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THE OPTIMAL CONDITIONS FOR THE MULTIPLICATION OF NEETHLING-TYPE LUMPY SKIN DISEASE VIRUS IN EMBRYONATED EGGS

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ABSTRACT

VAN ROOYEN, P. J., MUNZ, E. K. & WEISS, K. E. The optimal conditions for the multiplication of Neethling-type lumpy skin disease virus in embryonated eggs. *Onderstepoort J. vet. Res.*, 36 (2), 165-174, 1969.

Maximum yields of lumpy skin disease virus were obtained in the chorio-allantoic membranes of 5- to 7-day embryonated eggs incubated at 33.5° to 35°C for 5 to 6 days. The route of inoculation did not significantly affect the growth pattern of the virus.

There was no correlation between yield of virus and the appearance of lesions in the chorio-allantoic membranes. Lesions were only produced in the membranes of 7- to 9-day embryonated eggs inoculated onto the membrane and incubated at 33.5° and 35°C.

INTRODUCTION

Haig (1957) reported that on two separate occasions evidence was obtained that the agent of lumpy skin disease could be cultivated in the chorio-allantoic membrane (CAM) of embryonated eggs. In one instance, material from the 2nd passage in eggs was shown to be infectious for cattle, while in the other, material from the 4th but not from the 6th passage, produced a typical reaction.

In a preliminary note, Van Rooyen, Kümm, Weiss & Alexander (1959) reported the propagation of the Neethling strain in embryonated eggs for not less than 10 serial passages. Using the stab method of inoculation, it appeared that the virus multiplied equally well in the embryo and the CAM and that the virus was not lethal for embryos. Routine sub-inoculations were carried out on the 7th day in 7-day embryonated eggs, which were subsequently incubated at 33.5°C. No lesions could be detected on examination of infected eggs, but suspensions of CAM produced cytopathic effects, typical for the virus, in lamb kidney monolayer cultures. At the 19th passage level the virus was apparently modified to such an extent that a vaccine could be developed (Weiss, 1963).

The purpose of this paper is to record the results of investigations to determine the most suitable conditions for the multiplication of the virus in embryonated eggs including the age of the embryos, route of inoculation, temperature and period of incubation after inoculation of the virus and the optimal concentration of the virus in the embryonic tissues and fluids under these conditions.

METHODS AND MATERIALS

1. *Virus strain*

This particular strain of virus had previously been cultivated in monolayers of lamb kidney tissue cultures through 61 serial passages before serial propagation in the CAM of 7-day embryonated hens' eggs, using the stab method of inoculation (Van Rooyen & Weiss, 1959) commenced. During the serial passage in eggs an incubation temperature of 33.5°C was used and membranes were collected on the 7th day after infection. Suspensions of CAM of the 20th passage in embryonated eggs were passaged a further three times in lamb kidney monolayers. The infected tissue culture cells and fluid were stored at –80°C with an equal volume of Seitz filtered 10 per cent lactose. The TCID (tissue culture infective dose) 50 of this seed virus was $10^{4.5}$ per 0.2 ml.

2. *Inoculation of embryonated hens' eggs and harvesting of materials*

Eggs were obtained from the inbred flock of White Leghorn hens maintained at this Institute.

Prior to infection, eggs were incubated for the specified periods in a Gamble incubator with forced ventilation and controlled humidity as prescribed by the manufacturers. The eggs were incubated at 37° to 39°C and turned three times a day.

The fertile embryonated eggs were washed in a solution of 50 per cent alcohol, 25 per cent acetone, 25 per cent water and 0.1 per cent merthiolate before inoculation.

Unless otherwise stated, 0.2 ml of virus suspension was inoculated by one or another of the following routes or methods:

(i) Dropped CAM
(ii) Stab method
(iii) Yolk sac
(iv) Allantoic sac.

After inoculation openings in the shell were sealed with melted paraffin wax and the eggs were returned to an incubator set at the required temperature. The eggs were candled daily and those containing dead embryos were discarded.

After the required period of incubation, eggs were washed in tincture of merthiolate and then opened to harvest the following embryonic tissues and fluids:

(i) Allantoic fluid—this was aspirated with a syringe, care being taken to prevent contamination of allantoic fluid with yolk and blood
(ii) Yolk
(iii) Embryos
(iv) CAM

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The embryos as well as membranes were washed three times in sterile saline. The materials were stored at –18°C until used.

3. *Virus assay*

The membranes or embryos were ground from the frozen state to a fine paste with the aid of sterile pestles and mortars.

A diluting fluid containing Mycostatin (Squibb) (500 i.u. per ml), sodium penicillin (2000 i.u. per ml), streptomycin sulphate (2000 μg per ml) and neomycin sulphate (1250 μg per ml) in Hank's balanced salt solution (BSS) was added to the emulsified material in the proportion of two volumes of material to one volume of diluent.

