The Slow Decline in Quality of Vaccinia Virus at Low Temperatures (37°C to -62°C).* (29044)

D. G. SHARP, P. SADHUKHAN† AND G. J. GALASSO
Biophysics Laboratory, Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill

Studies of the kinetics of low temperature degradation of several viruses have indicated reactions of a kind quite different from those that prevail in the region of 50°C and higher. One excellent example of this is found in the work on Foot and Mouth Disease Virus (1) and others are presented in review by Woese(2). Kaplan(3) has indicated a complex behavior for vaccinia virus during heating in the temperature range 50° to 60°C. The present work, while providing some information on the storage life of the virus, shows also that the reaction in the range 37° down to —20°C and perhaps further, has an energy of activation much lower than that for degradation at 50-60°C.

None of the heating and freezing procedures employed here produced any change in the number of virus particles countable in the electron microscope. We will, therefore, express the state of the virus as its quality Q, the ratio of the number of plaque forming units, PFU, to the number of virus particles. While Q will have the same purpose as the usual infectivity term in such studies, it has added advantages which will be apparent through its use here. These advantages have been previously demonstrated(4,5).

Materials and methods. The WR (mouse neurotropic) strain of vaccinia virus was obtained from the American Type Culture collection and adapted to growth in L cells. Eight successive passages were made from single plaques. Progeny from the last of these were used in this work. Media composition and procedures for plaque titration on monolayer cultures of L cells have been published (4). Virus particle counting was done by the agar sedimentation procedure(7). The starting virus for most of these experiments produced about 38 plaques per thousand particles. All samples were treated for one minute with 9 kc sonic waves just prior to titration to avoid titer reduction through aggregation of the virus particles(6).

Sonic lysates of infected L cells containing an average of 5,000 to 10,000 particles per cell were made in and diluted further with complete growth medium(6) which contained 15% horse serum. Several screw capped test tubes, each containing one ml (about 10^9 virus particles) were set up for each temperature test. Samples from quick experiments, those in the 56°C water bath, were titrated all together but those from the long term ones required titration at various times when they were ready.

Experiments and results. Vaccinia virus preparations were kept in a Revco mechanical refrigerator at —62°C for 171 days. They were titrated weekly for 4 weeks, then at longer intervals. The 10 titrations of this series showed no detectable trend of change in virus quality.

One preparation was subjected to 3 successive cycles of freezing at —20°C and thawing in a water bath at room temperature. Titration data and particle counts show (Table I) that this virus was not measurably altered by the treatment. Other samples were kept at 6°, 26° and 37°C. These were observed for somewhat shorter periods, consistent with adequate determination of the rate of decline in quality (Fig. 1). The ordinates of these graphs are actually Log_{10} 1/Q and the significant property of each is the slope which is characteristic of the virus at one temperature. The one anomalous point is probably a mistake; we have not included it in calculations. The slope of a line drawn by the method of least squares through each set of data was used to calculate the time

* This work was supported by grant from Division of Research Grants, USPHS.

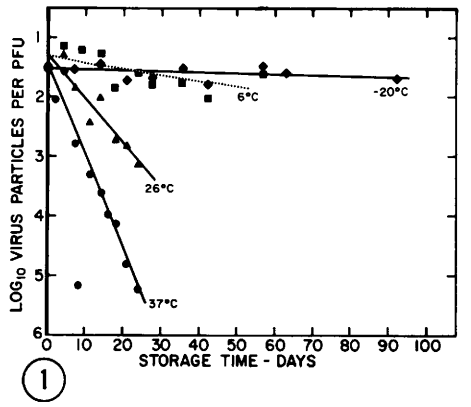
† On leave of absence from Biophysics Division, Saha Inst. of Nuclear Physics, Calcutta, India.

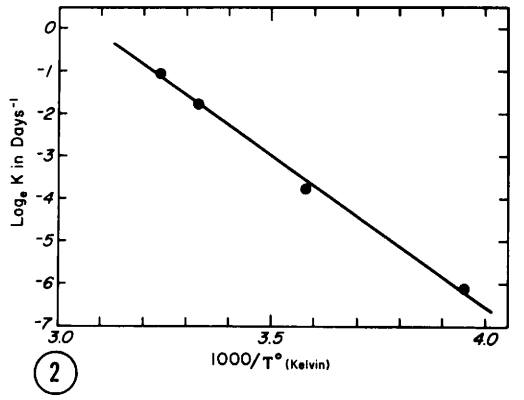
constant for each reaction. This constant, K, in Days^{-1} = 2.3 Δ Log$_{10}$ of the virus particles per PFU per day. These constants have, in turn, been plotted according to the empirical equation of Arrhenius (Fig. 2), in which they display a gratifying degree of consistency and reveal an activation energy of 14 K cal/mole for the reaction. Table II shows the corresponding times required for each of the reactions to reduce the quality of the virus by a factor of 1/e. Inasmuch as no detectable loss in titer was observed at −62°C, in the previous experiments, an extrapolation was made from the graph of Fig. 2 and the calculated time for this temperature is included in Table II. It is 372 years, a value considerably in excess of the life expectancy of the refrigerator.

