University of Rhode Island

DigitalCommons@URI

Open Access Master's Theses

1962

Chick Embryo Lethal Orphan Virus (CELO) as a Tissue Culture Contaminant

Dharam Vir Ablashi

University of Rhode Island

Follow this and additional works at: <https://digitalcommons.uri.edu/theses>

Recommended Citation

Ablashi, Dharam Vir, "Chick Embryo Lethal Orphan Virus (CELO) as a Tissue Culture Contaminant" (1962). *Open Access Master's Theses*. Paper 811.
<https://digitalcommons.uri.edu/theses/811>

This Thesis is brought to you for free and open access by DigitalCommons@URI. It has been accepted for inclusion in Open Access Master's Theses by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu.

636.50896

A664

CHICK EMBRYO LETHAL ORPHAN

VIRUS (CELO) AS A

TISSUE CULTURE CONTAMINANT

BY

DHARAM VIR ABLASHI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

POULTRY SCIENCE

UNIVERSITY OF RHODE ISLAND

1962

MASTER OF SCIENCE THESIS

OF

DHARAM VIR ABLASHI

Approved:

Thesis committee:

Chairman

Vance J. Gates

M. R. McClung

Philip L. Carpenter

Dean of the Graduate School

E. E. Hartung

William H. Wiley, Director

Agricultural Experiment Station

UNIVERSITY OF RHODE ISLAND

1962

ABSTRACT

In each of ten weekly trials, 105 eggs from mated White Leghorn hens were divided into sets of 15 eggs each. Yolks of some of these eggs carried CELO antibodies. Before incubation, the various sets of eggs were inoculated through the air cell into the albumen with decimal dilutions of the virus, 10^{-1} through 10^{-5} . In each trial two controls were maintained. One set was inoculated with normal allantoamniotic fluid, the other was not inoculated.

AAF harvested from CELO exposed embryos dying during incubation was found to carry the CELO virus. The higher concentrations of virus adversely affected embryo livability, and only a few receiving the virus diluted 10^{-1} survived until hatching. Embryos receiving the virus diluted 10^{-2} or 10^{-3} were also affected, but to a lesser degree. In the rest of the sets only normal embryo mortality occurred during incubation.

The kidneys were removed from the newly hatched chicks and were used as source tissue for cell cultures. Chicks from each of the seven sets were treated separately. In all cases the cells developed normally, and within 48 to 70 hours a complete sheet was observed. At this time, the starting medium was replaced with a serum-free maintenance medium. Within 36 to 150 hours of the

medium change cytopathogenicity developed in all but the control cells. The speed of development varied inversely with the concentration of the virus used to inoculate the eggs before incubation.

Presence of CELLO virus in tissue cultures was confirmed by inoculation of embryonating eggs and three-day-old baby chicks. The development of intranuclear inclusions in chick kidney cell cultures inoculated with the fluids was evident within 48 hours.

TABLE OF CONTENTS

	Page
I. <u>INTRODUCTION</u>	7
II. <u>REVIEW OF LITERATURE</u>	10
CELO VIRUS.....	10
TISSUE CULTURE.....	11
LATENCY AND MASKING.....	12
RECOVERY OF CONTAMINANTS.....	12
III. <u>MATERIALS AND METHODS</u>	15
EMBRYONATING EGGS.....	15
VIRUS (SEED CULTURE).....	15
SERUM FOR TISSUE CULTURE.....	16
SOURCE AND PREPARATION OF CHICK KIDNEY CELLS...	16
AGAR GEL MEDIUM AND PREPARATION OF AGAR PLATES.	17
PREPARATION OF ANTISERUMS AGAINST CELO.....	18
EXPERIMENTAL PROCEDURE (INOCULATION OF EGGS)...	18
REISOLATION OF THE VIRUS FROM EMBRYONATING EGGS	19
<u>Agar Gel Diffusion Test</u>	19
<u>Virus Neutralization Test</u>	20
REISOLATION OF THE VIRUS FROM TISSUE CULTURES..	20
STAINING OF TISSUE CULTURE MICROSLIDES.....	21
IV. <u>RESULTS</u>	23
V. <u>DISCUSSION</u>	40
VI. <u>CONCLUSIONS</u>	44

	Page
VII. <u>SUMMARY</u>	45
VIII. <u>ACKNOWLEDGMENTS</u>	46
IX. <u>BIBLIOGRAPHY</u>	47
X. <u>APPENDIX</u>	52

LIST OF TABLES

	Page
1. Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Summary of ten trials.....	24
2. Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 1.....	25
3. Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 2.....	26
4. Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 3.....	27
5. Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 4.....	28
6. Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 5.....	29
7. Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 6.....	30

8.	Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation.	
	Trial 7.....	31
9.	Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation.	
	Trial 8.....	32
10.	Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation.	
	Trial 9.....	33
11.	Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation.	
	Trial 10.....	34
12.	Results of intracerebral inoculation of tissue culture isolates into three-day-old chicks.....	39

LIST OF FIGURES

	Page
1. Time of CPE in kidney cells from embryos inoculated preincubation with various dilutions of CEL0 virus.	56
2. Effect of serial tissue passage on CEL0 virus isolates.....	57
3. Death time of embryos inoculated with CEL0 virus isolated from one-day-old chick kidney cells.....	58
4. Uninoculated one-day-old chick kidney cell cultures at 24 hours incubation.....	59
5. Cytopathogenicity at 24 hours incubation in one-day-old chick kidney cells inoculated preincubation with CEL0 virus.....	59
6. One-day-old uninoculated chick kidney cell culture at 48 hours incubation.....	60
7. Development of cytopathogenicity in kidney cell cultures at 48 hours of infection.....	60
8. Uninoculated chick kidney cells at 60 hours of incubation.....	61
9. Cytopathogenicity in kidney cell cultures at 60 hours of infection.....	61
10. Uninoculated chick kidney cell cultures at 72 hours of incubation.....	62

11. Cytopathogenicity in chick kidney cell cultures at 72 hours of infection.....	62
12. Intranuclear inclusion bodies produced after 72 hours of infection of CEL0 virus in one-day-old chick kidney cell cultures.....	63
13. Chick from control group I (uninoculated).....	64
14. Chick from control group II (inoculated with the diluent).....	64
15. Chick inoculated with pooled tissue culture material after five passages in tissue culture showing first stage of nervous symptoms.....	65
16. Second stage of nervous symptoms.....	65
17. Third stage of nervous symptoms.....	66
18. Fourth stage of nervous symptoms.....	66

I. INTRODUCTION

During the past five years, newer knowledge of mammalian cell culture has both resulted from and given rise to increased practical and theoretical application in virus research. Remarkable progress in virus propagation, isolation, and identification in tissue culture has been pointed out in a literature survey by Ross and Syverton (1957).

A biological system maintained under laboratory conditions is subject to contamination by extraneous agents. Living cells, regardless of type, are a potential source of masked and contaminating viruses. Moreover, accidental contamination of cultures in the laboratory is difficult to avoid. Such contaminants have been reported in normal tissue cultures. Hall and Minner (1957) isolated eight new viruses from normal monkey kidney tissue cultures while testing poliomyelitis vaccine for safety. Holmgren and Campbell (1960) demonstrated tissue culture contamination while investigating the conversion of pleuropneumonia-like-organisms to L-forms. These contaminants and others reported interfered with the multiplication of the original organism. In a two and one-half year period, Hall and Minner (1957) studied a

seasonal variation in the isolation of tissue culture contaminants. They observed the disappearance of some agents during certain months of the year. The significance of these results is obscure.

Latent viruses have been reported in tissue cultures from chick embryo cells. Some of these interfered with other viruses (Lennette and Koprowski, 1946; Sabin, 1954; Le Bouvier, 1954; Ackerman, 1957; Andrewes, 1942).

