gg Vo$, applicable to the whole reaction and not only the initial stages, of the form shown in Fig. 13B and given by the equation

$$C^* = Vo\left[1 + \frac{b - \sqrt{b^2 - 4c}}{2\sqrt{b^2 - 4c}} \cdot e^{\frac{-b-\sqrt{b^2-4c}}{2} \cdot t} - \frac{b + \sqrt{b^2 - 4c}}{2\sqrt{b^2 - 4c}} \cdot e^{\frac{-b+\sqrt{b^2-4c}}{2} \cdot t}\right] \quad (12)$$

where $b = k_1Co + k_2 + k_3$ and $c = k_1k_3Co$. This equation again predicts an initial lag before the second term, which diminishes rapidly with time compared with the third, becomes negligible. On expanding the exponentials in equation 12, again for t small,

$$C^* \leqq \frac{k_1 k_3 VoCo \cdot t^2}{2} \quad (13)$$

(iii) When $Vo \gg Co$, a solution of the same form as equation 12 is obtained (Fig. 13C) with Vo replacing Co throughout. Equation 13 applies for t small.

Sections i, ii, and iii should give valid approximate solutions when each cell has one and the same site for adsorption and penetration. For the virus of FMD, the problem is certainly more complex, since at least 20 to 30 pfu can be adsorbed per cell (Thorne and Cartwright, 1961) and presumably, therefore, each cell has many sites for adsorption. If all of these sites act independently as penetration sites, then at low virus concentrations similar equations describe the infection process, with the total number of sites replacing Co, and adsorption and penetration rate constants are derived as before. But other possibilities in which, for example, only some of the sites allow penetration or penetration at one site affects adsorption and penetration at others are too complex to warrant further examination at present.

Comparison of theory and experiment. For a two-stage reaction of the type postulated, the rate of the adsorption reaction should be proportional

TABLE 3. *Dependence of the parameters of the cell-infection curve on Vo at 25 and 37 C*

Expt[a]	*Co*	*Vo*	Slope[b]	$\bar{k}_1$[c]	$\bar{k}_3$[c]	$\bar{k}_1 \times \bar{k}_3$[c]	$\bar{k}_1\bar{k}_3$[d]
	cells/ml	*pfu/ml*					
1	1.7×10^5	3.7×10^5	1.1×10^3	1.7	0.2	3.4×10^{-9}	1.5×10^{-9}
		1.8×10^5	4.9×10^2	1.5	0.2	3.0×10^{-9}	1.3×10^{-9}
		9.2×10^4	2.3×10^2	1.4	0.2	2.8×10^{-9}	1.1×10^{-9}
		4.6×10^4	1.4×10^2	1.8	0.2	3.6×10^{-9}	1.3×10^{-9}
	4.3×10^5	3.7×10^5	1.3×10^3	0.8	0.2	1.6×10^{-9}	6.2×10^{-10}
		1.8×10^5	7.4×10^2	0.9	0.2	1.8×10^{-9}	7.0×10^{-10}
		9.2×10^4	5.2×10^2	1.3	0.2	1.6×10^{-9}	1.1×10^{-9}
		4.6×10^4	2.1×10^2	1.1	0.25	2.8×10^{-9}	9.0×10^{-10}
	8.7×10^5	3.7×10^5	2.1×10^3	0.7	0.3	2.0×10^{-9}	1.0×10^{-9}
		1.8×10^5	1.4×10^3	0.85	0.2	1.7×10^{-9}	1.0×10^{-9}
		9.2×10^4	7.2×10^2	0.65	0.25	1.6×10^{-9}	1.2×10^{-9}
		4.6×10^4	3.6×10^2	0.9	0.25	2.3×10^{-9}	1.0×10^{-9}
2	2.2×10^5	4.8×10^6	1.3×10^4	1.2	0.7	8.4×10^{-8}	3.9×10^{-9}
		2.4×10^6	1.2×10^4	2.2	0.6	1.3×10^{-8}	6.7×10^{-9}
		1.2×10^6	9.5×10^3	3.6	0.65	2.3×10^{-8}	1.1×10^{-8}
		6.0×10^5	4.3×10^3	3.2	0.6	1.9×10^{-8}	2.1×10^{-8}
3	4.8×10^5	1.1×10^6	1.4×10^4	2.7	1.7	4.6×10^{-8}	1.7×10^{-8}
		5.4×10^5	9.0×10^3	3.5	1.3	4.6×10^{-8}	1.6×10^{-8}
		2.7×10^5	4.6×10^3	3.6	1.0	3.6×10^{-8}	1.7×10^{-8}
		1.3×10^5	2.4×10^3	3.8	1.0	3.8×10^{-8}	2.3×10^{-8}

