Studies with Rinderpest Virus in Tissue Culture

III. The Stability of Cultured Virus and its Use in Virus Neutralization Tests

By

W. Plowright and **R. D. Ferris**

East African Veterinary Research Organization, Muguga, P. O. Box 32, Kikuyu, Kenya

With 2 Figures

(Received May 24, 1961)

In 1959 we described a method for the titration of culture-adapted rinderpest virus in monolayers of primary calf kidney cells (1). A similar system was also used for the detection and titration of virus-neutralizing (v/n) antibody (1—3). The present communication describes in detail various modifications of the original technique which have been found advantageous under some conditions. We also record data on the stability of cultured rinderpest virus at temperatures which may be employed in v/n tests, or in the production and storage of virus stocks. Results are given of experiments designed to investigate the relationship between the test-dose of virus and neutralizing titre of a serum, also the accuracy of replicate titrations of virus stocks and standard immune sera, carried out in different batches of calf kidney cells.

Finally, figures are presented which prove the great reliability of v/n antibody tests, performed in tissue cultures, for the detection of immune animals amongst experimental cattle, purchased as susceptible.

Materials and Methods

Virus

Stock virus preparations consisted of culture fluids which were harvested 5 to 8 days after seeding infected cell suspensions into Roux flasks or medical flat bottles of 20 oz. capacity. The virus used was the Kabete "O" strain, adapted to cultural growth in 1956 (4) and passaged 26 to 106 times in calf kidney cells. The maintenance medium was the usual LA:YE base (5) with 5% unheated serum from adult donor cattle which were known to be rinder-

pest-susceptible (O. S.). Fluid harvests were lightly centrifuged (2,000 r. p. m. for 5 minutes) and stored at $-25°$ or $-70°$ C in aliquots of 5 to 10 ml. in screw-cap containers, or as 2 ml. aliquots in glass sealed ampoules. Unused remnants of aliquots which had been thawed once were not used again. Ten-fold dilutions of virus were prepared in the culture growth medium already described (1) without the addition of serum.

Sera

Blood was collected from the jugular vein of cattle into chemically clean, screw-cap containers. The serum was allowed to separate overnight at room temperature and, after centrifugation, stored at $-25°$ C. Cattle sera were not filtered or inactivated prior to use and, employing the usual combination of antibiotics (*vide infra*), difficulties with fungal, bacterial or yeast contaminants were encountered very infrequently. Where observations have been made on human, laboratory-animal and various wild-animal sera they were inactivated at 56° C for 30 minutes. Dilutions of sera in 2, 4, 5 or 10-fold steps were prepared in culture growth medium without serum.

Treatment of Virus/Serum Mixtures

Serum or serum-dilutions were mixed with an equal volume of virus which had been diluted to yield an estimated $10^{1.8}$ to $10^{2.8}$ TCD_{50} in the final inoculum for each 160×15 mm. tube. Mixtures were commonly prepared in 5 ml. bijou bottles, closed by tightly-fitting screw-caps with rubber liners; in this manner the pH did not rise considerably before inoculation, as it was found to do when attempts were made to employ small, cotton-plugged tubes. The bottles were shaken well and left overnight, i. e. about 18 hours, at 4° C. In the lapinized virus system described by *Scott* and *Brown* (6) the neutralization reaction over 18 hours at 4° C was not found to be significantly different from that which took place over 1 hour at 37° C. Overnight treatment at 4° C was more convenient for tissue culture tests, since the preparation of v/s mixtures could be completed the day before that on which calf kidney cells were made ready.

All tests were performed in 160×15 mm. tubes which each received 0.1 ml. or 0.2 ml. v/s mixtures; the greater quantity was later considered desirable since the error in delivery was probably smaller. It was found more convenient to pipette the mixtures into the bottom of empty tubes to which cell suspensions were added later by automatic pipette, rather than dispense the cells first and then add the virus. However, this necessitated some system whereby the cells were distributed without contact being made between the tubes and the automatic pipette; in practice it was not difficult to ensure this by using a long cannula to direct a stream of cell suspension into the mouth of each tube without actually touching it. A similar technique was found to work reliably for changes of medium.

In quantitative v/n tests a separate pipette was used to inoculate each v/s mixture into tubes.

Preparation of Calf-Kidney Cell Suspensions and Maintenance of Cultures

Two types of cell were used, i. e. primary cells from the kidneys of calves (1) and serially-cultivated cells of the same origin (7). The former were always prepared by overnight trypsination at 4° C and used at a dilution of 1/150 of the packed cell volume; the latter were removed from the glass by a mixture of

versene (0.02%) and trypsin (0.01%) in Ca:Mg free saline (8) deposited by light centrifugation and resuspended in growth medium to give a final count of 2.0 to 3.0×10^5 per ml. Each 6—8 day Roux flask culture under these conditions yielded enough suspension for 100—150 tubes.