The suspensions were left at room temperature for one hour before centrifugation at 1000 rpm for 5 minutes. The supernatant materials, yolk or allantoic fluids were assayed for virus by preparing tenfold dilutions in BSS. At least four roller tube cultures of lamb kidney cells, from which the nutrient medium was removed, were seeded with 0.2 ml of each serial dilution. After adsorption for one hour at 37°C a modified maintenance medium consisting of BSS plus 2 per cent lactalbumin hydrolysate and 2 per cent normal bovine serum was added (Weiss & Geyer, 1959). The cultures were examined daily for 14 days and the cytopathic effects recorded. The TCID 50 endpoints were calculated according to the method of Reed & Muench (1937).

Results

1. *Determination of the optimal age of embryos and temperature of incubation for multiplication of the virus in the CAM*

In the first experiment, eggs pre-incubated for 5, 7 and 9 days were selected and each group was inoculated with 0.2 cc of undiluted virus suspension, with a titre of $10^{4.5}$ TCID 50, using the dropped CAM route of injection.

Each age group of embryos was divided into three lots and incubated for 7 days at temperatures of 33.5°C, 35°C and 37°C respectively. On the 7th day after infection eggs containing dead embryos were discarded and the CAM of the remainder were harvested and assayed for virus.

The results given in Table 1 showed that the virus concentration was greatest in the CAM of 5 and 7 day embryonated eggs incubated at 33.5°C and 35°C for 7 days.

TABLE 1.—*The effect of age of embryos and temperature of incubation on the concentrations of Neethling virus in the CAM of embryonated eggs*

Age of embryos in days at time of inoculation	TCID 50 per 0.2 ml after incubation at 33.5°C for 7 days	TCID 50 per 0.2 ml after incubation at 35°C for 7 days	TCID 50 per 0.2 ml after incubation at 37°C for 7 days
5	$10^{4.5}$ and $10^{5.0}$	$10^{5.0}$	$10^{1.0}$
7	$10^{5.0}$ and $10^{4.5}$	$10^{4.5}$	$10^{1.0}$
9	$10^{3.5}$	$10^{3.5}$	$10^{1.0}$
12	$10^{2.5}$		

In the next experiment 7-day embryonated eggs were inoculated onto the CAM and incubated at 33.5°C, 35°C, 37°C and 39°C. The membranes were harvested on the 7th day and assayed for virus in lamb kidney tissue cultures.

The results presented in Table 2 confirmed that the concentration of virus was greatest in the CAM of embryonated eggs incubated at 33.5 to 35°C. At higher incubation temperatures virus could not be detected in the CAM.

TABLE 2.—*The effect of temperature of incubation on the concentration of Neethling virus in the CAM of 7-day embryonated eggs*

Temperature of incubation	TCID 50 per 0.2 ml after incubation for 7 days
33.5°C	$10^{4.5}$
35°C	$10^{3.5}$
37°C	$10^{1.0}$
39°C	$10^{1.0}$

2. *Determination of the most suitable route of inoculation and the relative concentrations of virus in the component parts of the embryonated egg*

Groups of embryonated eggs, pre-incubated for 7 days were inoculated by the following routes and methods: dropped CAM, yolk sac, allantoic sac and stab method. After inoculation with a virus suspension containing $10^{4.5}$ TCID 50, the eggs were incubated at 35°C for 7 days. Yolk, allantoic fluid, embryos and membranes were collected separately from each group and assayed for virus.

The results, given in Table 3, showed that the greatest concentration of virus was present in the CAM of embryonated eggs irrespective of the route of inoculation. The CAM appeared to be the tissue of choice for virus multiplication, although a significant concentration of virus was also present in the embryos, following the stab method of inoculation. Insignificant amounts of virus were found in the yolk and allantoic fluids.

3. *Growth cycles of the virus in the CAM and embryos of 7-day embryonated eggs inoculated by various routes*

A number of 7-day pre-incubated eggs was divided into three groups and inoculated by the following routes respectively: dropped CAM, yolk sac and stab method. The inoculated eggs were incubated at 35°C. Each day the membranes and embryos of at least four eggs from each group were harvested separately and assayed for virus. The results are presented graphically in Fig. 1, 2 and 3.

