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The Propagation of Blue-tongue Virus in the Developing Chick Embryo with Particular Reference to the Temperature of Incubation.

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THE developing chick embryo may be regarded as the medium of choice for the propagation of a wide variety of viruses and the technique has been adapted to the study of numerous problems connected with virus diseases of man and animals. It is natural, therefore, that an effort should have been made to explore the possibilities of adapting strains of bluetongue virus to multiplication in fertile eggs.

Mason, Coles, and Alexander (1940) in a brief note reported, that after numerous attempts, by what may be termed the standard methods of injection and incubation, no evidence of multiplication had been obtained, but, using eggs produced by fowls maintained on a diet deficient in riboflavin, "virus has been carried through 21 egg to egg passages with irrefutable evidence of multiplication". Moreover, after only four passages through deficient eggs the adapted virus was able to multiply in eggs produced by hens on a complete balanced ration and had been maintained for 37 additional serial passages. The authors were careful to state that no proof existed that the vitamin deficiency of either the fowls or the eggs was the determining growth promoting factor, and indicated that critical experiments were under way to clear up the point. Fertile eggs were obtained from fowls on the riboflavin deficient diet and from a control group of similar birds on the same diet supplemented by an adequate ration of pure riboflavin given daily *per os*. It was soon apparent that there was no detectable difference in the multiplication of the virus in either the embryos or their membranes in eggs from either group of birds. This opportunity is taken of correcting any faulty deductions from the previously reported series of experiments.

The importance of vitamins or some imperfectly understood co-enzyme system for the multiplication of viruses is generally accepted as one explanation for failure to multiply in the absence of living cells. For instance as early as 1931 Zinsser, Castaneda and Seastone showed that guinea-pigs and rats maintained on a vitamin deficient diet to a point where symptoms of avitaminosis appeared, were considerably less resistant to typhus infection than normal animals. Pinkerton and Bessey (1939) showed that riboflavin deficiency was responsible for this lowered resistance and that vitamin A deficiency did not have a comparable effect. Singer (1941) concluded that several metabolites are necessary for the multiplication of the rickettsias of

European typhus and Rocky Mountain spotted fever and very briefly cited experiments to show that these rickettsias may be cultivated in a cell-free medium enriched with glutathione. This report is of prime importance but requires confirmation and elaboration. Sprunt (1942), working with vaccinia, found that if rabbits were deprived of food the lesions were either fewer or smaller and concluded that the virus was less able to multiply in the poorly nourished cell, though they stressed the possible influence of hydration and dehydration on the susceptibility of cells. In a later report Taylor & Sprunt (1943) confirmed the hypothesis that an increase of extracellular fluid decreases the susceptibility to vaccinia.

Similarly, the effect of temperature upon the multiplication of viruses both *in vivo* and *in vitro* has been the subject of investigation by a number of workers on a wide variety of different viruses. Castaneda (1937) found that guinea-pigs with rectal temperatures above 38° C. are not suitable for the growth of Mexican typhus within the serosal cells of the peritoneal cavity and obtained prolific multiplication in X-rayed guinea-pigs whose body temperature was kept below 38° C. by the use of veronal, or by repeated bathing of the shaved animals. Apart from this it is a common experience that strains of rickettsias of low infectivity for guinea-pigs are easier to maintain by serial passage in winter than in summer.

A review of the work on the cultivation of viruses by conventional tissue culture methods at temperatures lower than that normally used for bacteriological purposes would be out of place in this article which is confined to the use of the developing chick embryo. Sigurdsson (1943), in his report on the influence of age of host and temperature of incubation on infection of the chick embryo with vesicular stomatitis virus, reviewed the relevant literature. He pointed out that several authors had reported differences in the course of infections when different temperatures of incubation had been used, but apart from Cox (1938), little work of a definite quantitative nature had been done. Sigurdsson (1943) found that better multiplication of vesicular stomatitis virus was obtained at 35 to 36° C. than at 39 to 40° C. He (1944) demonstrated a similar effect of temperature upon the propagation of influenza virus a finding which was confirmed by Miller (1944). Finally, Beveridge (1944), in a study of the effect of the temperature of preliminary incubation on the susceptibility of the chick embryo to influenza virus, found that a decrease of this temperature from 39° C. to 36·6–37·8° C. had no influence on the susceptibility of eggs to influenza virus A or B inoculated into the amniotic cavity or the yolk sac and incubated at 35° C. after injection, but, using the allantoic route, obtained a two-fold increase in the volume of the allantoic fluid together with a decrease in the proportion of eggs which did not become infected. In the experimental work to be reported in this article the temperature of preliminary incubation has been kept constant at the level recommended by the manufacturers for maximum hatchability (cf. infra) but, after injection, not only the temperature of the incubator but also the actual temperature of the incubated eggs has been taken into consideration.

Technique and Materials.

1. *Virus*.—A single strain of virus has been used in this series of experiments—that known as "Bekker" virus (Bekker, de Kock and Quinlan, 1934). The bacteriologically sterile inocula for initiating the egg cultures were 860 mμ gradocol membrane filtrates* of 10 per cent. physiological saline emulsions of infective spleens from sheep

*The preparation of the gradocol membranes and all filtrations were carried out by Dr. A. Polson to whom the author expresses his indebtedness.

destroyed at the height of the febrile reaction to reconstituted stock preparations, desiccated from the frozen state *in vacuo* over anhydrous calcium sulphate and stored at $-10°$ C.

2. *Eggs.*—All the eggs used were obtained from the poultry section of the Institute where a large flock of selected high producing White Leghorns is maintained. The officers of that section carried out the preliminary incubation of the eggs in either a Jamesway or Buckeye incubator. These incubators, of the forced draught type, are the machines used by the poultry section for hatching their own chickens; they are adjusted to run at a temperature of 37·0-37·5° C. and as the hatching percentage in the plant is particularly high it is evident that the preliminary incubation was carried out under uniform optimum conditions.

3. *Inoculation of Eggs.*—Eggs were inoculated directly into the yolk sac by the method of Cox (1938). In the earlier experiments the eggs were not washed but were merely wiped with a clean cloth to remove gross extraneous material. From time to time, however, bacterial contaminants were picked up, so that in all the later work eggs were washed in $\pm 2\frac{1}{2}$ per cent. chloride of lime solution warmed to 40° C. after the preliminary incubation and prior to candling for the removal of dead-in-shells and those occasional eggs with misplaced air space or underdeveloped embryos. The holes in the shells over the air space were drilled with a No. 3 Dental Solila burr fitted to Dremel Moto Tool*, after disinfection with tincture of " Merthiolate ". Injections were made through $1\frac{1}{2}$-inch 23 gauge needles fitted to 1 c.c all glass tuberculin syringes. The dose of 0·1 c.c. was chosen since, with this amount, it was convenient to inject 6 eggs with one syringeful of emulsion and leave sufficient over for a bacterial sterility test. After injection the holes were sealed with paraffin wax applied with a sterile platinum loop.

