# Propagation of Fowl- and Pigeon-Pox Viruses in Avian Eggs

*and*

# Use of Egg-Cultivated Viruses for Immunization

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## FOREWORD

Abundant evidence has been presented in recent years to show that developing chick-embryo tissue is adapted to the propagation and concentration of a large number of filtrable viruses. As a result, extensive research in the field of filtrable-virus diseases has been made possible. For many years investigations have been under way at the Illinois Station on the nature of the virus causing pox in domestic fowl, with special reference to immunization procedures. These studies have made it apparent that the pox virus from chorio-allantoic tissue possesses certain advantages over the skin-lesion virus for immunization purposes.

This bulletin reviews the literature that has increased our knowledge of the production of viruses and of the value of the chorio-allantoic membrane for continuous passage of fowl- and pigeon-pox viruses. It also sets forth a propagation technic of proved value. This method, or an appropriate modification, may be employed not only in research but also in the production of virus for use in disease prevention and control.

Success in artificial propagation of viruses has already made possible much progress in the study of virus diseases of animals, but what has been accomplished appears to be only a beginning. It is hoped that further advances will follow discerning effort in the laboratory and in the field.

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# Propagation of Fowl- and Pigeon-Pox Viruses in Avian Eggs and Use of Egg-Cultivated Viruses for Immunization

By C. A. Brandly*

SINCE developing avian eggs have recently been utilized with valuable results in propagating the various so-called filterable viruses, the medium was used at the Illinois Agricultural Experiment Station for the passage of fowl- and pigeon-pox viruses. The present study, which is an outgrowth of investigations on fowl-pox immunization procedures conducted since 1926, describes the method used at this Station in propagating fowl- and pigeon-pox viruses in fertile chicken eggs and reports the results of using these viruses for the immunization of chickens against fowl pox. The monograph also gives a review of literature on previous uses of the egg-propagation method.

## PART I: REVIEW OF LITERATURE

Prior to its use in virus studies, the incubating egg had served as a fruitful subject for embryologic and biologic observation and experimentation as reviewed by Goodpasture (1938). As early as 1749 Beguelin, according to Gerlach (1886), made a window in the egg shell in order to observe the embryo during its development. Scymkiewicz (1815) and Gerlach (1886) made openings in the the egg shell as a means of manipulating and observing the embryo. Peebles (1898) experimented on the embryo thru a window in the shell, and the method was later adapted by Rous and Murphy (1911) for the propagation of certain virus agents in chicken, duck, and pigeon eggs. Murphy (1912, 1913) and Murphy and Rous (1912) produced tumors of the embryo and extra-embryonic membranes by injecting filtrates and suspensions of the Rous chicken sarcoma and similar agents. Juan and Staub (1920) carried fowl-pest virus thru six successive passages on developing eggs by inoculation into the yolk. Gay and Thompson (1929) found that vaccine virus injected into the yolk sac of incubating

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eggs could be recovered from the second but not the third transfer. It remained, however, for Goodpasture and co-workers to reveal the potentialities of the avian egg in the approach to various virus problems, and thus to stimulate much of the recent work in this direction.

In 1931 Woodruff and Goodpasture reported the cultivation of fowl-pox virus thru successive transfers on the chorio-allantoic membranes of chicken embryos. Their technic was based upon the method of Clark (1920) for embryological study. Later Goodpasture, Woodruff, and Buddingh (1931, 1932) cultivated vaccinia and herpes simplex viruses in developing eggs. Since then many viruses, as well as certain bacteria, fungi, spirochaetes, protozoa, and rickettsia, have been propagated in or upon the avian embryo and its membranes.

A number of significant observations and results have accrued in this field; but the scope of the work with the viruses is of such extent that for the purposes of this publication further reference will be limited largely to the methods employed and the results obtained in the propagation of those viruses which, under ordinary conditions, attack various species of birds.

## Cultivation of Viruses in Eggs

Rivers and Schwentker (1932) passed the virus of Pacheco's parrot disease thru six generations of developing eggs without altering its infectivity for experimental birds. The method of inoculation consisted of making a shell opening over the air sac, thru which the chorio-allantois was infected by puncture with a needle bearing the virus. Burnet (1933) carried Kikuth's canary virus thru several series of developing eggs. His technic is a variation of that of Woodruff and Goodpasture (1931) in that an artificial air sac is produced on the side of the egg to facilitate more satisfactory and uniform inoculation upon the membrane. More recent reports on the egg-cultivation of other viruses affecting poultry and birds include those on infectious laryngotracheitis (Burnet, 1934, and Brandly, 1935); fowl plague and Newcastle disease (Burnet and Ferry, 1934); psittacosis (Fortner and Pfaffenberg, 1935, and Burnet and Rountree, 1935); and avian-virus bronchitis (Beaudette and Hudson, 1937). Jarmai (1934) reported that chicken embryos could be infected after the 10th day with the agent of leukemia. McLennan (1935) inoculated suspension of nerve tissues and viscera from birds showing neurolymphomatosis into unincubated chicken eggs, as well as into the chorio-allantois of 10- and 14-day embryos. Definite evidence of transmission of the disease was not obtained. Gibbs (1936) reported experiments wherein suspensions

of neurolymphomatous cells were introduced "upon the embryo" of eggs incubated 1, 5, 10, 15, and 20 days. He observed that neurolymphomatosis may be transmitted in this way until the embryos were about 15 days old. At the Illinois Station a strain of erythroleukosis virus was carried thru several series of eggs inoculated 10 to 12 days after being placed in the incubator. In the Report of the U. S. Bureau of Animal Industry (1938) a case of generalized leukosis of the tumor type is reported in a chick 22 days old. This chick had been inoculated with leukotic-tissue suspension as a 6-day embryo in a group of 116 chick embryos.

Burnet (1936) stated that he had cultivated pigeon-pox and sparrow-pox viruses in the chicken egg. At the Illinois Station three strains of pigeon-pox virus, as well as the so-called "antidiphtherin" of De Blieck, were propagated in chicken eggs during 1936-1938. Beaudette and Hudson (1938) reported continued serial passage of pigeon-pox virus on the chorio-allantois of chicken and duck eggs. Higbie and Howitt (1935) cultivated the virus of equine encephalomyelitis on eggs, and this agent recently has been recovered from naturally affected ring-necked pheasants (Tyzzer and co-workers, 1938, and Van Roekel and Clarke, 1939) and also from pigeons (Fothergill and Dingle, 1938). The virus of rabies, altho rarely recognized as naturally affecting birds, has been reported to be cultivable in incubating chicken eggs by Peragallo (1937), Kligler and Bernkopf (1938), and Dawson (1939).