According to these results, the concentration of virus in the CAM increased at a linear rate from the first day to reach a maximum on the 5th to 6th day irrespective of the route of inoculation. Thereafter the virus titres declined, probably due to thermal inactivation. In the infected embryos there was a lag period of 48 hours before any increase in virus concentration could be detected. The maximum virus titres in the embryos were reached on the 4th

TABLE 3. — *Effect of the route of inoculation on the development of Neethling virus in 7-day embryonated eggs and the relative concentrations of virus in the component parts of the eggs harvested 7 days after inoculation*

Route and method of inoculation of embryonated eggs	TCID 50 of virus per 0.2 ml in the embryonic tissues and fluids after incubation at 35°C for 7 days			
	CAM	Allantoic fluid	Embryos	Yolk
Yolk sac	$10^{6.0}$	$10^{1.0}$	$10^{2.5}$	$10^{2.5}$
Allantoic sac	$10^{4.0}$	$10^{1.0}$	$10^{2.0}$	$10^{1.0}$
Dropped CAM	$10^{5.0}$	$10^{1.0}$	$10^{2.5}$	$10^{1.0}$
Stab method	$10^{6.0}$	$10^{1.0}$	$10^{4.5}$	$10^{1.5}$

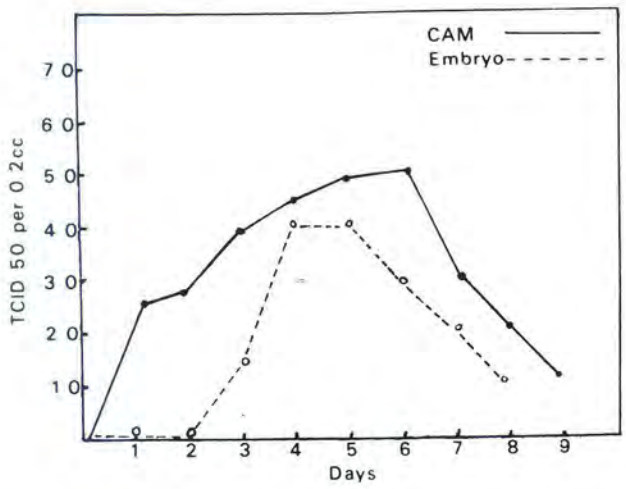

FIG. 1.—Growth curves for lumpy skin disease virus in the CAM and embryo of embryonated eggs inoculated onto the CAM

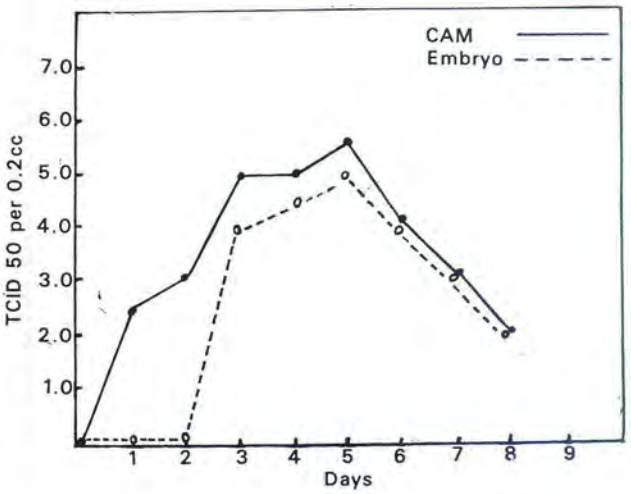

FIG. 3.—Growth curves for lumpy skin disease virus in the CAM and embryo of embryonated eggs inoculated by the stab method

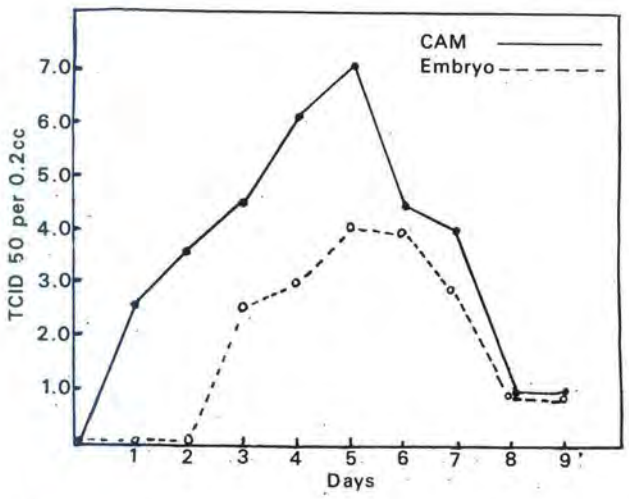

FIG. 2.—Growth curves for lumpy skin disease virus in the CAM and embryo of embryonated eggs inoculated into the yolk sac

to 6th day, but were lower than those obtained in the CAM. Multiplication of virus occurred in the embryos irrespective of the route of inoculation.

4. *The development of lesions in the CAM of embryonated eggs infected with Neethling virus*

During the course of this study it was observed that small opaque lesions developed in the infected CAM under certain conditions. Although the central portions of the lesions were distinctly white and opaque, they were not always clearly defined and were often surrounded by a zone of fading grey. Many of the lesions were pinpoint in size and were arranged in the form of streaks or stripes. None of the lesions showed evidence of necrosis. The macroscopic appearance of the lesions is illustrated in Plate I.