4. *Incubation of Eggs.*—After injection the eggs were packed in wooden trays of a size that would conveniently fit an ordinary Hearson bacteriological incubator. The trays were made of wooden strips, suitably spaced, to offer as little resistance as possible to the circulation of air and so constructed, that the eggs, placed end to end, were held in rows of six with a space of about 2 inches between each layer of eggs. By this arrangement it was possible to pack many dozens of eggs in a single electrically heated incubator.

In the later experimental work the trays of eggs were placed on shelves in large incubator rooms, These rooms are about 12 feet by 9 feet by 9 feet with adequately insulated floor, walls and ceiling and provided with double doors. The requisite heat is supplied by electric tubular heaters bolted to the walls on 3 sides about 1 foot from the floor. The temperature is controlled thermostatically by Negretti and Zambra low voltage relays, of the tilting mercury tube type, but there is no provision for ventilation or for distribution of heat by forced draughts. The thermostats are located under a shelf furthest from the door and about half way up the wall. A calibrated thermometer is placed in the vicinity of each thermostat for recording the temperature of the room The efficiency of the heat regulating mechanism of each room was tested both by thermographs and by daily records of National Physics Laboratory maximum and minimum thermometers. It was found that, in the vicinity of the thermostat, there was a fluctuation of less than 0·4° C. but that there was a variation of up to 3° C. in different parts of the rooms depending upon the shelf and the postition on the shelf, e.g. close to the wall, on the outer edge, immediately above a space between two tubular heaters or above an overlap of two heaters. Consequently, for this study, the temperature at precise points in each room had to be determined accurately and continually checked. It becomes obvious that for work of this nature a system of heating by forced draught is an absolute essential.

5. *Temperature of Incubation.*—All thermometers used were accurately calibrated against National Physics Laboratory standards but the variations in temperatures referred to above were such that some other method of recording had to be devised. Eventually it was decided to record the temperature not only of a room, but of a particular position in a room, as the temperature of the centre of an infertile egg which had been in position for not less than 48 hours. These temperatures were taken by means of a calibrated thermocouple needle and galvanometer according to the method described by Elder (1941). The needle was inserted through a hole previously drilled over the air space and sealed with paraffin wax. Exceedingly accurate temperature records of both fertile and infertile eggs were obtained by this method.

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*Dremel Manufacturing Co., Racine, Wisconsin, U.S.A.

6. *Harvesting Embryos.*—In the earlier experiments eggs were opened by cutting off the top of the shell with scissors sterilized by dipping into hot liquid paraffin maintained at a temperature of about 200° C. by suitable indirect heating. In spite of the precaution of dipping the eggs into sterile melted paraffin wax difficulty was experienced in preventing small fragments of shell from dropping into the eggs with the result that the percentage of bacterial contaminations was too high. The shells were then cut with an oxy-acetylene flame from a torch fitted with a No. 1 Nozzle, the egg being rotated, air space uppermost, in a cup attached to the spindle of an ordinary electrically driven gramophone turntable motor (*cf.* Penna, 1939). After removing the top of the shell each embryo is lifted out by means of a small platinum hook inserted round the neck after tearing the umbilical vessels. As speed of manipulation is essential for the maintenance of strict asepsis

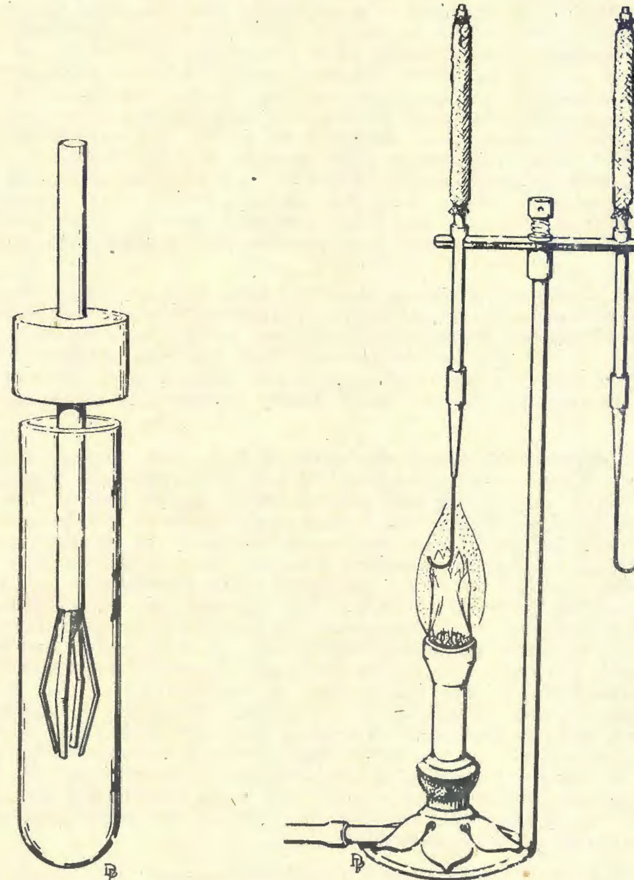

FIG. 1.

it was necessary to ensure that a sterile platinum hook was always at hand for the manipulator. A simple device overcame this difficulty. To the base of an ordinary bunsen burner was welded a metal upright to the top of which was attached a rotatable cross arm (*cf.* figure 1). Slits were cut in the cross arm to engage a flange on the handles of the hooks in such a way that each in turn could be suspended in the flame. While the egg shell was being seared one hook was being heated in the flame while the other was cooling before use. By an arrangement whereby the cross arm is rotated by depression of a foot control the attention of the operator need not be diverted from a simple but somewhat delicate task.

7. *Preparation of Emulsions.*—Since it was necessary to prepare a large number of different emulsions each day, provision had to be made for the disintegration of separate embryos, groups of embryos and/or their membranes with complete asepsis. A curved four fingered prong was welded to the end of a stainless steel rod which in turn passed through a central hole in the base of a cylindrical stainless steel hood to which it was firmly welded (*cf.* figure 1). The hood loosely fitted a hard glass tube of such a length that the tips of the prongs were close to the bottom. After sterilization in the autoclave, followed by drying in the hot air oven, these hooded tubes were used for collecting the harvested embryos which were disintegrated by attaching the rod to the spindle of a fractional horsepower variable speed motor by means of a flexible rubber connection. By high speed rotation the fragile embryo tissue can be reduced to a pulp sufficiently fine to be drawn into a pipette or syringe with or without the addition of any diluent. Large numbers of these socalled "mincer tubes" can be made easily and cheaply and are of the greatest value.