The media employed for the establishment and maintenance of primary calf kidney cells have already been described (1). The tubes were incubated sloped in a stationary position for 4 days at $36.5 \pm 0.5°$ C. At this time a good growth of cells, virtually covering the glass surface, was present and the medium was changed prior to transfer of the tubes to drums rotating at about 8 r. p. h. Until March, 1960 readings were terminated on the 8th day after seeding, but it was then noted that somewhat higher virus titres and lower S. $N._{50}$ titres were recorded if further changes of media were made on days 7 and 9 or 10, whilst microscopic observations were continued until days 11 to 12. This procedure has now become the standard one employed in this laboratory.

Serially-cultivated cells were grown in the usual Hanks' growth-medium supplemented with 10% O. S. and 10% tryptose phosphate broth*. On the 2nd or 3rd day after seeding the medium was changed to LA:YE with the same supplements and the tubes were transferred to roller drums. Observations were terminated on the 7th or 8th days.

All media employed contained 100 I. Us. of sodium penicillin, 100 μg. streptomycin sulphate and 50 units nystatin** per ml.

General Observations

For quantitative v/n tests five tubes were employed for each v/s mixture and the titre (S. $N._{50}$) of the serum was expressed as the reciprocal of the $\log_{10}$ of the serum dilution which completely neutralized the virus in 50% of the tubes. The serum dilutions referred to final concentrations in v/s mixtures, which were twice that of the series prepared initially, since an equal volume of virus had been added. End-points were calculated by the method of *Thompson* (9). In screening tests to detect immune animals only two tubes were used for each sample and hence it was quite simple to deal with 200 to 300 sera in a single run.

Where two or more sera from a single animal were to be tested every effort was made to do them simultaneously.

All tests were accompanied by a titration of the stock virus preparation, five tubes being employed per 10-fold dilution. In addition, in the great majority of instances, a titration of the virus was carried out using 10-fold dilutions which had been mixed and left overnight at 4° C with equal volumes of the undiluted serum (O. S.) which was employed in the media for cell growth and maintenance. In this manner a constant check was made on any non-specific inhibition of the virus by the serum in the medium. Where quantitative tests were performed they were usually accompanied by the titration of a standard immune serum, which was also included, though not titrated, in each series of screening tests.

Control primary cultures were observed for a minimum of 14 days to detect any contaminating virus which might have been present in the calf kidneys. Only one agent has been recovered to date from about 250 batches of cells, the great majority of which were derived from single calves.

---

* Difco Labs., Inc.

** "Mycostatin" of E. R. Squibb & Sons Ltd.

Results

Stability of cultured Virus in Maintenance Medium (LA: YE + 5% O. S.)

a) *At 4° ± 1° C*

Five experiments were conducted to determine the rate of decline in infectivity titre for undiluted fluid harvests stored at 4° C. The vessels used were screw-capped, universal containers which were opened at intervals to remove aliquots for titration. There was thus no adequate control

Table 1. Loss of Infectivity of Cultured Rinderpest Virus in Maintenance Medium at 4° C

Time of Storage in Days	Experiment No. and Passage Level of Virus		Time of Storage in Weeks	Experiment No. and Passage Level of Virus		
	I BK/39a	II BK/39b		III BK/95	IV BK/96	V BK/97
0	4.5*	4.5	0	5.8	6.2	5.6
4	4.5	—	1	—	5.2	4.9
11	—	3.9	2	—	4.2	4.8
14	3.7	—	3	4.4	5.0	5.2
18	—	3.1	4	4.2	4.4	4.0
21	3.3	—	5	4.6	3.8	4.0
22	—	3.3	6	4.8	3.9	—
25	4.0	—	7	3.8	—	—
28	3.5	—	8	3.6	—	3.4
31	—	2.9	9	—	3.0	—
36	—	3.3	11	3.4	—	3.2
39	3.5	—	12	—	2.0	—
50	—	2.5	14	3.2	—	—
53	3.1	2.3	15	—	2.0	—
63	2.5	—	17	3.0	—	—
—	—	—	19	—	$<10^{\circ}$	—
—	—	—	21	2.4	—	—
Half Life	10.95** days	8.40** days	—	17.65** days	7.79** days	9.79** days

* Titre as Log TCD_{50}/ml.

** Regression coefficient significant at less than 1% level

of pH, which did in fact tend to rise on repeated opening of the vessels, but did not go outside the approximate range of 7.4 to 7.8. For the first two experiments titration results were calculated on the 8th day; for the last three experiments end-points were recorded at 11 to 12 days. The 39th passage (BK/39) virus had been frozen once at −25° C and then thawed; the other samples had never been frozen.

From the regression equations calculated for each experiment the estimated half-life is shown in the last line of Table 1. In four experiments figures of approximately 8 to 11 days were obtained, whereas in the case

of the 95th calf kidney passage (BK/95) there were indications of the presence of a very stable virus fraction which caused an obvious departure from linearity and a considerable extension of the calculated half-life. This virus sample was not apparently produced under different conditions than those which obtained for the others, neither was the rise of pH on storage noticeably dissimilar.

From these results it was obvious that no significant loss of titre would be expected as a result of leaving virus or v/s mixtures overnight at 4° C, in the absence of neutralizing antibody.

