ENZYMATIC VARIANTS OF INFLUENZA VIRUS

I. Isolation and Characterization of Slowly Reacting Enzymatic Variants of Influenza B Virus*, ‡, §

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(Received for publication, March 11, 1957)

The influenza viruses are among the few animal viruses known to possess enzymatic activity. Since no agent which will inactivate the enzyme without destroying the infectivity of the virus has yet been found, it is considered that the enzyme is an essential part of the virus. However, the function of the enzyme has not been exactly defined although many possibilities have been suggested.

The earliest suggestion concerning the role of the influenza enzyme was that penetration of susceptible cells by virus was brought about by enzymatic activity (1). However, there is evidence that virus particles which have had their enzyme inactivated by heat can penetrate into cells and there interfere with the multiplication of active virus (2) or participate in genetic interactions with active virus (3). Other suggestions have been that the enzyme aids in the approach of the virus to susceptible cells by attacking the extracellular layer of mucin (4), or that it acts on an intracellular substrate resulting in the release of newly formed virus particles from an infected cell (5). Schlesinger and Karr (6) have recently correlated the breakdown of intracellular substrate with periods of active viral multiplication rather than with release of virus from the cell, and they suggest that the enzyme is somehow involved in the synthesis of new virus material.

Study of the function of the virus enzyme has been hampered by difficulties in devising an adequate experimental approach. Alteration of enzymatic activity by chemical or physical agents appears to be accompanied by changes in other viral activities. Although some influenza strains differ markedly in the characteristics of their enzymatic activity, they usually also differ widely in

* Data included in this report were submitted to the Graduate School of the University of Wisconsin by B. L. Padgett in partial fulfillment of requirements for the degree of Doctor of Philosophy.

‡ The opinions expressed in this article are solely those of the authors and do not necessarily reflect the viewpoint of the Navy Department.

§ This work was supported in part by the Research Committee of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

other respects that cannot clearly be attributed to their enzymatic differences. It appeared possible to us that variants selected for certain enzymatic characteristics from stable, standard, laboratory strains could profitably be used in this problem.

Björkman and Horsfall (7) found that Lee virus which had been exposed to ultraviolet irradiation or treated with lanthanum acetate had a decreased rate of elution from chicken erythrocytes but appeared in all characteristics tested other than enzymatic activity to be indistinguishable from the parent virus. While recognizing that the agents employed could have induced mutation, they felt that their results could be explained best on the basis of the selection of a naturally occurring mutant. This work suggested that naturally occurring mutants possessing markedly different and potentially useful enzymatic characteristics might exist within the population of the Lee strain of influenza B and that it might be possible to isolate these mutants by means less likely to be damaging or mutagenic than exposure to ultraviolet light or treatment with lanthanum acetate.

The present investigation has revealed that such mutants can be isolated using methods that are relatively uncomplicated but which markedly increase isolation efficiency over simple terminal dilution isolation. It will be shown that the characteristics of these variants are stable upon repeated subculture, that they remain very similar to the parent strain in their antigenic makeup and in several other characteristics, but that they differ markedly from the parent virus in their enzymatic activity.

Materials and Methods

Influenza Viruses.—The Lee strain of influenza B virus and the PR8 strain of influenza A virus were used. The Lee virus, a standard laboratory strain, has undergone an undetermined number of egg passages in this and other laboratories and is referred to in this paper as stock Lee virus.

Chicken Embryos.—Embryonated eggs were incubated at 39°C. for 9 to 12 days before inoculation. After allantoic inoculation with 0.2 ml. of the desired dilution of virus the embryos were incubated for 48 hours at 35°C. then chilled at 4°C. overnight and the allantoic fluids harvested. Infected allantoic fluids were stored at 4°C. or at −40°C. if not used within 7 days.

Saline.—A 0.15 M NaCl solution buffered at pH 7.2 with 0.01 M phosphate was used as routine as a diluent. Virus dilutions for egg inoculation were made in sterile saline containing 10 per cent by volume of sterile broth. When the inoculum contained erythrocytes, 500 units of penicillin and 2 mg. of streptomycin per ml. were added.

Erythrocytes.—Blood from various animals was stored in acid-citrate-dextrose solution (8). The erythrocytes were washed 3 times with saline and made up by volume to a concentration of 1 per cent.

Hemagglutination Titrations.—Serial twofold dilutions of allantoic fluid were made in saline. To 0.4 ml. of each dilution was added an equal volume of a 1 per cent suspension of chicken erythrocytes. The tubes were shaken and the erythrocytes allowed to settle for 1 hour at room temperature. Readings were based on the pattern of sedimented erythrocytes. The hemagglutination (HA) titer of the virus was expressed as the reciprocal of the highest dilution showing strong (2+) agglutination.

