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The Immunization of Horses against Horse-sickness by the use of Formalysed Virus—Part II.

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INTRODUCTION.

A progress report on the immunization of horses by the use of formalized virus was published in the Sixteenth Report of the Director of Veterinary Services and Animal Industry. At that time it had been established that a solid immunity can be set up by a series of subcutaneous injections of virulent spleen emulsion inactivated by progressively lower concentrations of formaldehyde. The concentration that will inactivate spleen virus to a degree that renders its injection into fully susceptible horses safe in practically every case was established, namely, 1 : 1000. Even repeated injections of this material did not produce an immunity sufficient to protect against 'O' virus but horses which are able to resist a vaccine containing 1 : 4000 formaldehyde in the vast majority of instances are immune to O virus. The present series of experiments were planned with the object of proceeding from the 1 : 1000 to the 1 : 4000 material with the smallest number of injections, in the shortest space of time. At the same time a watchful eye had to be maintained on the degree of immunity produced, the keeping qualities of the vaccine, and the possibility of an adverse local effect at the site of injection.

This work was indicated in the hope that sufficiently good results would be obtained to warrant the extension of the method to general immunization in the field in place of the serumvirus simultaneous method which has several serious disadvantages as indicated by Whitworth (1929).

Independent work on similar lines has been carried out by Whitworth and Walker. Generally, the results have been mutually confirmed with this exception, that it must be admitted that the results obtained by us do not warrant that confidence in the efficacy of the method that is apparent in the reports issued from Kenya.

Attention has been directed to the difficulty of producing a bacterially sterile formalized vaccine. Even though the aseptic removal and subsequent manipulation of an organ as bulky as the pathologically enlarged spleen of a reacting horse presents considerable difficulty, the possibility of bacterial contaminants within the spleen itself before removal could not be overlooked. Should any bacteria be present, a final sterile product cannot be anticipated

seeing that the only bactericide present in the completed vaccine is formalin in a concentration below its effective limit. No data are available as to the possible harmful effect of bacterial decomposition on the antigenic property of the formalized vaccine but, on several occasions injections have been followed by alarming local reactions and swellings accompanied by such extensive necrosis of the subcutaneous and muscular tissue as to necessitate extensive surgical treatment.

Two methods of sterilization were considered :

(1) The addition of 0·25 per cent. phenol to the saline used for preparing the emulsion.

(2) Filtration of the completed vaccine through a Seitz filter before bottling.

EXPERIMENTAL WORK.

Experiment 12 (S. 4111a).

Object.—To ascertain the effect on the antigenic property of spleen virus, of (1) adding phenol as a preservative, and (2) of filtration through a Seitz filter.

Method.—The method of vaccine production in this and subsequent experiments was identical with that described in the previous publications. Before use the Seitz filter was autoclaved for 30 minutes at 130°C. under positive pressure. The material was first clarified by aspiration through a thin layer of paper pulp placed over muslin on a Buchner funnel and was then quickly sucked through the filter under a negative pressure of 40 lb. per square inch. Immediately before filtration a small quantity of a fresh culture of *B. prodigiosus* was added and sterility tests aerobically and anaerobically were carried out on the filtrate : in every case the filtrate was found to be sterile. The actual time of filtration of the large bulk of material was approximately $1\frac{1}{2}$ hours.

At the same time advantage was taken of this experiment to confirm a previous observation that a commencing injection of 1 : 1500 formalized vaccine is dangerous ; incidentally the results would also indicate whether the phenol would have any additional attenuating effect upon the virus.

Group A.—This serves as a control group to indicate the efficacy of the particular batch of vaccine. Of the four horses, two died on the 7th day after the 1 : 2000 vaccine. Of the survivors one reacted severely and one mildly to an immunity test of ' O ' virus.

The seven horses in *Group B* received the vaccine to which phenol had been added, five commencing with a 1 : 1500 injection and two with a 1 : 2000 injection ; the latter two horses died on the 6th and 7th day respectively. Of the five that commenced with 1 : 1500, two died as a result of this injection, and 1 as a result of the 1 : 3000 material (third injection). The two survivors at no time reacted, and on immunity test were immune to ' O ' virus.

Results.—Details of the injections together with the results obtained are shown in Table I.

TABLE I.—*Experiment* 4111 (*a*).

Group.	D.O.B. No. of Horses.	Concentration of formaldehyde in spleen emulsion. 1 : 1500 Date and Dose. 30 c.c.	1 : 2000 Date and Dose. 30 c.c.	1 : 3000 Date and Dose. 30 c.c.	1 : 4000 Date and Dose. 30 c.c.	1 : 6000 Date and Dose. 30 c.c.	Interval in Days between Injections.	Result of Immunization.	Immunity Test. Date of Injection of O-virus.	Interval in days since last Injection.	RESULT.
A. Injection of spleen emulsion.	19800	30/1/30	13/2/30	27/2/30	13/3/30	27/3/30	14	No reaction.....	10/4/30	14	Mild febrile reaction 4th to 8th day.
	19732	30/1/30	13/2/30	27/2/30	13/3/30	27/3/30	14	No reaction.....	10/4/30	14	Severe reaction 6th to 15th day—recovered.
	19731	—	13/2/30	—	—	—	—	Reacted from 4th day and died 7th day after 1 : 2000 injection	—	—	—
	19793	—	13/2/30	—	—	—	—	Reacted 4th day and died 7th day after 1 : 2000 injection	—	—	—
	19734	30/12/30	—	—	—	—	—	Reacted from 3rd day and died 7th day after 1 : 1500 injection	—	—	—
	19795	30/12/30	—	—	—	—	—	Reacted from 4th day and died 7th day after 1 ; 1500 injection			
B. Injection of spleen emulsion to which 0·25 per cent. Phenol was added	19742	20/2/30	6/3/30	21/3/30	8/4/30	25/4/30	14	No reaction.....	9/5/30	14	Immune.
	19743	20/2/30	6/3/30	21/3/30	—	—	14	Reacted 4th day and died 7th day after 1 : 3000 injection	—	—	—
	19736	13/2/30	27/2/30	—	13/3/30	27/3/30	14	No reaction. Horse had received filtered spleen vaccine 1 : 2000 before 1 : 1500 injection	10/4/30	14	Immune.
	19733	—	30/1/30	—	—	—	—	Reacted 4th day and died 7th day after 1 : 2000 injection	—	—	—
	19797	—	30/1/30	—	—	—	—	Reacted 4th day and died 7th day after 1 : 2000 injection	—	—	—
C. Injection of spleen emulsion that had been passed through Seitz filter	19735	—	20 c.c. 30/1/30	30 c.c. 13/2/30	15 c.c. 28/2/30	20 c.c. 14/3/30	14	Reacted from 4th day and died 7th day after 1 : 6000 injection	—	—	—
	19794	—	20 c.c. 30/1/30	30 c.c. 13/2/30	30 c.c. 27/2/30	—	14	Reacted from 5th day and died 8th day after 1 : 4000 injection	—	—	—

Two horses in *Group C* received the Seitz filtrate. Both died as a result of the 1 : 4000 vaccine and one as a result of the 1 : 6000 vaccine.

Conclusions.—(1) It is difficult to offer any definite opinion as to the effect on the antigenic value of the spleen emulsion of adding 0·25 per cent. phenol to the saline, since two out of the four horses in the control group died during the immunizing process, and two out of five died in the experimental group. Possibly it may be concluded that the phenol has no harmful effect.

(2) A formaldehyde concentration of 1 : 1500 either in the presence or absence of phenol does not result in a safe vaccine.

(3) Phenol + formalin has no greater attenuating effect than formalin alone.

(4) Seitz filtration of the vaccine probably removes the greater portion of the antigenic fraction of the vaccine, but allows the passage of that virus which has not been inactivated. This result is of considerable interest because it indicates that the action of formaldehyde in decreasing concentration is not merely to inactivate decreasing amounts of virus, i.e., the immunity produced is not alone due to the repeated injection of increasing but still subinfective doses of virus. On the contrary, the action of the formalin probably is to destroy the infectivity of the virus without reducing its antigenic property; this inactivated virus is unable to pass the disc of a Seitz filter under the conditions of the experiment.

This observation on the low antigenic value of Seitz filtered vaccine was subsequently confirmed by a further test on twelve horses, with material prepared for a later series of experiments. Details of the injections are given in Appendix I. It is necessary only to state here that four of the horses survived the injection of 1 : 3500 or 1 : 4000 filtered vaccine and two of the four survivors were able to withstand an immunity test of either 5 c.c. of 'O' virus or 30 c.c. of unfiltered 1 : 4000 vaccine.

Previous work has indicated that the value of formalized vaccine is dependent on a high initial concentration of virus in the emulsion prepared. It is known that blood taken at the height of the febrile reaction is infective in as small an amount as ·0001 c.c. It was, therefore, considered that, if the virus found in the circulating blood is capable of being transformed into a potent vaccine its inclusion in future work might increase the value of the product.

Experiment S. 4111 b.

Object.—To determine the antigenic value of virulent blood treated with decreasing quantities of formalin in the presence or absence of spleen tissue.

Method.—(*a*) A horse was bled from the jugular vein at the height of its reaction to 'O' virus, formalin was added in varying amounts and injections were made as indicated in Table II.

(*b*) The horse was destroyed *in extremis*, the spleen was removed and pulped by passing it through a Latapi mincer. The contained virus was then inactivated by heating to 100°C. for thirty minutes in an Arnold's steam sterilizer on each of three successive days. The spleen tissue was then made up into a 20 per cent. emulsion, but the infective blood [as obtained for (*a*) above] was used to replace the isotonic saline. Formalin was added by the usual technique and injections made as indicated in Table II.

TABLE II.—*Experiment* 4111 *b*.

Group.	D.O.B. No. of Horse.	Concentration of formaldehyde in blood or blood-spleen emulsion.				Interval in Days between Injections.	Result of Immunization.
		1 : 1500 Date and Dose. 30 c.c.	1 : 2000 Date and Dose. 30 c.c.	1 : 3000 Date and Dose. 30 c.c.	1 : 4000 Date and Dose. 30 c.c.		
A. Injection of formalized blood	19738	30/1/30	—	—	—	—	Reacted from 4th day and died 6th day after 1 : 1500 injection.
	19783	30/1/30	—	—	—	—	Reacted from 5th day and died 7th day after 1 : 1500 injection.
	19739	—	30/1/30	13/2/30	27/2/30	14	Reacted from 8th to 11th day after 1 : 2000; and also reacted from 4th day and died on 8th day after 1 : 4000 injection.
B. Injection of formalized blood mixed with spleen to make a 20 per cent. emulsion. Heated for ½-hour on three successive days to 100°C.	19740	30/1/30	—	—	—	—	Too wild to temperature. Died 10th day after 1 : 1500 injection.
	19798	—	30/1/30	—	—	—	Reacted from 2nd day and died 6th day after 1 : 2000 injection.
	19792	—	30/1/30	—	—	—	Too wild to temperature. Died 11th day after 1 : 2000 injection.