[a] Experiments 1 and 2 were performed at 25 C, experiment 3 at 37 C.

[b] Infected cells per ml per min.

[c] $\bar{k}_1$, $\bar{k}_3$, and $\bar{k}_1 \times \bar{k}_3$ are from plots of infected cells vs. time; $\bar{k}_1$ expressed as $cm^3\ min^{-1}\ pfu^{-1} \times 10^8$ and $\bar{k}_3$ as min^{-1}.

[d] The $\bar{k}_1\bar{k}_3$ values are from plots of infected cells vs. t^2.

to the number of encounters between, and thus to the product of the concentrations of, virus particles and cells. At moderate concentrations, therefore, the infection rate also should be proportional to these concentrations. However, as the virus or cell concentration and thus the encounter rate is increased, the time taken by the second stage begins to have a greater relative influence on the over-all time of the reaction. As a result, the proportionality holds no longer; for very large concentrations, when adsorption becomes effectively instantaneous, the rate of reaction should become independent of the concentration and thus relate only to penetration time. The infection curves obtained are in agreement with these general predictions of the simple theory. By assuming the applicability of the theory, values of the rate constants can be determined if their relative magnitudes are such as to allow the use of equations 7, 8, or 9. In the absence of information on the numbers of adsorption and penetration sites and the possible relationships between them, the cell concentration has been used to represent the concentrations of these sites. The results of Thorne and Cartwright (1961) indicated that k_2 was very small or zero; since, from sections ii and iii the intercept, τ, was independent of virus and cell concentration, as required by theory up to 5×10^5 pfu or cells/ml, estimates of k_3, denoted $\bar{k}_3$, were calculated from the experimental curves from $\bar{k}_3 = 1/\tau$.

For concentrations below about 5×10^5 to 10^6/ml, the slope of the experimental curve was proportional to Vo and Co and, again neglecting k_2, a constant, $\bar{k}_1$, was calculated from $\bar{k}_1 = \frac{\text{slope}}{VoCo}$.

Finally, since the theoretically expected linear relationship was found between C^* and t^2 for the early stages of the reaction, values of k_1k_3 were calculated from $\bar{k}_1\,\bar{k}_3 = \frac{2 \times \text{slope}}{VoCo}$. Corresponding values of $\bar{k}_1$, $\bar{k}_3$, $\bar{k}_1 \times \bar{k}_3$, and $\bar{k}_1\,\bar{k}_3$ are given in the tables.

Adsorption rate constant ($\bar{k}_1$). The dependence of $\bar{k}_1$ on virus and cell concentration at 25 and 37 C is shown in Tables 3 and 4. In accordance with theory, the values are approximately constant at low concentrations but decrease at higher concentrations. Different values of $\bar{k}_1$ were obtained for different cell preparations. The ranges and means for virus concentrations less than 10^6 pfu/ml were 8×10^{-9}–1.1×10^{-7} (3.3×10^{-8}) $cm^3\ min^{-1}\ pfu^{-1}$ at 25 C and 3.5×10^{-8}–3.0×10^{-7}(7.8×10^{-8}) $cm^3\ min^{-1}\ pfu^{-1}$ at 37 C. Results for four separate preparations assayed with a single batch of monolayers are given in Table 5.

