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# The Attenuation of Bluetongue Virus by Serial Passage through Fertile Eggs.

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In an article by Alexander (in press) details are given of the influence of the temperature of incubation on the multiplication of one strain of bluetongue virus (" Bekker " strain) in fertile eggs. Before the importance of this factor was fully appreciated and serial passage had been reduced to a simple routine, that strain of virus had been passed successively through more than 100 egg to egg passages. It was stated that, by that time, the virus had become so attenuated as to produce little or no detectable clinical reaction in susceptible sheep and consequently the only certain index of infection was the development of a solid immunity to the homologous virulent virus. No opinion could be expressed as to whether this loss of virulence was merely a chance phenomenon, not repeatable with either the same or another strain of virus, whether attenuation took place slowly and progressively with passage or as the result of the sudden appearance of a stable mutant, or whether the lower temperature of propagation was the determining factor. For this reason a second strain of virus was adapted to propagation in eggs at three different temperatures and the virulence for sheep, together with the antigenicity, was tested at regular intervals. The significance of the findings are discussed in the light of the problem of mass immunization in the field.

## Technique and Materials.

The technique, conditions of incubation and the temperatures were those detailed in the previous report.

The strain of virus used was that known as " University Farm " (Neitz, in press) since this strain can be relied upon to produce severe clinical reactions in Merino sheep under stable conditions.

A sheep (64506) was destroyed 24 hours after the initial rise in temperature on the 8th day after infection with virulent blood. The spleen was removed with aseptic precautions, passed through a Latapie mincer and desiccated *in vacuo* from the frozen state over anhydrous calcium sulphate, after prefreezing in a dry ice-alcohol bath. The resulting powder was sealed in small glass ampoules in an atmosphere of dry nitrogen and stored at $-10^{\circ}$ C. to serve as a permanent stock of virus.

The bacteria-free inoculum for the eggs was prepared as a 660 m $\mu$ gradocol membrane filtrate of an approximate 2 per cent. broth emulsion of

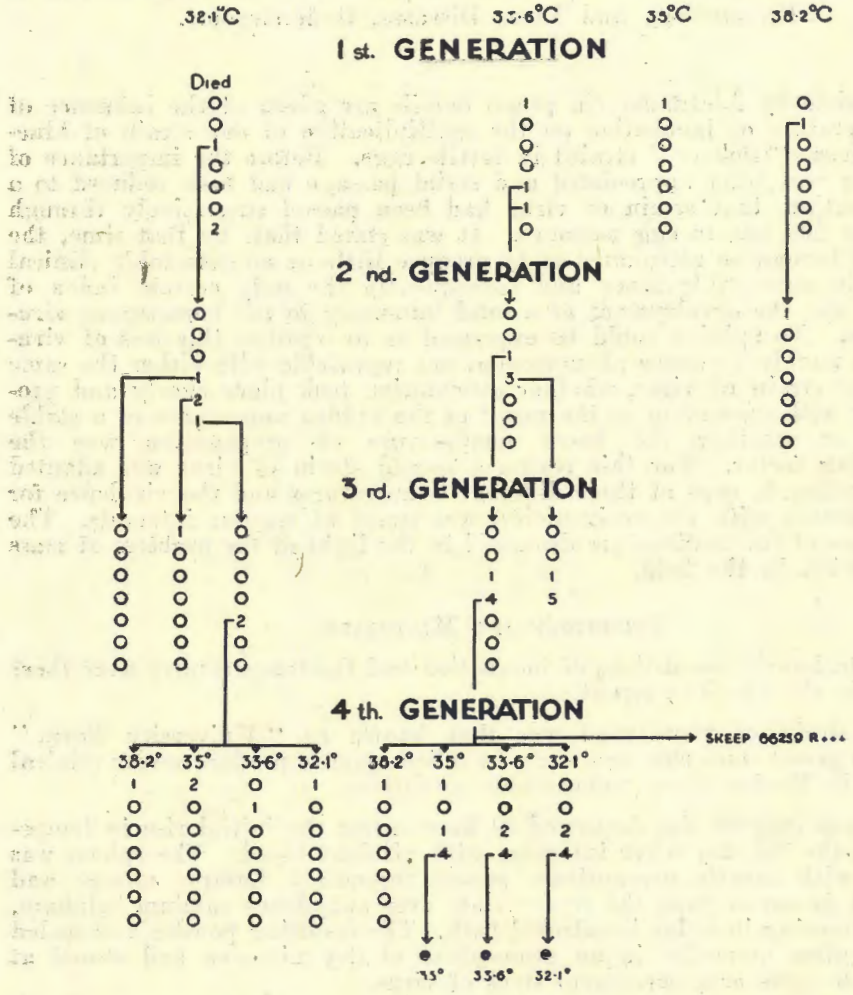
the dried spleen clarified by centrifugation at 3,000 revs. per min. for 2 hours in a Clay Adams angle centrifuge. The virulence of the filtrate was controlled by the subcutaneous injection of 2 c.c. into a sheep (64014). This sheep showed the usual severe reaction (††††) from the 7th to the 12th day after injection.