8. *Titration of Emulsions.*—Emulsions to be titrated for virus content were clarified by centrifugation in a Clay Adams angle centrifuge at 3,000 revs. per min. for 5 minutes. At this speed particulate matter is deposited but the virus particles are sufficiently small to ensure that there is no diminution of virus content in the supernatant fluid. Titrations are carried out by serial decimal dilution with full aseptic precautions and at least 6 eggs are injected with each dilution; 50 per cent. end points were calculated by the method of Reed and Muench (1938) and all titres are expressed logarithmically.

Experimental.

No good purpose would be served by detailing the disappointing negative results that were obtained from numerous modifications of technique that were tried in attempts to obtain uniformly good multiplication of the virus strains and to reduce serial passage to a simple routine. These modifications included variations in the dose and concentration of the inoculum, freezing and thawing tissue material to accelerate disintegration of the cellular elements, the use of a wide variety of diluents for emulsification, injection into the yolk sac, onto the chorioallantoic membrane and into the allantoic and amniotic cavity of eggs produced by fowls on sufficient and deficient diets and containing embryos of different ages. As passage proceeded the only constant observation was that it was possible to continue propagation with difficulty. Then it was noticed that if death of the embryo could be regarded as an index of virus multiplication there was a fairly well defined rhythm in the successes and failures. When the virus was growing well the number of eggs and trays of eggs in the incubator decreased and this would be followed by a period of poor growth when there was an accumulation of eggs containing embryos of all ages. A critical investigation of the temperatures at various levels inside the incubator showed that, when empty, the difference in temperature between the top and the bottom was fractional; when full, there was a difference of up to 4° C. Apparently the heating mechanism of the incubator was inadequate for the purpose for which it was being used and four incubator rooms, adjusted to run at 4 different temperatures, were brought into operation. It was soon apparent that the temperature of incubation was of prime importance and a detailed investigation was commenced. As a preliminary, large numbers of non-inoculated fertile and infertile eggs were incubated, the record of temperatures being given in detail in Table 1. All the fertile eggs had undergone 8 days' preliminary incubation before being used in the experiment.

Results.—An analysis of the records of temperatures in the 4 rooms brings out the following points:—

1. *Infertile eggs.*—There is a variation in the internal temperature of infertile eggs maintained under what are believed to be identical conditions in an artificially heated still atmosphere. This variation in the case of individual eggs shows a maximum deviation

TABLE 1.

The temperature of fertile and infertile eggs maintained in a still atmosphere at different temperatures.

Room.	Type of Egg.		Temperature °C.						
			Day I.	Day II.	Day III.	Day IV.	Day V.	Day VI.	Day VII.
A (32·08) 32·1 °C.	Infertile	Highest	32·13	32·15	32·21	32·20	32·23	32·23	32·14
		Lowest	32·01	32·07	31·85	32·02	31·99	32·03	32·00
		Average	32·07	32·11	32·03	32·06	32·10	32·08	32·12
	Fertile	Highest	32·45	32·55	32·49	32·56	32·79	32·79	33 00
		Lowest	32·05	32·07	32·25	32·20	32·39	32·39	32·40
		Average	32·33	32·36	32·37	32·37	32·53	32·63	32·66
	Mortality	—	2/35	0/26	0/22	0/37	1/48	1/30	8/54
B (33·59) 33·6 °C.	Infertile	Highest	33·72	33·68	33·70	33·70	—	—	33·80
		Lowest	33·46	33·46	33·48	33·42	—	—	33·40
		Average	33·61	33·59	33·60	33·58	—	—	33·59
	Fertile	Highest	34·00	34·00	34·10	34·32	34·40	34·84	34·84
		Lowest	33·56	33·56	33·60	33·90	33·92	34·28	34·44
		Average	33·79	33·88	33·82	34·13	34·36	34·56	34·63
	Mortality	—	0/18	1/40	0/20	1/15	1/60	3/38	6/52
C (35·00) 35·0 °C	Infertile	Highest	35·04	35·01	35·01	35·10	35·04	35·01	35·02
		Lowest	34·98	34·92	34·93	34·92	34·96	34·94	34·96
		Average	35·00	34·99	35·00	35·02	35·00	35·01	35·00
	Fertile	Highest	35·49	35·60	35·96	35·90	36·00	36·18	36·34
		Lowest	34·98	35·08	35·18	35·43	35·42	35·50	35·54
		Average	34·95	35·25	35·43	35·54	35·67	35·82	36·08
	Mortality	—	2/27	2/29	0/24	1/18	0/32	1/30	2/36
D (38·21) 38·2 °C	Infertile	Highest	38·33	—	—	—	—	—	—
		Lowest	38·10	—	—	—	—	—	—
		Average	38·21	—	—	—	—	—	—
	Fertile	Highest	38·40	38·50	39·14	39·66	40·82	42·34	—
		Lowest	38·10	38·38	38·98	39·30	40·34	41·62	—
		Average	38·36	38·45	39·05	39·54	40·60	41·87	—
	Mortality	—	0/12	1/12	0/12	0/12	1/12	0/12	—

Fertile eggs had been incubated in chick incubators for 8 days before being placed in incubator rooms.
In mortality figures numerator = No. dead embryos, denominator = total eggs examined.

from the mean of 0·23° C. and is believed to be due to such factors as density and porosity of the shell, under conditions where the humidity of the atmosphere was not accurately controlled. The average temperature of groups of not less than 12 eggs shows a maximum deviation of 0·05° C (usually 0·01° C.) about the mean, a figure within the limits of experimental error of the technique, Consequently, for the purposes of this study the temperatures of incubation in the 4 rooms have been taken as 32·1° (actually 32·08°), 33·60° (actually 33·59°), 35·0° and 38·° C. (actually 38·21°).

2. *Fertile eggs.*—After 8 days' preliminary incubation in an efficient commercial chick incubator followed by 24 hours at the lower temperatures the internal temperature of a fertile egg is not the same as the air temperature but is somewhat higher. At 32·1° the average increase was 0·25°; at 33·6°, 0·20°; at 35°, 0·05°; and at 35·0°, 0·15° C. Moreover, in individual eggs the deviation from the mean was far greater than was the case with the infertile eggs. After incubation for a further period of 6 days the average temperatures were respectively 0·58°, 1·04°, 1·08°, and 3·66° C. above the air temperature (the latter at 38·2° for 5 days), i.e., the higher the temperature of incubation the greater the difference between the air temperature and the egg temperature. During the first few days, i.e., the 9th, 10th and 11th day of incubation the daily increase in temperature was not marked but it gradually became greatly accentuated during the last two days, i.e., 14th and 15th days.

During the first 5 days of incubation there was no significant difference in the rate of survival of the embryos at any of the 4 temperatures but on the 7th day the mortality, particularly at the lowest temperature, was significantly higher.