Virus Infectivity Titrations.—Infectivity titrations in chicken embryos were carried out by inoculating 0.2 ml. of falling tenfold dilutions of virus into a group of 5 embryos for each dilution. After 48 hours of incubation, the allantoic fluids were harvested individually and tested for virus by the hemagglutination technique. The EID_{50} was calculated by the method of Reed and Muench (9).

Infectivity titrations in mice were carried out by the intranasal inoculation of 0.05 ml. of falling tenfold dilutions of virus into groups of 8 mice per dilution while the mice were under light ether anesthesia. The mice were observed for a period of 10 days at which time all surviving mice were sacrificed and their lungs examined for the presence of pulmonary consolidation. The degree of consolidation was scored from 0 to 4+ and the 50 per cent maximum score end-point (10) was calculated.

Immune Sera.—Adult albino rabbits were given an intravenous injection of 10 ml. of undiluted infected allantoic fluid pools. The pools were obtained by passing the desired virus in chicken embryos and pooling the infected allantoic fluids at the end-point of infectivity. The rabbits were bled 2 weeks later and given an intraperitoneal injection of 10 ml. of the same virus pool diluted 1:2. The rabbits were exsanguinated 2 weeks after the 2nd injection. The sera were stored at −40°C. and inactivated at 56°C. for 30 minutes before use.

Antibody Titrations.—Hemagglutination-inhibition titrations were done in the following manner. Serial twofold dilutions of the inactivated serum were made in saline. To 0.2 ml. of each dilution was added an equal volume of infected allantoic fluid containing 8 hemagglutinating doses (AD) of virus and 0.4 ml. of a 1 per cent suspension of chicken erythrocytes. The erythrocytes were allowed to settle at room temperature for 1 hour. The hemagglutination-inhibition titer of the serum was expressed as the reciprocal of the highest dilution of antiserum which completely inhibited agglutination of the erythrocytes.

Ovomucin.—Ovomucin was prepared from egg white according to the method of Gottschalk and Lind (11). Following dialysis against 1 per cent NaCl, the suspension was adjusted to pH 7.2 and stored at 4°C.

Indicator Viruses.—Indicator viruses were prepared by heating the appropriate infected allantoic fluids at 56°C. for 30 minutes.

Determination of Elution Rate.—The procedure employed to determine elution rate was a modification of the stepwise elution technique of Björkman and Horsfall (7). One ml. of infected allantoic fluid having an HA titer of 512 or higher was added to the packed erythrocytes from 5 ml. of a 1 per cent suspension of chicken erythrocytes. The mixture was held at 4°C. for 30 minutes with occasional agitation to facilitate maximal adsorption of virus to the erythrocytes. The erythrocytes 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 erythrocytes were saturated with virus. The sedimented erythrocytes were washed twice with 10 ml. cold saline, suspended in 1 ml. warm (37°C.) saline, and placed in a water bath at 37°C. Periodically they were removed and centrifuged at 2500 R.P.M. for approximately 1 minute. The supernate (eluate) was removed and saved. The erythrocytes were resuspended in a new 1 ml. volume of warm saline and returned to the water bath. The HA titer of each eluate was determined. When the HA titers of the eluates are plotted against time, an elution curve is obtained.

EXPERIMENTAL

Isolation of Slowly Reacting Enzymatic Variants

In order to isolate the desired variants, presumed to be present in low concentration among the stock Lee virus particles, some method had to be employed which would concentrate the slowly reacting fraction of the population without seriously damaging the virus particles. Procedures were designed to

accomplish this concentration by permitting the rapidly reacting particles to elute from erythrocytes. After a period of elution the rapidly reacting particles would be present in the eluate and could be discarded. The slower reacting particles still attached to the erythrocytes could then be passed in chicken embryos. The resulting infected allantoic fluid would be expected to contain a higher concentration of slowly reacting particles provided that stable, slowly reacting genetic variants were present in the initial population and were not at a serious disadvantage when multiplying together with non-variant virus particles in eggs.

The following procedures were designed to isolate a slowly reacting variant of stock Lee virus.