## Propagation in Eggs.

The procedure adopted for the initial adaptation to eggs is shown schematically in Chart I.

### Chart No. I.

*Adaptation to Serial Passage in Eggs at* 38·2°, 35·0°, 33·6° and 32·1° C.




Note.—0=no deaths, numeral=number of embryos which died on that day after injection.

The 24 eggs containing 8 day embryos which had received an injection of 0·1 c.c. of the virulent filtrate were divided into 4 groups of 6 eggs each for incubation at each of the 4 temperatures, viz., 38·2°, 35·0°, 33·6° and 32·1° C. These, and every subsequent group of subinoculated eggs were kept under observation for 7 days after which they were discarded. Death of an embryo was taken as the index of virus multiplication, and subinoculations were made from the dead embryos except those which were found dead within 24 hours; these were assumed to have died of traumatic injury, and were discarded. The results may be summarized as follows:—

*A. at* 38·2° *C.*

Only one of the 6 embryos died—on the second day. This was harvested and subinoculated into 6 eggs of which one embryo died within 24 hours.

*Result.*—No multiplication of virus took place.

*B. at* 35·0° *C.*

Only one embryo died within 24 hours.

*Result.*—No multiplication of the virus.

*C. at* 33·6° *C.*

*Generation* 1.—One embryo died within 24 hours and was discarded.

Two embryos died, one each on the 5th and 6th day. These were pooled, the first after storage overnight in the refrigerator, and subinoculated into 6 eggs to constitute generation 2.

*Generation* 2.—One embryo died on the 3rd day and 3 on the 4th day. These were harvested as 2 separate groups and each subinoculated into 6 eggs of generation 3.

*Generation* 3.—One of each group died on the 3rd day, a total of 9 died on the 4th day and the remaining embryo survived for 7 days. Four of the 4th day dead embryos were pooled for subinoculation into 24 eggs of generation 4. A sheep (66259) was given 1 c.c. of this emulsion subcutaneously. It reacted severely from the 5th to the 13th day with clinical lesions of bluetongue. On immunity test after an interval of 38 days it was proved to be solidly immune.

*Generation* 4.—The 24 eggs were again divided into 4 groups of 6 each, for incubation at each of the 4 temperatures. At 38·2° C. there were no deaths except one within 24 hours. At each of the other temperatures all the embryos died on the 3rd and 4th day. The 4th day dead embryos were harvested for subinoculation and the strain has been maintained by serial passage at those temperatures until the present time.

*Result.*—The virus was adapted to propagation in the developing chick embryo by incubation at 33·6° C. for 3 generations. After this initial adaptation the virus continued to multiply when incubated at 32·1° 33·6° and 35·0° C. but not at 38·2° C.

*D. at* 32·1° *C.*

*Generation* 1.—One embryo died on the 3rd day and was subinoculated into 6 eggs of generation 2. Two embryos died on day 7 but on examination were found to be pale and underdeveloped so that death was regarded as non-specific (see previous report) and they were discarded.

*Generation* 2.—One embryo died on the 4th day, 2 on the 5th day and one on the 6th day. The dead embryos on each day were harvested as groups and each was subinoculated into 6 eggs of generation 3.

