The Possible Nature of the Chance-event in Initiation of Virus Infection at the Cellular Level

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SUMMARY
A direct correlation was found between the affinity of certain cardioactive sterols for the binding site of (Na+, K+)-dependent ATPase and their capacity to raise poliovirus yields in single cycles of infection in suspended monkey kidney cells. The latter effect was due to an improved efficiency of infection by the attached particles rather than to higher yields/cell.

The average number of p.f.u. attached/cell within a certain time was directly proportional to the number of free p.f.u. initially present. In untreated systems, the efficiency of initiation of infection was low and was not directly related to the average number of p.f.u. attached/cell. The addition of digitoxin exalted the efficiency of the initiation of infection to a constant level at any number higher than one of free p.f.u. initially present.

A brief reference is made to the mathematical treatment of these findings.

As a possible explanation of the phenomenon we propose that the polio receptor is a metastable pattern of macromolecules. The time of existence of a structure fully complementary to the attachment site(s) for virus may control the chance of reception and initiation of infection. The stabilization of a given conformational state for all or most (Na+, K+)-dependent ATPase molecules is believed to prolong the time of existence of the specific structural state required for reception and infection.

INTRODUCTION
Virus attachment to susceptible cells has been found generally to follow the kinetics of the pseudo-first-order, irreversible reaction. However, the relation of the efficiency of attachment to the efficiency of initiation of infection is poorly understood.

Cooper (1964) demonstrated that the multiplicity of infection considerably affected the synchrony and the ‘chance-delay’ of initiation of infection by poliovirus in ERK cells. He stated that ‘much delay must be present during penetration’. Using multiplicities higher than 3 p.f.u./cell he succeeded in eliminating ‘chance-delay’ and in reducing the ‘eclipse period’ to an apparent minimum of 2·6 to 2·7 h. At high multiplicities, however, there was an inverse relation between the ‘multiplicity of infection’ and the final virus yield. Nevertheless, it is still a general practice to use high multiplicities if a high efficiency of infection is required.

Earlier studies on a poliovirus-host cell system showed that the addition of certain cardioactive sterols immediately after virus particle attachment enhanced the final virus yield and synchronized the initiation of infection (Koch & György, 1969). This effect was utilized in the present study of the factors controlling the efficiency of infection by virus already attached. A theoretical treatment is offered of some of the factors involved.
METHODS

Cells. A rhesus monkey kidney cell line (PMK III/1), generally used in Hungary for enterovirus work (Ruzicska, 1964) was used throughout. Cells were grown in 2 l Roux flasks in a growth medium (GM) consisting of 40 parts Parker's no. 199 medium, 45 parts Hanks' balanced salt solution (HBS), 10 parts inactivated calf serum and 5 parts of a solution of 5 g lactalbumin hydrolysate in 100 g distilled water. Absence of contamination by PPLO was checked regularly by the arginase test (Schlimke, 1962).

Poliovirus. The type 1 (MAHONEY) standard strain of poliovirus was supplied by the Department of Virology, National Institute of Public Health, Budapest. Stock virus was prepared in this laboratory on PMK III/1 monolayers in 2 l Roux flasks. The culture was washed by three changes of HBS and inoculated with 1 ml of a suspension of $2.5 \times 10^7$ p.f.u./ml in Parker no. 199 medium, enriched with 0.5 g bovine albumin (Cohn fraction V)/100 ml medium. Attachment proceeded for 30 min at 37 °C. Finally 100 ml of warmed HBS was added to the culture which was reincubated for 18 to 24 h until specific cell destruction was complete. Cell-associated virus was released by five cycles of freezing and thawing and debris was removed by centrifuging at low-speed. No further purification of the virus was made before ampouling and storage at $-50^\circ$ C.

Attachment. Monolayers grown in GM were washed in three changes of Ca$^{2+}$Mg$^{2+}$ free HBS. Cells were suspended by treatment with EDTA, washed in HBS and counted. A final sediment of $10^7$ cells was prepared. This was suspended in 0.2 ml of virus suspension to give a predetermined initial number of p.f.u./cell: this was designated $V_i$. Attachment proceeded for 15 min at 25 °C under continuous mixing. The cells were then rapidly diluted in 10 ml ice-cold HBS and centrifuged. After one more similar washing both supernatant fluids of 10 ml were collected and titrated by plaque counting on PMK III/1 monolayers. The number of unattached p.f.u./cell in the total 20 ml volume is designated as $V_u$. The difference $V_a = V_i - V_u$ is the average number of p.f.u. attached/cell.

