serum magnesium concentrations increased significantly.

In patients, amphotericin B was given during infusions of 2 hours' duration to patients who previously had received drug for several months. In most cases the serum concentration and renal clearance of electrolytes were similar to control infusions without drug. However, the serum magnesium concentration tended to decrease after drug infusion more so than after control infusion. There was no measurable increase in urinary magnesium clearance. One patient with hypokalemia showed excessive urinary potassium excretion which increased further during infusion of amphotericin B.

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Penetration of Herpes Simplex Virus into Human Epidermoid Cells.* (29392)

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There is general acceptance of the thesis that all viral infections are initiated by two sequential events: adsorption and penetration. The mechanisms by which virions attach to cell surfaces have been thoroughly studied and carefully reviewed(1,2,3). When cellular receptor sites greatly exceed numbers of virions, adsorption rates approximate first order kinetics. Temperatures between 0° and 37° influence the rate of virion attachment only by virtue of slight changes in viscosity of the suspending medium which, in turn, slightly alters the frequency of virion-cell collisions. DNA-containing bacterio-

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phages are equipped with special attachment organs located in the tip of the tail(4), whereas RNA-containing bacteriophages(5) and animal viruses have multiple attachment sites on capsids or envelopes. Penetration of DNA phages takes place in a complicated series of temperature-dependent steps, but the essential event is injection of phage DNA, which is probably controlled by contractile tail proteins supplied with energy-rich nucleotide triphosphates and adenosine triphosphatase(6). The phage protein coat is left outside the cell.

Penetration of animal viruses apparently occurs by the very different process of pinocytosis in which the cell plays the active role. As viewed by electron microscopy, the whole virion, or at least a major portion, is transported through the cytoplasmic membrane of the host animal cell(7,8,9,10). Although some virions may be altered on attachment to the cytoplasmic membrane(10, 12), viral nucleic acid is finally stripped of its capsid only after entering the cell(11). Careful kinetic analyses of the penetration process have been made with several viruses, particularly the small RNA-containing poliovirus(12,13) and foot-and-mouth disease (FMD) virus(14). These studies reveal that penetration is a temperature-dependent process that begins soon after virion attachment. The present experiments with the large DNA-containing herpes virus were designed to study penetration as an isolated process distinct from attachment.

Materials and methods. The basic properties of the virus-cell system have been described by Roizman and his colleages(15). The solution used for suspending cells and for diluting virus was phosphate-buffered saline containing 1% crystalline bovine serum albumin (PBS-A). The source of herpes virus antibody was a commercial preparation of human gamma globulin (γ-G) (Lederle).

Cells. Human epidermoid carcinoma #2 (HEp-2) cells were used both for virus assay and for studies of virus adsorption and penetration. Cells were grown at 37° in medium 199 supplemented with 10% calf serum (Microbiological Associates, Bethesda, Md.) until confluent sheets formed on the flat surfaces of 32-oz prescription bottles. The monolayers were detached from the glass and dispersed into uniform suspensions of single cells by treatment with 0.2% ethylenediaminotetraacetic acid (EDTA). The cells were then packed by centrifugation at 1500 RPM for 2 minutes, suspended in PBS-A at 4° and repacked immediately prior to use in studies of virus adsorption and penetration.

Virus. Herpes simplex virus (HSV), the macroplaque variant, was originally obtained from Dr. Bernard Roizman; the properties of this strain have been described elsewhere (16). A single pool of HSV cultivated in HEp-2 cells for 24 hours at 34° was used in all experiments. At the end of the incubation period infected cells were disrupted by alternate freezing and thawing to release intracellular virus, which was combined with extracellular virus in the culture medium. This stock HSV suspension maintained a titer of 2×10^6 polykaryocyte-forming units (PoFU) for several months when stored at −60° in a stabilizing medium of 16% sterile milk. In view of a later finding that HSV could be disaggregated with resultant doubling in titer, certain stock virus suspensions were subjected to ultrasonic vibration for 30 seconds in a Raytheon 10 kc oscillator prior to use.