## Other Applications of the Method

That differentiation of certain virus diseases of fowls may be accomplished on the basis of gross and microscopic pathology produced in the chorio-allantoic membrane was demonstrated with fowl plague and Newcastle disease (Burnet and Ferry, 1934), with canary and fowl pox (Burnet, 1933), and with fowl and pigeon pox (Brandly and Dunlap, 1939). Burnet (1936) observed gross differences in chorionic lesions among strains of laryngotracheitis virus, and similar variations were seen by Brandly (1936). The observation that laryngotracheitis virus introduced by the methods employed (Brandly, 1936) may induce gross lesions in chicken and turkey eggs but not in duck, guinea fowl, and pigeon eggs suggests the possible use of egg propagation as a means of distinguishing laryngotracheitis from other viruses.

The developing egg has been found superior to the baby chick or older bird for the titration of certain viruses as well as for detection of minute quantities of viruses naturally attacking birds. Satisfactory titrations have been accomplished with the chick embryo in the cases

of viruses rapidly lethal for the embryo, e.g. fowl plague and Newcastle disease (Burnet and Ferry, 1934), equine encephalomyelitis (Higbie and Howitt, 1935); and also with viruses which produce plaques or pock-like foci on the chorio-allantois, e.g. avian-pox viruses (Burnet, 1936, and Burnet and Lush, 1936), and infectious laryngotracheitis (Burnet, 1936A). The suitability of the egg for determination of virus neutralization was demonstrated with equine encephalomyelitis by Higbie and Howitt (1935) and as a means for serological differentiation between canary pox and fowl pox by Burnet and Lush (1936). In work with laryngotracheitis Burnet (1936) found the chorio-allantoic membrane a satisfactory medium for elucidation of the mechanism of the virus-serum reaction. By use of the egg Burnet (1936A) was able to explain the epizoology of laryngotracheitis in Australian territories.

### Egg-Propagated Viruses for Immunization

Because of certain obvious advantages, pure-culture egg-propagated viruses have aroused much interest from the standpoint of immunization in poultry as well as in man and other mammals. Goodpasture and Buddingh (1933, 1935), Lehmann (1934, 1936, 1937), Godinho (1934), Herzberg (1935), and Gallardo and Sanz (1937) have utilized egg-cultivated vaccinia virus successfully in immunization of man against variola. Brandly (1935, 1936) obtained satisfactory results in fowl-pox and laryngotracheitis immunization of chickens with the respective egg-propagated viruses. Beaudette and Hudson (1937A) reported the use of egg-propagated laryngotracheitis virus in immunization. Egg-cultivated fowl- and pigeon-pox viruses were employed in immunization studies by Dunlap (1938) and by Brandly and Dunlap (1939). Pigeon-pox virus propagated in eggs was reported by Beaudette and Hudson (1938) to have given good results in vaccination of chickens under flock conditions.

## PART II: ILLINOIS STATION EXPERIMENTS

### Propagation of Fowl- and Pigeon-Pox Viruses in Chicken Eggs

The experimental work undertaken at the Illinois Station on the egg-propagation of fowl- and pigeon-pox viruses is an extension of the study on immunization against pox in domestic fowl reported by Graham and Brandly (1940). Results and observations on the immunizing value of the egg-produced virus are given in this section.

**Source of viruses.** Three strains of pigeon-pox virus were used in these studies. Strain $P_1$ was believed to have originated from pigeons in England. This strain has been passaged thru 60 series of chicken eggs since March, 1937. The origin of Strain $P_2$, which was used only in the later work and was passaged thru 8 series of eggs, was not definitely known. A third strain, $P_3$, was obtained during 1937 from an outbreak among pigeons in a loft in Illinois. It was carried thru 25 chicken-egg passages. Each of these three strains was pathogenic for pigeons and for chickens.

Pox virus was also studied in the form of the so-called "antidiphtherin" supplied by Dr. L. de Blieck of Holland. Seventeen egg passages were made with this virus.

Four strains of fowl-pox virus were propagated in developing eggs. All strains but one ($FP_3$) were obtained from apparently unrelated field outbreaks in Kansas and Illinois. Strain $FP_3$ was isolated from commercial "fowl pox" vaccine purchased on the open market and subsequently carried thru 43 egg passages. This virus was pathogenic both for chickens and pigeons and, as determined by repeated serial passage on chicken eggs, chickens, and pigeons, appeared to represent an original bipathogenic strain and not a mixture of pigeon and fowl viruses. The other three strains of fowl-pox virus were pathogenic for the fowl, but not for the pigeon. The first strain ($F_1$) was isolated in March, 1935, and up to February, 1939, had been passaged on 90 series of eggs, including one series of duck, one of guinea fowl, and two of turkey. Strain $F_2$ was passaged on 45 series of eggs, and Strain $F_4$ on 19 series.

**Methods of freeing pox viruses of bacteria.** Several methods were successfully employed for freeing pox skin-lesion material from bacteria. Attempts to obtain bacteria-free virus from a suspension of fowl-comb lesion material by filtration thru Berkefeld N and V candles and coarse acetic-acid collodion membranes almost invariably resulted in failure. Virus filtration was successful, however, in a number of trials with fowl-pox-infected chorio-allantoic membrane suspension in broth (Brandly, 1937). At the outset susceptible chickens were infected by skin puncture and, upon development of early skin pocks, the deeper portions of the lesion were excised aseptically according to a method described by Woodruff and Goodpasture (1931). If free of bacteria, as determined by suitable cultural methods, this tissue was used for egg inoculation. Several attempts to isolate a pure virus from turkey-pox lesion material were unsuccessful. Possibly the turkey-pox virus possessed low virulence for the chicken (Brandly and Dunlap, 1938).

For freeing pigeon-pox lesion material from associated bacteria, intracerebral inoculation passage in pigeons was utilized with good results. This method, described by Bierbaum and Gaede (1935), depends upon the neurotropic properties of the pox virus for its sur-


FIG. 1.—PROPAGATING FOWL-POX VIRUS IN EGGS

(A) Candling egg to determine viability of embryo and to mark position of air sac and window. (B) Cutting the window opening. (C) Inoculating egg with a small-gage glass tuberculin syringe. (D) Sealing egg with cellophane window and paste. (E) Inoculated eggs returned to the incubator. (F) Harvesting lesion. (G) Drying lesion material in a vacuum over calcium chlorid and sulfuric acid.

vival in the brain. The adventitious bacteria invariably present in such material are reduced by dilution of the inoculum, and when introduced with the virus they may fail to survive one or more brain passages. In two instances intracerebral inoculations of pigeons with skin-lesion material yielded a pure pox virus on first passage. Rarely were more than three brain-to-brain passages necessary to free the virus from bacteria. However, one sample of pigeon-skin virus was found to be contaminated heavily with an organism indistinguishable from *Salmonella pullorum*. This organism, even in diluted suspensions, caused death of all birds inoculated, and several attempts to isolate the pigeon-pox virus by intracerebral inoculation resulted in failure.