Table 2. Loss of Infectivity of Cultured Rinderpest Virus in Maintenance Medium at 36.5° C

Time of Exposure in hours	Passage Level and Titre* of Virus		
	BK/40	BK/43	BK/106
0	5.8	4.7	5.4
2	5.3	—	—
4	5.3	—	—
6	—	4.3	5.2
8	4.9	—	—
12	4.5	4.1	—
13	—	—	4.2
18	—	—	4.0
24	3.5	3.5	3.2
30	—	2.5	3.0
36	—	1.9	1.4
42	—	—	0.0
48	0.0	$<10^{\circ}$	0.0
Half Life	2.6** hours	3.2** hours	2.45** hours

* Log TCD_{50}/ml.
** Regression coefficient significant at less than 1% level

b) *At 36.5 ± 0.5° C*

Two experiments similar to those described above were performed with freshly-harvested virus held immersed in screw-cap containers in a water bath at 36.5° C. Again the pH was not adequately controlled.

In a third experiment virus of the 106th calf-kidney passage (BK/106) was dispensed in 2 ml. quantities into glass-sealed ampoules which were shell-frozen for storage at −70° C. They were thawed rapidly and exposed by complete immersion in a water bath at 36.5° C. The reaction in this instance was constant at about pH 7.5. Ampoules were removed at intervals and immersed immediately in iced water. All aliquots were stored at 4° C and titrated simultaneously in a single batch of calf kidney cells, final readings being made on the 12th day.

The results are also shown in Table 2. It was clear that there had been no change in the heat-stability of the virus between the 40th and 106th culture passages and that holding virus or v/s mixtures for 1 hour at 36.5° C would have no significant effect on the titre.

c) *At 56° C*

Two experiments were performed with the glass-sealed ampoules of virus mentioned in the previous section. Again, thawing was carried out rapidly at 37° C and the ampoules were completely immersed in a water

Table 3. Loss of Infectivity of Cultured Rinderpest Virus in Maintenance Medium at 56° C

Time of Exposure in Minutes	Titre* of Virus (BK/106)	
	Experiment I	Experiment II
0	5.4	5.2
5	5.6	—
10	4.2	—
15	4.0	3.2
20	3.8	2.4
25	3.0	—
30	2.4	0.8
40	—	2/5 tubes positive at 10^{-1}; 1/5 at 10^{0}
50	—	2/5 tubes positive at 10^{-1}; 0/5 at 10^{0}
60	0.6	0.0
70	—	0.0
Half Life, Mins.	3.57***	3.37**

* Log TCD_{50}/ml.
** The results of the 40 and 50 minute titrations were not included in this calculation. Regression coefficient significant at less than 5% level
*** Regression coefficient significant at less than 1% level

bath at 56° C. After removal at successive intervals each was cooled rapidly in iced water and held at 4° C until all were titrated simultaneously at the end of the experiment. Table 3 gives the figures obtained.

In the first experiment a small quantity of infective virus remained after 60 minutes but in the second all had disappeared at this time. The irregular titration results on the 40- and 50-minute samples in the latter may have been due to auto-interference by inactivated virus.

d) *The Effect of Freezing Newly-Harvested Virus at —25° C and —70° C*

Aliquots of 5 to 10 ml. of virus at three successive passage levels were distributed in screw-cap containers and placed in mechanically-refrigerated cabinets at the desired temperature. There were minor differences

in the rate of freezing, according to whether or not the bottles were placed in direct contact with the lining of the cabinet or other frozen material. These differences were not taken into account. Frozen samples were first removed for titration after periods of 1—3 weeks, it being assumed that all losses of infectivity occurred during the freezing process (*vide infra*).

Table 4. Loss of Infectivity of Cultured Rinderpest Virus on Freezing

Passage Level	Titre* before Freezing	Titre after Freezing at −25° C (a)	Titre after Freezing at −70° C (b)	Difference between (a) and (b)
BK/95	5.8	5.2	5.4	−0.2
BK/96	6.2	5.2	5.4	−0.2
BK/97	5.6	5.6	5.2	+0.4

* Log TCD_{50}/ml.

It can be seen that losses on freezing were variable between samples but that no significant difference was detected between freezing at the two temperatures.

Table 5. Storage of Low-Passage Virus Stocks at −25° C

Stock E (BK/28)		Stock F (BK/28)		Stock M (BK/48)	
Storage Time	Titre*	Storage Time	Titre	Storage Time	Titre
Nil (prefreezing)	5.1	Nil (prefreezing)	4.9	Nil (prefreezing)	>5.5
114 days	4.5	34 days	3.5	40 days	5.9
129 days	4.7	40 days	3.5	46 days	5.0
142 days	4.5	53 days	3.6	50 days	5.2
154 days	3.9	60 days	3.5	53 days	5.0
160 days	4.7	68 days	3.3	59 days	5.4
163 days	4.5	—	—	71 days	5.5
Mean after freezing	4.47 ±0.29	Mean after freezing	3.48 ±0.11	Mean after freezing	5.33 ±0.31

* Log TCD_{50}/ml.

e) *The stability of Culture Virus Stored Frozen at −70° C and at −25° C*

Two high passage-level stocks, designated Z and A, were titrated on 7 and 9 occasions respectively in different batches of primary calf kidney cells. The time of storage at −70° C varied from one to 16 or 17 weeks during which time there was no significant fall of infectivity. The means

and standard deviations for the titres of the two stocks were 5.23 ± 0.14 and 5.56 ± 0.35 respectively, final readings being taken on the 11th or 12th days after seeding of the cultures.

