J. Hyg., Camb. (1963), **61**, 205
Printed in Great Britain

Studies in tissue culture on the pH-stability of rinderpest virus

By B. LIESS* and W. PLOWRIGHT
East African Veterinary Research Organization, Muguga, P.O. Box 32, Kikuyu, Kenya

(*Received* 23 *January* 1963)

INTRODUCTION

Although information on the pH-stability of rinderpest virus has considerable practical importance and a comparative interest for those working with the measles–rinderpest–distemper group of viruses, we know of no adequate published data on this subject. An early report by Maurer (1946) is concerned with the stability of rinderpest virus, which had previously undergone several alternating calf-rabbit passages. The source of virus was spleen tissue from an infected calf and 1% (w/v) suspensions in phosphate buffers of pH 6·0, 7·0 and 8·0 were maintained at 36° F. Infectivity was demonstrated by inoculating rabbits and recording the characteristic pyrexia which followed successful infection.

The introduction of tissue culture techniques for the propagation and titration of rinderpest virus *in vitro* has provided a cheaper, more accurate system for investigations of this problem. The results of our experiments with a tissue culture system are presented in the following report.

MATERIALS AND METHODS

Virus strains

In an initial experiment the 95th bovine kidney passage of the Kabete 'O' strain of rinderpest virus (RBOK) was employed. Culture fluids were harvested 7 days after seeding an infected cell suspension into 20 oz. medical flat bottles (Plowright & Ferris, 1959). They were clarified by light centrifugation (2000 r.p.m. for 5 min.) and stored at −70° C. The maintenance medium was that designated LA:YE by Franklin, Rubin & Davis (1957), with the addition of 5% normal, unheated ox serum.

In subsequent experiments two virulent field strains of virus were used, in addition to the RBOK strain which is highly attenuated. The origin of the RBT/1 isolate has already been described (Plowright & Ferris, 1962); the material employed consisted of a 9-day fluid harvest from its 3rd passage in bovine kidney cells. The third virus strain, RGK/1, was isolated in 1962 from lymph node tissue of a sick giraffe which was shot in the Northern Frontier District of Kenya. It produces an 80% mortality in experimental cattle and very high levels of virus can be demonstrated in the tissues of sick animals by direct inoculation of tissue cultures. The RGK/1 strain was used for pH studies as a 10-day fluid harvest from the 2nd culture passage.

* Aided by a Research Fellowship Grant from the Deutsche Forschungsgemeinschaft.

Buffer solutions

The isotonic veronal-acetate buffers of Michaelis (Hull, 1943) were used throughout. Solutions ranging from pH 2·6 to 9·0 were prepared according to the original formulae. For pH 10·0 and 10·7, which were not covered in the original publication, adjustment from pH 9·6 was effected with 0·1 N or 1 N sodium hydroxide. The final pH was checked in each instance by using a meter with a glass electrode and thermal control element.

With the exception of the first experiment highly alkaline or very acid virus-buffer mixtures were adjusted to pH 7·6, at the end of the period of exposure; for this purpose an equal volume of a suitable complementary buffer was added.

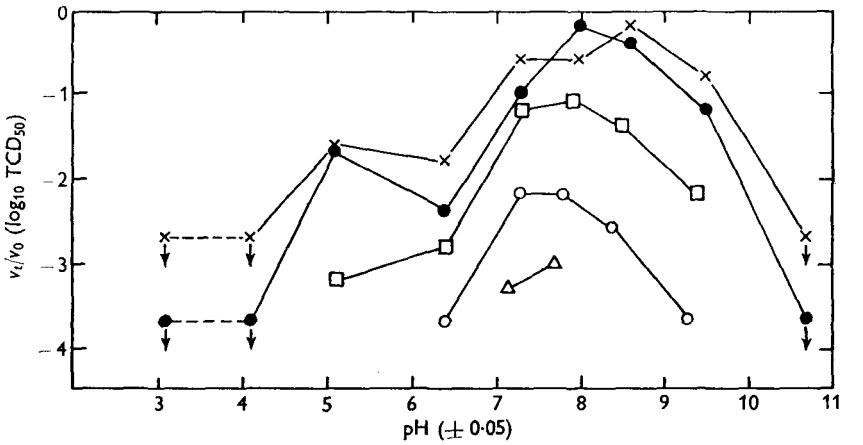

Fig. 1. Inactivation of rinderpest virus (strain RBOK) in culture fluids diluted tenfold in Michaelis's buffers at 4° C. Exposure: ×, 1 week; ●, 2 weeks; □, 3 weeks; ○, 6 weeks; △, 7 weeks.