**Methods of egg inoculation.** The method of egg inoculation described by the writer (Brandly, 1935, 1936) was used for a large part of the work. This method utilizes the principle of Rous and Murphy and of Goodpasture and coworkers, but differs in the location of the shell opening as well as the method and site of the introduction of inoculum. The incidence of contamination was materially less and accurate candling was greatly expedited by this method. Furthermore the turning of the eggs several times daily, which is highly desirable for normal development of the embryo, need not be dispensed with. The procedure of preparation and manipulation of the egg is briefly as follows:

To provide a continuous supply of eggs for repeated passage of the virus, eggs from healthy, vigorous breeding flocks of chickens are obtained at regular intervals for setting. White-shelled eggs offer the advantage of easier transillumination to determine the vigor, activity, and continued viability of the embryo. Eggs which, after 12 days of incubation at a dry-bulb reading of 37° to 38° C. and a wet-bulb temperature of 27° to 31° C., show actively developing, vigorous embryos, are utilized for inoculation. Altho incubation periods of 10 and 14 days were also satisfactory, the 12-day interval was largely used. The position of the air sac is marked (Fig. 1, A). A circular piece of shell (approximately 1.5 cm. in diameter) with the margin at the line delimiting the air sac is removed by cutting thru the shell only with a dental motor and an abrasive disk (Fig. 1, B). The outer shell membrane is left intact. The area is then swabbed with alcohol, and just before inoculation the circular piece of shell and the outer shell membrane are removed with a sharp-pointed instrument. The inoculation of the chorio-allantois is done by means of a small-gage glass tuberculin syringe graduated in .01 cc. and fitted with a sharp 1-inch 22- to 24-gage needle. The point of the needle is introduced thru the shell opening, bevel down, and maintained almost parallel with the inner shell membrane or it may pass thru it (Fig. 1, C). The inoculum, in amounts varying from .05 to .5 cc., is slowly injected. Separation of the inner shell membrane and the chorio-allantois is indicated by the appearance of a definite

elevation and slight wetting of the shell membrane in the area. The distribution of the lesions induced is in part dependent upon whether the virus is introduced upon or beneath the chorio-allantois (Fig. 2).

After inoculation, the shell opening or window is closed by means of cellulose tape or a disk of cellophane applied with library paste (Fig. 1, D).

The inoculated egg is then returned to the machine for further incubation (Fig. 1, E). Daily candling is essential to determine impaired develop-

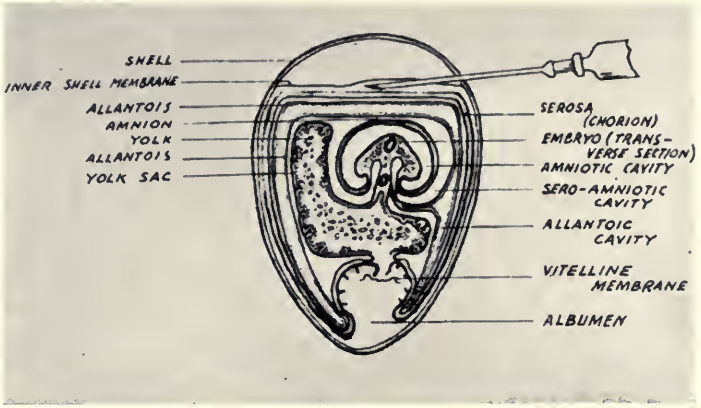


FIG. 2.—POINT OF INOCULATION ON CHORIO-ALLANTOIC MEMBRANE OF A 12-DAY EMBRYO

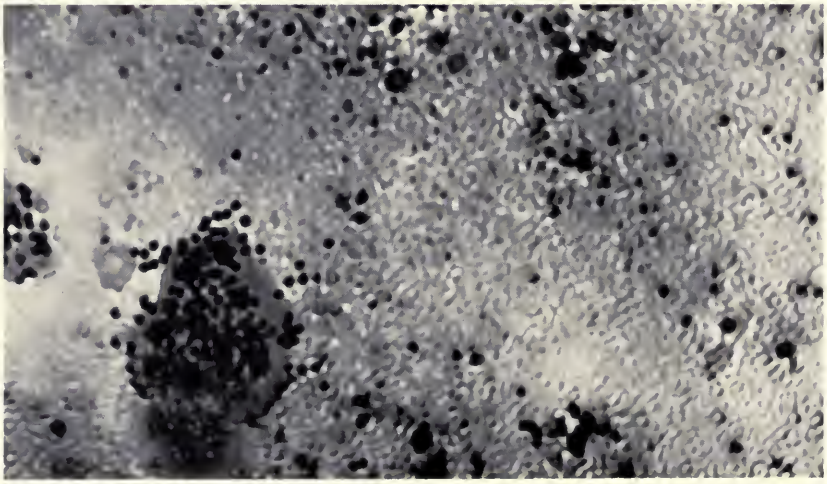

FIG. 3.—BORREL BODIES IN CHORIO-ALLANTOIC LESIONS OF FOWL POX (*Magnification* × *1200*)

ment or death of the embryo. As a rule, eggs inoculated for the culture and passage of pox virus are removed from the incubator after a further incubation period of 3 to 5 days.

Membranes showing lesions (Figs. 5, A, B, and C) are removed aseptically (Fig. 1, F) and tested for sterility. If desired for indefinite storage, the tubes containing virus are placed on a rack in a desiccator which holds a suspension of carbon-dioxid ice in glycerin (Fig. 1, G) and are dried in a vacuum of less than 10 microns of mercury at a low temperature. The individual tubes of dried virus are then sealed *in vacuo* and stored at 0° to 4° C. To better preserve the potency of the virus for use over an extended period, the infected membranes are dried and stored in bulk. Trituration and suspension are effected just before use of each lot. Virus to be used within 2 to 4 weeks may be stored at 0° to 4° C. without drying or evacuation. Staining of suspensions to demonstrate Borrel bodies may be accomplished by the method of Morosow (1926) (Fig. 3), or that of Herzberg (1934).

