Onderstepoort Journal of Veterinary Research,
Volume 25, Number 1, January, 1951.

The Government Printer, Pretoria.

The Use of Egg Attenuated Bluetongue Virus in the Production of a Polyvalent Vaccine for Sheep. A.—Propagation of the Virus in Sheep.

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In a previous publication (Alexander, Haig and Adelaar, 1947) various aspects of the problem of producing from embryonated hen eggs sufficient polyvalent vaccine to meet the present annual demand of some 2 million doses were discussed. The opinion was expressed that such a procedure is entirely practical and could be brought into operation as soon as adequate data had been collected on a number of subsidiary details connected with mass vaccine production. Progress has been made with the investigations and now that it has been decided to produce, solely from eggs, all the virus necessary for vaccine for the 1948-49 season it is essential to record the reasons for taking that decision.

Very brief reference has been made to some of the factors involved in a review of the bluetongue problem in South Africa (Alexander, Neitz, Adelaar and Haig, 1947) published chiefly for the information of practising veterinarians. For the sake of clarity it is essential that some amplification should be made.

It must be borne in mind that a re-investigation of the whole problem of bluetongue was prompted by an appreciation of the fact that the reaction produced by the previous vaccine was on occasion so severe as to simulate an outbreak of the natural disease, and that frequently the resultant immunity was inadequate to protect against natural virulent infection. It had also become imperative to take into account the plurality of strains of virus (Neitz 1948).

It has been shown quite conclusively that bluetongue virus, contrary to the previously accepted conception, is not amenable to attenuation by serial passage through susceptible sheep (Neitz 1948). Full details of the propagation of the virus in fertile eggs has been reported and the prime importance of the temperature of incubation stressed (Alexander 1947). Additional data on the attenuation resulting from serial passage through eggs have been published (Alexander, Haig and Adelaar 1947) and the conclusions drawn have been confirmed and extended during the course of work which is in progress at present (Haig, personal communication). The adaptation of this work to routine vaccine production by propagation of the attenuated virus in susceptible Merino sheep is the object of this report.

For the sake of completeness, but also to explain step by step the modifications that were introduced until the present method of vaccine preparation was adopted, it is necessary briefly to outline the former technique which was introduced by Theiler in 1907 and has been used with minor modifications from that time.

Received for publication on 28th January, 1949.—Editor.

Theiler's method of vaccine preparation.

Sheep presumed to be susceptible to bluetongue were purchased annually from selected breeders in an area of the Karroo where the disease does not normally occur. These sheep, approximately 1200 in number, were transported by rail to Onderstepoort towards the end of May each year so that they would arrive after the first heavy frost of winter when the danger of exposure to natural infection in an enzootic area had passed. After allowing a period of some weeks for the animals to settle down to their new environment two groups of 25 sheep each day were given 1 c.c. subcutaneously of infective preserved sheep blood containing the vaccine strain of virus. A daily record of temperatures was kept. Quite regularly a febrile reaction commenced on the 6th or 7th day after injection and reached its peak on the 9th day, (temperature above 106°F). On that day any sheep which had not reacted or which showed an indefinite or abnormal reaction was discarded. From each of the remainder, 700 c.c. of blood was tapped from the jugular vein into an equal volume of an anticoagulant preservative solution consisting of 5 grams of potassium oxalate, 5 grams of phenol, 500 c.c. of distilled water and 500 c.c. of glycerine, known locally as O.C.G. Thus 1400, 1 c.c. doses of vaccine were obtained from each virus donor. The diluted blood from each batch of sheep was pooled, mixed and bottled to produce on an average, when allowing for wastage and failure of individual sheep to react normally, some 33,000 doses of vaccine per batch. This vaccine was stored initially in a cool basement, in later years in refrigerated rooms at about 6°C. When the requisite amount to meet the estimated requirements for the year had been prepared a random sample from each batch, including those batches remaining over from the previous year, was tested by injection into not less than two susceptible sheep. If a febrile reaction deemed to be satisfactory was produced the vaccine was released for issue. No bacteriological sterility test nor estimation of virus titre was made, the *in vivo* biological test being considered adequate.