Virus exposure and infectivity titrations

In the first experiment, designed to determine the rate of virus inactivation over a long period, culture fluid infected with the strain RBOK was diluted 1:10 in buffers to give a final pH of 3·1 to 10·7 (Fig. 1). Virus-buffer mixtures were kept in tightly stoppered, screw-capped bottles and the pH was checked on the occasion of each sampling. Minor changes of pH were noted and not readjusted but the fall in pH levels higher than 7·0 was taken into account in preparing Fig. 1. Below pH 7·0 no change of more than 0·05 units could be detected over the test period of 7 weeks.

Judging from the results of the initial experiment a rapid fall of infectivity was not to be expected except between pH 3·0 and 5·0 and between pH 9·5 and 11·0. Subsequent experiments within these ranges were therefore designed with short exposure times, ranging from seconds to not more than 24 hours. The more refined technique of Bachrach *et al.* (1957) was adopted to ensure accuracy of exposure. Virus preparations were added (1:10, v/v) to buffers of the required pH, using a magnetic stirrer to ensure instantaneous and uniform distribution of the virus. At suitable intervals aliquots of the virus-buffer mixtures were withdrawn and

added to an equal volume of the required neutralizing buffer. All buffers and virus-buffer mixtures were precooled and kept at 4° C. throughout the duration of all except the shortest experiments.

Virus assay was carried out by inoculating serial tenfold dilutions in culture growth medium into tubes containing trypsin-dispersed calf kidney cells (Plowright & Ferris, 1959) or into established cultures of the same cell type (Plowright & Ferris, 1962). Five tubes were inoculated per dilution and titres were expressed as $\log_{10}$ TCD 50 per ml. calculated by the method of Thompson (1947).

RESULTS

The first experiment was designed to show the effect of a wide range of pH values (3·1–10·7), on the inactivation of rinderpest virus over a period of 7 weeks at 4° C. There was no necessity to neutralize very acid or alkaline suspensions prior to inoculating them undiluted into culture tubes. The buffering capacity of 1 ml. of growth medium was sufficient to prevent any detectable effects of the virus inoculum on the growth potential of the cells.

The results of this experiment are expressed graphically in Fig. 1, which shows the surviving virus fractions, relative to the titre of the original culture fluid. The virus was quite stable from pH 5·0 to 9·6 but it is clear that relatively rapid inactivation of the infectivity occurred outside this range. Further experiments were therefore carried out to determine the rate of inactivation in the two critical zones and the results are given in Table 1.

Table 1. *Half-life of attenuated and virulent strains of rinderpest virus diluted tenfold in Michaelis's Buffers at* 4° C.

	Virus strain		
pH (±0·1)	RBOK	RGK/1	RBT/1
3	24·0 sec.	12·5 sec.	—
4	2·3 hr.	—	2·2 min.
5	25·0 hr.	4·1 hr.	42·3 min.
7·2	3·68 days*	—	—
7·8–7·9	3·72 days*	—	—
9	50·1 hr.	8·7 hr.	16·7 hr.
10·2	2·6 hr.	—	5·5 hr.
10·7	5·0 min.	8·3 min.	—

* Results from initial experiment only.

Figure 2 presents data for two strains at pH 3·0. There were clear indications that the rate of loss of infectivity was higher for the recently isolated, highly virulent strain (RGK/1) than for the RBOK strain at high passage level. The difference between the regression lines as judged by the covariance test (Snedecor, 1959) was significant and the half-life periods were 12·5 and 24 sec., respectively (Table 1). It is also of interest to note that the inactivation rate at pH 4·0 and 5·0 for the other recent isolate, RBT/1, was considerably higher than that for the RBOK strain.

Figure 3 shows that the strains RBOK and RGK/1 were considerably less sensitive to exposure at pH 10·7 than at pH 3. It was barely possible to demonstrate a decline of titre over a period of 5 min. for the strain RGK/1.

