A STUDY OF THE pH STABILITY OF VACCINIA VIRUS*

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The interest in the action of chemical agents upon vaccinia virus is not of particularly recent origin. Jenner[16] suggested that zinc sulphate and copper sulphate could be used to mitigate or stop the progress of vaccinia infection. However, it was not until much later that Copeman[11, 12] began a thorough study of the action of various chemical agents upon virus-containing calf lymph. This study was aimed primarily at discovering an agent for the preservation of the infective agent of cowpox, in order that it might be kept for long periods of time while being freed of contaminating bacteria. Since this early work of Copeman, there have been many studies with this same purpose in mind, but since they show the attempts to preserve rather than destroy vaccinia virus, they are only of passing interest in the study of the dynamic action of chemical agents upon the virus. Nevertheless, in not a few of these studies the deleterious action of the H^+ and OH^- ions was sufficiently demonstrated to stimulate speculation on the pH stability of vaccinia virus. Hence it was with the aim of demonstrating the behavior of vaccinia virus at various pH levels that this study was undertaken.

The many attempts to purify vaccinia virus offer a great amount of evidence that the stability of the virus decreases with increased acidity. Since these studies were made with an attempt to retain the infectivity of the virus, there is actually little revealed regarding virus survival time at any of the pH levels studied.

Employing selective adsorption and elution as a means of purifying vaccinia virus, Yaoi and Kasai[22] and Yaoi[21] found that the virus sustained very little injury when exposed for only short periods of time to pH 4.6 and to N/25 NH_4OH. However, virus titers were not determined nor were they used as criteria of virus activity.

In a long series of investigations, Behrens and Morgan,[5] Behrens and Nielson,[6, 7, 8] and Behrens and Echelbarger[3] studied methods of separating vaccinia virus from associated foreign protein. They

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treated dermal vaccine with a variety of weak acids, which, in the concentration used, gave pH values of between pH 4.4 and 5.5. Although the treatment of the material rarely lasted longer than 20 minutes at between 20° and 25° C., the virus retained activity, though it was attenuated.

In the course of studies on the iso-electric point of virus-tissue suspensions, Moriyama[18] found that if salts were added to a tissue-virus suspension, a higher concentration of acid was necessary to precipitate the tissue. However, the salts and the hydrogen ion were complementary in their action, and in the presence of salts the virus became inactive in extremely dilute acid solutions. The salts of mercury and copper in conjunction with the hydrogen ion were the most active in effecting a destruction of the virus.

In an attempt to find a pH at which vaccinia virus gave sharp sedimentation boundaries in an ultracentrifuge, Beard, Finkelstein, and Wyckoff[1, 2] carefully studied the pH stability of vaccinia virus in terms of physical changes. On the acid side marked and rapid changes took place in the virus below pH 2.5; on the alkaline side changes markedly different from those occurring on the acid range quickly altered the virus at pH levels above 11.5. They conjectured that the virus was very rapidly inactivated at these levels and that the range of pH stability for vaccinia virus was between pH 5.5 and pH 10.0. This particular study is of especial interest, since it had as its aim the determination of the physical or chemical changes which took place in the virus particle when treated with acid or base, and, although rabbits were used as test animals in determining the activity of the virus, the chief evidence submitted was of alteration of the protein particles. It did not reveal the actual rate at which activity was lost with reference to the concentration of the hydrogen ion.

Methods and procedure

1. *The virus.* The strain of vaccinia virus used in this study was originally obtained from Dr. T. M. Rivers of the Rockefeller Medical Center. This virus had been adapted to tissue cultures of minced chick embryo by Rivers and Ward[19] in 1932 and was called the "revived strain of culture vaccine virus." Without difficulty, this virus was adapted to the chorio-allantoic membranes of developing chick embryos, and thereafter was carried in the developing chick embryo until this investigation had been completed. The method used in this serial transfer of the virus and in the preparation of

the fertile hens' eggs were those of Woodruff and Goodpasture,[20] and Goodpasture, Woodruff, and Buddingh[15] as modified by Burnet.[9, 10] At no time during the investigation, lasting about 18 months and through approximately 50 serial transfers, were any changes noted in the virus.