CELO virus was identified while attempting to isolate laryngotracheitis virus in tissue culture (Chomiak et al., 1961). Hwang et al. (1959) reported isolation of a CELO-like virus during attempts to propagate avian encephalomyelitis (AE) virus in tissue culture. This isolate was closely related serologically to AE virus and avian enteric #89 virus (Luginbuhl and Ablashi, 1960; Burke et al., 1959). CELO virus has been established as the cause of an inapparent infection of the chick embryo (Yates and Fry, 1957). Du Bose (1958) isolated CELO strains from uninoculated embryonating chicken eggs.

Yates et al. (1960) demonstrated that CELO virus can be carried in embryonating eggs and the resulting chicks show no detrimental effects when the virus is introduced before incubation. These findings suggested that at least one method of spread of CELO virus was from the dam to the progeny via the fertilized eggs.

Observations were made to determine if the CELO virus, when introduced into the eggs before incubation,

II. REVIEW OF LITERATURE

CELO VIRUS

The CELO virus has been found in two different host-parasite situations, one as a causative agent of highly fatal respiratory disease in quail and possibly of other birds and, secondly, as the cause of an inapparent infection in chickens. Yates et al. (1954) reported the isolation of the CELO virus from fertile chicken eggs used for diagnostic studies in a research laboratory. Du Bose (1958), also, isolated CELO virus from fertile eggs. The hens producing the eggs were housed near the area where CELO virus was being studied. Yates and Fry (1957) suggested that the CELO virus was sometimes found in the fertile eggs of apparently normal chickens and that it was revealed when the normal physiology of embryonated eggs was altered either by inoculation or by some other means. Yates et al. (1960) demonstrated that CELO virus introduced before incubation was carried in the embryonated eggs and in the resulting chickens without producing outward effects. It was further shown that antibodies for CELO virus were commonly found in chickens and that laboratory diagnosis of infectious bronchitis, laryngotracheitis, avian encephalomyelitis and poultry enteric viruses may be confused by the CELO virus.

TISSUE CULTURE

In recent years tissue culture techniques have made significant contributions in the study of microorganisms, especially viruses. Cell monolayers on glass, cellophane, or similar substrates are being used more extensively than other cultural methods in viral research. Dulbecco (1952) and Dulbecco and Vogt (1954) demonstrated that cytocidal viruses produced discrete areas of dead cells in monolayer cultures. Madin (1959) reviewed the application of tissue culture in isolation, propagation, and identification of microorganisms. He further discussed the preparation of virus vaccines of human and veterinary importance. Tissue culture has also been used in the study of phagocytosis and in testing toxicity of drugs. Pomerat and Leake (1954) studied carcinogens and the biochemical synthesis of viruses. Initiation of infection by ribonucleic acid (RNA) has been demonstrated in tissue cultures by these authors. Cell cultures have contributed to the study of viral genetics (Madin, 1959).

There is no doubt that tissue culture systems have facilitated the study of diseases, but at the same time problems have arisen as a result of latent viruses. These have led to confusion, especially in the identification of viruses.

LATENCY AND MASKING

Latency and masking may confuse the propagation, isolation, and cultivation of viral agents. The latent infection does not show up until it is unmasked by certain environmental conditions, e.g., long incubation period and/or suitable growth medium. Ginsberg (1958) studied latent infections in tissue culture over the period 1956 to 1958. He concluded that latent infections can be regarded as a laboratory artefact, relevant only to the basic nature of the cell-virus interaction. Ackerman (1957) experimentally showed the persistence of virus without replication. He further demonstrated that masking caused by an inhibitor could be reversed, producing a consuming infection of the cells in a culture. This was brought about by the addition of neutral mixtures of virus and antibody. These cells will yield the virus when other techniques are employed.

Bader et al. (1961) maintained cells for two or three days in phenylalanine or isoleucine deficient mediums which inhibited the synthesis of psittacosis virus. Virus was recovered from these cultures only after the addition of a complete medium. Extension of the interval between infection and addition of complete medium beyond eight days resulted in a permanent disappearance of infectious virus.

RECOVERY OF CONTAMINANTS

The sudden appearance of an unknown agent in normal tissue cultures confuses the identification of

certain viral and bacterial agents. The viruses that Hall and Minner (1957) recovered from normal monkey kidney cultures produced cytopathogenicity and were identified and classified on the basis of their serologic properties. Uninoculated tissue cultures prepared from monkeys with diarrheal disease produced eighteen unknown agents. Lundholm et al. (1959) recovered large motile bodies somewhat resembling vibrios from cell cultures prepared from fetal tissues of cattle and sheep. Later, the cultures showed mature Trypanosoma theileri, and these findings were confirmed by critical morphologic studies.

Fontes et al. (1958) and Sharpless et al. (1958) reported the cultivation of a viral agent (PRL12) from the tissues of chickens suffering from avian lymphomatosis. Later, Burmester et al. (1960) studied the pathogenicity of this agent by various routes of inoculation in chicks. They also demonstrated that the virus isolated was antigenically unrelated to lymphomatosis and did not cause the disease. Immunological and serological tests indicated that the starting material contained a mixture of viruses.

Macpherson (1960) studied the above agent by the plaque assay method. He found that high titer tissue culture material did not cause the formation of tumors in chicks during a six months observation period. On challenge with Es₄ strain of avian lymphomatosis, the chicks were completely susceptible. It was noted that a high dilution of a pool of normal serum from an Es₄ susceptible flock

neutralized plaque formation of the (PRL12) virus in tissue cultures. Macpherson stated that the virus he studied was an orphan virus present in the poultry stock.

Rothblat (1960) suggested that PPLO may be present as a contaminant in tissue cultures as a result of (1) PPLO infection of the animal from which the tissue was derived or (2) a break in aseptic technique when cell lines undergo numerous passages. Holmgren (1960) proposed that the source of infection from PPLO in tissue cells is the conversion of bacterial contaminants to stable L-form by the common use of antibiotics.

Chomiak et al. (1961) isolated an agent while serially passing laryngotracheitis in tissue culture. It differed from laryngotracheitis virus in production of CPE. The agent was recognized as a CELV virus in the 17th serial passage.

Luginbuhl and Ablashi (1960) showed that the AE virus propagated by Hwang et al. (1959) in chick kidney cells produced a very high level of antibodies in ten weeks but on challenge with an embryo strain of AE the birds developed muscular paralysis and other signs. Later, this tissue culture agent was shown to be serologically related to CELV and avian enteric (#89) viruses.

III. MATERIALS AND METHODS

EMBRYONATING EGGS

The eggs used were from Single Comb White Leghorns. A few of these eggs carried yolk antibodies against the CEL0 agent. The birds were under the care of the Poultry Science Department. The eggs were stored between 55° and 60°F. with the relative humidity of approximately 60 per cent. The eggs were inoculated every Friday and then immediately placed in a commercial incubator.

The post-inoculation temperature was about 37.5°C. with a relative humidity of 86 per cent. On the ninth day the eggs were taken to the laboratory, candled to remove the infertile and dead eggs, and then placed in a laboratory incubator. The embryonating eggs in this incubator were not turned except at the time of candling. The dead embryos were put in a refrigerator to chill for an hour and the AAF was collected to test for the presence of the virus.

VIRUS (SEED CULTURE)

The stock culture used in the study reported here was the Phelps strain of the CEL0 virus dated November 21, 1955. The stock culture was prepared by inoculating nine-day-old embryonating eggs with 0.1 ml. of 10^{-1} dilution of the virus. The eggs were candled daily, and

all embryos dying early were discarded. Generally 50 to 60 per cent of the eggs died the third day. These and the rest of the eggs were chilled at 4°C . before harvesting. The AAF was harvested aseptically and pooled. It was tested on nutrient agar and in nutrient broth for sterility and was then distributed in five to eight ml. quantities in vacutainers (Becton-Dickenson) and stored in a mechanical freezer at -20°C .