Two additional points which were readily apparent but which cannot be indicated in the table were the marked difference in the development of the embryos at the different temperatures—being retarded with each additional decrease— and the observation that even before death of the embryo the temperature of an egg started to drop to that of an infertile egg; in fact the temperature of an egg frequently, but not invariably, was an index of the vigour and viability of the embryo.

Comment.—An attempt was made to investigate the effect of incubation at temperatures lower than 32° C. but it had to be abandoned for two reasons. In the first place, after the first few days of incubation the death rate amongst the embryos was so high that the results were confusing and of little practical value. Secondly the outside temperature was so frequently higher than that inside the incubator room that accurate control with the facilities available would have been difficult.

It must again be emphasized that under conditions of incubation in a still atmosphere such as is found in a large incubator room recorded temperatures apply only to particular positions in the room and consequently throughout this study great care was taken to keep this point in view. It is stressed that in work of this nature provision must be made for artificial heating by some system of dispersed forced draughts.

The results of this study show clearly that for the cultivation of viruses in the developing chick embryo it is necessary to take into consideration the conditions of preliminary incubation and the age of the embryo, as well as the experimental temperature of incubation since it is possible for each of these factors to play an important rôle. In any case, with the possible exception of embryos below the age of 7 days which are seldom used, it must be realized that the air temperature of incubation is not the temperature to which the virus is being submitted.

In passing it may be stated that a record of the temperatures of a large number of eggs infected with the virus of bluetongue was kept but no significant difference from uninoculated eggs could be determined.

By this time the strain of Bekker virus which was being passaged serially through eggs had been divided so that a duplicate series was being maintained at each of the 4 temperatures. As great difficulty was experienced in continuing propagation at 38·2° C. that series was abandoned and all the subsequent quantitative work was carried out with the strains kept at 32·1°, 33·6° and 35·0° C.

TABLE 2.

The effect of distilled water, physiological saline, phosphate buffer, and broth as a vehicle and diluent in titration experiments.

Vehicle.	Dilution	Day 2.	Day 2.	Day 3.	Day 4.	Day 5.	Day 6.	Day 7.	
Distilled water	$1/10$	1—5	0—5	2—3	2—1	1—0	—	—	Titre 3·5555
	$1/10^{-2}$	0—6	0—6	1—5	1—4	2—2	1—1	0—1	
	$1/10^{-3}$	0—6	1—6	1—5	1—4	4—0	—	—	
	$1/10^{-4}$	0—6	‡1—5	‡1—4	0—4	1—3	0—3	0—3	
	$1/10^{-5}$	1—5	0—5	0 5	0—5	0—5	0—5	0—5	
	$1/10^{-6}$	0—6	‡1—5	0—5	0—5	0—5	0—5	0—5	
0·85 % Saline	$1/10$	2—4	0—4	2—2	2—0	—	—	—	Titre 4·3637
	$1/10^{-2}$	1—5	0—5	1—4	3—1	0—1	1—0	—	
	$1/10^{-3}$	1—5	1—4	0—4	3—1	0—1	1—0	—	
	$1/10^{-4}$	0—6	0—6	0—6	3—3	0—3	1—2	0—2	
	$1/10^{-5}$	1—5	0—5	0—5	0—5	0—5	0—5	†2—3	
	$1/10^{-6}$	0—6	0—6	1—5	0—5	1—4	0—4	0—4	
M/50 Phosphate Buffer	$1/10$	0—6	0—6	4—2	1—1	0—1	1—0	—	Titre 5·0000
	$1/10^{-2}$	0—6	0—6	3—3	2—1	1—0	—	—	
	$1/10^{-3}$	1—5	0—5	0—5	2—3	3—0	—	—	
	$1/10^{-4}$	0—6	0—6	1—5	1—4	2—2	1—1	0—1	
	$1/10^{-5}$	0—6	1—5	0—5	0—5	1—4	0—4	*2—2	
	$1/10^{-6}$	0—6	0—6	0—6	0—6	0—6	0—6	†1—5	
Broth........	$1/10$	0—6	0—6	3—3	2—1	1—0	—	—	Titre 5·3750
	$1/10^{-2}$	0—6	0—6	1—5	5—0	—	—	—	
	$1/10^{-3}$	2—4	0—4	0—4	2—2	2—0	—	—	
	$1/10^{-4}$	0—6	0—6	0—6	2—4	4—0	—	—	
	$1/10^{-5}$	0—6	0—6	0—5	1—5	1—4	*2—2	†1—1	
	$1/10^{-6}$	0—6	1—5	0—5	0—5	0—5	0—5	0—5	

NOTE.

In this and all further titrations 0— 6 = no deaths, 6 alive ; 3—3 = 3 dead and 3 alive etc.

The * or † sign above the indicated dead embryos means the embryos were harvested and subinoculated into other eggs with positive or negative results for presence of virus thus * death due to to specific action of virus and † death due to some other cause. ‡ Means death is regarded as nonspecific but not proved by subinoculation. Unless otherwise stated eggs after 8 days preliminary incubation were used.

Since it had been decided to carry out the work on a quantitative basis, the first essential now was to determine the most suitable diluent for titration experiments and then to test the validity of such titrations by ascertaining whether they could be replicated within the limits of experimental error of a technique which demands complete asepsis and, finally, whether the results in eggs are applicable to sheep.

EXPERIMENTAL.

Bekker virus egg generation 146 cultivated at 32·1°C. was used to inoculate 6 eggs. Of these 2 were dead on day 3, 3 an day 4 and 1 on day 5. The 3 embryos which died on the 4th day were harvested and after thorough disintegration without the addition of any diluent 0·5 c.c. was added to each of 4 tubes containing respectively sterile distilled water, 0·85 per cent. saline, M/50 phosphate buffer pH 7·3 and horse flesh infusion broth in 4·5 c.c. amounts. The contents of each tube were again thoroughly emulsified and, after clarification in the angle centrifuge, were titrated by decimal dilution in the same diluent. The egg injections were made 4 hours after the serial dilutions had been completed and for reasons which will become apparent later the eggs were incubated at 33·6° C. The results are given *in extenso* in Table 2.

TABLE 3.

The Apparent Virus Titre of Emulsions on Incubation at Various Temperatures.

Virus Emulsion.			Virus Titre.			
Egg Generation.	Temp. of Incubation.	Deaths.	32·1 °C.	33·6 °C.	35·0 °C.	38·2 °C.
136	32·1	0/1, 1/2, *4/3, 1/4, 1/0	5·63	4·83	4·00	—
147	32·1	*3/3, 2/4, 1/0	5·40	4·00	3·73	<1·00
149	32·1	2/3, *4/4	5·00	4·12	2·86	—
156	32·1	*4/3, *2/4	5·50	4·69	4·00	—
			5·00	4·30	3·63	—
134	33·6	*6/4	4·64	4·00	2·60	<1·00
151	33·6	1/2, 2/3, *3/4	3·87	3·00	2·75	—
139	35·0	*5/3, 1/4	4·83	4·21	3·69	<1·00
151	35·0	*6/3	4·12	3·50	2·50	—
159	35·0	*3/3, 2/4, 1/0	3·63	3·24	1·75	—
160	35·0	*10/3, 3/4, 2/5, 1/0	3·12	2·75	2·00	—

NOTE.