Microscopic examination of histological sections of the lesions stained with haematoxylin-phloxin presented some evidence regarding the specificity of these lesions. The microscopic changes are illustrated in Plates 2, 3 and 4. Multiplication of the virus was evidently accompanied by a focal progressive thickening of the ectoderm, the cells of which were swollen and vacuolated. Some cells contained large, homogeneous inclusion bodies (Plate 2). In older lesions

PLATE 1.—Macroscopic appearance of CAM lesions produced by lumpy skin disease virus

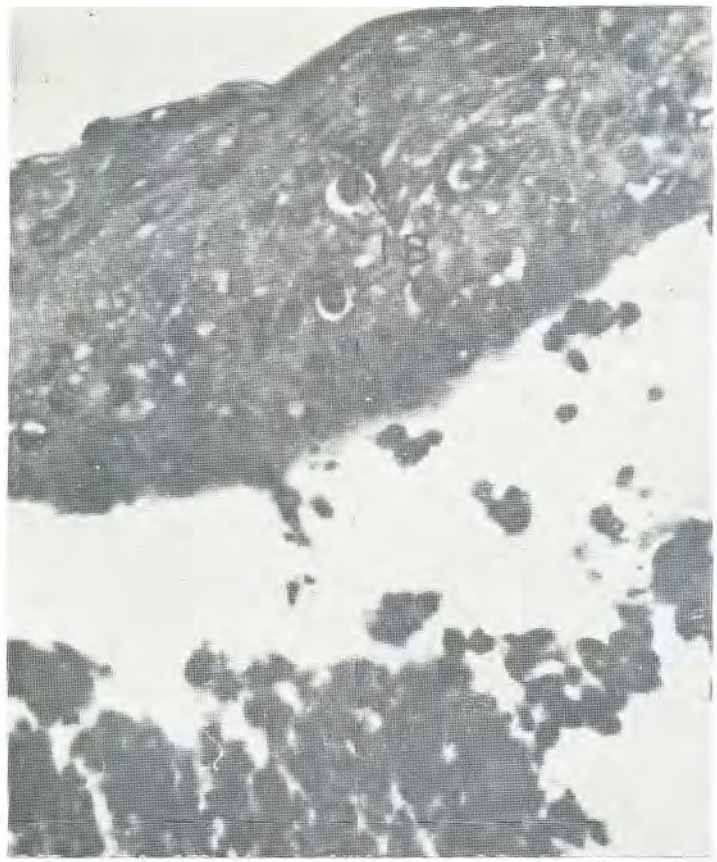

PLATE 2.—Inclusion bodies in the ectoderm of a CAM, 7 days after infection with Neethling virus. (450×)
IB = inclusion bodies

examined on the 10th day after infection the superficial cell-layer was necrotic and became detached (Plate 3). The changes in the mesoderm consisted of a considerable oedematous thickening followed by an infiltration of leucocytic cells (Plate 4). Inclusion bodies were not found in the mesoderm. The entoderm was relatively unaffected.

In order to determine the optimal conditions for the development of lesions and their relationship to multiplication of the virus, embryonated eggs preincubated for 5, 7 and 9 days were inoculated onto the dropped CAM with Neethling virus as previously described. Embryonated eggs from each group were then incubated at 33.5°, 35° and 37°C for 7 days. The membranes were harvested and assayed for virus after having been examined for the presence of lesions. The results given in Table 4 showed that lesions failed to develop in the CAM of 5-day embryonated eggs incubated at 33.5°, 35° and 37°C, despite the fact that considerable multiplication of the virus had occurred in these membranes at incubation temperatures of 33.5° and 35°C. It is apparent that 7- and 9-day embryonated eggs incubated at either 33.5° or 35°C were more suitable for the production of lesions, although infective virus titres were lower. In 13-day embryonated eggs only indistinct lesions were observed.

In the following experiment a number of 7-day embryonated eggs was divided into two groups. One group was inoculated onto the dropped CAM and the other by the yolk sac route. Following incubation at 35°C for 7 days the membranes were harvested, examined for lesions and assayed for virus. The results presented in Table 5 showed that development was markedly influenced by the route of inoculation. Following inoculation by the yolk sac route, considerable virus multiplication occurred in the CAM in the absence of any visible lesions, whereas direct inoculation of the virus onto the membranes resulted in the development of lesions as well as high titre infective virus.