With a view to more economic utilization of the virus donors Du Toit (1929) investigated the effect of higher dilution of the infective blood. From the results of a series of experiments he concluded that an effective vaccine with no impairment of keeping quality could be produced by diluting the blood 1:2 instead of 1:1, provided the final concentration of oxalate, phenol, and glycerine remained unaltered. He therefore increased the water component of the diluent from 500 to 833 c.c., and added 700 c.c. of blood to 1400 c.c. of this fluid, thus producing 2100 doses of vaccine from each donor (about 45,000 doses per batch). This formula was used from 1927 onwards. At the same time, fearing that Theiler's original strain of virus, which had been in use for more than 20 years, might have become progressively more attenuated, and possibly antigenically less potent by repeated passage through sheep, he substituted a new strain of virus. This, the Veglia strain, was reported to have been attenuated by passage through sheep thus confirming Theiler's original conception (Du Toit 1929).

At a later stage (unpublished experiments Alexander and Neitz) it was found that if the vaccine virus donors received a larger infecting dose of virus by the intravenous route the incubation period of the resulting febrile reaction could be reduced by two days without making any apparent difference to the final product. Therefore, purely in the interests of economy and as a means of simplifying the daily routine, i.e. sheep injected on one day of the week could be bled on the same day of the following week, the donor sheep were given 5 c.c. of blood virus intravenously and were bled as before but on the 7th day after injection. This day coincided almost invariably with the height of the thermal reaction, but no

data were available to indicate whether the highest titre of virus was circulating in the peripheral blood at that stage, nor what the virus titre of the final vaccine might be. An experiment was therefore made to clear up the latter point.

Experimental.—Serial ten-fold dilutions of vaccine were made in broth and 1 c.c. of each dilution was injected subcutaneously into each of two susceptible sheep. A careful record was kept of the reactions produced and after an interval of 28 days the survivors were challenged with 1 c.c. of the homologous virus given subcutaneously. The results are shown in Table 1.

TABLE NO. 1.

Estimation of the Virus Titre of Routine Vaccine in Sheep.

Sheep.	Vaccine 1 c.c. Subcut.		Immunity Test.	
	Dilution.	Reaction.	Interval.	Reaction.
			days.	
57826	10^{-1}	R++ day 7/6	28	No Reaction
57961	,,	R+++ day 5/9 † day 29		
57942	10^{-2}	R indefinite	28	No reaction
57964	,,	R++++ 7/7 † day 14		
58074	10^{-3}	R++ day 7/8	28	No reaction
58008	,,	R+++ day 7/6	28	No reaction
57896	10^{-4}	R indefinite	28	R indefinite
57949	,,	No reaction	28	R+++ day 7/7
58068	10^{-5}	No reaction	28	R++ day 8/4
58093	,,	No reaction	28	R+++ day 8/7

R+ to ++++=estimated severity of clinical symptoms.

7/6=incubation period of 7 days and 6 days duration of febrile reaction.

†=died.

Result.—From Table 1 it is apparent that a dilution of 10^{-4} corresponds to the 50 per cent. infective end point (Id 50). Therefore each dose of vaccine contained 10,000 infecting doses of virus for sheep.

Comment.—This experiment has been repeated and confirmed on several occasions during the course of other work. On no occasion has the Id 50 of freshly prepared vaccine been found to be lower than 10^{-3}. In the experiment detailed it is interesting to note that, of the 10 susceptible sheep which received an infecting dose of virus, 2 died, the one (57964) on the 14th day showing lesions on post mortem examination comparable with those of virulent bluetongue, the other (57961) on the 29th day showing general cachexia.

In 1942, in view of the continued reports of severe reactions produced by the vaccine, the Veglia strain of virus, substituted in 1929, was replaced by the original Theiler strain. This strain had been isolated from a bottle of vaccine which had been misplaced and lain behind a cupboard in the office of the Government Veterinary Officer, Vryheid, for a period of no less than 25 years (Neitz 1948).