The efficiency of infection (IC). Earlier studies (Koch et al. 1970) showed that IC attained its final value after incubation of the system at 37 °C for 1 h. In this study, the cell sediment obtained on the second washing after attachment was made up to a concentration of $10^8$ cells/ml in cold HBS. One ml of this suspension was added to 9 ml HBS or HBS + $10^{-8}$ M final concentration of digitoxin in 100 ml siliconed, airtight bottles in an atmosphere of air 5 % CO$_2$. The suspensions were incubated under gentle rotation in a water bath for 1 h at 37 °C. A sample was then withdrawn and diluted in cold HBS to contain an average of 50 cells/ml. At least 15 PMK III/1 monolayers, grown in 6 cm Petri dishes, were each seeded with 0.2 ml (~ 10 cells) of the above dilution. The number of plaques/Petri dish, counted after incubation for 72 h was totalled and divided by the calculated total number of cells seeded to obtain the efficiency of infection at 60 min (IC$_{60}$).

Single cycles of infection. These were performed as described above, but incubation was extended from 1 to 7 h. Samples were taken at 5, 6 and 7 h for determination of the total p.f.u. content after disruption of the cells. The yield (p.f.u./cell) attained its maximum in the 6th h of the cycle. This was regarded as the average final virus yield/cell (FY).

Chemicals. The analytical grade salts were obtained from REANAL (Budapest, Hungary); other components of the medium were the products of NBC (Cleveland, Ohio, U.S.A.). The cardioactive sterols were kindly supplied by Dr G. Fekete (Chemical Works of Gedeon Richter Ltd., Budapest).
RESULTS

Structure and activity of cardioactive sterols

Earlier studies showed cardioactive sterols to be non-toxic for PMK III/1 cells, when applied in final concentrations lower than $10^{-6}$ M (Koch et al. 1970). The ratios of average final virus yield in p.f.u./cell (FY) in the presence and absence of $10^{-8}$ M concentrations of different cardioactive sterols were determined. These ratios are compared in Table 1 with the affinity coefficients of the same compounds for the A and B components of (Na$^+$, K$^+$)-dependent ATPase (ATP-phosphohydrolase, EC 3.6.1.3) binding site, as described by Wilson, Sivitz & Hanna (1970).

Table 1. Relative virus yield and affinity to the (Na$^+$, K$^+$)-ATPase binding site of some cardioactive sterols

<table>
<thead>
<tr>
<th>Sterol added to $10^{-8}$ M-</th>
<th>Aglycone</th>
<th>Binding site component A.</th>
<th>Glycoside-acetylglycoside</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative affinity coefficient</td>
<td>Relative virus yield</td>
<td>Relative affinity coefficient</td>
</tr>
<tr>
<td>3β, 14-Dihydroxy-5β-card-20(22)-enolide (digitoxigenin)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.10</td>
</tr>
<tr>
<td>3β, 12β, 14-Trihydroxy-5β-card-20(22)-enolide (digoxigenin)</td>
<td>0.54</td>
<td>0.30</td>
<td>0.66</td>
</tr>
<tr>
<td>3β, 14, 16β-Trihydroxy-5β-card-20(22)-enolide (gitoxigenin)</td>
<td>0.3</td>
<td>not done</td>
<td>0.41</td>
</tr>
</tbody>
</table>

The relative virus yield is presented as the optimum virus yield (p.f.u./cell) obtained in the presence of the sterol divided by that in its absence. The relative affinity coefficients are calculated from the data of Wilson et al. (1970) by dividing the affinity coefficients for each cardioactive sterol by that of digitoxigenin.

The affinity coefficients for the C component of the binding site are not included in Table 1. This component represents the active site reacting with the 17β-lactone-(card-20[22]-enolide) group of the molecule, which is identical in all compounds tested. The 16β-hydroxyl substituent of gitoxigenin is known to interact intramolecularly with the 17β-lactone ring. This affects unfavourably the specific binding to the C component and causes gitoxigenin and gitoxin not only to be poor inhibitors of the enzyme (Wilson et al. 1970), but also to fail in exalting virus yield (FY).