The physical method of purification which was used throughout this investigation was basically that designed by Eagles and Ledingham,[14] and depended entirely upon differential centrifugation. The virus was freed from the chorio-allantoic membrane by grinding with pulverized "Kimball" heat-resistant glass. The resultant paste was diluted with four times its volume of a 1:50 dilution of McIlvaine's standard phosphate-citric acid buffer adjusted to pH 7.2. After the grinding and dilution, the diluent and tissue were thoroughly mixed and immediately centrifuged at approximately 1000 revolutions per minute (r.p.m.) in a horizontal centrifuge. The supernatant was saved and the sediment returned to the sterile mortar and ground, diluted, and centrifuged again. This was repeated three times, after which the sediment was discarded and the supernatants from the several washings were pooled. This pooled material contained both virus and finely dispersed tissue and foreign protein. The pooled tissue washings were then divided among several conical centrifuge tubes and centrifuged for 2 hours at 4000 r.p.m. in an "Adams Senior" angle-head centrifuge. The supernatants became relatively free of virus and tissue particles, but contained the soluble protein carried over with the original tissue, hence it was discarded. The sediment contained the virus and the small tissue particles. This was again resuspended in the dilute buffer and the washing process was repeated twice more. After the third resuspension very little of the soluble protein was left, but the full complement of insoluble tissue remained. However, the tissue particles had become increasingly difficult to disperse due, supposedly, to agglutination, and when this final preparation was centrifuged for 30 minutes at approximately 1000 r.p.m. in a horizontal centrifuge the supernatant contained well-dispersed vaccinia particles and under the microscope it appeared to be homogeneous. This final supernatant, consisting of thrice washed and resuspended virus, constituted the purified virus used throughout this investigation.

An effort was made to demonstrate the amount of foreign protein contained in the purified virus suspension through the use of the precipitin test, using anti-chick embryo rabbit sera, but no precipitate could be demonstrated. However, the skin of rabbits which had been sensitized to the ground chorio-allantois reacted violently within 30 minutes of the intracutaneous injection of 0.1 cc. of the suspension. This showed that the suspension was not entirely free of the foreign protein derived from the embryo.

Although the entire preparation of the virus suspension was carried out under aseptic conditions, samples were plated out with liver infusion agar and run into meat tubes. Whenever a virus suspension showed that it had become contaminated, it was discarded.

No attempt was made to preserve the virus, other than storage in an ice-box at a temperature between 6° and 10° C., in the original diluted buffer solution. The suspension was titrated immediately before storage and, since the drop in titer at this temperature appeared to be very slow, the value obtained was used with very little adjustment when planning experiments during the following 1 or 2 weeks. The slow drop in titer that did occur could probably be explained by iso-agglutination as evidenced by the precipitate which eventually accumulated in the bottom of the bottle containing the virus suspension. Whether the loss in titer was actual or apparent could not be determined. A typical protocol resulting from a virus titration is given in Table 1.

TABLE 1

TITRATION OF THE VIRUS SUSPENSION

Dilution	*Inoculum*	*Pocks counted on each egg membrane*						*Total*	*Average*
1:1000	0.2 cc.	+	+	D	D	D	D	+	+
1:10,000	0.2 cc.	200+	112+	180+	D	D	D	942+	164+
1:100,000	0.2 cc.	90	75	124	112	98	D	499	99
1:1,000,000	0.2 cc.	12	7	7	15	4	9	54	9

D = dead embryos; + = Confluent pocks; 200+ = 200 pocks plus an area impossible to count. Titer approximately 45 million to 50 million infective units per cubic centimeter.

2. *Experimental methods.* Since it had been shown by Moriyama[18] that the influence of the hydrogen ion upon the physical nature of vaccinia virus is complemented by the presence of various salts, it was deemed wise to establish the various pH values, at which the virus was to be treated, through the use of distilled water and either hydrochloric acid or sodium hydroxide. Nevertheless, it was recognized that the buffering capacity of both these substances decreased rapidly when changes were made from the extreme toward the center of the pH range. Therefore, in order to maintain a selected pH value without altering it too greatly, due to the action of the diluted buffer in the virus suspension, the following procedure was used in setting up each experiment described in this investigation: A number of time intervals were chosen for the titration of the virus after treatment at a particular pH level. These time intervals were selected by observing the action of the virus under similar conditions during preliminary experiments. With this knowledge in mind, 10 cc. of the test solution were run into one more sterile tube than the number of time intervals selected. The solution was allowed to remain in the tubes for 10 minutes during which time they were repeatedly shaken. The tubes were then emptied of the solution, snapped free of the remaining droplets, and 9 cc. of the test solution were placed in each tube. Thus prepared, the tubes were

ready for the virus. The purpose of these precautions rested in the hope that the interval of soaking would dissolve any foreign salt having an unfavorable buffering action upon the test material.