The above was the method of bluetongue vaccine preparation in use when the optimum requirements for the propagation and attenuation of several strains of bluetongue virus in fertile hen eggs had been worked out and confirmed (Alexander 1947, Alexander, Haig and Adelaar 1947). It was decided to investigate the

possibility of eliminating one major defect of the vaccine, i.e. the severity of the reaction, by replacing the comparatively avirulent sheep virus by an attenuated egg passage virus.

The Propagation of Egg Attenuated Bluetongue Virus in Susceptible Merino Sheep.

The current technique of vaccine preparation was not to be modified in any way other than by substitution of the strain of virus. Seeing that an egg adapted strain of virus was to be used, it was anticipated that the entire procedure could be controlled by quantitative determinations in eggs. Since it had been shown that the estimation of the virus titre of an emulsion, by titration in eggs containing 8 day embryos and then incubated in a forced draught incubator at 33·5°C, corresponds closely to the estimation by titration in Merino sheep, all the work was to be carried out at 33·5°C. in the expectation that the results in eggs would be transposable directly to sheep. It would then be necessary, merely for confirmatory purposes, to check the salient features of the egg work on sheep.

Method and materials.

The virus selected at random for the experiment happened to be that known as V2 which had been attenuated by 70 serial passages through eggs. A number of eggs (42) containing 8 day embryos preincubated at 37·5°C were infected via the yolk sac with a diluted emulsion known to have a titre of approximately 3·0. The eggs were incubated at 35° for 24 hours and then transferred to 32°C. Eggs containing embryos that died up to 48 hours after injection were discarded (2 after 24 hours and 2 after 48 hours). Of the remaining 38, 27 were dead on the 3rd day, and 11 on the 4th day, on which days they were harvested, pooled and emulsified without the addition of any diluent. The emulsion was centrifuged in a Clay Adams angle centrifuge at 3,500 r.p.m. for 15 minutes, and the supernatant fluid was decanted for storage at 4°C; aerobic and anaerobic sterility tests proved the absence of any ordinary bacterial contaminant. Titration of this reddish opalescent fluid in 8 day embryos at 33·5°C showed the Ld 50 to be slightly greater than 6·0. A dilution of 1:1000 was made in broth, titrated in eggs (titre 3·2) and injected intravenously into 6 sheep from the available bluetongue susceptible stock i.e. each sheep received approximately 5000 M.I.D's of virus. In addition to recording the daily temperatures the sheep were examined clinically every day. Three showed neither a febrile nor any other clinical reaction. Three showed a slight febrile reaction of 1 to 2 days duration after an incubation period of 6 days, the highest recorded temperature being between 105 and 106°F. The only other clinical reaction was a slight transient buccal hyperaemia. After an interval of 21 days all 6 sheep proved to be solidly immune to a challenging dose of the homologous unattenuated virus to which a susceptible control reacted and died. On the 5th, 7th, 9th and 11th day after infection with the attenuated virus blood samples were collected from each of the 6 sheep as follows:—

(1) A 7 c.c. sample in 14 c.c. of the O.C.G. mixture used as the diluent for routine vaccine. This was taken to represent as near as possible a sample of routine vaccine and it was assumed that the small quantity of blood withdrawn would not influence in any way the normal course of multiplication of the virus in the animals.

(2) A 9 c.c. sample in 1 c.c. of 10 per cent. sodium citrate to be used for determination of the virus titre by titration in eggs. Sodium citrate was used as the anti-coagulant because of the lethal effect of phenol glycerine in high concentration on egg embryos.

1. *The virus titre of the blood as determined by titration of the "vaccine" samples in sheep.*—Sheep were given 1 c.c. of decimal dilutions of the pooled daily samples subcutaneously and 28 days later were challenged with the homologous virulent virus. The results are given in tabular form in Table No. 2.

TABLE NO. 2.