Affinity for component B and enhancement of virus yield decreased similarly with the increase in the polarity of the molecule's sterol skeleton. The decrease in the affinity for component A was attributed by Wilson et al. (1970) to the increase in the bulk of the 3β substituent on adding further sugar molecules. Otherwise they found it difficult to rationalize a diminution in enzyme-inhibitory responsiveness to additional hydrogen-bonding capacity. However, these factors improved virus yields, apparently by exalting efficiency of infection.

Action of digitoxin on the efficiency of infection (IC$_{60}$) and virus yield (FY)

Of the cardioactive sterols tested, digitoxin was selected for further study. Cell suspensions were exposed to different numbers of free p.f.u./cell ($V_i$) as described above. The cell suspensions were then diluted appropriately and transferred to HBS or to HBS + $10^{-8}$ M final concentration of digitoxin. The efficiency of infection (IC$_{60}$) and virus yield (FY) were determined in both systems. As shown in Fig. 1, at any number of free initial p.f.u.
the virus yield was higher in the presence than in the absence of digitoxin. The FY/IC₆₀ ratio appeared to be constant and independent of the presence or absence of digitoxin. From this it was concluded that the improved yields in the presence of digitoxin were due to higher efficiencies of infection by the already attached virus, rather than to stimulation of intracellular replication and higher yield/cell.

Relation of efficiency of infection to numbers of initial free and attached p.f.u./cell

The average number of p.f.u. attached/cell ($V_a$) in a certain time was proportional to the initial number of free p.f.u./cell ($V_i$) as anticipated for virus attachment as a pseudo-first-order irreversible reaction (Schlesinger, 1933a, b; Delbrück, 1940). However, the efficiency of infection (IC₆₀) was low and decreased rapidly when $V_i$ was above unity. The relationship of these three parameters was examined in greater detail.

As shown by Marcus & Puck (1958) it is possible to calculate, on the basis of the Poissonian distribution, the frequency of cells having a certain number of attached p.f.u., or at least a certain number of attached p.f.u., for any average number of attached p.f.u./cell ($V_a$). Clearly, the higher the average attached p.f.u./cell, the higher the frequency ($p_z$) of cells carrying attached virus(es), and the higher the frequency of potentially infected cells.

The values of $p_z$ were obtained from Poisson’s formula. The suffix $z$ defines the only or the least number of p.f.u./cell for which the frequency $p_z$ was calculated. The frequency of cells infected is IC₆₀ by definition. Therefore, if the efficiency of infection is a function only of the frequency of particle attachment, $p_z$, then the influence of $V_a$ or $V_i$ are expressed through $p_z$. However, if IC₆₀ is determined independently by $V_a$ or $V_i$ and $p_z$, then factors other than the frequency of attachment are involved.

To facilitate comparison, the results in Fig. 2 are expressed as ratios with the corresponding $V_i$ as denominator.

The ratio $V_a/V_i$ appears to be constant ($\sim 0.83$) at all values of $V_i$ examined. However,
The chance-event in virus-cell interaction

Fig. 2. Ratios of the proportion of cells infected (IC60) and of the average number of p.f.u. attached/cell at given time (Va) with the initial number of free p.f.u./cell (Vf). For comparison are shown ratios to Vf of the calculated values of the frequency of attachment of a certain number z of p.f.u./cell (pz). Ratio Va/Vf = ●; IC60/Vf = ▲. Ratio pz/Vf for values of z as follows:

curve a: \( z = \text{only 1}, p_z = \frac{\bar{V}_a \exp(-\bar{V}_a)}{2} \),

\( b: \quad 2, p_z = \frac{\bar{V}_a^2 \exp(-\bar{V}_a)}{2} \),

\( c: \quad 3, p_z = \frac{\bar{V}_a^3 \exp(-\bar{V}_a)}{6} \),

\( d: \quad z = \text{at least 1}, p_z = 1 - \exp(-\bar{V}_a) \),

\( e: \quad 2, p_z = 1 - \exp(-\bar{V}_a)(1+\bar{V}_a) \),

\( f: \quad 3, p_z = 1 - \exp(-\bar{V}_a)(1+\bar{V}_a+\frac{\bar{V}_a^2}{2}) \).
Fig. 3. Effect of digitoxin on the proportion of cells infected (IC\textsubscript{60})/initial number of free p.f.u. (V\textsubscript{i}). Ratios of IC\textsubscript{60}/V\textsubscript{i} in the absence (▲) and in the presence (●) of 10^{-8} M digitoxin. The calculated ratio IC\textsubscript{60}/V\textsubscript{i} for theoretical constant values of IC\textsubscript{60} = 1.0 (a); 0.75 (b); 0.50 (c); 0.45 (d); 0.25 (e); and 0.10 (f), are shown for comparison.

