THE BEHAVIOR OF POX VIRUSES IN THE RESPIRATORY TRACT

II. The Response of Mice to the Nasal Instillation of Variola Virus

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Plates 8 and 9

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Variola virus introduced cutaneously in the monkey establishes itself in the skin where it produces a characteristic vesicular reaction and apparently retains its identity. Other animals which are highly susceptible to vaccinia, as the calf and the rabbit, are peculiarly refractory to variola. In the absence of an initial reaction it is claimed that successive passages in these animals, either cutaneously or intratesticularly, may eventually be followed by vesiculation. The virus recovered in this event, however, has invariably been vaccinia and not variola. The literature on this so called transformation of variola to vaccinia has recently been reviewed by Horgan (1) who also contributed an example of such a change following testicular passage in the rabbit. The experimental observations on variola in animals have been chiefly confined to these two loci, namely, the skin and testis. In view of the rapid multiplication which vaccinia virus can undergo when implanted on the unbroken nasal mucosa of the white mouse, it seemed of interest to inquire into the behavior of variola virus in the same location (2).

Source of the Virus

The virus used in the following experiments was isolated from crusts[1] obtained from a case of smallpox in a 13 year old boy, a resident of Minnesota.

The history of this case is as follows: Symptoms appeared on Jan. 18, 1938, malaise, headache, nausea, vomiting, and backache, and were followed on Jan. 23 by a macular eruption. Crusts were collected on about Feb. 5 and were shipped to us shortly after in 50 per cent glycerin.

[1] We are pleased to acknowledge the cooperation of Dr. A. J. Chesley, Executive Officer of the Minnesota Department of Health, and of Dr. R. B. J. Shoch, Health Officer of Saint Paul, in arranging for the collection and shipment of the smallpox material, and express our indebtedness to them.

Cultivation of the Virus in Embryonated Eggs

A virus similar in characteristics to the known strains of variola was readily obtained from the glycerinated crusts by transfer to embryonated eggs. The first isolation was made on March 23, 1938, approximately 45 days after collection of the scabs. With the exception of several weeks during the summer when it was maintained in a dried state, the virus has been transferred once or twice a week, 85 passages having been completed. Development of the virus in the egg is characterized by the appearance of focal areas of cellular proliferation and necrosis in the chorioallantoic membrane. If numerous, these areas coalesce forming a solid mass of seminecrotic tissue, sharply demarcated from the normal portions of the membrane. The embryo is not affected.

We have found only two references in the literature to the cultivation of variola virus by the egg technique: one reported by Torres and Teixeira (3) in 1935; the other by Lazarus, Eddie, and Meyer (4) in 1937. Hence it seems desirable to report our own observations in some detail.

One small crust was washed in water, finely ground, and suspended in 2 cc. of saline. 3 embryonated, 10-day, hen eggs were inoculated with small unmeasured amounts of the suspension and incubated at 37°C. The method of Burnet (5) was used, withdrawing the chorioallantoic membrane from the shell membrane and creating a new air sac thereby. Membranes removed on the 2nd and 3rd days from these eggs were normal, but on the 4th day a central cluster of 10 small white foci was observed in the membrane of the remaining egg. This portion of the membrane was removed, suspended in saline after grinding, and inoculated as before. One of the 2nd passage eggs showed focal areas of reaction in the membrane on the 3rd day.

The virus was successfully maintained thereafter in embryonated eggs by inoculation with small amounts of approximately 10 per cent membrane suspensions in saline. To date 85 passages have been made, in most of which a membrane reaction was apparent on the 3rd day after inoculation. Up to the 50th transfer the severity of the reaction fluctuated considerably in eggs inoculated with any one suspension. Thus, in 13 of the 25 passages made prior to the 50th transfer, the number of foci in the membranes of paired eggs varied from few to innumerable. From the 50th passage on the membrane reaction was more uniformly severe.

In inoculated eggs opened on the 3rd day it was customary to find a thickened and heavy membrane with a large oval area, up to 4 × 3 cm., studded with minute white nodules either discrete or coalesced into small groups. Congestion and hemorrhage were rarely conspicuous. On continued inoculation these focal areas tended to merge forming a solid plateau of seminecrotic tissue. Unless bacterial contaminants were accidentally introduced the embryo was invariably active. On two occasions, however, virus was recovered from washed and ground embryos on subinoculation. If incubation was continued the embryos developed normally, up to the point of hatching, and retrogressive changes were apparent in the involved membrane. Virus was not recovered from the membrane of one egg removed on the 10th day.

