

LYMPH NODES AS A SOURCE OF NEUTRALIZING PRINCIPLE FOR VACCINIA

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Previous papers from this laboratory have shown that every intradermal injection is truly intralymphatic (1-3). Dyes introduced in this way enter the superficial plexus of lymphatics through channels torn by the needle; and particulate substances which are punctured, scratched or even "vaccinated" into the skin pass in some part directly into these channels and are carried to the regional nodes (4, 5). Torn lymphatic capillaries remain open for hours (5), whence it follows that infectious agents introduced by way of a skin wound may reach the lymphatic glands directly. All this being the case, the possibility suggests itself that lymph nodes may do more in defense of the body than act as barriers in the way of invading organisms. Their activity in the latter relation is attested by their secondary involvement in infections of the skin and mucous membranes. Recent work has demonstrated the formation of specific bacterial agglutinins in lymph nodes draining the ears of mice into which killed cultures of bacteria had been injected intradermally (4). Experiments have now been carried out to determine whether the lymph nodes of rabbits elaborate a neutralizing principle effective against vaccine virus draining to them.

Methods and Preliminary Experiments

The lymphatics of the rabbit's ear converge into one or two trunks which drain into a large lymph node in the region of the parotid gland and into a group of 2 or 3 nodes situated at the junction of the internal and external jugular veins. India ink and vital dyes can be injected intradermally in the outer fourth of the rabbit's ear in such a way that several lymphatics are invariably torn by the needle, with the consequence that some part of the injected material drains to these nodes. Furthermore, it seems probable that much of the dye lying intersti-

tially after such an injection, later passes along the same channels (4). Dissections of the head and neck of 4 rabbits that had been injected into the ear with 0.05 to 0.4 cc. of dye intradermally showed that the colored fluid always reached the nodes in a few minutes. On the basis of this knowledge an experiment was carried out as follows:

A suspension of vaccine virus, prepared as described below, was faintly colored by adding to each 50 cc., 0.3 cc. of an isotonic 11 per cent aqueous solution of an innocuous vital dye, patent blue V¹ (1, 2). 0.1 cc. of the colored suspension was injected intradermally in the outer fourth of the left ears of 3 rabbits. Almost at once the lymphatics at the base of the ear became visible because of a content of stained fluid. 2 hours later, to prevent further possible drainage to the nodes of the injected side, the ear on this side was amputated under ether, and so too was its fellow. One of the animals was killed at once, the others after 24 and 48 hours. Dissection showed the draining lymphatics in the neck and the parotid and cervical lymph nodes to be very faintly colored with dye in each instance. The left cervical lymph nodes of the two animals killed last were enlarged 4-fold, pale blue and hemorrhagic, whereas the right parotid and cervical nodes appeared normal. All were removed with precautions for asepsis, extracted by a method to be detailed shortly, and the extracts injected into the shaved skin of normal rabbits. In each instance extracts of the nodes from the side injected with vaccine virus yielded typical vaccinia lesions, whereas those from the uninjected side gave none.

Having determined in this way that vaccinia entering the peripheral lymphatics can be demonstrated in the regional lymph nodes 48 hours later, we next sought evidence of its fate within the glands. Does it increase or decrease in the nodes with the passage of time? Do antiviral principles appear in the nodes? And if so, are these formed within them?

To answer some of these questions experiments were begun involving the injection of suspensions of vaccine virus and its recovery from various tissues or body fluids as well as its subsequent titration. The techniques used will be described before detailing the experiments.

Preparation of Vaccine Virus Suspensions.—Vaccine virus of the New York City Board of Health strain was provided² as a 28th subculture with chick embryo tissue and Tyrode's solution, cultivated *in vitro*. The pathogenicity of the strain had been revived twice by inoculation into susceptible rabbits (6) and it was propa-

¹ General Dyestuff Corporation.

² This virus was kindly provided by Dr. T. M. Rivers.

gated further by us in the testicles of rabbits. For the purposes of this work the testicles of one rabbit, 3 days after inoculation, were minced with scissors, well mixed and passed through a Latapie grinder. 1 gm. portions were sealed in small tubes and placed in a freezing chamber at -20° to -22°C . Later, when needed, active virus suspensions were obtained by grinding the contents of one of these tubes with sand in a TenBroeck grinder, diluting with 100 cc. of Tyrode's solution, and centrifuging at 1800 R.P.M. for 10 minutes. The supernatant fluid was transferred to small tubes and again centrifuged at 2500 to 3000 R.P.M. for 20 minutes. To exclude cells that might conceivably still be living (7), a monel metal disc, attached to a stout wire and heated red-hot was held 2 or 3 mm. above the level of the fluid in the tubes until the surface layer boiled, thus killing such cells as might have come to the surface. The clear fluid of the middle layer was aspirated into a sterile syringe through a long lumbar puncture needle which was not allowed to touch the glass. The needle was removed and the aspirated fluid expelled into a fresh tube. A separate syringe and needle were used for each aspiration. The clear fluids thus obtained were centrifuged at high speed and again "de-celled" in the same way. On inspection under the microscope the 1 per cent suspension obtained in this way appeared cell-free. It was employed as such or diluted 10-fold before injection.

Methods of Inoculation.—In about half the instances, 0.3 to 0.4 cc. of the 1 per cent virus suspension was injected intradermally, 0.05 to 0.1 cc. at each of 4 sites near the shaved tip of the ear. Before expelling the fluid, the injecting needle was thrust 3 or 4 times into each area to tear many lymphatics, thus assuring the introduction of some of the inoculum and the consequent transport of virus to the cervical lymph nodes. Under ether anesthesia the injected ears were amputated at the base, that is to say several centimeters from the nearest injection site, 2 to 4 hours later. This was done to prevent the development of large vaccinia lesions at the site of injection, with consequent drainage of much virus to the nodes. In most instances the ear stumps healed without evidence of vaccinia infection there, but in a few animals local lesions developed. Because of this, other experiments were done without ear amputation, but with the dose of virus greatly reduced. In these instances the original 1 per cent virus suspension was diluted 10-fold, and only 0.1 cc. injected, at one site, that is to say approximately 1/40 of the dose used in the other experiments. Rabbits so injected developed typical vaccinia lesions at the injection sites, and no doubt virus drained to the neighboring lymph nodes during a period of several days.

In all of the animals virus was injected into one ear only, with the addition in the earlier experiments of the small amount of dye already described. The dye served to demonstrate, not only that the fluid entered the lymphatics at the time of injection, but also showed at autopsy to which of the lymph nodes it had come. In later experiments the use of dye was abandoned because we had found that virus never failed to appear in the chain of lymph nodes draining the injected side. The omission did not entail any differences in the findings.

As result of the virus injections the cervical lymph nodes on the injected side became enlarged, inflamed and often hemorrhagic. In all the experiments the cervical nodes from the uninjected side as well as nodes from other portions of the body served as controls. To control the possibility that the inflamed nodes might in some manner take up from the blood protective principles formed elsewhere in the body, the cervical nodes of the side not injected with virus were experimentally inflamed in more than half the instances. This was done by injecting into the ear of that side New York City Board of Health typhoid bacterin containing 1000 million killed *B. typhosus* organisms to the cubic centimeter together with 750 million of paratyphoid α and β . The bacterin was diluted 4-fold with Tyrode's solution colored, as was the virus suspension, with patent blue V, and injected intradermally in the ear in the same amounts as the virus suspension in the other ear. The bacterin caused the cervical lymph nodes to become enlarged, edematous and inflamed, the gross changes in these respects being roughly equivalent to those on the virus-injected side. They were tinged pale blue by the dye.

In many experiments the injection of typhoid bacterin on the control side was omitted in order to compare the size of approximately normal lymph nodes with that of the enlarged ones on the virus-injected side. This was done only after several experiments had shown that virus could not be demonstrated in the nodes of the typhoid-injected side even after they had become inflamed, as further that extraction of them failed to yield a neutralizing fluid for vaccinia until many days after this had become the case with the corresponding nodes of the virus-injected side.

At varying intervals from a few minutes to 15 days after introduction of the virus, the injected animals were etherized and samples of blood obtained aseptically for serum. The organs for study were then removed with aseptic technique. The lymph nodes from the parotid region and those lying at the junction of the internal and external jugular veins of the control side were first removed and weighed as a group. They will be termed the cervical nodes. The axillary nodes and one large inguinal gland were then taken from the same side and, if the experiments required it, samples of femur or tibia marrow, spleen or liver. To avoid possible contamination of other tissues with virus the cervical lymph nodes of the virus-injected side were removed last. All these tissues were weighed, placed in small Petri dishes and kept moist with filter paper saturated with Tyrode's solution. After tracings had been made of the size of the nodes the dishes were put in the freezing chamber at -20° to -22°C . and 1 to 10 days later the frozen material was ground aseptically with sand, and Tyrode's or Locke's solution added to form 10 per cent suspensions. These were cleared in the centrifuge at 2500 R.P.M. for 10 minutes and the supernatant fluids were then tested.