Results.—(*a*) Of three horses which received formalized infective blood two died as a result of the injection of 1 : 1500 fluid, and one which had received 1 : 2000 and 1 : 3000 died as result of the 1 : 4000 blood.

(*b*) Of three horses which received the blood virus formalized in the presence of inactivated spleen tissue one died as a result of the 1 : 1500 concentration and two as a result of the 1 : 2000 concentration.

Conclusion.—Although by no means conclusive, it would appear that the virus found in the blood is not of high antigenic value when formalized in the presence or absence of heat inactivated spleen tissue. A considerable amount of additional work would be required definitely to clear up this point but the results obtained with six horses were so discouraging that the risk of sacrificing any more animals could not be justified. This aspect of the work has, therefore, been discontinued.

Up to the present the major portion of the work had been directed towards the elaboration of a method of immunization based on the injection of virus inactivated by progressively decreasing concentrations of formaldehyde. No serious attempt had been made to ascertain the effect of repeated injections of spleen virus inactivated by the same safe formaldehyde concentration. A series of experiments were, therefore, planned to clear up this point. It was decided to make the interval between injections seven days the details of treatment being as follows :—

1. 1 horse (20307) received 4 injections of 1/1250 vaccine in 60 c.c. doses.
2. 1 ,, (20308) ,, 8 ,, 1/1250 ,, ,,
3. 1 ,, (20325) ,, 4 ,, 1/1500 ,, ,,
4. 1 ,, (20327) ,, 8 ,, 1/1500 ,, ,,
5. 1 ,, (20341) ,, 2 ,, 1/1500 ,, 250 c.c. doses.
6. 1 ,, (20340) ,, 1 injection of 1/1500 ,, 500 c.c. dose.

There were no reactions to the injections but the horses given the 250 c.c. and 500 c.c. doses developed local abscesses requiring surgical treatment.

On applying an immunity test after an interval of ten to fourteen days after the last injection, all the horses except 20308, which had received eight injections of 1/1250 vaccine were found to be fully susceptible to the intravenous injection of 5 c.c. of 'O' virus and died of horse-sickness. The one exception, 20308, showed a very severe reaction but survived.

Conclusion.—The repeated injection of moderate doses of spleen virus inactivated to a safe degree by an adequate concentration of formaldehyde or the single injection of a massive dose of the same vaccine produces very little or no immunity.

Previous work has shown that the lowest limit of safe formaldehyde concentration is 1 : 1000. In addition it had been found that horses which withstand an injection of 1 : 4000 material are subsequently immune to 'O' virus. The dose used was fixed arbitrarily at 30 c.c. It was, therefore, decided to carry out a further set of experiments with the object :

(1) Of confirming the conclusions drawn as to the effective limits of formaldehyde concentration.

(2) Of determining whether a smaller dose, which would make for greater convenience in practice, would be equally effective.

(3) Of ascertaining the smallest number of injections required to produce immunity with the smallest risk.

(4) Of ascertaining any difference in antigenic value between individual spleens and batches of different pooled spleens.

(5) Of determining the probable duration of immunity produced by formalized vaccines.

Full details of the injections will be found in Appendix II, of which a summary is shown in Table III of the text.

TABLE III.—EXPERIMENT S. 4193.

Group.	No. of Horses in each Group.	Concentration of formaldehyde in spleen emulsion.					Interval in Days between Injections.	Result of Immunization.	Immunity Test.		
		1 : 1000 Dose.	1 : 2000 Dose.	1 : 3000 Dose.	1 : 4000 Dose.	1 : 6000 Dose.			Injection of O-virus.	Interval in days since last Injection.	RESULT.
A1. Spleen emulsion prepared from Horses 19882, 19883 and 19907	7	30 c.c.	30 c.c.	30 c.c.	30 c.c.	—	14	(*a*) One horse reacted after 1 : 3000 injection and died (*b*) Two horses reacted to 1 : 4000 injection and recovered (*c*) Four horses showed no reactions	— 5 c.c. 5 c.c.	— 14 and 168 14, 28, 83, and 168	— (*b*) Both horses proved to be immune. (*c*) Two horses (14 and 168 days interval) proved to be immune. Two horses (83 and 28 days interval) reacted and recovered.
A2.....	2	20 c.c.	20 c.c.	20 c.c.	20 c.c.	—	14	Two horses did not react	5 c.c.	28, 83	Both horses proved to be immune.
A3.....	2	15 c.c.	15 c.c.	15 c.c.	15 c.c.	—	14	One horse reacted and died after 1 : 3000 injection; other horse reacted after 1 : 4000 injection and recovered	5 c.c.	13	Remaining horse proved to be immune.
A4.....	2	10 c.c.	10 c.c.	10 c.c.	10 c.c.	—	—	Two horses did not react	5 c.c.	28 and 168	Two horses reacted and recovered.
A5.....	2	5 c.c.	5 c.c.	5 c.c.	5 c.c.	—	14	One horse died after 1 : 3000 injection; other horse reacted after 1 : 4000 injection and recovered	5 c.c.	13	Remaining horse proved to be immune.
B1. Spleen emulsion prepared from Horse 19882	2	30 c.c.	30 c.c.	30 c.c.	30 c.c.	—	14	One horse died after 1 : 2000 injection and the other reacted to 1 : 3000 injection and recovered	5 c.c.	13	Remaining horse proved to be immune.

TABLE III—(*continued.*)

Group.	No. of Horses in each Group.	Concentration of formaldehyde in spleen emulsion.					Interval in Days between Injections.	Result of Immunization.	Immunity Test.		
		1 : 1000 Dose.	1 : 2000 Dose.	1 : 3000 Dose.	1 : 4000 Dose.	1 : 6000 Dose.			Injection of O-virus.	Interval in days. since last Injection.	RESULT.
B2. Spleen emulsion prepared from Horse 19883	2	30 c.c.	30 c.c.	30 c.c.	30 c.c.	—	14	One horse died after 1 : 2000 injection and the other horse did not react	5 c.c.	13	Remaining horse proved to be immune.
B 3. Spleen emulsion prepared from Horse 19907	2	30 c.c.	30 c.c.	30 c.c.	30 c.c.	—	14	Two horse reacted after 1 : 4000 injection, one died and the other recovered	5 c.c.	13	Remaining horse proved to be immune.
C1. Spleen emulsion prepared from Horses 19882, 19883, 19907	3	30 c.c.	—	30 c.c.	30 c.c.	30 c.c.	14	Two horses did not react, one horse reacted after 1 : 4000 injection and recovered	5 c.c.	13, 28, 83	Horses proved to be immune.
C2.....	2	30 c.c.	—	30 c.c.	30 c.c.	—	14	One horse reacted to 1 : 4000 injection and recovered : other horse showed no symptoms	5 c.c.	14	Horses proved to be immune.
C3.....	5	—	30 c.c.	30 c.c.	—	30 c.c.	14	(*a*) Four horses reacted after 1 : 3000 injection of which three died (*b*) The other horse did did not show any symptoms	5 c.c.	14	(*a*) Remaining horse proved to be immune. (*b*) Horse proved to be immune.

Results : Group A 1.—Seven horses received 4·30 c.c. injections of 1 : 1000, 1 : 2000, 1 : 3000 and 1 : 4000 material ; one commenced to react from day 11 after the 1 : 3000 injection and died on day 17 ; after the 1 : 4000 injection one reacted fairly severely from the 6th day to the 10th day, one from the 5th day to the 10th day, but both recovered. The six survivors were solidly immune to ' O ' virus, the immunity test being made in different individuals on the 14th, 28th, 83rd and 168th day after the last immunizing injection.

Group A 2.—Two horses which received injections in 20 c.c. amounts showed no reactions, one being immune on the 28th day, one on the 83rd day.

Group A 3.—Two horses each received 15 c.c. doses ; one commenced to react on the 10th day after the 1 : 3000 injection and died on the 14th day (a delayed reaction), one did not react and was immune on the 13th day.

Group A 4.—Two horses received 10 c.c. amounts. There were no reactions to the immunizing injections. On immunity test after an interval of 28 days the one horse reacted severely from the 2nd day to the 10th day and recovered ; the other horse after an interval of 168 days commenced to react on the 2nd day and died on the 6th day.

Group A 5.—Two horses received 5 c.c. amounts ; the one horse commenced to react on the 6th day after the 1 : 2000 injection and died on the 11th day ; the other survived immunization and fourteen days later was solidly immune to ' O ' virus.

Conclusions.—The results are by no means clear-cut but it would appear to be inadvisable to reduce the dose of vaccine below 20 c.c. This or a larger dose appears to be safe in the vast majority of cases (8 out of 9), and produces an immunity capable of protecting against ' O ' virus for as long as 168 days. Doses smaller than 20 c.c. appear to possess a less favourable balance between antigenic non-virulent fraction and living virus and is less safe (4 out of 6 survived). Moreover, the immunity is of shorter duration since one horse reacted severely after 28 days, one horse died after 168, although two survivors were solidly immune after 13 days.

The material used in the above five groups comprised the pooled emulsion from three spleens. The six horses which make up Group B received injections in 30 c.c. amounts of vaccine prepared from each of the individual spleens.

Results.—Two horses received material from each spleen and of each Group B1 and B2 one died as a result of the 1 : 2000 and in Group B3 one died as result of 1 : 4000 vaccine. In addition one horse reacted severely to the 1 : 4000 material but recovered.

Conclusion.—The results are in striking contrast to those obtained in Group A above. It must be concluded definitely that a safer vaccine can be obtained from the pooled mixture of several spleens than from a single spleen.

Concurrently with the above two groups eight horses were given the series of injections indicated in Groups C1, C2 and C3, with the main object of determining whether a better immunity could be set up by completing the course of injections with 1 : 6000 material, and also of determining whether three injections would not be as effective as four. The final injection of 1 : 6000 was not given in the two horses of C2.

Results.—In Group C1 which were commenced with 1 : 1000 vaccine and then received 1 : 3000, 1 : 4000, and 1 : 6000 vaccine all the animals survived and even after 83 days one horse was still solidly immune.

In Group C2 three injections of 1 : 1000, 1 : 3000 and 1 : 4000 were given. Only one of the horses reacted, viz., to 1 : 4000 vaccine but recovered. Both were found to be immune on the 14th day.