the ratio IC\textsubscript{60}/V\textsubscript{i} fell steeply with the increase of V\textsubscript{i} above unity. Six additional curves representing ratios p\textsubscript{z}/V\textsubscript{i} for z only 1, 2 or 3 and for z at least 1, 2 or 3 are included in Fig. 2 for comparison. The curve for IC\textsubscript{60}/V\textsubscript{i} clearly does not fit any of the curves calculated for p\textsubscript{z}/V\textsubscript{i} at any value of z. Moreover, the curve for IC\textsubscript{60}/V\textsubscript{i} intersects all curves for p\textsubscript{z}/V\textsubscript{i} except that in which z specifies only one p.f.u./cell. It follows from this that the efficiency of infection is a different function of V\textsubscript{i} than is the frequency p\textsubscript{z} of cells having attached virus particle(s).

Effect of digitoxin on the ratio IC\textsubscript{60}/V\textsubscript{i}

If the efficiency of infection is constant then its value (IC\textsubscript{60} const.) can only be determined if there is available at least a critical minimum initial number of free p.f.u./cell. At lower values of V\textsubscript{i} the constancy of IC\textsubscript{60} cannot be detected. At values of V\textsubscript{i} above the critical minimum, the ratio IC\textsubscript{60} const./V\textsubscript{i} should be inversely proportional to V\textsubscript{i}. The maximum value of IC\textsubscript{60} is unity, when all cells are infected. This can not be achieved experimentally since p\textsubscript{z} approaches unity asymptotically. However, it should be noted that an average of 3 p.f.u. attached/cell (\overline{V\textsubscript{o}}) ensured attachment of at least one p.f.u. to 95% of the cells.
The IC₆₀/Vᵢ ratios in the presence and absence of digitoxin are shown in Fig. 3 with ratios calculated for various values of IC₆₀ const. Clearly, the efficiency of infection is not constant in the control system. However, in the digitoxin-treated system, IC₆₀ not only increased but became constant at ~ 0.45. Thus the theoretical minimum value of Vᵢ is 0.72 when \( \bar{V}_a = 0.60 \) and \( p_z = 0.45 \), for a \( z \) value of at least one.

**Theoretical considerations**

Although it is trivial to state that virus attachment is an essential condition of infection, it is useful to emphasize that attachment is not the only condition of infection. Phenomenologically, attachment proceeds as a pseudo-first-order irreversible reaction. Therefore, as shown also in this study, the average number of p.f.u. attached/cell at a given time is proportional to the number of free p.f.u./cell. This implies that the attachment is diffusion limited and the probability of successful collisions (\( p \)) between adsorbate and adsorbent is constant (Collins & Kimball, 1949; Collins, 1950). However, for most virus systems, this constant probability is not maximal at \( p = 1 \). Thus, the application (Valentine & Allison, 1959) of the Smoluchowsky solution of Fick’s equations may lead to erroneous conclusions, as shown by Koch (1960) and Ogston (1963). None of the equations available on diffusion limited reactions permits estimation from the rate of attachment of the probability of successful collisions. Nevertheless, the boundary condition suggested by Collins & Kimball (1949) and Collins (1950) allow consideration of the probability parameter. Their equation giving the flux of attachment (\( \phi \)) can be written in the general form

\[
\phi = C_\infty f(D, r_0, t, p),
\]

where \( C_\infty \) is the constant concentration of the diffusing species at infinite distance from the adsorbent, \( r_0 \) is the adsorbent’s radius, \( t \) is time and \( p \) the probability of a successful collision. When \( p \) is constant, then at constant time \( t = \tau \)

\[
\frac{1}{C_\infty} \int_0^\tau \phi dt = \int_0^\tau f(D, r_0, t, p) = \text{constant},
\]

but

\[
\int_0^\tau \phi dt = C_0(\tau)
\]

is the concentration of adsorbate removed from the bulk phase in \( \tau \) time. Thus we have

\[
\frac{C_0(\tau)}{C_\infty} = \text{constant}.
\]