*Generation* 3.—Of the 12 eggs which received emulsion prepared from the 4th and 5th day dead embryos one died within 24 hours and the remaining 11 survived for 7 days when they were discarded. Of the group which were given emulsion from the previous 6 day death, 2 died after 5 days incubation. These were pooled and subinoculated into 24 eggs of generation 4.

*Generation* 4.—Four groups were again incubated at the 4 temperatures. Four embryos died within 24 hours and one, incubated at 33·6° C., died on the 2nd day. As no further embryos died the series was discontinued after 7 days.

*Result.*—Although it appeared as if the virus was becoming adapted to multiplication at 32·1° C. as early as the 2nd serial passage, though this was not confirmed by injection into sheep, no multiplication took place on further subculture.

*Comment.*—The extreme importance of the temperature of incubation to the adaptation of this strain of virus to multiplication in the developing chick embryo is clearly demonstrated. Using death of the embryo as an index of virus multiplication, and, at the present time there appears to be no other index, the only temperature of the 4 under consideration at which continued multiplication took place was 33·6° C. Had this temperature not been used it might have been concluded, quite justifiably, that the strain of virus could not be adapted to serial egg passage. After the virus had been passed through 3 generations of embryos of 33·6° C. no difficulty was experienced in continuing propagation at that temperature. In addition it could then be cultivated with ease at both 35·0° and 32·1° C. but no evidence of multiplication at 38·2° C. was obtained. In passing it may be stated that after an additional 50 passages at 33·6° C. a further unseccesful attempt was made to propagate the virus at 38·2° C.

From the above observations it was concluded that the " University Farm " strain of virus differed from the one previously studied (Bekker strain) in that 33·6° C. and not 32·1° C. appeared to be the optimum temperature for cultivation. As facilities were not available to duplicate the previous detailed investigation, merely the salient features of that work were studied in order to develop a procedure for obtaining the highest titre of emulsion with the greatest economy of material.

While passage was proceeding at each of the 3 temperatures, a record was kept of the fate of all eggs over a period of 4 days after infection. For the sake of brevity only details of generations 11 to 20, 41 to 50, 91 to 100, 121 to 130 are shown in the summary of results in Table 1. These were selected at random as representing early, medium, and late passages, and because the conclusions would not be affected by detailing the whole series.

*Result.*—A general consideration of the results shows that, throughout the course of the experiment, the technique of handling and injecting the eggs must have been uniform because the number of deaths amongst the embryos during the first 24 hours, i.e., due to traumatic injury, etc., was remarkably constant. Further, there appears to be no significant difference

in the daily mortality at any particular period in either the early or the late passage series with two possible exceptions:—

1. In the series generation 91 to 100 at 33·6 the mortality on the 2nd and 3rd days increased somewhat with a consequent decrease in mortality on the 4th day and survivors beyond that time. This was not repeated in the 121 to 130 generation series so the occurrence was probably fortuitous.
2. The mortality figures for the 3rd day at 35° in the 121 to 130 generation series was low. Since this was compensated by a corresponding increase in the number of deaths on the 4th day, with the number of survivors only slightly higher, this again appears to be merely a chance occurrence.

TABLE 1.

*The Fate of Embryos on Passage of Virus at* 32·1°, 33·6° *and* 35·0° *C.*

| INCUBATION. | | GENERATION. | | | | TOTAL. |
|---|---|---|---|---|---|---|
| Temp. °C. | Period | 11–20. | 41–50. | 91–100. | 121–130. | |
| 32·1 | 1 | 4–116 | 9–111 | 7–112 | 2–118 | 22–457 |
| | 2 | 3–113 | 2–109 | 0–112 | 3–115 | 8–449 |
| | 3 | 51–62 | 52–57 | 40–72 | 44–71 | 187–262 |
| | 4 | 43–19 | 43–14 | 51–21 | 52–19 | 189–73 |
| 33·6 | 1 | 4–114 | 5–114 | 5–115 | 6–114 | 20–457 |
| | 2 | 1–113 | 2–112 | 16–99 | 4–110 | 23–434 |
| | 3 | 58–55 | 62–50 | 81–18 | 73–57 | 274–160 |
| | 4 | 32–23 | 35–15 | 13—5 | 24–13 | 104–56 |
| 35·0 | 1 | 8–111 | 3–115 | 10–110 | 6–114 | 27–450 |
| | 2 | 3–108 | 5–110 | 7–103 | 2–112 | 17–43 |
| | 3 | 63–45 | 57–53 | 58–45 | 35–77 | 213–220 |
| | 4 | 25–20 | 31–22 | 12–33 | 39–38 | 107–113 |

NOTE.—4–116, 9–111, etc., means 4 dead, 116 alive; 9 dead, 111 alive, etc.