To the 9 cc. of reagent in tube No. 1 was added 1 cc. of the virus suspension. In order to mix the virus and the reagent thoroughly, the two were drawn into and discharged from a 10 cc. pipette six times, a procedure used in making all of the virus dilutions. This 1:10 dilution of the virus in test material was distributed in 1 cc. amounts among the remaining prepared tubes. Tube No. 1 was then discarded. In all of the remaining tubes, the contents were mixed as had been the case with tube No. 1, and with the exception of tube No. 2 they were all placed in a 25° C. water-bath along with an equally diluted saline-virus control. In testing the action of the hydroxyl ion, this procedure was varied slightly. Since the carbon dioxide of the operator's breath had a tendency to alter the pH in the presence of sodium hydroxide, all of the tubes were covered with parafilm and were shaken 100 times wherever pipetting had been used to mix virus and reagent.

The contents of tube No. 2, although these preparations had taken approximately 6 minutes, represented the 0-hour titration. At this point the virus had been diluted 1:100. This was immediately diluted with saline so that were the virus still infective an inoculum of 0.2 cc. placed on the chorio-allantoic membrane of a 12-day-old chick embryo would result in a countable membrane, and six chick embryos were then inoculated. Without delay the pH of the undiluted test mixture was determined with a Beckman glass electrode pH meter. At the end of each time interval, one of the tubes was taken from the water-bath, similarly diluted 0.2 cc. amounts inoculated into a series of six eggs, and the pH determination made. The final tube taken from the water-bath was always the saline-virus control which was similarly treated.

3. *The method of virus titration.* Fresh fertile hens' eggs were obtained from a reliable commercial hatchery. These were incubated in a 300-egg Montgomery Ward and Company incubator. On the eleventh day of incubation, approximately a tenth more eggs than would be needed for the planned experiment were removed from the incubator, candled, and prepared for inoculation as described by Burnet.[9, 10] These eggs were then placed in a standard bacteriological incubator and allowed to rest for 24 hours. This rest period allowed those embryos which had been seriously injured either to recover from the injury or to die before the experiment was continued.

When a single titration was to be made, 6 eggs were removed from the incubator and with a tuberculin syringe, equipped with a 27-gauge needle, 0.2 cc. of the diluted virus were distributed widely over the surface of the chorio-allantois of each egg. After the window, over the shell opening, had been replaced, each egg was rocked vigorously from end to end, from side to

side, and with a circular motion not less than six times. The eggs were then labeled and returned to the incubator.

Forty-eight hours later, the eggs were removed from the incubator, the dead embryos discarded, and the remaining chorio-allantoic membranes harvested as outlined by Burnet.[9, 10] Each membrane was then washed in saline and floated out flat in a petri dish; the pocks were counted against a black background. When only confluent or non-specific lesions developed, these membranes were ground, and without further treatment other than dilution they were inoculated into a second series of eggs in order that the presence or absence of virus might be demonstrated by definite pock formation.

4. *The evaluation of data.* Although there is good agreement between the last two dilutions of the virus titration illustrated in Table 1, it was found early in this investigation that the values obtained from most series of dilutions varied greatly, as shown in Table 2. Since the 1:1000 dilution produced

TABLE 2

ELEMENTARY BODY TITRATION

Egg No.	*1*	*2*	*3*	*4*	*5*	*6*	*Total*	*Average*
Series and dilution								
A–1:1000	100+	72+	+	+	D	D	172+	86+
B–1:10,000	7	21	12	17	+	D	57	14.25
C–1:100,000	3	1	3	0	2	2	11	1.8
D–1:1,000,000	0	2	0	0	0	1	3	0.5

100+ = 100 pocks and confluent area; + = confluent lesions.
D = dead embryos; 0 = no lesions.

confluent areas on all of the membranes harvested from living embryos, the value obtained, in the average, must be disregarded; likewise, the value obtained from the 1:1,000,000 dilution becomes invalid, since only two membranes out of the series of six eggs developed pocks. The 1:10,000 dilution would lead one to believe that there were approximately 600,000 infective units in 1 cc. of the original virus suspension; the 1:100,000 dilution would indicate that there were 900,000 infective units. Therefore, between the two dilutions there exists a difference of approximately 300,000 units. This led to speculation as to whether the pocks resulting, after the virus had been treated at a specific pH, should be recorded merely as evidence of virus activity or as a definite number of pocks. If the actual pock counts from a six-egg series could be used with security, it would be possible not only to determine the time at which the virus became inactive, but also the rate at which inactivation took place.