Virus assay. All HSV titrations were performed by making 3-fold dilutions of the test suspension in PBS-A and plating 0.1-ml aliquots on each of two HEp-2 cell monolayers in plastic petri plates (35 mm in diameter). Virus was permitted to adsorb on the cell sheet for one hour at 37° prior to adding 2 ml of liquid antibody overlay consisting of medium 199 with 1% calf serum and 0.2% human gamma globulin. After further incubation for 2 days in a humidified atmosphere of 5% CO_2, the cell monolayers were stained with Giemsa and the polykaryocytes (plaques) scored. A similar procedure was followed for determining the number of infective centers by diluting HSV-infected cell suspensions in PBS-A with 1% γ-G and plating 0.1 ml of each dilution on duplicate monolayers of HEp-2 cells.

Experimental procedures. Penetration of HSV into suspended HEp-2 cells was determined by measuring the rate at which cell-attached virus became resistant to inactivation by antiviral antibody or by brief exposure to an acid environment. In all experiments, HSV was first adsorbed on cells at 4° in order to minimize penetration until virus attachment was complete. This was accomplished under standardized and reproducible conditions by mixing prechilled packed cells (2×10^7) with 1 ml of prechilled virus to yield adsorption multiplicities of 0.1-0.3 for the experiments in which cell-attached virus was inactivated by acid and 0.02 for the experiments in which inactivation was by antibody. The low multiplicities insured that the vast majority of infected cells would each be infected with only a single PoFU.

HSV formed a stable union with cell surface at 4° as evidenced by the finding that less than 2% of attached virus could be dissociated by repeated washing with PBS-A at 4° or 37°. However, viable and intact cells were essential for firm binding of virus. Prior fixation of cells in absolute methanol did not appreciably reduce adsorption but virtually all the virus could be physically dissociated from the cell surface by washing.

These and other preliminary experiments furnished evidence that almost all adsorbed HSV remained unaltered at the surface of intact HEp-2 cells for at least one hour at 4°. Under optimal conditions the number of infective centers that eventually formed equalled the total attached virus. Penetration was initiated (time 0) by making a 10-fold dilution of suspended cells with attached HSV into PBS-A prewarmed in a water bath to the desired reaction temperature. In each experiment a sample of the original cell suspension diluted into PBS-A at 4° served as a control.

A virus particle was considered to have penetrated into a cell when an infective center would form despite the addition of antibody or acid. In one series of experiments, penetration was stopped at intervals after warming the suspended cells by diluting them 10-fold in PBS-A with 1% γ-G; serial 3-fold dilutions were then made in 1% γ-G and the suspended cells plated on HEp-2 monolayers. In another series of experiments, virus penetration was terminated by diluting the suspended cells 20-fold with PBS-A previously adjusted at pH 3 with HCl. After exposure to the acid environment for 1 minute at room temperature, the medium was neutralized with equimolar NaOH. The suspended cells were then diluted in 3-fold steps in PBS-A without antibody and 0.1-ml aliquots were plated on duplicate HEp-2 cell monolayers under liquid medium containing antiserum to determine the number of infective centers.

To establish the validity of these procedures as criteria of virus penetration, control experiments were performed to determine the effect of antiviral antibody and an acid environment on free HSV and uninfected HEp-2 cells. When free HSV at a titer of 3.94×10^6 PoFU/ml was diluted serially in 1% γ-G, in a manner similar to the procedure for assaying infective centers, 99.94% of the virus was neutralized. A kinetic neutralization test of the γ-G preparation against HSV yielded a k value of 49.5 $min.^{-1}$.

Exposure to pH 3 for 1 minute at room temperature was also a highly efficient method for inactivating free HSV. In 7 separate control experiments, virus titers of $1.44\text{-}2.51 \times 10^5$ PoFU/ml were reduced by at least 99.8%. Moreover, in 2 additional experiments inactivation occurred to the same degree despite the presence of 5×10^5 HEp-2 cells in the acid medium prior to addition of virus. This latter finding suggests that HEp-2 cells do not protect HSV from acid inactivation simply by a buffering effect. However, acid treatment did affect the capacity of uninfected cells to serve as infective centers without appreciably altering surface binding of virus. After being suspended in PBS-A at pH 3 for 1 minute, formation of infective centers was reduced by 30-50% when compared with equal numbers of HEp-2 cells not treated with acid. However, the acid-inactivation method still proved to be a reliable index of penetration because, in any single experiment, the same proportion of suspended cells in each sample failed to