On the second day there was hardly any significant difference in the number of deaths at any temperature with the possible exception of a slightly increased rate at 33·6° C. It was only during the 3rd and 4th days that differences became apparent. At 32·1° and 33·6°, 376 and 378 embryos respectively died on the 3rd and 4th days, but, whereas the deaths at 32·1° C. were evenly divided between the two days, a significantly larger number died on the 3rd day at 33·6° C. (274 as compared with 104). At 35·0° almost exactly twice as many embryos died on the 3rd day as on the 4th day, and the total number of survivors was significantly higher than at either of the other two temperatures.

*Conclusion.*—From these figures, using death of the embryo as an index of virus multiplication, it appears that there is little difference in the multiplication at 32·1° and 33·6° except for slight acceleration at the higher temperature. At 35·0° C. conditions for propagation are less satisfactory,

particularly in the case of eggs which survive for 72 hours. In this connection it should be borne in mind that the actual temperatures of these embryos on the 4th day of incubation were on the average 32·37°, 34·13° and 35·54° C. i.e., proportionally higher than the temperature of incubation in each case. (Alexander.)

These results do not throw very much light on the optimum conditions for virus multiplication to that, concurrently, a number of quantitative determinations were made on the virus titre of various emulsions. The general scheme was to take passage material prepared at any one temperature and, as eggs became available in adequate quantities, to titrate that material at each of the 3 temperatures. The results are given in Table 2.

TABLE 2.

*The Virus Titre of Embryo Emulsions Cultivated and Titrated at Three Different Temperatures.*

| Virus. | | LD (50) at °C. | | |
|---|---|---|---|---|
| Generation. | Cultivated at. | 32·1. | 33·6. | 35·0. |
| 10 | 32·1 | 4·0000 | 2·6990 | 2·0000 |
| 13 | 32·1 | 4·7570 | 3·4770 | 2·6642 |
| 64 | 32·1 | 4·7094 | 4·9243 | 2·7477 |
| 70 | 32·1 | 4·9208 | 4·4949 | 4·0000 |
| 64 | 33·6 | 4·6000 | 5·0000 | 4·5925 |
| 80 | 33·6 | 5·0000 | 4·0000 | 3·5173 |
| 68 | 35·0 | 5·0968 | 4·8750 | 4·8561 |
| 80 | 35·0 | 5·0000 | 4·3979 | 4·0865 |
| 132 | 35·0, 32·1* | — | (1) 6·0000<br>(2) 5·6990 | —<br>— |

NOTES.—* 24 hours at 35·0 then transferred to 32·1.
(1) Empryos dead on day 3.
(2) Embryos dead on day 4.
LD(50) calculated according to method of Reed & Muench (1938) and expressed as the logarithm of the 50% death end point.

*Result.*—Virus propagated at 32·1° C. showed the anticipated decrease in apparent titre as the temperature of incubation for the titration test increased; the decrease was not marked in the case of generation 70. In only one of two experiments with material incubated at 33·6° was the same tendency shown, and with material left at 35·0° this tendency was even less pronounced. Consideration of all the virus titres at 33·6° shows that, after sufficiently prolonged passage to warrant the assumption of full adaptation of the virus to chick embryos, there was no significant difference in virus multiplication no matter at which of the 3 temperatures the eggs were incubated. It is worthy of note, however, that by incubating for 24 hours at 35° and then transferring the eggs to 32° the highest titre emulsions were obtained, with rather more virus in the embryos that died on the 3rd day than those which survived until the following day. Moreover, by adopting that procedure, 16 out of 24 eggs injected died on the 3rd day and all the remainder were dead by the following morning.