Table 5 shows the results obtained in freezing three earlier virus stocks at −25° C and of replicate titrations carried out at intervals in different batches of serially-cultivated or primary calf kidney cells, which were observed for 7 to 8 days only.

There were indications of a variable loss in freezing similar to those recorded in the previous section.

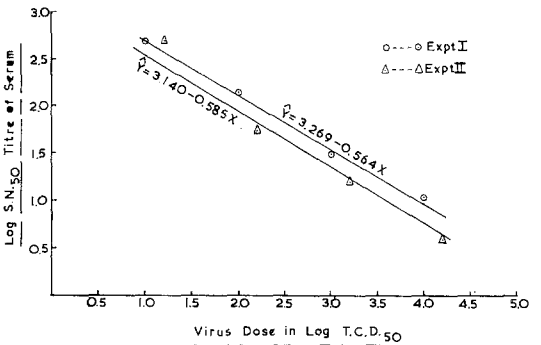

Fig. 1. Box titrations of neutralizing antibody in standard immune serum.

Box Titrations of a Standard Rinderpest-Immune Serum (Ox 2841)

Four experiments were performed with the object of establishing the relationship between the dose of virus employed and the SN_{50} titre of a standard immune serum, collected from an ox (No. 2841) convalescent from virulent rinderpest virus infection. This serum was employed as a reference in many later "screening" and quantitative neutralization tests. It was stored frozen in aliquots at −25° C and it was assumed that a reasonable number of freezing and thawing cycles did not affect neutralizing antibody. The reference serum was never heated.

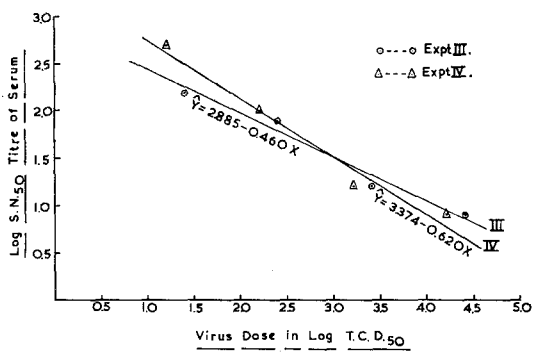

Fig. 2. Box titrations of neutralizing antibody in standard immune serum.

The results of the four experiments using two stock virus preparations (Z and A) and 5-fold dilutions of the serum are given in Table 6 and Figures 1 and 2.

The v/s mixtures, as usual, were left overnight at 4° C and the inoculum for each tube was 0.2 ml. The figures recorded for experiments 1a, 2a, etc., were calculated from 8-day readings, those for 1b, 2b, etc., from 12-day readings on the same tubes. Taken together, they show that the virus

Table 6. Box Titrations of a Standard Immune Serum (Ox 2841)

Experiment No.	Virus Stock	Titre* of Stock	Log SN_{50} Titre of Serum Against Virus Dilution				Regression Equation**	Significance of Regression Coefficient
			10^{0}	10^{-1}	10^{-2}	10^{-3}		
1a	Z	3.8	1.05	1.8	2.3	3.15	—	—
1b	Z	4.0	1.05	1.5	2.2	2.7	$y = 3.269 - 0.564$***	$P < 0.01$
2a	Z	3.6	1.05	1.5	2.3	3.15	—	—
2b	Z	4.2	0.6	1.2	1.75	2.7	$y = 3.40 - 0.585$***	$P < 0.05$; > 0.02
3a	A	4.2	1.05	1.75	2.2	2.9	—	—
3b	A	4.4	0.9	1.2	1.9	2.2	$y = 2.885 - 0.460$***	$P < 0.02$; > 0.01
4a	A	3.6	1.05	1.75	2.3	2.9	—	—
4b	A	4.2	0.9	1.2	2.0	2.7	$y = 3.374 - 0.620$***	$P < 0.05$; > 0.02

* Log TCD_{50}/0.1 ml. ** y = Log SN_{50} titre of serum *** Log TCD_{50} of virus per tube

titration results were always 0.2 to 0.6 log units higher at the 12th than at the 8th day and that serum titres nearly always dropped during the same time interval, sometimes by up to 0.7 units. From the slope of the regression lines shown in the penultimate column of Table 6 it can be seen that a 1-log increase in the test dose of virus may be expected to result in approximately a 3- to 4-fold decrease in the arithmetic titre of the serum (0.46 to 0.62; mean 0.56 log units). Estimates of the standard deviation of $y \cdot x$ in the four experiments were 0.17, 0.13, 0.14 and 0.03, hence in any one run of serum titrations differences of the order of 0.6 log units were probably significant.

