

STUDIES ON THE ETIOLOGY OF RABBIT POX

I. ISOLATION OF A FILTERABLE AGENT: ITS PATHOGENIC PROPERTIES

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The investigations reported in this and succeeding papers (1-4) had their inception in a highly contagious and fatal epidemic which broke out with explosive violence in a rabbit breeding colony at The Rockefeller Institute in December, 1932. Within a month practically every animal in the colony of over 1,400 rabbits had been infected. The disease resembled small pox of man in so many respects that it was called rabbit pox. Its clinical and pathological aspects have been described by Greene (5, 6). Suffice it to say here that it was characterized by a pock-like eruption in the skin and mucous membranes, lymphadenitis, eye involvement, coryza, an orchitis in male animals, and various constitutional symptoms including fever. The incubation period varied from 5 to 14 days. The course was variable. Death frequently occurred within a few hours after the first signs of the infection were noted or after a period of a few days or weeks. On the other hand, recovery from a severe infection took place in many instances, visible lesions of the skin frequently healing with scar formation. Mild and asymptomatic cases were also seen. Although the mortality rate was variable, the disease was extremely destructive and 46 per cent of the colony succumbed. The rate was highest in young animals 4 to 8 weeks old, while in those less than 14 weeks of age it was 72 per cent. In adults the mean mortality was about 15 per cent, but the rate in different breeds varied widely. The infection produced the severest symptoms in pregnant and lactating does, and pregnancy was commonly terminated by abortion. During the termi-

nal stages of the epidemic, 6 to 8 weeks after its onset, mild and abortive types of infection were common and a large proportion of animals of the most susceptible breeds and age groups survived infection contracted at that time.

The literature contains little information which throws any light on the situation with which we were confronted. Mahlich (7) in a handbook for rabbit breeders and fanciers published in 1919 mentions a disease *Pocken* which "fortunately occurs very rarely in our rabbit population." The brief description of the generalized cutaneous eruption, together with the statement that the best remedy is killing affected animals, strongly suggests that the disease is the same as or very like the epidemic disease which we encountered.

The pock-like character of the cutaneous eruption in the epidemic disease in our colony was extremely striking and on this basis alone, the condition would unquestionably be classified in the pock group of diseases. The question naturally arose as to whether vaccine virus was concerned. At the time of the outbreak, however, there was no evident source of vaccine virus. Furthermore, vaccinal infection arising from exposure or contact is generally considered to be a mild or asymptomatic condition although there have been instances of severe epidemics which have been ascribed to neurovaccine virus. Investigations of these epidemics, however, particularly from the standpoint of comprehensive experiments to determine the precise nature of the etiological agents were apparently not carried out. It was subsequently learned that in at least three other laboratories of the Institute rabbits had been inoculated with vaccine virus prior to the epidemic and that in two of these neurovaccine had been used. The possibility of conveyance of a highly contagious infection from one group of animals to another must be considered, but if a spread of vaccinia among the experimental animals in these other laboratories took place, it apparently was not detected before the outbreak of the present epidemic in the breeding colony. In this connection, it should be mentioned that it was not the first time we have encountered this disease. In the spring of 1930 there was a small and much less severe epidemic of a disease presenting the same clinical and pathological picture. Its effects were comparatively mild and with the recovery or elimination of affected animals, fresh cases ceased to develop.

Early in the course of the epidemic, attempts to transmit the infec-

tion experimentally were made for the purpose of identification of the causative agent with the hope that means might be found to prevent future outbreaks. Experimental transmission of the infection was readily accomplished by rabbit inoculation of Berkefeld V filtrates or of unfiltered emulsions of tissues derived from spontaneous cases. The causative agent was easily filterable through Berkefeld V candles and was transmitted by tissue filtrates for 15 consecutive rabbit passages. The characteristic features of the virus persisted throughout the 9 months from January to October, 1933, in which the experimental investigations were carried out. The results of various tests showed that although the virus was related to vaccine virus, it was not completely identical with two specimens of dermovaccine and one of neurovaccine used for comparison. The results of the experiments which demonstrated this relationship led to the opinion that vaccination with dermovaccine might constitute an efficient prophylaxis against the spontaneous disease.

The results of the investigation on the filterable agent and the experimental infection which have been referred to in various preliminary communications (8) are now reported in detail. The study is obviously incomplete, but after information on certain major points had been obtained it was decided that further work should not be undertaken because at this time the necessary isolation facilities were not available. Many phases of the problem as for example, a study of the filtration properties of the virus, particularly from the standpoint of comparisons with other viruses, were only touched upon or were omitted altogether. Furthermore, certain aspects of the work, such as complete histological examinations of post mortem material had to be omitted because of the demands of other experiments. In the present paper the experiments dealing with the isolation, serial passage, and certain pathogenic properties of the virus are reported, together with a description of the acute fulminating and rapidly fatal type of infection, associated with the routine serial passage of the virus. Subsequent papers deal with the clinical manifestations and course of the less acute type of the experimentally induced disease which was indistinguishable from the spontaneous condition (1); with the results of various experiments, chiefly of an immunological character, in which the virus was compared with other filterable

viruses (2, 3); and finally, with experiments in which the susceptibility of certain other animal species to inoculation of the virus was studied (4). The last paper also contains a general discussion of the work on experimental pox with special reference to the nature of the virus, and the significance of rabbit pox to other pock diseases.

Materials and Methods

The successful experimental transmission of rabbit pox was made from spontaneous cases by the injection of both filtered and unfiltered emulsions of tissues into the testicles of normal rabbits. 4 strains of the virus were thus obtained. For a short period, the strains were carried by serial rabbit transfer, but as soon as it was certain that there was no difficulty in preserving an active agent by rabbit passage, only 1 strain (Xy171) was retained for routine work. Later in the course of the epidemic, additional specimens of virus were recovered from other spontaneous cases.