Elementary bodies were usually demonstrable in membrane films by the silver method of Morosow. They were rarely as numerous, however, as in membranes from eggs inoculated with vaccinia virus but were indistinguishable otherwise.

One titration was made using a membrane removed on the 4th day from a 15th passage egg. The membrane was weighed, ground, and diluted serially so that the concentration inoculated (0.05 cc.) varied by intervals of 10 beginning with 10^{-2}. A reaction was obtained with all dilutions through 10^{-7}; of the 2 eggs inoculated with this dilution, the membrane of one showed 6 foci on the 3rd day and the other 12. Subinoculation from these 2 membranes was attended by a moderate reaction.

Cutaneous Inoculation of the Virus in Monkeys

Two *Macacus rhesus* monkeys were inoculated into the scarified skin with suspensions of membranes from the 13th and 40th egg passages of the virus. Both animals showed a typical eruption, papules appearing on the 5th day after inoculation. After the local reaction had subsided both animals were tested for susceptibility to variola and vaccinia.

The hair over the sides was removed with an electric clipper, a criss-cross of scratches made in the skin, and the virus suspensions rubbed into the abraded areas with a glass rod. The first monkey received a single dilution spread over a wide area, approximately 0.05 cc. of a 10 per cent suspension being used. Four dilutions, 10^{-2} through 10^{-5}, were inoculated into small squares in the skin of the 2nd monkey. The volume of inoculum, 0.02 cc., was included in the dilution figure. The inoculated animals were held under strict quarantine in an isolation unit and examined daily.

The monkey inoculated with a single dilution of variola virus showed small papules along the lines of scarification on the 5th day. These nodules increased in size and number, reaching a maximum of 20 with a diameter of 5 mm. on the 12th day. At this time some of the nodules were discrete, others confluent. Most of them showed an inconspicuous dirty white tip surmounting a red base. Retrogressive changes with scab formation set in promptly and by the 20th day the area was flat. The virus was active through a dilution of 10^{-4} as indicated by the reaction in the 2nd monkey. Papules were again visible on the 5th day but reached a peak earlier, on the 8th day. At this time the 10^{-2} area showed confluent nodules; the 10^{-3} area, coalesced and discrete ones; the 10^{-4} area, 8 discrete nodules; and the 10^{-5} area, no reaction. Retrogressive changes began on the 9th day and progressed rapidly.

Six weeks after the primary inoculation both monkeys were retested with variola virus and also vaccinia virus, 10^{-2} and 10^{-3} dilutions being introduced cutaneously. There was no reaction to the variola virus in either dilution, indicative of a fairly complete immunity. The vaccinia virus, however, produced a reaction in both animals. The first monkey showed discrete papules after 3 days, on the area which received the 10^{-2} dilution. The 10^{-3} dilution was inactive. Both dilutions produced a reaction in the 2nd monkey, confluent nodules appearing on the 2nd day.

Cutaneous and Testicular Inoculation of the Virus in Rabbits

Four rabbits were inoculated cutaneously, following scarification, with 0.02 to 0.05 cc. of 10 per cent membrane suspensions prepared from the 10th, 13th (2 rabbits), and 34th

egg passages, respectively, and were kept under observation for 3 to 6 weeks. Aside from a transient erythema along the lines of scarification there was no visible reaction. During the 6th week the rabbit which had received the 34th passage suspension was inoculated cutaneously with vaccinia virus diluted 10^{-3} through 10^{-8}. All of the virus dilutions through 10^{-6} gave rise to typical vaccinial reactions, indicative of normal susceptibility.