The Virus Content of the Blood of Sheep infected with Egg Attenuated Virus.

Interval in Days.	Dilution.								
	Undiluted.			1/200			1/2,000		
	Sheep.	Reaction.	Immunity Test.	Sheep.	Reaction.	Immunity Test.	Sheep.	Reaction.	Immunity Test.
5	74701 74703	NR R6/2 M+Fo	NR NR	— —	— —	— —	— —	— —	— —
7	74692	NR	NR	74695	R11/2 MoFo	NR	74698	NR	NR
	—	—	—	74697	NR	NR	74702	NR	R 5/6 M+++ F++++
9	74684	NR	NR	74699	NR	R 6/3 M++F?	74677	NR	R 6/4 M+++ F+++
	—	—	—	74678	NR	NR	74686	NR	R 6/7 M++++ F++++
11	74700	NR	R7/4 M++ F++	—	—	—	—	—	—

NOTE.—NR=No reaction.

R6/2 MoF++=Febrile reaction commenced day 6 and lasted 2 days. Severity of mouth (M) and foot (F) lesions denoted by o. . . ++++

Result.—From the data in Table 2, it will be seen that of the 13 sheep used in the experiment 11 showed no reaction whatever to the egg attenuated sheep blood virus. The 2 doubtful reactors showed merely a mild fever of short duration and slight accompanying hyperaemia of the buccal mucosa. It is apparent therefore that evidence of infection is dependent entirely on the production of immunity. The immunity tests show that virus was present in the blood 5 days after infection, had reached a titre of approximately 1:1000 on the 7th day, had decreased to a titre of 1: 100 on the 9th day, and its presence could not be demonstrated on the 11th day.

Comment.—It is unfortunate that in this experiment a 50 per cent. end point was not reached with the 5 day sample, but other determinations have shown that the quantity of virus present at that stage is small and seldom reaches a titre higher than 10^{-1}. It may be concluded therefore that virus appears in the peripheral blood slightly before the 5th day after intravenous injection with 1000

M.I.D's of egg attenuated virus, reaches the highest titre on or about the 7th day and then decreases rapidly in amount until it has disappeared by the 11th day. This conclusion has been confirmed by several other experiments and in addition it has been shown that the highest titre of virus found in the blood has been rather less than 10^{-4}. A rational procedure for the production of routine vaccine therefore would be to infect the donors by the intravenous route with not less than 1000 M.I.D.'s of virus and to tap the requisite blood virus on the 7th day.

2. *The virus content of the blood as determined by titration in eggs.*—The citrated blood samples were diluted with an equal volume of distilled water to haemolize the cells and to bring the blood concentration approximately to that of the "vaccine" samples. The diluted material was centrifuged at 3500 r.p.m. for 15 minutes in an angle centrifuge, to deposit any particulate matter and the supernatants were collected for titration in eggs by the usual technique.

Result.—It would be superfluous to give the results in detail because of all the eggs used, less than 20 per cent. of embryos that received the highest concentration of blood died. All the remainder except the few that succumbed to injury at the time of injection survived for 7 days.

Conclusion.—The virus titre of the blood of sheep reacting to egg adapted attenuated virus cannot be determined by titration in fertile hen eggs.

This result appeared to be so remarkable that the experiment was repeated on individual sheep as well as on groups of sheep using the same as well as two other egg attenuated strains of virus (Cyprus and Mimosa Park) with similar results. Ultimately the only justifiable conclusion appeared to be that an egg adapted strain of bluetongue virus (A strain), after even a single passage through sheep, reverts to an apparently original (O) strain. It is necessary to state that no difficulty whatever was experienced in readapting such a strain to eggs, and that after no more than 3 egg passages it appears to have regained essential characteristics of an A strain.

Although inability to carry out the quantitative aspects of vaccine production in eggs is a decided handicap, nevertheless it appeared to be an entirely practical procedure to replace the O type of relatively avirulent virus by an attenuated A type. However, the reversion from the A to the apparent O phase made it essential to determine whether there might not be a simultaneous reversion to original virulence. Therefore blood collected on the 7th day in the experiment detailed above was passaged for 8 generations in susceptible sheep in the manner and with the results shown in Table 3.