Procedure I.—Infected allantoic fluid was added to packed chicken erythrocytes in sufficient quantity to saturate the cell surfaces with virus at 4°C. The cells were washed and resuspended in fresh diluent and held at 37°C. to allow elution of virus. Since all receptor sites were presumably saturated initially, virus particles capable of rapid elution could not be readsorbed and could be eliminated in the supernatant fluid. Virus detaching from the cells within 240 minutes was discarded. The erythrocytes with attached, uneluated virus were suspended in a small volume of diluent and inoculated into a group of chicken embryos. After incubation for 48 hours at 37°C. the allantoic fluids were harvested individually and their hemagglutination titer determined. One infected allantoic fluid having an HA titer of 512 or higher was used for the next passage which was a repetition of the first. After four such serial passages, the rate of elution of each infected 4th passage allantoic fluid was determined.

The elution curves of the 4th passage allantoic fluids did not differ significantly from that of stock Lee virus. It appeared, therefore, that a slowly reacting variant could not be isolated in a few passages by so simple a method as this. Another series of passages of Lee virus was initiated employing a procedure that combined the concentration technique used previously with limiting dilution passage of the erythrocytes with attached virus.

Procedure II.—Erythrocytes were saturated with virus and the virus was allowed to elute as in Procedure I with the difference that at the completion of the elution period the erythrocytes were serially diluted in fivefold steps. Each dilution was inoculated into a group of 10 to 25 embryos. After 48 hours of incubation the allantoic fluids were harvested individually. The infected fluids from the group injected with the highest infective dilution of inoculum were stored at −40°C. for later testing. The fluids from the group injected with the 10^{-5} dilution of inoculum were pooled and an aliquot of this pool was used for the next passage. Four serial passages were made in this way, the concentration procedure being carried out between each passage.

In the 4th passage of this series the highest infective dilution of inoculum resulted in 11 infected allantoic fluids. The virus in each of these fluids was tested for its enzymatic activity and one of these virus populations appeared to have a reduced rate of elution from chicken erythrocytes. This virus was passed once in chicken embryos at limiting dilution. The infected allantoic fluids at the end-point were pooled. The virus in this pool and in all infected allantoic fluids derived from it is referred to as Variant 1.

A new series of passages was started with stock Lee allantoic fluid in an attempt to repeat the isolation of a slowly reacting variant. The procedure employed was a simplified version of the preceding one.

Procedure III.—Erythrocytes were saturated with virus at 4°C. and, after washing with cold saline, were suspended in saline and held at 37°C. In this procedure 500 units of penicillin and 2 mg. of streptomycin per ml. were added and the erythrocytes were incubated in the water bath for 20 hours. Following this period of elution the erythrocytes were sedimented by centrifugation at 4°C., washed 6 times with 15 ml. of cold saline, and serially diluted in fivefold steps. Each dilution was inoculated into a group of 5 to 8 embryos. When harvested, after 48 hours' incubation, the infected allantoic fluids at the end-point were pooled. A portion of the

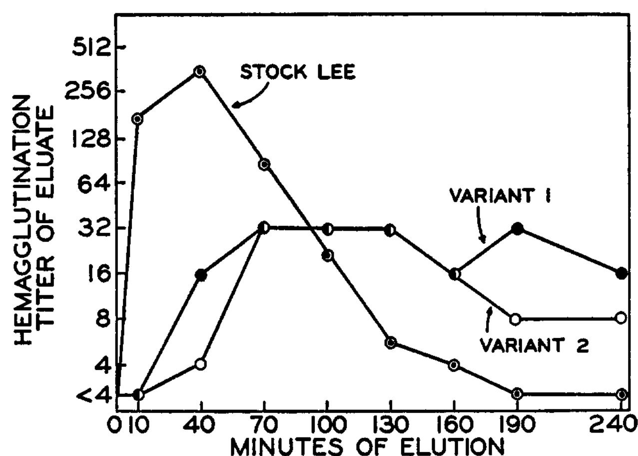

FIG. 1. Typical elution curves of stock Lee, Variant 1, and Variant 2 viruses from chicken erythrocytes. The hemagglutination titers of successive eluates are plotted against the time at which the eluate was removed.

pool was used for the next passage which was a repetition of the first. The remainder of the pool was stored at −40°C. for later testing. Four such serial passages were made.

After completion of the four passages the elution rate of the 3rd and 4th passage pools was determined. The elution rate of the 3rd passage pool was not significantly different from that of stock Lee virus. However the elution rate of the 4th passage pool was quite different. The virus in this pool and in all infected allantoic fluids derived from it is referred to as Variant 2.

