and opened under aseptic conditions. Through an intercostal space (for auricular implantation the 3rd, and for ventricular the 5th, were most suitable) the pleura was opened and the ribs were retracted. The pericardium was slit over the site of the implantation and stay sutures were passed for retraction. With the aid of a 20 mm Carrel arterial needle, the sutures (000,000 silk) were inserted as follows: the needle was passed through one distal hole of the plate, then through the superficial epicardium, out the other distal hole, and tied over the plate. The proximal holes were then similarly treated. Thus the electrode was secured by only 2 sutures.

The pericardium was closed and the free ends of the electrodes brought out through an interspace anterior or posterior to the incision. The ribs were approximated and a fine rubber tubing was temporarily inserted in the pleural space for subsequent evacuation of air. The chest wall was closed in layers, the intrapleural space evacuated, and the rubber tubing removed after which the skin was sutured.

The free end of the electrode outside the body was covered with a gauze square the edges of which were sealed to the skin by means of collodion, thus allowing easy access to the electrodes, and at the same time protecting them from breakage due to the animals' activities.

Comment. The results of studies using this technic have been reported(9). The presence of the electrodes does not interfere either with the movements nor with the health of the subject. Microscopic examination of the hearts of animals autopsied at varying intervals after implantation revealed no reaction except a slight thickening of the pericardium overlying the plate of the electrode.

The excitability determinations may be carried out by the methods described(10) without the use of any anesthetic agents provided the animals are trained to lie quietly. In addition to fulfilling the advantages stated above, this technic also makes it possible to study the conduction velocities of the different regions and the effects of pharmacological agents on both conduction velocities and excitability cycle determinations.

Summary. A technic has been described for the study of the excitability cycle of the heart employing permanent implantation of electrodes.

1. Trendelenburg, W., *Arch. ges. Physiol.*, 1911, v141, 378.

2. Adrian, E. D., *J. Physiol.*, Lond., 1920-21, LIV, 1.

3. Lewis, T., Feil, H. S., and Stroud, W. D., *Heart*, 1918-20, v7, 191.

4. Wiggers, C. J., and Wegria, René, *Am. J. Physiol.*, 1939-40, v128, 500.

5. Andrus, Cowles E., and Carter, Edward P., *J. Exp. Med.*, 1930, v51, 357.

6. Suckling, E. E., Brooks, C. McC., Orias, O., Gilbert, J. L., and Siebens, A. A., *Am. J. Physiol.*, 1950, v162, 213.

7. Delgado, José M. R., *Yale J. Biol.*, 1952, v24, 351.

8. Riley, J. A., *Science*, 1949, v109, 281.

9. Sikand, Rajinder S., and Nahum, L. H., *Fed. Proc.*, 1952, v11, 148.

10. Sikand, Rajinder S., Nahum, L. H., Levine, H., and Geller, H., *Yale J. Biol.*, 1952, v24, 366.

Received July 14, 1952. P.S.E.B.M., 1952, v81.

Propagation of Coxsackie Virus on the Chorioallantoic Membrane of Embryonated Eggs.* (19784)

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In the course of efforts to determine convenient laboratory methods for differentiating

* Aided by a grant from the National Foundation for Infantile Paralysis and by a grant from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

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between strains of herpes simplex virus and Coxsackie viruses isolated in suckling mice (1), the possibility was investigated that viruses of the Coxsackie group might propagate on the chorioallantoic membrane of embryonated hens' eggs. In previous attempts to grow Coxsackie or C viruses in eggs, positive results were obtained only with Type 2 strains and only when the yolk sac route of inoculation was employed(2,3). The capacity of herpes simplex virus to multiply on the chorioallantoic membrane has been well established. This agent resembles viruses of the Coxsackie group in that it causes a fatal disease in suckling mice and can be readily isolated in these animals(1,4-7). The purpose of this report is to present the results obtained with 17 strains of C virus, representing 16 different antigenic types, following inoculation on the chorioallantoic membrane. In this study, the technic employed with C viruses was similar to that commonly used for the propagation of herpes simplex virus.

Materials and methods. Viruses. The C viruses used were designated as follows: Types 1, 2, and 3(8); Texas-1, Texas-12, Texas-14, Texas-15, Ohio-1, Conn.-5, Israel-7, Easton-10, Easton-14 (strains L.O. and M.K.), Alaska-5, and Nancy(9); Belgium-1 and Belgium-2(10). The identity and specificity of each strain used were determined and confirmed by neutralization tests carried out in suckling mice with specific immune mouse or hamster sera. Two strains of the Easton-14 type were tested; otherwise, each of these agents was antigenically unrelated to the others. The Belgian strains were isolated in this laboratory from specimens collected during the summer of 1951 from patients in Brussels. Five of the other strains used were also recovered and identified in this laboratory.