To collect embryos for the preparation of the various emulsions 6 eggs were injected in each case except generation 160 at 35° when 16 were used.

0/1 = 0 deaths on day 1, 1/2 4/3 etc. = 1 and 4 deaths on day 2 and 3 respectively.

1/0 = survival for 7 days.

* = indicates the particular embryos titrated.

Result.—The titre with phosphate buffer or broth as diluent was higher than that obtained with saline or water. Either more virus was dispersed into the supernatant fluid when broth or phosphate buffer was used or less inactivation of virus took place in the 4-hour period which elapsed between emulsification and injection. In any case the superiority of broth and buffer solution was readily apparent. As the result was confirmed in a second experiment it was decided that in all future work broth would be used as a routine. There is an added advantage in that the residual fluid in each tube of a series of titrations constitutes a suitable gross bacterial sterility test.

In a second series of experiments it was found that the 50 per cent. lethal end-points (L.D. 50) as calculated by the method of Reed and Muench are easily repeatable within the range $\pm \cdot 2$ so that the validity of expressed titres is not open to doubt.

The next steps in the investigation were to determine the optimum temperature of incubation (1) for the production of the highest titre of virus, and (2) for the initiation of infection after the injection of the smallest dose of virus. The general scheme for this investigation was to passage the strain of virus at each of the selected temperatures and then to titrate each series of emulsions at the same temperatures. A comprehensive series of experiments were completed, the results of which are given in Table 3.

Results.—Consideration of the results given in Table 3 justifies the following conclusions:—

1. The apparent virus titre of a given emulsion is dependent on the temperature of incubation of the eggs in the titration test.
2. For any given emulsion a consistently higher titre is obtained at 32·1° C. than at the two higher temperatures and if a temperature of 38·2° C. alone is used it would be concluded that no active virus was present. Alternately the M.I.D. of an emulsion appears to be smaller at 32·1° C. than at the other temperatures.
3. The apparent decrease in titre is somewhat less than 10 fold (ca. log 0·8) for each 1·5° rise in temperature in the range 32·1° to 35°C. Whether this decrease continues at the same rate above 35° C. was not determined.
4. The titre of virus propagated at 32·1° C. is consistently higher than those obtained by propagation at the higher temperatures, no matter at what temperature the titration was carried out. There is only a tendency for titres produced by incubation at 33·6° C. to be higher than those obtained at 35° C.

It is emphasized that throughout the entire series of tests the corresponding group of eggs incubated at each temperature was injected with fluid obtained from the same tube of diluted emulsion so that there is no possibility of a recurrent error of technique being responsible for the constant results. As the experiments did not give any indication of any correlation between the apparent and the true titre of a given emulsion it was necessary to compare the titres obtained in eggs with the infectivity for Merino sheep. The dose for eggs was kept at 0·1 c.c. while 1·0 c.c. of the same virus dilution was given subcutaneously to sheep. By the time this work was commenced the virus strain had become so attenuated by serial passage that the production of a febrile reaction could no longer be relied upon as an index of infection, and conclusions could be drawn only from the results of immunity tests with the homologous virulent strain applied after

an interval of approximately 4 weeks. The dose of virus for these immunity tests was 2·0 c.c. of preserved blood given subcutaneously. It must be mentioned that before the influence of temperature of incubation had been appreciated several attempts had been made to correlate the infectivity for eggs with that for sheep, without success. For the sake of completeness those attempts are included in the results shown in Table 4 but the temperature of incubation is shown as a query because it had not been accurately controlled or determined.

TABLE 4.

Relation between Infectivity for Sheep and the Apparent Titre in Eggs Incubated at Various Temperatures.

Virus.			Titration of Infectivity.					
			Eggs.		Sheep.			
Exp. No.	Egg Generation.	Temp. of Incubation.	Temp.	Titre.	Sheep No.	Dilution Log.	Reaction.	Immunity Test.
					60811	0	++	—
					62209	1	?	—
					61239	2	—	—
1	46	?	?	2·47	62159	3	—	+++
					61459	4	—	+++
					62150	5	—	+++
					60850	1	—	+++
					62238	2	—	+++
					62266	3	—	+++
2	53	?	?	1·83	62266	3	—	+++
					60429	4	—	+++
					60914	5	—	+++
					60630	5	—	+++
					60891	1	?	?
					60510	2	—	—
					60348	3	—	?
3	77	?	?	2·50	62278	4	—	+++
					61658	5	öö	+++
					62050	6	—	+++
					60802	1	+	—
4	70	?	?	2·50	61343	2	—	—
					60488	3	—	—
					61313	4	?	—
					66238	1	—	?
5	151	35	32·1	4·12	66288	2	—	—
			33·6	3·50	66129	3	—	—
			35·0	2·50	66277	4	—	+++
					67855	1	—	—
					67904	2	—	?
					67521	3	++	—
6	176	32·1	33·6	4·25	67425	4	—	—
					67844	5	?	+++
					67950	6	—	+++
					67829	7	—	+++

Results.—In the first 4 experiments where the temperature of incubation was not known accurately, the titre of the virus emulsions was particularly low as indicated by the results of titrations in eggs. Moreover, the titre in eggs did not correspond to the infectivity of the serial dilutions for sheep with the exception of experiment 1 and, to a lesser extent, experiment 2.

In experiment 5, the egg titre at 35° C. was very similar to the titre in the previous 4 tests but the indicated titre at 33·6° C. corresponded to the infectivity for sheep.

In experiment 6 the titre at 33·6° C. only was determined and this corresponded to the infectivity for sheep.

Conclusion.—It is concluded that the apparent titre of a given embryo emulsion, as determined by titration in eggs incubated at 33·6° C., corresponds to the infectivity for sheep, the respective doses being 0·1 c.c. injected into the yolk sac of 8-day embryos, and 1 c.c. subcutaneously. Consequently, in all subsequent work, egg titrations were conducted at 33·6° C. only, since this served to reduce the amount of work considerably and permitted a fairly accurate estimate of what the results in sheep would have been.