Comparison of Elution Rates of Stock Lee and Variant Viruses from Chicken Erythrocytes.—Typical elution curves of stock Lee, Variant 1, and Variant 2 allantoic fluids are shown in Fig. 1. It can be seen that stock Lee virus exhibited a rapid dissociation from the erythrocytes during the first 40 minutes. After the peak of elution was reached there was a progressive decrease in the amount of virus which eluted during each succeeding time interval. No virus detectable by hemagglutination eluted after 160 minutes. The elution curves of

the two variant strains were quite different from that of stock Lee. The 10 minute eluate contained little or no virus and there was no sharp peak of elution. After the first 10 to 40 minutes virus appeared in the supernatant fluid at a low and fairly constant level and the rate of elution was changed very little at the end of the 4 hour observation period. From these results it is evident that the last two procedures outlined above were successful in isolating slowly eluting variants from the population of stock Lee virus.

Action of Stock Lee and Variant Viruses on Ovomucin.—The elution of a virus from erythrocytes is considered to be the result of the enzymatic degradation of virus receptors present on the surface of the erythrocyte. The stepwise elution technique, however, does not permit measurement of the substrate but only of virus which has separated from the erythrocyte for one reason or another. It was decided, therefore, to investigate the action of the variant viruses on one of the soluble mucoproteins which are also substrates for the virus

TABLE I

Comparison of the Destructive Activity of Stock Lee and Variant Viruses on the Inhibitory Titer of Ovomucin

Virus	Incubation	HI titer of ovomucin	
		Initial	Residual
Stock Lee	1 hr. at 25°C.	20,480	1,280
Variant 1	1 " " 25°C.	20,480	10,240
Variant 2	1 " " 25°C.	20,480	10,240

enzyme. This would allow measurement of the substrate and relate differences in viral activity more specifically to differences in substrate degradation. Ovomucin was chosen for this purpose as it is obtained from the same species of animal as that supplying the erythrocytes used in the isolation procedures.

Serial twofold dilutions of ovomucin were made in buffered saline. To 0.2 ml. of each dilution was added an equal volume of active virus containing 4 hemagglutinating doses (AD) of virus. After an incubation period of 1 hour at room temperature the tubes were heated at 65°C. for 30 minutes. Then they were cooled to room temperature and 5 AD of indicator stock Lee in a volume of 0.2 ml. was added to each tube. After 30 minutes 0.4 ml. of a 1 per cent suspension of chicken erythrocytes were added to each tube. One hour later the pattern of erythrocyte sedimentation was recorded. The hemagglutination-inhibition (HI) titer of the residual ovomucin was expressed as the reciprocal of the highest dilution of ovomucin that inhibited 2 + hemagglutination.

The results of a typical experiment using stock Lee, Variant 1, and Variant 2 viruses are shown in Table I. It can be seen that stock Lee reduces the HI titer of ovomucin 16-fold in 1 hour at room temperature while the two variants reduce the titer only twofold in the same period of time. It appears therefore

that the variant viruses have a decreased capacity to degrade ovomucin as well as a slower rate of elution from chicken erythrocytes.

Additional information regarding the rate of ovomucin alteration by Variant 1 and stock Lee viruses and the effect of temperature on the reaction rate were obtained with the following experiment.

Ovomucin in a volume of 1.5 ml. was mixed with 128 AD of the desired virus (0.4 ml.) or with 0.4 ml. of saline. Duplicate mixtures were held at 25°C. and at 37°C. After various time intervals aliquots were removed and heated at 65°C. for 30 minutes. Residual ovomucin was measured using 4 AD of indicator stock Lee virus.

It is apparent from the results shown in Table II that stock Lee degraded ovomucin very rapidly. After 2 hours' incubation at either temperature the

TABLE II

Effect of Temperature on the Reduction in Ovomucin Inhibitory Titer by Stock Lee and Variant 1 Viruses

Temperature of incubation	Virus	Residual ovomucin HI titer after indicated time (hrs.) of incubation		
		½	2	4
°C.				
	Stock Lee	16,384	<8	<8
24–25	Variant 1	16,384	128	32
	None	16,384		16,384
	Stock Lee	8,192	<8	<8
37	Variant 1	16,384	16,384	8,192
	None	16,384		16,384

HI titer of ovomucin was reduced below the level of measurement. While Variant 1 was much less active it is interesting to note that it had greater activity at 25°C. than at 37°C. After 4 hours' incubation at 37°C. the variant had reduced the HI titer of ovomucin only twofold whereas at 25°C. the titer was reduced over 500-fold. The variant virus therefore appears to be capable of degrading ovomucin, but its rate of action is much slower than that of stock Lee and the variant is more active at 25°C. than at 37°C. In confirmation of this it has been found that Variant 1 virus elutes from erythrocytes more readily at 25°C. than at 37°C. More detailed studies of the influence of temperature upon this reaction are in progress.