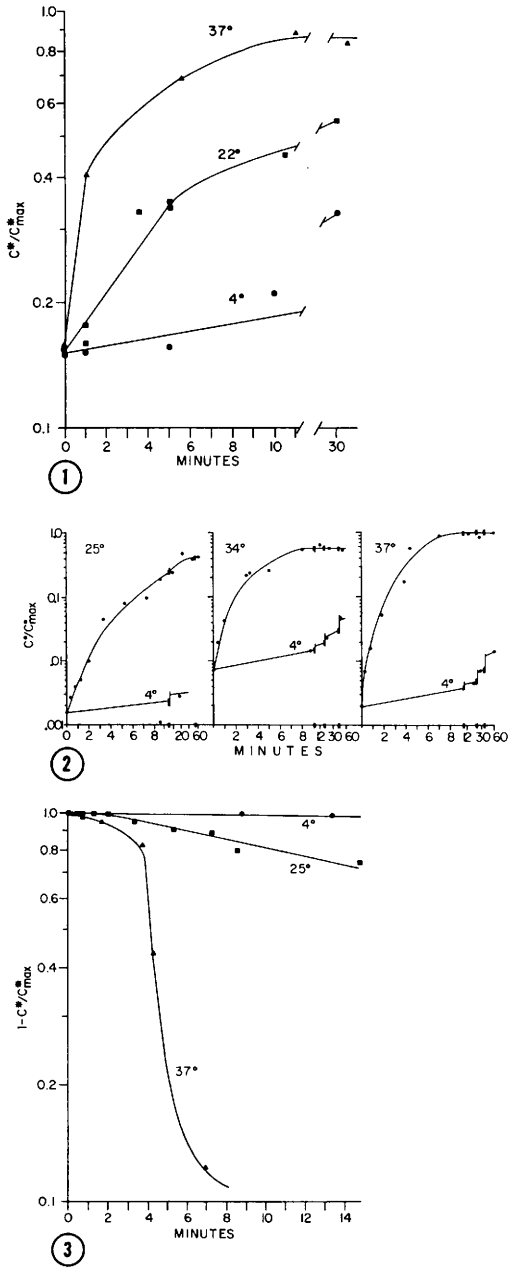

FIG. 1. Rate at which cell-attached herpes virus becomes resistant to antibody at various temperatures. Results are expressed as fraction of attached virus (C^*_{max}) that formed infective centers (C^*) at each time interval.

FIG. 2. Rate at which cell-attached virus becomes resistant to inactivation by acid. C^* = infective centers; C^*_{max} = total attached virus capable of forming infective centers at 37°. Data are corrected for cells killed by the short exposure to pH 3.

FIG. 3. Disappearance of acid-sensitive herpes virus from cell surface as functions of temperature and time. Data in Fig. 2 for fractions of acid-resistant virus (C^*/C^*_{max}) are transposed here to acid-sensitive virus ($1 - C^*/C^*_{max}$).

register as infective centers and could be discounted.

Results. Antibody resistance of cell-attached virus. Fig. 1 summarizes the results of several experiments designed to determine the rate at which cell-attached HSV becomes resistant to antibody at different temperatures. The data are plotted as fraction of cell-attached virus that formed infective centers at intervals after initiation of penetration at the desired temperature. Penetration, defined in this narrow sense, was clearly a temperature-dependent reaction. Virtually all the virus that could be recovered as infective centers, approximately 90% of attached virus, had become resistant to antibody within 10 minutes of incubation at 37°. Resistance to antibody developed less rapidly and less completely at 22°. However, when cells incubated for 30 minutes at 22° or 4° were transferred to 37°, formation of infective centers resumed and achieved levels expected at the higher temperature. These data can be interpreted as evidence that virus attached on the cell surface retains its infectious potential at temperatures suboptimal for penetration. As would be expected, once virus had become resistant to antibody at 37°, the process could not be reversed by lowering the temperature.

Also evident from the data in Fig. 1 are the limitations of the antibody method as a criterion of penetration. Even at 4°, approximately 15% of cell-attached virus resisted neutralization by antibody after the period of adsorption (penetration time 0), and infective center formation increased to 30% on further incubation at 4°. It was essential, therefore, to use a more efficient method for inactivating "unpenetrated" virus.