Since the ultimate object of this investigation was the development of a technique for the routine preparation of bluetongue vaccine on a large scale it now became necessary to determine under what conditions a consistently high titre of virus could be produced. It was argued that, as bluetongue is essentially a disease of sheep which have a normal temperature of approximately 39° C., and, as the highest titre of virus in the blood of a sheep is found at the height of the febrile reaction (frequently above 41·5° C.), therefore, the temperature *per se* could not be the determining factor. More probably the lower temperature of incubation of the eggs retarded the development of the embryos and made available to the virus certain hypothetical metabolites which would normally be utilized exclusively by the embryo. Alternately incubation at a low temperature immediately after inoculation might result in rapid initial multiplication, corresponding say to the injection of an exceedingly high titre inoculum, and that subsequent incubation at a higher temperature would accelerate the multiplication which had been initiated. A preliminary experiment was planned to throw some light on this point.

Experimental.

Each of the 30 eggs containing 8-day embryos were seeded with 0·1 c.c. of virus emulsion representing a 10 per cent. emulsion of embryos, egg generation 160, at 32° C.; 6 eggs were incubated at 38·2° C. to serve as controls and the remaining 24 were placed at 32·1° C. Each day after candling, 6 eggs containing live embryos were transferred from the 32·1° C. room to 38·2°. As the embryos died the various groups of dead embryos were pooled and immediately titrated at 33·6° C. The results are given in Table 5.

Results.—The adverse effect of incubation at the higher temperature is readily apparent from the progressive decrease in titre as the length of time the eggs were maintained at 32·1° C. decreased, and the period at 38·2° increased.

To confirm this result, and at the same time to investigate the effect of intermediate temperatures, a more comprehensive experiment was planned. In addition, bearing in mind the progressive increase in the temperature of

a fertile egg under normal conditions of inoculation (cf. Table 1), a further series was included in which the temperatures were decreased. The general plan of the experiment is obvious from the results given in Table 6, where the actual deaths of the embryos are shown, because uniformity, not only of high titre, but also deaths, would be of extreme importance in vaccine production.

TABLE 5.

The Effect of increasing the Temperature of Incubation from 32·1° C. to 38·2° C.

Temperature of Incubation.	Titre.
32·1° for 3 days	5·57
32·1° for 3 days, then 38·2° for 1 day	4·41
32·1°, for 2 days, then 38·2° for 1 day	3·75
32·1° for 2 days, then 38·2° for 2 days	2·62
32·1° for 1 day, then 38·2° for 5 days	0
38·2° for 5 days	<1·00

NOTE.—Of the control eggs at 38·2 only 2 embroyos died, both on day 5.

TABLE 6.

The Effect of both Increasing and Decreasing the Temperature of Incubation on Virus Multiplication.

Temperature of Incubation.	Deaths of Embryos on Day.							Virus Titre at 33·6 C.
	1	2	3	4	5	6	7	
32·1° for 1 day, then at 38·2°	0—6	0—6	1—5	0—5	0—5	0—5	0—5	—
32·1° for 2 days, then at 38·2°	0—6	0—6	5*—1	0—1	0—1	0—1	0—1	3·50
32·1° for 2 day, then at 35·0°	0—6	0—6	4*—2	1—1	0—1	0—1	0—1	4·75
32·1° for 2 days, then at 35·0°	0—6	0—6	3*—3	1—2	2—0	—	—	6·00
32·1° for 1 day, then at 33·6s	0—6	0—6	2—4	3*—1	1—0	—	—	4·75
32·1° for 2 days, then at 33·6s	0—6	0—6	2—4	4*—0	—	—	—	4·50
32·1° only	1—23	0—23	17*—6 (1)	6*—0 (2)	—	—	—	(1) 4·84 (2) 4·57
33·6° for 1 day, then at 32·1°	0—6	0—6	4*—2	1—1	1—0	—	—	4·33
33·6° for 2 days, then at 32·1°	0—6	1—5	4*—1	1—0	—	—	—	4·87
35·0° for 1 day, then at 32·1°	0—6	0—6	4*—2	1—1	1—0	—	—	5·75
35° for 2 days, then at 32·1°	0—6	1—5	5*—0	—	—	—	—	4·75
38.2° for 1 day, then at 32·1°	0—6	0—6	4*—2	1—1	0—1	0—1	0—1	4·62
38·2° for 2 days, then at 32·1s	0—6	0—6	0—6	1—5	0—5	0—5	3*—2	0

NOTE.—* Denotes embryos titrated in eggs.

Results. 1. A general survey of the results shows that when 38·2° C. was included in the scheme of incubation there was a significant tendency for the virus titre to be reduced and, in addition, deaths of the embryos were either irregular or there were many survivors. The number of survivors increased with the length of time the eggs were held at 38·2° C., before being transferred to a lower temperature.

2. The highest titre emulsion was obtained after two days' incubation at 32·1° C. followed by transfer to 35·0° C. (6·00) though this titre was hardly significantly higher than that obtained by the reverse procedure, viz., 1 day at 35° C. followed by transfer to 32·1° C. (5·75). Moreover, by both procedures all the embryos died between the 3rd and the 5th day, the majority on the 3rd day.

3. Continued incubation at 32·1° C. resulted in all the embryos dying on the 3rd and 4th day. There was no significant difference in the titres of virus obtained from the dead embryos on those two days, but that titre was approximately ten fold lower than was the case after preliminary 24 hours' incubation at 35·0° C.

4. The titre of virus from all other combinations of temperature excluding the 38·2° C. were remarkably constant at a lower level.

In view of these results it was decided to run a further experiment to determine whether preliminary incubation at 32·1° C. for 24 hours followed by transfer to 35·0° C. or the reverse procedure produces the best results.

Experimental.

Virus, egg generation 169 at 32·1° C., titre 4·50 at 33·6° C., was used as the inoculum. All the embryos found dead each day were harvested, pooled in their respective groups, emulsified without the addition of any diluent, and titrated with broth as diluent on the day that they were harvested. The method of incubation and the titres of the various emulsions are shown in Table 7.

Table 7.

The Effect of Incubation at a Combination of 32·1° C. *and* 35·0° C.

Temperature of Incubation.		Day.						
		1	2	3	4	5	6	7
At 32·1° C.	Deaths	2—20	1—19	3—16	11—5	5—0	—	—
	Titre	—	—	5·30	5·45	4·22	—	—
1 day at 32·1°, then at 35°	Deaths	0—20	1—19	3—16	3—13	10—3	0—3	0—3
	Titre	—	—	5·00	4·84	3·28	—	—
1 day at 35°, then at 32·1°	Deaths	0—21	0—21	13—8	5—3	2—1	1—0	—
	Titre	—	—	5·57	5·00	3·40	—	—
At 35°	Deaths	0—20	1—19	3·16	6—10	2—8	0—8	0—8
	Titre	—	—	4·30	3·83	2·00	—	—

Note.—Virus titres were determined by incubation at 33·6° C.

Results.—The results of this experiment may be conveniently considered under two headings, viz., the deaths of the embryos and the virus titre of the emulsions.