Result.—Although 9 of the 15 sheep used for serial passage of the virus showed a modified febrile reaction with slight buccal hyperaemia there was never any involvement of the feet in the form of the pathognomonic coronitis nor was there any sign of a general reaction with loss of condition. All the sheep were subsequently found to be immune to the homologous virulent virus.

Conclusion.—It is believed that the slight variation in the very mild reactions is due to variation in the susceptibility of individual sheep, an observation constantly recorded in work with attenuated A strains of virus. Therefore it is concluded that with the apparent reversion to the O phase of virus there is no tendency for simultaneous reversion to the original virulent phase. In other words once an attenuation has been brought about by suitable serial egg passage, that attenuation is retained on passage through susceptible sheep at least for a limited number of sheep generations.

TABLE No. 3.

Passage of egg attenuated virus through sheep.

Generation.	Sheep.	Donor.	Interval days.	Reaction.
1....................	Pooled blood of 6 donors	Egg virus	—	See text.
2....................	74701	74708 *	5	R 6/2. T 102. Mo Fo
	74703	74708	5	R7/3. T106. M++ F ?
	74695	74708	7	R11/2. T106. Mo Fo.
3....................	74681	74703	9	NR.
	74687	74703	9	NR.
	74680	74695	12	NR
4....................	74690	74687	9	R7/4. T106. M++ Fo.
	74693	74680	8	NR.
5....................	74673	74690	7	R6/4. T106. Mo Fo.
	74664	74690	7	R 6/2. T 104·6 M++ Fo.
6....................	74666	74673	7	R 6/4. T 107 M++ Fo.
	74659	74664	8	R 7/2. T 104. Mo Fo.
7....................	74649	74666	7	NR.
	74642	74659	9	R 8/4. T 106. M+ Fo.
8....................	74655	74649	7	NR.

NR.=No reaction.

R6/1. T105·4 M+ F++.=Reacted, febrile reaction commenced after an incubation period of 6 days lasting for 1 day with maximum temperature 105/4. Clinical involvement of mouth and feet indicated by + with ++++ very severe.

*1 of 6 donors of Gen. 1.

With this information available it was decided to prepare a number of batches of vaccine for trial in the laboratory, as well as in the field, on an experimental basis. In addition, instead of a monovalent vaccine based upon the use of a single strain of egg adapted virus a trivalent and later a quadrivalent vaccine was prepared from attenuated strains showing pronounced antigenic differences.

The procedure adopted was simple. Sheep in batches of 6 were infected intravenously with egg embryo emulsions suitably diluted to contain not less than 1000 M.I.D's of virus, each strain being maintained separately and in pure culture in eggs and in sheep. Whether a clinical reaction or not was produced the sheep were bled in the usual way on the 7th day and the total harvest pooled. After storage for about 14 days at 5°C. to permit fragmentation of the cells, samples were taken at random and tested on susceptible sheep. No severe reactions were produced and in the laboratory immunity to each of the 4 strains, separately and simultaneously, was solid. In addition quantitative virus estimations in sheep showed that a minimum of 400 infecting doses of each virus strain was present in the experimental batches.

Approximately 40,000 doses of this vaccine were issued to the field, for use by breeders, although it is appreciated that the results of uncontrolled trials of this nature are notoriously unsatisfactory. No information was available as to the full susceptibility or otherwise of the sheep injected, but at least no severe reactions were reported. In fact a number of users expressed considerable concern at their failure to detect any reaction and therefore doubted whether any immunity had been produced. Unfortunately the season 1945-46 turned out to be a " poor " bluetongue year, so that even though no breakdowns were reported the natural immunity test can be regarded only as inconclusive.

While preparations were being made to produce the bulk, if not all, the vaccine for the following season by this method routine exploratory investigations were carried on with the result that two serious objections were brought to light.