Since the titration methods used had shown the spread between counts

to be relatively wide, an experiment was designed to demonstrate the differences that would develop when several counts were made of pocks developing from the same virus suspension each time the operator carried out the dilution process. Starting each time with the same virus suspension, a 1:10,000 dilution was prepared at six different times during an 8-hour period. Each independent dilution was inoculated into a series of six embryos. Forty-eight hours later, the membranes were harvested and counted. None of the membranes showing a confluent area was considered in striking the totals and the averages. The results of this experiment are given in Table 3.

TABLE 3

THE RELIABILITY OF POCK COUNT METHODS IN THE ENUMERATION OF ELEMENTARY BODIES

Series	*A*	*B*	*C*	*D*	*E*	*F*
Egg No.						
1	36	12	20	3	15	3
2	21	17	13	6	5+	20
3	5	15	0	11	10+	+
4	19	19	+	1	4+	4+
5	4+	D	5+	7+	4+	1+
6	4+	D	+	1+	4–	D
Total	81	63	33	21	15	23
Average	20	16	11	5	15	11

D = dead embryos.
+ = confluent lesions.
4+ = pocks and confluent area.
Virus diluted 1:10,000 in each series.

Although each series of dilutions was made with extreme care and with a conscious effort to keep the technic unvaried, the highest count-average was four times greater than the lowest count-average. This evidence, coupled with the counts taken from membranes studied after preliminary pH-virus experiments, demonstrated that it was impractical to attempt to determine inactivation rates with a six-egg series, and that only the time of complete inactivation could be determined with security. To insure validity in data of this kind, a standard was decided upon for the virus which was to be used. Any virus which did not cause the development of 100 or more pocks, when diluted 1:5000 and inoculated in 0.2 cc. amounts, was considered unsatisfactory. Therefore, in reporting the results of these experiments counts will rarely be mentioned. Nevertheless, if only one pock developed in a series of six-eggs, the virus was considered active; likewise, if no pocks developed, the

virus was considered inactivated. All lesions which had any similarity to pocks were tested for active virus through re-inoculation into another series of eggs.

Experimental results and discussion

1. *The influence of the sodium ion and the chloride ion upon vaccinia virus.* Since very little is positively known regarding the influence of various ions upon vaccinia virus, the question of the toxicity of those ions, which from necessity would be associated with the hydrogen or the hydroxyl ions, arose early in this investigation. Sodium chloride, therefore, was chosen as a test salt, with the hope that the chloride ion and the sodium ion in themselves would prove innocuous, thus enabling the investigation to be carried out with hydrochloric acid and sodium hydroxide.

A suspension of vaccinia virus, containing approximately 2,500,000 infective units per cubic centimeter, was used in this experiment to demonstrate the effect of the sodium ion and the chloride ion. Physiological saline, with a concentration of 0.85 per cent sodium chloride, was used as a test reagent. The actual ionic concentration of either the sodium ion or the chloride ion in this solution was greater than would be the case in the hydrochloric acid or the sodium hydroxide solutions to be tested, the sodium chloride solution being approximately 0.15 *N*, while neither the hydrochloric acid nor the sodium hydroxide would be used in concentrations greater than 0.1 *N*. As a control, and also to test the influence of distilled water, the virus was treated in parallel with twice-distilled water. Time intervals for the titration of the virus with these reagents were fixed at values that might prove convenient in subsequent titrations of the virus treated with acid and alkali. These time intervals were 6 minutes, 1, 2, 4, 8, and 17 hours. The method of setting up the experiment was exactly as described earlier in this paper.

The pH of the saline throughout this experiment was 6.9; the pH of the distilled water was 7.1.

Forty-eight hours after the embryos had been inoculated, the membranes were harvested and the pocks counted. From a previous titration of this particular virus, an average of approximately 100 pocks per membrane was to be expected if either the distilled water or the saline were without influence upon the virus. A study of the averages, in Tables 4 and 5, shows that the counts averaged very

close to 100 pocks per membrane at any time interval. The lowest count obtained was with the distilled water at the end of 17 hours, but the other variations among the averages, as well as a comparison with Table 3 discussed earlier, would indicate that this low count was due to experimental error and not to the virucidal action of distilled water.

