THE ROLE OF HORMONES IN VIRAL INFECTIONS. I. SUPPRESSION OF VIRAL ADSORPTION AND PENETRATION INTO CELLS TREATED WITH PARATHYROID HORMONE IN VITRO\textsuperscript{*}

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It is evident that if an organism is at all sensitive to a virus some of its cells must be susceptible to infection. In most viral infections, the severity of the disease reflects necessarily the number and nature of the cells that are infected and destroyed. The observations that free poliovirus RNA infects and produces viral progeny in cells incapable of becoming infected with poliovirus virions\textsuperscript{1} suggest that more cells are capable of supporting viral reproduction than there are cells capable of becoming infected. It would appear that the competence of cells to become infected is one of the factors limiting the number of infected cells and perhaps determining the survival of the nonimmune host. The results of studies presented here show that some hormones modify the ability of cells in vitro to become infected.

The idea that hormones play an important, and perhaps determinant, role in viral infections is not new. It is well known that administration of hormones, notably steroids and the adrenocorticotropic factor (ACTH), may alter the course of viral infection to the benefit or detriment of the host. The mechanism by which most hormones affect the course and outcome of viral infection is not settled. It has been held by various authors\textsuperscript{2} that the effects of ACTH and corticosteroids are the result of the suppression of the manifestations of inflammation, involution of lymph nodes and thymus, diminished migration of leucocytes and ability of reticulo-endothelial cells to remove foreign matter, and inadequate specific (antibody) and nonspecific (interferon) response. To paraphrase the conclusions stated in different ways by many investigators, hormones alter the probability that susceptible cells come in contact with the virus by making vital cells more or less accessible and by delaying or reducing the immune response. It seems pertinent, however, to raise the question whether hormones alter the ability of the "susceptible" cells to become infected and whether, in many instances, this effect of hormones, more than any other, determines the outcome of a natural or experimental challenge with a virus.
The hypothesis that hormones alter the ability of cells to become infected may be inferred from current concepts of the mechanism by which these substances modify cell function and structure. It has been suggested\textsuperscript{6-8} that hormones act at cytostuctural barriers, namely membranes of cells, nuclei, or mitochondria, and affect the transfer of specific molecules through the barriers by causing new receptor-carriers or permeases to become exposed to the surface of the membranes. It is conceivable that a structural alteration in the cytoplasmic membrane may bring about a drastic reduction in its ability to absorb virus without impairing the competence of the cell to support viral multiplication provided it becomes infected. It is conceivable also that hormones bring about a specific state of differentiation that enables cells to become infected or the specific effect of some viruses, notably tumorigenesis, to become manifest.

Experimental evidence in support of this hypothesis is meager, largely because there have been no studies specifically designed to test it. Moreover, in the \textit{in vivo} studies it is impossible to differentiate with certainty direct effects of hormones on cells which become infected from collateral effects on the organism (blood-brain barrier, antibody formation, etc.) which might affect the probability and duration of viral contact with susceptible cells. A few observations, however, are very suggestive. It was noted, for example, that estrogens increase the resistance of rabbits to vaccinia virus administered intradermally.\textsuperscript{8, 10} Estrogens were also shown to increase the resistance of monkeys and mice given neurotropic viruses by intranasal route.\textsuperscript{11, 12} Progesterone, stilbestrol, and testosterone propionate abolished or diminished mortality in mice fed MM virus.\textsuperscript{13} The beneficial effects of ACTH and cortisone in viral hepatitis might be due to suppression of inflammation as well as to an alteration of the ability of liver cells to become infected. It was noted by Shwartzman\textsuperscript{14} and by Kilbourne and Horsfall\textsuperscript{15} that mice become very susceptible to poliovirus and coxsackie viruses, respectively, following administration of cortisone. In these studies, the virus was injected directly into the tissues containing the susceptible cells; breakdown of "blood" barriers was apparently not involved.

It is of interest to recall that in embryonated eggs, administration of cortisone affects the multiplication of influenza virus in two ways: First, the latent phase of viral development is significantly longer in treated eggs.\textsuperscript{17} Second, treated eggs yield more virus than untreated ones.\textsuperscript{18} No explanation has been offered for the prolongation of the latent phase; high yields in treated eggs were attributed to inhibition of auto-interference. It could be, however, that both effects are the result of alteration in the structure of the cellular membranes caused by the hormone.

