CULTURE ON THE CHICK CHORIO-ALLANTOIS AS A TEST OF INACTIVATION OF VACCINIA VIRUS

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There is considerable evidence of an empirical nature to indicate that the habitual use as a gargle and mouth wash, of an antiseptic solution so mild that it may be tolerated in contact with the mucous membrane, affords some degree of protection against upper respiratory infections. Thomson and Thomson (1932) writing in regard to prevention of colds have said "Probably one of the most important methods of reducing the chance of infection is by means of gargling the throat and treating the nasal passages with some mild antiseptic once or more times daily, more especially during epidemics. There has been a great deal of controversy over this procedure, but it seems to us extremely reasonable to suppose that just as we can prevent infection with venereal diseases through the application of antiseptics soon after exposure so we should be able to prevent droplet infection from respiratory diseases by gargling and washing out the nasal cavities with weak antiseptics." (Page 137.) This supposed favorable effect may be ascribed in part to the mechanical removal of microbes and their toxic products, in part to a favorable influence upon the blood supply to the mucous membranes and in part to a direct germicidal or restraining effect upon the micro-organisms themselves. It is possible that similar effects come into play when a gargle is employed by the physician to treat the early stages of a pharyngeal inflammation.

In immediate relation to the problem of preventing infections of the upper respiratory tract is the question whether such mild antiseptics may or may not exhibit an inactivating or restraining effect upon filterable pathogenic agents known as viruses, which are now generally regarded as important causative factors in some of these upper respiratory infections. Without experimental study, a mere survey of the known characters of these viruses suggested to us that an appreciable effect upon them by any such mild antiseptic was hardly to be expected. However, the experimental observations of others already recorded served to put us on guard against prophetic assumptions in regard to this matter.

In order to avoid, as far as might be, the theoretical entanglements and uncertainties which tend to confuse virus research, it seemed wise to initiate our studies by utilizing one of the best known of these agents giving rise to lesions in man, namely the virus of vaccinia. Our knowledge of this virus rests upon numerous repeated scientific observations by many workers over a con-

1 Aided in part by the Virus Research Fund of the Lambert Pharmaceutical Company, St. Louis, Missouri.
siderable period of years in contrast to the somewhat insecure and unconfirmed doctrines which are current in respect to some other viruses. The individual virus particle of vaccinia, the Paschen body, may be accepted as a definite morphological entity. The lesions resulting from inoculation of the virus to man, calf, rabbit and to the membranes of developing chick embryos are well known. The latter lesions are distinctly visible and possess characteristics which permit them to be recognized with a fair degree of accuracy by the eye and sufficiently differentiated so that they can be recorded by photographs and preserved as permanent specimens in plastic mounts (Dunham, 1941). Vaccinia virus is apparently an altered type of the virus of variola and variola is a disease of man which may be acquired by inhalation of dry material harboring the virus or by the intentional application of such material to the mucous membrane of the upper respiratory region, as in the ancient practise of variolation. Another advantage is found in the harmless quality of this virus in relation to vaccinated human individuals, a matter of some importance in conserving laboratory personnel for work upon viruses. Furthermore the effect of antiseptics on vaccinia virus has already been studied to a considerable extent and further investigation of this matter cannot be expected greatly to alter the habits of the general population nor to revolutionize the practise of physicians. Hence the examination of this question may be undertaken in a quiet atmosphere of scientific enquiry sometimes referred to as academic.

Two strains of vaccinia virus have been used: (1) the glycerinated vaccine of the Department of Health of New York City, distributed for practical use in the Jennerian vaccination of the population and (2) an egg-adapted vaccinia virus, Strain CAEB, kindly supplied to us by Doctor Joseph F. Smadel of the Rockefeller Institute, New York City. This latter strain was received in the form of two specimens of virus-infected tissue. One was a chorio-allantoic membrane which had been preserved in the frozen state for more than a year. The other was a piece of similar tissue which had been dried rapidly while frozen and then preserved in the dry state. In our hands this virus has been propagated by serial inoculation onto the chorio-allantois of fertile eggs or by inoculation into the yolk of such eggs.