TABLE 4

THE INFLUENCE OF PHYSIOLOGICAL SALINE, AT 25° C. AND pH 6.9, UPON VACCINIA VIRUS

Series	*0.1 Hr.*	*1 Hr.*	*2 Hrs.*	*4 Hrs.*	*8 Hrs.*	*17 Hrs.*
Egg No.						
1	+	65	250	85	96	221
2	22+	163	151	116	60	75
3	61+	55	142	70+	40	73
4	D	115	60+	50+	D	+
5	D	58	50	3+	D	D
6	D	110	D	D	D	—
Total	83+	566	653	324	196	369
Average	28+	94	131	65	85	123

D = dead embryos; + = confluent lesions; 22+ = confluent area and 22 pocks.

TABLE 5

THE INFLUENCE OF DISTILLED WATER, AT 25° C. AND pH 7.1, UPON VACCINIA VIRUS

Series	*0.1 Hr.*	*1 Hr.*	*2 Hrs.*	*4 Hrs.*	*8 Hrs.*	*17 Hrs.*
Egg No.						
1	184	63+	44	75+	90	21
2	162	150+	46	100	235	98
3	113	71+	166	73	141	110
4	141	45	77	56	100+	13
5	7	45+	140	45	D	55
6	D	D	D	96	D	—
Total	607	375+	473	445	566	297
Average	122	75+	95	74	142	60

D = dead embryos; + = confluent lesions; 63+ = confluent area and 63 pocks.

This experiment indicates, therefore, that neither the sodium ion nor the chloride ion in concentrations of 0.85 per cent nor

the distilled water has, at neutrality, an influence upon vaccinia virus that can be demonstrated within a 17-hour period of treatment at 25° C.

2. *The influence of various pH values at 25° C. upon vaccinia virus.* There are two basic methods of presenting the data which will demonstrate the action of various concentrations of acid or base upon vaccinia virus; first, the longest time of survival as in Table 6; second, the time of complete inactivation as in Figure 1.

TABLE 6

THE TIME AND pH AT 25° C. NEEDED TO INACTIVATE VACCINIA VIRUS

Time	*0.1 Hr.*	*0.5 Hr.*	*1 Hr.*	*2 Hrs.*	*4 Hrs.*	*8 Hrs.*	*17 Hrs.*
pH Value Tested							
1.05	—	—	—	—	—	—	—
2.05	+	—	—	—	—	—	—
2.20	+	—	—	—	—	—	—
2.40	+	+	—	—	—	—	—
2.50	+	+	+	—	—	—	—
2.70	+	+	+	—	—	—	—
3.50	+	+	+	+	+	+	—
11.45	+	+	+	+	+	+	—
11.55	+	+	+	+	+	—	—
11.60	+	+	+	+	+	—	—
11.75	+	+	+	—	—	—	—
13.00	—	—	—	—	—	—	—
Saline-Virus Control	+	+	+	+	+	+	+

+ = Active; — = Inactive.

It is shown in Table 6 that no survival time was found for vaccinia virus at either pH 1.05 or pH 13.0, six minutes being the shortest time interval studied. As will be shown later 6 minutes probably far exceeds the survival time of the virus at both of these pH levels. At pH 2.05 only 3 pocks in one experiment and 6 pocks in another, out of a possible 600, developed as evidence of virus activity after an interval of 6 minutes. Six minutes also proved to

be the longest interval of survival of active virus at pH 2.20. The activity of the virus persisted only 30 minutes at pH 2.40; 60 minutes at pH 2.50, pH 2.70, and pH 11.75. Vaccinia virus withstood the action of pH 11.60 and pH 11.55 for 4 hours, at which time it was still active. Finally, at pH 3.5 and pH 11.45, virus activity remained for a full 8 hours.

The virucidal action of hydrochloric acid, as shown in Figure 1, is spread over a wide pH range and apparently depends upon the concentration of the hydrogen ion in the virus-reagent mixtures, since the data show that the virus is inactivated quickly and completely at pH 1.0 and 2.05, and between 8 and 17 hours at pH 3.5. In mixtures of virus and acid at pH 2.05 and pH 2.20, the virus was inactivated within 30 minutes. Sixty minutes were needed to inactivate the virus at pH 2.4, and 90 minutes were necessary to bring about complete inactivation of the virus at pH 2.5. Two hours were required to complete the process of virus inactivation at pH 2.7. With a decrease in the concentration of the hydrogen ion, the time for complete inactivation became progressively longer, until at a pH of 3.4 inactivation was not complete until the 17th hour. Since it has been shown previously that the chloride ion did not exert a virucidal action toward the virus, it is probable that this inactivation is due entirely to the hydrogen ion and is directly related to its concentration.