Table 7. Replicate Titration of a Standard Immune Serum (Ox 3594) in Serially Cultivated Calf Kidney Cells

Cell Designation	Test Dose of Virus*	Log $S.N._{50}$ Titre of Serum
BK/146	2.0, 2.2, 2.2, 2.4	2.7, 2.5, 2.4, 2.7
BK/153	2.0, 2.0, 2.2	2.7, 2.6, 2.7
BK/154	1.8, 2.2	2.8, 2.7
BK/155	2.2, 2.6	2.4, 2.8
BK/156	2.0, 2.6	2.8, 2.8

* $\text{Log}_{10}\ TCD_{50}$ per tube

The Accuracy of Replicate Titrations of a Standard Immune Serum

As stated in the Section on "Materials and Methods", the majority of quantitative neutralization tests included the titration of a standard immune serum. It was of interest to determine the variation which occurred as a result of using different batches of primary calf kidney cells or different lines of serially-cultivated cells of similar origin (7). In addition there were the normal variations in the precise dose of virus employed, as calculated by simultaneous titration. The results for serially-cultivated cells, when the test virus dose fell between $10^{1.8}$ and $10^{2.6}$ TCD_{50} are shown in Table 7. In these instances all results were read finally on the 7th or 8th days after seeding.

There were obviously no significant differences between the results obtained with different lines of cultured cells, the mean and standard deviation of all 13 titres recorded being 2.66 ± 0.14 log units.

Using different batches of primary calf kidney cells, derived from individual calves, 8 day and 11—12 day readings for another standard immune serum (Ox 2841) are given in Table 8. The experiments involved 10-fold, not 5-fold serum dilutions as in the "box" titrations. They show again that virus titres may be fractionally higher and serum titres frequently lower at 11—12 days than at 8 days.

Taken together with the results for the 10^{-2} dilutions of stock virus preparations, recorded in Table 6, there were 12 titrations of this serum against $10^{2.0}$ to $10^{2.8}$ TCD_{50} of virus in 12 different lots of cells. The mean 11—12 day titre of the serum was 2.24 ± 0.28 log units.

The Effect of Pooled Normal Ox Serum on Cultured Rinderpest Virus

As noted in the Section on "Materials and Methods", many tests included a titration of virus where separate 10-fold dilutions had been exposed overnight to an equal volume of undiluted serum used for the growth and maintenance media. This served as a constant check on any non-specific inhibitory factors in the serum which might have influenced

Table 8. Replicate Titrations of a Standard Immune Serum (Ox 2841) in Primary Calf Kidney Cells

Culture Reference	Virus Stock	Virus Dose* at 8 days	Serum Titre at 8 days	Virus Dose* at 11/12 days	Serum Titre at 11/12 days
BK/232	A	2.2	2.6	2.2	2.4
BK/234	A	2.0	2.8	2.0	2.4
BK/231	C	2.3	2.8	2.7	2.6
BK/216	W	2.8	2.0	2.8	2.0
BK/220	Z	2.0	2.8	2.2	2.8
BK/222	Z	2.2	2.6	2.2	2.2
BK/223	Z	2.2	2.6	2.2	2.4
BK/225	Z	2.2	2.2	2.4	2.2

* Log TCD_{50} per tube, estimated by simultaneous reading of virus titration

the effective virus dose. In the majority (12/16) of recent experiments for which 11—12 day readings were available, the titre of virus, following exposure to undiluted normal serum, was reduced by 0.3 to 0.9 log units, whilst in the remaining instances (4/16) it was higher by 0.1 to 0.3 log units. Table 9 shows six such comparative titrations with one virus stock (Z) in six different batches of calf kidney cells. The results with other virus stocks and different batches of normal, unheated ox-serum were similar.

On the basis of these results it would be expected that normal, unheated ox-serum derived from rinderpest-susceptible cattle, might neutralize as much as 10 TCD_{50} of culture virus but would never be capable of completely neutralizing 100 TCD_{50}. The next section shows how this expectation has been shown to be justified over the last three and a half years or so.

"Screening" Tests on the Sera of Experimental Cattle

Since August, 1957, v/n tests have been performed on the sera of virtually all the many hundreds of cattle which are purchased annually by this institute for experimental use, particularly in the potency testing of living rinderpest vaccines. One of these vaccines, the "K. A. G." or goat-adapted strain, produces detectable temperature reactions in over 95% of the susceptible grade cattle which are successfully immunized with it (10). The other vaccine strain, the Nakamura III lapinized virus (11), does not normally produce any recognizable temperature or other clinical reaction in inoculated grade cattle (12), hence for these animals no effective check on the accuracy of the "screening" test was available. Each batch of animals used in potency and safety-testing of vaccines includes

Table 9. The Effect of Normal Pooled Ox Serum on the Titre of Rinderpest Virus (Stock Z)

Cell Batch	A Titre without Serum	B Titre with Serum	Difference A − B
BK/220	5.2	4.9	+0.3
BK/222	5.2	4.5	+0.7
BK/223	5.2	5.5	−0.3
BK/224	5.0	4.2	+0.8
BK/225	5.4	4.5	+0.9
BK/226	5.2	4.9	+0.3

The depression due to overnight exposure to normal serum just failed to be significant in this instance at the 5% level (P = 0.06)

an uninoculated control which is subsequently challenged with the virulent Kabete "O" strain, at the same time as the inoculated cattle. The clinical reaction to the latter is rapid, severe and easily recognized clinically.