Culture technic. The technic of culture on the chorio-allantois is essentially that of Goodpasture, Woodruff and Buddingh (1932) as modified by Burnet (1936). However some new instruments have been devised and some technical modifications introduced (Dunham 1941, 1942.) An adequate supply of fertile eggs is obtained at regular intervals from chickeries which deal in eggs for hatching. Upon arrival at the laboratory each egg is numbered, recorded under the proper date and then placed in the egg incubator at a temperature of 100 to 101 degrees Fahrenheit (37.7 to 38.3 degrees C.). After development for 10 to 13 days, with daily turning, each egg is candled and the unsatisfactory sterile eggs or those with feeble or otherwise abnormal embryos are discarded. The side of the shell nearest the contained embryo is marked and this spot is held uppermost during the procedure of inoculation.

The uppermost surface of the shell is disinfected with 95 per cent alcohol and,
with aseptic precautions, is cut with a dental carborundum disc so as to separate a triangular segment of the shell measuring about 12 millimeters on each side. One is careful to avoid injuring the delicate underlying shell membrane. This operation is best done in or near an open funnel through which the dust is drawn away by adequate suction. The blunt end of the egg is then disinfected with alcohol and a minute opening is made with a small dental drill through the shell at this end so as to enter the normal air sac. The egg is then placed in our egg inoculator (fig. 1) so that the opening into the air space is covered by the suction disc which is connected to a continuous suction giving a negative pressure of about 50 millimeters of water. The suction line is provided with a safety by-pass to ensure that the negative pressure shall not become excessive. While the air space and, through it, the interior of the egg is subjected to this slight negative pressure, the loosened triangular piece of shell is lifted off with sterile forceps and placed in a petri dish. Then by means of our shell-membrane teaser (fig. 2) a slit is produced by gently separating the diagonal fibers of the shell membrane; at either end of the slit a short incision at right angle to it is cut with sterile scissors and the membrane is torn by gentle traction so as to remove an approximately rectangular portion. At the initial perforation of the shell membrane air passes through and the immediately underlying vascular chorio-allantois falls away so that the risk of technical injury to this delicate and important structure is practically eliminated. The previously prepared inoculum is now dropped through the opening onto the chorio-allantois and the final droplet touched off by gentle contact with this membrane. The inoculum is conveniently handled in a tuberculin syringe with a 2 inch 20 gauge needle attached. After inoculation of the egg the triangular fragment of shell is replaced and sealed with liquid adhesive.2 The hole at the blunt end is likewise sealed and the egg is returned to the incubator.

The subsequent period of incubation may be two or more days as desired. Ordinarily the inoculation of stock or control vaccine results in the formation of

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2 The liquid adhesive is obtainable from Johnson and Johnson Company, New Brunswick, N. J.
small opaque lesions on the chorio-allantois, which are about 0.5 millimeter in diameter at the end of two days and, after three days, 1 to 1.5 millimeters in diameter with visible necrotic centers. They tend to become confluent as they get older. For propagation of the stock vaccinia a period of three days seems best. For experimental tests of viability after possible inactivation somewhat longer incubation may be preferred. At the end of this period of incubation the membrane is harvested.

To harvest the membrane, one first cuts the shell with the carborundum disc, entirely around, just above the level of the chorio-allantoic membrane, and removes the upper portion of the shell so as to expose the inoculated area. By use of fine sterile scissors and forceps this portion of the chorio-allantois is removed and placed in sterile saline solution in a petri dish, where it is spread out flat for study with a hand lens. Discrete lesions are counted and the approximate number of lesions in confluent areas estimated. The membranes are described for the record and sometimes are photographed in the salt solution or are dehydrated and embedded in plastic for permanent preservation. A diagrammatic drawing of the membrane is also made in the protocol as an aid to identification, thus permitting one dish to serve for holding several specimens. Sometimes the chorio-allantois is found thin and partly disintegrated by autolysis. Such eggs have evidently died shortly after inoculation, possibly because of operative trauma or some undetermined cause. They are discarded.