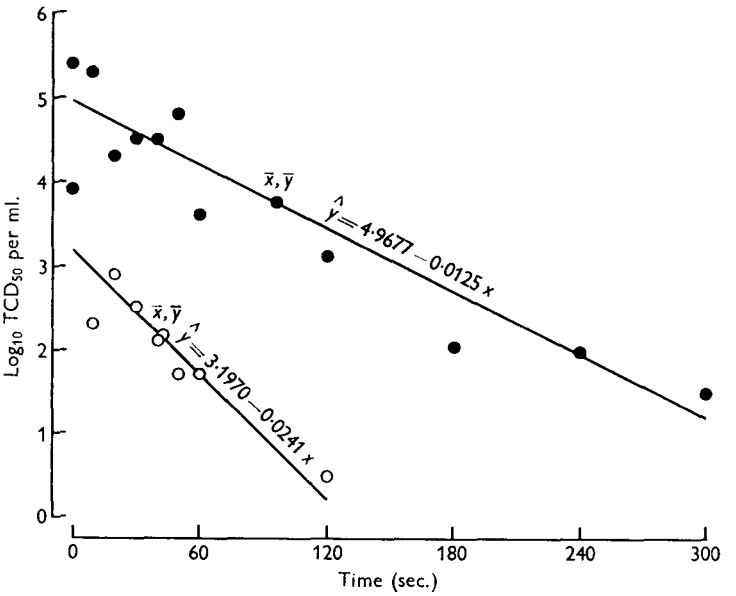

Fig. 2. Inactivation of virulent and attenuated strains of rinderpest virus at pH 3 and at 4° C. ●, Attenuated strain, Kabete 'O', 95 passages in bovine kidney cells; ○, virulent strain, RGK/1, 2 passages in bovine kidney cells.

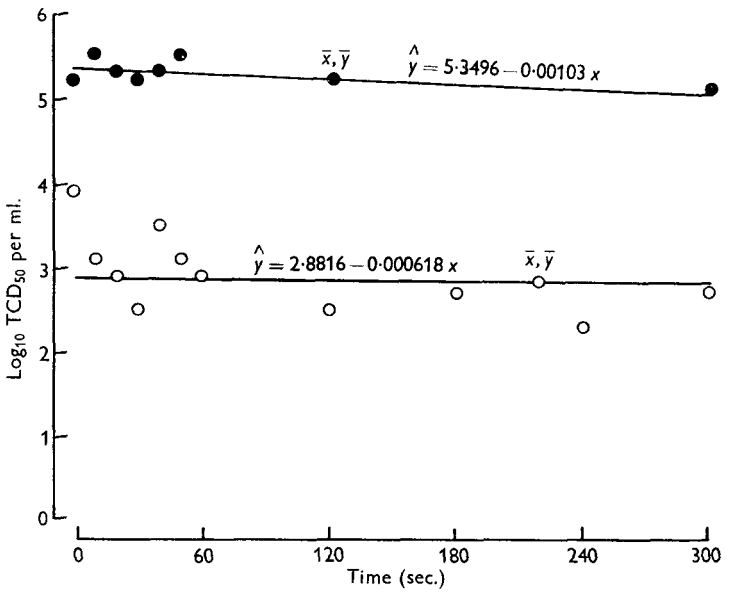

Fig. 3. Inactivation of virulent and attenuated strains of rinderpest virus at pH 10·7 and at 4° C. ●, Attenuated strain, Kabete 'O', 95 passages in bovine kidney cells; ○, virulent strain, RGK/1, 2 passages in bovine kidney cells.

https://doi.org/10.1017/S002217240002091X Published online by Cambridge University Press

DISCUSSION

There appear to be no generally accepted criteria in assessing the pH stability of viruses and much of the published information has been obtained in experiments which failed to determine the initial concentration of virus or the rate of inactivation, as opposed to the mere presence or absence of infectivity. Other variables which need to be taken into consideration are the composition of the suspending medium, the origin and passage history (Gorham, 1960) of the virus isolate, and the temperature of exposure. Variations in the procedures which have been employed are matched in numbers by the methods used to express the results. In this respect there can be little doubt that the most useful abstraction is the half-life period derived from the regression coefficient, calculated from an adequate series of observations over a significant period of time for each pH value.

The utility of the half-life period will be limited, of course, if a virus population is heterogeneous with respect to its pH stability, as shown for example by Bachrach *et al.* (1957) for foot-and-mouth disease virus at pH 5 and 6. We did not obtain any evidence that rinderpest virus exhibits any such heterogeneity.