At various times experiments have also been carried out with field strains of rinderpest virus which produce an easily recognizable syndrome and serological conversion, even though they do not produce a high mortality. Finally, several hundreds of cattle have been used in experiments with attenuated culture virus; these have always included neutralization tests on the sera of individual animals before and after inoculation. In all such experiments, therefore, the susceptibility of animals which passed the screening test could be readily confirmed.

In general it can be said that of all the cattle bled on arrival at the laboratory, the subsequent history in 70—75% gave unequivocal indications of their rinderpest susceptibility or otherwise. Whenever possible, cattle which were found to be serologically positive were challenged with

virulent virus immediately after completion of the test. The challenge dose probably contained at least 10^4 cattle ID_{50} of the Kabete "O" strain, in the form of a spleen suspension.

Table 10 shows the results of all screening tests performed from August, 1957 to March, 1961. The vast majority of v/s mixtures were only inoculated into two tubes and the complete protection of any tube was regarded as evidence of immunity. Cultures were retained for 11—12 days in the case of the last 923 sera, prior to this for 7—8 days only. Both primary and serially-cultivated calf kidney cells were employed. It is noteworthy in the case of three out of 26 serologically-positive animals which were challenged and found to be immune that their sera contained sufficient antibody to protect only one of the two tubes inoculated. Hence very low levels of neutralizing antibody indicated solid immunity in animals within the age range of 18 to 36 months.

Table 10. Detection of Rinderpest-Immune Amongst Presumed-Susceptible Cattle: Culture Screening Tests

Time of Reading days	Number of Cattle Tested	Number Positive	Result of Challenge of Positives
7/8	2188	21	17/17 resistant
11/12	923	10	9/9 resistant
Totals	3111	31	26/26 resistant

Amongst 800 serologically-negative cattle, which were used in vaccine testing and received "K. A. G." or virulent Kabete "O" virus only two animals (0.25%) behaved in such a manner as to indicate a clearcut immunity existing at the time of their first arrival and bleeding. It is known (*Plowright* and *Ferris* to be published) that some cattle immune to challenge following the inoculation of small doses of culture-attenuated virus may not have demonstrable neutralizing antibody in their serum. If there is a comparable situation following immunization with, for example, minimum quantities of lapinized virus vaccine, then culture tests may sometimes be expected to fail in detecting all Kenyan cattle which would resist challenge.

Of the several hundreds of serologically negative cattle which were used in experiments on attenuated culture vaccine or with field strains of rinderpest, not one showed any evidence of having had previous experience of the virus.

Discussion

Very little quantitative information is available on the stability of rinderpest virus at various temperatures, but the data recorded here may be compared with those recorded by *Scott* (13). He found that the Kabete

"O", caprinized and lapinized strains of virus, produced in and stored as unprocessed cattle tissues, behaved in a uniform manner, all being inactivated at the same rate at temperatures of 56° C, 7° C and —15° C (see Table 11). At 37° and 25° C the inactivation rate in blood was significantly slower. Whilst the rate of inactivation of culture virus at 56° C was essentially the same as in *Scott*'s systems, and not markedly dissimilar at 37° C from his results using spleen and lymph gland, there were considerable differences from his results as obtained with blood at 37° C and all tissues maintained at 7° C. It is very difficult to account for an 8-fold advantage to the first three viruses in blood at 37° C and, at the same time, a 4-fold disadvantage as compared with cultured virus within the range 4°—7° C. The small temperature differential (3° to 4° C) could hardly have accounted for the latter. It is possible that, in blood stored at 25° and 37° C, much of the infectivity is in the form of virus within viable leucocytes and that the longer survival time for the virus in this temperature range reflects particularly favourable conditions for cell survival. In this case the so-called "heat inactivation" rate may be compounded of two groups of factors — those which influence cell survival and indirectly protect virus and those which directly influence the survival of free virus.

Our results for rinderpest virus may usefully be compared with those available for the serologically-related measles virus (14, 15). *Black*, *Reissig* and *Melnick* (16) summarized the information obtained up to 1959, giving figures from their own and other work which indicated a variable loss in 24 hours at 37° C of 3.2, 2.0 and 1.2 logs respectively. *Musser* and *Underwood* (17) presented more extensive personal data, which showed inci-

Table 11. A Comparison of Scott's (1959) and Present Data on Heat Stability of Rinderpest Virus

Strain of Virus	Storage Medium	Half-Life at Stated Temperature			
		56° C	37° C	25° C	4–7° C
Kabete "O"	Cattle spleen fragments	5 mins	105 mins	6.4 hrs	2.3 days
Caprinized	Cattle gland fragments	5 mins	105 mins	6.4 hrs	2.3 days
Lapinized	Cattle blood	5 mins	21 hrs	36 hrs	2.3 days
Tissue culture	Maintenance medium	3.46 mins*	165 mins**	—	9.2 days***

* Mean of 2 experiments ** Mean of 3 experiments *** Mean of 4 experiments (See Table 1, excluding Exp. III)

dentally that the addition of 5% calf serum to the storage medium considerably improved the storage properties of cultured measles virus, so that, for example, at 6° C there was a fall of titre from 5.2 to 2.8 over a period of 54 weeks. Assuming a straight line relationship this would correspond with a very long half-life of about 6—7 weeks, and, indeed, storage at —30° C "offered little advantage over +6° C". At 56° C the titre of the same virus preparation fell from 5.0 to nil over a period of 47 minutes, the results being closely comparable to those of our experiment II at this temperature. At 37° C, virus without calf serum and an initial titre of 3.8, lost 2.1 logs over 24 hours but was still detectable after 72 hours. No figures were recorded for virus in medium containing 5% calf serum at this temperature. In general, however, it seems that cultured measles virus is more resistant than cultured rinderpest virus.