Rate of Enzymatic Activity on Ovomucin as Measured with Various Indicator Viruses.—In all previous experiments with ovomucin the indicator was heated stock Lee virus. Since it appeared possible that the variant virus could alter the substrate in a manner not detected by stock Lee indicator virus, it was of interest to see if additional enzymatic activity by the variant could be demon-

strated using the homologous virus as indicator. PR8 virus was included as a representative strain of type A influenza virus.

To 2.5 ml. aliquots of ovomucin were added 128 AD of stock Lee, Variant 1, or PR8 viruses in 0.4 ml. saline. A control consisting of 2.5 ml. ovomucin and 0.4 ml. saline was included. The mixtures were held at 37°C. and aliquots were removed at various time intervals and treated as in the previous experiment. The residual ovomucin in each aliquot was measured with indicator stock Lee, Variant 1, and PR8 viruses.

From the results shown in Table III it is evident that stock Lee virus was active in degrading inhibitor for indicator stock Lee and Variant 1 viruses

TABLE III

Comparison of Three Indicator Viruses in the Measurement of Residual Ovomucin after Incubation with Active Stock Lee, Variant 1, or PR8 Virus at 37°C.

Active virus	Indicator virus	Residual ovomucin HI titer after indicated time (hrs.) of incubation			
		1	2	8	12
Stock Lee	Stock Lee	1,024	64	32	<8
	Variant 1	2,048	128	8	<8
	PR8	16,384	4,096	2,048	2,048
Variant 1	Stock Lee	8,192	8,192	4,096	2,048
	Variant 1	4,096	2,048	2,048	1,024
	PR8	16,384	4,096	4,096	8,192
PR8	Stock Lee	2,048	1,024	2,048	256
	Variant 1	2,048	2,048	128	32
	PR8	16,384	4,096	8,192	512
None	Stock Lee	4,096			4,096
	Variant 1	2,048			2,048
	PR8	16,384			8,192

but less active against the inhibitor of indicator PR8 virus. Variant 1 virus had little activity on ovomucin regardless of the indicator virus used to measure residual ovomucin and homologous indicator virus was not more sensitive than stock Lee indicator virus in detecting inhibitor alteration. PR8 virus, although slower acting than stock Lee virus, was active against inhibitor of all three indicator viruses. It is notable that after PR8 action indicator Variant 1 was less inhibited by residual ovomucin than indicator stock Lee virus, suggesting that in the indicator state these viruses may differ somewhat in their affinity for certain combining sites.

Stability of Enzymatic Characteristics of the Variant Strains on Serial Passage.—The stability of the enzymatic characteristics of the variant strains was in-

vestigated by serial passage of the viruses in chicken embryos at low dilution. Two series of passages were begun with Variant 1 virus: one at end-point dilution and the other at a dilution of 10^{-4}. Four passages were made in each series. The 4th 10^{-4} passage allantoic fluid was then used to start a series of four passages at a dilution of 10^{-1}. No change in the rate of elution of the virus from chicken erythrocytes was noted at the completion of these passages. Variant 2 virus also received four serial passages at end-point dilution and at a dilution of 10^{-4}. Again no change occurred in the rate of elution of the virus during these passages.

The Concentration of Enzymatic Variants in the Stock Lee Virus Population.—Isaacs and Edney (12) were able to isolate an enzymatic variant of the MEL strain of Type A influenza virus by simple limiting dilution selection in chicken embryos. They found that the variant was present in the stock MEL population in a concentration of roughly 50 per cent. In the present case, isolation of the variants of Lee virus was accomplished only when a concentration technique was combined with passage of the concentrated material at high dilution and, therefore, it was felt that the variant was present in stock Lee preparations in low concentration. An estimation of the concentration of the variant in the stock Lee population could be obtained by analysis of data furnished by attempts to isolate the variant using the limiting dilution technique.

As a preliminary step the EID_{50} of stock Lee virus was determined. A dilution of the virus beyond the calculated EID_{50} was inoculated into a large group of embryos and the infected allantoic fluids harvested individually after 48 hours' incubation. The enzymatic activity of each virus clone was determined. Five such experiments were performed resulting in 76 end-point isolations at a level below 50 per cent infection. In no instance was a slowly reacting variant isolated. Analysis of these results using the inverse solutions of the incomplete beta function ratio at a probability of 0.9 indicated that the chances were 9 in 10 that the true percentage of slowly reacting variants in the stock Lee virus population did not exceed 2.95 per cent. Although the actual incidence of slowly reacting variants in the stock virus population may have been much below this figure, this could not be determined experimentally without many additional end-point dilution selections.