Finally, the role of hormones in the induction of tumors by viruses is well known.\textsuperscript{19} It may be recalled that, in mice of resistant strains, estrone administration is required for the induction of mammary tumors by a virus.\textsuperscript{20} Resistance to lymphomatosis in chickens may be diminished by estrone\textsuperscript{21} and increased by diethylstilbestrol and testosterone propionate.\textsuperscript{22} Although the thymus may consist largely of lymphoid tissue and its functions are not entirely clear, it may be considered an endocrine organ.\textsuperscript{23} In mice, thymectomy leads to a considerable reduction in the incidence of leukemias occurring spontaneously\textsuperscript{24} or triggered by radiation\textsuperscript{25} or carcinogens.\textsuperscript{26} A virus capable of inducing leukemias in intact, but not thymectomized, mice\textsuperscript{27} was demonstrated in thymic filtrates of irradiated mice.\textsuperscript{28, 29}
It is conceivable that the thymus elaborates a factor, potentially the lymphomasis-stimulating factor of Metcalfe\(^{20}\) which brings about the specific stage of cellular differentiation required for infection and transformation.

In essence, each of these observations is in accord with the hypothesis that endocrine secretions may alter the ability of cells to become infected. Adequate supporting evidence, however, is lacking. This and succeeding communications deal with in vitro studies designed specifically to test this hypothesis.

**Materials and Methods.**—Cells: Human epidermoid carcinoma no. 2 (HEp-2) cells were grown in disposable plastic flasks (Tissue Culture Flasks, Falcon Plastics, Inc., Los Angeles, California) in a medium consisting of Mixture 199 with 10% calf serum.

**Virus:** *Herpes simplex* virus (HSV) strain MP\(^{32}\) was prepared in HEp-2 cells as described elsewhere\(^{33}\) and stored in sterile skimmed milk at \(-60^\circ\text{C}\).

**Hormones:** The thyroid hormone was obtained in the form of lyophilized thyroid gland extract from “Société industrielle de Biologie, Nice.” Dosage is expressed in terms of milligrams of lyophilized preparation per ml of medium. The parathyroid gland extract (Parathyrone, “Laboratoires Byla, Paris”) titered 100 USP units per ml of solution.

**Solutions:** Maintenance medium (MM) consisted of Mixture 199 with 1% calf serum. MM-\(\gamma\)G was prepared by adding 0.3 ml of pooled human gamma globulin (Lederle Laboratories, Pearl River, New York) to 100 ml of MM. Phosphate buffered saline (PBS) was prepared according to Dulbecco and Vogt.\(^ {42}\) PBS-A was prepared by adding sufficient bovine albumin (Armour Co., Chicago, Illinois) to PBS to make 0.2 per cent.

**Experimental design:** In most experiments the HEp-2 cells were exposed to the hormones before infection. This was done as follows: Growth medium was removed from HEp-2 monolayer cultures prepared in disposable plastic flasks. Each flask then received 5 ml of MM containing the appropriate dilution of the hormones. The number of cultures used for each dilution varied between 2 and 4. After incubation at 37\(^\circ\)C for various intervals, as stated in the text, the MM was removed and the cells were washed with PBS. Each flask then received 1 ml of PBS-A containing 300–500 polykaryocyte-forming units of HSV. The flasks were then attached to an arm of a rotary shaker (80 rpm) and inserted inside a water bath at 36\(^\circ\)C. At intervals, as stated in the text, the flasks were removed from the shaker, the inoculum was aspirated, and 5 ml of MM-\(\gamma\)G added to each flask. The cultures were then incubated at 37\(^\circ\)C for 40–48 hr. At that time the tissue-culture fluid was aspirated and discarded, the cells were fixed with methyl alcohol, stained with Giemsa and air-dried. The polykaryocytes were counted with the aid of a stereomicroscope and a pen attached to an electric counter. This procedure was used to estimate the amount of virus which became adsorbed, and penetrated into cells, i.e., became inaccessible to antibody in the interval between virus inoculation and addition of MM-\(\gamma\)G.