1. *Deaths of the embryos.*—At 32·1° C. there was a tendency for the time of survival of the embryos to be prolonged; only 3 out of 19 died on the 3rd day, 11 out of the remaining 16 died on the 4th day, and the balance were all dead on the 5th day. This tendency was more pronounced after one day's incubation at 32·1° C. before being transferred to 35° C., and, at 35° C., only 12 out of 20 (=60 per cent.) of the embryos died. When the eggs were first incubated at 35° C. for 1 day and then transferred to 32·1° C. 13 out of 21 died on the 3rd day, 5 of the remaining 8 were dead on the 4th day, but 1 embryo survived to the 6th day.

2. *Virus titre.*—The highest titre of virus, was obtained by incubating for one day at 35·0° C. and two days at 32·1° C. (5·57). This titre was only slightly and possibly not significantly higher than that obtained from almost the same number of dead embryos on the 3rd and 4th day of incubation at 32·1° C. All the other titres were considerably lower. Further it should be noted that the longer the embryos survived at the higher temperature the more rapidly the virus titre diminished; the same, but far less pronounced, tendency was shown by the survivors to the 5th day at 32·1° C.

These results were confirmed by several experiments planned on similar lines.

Having obtained adequate data on the effect of temperature on virus multiplication it was necessary to investigate the rôle of the concentration of virus in the inoculum. This was very conveniently done by harvesting the dead embryos in the titration experiments referred to above and then to titrate the emulsions thus obtained. In this way the titres of the inocula were accurately known in each case but, since all the initial titrations were carried out only at 33·6° C., the results apply to that temperature only. It was found that the titres were remarkably constant so the average of 3 strictly comparable experiments are given in Table 8.

TABLE 8.

The Effect of Concentration of Inoculum on Virus Titre at 33·6° C.

Inoculum M.I.D.'s.	Titre of Embryos.			
	Day 3.	Day 4.	Day 5.	Day 6.
5	—	4·84	4·00	2·84
50	4·50	4·41	4·76	3·00
500	5·12	4·37	3·43	—
5,000	4·50	4·56	3·50	—
50,000	4·33	2·36	—	—

Results.—The results show that as the concentration of virus in the inoculum increased so the time of survival of the embryos decreased. For instance when the inoculum contained only 5 M.I.D.'s of virus the number of embryos which died on the 3rd day of incubation was too small to warrant an estimation of the virus titre. On the other hand when the inoculum contained 50,000 M.I.D.'s of virus a similar insignificant number of embryos survived to the 5th day. Apparently, no matter the virus titre of the inoculum, multiplication of the virus continues to a constant maximum for that temperature, the length of time it takes to reach that maximum being

dependent on the initial virus concentration. For instance, embryos that received an infecting dose of 5 M.I.D.'s attained this maximum on the 4th and 5th day, survivors to the 6th day containing approximately 100 fold less virus; eggs which received 50 M.I.D.'s succumbed when they had reached the maximum titre on the 3rd, 4th and 5th days probably depending on individual variation of different eggs; eggs which received 50,000 M.I.D.'s rapidly reached maximum titre on day 3 and any survivors to the 4th day again showed an approximate 100 fold decrease in virus content.

Conclusion.—It is concluded that the concentration of virus in the inoculum does not affect the ultimate virus content of the embryos but that the larger the dose the quicker will this constant maximum be obtained. In embryos which are able to survive the presence of the maximum amount of virus attainable at 33·6° C., the titre rapidly decreases. Consequently, with a view to the economy of antigen it is advisable to dilute an emulsion so that it contains approximately 500 M.I.D.'s of virus, and to harvest the embryos at death on the 3rd and 4th day. This conclusion is valid only for incubation at 33·6° C. and need not necessarily apply to incubation at higher or lower temperatures. There is some evidence to show that particularly with inocula prepared from embryos incubated at 35° C. variations in virus titre of the inoculum have a marked effect at least on the time of survival of the embryos.

General Conclusions.

From the results of this series of experiments considered to be sufficiently comprehensive to warrant generalization, it is believed that, to produce the maximum titre of virus in fertile hens' eggs injected directly into the yolk sac after 8 days' preliminary incubation, the inoculum should contain approximately 500 M.I.D.'s of virus, the eggs should be incubated at 35° C. for 24 hours and then at 32° C. The great majority of embryos will succumb on the 3rd and 4th day of incubation, a 100 per cent. mortality may be anticipated but survivors beyond the 4th day should be discarded since the virus content is lower.

Influence of the Age of Embryo.

Many titrations were replicated in embryos which had undergone 7, 8, and 9 days' preliminary incubation. As the investigation was confined to this narrow range the results are not given in detail, but, it may be stated that no significant difference in the susceptibility of the embryos nor of the virus titre attained could be established. Embryos, harvested on the 4th day after inoculation and incubated for more than 9 days before being seeded, are somewhat difficult to handle with complete asepsis and, in addition, the commencing development of feathers is something of a nuisance, so that 8-day old embryos are constantly used.

Keeping Qualities and Storage of Virus.

Bluetongue virus is characterized by remarkable longevity. Blood from a reacting sheep usually retains infectivity for periods up to two years merely on storage at ±5° C. and it has been recorded (Neitz 1945) that a bottle of vaccine consisting of infective blood diluted 1:3 in an anti-coagulant preservative mixture of potassium oxalate-phenol-glycerine and saline was infective after storage for 25 years at room temperature. In view of this it is desirable to mention two phenomena which were encountered during the course of this work.

1. At one stage of the investigation it became necessary to suspend the routine passage of all virus strains for a period of about 3 weeks. To ensure the continuity of the work at a later stage, the various groups of embryos were made up as approximate 10 per cent. emulsions in broth in the ordinary way, and stored in cotton wool stoppered glass tubes in 5 c.c. amounts in an ordinary household refrigerator running at ± –10° C. At the end of 3 weeks the tubes of frozen emulsions at –10° C. were removed, rapidly thawed by immersion in tap water, clarified by spinning in an angle centrifuge at 3,000 revs. per minute for 5 minutes and then injected into eggs. All the emulsions proved to be inactive. The tubes stored unfrozen were then clarified in the same way; all proved to be fully infective and routine passage was continued. A number of other emulsions which had been stored in the cabinet at –10° C. were then tested and from only one was active virus isolated. To investigate the occurrence more closely the following experiment was run:—

Experimental.—Of 18 eggs that received Bekker virus, generation 153 at 32·1° C., 8 were dead on the 3rd day; four embryos were selected at random and emulsified without the addition of any diluent. To each of 3 tubes containing 4·5 c.c. of sterile broth 0·5 c.c. of the emulsion was added and each tube thoroughly shaken. The contents of one tube were frozen rapidly by immersion in an alcohol-carbon dioxide snow mixture and then placed at –10° C.; one tube was placed directly in the cabinet at –10° C. and it was noticed that the contents were still fluid 5 hours later but had frozen after 18 hours; one tube was stored at 5° C., and one tube at room temperature (maximum temperature 29·4° C.). After an interval of 48 hours the frozen emulsions were rapidly melted, all were clarified by angle centrifugation and titrated in eggs by decimal dilution at 33·6° C. with the following results:—

Stored at room temperature ... ... titre 4·31
Stored at +5° C. ... ... titre 4·43
Stored at –15° C. (after rapid freezing) ... ... titre 4·31
Stored at –15° C. (slow freezing) ... ...titre 1·00

Result.—There was no difference in virus titre after storage for 48 hours at room temperature or at 5° C., nor any decrease on storage at –10° C. provided the material has been rapidly pre-frozen in an alcohol-dry ice mixture, but, on storage at –10° C., with the very slow freezing at that temperature, the drop in titre was marked.