SERUM FOR TISSUE CULTURE

The serum used in the growth medium was obtained from calves in the University of Rhode Island dairy herd. After the serum was collected, it was filtered through a Seitz EK pad under positive pressure and stored at -20°C .

SOURCE AND PREPARATION OF CHICK KIDNEY CELLS

Chicks were hatched from eggs which had been inoculated before incubation with a decimal dilution (10^{-1} through 10^{-5}) of the virus. Chicks from control eggs which were inoculated with 10^{-1} dilution of normal allantoamnionic fluid (AAF) and from uninoculated eggs were also used for the preparation of kidney tissue cultures.

The kidneys were removed, minced thoroughly, washed at least three times in phosphate buffered saline (PBS), and then put in trypsin. Ten milliliters of trypsin were used for each pair of kidneys.

The time employed for trypsinization was either one hour at room temperature (18° - 20°C .) or 45 minutes at

37°C. The whole mixture was shaken thoroughly until the fragments of kidneys were broken down into a cellular suspension.

The suspended cells were centrifuged at 800-900 r.p.m. for ten minutes. The supernatant fluid was discarded, and the cells were resuspended in growth medium to obtain a cell concentration of 300,000 per ml. (i.e., 25-30 ml. of growth medium was required per pair of kidneys). The cells were dispensed in bottles in the amount of ten ml. per bottle. A complete sheet of cells was produced in 48-60 hours. At this time the medium had a pH of 6.8, and it was replaced by a serum-free maintenance medium. The cells could be maintained for seven to nine days, depending upon the incubation time and temperature and the metabolic activity of the cells.

AGAR GEL MEDIUM AND PREPARATION OF AGAR PLATES

The contents of the agar gel medium was : (1) iso-agar (Difco Laboratory, Detroit, Michigan), (2) sodium chloride, (3) thimerosal (sodium-ethyl-thiosalicylate). Six-tenths per cent of iso-agar and eight per cent sodium chloride were used with 100 ml. of demineralized distilled water. After autoclaving the mixture at 15 pounds pressure for 15 minutes, the pH was adjusted to 7.0-7.2 with one normal sodium hydroxide. To control bacterial contamination, 0.0002 per cent of 1.0 per cent thimerosal was added to the agar before pouring the plates (Ablashi et al., 1961).

The plates were allowed to solidify for an hour at room temperature and then stored at 4°C. (at least 24 hours) before use.

PREPARATION OF ANTISERUMS AGAINST CELO

Antiserum against the Phelps strain of CELO virus was prepared in rabbits and chicks (six-weeks-old) by a series of intravenous and intraperitoneal injections. Every week, trial bleedings were made until a high titer was achieved. After this, the animals and birds were exsanguinated. The serum was separated, centrifuged, and stored at -20°C.

EXPERIMENTAL PROCEDURE (INOCULATION OF EGGS)

The experiment consisted of ten weekly trials. In each trial 105 eggs from mated White Leghorn hens were divided into sets of 15 eggs each. After marking, they were cleaned at the site of the air cell with 1:1,000 solution of tincture of Zephiran chloride (Winthrop-Stearns). A hole was punched in the shell over the air sac with a sharpened 20d. spike. The inoculum, i.e., decimal dilution of virus, 10^{-1} through 10^{-5} (made in nutrient broth), was deposited in the amount of 0.1 ml. per egg using a tuberculin syringe fitted with a 25 or 26 gauge needle. After inoculation, the eggs were wiped with 95 per cent ethyl alcohol and sealed with glue (DuPont's Duco Cement). They were incubated for nine days in a Jamesway 2940 incubator and then removed to the laboratory incubator. At the time

of transfer all eggs were candled. Infertile eggs were recorded and discarded, dead ones were recorded and put in the refrigerator for chilling. After chilling, AAF was collected for the agar gel diffusion test as well as virus neutralization tests to detect the CEL0 virus.

From the ninth day embryonating eggs were candled every day and dead embryos as well as a few live ones from each batch were used for detecting the virus by the two methods mentioned above.

On the 20th day, the eggs were put in pedigree boxes for hatching. Four or five chicks from each of two sets of virus inoculated eggs and also from one set of controls (inoculated or uninoculated) were taken for the preparation of chick kidney cells in each of the ten trials.

REISOLATION OF THE VIRUS FROM EMBRYONATING EGGS

After the collection of AAF from the embryos, tests for the presence of CEL0 virus were carried out by the following two methods.

Agar Gel Diffusion Test

Three parallel lines of holes were cut in the agar plate with a steel tube sharpened at one end. The holes were approximately 1.0 cm. in diameter and 0.5 cm. apart. The holes in the central line were offset 0.5 cm. from the centers of the outside holes. The outside lines contained five holes whereas the central line contained six holes. Two-tenths ml. of AAF from each of the samples was deposited

in the side holes. Two-tenths ml. of anti-CELO serum, prepared in rabbits or chicks, and anti-AAF serum were placed in the center holes alternately.

The plates were incubated at 37°C. and examined for the formation of precipitation bands over a period of 120 hours. A lamp was used to illuminate the plate from below so as to obtain better visibility.

Neutralization Test

The test was performed as described by Cunningham (1960). One-day-old chick kidney cell cultures were used. Fluids from embryonating eggs or tissue cultures were mixed with rabbit or chicken anti-CELO serum and incubated for one hour at room temperature before inoculating tissue cultures. The readings, i.e., production of cytopathogenic effect (CPE) in tissue cultures, were recorded up to seven days. The TCID₅₀ was computed by the Reed and Muench method (1938).

REISOLATION OF THE VIRUS FROM TISSUE CULTURES

Tissue culture fluid collected from bottles showing CPE was tested for virus by the following methods: (1) agar gel diffusion, (2) virus neutralization, (3) inoculation into ten-day-old embryonating eggs, (4) bird inoculation.

The eggs used for inoculation were from the flock used for the previous study. They were inoculated with 0.1 ml. of the tissue culture material by the chorioallantoic sac (CAS) route. Deaths within 48 hours were considered the

result of something other than virus.

A pool of the tissue culture material from all dilutions was inoculated into one-day-old chick kidney cells and passed serially. Decimal dilutions (10^{-1} through 10^{-5}) of the fifth tissue culture passage fluid were inoculated into three-day-old chicks. For each dilution 0.05ml. was inoculated intracerebrally into each of five chicks. Three control groups were also used. Two groups were inoculated with the same quantity and by the route mentioned above. The first group was inoculated with the diluent, and the second group was inoculated with tissue cultures prepared from the chicks hatched from eggs inoculated with normal AAF. The third group served as uninoculated controls.

All the chicks were housed in the same battery. They were observed daily for the nervous signs described by Yates et al. (1960).

STAINING OF TISSUE CULTURE MICROSLIDES

The microslides were prepared in Leighton tubes especially designed for this purpose. These microslides were from tissue cultures prepared of chicks hatched from eggs inoculated with various dilutions of CEL0 virus, and from AAF inoculated and uninoculated controls. Microslides showing various stages of CPE were fixed and stained according to the following technique, modified from Johansen (1940): (1) Maintenance medium was discarded from the tubes and microslides were washed with PBS three times.

(2) Tubes containing the microslides were placed in methyl alcohol for five minutes. (3) Giemsa solution was poured into the tubes and left for 40 minutes at room temperature. (4) Giemsa solution was discarded and the microslides were washed two times with acetone for two and one-half seconds. (5) Microslides were washed for five seconds in a mixture of equal parts of acetone and xylol. (6) Microslides were cleared in xylol. (7) The stained and cleared microslides were mounted on glass slides with Clarite. (8) They were placed in an incubator to dry and to remove air bubbles.

IV. RESULTS

Ten trials were carried out to determine if the CELO virus survived when inoculated into eggs before incubation and contaminated cell cultures made from the tissue of hatching chicks. Table 1 summarizes the individual results presented in Tables 2 to 11.