Resistance to acid inactivation of cell-attached virus. The data plotted in Fig. 2 again demonstrate that the rate and extent of infective center formation are a function of temperature of incubation. In these experiments cell-attached virus was inactivated by acid at intervals after incubating cell suspensions at 25°, 34°, or 37°. Exposure to acid

was clearly superior to antibody neutralization as a method for inactivating "unpenetrated" virus. After adsorption at 4° for 1 hour (time 0 in Fig. 2), only 0.1-0.7% of total cell-attached virus resisted inactivation by acid in 3 separate experiments. Therefore, greater than 100-fold increases in formation of infective centers could be detected by this method compared with maximal 6-fold increases by development of resistance to antibody. It is of interest, nevertheless, that approximately 90% of cell-attached virus became resistant to inactivation by acid or antibody within 7-10 minutes of incubation at 37°. Moreover, the same fraction of cell-attached virus resisted successive exposure to acid and antibody.

The rapidity with which cell-attached virus became resistant to acid is particularly noteworthy. The times required to reach maximal levels of "penetration" were no greater than 7 minutes at 37°, 8 minutes at 34° and 20 minutes at 25°. The curvilinearity of the graphs suggests that the rates of infective center formation do not conform to first order kinetics, as has been suggested for penetration of other viruses(17,18).

To analyze the nature of the penetration reaction in greater detail, data in Fig. 2 were transposed to express the results as disappearance of acid-sensitive virus from the surface of HEp-2 cells at 4°, 25° and 37° (Fig. 3). Even when expressed in this more conventional manner, penetration rates clearly do not conform to simple first order kinetics. At least 80% of attached virus remained sensitive to acid during the first 4 minutes of incubation at 37°. Following this initial lag period, the rate of decrease was more nearly exponential both at 37° and 25°.

Assuming a 2-stage reaction, rate constants (k_1 and k_2) were calculated from the initial and secondary slopes for the disappearance of acid-sensitive virus from the surface of cells at 25° and 37°. The calculations are based on first order kinetics for the linear portions of the curves on the assumption that the rates are proportional to the concentration of residual herpes virus on the cell surface. The results, shown in Table I, again demonstrate the marked temperature dependence and complex nature of the penetration reaction. The differences in the values for k_1 and k_2 suggest that development of acid resistance may be a function of the time required for virus to move from an exposed position on the cell surface to a protected position of envelopment by cytoplasmic membrane.

TABLE I. Initial (k_1) and Secondary (k_2) Rate Constants for Penetration of HSV.

Temperature	k_1 (min^{-1})*	k_2 (min^{-1})*
25°	.0058	.025
37°	.0406	1.37

* Calculated from the equation $dC^*/dt = k(C^*_{max} - C^*)$ and based on the data for development of resistance to acid inactivation shown in Fig. 2 and 3. Values for k_1 correspond to the first 3 minutes of the reactions at each temperature; values for k_2 are calculated from data for the periods 4-5 min at 37° and 4-15 min at 25°.

Discussion. Recent studies by electron microscopy have demonstrated unequivocally that large and morphologically identifiable viruses, such as vaccinia(7) and herpes simplex(8,9) are engulfed by cytoplasmic membrane and pass into the cell in pinocytotic vesicles. The process probably does not differ in any essential respect from pinocytosis of particulate inorganic matter such as thorium dioxide(9) and colloidal gold(8). Active participation of the cell is implicit in the finding that transport of particles across the cytoplasmic membrane is temperature dependent. However, even when virus-cell interaction is partially synchronized by prior adsorption at 0° before initiation of penetration by warming to 37°, herpes simplex virions can be seen for at least 30 minutes on both sides of the cytoplasmic membrane (8,9). Although recent photographs are remarkable in their clarity and detail, electron microscopy can offer only partial explanations of the events that lead to virus penetration. Among the intrinsic limitations of the method are requirements for extremely high multiplicities of infection, lack of assurance that observations are being made on representative virus and cell populations, and the relatively low ratio of infectious units to physical virus particles. It still seems essential for a more complete biological explana-

tion of virus penetration to correlate direct observation with detailed analyses of the kinetics of the reaction.