The considerable variations between the results of different observers working with measles virus may be partially attributed, as noted by *Black* et al. (16), to lack of uniformity in the medium used, the absence of control over pH in some instances and to differences in the passage and storage history of the lines studied. *Musser* and *Underwood* (17) also stated, however, "we have observed considerable variation in rates of inactivation between different virus stocks", when by inference the strain of virus and storage medium were identical. They also noted a "tailing effect" at 25° C and 37° C which disappeared at 45° C and 56° C, whilst *Black* et al. (16) deduced that "all these studies give some indication of a decreasing rate of inactivation (at 22° and 37° C) with increasing time". In this connection we would draw attention to our experiment III at 4° C; there was apparently no reason for the marked "tailing effect" with consequent extension of the calculated half-life. The next passage level of virus produced under the same conditions, showed no such evidence for a fraction of virus particles of different stability (experiment IV). Further studies with cloned virus populations, a rigid control of physico-chemical factors in the medium and replicate titrations in the most accurate systems available are clearly essential.

One of the most important objectives in attempting to develop tissue-culture techniques applicable to rinderpest virus was to produce cheap, sensitive and accurate methods for the detection and titration of v/n antibody. In 1958, *Scott* and *Brown* (6) reported their results with a method based on the use of the Nakamura III strain (11) of lapinized virus in rabbits; their technique was essentially similar to that of *Nakamura* (18) and, for titrations, to quote these workers "... at Kabete each unknown serum required 21—35 rabbits. At Muguga 33—35 rabbits were used". Admittedly, economies can be effected by running several unknown sera at the same time, but in the majority of under-developed countries where rinderpest research is of immediate practical importance, such prodigal

expenditure of laboratory animals for serological tests is not to be envisaged and, indeed it is difficult to imagine how any very extensive serological research or vaccine control could be founded on the use of rabbits.

Japanese workers not only introduced the rabbit for neutralization tests with rinderpest, but have since been most active in the search for alternative, cheaper techniques. Thus *Nakamura* et al. (19) reported on tests carried out *in ovo*, by making use of the lapinized-avianized (L. A.) strain of virus (20) which unfortunately had to be detected, in each group of eggs given a single v/s mixture, by complement fixation (c/f) tests. Later, when the L. A. virus had been adapted to growth in suspended-fragment cultures of chick-embryo tissue (21), the latter were also used with the suggestion that "un progrès encore plus grand peut être espéré dans ce domaine, particulièrement du point de vue économique..." (22). But, since the L. A. virus had also to be detected in each group of tissue cultures by c/f tests, it was evident that the technique was still somewhat cumbersome and of limited accuracy.

It is of interest to compare neutralization tests carried out in rabbits and monolayer tissue-cultures, ignoring the obvious economic advantage of the latter. Rabbit tests (6, 23) require larger quantities of sera since the inoculum for each rabbit contains 0.5 ml. contrasted with 0.1 ml. for each tube culture. Where large numbers of sera have to be stored, sometimes for long periods, any increase in volume required is a disadvantage. Secondly, the amount of immune globulin for each reaction mixture in the rabbit system is approximately five times that employed in the tissue culture technique and yet the number of infective units to be neutralized is roughly-speaking three times smaller, $10^{1.3}$ to $10^{2.3}$ RID_{50} compared with $10^{1.8}$ to $10^{2.8}$ TCD_{50}. It was expected, therefore, that serum titres recorded in the culture system, would be somewhat lower than those obtained in rabbits and this expectation has been fulfilled in practice (3).

Thirdly, for rabbit tests (6, 23) all cattle sera were inactivated for 30 minutes at 56° C, whereas this procedure has been found to be unnecessary in the culture technique for cattle sera. The results of our screening tests show quite clearly that any significant neutralizing activity in unheated sera from adult cattle can be reliably related to immunity as shown by resistance to challenge. With other species such as man and various game animals, we have always inactivated their sera, since no comparable data are available on the relationship between neutralizing activity of fresh sera and resistance to infection.

Lastly, from the information provided by *Scott* and *Brown* (6), it may be calculated that each 1 log increase in the dose of virus employed in rabbits should result in a fall of either 1.59 log:units (Kabete method) or 0.97 log:units (Muguga method) in the serum titre. The difference in

slope of the regression lines for the two methods was not, however, significant. Since the permissible variation in virus dose was stated to be 20 to 200 RID_{50}, it follows that large differences in neutralizing titre, reaching 0.97 to 1.59, can be expected to occur between repeated titrations of the same serum. In fact, *Brown* mentions in his study of rinderpest immunity in calves (24), that "standardization of results in different tests" was made possible by reference to simultaneous titrations of a standard immune serum. In "box-titration" tests in tissue cultures we found that each 1 log increase in virus dose could be expected to result in a mean 0.56 log decrease in serum titre; this relationship is very close to that reported by *Capstick, Sellers* and *Stewart* (25), in a foot-and-mouth disease system where plaques on monolayers were used to measure virus. For rinderpest, therefore, variations in the actual dose of virus recorded for each test are of less significance in tissue culture than when rabbits are employed.

