ENZYMATIC VARIANTS OF INFLUENZA VIRUS

II. EFFECT OF ENVIRONMENTAL FACTORS ON ENZYMATIC CHARACTERISTICS OF A VARIANT OF INFLUENZA B VIRUS*

BY BILLIE L. PADGETT, PH.D., AND DUARD L. WALKER, M.D.

(From the Department of Medical Microbiology, University of Wisconsin Medical School, Madison)

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INTRODUCTION

The enzymatic elution of influenza viruses from red blood cells was reported by Hirst in 1942 (1). The possession of this mucolytic enzyme by members of the mumps-Newcastle-influenza group of viruses is one of its unique characteristics. Although a great deal is known concerning the interaction of this viral enzyme with a variety of mucoprotein substrates in vitro (see reviews by Gottschalk (2) and Burnet (3)), the function of the enzyme in the multiplication of the virus has not been clearly demonstrated.

We recently reported (4) the isolation of a stable variant from the population of influenza B, strain Lee, virus particles which possessed enzymatic characteristics distinct from those of the parent virus. The techniques used to isolate the variant would be expected to select virus particles having a decreased rate of enzymatic activity. In comparative tests with the parent Lee virus the variant was shown to have a markedly slower rate of elution from chicken red blood cells, decreased activity against the soluble mucoprotein ovomucin, and a more heat-labile enzyme. In all characteristics examined other than those related to the mucolytic enzyme, the variant and parent were indistinguishable. Preliminary examination of the enzymatic characteristics of the variant showed that its activity could be markedly enhanced by adding calcium to the medium and inhibited by a calcium-chelating agent. It was also noted that the variant appeared to be more active in degrading ovomucin at 25°C than at 37°C. Since these characteristics were quite different from those of the parent virus and from the usual enzymatic behavior of influenza viruses, a more detailed study of the effect of these and other environmental factors was undertaken.

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Materials and Methods

The major techniques and procedures employed in this study have been described in detail previously (4).

Influenza Viruses.—The Lee strain of influenza B virus was used and is referred to in this paper as stock Lee virus. The slowly reacting enzymatic variant of Lee virus was isolated in this laboratory. The general characteristics of the variant virus and techniques used in its isolation have been described (4). Only Variant 1 virus was used in the present experiments.

Red Blood Cells.—Blood was obtained from adult chickens. The red blood cells (RBC) were washed and made up by volume to a concentration of 1 per cent in buffered saline.

Virus Titrations.—Hemagglutination titrations were performed in tubes and the hemagglutination (HA) titer was based on the pattern of sedimented RBC.

Infectivity titrations were done in ovo with a postinoculation incubation time of 72 hours. The EID₉₀ was calculated by the method of Reed and Muench (5).

Buffers.—Buffered saline contained 9 gm. NaCl in one liter and was buffered at pH 7.2 with 0.01 M phosphate. To obtain phosphate-buffered saline of different pH values the ratio of Na₂HPO₄ to NaH₂PO₄ was altered. All pH values were determined using a Beckman, model G, pH meter with a glass electrode.

Calcium acetate buffer contained 12.3 gm. sodium acetate, 5.0 gm. NaCl, 1.0 gm. CaCl₂ in one liter of water and 2 N acetic acid was added to pH 6.2.

Calcium borate buffer contained 9.0 gm. NaCl, 1.0 gm. CaCl₂, 1.203 gm. H₃BO₃, and 0.052 gm. Na₂B₄O₇·10H₂O in one liter. The pH was approximately 7.0. To obtain borate-buffered saline solutions of higher pH the ratio of Na₂B₄O₇·10H₂O to H₃BO₃ was increased. No CaCl₂ was added to these solutions unless stated.

Glucosol.—The modified glucosol of Fulton and Armitage (6), consisting of glucose and sodium, magnesium, and calcium chlorides buffered at pH 7.28 with a phosphate buffer, was used.