In several experiments, the inocula removed from cultures of pretreated and untreated cells were assayed for the amount of residual virus. This was done as follows: The inocula from 3 replicate cultures treated in the same manner were pooled and reinoculated into fresh, untreated cultures. An aliquot of the original inoculum was also retitre at the same time. After 150 min of shaking in the water bath, the inoculum was aspirated and discarded and 5 ml of MM-\(\gamma\)G were added to each culture. This procedure was used to estimate the amount of virus absorbed by cells.

Finally, in a few experiments the cells were exposed to the hormone after infection. This was done as follows: One HEp-2 culture containing 1–2 \(\times\) \(10^6\) cells was inoculated with 2 ml of medium containing 8 \(\times\) \(10^8\) polykaryocyte-forming units. After two hr of shaking in a water bath at 37\(^\circ\)C, the cells were suspended with trypsin, washed and resuspended in MM-\(\gamma\)G to yield a concentration of 25–30 infected cells per ml. Two ml of the suspended-infected cells and 2 ml amounts of the hormone diluted so as to give twice the desired final concentration were added to each of two replicate monolayer cultures. The cultures were incubated for 40–48 hr, then fixed and stained as usual. This procedure was used to determine the effects of hormones on polykaryocytosis.

The virus assay method used in this study evolved from the earlier observations that in the presence of antiviral antibody, furnished in the form of pooled human globulin, the number of polykaryocytes produced by the MP strain of HSV is proportional to virus concentration or equal to the number of infected cells seeded on the monolayer culture.\(^ {34}\) The polykaryocytes appear as
plaquelike lesions with distinct staining properties and are easily countable. In the assay system, the function of the antibody is to prevent the spread of virus from cell to cell via the fluid phase; it does not prevent the formation of polykaryocytes. In this study, the antibody had an additioned function, namely to neutralize the virus which adsorbed but did not penetrate into cells. A recent analysis of the distribution of polykaryocytes in replicate cultures indicates that the mean variance may be estimated by the mean plaque count as expected from the Poisson distribution.

Results.—The effect of thyroid and parathyroid extracts on the formation of polykaryocytes induced by preinfected cells: Replicate monolayer cultures each received 2 ml of suspended infected cells and 2 ml of thyroid or parathyroid extracts at twice the final concentrations shown in Figure 1. The cultures were incubated for 48 hr, then fixed and stained. At this time the polykaryocytes were counted and the diameter of each measured. The average area of polykaryocytes shown in Figure 1 was calculated as described elsewhere. In cultures exposed to 10 units/ml of parathyroid hormone, the polykaryocytes were barely discernible. At all other concentrations of thyroid or parathyroid extracts tested, the number of polykaryocytes in treated and untreated cultures were countable and found to correspond in number to the polykaryocytes found in untreated cultures.

It is evident that once the cells are infected addition of thyroid or parathyroid extracts does not stop the development of HSV. However the size of polykaryocytes, that is, the ability of the infected cells to form polykaryocytes by recruiting new cells is markedly diminished in the presence of parathyroid extract and slightly diminished in the presence of thyroid extract.

The effect of pretreatment with parathyroid hormone on the number of polykaryocytes induced by HSV: In two experiments, HEp-2 cells were exposed to parathyroid hormone at a concentration of 1 unit/ml for 60, 20 and 5 min, immediately preceding infection. In most experiments, cells were exposed to parathyroid hormone (10-
Fig. 2.—Formation of polykaryocytes in HE-p-2 cell cultures pretreated with parathyroid hormone. Medium containing hormone was added 24 hr (A), 3 hr (B), or both 24 and 3 hr (C) before infection. Each symbol represents a different experiment.

0.015 unit/ml) for the 3 or 24 hr immediately preceding infection. In all of these experiments the cells were exposed to the virus inoculum for 30 min.