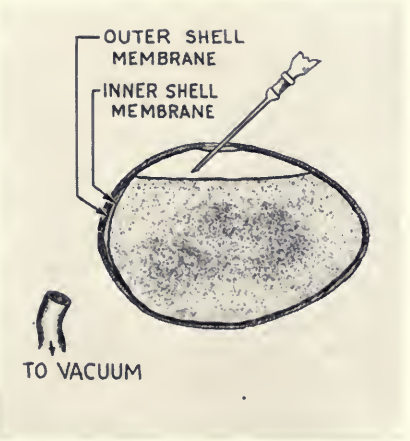


FIG. 4.—BURNET METHOD OF INOCULATING THE CHORIO-ALLANTOIC MEMBRANE WITHIN THE ARTIFICIALLY PRODUCED AIR SAC

The procedure of preparation and inoculation of eggs as developed by Burnet (1933, 1936) was also utilized. In this method an artificial air sac is formed at the side of the long axis of the egg. Since the base of this sac is a relatively large area freed from the rather bibulous shell membrane normally in apposition to it, the suspension may be distributed on the chorio-allantois with relative uniformity (Fig. 4).

**Observation and collection of membranes.** The inoculated eggs are examined at the end of the desired period of incubation or prior to

this time if the embryos appear moribund or dead, as determined by daily candling. Eggs inoculated by way of an opening in the air sac are placed with the large pole uppermost in an improvised holding rack. The cellulose tape or disk of cellophane over the window is removed and the opening is enlarged by breaking away more of the shell and outer shell membrane. With sterile forceps the inner shell membrane is stripped away to expose the chorio-allantois at the large pole of the egg. The nature and extent of lesions or alterations may be observed in the exposed extra-embryonic tissues and fluids and in the embryo. The aseptic collection of obviously affected, as well as questionably affected membranes, is desirable at this time. Membranes for histologic studies are preferably first fixed *in situ* by immersing them in a desirable fixative and are then cut away. Allantoic or amniotic fluid may be removed before or after removal of a section of the chorio-allantoic membrane. The collection of blood from the allantoic vessels of living embryos, as suggested by Burnet (1936), may be done at this stage. The egg may then be inverted and the contents expelled into a sterile Petri dish for further examination. The chorio-allantoic membrane may be removed from the shell membrane. Early and small pocks may often be observed when the membrane is still in the shell, but observation is usually facilitated by removing the membrane and holding it up to a strong light. Membranes to be photographed may be stretched over a moist piece of black blotting paper as was done with the membranes shown in Figs. 5, A and C. The blotting paper serves as a satisfactory contrasting background and the material adheres to it even when it is immersed in water to make a photograph.

The skin of the embryo may show alterations resulting from fowl-pox virus introduced directly thru the amniotic sac. Dysfunction of the pox-involved chorio-allantois may account for delayed development, as well as early death of the embryo, sometimes observed with fowl-pox virus strains, particularly those which have been passaged thru many series of eggs. The lower average weight of a representative number of embryos from pox-inoculated eggs compared with the weight of normal embryos of the same age may be indicative of the influence of the virus on the growth of the embryo. Necrotic foci were seen in the liver of a small percentage of embryos which died following fowl-pox inoculation and subsequent extensive involvement of the chorio-allantoic membrane. However, the extent and significance of virus metastasis from the chorio-allantoic membrane, particularly as regards the lesions noted, is not known since histological examinations of the embryo were not carried out. Because of apparent failure of the

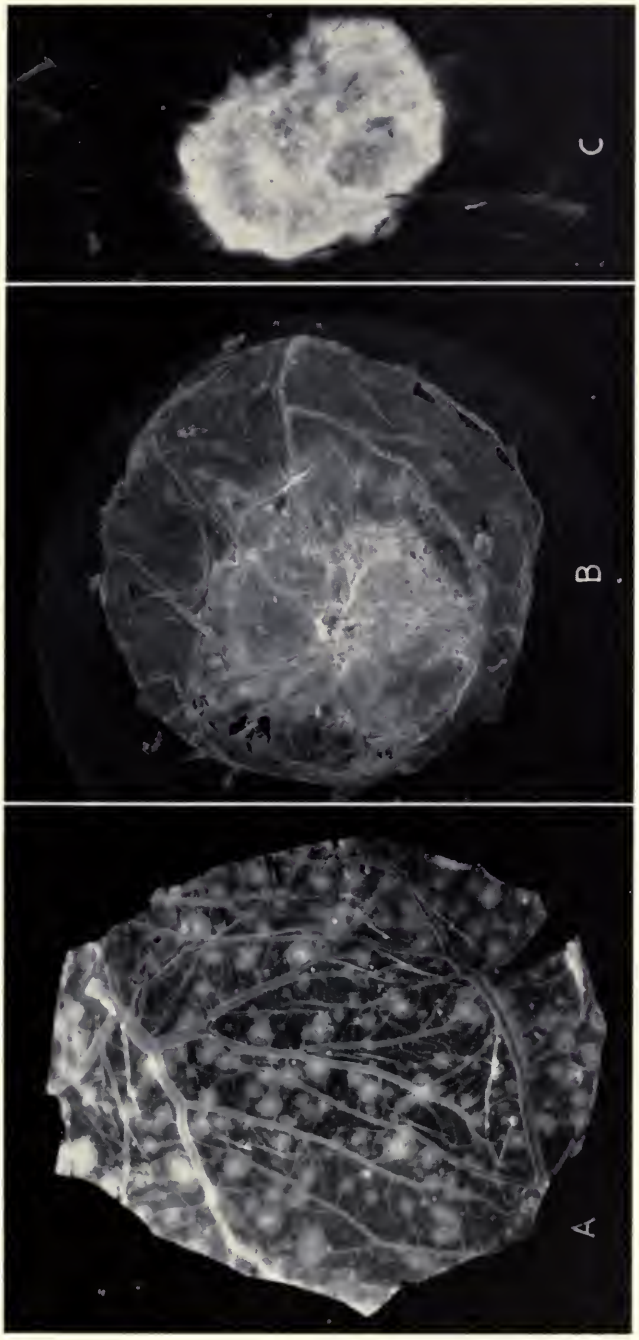


FIG. 5.—FOWL- and PIGEON-POX LESIONS ON CHORIO-ALLANTOIC MEMBRANE
(A) Metastic fowl-pox lesions. (B) Diffuse fowl-pox lesions. (C) Focal pigeon-pox lesion.

pigeon virus to spread from the point of inoculation, embryo mortality could not be attributed to pigeon virus even in eggs inoculated as early as the 9th day of incubation.