Inactivation of the virus proceeded so rapidly at 56°C that it was not practical to include it on the time scale of Fig. 1. Two samples were tested for comparison with those observed at lower temperatures. The first was the same virus that was used in the other experiments, while the second was early passage material prepared in the course of adapting the virus to growth in L cells. Its starting quality was about 1/1000. Both can be seen to decline in quality at equal rates as shown by the least squares lines of Fig. 3. The time constant, K min^{-1} = 0.302, is not included in the Arrhenius plot (Fig. 2) because it would be far above the line formed by all the observations at the lower temperatures.

When the many electron micrographs which have been made for the counting of heated virus particles in this and other work (8) are mixed with others made from fresh virus it is difficult to separate them by inspection. Fresh virus is shown in Fig. 4A, while that of Fig. 4B and C has been heated at 56° for 30 minutes. There is some raggedness in the particles shown in picture B, but this is not always apparent, as in picture C. One thing, however, is certain. The particle count is the same for fresh virus as that for virus heated at 56° for 30 minutes.

Discussion. These experiments provide further encouragement to those wishing to maintain vaccinia virus without measurable change in quality for long periods. They indicate that it should remain fully active at





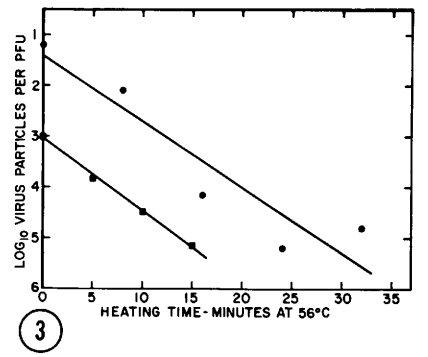


FIG. 1. Ratios of decline in quality of vaccinia virus heated in complete growth medium at several temperatures.

FIG. 2. Dependence of rate constants on temperature, plotted according to Arrhenius. The indicated activation energy is 14 K cal/mole over the range 37° to −20°C.

FIG. 3. Rate of decline in quality of vaccinia virus heated at 56°C. Circles, same virus as that used, Fig. 1; squares, early passage, incompletely adapted virus of low plaque forming quality. Values of K in min^{-1} are 0.302 and 0.298.

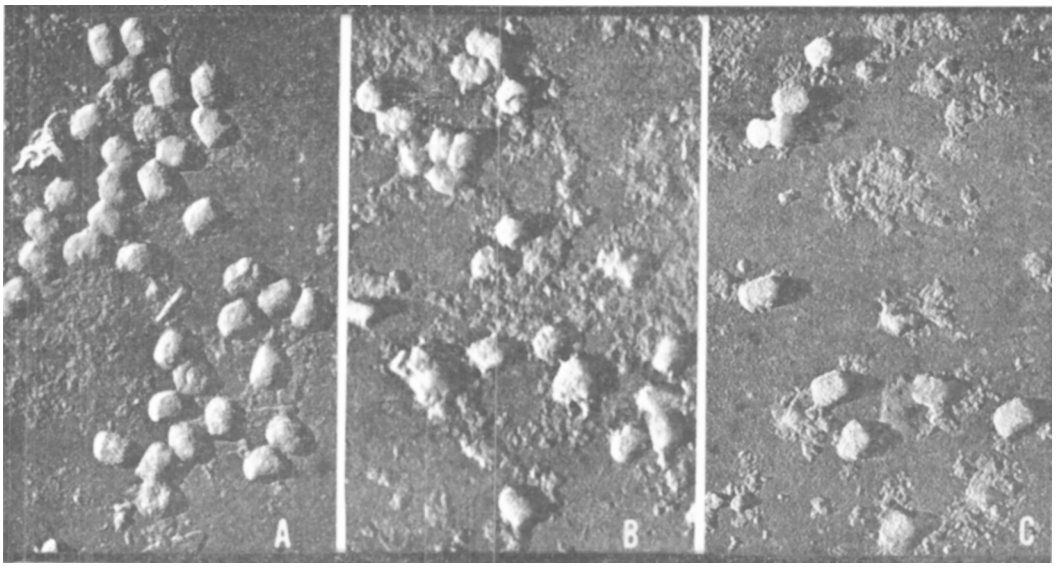


FIG. 4. It is difficult to distinguish fresh virus (a) from heated (56°C–30 min) virus in electron micrographs. Pictures (b) and (c) were made from different heated preparations. Raggedness seen in (b) is not always seen, (c). Neither positive nor negative staining has proved helpful.

TABLE I. Effect of Repeated Freezing (–20°C) and Thawing on Vaccinia Virus.