For the preservation of viruses it is common knowledge that very rapid freezing is an essential. It might be believed that this applied only to the more fragile viruses but it has been shown experimentally that rapid freezing and not merely the temperature of storage is a factor of importance even with a resistant virus like that of bluetongue.

2. *Hypothetical Interference Phenomenon.*

In Table 8 it has been shown that within certain limits the virus titre of an infected embryo is independent of the number of infecting doses in the inoculum. In addition to the conditions cited, further reservations must be made.

It has frequently been found in routine titrations that eggs which were seeded with concentrated inocula died less regularly than those which received higher dilutions. A typical example of such a titration is given in Table 9, the emulsion investigated being 4th day dead embryos of egg generation 160, at 35° C. The titration as usual was carried out at 33·6° C.

TABLE 9.

Inoculum.	Dilution.	Day.						
		1	2	3	4	5	6	7
Egg generation 160 at 35° C.	10^{-1}	0—6	0—6	2—4	1—3	0—3	0—3	0—3
	10^{-2}	0—6	0—6	3—3	2—1	0—1	0—1	0—1
	10^{-3}	0—6	0—6	6—0	—	—	—	—
	10^{-4}	0—6	0—6	1—5	1—4	0—4	0—4	1—3
	10^{-5}	1—5	0—5	0—5	0—5	0—5	0—5	0—5
	10^{-6}	0—6	0—6	0—6	0—6	1—5	0—5	0—5

Result.—It is seen that the 10^{-3} dilution provided the inoculum with the maximum lethal effect and that there were the same number of survivors amongst the embyros that received the 10^{-1} dilution as those that received the 10^{-4} dilution. Had the dilutions 10^{-1} to 10^{-3} been reversed in order the result would have been more nearly that which might have been anticipated.

A somewhat similar phenomenon was encountered on checking the results of estimations of virus content by retitration of material which had been stored at 5° C. in the refrigerator. It has been clearly stated that the results of titrations may be replicated within the limits of the experimental error of a somewhat exacting technique and that there may be no decrease in titre on storage even for considerable lengths of time. This statement must be qualified by saying that it appears to apply to diluted material only as shown by a series of results given in Table 10.

TABLE 10.

Retitration of Undiluted Embryo Emulsions stored at 5° C.

Undiluted Embryo Emulsion.		Virus Titre.	Retitration.		Difference.
Temperature of Incubation.	Death of Embryo Day.		Interval Days.	Virus Titre.	
32·1° C.	3	4·33	8	4·40	0·06
	3	5·57	8	5·40	—0·17
	4	5·45	8	5·14	—0·31
33·6° C.	3	4·30	11	3·75	—0·45
	3	5·00	8	4·70	—0·30
	4	2·50	11	1·78	—0·71
35·0° C.	3	3·83	8	1·50	—2·33
	3	4·00	6	2·33	—1·66
	3	3·51	2	2·82	—0·69
	4	2·30	8	0·50	—1·80

Results.—It is seen that the virus titre of the emulsions as determined by titration of decimal dilutions decreased on storage. This decrease was greater in the case of material obtained from embryos incubated at 35° C. and, at the lower temperatures was more apparent in embryos which survived to the 4th day of incubation. This finding is in striking contrast to the longevity of bluetongue virus referred to previously and the constant titre

of diluted emulsions stored for periods up to many months, details of which will be given in a future report on the use of the egg attenuated virus for routine immunization.

At this stage no explanation can be given for these two phenomena which were encountered so frequently that a brief report is warranted. The problem is being investigated in the light of what appears to be a closely related finding recorded by Henle and Henle (1944) in their work on interference between inactive and active viruses of influenza. In this work an appreciation of the existence of the phenomenon has been of the greatest importance and has necessitated taking the greatest care to carry out all titrations immediately after harvesting the embryos or alternately to dilute emulsions at least 100 fold before storage.

Summary.

1. The technique used for the propagation of the Bekker strain of bluetongue virus in the developing chick embryo is described in detail.

2. The important rôle played by the temperature of incubation on the multiplication of the virus is stressed.

3. A convenient and accurate method of determining the air temperature of incubation is to take an average of the temperatures of 6 fertile eggs.

4. The importance of an accurately controlled system of incubation by dispersed forced draughts is stressed for all work of this nature.

5. The temperature of a developing fertile egg between the 8th and the 15th day of incubation is higher than the air temperature of incubation. The older the embryo, and the higher the temperature of incubation in the range 32·1° C. to 38·2° C., the greater the difference between the egg and the air temperature.

6. The apparent virus titre of a given emulsion is dependent on the temperature of incubation of the eggs used for the titration test. The lower the temperature the higher the apparent titre.

7. The titre obtained on incubation at 33·6° C. corresponds to the infectivity of the emulsion for sheep.

8. Using fertile eggs after 8 days' preliminary incubation, virus titre of an emulsion prepared from embryos incubated at 32·1° C. is consistently higher than that obtained by incubation at higher temperatures.

9. At 32·1° C. the majority of embryos in injected eggs are dead by the 3rd and 4th days and the remainder invariably are dead on the 5th day. There is little difference in the titre of virus in the 3rd and 4th day dead embryos but a significant decrease occurs on the 5th day.

10. At 35·0° C. a significant number of embryos survive for longer than 5 days and there is a rapid decrease in titre after the 3rd day.

11. The longer injected eggs are left at a higher temperature before transfer to, or transfer from, a lower temperature the lower the virus titre in the embryos at death.

12. The optimum conditions for maximum multiplication of this strain of virus is to use an inoculum containing 500 M.I.D.'s of virus, to incubate at 35·0° C. for 24 hours, then at 32·1° C. and to harvest the dead embryos on the 3rd and 4th day after injection.

13. The harmful effect of storage at ca. $-10°$ C. is noted.

14. Attention is directed to the apparent poor viability of virus in certain undiluted embryo emulsions and to a hypothetical interference phenomenon.

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