The virus suspension. For the preparation of a virus suspension to be used in testing antiseptics, several egg membranes with abundant vaccinia lesions are placed in a mortar and ground with sand. Tyrode solution, 2 milliliters for
each membrane used, is added to suspend the material and this suspension is then sedimented in the centrifuge at about 1,000 revolutions per minute for ten minutes. The supernatant, translucent but cloudy, light brown suspension is siphoned off and is used as the stock virus suspension. A freshly prepared suspension has been used for each experiment in the testing of the antiseptics.

**Antiseptic agents.** Several different chemical preparations were tested simultaneously against the same virus suspension and several eggs, usually six, were used to test the resulting possible inactivation of the virus by each antiseptic solution. The antiseptics were employed in their original state as supplied in the drug trade or were prepared in the laboratory from chemicals of high purity.

**Technic of the tests.** The antiseptic solution to be tested, 9 ml., was vigorously mixed with 1 ml. of the virus suspension and then, before sedimentation, a sample of this mixture was withdrawn into a tuberculin syringe fitted with a two-inch, gauge 20 needle. This syringe was inserted into a test tube which was immersed in ice water. The moment of mixing the virus with the antiseptic was recorded and the time of subsequent inoculation of this mixture into each egg was noted. The eggs were inoculated in rotation. Thus, in making a test of five antiseptic solutions and one control suspension the first egg received solution No. 1, the second solution No. 2 and so on, until each of the six had been inoculated into an egg. Then the seventh egg would be inoculated with solution No. 1. By this schedule solution No. 1 would be introduced into the first, seventh, thirteenth, nineteenth, twenty-fifth and thirty-first egg of the series. Thus, the time interval from the moment of mixing the antiseptic with the virus would be different for each egg but each of the solutions tested would be represented by an egg in each group of six eggs in the consecutive series.

**The tests and their results.** The most immediate practical question involved in these studies has concerned the possible virus-inactivating effect of Liquor antisepticus, (National Formulary, 1935) a solution which is extensively used in hospitals as a mouth wash and throat gargle. It is only fair to state that our experiments were undertaken with the expectation that the virus of vaccinia would not be perceptibly influenced by contact with this preparation. Hence a preliminary experiment was performed to demonstrate this assumed lack of inactivating power. A virus suspension was prepared from an egg membrane rich in lesions of vaccinia strain of the Health Department. Three solutions were tested (1) Tyrode solution as a control (2) Ethyl alcohol 25 per cent and (3) Liquor antisepticus. The eggs had been incubated eleven days; then inspected by candling and marked in the usual way. The mixture of Tyrode 9 ml. plus virus suspension 1 ml. after standing 21 minutes was inoculated to the chorio-allantois of egg 7627. In the same way egg 7608 was inoculated with the mixture of Alcohol 9 ml. plus virus suspension 1 ml. after this mixture had stood 26 minutes and egg 7620 was inoculated with the mixture of Liquor antisepticus, 9 ml. plus virus suspension 1 ml., after this mixture had stood 57 minutes. Additional eggs were inoculated at somewhat longer intervals as shown in table 1. The result, somewhat contrary to expectations, indicated a moderate degree of inactivation of vaccinia virus by the alcohol and complete
inactivation by the Liquor antisepticus in the exposure periods of 57 and 65 minutes.

Several repetitions of this experiment, exposing the virus for shorter time intervals, have consistently shown a greater degree of inactivation by the Liquor antisepticus as compared with the alcohol, although complete inactivation was not always attained. The protocol of one large experiment may serve as an example (table 2). In this experiment an attempt was made to test some of the individual constituents of Liquor antisepticus in concentrations of approximately the strength in the official solution. The Boric acid was dissolved in water. A mixture of Alcohol, 1 part, and Tyrode solution, 3 parts was employed to dissolve the Menthol, Thymol, Eucalyptol and Methyl salicylate. Along with these, Tyrode solution, Distilled water, Alcohol, 25 per cent in Tyrode solution, Alcohol 25 per cent in Distilled water and Liquor antisepticus were tested against the same virus suspension. The experiment required the inoculation of sixty eggs.