One series of testicular passages was also carried out in rabbits. The first animal was injected intratesticularly with 0.25 cc. of a 10 per cent membrane suspension from the 34th egg passage. On the 4th day the skin of the scrotum was somewhat thickened and congested at the site of inoculation. The testis was removed on the 5th day and was normal macroscopically and microscopically. Portions of the removed testis and the thickened skin of the scrotum were ground, suspended in several cc. of saline, and 0.25 cc. injected into a second rabbit. 4 subsequent testicular passages were made at intervals of 5 to 7 days. The scrotum and testis showed no visible nor histologic indication of involvement in any rabbit save the first. The rabbits injected intratesticularly with the 3rd, 4th, and 5th passage suspensions were also inoculated cutaneously with the same material. Aside from the initial trauma there was no response. 4 weeks after inoculation the 6th rabbit was tested with vaccinia virus to which it reacted with all dilutions through 10^{-6}.

Cutaneous Inoculation of the Virus in Mice

Five mice were inoculated cutaneously with a membrane suspension from the 64th egg passage. The skin over the abdomen was shaved, scarified, and approximately 0.02 cc. of the suspension rubbed into the abraded area. There was no specific response to the inoculated virus.

Nasal Instillation of the Virus in Mice

Young mice in groups of 5 each were inoculated intranasally with the original suspension of smallpox crusts and with suspensions of the 3rd, 10th, 13th, and 34th egg membrane passages. Titration of the virus content of the several membranes was not made, but judged from the severity of the reaction in the membrane and the number of elementary bodies the concentration was high.

In these and the subsequent experiments white mice weighing 15 to 20 gm. were well etherized and their muzzles were dipped in the test suspension. By this method of administration between 0.05 and 0.1 cc. of fluid is taken up on inhalation. Unless otherwise specified approximately 10 per cent saline suspensions of ground egg membranes were used.

The disposal of the inoculated mice varied from group to group: some were autopsied on the 2nd to the 10th day after inoculation; others were held under observation for a month or longer and injected intranasally with vaccinia virus.

None of the mice injected with variola virus showed either a local or a general response. They gained weight normally and retained their original sleek appearance, quite in contrast to the appearance of mice similarly injected with vaccinia. The nasal passages

were invariably normal at autopsy and elementary bodies were not demonstrable in films. The lungs showed no obvious pathological changes on macroscopic examination. There was some indication that the mice held for a month and then injected with vaccinia were less susceptible than normal individuals, but the observations were too few to be conclusive.

Additional nasal injections were not made in mice until the virus had been carried through 64 egg passages. At this time mice were injected with membrane suspensions from 3 consecutive transfers and killed at daily intervals thereafter to determine whether virus was present in the blood. 2 to 3 mice were bled from the heart under ether on the 1st through the 5th day after nasal instillation of the virus and approximately 0.05 cc. of a 1:10 dilution of blood in saline solution inoculated into 2 embryonated eggs. There was no indication that variola virus was present in the blood in amounts detectable by the egg technique during this period. With the exception of 2 contaminated eggs the membranes were invariably normal when removed on the 3rd or 4th day after inoculation.

While this work was in progress the lungs of 3 mice, killed on the 4th day, were examined by lower power microscopy, using a binocular dissecting microscope which magnified 7 diameters. We had found somewhat earlier in studying a disease of guinea pigs that low power magnification brought out pathological changes in the lung which were not clearly defined by visual inspection. Each specimen showed small but unmistakable areas of consolidation in one or more lobes. A suspension was made from one involved lobe and inoculated into an embryonated egg which was opened on the 3rd day. The embryo was active but the membrane was studded with a fair number of discrete foci.

Additional lung cultures were made from 48 mice injected intranasally with membrane suspensions from the 65th through the 82nd egg passages of the virus and killed at varying intervals through the 7th day.

The first isolations were made from etherized mice killed by decapitation. The lungs of mice killed in this way often showed congestion and hemorrhage which interfered seriously with microscopic examination. Later, the mice were deeply etherized and the lungs removed aseptically with practically no attendant trauma. The separated lobes, in a sterile Petri dish, were examined microscopically under a dissecting microscope at a magnification of 7 diameters. With mice killed on the 1st through the 5th day a suspension was made from one lobe, usually the left, by grinding in the presence of 1 to 2 cc. of saline. All 5 lobes were used in the preparation of lung suspensions from mice killed on the 7th day. Small unmeasured amounts of each suspension were inoculated into 3, 10-day embryonated eggs which were incubated at 37°C. and opened on the 3rd or 4th day.