Comparison of Other Characteristics of Variant and Stock Viruses

A. Serologic Relationship of the Variant Viruses to Stock Lee Virus.—Cross hemagglutination-inhibition titrations were carried out with stock Lee, Variant 1, and Variant 2 allantoic fluids. Anti-stock Lee, anti-Variant 1, and anti-Variant 2 immune sera were used. There were no significant differences between the HI titers obtained with stock Lee and the variants isolated from it. The results indicated that the two variants had the same immunological specificity as the strain from which they were isolated.

B. Infectivity for Chicken Embryos and Mice.—Infectivity titrations in chicken embryos and in mice were carried out using Variant 1 and stock Lee virus preparations of comparable hemagglutination titer. It was found that there was no significant difference between the final infectivity end-points obtained with Variant 1 and stock Lee viruses in mice or in chicken embryos incubated at 35°C.

C. Hemagglutination of Erythrocytes of Various Species.—The capacity of Variant 1 and stock Lee viruses to agglutinate the erythrocytes of various species was determined in parallel. Hemagglutination titrations were performed using 1 per cent suspensions of chicken, guinea pig, human group O, sheep, and mouse erythrocytes. The latter two titrations were carried out at 4°C.; the others at room temperature. From the results shown in Table IV it is apparent that Variant 1 and stock Lee virus suspensions which were similar in their capacity to agglutinate chicken erythrocytes were also similar in their capacity to agglutinate erythrocytes of other species.

TABLE IV

Hemagglutination Titers of Stock Lee and Variant 1 Viruses with Erythrocytes of Various Species

Erythrocytes	Stock Lee	Variant 1
Chicken	2048	1024
Guinea pig	512	512
Human group O	2048	1024
Sheep (at 4°C.)	256	256
Mouse (at 4°C.)	8	16

D. Thermal Inactivation of the Mucolytic Enzyme.—Aliquots of undiluted stock Lee and Variant 1 allantoic fluids were heated at various temperatures for 30 minutes. Residual enzymatic activity was determined in the following manner:—

Heated allantoic fluid was added to packed chicken erythrocytes in sufficient quantity to saturate the cell surfaces with virus at 4°C. The erythrocytes were washed, resuspended in 1 ml. of fresh saline, and held at 37°C. for 3 hours to allow elution of virus. After this time the erythrocytes were centrifuged and the supernate removed. The HA titer of the supernate was determined. The virus enzyme was considered to have been inactivated if the 3 hour eluate had an HA titer of less than 4.

It was found that the enzyme of stock Lee virus was inactivated by heating for 30 minutes at a temperature between 53°C. and 54°C. In contrast, the enzyme of Variant 1 virus was inactivated in 30 minutes by heating at a temperature between 46°C. and 48°C. indicating that it was markedly more susceptible to thermal inactivation than was the enzyme of stock Lee virus.

E. Thermal Inactivation of the Hemagglutinin.—As it was found that the enzymatic activities of Variant 1 and stock Lee viruses were inactivated at different temperatures it was of interest to determine the temperatures at which the capacity of these viruses to combine with receptor sites and cause agglutination of erythrocytes was destroyed.

Aliquots of partially purified suspensions of Variant 1 and stock Lee viruses in 0.1 M sodium phosphate (pH 7.5) were distributed in 0.5 ml. volumes in small, rubber stoppered tubes and heated at various temperatures for 30 minutes. Duplicate hemagglutination titrations were then carried out and the hemagglutinin was considered to have been inactivated when the hemagglutination titer was reduced to less than 8.

No significant difference was found in the thermal stability of the hemagglutinin of these viruses since the temperature at which the hemagglutinin was destroyed by 30 minutes exposure was found to be 64.5°C. for both Variant 1 and stock Lee viruses.

The Effect of Calcium Ion on the Elution of Variant 1 from Chicken Erythrocytes

Briody (13) and Edney (14) have each reported enhancement of the enzymatic activity of influenza viruses by calcium ion under certain conditions. On the possibility that the strikingly reduced enzymatic activity of these slowly reacting variants might be altered by additional calcium ion, experiments were designed to follow the elution of virus from chicken erythrocytes under conditions of controlled calcium ion concentration.