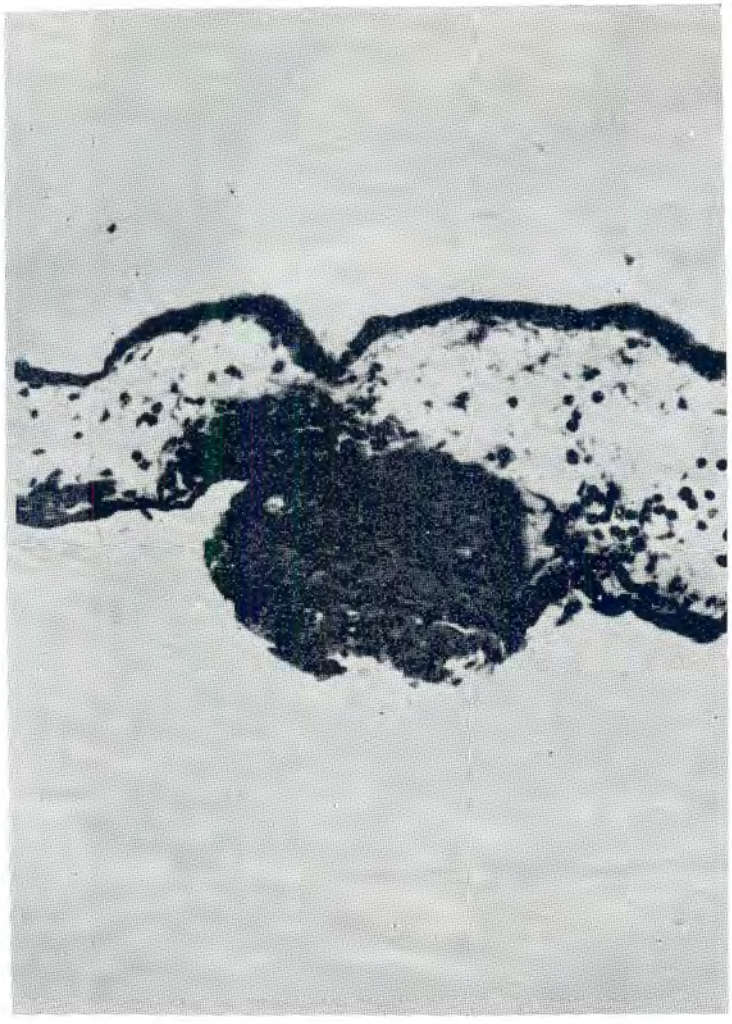

PLATE 3.—Histological section of a lesion in the CAM on the 10th day after infection. (175×)

TABLE 4. — *The effect of age of embryos and temperature of incubation on virus multiplication and the development of lesions in the CAM*

Age of embryos	Temperature of incubation of embryonated eggs after inoculation					
	33.5°C		35°C		37°C	
	Lesions in the CAM	TCID 50 of virus per 0.2 ml CAM	Lesions in the CAM	TCID 50 of virus per 0.2 ml CAM	Lesions in the CAM	TCID 50 of virus per 0.2 ml CAM
5	0	$10^{5.0}$	0	$10^{5.0}$	0	$10^{1.0}$
7	+++	$10^{4.5}$	+++	$10^{4.5}$	+	$10^{1.0}$
9	+++	$10^{3.5}$	+++	$10^{3.0}$	0	$10^{1.0}$
13	—	—	+	$10^{2.5}$	—	—

0 — no CAM lesions in any of the inoculated eggs
+++ — marked CAM lesions in all inoculated eggs
\+ — slight CAM lesions in some of the inoculated eggs

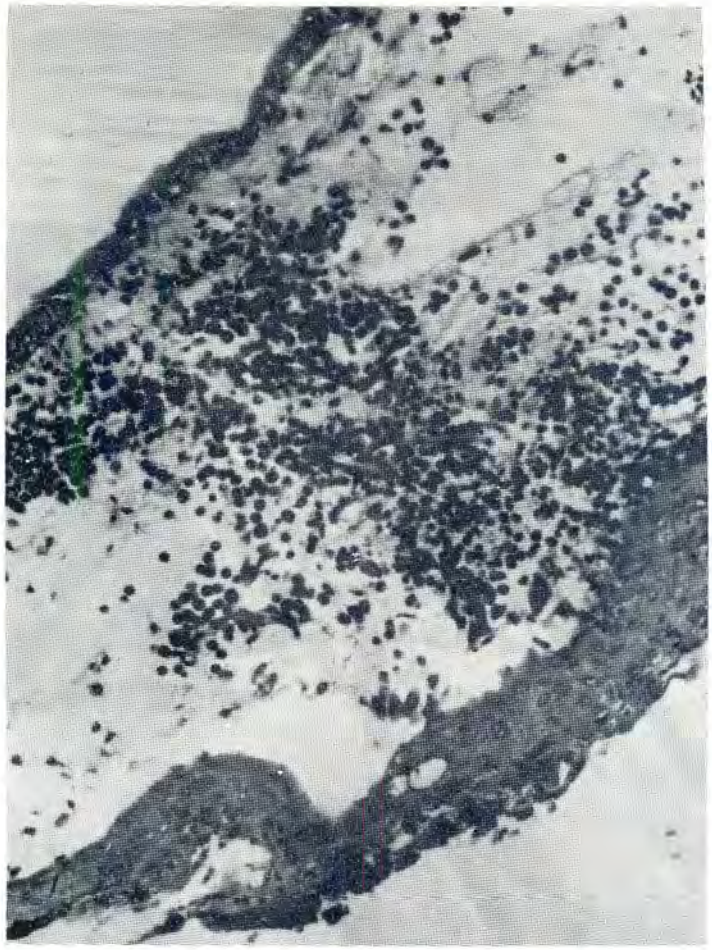

PLATE 4.—Proliferation of the ectoderm and infiltration of leucocytes in the mesoderm of a CAM on the 10th day after infection (175×)