The results of these examinations are summarized in Table I. 36 of the 48 mice were killed on the 1st through the 5th day after injection. Variola virus was demonstrable in the egg cultures from all of these mice. Three

isolations of the virus were made from the 12 animals that were killed on the 7th day.

Comparatively few bacterial contaminations were encountered in the inoculated eggs even when the entire lung was used. In no case was the embryo affected in the absence of bacteria. The reaction in the membranes of eggs inoculated with suspensions of lungs removed on the 1st through the 5th day varied appreciably from group to group, according to the passage, and also within the different groups. The reaction was more commonly like that in eggs inoculated with a moderate concentration of the virus, characterized by a central oval area studded with discrete foci or small groups of coalesced ones. Short lines or streaks as illustrated in Fig. 4 were frequently seen. Elementary bodies were usually demonstrable in films. In some membranes, however, the reaction

TABLE I

The Examination of Lungs from Mice Injected Intranasally with Variola Virus

Time between injection and examination	Number of mice examined	Number of virus isolations from the lung	Number of lungs with pathological changes
days			
1	11	11	4
4	11	11	8
5	14	14	13
7	12	3	12

was limited to widely separated discrete foci, characteristic of a very low concentration of virus. Sparsely distributed foci, rarely more than 10 in number, were also present in the membranes of the 3 groups of eggs inoculated with 7-day lung suspensions.

Two virus titrations were made in embryonated eggs using whole lung removed 1 hour after nasal instillation and again after 4 days. The end-point was the same for both time intervals in each determination, the limiting dilution being 10^{-5}.

The mice in the above experiment were injected with the 72nd and the 77th egg passages of the virus, respectively. The lungs were weighed immediately after removal, ground, and 20 per cent saline suspensions made. Serial dilutions varying by intervals of 10 were set up and 2, 10-day eggs inoculated with 0.5 cc. of each dilution, the final concentrations ranging from 10^{-3} through 10^{-7}. The inoculated eggs were opened on the 3rd day.

To determine whether any change was detectable in the activity of the virus as the result of its residence in the lung of the mouse, a membrane, showing some 50 foci after inoculation from a lung removed on the 5th day, was carried through 2 successive passages in eggs and then injected cutaneously in a rabbit. In the egg there was no departure from the reaction

characteristic of the long continued passage series. A large area of confluent foci was present in each membrane on the 4th day with no involvement of the embryo. A 10 per cent suspension of the 2nd passage membrane on cutaneous inoculation in a rabbit produced no reaction aside from a transient reddening along the lines of scarification.

The reaction observed by low power microscopy in the lungs of the mice injected with variola virus was progressive through the 7th day in respect to the amount of tissue involved as well as the number of animals affected. Pathological changes were observed as early as 24 hours after injection, but only in 4 of the 11 mice examined. 8 of the 11 animals killed on the 4th day, 13 of the 14 killed on the 5th day, and all of the 12 killed on the 7th day showed pulmonary abnormalities.

The normal lung at a magnification of 7 diameters resembles a confined suspension of tiny, closely packed droplets, white or faintly tinged with pink. Injected mice show patchy translucent areas of consolidation which are sharply demarcated from the normal tissue. At first these are small and colorless or grayish, blending perfectly with the involved portions of the lung on visual examination, and commonly found at the attachment of the lobes. Later they increase in size and number, coming to occupy a considerable portion of the lung and giving it a piebald appearance. By the 5th to the 7th day the consolidated areas may be congested and pink in color, and if extensive barely detectable visually. Unmagnified, however, the change in the appearance of the lung is slight and very different from the frank reaction characteristic of vaccinial pneumonia and the native pneumonias of the mouse. As already noted the lungs were sometimes normal in appearance even though virus was present. At the height of the reaction, on the 7th day, virus was no longer recoverable from most of the animals. There was no indication that the lung reaction was referable to the foreign cells or proteins of the egg membrane, the lungs of mice injected with membranes in the absence of virus being normal microscopically.