Chicken erythrocytes were saturated with Variant 1 and stock Lee viruses at 4°C. These erythrocytes were then washed and aliquots were resuspended in the following solutions adjusted to pH 6.9: (*a*) borate buffered saline with 0.009 M (0.1 per cent) calcium chloride, (*b*) borate buffered saline brought to an ionic strength equivalent to solution described under (a) with sodium chloride, (*c*) borate buffered saline with 0.009 M calcium chloride plus the sodium salt of ethylenediaminetetraacetic acid (EDTA) in a concentration of 0.018 M. These suspensions were placed at 37°C. to allow elution of the virus from the erythrocytes.

The results of such an experiment are shown in Fig. 2. It is evident that the elution rate of stock Lee virus was not affected by additional calcium ions. The elution curve obtained in the presence of added calcium is superimposable at all points on that obtained in saline alone. The addition of EDTA resulted in a moderate increase in elution of virus during each interval. In contrast, the elution rate of Variant 1 virus was greatly accelerated by the presence of additional calcium ions and depressed by removal of free calcium ion by EDTA. The elution curve obtained in 0.009 M calcium chloride is almost indistinguishable from that of stock Lee virus in saline.

It appears that calcium ions accelerate the rate of enzymatic activity of Variant 1 virus. This effect is not attributable to changes in ionic strength and it can be overcome by the addition of the calcium chelating compound EDTA.

Sodium ions cannot substitute for calcium ions in this respect. Investigations are underway to determine the minimum concentration of calcium required for maximum activity of Variant 1 virus and to determine whether other divalent cations can substitute for calcium.

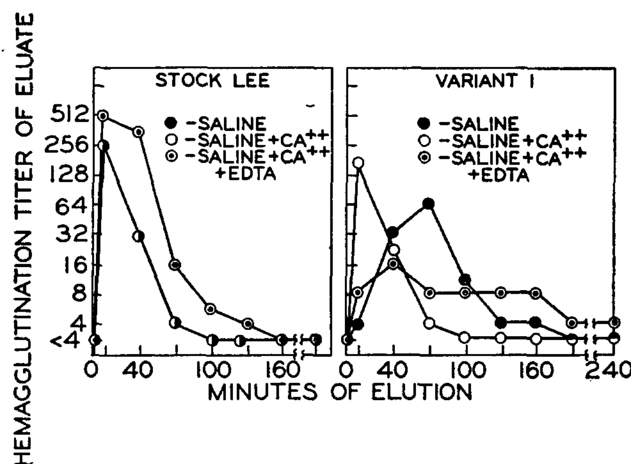

FIG. 2. The effect of calcium ion concentration on the rate of elution of stock Lee and Variant 1 viruses from chicken erythrocytes. The hemagglutination titers of successive eluates are plotted against the time at which the eluate was removed. Each point represents the geometrical mean of duplicate experiments. The suspending fluids were borate buffered saline + 0.009 M $CaCl_2$ (open symbols), borate buffered saline brought to equivalent ionic strength with NaCl (solid symbols), and borate buffered saline + 0.009 M $CaCl_2$ + 0.018 M EDTA (dotted symbols).

DISCUSSION

It is apparent from the results obtained in this study that enzymatic variants can be isolated from the population of Lee virus particles. That such variants can be isolated by means of relatively simple procedures which do not involve the use of mutagenic agents supports the assumption that the variants are naturally present in the Lee population and that the methods used have simply selected from the population certain preexisting variant virus particles. This selection can be repeated with ease. The procedures employed were so designed that, even though present in low concentration, variants with certain desired characteristics were isolated; namely, those having decreased enzymatic activity on the mucoprotein of chicken erythrocytes. Advantage was taken of the property by which the desired variants differed from the other virus particles to effect their concentration and eventual isolation. Now that more is known concerning the enzymatic characteristics of the variant more efficient methods, utilizing control of calcium ion concentration and temperature, could be devised to effect its concentration. A concentration procedure was neces-

sary since the variant was present in the initial population in such a low concentration as not to be readily isolated by the terminal dilution method. The same general approach could be applied to the isolation of other variants.

The variant differs strikingly from stock Lee virus in certain characteristics of its enzymatic activity. The variant has a much slower rate of enzymatic activity than has stock Lee virus as evidenced by the marked differences in their patterns of elution from chicken erythrocytes and the decreased percentage of variant virus which dissociates from the erythrocytes within 4 hours. Approximately 70 per cent of the stock Lee virus particles adsorbed to chicken erythrocytes elute within 4 hours at 37°C. whereas only about 20 per cent of the variant elutes during the same time interval. That the altered elution characteristics of the variant are in fact due to a slower rate of enzymatic activity is indicated by the decreased action of the variant on the soluble substrate ovomucin.