Poliovirus has served as the model for many recent studies of the early stages of infection, partly because it is far more resistant to inactivation by physical and chemical agents than are larger viruses. However, interaction of poliovirus and isolated fragments of cytoplasmic membranes causes partial degradation of virus capsid that results in reduced infectivity and loss of reactivity with antiserum(12). This reaction is considered to be temperature dependent and to proceed by first order kinetics(12). Two parallel methods have been used to study interaction of poliovirus and intact HeLa cells. In one, residual infectious virus is detached from cells at intervals after infection by chemical or physical agents and, in the other, resistance to antibody is determined simultaneously by rates of infective center formation. First order kinetics at similar rates have been reported for formation of infective centers and loss of infectious virus detachable from cells with lithium chloride or urea(18). However, Mandel(17) has shown that fully infectious poliovirus can be recovered from HeLa cells by disrupting them with sodium dodecyl sulfate long after the virus has become resistant to antibody. These data suggest that antibody insensitivity is only an early stage in a series of sequential events that leads to penetration and eclipse. The question arises whether any biological methods currently in use can distinguish between virus physically located on the cell surface or within intracytoplasmic pinocytotic vesicles. The classical method of antibody neutralization is far from ideal for determining when cell-attached virus is no longer influenced by the extracellular environment. The present studies confirm many earlier observations that virus on the cell surface is far more resistant to antibody than free virus.

The method of resistance to acid inactivation of cell-attached virus(14), which was adapted for use in our experiments, is a sensitive and reliable procedure for measuring rates of infective center formation. Thorne(14) analyzed his extensive data on infection of pig kidney cells with FMD virus using this parameter of virus penetration. By assuming Michaelis-Menten kinetics for the combined reactions of adsorption and penetration, rate constants were calculated for attachment of virus and the temperature-dependent step of penetration. These assumptions appear to be valid as an expression of the overall process of infection, but it is less certain that penetration, as an isolated event, can be described as a first order reaction. When FMD virus was previously adsorbed on cells at 10°, curves of infective center formation at higher temperatures appear strikingly similar to those reported herein for herpes virus. Under the conditions of our acid inactivation experiments, penetration of HSV is clearly not a first order reaction. There are at least two distinct kinetic rates for the formation of infective centers from the time of the first observations at 15-20 seconds. The curves may also reflect heterogeneity of cell population as well as virus population. Holmes and Watson(9) have shown by electron microscopy that enveloped herpes virus adsorbs more readily to cell surfaces than do naked virus particles. The presence of adenosinetriphosphatase in enveloped herpes virus(19) may also contribute to the process of penetration. In fact, the suggestion has been made that nonenveloped herpes virus is not infectious(20).

It is of interest to compare rates of penetration for FMD virus(14) and HSV studied by similar methods of resistance to acid inactivation. Based on an index of 90% formation of infective centers, penetration of FMD virus required about 3 minutes at 37° and about 20 minutes at 25°; in our experiments, equivalent penetration of HSV occurred in 4-7 minutes at 37° and in less than 20 minutes at 25°. In striking contrast to these results, Farnham and Newton(21) reported a time of about 2 hours for penetration of HSV into HeLa cells as studied by resistance to antibody. The discrepancy may be due to strain differences in viruses and cells, but it seems more likely that methods used in earlier experiments were relatively insensitive and not sufficiently accurate for detecting small increments in numbers of infective cen-

ters. In our experience, the reliability and reproducibility of virus penetration studies are considerably enhanced if the following experimental conditions can be satisfied: low multiplicity infection, ability to make separate measurements of attachment from penetration, efficient methods for inactivating virus on the cell surface, and at least 100-fold increments in formation of infective centers at optimal conditions.

Summary. The kinetics of infective center formation was studied as a parameter of herpes simplex virus penetration into suspended HEp-2 cells. Similar rates of penetration were obtained when antibody or acid was used to inactivate virus attached to the cell surface, although the acid method was far more efficient and reliable. The data indicate that virus penetration is strictly temperature dependent and is virtually complete by 7 minutes at 37°, but the reaction does not conform to simple first order kinetics.

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Muramidase Activity of Kidney and Spleen in Swiss Mice Challenged with B.C.G., Zymosan and Bacterial Endotoxins.* (29393)

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Increased muramidase activity in the kidneys and spleens of tumor-bearing mice, rats and hamsters has been demonstrated in our laboratory(1,2,3). Elevated enzyme levels in tissues other than kidney have been reported for non-neoplastic pathological conditions(4, 5,6). Granulomatous tissue of the lungs of rabbits infected with *Bacillus Calmette-Guerin* (BCG)(7), plasma of animals infected with Newcastle's disease virus(8), and blood of animals treated with bacterial endotoxins (9), also show increased muramidase activity. It is our purpose to demonstrate that after specific challenges to the host defense mechanism, by BCG, zymosan and bacterial endotoxins, muramidase activity in the kidney and spleen is elevated.

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