Histologically the reaction in the lung was characterized chiefly by an interstitial congestion and an infiltration of lymphocytes and mononuclear cells both as cuffs around bronchioles and as solid accumulations or islands, often adjacent to the pleural surface. In general there was little involvement of the alveoli, although in some sections groups of air sacs were filled with erythrocytes or fluid with mononuclear cells. These changes were most noticeable in lungs removed on the 4th through the 7th day. In sections from lungs removed about the 4th day there was sometimes an indication of a proliferation of the bronchial epithelium, solid plugs of necrotic cells being present in the lumen. Polynuclear leucocytes were rarely present at any time and inclusion bodies were never demonstrable.

DISCUSSION

The activity of the virus isolated by us from a human skin eruption diagnosed as smallpox is similar to that of variola strains studied by others. In embryonated eggs it behaves like the virus propagated by Lazarus,

Eddie, and Meyer (4). They commented on the inconstancy of the lesions produced in the membrane by their strain which was carried through 45 egg passages. A similar variability was noted with the present virus up to the 50th passage. The inactivity of the virus on the skin of the rabbit on primary injection is in agreement with the observations of Gordon (6), Horgan (1), and others. There was no indication, however, of a transformation to vaccinia virus on passage through the testis of the rabbit, but that variola virus behaves in this way is still uncertain. The cutaneous eruption which the virus produces in the monkey is essentially like that of the alastrim strains reported by Gordon (6) but apparently less severe than the reaction of his virus from confluent smallpox. The outcome of the protection tests in the monkey agrees with Gordon's (6) finding that recovery from variola affords complete protection against the homologous virus but only partial protection against vaccinia. We believe that these characteristics are sufficient to establish the virus as variola and hence designate it as such.

The present strain of variola virus was also inert in the skin of the mouse and on nasal instillation was much less active than vaccinia virus. Unlike the latter it provoked no symptoms and regularly failed to become established on the nasal mucosa. Beginning with the 64th egg passage it was found that the virus was recoverable from the lung through the 5th day after introduction by the nasal route, and that its residence in the lung coincided with the appearance of well marked pathological changes. These changes were progressive, reaching their height on the 7th day at which time the incidence of recovery of the virus was low.

Whether or not the virus actually multiplied in the lung during its period of residence cannot be said from the present evidence. The results of two titrations indicate that there was no increase in titer beyond that originally introduced. The limiting dilution on subinoculation in embryonated eggs was 10^{-5}, 1 hour after injection, indicative of the amount actually drawn into the lung, and was still 10^{-5} on the 4th day. That the virus did multiply to a limited extent seems a reasonable assumption in view of the stationary titer, particularly since this was maintained under adverse conditions, but there is no actual proof. Whether the maintenance of the virus in the lung is due in part to multiplication or solely to survival, its establishment there is accomplished only when the initial concentration is high.

It is also uncertain whether this short maintenance of the virus in the lung was characteristic of it from the beginning or was a property acquired by adaptation in the egg as the result of long continued passage. Adaptive changes referable to egg cultivation are commonly retrogressive at least in respect to virulence. It is true, however, that the virus did undergo a

change on continued passage as indicated by the more uniform reaction in the membrane after the 50th transfer. Whatever the nature of this change, it was in no sense a transformation. The virus is still basically the same after 85 passages in embryonated eggs and is quite unlike vaccinia virus. There is no indication either that the nature of the virus was altered by its residence in the lung of the mouse. It is planned to continue the egg passages and mouse transfers to determine whether its present identity will be preserved.

SUMMARY

Variola virus was cultivated in embryonated eggs from smallpox crusts and maintained through 85 passages. Therein it produced foci of cellular proliferation and necrosis on the chorioallantoic membrane but did not affect the embryo.

The virus from egg cultures was inactive in the skin of the rabbit on primary injection and in the testis both initially and on passage. In the monkey it provoked a cutaneous eruption of short duration after an incubation period of 5 days.

On nasal instillation in the mouse the virus caused no symptoms and failed to survive on the mucous membrane of the upper air passages. Beginning with the 64th egg passage it was regularly recoverable from the lung, on subinoculation in eggs, through the 5th day and occasionally through the 7th day. Its presence in the lung was attended by progressive pathological changes.