Presence of the Virus.—To determine the presence of the virus in the extracts, they were diluted in multiples of 10 to 10^{-5} or in some experiments to 10^{-7} . Node material from the control side was diluted in this way but only to 10^{-1} , 10^{-2} and 10^{-3} . The diluted suspensions were then inoculated intradermally (0.2 cc.) into the shaved sides of at least 3 normal brown-gray rabbits, varying the site of in-

jection from animal to animal. In each of these animals vaccine virus was inoculated as well, using for the purpose a freshly made de-celled 1 per cent suspension diluted to 10^{-5} , 10^{-6} and 10^{-7} . This was obtained from another frozen sample of the same testicular material originally employed for the ear injections and kept at -30°C . in the freezing chamber, like the original material before its injection.

Neutralization Tests.—These tests were employed to demonstrate the presence of antiviral principles in the serum and in the tissue extracts. Some of the freshly made 1 per cent vaccine virus suspension was diluted 50,000, 500,000 and 5,000,000 times. The resulting fluids were mixed in equal parts with the cleared, centrifuged 10 per cent tissue extracts or with whole serum or serum diluted 1 in 10 with Locke's solution, or both. There resulted 5 per cent tissue or serum mixtures with vaccine virus in dilutions of 10^{-5} , 10^{-6} and 10^{-7} . These mixtures were allowed to stand at room temperature for 1 to 3 hours before they were employed. The differences in interval caused no observable difference in the findings. This was to have been expected from the work of Andrewes (8), who found that immune serum incubated with vaccine virus before testing (within the limits imposed by the tendency of the virus to deteriorate) showed no better neutralization upon inoculation into animals than did freshly made mixtures. Intradermal inoculations of 0.2 cc. of each of the virus-extract mixtures were then made upon at least 3 rabbits which also received the plain vaccine virus suspensions on the sides. Daily, for 7 days, these animals were examined and the size and character of the lesions traced and noted.

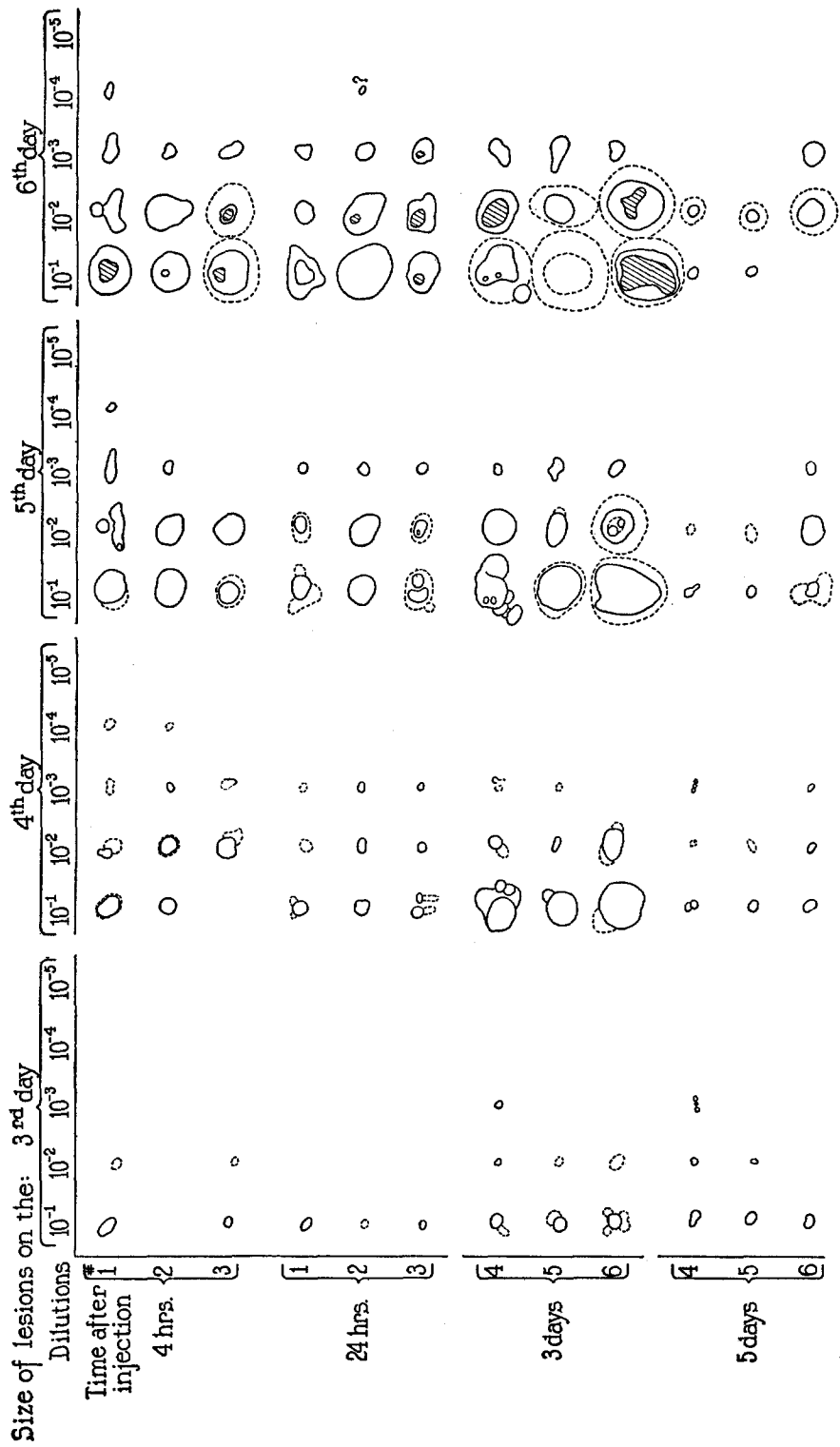
The Fate of Vaccine Virus in the Lymph Nodes

How long does vaccinia persist in the cervical lymph nodes following intradermal inoculation in the ear?

An experiment to answer this question disclosed outstanding differences in the amount of virus present in the cervical lymph nodes of the injected side in animals which were allowed to survive for differing periods of time after amputation of the ears.

In the manner already described, vaccine virus suspension, prepared in the usual manner, was injected near the tip of the shaved left ears of 6 rabbits. Under ether anesthesia these ears were amputated 4 hours later. After another hour one of the animals was killed and the cervical lymph nodes on the injected and uninjected sides were separately removed and placed in the freezing chamber. On the next day and upon the 3rd, 5th, 7th and 11th days, respectively, a single animal of the lot was sacrificed and the material was treated in the same way.

The de-celled extracts of cervical nodes in dilutions of 10^{-1} to 10^{-5} with Tyrode's solution were then intradermally injected into the shaved sides of 3 brown-gray rabbits. The sites of injection were varied as usual. Only the extracts of the cervical nodes from the virus-injected side yielded lesions. The more concentrated



TEXT-FIG. 1 a. The vaccine virus content of cervical lymph nodes at intervals after injection in the ear.

extracts developed characteristic vaccinia lesions by the 3rd day, the more dilute extracts 24 to 48 hours later. These ran the characteristic course and daily for a week their size was recorded, either by tracing it on a piece of transparent celluloid or by transferring caliper measurements to paper. The tracings taken from the test animals on the 3rd, 4th and 5th days are shown in Text-fig. 1. In this and in subsequent charts the continuous lines represent the raised, indurated border of the lesion itself. The dotted lines show the boundary of the edematous swelling about the lesion, when present. Areas of beginning necrosis have been shown by hatching, advanced necrosis by cross-hatching, and crater formation in regressing lesions by solid black areas. The tracings in all the text-figures have been reduced to the same extent and are therefore comparable.

As shown in Text-fig. 1 the dilutions of the lymph node extracts recovered 4 and 24 hours after injecting rabbits in the ear with vaccine virus gave rise to similar lesions. Material from the animals killed on the 3rd day yielded larger lesions; that obtained 5 days after virus injection yielded smaller lesions again, and only in the lower dilutions. After 7 days still smaller lesions were obtained and the material taken on the 11th day yielded practically none. The findings from the last 2 animals have not been included in Text-fig. 1, since they obtain abundant illustration in the charts of subsequent experiments.

For about 3 days after the virus had been injected intradermally in the ears of the rabbits of this experiment, it appeared to increase within the cervical lymph nodes, as Text-fig. 1 shows, and this although the injected ears had been amputated 4 hours after the injection. By the 5th day less virus was demonstrable, by the 7th day still less, and on the 11th day, none.