Partially Purified Virus Preparations.—The virus in infected allantoic fluid was partially purified by differential centrifugation. Allantoic fluid was centrifuged at 6000 g for 16 minutes. The supernatant fluid was then centrifuged at 81,000 g for 30 minutes. The supernatant fluid was discarded and the pellet was resuspended and centrifuged again at 6000 g for 15 minutes. The supernatant fluid from this final centrifugation was used.

Determination of Elution Rate.—A stepwise elution technique was used. One ml. of a virus suspension having an HA titer of 512 or 1024 was added to the packed RBC from 5 ml. of a 1 per cent suspension of chicken RBC. The mixture was held at 4°C. for 30 minutes with occasional agitation to facilitate maximal adsorption of virus to the RBC. The RBC were then sedimented by centrifugation in the cold. The supernate was removed and its HA titer subsequently determined. The presence of virus in this supernate was taken as an indication that the RBC were saturated with virus. The sedimented RBC were washed twice with cold buffered saline, suspended in 1 ml. buffered saline, and placed in a water bath at the desired temperature. Periodically they were removed and centrifuged at 2500 r.p.m. for approximately 1 minute. The supernate (eluate) was removed and saved. The RBC were resuspended in a new 1 ml. volume of buffered saline and returned to the water bath. When the HA titers of the eluates are plotted against time, an elution curve is obtained.

Experimental

Effect of Temperature on the Enzymatic Activity of Stock Lee and Variant Viruses.—Previous work (4) had shown that the variant virus degraded ovo-mucin more rapidly at 25°C. than at 37°C. whereas stock Lee virus was very
active at both temperatures. This unexpected influence of temperature on the rate of enzymatic activity of the variant was studied in detail by determining elution rates from chicken RBC held at various temperatures. Elution of stock Lee and variant virus from chicken RBC incubated at 25, 30, 37, 40, and 45°C, was measured.

Typical elution curves are shown in Fig. 1. As the temperature of incubation was increased from 25 to 40°C, both the amount of stock Lee virus eluting from the RBC and the rate at which it eluted were increased, resulting in elution curves which show a progressive shift upward and to the left, and maximal elution of stock Lee virus occurred at 40°C. In marked contrast to this the variant was most active at 25°C, and as the temperature was increased above 25°C the amount of variant virus eluting from the RBC decreased, resulting in elution curves which show a progressive shift downward. Although the stock virus eluted at a maximal rate at 40°C, this rate was considerably reduced at 45°C. Only insignificant quantities of the variant virus eluted from the RBC at 45°C.

Mechanism of Temperature Effect on Enzymatic Activity of the Variant Virus— Inactivation or Inhibition?—In general, the rate of an enzymatic reaction increases with increasing temperature until an optimal temperature is reached. Above this temperature the rate of the reaction decreases due to thermal inactivation of the enzyme molecules. The results of the previous experiments indicated that the optimal temperature for the variant enzyme was much lower than that for the stock Lee enzyme. We had found (4) that the variant enzyme
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was inactivated by heating infected allantoic fluid at 46 to 48°C., but the decreased activity of the variant at temperatures as low as 30°C. suggested that some mechanism other than thermal inactivation might be operating in this system. This possibility was investigated by the following experiment.

Three aliquots of chicken RBC were saturated with variant virus. Elution of virus in one aliquot was followed at 25°C. and in a second at 40°C. The third aliquot was incubated first at 40°C. and the elution of the virus followed for 70 minutes; then, after removal of the 70 minute eluate, this aliquot was resuspended in saline at 25°C. and the elution of the virus at this lower temperature was followed for the remainder of the 4 hour observation period.

![Graph showing the elution of variant virus from chicken RBC incubated at indicated temperatures.](image)

**Fig. 2.** Elution of variant virus from chicken RBC incubated at indicated temperatures. After removal of the 70 minute eluates one of the aliquots at 40°C. was resuspended in saline at 25°C. and the virus was allowed to elute at this lower temperature for the remainder of the 4 hour period. Each point represents the geometric mean of duplicate experiments.