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EXPLANATION OF PLATES

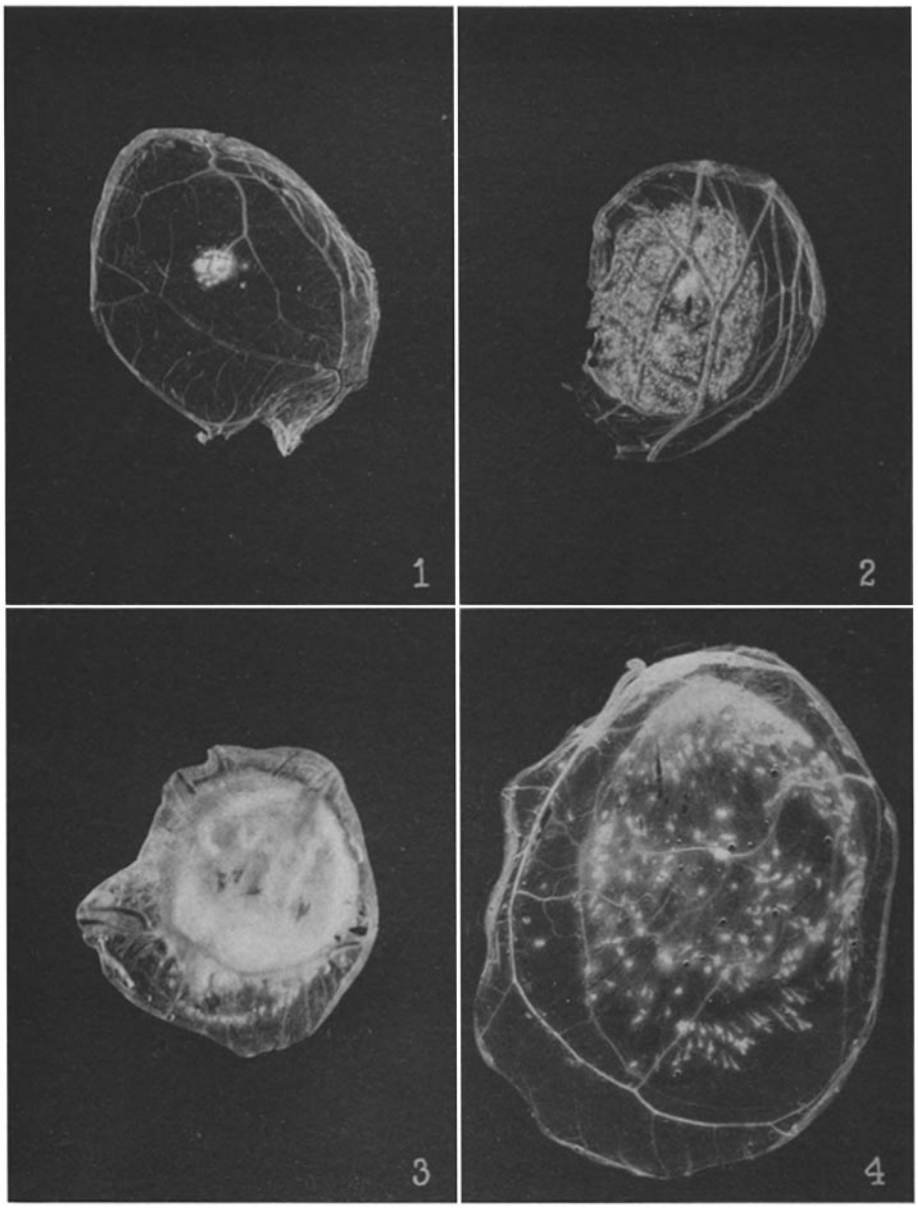
PLATE 8

FIG. 1. Small cluster of foci in membrane from egg inoculated with variola virus, type of reaction often encountered in early passages.

FIG. 2. Discrete foci in membrane removed on the 3rd day after inoculation.

FIG. 3. Coalesced foci in membrane removed on the 4th day after inoculation.

FIG. 4. Streaky foci in membrane from an egg inoculated with a 4-day lung suspension. All of these membranes are approximately normal size.