<p>| TABLE 1 |</p>
<table>
<thead>
<tr>
<th>Inoculations of vaccinia onto chorio-allantois. Preliminary test</th>
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<tbody>
<tr>
<td>Tyrode solution, 9 parts, plus Virus suspension, 1 part</td>
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<tr>
<td>Alcohol 25 per cent, 9 parts, plus Virus suspension, 1 part</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Liquor antisepticus, 9 parts, plus Virus suspension, 1 part</td>
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To prepare the virus suspension, the membranes of three eggs with abundant vaccinia lesions, representing the virus of the Health Department, were used. Each final mixture was found free from ordinary bacteria by culture control. The inoculation dose for each egg was 0.025 ml. dropped onto the chorio-allantoic membrane. Results are shown in table 2.

In this experiment a long time was required for the technical procedure of inoculating the eggs. However, it is evident that the virus retained its potency in the Tyrode solution (391 minutes), in the Distilled water (392 minutes), in the Alcohol 25 per cent in Tyrode (390 minutes) and in the Alcohol 25 per cent in Distilled water (387 minutes). On the other hand, the virus was evidently inactivated by the Liquor antisepticus and apparently by the longer exposures to Boric acid. The Menthol, Thymol and Eucalyptol also seemed to have some inactivating effect, but the results were so irregular as to appear uncertain. For the Liquor antisepticus and the Boric acid it seemed that inactivation might be accomplished within a period shorter than any used in this experiment. Hence further tests were done at briefer exposure intervals for these two.

Six eggs were opened, ready for inoculation for each of these two antiseptic mixtures (Boric acid and Liquor antisepticus) and covered with sterile paper.
The virus suspension was then quickly mixed with the antiseptic. These eggs were inoculated after elapsed time of 1, 3, 5, 8, 10 and 12 minutes for the Boric acid and after 2, 3 1/2, 4, 5, 6 and 7 minutes for the Liquor antiseptics. Longer periods of exposure were allowed for the other antiseptics and for the control solutions. The time intervals and the results are shown in table 3. In this situation...
experiment there was again a striking indication of rapid inactivation of the virus after it was mixed with the Liquor antisepticus.

The apparent inactivation of the virus by Liquor antisepticus was subjected to a further check. The chorio-allantoic membrane of an egg which had been inoculated with this mixture after an exposure period of 5 minutes, was preserved in Tyrode solution in the refrigerator for 48 hours, then ground with sand, suspended in 3 ml. Tyrode solution and inoculated onto the chorio-allantoic membranes of three more eggs. Two of these were opened after 48 hours and the third after four days. All three were alive and free from recognizable lesions of vaccinia.

### TABLE 4

<table>
<thead>
<tr>
<th>Mixture and Exposure</th>
<th>Number of Lesions</th>
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<tr>
<td>Liquor antisepticus, N.F. VII, 90 per cent, 9 parts, plus Virus suspension, 1 part; after standing 30 seconds this was further diluted with Distilled water, 90 parts; then inoculated to four eggs</td>
<td>0</td>
</tr>
<tr>
<td>Liquor antisepticus, N.F. VII, 80 per cent, 9 parts, plus Virus suspension, 1 part; after standing 30 seconds this was further diluted with Distilled water, 90 parts; then inoculated to four eggs</td>
<td>0</td>
</tr>
<tr>
<td>Liquor antisepticus, N.F. VII, 10 per cent, 9 parts, plus Virus suspension, 1 part; after standing 20 minutes this was further diluted with Distilled water, 90 parts; then inoculated to four eggs</td>
<td>68</td>
</tr>
<tr>
<td>Distilled water, 9 parts, plus Virus suspension, 1 part; this was further diluted with Distilled water, 90 parts; then inoculated to four eggs</td>
<td>109</td>
</tr>
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</table>

Repeated experiments of this type have shown some variations, particularly when much more dilute virus was used. In such a case the inactivation by Boric acid became evident after an exposure of 9 minutes and the Liquor antisepticus caused complete inactivation at 2 minutes while the virus remained active for two hours or more in the other solutions.