Embryo mortality was increased in the eggs inoculated with 10^{-1} and 10^{-2} dilutions of CELO virus. The other dilutions did not adversely affect embryo livability. The chicks that hatched from the CELO inoculated, as well as from normal AAF inoculated eggs, were clinically healthy.

AAF was collected from embryos dying from the first to the 20th days as well as from a few killed between the ninth to the 20th days of incubation. The titer was low in one-half of the AAF samples collected from the embryos that carried 10^{-5} dilution of virus. These samples of AAF were neutralized by chick anti-CELO serum.

TABLE 1.--Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Summary of ten trials.

Virus Dil.	No. Eggs Inoculated	No. Eggs Infertile	% Dying During Incubation	CEL0 Reisolation	
				From AAF*	From Tissue Cultures**
10-1	150	44	76.4	29/29	3/3
10-2	150	38	55.4	26/26	6/6
10-3	150	35	22.6	20/20	4/5
10-4	150	49	8.9	10/10	4/4
10-5	150	42	16.7	14/14	3/3
AAF Inoculated Control	150	42	4.6	0/5	0/8
Uninoculated Control	150	43	4.6	0/4	0/3

* The presence of virus in AAF from embryos dying during incubation was confirmed by the agar gel diffusion test and by the virus neutralization test. Numerator = number of positive results; denominator = number of samples tested.

** The presence of virus in tissue cultures of kidney cells from chicks hatched from inoculated eggs was confirmed by the agar gel diffusion test, the virus neutralization test, inoculation into embryonating eggs and bird inoculation. Numerator = number of positive results; denominator = number of samples tested.

TABLE 2.--Reisolation of CELO virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 1.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos			Tissue Culture		
				% Dying		CELO Reiso-lation*	Chicks Used**	CPE	CELO Reiso-lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	3	12	83.3	16.7	3/3	---	---	---
10 ⁻²	15	4	11	54.5	27.3	6/6	---	---	---
10 ⁻³	15	6	9	11.1	44.5	3/3	---	---	---
10 ⁻⁴	15	9	6	0	16.6	1/1	3	+	+
10 ⁻⁵	15	8	7	0	14.2	1/1	---	---	---
AAF	15	3	12	0	0	---	5	---	---
Uninoc- ulated	15	7	8	0	0	---	---	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route.

* Virus isolations were made by collecting AAF from live embryos or those dying during incubation. AAF was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

TABLE 3.--Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 2.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos		CELO Reiso- lation*	Tissue Culture		
				% Dying			Chicks Used**	CPE	CELO Reiso- lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	8	7	28.6	28.6	2/2	3	+	+
10 ⁻²	15	8	7	42.9	14.3	2/2	---	---	---
10 ⁻³	15	7	8	0	12.5	1/1	7	+	+
10 ⁻⁴	15	11	4	0	0	---	---	---	---
10 ⁻⁵	15	8	7	0	0	---	---	---	---
AAF	15	14	1	0	0	---	---	---	---
Uninoc- ulated	15	10	5	0	0	---	5	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route.

* Virus isolations were made by collecting AAF from live embryos or those dying during incubation. AAF was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

TABLE 4.--Reisolation of CELO virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 3.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos			Tissue Culture		
				% Dying		CELO Reiso-lation*	Chicks Used**	CPE	CELO Reiso-lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	9	6	33.3	16.7	3/3	3	+	+
10 ⁻²	15	9	6	16.7	0	1/1	6	+	+
10 ⁻³	15	6	9	0	22.2	1/1	---	---	---
10 ⁻⁴	15	11	4	0	0	---	---	---	---
10 ⁻⁵	15	7	8	12.5	12.5	2/2	---	---	---
AAF	15	10	5	0	0	0/1	5	---	---
Uninoc-ulated	15	2	13	0	0	---	---	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route.

* Virus isolations were made by collecting AAF from live embryos or those dying during incubation. AAF was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

TABLE 5.--Reisolation of CELO virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 4.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos			Tissue Culture		
				% Dying		CELO Reiso- lation*	Chicks Used**	CPE	CELO Reiso- lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	5	10	60.0	20.0	2/2	0	---	---
10 ⁻²	15	2	13	15.3	30.6	3/3	3	+	+
10 ⁻³	15	4	11	9.1	18.2	3/3	0	---	---
10 ⁻⁴	15	4	11	0	0	1/1	0	---	---
10 ⁻⁵	15	5	10	0	20.0	1/1	5	+	+
AAF	15	2	13	0	7.6	0/1	7	---	---
Uninoc- ulated	15	5	10	0	0	---	0	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route.

* Virus isolations were made by collecting AAF from live embryos or those dying during incubation. AAF was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

TABLE 6.--Reisolation of CELO virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 5.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos			Tissue Culture		
				% Dying		CELO Reiso- lation*	Chicks Used**	CPE	CELO Reiso- lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	2	13	69.2	7.7	4/4	0	---	---
10 ⁻²	15	2	13	46.1	30.8	3/3	4	---	contaminated
10 ⁻³	15	1	14	7.1	7.1	2/2	4	+	+
10 ⁻⁴	15	2	13	0	0	1/1	4	+	+
10 ⁻⁵	15	5	10	0	30.0	2/2	0	---	---
AAF	15	1	14	0	0	0/1	7	---	---
Uninoc- ulated	15	2	13	0	0	0/1	0	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route.

* Virus isolations were made by collecting AAF from live embryos or those dying during incubation. AAF was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

TABLE 7.--Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 6.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos			Tissue Culture		
				% Dying		CELO Reiso- lation*	Chicks Used**	CPE	CELO Reiso- lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	4	11	63.6	36.4	2/2	0	---	---
10 ⁻²	15	2	13	69.2	15.4	3/3	0	---	---
10 ⁻³	15	2	13	7.6	15.2	2/2	5	+	+
10 ⁻⁴	15	3	12	16.6	8.3	1/1	5	+	+
10 ⁻⁵	15	0	15	0	26.6	4/4	0	---	---
AAF	15	3	12	0	0	---	5	---	---
Uninoc- ulated	15	5	10	0	0	---	0	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route

* Virus isolations were made by collecting AAF from live embryos or those dying during incubation. AAF was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

TABLE 8.--Reisolation of CELO virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 7.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos			Tissue Culture		
				% Dying		CELO Reiso-lation*	Chicks Used**	CPE	CELO Reiso-lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	3	12	50.0	25.0	3/3	0	---	---
10 ⁻²	15	3	12	16.6	25.0	2/2	4	+	+
10 ⁻³	15	5	10	10.0	10.0	2/2	4	+	+
10 ⁻⁴	15	6	9	22.2	0	2/2	5	+	+
10 ⁻⁵	15	0	15	6.6	0	---	0	---	---
AAF	15	4	11	9.9	0	0/1	0	---	---
Uninoc- ulated	15	2	13	0	0	---	5	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route.

* Virus isolations were made by collecting AAF from live embryos or those dying during incubation. AAF was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

TABLE 9.--Reisolation of CELO virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 8.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos			Tissue Culture		
				% Dying		CELO Reiso-lation*	Chicks Used**	CPE	CELO Reiso-lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	3	12	75.0	0	4/4	0	---	---
10 ⁻²	15	3	12	33.3	25.0	3/3	4	+	+
10 ⁻³	15	2	13	15.4	30.8	4/4	0	---	---
10 ⁻⁴	15	1	14	0	7.1	2/2	0	---	---
10 ⁻⁵	15	3	12	0	8.3	1/1	5	+	+
AAF	15	0	15	0	13.3	0/1	4	---	---
Uninoc- ulated	15	5	10	0	0	---	4	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route.