5. *The specificity of the lesions*

In a preliminary experiment, 7-day embryonated eggs were inoculated onto the CAM and incubated at 35°C. After 7 days the membranes were harvested and assayed for virus in lamb kidney tissue cultures as previously described but using 0.1 ml as inoculum, as well as in embryonated eggs by inoculating 0.1 ml of the serial tenfold dilutions onto the CAM of 7-day embryonated eggs. After incubation at 35°C for 7 days the membranes were examined for lesions and the EID (egg infective dose) 50 was calculated. In this comparative titration the TCID 50 was $10^{3.5}$ and the EID 50 $10^{2.6}$ per 0.1 ml.

TABLE 5.—*The effect of the route of inoculation on the multiplication of virus and the development of lesions in the CAM of 7-day embryonated eggs incubated at 35°C for 7 days*

Route of inoculation	Lesions in the CAM	TCID 50 of virus per 0.2 ml CAM suspension
Yolk sac	0	$10^{6.0}$
CAM	+++	$10^{6.0}$

By using this information, a neutralization test was set up to determine the specificity of the lesions by inhibition with specific immune serum. In this experiment serial tenfold dilutions of the virus were mixed with equal amounts of bovine antiserum to lumpy skin disease and normal bovine serum respectively. The serum-virus mixtures were incubated at 37°C for one hour and then inoculated in 0.2 ml amounts onto the CAM of groups of 7-day embryonated eggs. After incubation at 35°C for 7 days, the membranes were examined for lesions and the endpoints calculated on the basis of extinction of lesions. The same serum-virus mixtures were also inoculated in 0.2 ml volumes into roller tube cultures of lamb kidney cells. After adsorption for 1 hour at 37°C, 1.0 ml of maintenance medium, previously removed, was replaced. The cultures were incubated at 37°C and examined daily for 14 days for cytopathic effects. The endpoints were calculated on the basis of presence or absence of cytopathic changes. From the results given in Table 6, it is apparent that the antiserum which neutralized more than 3.5 logs of virus in tissue culture, inhibited the development of lesions in the CAM of embryonated eggs.

TABLE 6.—*Neutralization of the effects of lumpy skin disease virus in embryonated eggs and tissue culture by specific antiserum*

Item	TCID 50 per 0.1 ml in lamb kidney cultures	EID 50 per 0.1 ml based on the development of lesions in the CAM
Virus + normal control serum	$10^{4.0}$	$10^{2.4}$
Virus + lumpy skin disease antiserum	$10^{0.5}$	$10^{0.5}$

6. *Electron microscopy of the lesions*

In order to gain further evidence of the nature and specificity of the lesions, ultra-thin sections of CAM lesions were prepared with a Porter-Blum ultramicrotome, stained with uranylacetate and examined in a Siemens Elmiskop I electron microscope.

Plate 5 shows an infected cell with a number of immature virus particles embedded in a zone of electron dense material (matrix) in the cytoplasm. These virus particles, as well as those presented in Plate 6, have the typical shape of pox viruses. Immature particles are homogeneous, more or less round in shape and sometimes surrounded by a membrane. A nucleoid region can be recognised in some particles. Mature virus particles have a double membrane and show an inner core and lateral bodies depending on the section.

DISCUSSION

The results of the investigations reported in this paper have conclusively shown that there are certain optimal conditions for the multiplication of lumpy skin disease virus in embryonated eggs. This virus, which is considered a member of the pox group (Weiss, 1963; Munz & Owen, 1966), appears to differ considerably in its growth characteristics in embryonated eggs compared with the other mammalian pox viruses.

The age of embryos and the temperature of incubation were found to be important factors influencing the multiplication of lumpy skin disease virus. The highest concentrations of virus were found in the CAM of 5- to 7-day embryonated eggs incubated at 33.5 to 35°C. With an inoculum of 0.2 ml containing $10^{4.5}$ TCID 50 of virus and an estimated dilution factor of at least 1:10, it is apparent that only limited virus multiplication occurred in 9- and 12-day embryonated eggs at the lower incubation temperatures, and none at all in any of the age groups incubated at temperatures of 37° and 39°C.

In their experiments with variola virus, Hahon, Ratner & Kozikowski (1958) likewise found that an incubation temperature of 35°C was optimal for virus multiplication, but that the highest yields were obtained in 9- to 13-day embryonated eggs.