**Lesions of the chorio-allantoic membrane.** Definite gross lesions of the chorio-allantois were visible as early as 48 hours after inoculation with the virus. The nature of the lesions induced in developing chicken eggs by fowl-pox and by pigeon-pox viruses differed considerably among the strains studied. Grossly, the pigeon-virus lesions were typically pale yellow to white, with a nacre or pearl tint, whereas the fowl-virus infected membranes were usually reddish gray and quite heavily congested. With both viruses varying quantities of free clear fluid transudate might lie upon the ectodermal surface. Individual pocks, which may be considered the product of infection and colonization from one virus particle or elementary body, were globular in form in the case of pigeon virus, compared with the somewhat thinner and relatively flat fowl pocks. Heavy infection with pigeon virus on the free or covered chorio-allantoic membrane was seen to induce a coalescent lesion with a marked thickening and elevation, sometimes virtually hemispherical in form and 10 to 20 mm. or more in diameter. The entodermal aspect of the involved membrane was usually flat or indented, altho occasionally it was slightly convex. Extensive primary involvement with the strains of fowl virus usually produced at the most only a slight thickening. However, in the case of some eggs inoculated with newly isolated strains of fowl-pox virus, infected membranes 5 to 6 mm. in thickness were encountered. These usually showed a marked cellular proliferation and swelling and a limited degree of necrosis.

Other distinctions were readily apparent in the tendency of the pigeon virus to remain localized over the area inoculated (Fig. 5, C), whereas the fowl-pox infection tended to metastasize rapidly (Fig. 5, A). Macroscopic pigeon-virus lesions were not observed in the membrane of the small pole except when the inoculum had been introduced deeply beneath the chorio-allantois, that is, into the allantoic or amniotic sacs. With the pigeon viruses the tendency to remain quite sharply localized at the point of inoculation on the chorio-allantois was strikingly shown by tests for distribution of the virus within the infected membranes (Table 1). In direct contrast was the ready metastasis observed with strains of the fowl-pox virus. Eggs inoculated on the 12th day with fowl or with pigeon virus were incubated for an additional period of 4 to 5 days. The typical relatively thick lesions developing in the inoculated area of the chorio-allantois were excised aseptically, rinsed in sterile physiological salt solution and immediately ground

TABLE 1.—DISTRIBUTION OF FOWL- AND PIGEON-POX VIRUSES IN THE EMBRYO AND IN CHORIO-ALLANTOIC MEMBRANES OF INOCULATED CHICKEN EGGS

| Source of virus | Tissues tested | Titre (via feather follicles) |
|---|---|---|
| Fowl—$F_1$ (E2069-70-71) | Chorio-allantoic membrane with primary focal lesions | 1–1,000,000[a] |
| | Chorio-allantoic membrane without primary focal lesions | 1–1,000,000[a] |
| | Embryo (entire) | 1–1,000 |
| Pigeon—$P_3$ (E2076-79-80) | Chorio-allantoic membrane with primary focal lesions | 1–1,000 |
| | Chorio-allantoic membrane without primary focal lesions | 1–10 |
| | Embryo (entire) | 0 |

[a]Highest dilution tested.

for testing or dried for storage. The balance of the chorio-allantoic membrane was handled similarly as a separate portion. The yolk sac was detached and discarded, and the entire embryo was also washed in sterile physiological salt solution and prepared separately for testing. Three eggs were used for each virus. The fowl strain ($F_1$) had previously been passed thru 86 series of eggs, the pigeon strain ($P_3$) thru 21 series. The three lots of tissues for each virus were finely ground and suspended in nutrient broth to make decimal dilutions of

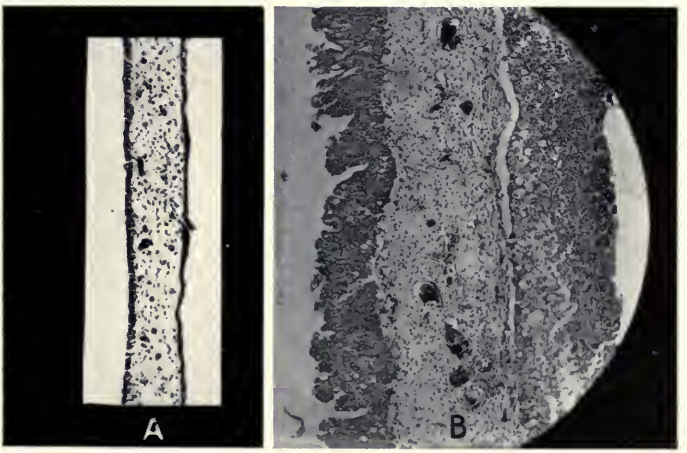


FIG. 6.—DIFFERENCE BETWEEN CHORIO-ALLANTOIC MEMBRANE OF A NORMAL CHICKEN EMBRYO AND A FOWL-POX-INFECTED EMBRYO

(A) Chorio-allantoic membrane of a normal chicken embryo 12 days old (*magnification* × *130*). (B) Chorio-allantoic membrane of a fowl-pox-infected chicken embryo 16 days old after inoculation at 12 days old (*magnification* × *100*).

the moist tissues by weight from 1-10 to 1-1,000,000. Each dilution was applied to 6 to 8 open feather follicles on the skin of the thigh of pox-susceptible chickens 5 to 6 weeks old. Two dilutions were tested on a single bird (1-10 on the left, for example, and 1-100 on the right), and each dilution was used on two birds. Observations for focal lesions were made 4, 6, 8, 10, and 12 days following application of the suspensions.

Both fowl- and pigeon-pox viruses produced microscopic retrograde changes in all three germ layers of the chorio-allantois (Figs. 6, A and B, and Fig. 7, A). Fowl-pox strains produced more severe alterations in the ectodermal and entodermal layers, while with the pigeon virus the reactions were more marked in the mesoderm (cellular infiltration and edema). The development of cytoplasmic inclusions (Bollinger bodies) produced by fowl strains appeared to be associated with necrosis of the limiting cellular layers. As a rule these inclusions in

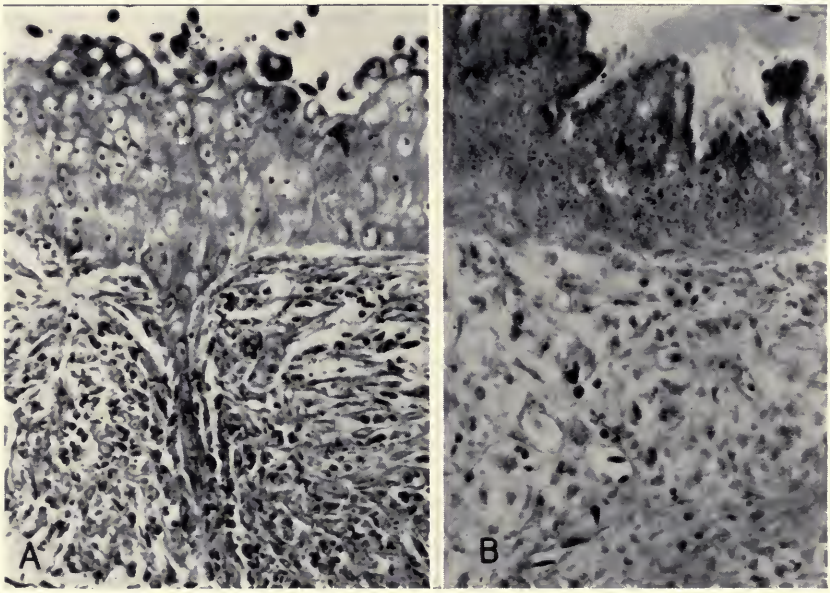


FIG. 7.—CROSS-SECTIONS OF CHORIO-ALLANTOIC MEMBRANE INFECTED WITH PIGEON POX AND WITH "ANTIDIPHTHERIN" IN CHICKEN EMBRYOS 16 DAYS OLD AFTER INOCULATION AT 12 DAYS OLD