At constant time, analogues of this equation may be derived from the Smoluchowsky equation and for pseudo-first-order irreversible reactions. In animal virus systems, \( C_0(\tau) \) is analogous to \( \bar{V}_a \) and \( C_\infty \) to \( V_0 \), because the equation is valid for both open and closed physical systems. Now if the probability of a successful collision (\( p \)) and the time of attachment (\( \tau \)) are constant

\[
\int_0^\tau \phi dt = \bar{V}_a = f_1(p_z)
\]

and

\[
\frac{f_1(p_z)}{V_0} = \int_0^\tau f(D, r_0, t, p) = \text{constant}.
\]

Thus in this case the frequency of potentially infected cells (\( p_z \)) is a function only of the diffusion limited rate of attachment.
Now because $p_z$ is diffusion limited and the efficiency of infection is not, a strict distinction should be made between the probability of a 'successful collision' which result in attachment and the probability of a 'successful hit' which result in infection. The collisions of virus particles with quasi-complementary receptors may result in attachment, whereas hits leading to infection require full complementarity and optimal fitting and intermolecular bonding between virus and receptor.

**DISCUSSION**

It is remarkable that essentially the same structural characteristics of the cardioactive sterols tested are required for the optimal inhibition of $(\text{Na}^+, \text{K}^+)$-dependent ATPase and the improved efficiency of virus infection. This led us to postulate a causal correlation between the two effects. According to Opit & Charnock (1965) and Lowe (1968), the inhibitory effect of cardioactive sterols consists of stabilization of the $(\text{Na}^+, \text{K}^+)$-dependent ATPase in one of its possible conformations. In a metastable macromolecular structure like the cytoplasmic membrane, it is difficult to consider such a change in one component without effect on neighbouring components.

In our own poliovirus–monkey kidney cell system, the digitoxin ratio was more than $10^7$ molecules/cell. In several different cell systems, the rate of specific cardioactive sterol binding was reported to be very high even at lower concentrations (Ellory & Keynes, 1969). Therefore, it was anticipated that in our own system, most if not all $(\text{Na}^+, \text{K}^+)$-dependent ATPase sites were saturated. We can offer no direct evidence of this, because more than 90% of cellular ATPase activity was sterol insensitive and measurements on intact cells were unreliable.

As can be seen from Figs. 2 and 3, the efficiency of infection ($IC_{60}$) was neither constant, nor a function of the initial number of free p.f.u./cell ($V_i$) as expressed through the diffusion-limited parameters of attachment ($V_a$ and $p_z$). The presence of digitoxin stabilized the efficiency of infection at an elevated level, which was constant at all values of $V_i$ higher than unity ($p_z > 0.45$). Since digitoxin acted on the system in the post-attachment, pre-penetration phase of interaction (Koch et al. 1970), it was supposed to affect the frequency of 'successful hits' required for a successful infection.

An interpretation of these findings may be offered if it is supposed that the cellular receptors for poliovirus are metastable structures affected directly or indirectly by the actual conformational state of the $(\text{Na}^+, \text{K}^+)$-dependent ATPase. The chance of successful infection depends on several parameters, including the number and orientation of virus particles in the proximity of receptors in the 'active' or complementary structural state and the duration of this state which may or may not be sufficient for a successful hit. The chance of a successful collision, leading to attachment but not necessarily infection, is much higher since this may occur also at receptors in a 'quasi-complementary' structural state.

Inhibition by digitoxin of the enzyme's conformational changes would 'immobilize' the receptors in their active structural state. Such receptors may be expected to exist at given sites for prolonged periods during which the efficiency of infection depends only on the biological integrity of the cell (Eremenko, Benedetto & Volpe, 1972) and on the chance for an attached virus particle to hit the active receptor.

It should be noted that for enveloped viruses and $10^{-5} + 10^{-6}$ M final concentrations of cardioactive sterols, only reduced virus yields have been detected (Link et al. 1966; Nagai et al. 1972). In our own system, too, virus yields were considerably reduced at cardioactive sterol concentrations higher than $10^{-7}$ M.
REFERENCES


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