In the growth studies with lumpy skin disease virus reported in this paper, it was found that the virus has a predilection for the CAM. Peak concentrations of virus were found in the membranes on the 5th to 6th day, irrespective of the route of inoculation. Multiplication of virus also occurred in the embryos after a significant lag phase of 48 hours to reach a somewhat lower peak of virus concentration on the 4th to 6th day. There was no evidence of virus in the allantoic fluid even after allantoic sac inoculation and only limited survival of virus in the yolk following yolk sac and the stab method of inoculation. Hahon *et al.* (1958) and Mahnel & Herlich (1961) studied the concentrations of variola virus in the component parts of embryonated eggs inoculated by various routes. They found that the highest virus yields were obtained in the CAM following inoculation onto the membrane and lower concentrations in the embryo, yolk sac and other parts in this descending order. Their results with variola virus, however, differ from those with lumpy skin disease virus in that they failed to demonstrate multiplication or dissemination of virus by allantoic sac inoculation. With yolk sac inoculation the highest concentration of virus was present in the yolk, but the evidence suggested that there was only limited multiplication.

The production of lesions in the CAM of embryonated eggs infected with lumpy skin disease virus has been a matter of controversy in the past. Haig (1957) and Alexander, Plowright & Haig (1957) claimed that the virus produced distinct "pocks" in the CAM, whereas van Rooyen & Weiss (1959) and Weiss (1963) failed to observe these lesions. Haig (1957) and Alexander *et al.*, (1957), however, omitted to mention the age of embryos and temperature of incubation employed by them. It is apparent from the results presented in this paper that lumpy skin disease virus is capable of producing lesions in the CAM under certain conditions, but the presence or absence of lesions is apparently not a reliable indication of virus multiplication. Using tissue cultures for the assay of virus, the highest yields of virus were obtained in the CAM of 5-day embryonated eggs incubated at 33.5° and 35°C without any evidence of lesions in the membranes. Lesions could only be demonstrated in the membranes of 7- and 9-day embryonated eggs incubated at 33.5° and 35°C, which yielded lower virus titres. By yolk sac inoculation high concentrations of virus were demonstrated in the CAM in the absence of any lesions, whereas direct inoculation of virus onto the membranes favoured the development of lesions.

Bedson & Dumbell (1961) studied the effect of temperature of incubation on the capacity of various mammalian pox viruses to produce lesions in the CAM of embryonated eggs. They found that all viruses were capable of producing "pocks" at 35°C and that the "ceiling temperatures" were as follows: Variola minor, 37.5°C; Variola major, 38.5°C; ectromelia and monkey pox, 39°C; cowpox, 40°C and vaccinia and rabbit pox in the region of 41°C. Although such critical studies were not conducted with lumpy skin disease virus, it would appear that, in addition to the incubation temperature, of which the "ceiling" is in the region of 35°C, the age of the embryo and the route of inoculation are important factors which influence the development of lesions.

It is quite clear that the failure of previous workers (Haig, 1957; Alexander *et al.*, 1957) fully to adapt and maintain the virus of lumpy skin disease in embryonated eggs, was due to the lack of a suitable assay system to determine the optimal conditions of growth of the virus in eggs as well as the poor