In Group C3 which commenced with 1 : 1500 vaccine and then received 1 : 3000 and 1 : 6000 vaccine four hourses reacted severely to 1 : 3000 vaccine and three died. The two survivors were solidly immune.

Conclusion.—(1) There is no evidence that 1 : 6000 vaccine produces an immunity greater than that set up by 1 : 4000.

(2) It is unsafe to commence with a dilution greater than 1 : 1000 and to jump from 1 : 1500 to 1 : 3000 vaccine may be followed by disaster.

Encouraged by the excellent results obtained in Group C1 above, and bearing in mind that it is apparently not necessary to proceed beyond an injection of 1 : 4000 vaccine an experiment was carried out as shown in Tabel IV.

Object of experiment.—(1) To ascertain whether three injections of 1 : 1000, 1 : 2500 and 1 : 4000 vaccine are both safe and adequate.

(2) To collect data on the keeping quality of vaccine.

Method.—A fresh batch of vaccine was prepared from two spleens and injected into the eight horses in Group A while the same material that was used in Group C of the previous experiment was injected into four horses in Group B after being stored at room temperature for slightly more than four months.

Results.—In Group A, five out of the eight horses reacted to 1 : 4000 vaccine ; of these three died. Of the five survivors four were solidly immune but one reacted severely and recovered. In Group B the two horses that received 1 : 1000, 1 : 2500 and 1 : 4000 vaccine both died as a result of the last injection. Of the two horses that had received 1 : 4000 alone one did not react and one died. The non-reactor proved to be immune.

Conclusion.—(1) The three injections of vaccine are not safe but horses which survive 1 : 4000 vaccine are immune.

(2) There appears to be a definite decrease in antigenic potency after storage for four months at room temperature.

(3) The dose of 10 c.c. apparently is not large enough.

The results obtained in this experiment both as regards the keeping quality of the vaccine, the dose, and the efficacy of three injections were so inconsistent with what had been anticipated that it was decided to carry out an experiment on as large a number of horses as were available, in order to obtain some finality.

Object of the experiment.—(1) To confirm the efficacy of four injections of 1 : 1000, 1 : 2000 and 1 : 3000 and 1 : 4000 vaccine.

(2) To obtain data as to the duration of immunity produced by formalized vaccines.

(3) To ascertain if three injections could not be substituted for the four given in (1) above.

(4) To determine of a concentration of formaldehyde higher than 1 : 4000 which would be safer to include in a course of three injections would still produce a solid immunity to O-virus.

TABLE IV.—EXPERIMENT S. 4271.

Group.	D.O.B. No. of Horses.	Concentration of formaldehyde in spleen emulsion. 1 : 1000 Dose 10 c.c. and Date.	1 : 2500 Dose 10 c.c. and Date.	1 : 4000 Dose 10 c.c. and Date.	Interval in Days between Injections.	Result of Immunization.	Immunity Test. Date of Injection of O-virus.	Interval in days since last Injection.	Result.
A. Spleen emulsion prepared from Horses 19965, 19787	19890	7/11/30	21/11/30	5/12/30	14	Reacted from 5th day to 12th day after 1 : 4000 injection	19/12/30	14	Immune.
	19891	7/11/30	21/11/30	5/12/30	14	No reaction (too wild to be temperatured)	19/12/30	14	Immune.
	19892	14/11/30	28/11/30	12/12/30	14	Reacted from 6th to 13th day after 1 : 4000 injection	29/12/30	17	Immune.
	19893	14/11/30	28/11/30	12/12/30	14	Reacted from 3rd day and died 7th day after 1 : 4000 injection	—	—	—
	19894	14/11/30	28/11/30	12/12/30	14	Reacted from 5th day and died 8th day after 1 : 4000 injection	—	—	—
	19895	14/11/30	28/11/30	12/12/30	14	No reaction.....	29/12/30	17	Immune.
	19896	14/11/30	28/11/30	12/12/30	14	No reaction.....	29/12/30	17	Reacted from 4th to 10th day and recovered.
	19897	14/11/30	28/11/30	12/12/30	14	Reacted from 3rd day and died 7th day after 1 : 4000 injection	—	—	—
B. Spleen emulsion prepared from Horses 19882, 19883, 19907	19898	14/11/30	28/11/30	12/12/30	14	Reacted from 5th day and died 9th day after 1 : 4000 injection	—	—	—
	19899	14/11/30	28/11/30	12/12/30	14	Reacted from 5th day and died 10th day after 1 : 4000 injection	—	—	—
	19992	—	—	17/12/30	—	No reaction.....	29/12/30	12	Immune.
	19993	—	—	23/12/30	—	Reacted from 4th day and died 7th day after 1 : 4000 injection	—	—	—

Method.—The details of injections together with the results will be found in Appendix III, of which a summary is given in Table V of the text. The vaccine was prepared from three spleens and was pooled before bottling or injection. The dose was fixed at 30 c.c. since the smaller dose given above may in part have been responsible for the bad results.

Results: Group A (*A*1–*A*5).—Ten horses received 1 : 1000, 1 : 2000, 1 : 3000 and 1 : 4000 vaccine at 14 or 21 day intervals as indicated. During this course of immunization one horse reacted very mildly to 1 : 1000 vaccine one reacted mildly to 1 : 2000 vaccine, two reacted severely and one died after 1 : 4000 vaccine, while the remaining five showed no reactions. On immunity test after 14 days, two horses were immune, after 21 days two horses appeared to have acquired either no immunity or to have lost it because both died, the one with a slightly prolonged reaction. After 138 days the reaction to O-virus was that which would have been anticipated in fully susceptible horses. Owing to this loss of immunity the remaining three horses were given virus + hyperimmune serum as practised in the simultaneous method of immunization. Only one horse showed a very mild reaction, although at least two severe reactions would have been anticipated in susceptible animals.

*Group A*6.—Ten horses received a course of injections identical with those given in A1–A5 seven months later, i.e., the vaccine had been stored for seven months at room temperature. On this occasion two horses showed a mild febrile reaction after 1 : 2000 vaccine, two reacted more severely to 1 : 3000 vaccine and of two severe reactors to 1 : 4000 vaccine one died and one recovered. An immunity test with O-virus was made 28 days after the last injection when two died, three showed reactions of varying severity and the remaining five were immune. It is worthy of note that the two deaths and three reactors occurred among six horses that had shown no febrile reaction at any time during the process of immunization. The results are not dissimilar to those obtained in the first instance, the vaccine having been stored for approximately seven months at room temperature.

*Group B*1 *and B*2.—Four horses received 1 : 1000, 1 : 2500 and 1 : 3000 vaccine; two reacted severely to the 1 : 3000 injection one dying and the other being destroyed *in extremis*. Of the two survivors one died on testing the immunity after an interval of 21 days, the other animal was given serum and virus 149 days later and showed a mild reaction.

*Group C*1–*C*3.—Four horses received 1 : 1000, 1 : 2500 and 1 : 4000 vaccine, three horses showed no reaction, one reacting severely to 1 : 3500 vaccine and was found to be immune 71 days later. After an interval of 21 days, of the three non-reactors two were found to possess little immunity to O-virus (one died, one reacted severely and recovered); the other was given serum and virus after 149 days and showed no clinical reaction.

*Group D*1–*D*3.—Nine horses received 1 : 1000, 1 : 2500 and 1 : 4000 vaccine; five horses did not react, but four reacted severely to 1 : 4000 vaccine and one died, the survivor being solidly immune after 21 days. Of the non-reactors two showed a severe reaction to O-virus after 21 days but recovered; one died after 146 days; three received serum and virus after 149 days, one showing no reaction.

Group E.—One horse received 1 : 1000, 1 : 2500, 1 : 3000 and 1 : 4000 vaccine. There was no reaction during immunization but O-virus after 14 days produced Dikkop horse-sickness though the animal recovered.

Group F1–F7.—Served as controls for the infectivity of the various formalin dilutions. It will be seen that there was sufficient free virus in 30 c.c. of 1 : 3000, 1 : 3500 and 1 : 4000 vaccine to set up fatal infections in horses. In 1 : 1000 and 1 : 2000 the amount would appear to be on the border line of a single infective dose, since three horses did not react; several horses in the experimental groups, however, undoubtedly reacted to 1 : 2000.

Conclusions.—(1) Possibly the most striking conclusion to be drawn from this experiment is the great variation in antigenic potency of different batches of pooled spleens even though the greatest care is taken to standardize each process in the production of the vaccine. In this experiment it would appear that the vaccine was of low potency, since one horse died after 1 : 4000 injection, and though two horses were immune after 14 days, an immunity sufficient to withstand 5 c.c. of O-virus had disappeared after 28 days. However, even after 149 days there must have been some residual immunity since survivors which received serum and virus reacted at most with a mild fever whereas a mortality of 5 per cent. and a fairly severe reaction in the majority of horses would not have been surprising.

(2) The keeping quality of even a poor batch of vaccine is good since after storage for approximately seven months at room temperature practically no difference in potency could be ascertained in two groups of ten horses.

(3) A formaldehyde concentration of 1 : 1000 is safe as a commencing dose.

(4) It must be concluded that only with a vaccine of high potency are survivors of a 1 : 4000 injection immune to O-virus.

(5) A previous observation that immunity produced by formalized vaccine may last for at least 168 days must be modified, since in this experiment there was a marked decrease in immunity after 28 days though some immunity could be detected after 142 days.

(6) There appears to be a better immunity produced in those animals which show a definite clinical reaction during immunization than in animals which show no reaction.

(7) To complete a series of injections with a formaldehyde concentration greater than 1 : 4000 results in an immunity of lower grade.

(8) The best results are obtained with four injections of 1 : 1000, 1 : 2000, 1 : 3000 and 1 : 4000 vaccine.

(9) Apart from exceptional vaccines of high potency the possibility of reducing the number of vaccinations from four to three does not appear to be good.

(10) The best results obtained with three injections were with 1 : 1000, 1 : 2500 and 1 : 4000 vaccine. Making the second of three injections either 1 : 2000 or 1 : 3000 is not safe.

At this stage attention must be directed to the fact that the injection of 5 c.c. of O-virus intravenously is an exceeding severe immunity test, possibly more severe than a test of immunity by exposure to natural infection. No data are available to substantiate this expression of opinion, however, since the transmitter of the virus under field conditions is not known. Still, horses which have received O-virus together with hyperimmune serum have reacted in a manner certainly indicative of the presence of some immunity. Consequently, it was decided to run a further series of experiments to ascertain whether it would not be possible to combine the simultaneous serum virus method with the formalized spleen virus method in the hope :—

(1) That the amount of serum required for each injection could be considerably reduced.

(2) That the margin of safety of the formalized vaccine method might be increased.

(3) That the danger of the consolidating dose of virus might be minimized by the injection of serum simultaneously.