It is evident from the survey of the technique already given that the interpretation of our results depended upon the finding of differing amounts of virus or of protective principle in the corresponding lymph nodes of the two sides. We desired to know whether the various amounts of virus found in the lymph node extracts could be ascribed to differences in the amounts which had reached the cervical lymph nodes immediately after intradermal injection into the ear. An experiment was done to test the point.

Near the shaved tip of the left ear of each of 4 normal brown-gray rabbits 4 intradermal injections were made into the same approximate situations, of 0.1 cc. each of freshly de-celled 1 per cent vaccine virus suspension. In the right ears typhoid bacterin was similarly injected at 4 sites, 0.1 cc. in each. Approximately 4 hours later the left ears of these animals were amputated, under ether, to render the technique comparable with that of other experiments to be reported below. They were then killed with chloroform and the right and left cervical lymph nodes

were aseptically removed and separately extracted with Locke's solution as already described. Dilutions of the extracts, to 10^{-1} to 10^{-5} , were then injected separately in amounts of 0.2 cc. intradermally into the shaved sides of 3 normal brown-gray rabbits.

In the meantime the left ears of 2 other animals were injected but once, with 0.1 cc. of the virus suspension diluted 10-fold and the right ears injected with typhoid bacterin as described above. The ears were not removed later. The following day these 2 rabbits were killed and similar titrations made with extracts of the cervical lymph nodes. The lesions resulting from both sets of titrations were charted daily for 7 days. No growth resulted from the fluids obtained from the right nodes, the side injected with typhoid bacterin. The left node material of all the rabbits yielded typical vaccinia lesions at dilutions of 10^{-1} , 10^{-2} and 10^{-3} in all instances and at 10^{-4} in only one.

Only slight differences appeared among the lesions developing from the node extracts taken from the 4 animals with amputated ears. In size and in rate of development the lesions were like those yielded in the preceding experiment by node extracts made 4 and 24 hours after injecting virus in the ear. (Tracings in the first two rows of Text-fig. 1.) The differences were in no way as great as those shown in the remaining portions of Text-fig. 1. Obviously the cervical lymph nodes of the 4 rabbits contained similar amounts of virus 4 hours after the injection. Differences in the virus content of cervical lymph nodes as obtained in the previous experiment and in those to be described below may therefore be attributed to changes in the amount of virus after it had reached the nodes. The titration tests for the 2 rabbits given small doses of vaccine virus and allowed to live for 24 hours showed also no differences, indicating that similar quantities of virus reached the nodes in both animals. By chance the lesions were strikingly similar to those of the previous titration tests. Similar amounts of virus were present in the cervical nodes of the two groups of animals, one 24 hours after allowing a small dose of virus to drain in an intact ear, the other 4 hours after making the larger injection.

In the preceding experiment (of Text-fig. 1) the virus content of the cervical lymph nodes was greatest in the animal injected 3 days previously. Nodes removed on the 5th and 7th days yielded less and less virus and by the 11th day gave almost no evidence of its presence. Was the virus destroyed within the nodes, and if so, how? To answer this question neutralization tests were done, to compare the lesions produced in the skin of healthy rabbits, by vaccine virus at certain Tyrode dilutions, with those resulting when it had been diluted instead with extracts of lymph nodes to which vaccinia had been carried on the lymph some days before. But first we determined the effect of extracts of normal lymph nodes.

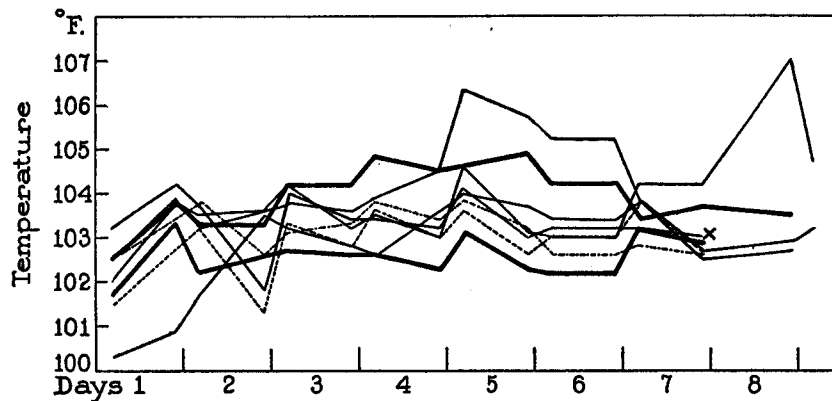
Duran-Reynals (9) has shown that certain tissue extracts, notably those of the testicle, enhance the lesions produced by vaccine virus, whereas other extracts inhibit it. He found the effect of lymph node extract to be very slightly inhibitory (9). It seemed wise to make further tests under the conditions of our experiments. They were carried out as a matter of general information despite the fact that we employed in all of our subsequent experiments control mixtures of vaccine virus and of extracts of lymph nodes from the side not injected with virus.

The cervical lymph nodes were removed aseptically from a chloroformed normal rabbit, and 10 per cent extracts were made in the usual manner. A freshly prepared, de-celled vaccine virus suspension was mixed with equal parts of the lymph node suspensions to yield final dilutions of 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} . Each of these fluids was injected intradermally (0.2 cc.) at 2 sites in the shaved skin of each of 3 normal brown-gray rabbits. The resulting lesions were observed and traced daily for 10 days. For the sake of brevity no charts will be given, since in the charts of subsequent experiments comparable tracings are shown of the lesions arising from inoculations of plain virus and of virus mixed with extracts of normal and pathological lymph nodes. The extracts of normal lymph nodes exerted only the slightest inhibitory action, if any, upon the development of vaccine virus lesions, as shown by a comparison with the control lesions caused by plain virus at similar dilutions.

Before searching for antiviral principles in the lymph nodes it was necessary to cope with one other possibility. It is well known that antibodies can be demonstrated in many organs and body fluids. In an animal flooded with antigen, they may appear in such quick succession in the various tissues and body fluids that one is unable to determine in which they first occurred. The satisfactory demonstration of the presence of a neutralizing principle for vaccine virus in one tissue or body fluid prior to its appearance elsewhere in the body, after injection in the ear, called for a dose of the virus so gauged that the rapid development of generalized vaccinia did not happen, although the animals became immune. We determined the dose by observing the clinical course of the disease for 10 days in rabbits injected in one ear with various amounts of the virus. The temperature of each animal was noted twice daily and the shaved skin of both sides of the body was watched for the appearance of pocks. Furthermore sterile testicular stabs were made, as hereafter described, with a view to inducing localization of vaccinia which would disclose its presence in the circulation. It is well known that generalized skin pocks develop in rabbits receiving large doses of vaccine virus intra-

dermally. We have noted the almost invariable appearance of skin pocks, usually on the 5th or 6th day after inoculation in our titration rabbits which necessarily received large amounts of virus. We therefore examined the rabbits injected in the ear with small doses of virus for a period of 7 days to see if they too developed generalized pocks. The final experiment to ascertain the dose selected for the purposes of the present work will be briefly described.

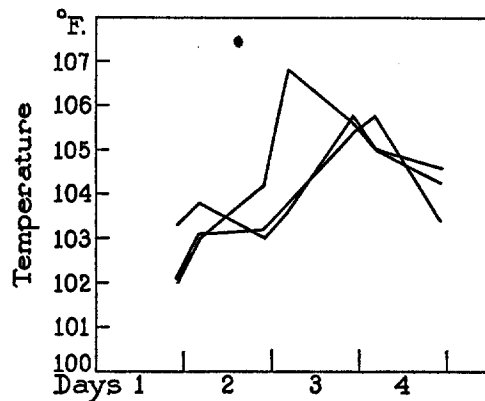
Six normal brown-gray rabbits were injected in one ear with 0.1 cc. of fresh vaccine virus suspension diluted to 10^{-2} . 4 hours later both ears were amputated



TEXT-FIG. 2. Temperature curves of 6 rabbits injected in one ear with 0.1 cc. of a fresh vaccine virus suspension at a dilution of 10^{-2} . The two heavy continuous lines record the temperatures of 2 of these animals in which, as described in the text, sterile testicular stabs were made. No localized vaccinia lesions developed in these. The broken lines are the temperature curves of 2 uninoculated control rabbits. None of the animals receiving the virus developed generalized vaccinia.