*Comment.*—The result of these two series of experiments do not clear up the question of the most suitable temperature at which to propagate this strain of virus, a finding which is rather remarkable in view of the fact that only at 33·6° was it possible to adapt the strain to multiplication in eggs at all. Comparing these results with the very clear cut results obtained with the Bekker strain of virus, shows that there may be considerable differences, in addition to antigenic structure, between different strains of virus.

### Attenuation by Serial Passage.

As passage proceeded, material from each successive tenth generation of each series of eggs at the three temperatures was injected into susceptible Merino sheep, the dose being 1 c.c. subcutaneously. Unfortunately there were a few unavoidable omissions but these in no way invalidate the final conclusions. The material used in each case was that used for subinoculation into the next group of eggs so that each sheep received not less than 1,000 M.I.D.'s of virus. It was the original intention to determine the titre of virus in every inoculum by titration in eggs at 33·6° C. From time to time the shortage of eggs made it quite impossible to complete this phase of the investigation. The incomplete results of titration have been omitted since it was early apparent that, within the limits of this method of virus propagation, the severity of the reaction was in no way correlated with the number of M.I.D.'s injected.

The sheep were maintained under stable conditions without exposure to direct sunlight. In addition to carefully controlled daily temperatures they were inspected each morning to estimate the severity of the reactions (c/f. Neitz). The results are shown in tabular form in Table 3.

Table 3.

*Attenuation of the Virus by Serial Passage through Fertile Eggs.*

| Egg Generation. | Temperature of Incubation. | | |
|---|---|---|---|
| | 32·1 °C. | 33·6 °C. | 35·0 °C. |
| 3 | — | 66259. R+++(+) (6–13) | — |
| 10 | — | 66228. R++ (7–12) | — |
| 20 | 66521. R++ (6–9) | — | — |
| | 66519. R+++ (6–9) | — | — |
| 30 | 66665. R (?) (8–9) | — | 66647. R+ (6–10) |
| 40 | 68659. R+ (6–9) (2) | 68948. R++ (6–9) (1) | 68715. R+++ (5–10) |
| 50 | 67604. NR. | 68916. R (?) (8–9) | 68038. R+++ (4–9) |
| 60 | 69172. R++ (6–10) | 69171. R+ (7–11) | 69146. R+++ (6–11) |
| | 69176. R+ (10–13) | | |
| 70 | 69222. R+ (7–8) | 69200. NR. | 69235. R++ (6–11) |
| 80 | 70129. R+ (7–10) | 69215. R (?) (8) | 70056. R++(+) (5–8) |
| 90 | 71174. R++(+) (7–9) | 69236. R++(+) (7–14) | 70066. R+++ (7–10) |
| 100 | 71962. R+ (7–9) | 71982. NR. | 71986. R++ (6–9) |
| 110 | 72250. NR. | 72071. R(+) | 72148. R+ (6–13) |
| 120 | 72055. R+ (6–7) | 72224. NR. | 72171. R+ (6–8) |
| 130 | 72046. R+ (5–7) | 72200. R (?) | 72023. R+ (5–8) |

Note.—R+ . . . ++++ indicates varying degrees of severity of reaction.
R+ and R++ denote febrile reaction only.
R+++ and R++++ febrile reaction and clinical lesions. N.R.=No reaction
(1) and (2)—See text on immunity.
6–9, etc Incubation period 6 days, duration febrile reaction 9 days etc.

*Results at* 32·1° *C.*—By the 20th subculture it was apparent that some attenuation of the virus had taken place since the reactions produced in two sheep were unmistakably milder than those seen in a number of animals reacting to infective blood. From the 30th generation onward this attenuation was well marked in spite of the fact that one sheep (71174 generation 90) showed a well defined febrile reaction accompanied by slight hyperaemia of the buccal mucosa. The remainder of the sheep either showed no detectable reaction at all or an indefinite fever lasting for not more than 48 hours, without any other symptoms. Further, it was apparent that although the period of incubation remained unaltered, the course of any reaction was considerably decreased.

*Results at* 33·6° *C.*—It is unfortunate that generations 20 and 30 were not injected into sheep, more particularly since generation 40 produced a reaction comparable in every respect with that produced by generation 20 at 32·1° C. From then on the reactions were mild and of short duration or practically indetectable.