* Virus isolations were made by collecting AAF from live embryos or those dying during incubation. AAF was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

TABLE 10.--Reisolation of CELO virus from tissues (AAF^F and embryo kidney) of eggs exposed before incubation. Trial 9.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos			Tissue Culture		
				% Dying		CELO Reiso-lation*	Chicks Used**	CPE	CELO Reiso-lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	6	9	44.4	22.2	2/2	0	---	---
10 ⁻²	15	2	13	15.3	23.2	1/1	4	+	+
10 ⁻³	15	1	14	0	7.1	1/1	0	---	---
10 ⁻⁴	15	1	14	0	7.1	1/1	0	---	---
10 ⁻⁵	15	4	11	0	18.1	2/2	5	+	+
AAF ^F	15	3	12	0	0	---	5	---	---
Uninoc- ulated	15	4	11	0	0	---	0	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route.

* Virus isolations were made by collecting AAF^F from live embryos or those dying during incubation. AAF^F was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

TABLE 11.--Reisolation of CEL0 virus from tissues (AAF and embryo kidney) of eggs exposed before incubation. Trial 10.

Virus Dil.#	No. Eggs	No. Infertile	No. Embryos	Embryos			Tissue Culture		
				% Dying		CEL0 Reiso-lation*	Chicks Used**	CPE	CEL0 Reiso-lation***
				To 10 Days	11-20 Days				
10 ⁻¹	15	1	14	50.0	14.3	4/4	4	+	+
10 ⁻²	15	3	12	16.6	16.6	2/2	5	+	+
10 ⁻³	15	1	14	0	7.1	1/1	0	---	---
10 ⁻⁴	15	1	14	0	7.1	1/1	0	---	---
10 ⁻⁵	15	2	13	7.6	7.6	1/1	0	---	---
AAF	15	2	13	0	7.6	0/1	7	---	---
Uninoc- ulated	15	1	14	0	0	0/3	0	---	---

Virus diluted in nutrient broth. Inoculum 0.1 ml. via air cell route.

* Virus isolations were made by collecting AAF from live embryos or those dying during incubation. AAF was diluted 10⁻¹ in nutrient broth and inoculated into 7-day-old chick kidney cells. Numerator = number of isolations. Denominator = number of samples tested.

** Chick kidney cells prepared from hatching chicks.

*** Virus isolation from inoculated chick kidney cells prepared from hatching chicks. TC fluid from bottles showing CPE was used to inoculate 10-day-old embryonating eggs.

Agar gel diffusion tests using virus samples against rabbit anti-CELO serum developed double, sharp or diffuse precipitation bands after an incubation period of 24-48 hours at 37°C. A single sharp well defined band developed in 24-48 hours when chick anti-CELO serum was used. In a few instances AAF collected from eggs inoculated with 10^{-5} dilution of CELO virus formed a confusing thick band of precipitate around the hole in which it was placed.

In all ten trials, the chick kidney cells from the virus inoculated eggs (Group I) and from chicks of normal AAF inoculated as well as uninoculated eggs (Group II) developed a complete sheet in 48 hours of incubation. The cells from both these groups appeared healthy. After the replacement of growth medium by a serum-free maintenance medium, the following observations were made: (1) In all trials cells prepared from chicks hatched from eggs inoculated with 10^{-4} dilution of CELO showed degeneration in 30-36 hours. (2) Cells from 10^{-3} dilution chicks showed degeneration after 48 hours. (3) Degeneration patterns in cells prepared from 10^{-5} , 10^{-2} , and 10^{-1} dilutions started after 75, 138, and 150 hours respectively.

In general, 24 hours after the onset of degeneration, the maintenance medium in these tissue cultures had a pH range of 7.2-7.0. At 36 hours, distinct, scattered dark areas were observed in the sheet of cells. At 48 hours, CPE was very distinct. The cells were reduced in length

and width. The nuclei were enlarged, irregular in shape, and their margins were rough. The pH was 7.0-6.8. After this stage, the cytological changes occurred rapidly. At 60 hours, more than half the cells were gone. The cells appeared round and granular. At 72 hours, only a few cells were left, more than 90 per cent containing nuclei of irregular shape. Intranuclear inclusion bodies were distinct when the cells were stained with Giemsa. Various stages of CPE in chick kidney cell cultures are shown in Figures 5, 7, 9, 11, and 12.

The second group of cells, prepared from chicks hatched from eggs inoculated with normal AAF as well as uninoculated eggs, were found healthy. A change of pH 7.4 to 7.2 appeared 48 hours after replacement of the growth medium by maintenance medium. The cell sheet was complete with very slight shrinkage in cell length. A slight change in cell length occurred at 60 hours that might have been due to change in pH. Most of the cells were flat, elongated, and thick. The nuclei were round or oval with regular margins and not enlarged. At 72 hours, some of the cells showed increased shrinkage and, at a few places, the cells started peeling off the glass. Photomicrographs of the second group of cells are shown in Figures 4, 6, 8, and 10.

The tissue culture fluids from both groups, i.e., those which showed CPE and those which did not, were harvested separately after 72 hours. In harvesting, the tissue

culture bottles were frozen for one hour at $-20^{\circ}\text{C}.$, the bottles were partially thawed, and the slush was shaken to dislodge any cells still adhering to the glass.

The material from each group was pooled and inoculated via the CAS route into ten-day-old embryonating eggs. Embryos inoculated with AAF from the first group died on the third, fourth, and fifth day. There were variations in the death pattern from dilution to dilution. The pool from the 10^{-1} dilution caused deaths at 120 hours, whereas that from the 10^{-2} dilution killed at 96 hours. Deaths occurred in 72, 80, and 90 hours from 10^{-3} , 10^{-4} , and 10^{-5} dilutions, respectively.

No death occurred in the second (control) group of inoculated eggs. The tissue culture material collected from the first group was sterile and not toxic. The tissue culture material from the first group was passed serially six times in chick kidney cell cultures (Figure 2). Serial tissue culture passage of the CEL0 isolates increased their pathogenicity for kidney cells. After six passages CPE developed 25 to 40 hours after inoculation with virus isolated from cells of eggs receiving 10^{-1} through 10^{-5} virus dilutions (Figure 2). On inoculation of this material into ten-day-old embryonating eggs, deaths occurred in 72 to 96 hours (Figure 3). Pools of isolates were neutralized by chick and rabbit anti-CEL0 serums. Virus titers in these pools were $10^{9.5}$ and $10^{8.0}$ in tissue cultures and embryonating eggs, respectively.

The results of the virus neutralization and titration tests indicated that the isolates were CEL0 virus.

The results of bird inoculations are summarized in Table 12.