In addition to real differences in adsorption rate between preparations (Thorne and Cartwright, 1961), errors in C^*, Vo, and the effective Co contribute to the variations. Vo probably makes the greatest contribution to variation, since the plating efficiency for virus on different batches of monolayers varies in the extreme as much as tenfold. Variations in plating efficiency for infected cells (C^*) are much less than for virus (Thorne and Cartwright, 1961), probably because each cell provides many particles capable of plaque initiation. The cell count Co may include varying proportions of nonviable or insusceptible cells. Staining indicated that variations due to nonviability were probably small, but the variable proportion of cells infected (20 to 70%) by high virus concentrations (Thorne and Cartwright, 1961) indicated heterogeneity of the cell populations.

TABLE 4. *Dependence of the parameters of the cell-infection curve on Co at 25 C*

Co	*Vo*	Slope[a]	$\bar{k}_1$[a]	$\bar{k}_3$[a]	$\bar{k}_1 \times \bar{k}_3$[a]	$\bar{k}_1\bar{k}_3$
cells/ml	*pfu/ml*					
2.0×10^6		6.6×10^3	1.9	0.33	4.4×10^{-9}	2.6×10^{-9}
1.3×10^6		4.4×10^3	1.9	0.4	3.8×10^{-9}	3.7×10^{-9}
1.0×10^6		3.8×10^3	2.2	0.3	4.4×10^{-9}	2.7×10^{-9}
5.0×10^5	1.7×10^5	2.9×10^3	3.3	0.25	8.0×10^{-9}	4.2×10^{-9}
2.0×10^5		1.6×10^3	4.6	0.25	1.2×10^{-8}	4.9×10^{-9}
5.0×10^4		3.1×10^2	3.6	0.2	7.2×10^{-9}	3.3×10^{-9}
1.0×10^4		8.1×10^1	4.7	0.2	9.4×10^{-9}	4.7×10^{-9}

[a] See footnotes to Table 3 for explanation.

TABLE 5. *Parameters of the cell-infection curve for different pig-kidney cell preparations at 37 C*

Prep.	*Co*	*Vo*	Slope[a]	$\bar{k}_1$[a]	k_3[a]	$\bar{k}_1 \times \bar{k}_3$[a]	$\bar{k}_1\bar{k}_3$[a]
	cells/ml	*pfu/ml*					
1	4×10^5	2.1×10^5	9.0×10^3	10.7	2.0	2.1×10^{-7}	1.0×10^{-7}
2			9.6×10^3	11.4	1.3	1.5×10^{-7}	5.4×10^{-8}
3			7.8×10^3	9.2	2.0	1.8×10^{-7}	1.0×10^{-7}
4			4.6×10^3	5.5	1.4	7.7×10^{-8}	3.7×10^{-8}

[a] See footnotes to Table 3 for explanation.

If the simple theory for one adsorption site applied, or if all sites allowed penetration, the values of $\bar{k}_1$ and those of the rate constant from adsorption measurements should agree. Values obtained in two experiments at 25 and 37 C, using cells from a single kidney pool, are in reasonable agreement (Table 6), as also are the mean values from adsorption and cell infection at 25 C, 6.3×10^{-8} cm^3 min^{-1} cell^{-1} (Thorne and Cartwright, 1961) and 3.3×10^{-8} cm^3 min^{-1} pfu^{-1}, respectively. This suggests, since each cell can adsorb at least 20 to 30 pfu, that cells may possess many sites, each capable of adsorption and penetration.