The effect of the suspending medium, at least so far as long term experiments are concerned, was shown by a comparison of results in this study with those obtained by Plowright & Ferris (1961). These authors found that the mean half-life at 4° C. of virus in the form of undiluted culture fluids was 9·2 days in four experiments with virus of the strain RBOK in its 39th to 97th culture passages; the pH of these fluids varied from approximately 7·3 to 7·6 and they contained 5 % ox serum. Similar fluids diluted tenfold in Michaelis buffer, i.e. with 0·5 % serum and a tenfold reduction in other medium constituents, had a half-life in the present experiments of 3·72 days at pH 7·7–8·0 and 3·68 days at pH 7·2–7·3.

The differences between the pH stability of the three strains of rinderpest virus employed here illustrate the dangers of generalizations from experience limited to a single isolate. The strain RBOK, for example, was relatively stable at pH 5·0 and 9·0 and a significant fall of titre would not be expected over a 24-hr. exposure period (half-life periods of 25·0 and 50·1 hr., respectively). The isolate RBT/1, on the other hand, was unstable at pH 5·0, with a half-life of 42·3 min., but more stable than the strain RBOK at pH 10·1 (half-life 5·5 hr., compared with 2·6 hr.). As already noted, RGK/1 virus had a somewhat lower stability than RBOK virus at pH 3·0 but was at least equally stable in the higher pH range up to 10·7. These differences may be accounted for by selection pressure or mutation within the RBOK virus population during prolonged culture passage; as described in the section on Materials and Methods the other two strains were in their second and third culture generations only, following isolation from field outbreaks of the disease.

Maurer (1946) concluded that the optimal pH for survival of his strain of rinderpest virus lay between 6·5 and 7·0, using M/10 phosphate buffers. The results recorded here clearly indicate that the optimal pH for the RBOK strain of virus was between 7·5 and 8·0. A detailed comparison of the two studies is not possible, however, since Maurer neither stated the initial titre of his preparation nor the number of rabbits used for each aliquot.

Of the other members of the measles–rinderpest–distemper group (Warren, 1960), the pH-stability of measles virus was investigated by Black (1959) and Black, Reissig & Melnick (1959) who reported complete inactivation within 3 hr. at 0° C. and pH 4·4 or below; there was little loss of infectivity between pH 6·0 and 10·5 but no point between pH 6·0 and 4·4 was investigated. The initial titre in this experiment was about $10^{3·5}$ p.f.u./ml. Essentially similar results were reported by Musser & Underwood (1960), but they found complete inactivation of a comparable virus preparation within 3 hr. at pH 10·0 and at 25° C. The optimal pH for virus survival was 7·0–8·0. The virulent rinderpest strains, RGK/1 and RBT/1, were shown to have a wider range of relative pH-stability than measles virus since they would not have shown a significant depression of titre over a period of 3 hr. at 4° C., if exposed to buffers of pH 5·0–10·2 (see Table 1). Similarly samples with an initial titre of $10^{3·5}$ would not have been completely inactivated by a 3-hr. treatment at pH 4·0 and 10·7, since the half-life under these conditions varied between 1·5 and 4·6 hr. The attenuated strain (RBOK) showed an even higher resistance at pH 4, with a half-life of 7·3 hr.

Bindrich (1951) investigated the pH-stability of a virulent strain of canine distemper virus in the form of infected serum and cellular washings from the blood of dogs. He diluted the virus in phosphate buffers of pH 3·0–12·0 and exposed the mixtures for 20 or 30 min. in the refrigerator. Infectivity was detected by inoculation of dogs and he concluded that the virus was relatively stable in acid media down to pH 3·6, but partially or completely inactivated at pH levels of 9·0 or higher. Celiker & Gillespie (1954) showed that the Onderstepoort strain of avianised distemper virus, as a suspension of infected chorio-allantoic membranes, survived 24 hr. treatment at 4° C. in the pH range of 4·4–10·4; it was inactivated within 2 hr. at pH 4·2 or 10·9.

In conclusion, it appears that all members of the measles–rinderpest–distemper group of viruses show a relatively high stability at pH 5–10. This, for example, may be compared with the different stabilities of small RNA viruses—high in the case of polioviruses (Bachrach & Schwerdt, 1952) and low in the case of foot-and-mouth disease (Bachrach *et al.* 1957).