The resulting elution curves are shown in Fig. 2. The elution curves at 40°C. and 25°C. were quite similar for the first 40 minutes, but thereafter elution at 40°C. fell much below that at 25°C. and the virus at 40°C. continued to elute at a generally decreasing rate. When, however, after 70 minutes at 40°C. an aliquot was shifted to 25°C., the viral elution rate increased and large amounts of virus were eluting at the end of the observation period. At 40°C. about 7 per cent of the virus initially adsorbed to the RBC eluted after 70 minutes, whereas about 23 per cent of the adsorbed virus in the third aliquot eluted from the RBC after they were shifted to 25°C.

These results suggested that the enzyme of the variant virus was actually inhibited in some manner at temperatures above 25°C. However, the process of thermal inactivation of the enzyme would be continuous throughout the entire 4 hour period at 40°C., while virus in the third aliquot was exposed to this
temperature for only 70 minutes. The total amount of virus that eluted in the third aliquot was greater than that which eluted from the RBC at 40°C, but did not equal the amount eluted in the aliquot at 25°C. Large amounts of virus, however, were still eluting in the third aliquot when the experiment was terminated at the end of 4 hours.

A second experiment was performed using a different experimental approach. It has been found previously (4) that the addition of calcium ions to the eluting system greatly accelerated the rate of elution of the variant virus. If the decreased elution of the variant virus at 40°C. were due to inactivation of the viral enzyme, the addition of calcium to the system after a period of incubation at 40°C. should have little effect on the amount of virus which subsequently elutes.

Three aliquots of chicken RBC saturated with variant virus were prepared. One aliquot was held at 25°C and the other two at 40°C. The elution of the virus into borate-buffered saline (pH 6.98) was followed for 130 minutes. After removal of the 130 minute eluates one of the aliquots at 40°C. was resuspended in calcium-borate buffer and the virus was allowed to elute into this medium for 240 minutes. Each point represents the geometric mean of duplicate experiments.

The resulting elution curves are shown in Fig. 3. Prior to the addition of CaCl₂ the shapes of the elution curves at the two temperatures were similar to those obtained with phosphate-buffered saline, although slightly more virus eluted in the borate system. After the addition of CaCl₂ to one of the aliquots at 40°C, the elution of the virus in this system was markedly enhanced and the...
shape of the elution curve thereafter resembled that at 25°C. in the absence of
CaCl₂. About three times as much virus eluted at 40°C. in the presence of
CaCl₂ than in its absence in spite of the fact that both systems had been in-
cubated at this temperature for 130 minutes prior to the addition of calcium.
The total amount of virus which eluted in the aliquot at 40°C. to which calcium
was added (424 HA units) was similar to that which eluted at 25°C. (520 HA
units). It appears, therefore, that little inactivation of the enzyme occurred at
40°C. during the 130 minutes prior to the addition of calcium although during
that time elution of virus at 40°C. was less than that at 25°C. These results
indicate that inhibition of enzyme activity rather than inactivation is the
major cause of decreased elution of the variant virus at temperatures above
25°C.

Influence of Divalent Cations on Enzymatic Activity of Variant Virus.—Since
0.009 M CaCl₂ had a marked stimulatory effect on the elution of variant virus
and was without such an effect on stock Lee virus, the effect of various con-
centrations of calcium and other divalent cations on the elution process of the
variant was studied in some detail.

Aliquots of chicken RBC were saturated with variant virus at 4°C. After being washed,
the aliquots were resuspended in various concentrations of the following chlorides: calcium,
strontium, barium, magnesium, and manganese, dissolved in borate-buffered saline (pH
6.9-7.1). Elution of the virus was allowed to proceed for 10 minutes at 37°C. The eluates were
then removed and their HA titer determined.

The results are shown in Fig. 4. Of the divalent cations tested, strontium and
calcium had the greatest stimulatory effect on the elution of the variant,
Strontium being even more effective than calcium in certain concentrations. Barium was less effective than calcium, and manganese resulted in only minimal stimulation in the highest concentrations tested. Magnesium had no effect in any of the concentrations tested. The range of concentrations which resulted in enhanced elution varied from ion to ion with strontium having the widest stimulatory range. From these results it is apparent that there is a certain specificity involved in the enhancement of variant enzyme activity by divalent cations.