A. Change in antigenic structure of an attenuated virus on reversion from the A to the O phase.—A single sheep (74676) received intravenously 5 c.c. embryo emulsion egg generation 71 strain V2 having a titre in eggs of 4·2. The temperature of the sheep rose to 105°F. on the 6th day but had returned to normal 2 days later; no other symptoms were detected. Blood collected in O.C.G. on the 7th day after infection was injected subcutaneously into sheep in dilutions from 2×10^{-1} to 2×10^{-6}. The reactions to the immunizing injection and to the subsequent immunity test of fully virulent virus are given in Table No. 4.

TABLE No. 4.

Change in antigenic structure of attenuated virus V2 on reversion from the A to the O phase.

Sheep No.	Immunization.		Immunity Test—Reaction.
	Blood Dilution.	Reaction.	
76195	2×10^{-1}	R 8/2. T105·0. Mo Fo	NR.
76087		R 7/3. T105·4. M ? Fo	R 6/5. T107. M+ Fo.
76200	2×10^{-2}	NR.	R 6/3. T106. M++ F+.
75421		R 8/3. T106. Mo Fo	R 6/3. T106·2. M ? F+.
76101	2×10^{-3}	R 7/2. T106. M ? Fo	R 6/3. T106·4. M++ F+.
76244		R 8/2. T105. Mo Fo	R 6/3. T105. Mo Fo.
76077	2×10^{-4}	NR.	R 6/3. T 107. M+++ F+++.
76083		R ? 12/3. T 105. Mo Fo	R 5/6. T 107·8. M++++ F++. Day 18†
76174	2×10^{-5}	R ? 10/1. T 106. Mo Fo	R 6/5. T 107. M+++ F++.
76198		NR.	R 4/5. T 107·8. M++++ F++++. Day 12†
76141	2×10^{-6}	NR.	R 5/6. T 107·6. M+++ F+++.
76216		NR.	R 6/5. T 106. M+++ F+++.
76907	—	Control not injected.	R 5/3. T 107. M++++ F+++. Day 14†
74676 =donor	Egg generation 7 Titre 4·2	R 6/2. T 105. Mo Fo	NR.

Results.—The reactions in the sheep are strictly comparable to that shown by the blood virus donor (74676) that received the egg attenuated virus, the slightly longer periods of incubation being due to the route of infection and the progressively smaller dose of virus. The transient thermal reaction in two of the sheep (76083 and 76174) which subsequently were found to have no immunity is inexplicable but is regularly encountered by anyone who has maintained a temperature record of even so-called normal sheep under laboratory stable conditions.

On immunity test only 2 sheep were solidly immune, the original virus donor and one of the two that received the highest concentration of blood virus. All the remainder reacted but a very marked difference was noted in the reactions of those sheep which received blood dilutions up to 2×10^{-3} and those from 2×10^{-4} upwards. This difference was far more marked clinically than can be depicted with strict accuracy, in a table. In fact up to 2×10^{-3} the reactions were almost, but not quite so severe as those which experience has shown would have been obtained on applying a test with a strain of somewhat different antigenic structure. The reactions from 2×10^{-4} upwards were those to be anticipated in fully susceptible animals maintained under stable conditions.

Conclusion.—It is concluded that virus was present in the blood dilutions up to 2×10^{-3} and absent in higher dilutions but the virus had failed to afford full protection against the original unattenuated virus. A change in antigenic structure had not taken place as a result of serial egg passage to this level, as shown by the solid immunity of the sheep that received egg virus, a finding confirmed by repeated routine immunity tests during serial egg passage. Therefore the antigenic modification could only have occurred concurrently with the change from the A phase of virus.

This antigenic reorientation was equally pronounced in another experiment with the same strain of virus (V2), was observed to a lesser extent with a second strain (Bekker) but up to the present has not been encountered with two other strains (Estantia and Mimosa Park).

From the point of view of routine vaccine production this change in antigenic structure introduces a complicating difficulty but it is believed that it could be overcome by careful selection of the virus strains.