near the base. Rectal temperatures were taken at 10 a.m. and 4 p.m. daily. The following day a wide area was shaved over the abdomen and both sides of each animal, not only to facilitate the finding of pocks should they develop but to cause abrasions and slight injuries of the skin in which virus might become localized. Under ether, aseptic testicular stabs were made in 2 of the rabbits, using 19 gauge needles. The organs were examined daily thereafter for evidence of lesions. On the 2nd, 3rd and 4th days the ear stumps showed only a slight amount of soft edema; none was red, warm or inflamed. Subsequent work has shown that the virus can be obtained from such stumps. On the 5th day the stump of the injected ear of one rabbit showed an irregular area of dry necrosis 1 cm. in diameter overlying the central artery and lymphatic trunks. Surrounding the necrosis

brawny induration extended for 2 cm. At no time was this animal's temperature markedly elevated (Text-fig. 2, the line marked with an x), nor did the abdominal area show pocks. In later experiments about one-third of the animals so injected showed vaccinia lesions in the ear stumps. No pocks developed on the shaved areas of skin. In Text-fig. 2 the temperature curves of these animals are plotted. There was no exceptional increase in the animals with testicular stabs (heavy lines) and no localized lesions developed. It will be seen that the rabbits injected in this way failed to show the clinical signs of generalized vaccinia. Text-fig. 3 shows as contrast the temperature curves (for the first 4 days only) of 3 rabbits used for routine propagation of the virus, and which received 1 intratesticular



TEXT-FIG. 3. The temperature curves of 3 rabbits inoculated intratesticularly with 0.75 cc. of a 1 per cent suspension of the same virus-containing material used with the animals of Text-fig. 2. Generalized vaccinia developed.

injection of 0.75 cc. of a 1 per cent suspension of the same virus-containing material used to inoculate the animals charted in Text-fig. 2. These animals exhibited generalized pocks.

On the 7th day after inoculation 2 of the group of experimental rabbits were killed and the cervical lymph nodes of the injected and intact side were removed, as also samples of spleen, bone marrow and serum. Extracts of these tissues and specimens of the blood serum as well were utilized in titration and neutralization tests. The latter disclosed the presence of a neutralizing principle in the serum and in the extracts of the cervical lymph nodes of the virus-injected side, as will be evidenced further on. The animals that were let live and others similarly injected in later experiments proved refractory to infection when reinoculated with 0.2 cc. of the 1 per cent vaccine virus suspensions 2 weeks after the original injection.

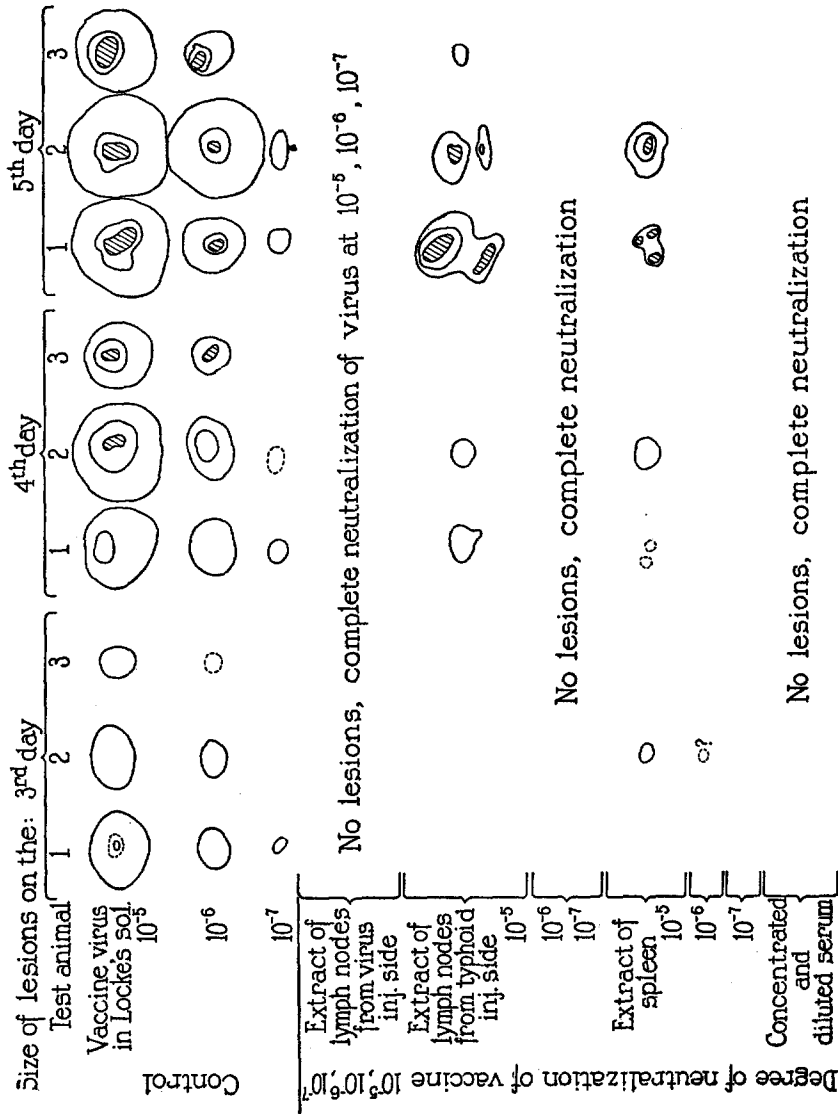
The amount of vaccinia virus employed in this experiment gave rise to a localized infection which resulted in generalized immunity. In the later experiments the same dosage was employed.

The Local Development of an Antiviral Principle

The foregoing results provided a basis for neutralization tests to determine whether or not the lymph nodes are capable of elaborating an antiviral principle. At various time intervals serum, cervical and other lymph nodes and portions of the bone marrow and spleen were procured from 36 rabbits and titration and neutralization tests were carried out. In 6 instances the materials were taken 4 to 6 hours after inoculation of vaccine virus into the ear, in another instance after 24 hours, in 1 after 2 days, in 5 after 3 days, from 6 animals upon the 4th day, from 6 on the 5th day, 2 on the 6th day, 4 on the 7th day, 2 on the 11th and 3 on the 15th day. The outcome of these experiments is best shown by presenting first the findings in the animals that had become highly resistant to vaccinia 15 and 11 days after inoculation and then describing the results obtained from animals showing less and less neutralizing principle in the serum and organ extracts, 7, 6, 5, 4 and 3 days after inoculation, respectively.

15 days after the inoculation of virus in the left ears of 7 rabbits in the manner described, 3 were killed. Specimens of the serum were taken and extracts made of the cervical lymph nodes from both sides, separately, from axillary and inguinal lymph nodes, bone marrow and spleen. Titration tests and neutralization tests were carried out on 3 normal brown-gray rabbits, varying the injection sites as usual. The titration tests, even those done with the extracts of cervical lymph nodes from the virus-injected side, yielded no lesions. The neutralization tests, employing the extracts mentioned above mixed with vaccine virus suspension diluted 10^{-5} , 10^{-6} and 10^{-7} , showed either complete neutralization, or marked inhibition of the ability of the virus to form lesions. In Text-fig. 4 are given the tracings of the few scanty lesions which appeared in these tests. Tracings of other lesions produced in the same test animals by suspension of virus alone are shown for comparison. It is to be noted that no lesions appeared when virus was mixed at a dilution of 10^{-5} with the extract from the lymph nodes of the virus-injected side or with the serum. Obviously an antiviral principle was widely present in the animals.

The remaining 4 rabbits inoculated 15 days previously were intradermally injected in their shaved sides with 1000 infective doses of our virus and were found to be refractory. They served as controls to the state of the 3 animals which were killed to furnish material for the titration and neutralization tests.



TEXT-FIG. 4. Neutralization tests with tissue extracts and serum. Material procured after 15 days.