Vaccinia virus treated with sodium hydroxide at 25° C. was inactivated completely within various intervals of time between 6 minutes and 16 hours, depending upon the concentration of the hydroxyl ion in the virus-reagent mixture. In a solution of pH 13.0 the virus became completely inactivated within 6 minutes; it was inactivated within 2 hours at pH 11.75, within 8 hours at pH 11.60 and pH 11.55, and within 17 hours at pH 11.45.

Except at pH 3.5 and at values near this pH level, all of the pocks that did develop, after treatment with acid or alkali, on the chorio-allantois were typical. However, at those pH values where the inactivation was slow, and at the time when inactivation was nearly completed, lesions resulted that appeared to be caused by nonspecific injury rather than from vaccinia infection. These lesions were not the typical discrete pocks, but were one or more confluent areas, and it was only upon the re-inoculation of this material into another series of eggs that typical pock formation resulted.

3. *Discussion of experimental results.* The two pictures presented by the data, the one showing the action of acid on vaccinia virus, the other showing the action of alkali, differ markedly. The range of pH over which the H ion is virucidal is relatively broad when compared to the range over which the OH ion displays a virucidal action. Figure 1 presents this relationship.

This shows graphically that the hydrogen ion is much more active as a virucide than is the hydroxyl ion and acts much more rapidly at lower concentrations. At approximately pH 1 and pH 13 the time needed in both the acid and the alkaline solutions is 6 minutes, or in all probability much less. At pH 11.75 the time needed to inactivate the virus has increased to 60 minutes, while at a comparable acid pH, 2.2, the time needed is only 30 minutes. Again at comparable pH levels, pH 2.4 and pH 11.6, the values of time are widely different; pH 2.4 demanding only 60 minutes, but pH 11.6 demanding 8 hours. Only 120 minutes are necessary for the inactivation of the virus at pH 2.6, but at a comparable value of pH 11.4 complete inactivation does not occur until near the 17th hour.

Fig.1
Time of Complete Inactivation of Vaccinia Virus at Comparable H^+ and OH^- Concentrations

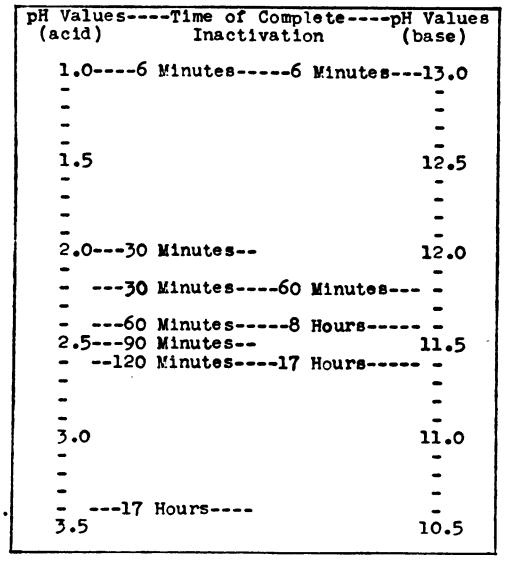

Figure 1 also suggests that had complete inactivation time been determined for pH 12.0, it would have fallen below the inactivation time of the corresponding pH value, pH 2.0, which was 30 minutes. It is also indicated that the 6 minutes, placed opposite pH 1.0 and pH 13.0, far exceed the time needed for the complete inactivation of the virus at these particular pH levels.

Whether this difference in the limits of the effective range is due to the differences in the size and the rate of movement that

exist between the two ions or to the inherent nature of the ions and the virus particles can only be a matter of conjecture, but both factors may play very definite rôles in the inactivation-rate differences that can be observed. Recognizing, however, that these differences do exist, it becomes necessary to study these two sets of values separately.