Dependence of $\bar{k}_1$ on temperature. The dependence of the slope of the cell-infection curve on temperature was described in section i. The calculated values of $\bar{k}_1$ are given in Table 7, and Arrhenius plots of log $\bar{k}_1$ vs. the reciprocal of the absolute temperature for two experiments are shown in Fig. 14. Both plots are linear down to 20 C and give activation energies with a mean of 14,000 cal/mole. The break below 20 C is probably due to the fact that here penetration becomes the slowest reaction and therefore rate-limiting, and the slope no longer gives the value of k_1. The adsorption-rate constant from measurements of virus adsorption (Thorne and Cartwright, 1961) with an activation energy of 6,000 cal/mol ($Q_{10} = 1.5$) was less affected by temperature than that from cell-infection rate ($Q_{10} = 2.3$). This discrepancy could possibly be explained by the existence of temperature-dependent reactions after attachment, before the cell becomes infected by the penetration reaction of the simple theory. Or, measurement of virus adsorption by determination of virus remaining in the supernatant predominantly may measure adsorption reactions which are unrelated to the adsorption reaction preceding infection and have a different temperature dependence. The loss of infectivity of much of the virus adsorbed to pig-kidney cells at 4 C, as well as at higher temperatures (Thorne and Cartwright, 1961), would be consistent with the second of these possibilities.

TABLE 6. *Comparative values for adsorption rate constant from adsorption and cell-infection experiments*

Temp	Cell infection ($\bar{k}_1$)[a]	Adsorption (k)[b]
C		
37	3.8	9.8
25	2.7	4.7

[a] Expressed as cm^3 min^{-1} pfu^{-1} $\times 10^8$.
[b] Expressed as cm^3 min^{-1} cell^{-1} $\times 10^8$.

TABLE 7. *Influence of temperature on $\bar{k}_1$*[a]

Temp	$\bar{k}_1$[b]	
	Expt 1	Expt 2
C		
37	5.0	10.7
30	2.6	4.1
25	2.8	2.7
20	1.5	1.8
15	0.11	0.04

[a] Expt 1: 5.3×10^5 cells/ml; 1.3×10^5 pfu/ml; expt 2: 5.2×10^5 cells/ml. 8.0×10^4 pfu/ml.
[b] Expressed as cm^3 min^{-1} pfu^{-1} $\times 10^8$.

Penetration rate constant ($\bar{k}_3$). The typical values given in Tables 3 and 4 show that $\bar{k}_3$ remained substantially constant at virus and cell concentrations below 5×10^5 pfu or cells/ml, The means of 30 values of $\bar{k}_3$ at 25 C and 25 values at 37 C were 0.25 ($\pm$ 0.1) min^{-1} and 1.5 ($\pm$ 0.5) min^{-1}, respectively. The $\bar{k}_3$ showed a much smaller range of variation from one experiment to another than did the $\bar{k}_1$, most values lying in the 0.2 to 0.25 min^{-1} range at 25 C.

At high values of *Vo* or *Co*, the intercept decreased and the values of $\bar{k}_3$ increased; e.g., in

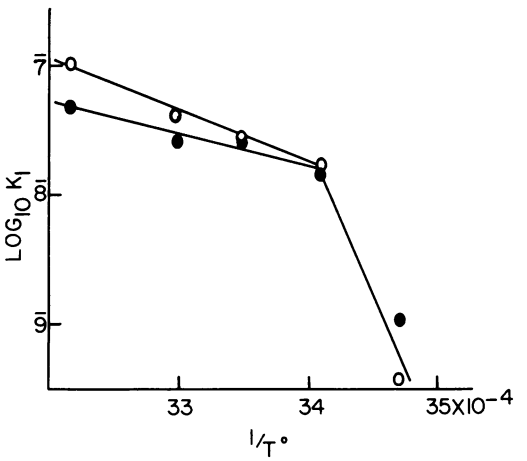

FIG. 14. *Arrhenius plot for* $\bar{k}_1$ *(data of Table 7).*

TABLE 8. *Estimation of* k_3 *from cell-infection rates at high virus concentrations*

Vo	*Co*	k_3	Temp
pfu/ml	*cells/ml*	min^{-1}	*C*
6.0×10^5	2.2×10^5	0.12	25
1.2×10^6	2.2×10^5	0.15	25
2.4×10^6	2.2×10^5	0.15	25
4.8×10^6	2.2×10^5	0.12	25
9.6×10^6	2.2×10^5	0.21	25
2.2×10^7	2.2×10^5	0.22	25
1.3×10^6	4.2×10^5	0.19	37
2.5×10^6	4.2×10^5	0.19	37
5.0×10^6	4.2×10^5	0.44	37
1.0×10^7	4.2×10^5	0.70	37

one experiment at 25 C with 2×10^5 cells/ml, $\bar{k}_3$ was 0.2 for 5×10^5 pfu/ml and 0.5 for 10^7 pfu/ml.