B. Inactivation of vaccine virus by the inclusion of immune sheep amongst the donors.—A small batch of monovalent vaccine had been prepared for experimental purposes. The 3 blood virus donors 75447, 76153, 76116 received intravenously 5 c.c. of a 1/100 dilution of egg embryo emulsion strain Cyprus, egg generation 126, the titre in eggs being 3·3. No reaction was produced and after an interval of 21 days the sheep were shown to be solidly immune. Blood samples (20 c.c.) in O.C.G., to make a final dilution of 1 in 3, were collected from each sheep on day 5, 7, 9 and 11 after injection. Equal volumes of the samples on each day were pooled, decimal dilutions were made in broth and injected subcutaneously in 1 c.c. amounts into other sheep from the available susceptible stock, to check once more the rise and fall in virus titre of the blood during the course of infection. None of the sheep showed any clinical reaction, so that again determination of infection with virus was dependent solely upon the results of the immunity test. These results are given in Table No. 5.

TABLE No. 5.

Reaction to immunity tests of sheep which received serial dilutions of a pooled vaccine.

Day after Injection Sheep Bled.	VACCINE DILUTION.				
	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
5............	76903 R++++ day 20†	76876 R++++ day 9†	76812 R++++	—	—
	76751 R++++ day 10†	76745 R+++	76870 NR.	—	—
7............	76816 R ?	76671 R+++	76820 R+++	76852 R++++ day 17†	76954 R+++
	76930 R+++	76853 R++++ day 25†	76855 R+++	76793 R++++ day 11†	76673 R++++ day 17†
9............	76872 R+++	76776 R+++	76714 R+++	76956 R++++ day 11†	—
	76794 R+++	77232 R++++	76845 R++++ day 15†	76803 R+++	—
11..........	76840 R+++	76963 R+++	76962 R+++	—	—
	76770 R+++	76746 R++	76938 R++++ day 20†	—	—

Result.—On immunity test, of the 30 sheep used in the experiment, 10 died, 18 reacted very severely but recovered, 1 (76816 vaccine dilution 10^{-1} day 7) showed a doubtful febrile reaction only, and 1 (76870 vaccine dilution 10^{-3} day 5) did not react. As an additional check this sheep proved to be solidly immune to a second test applied 14 days later.

Conclusion.—The obvious conclusion from this experiment is that the pooled samples of blood collected on the 5th, 7th, 9th and 11th day after infection with egg attenuated virus contained no virus at all or at most 10 infecting doses on day 7, a result completely at variance with all previous experience. The observation that one of the two sheep that received the 10^{-3} dilution of the 5th day sample failed to react to the immunity test, a result completely out of place, led to the suspicion that at least an odd individual amongst the carefully selected susceptible sheep might be immune. Therefore the various samples of blood which had been stored at 5°C. were diluted separately and injected into sheep as shown in Table 6. No reactions were produced. For the sake of economy only 1 of each pair of sheep were given an immunity test on the 21st day; 7 days later, by which time the probable outcome of the experiment was apparent, the balance of the sheep were tested or discharged to other experiments. The results are given in Table 6.

TABLE NO. 6.

Reactions to immunity test of sheep which received vaccine prepared from 3 separate sheep.

Donor.	Day after Injection Sheep Bled.	Vaccine Dilution.			
		Undiluted.	10^{-2}.	10^{-3}.	10^{-4}.
75447........	5	76976 R++++† 77026	76984 — 79119 —		
	7	77039 R+++ 77074 R+++	77052 R++++† 77070 R++++	—	—
	9	77005 R+++ 77105 —	76759 — 77158 —		
	11	76937 R++ 76749 —	77078 — 76764 —		
76153........	5	76929 R++++ 76782 —	76815 — 76970 —		
	7	76934 R++++ 76845 R++++	76787 NR. 76810 R+++	— —	— —
	9	77218 R+++ 76823 —	76685 — 77001 —		
	11	76948 R+++ 76698 —	77015 — 76730 —		
76116........	5	75793 NR. 75876 NR.	76988 R+++ 76904 R++++		
	7	75751 NR. 75937 NR.	75927 NR. 75893 NR.	77119 NR. 76782 R ?	77158 R+++ 76970 R++++†
	9	75882 NR. 75863 NR.	75748 NR. 75970 NR.		
	11	76960 R+++ 76939 R+++	76890 R+++ 76780 R+++		