Isolate	No. of birds inoculated	Days after inoculation										Per cent of birds showing symptoms
		1	2	3	4	5	6	7	8	9	10	
10-1	5	1	0	0	0	0	0	0	0	0	0	20
10-2	5	0	0	0	0	0	0	0	0	0	0	0
10-3	5	1	0	0	0	0	0	0	0	0	0	20
10-4	5	1	0	0	0	0	0	0	0	0	0	20
10-5	5	1	0	0	0	0	0	0	0	0	0	20
10-6	5	1	0	0	0	0	0	0	0	0	0	20
10-7	5	1	0	0	0	0	0	0	0	0	0	20
10-8	5	1	0	0	0	0	0	0	0	0	0	20
10-9	5	1	0	0	0	0	0	0	0	0	0	20
10-10	5	1	0	0	0	0	0	0	0	0	0	20
10-11	5	1	0	0	0	0	0	0	0	0	0	20
10-12	5	1	0	0	0	0	0	0	0	0	0	20
10-13	5	1	0	0	0	0	0	0	0	0	0	20
10-14	5	1	0	0	0	0	0	0	0	0	0	20
10-15	5	1	0	0	0	0	0	0	0	0	0	20
10-16	5	1	0	0	0	0	0	0	0	0	0	20
10-17	5	1	0	0	0	0	0	0	0	0	0	20
10-18	5	1	0	0	0	0	0	0	0	0	0	20
10-19	5	1	0	0	0	0	0	0	0	0	0	20
10-20	5	1	0	0	0	0	0	0	0	0	0	20
10-21	5	1	0	0	0	0	0	0	0	0	0	20
10-22	5	1	0	0	0	0	0	0	0	0	0	20
10-23	5	1	0	0	0	0	0	0	0	0	0	20
10-24	5	1	0	0	0	0	0	0	0	0	0	20
10-25	5	1	0	0	0	0	0	0	0	0	0	20
10-26	5	1	0	0	0	0	0	0	0	0	0	20
10-27	5	1	0	0	0	0	0	0	0	0	0	20
10-28	5	1	0	0	0	0	0	0	0	0	0	20
10-29	5	1	0	0	0	0	0	0	0	0	0	20
10-30	5	1	0	0	0	0	0	0	0	0	0	20
10-31	5	1	0	0	0	0	0	0	0	0	0	20
10-32	5	1	0	0	0	0	0	0	0	0	0	20
10-33	5	1	0	0	0	0	0	0	0	0	0	20
10-34	5	1	0	0	0	0	0	0	0	0	0	20
10-35	5	1	0	0	0	0	0	0	0	0	0	20
10-36	5	1	0	0	0	0	0	0	0	0	0	20
10-37	5	1	0	0	0	0	0	0	0	0	0	20
10-38	5	1	0	0	0	0	0	0	0	0	0	20
10-39	5	1	0	0	0	0	0	0	0	0	0	20
10-40	5	1	0	0	0	0	0	0	0	0	0	20
10-41	5	1	0	0	0	0	0	0	0	0	0	20
10-42	5	1	0	0	0	0	0	0	0	0	0	20
10-43	5	1	0	0	0	0	0	0	0	0	0	20
10-44	5	1	0	0	0	0	0	0	0	0	0	20
10-45	5	1	0	0	0	0	0	0	0	0	0	20
10-46	5	1	0	0	0	0	0	0	0	0	0	20
10-47	5	1	0	0	0	0	0	0	0	0	0	20
10-48	5	1	0	0	0	0	0	0	0	0	0	20
10-49	5	1	0	0	0	0	0	0	0	0	0	20
10-50	5	1	0	0	0	0	0	0	0	0	0	20
10-51	5	1	0	0	0	0	0	0	0	0	0	20
10-52	5	1	0	0	0	0	0	0	0	0	0	20
10-53	5	1	0	0	0	0	0	0	0	0	0	20
10-54	5	1	0	0	0	0	0	0	0	0	0	20
10-55	5	1	0	0	0	0	0	0	0	0	0	20
10-56	5	1	0	0	0	0	0	0	0	0	0	20
10-57	5	1	0	0	0	0	0	0	0	0	0	20
10-58	5	1	0	0	0	0	0	0	0	0	0	20
10-59	5	1	0	0	0	0	0	0	0	0	0	20
10-60	5	1	0	0	0	0	0	0	0	0	0	20
10-61	5	1	0	0	0	0	0	0	0	0	0	20
10-62	5	1	0	0	0	0	0	0	0	0	0	20
10-63	5	1	0	0	0	0	0	0	0	0	0	20
10-64	5	1	0	0	0	0	0	0	0	0	0	20
10-65	5	1	0	0	0	0	0	0	0	0	0	20
10-66	5	1	0	0	0	0	0	0	0	0	0	20
10-67	5	1	0	0	0	0	0	0	0	0	0	20
10-68	5	1	0	0	0	0	0	0	0	0	0	20
10-69	5	1	0	0	0	0	0	0	0	0	0	20
10-70	5	1	0	0	0	0	0	0	0	0	0	20
10-71	5	1	0	0	0	0	0	0	0	0	0	20
10-72	5	1	0	0	0	0	0	0	0	0	0	20
10-73	5	1	0	0	0	0	0	0	0	0	0	20
10-74	5	1	0	0	0	0	0	0	0	0	0	20
10-75	5	1	0	0	0	0	0	0	0	0	0	20
10-76	5	1	0	0	0	0	0	0	0	0	0	20
10-77	5	1	0	0	0	0	0	0	0	0	0	20
10-78	5	1	0	0	0	0	0	0	0	0	0	20
10-79	5	1	0	0	0	0	0	0	0	0	0	20
10-80	5	1	0	0	0	0	0	0	0	0	0	20
10-81	5	1	0	0	0	0	0	0	0	0	0	20
10-82	5	1	0	0	0	0	0	0	0	0	0	20
10-83	5	1	0	0	0	0	0	0	0	0	0	20
10-84	5	1	0	0	0	0	0	0	0	0	0	20
10-85	5	1	0	0	0	0	0	0	0	0	0	20
10-86	5	1	0	0	0	0	0	0	0	0	0	20
10-87	5	1	0	0	0	0	0	0	0	0	0	20
10-88	5	1	0	0	0	0	0	0	0	0	0	20
10-89	5	1	0	0	0	0	0	0	0	0	0	20
10-90	5	1	0	0	0	0	0	0	0	0	0	20
10-91	5	1	0	0	0	0	0	0	0	0	0	20
10-92	5	1	0	0	0	0	0	0	0	0	0	20
10-93	5	1	0	0	0	0	0	0	0	0	0	20
10-94	5	1	0	0	0	0	0	0	0	0	0	20
10-95	5	1	0	0	0	0	0	0	0	0	0	20
10-96	5	1	0	0	0	0	0	0	0	0	0	20
10-97	5	1	0	0	0	0	0	0	0	0	0	20
10-98	5	1	0	0	0	0	0	0	0	0	0	20
10-99	5	1	0	0	0	0	0	0	0	0	0	20
10-100	5	1	0	0	0	0	0	0	0	0	0	20

Results of virus neutralization tests indicated that the isolates were CEL0 virus. The results of bird inoculations are summarized in Table 12.

TABLE 12.--Results of intracerebral inoculation of tissue culture isolates into three-day-old chicks.

Source Of Isolate*	No. Chicks Inoc.	Days After Inoculation									Per cent Nervous Symptoms	Per cent Dying**
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th		
10-1	5	--	2d	2N	--	1D	1D	2N	--	1N	100	40
10-2	5	--	2d	1d 2N	1N	2D	--	2N	--	--	100	40
10-3	5	--	--	--	--	2D	--	1N	--	1N	80	40
10-4	5	--	--	4d	3N	--	2N	--	--	--	100	0
10-5	5	--	--	4d	3N	--	2N	--	--	--	100	0
Control Groups I,II,III	15	--	--	--	--	--	--	--	--	--	0	0

*CELO isolates were passed serially in 1-day-old kidney cells. The fluid from the 5th passage was collected for inoculation into chicks. The inoculum was 0.05 ml. per chick.

**Death during 15 day observation period.

N = Nervous symptoms (shaking of head, stretching of neck, reluctance to move and when disturbed runs backward and falls over suddenly on the side).

d = Dullness and unsteady gait. D = Death.

V. DISCUSSION

The developing embryo was adversely affected by the higher concentrations of the virus; however, the lethal effect of the virus was considerably reduced when it was introduced prior to incubation. The CELO virus was consistently present in the inoculated eggs through the 19th day of incubation. The above results could be expected since the virus has been known to survive for 42 days at 37.5°C. (Yates, 1960). There was no evidence of virus multiplication.

The chicks that hatched from virus inoculated eggs as well as those hatched from the control groups appeared clinically healthy. The observations did not agree with those reported by Yates et al. (1960). The present investigation did show the persistence of virus, as it was isolated from the kidney cells harvested from chicks at hatching.