At high virus concentrations, an alternative means of estimating k_3 is theoretically possible, if the rate of adsorption can be increased so that penetration rate becomes the rate-limiting factor and the time for adsorption can be neglected. Then $\frac{dC^*}{dt} = k_3 [VC]$, where $[VC]$ represents the concentration of complexes capable of reacting to give infected cells. This concentration will be given by the total number of sites at which penetration can occur, assuming that all such sites will be occupied. But to allow for insusceptible cells, the final number of cells infected has been used as an estimate of this concentration. Estimates of k_3 have therefore been calculated from the formula $k_3 = \frac{1}{C^* \max} \frac{dC^*}{dt}$ and are given in Table 8. At high virus concentrations, these values are in good agreement with the values obtained by the intercept method.

Dependence of $\bar{k}_3$ *on temperature.* The values of $\bar{k}_3$ showed a greater dependence on temperature than those of $\bar{k}_1$ (Table 9). There was an increase in $\bar{k}_3$ of about fivefold between 25 and 37 C ($Q_{10} = 4$); below 25 C, the values decreased rapidly. At 10 C, the amount of penetration as measured by the number of cells infected was 0.2% of that at 37 C. The Arrhenius plots (Fig. 15) corresponding to those for $\bar{k}_1$ were linear throughout, with a mean activation energy of 24,000 cal/mole.

Composite constant $\bar{k}_1 \bar{k}_3$ *from* t^2 *plots.* Values of $\bar{k}_1 \bar{k}_3$, although independent of *Co* and *Vo* over a wide range for a single preparation, varied between preparations in a similar manner to $\bar{k}_1$. In Tables 3, 4, and 5, $\bar{k}_1 \bar{k}_3$ values and the products of $\bar{k}_1$ and $\bar{k}_3$ obtained with the same cell suspensions are given. It is evident that there is a fairly constant ratio between the two values, $\bar{k}_1 \times \bar{k}_3$ being about twice $\bar{k}_1 \bar{k}_3$. Since theoretically

TABLE 9. *Influence of temperature on* $\bar{k}_3$[a]

Temp	$\bar{k}_3$ (min^{-1})		Mean half-time
	Expt 1	Expt 2	
C			*min*
37	1.0	1.25	0.6
30	0.55	0.6	1.2
25	0.25	0.25	2.7
20	0.09	0.13	6.3

[a] Expt 1: 5.3×10^5 cells/ml; 1.3×10^5 pfu/ml; expt 2: 5.2×10^5 cells/ml; 8.0×10^4 pfu/ml

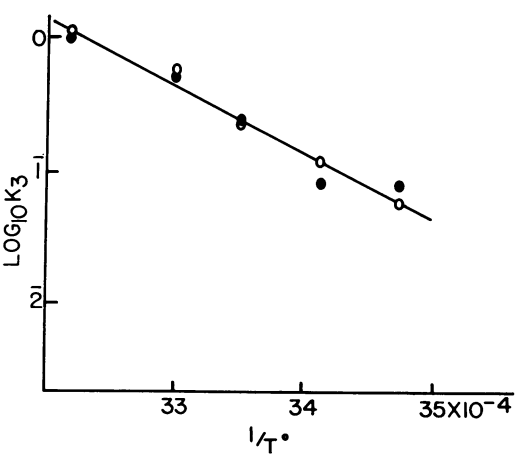

FIG. 15. *Arrhenius plot for* $\bar{k}_3$ *(data of Table 9).*

TABLE 10. *Estimation of k_3 from cell-infection rates after adsorption at 10 C*[a]