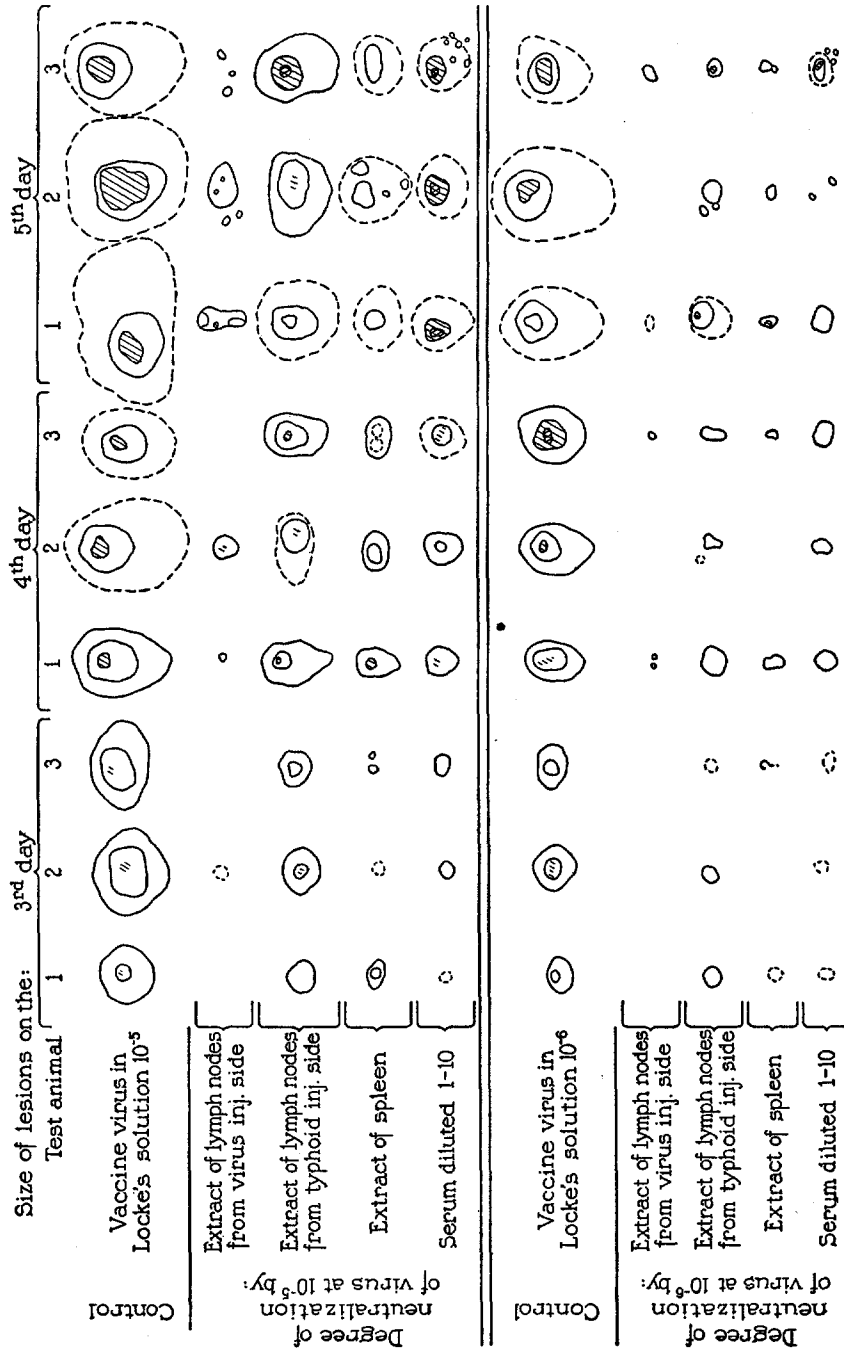
In another experiment 2 rabbits were inoculated with vaccinia in one ear and with typhoid bacterin in the other, as described. On the 11th day thereafter, titration tests with serum, and the usual lymph node, spleen and marrow extracts failed to yield evidence of the virus. Neutralization tests showed neutralization of the virus almost equal to that after 15 days. Obviously in these animals too the antiviral principle was widespread in the body and very effective.

Four rabbits injected 7 days previously with virus in one ear and with typhoid bacterin in the other served as the source for serum and tissue extracts procured in the usual way. In 2 of these instances the ears were amputated, in the others left intact. Each extract was titrated upon 3 rabbits and neutralization tests carried out on 3 more with each of the extracts against vaccine virus at dilutions of 10^{-5} , 10^{-6} and 10^{-7} , varying the injection sites. The findings in all were similar. Text-figs. 5 and 6 of typical instances show the results of the neutralization tests with virus at dilutions of 10^{-5} and 10^{-6} , as they appear in the test animals on the 3rd, 4th and 5th days after inoculation. In the instance used for Text-fig. 5 the serum was diluted 1 in 10, that is to say to the same extent as the tissues from which the extracts were made. In the instance shown in Text-fig. 6 whole serum was used.

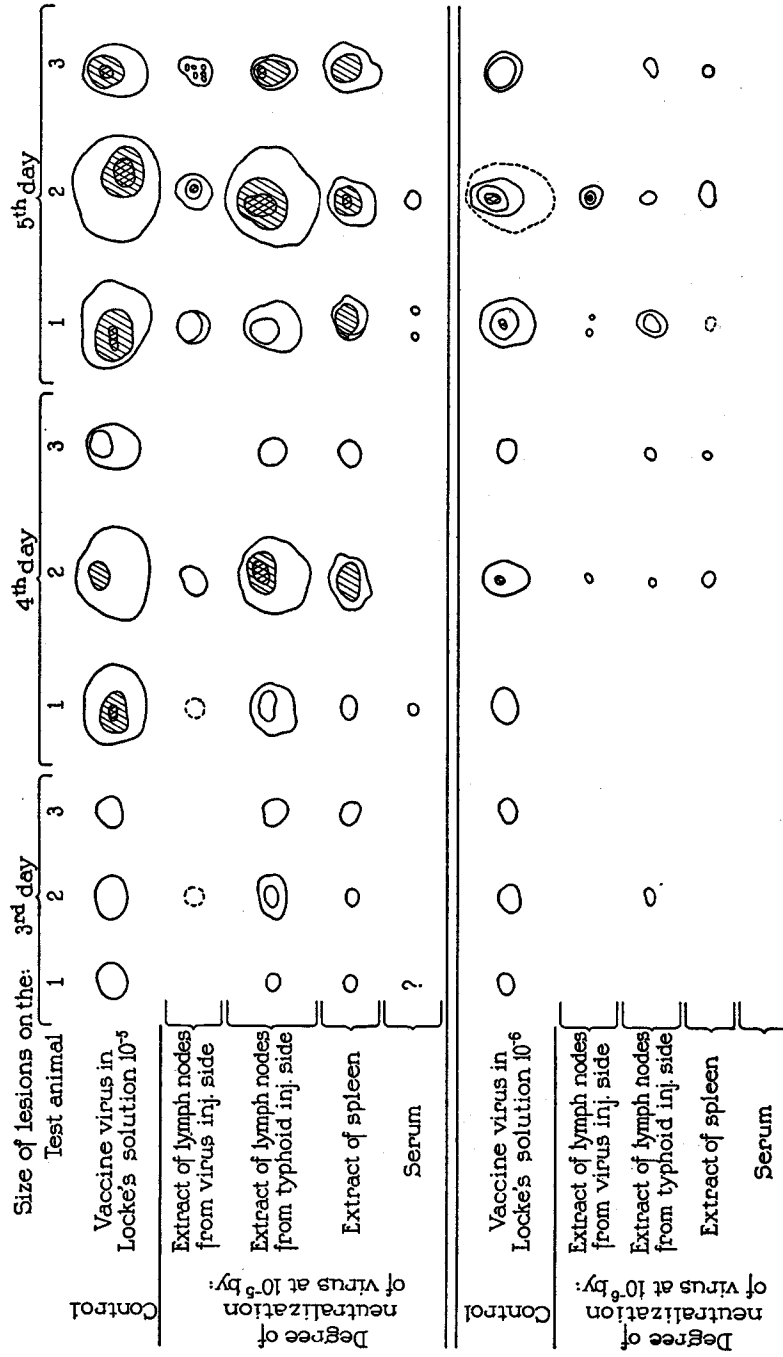
7 days after inoculation of virus in one ear and typhoid bacterin in the other the serum and all the tissue extracts showed some neutralizing ability, but far less than that of similar material taken from animals 11 days after inoculation. The highest concentration of antiviral principle was found in whole serum and in the extracts of the cervical lymph nodes of the injected side. Serum, diluted to the same extent as the lymph node tissue, and spleen extract exhibited less effectiveness in an almost equal degree. As Text-fig. 5 shows the extract of the lymph nodes of the typhoid-injected side showed least neutralizing power. By titration tests, not shown in the chart, virus was demonstrated to be still present in the nodes of the virus-injected side but there only.

Text-fig. 6 demonstrates the strong neutralizing power of whole serum 7 days after inoculation in the ear, as compared with that of the diluted serum (Text-fig. 5). In making this comparison it is to be recalled that the lymph node tissue was diluted 10-fold and a direct comparison of its neutralizing power with that of whole serum cannot be made. One can only say that whole serum neutralized virus more readily than did the diluted lymph node extract. The neutralizing power of serum procured on the 7th day is not found in animals sacrificed earlier, for example on the 4th day, and the results in Text-fig. 6 should be compared with those in Text-figs. 8 and 9 in which the neutralizing effect by extract of the lymph nodes from the injected side proved to be greater than that of whole serum. The significance of this will be discussed below.