Suckling mice. Suckling Swiss albino mice less than 48 hours of age were used for propagation of virus, for titrations of viral activity, for neutralization tests and in tests for the presence of virus in egg membranes. The number of baby mice in each litter was approximately eight.

Ten per cent suspensions consisting chiefly of muscle and bone were prepared in broth from infected suckling mice which had been killed with ether, decapitated, skinned and eviscerated. The suspensions were clarified by centrifugation first in an International Centrifuge at 2,500 RPM for 10 minutes and then in an angle rotor at 8,000 RPM for 10 minutes. The supernatant fluids prepared in this manner were treated with a mixture of penicillin and streptomycin to resist bacterial contamination and were used as a source of virus for the initial inoculation of egg membranes. Mice used for propagation of virus were injected with 0.02 ml of a suspension of infected baby mice by the intraperitoneal route and were sacrificed after signs of disease had appeared. Titrations of viral activity were carried out by injecting suckling mice by the intraperitoneal route with 0.02 ml of decimal dilutions of the various tissues to be tested. Tests for the presence of virus in egg membranes were performed by injecting suckling mice with 0.01 ml of a suspension of membranes by the intracerebral route. Following injection, the animals were observed daily during a period of 12 days for signs of disease or death. At the end of this period of observation the results were considered positive if all of the animals had developed fatal disease and negative if all of the animal remained healthy. Mice which died within 24 hours of the time of injection were discarded and not included in the test.

Embryonated eggs. Fertile hens' eggs which had been incubated at 36.6°C for 11 or 12 days were candled and those with active embryos were used in groups of 5 for each specimen or dilution tested. An inoculation of 0.1 ml was made on the chorioallantoic membrane of each egg. The eggs were then incubated again at 36.6°C for 72 hours. They were candled daily during this period and those in which the embryos had died were discarded.

The membranes of surviving embryos were harvested aseptically, washed in sterile saline, and ground in a mortar. They were then made into a 10% suspension in broth which was clarified by centrifugation for 10 minutes at 2,500 RPM. The suspensions were treated with a mixture of penicillin and streptomycin and stored in an electric refrigerator at —20°C. Cultures were made of all suspensions prior to inoculation of mice or eggs to