Group.	No. of Horses in each Group.	Concentration of formaldehyde in spleen emulsion.										
		1 : 1000 Dose.	Interval in Days.	1 : 2000 Dose.	Interval in Days.	1 : 2500 Dose.	Interval in Days.	1 : 3000 Dose.	Interval in Days.	1 : 3500 Dose.	Interval in Days.	1 : 4000. Dose.
A1	2	30 c.c.	14	30 c.c.	—	—	14	30 c.c.	—	—	14	30 c.c.
A2	2	30 c.c.	14	30 c.c.	—	—	14	30 c.c.	—	—	14	30 c.c.
A3	1	30 c.c.	14	30 c.c.	—	—	14	30 c.c.	—	—	14	30 c.c.
A4	3	30 c.c.	14	30 c.c.	—	—	14	30 c.c.	—	—	21	30 c.c.
A5	2	30 c.c.	14	30 c.c.	—	—	14	30 c.c.	—	—	21	30 c.c.
A6	10	30 c.c.	14	30 c.c.	—	—	21	30 c.c.	—	—	21	30 c.c.
B1	3	30 c.c.	—	—	14	30 c.c.	21	30 c.c.	—	—	—	—
B2	1	30 c.c.	—	—	14	30 c.c.	21	30 c.c.	—	—	—	—
C1	2	30 c.c.	—	—	14	30 c.c.	—	—	21	30 c.c.	—	—
C2	1	30 c.c.	—	—	14	30 c.c.	—	—	21	30 c.c.	—	—
C3	1	30 c.c.	—	—	14	30 c.c.	—	—	21	30 c.c.	—	—
D1	4	30 c.c.	—	—	14	—	—	—	—	—	21	30 c.c.
D2	2	30 c.c.	—	—	14	30 c.c.	—	—	—	—	21	30 c.c.
D3	3	30 c.c.	—	—	14	30 c.c.	—	—	—	—	21	30 c.c.
E1	1	30 c.c.	—	—	14	30 c.c.	21	30 c.c.	—	—	41	30 c.c.
F1	1	—	—	30 c.c.	—	—	—	—	—	—	—	—
F2	1	—	—	—	—	30 c.c.	—	—	—	—	—	—
F3	2	—	—	—	—	—	—	30 c.c.	—	—	—	—
F4	1	—	—	—	—	—	—	—	—	30 c.c.	—	—
F5	1	—	—	—	—	—	—	—	—	—	—	30 c.c.
F6	1	—	—	30 c.c.	—	—	—	—	—	—	22	30 c.c.
F7	1	—	—	—	—	30 c.c.	—	—	—	—	22	30 c.c.

Result of Immunization.	Interval in Days since last Injection.	Injection of hyperimmune serum and N.-virus.	Interval in Days.	Injection of hyperimmune serum and O.-virus.	Injection of O.-virus.	Result.
One horse showed a mild febrile reaction after 1 : 1000 injection and the other showed no symptoms	21	—	—	—	5 c.c.	Both horses died from horse-sickness.
No reactions observed.....	138	—	—	—	5 c.c.	Both horses died from horse-sickness.
Febrile reaction after 1 : 2000 injection	142	400 + 5 c.c.	3	400 + 5 c.c.	—	Very mild febrile horse-sickness reaction and recovered.
Two horses reacted after 1 : 4000 injection, and recovered. One horse died after 1 : 4000 injection.	14	—	—	—	5 c.c.	Remaining two horses proved to be immune.
No reactions observed.....	136	400 + 5 c.c.	3	400 + 5 c.c.	—	Horses proved to be immune.
(*a*) No reactions observed in four horses (*b*) Febrile reactions observed in two horses after 1 : 2000 injection (*c*) Febrile reactions observed in two horses after 1 : 3000 injection (*d*) Febrile reactions observed in two horses after 1 : 4000 injection, one of which died	28	—	—	—	5 c.c.	(*a*) One horse reacted and died from horse-sickness. Two horses reacted and recovered. One horse proved to be immune. (*b*) One horse reacted and recovered. One horse reacted and died from horse-sickness. (*c*) Both horses proved to be immune. (*d*) Remaining horse proved to be immune.
(*a*) Mild febrile reaction observed in one horse after 1 : 1000 injection (*b*) Two horses showed reactions after 1 : 3000 injection ; one horse died and the other was killed *in extremis*	21	—	—	—	5 c.c.	(*a*) Horse died from horse-sickness.
No reaction observed.....	149	400 + 5 c.c.	3	400+ 5 c.c.	—	Mild febrile horse-sickness reaction and recovered.
No reactions observed.....	21	—	—	—	5 c.c.	Both horses reacted to horse-sickness. One recovered and one died.
Reacted after 1 : 3500 injection and recovered	71	—	—	—	5 c.c.	Horse proved to be immune.
No reaction observed.....	149	400 + 5 c.c.	3	400 + 5 c.c.	—	Horse proved to be immune.
(*a*) One horse showed no reaction (*b*) Two horses reacted after 1 : 4000 injection, one of which died (*c*) One horse showed febrile reaction after 1 : 1000	21	—	—	—	5 c.c.	(*a*) Mild febrile reaction and recovered. (*b*) Horse proved to be immune. (*c*) Reacted severely and recovered.
No reactions observed.....	145	—	—	—	5 c.c.	One horse proved to be immune and the other died from horse-sickness.
(*a*) One horse did not react... (*b*) Two horses reacted after 1 : 4000 injection and re-recovered	149	400 + 5 c.c.	3	400 + 5 c.c.	—	Three horses proved to be immune.
No reaction observed.....	15	—	—	—	5 c.c.	Reacted severely and recovered.
No reaction observed.....	21	—	—	—	5 c.c.	Died from horse-sickness.
No reaction observed.....	21	—	—	—	5 c.c.	Died from horse-sickness.
One horse showed no reaction, and the other died	26	—	—	—	—	Died from horse-sickness.
Died from horse-sickness.....	—	—	—	—	—	—
Died from horse-sickness.....	—	—	—	—	—	—
No reaction.....	20	—	—	—	5 c.c.	Died from horse-sickness.
Died from horse-sickness.....	—	—	—	—	—	—

TABLE V.—EXPERIMENT S. 1496.

Group.	No. of Horses in each Group.	Concentration of formaldehyde in spleen emulsion. 1 : 1000 Dose.	Interval in Days.	1 : 2000 Dose.	Interval in Days.	1 : 2500 Dose.	Interval in Days.	1 : 3000 Dose.	Interval in Days.	1 : 3500 Dose.	Interval in Days.	1 : 4000 Dose.	Result of Immunization.	Interval in Days since last Injection.	Injection of hyperimmune serum and N.-virus.	Interval in Days.	Injection of hyperimmune serum and O.-virus.	Injection of O.-virus.	RESULT.
A1	2	30 c.c.	14	40 c.c.			14	30 c.c.		—	14	30 c.c.	One horse showed a mild febrile reaction after 1 : 1000 injection and the other showed no symptoms	21				5 c.c.	Both horses died from horse-sickness.
A2	2	30 c.c.	14	30 c.c.			14	30 c.c.			14	30 c.c.	No reactions observed.....	138				5 c.c.	Both horses died from horse-sickness.
A3	1	30 c.c.	14	30 c.c.			14	30 c.c.			14	30 c.c.	Febrile reaction after 1 : 2000 injection	142	100 + 5 c.c.	3	100 + 5 c.c.		Very mild febrile horse-sickness reaction and recovered.
A4	3	30 c.c.	14	30 c.c.			14	30 c.c.			21	30 c.c.	Two horses reacted after 1 : 4000 injection, and recovered. One horse died after 1 : 4000 injection.	14				5 c.c.	Remaining two horses proved to be immune.
A5	2	30 c.c.	14	30 c.c.			14	30 c.c.		—	21	30 c.c.	No reactions observed.....	136	100 + 5 c.c.	3	100 + 5 c.c.		Horses proved to be immune.
A6	10	30 c.c.	14	30 c.c.			21	30 c.c.		—	21	30 c.c.	(*a*) Not all reactions observed in four horses (*b*) Febrile reactions observed in two horses after 1 : 2000 injection (*c*) Febrile reactions observed in two horses after 1 : 3000 injection (*d*) Febrile reactions observed in two horses after 1 : 4000 injection, one of which died	28				5 c.c.	(*a*) One horse reacted and died from horse-sickness. Two horses reacted and recovered. One horse proved to be immune. (*b*) One horse reacted and recovered. One horse reacted and died from horse-sickness. (*c*) Both horses proved to be immune. (*d*) Remaining horse proved to be immune.
B1	3	30 c.c.			14	30 c.c.	21	30 c.c.					(*a*) Mild febrile reaction observed in one horse after 1 : 1000 injection (*b*) Two horses showed reactions after 1 : 3000 injection; one horse died and the other was killed *in extremis*	21				5 c.c.	(*a*) Horse died from horse-sickness.
B2	1	30 c.c.			14	30 c.c.	21	30 c.c.					No reaction observed.....	149	100 + 5 c.c.	3	100 + 5 c.c.	—	Mild febrile horse-sickness reaction and recovered.
C1	2	30 c.c.			14	30 c.c.			21	30 c.c.			No reactions observed.....	21				5 c.c.	Both horses reacted to horse-sickness. One recovered and one died
C2	1	30 c.c.			14	30 c.c.			21	30 c.c.			Reacted after 1 : 3500 injection and recovered	71				5 c.c.	Horse proved to be immune.
C3	1	30 c.c.			14	30 c.c.			21	30 c.c.		—	No reaction observed.....	149	100 + 5 c.c.	3	100 + 5 c.c.	—	Horse proved to be immune.
D1	4	30 c.c.			14						21	30 c.c.	(*a*) One horse showed no reaction (*b*) Two horses reacted after 1 : 4000 injection, one of which died (*c*) One horse showed febrile reaction after 1 : 4000	21				5 c.c.	(*a*) Mild febrile reaction and recovered. (*b*) Horse proved to be immune. (*c*) Reacted severely and recovered.
D2	2	30 c.c.			14	30 c.c.					21	30 c.c.	No reactions observed.....	145				5 c.c.	One horse proved to be immune and the other died from horse-sickness.
D3	3	30 c.c.			14	30 c.c.					21	30 c.c.	(*a*) One horse did not react... (*b*) Two horses reacted after 1 : 4000 injection and recovered	149	100 + 5 c.c.	3	100 + 5 c.c.		Three horses proved to be immune.
E1	1	30 c.c.			14	30 c.c.	21	30 c.c.		—	41	30 c.c.	No reaction observed.....	15				5 c.c.	Reacted severely and recovered.
F1	1			30 c.c.	—	—	—	—					No reaction observed.....	21				5 c.c.	Died from horse-sickness.
F2	1			—	—	30 c.c.	—	—					No reaction observed.....	21				5 c.c.	Died from horse-sickness.
F3	2			—	—	—	—	30 c.c.					One horse showed no reaction, and the other died	26					Died from horse-sickness.
F4	1								—	30 c.c.	—		Died from horse-sickness.....						—
F5	1										—	30 c.c.	Died from horse-sickness.....						—
F6	1			30 c.c.	—	—	—	—	—	—	22	30 c.c.	No reaction.....	20				5 c.c.	Died from horse-sickness
F7	1					30 c.c.	—	—	—	—	22	30 c.c.	Died from horse-sickness.....	—				—	—

A summary of the injections in this experiment is given in Table VI, while full details of the reactions to each injection are given in Appendix IV.