Summary

The stability of cultured rinderpest virus, in maintenance medium containing 5% normal ox serum, was studied at 4°, 37°, and 56° C. The half-life at these temperatures was calculated and the results compared with figures available for other strains of rinderpest virus in cattle tissues and for measles virus in tissue culture fluids.

Data were also provided on the freezing of the same virus at −25° C and −70° C, with storage for periods of up to four months. The accuracy of replicate virus titrations, in primary or serially-cultivated calf-kidney cells, was determined.

Details were given of tissue culture techniques for the detection and titration of neutralizing antibody to rinderpest virus in the sera of animals, especially cattle.

Box titrations of a standard ox immune serum showed that a 1 log increase in virus dose lowered the SN_{50} titre of the serum by a mean 0.56 log units. The error in replicate titrations of two standard immune sera, using different batches of calf-kidney cells as substrate, was determined.

The effect of normal ox serum on rinderpest virus was investigated and the sera of over 3.000 experimental cattle were examined by a "screening" test for immunity. There were no false positives and only 0.25% of the serologically-negative cattle gave later evidence of resistance to challenge.

Tissue culture techniques for the detection and titration of rinderpest neutralizing antibody are briefly compared with the methods hitherto available.

Acknowledgements

We are grateful to Mr. *S. A. Evans* and Dr. *G. R. Scott* of this Organization for providing details of the subsequent behaviour of many cattle subjected to "screening" tests. This paper is published by permission of the Director, E. A. V. R. O., to whom we are indebted for the provision of excellent working facilities.

References

1. *Plowright, W.*, and *R. D. Ferris:* J. comp. Path. Therap. **69,** 152 (1959a).
2. *Plowright, W.*, and *R. D. Ferris:* J. comp. Path. Therap. **69,** 173 (1959b).
3. *Plowright, W.:* Proceedings XVIth Int. vet. Congr., Madrid. Vol. II, 451 (1959).
4. *Plowright, W.*, and *R. D. Ferris:* Nature **179,** 316 (1957).
5. *Franklin, R. M., H. Rubin,* and *C. A. Davis:* Virology, **3,** 96 (1957).
6. *Scott, G. R.*, and *R. D. Brown:* J. comp. Path. Therap. **68,** 308 (1958).
7. *Ferris, R. D.*, and *W. Plowright:* to be published.
8. *Westwood, J. C. N., I. A. Macpherson,* and *D. H. J. Titmuss:* Brit. J. exp. Path. **38,** 138 (1957).
9. *Thompson, W. R.:* Bact. Rev. **11,** 115 (1927).
10. *Daubney, R.:* Rinderpest Vaccines. Their Production and Use in the Field. F. A. O. Agricultural Series No. 8, Washington, U. S. A. (1949).
11. *Nakamura, J. S., S. Wagatuma,* and *K. Fukusho:* J. Jap. Soc. vet. Sci. **17,** 185 (1938).
12. *Brotherston, J. G.:* J. comp. Path. Therap. **61,** 289 (1951).
13. *Scott, G. R.:* Nature **184,** 1948 (1959).
14. *Imagawa, D. T., P. Goret,* and *J. M. Adams:* Proc. Nat. Acad. Sci. **46,** 1119 (1960).
15. *Warren, J.:* Advances in Virus Research, Vol. **7,** 27. Academic Press Inc., New York, U. S. A. (1960).
16. *Black, F. L., M. Reissig,* and *J. L. Melnick:* Advances in Virus Research, Vol. **6,** 218. Academic Press Inc., New York, U. S. A. (1959).
17. *Musser, S. J.*, and *G. E. Underwood:* J. Immunol. **85,** 292 (1960).
18. *Nakamura, J.:* Jap. J. vet. Sci. **2,** 567 (1940).
19. *Nakamura, J.*, S. *Kishi, J. Kiuchi,* and *R. Reisinger:* Am J. vet. Res. **16,** 71 (1955).
20. *Nakamura, J.*, and *T. Miyamoto:* Am. J. vet. Res. **14,** 307 (1953).
21. *Nakamura, J., T. Motohashi,* and *S. Kishi:* Am. J. vet. Res. **19,** 174 (1958).
22. *Nakamura, J.:* Bull. Off. Int. Epizoot. **47,** 555 (1957).
23. *Brown, R. D.*, and *G. R. Scott:* Bull. epiz. Dis. Afr. **7,** 169 (1959).
24. *Brown, R. D.:* J. Hyg. (Camb.) **56,** 427 (1958).
25. *Capstick, P. B., R. F. Sellers,* and *D. L. Stewart:* Arch. Virusforsch. **9,** 606 (1960).