No reduction or increase in polykaryocyte count was noted in cultures of cells exposed to parathyroid hormones for less than one hr and only a slight reduction (10%) was noted in cultures of cells treated for one hr. The dose-response relationship between the concentration of hormone added 3 and 24 hr before infection, and the formation of polykaryocytes is shown in Figure 2. It is of interest that cultures of cells treated for 24 hr with more than 0.05 unit/ml of parathyroid hormone contain fewer polykaryocytes than untreated cultures. On the contrary, cultures of cells pretreated with less than 0.5 but more than 0.05 unit/ml show consistently more polykaryocytes than untreated cultures. This dual effect of the parathyroid hormone is not seen in cultures of cells treated for 3 hr before infection. It was ascertained that the cells treated for 24 hr with less than 0.5 unit of parathyroid hormone are as sensitive as previously untreated cells (C, Figure 2). The data, however, also show that in cultures in which the hormone is still effective, i.e. the polykaryocyte count is low, replacement of the culture fluid with fresh medium containing an equal amount of parathyroid hormone brings about the same effect as would a single exposure to the hormone at a much greater concentration. This is evident from the observation that the polykaryocyte count in cultures exposed for 24 hr to 1 unit/ml is usually 55% of the count obtained in untreated cultures. In cultures of cells re-exposed to fresh parathyroid hormone 3 hr before infection, the polykaryocyte count was only 5% of the control. A 95% reduction is seen only in cultures of cells exposed to 3–4 units/ml for 24 hr. The data suggest that the maintenance of the hormonal effect requires less hormone than its initiation.

*The mechanism by which parathyroid hormone effects a decrease in polykaryocyte*
count: Two series of experiments were made. In one series of experiments, untreated cells and cells treated for 24 hr with 3 units/ml of parathyroid hormone and 9 untreated cultures were washed and exposed to HSV. At 7, 15, and 30 min, respectively, the inoculum was removed for assay and MMγG was added to each of 3 treated and 3 untreated cultures. The results of one of these experiments are shown in Figure 3. It may be seen that parathyroid hormone had 3 effects: First, the number of plaques formed in the pretreated cultures was reduced, as might be expected from the data shown in Figure 2. Second, pretreated cells removed considerably less virus from the inoculum than untreated cells. Finally, the small amount of virus removed by pretreated cells was still considerably greater than the number of polykaryocytes formed in the pretreated cultures. In order to explain these results, it may be recalled that even in the presence of hormone at this concentration, the number of polykaryocytes equaled the number of preinfected cells. It may be deduced therefore that in pretreated cultures the number of polykaryocytes corresponds to the number of cells which became infected by absorbing virus and effecting its penetration. Impaired adsorption of virus, as evidenced by the recovery of most of the virus in the inocula, accounts to a large extent for the failure of pretreated cells to become infected. To a lesser extent, the failure of pretreated cells to become infected may be due to impaired penetration of virus into cells. This conclusion is supported by the discrepancy between the amounts of virus removed from the inocula and the number of polykaryocytes formed in pretreated cultures.

Pretreatment with parathyroid hormone decreases the rate, but does not prevent ultimately the HEp-2 cells from becoming infected. Moreover, the rate at which cells become infected increases rapidly once the hormone is withdrawn. These conclusions emerged from another series of experiments.

Each culture in a group of 14, designated A, received 5 ml of MM. Twenty
cultures divided into two groups, B and C, received each 5 ml of MM containing 2 units/ml of parathyroid hormones. Two hours before infection the medium containing the hormone was removed from the cultures in group B; the cells were washed with PBS and fresh MM was added to each culture. The cultures were infected as usual. At intervals after exposure to virus, as shown in Figure 4, the inocula from two cultures of each group were aspirated and replaced with MMγG. The results (Fig. 4) show that the polykaryocyte count in untreated (A) cultures levels off after 90-120 min of exposure to HSV. The polykaryocyte counts in pretreated cultures (B and C) increase steadily throughout the 150 min of exposure, but at lower rates. The comparison of polykaryocyte counts obtained in groups B and C is most revealing. It is clear that once the hormone is withdrawn its effects rapidly wear off and would entirely disappear within a few hours.