Result.—Blood of only one of the three donors (76116) produced any immunity in the sheep; therefore only in this sheep did virus find its way into the circulation in demonstrable amount. Also, in this sheep the rise and fall of virus titre followed the anticipated pattern in that virus was present in small amounts on the 5th day, attained a titre of 10^{-3} on the 7th day and had disappeared by the 11th day. From the result of the previous experiment in which this infective blood was diluted 1 in 3, even though the 5th, 7th and 9th day samples would be infective in higher dilution, the mixtures were inactive. Therefore the only reasonable conclusion is that the virus was neutralized by antibodies present in the other samples. That it is possible for this to happen has been shown experimentally by mixing infective blood with blood from a known immune and a known susceptible animal; it is quite unnecessary to give full details of that experiment here. Again it must be pointed out that in the experiment under consideration one sheep (76787) that received dilution 10^{-2} of non-infective 7th day sample 76153 was found to be immune, a finding which is quite out of place.

It is concluded therefore that in spite of the greatest care in selecting what are believed to be fully susceptible animals, a small number, estimated at about 1 per cent may be found to be immune. Inadvertently two such immune sheep were selected quite at random for vaccine virus production.

Discussion.

For more than 20 years a bluetongue vaccine has been produced by a method based upon the propagation of an avirulent strain of virus in susceptible sheep. Objections to the continued use of this vaccine have been enumerated elsewhere (Neitz 1948). But, as regards the actual method of production, the vaccine probably never contained virus in a lower titre than 10^{-2}. It appears to be certain that this was possible because any immune sheep used for virus propagation were eliminated by discarding all animals which did not respond to infection by what was considered a normal febrile reaction. When the method is adapted to the propagation of virus attenuated to such a degree that they produce no clinical reaction, detection of the immune animal becomes quite impossible. To produce the requisite amount of vaccine at least 1,000 donors would be required each year. Detection and elimination of any immune animals whose sera would neutralize large quantities of prepared vaccine would be possible by the application of a test based upon *in vitro* serum virus neutralization of egg adapted virus strains. Such a procedure would involve considerable expense, not the least of which would be the housing, isolation and maintenance of the sheep for considerable periods while the tests were being carried out. Since it appeared to be quite impossible to devise any system of selecting only fully susceptible sheep by any other method, it was decided that, what must be regarded as the classical method of vaccine production, must be abandoned, and replaced by one dependent upon the propagation of all the requisite virus in eggs. An exact description of the technique developed will form the basis of the second publication in this series.

Summary.

(1) A brief description of Theiler's classical method of bluetongue vaccine production is given and various modifications introduced from time to time are recorded.

(2) In an attempt to adapt the recognized technique to the routine mass production of a polyvalent vaccine using fully attenuated egg adapted (A) strains of virus three difficulties were encountered:

(*a*) As a result of even a single passage through susceptible sheep the adapted (A) strains were transformed at least partially into apparently original or sheep strains (O) so that quantitative control could not be carried out in eggs.

(*b*) During the course of change from the A to the pseudo-O phase at least some strains show a marked change in antigenic structure.

(*c*) Since the majority of sheep infected with egg attenuated virus show no clinical reaction it is impossible to eliminate animals included by accident amongst the virus donors. In the final mixture specific antibodies in the serum of such immune sheep neutralize the virus propagated in the susceptible donors, thus making the vaccine inert. This has proved an insurmountable difficulty.

(3) Though passage through sheep causes a change from the A to the pseudo-O type of virus there is no reversion to original virulence.

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