*Results at* 35·0° *C.*—The omission to inject material prior to generation 30 into sheep is of no consequence since every animal which received material from generation 40 to 100 showed marked reactions with well defined mouth and foot lesions. In addition the duration of the reactions were protracted and convalescence was prolonged. After generation 100 a decrease in the severity of the reactions became noticeable so that they approximated those produced by the earlier passage material at the lower temperatures.

*Conclusion.*—This strain of virus was attenuated by serial passage through eggs. Attenuation took place rapidly on cultivation at 32·1° C. being well pronounced after 30 passages. If attenuation was somewhat slower at 33·6° C. it is not apparent from the somewhat incomplete data presented; the opinion is held that no difference could be determined. At 35·0° C. the virus eventually did become attenuated but a well defined decrease in virulence was not apparent before the 90th or 100th passage. The attenuation showed itself as a decrease in the severity of the febrile reaction, the absence of specific lesions of the buccal mucosa and coronets, a shortened course of any detectable reaction without any adverse sequelae, all without any alteration of the period of incubation.

## Immunity.

All the sheep referred to in Table 3 were given an immunity test in the form of 2 c.c. of virulent blood subcutaneously at intervals varying from 14 days to 46 days after receiving egg propagated virus. All were found to be solidly immune, but attention must be directed to two anomalies. Sheep 68948 which received material from generation 40 at 33·6° (marked 1 in Table 3) showed a well defined febrile reaction, without clinical lesions, from the 6th to the 9th day after injection. On application of the immunity test after an interval of 32 days a well marked febrile reaction commenced on the 4th day and lasted for 5 days (maximum temp. 106·8° F.). No clinical lesions of blue tongue were observed and the relation of the reaction to bluetongue remains obscure. Sheep 68659 received attenuated virus generation 40 at 32·1° C., and reacted only with slight fever from the 6th to the 9th day. On immunity test after an interval of 46 days, a severe febrile reaction commenced after 48 hours and persisted for 48 hours.

(Maximum temperature 107·2° F.) Obviously this reaction was not connected with a specific bluetongue reaction and is believed to be something of the nature of " protein shock ".

*Conclusion.*—In spite of the two irregularities quoted it is concluded that whether a sheep shows a clinical reaction or not to the injection of an infecting dose of egg attenuated virus it responds with the production of a solid immunity, which may develop as early as 14 days after injection.

## Discussion.

The results of the limited amount of experimental work on the optimum conditions for the propagation of this strain of bluetongue virus are not conclusive. However, two points of extreme importance are apparent, viz.:

1. Using the yolk sac method of infecting fertile hens' eggs after 8 days' preliminary incubation, it was possible to adapt the strain to multiplication in the embryos only by incubation at 33·6° C. Thus the vital rôle played by the temperature of incubation, as previously reported, was confirmed.

2. Adaptation to growth in the embryo occurred rapidly because after only 3 passages at 33·6° C. no difficulty was experienced in starting and continuing propagation at 32·1° C. and 35·0° C., though the range could not be extended to include 38·2° C. This rapid adaptation would seem to be analogous to that reported by Burnett and Bull (1943) from their work on influenza virus.

After the initial adaptation there was little difference in the titre of virus produced by incubation at other of the 3 temperatures, a finding not in accordance with that reported from work with the Bekker strain of virus. It was confirmed, however, that the highest titre of virus together with the greatest mortality on the 3rd day after infection was obtained by incubation at 35·0° C. followed by transfer to 32·1° C. It is apparent, therefore, that a considerable amount of additional work is necessary to elucidate many points on the influence of temperature which, at present, are obscure. This investigation is being carried out in conjunction with the serial passage of other antigenically different strains of virus and will be reported on at a later date.