In Figure 2, the logarithm of the time for complete inactivation of vaccinia virus by hydrochloric acid is plotted against pH. It is of interest to note that these values fall along a straight line having a slope of approximately 1.0. Such a relationship between the time of complete inactivation and the concentration of the hydrogen ion indicates that the concentration of the reagent is inversely proportional to the time and can be expressed mathematically as,

$$ct = k$$

where c is the concentration of the hydrogen ion, t is the time necessary for complete inactivation, the exponent of c is 1 (the slope of the graphed log functions of c and t) and k is the resulting constant. This means that for each decrease in concentration the time needed to inactivate the virus becomes proportionally longer. Halving the concentration, therefore, doubles the time of survival. It can be

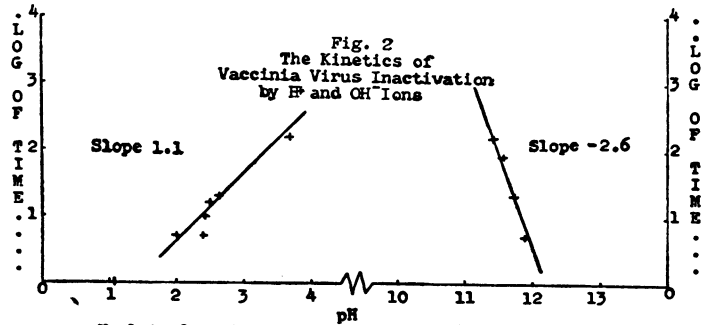

Fig. 2
The Kinetics of Vaccinia Virus Inactivation by H^+ and OH^- Ions

pH plotted against the log of time; 6 minutes = 1 time unit, 1 hour = 10 time units.

seen from such a graph as Figure 2, that a pH of 5.0 should permit the survival of the virus for at least 1000 hours were no other factors than the hydrogen ion exerting a detrimental influence. Without doubt, other factors influencing the viability of the virus would appear at this pH, and these might be aided by time much more effectively than would the hydrogen ion. This seems evident, since the work done on the purification of viruses indicates that pH

4.4 tends to weaken the virus (Behrens et al.[3, 4, 5, 6, 7, 8]; Yaoi and Kasai[22]).

Figure 2 also presents the logarithm of the time required for complete inactivation of vaccinia virus by sodium hydroxide plotted against the pH. Although the course of the plot indicates that the time necessary for complete inactivation is proportional to the concentration, it is apparent that the relationship is not a direct one. The slope of the line, representing the relationship of time to concentration, measures 2.6. (This measurement was made in full recognition that an insufficient number of points had been determined to plot the line accurately.) This means that with every decrease in the concentration of the hydroxyl ion the time necessary for complete inactivation of the virus will be proportionally increased by approximately six times, since the relationship can be expressed mathematically as,

$$c^{2.6}t = k$$

where c is the concentration of the hydroxyl ion, 2.6, the exponent of c, is obtained from the log time-pH curve, and k is the constant for the action of the hydroxyl ion on vaccinia virus at 25° C.

It is evident from this study that though the virus may show a decrease in titer at a pH of 11 or even at pH 10, the inactivation is not completed between these values in such a sudden fashion as has been suggested by Beard, Finkelstein, and Wyckoff[1, 2] in their report on the pH stability range of the elementary bodies of vaccinia. The sudden loss in viability, which they assumed should occur between pH 10 and 11, must occur some place between pH 12 and 13. Theoretically, the virus ought to remain viable at a pH of 11 for more than 1000 hours, although it should progressively lose in titer over this interval.

That there are differences in the mode of action of the two reagents much more fundamental than merely the rate of inactivation is seen in the nature of the lesions resulting after the virus has been treated for an interval with either one or the other chemical agent. The lesions resulting after treatment with sodium hydroxide did not differ from those induced by untreated virus. Apparently the surviving virus, in its capacity to cause a typical infection, had not been altered, although its titer may have been greatly reduced. No alkaline pH value was discovered that so altered the virus that the

nature of the lesions differed from that of lesions resulting from virus treated at other alkaline pH levels. This evidence indicates that there was no process of attenuation which would leave a viable virus particle with the ability to cause an infection differing from the infection caused by an unattenuated virus particle. This evidence also indicates that no products were formed by the sodium hydroxide inactivation of the virus which might have a detrimental and injurious effect upon the chorio-allantois of the developing chick. The inactivation of vaccinia virus by sodium hydroxide, at various pH levels at 25° C., was an all-or-none reaction.