In the experiments so far, the briefest period of exposure of the virus to the antiseptic before inoculation into the egg was one minute. In order to test the possible effect of an even more brief exposure and at the same time to test the effect of dilution, a modification of the technic was introduced. Liquor antisepticus was prepared in the laboratory according to the officially revised formula (National Formulary, Interim Revision, 1939) effective July 1, 1940, a preparation to be designated as Liquor antisepticus, N.F. VII. Preliminary
tests showed that when this solution was diluted with nine parts of water it had no appreciable inactivating effect upon vaccinia virus. Hence it was possible to utilize this fact and to stop the action of the antiseptic on the virus at the end of any predetermined period of exposure, merely by diluting the test mixture. Subsequent inoculation onto the egg membranes was then carried out without undue haste. The results of a typical experiment of this kind, in which vaccinia virus CAEB was used, are shown in table 4. It is evident that 90 per cent and 80 per cent dilutions (actually reduced to 81 per cent and 72 per cent, respectively, by addition of the Virus suspension) of Liquor antisepticus, N.F. VII, inactivated the vaccinia virus in thirty seconds, at which time further action was halted by tenfold further dilution of these mixtures. The lack of potency after dilution is indicated by the behavior of the mixture in which 10 per cent Liquor antisepticus was tested against the virus for a period of 20 minutes, a much longer time than had been required to inoculate the eggs with the preceding test mixtures. Here the numbers of lesions developing on the inoculated egg membranes were of the same order as observed in the
control preparation using Distilled water. Photographs of membranes from this experiment, preserved in plastic, are shown in figures 3, 4 and 5.

Discussion. The criterion for inactivation of vaccinia virus employed has depended upon the production of vaccinia lesions upon the chorio-allantoic membrane of the chick embryos inoculated. Not too much should be claimed for the precision of the method. It may be assumed that the tissues of the chick have some resistance to the virus. Hence one is justified in concluding that inactivation of the virus for the chick embryo may not signify complete destruc-

![Figure 4: Egg Membranes with Numerous Lesions of Vaccinia, Taken from Two Eggs Listed in Table 4. Liquor Antisepticus, 10 Per Cent. Mounted in Plastic.](http://jb.asm.org/)

tion of the virus. There is no reason to believe that the tissues of the chick embryo are more resistant in this respect than the tissues of other animals and it would seem fair to assume that the results have comparative value.

The experimental results were not entirely uniform. Irregularities in such work are, however, to be expected. We believe that they are in part due to the character of the virus preparation. This is a suspension of embryonic tissue elements containing the virus and not a suspension of separated virus particles. Hence it lacks uniformity of composition. To be sure, the gross bits of tissue
are thrown down by the centrifuge but it is by no means certain that small
groups of tissue cells may not remain in the final suspension and thus offer a
relative protection to virus particles in their interior. More exact and more
regular results might be obtained by employing specially purified suspensions
of the virus particles as used by Wilson Smith (1939) but such results would be
of a less practical value because the natural dissemination of the virus takes
place in association with tissue elements. For practical significance, therefore,
the virus suspension as prepared would appear to possess some advantages over

![Image of egg membranes with numerous lesions of vaccinia, taken from two eggs listed in Table 4. Distilled water control. Mounted in plastic.]

The results show that vaccinia virus remains active for several hours at least
when suspended in Tyrode solution, Distilled water, Alcohol 25 per cent in
Tyrode solution, and in Alcohol 25 per cent in Water. On the other hand the
virus is inactivated, as far as concerns its ability to produce lesions on the
chorio-allantois of chick embryos, by Liquor antisepticus in less than a minute.
Some individual constituents of Liquor antiseptics, when employed separately, exhibit some tendency to inactivate the virus after longer periods of time. This seemed to be true particularly of the Boric acid, Thymol, Eucalyptol and Menthol. The action of each of these was however much less potent than the action of the Liquor antiseptic itself.

SUMMARY

1. The ability of Liquor antisepticus and of some of its constituents to inactivate vaccinia virus has been tested by inoculation onto the chorio-allantoic membranes of developing chick embryos.

2. When tested in this way it was found that the virus retains its activity very well when suspended in Tyrode solution, in Distilled water or in Alcohol 25 per cent. On the other hand it is quickly inactivated by Liquor antisepticus and appears to deteriorate less rapidly in solutions of Boric acid, Menthol, Thymol and Eucalyptol.

REFERENCES


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