Photographed by J. A. Carlile

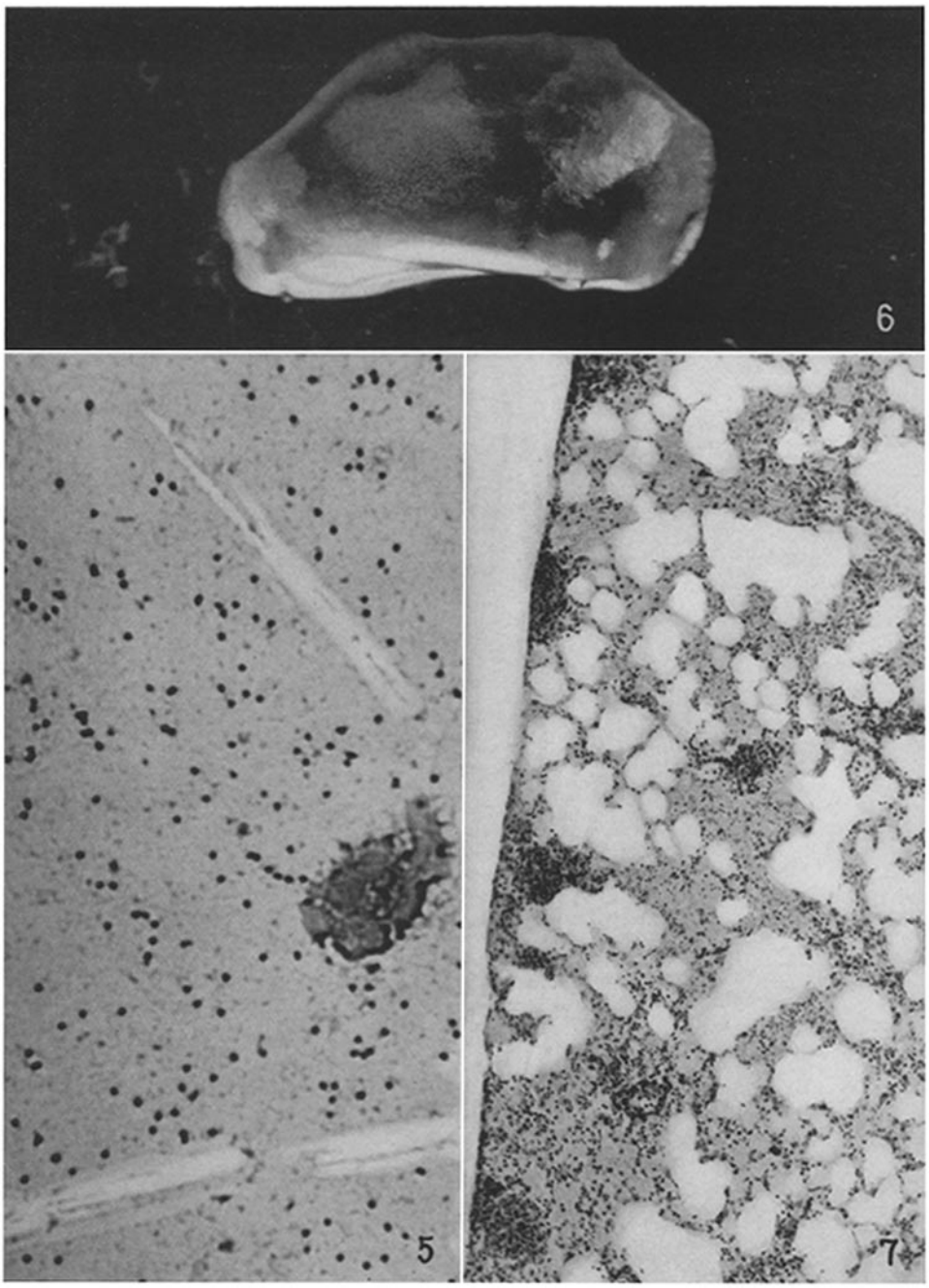
(Nelson: Pox viruses in respiratory tract. II)

PLATE 9

FIG. 5. Elementary bodies in membrane removed on the 3rd day after inoculation. Morosow preparation. × 2100.

FIG. 6. Left lobe of lung removed on the 5th day from a mouse inoculated with variola virus. × 6.

FIG. 7. Section of lung removed on the 7th day from an inoculated mouse. Phloxin-methylene blue stain. × 120.



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