TABLE VI.

Group.	No. of Horses.	Concentration of formaldehyde in spleen emulsion.								N.-virus + Hyper-immune serum Dose.	Interval in Days.	O.-virus + Hyper-immune serum Dose.	Interval in Days.	O.-virus. Dose.	RESULT.
		1 : 1000 Dose.	Interval in Days.	1 : 2000 Dose.	Interval in Days.	1 : 3000 Dose.	Interval in Days.	1 : 4000 Dose.	Interval in Days.						
I.	10	—	—	—	—	—	—	—	—	5 + 400 c.c.	3	5 + 400 c.c.	—	—	Two horses died ; eight showed severe reactions.
II.	10	60 c.c.	—	—	—	—	—	—	14	5 + 400 c.c.	3	5 + 400 c.c.	—	—	Following serum + virus two horses died, five reacted severely but recovered, two reacted mildly and one did not react.
III.	2	60 c.c.	—	—	—	—	—	—	14	5 + 200 c.c.	3	5 + 200 c.c.	—	—	One horse showed a moderate reaction, the other a mild reaction.
IV.	7	60 c.c.	—	—	14	30 + 400 c.c. serum	—	—	—	—	3	5 + 400 c.c.	—	—	Three horses showed severe and four moderate reactions. All recovered.
V.	2	60 c.c.	—	—	14	30 + 200 c.c. serum	—	—	—	—	3	5 + 200 c.c.	—	—	Both horses reacted severely but recovered.
VI.	2	60 c.c.	14	60 c.c.	—	—	—	—	—	—	14	5 + 400 c.c.	—	—	One horse reacted severely and recovered ; one showed no reaction.
VII.	2	60 c.c.	14	60 c.c.	—	—	—	—	—	—	14	5 + 200 c.c.	—	—	One horse succumbed to the 1 : 1000 formalized spleen emulsion, the other died after serum-virus injection.
VIII.	2	60 c.c.	14	60 c.c.	14	60 c.c.	—	—	—	—	14	5 + 200 c.c.	—	—	One horse reacted severely, the other mildly.
IX.	2	30 c.c.	14	30 c.c.	14	30 c.c.	—	—	—	—	14	5 + 200 c.c.	—	—	One horse died the other reacted severely but recovered.
X.	5	30 c.c.	14	30 c.c.	14	30 c.c.	14	30 c.c.	—	—	14	5 + 400 c.c.	—	—	Three reacted mildly ; two did not react ; one horse reacted severely after 1 : 4000 injection.
XI.	5	30 c.c.	14	30 c.c.	14	30 c.c.	14	30 c.c.	—	—	14	5 + 200 c.c.	—	—	One horse died, two reacted, severely, one reacted mildly one did not react.
XII.	5	30 c.c.	14	30 c.c.	14	30 c.c.	14	30 c.c.	—	—	—	—	14	5 c.c.	Two horses died, two reacted severely, one reacted mildly.

It will be observed that the experiment was planned in such a way as to vary from a group of horses immunized by the simultaneous serum virus method to a group of horses immunized by what is considered the safest and most efficient formalized vaccine method, namely, four injections of 1 : 1000, 1 : 2000, 1 : 3000, 1 : 4000 vaccine at 14 day intervals. Intermediate groups were included to constitute a gradation from the one extreme to the other, and at the same time to attempt to reduce the amount of hyperimmune serum necessary to control the reactions to the fully virulent virus.

Results.—Group I.—Of the ten horses immunized by the serum virus method two horses died of Dikkop horse-sickness on the 12th and 13th day respectively, while the remaining eight recovered after undergoing severe clinical reactions.

Group II.—When 60 c.c. of 1 : 1000 vaccine was given as a preliminary immunizing injection 14 days prior to the serum virus method the result was slightly better, though the percentage mortality was the same, i.e., two out of ten died, one horse showed no symptoms, one reacted mildly and five survived severe reactions.

Group III.—This group differed from II above only in respect of the dose of serum which was reduced by half to 200 c.c.; both horses survived, one after a severe, the other after a mild reaction.

Group IV.—A preliminary injection of 60 c.c. of 1 : 1000 vaccine was given, but the " N " virus in the first of the serum virus injections was replaced by 1 : 3000 vaccine a " strength " of vaccine to which at least a very severe reaction would have been expected. Out of seven horses three showed mild reaction, and four reacted severely but recovered.

Group V.—This group differed from IV above by the reduction of the dose of serum to half. Of two horses both reacted severely but recovered.

Groups VI and VII received two injections of vaccine (1 : 1000 and 1 : 2000) followed by O-virus + serum. When the dose of the serum was 400 c.c. both horses recovered, although one reacted severely. When the dose of serum was 200 c.c. the one horse that survived vaccination died.

Groups VIII and IX, received three injections of vaccine (1 : 1000, 1 : 2000, 1 : 3000) followed by O-virus and a half dose of serum. When the dose of vaccine was 60 c.c. both horses recovered, although one reacted severely. When the dose was 30 c.c. one reacted severely and recovered, the other died.

Groups X, XI, and XII received the ordinary course of four formalized spleen injections but five horses received a consolidating or immunity test injection of O-virus, five received O-virus + half dose of hyperimmune serum, and five received O-virus + a full dose of hyperimmune serum. Only one horse showed a reaction to vaccination. When O-virus alone was given two horses died, one recovered after a severe reaction, and two reacted mildly. With the addition of 200 c.c. of serum, one died, two reacted severely, one mildly and one was solidly immune. But with 400 c.c. of serum there were no deaths, three reacted very mildly and two showed no clinical disturbance.

Conclusions.—A direct comparison of the two methods of immunization is possible from this series of experiments, though it must be stated that the results obtained with the serum virus method were not good since the reactions were more severe and the mortality was higher than usually seen in the field.

On the other hand, the antigenic value of the batch of three pooled formalized spleens used was poor since two out of five horses which survived the 1 : 4000 injection developed an immunity insufficient to protect against 5 c.c. of O-virus intravenously.

The four injections of formalized vaccine caused a severe reaction in only one out of fifteen horses, but a degree of immunity was produced which could protect against O-virus only when modified by the simultaneous injection of a massive dose of hyperimmune serum. Previous work has shown that formalized virus sets up an immunity which may decrease to a low level is as short a time as 28 days so that the consolidating dose of virus must be regarded not as an immunity test but as an integral part of the prophylactic treatment. Consequently, unless massive doses of hyperimmune serum are used the formalized vaccine method has no advantage over the serum virus method in respect of safety. No data are available as to the incidence of alarming sequelae such as staggers, but in view of the necessity for a massive dose of serum and the suspected relationship between large doses of serum and the incidence of staggers, any advantage in this respect is doubtful. A comparison between the degree of protection to natural infection in the field could only be made after several seasons exposure of immunized horses, but since a single virus has been used in the vaccine method, and since two antigenically different strains are used in the serum virus method, it is not anticipated that any advantage will rest with the vaccine method.

For the rest it would appear that no striking results can be expected from a more intimate fusion or combination of the two methods.

DISCUSSION.

The previous observation that horses may be immunized by a series of injections of spleen virus inactivated by progressively decreasing concentrations of formaldehyde has been confirmed. When due regard is paid to the practical necessity of reducing the number of injections to the minimum commensurate with safety and efficacy, the best results have been obtained with four injections of spleen virus inactivated by concentrations of 1 : 1000, 1 : 2000, 1 : 3000, and 1 : 4000 formaldehyde. To commence immunization with a vaccine containing a formaldehyde concentration less than 1 : 1000 is definitely dangerous; repeated injections of 1 : 1000 vaccine has been shown previously not to produce an immunity of high order, but horses which survive a 1 : 4000 vaccine after suitable preliminary treatment are in the vast majority of instances solidly immune to O-virus. The progression from 1 : 1000 to 1 : 4000 vaccine must be slow and gradual if the danger of breakdowns to horse-sickness is to be avoided.

The antigenic value of different individual spleens varies within wide limits. To obtain a final product of high potency it is essential to pool the material prepared from three spleens at the very least. Preferably batches of vaccine should be made up to comprise the spleens of a greater number of horses, since the vaccine from three has been shown to vary greatly in value.

Phenol in a concentration of 0·25 per cent. may be added as a preservative either before or after formalizing as it appears to have no detrimental effect upon the potency of the vaccine. At the same time it must be remembered that phenol neither increases nor decreases the attenuating action of formalin. Filtration through Seitz discs as an aid to the production of a sterile vaccine is definitely contra-indicated since the living virus appears partly to pass through with the filtrate while the avirulent antigenic portion is retained.

When the potency of a particular batch of vaccine is higher than the average the immunity produced may be of considerable duration, it has been proved to be of a high order, as long as six months after the last injection. On the other hand, even after as short an interval as 28 days immunity has been found to fall to such a level that it was unable to protect against O-virus. Consequently, the injection of fully virulent virus should be regarded not so much as an immunity test but rather as an integral portion of the process of immunization. Exposure to nature infection may be considered as an alternative, but in South Africa the seasonable occurrence of horse-sickness is so variable and so irregular, that, in the absence of any definite knowledge of the natural transmitter, this procedure is too uncertain to be of practical value.

In all the work reported here the strain of virus which has been used is O-virus. This strain has been maintained in horses by serial passage for more than 250 generations over a period of approximately twenty-five years. Consequently, due consideration should be given to the possibility of this virus having become 'fixed' for horses without diminition of antigenic value. If this is conceded it will indicate that the injection of O-virus with its enhanced infectivity constitutes too serious a test on the immunity of the horse. This is borne out by the observation that, if a dose of hyperimmune serum is given simultaneously with the virus the reaction is almost completely blocked, although this would certainly not be the case with fully susceptible horses. There exists a possibility that a similar end result would be obtained if a less virulent and antigenically similar strain of virus was used for the consolidating injection. If it is found to be essential to use hyperimmune serum then one of the chief advantages of the formalized vaccine disappears.