Stock Lee virus is most active enzymatically at a temperature of 37°C. or slightly higher while in contrast the variant is much more active at 25°C. than at 37°C. It is generally considered that enzymes become more active as the temperature is increased until an optimum temperature is reached beyond which activity decreases owing to heat denaturation of the enzyme. It would appear that the variant enzyme has a lower optimum temperature than that of stock Lee and it is noteworthy that the variant enzyme was inactivated at a lower temperature than that of stock Lee. More detailed studies of the thermal stability of these enzymes and of the effect of reaction temperature on their activity are in progress and preliminary evidence suggests that the decreased activity of the variant at 37°C. is not due solely to heat denaturation of the enzyme.

While it has been suggested (13, 14) that calcium increases the rate of enzymatic activity of influenza viruses, such an influence has been hard to demonstrate experimentally with Lee virus and when demonstrated has been of small magnitude. Often no positive effect of calcium could be demonstrated but its influence was inferred from the decreased activity of the virus in the presence of calcium deionizing agents. However Porterfield (15), using virus suspensions and reagents which had been decalcified by an ion exchange resin, found no change in the associative or enzymatic characteristics of the viruses. She attributed the decrease in virus activity observed following the addition of calcium deionizing agents to changes in the ionic strength of the system. The enzymatic activity of the variant isolated in this study however exhibits marked changes with alteration of calcium ion concentration. When 0.1 per cent $CaCl_2$ is added to the system the rate of elution of the variant from chicken erythrocytes is increased to such an extent that it can no longer be distinguished from stock Lee virus. The activity of stock Lee virus is unaffected by this concentration of calcium. It is noteworthy that elution experiments are carried out as

routine in the presence of 0.85 per cent NaCl suggesting that the enzyme of the variant virus may have a specific requirement for calcium or divalent cations.

It is apparent from the effect of temperature upon the reaction rates, from the differences in thermal stability of the enzymes, and from differences in their sensitivity to calcium ion concentration that there are qualitative differences in the mucolytic enzymes of stock Lee and variant viruses. Whether there are also quantitative differences in the number of enzyme molecules present on the virus particles cannot be stated at this time. Burnet and Edney (16) have suggested that influenza viruses carry calcium ions firmly integrated into their enzyme groupings and Briody (13) has reported that in the presence of calcium ions the temperature at which the influenza enzyme is inactivated by heat is increased. Whether a deficiency in calcium is the basis for the qualitative differences observed in the enzymatic characteristics of variant and stock Lee viruses, particularly with respect to the influence of temperature, remains to be determined.

It is notable that in the characteristics of immunological specificity, infectivity and pathogenicity for mice, infectivity in chicken embryos, hemagglutination of erythrocytes of various species, and heat stability of the hemagglutinin the variant and parent viruses were indistinguishable. These represent all characteristics studied other than those related to the virus enzyme. Although the combining reaction of enzyme and substrate has not been studied specifically, no differences have been observed in the rate or extent to which variant and stock Lee virus combine with substrate. This clear separation of enzymatic characteristics from other viral properties lends support to the possibility that such variants might be useful in the study of the role of the viral enzyme in the life cycle of the virus.

Since the preparation of this report a paper by Smith and Cohen (17) has appeared describing an attempt to obtain stable, homogeneously eluting fractions of influenza A and A prime viruses. The methods employed to fractionate allantoic fluid into rapidly and slowly eluting components were somewhat similar to the ones used in the present study. Smith and Cohen however were unable to obtain fractions with different enzymatic activities which were stable on passage.

SUMMARY

Slowly reacting enzymatic variants of the Lee strain of influenza B virus were isolated by the serial application of a technique which concentrated the fraction of the virus population obtained by slow elution followed by passage of this fraction at high dilution in chicken embryos. The variants were stable upon serial passage in chicken embryos and did not differ significantly from

the parent virus in any of the characteristics tested other than those relating to the virus enzyme.

Enzymatically, the variants had a much slower rate of activity on chicken mucoproteins than the parent virus. Their activity was greater at 25°C. than at 37°C. and was much enhanced by calcium ions and depressed by a calcium chelating agent. The enzyme of the variant virus was more labile to heat than that of the parent virus although their hemagglutinins were equally stable. It was estimated that the variants comprised less than 3 per cent of the original population of the Lee strain of influenza B virus particles.

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