(*Magnification* × *230*)

(A) Pigeon-pox-infected membrane. Note fibroblasts and edema in the mesoderm. (B) "Antidiphtherin" lesions. Note mesodermal involvement resembling that of the pigeon-pox-infected membrane.

the ectoderm and entoderm were larger and more readily demonstrated than those occurring in pigeon-virus lesions. Entodermal involvement by pigeon virus was invariably slight.

The gross and microscopic appearance of "antidiphtherin" lesions of the chorio-allantois resembled quite closely those produced by pigeon virus (Fig. 7, B). However, a lesser degree of edema and thickening of the involved membrane and a somewhat greater degree of ectodermal separation were noted with the "antidiphtherin" virus. This virus, like the pigeon strains, lacked the metastatic character of the fowl strains of pox virus. These observations are of interest in connection with the statements of Baumann (1926), De Blieck (1927), and others regarding the nature of "antidiphtherin."

## Egg-Propagated Fowl- and Pigeon-Pox Viruses in Immunization Studies

The use of egg-propagated fowl-pox virus for immunization against fowl pox was first reported by the writer in 1936. This work was prompted in part by the encouraging reports from the use of egg-propagated vaccinia virus in vaccination against smallpox in man and by the successful utilization of egg-cultivated laryngotracheitis virus for vaccination of fowl (Brandly, 1935). In the first trials fresh fowl-pox-infected chorio-allantoic membrane virus (3d to 8th egg passage of a recently isolated strain) was compared with fresh dried comb-lesion virus for capacity to produce takes and immunity in chickens ranging in age from 4 to 20 weeks. The results obtained indicated that the egg virus was just as satisfactory as, if not superior to, the comb virus. In later observations (Brandly, 1937) it was concluded that 20 successive serial passages on eggs did not change the virus perceptibly in so far as infectivity for the skin and the appearance of skin lesions of chickens were concerned. In subsequent laboratory and field work (Brandly and Dunlap, 1939) it was observed that continued passage thru 68 series of eggs during a period of two years had not induced marked alterations in the capacity of the original fowl strain to infect chickens. It was noted, however, that with continued egg passage there appeared to be an increase in necrotic and metastatic activity in the chorio-allantois and an earlier and higher embryo mortality. The antigenic capacity was retained and the virus content of the infected egg membranes was invariably higher than that of the skin-lesion or tissue-cultured material titrated with it. Table 2 shows the comparative ability of the egg- and skin-grown viruses to produce takes.

TABLE 2.—COMPARATIVE ABILITY OF EGG- AND SKIN-GROWN FOWL VIRUS TO PRODUCE VACCINATION TAKES WHEN APPLIED TO REPRESENTATIVE FLOCKS OF CHICKENS (1936)

| Flock | Age at vaccination | Source of virus | Number vaccinated | Number showing takes | Takes |
|---|---|---|---|---|---|
| | | | | | *perct.* |
| 1 | 8-14 weeks | Egg | 225 | 219 | 97.3 |
| | | Skin | 158 | 138 | 87.4 |
| | 1-3 years (hens) | Egg | 156 | 136 | 90.0[a] |
| | | Skin | 68 | 38 | 56.7[a] |
| 2 | 4 months | Egg | 305 | 302 | 99.3 |
| | 4 months | Comb[b] | 104 | 84 | 80.7 |
| | 3 months | Egg | 211 | 206 | 97.6 |
| | 3 months | Comb[b] | 130 | 118 | 90.8 |
| 3 | 3-4 months | Egg | 2 516 | 2 316 | 92.0 |
| | | Skin | 2 048 | 1 874 | 91.5 |
| 4 | 7-10 weeks | Egg | 108 | 105 | 94.3 |
| | | Skin | 108 | 101 | 93.5 |
| 5 | 9 weeks | Egg | 150 | 146 | 98.2[a] |
| | | Skin | 107 | 102 | 97.1[a] |
| 6 | 3 months | Egg | 289 | 288 | 100.0[a] |
| | | Skin | 304 | 301 | 99.0 |
| 7 | 8-14 weeks | Egg | 2 895 | 2 873 | 99.2 |
| | | Skin | 1 990 | 1 906 | 95.8 |

[a]Exclusive of mortality during first week. Results otherwise calculated on basis of number vaccinated.
[b]Commercial fowl-pox vaccine was used.

As determined by reactions following dermal vaccination (constitutional disturbance, secondary pocks, and mortality), the egg-grown virus was similar in effect to the tissue-cultured and skin-lesion virus.

In further laboratory experiments (Brandly and Dunlap, 1939) the bacteria-free viruses of egg-cultivated fowl and pigeon pox, when given intramuscularly in relatively massive quantities, showed more antigenicity than comparable dosages of the skin-lesion viruses, because they induced somewhat milder responses. Comparative titrations of potency of pigeon virus also revealed a higher concentration in the egg-cultivated virus than in the tissue-cultured and skin-lesion viruses. Since it is essential that the pigeon virus be highly potent for vaccination of chickens if satisfactory takes and a maximum degree of immunity are to be secured, it is obvious that the egg-grown virus would be superior for this purpose. The pigeon virus, as was true of fowl strains, is apparently not altered in antigenic function after successive passage thru chicken eggs. Strain $P_1$ went thru 60 passages, Strain $P_3$ thru 25 passages.

**Double vaccination.** The control of fowl pox would be definitely expedited if double or multiple vaccination of chickens with pigeon and fowl viruses would reduce or eliminate the shortcomings of either used alone and still give a degree of immunity comparable to that obtainable with the fowl virus.