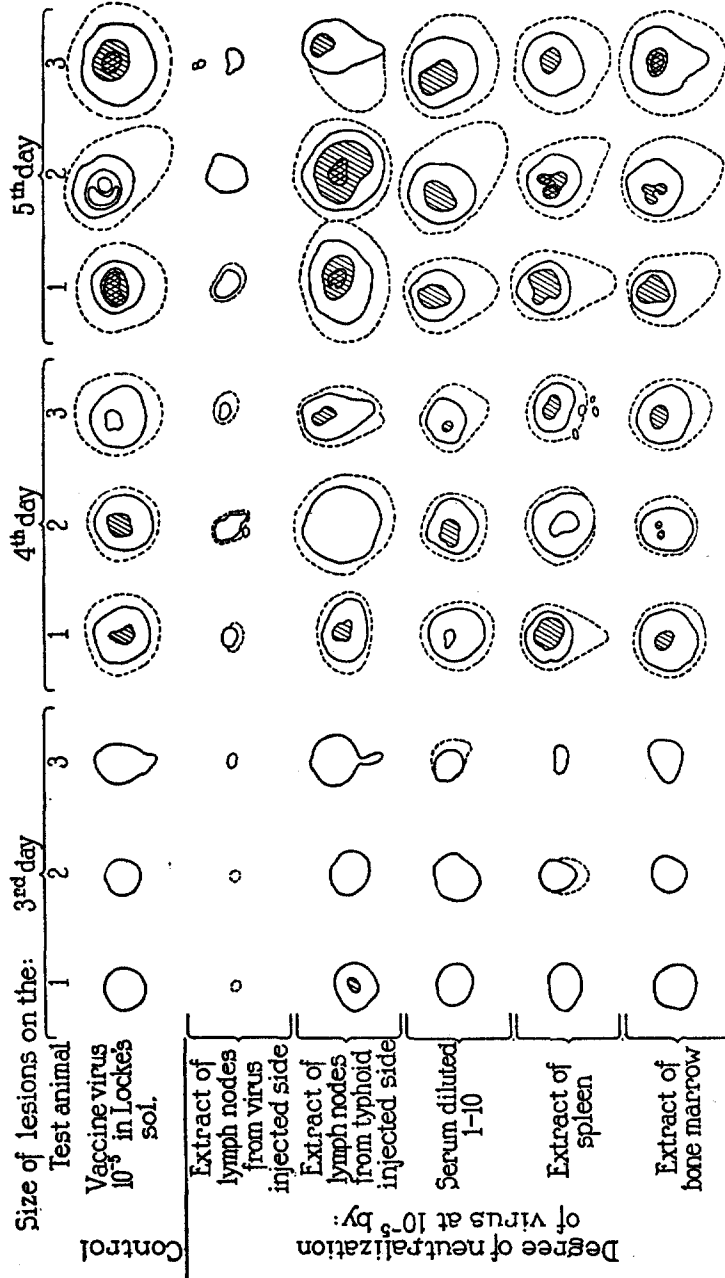
Two similar experiments were done, using material from animals sacrificed 6 days after inoculation of virus in one ear, in the usual manner. Text-fig. 7 shows the results of the neutralization tests of one of these instances. Vaccine virus suspension at 10^{-5} and 10^{-6} was much inhibited by the extract of lymph nodes



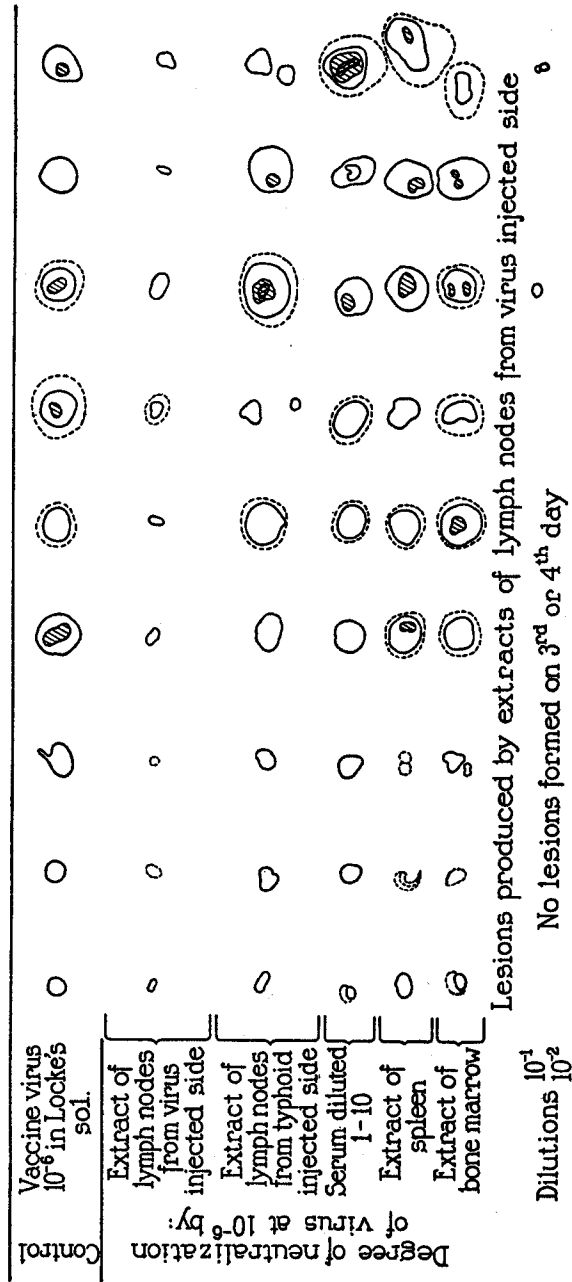
TEXT-FIG. 5. Neutralization tests with tissue extracts and serum. Material procured after 7 days.



TEXT-FIG. 6. Neutralization tests with tissue extracts and serum. Material procured after 7 days.



TEXT-FIG. 7—Continued on Next Page



TEXT-FIG. 7—Concluded

TEXT-FIG. 7. Neutralization tests with tissue extracts and serum. Material procured after 6 days.

from the virus-injected side, but not by serum similarly diluted or by lymph node material from the uninjected side or by the spleen or bone marrow extracts.

The findings after 5 days yielded still more instructive results. 6 rabbits received virus inoculations in one ear as already described, following which the injected ear was amputated in 3 cases while in the others the injected ear was left intact. All were sacrificed on the 5th day. These experiments were done at different times, and in each necessarily a different frozen specimen of testicular material from the same general batch was used as a source of vaccine virus. Of the 6 animals only one with ear intact and one with the ear amputated received injections of typhoid bacterin into the ear not inoculated with virus. In these instances the nodes were equally enlarged at autopsy, edematous and hemorrhagic. In the other 4, only the cervical lymph nodes of the injected side were inflamed and 6 to 8 times heavier than their companion nodes. The latter seemed in every way normal.

In all 6 experiments the cervical nodes on the virus-injected side showed the presence of antiviral principle as evidenced by some neutralization of the virus on test. In 2 of the 6 instances the extract completely neutralized vaccine virus at a dilution of 10^{-7} and did so largely at 10^{-6} , while the serum in corresponding dilutions showed far less antiviral power, when any, and the other extracts none whatever. The extracts of the cervical lymph nodes of the other 4 animals manifested less antiviral activity and their diluted serum had none at all. In one of these instances the spleen extract neutralized the virus slightly. By titration tests virus was found only in the extracts of the nodes of the virus-injected side. The findings were so similar to those already described that no charts need be given.

3 days after inoculation of virus in one ear an antiviral principle could be detected in the extracts of the cervical lymph nodes of the virus-injected side.

Five experiments of the sort were carried out in the usual way with amputation of the injected ears in three. Typhoid bacterin was injected in the control ears in 2 instances. In these cases the cervical lymph nodes of both sides proved to be greatly and about equally enlarged and hemorrhagic. The findings differed not at all from those in instances in which the bacterin had not been introduced.

Tests showed the presence of virus only in the lymph nodes of the virus-injected side, and the same extract in which it was demonstrated had slight neutralizing power. This was shown by a faint and irregular inhibition of the vaccinia virus in neutralization tests which were made in the usual way. There was no neutralization by the serum and none by extracts of cervical lymph nodes from the uninjected side or by bone marrow or spleen. The tracings of the neutralization tests from this experiment are not shown. The neutralization of virus by the extract of lymph nodes from the virus-injected side was less striking than that of similar node extracts removed on the 4th day after injection of virus in the ear, as described in a following experiment and traced in Text-figs. 8 and 9.