Temp	k_3 (min^{-1})	
	Expt 1	Expt 2
C		
37	1.0	0.8
30	0.6	
25		0.12
20	0.2	

[a] Expt 1: 1.0×10^6 cells/ml; 5.3×10^6 pfu/ml; expt 2: 8.0×10^5 cells/ml; 4.5×10^6 pfu/ml.

both of these products give k_1k_3 irrespective of the relative magnitudes of Co, Vo, k_1, k_2, or k_3, the ratio should be unity. The discrepancy probably arises from the oversimplification of the theoretical model.

Determination of k_3 from cell infection after adsorption at low temperature. For adsorption at low temperature followed by dilution to the required temperature, the equation describing the cell-infection curve becomes $C^* = C^*_{max}(1 - e^{-k_3t})$, and a penetration rate constant can be derived from the initial slope, where $C^* = C^*_{max}k_3t$, or from $\log e(C^*_{max} - C^*)/C^*_{max} = -k_3t$.

The values of k_3 in Table 10 are in fair agreement with the range of values derived from intercept measurements. This suggests that the assumptions as to the relative magnitudes of k_1, k_2, and k_3 employed in approximating the theoretical equations were reasonable.

DISCUSSION

The lability of the virus of FMD at acid pH has made possible the study and tentative interpretation of relationships between cell and virus concentrations and the kinetics of cell infection. The experimental results clearly establish a multistep process for infection and can be semiquantitatively accounted for by a simple model involving only a second-order adsorption reaction and a first-order penetration reaction. At temperatures above 20 C, the virus and cell concentrations are predominant in the determination of the over-all infection rate. The penetration reaction which determines the temperature dependence of the over-all reaction is very fast at 37 C (half-time about 30 sec), resulting in 90% completion of cell infection in about 3 min, but rapidly decreases in rate at lower temperatures. Further investigation is required to determine whether viral or cellular enzymes (or both) function in this reaction.

The few reported estimates of penetration rate for other animal viruses were obtained using antiserum to inactivate free virus. However, several viruses have been shown to be only partly neutralized when adsorbed to cells and, unless it is shown that the adsorbed virus escaping neutralization is negligible, the distinction between adsorption and penetration becomes blurred and the penetration rate can be overestimated. It is nevertheless of interest to compare the values for the rate at which antiserum resistance is acquired for different viruses. For HeLa cells at 37 C, the half-time is about 30 min for polio virus (Payne, Kurtz, and Ackermann, 1958), 2 hr for herpes virus (Farnham and Newton, 1959), and 5 min for Newcastle disease virus (Marcus, 1959). Whether these differences for the same host cell arise from different viral properties or distinct cellular sites (or both) cannot yet be decided. Accurate studies of the temperature dependence of these reactions, and determination of the energy involved, could help to clarify the problem. There appears to be a correlation between the time for penetration and the length of the latent period, since the virus of FMD with the shortest penetration time has a latent period of 90 min, compared with about 4 hr for polio virus and 12 hr for herpes virus. These differences in penetration rate and latent period may result from differing complexities of the respective reactions for different viruses.

Current conceptions of viral infection mechanisms would suggest that the penetration reaction studied represented penetration of the virus nucleic acid. A comparison of the kinetics and thermodynamic constants for infection by free infectious nucleic acid and intact virus might help to decide whether these processes are equivalent and occur at the same cellular site. Since cell infection was unchanged in rate or extent in the presence of ribonuclease, the occurrence of an intermediate stage in which ribonucleic acid (RNA) is released at the external surface of the cell appears unlikely. Even if ribonuclease were unable to attack RNA adsorbed in this way, the absence of infection after adsorption at low temperature followed by mild acidification indicates that external breakdown of the infecting virus to RNA, with continuing attachment before penetration, is an unlikely mechanism.

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