The literature reveals numerous observations on duration of immunity following infection with active fowl- and pigeon-pox viruses, but few references deal with the time required for appearance of immune reactions and with double vaccination with these viruses. Henseval and Convent (1910) reported that by inoculating a fresh area of skin daily a certain degree of immunity to pox was demonstrable by the 4th day. Findlay (1928) confirmed this observation with both chickens and pigeons. He found a certain degree of immunity present at 4 days, but the immunization was not adjudged complete until the 20th day. Doyle and Minnett (1927) observed that a strong concentration of fowl-pox virus applied to one side of the comb decreased the incubation period of a weak virus applied to the other side. Komarov and Kligler (1936) employed skin-lesion fowl and pigeon viruses for cutaneous vaccination of chickens and found no advantage in using a double (mixed) vaccine or a double method of vaccination.

In a series of experiments at this Station the egg-propagated pigeon-pox virus was used for preliminary cutaneous vaccination in order to ascertain how it would influence the reaction to subsequent exposure to fowl-pox virus.

In Experiment 1, 82 White Rock chickens 5 to 6 weeks old were employed. Fifty-two of these birds, comprizing Lot 1, were vaccinated with a 5-percent suspension of fresh finely ground pigeon-pox-infected chorio-allantoic membranes in broth. This virus represented the 21st egg passage of Strain $P_1$. The virus suspension was applied with a small bristle brush to a scarified area about 2 cm. square on the feather-free skin of the right anterior pectoral region and also to 5 to 10 open follicles of the feather tract immediately anterior. On the day the birds were vaccinated with pigeon virus and at each of 12 subsequent two-day intervals, 4 birds were exposed to fowl-pox virus in the form of a 1-percent suspension of fresh, infected chorio-allantoic membranes in broth. The fowl virus represented the 34th egg passage of Strain $FP_3$. Two birds of each group were inoculated intravenously with .1 cc. of the suspension and 2 were inoculated on the left shoulder by 4 punctures of the skin with a 20- or 22-gage hypodermic needle. Lot 2 was made up of 26 birds divided into seven groups and inoculated with fowl virus by skin sticks or intravenously, as was done with Lot 1. A group from Lot 2 was inoculated at the beginning of the experiment and others at subsequent 4-day intervals. Four untreated birds comprized a control pen (Lot 3). With one exception the pairs of males and females comprizing each group were of similar weight. Each group was held in a separate wire cage.

TABLE 3.—RESULTS OF DOUBLE VACCINATION WITH PIGEON- AND FOWL-POX VIRUSES AND OF SINGLE VACCINATION WITH FOWL-POX VIRUS IN YOUNG WHITE ROCK CHICKENS (EXPERIMENT 1)

| Lot | Number of chickens | Vaccination treatment | Average time from vaccination to appearance of fowl pox | Average time from appearance of fowl pox to recovery | Cases of generalized fowl pox | Average individual weight gains, 30 days | Number of deaths, 30 days |
|---|---|---|---|---|---|---|---|
| | | | *days* | *days* | | *gm.* | |
| 1..... | 52 | Double (pigeon and fowl pox) | 4.7 | 15.6 | 20 | 138 | 3 |
| 2..... | 26 | Single (fowl pox) | 6.8 | 25.9 | 17 | 126 | 2 |
| 3..... | 4 | None | ... | .... | .. | 210 | 0 |

Observations were made on the time of appearance and disappearance of the focal lesions, both of pigeon and fowl pox, as well as on the occurrence of generalized pox and of mortality. Group weights were taken on alternate days for 30 days following exposure to fowl pox. All 52 birds of Lot 1, which were treated with pigeon virus, gave a well-marked positive reaction at the site of inoculation.

Significant differences in average individual weight gains failed to develop between the chickens given double vaccination (Lot 1) and those treated with fowl virus only (Lot 2), altho both lots showed appreciably lower gains than the unvaccinated birds of Lot 3 (Table 3). Evidence that preliminary treatment with pigeon virus had induced a degree of protection to subsequent exposure to fowl virus was shown

TABLE 4.—EFFECT OF THE INTRAVENOUS AND CUTANEOUS ROUTES OF INOCULATION WITH FOWL-POX VIRUS UPON THE REACTION OF YOUNG WHITE ROCK CHICKENS IN A SINGLE AND IN A DOUBLE VACCINATION TREATMENT (EXPERIMENT 1)

| Lot | Vaccination treatment | Route of first inoculation | Route of second inoculation | Average time from vaccination to appearance of fowl pox | Cases of generalized fowl pox | Number immune among those alive 2 months later |
|---|---|---|---|---|---|---|
| | | | | *days* | | |
| 1A..... | Double (pigeon and fowl pox) | All cutaneous | 8 intravenous<br>8 cutaneous | 6.0<br>... | 8<br>3 | All of 5<br>5 of 6 |
| 1B..... | Double (pigeon and fowl pox) | All cutaneous | 18 intravenous<br>18 cutaneous | 4.2<br>... | 8<br>1 | All of 12<br>13 of 17 |
| 2..... | Single (fowl pox) | 13 intravenous<br>13 cutaneous | .....<br>..... | 6.8<br>... | 13<br>4 | All of 7<br>All of 13 |
| 3..... | None | ..... | ..... | ... | .. | 1 of 3 |

by the more rapid development of focal pox lesions or takes in Lot 1, probably because of an enhanced capacity for local fixation of pigeon virus. A significant reduction in the average individual recovery time (period from appearance to healing of pocks) of surviving birds of the first lot, compared with the second, may also be taken as evidence of an acquired resistance adequate to somewhat abort the reaction to fowl-pox virus. Still greater differences between the first two lots are shown by excluding from Lot 1 the four groups of birds vaccinated with fowl virus during the 6 days following administration of pigeon virus (Lot 1A, Table 4). Not until the birds were tested 8 days after exposure to the pigeon virus was there apparent a definitely altered response to the fowl virus. In the four groups of Lot 1A the average time for appearance of lesions after fowl-virus inoculation was significantly greater than in the nine groups (Lot 1B) exposed to the fowl virus 8 to 24 days after pigeon-virus vaccination.

Tests for immunity of all surviving chickens in Lots 1A and 1B two months after the beginning of the experiment showed that when enormous doses of fowl virus were given intravenously, the primary pigeon-virus vaccination produced immunity within a very short time (Table 4). The only birds in the lot not completely immune were among the skin-treated series. This would suggest that revaccination by the cutaneous route employing fowl virus may fail to augment the immunity induced by the first cutaneous vaccination with pigeon virus.