The neutralization tests showed the presence of antiviral principle spread widely through the body of rabbits injected intradermally in

one ear with virus 11 or 15 days before. Extracts of the serum and of various tissues obtained at shorter intervals of time following injection of virus in the ear showed less and less of the neutralizing principle. 1 week after such an injection whole serum, when mixed with vaccine virus suspension, neutralized the virus more than did a 10 per cent extract of the lymph nodes of the virus-injected side, but the latter showed more neutralizing power than the serum when similarly diluted. At an interval of 5 and 6 days after injecting virus in one ear the lymph node extract of the virus-injected side showed more neutralizing power than similarly diluted serum. Even after an interval of 3 days, the lymph node extract showed some neutralizing power and the diluted serum none. An experiment to be described below shows further that after an interval of 4 days the lymph node extracts from the virus-injected side showed more neutralizing power than whole serum.

In these experiments virus could be demonstrated by titration tests in the extracts of lymph nodes from the virus-injected side at the same time that antiviral principle could be demonstrated in the same extracts by the neutralization tests. This finding was obtained in every experiment in which these extracts were made 3 to 7 days after injecting virus in the ear.

Is the Antiviral Principle Developed within Lymph Nodes or Merely Concentrated There?

The lack of neutralizing power of the extracts of the inflamed nodes draining the ear injected with typhoid bacterin would seem to exclude the possibility that antiviral principle, formed elsewhere in the body and circulating in the blood, had collected or been concentrated in the nodes of the virus-injected side. There remained another possible source of the antiviral principle other than the lymph nodes themselves, namely the tissues of the injected ears. The possible formation of bacterial antibodies in the skin has been stressed by Fernbach and Hässler (10), by Cannon and Sullivan (11), Cannon and Pacheco (12) and others. An antiviral principle, formed locally in the ear tissue, might have drained directly by the lymphatics to the lymph nodes, and there accumulated. For virus often remains present for some time in the stump of an ear, injected with virus at its

tip and amputated a few hours later, as shown by the fact that about one-third of our animals so treated developed a definite vaccinia lesion in the ear stump. In about half our experiments the ears injected with relatively small amounts of virus were left intact, with the result that this could drain freely to the cervical nodes. For these reasons an antiviral principle was sought in the ear tissues.

Two rabbits were injected in the usual manner with virus and typhoid bacterin, after which the ears of one were amputated. 4 days later serum and tissue extracts were procured from the tissues of both animals as usual, and in addition ear tissue from the intact ears or stumps on both sides extracted. To obtain the latter the skin and all subcutaneous soft tissue above the cartilage on the upper side of the ear was removed, from its tip to a point three-quarters of the way to the base of the ear. This included all inflamed tissue, the vaccinia lesion on one side, and the area inflamed by the typhoid vaccine on the other side. In the instance in which amputation of the ear had been done, all soft tissue above the cartilage was removed from the tip of the stump to the base of the ear. The material thus obtained from the etherized, living animal or from one just killed, held practically no blood, for the latter was squeezed out when skin and subcutaneous tissue were stripped from the cartilage. The material was ground with sand and 10 per cent extracts made with Tyrode's solution, as with the other tissues removed. Titration and neutralization tests were carried out as usual with all the extracts and the blood serum. The lymph node extract from the virus-injected side markedly neutralized the virus, that of the typhoid side did not. The ear tissue of the typhoid-injected side yielded no evidence of the presence of virus or of a neutralizing principle, whereas that of the virus-injected side, when injected into the test animals at dilutions of 10^{-5} , 10^{-6} and 10^{-7} , gave rise to large lesions, and when mixed with virus, as in neutralization tests, caused even greater ones.

In the foregoing experiment the procedure employed failed to rule out the possibility that the action of an antiviral principle formed locally in the virus-injected ear had been masked by the presence of much virus. To overcome this difficulty we sought for an antiviral principle in the extract of the tissue of the virus-injected ear by a method known to separate it from virus when both are present together. We resorted to filtration of the greater part of each of the ear and lymph node extracts through Seitz pads. The filtrates of ear and cervical node extracts thus obtained and the extracts themselves were titrated on normal animals to see whether the virus had actually been filtered out, and in addition both extracts and filtrates

were used for neutralization tests. A description of the experiments follows.

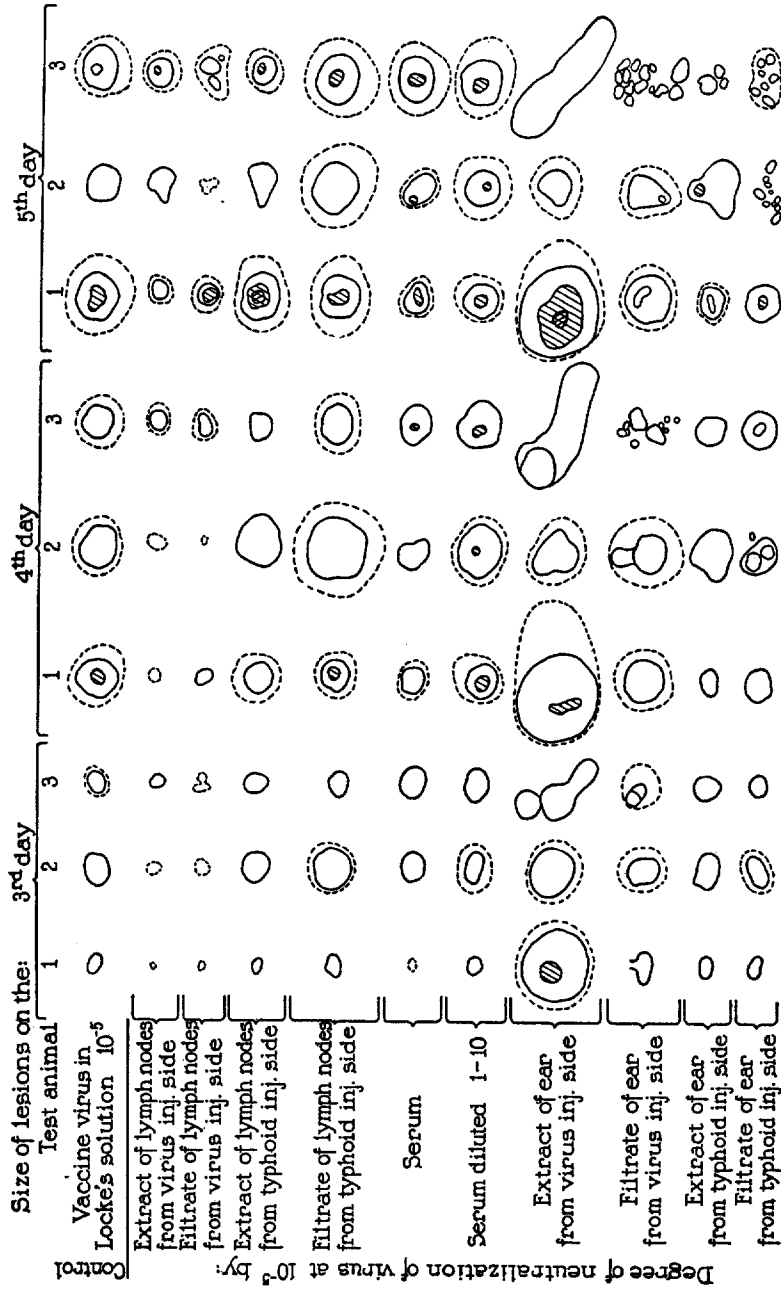
Four normal brown-gray rabbits were injected in the left ears with vaccine virus suspension in the manner already described, 2 receiving 0.3 to 0.4 cc. of the virus suspension, the others 0.1 cc. In the right ears typhoid bacterin was injected. 3 hours later the left ears of the 2 receiving the larger dose were amputated under ether. After 4 days the animals with ears intact showed small vaccinia lesions at the injection site. Of the 2 with amputated ears one showed a single small lesion in the ear stump. Serum was procured and the usual tissue extracts made, with, in addition, an extract of the tissue of the ears as in the preceding experiment. It will suffice to give the results in one experiment, in which materials were procured from an animal with the virus-injected ear intact, the ear likely to harbor antiviral principle in its tissue. The other 3 experiments yielded similar results.

Two 10 per cent extracts of ear tissue, one including vaccinia lesions, the other inflamed tissue of the typhoid-injected ear, and the extracts of the cervical lymph nodes of each side as well were filtered through Seitz filters. Enough of each extract was saved so that titration and neutralization tests could be performed with both the unfiltered and filtered material.