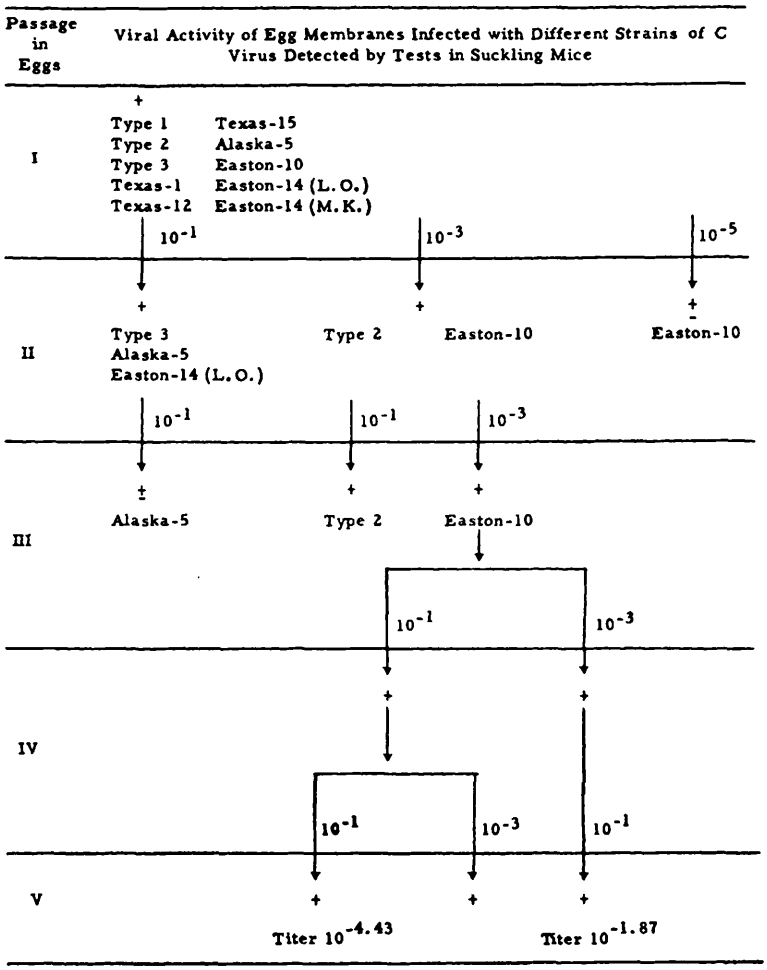

FIG. 1. Results obtained with 17 strains of 16 different antigenic types of C virus following inoculation on the chorioallantoic membrane of embryonated eggs.
Results: + = all of mice died; ± = approximately half of mice died.

assure absence of bacterial contamination.

An aliquot of each suspension of membrane was tested in suckling mice for viral activity. The occurrence of paralysis with fatal termination in these animals during the 12-day period of observation was taken to indicate the presence of virus. When a suspension of membranes was thus found to have viral activity, an additional serial passage was made in different groups of eggs using as inocula the 10% (10^{-1}) suspension and 2 additional dilutions (10^{-3} and 10^{-5}) calculated as dilutions of the egg membranes themselves. This was done in order to obtain as promptly as possible an indication of whether viral activity was attributable to a residue of virus from the original inoculum (passive transfer) or the result of actual multiplication of virus on the egg membrane. After the eggs used for this passage had been incubated for 72 hours, the membranes of each group were collected, made into a suspension and tested in suckling mice. Further passages in eggs were made in a similar manner with each of the membrane suspensions which was found to have viral activity. It should be emphasized that in this study all passages were made in eggs and that suckling mice were used merely as indicators in tests for the presence of virus.

Results. The results obtained with the 17

strains of C virus following inoculation on the chorioallantoic membranes of embryonated eggs are summarized in Fig. 1. The suspensions of infected baby mice which were used to inoculate first passage egg membranes had LD50 titers greater than 10^{-5} and less than 10^{-8} excepting suspensions of the Israel-7, Belgium-1 and Belgium-2 strains which had titers of 10^{-4} or less. In Fig. 1, only those strains which yielded positive results are indicated. Excepting the Easton-10 virus, each strain is recorded only once per passage in such a manner as to show the highest dilution of inoculum which transmitted viral activity. Higher dilutions and an additional passage of membranes which showed viral activity were tested but, except as indicated, the results were negative. Negative results were also obtained with suspensions prepared from uninoculated membranes and from membranes inoculated with sterile broth.

As can be seen in Fig. 1, after one egg passage, positive results were obtained in tests for viral activity with suspensions of membranes inoculated with each of 10 strains representing 9 different antigenic varieties of C virus. No evidence of viral activity was found in suspensions of membranes inoculated with the other 7 strains. In this and in subsequent passages most of the embryos remained alive during the 72 hours of incubation following inoculation. This was true both with eggs which showed viral activity and those which did not.

A second egg passage was made with each of the membrane suspensions which showed viral activity on the first passage. In tests for activity with membranes of the second passage, the results were positive with 5 strains and negative with 5 strains. Among the viruses which remained active on the second egg passage 3 were demonstrable only in membranes inoculated with a 10^{-1} suspension and not in those which received greater dilutions of the first passage material. The Type 2 and Easton-10 agents were present in membranes inoculated with a 10^{-1} and a 10^{-3} dilution, and some activity of the Easton-10 strain was also detected in membranes which had received a dilution of 10^{-5}.

In tests for the presence of virus in membranes of the third egg passage, the results were positive with the Type 2 and Easton-10 strains; some activity was also detected with the Alaska-5 virus. On the fourth egg passage only membranes inoculated with the Easton-10 strain were found to be active.

On the fifth consecutive egg passage of the Easton-10 strain, membranes inoculated with 3 different preparations of fourth passage material gave positive results in tests for the presence of virus. In order to confirm the identity of the viral agent in this passage one of the suspensions was tested with and found to be neutralized by specific immune mouse serum against the Easton-10 virus. Decimal dilutions of 2 of the fifth passage membrane suspensions were injected into suckling mice in order to estimate the amount of virus present in each of them. As indicated in Fig. 1, LD50 titers of $10^{-4.43}$ and $10^{-1.87}$ were obtained.