The observations on the attenuation of the virus are important. In the first place it is shown conclusively that a second strain of virus has been attenuated to a point where it may be injected into susceptible sheep under stable conditions with the production of practically no detectable reaction, followed by the development of a solid immunity to the homologous virulent virus. This attenuation was brought about rapidly, after not more than 20 passages, at 32·1° C., with almost equal rapidity at 33·6° C., but at 35·0° C. it was so retarded that full attenuation was obtained only after about 100 passages. No opinion whatever can be expressed on the mode of action of the temperature of incubation in the production of avirulence. It is reasonable to assume, however, that, if it has been possible to attenuate two strains, it will be possible to attenuate more of the antigenic variants that have been recognized and isolated recently. This assumption is being borne out by the work at present under way, and when considered in conjunction with the solid immunity production, has an important bearing upon the whole problem of the control of the disease in the field.

Neitz showed that the method of immunization at present in use has two major defects:—

1. The attenuated, but comparatively avirulent natural strain of virus, which constitutes the essential portion of that vaccine, produces severe reactions in the field and under unfavourable climatic conditions may be the cause of mortality and serious economic loss.
2. The use of a single strain of virus, with a narrow antigenic range, results in the production of an immunity which is inadequate to protect against the plurality of virus strains which are now known to exist.

There remains a great deal more work to be carried out on the polyvalent immunity produced by a combination of two or more strains of egg attenuated virus, but even at this early stage it is apparent that the above two major defects may be eliminated. It only remains to indicate the possibility of using the developing chick embryo to produce the large quantities of vaccine that are required annually.

At present rather more than 2½ million doses of vaccine are issued each year, and of that amount rather more than half during the 3 months period October to January. With the introduction of a safe and more efficient product it is estimated that the demand would be approximately doubled so that provision would have to be made for the production of at least 5 million doses per annum. Fortunately the virus possesses remarkable keeping qualities on storage at $\pm 5°$ C. so that provision could be made to meet the peak demand during the spring or early summer by continued production throughout the year and storage either in bulk or as the final product. It has been shown that under the conditions described it is possible constantly to produce embryo emulsions with a titre of not less than $10^{-5}$, and it is known that 10 c.c. of emulsion is obtained from 3 embryos. If the emulsion is diluted 1 to 500 then 6 eggs would be required to produce 100 litres of vaccine containing 200 M.I.D.'s of virus per dose of 1 c.c. That means that the entire annual output of monovolent vaccine could be obtained from 3,000 embryos which would necessitate the injection of not more than 12 eggs per working day. Naturally additional fairly large numbers of eggs would be required for the quantitative determinations which are inseparable from routine vaccine production, but it is confidently believed that the entire task could be carried out by two trained technicians provided with very simple equipment and accommodation.

There still remains some research to be carried out to determine such points as the most suitable diluent to serve as a vehicle for the virus, the most suitable bacterial preservative and the keeping qualities of the final vaccine under field as well as laboratory conditions. These, however, are comparatively minor, though important points, upon which considerable data have been collected so that it is believed that the production of an efficient vaccine against bluetongue is in sight.

## Summary.

1. A strain of virus (University Farm strain) was adapted to propagation in the developing chick embryo by incubation of infected eggs containing 8 day embryos at 33·6° C. but not at 32·1° C., 35·0° C. or 38·2° C.

2. After 3 serial passages at 33·6° C. it was possible to continue propagation at 32·1° C. and 35·0° C. but not at 38·2° C.

3. Using death of the embryos as an index of multiplication of egg-adapted virus there was little difference in the results obtained from incubation at 32·1° C. or 33·6° C. except that multiplication was slightly retarded at the lower temperature. At 35·0° C. the number of survivors beyond the 4th day of incubation was significantly increased.

4. There was little variation in the titre of emulsions produced from dead embryos at either of the temperatures after adaptation to eggs by serial passage.

5. The highest titre emulsions (not less than $10^{-5}$) together with the highest death rate on the 3rd day were produced from eggs incubated for 24 hours at 35·0° C. and then transferred to 32·1° C.

6. The virulent strain of virus was attenuated by serial egg to egg passage. At 32·1° C. attenuation took place rapidly after approximately 20 passages, at 33·6° C. at approximately the same rate, but at 35·0° C. it was delayed until about the 100th subculture.

7. Whether the attenuated virus produces a clinical reaction or not a solid immunity is produced against the homologous strain of virus.

8. The application of the results to the production of large quantities of vaccine for the mass immunization of sheep in the field is discussed.

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