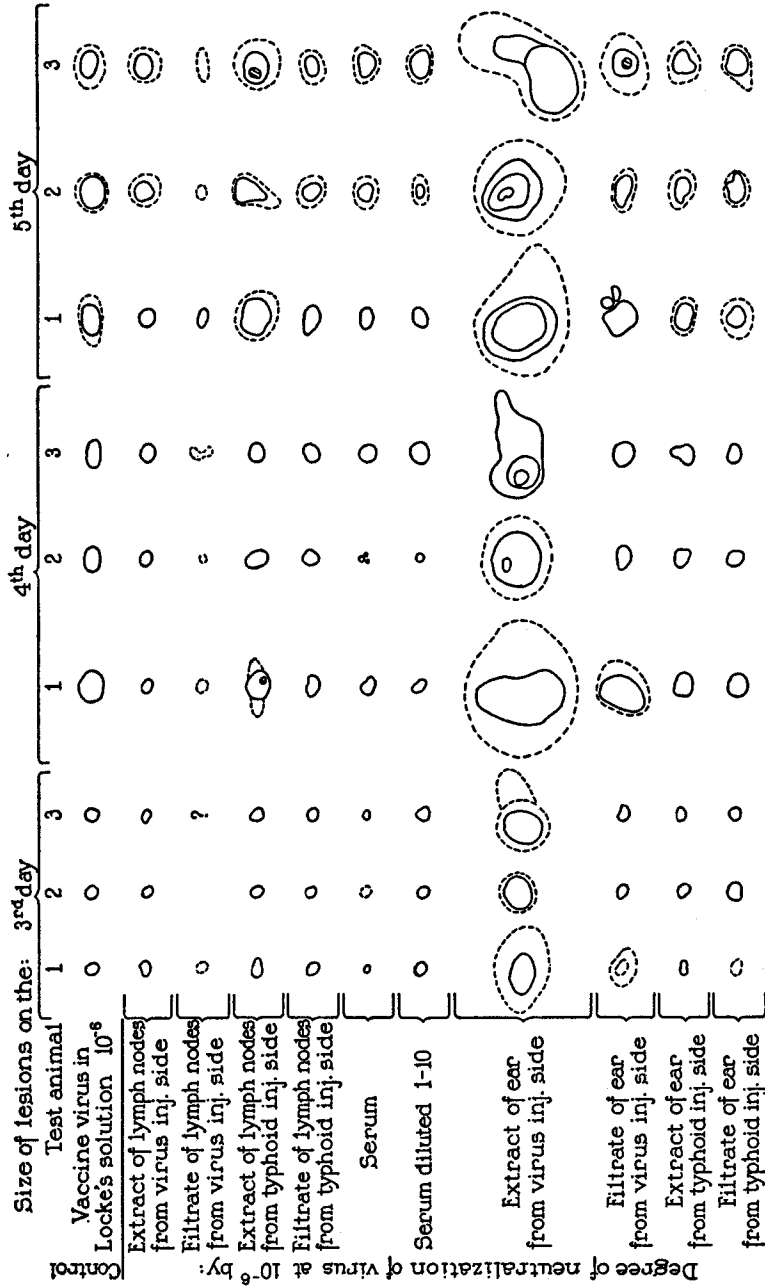
The neutralization tests were carried out upon 6 normal rabbits in the usual way. For comparative purposes injections were also made of ordinary virus suspension at the same dilutions, 10^{-5} and 10^{-6} . The employment of so many test animals in this experiment avoided the injection of excessive amounts of vaccine virus in any one individual, which might have led to secondary activation of lesions produced by the neutralization mixtures. The experiment carried out in this way contained its own control as concerned the efficacy of the filtration method to separate vaccine virus from an antiviral principle when both were present in the ear extract. For both were known to be present in the lymph node extract and they were successfully separated.

Titration tests on some additional animals disclosed the presence of virus only in the extracts of the cervical nodes and of the ear tissue on the virus-injected side. It could not be found in the filtrates of these extracts nor in any of the other extracts or filtrates.

Text-figs. 8 and 9 show the results of the neutralization tests made with vaccine virus diluted 10^{-5} and 10^{-6} respectively. The Seitz filtration had effectively held back virus and allowed the passage of the antiviral principle in the extract of cervical lymph node of the virus-injected side; for this filtrate, which on titration test yielded no vaccinia lesions, markedly neutralized vaccine virus. Indeed the filtrate showed greater neutralizing power than the unfiltered extract, a fact explainable by the finding of some free virus in the



TEXT-FIG. 8. Neutralization tests with tissue extracts, filtrates and serum. Material procured after 4 days.



TEXT-FIG. 9. Neutralization tests with tissue extracts, filtrates and serum. Material procured after 4 days.

unfiltered extract, and this when added to that used in the neutralization test gave rise to slightly larger lesions.

In contrast to such findings the filtrate of the extract of the virus-injected ear itself effected no neutralization of virus. It follows that there was no evidence of an antiviral principle in the ear tissue which might have been transmitted to the cervical lymph nodes. The extract of this ear contained much virus as shown by the titration tests, and when mixed with vaccine virus in neutralization tests yielded greater lesions than did the latter alone. Neither the node nor ear extracts or filtrates from the typhoid-injected side caused any neutralization of virus.

The results of tests with whole serum and with serum diluted 1 in 10 have also been charted. Neither show as much neutralization of virus as that caused by the extract of lymph node from the virus-injected side, the former showing some influence, the latter somewhat less. The finding is striking when compared with those described in the 7 day experiment of Text-figs. 5 and 6.

DISCUSSION

The data prove that the regional lymph nodes elaborate an antiviral principle when virus is brought to them by way of the lymphatics from the injected ear, and that this is demonstrable within 4 days after the virus inoculation. The experiments indicate that it could probably be demonstrated even after a shorter time, following an injection of virus in the ear, were better testing methods utilized, as for example if protective substances and virus were separated by filtration or by electrophoresis (13). The antiviral principle was present in greater concentration in the extract of the lymph nodes of the virus-injected side than in the undiluted blood serum procured at the same time, and in far greater concentration than in the serum diluted equally with the node extract. The possibility would seem to have been excluded that an antiviral principle developing in the injected ear or elsewhere in the body accumulated in the lymph nodes, thus accounting for the findings. The control injections of typhoid vaccine inflamed both the injected ear and the nodes draining it, but no antiviral principle was ever found in either until long after their appearance in the nodes of the virus-injected side.

It may well be that under the conditions of the experiments, in which virus reached the nodes on the lymph stream in small amounts and generalized vaccinia did not occur, the lymph nodes played a major rôle in producing the neutralizing principle found in the serum. Certainly the antiviral principle developed first in the nodes. The circumstances were much like those of natural infection through cuts or abrasions in the skin or mucous membranes, with retention of the infecting agent by the draining nodes, and they were so like those of artificial vaccination that there is no need to stress this point. The immunity following clinical vaccination may well be of lymph node origin in great part.

The relative amounts of antiviral principle present in the various organs or body fluids change much with the lapsed time after virus infection. For example the extracts and filtrates from cervical lymph nodes of the virus-injected side, procured 4 days after injection and diluted 10-fold, inhibited the activity of vaccine virus slightly more than did whole serum. Serum diluted 10-fold had little or no effect upon the activity of virus. After 7 days serum and node extract equally diluted show almost equal inhibitory powers, with the serum slightly the weaker. Whole serum showed definitely more neutralizing ability than did gland extract. One may infer that some at least of the antiviral principle now present in the serum was derived from the nodes. Whether it all came from them has still to be determined. The results in any individual case may depend largely upon the portal of entry of the virus, upon the quantity entering and upon whether it spreads rapidly or is retained by one tissue or organ.

We have not sought to ascertain the earliest moment at which the antiviral principle appears in the lymph nodes. Much must depend upon the amount of virus reaching these organs.

A word should be said concerning the methods employed. In our earlier experiments (4) on the formation of agglutinins in lymph nodes of mice, killed cultures or organisms were intradermally injected into ears which were later amputated. The amputation was done to exclude a seepage of antibodies from the dilated blood vessels of the inflamed ear to the interstitial tissue from which they might be drained by lymphatics to the lymph node. The procedure also prevented the possibility of drainage to the nodes of antibodies which might later

have been formed locally. The bacterin primarily introduced was entirely removed save for an insignificant amount present in lymph capillaries cut across at the time of amputation; and antibodies were not found in the tissue of intact, inoculated ears. The employment of active virus introduced a special difficulty. Amputation of the ear largely prevented seepage of antiviral principle into the inflamed tissue of the ear and thence to the nodes, but it did not prevent an increase of virus in the ear stump and the not infrequent occurrence of vaccinia lesions there. The presence of virus might well have acted to stimulate the local development of antiviral properties, were this possible, and the possibility would have existed even if intra-lymphatic injections had been made. It would have been ideal for the purposes of our work if one had been able to instil a known quantity of virus into the afferent channel of a lymph node without the infection of other tissues. But even under such circumstances virus would doubtless have passed from the lymphatic channel to proliferate in the tissues.

SUMMARY

An antiviral principle is elaborated within the regional lymph nodes draining skin into which vaccinia is injected. The immunity conferred by clinical Jennerian vaccination may be largely of lymph node origin.

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