**Adsorption and penetration of HSV into HEp-2 cells pretreated for 24 hours with low concentrations of parathyroid hormone:** Untreated cells and cells exposed for 24 hr to 0.37 unit/ml of parathyroid hormone were washed and exposed to HSV. At 15 and 30 min, respectively, the virus inoculum in each of 3 pretreated and 3 untreated cultures was removed for assay and replaced with 5 ml of MMγG. As shown in Table 1, the number of polykaryocytes formed in pretreated cultures was consistently higher than in untreated cultures. At the same time, the amount of virus absorbed by the pretreated cells is only slightly greater in experiment I, and actually smaller in experiment II than the amount of virus absorbed by untreated cells. It is tempting to conclude that the increase in the number of polykaryocytes formed in cultures pretreated with less than 0.5 unit/ml of parathyroid hormone is due to an increase in the rate of penetration of HSV into cells.

**Summary.**—Experiments were designed to test the hypothesis that hormones may affect the outcome of viral infections by altering the ability of cells to become
infected. It was shown that thyroid or parathyroid hormone added immediately after infection of HEp-2 cells with *herpes simplex* virus (strain MP) did not decrease the number of polykaryocytes and therefore were ineffective in preventing viral development. However, cells pretreated for 3 to 24 hr with 0.5 or more units per ml of parathyroid hormone were shown to absorb and effect intracellular entry of virus more slowly than untreated cells.

The significance of the findings with respect to the role of hormones in viral infections will be discussed in a succeeding paper.\(^{26}\)

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† Scholar in Cancer Research of the American Cancer Society during leave of absence, July 1961 through June 1962, from the Department of Microbiology, School of Medicine, and School of Hygiene and Public Health, The Johns Hopkins University.

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**TABLE 1**

**Adsorption and Penetration of HSV into HEp-2 Cells Pretreated for 24 Hr with 0.37 Unit/ml of Parathyroid Hormone**

<table>
<thead>
<tr>
<th>Amount of virus</th>
<th>Polykaryocyte-forming units</th>
</tr>
</thead>
<tbody>
<tr>
<td>In the inoculum</td>
<td>Exper. I</td>
</tr>
<tr>
<td>Absorbed by pretreated cells</td>
<td>492</td>
</tr>
<tr>
<td>Absorbed by untreated cells</td>
<td>274</td>
</tr>
<tr>
<td>Inaccessible to antibody in pretreated cultures</td>
<td>196</td>
</tr>
<tr>
<td>Inaccessible to antibody in untreated cultures</td>
<td>163</td>
</tr>
</tbody>
</table>

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\(^{26}\)
NOTE ON SECOND-ORDER W. K. B. PHASE SHIFTS*

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The phase shifts for elastic scattering by a spherical potential are obtained by the W. K. B. approximation of the radial wave function up to and including the second-order approximation. Langer's transformation of the radial equation is used and the second-order terms of the phase shift thus obtained differ from Kahn's results even on substitution of \((l + \frac{1}{2})^2\) for \(l(l + 1)\).

The W. K. B. method as normally stated\(^1\) is an approximation to the solution of the Schrödinger equation in one-dimensional Cartesian coordinates. As Langer has pointed out,\(^2\) there are difficulties associated with the direct application of this method to the radial Schrödinger equation in spherical coordinates because of the difference in range of coordinates \((-\infty < y < \infty\) for the Cartesian coordinate \(y\), against \(0 < r < \infty\) for radial coordinate \(r\)) and the consequent difference in behavior of the wave functions at the lower boundary in the two coordinate systems. These difficulties can be overcome simply by a change of variables in the radial Schrödinger equation

\[
\frac{d^2R}{dr^2} + \left(\frac{Q}{\mu^2}\right)R = 0 \tag{1a}
\]

\[
Q \equiv 2\mu \left[ E - l(l + 1)\frac{\mu^2}{2r^2} - V(r) \right] \tag{1b}
\]

from \(r\) to \(x\) according to the relations

\[
r = \exp{(x)}, \quad R(r) = U(x) \exp{(x/2)}. \tag{2}
\]

The transformed equation

\[
\frac{d^2U}{dx^2} + \frac{\mu^2}{2\mu} q(x) U = 0 \tag{3a}
\]

\[
q(x) \equiv 2\mu \left[ E - \frac{\mu^2}{2\mu} (l + \frac{1}{2})^2 e^{-2x} - V(e^x) \right] e^{2x} \tag{3b}
\]

is in suitable form for the W. K. B. method. Langer found that the only difference between the first-order W. K. B. solution of equation (3a) and the same method