Influence of pH on the Elution Rate of Stock Lee and Variant Viruses from Chicken RBC.—Davenport and Horsfall (7) reported that the rate of elution of influenza virus from chicken RBC was not significantly affected by saline buffered with phosphate at pH 6, 7, or 8. However, as the activity of the variant was found to be influenced markedly by temperature and the presence of certain ions, the possible effect of pH on the activity of the variant enzyme was investigated.

Aliquots of packed chicken RBC were saturated with either stock Lee or variant-infected allantoic fluid at 4°C. After a 30 minute adsorption period each aliquot was washed twice with unbuffered saline to remove unadsorbed virus. The packed RBC with attached virus were re-suspended in one of the following solutions: saline buffered at pH 6, 7, 7.5, and 8 with phosphate buffer, saline buffered at pH 7, 7.5, and 8 with borate buffer, or saline containing 0.009 M CaCl₂ buffered at pH 7.5 with borate buffer. Each aliquot was incubated at 37°C and the elution of the virus followed for 70 minutes.

Representative results are shown in Fig. 5. The elution of stock Lee virus from chicken RBC was not appreciably affected by the pH of the suspending buffer through the range of pH values tested, nor was it influenced by the
choice of buffer or the presence of calcium. In contrast, the elution of the variant virus was progressively inhibited at increasing pH values above 7, and at pH 8 no virus detectable by HA eluted from the RBC. A comparison of phosphate and borate buffers of equivalent pH revealed that slightly more variant virus eluted in the borate-buffered system. The addition of 0.009 M CaCl₂ to a system buffered with borate at an adverse pH resulted in marked stimulation of elution of the variant virus.

Similar experiments were then carried out at different temperatures to investigate the interactions of the following environmental factors: temperature, pH, and the presence of calcium ions.

![Fig. 6. Elution of variant virus from chicken RBC under various conditions of pH, temperature, and calcium concentration.](image)

Aliquots of chicken RBC were saturated with variant virus at 4°C. After being washed to remove unadsorbed virus, the aliquots were resuspended in the following solutions: calcium acetate buffer (pH 6.17), phosphate buffer (pH 6.04), and phosphate buffer (pH 7.2). Duplicate experiments were carried out at 25°C. and 40°C. Elution of virus from the RBC was followed for 4 hours.

The resulting elution curves are shown in Fig. 6. At 25°C. in the absence of calcium the variant elutes more rapidly at pH 7.2 than at pH 6.04, but at 40°C. elution is much more complete and rapid at pH 6.04. At pH 6.04 in the absence of calcium elution of the variant is more rapid at 40°C. than at 25°C., while the reverse is true at pH 7.2. Indeed, as previously noted, the elution of the variant at pH 7.2 is greatly inhibited at 40°C. A similar decrease in elution at 40°C. was observed with a borate buffer (pH 7.01). The presence of calcium (pH 6.17) accelerates the elution of the variant at both temperatures. Elution at 40°C. in the presence of calcium is even more rapid than at 25°C. although the total quantity of virus eluting from the RBC during 4 hours is about the same.

*Is Calcium Bound in the Union Between Variant Virus and RBC?*—The activity of many enzymes is dependent on the presence of certain accessory
metal ions and, in some cases, the metal ion is thought to act as a link between
the enzyme and substrate molecules. It was considered possible that calcium
ions might exert their influence in this manner in the system under investigation.
In previous experiments, however, calcium was not present during the initial
union between virus and RBC which took place in allantoic fluid at \(4^\circ\text{C}\). Therefore it was decided to determine whether the presence of calcium during the
adsorption phase of the reaction would influence the subsequent elution of the
virus in a calcium-free medium.