When the virus was inactivated by acid solutions a certain number of atypical lesions appeared on the membranes. If the virus was completely inactivated by an acid pH, no typical lesions capable of transferring the infection were produced. Here, too, the process of inactivation was an all-or-none reaction leaving behind no residues injurious to the chorio-allantois. However, when the acidity of the virus-acid mixtures had been lowered by dilution and the complete inactivation of the virus was protracted beyond the 8-hour interval, materials treated at this pH for only 4 hours were capable of causing atypical lesions. This suggests that when the action of the acid has been slowed down by dilution the process of inactivation is revealed as taking place in several steps, involving perhaps a sequence of events which gradually alter the inherent nature of the virus before it becomes totally inactive. Another possibility that seems more reasonable is that during the process of inactivation products are formed which are injurious to the chorio-allantoic membrane of the developing chick; if the concentration of the acid is great enough these products are destroyed at the time they are formed, and if the concentration of the acid is low these products are destroyed less rapidly than are the virus particles. The evidence supporting this theory is that when membranes were inoculated with a virus-acid mixture of pH 3.4, which had incubated at 25° C. for 18 hours, two of the six membranes showed non-specific lesions which could have been mistaken for the virus-carrying atypical lesions which had been seen earlier. However, these non-specific lesions did not carry the virus, as was demonstrated by inoculating them into a fresh series of embryos.

That the rate of the action of the hydrogen ion upon vaccinia virus does not differ greatly from the rate of action of the ion on bacteria appears from tables presented by McCulloch.[17] These tables

show that as the concentration of formic acid decreases, the survival time of *Eberthella typhi* increases and that this relationship between survival time and the concentration of the acid is one of inverse proportion. Each time the concentration of the acid was halved, the survival time was roughly doubled. McCulloch believes that the disinfectant action of formic acid is due entirely to the hydrogen ion. The fact that acids are bactericidal in direct proportion to the concentration of hydrogen ions has confirmation in a report by Cowles.[13] He states that the survival time of *Staphylococcus aureus* is inversely proportional to the hydrogen ion concentration. However, the exponent of the concentration of the hydrogen ion against the colon bacillus was 1.75.

The limits of H-ion concentration over which this phenomenon can be observed may differ from organism to organism, depending upon the inherent acid tolerance of the bacterium; thus, the plotted logarithmic functions of ion and time might not exactly coincide, but they would run approximately parallel to each other and to the plotted logarithmic functions of time and hydrogen ion concentration for vaccinia virus (Fig. 2).

The data on the rate of action of the hydroxyl ion upon bacteria are relatively meager, but McCulloch[17] presents two bits of evidence from the work of others that serve to shed light on the rate at which bacteria are killed in solutions of sodium hydroxide. *Eberthella typhi* was killed in 3 minutes by a 1:625 solution of sodium hydroxide, in 20 minutes by a 1:1250 dilution, and in 45 minutes by a 1:2500 dilution of the alkali. The second time interval doubles with the halving of the concentration, but the first time interval more than doubles with the halving of the concentration. When *Staphylococcus aureus* was the test organism and solutions of 1:100, 1:150, and 1:200 of sodium hydroxide were the test reagents, the first concentration of alkali killed the organism in 10 minutes; the second killed in 15 minutes; the third killed in 20 minutes. From these limited data the only conclusion that can be drawn is that the rate at which the hydroxyl ion kills bacteria is apparently in direct proportion to its concentration; that is, the concentration exponent is 1. This does not parallel the action of the hydroxyl ion upon the virus of vaccinia, where, as has been shown, the concentration exponent is 2.6.

Conclusions

1. Neither sodium chloride, in a concentration of 0.85 per cent, nor distilled water is virucidal over a period of 17 hours at 25° C., and neither has a tendency to reduce the titer of the virus during this interval.

2. Neither the sodium ion nor the chloride ion by itself exerts a virucidal action upon vaccinia virus when in 0.15 molar solutions at 25° C.

3. Either the hydroxyl ion, or the hydrogen ion, when present in a sufficient concentration to produce a 0.1 molar solution, is actively virucidal.

4. As indicated by the time needed for complete inactivation, the virucidal action of hydrochloric acid is directly related to the concentration of the hydrogen ion.

5. Each time the concentration of the hydrogen ion is halved, the time needed for complete inactivation is doubled.

6. As indicated by the time of complete inactivation, the virucidal action of sodium hydroxide is related, but not directly, to the concentration of the hydroxyl ion.

7. Each time the concentration of the hydroxyl ion is halved, the time needed for complete inactivation is increased six times, the exponent of concentration in a concentration-time equation being 2.6.

8. This study, when compared with the reports dealing with the action of the H ion on bacteria, reveals that no great difference exists between this and its action on vaccinia virus.

9. When the time for the complete inactivation of vaccinia virus by the hydroxyl ion is compared with the time of bacterial sterilization by that ion a distinct difference is noted. In the first case the concentration exponent is 2.6, while with bacteria it appears to be 1.

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