On the whole the results with formalized vaccine have been disappointing. The margin of safety appears to be too narrow to permit of application of the method to the field on a large scale. But what is of equal importance is the fact that by the methods investigated so far there does not appear to be a reasonable possibility of advancing to the production of a polyvalent vaccine, so that the number of subsequent breakdowns cannot be less than is experienced by the present simultaneous serum virus method. Consequently, it must be concluded reluctantly that in the present state of our knowledge the application of the formalized spleen virus method holds out little hope of solving many of the difficulties encountered in the immunization of horses against horse-sickness.

SUMMARY.

(1) The antigenic value of individual spleens varies greatly.

(2) The product of several pooled spleens is more potent than that obtained from a single spleen.

(3) The potency of different batches of pooled spleens varies.

(4) The keeping quality of formalized vaccines for four months is good. After that time there is evidence of decrease in value (not tested after seven months).

(5) The addition of phenol as a bactericide has no detrimental effect.

(6) Filtration through a Seitz filter is contra-indicated.

(7) The lowest concentration of formaldehyde required to produce a safe vaccine is 1 : 1000.

(8) The lowest concentration of formaldehyde able to produce a solid immunity to O-virus is 1 : 4000.

(9) It is not safe to proceed from a 1 : 1000 vaccine to a 1 : 4000 vaccine n less than two intermediate steps.

(10) The best results have been obtained with four injections of 1 : 1000, 1 : 2000, 1 : 3000 and 1 : 4000 vaccine.

(11) The interval between injections should not be less than 14 days, or greater than 21 days.

(12) The dose of vaccine should not be smaller than 20 c.c. A dose of 30 c.c. has given the most constant results.

(13) The immunity produced by the vaccine may last for as long as six months. Usually it is transient and on accasion has markedly decreased in 28 days.

(14) It is necessary to complete immunization by a dose of fully virulent virus.

(15) The margin of safety of the method is narrow.

(16) The application of the method to the field cannot be recommended in the present state of our knowledge.

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APPENDIX I.—EXPERIMENT 4351.

Group.	D.O.B. No. of Horses.	Concentration of formaldehyde in spleen emulsion. Material passed through Seitz filter before injection.											Result of Immunization.	Immunity Test.		
		1 : 1000 Dose 30 c.c. and Date.	Interval in Days.	1 : 2000 Dose 30 c.c. and Date.	Interval in Days.	1 : 2500 Dose 30 c.c. and Date.	Interval in Days.	1 : 3000 Dose 30 c.c. and Date.	Interval in Days.	1 : 3500 Dose 30 c.c. and Date.	Interval in Days.	1 : 4000 Dose 30 c.c. and Date.		Date of Injection of O.-virus.	Interval in days since last Injection.	RESULT.
A.	20054	10·2·31	14	24·2·31	—	—	14	10·3·31	—	—	21	31·3·31	Reacted from 5th day and died 9th day after 1 : 4000 injection	—	—	—
	20055	10·2·31	14	24·2·31	—	—	14	10·3·31	—	—	21 28	31·3·31 30 c.c. unfiltered 28·4·31	Reacted from 15th to 20th day after 1 : 2000 injection	13·5·31	14	Severe reaction 3rd to 11th day and recovered.
	20056	10·2·31	14	24·2·31	—	—	14	10·3·31	—	—	21 28	31·3·31 30 c.c. unfiltered 28·4·31	No reaction..... Reacted 5th day and died 8th day after 1 : 4000 injection	—	—	—
B.	20057	10·2·31	—	—	14	24·2·31	—	—	21	17·3·31	—	—	Reacted from 6th day and died 8th day after 1 : 3500 injection	—	—	—
	20058	10·2·31	—	—	14	24·2·31	—	—	21	17·3·31	—	—	Reacted from 6th day and died 9th day after 1 : 3500 injection	—	—	—
	20059	10·2·31	—	—	14	24·2·31	—	—	21	17·3·31	—	—	Reacted 6th day and died 9th day after 1 : 3500 injection	—	—	—
C.	20060	10·2·31	—	—	14	24·2·31	21	17·3·31	—	—	22	8·4·31	Reacted from 8th day and died 13th day after 1 : 4000 injection	—	—	—
	20061	10·2·31	—	—	14	24·2·31	21	17·3·31	—	—	22	8 4 31	Reacted from 5th day and died 9th day after 1 : 4000 injection	—	—	—
	20062	10·2·31	—	—	14	24·2·31	21	17·3·31	—	—	22	8·4·31	Reacted from 6th day and died 9th day after 1 : 4000 injection	—	—	—
D.	20063	10·2·31	—	—	14	24·2·31	—	—	—	—	21	17·3·31	Severe reaction 6th to 11th day after 1 : 4000 injection	8·4·31	22	Reacted from 3rd day and died 6th day.
	20064	10·2·31	—	—	14	24·2·31	—	—	—	—	21	17·3·31	Reacted from 2nd day to 7th day after 1 : 2500 injection	8·4·31	22	Severe reaction 3rd to 9th day and recovered.
	20065	10·2·31	—	—	14	24·2·31	—	—	—	—	21	17·3·31	Reacted from 4th day and died 8th day after 1 : 4000 injection	—	—	—

Appendix II.—Experiment 4193.

Group.	D.O.B. No. of Horses.	Concentration of formaldehyde in spleen emulsion. 1 : 1000 Dose and Date.	1 : 1500 Dose and Date.	1 : 2000 Dose and Date.	1 : 3000 Dose and Date.	1 : 4000 Dose and Date.	1 : 6000 Dose and Date.	Interval in days between Injections.	Result of Immunization.	Immunity Test. Date of Injection of O. virus.	Interval in days since last Injection.	Result.
A1. Spleen emulsion prepared from Horses 19907, 19883, 19882	19853	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	Reacted severely from 6th to 10th day after 1 : 4000 injection	3/9/30	14	Proved to be immune.
	19854	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	No reaction (too wild to be temperatured)	3/9/30	14	Proved to be immune.
	19856	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	No reaction (too wild to be temperatured)	18/9/30	28	Reacted from 5th to 10th day and recovered.
	19857	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	No reaction (too wild to be temperatured)	12/11/30	83	Reacted from 4th to 8th day and recovered.
	19858	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	No reaction (too wild to be temperatured)	4/2/31	168	Proved to be immune.
	19859	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	Reacted from 11th and died 17th day after 1 : 3000 injection	—	—	—
	19860	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	Reacted severely from 5th to 10th day after 1 : 4000 injection and recovered	4/2/31	168	Proved to be immune.
A2.	19861	20 c.c. 9/7/30		20 c.c. 22·7·30	20 c.c. 6/8/30	20 c.c. 21·8·30	—	14	No reaction (too wild to be temperatured)	18/9/30	28	Proved to be immune.
	19862	20 c.c. 9/7/30	—	20 c.c. 22·7·30	20 c.c. 6/8/30	20 c.c. 21·8·30	—	14	No reaction (too wild to be temperatured)	12/11/30	83	Proved to be immune.
A3.	19863	15 c.c. 9/7/30	—	15 c.c. 22·7·30	15 c.c. 6/8/30	—	—	14	Reacted from 11th and died 14th day after 1 : 3000 injection	—	—	—
	19864	15 c.c. 9/7/30	—	15 c.c. 22·7·30	15 c.c. 6/8/30	15 c.c. 21·8·30	—	14	Mild reaction 6th to 12th day after 1 : 4000 injection	3/9/30	13	Proved to be immune.
A4.	19865	10 c.c. 9/7/30	—	10 c.c. 22·7·30	10 c.c. 6/8/30	10 c.c. 21·8·30	—	14	No reaction.....	18/9/30	28	Reacted from 3rd to 9th day and recovered.
	19866	10 c.c. 9/7/30	—	10 c.c. 22·7·30	10 c.c. 6/8/30	10 c.c. 21·8·30	—	14	No reaction.....	4/2/31	168	Reacted from 2nd and died on 6th day.
A5.	19867	5 c.c. 9/7/30	—	5 c.c. 22·7·30	5 c.c. 6/8/30	—	—	14	Reacted from 7th and died on 11th day after 1 : 3000 injection	—	—	—
	19868	5 c.c. 9/7/30	—	5 c.c. 22·7·30	5 c.c. 6/8/30	5 c.c. 21·8·30	—	14	Reacted from 8th to 16th day after 1 : 4000 injection	3/9/30	13	Proved to be immune.

APPENDIX II—(*continued.*)

Group.	D.O.B. No. of Horses.	Concentration of formaldehyde in spleen emulsion. 1 : 1000 Dose and Date.	1 : 1500 Dose and Date.	1 : 2000 Dose and Date.	1 : 3000 Dose and Date.	1 : 4000 Dose and Date.	1 : 6000 Dose and Date.	Interval in days between Injections.	Result of Immunization.	Immunity Test. Date of Injection of O.-virus.	Interval in days since last Injection.	Result.
B1. Spleen emulsion prepared from Horse 19882	19869	30 c.c. 9/7/30	—	30 c.c. 22·7·30	—	—	—	14	Reacted from 6th and died on 9th day after 1 : 2000 injection	—	—	—
	19870	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	Reacted from 5th to 12th day after 1 : 3000 injection	3/9/30	13	Proved to be immune.
B2. Spleen emulsion prepared from Horse 19883	19873	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	No reaction.....	3/9/30	13	Proved to be immune.
	19874	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	—	—	14	Reacted from 7th and died 17th day after 1 : 2000 injection	—	—	—
B3. Spleen emulsion prepared from Horse 19907	19875	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	Reacted from 6th and died 8th day after 1 : 4000 injection	—	—	—
	19876	30 c.c. 9/7/30	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	—	14	Reacted from 6th to 13th day after 1 : 4000 injection	3/9/30	13	Proved to be immune.
C1. Spleen emulsion prepared from Horses 19882, 19883, 19907	19885	30 c.c. 9/7/30	—	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	14	No reaction.....	3/9/30	13	Proved to be immune.
	19886	30 c.c. 9/7/30	—	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	14	Reacted from 12th to 18th day after 1 : 4000 injection	18/9/30	28	Proved to be immune.
	19887	30 c.c. 9/7/30	—	—	30 c.c. 22·7·30	30 c.c. 6/8/30	30 c.c. 21·8·30	14	No reaction. Too wild to be temperatured	12/11/30	83	Proved to be immune.
C2.	19888	30 c.c. 9/7/30	—	—	30 c.c. 22·7·30	30 c.c. 6/8/30	—	14	No reaction. Too wild to be temperatured	20/8/30	14	Proved to be immune.
	19889	30 c.c. 9/7/30	—	—	30 c.c. 22·7·30	30 c.c. 6/8/30	—	14	Reacted from 5th to 11th day after 1 : 4000 injection	20/8/30	14	Proved to be immune.
C3.	19877	—	30 c.c. 9/7/30	—	30 c.c. 22·7·30	—	30 c.c. 6/8/30	14	Reacted from 15th to 23rd day after 1 : 3000 injection	20/8/30	14	Proved to be immune.
	19878	—	30 c.c. 9/7/30	—	30 c.c. 22·7·30	—	30 c.c. 6/8/30	14	Reacted from 6th and died 9th day after 1 : 3000 injection	—	—	—
	19879	—	30 c.c. 9/7/30	—	30 c.c. 22·7·30	—	30 c.c. 6/8/30	14	No reaction.....	20/8/30	14	Proved to be immune.
	19880	—	30 c.c. 9/7/30	—	30 c.c. 22·7·30	—	—	14	Reacted from 5th and died 9th day after 1 : 3000 injection	—	—	—
	19884	—	30 c.c. 9/7/30	—	30 c.c. 22·7·30	—	30 c.c. 6/8/30	14	Reacted from 14th and died 20th day after 1 : 3000 injection	—	—	—