Aliquots of partially purified variant virus were resuspended in glucosol and in glucosol
without CaCl\(_2\). Four aliquots of chicken RBC were washed repeatedly with unbuffered saline

<table>
<thead>
<tr>
<th>Calcium content of system</th>
<th>Calcium content of system</th>
<th>HA titer of eluates removed after indicated time (min.) of elution at (37^\circ\text{C}).</th>
</tr>
</thead>
<tbody>
<tr>
<td>during adsorption</td>
<td>during elution</td>
<td>10</td>
</tr>
<tr>
<td>0.0009 M CaCl(_2)</td>
<td>No calcium</td>
<td>4</td>
</tr>
<tr>
<td>0.0009 M CaCl(_2)</td>
<td>0.0009 M CaCl(_2)</td>
<td>32</td>
</tr>
<tr>
<td>No calcium</td>
<td>No calcium</td>
<td>4</td>
</tr>
<tr>
<td>0.0009 M CaCl(_2)</td>
<td>0.0009 M CaCl(_2)</td>
<td>32</td>
</tr>
</tbody>
</table>
* After removal of the 70 minute eluates all aliquots were resuspended in glucosol containing 0.0009 M CaCl\(_2\).

and the RBC packed after the final wash. Two of the aliquots were saturated with variant
virus in glucosol; the other two with variant virus in glucosol without CaCl\(_2\). After the 30
minute adsorption period at \(4^\circ\text{C}\), the RBC were washed with unbuffered saline. One aliquot
from each set was then resuspended in glucosol, the others in glucosol without CaCl\(_2\), and the
tubes placed at \(37^\circ\text{C}\). Elution of the virus was followed for 70 minutes. After removal of the
70 minute eluates, all four aliquots were resuspended in glucosol and elution was allowed to
proceed for another 30 minutes. The HA titer of all eluates was determined.

The results are shown in Table I. It is evident that the elution of the variant
virus in glucosol without CaCl\(_2\) is the same whether calcium was present during
adsorption or not. Also there is no indication that the presence of calcium during adsorption accelerated the subsequent elution of the variant in glucosol.
Similar results were obtained using 0.009 M CaCl\(_2\) in a borate buffer. It appears
that calcium was not bound firmly into the virus-RBC union during adsorption
at \(4^\circ\text{C}\). or at least was not bound in an amount sufficient to stimulate subsequent elution of the virus.

*Effect of Calcium on the Thermal Inactivation of the Enzymatic Activity of Stock
Lee and Variant Viruses.*—It was found previously (4) that the enzyme of the
variant virus was inactivated at a much lower temperature than that of stock Lee virus. As it has been reported (8) that calcium inhibits the thermal destruction of the influenza virus enzyme, experiments were performed to determine the effect of calcium on the unusual heat sensitivity of the variant as compared to stock Lee virus. Because of the lower inactivation temperature of the variant enzyme it was not feasible to compare the destruction of the two enzymes at the same temperature.

### TABLE II

<table>
<thead>
<tr>
<th>Virus</th>
<th>Temperature</th>
<th>Calcium content of suspending fluid</th>
<th>HA titer of 3 hr. eluate of virus previously heated for indicated time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C.</td>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td>Stock Lee</td>
<td>46</td>
<td>none</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0009 M CaCl₂</td>
<td>128</td>
</tr>
<tr>
<td>Variant</td>
<td>40.4</td>
<td>none</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0009 M CaCl₂</td>
<td>32</td>
</tr>
</tbody>
</table>

Pellets of partially purified stock Lee and variant virus were suspended in glucosol and in glucosol without CaCl₂. Aliquots (0.5 ml.) of the viruses were distributed in acid-cleaned Wassermann tubes. The tubes were sealed with rubber stoppers and placed in a water bath with the meniscus at least 5 cm. below the water level. The temperature of the water bath was 46.0 ± 0.1°C. in the case of stock Lee virus and 40.4 ± 0.1°C. in the case of the variant virus. At intervals, one tube of each set was transferred from the water bath to an ice bath. As a control one aliquot of each set was held in the ice bath during the total heating period. Residual enzymatic activity was determined in the following manner: 1 ml. of a 1 per cent suspension of chicken RBC was added to each aliquot of virus in the ice bath. After a 30 minute adsorption period, the RBC were washed and resuspended in 1 ml. of buffered saline at 37°C. and the virus was allowed to elute for 3 hours at 37°C. At the end of the elution period the eluates were removed and their HA titer determined.