The suckling mouse material infected with the Easton-10 virus which was used to inoculate membranes of the first egg passage had an LD50 titer of $10^{-6.58}$. On the basis of dilutions made deliberately in the subsequent passages, quite apart from possible additional dilution within the eggs used, the inocula for the 3 groups of fifth passage membranes shown from left to right in Fig. 1 can be estimated to have contained as residue from the initial (first passage) inoculum not more than 0.01 LD50 for the first and 0.0001 LD50 for the second and third. The LD50 titers of $10^{-4.43}$ and $10^{-1.87}$ obtained with the first and third membrane suspensions of the fifth passage represent in both instances approximately one million times the calculated infective residue in the respective fifth passage inocula. These findings appear to indicate that in the serial egg passages, actual multiplication of the Easton-10 virus occurred.

Discussion. In this investigation it was found that the behavior of Coxsackie viruses following inoculation on the chorioallantoic membrane is not uniform and that several different antigenic varieties can remain active after more than one serial passage by this route. Variation in the quantity of virus contained in the suspensions used to inoculate membranes in the first egg passage and pos-

sibly also strain differences(3) may have affected the results which were obtained. In all but one instance, it seems possible to account for the presence of virus as residue from the initial inoculum. It is noteworthy, however, that the Type 2 virus, one of those which remained active through 3 consecutive egg passages is the only Coxsackie virus previously found to propagate in eggs(2,3).

Evidence was obtained that, in the case of the Easton-10 agent which remained active through 5 consecutive passages, actual multiplication of virus had occurred. The possibility that this agent might be a strain of herpes simplex virus and not a C virus seems unlikely in view of the procedures employed in its original isolation and identification(11) and would appear to be excluded by the results of other studies. These showed that, unlike herpes virus, the Easton-10 agent was resistant to treatment with ether and failed to cause apparent disease in adult mice inoculated by the intracerebral route or lesions following inoculation on the scarified cornea of the rabbit's eye(1).

It should be pointed out that in the present study possible variations in the procedure used were not explored. Only embryos of 11 or 12 days of age, only one route of inoculation, and only one interval of incubation following inoculation were employed. The chorioallantoic membrane was the only component of the embryonated egg which was tested for the presence of virus. Optimal conditions for the propagation of Coxsackie viruses in eggs were not investigated. It is quite possible that for this latter purpose better results could be obtained by varying the procedures which were used.

In view of the dissimilar results obtained with different Coxsackie viruses, the technic of inoculation on the chorioallantoic membrane appears to be of limited value for the purpose of differentiating between these agents and herpes simplex virus.

Summary. Seventeen strains of C virus representing 16 different antigenic varieties were tested for their capacity to remain active or multiply following consecutive passages in embryonated eggs inoculated on the chorioallantoic membrane. Viral activity was demonstrated in suckling mice with membranes infected by each of 10 strains after one egg passage, by 5 strains after an additional passage, by 3 strains after 3 passages and by one strain (Easton-10) after 5 passages. The findings indicated that multiplication of the Easton-10 virus had occurred.

The assistance of Miss Eru Tanabe is gratefully acknowledged. Dr. J. L. Melnick kindly provided the Easton-10 strain of virus and homologous antiserum as well as some of the other strains and immune sera used in this study.

1. Curnen, E. C., and Godenne, M. O., *Abst. Soc. Pediatric Research,* 1952, v22, 8.

2. Huebner, R. J., Ransom, S. E., and Beeman, E. A., *Pub. Health Rep.,* 1950, v65, 803.

3. Shaw, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 718.

4. Slavin, H. B., and Berry, G. P., *J. Exp. Med.,* 1943, v78, 305.

5. Dalldorf, G., *Am. J. Pub. Health,* 1950, v40, 1508.

6. Stanley, N. F., Hayes, L., and Dorman, D. C., *Aust. J. Exp. Biol. and Med. Sc.,* 1951, v29, 367.

7. Kilbourne, E. D., and Horsfall, F. L., Jr., *J. Immunol.,* 1951, v67, 321.

8. Dalldorf, G., *Bull. N. Y. Acad. Med.,* 1950, v26, 329.

9. Contreras, G., Barnett, V. H., and Melnick, J. L., *J. Immunol.,* 1952, v69, 395.

10. Godenne, M. O., and Curnen, E. C., Unpublished data.

11. Melnick, J. L., Kaplan, A. S., Zabin, E, Contreras, G., and Larkum, N. W., *J. Exp. Med.,* 1951, v94, 471.

Received July 16, 1952. P.S.E.B.M., 1952, v81.