In all the ten trials, the kidney cell cultures of chicks from the uninfected eggs, those inoculated before incubation with normal AAF as well as those which were not inoculated, produced a complete cell sheet. The cells looked normal and healthy. After the starting medium was replaced with a serum-free maintenance medium, the cell sheet

showed cytopathologic changes which varied in time of appearance. Melnick et al. (1957) stated that alteration in the composition of the medium may influence markedly the multiplication and cytopathogenicity of animal viruses. The speed of cytopathogenicity development varied inversely with the concentration of the virus used to inoculate the preincubation eggs. The reason for this is unknown. It may be due to the presence of incomplete virus particles which delayed the development of CPE. It would be interesting to observe whether in some situations the unmasking of the CELV virus could be delayed sufficiently to interfere with the use of the cells in propagation or identification of viruses. The new virus may not grow at all or the CELV virus may only inhibit the activity of the virus in question.

Isolates secured from each viral dilution showed a rapid or gradual change in time of CPE appearance, depending to some extent upon the source of the virus used in the first passage. On the sixth serial passage all developed CPE within 25 to 40 hours. It was interesting to observe that with passage isolates from tissues previously exposed (as eggs) to 10^{-1} to 10^{-2} viral dilutions showed a drop in CPE time from 140 to 63 hours and from 131 to 63 hours respectively. The postulated incomplete viral particles could have played a part in these results. Von Magnus (1954) pointed out in his experiments that cells heavily infected with virus often produced incomplete virus,

which interfered with complete (infective) virus particles. Later he demonstrated that the varying decimal dilutions of influenza PR8 strain showed an increase in hemagglutinin titer with serial passage in embryos. It is evident that incomplete CEL0 virus particles may have infected cells at the same time as the standard virus particles. The first two serial passages of the 10^{-1} and 10^{-2} CEL0 isolates may have carried equal amounts of complete and incomplete particles. The incomplete particles would interfere with the complete (infective) particles and delay CPE. In the later serial passages the amount of incomplete virus particles likely decreased hence the CPE time was shortened considerably. The serial passages of the 10^{-3} to 10^{-5} CEL0 isolates were more consistent in CPE time, indicating that the number of mature viral particles (infective) was higher than the number of incomplete particles (Figure 2).

Photomicrographs of tissue culture infected with the isolates showed intranuclear inclusion bodies. It was suggested by Bunting (1953) that these bodies in the last stages of infection become converted to virus particles and fill the nucleus. Only a few animal viruses produce inclusion bodies in the cytoplasm or nucleus of a cell. The presence of intranuclear inclusion bodies should be helpful in further differentiating CEL0 from other viruses. The inclusions may be Feulgen positive or negative, basophilic or acidophilic and crystalline or non-crystalline.

All the chicks inoculated with serial decimal dilutions of the fifth tissue culture passage of the isolates developed nervous symptoms. There was a difference in the severity and time of appearance of symptoms. Chicks which were used as controls remained healthy and normal. The controls and infected chicks were together for more than 15 days, but no clinical signs developed in the controls.

VI. CONCLUSIONS

It is concluded that the virus behaved as a masked infection in embryonated eggs and in the chicks that hatched from these eggs. The virus which was present in the host (kidney cells) was revealed under the conditions of these trials.

The virus-host relationship demonstrated in this study should be valuable as a research procedure for the investigation of viral latency and interference.

VII. SUMMARY

The inoculation of CEL0 virus into fertile eggs before incubation resulted in embryo mortality only when the inoculum contained a high concentration of the virus. CEL0 virus was consistently reisolated from allantoamnionic fluid of embryos exposed to the virus prior to incubation. Cytopathogenicity developed in uninoculated tissue culture made from one-day-old embryo kidney tissues of eggs exposed to the CEL0 virus before incubation. Reisolation of CEL0 virus from tissue culture was confirmed by serological tests and by inoculation in three-day-old chicks.

VIII. ACKNOWLEDGMENTS

The author acknowledges with gratitude the help extended to him by Dr. Vance J. Yates in planning this research project as well as in securing a National Institute of Health Grant for the Study. Appreciation is, also, expressed to Dr. M. R. McClung, Dr. P. L. Carpenter and Dr. A. H. Dardiri for their valuable suggestions.

In addition, the author wishes to thank Miss Sheryl Palmatier for her help in typing, reading, and rearranging the material of this thesis. Thanks are due to Mr. Elot J. Hairr for his valuable assistance in the photomicrographs.

Last but not the least, gratitude is extended to the members of the Animal Pathology Department for their co-operation and assistance during this study.

IX. BIBLIOGRAPHY

- Ablashi, D. V., Yates, V. J., Chang, P. W., and Fry, D. E. 1961. Study of a chick-embryo-lethal-orphan (CELO) virus by agar gel diffusion test. *Avian Dis.*, 5: 456.
- Ackermann, W. W. 1957. Mechanism of persistent and masked infection in tissue culture. *Ann. N. Y. Acad. Sci.*, 67: 392-402.
- Andrewes, C. H. 1942. Interference by one virus with the growth of another virus in tissue culture. *Brit. Jour. Exptl. Pathol.*, 23: 214-220.
- Bader, J. P., and Morgan, H. R. 1961. Latent viral infection of cells in tissue culture. IX. Abortive infection with psittacosis virus. *Proc. Soc. Exptl. Bio. and Med.*, 106: 311-313.
- Bunting, H. 1953. Close-packed array of virus-like particles within cells of a human skin papilloma. *Proc. Soc. Exptl. Biol. and Med.*, 84: 327-332.
- Burke, C. N., Luginbuhl, R. E., and Jungherr, E. L. 1959. Avian enteric cytopathogenic viruses. I. Isolation. II. Characteristic of a prototype. *Avian Dis.*, 3: 413-419.
- Burmester, B. R., Sharpless, G. R., and Fontes, A. K. 1960. Virus isolation from avian lymphomas unrelated to lymphomatosis virus. *Natl. Cancer Inst.*, 24: 1443-1447.

Chomiak, T. W., Luginbuhl, R. E., and Helmboldt, C. F.

1961. Tissue culture propagation and pathology of CEL0 virus. Avian Dis. 5: 313-320.

Cunningham, C. H. 1960. A laboratory Guide in Virology.

Burgess Publishing Company, Minneapolis, Minnesota.

Du Bose, R. T. 1958. The identification and characterization of a virus isolated from Bob White quail with a respiratory disease. M. S. Thesis, A. & M. College of Texas.

Dulbecco, R. 1952. Production of plaques in monolayer tissue cultures by single particle of an animal virus. Proc. Natl. Acad. Sci., 38: 747-752.

_____, and Vogt, M. 1954. Plaque formation and isolation of pure lines with poliomyelitis virus. Jour. Exptl. Med., 99: 167-182.

Fontes, A. K., Burmester, B. R., Walter, W. G., and Iseler, P. E. 1958. Growth in tissue culture of cytopathogenic agent from strain of virus which produces avian lymphomatosis. Proc. Soc. Exptl. Bio. and Med., 97: 854-857.

Ginsberg, H. S. 1958. The significance of the viral carrier state in tissue culture systems. Progr. Med. Virol., 1: 36-58.

Hall, N. R., and Minner, J. R. 1957. New viral agents recovered from tissue culture of monkey kidney cells.

II. Problems of isolation and identification. Ann.

N. Y. Acad. Sci., 67: 413-423.

- Holmgren, N. B., and Campbell, W. E., Jr. 1960. Tissue cell culture contamination in relation to bacterial pleuropneumonia-like organisms L-form conversion. Jour. Bact., 79: 869-874.
- Hwang, J., Luginbuhl, R. E., and Jungherr, E. L. 1959. Simplified way to cultivate chick kidney cells and maintain the cultures without serum. Science, 130: 793.
- Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill Book Company, Inc., New York.
- Le Bouvier, G. L. 1954. Interference and cell protection by poliomyelitis virus in tissue culture. Nature, 174: 649-650.
- Lennette, E. H., and Koprowski, H. 1946. Interferences between viruses in tissue culture. Jour. Exptl. Med., 83: 195-219.
- Luginbuhl, R. E., and Ablashi, D. V. 1960. The serologic response of chickens inoculated with tissue culture propagated avian encephalomyelitis like-virus. Proc. A. V. M. A. Meeting, Denver, Colorado.
- Lundholm, B. D., Johannes, S., and McKercher, D. G. 1959. Trypomonosoma theileri as a contaminant of tissue origin in cultures of fetal kidney cells in vitro. Virology, 8: 394-396.
- Macpherson, I. A. 1960. Plaque formation by an orphan virus of fowl. Nature, 188: 1213-1214.
- Madin, S. H. 1959. Tissue culture in veterinary medical research. Advances Vet. Sci. V. Academic Press Inc., New York.