The results are shown in Table II. It is evident that the presence of calcium during the heating period protected the enzyme of both stock and variant viruses from inactivation. It was not determined whether the presence of calcium increased the heat stability of the variant enzyme to a level comparable with that of stock Lee virus.

**Effect of Calcium on the Thermal Inactivation of Stock Lee and Variant Virus Hemagglutinin.**—It was found previously (4) that there was little difference in the heat stabilities of the hemagglutinins of stock Lee and variant virus in phosphate buffer at pH 7.5. Both were inactivated by heating at 64.5°C. for 30 minutes. Briody (8) reported that the heat inactivation of the hemagglutinin of Lee virus was accelerated by 0.1 per cent CaCl₂. Since calcium enhanced the
heat stability of the viral enzyme, experiments were undertaken to investigate its effect on the hemagglutinin of the variant virus.

Partially purified stock Lee and variant viruses were suspended in glucosol and in glucosol without CaCl₂. Aliquots (0.5 ml.) were distributed and heated as in the previous experiment except that the temperature of the water bath was 65.0 ± 0.1°C. Similar controls were included. At the end of the heating period the HA titer of each aliquot was determined in duplicate.

The results of this experiment are shown in Table III. It is evident that in glucosol medium in the absence of calcium the variant hemagglutinin was inactivated at a faster rate than that of stock Lee virus. The presence of calcium had little effect on the inactivation of stock Lee hemagglutinin but did retard the inactivation of the variant hemagglutinin. The magnitude of this effect was not great and might be increased by higher concentrations of calcium, but the phosphate buffer in the glucosol medium limited the calcium concentration.

TABLE III

<table>
<thead>
<tr>
<th>Virus</th>
<th>Calcium content of suspending fluid</th>
<th>HA titer* of aliquots after indicated time (min.) of heating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Stock Lee</td>
<td>None</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>0.0009 M CaCl₂</td>
<td>512</td>
</tr>
<tr>
<td>Variant</td>
<td>None</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>0.0009 M CaCl₂</td>
<td>256</td>
</tr>
</tbody>
</table>

* Geometric mean of duplicate determinations.

**Comparison of Thermal Inactivation of Stock Lee and Variant Virus Infectivity.**

Since there was a great difference in the heat stabilities of the enzymes of stock Lee and variant virus but only a slight difference in the heat stabilities of their hemagglutinins, the thermal inactivation of the infectivity of these viruses and the effect of calcium thereon was investigated.

Partially purified stock Lee and variant virus preparations were suspended in borate-buffered saline (pH 7.6). Aliquots (0.5 ml.) of the viruses were distributed and heated as in the previous experiments. The temperature of the water bath was 51.0 ± 0.1°C. At intervals one tube of each set was transferred from the water bath to an ice bath. As a control one aliquot of each set was held in the ice bath during the total heating period. The infectivity titer of each aliquot was then determined.

The results are shown in Fig. 7. It is evident that in the absence of calcium the variant was inactivated at a faster rate than was the stock virus. The infectivi-
ties of both viruses decreased at a constant rate for at least 25 minutes. The break in the line between 25 and 40 minutes seen with the variant is typical of the results obtained in several experiments, and may be due to aggregation of virus particles or to a heterogeneous viral population, but the point was not investigated further. In the presence of 0.009 M CaCl₂ the rate of inactivation of both viruses was decreased but the effect on the variant was more marked. Indeed, the line representing the variant became roughly parallel to that of the stock virus.

DISCUSSION

It is apparent from the results of these studies that the enzymatic activity of the variant is affected differently by changes in various environmental factors than is that of stock Lee virus. In contrast to the parent virus, the activity of the variant is increasingly inhibited at temperatures above 25°C. and at pH values above 7, but it is enhanced markedly by the presence of calcium or certain other divalent cations.