Group.	D.O.B. No. of Horse.	Concentration of formaldehyde in spleen emulsion.										Result of Immunization.	Interval in days since last injection.	Injection of 400 c.c. hyper-immune serum + 5 c.c. N.-virus.	Interval in days.	Injection of 400 c.c. hyper-immune serum + 5 c.c. O.-virus.	Injection of 5 c.c. O. virus.	RESULT.
		1 : 1000 dose 30 c.c. Date.	Interval in days.	1 : 2000 dose 30 c.c. Date.	Interval in days.	1 : 2500 dose 30 c.c. Date.	Interval in days.	1 : 3000 dose 30 c.c. Date.	Interval in days.	1 : 3500 dose 30 c.c. Date.	Interval in days.	1 : 4000 dose 30 c.c. Date.						
A1.	20017	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	14	25/3/31	Mild febrile reaction from 12th to 16th day after 1 : 1000 injection	21	—	—	—	15/4/31	Reacted from 5th day and died 10th day from horse-sickness.
	20019	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	14	25/3/31	No reaction.....	21	—	—	—	15/4/31	Reacted from 3rd and died 6th day from horse-sickness.
A2.	20020	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	14	25/3/31	No reaction. Exposed to natural infection. During that period no symptoms of horse-sickness were observed	138	—	—	—	10/8/31	Reacted from 2nd and died 5th day from horse-sickness.
	20021	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	14	25/3/31	No reaction. Exposed to natural infection. During that period no symptoms of horse-sickness were observed	138	—	—	—	10/8/31	Reacted from 2nd and died 6th day from horse-sickness.
A3.	20022	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	14	25/3/31	Febrile reaction from 8th to 16th day after 1 : 2000 injection. Exposed to natural infection	142	14/8/31	3	17/8/31	—	Very mild febrile reaction from 7th to 13th day. Immune to horse-sickness.
A4.	20023	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	21	1/4/31	Severe horse-sickness febrile reaction from 6th to 10th day after 1 : 4000 injection and recovered	14	—	—	—	15/4/31	Immune to horse-sickness.
	20024	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	21	1/4/31	Mild horse-sickness febrile reaction from 7th to 11th day after 1 : 4000 and recovered	14	—	—	—	15/4/31	Immune to horse-sickness.
	20027	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	21	1/4/31	Severe horse-sickness reaction from 5th and died 10th day after 1 : 4000	—	—	—	—	—	—
A5.	20025	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	21	1/4/31	No reaction. Exposed to natural infection	136	14/8/31	3	17/8/31	—	No reaction. Immune to horse-sickness.
	20026	11/2/31	14	25/2/31	—	—	14	11/3/31	—	—	21	1/4/31	No reaction. Exposed to natural infection	136	14/8/31	3	17/8/31	—	No reaction. Immune to horse-sickness.
A6.	20186	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	No reaction.....	28	—	—	—	27/11/31	Reacted from 3rd and died 8th day from horse-sickness.
	20187	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	No reaction.....	28	—	—	—	27/11/31	Severe reaction 3rd to 10th day and recovered.
	20188	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	Severe reaction 5th to 11th day after 1 : 3000 injection, recovered	28	—	—	—	27/11/31	Immune to horse-sickness.
	20189	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	No reaction.....	28	—	—	—	27/11/31	Severe reaction 3rd to 12th day and recovered.
	20190	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	Reacted severely from 5th and died 9th day after 1 : 4000 injection	—	—	—	—	—	—
	20191	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	Reacted severely from 11th to 18th day after 1 : 4000 injection and recovered	28	—	—	—	27/11/31	Immune to horse-sickness.
	20192	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	Reacted severely from 17th to 29th day after 1 : 2000 injection	28	—	—	—	27/11/31	Mild febrile reaction 3rd to 7th day and recovered.
	20193	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	No clinical symptoms seen. Too wild to be temperatured	28	—	—	—	27/11/31	No clinical symptoms seen. Immune to horse-sickness.
	20194	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	Marked reaction 21st to 30th day after 1 : 2000 injection. The 1 : 3000 superimposed a reaction	28	—	—	—	27/11/31	Reacted from 3rd and died 7th day from horse-sickness.
	20195	4/9/31	14	18/9/31	—	—	21	9/10/31	—	—	21	30/10/31	Reacted moderately 8th to 16th day after 1 : 3000 injection	28	—	—	—	27/11/31	Immune to horse-sickness.
B1.	20028	11/2/31	—	—	14	25/2/31	21	18/3/31	—	—	—	—	Mild febrile reaction from 5th to 10th day after 1 : 1000 injection and recovered	21	—	—	—	7/4/31	Reacted from 2nd and died 8th day from horse-sickness.
	20029	11/2/31	—	—	14	25/2/31	21	18/3/31	—	—	—	—	Reacted from 6th and died 10th day after 1 : 3000 injection	—	—	—	—	—	—

Appendix III -- Experiment S. 4496 (*continued*)

B1.	20028	11/2/31	—	—	14	25/2/31	21	18/3/31	—	—	—	—	Mild febrile reaction from 5th to 10th day after 1 : 1000 injection and recovered	21	—	—	—	7/4/31	Reacted from 2nd and died 8th day from horse-sickness.
	20029	11/2/31	—	—	14	25/2/31	21	18/3/31	—	—	—	—	Reacted from 6th and died 10th day after 1 : 3000 injection	—	—	—	—	—	—
	20030	11/2/31	—	—	14	25/2/31	21	18/3/31	—	—	—	—	Reacted severely from 6th to 11th day after 1 : 3000 injection, and was killed *in extremis* 19th day	—	—	—	—	—	—
B2.	20031	11/2/31	—	—	14	25/2/31	21	18/3/31	—	—	—	—	No reaction. Exposed to natural infection	149	14/8/31	3	17/8/31	—	Mild febrile reaction 11th to 16th day. Immune to horse-sickness.
C1.	20040	11/2/31	—	—	14	25/2/31	—	—	21	18/3/31	—	—	No reaction.....	21	—	—	—	8/4/31	Reacted Dikkop horse-sickness 4th to 13th day. Recovered.
	20042	11/2/31	—	—	14	25/2/31	—	—	21	18/3/31	—	—	No reaction.....	21	—	—	—	8/4/31	Reacted from 3rd and died 8th day from horse-sickness.
C2.	20041	11/2/31	—	—	14	25/2/31	—	—	21	18/3/31	—	—	Reacted to Dikkop horse-sickness from 9th to 14th day after 1 : 3500 injection and recovered	71	—	—	—	28/5/31	Immune to horse-sickness.
C3.	20043	11/2/31	—	—	14	25/2/31	—	—	21	18/3/31	—	—	No reaction. Exposed to natural infection	149	14/8/31	3	17/8/31	—	Immune to horse-sickness.
D1.	20044	11/2/31	—	—	14	25/2/31	—	—	—	—	21	18/3/31	Reacted severely 4th to 11th day after 1 : 4000 injection	21	—	—	—	8/4/31	Immune to horse-sickness.
	20045	11/2/31	—	—	14	25/2/31	—	—	—	—	21	18/3/31	No reaction.....	21	—	—	—	8/4/31	Mild febrile reaction 12th to 14th day. Immune to horse-sickness.
	20046	11/2/31	—	—	14	25/2/31	—	—	—	—	21	18/3/31	Reacted 12th to 18th day after 1 : 1000 injection	21	—	—	—	8/4/31	Reacted severely 2nd to 10th day and recovered.
	20051	11/2/31	—	—	14	25/2/31	—	—	—	—	21	18/3/31	Reacted from 5th day and died 10th day after 1 : 4000 injection	—	—	—	—	—	—
D2.	20048	11/2/31	—	—	14	25/2/31	—	—	—	—	21	18/3/31	No reaction. Exposed to natural infection	145	—	—	—	10/8/31	Reacted from 2nd and died 9th day from horse-sickness.
	20049	11/2/31	—	—	14	25/2/31	—	—	—	—	21	18/3/31	No reaction. Exposed to natural infection	145	—	—	—	10/8/31	Immune to horse-sickness.
D3.	20050	11/2/31	—	—	14	25/2/31	—	—	—	—	21	18/3/31	Mild febrile reaction from 10th to 16th day after 1 : 4000 injection and recovered. Exposed to natural infection	149	14/8/31	3	17/8/31	—	Immune to horse-sickness.
	20052	11/2/31	—	—	14	25/2/31	—	—	—	—	21	18/3/31	Severe reaction from 5th to 10th day after 1 : 4000 injection and recovered. Exposed to natural infection	149	14/8/31	3	17/8/31	—	Immune to horse-sickness.
	20053	11/2/31	—	—	14	25/2/31	—	—	—	—	21	18/3/31	No reaction. Exposed to natural infection	149	14/8/31	3	17/8/31	—	Immune to horse-sickness.
E1.	20032	11/2/31	—	—	14	25/2/31	21	18/3/31	—	—	41	28/4/31	No reaction.....	41	—	—	—	13/5/31	Severe Dikkop horse-sickness reaction from 4th to 11th day and recovered.
F1. Controls	20009	—	—	11/2/31	—	—	—	—	—	—	—	—	No reaction.....	21	—	—	—	4/3/31	Reacted from 3rd and died 7th day from horse-sickness.
F2.	20010	—	—	—	—	11/2/31	—	—	—	—	—	—	No reaction.....	21	—	—	—	4/3/31	Reacted from 2nd and died 6th day from horse-sickness.
F3.	20011	—	—	—	—	—	—	11/2/31	—	—	—	—	Reacted from 6th to 11th day and died from horse-sickness after 1 : 3000 injection	—	—	—	—	—	—
	20071	—	—	—	—	—	—	13/3/31	—	—	—	—	No reaction.....	26	—	—	—	8/4/31	Reacted from 3rd and died 7th day from horse-sickness.
F4.	20012	—	—	—	—	—	—	—	—	11/2/31	—	—	Reacted from 3rd and died 7th day from horse-sickness after 1 : 3500 injection	—	—	—	—	—	—
F5.	20013	—	—	—	—	—	—	—	—	—	—	11/2/31	Reacted from 4th and died 8th day from horse-sickness after 1 : 4000 injection	—	—	—	—	—	—
F6.	20036	—	—	25/2/31	—	—	—	—	—	—	22	19/3/31	No reaction.....	20	—	—	—	8/4/31	Reacted from 2nd and died 7th day from horse-sickness.
F7.	20037	—	—	—	—	25/2/31	—	—	—	—	22	19/3/31	Reacted 4th and died 8th day from horse-sickness after 1 : 4000 injection	—	—	—	—	—	—