SUMMARY

The pH-stability of three strains of rinderpest virus, propagated in tissue cultures, was investigated at 4° C. in veronal-acetate buffers (Michaelis) of pH 3·0–10·7.

An attenuated laboratory strain, in its 95th culture passage (RBOK), showed maximal stability in the pH range 7·2–8·0, the half-life being about 3·7 days. It was relatively stable from pH 4·0 to 10·2, the half-life at the extremes of this range being over 2 hr. At pH 3·0 the infectivity declined very rapidly, the half-life period being 24·0 sec.

One virulent strain (RGK/1) showed a significantly lower resistance than strain RBOK at pH 3·0, probably also at pH 5·0. The inactivation rates for these two strains were, however, not greatly different at pH 10·7. The other virulent strain (RBT/1) was even less stable than RGK/1 at pH 4·0 or 5·0 but of a comparable stability at pH 9·0 and 10·2.

These findings are discussed in comparison with published data on the related viruses of measles and canine distemper. The importance of strain differences in studies of this kind is stressed.

We are grateful to Mr C. S. Rampton, A.I.M.L.T., and Mr L. W. Rowe, F.I.M.L.T., for help in the preparation of the diagrams. This paper is published with the permission of Mr H. R. Binns, C.M.G., O.B.E., Director, E.A.V.R.O.

REFERENCES

Bachrach, H. L. & Schwerdt, C. E. (1952). Purification studies on Lansing poliomyelitis virus: pH stability, CNS extraction and butanol purification experiments. *J. Immunol.* **69**, 551–61.

Bachrach, H. L., Breese, S. S. Jr., Callis, J. J., Hess, W. R. & Patty, R. E. (1957). Inactivation of foot-and-mouth disease virus by pH and temperature changes and by formaldehyde. *Proc. Soc. exp. Biol., N.Y.*, **95**, 147–52.

Bindrich, H. (1951). Untersuchungen über die pH-Resistenz des Virus der Hundestaupe. *Arch. exp. Vet. Med.* **4**, 120–6.

Black, F. L. (1959). Growth and stability of measles virus. *Virology*, **7**, 184–92.

Black, F. L., Reissig, M. & Melnick, M. L. (1959). Measles virus. In *Advances in Virus Research*, **6**, 205–27. New York: Academic Press Inc.

Celiker, A. & Gillespie, J. H. (1954). The effect of temperature, pH, and certain chemicals on egg-cultivated distemper virus. *Cornell Vet.* **19**, 276–80.

Franklin, R. M., Rubin, H. & Davis, C. A. (1957). The production, purification, and properties of Newcastle Disease Virus labelled with radiophosphorus. *Virology*, **3**, 96–114.

Gorham, J. R. (1960). Canine Distemper (La Maladie de Carré). In *Advances in Veterinary Science*, **6**, 287–351. New York: Academic Press Inc.

Hull, H. B. (1943). *Physical Biochemistry*, pp. 122–3. New York: John Wiley and Sons Inc.

Maurer, F. D. (1946). Rinderpest. XI. The survival of rinderpest virus in various mediums. *Amer. J. vet. Res.* **7**, 193–5.

Musser, S. J. & Underwood, G. E. (1960). Studies on measles virus. II. Physical properties and inactivation studies on measles virus. *J. Immunol.* **85**, 292–7.

Plowright, W. & Ferris, R. D. (1959). Studies with rinderpest virus in tissue culture. I. Growth and cytopathogenicity. *J. comp. Path.* **69**, 152.

Plowright, W. & Ferris, R. D. (1961). Studies with rinderpest virus in tissue culture. III. The stability of cultured virus and its use in virus neutralization tests. *Arch. ges. Virusforsch.*, **11**, 516–33.

Plowright, W. & Ferris, R. D. (1962). Studies with rinderpest virus in tissue culture. A technique for the detection and titration of virulent virus in cattle. *Res. vet. Sci.* **3**, 94–103.

Snedecor, G. W. (1959). *Statistical Methods*, 5th edn. Ames, Iowa: The Iowa State College Press.

Thompson, W. R. (1947). Use of moving averages and interpolation to estimate median-effective dose. I. Fundamental formulas, estimation of error and relation to other methods. *Bact. Rev.* **11**, 115–45.

Warren, J. (1960). The relationships of the viruses of measles, canine distemper and rinderpest. In *Advances in Virus Research*, **7**, 27–60. New York: Academic Press Inc.