- Melnick, J. L., Hsiung, G. D., Rappaport, C., Howes, D., and Reissing, M. 1957. Factors influencing the proliferation of viruses. *Texas Rep. Biol. and Med.*, 15: 496-533.
- Pomerat, C. M., and Leake, C. D. 1954. Short term cultures for drug assays. *Ann. N. Y. Acad. Sci.*, 58: 1110-1124.
- Reed, L. F., and Muench, H. 1938. A simple method of estimating fifty per cent endpoint. *Amer. Jour. Hyg.*, 27: 493-497.
- Ross, J. D., and Syverton, J. T. 1957. Use of tissue culture in virus research. *Ann. Rev. Microbiol.*, 11: 459-508.
- Rothblat, G. H. 1960. PPL0 contamination in tissue culture. *Ann. N. Y. Acad. Sci.*, 79: 430-432.
- Sabin, A. B. 1954. Noncytopathogenic variants of poliomyelitis virus and resistance to superinfection in tissue culture. *Sci.*, 120: 357.
- Sharpless, G. R., Defendi, V., and Cox, H. R. 1958. Cultivation in tissue culture of the virus of avian lymphomatosis. *Proc. Soc. Exptl. Biol. and Med.*, 97: 755-757.
- Von Magnus, P. 1954. Incomplete forms of influenza virus. *Advances Virus Res.*, 2: 59-79.
- Yates, V. J. 1960. Characterization of the chicken-embryo-lethal-orphan (CELO) virus. Ph. D. Thesis, University of Wisconsin, Madison, Wisconsin.

Yates, V. J., Chang, P. W., Dardiri, A. H., and Fry., D. E.
1960. A study of the epizootiology of the CEL0 virus.
Avian Dis., 4: 500-505.

_____, and Fry, D. E. 1957. Observations on a
chicken-embryo-lethal-orphan (CELO) virus. Amer.
Jour. Vet. Res., 18: 687-690.

_____, _____, and Wasserman, B. 1954. A preliminary
report on an apparently new virus disease of chickens.
Proc. Ann. Conf. Lab. Workers in Pullorum Dis. Control,
unpaginated.

X. APPENDIX

Reagents used in Standard Tissue Culture Medium

(The following alterations were made by the Virus Lab., Dept. of Animal Disease, University of Connecticut, Storrs, Conn., and the Dept. of Animal Pathology, University of Rhode Island, Kingston, R. I.)

Phosphate Buffered Saline (PBS)

In the first four trials of the experiment one ml. of penicillin and streptomycin was added to 100.0 ml. of PBS.

Penicillin Solution 0.25 per cent (Difco Lab.)

Trypsin	0.75 g.
---------	---------

Hanks IX	300.00 ml.
----------	------------

Sterilized by Seitz EK filter and stored at -10°C .

Balanced Salt Solution 10X Stock (HBSS)

Solutions A and B were prepared separately and autoclaved for ten minutes at ten pounds pressure.

Solution A

NaCl	160.0 g.
------	----------

KCl	8.0 g.
-----	--------

MgSO ₄ · 7H ₂ O	4.0 g.
---------------------------------------	--------

H ₂ O	800.0 ml.
------------------	-----------

The salts were dissolved in H₂O.

CaCl ₂	2.8 g.
-------------------	--------

H ₂ O	100.0 ml.
------------------	-----------

Solution B

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	3.04 g.
KH_2PO_4	1.20 g.
Glucose	20.00 g.
H_2O	800.00 ml.
Phenol red solution 0.5%	80.00 ml.

Solutions A and B cooled at room temperature, mixed, and stored at 4°C .

Glutamin Hydrolyzate (LAH)

A five per cent solution in demineralized water was prepared, autoclaved at ten pounds for ten minutes, cooled to room temperature, and 1.0 ml. of four per cent NaOH added to every 100 ml.

Tryptose

A five per cent solution in demineralized water was prepared, autoclaved at ten pounds for ten minutes, and then chilled to room temperature. One ml. of four per cent NaOH was added to every 100 ml.; the resulting solution was stored at 4°C .

For Seeding Medium

I. Hanks 10X	10 ml.
Sterilized demineralized distilled water	80 ml.
5% LAH	10 ml.

II. Powdered antibiotics as follows:

Penicillin (1,000,000 units)

Streptomycin (1 gram)

Mycostatin (500,000 units)

Dissolved in 5 ml. sterilized, demineralized,
distilled water; 0.1 ml. was added to the above
mixture.

III. Sterile NaHCO_3 (7.5%): one ml. was added to the
mixture of I and II if four per cent NaOH was
used in preparation of five per cent LAH.

IV. Calf serum: ten ml. added to the above.

Maintenance Medium

Hanks 10X	10	ml.
-----------	----	-----

LAH (5%)	10	ml.
----------	----	-----

Tryptose (5%)	10	ml.
---------------	----	-----

NaHCO_3 (7.5%)*	1	ml.
--------------------------	---	-----

Penicillin, Streptomycin,		
---------------------------	--	--

Mycostatin (PSM)**	0.1	ml.
--------------------	-----	-----

Sterile demineralized		
-----------------------	--	--

distilled water	Q.S.	100 ml.
-----------------	------	---------

Stirred thoroughly and stored at 4°C .

* When LAH and Tryptose had one ml. each four per cent NaOH
to 100 ml.

** Preparation of PSM is described in Step II of Seeding
Medium.

Giemsa StainingGiemsa stain (stock solution)

A ten per cent solution was prepared in distilled water. This stock solution was further diluted 1:10 with distilled water for use.

Figure 3.4

Figure 1.

Figure 2.

DEATH TIME OF EMBRYOS INOCULATED WITH CELO VIRUS
ISOLATED FROM ONE-DAY-OLD CHICK KIDNEY CELLS

Figure 3.

Figure 4.

Figure 5.

Figure 4. Uninoculated one-day-old chick kidney cell cultures at 24 hours incubation.

Figure 5. Cytopathogenicity at 24 hours incubation in one-day-old chick kidney cells inoculated preincubation with CELO virus.

Figure 6.

Figure 7.

Figure 6. One-day-old uninoculated chick kidney cell culture at 48 hours incubation.

Figure 7. Development of cytopathogenicity in kidney cell cultures at 48 hours of infection.

Figure 8.

Figure 9.

Figure 8. Uninoculated chick kidney cells at 60 hours of incubation.

Figure 9. Cytopathogenicity in kidney cell cultures at 60 hours of infection.

Figure 10.

Figure 11.

Figure 10. Uninoculated chick kidney cell cultures at 72 hours of incubation.

Figure 11. Cytopathogenicity in chick kidney cell cultures at 72 hours of infection.

Figure 12.

Figure 12. Intranuclear inclusion bodies produced after 72 hours of infection of CEL0 virus in one-day-old chick kidney cell cultures.

Figure 13.

Figure 14.

Figure 13. Chick from control group I (uninoculated).

Figure 14. Chick from control group II (inoculated
with the diluent).

Figure 15.

Figure 16.

Figure 15. Chick inoculated with pooled tissue culture material after five passages in tissue culture showing first stage of nervous symptoms.

Figure 16. Second stage of nervous symptoms.

Figure 17.

Figure 18.

Figure 17. Third stage of nervous symptoms.

Figure 18. Fourth stage of nervous symptoms.