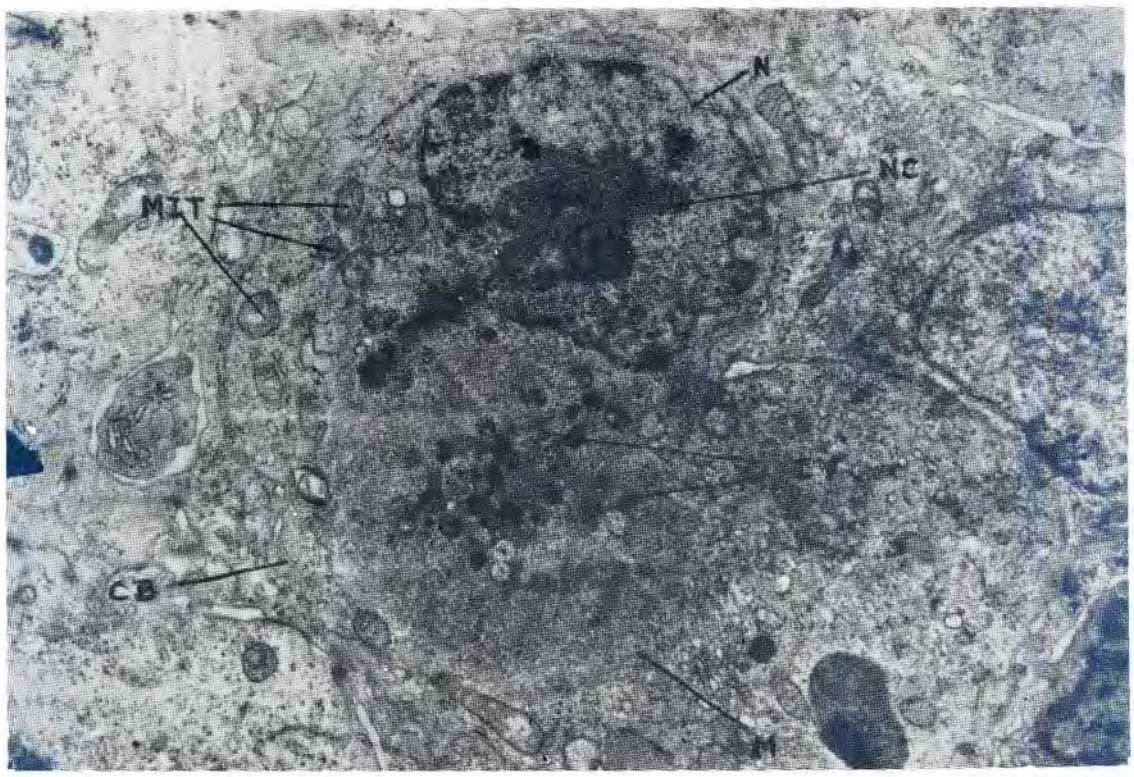

PLATE 5.—Ultrasection of a CAM lesion on the 7th day after infection. Cell showing intracytoplasmic situation of immature virus particles. (1800×)
MIT = Mitochondria
N = Nucleus
NC = Nucleolus
M = Matrix
IV = Immature virus particles
CB = Cell border

correlation between virus multiplication and appearance of lesions in the CAM under certain conditions.

The electron microscopic study of infected chorioallantoic membranes, presented in this paper, substantiates the observations of Munz *et al.* (1966) on the morphology of lumpy skin disease virus and provides further evidence for the classification of the virus as a member of the pox group.

SUMMARY

The effect of the age of the embryo, temperature of incubation and route of inoculation on the multiplication of lumpy skin disease virus in embryonated eggs was studied. The greatest yields of virus were obtained in the CAM of 5- to 7-day embryonated eggs incubated at 33.5° to 35°C for 5 to 6 days. Virus multiplication also occurred in the embryos to a lesser extent. The route of inoculation did not significantly affect the growth pattern of the virus.

"Pock" lesions in the CAM only appeared in 7- to 9-day embryonated eggs inoculated onto the membrane and incubated at 33.5° and 35°C. The presence or absence of lesions does not appear to be a reliable indication of virus multiplication.

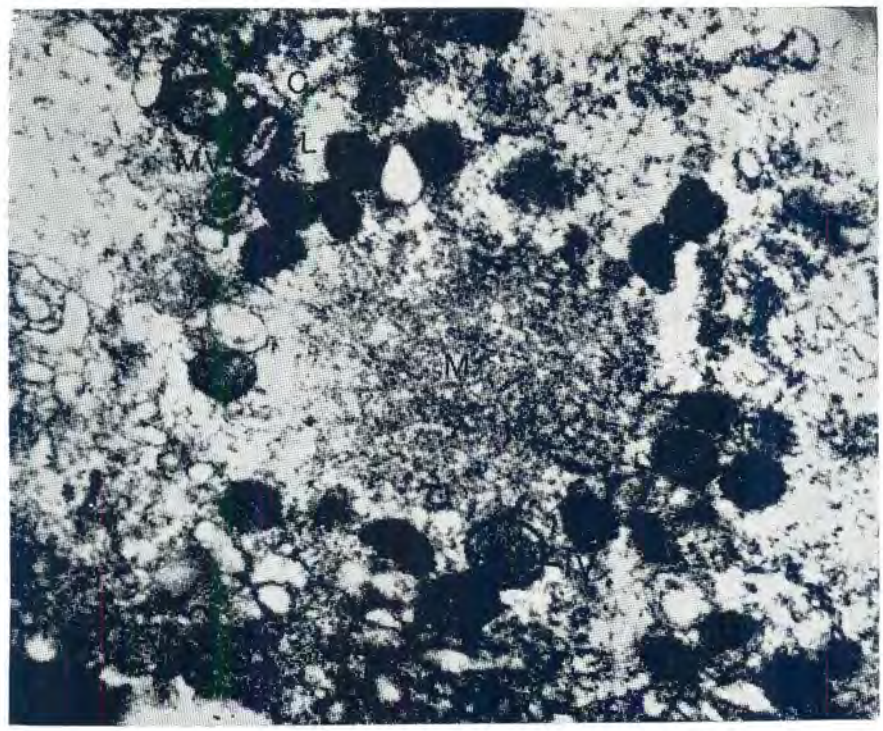

PLATE 6.—Ultrasection of a CAM lesion on the 7th day after infection. Cell showing mature as well as immature virus particles (35,000×)
IV = Immature virus particle
MV = Mature virus particle
L = Lateral bodies
C = Core
M = Matrix

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