	Time	Virus particles per dish	Plaques per dish	Quality (plaques per 1000 particles)
Control	0	4000	94	23.3
Freeze-thaw once	1 hr	"	86	21.7
Freeze-thaw twice	2 hr	"	106	29.4
Freeze-thaw thrice	3 hr	"	88	21.7

Initial particle count 4.4×10^9 per ml. Final particle count 4.2×10^9 per ml.

—62° or —70°C for many years and that it can successfully withstand several cycles of alternate freezing and thawing at —20°C.

Probably the most pertinent comparative work with vaccinia is found in the papers of Kaplan(3) and Woodroofe(9). The rate constant for Kaplan's "fast" reaction at 55°C was given as 0.87 min^{-1} and 0.22 min^{-1} at 50°C. We have calculated the corresponding values from the slopes of the lines on Woodroofe's graphs of virus treated at the same temperatures. They are 0.38 and 0.22 respectively. Our own values were taken at 56°C but they resemble more closely those of Woodroofe, being 0.30 and 0.31 in the present work and 0.34 in an earlier determination(8). It is not surprising that our constants are somewhat lower than the others because our virus was kept in buffered growth medium containing serum while theirs was in buffer solution only. It is notable that none of these reaction constants fits even approximately on the Arrhenius plot of the low temperature experiments (Fig. 2). Apparently the kind of reaction in the 2 tem-

TABLE II. Time for Virus Quality to Fall by the Factor 1/e.

Temp (°C)	Time
56	3.4, 3.3 min.
37	2.8 days
26	5.9 "
6	44 "
–20	440 "
–62	372* years

* This value was obtained from extrapolation of the line of Fig. 2.

perature ranges is quite different. Besides providing a means of extrapolating the expected life of virus at —62° storage, Fig. 1 also indicates an activation energy of 14 K cal/mole for inactivation of the virus. This is lower than all of those reviewed by Woese (2) for animal viruses, being comparable only with the values 18 K cal/mole listed for the low temperature inactivation of measles virus.

Conclusions. Vaccinia virus is exceedingly stable, with an indicated half-life of many years at —70°C. It may lose virtually all infectivity (PFU) in the temperature range 56° to —20°C without loss in countable particles. In the range 37°C to —20°C the activation energy for the process is 14 K cal/mole, a value which is lower than that of most viruses and much lower than that for the reaction at 56°C. Apparently the process of degradation of the virus is quite different in the 2 temperature regions.

1. Bachrach, H., Breese, S., Callis, J., Hess, W., Patty, R., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 147.

2. Woese, Carl, *Ann. N. Y. Acad. Sci.*, 1960, v83, 741.

3. Kaplan, C., *J. Gen. Microbiol.*, 1958, v18, 58.

4. Galasso, G. J., Sharp, D. G., PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 43.

5. Sharp, D. G., *Ergeb. der Mikrobiol.*, 1963, v36, 214.

6. Galasso, G. J., Sharp, D. G., *J. Immunol.*, 1962, v88, 339.

7. Sharp, D. G., *Proc. 4th Int. Cong. for Electron Microscopy*, Springer-Verlag, Heidelberg, 1960, 542.

8. Galasso, G. J., Sharp, D. G., *Virology*, 1963, v20, 1.

9. Woodroofe, G. M., *ibid.*, 1960, v10, 379.

Received November 20, 1963. P.S.E.B.M., 1964, v115.

Relationship Between the Envelope and the Infectivity of Herpes Simplex Virus.* (29045)

KENDALL O. SMITH (Introduced by Joseph L. Melnick)
Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas

Pirie(1) raised the question of whether the herpes virus particle requires an envelope in order to be infective. Wildy *et al*(2), using the negative staining technique, clearly differentiated between 2 morphological types of herpes virus: the naked particles (possessing cubic symmetry) and enveloped particles. Recently Watson and Wildy(3) suggested that the envelope is *not* essential for infectivity, although data to support this view were not presented. Holmes and Watson(4) found that enveloped particles are more readily absorbed to cells than are naked particles. We observed the same phenomenon, which suggested to us that enveloped particles might be associated with infectivity. Brief ether treatment removes enveloped particles from aqueous suspension, drastically reduces the infectious titer, but does not noticeably alter the fine structure of naked particles(5). Although suggestive, none of the above experiments clearly answers whether or not naked particles are infectious.

Materials and methods. A physical separation of naked from enveloped particles was attempted, using the method described by Roizman and Roane(6) for fractionating particles on the basis of differences in their bouyant densities in cesium chloride density gradients. Freshly grown virus was sonically treated briefly(5), centrifuged at 2,000 × *g* for 5 minutes to remove large viral aggregates, and the supernatant suspensions layered over 4.0 ml cesium chloride having a density of 1.34. Tubes were centrifuged for 24 hours at 40,000 rpm and fractionated by the bottom drip method. Plaque titrations,

* This investigation was supported in part by grant from Nat. Inst. of Allergy and Infect. Dis., Nat. Inst. Health, U.S.P.H.S.