The adverse effect of temperatures above 25°C. on the rate of elution of the variant was unexpected. The experiments reported herein support the conclusion that inhibition of enzyme activity rather than its inactivation is the major cause of the decreased activity. The mechanism of this inhibition has not been determined. It is noteworthy, however, that in the presence of calcium the response of the variant to increasing temperature resembles that of stock Lee virus. Fig. 6 shows that in calcium acetate buffer the variant eluted at a faster rate at 40°C. than at 25°C. If, as seems most likely, the decreased activity of the variant is due to a deficiency of calcium the inhibition seen with increasing temperature may have its basis in one of the following mechanisms:

1. Although calcium is not ordinarily added to the system when elution rate determinations are made, a certain low concentration of calcium may be present initially. It is possible that as the temperature of incubation is increased the amount of available calcium is decreased through some competing reaction
which is accelerated or has its equilibrium shifted with increasing temperature. As this phenomenon was observed in borate- as well as phosphate-buffered systems, it is apparent that the hypothetical reaction does not involve the buffering system.

2. Another possibility is that calcium is not essential to the enzyme action but serves to stabilize the spatial orientation between enzyme and substrate which is necessary for the enzymatic hydrolysis of the substrate. In the absence of calcium the probability that the enzyme groupings will attain the necessary orientation is decreased, and with increasing temperature there will be a concomitant increase in the thermal agitation of the particles which will further decrease the probability of forming and holding the necessary orientation.

Burnet and Edney (9) have suggested that the enzyme groupings of influenza virus carry bound calcium ions and that the cation serves to bring the enzyme and substrate into primary union. Then, if the primary adsorption is followed by the correct orientation, a specific activating union can develop which permits hydrolysis of the substrate. Experimentally they found that cations influenced the initial adsorption between virus and substrate more strikingly than they influenced the enzymatic reactions. However, they found no specific requirement for calcium ions; that is, calcium could be replaced by sodium ions.

We have not found calcium necessary for the initial adsorption of virus to RBC and were unable to demonstrate that calcium was bound into the initial union between enzyme and substrate. This possibility, however, still exists, for, under the conditions of our experiment, although the RBC were "saturated" with virus it is possible that repeated associations and dissociations of reactive groups are necessary before sufficient substrate is degraded to result in virus release. Calcium may have been bound into the initial union but released after the splitting of the first substrate molecule and diluted out in the calcium-free medium. The enzymatic activity of the variant, on the other hand, is increased strikingly by calcium and the related ions strontium and barium, but it is unaffected by certain other divalent cations or by even much higher concentrations of sodium ions.

Can all of the differences in the enzymatic characteristics of parent and variant viruses be explained on the basis of a differential requirement for calcium or, in Burnet’s terms, a deficiency of calcium in the enzyme groupings of the variant? This seems possible because in the presence of sufficient calcium the rate of elution of the variant is indistinguishable from that of stock Lee virus and the response of the variant to increasing temperature resembles that of the parent virus. Calcium also overcomes the effects of adverse pH on the elution rate of the variant. The effects of pH in the absence of calcium (no elution at pH 8 at 37°C., inhibition of elution at pH 7.2 at 40°C., but good elution at pH 6.04 at 40°C.) may indicate the importance of ionized amino
groups in the reaction. Calcium also increases the heat stabilities of the variant enzyme, hemagglutinin, and infectivity. However, except for the property of infectivity, it has not been determined whether calcium increases the heat stability of the variant to a level strictly comparable with that of the parent virus.

**SUMMARY**

The rate of elution of the variant virus from chicken RBC is progressively decreased as the temperature of incubation is increased above 25°C. The activity of the parent virus, on the other hand, is increased as the temperature is increased up to 40°C. The major cause of the decreased activity of the variant at temperatures above 25°C is an inhibition of the variant enzyme rather than its inactivation.

The activity of the variant enzyme is stimulated in the presence of strontium, calcium, and barium ions. Manganese has only a slight effect, and magnesium has no stimulatory effect on the elution of the variant virus.

Elution of the variant at 37°C is progressively inhibited at pH values above 7, while the parent virus is still active at pH 8.

In the absence of calcium the variant enzyme, hemagglutinin, and infectivity are more heat labile than those of the parent virus. The addition of calcium increases the heat stability of all three properties of the variant, and in the presence of calcium the infectivity of the variant is as stable as that of the parent virus.

**BIBLIOGRAPHY**