Experiment 2 (Tables 5 and 6) was set up in duplication of Experiment 1. The pigeon virus used represented the 31st egg passage of Strain $P_1$; the fowl virus was the 69th egg-passage virus of Strain $F_1$ admixed with the 19th passage virus of Strain $F_4$. Another control lot with single pigeon-pox vaccination (Lot 4) was included to check on the reaction to fowl-pox exposure after two months. The results of this study closely parallel those of Experiment 1. The heavier mortality in Experiment 2 was probably associated with an outbreak of cecal coccidiosis, but the protective response from the primary pigeon-virus inoculation, as determined by reduced incubation and recovery time following fowl-pox exposure, was not so marked as in Experiment 1. In Experiment 2 the average time from vaccination to appearance of fowl pox was 4.5 days for Lot 1, which was given a primary pigeon-pox inoculation, and 4.7 days for Lot 2, which received no prior inoculation treatment. In Experiment 1 the average time for incubation was 4.7 days for Lot 1 and 6.8 days for Lot 2. In Experiment 2 the average recovery time for the lot given the primary pigeon-pox inoculation was only 6.1 days less than for the lot given the single

TABLE 5.—RESULTS OF DOUBLE VACCINATION WITH PIGEON- AND FOWL-POX VIRUSES AND OF SINGLE VACCINATION WITH FOWL- OR WITH PIGEON-POX VIRUS IN YOUNG WHITE ROCK CHICKENS (EXPERIMENT 2)

| Lot | Number of chickens | Vaccination treatment | Average time from vaccination to appearance of fowl pox | Average time from appearance of fowl pox to recovery | Cases of generalized fowl pox | Average individual weight gains, 30 days | Number of deaths, 30 days |
|---|---|---|---|---|---|---|---|
| | | | *days* | *days* | | *gm.* | |
| 1..... | 52 | Double (pigeon and fowl pox) | 4.5 | 14.1 | 10 | 152 | 11 |
| 2..... | 26 | Single (fowl pox) | 4.7 | 20.2 | 13 | 166 | 11 |
| 3..... | 4 | None | ... | .... | 0 | 168 | 0 |
| 4..... | 4 | Single (pigeon pox) | ... | .... | 0 | 186 | 0 |

fowl-pox inoculation; in Experiment 1 the reduction was 10.3 days. In both experiments the least reaction to fowl pox (i.e., earlier appearance and recession of focal pocks, absence of cases of generalized fowl pox) among the 9 groups of Lot 1B, that were subjected to double vaccination, appeared in those groups given the fowl virus 10 and 12 days after the first vaccination with pigeon-pox virus.

In spite of some variation between the two experiments the observation of Experiment 1, that prior infection of the skin of chickens with pigeon virus may ameliorate a subsequent infection of this tissue with

TABLE 6.—EFFECT OF THE INTRAVENOUS AND CUTANEOUS ROUTES OF INOCULATION WITH FOWL-POX VIRUS UPON THE REACTION OF YOUNG WHITE ROCK CHICKENS IN A SINGLE AND IN A DOUBLE VACCINATION TREATMENT (EXPERIMENT 2)

| Lot | Vaccination treatment | Route of first inoculation | Route of second inoculation | Average time from vaccination to appearance of fowl pox | Cases of generalized fowl pox | Number immune among those alive 2 months later |
|---|---|---|---|---|---|---|
| | | | | *days* | | |
| 1A..... | Double (pigeon and fowl pox) | All cutaneous | 8 intravenous<br>8 cutaneous | 6.7<br>6.7 | 4<br>1 | All of 4<br>All of 5 |
| 1B..... | Double (pigeon and fowl pox) | All cutaneous | 18 intravenous<br>18 cutaneous | 4.2<br>... | 5<br>0 | All of 9<br>6 of 11 |
| 2..... | Single (fowl pox) | 13 intravenous<br>13 cutaneous | .....<br>..... | 4.7<br>... | 12<br>1 | All of 5<br>All of 7 |
| 3..... | None | ..... | ..... | ... | .. | None of 4 |
| 4..... | Single (pigeon pox) | 4 cutaneous | ..... | ... | .. | None of 4 |

fowl-pox virus, was apparently verified. It also appears, from a comparison of Lots 1A and 1B with Lot 2, that a degree of immunity comparable to that resulting from a single cutaneous vaccination with fowl pox may be secured if, after pigeon-virus vaccination, the fowl virus is introduced by the intravenous route. This situation would support the observation that the mechanism or function responsible for the acquiring of an active immunity to fowl pox is by no means limited to the skin.

The results of these experiments would suggest further study of routes for introduction of the egg-cultivated virus in the immunization of chickens against fowl pox. Double vaccination in which fowl virus is employed after a preliminary cutaneous vaccination with strains of pigeon virus, or with other strains possessing similar characters, would seem to warrant further investigation.

## SUMMARY

Reference to the literature on the embryonated avian egg discloses its value for use in the investigation of various viruses affecting birds. It is a convenient and economical medium with a measure of protection against cross-infection. It is also pointed out in the literature that propagating fowl- and pigeon-pox viruses on the developing egg makes possible: (1) a pure culture; (2) detection of viruses even in small quantities, as well as titration of viruses and virus antiserums; (3) pathologic and serologic study in differentiation of various viruses; and (4) development of virus material suitable for experimental and field immunization.

Experiments conducted at the Illinois Station over an extended period confirmed the literature summarized above. A number of strains of fowl- and pigeon-pox viruses were propagated serially on the chorio-allantoic membranes of embryonated chicken eggs without causing apparent alteration in infective and immunizing capacity of these viruses for chickens. The egg-cultivated viruses in the form of fresh, or properly preserved, infected chorio-allantoic membranes proved to be a concentrated and refined product suitable for use in the field with the current methods of vaccination. It was also found that the hazards of contamination by other viruses and by bacteria are minimized in the egg-grown virus, which may be administered by various parenteral routes. In spite of the findings of Dettwiler and Markham (1938) with a series of twenty commercial skin-lesion fowl-pox vaccines, the results obtained at this Station in this and unpublished work and the

study previously cited (Brandly and Dunlap, 1939) emphasize the possible advantages of egg-propagated virus vaccines over the skin-lesion pox-virus vaccines even when applied cutaneously.

The results obtained in comparisons of single fowl-pox vaccination and double vaccination with pigeon- and fowl-pox viruses suggest the desirability of further study of the multiple use of pigeon- and fowl-pox viruses for immunization against pox in chickens. Following preliminary vaccination with pigeon virus, the administration of fowl-pox virus by the intravenous route apparently induced a greater degree of resistance to fowl-pox exposure than did cutaneous administration.

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## ADDENDUM

Since this report was written R. E. Glover (1939), in Jour. Comp. Path. and Ther. **52**, 29, has emphasized the possibilities and advantages of egg-propagated fowl- and pigeon-pox viruses for use in immunization against the disease in both fowl and pigeons.

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