Group.	D.O.B. No. of Horse.	Concentration of formaldehyde in spleen emulsion. 1 : 1000 Dose and Date.	Interval in Days.	1 : 2000 Dose and Date.	Interval in Days.	1 : 3000 Dose and Date.	Interval in Days.	1 : 4000 Dose and Date.	Interval in Days.	Injection of N.-virus + hyper-immune serum.	Interval in Days.	Injection of O.-virus + hyper-immune serum.	Interval in Days.	Injection of O.-virus alone.	REMARKS.
I. Experiment 4892	20197	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	* Severe Dikkop reaction from 6th day after first injection. Died 13th day. Reaction complicated with haemoglobinuria from 10th to 13th day.
	20198	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	Severe Dikkop reaction from 9th to 16th day. Recovered.
	20199	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	Moderate reaction from 8th to 16th day. Recovered.
	20201	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	Severe Dikkop reaction from 6th to 16th day. Recovered.
	20202	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	Severe Dikkop reaction from 6th day. Died 12th day.
	20203	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	Severe Dikkop reaction from 6th to 17th day. Recovered.
	20204	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	Severe Dikkop reaction from 6th to 20th day. Recovered.
	20206	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	Severe Dikkop reaction from 7th to 17th day. Recovered.
	20207	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	Severe Dikkop reaction from 7th to 16th day. Recovered.
	20208	—	—	—	—	—	—	—	—	5 + 400 c.c. 11/10/32	3	5 + 400 c.c. 14/10/32	—	—	Severe Dikkop reaction from 7th to 21st day. Recovered.
II. Experiment 4860	20230	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	Severe Dikkop reaction after serum virus injection. Too wild to temperature. Died 13th day.
	20231	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	Severe Dikkop reaction from 6th to 17th day. Recovered.
	20232	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	Moderate febrile reaction from 6th to 17th day. Recovered.
	20233	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	Very mild febrile reaction from 5th to 9th day. Recovered.
	20234	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	Severe reaction from 7th to 17th day. Recovered.
	20356	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	Very mild febrile reaction from 7th to 11th day. Recovered.
	20361	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	Severe Dikkop reaction from 6th to 16th day. Recovered.
	20363	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	Severe Dikkop reaction from 6th day. Died, 14th day.
	20373	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	No reaction.
	20375	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 29/11/32	3	5 + 400 c.c. 2/12/32	—	—	No reaction.
III. Experiment 4861	20332	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 200 c.c. 29/11/32	3	5 + 200 c.c. 2/12/32	—	—	Moderate reaction 6th to 15th day after serum and virus. Recovered.
	20443	60 c.c. 15/11/32	—	—	—	—	—	—	14	5 + 200 c.c. 29/11/32	3	5 + 200 c.c. 2/12/32	—	—	Mild reaction from 9th to 13th day. Recovered.
IV. Experiment 4862	20312	60 c.c. 15/11/32	—	—	14	30 c.c. + 400 serum 29/11/32	—	—	—	—	3	5 + 400 c.c. 2/12/32	—	—	Moderate reaction from 9th to 16th day after second injection. Recovered.
	20336	60 c.c. 15/11/32	—	—	14	30 c.c. + 400 serum 29/11/32	—	—	—	—	3	5 + 400 c.c. 2/12/32	—	—	Moderate reaction from 9th to 17th day after second injection. Recovered.
	20388	60 c.c. 11/1/33	—	—	14	30 + 400 c.c. 25/1/33	—	—	—	—	3	5 + 400 c.c. 28/1/33	—	—	Severe reaction from 6th to 16th day after second injection. Recovered.
	20390	60 c.c. 11/1/33	—	—	14	30 + 400 c.c. 25/1/33	—	—	—	—	3	5 + 400 c.c. 28/1/33	—	—	Severe Dikkop reaction. Too wild to be temperatured. Recovered.
	20391	60 c.c.	—	—	14	30 +	—	—	—	—	3	5 + 400 c.c.	—	—	Moderate reaction from 5th to 16th day.

	20388	60 c.c. 11/1/33	—	—	14	30 + 400 c.c. 25/1/33	—	—	—	—	3	5 + 400 c.c. 28/1/33	—	—	Severe reaction from 6th to 16th day after second injection. Recovered.
	20390	60 c.c. 11/1/33	—	—	14	30 + 400 c.c. 25/1/33	—	—	—	—	3	5 + 400 c.c. 28/1/33	—	—	Severe Dikkop reaction. Too wild to be temperatured. Recovered.
	20391	60 c.c. 11/1/33	—	—	14	30 + 400 c.c. 25/1/33	—	—	—	—	3	5 + 400 c.c. 28/1/33	—	—	Moderate reaction from 5th to 16th day. Recovered.
	20396	60 c.c. 11/1/33	—	—	14	30 + 400 c.c. 25/1/33	—	—	—	—	3	5 + 400 c.c. 28/1/33	—	—	Severe Dikkop reaction from 5th to 16th day. Recovered.
	20466	60 c.c. 11/1/33	—	—	14	30 + 400 c.c. 25/1/33	—	—	—	—	3	5 + 400 c.c. 28/1/33	—	—	Moderate Dikkop reaction from 6th to 14th day. Recovered.
V. Experiment 4863	10314	60 c.c. 15/11/32	—	—	14	30 + 200 c.c. 29/11/32	—	—	—	—	3	5 + 200 c.c. 2/12/32	—	—	Severe Dikkop reaction from 8th to 18th day. Recovered.
	20424	60 c.c. 15/11/32	—	—	14	30 + 200 c.c. 29/11/32	—	—	—	—	3	5 + 200 c.c. 2/12/32	—	—	Severe Dunkop reaction from 7th to 21st day. Recovered.
VI. Experiment 4864	20328	60 c.c. 15/11/32	14	60 c.c. 29/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 13/12/32	—	—	Severe clinical reaction from 4th to 11th day. Recovered.
	20462	60 c.c. 15/11/32	14	60 c.c. 29/11/32	—	—	—	—	—	—	14	5 + 400 c.c. 13/12/32	—	—	No clinical reaction. Too wild to be temperatured.
VII. Experiment 4865	20279	60 c.c. 15/11/32	14	60 c.c. 29/11/32	—	—	—	—	—	—	14	5 + 200 c.c. 13/12/32	—	—	Severe Dikkop reaction, died 9th day. Too wild to be temperatured.
	20454	60 c.c. 15/11/32	14	—	—	—	—	—	—	—	—	—	—	—	Severe Dunkop reaction from 7th day and died 9th day after 1 : 1000 injection.
VIII. Experiment 4866	20294	60 c.c. 15/11/32	14	60 c.c. 29/11/32	14	60 c.c. 13/12/32	—	—	—	—	15	5 + 200 c.c. 28/12/32	—	—	Slight Dikkop reaction from 6th to 11th day. Recovered.
	20284	60 c.c. 15/11/32	14	60 c.c. 29/11/32	14	60 c.c. 13/12/32	—	—	—	—	15	5 + 200 c.c. 28/12/32	—	—	Mild febrile reaction 6th to 8th day. Recovered.
IX. Experiment 4867	20264	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	—	—	—	—	15	5 + 200 c.c. 28/12/32	—	—	Severe Dikkop reaction from 3rd day. Died 8th day.
	20445	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	—	—	—	—	15	5 + 200 c.c. 28/12/32	—	—	Moderate reaction from 7th to 11th day. Recovered.
X. Experiment 4868	20210	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	14	5 + 400 c.c. 11/1/33	—	—	No clinical reaction, too wild to be temperatured.
	20229	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32		—	14	5 + 400 c.c. 11/1/33	—	—	No reaction.
	20348	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	14	5 + 400 c.c. 11/1/33	—	—	Mild febrile reaction from 5th to 10th day. Recovered.
	20349	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	14	5 + 400 c.c. 11/1/33	—	—	Mild febrile reaction from 7th to 12th day. Recovered.
	20350	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	14	5 + 400 c.c. 11/1/33	—	—	Mild febrile reaction from 2nd to 7th day. Recovered.
XI. Experiment 4869	20351	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	14	5 + 200 c.c. 11/1/33	—	—	No clinical reaction. Too wild to be temperatured.
	20352	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	14	5 + 200 c.c. 11/1/33	—	—	Severe Dunkop reaction from 2nd day. Died 8th day.
	20353	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/13/32	15	30 c.c. 28/12/32	—	—	14	5 + 200 c.c. 11/1/33	—	—	Severe Dikkop reaction from 5th to 9th day. Recovered.
	20368	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	14	5 + 200 c.c. 11/1/33	—	—	Severe Dikkop reaction from 2nd to 9th day. Recovered.
	20437	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	14	5 + 200 c.c. 11/1/33	—	—	Mild febrile reaction from 5th to 9th day. Recovered.
XII. Experiment 4870	20364	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	—	—	14	5 c.c. 11/1/33	Severe reaction from second day. Died 6th day.
	20365	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	—	—	14	5 c.c. 11/1/33	Severe reaction from second day. Died 7th day.
	20372	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	—	—	14	5 c.c. 11/1/33	Severe reaction from 5th to 10th day. Recovered.
	20389	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	—	—	14	5 c.c. 11/1/33	Mild reaction from 5th to 11th day. Recovered.
	20393	30 c.c. 15/11/32	14	30 c.c. 29/11/32	14	30 c.c. 13/12/32	15	30 c.c. 28/12/32	—	—	—	—	14	5 c.c. 11/1/33	Severe reaction from 5th to 11th day. Recovered.

* *Unless otherwise stated, day of reaction counted from injection of N. virus or O. virus if N. virus was not given*