Organ Source of Virus.—The agent was recovered from the following organs and tissues of cases of the spontaneous disease: testicle, ovary, liver, spleen, popliteal and inguinal lymph nodes, brain, spinal cord, whole blood, defibrinated blood, and skin.

Animals.—Male rabbits in most instances obtained from dealers were used for inoculation. There was no evidence at any time that they had been affected by pox. They were approximately 4 to 6 months old and were of the usual hybrid type, the common greys, browns, Flemish crosses, and Chinchillas predominating. In some experiments younger rabbits were used.

Routines of Inoculation.—The initial injections were made intratesticularly and this method was followed for the routine passage of virus. Other routes employed in various experiments were: intravenous, intracutaneous, subcutaneous, intramuscular, intraperitoneal, intracerebral, nasal, and conjunctival instillation, and the application of tissue-virus emulsions to scarified areas of skin and cornea. Exposure in the rooms and in cages in which experimental animals were kept was also made.

Dosage.—In most experiments an involved testicle was used for inoculation and both filtered and unfiltered emulsions were employed. Emulsions were prepared in the usual manner with Locke's solution by grinding with alundum and a 10 or 15 per cent suspension by weight was ordinarily made. For the routine passage of virus, rabbits were injected in one or both testicles and in most instances with a dose of 0.5 cc. With other routes various dilutions of tissue-virus suspension as well as variable amounts were injected; these will be mentioned in connection with the particular experiments.

Filtration.—The virus in a Locke solution tissue emulsion was easily filtered through Berkefeld V candles. In certain experiments Berkefeld N and W candles and Seitz filters were used. All filtrations were made under negative pressure (air vacuum) varying from 100 to 500 mm. of Hg in from 2 to 8 minutes. The

procedure was controlled by adding the saline washing from a 24 hour agar slant culture of *B. prodigiosus* to the tissue emulsion before filtering in an amount equivalent to 10 per cent of the emulsion.

Examination of Tissues.—The majority of autopsies were performed immediately after the animals had died or after they had been sacrificed by means of an injection of air into a marginal ear vein. Aerobic and anaerobic cultures in broth and other media for the detection of ordinary bacteria were made of tissues used for inoculation and in many instances from other organs as well. Tissues were fixed in Zenker's fluid, Helley's fluid, and 10 per cent formalin, and sections were stained with hematoxylin and eosin and with Giemsa's stain.

Recovery of Virus from Spontaneous Cases

The results of bacteriological examinations of organs and tissues from spontaneous cases revealed no organism with which the disease could be induced. The intratesticular injection of Berkefeld V¹ filtrates from a variety of such tissues, however, produced an acute reaction in rabbits which was characterized by fever, a marked hemorrhagic orchitis, and death. Furthermore, this reaction was regularly reproduced in a consecutive series of rabbit to rabbit passages by means of Berkefeld V filtrates of tissue emulsions as well as by unfiltered emulsions. The less acute type of reaction also induced by Berkefeld V filtrates was indistinguishable from spontaneous cases of pox. This type of disease was associated particularly with routes of injection other than the intratesticular and with a smaller dosage as is described in the following paper (1). In all cases the filtrates were cultured aerobically and anaerobically in broth. In the following discussion all references to filtrates used are understood to indicate sterile filtrates.

The spontaneous cases from which tissues were obtained for inoculation will first be briefly described.

All inoculations were made intratesticularly. To avoid repetition, the results are simply stated as "positive" or "successful," "negative" or "unsuccessful." The positive results refer to the development of an acute orchitis and fever and a fatal outcome. These features are fully discussed in the following section in which the results of the serial passage of the virus are taken up. In the absence of these clinical features, the results of inoculation were considered to be negative.

¹ Experiments with other filters were limited to a few preliminary tests. The results indicated that the virus was filterable through Berkefeld N and Seitz filters. In the case of Berkefeld W candles the results were uncertain.

Rabbit Xy171.—Sable Marten hybrid male, 8 months old. Well marked acute symptoms including diffuse orchitis, popliteal adenitis, mucopurulent nasal and conjunctival discharge, slight papular cutaneous eruption over body and ears; general weakness and listlessness. Killed. 0.5 cc. of the Berkefeld V filtrate of the right testicle injected in each testicle of 3 rabbits and 1 cc. in one testicle of a 4th rabbit; in 2 of these animals 0.5 cc. was dropped in the right nostril. 0.5 cc. of the Berkefeld V filtrate of defibrinated heart's blood was injected in each testicle of 2 rabbits and 0.5 cc. was injected into one testicle of a 3rd rabbit. All 4 rabbits injected with testicular emulsion and 1 rabbit injected with defibrinated blood were successfully inoculated.

The majority of experiments were carried out with the virus obtained from an animal inoculated with testicular emulsion.

Rabbit 782.—Himalayan hybrid breeding doe 11 months old. Marked weakness and prostration of a few days' duration, profuse blood stained mucopurulent discharge, mucoserous conjunctival discharge, mucous anal discharge, diarrhea, sparse cutaneous maculopapular eruption on ears and body, emaciation. Found dead. 0.5 cc. of the Berkefeld V filtrate of an emulsion of liver, spleen, and ovaries was injected in both testicles of 2 rabbits with positive results.

Rabbit AB18.—American Blue doe 9 weeks old. Very recent widespread papular eruption on ears, eyelids, nose, lips, genital region, and body; pronounced cutaneous edema of the hind feet; enlargement and induration of the superficial lymph nodes, especially of the popliteals; no nasal or conjunctival discharge. Killed. 2 rabbits were injected with doses of 0.5 cc. of an unfiltered suspension of popliteal lymph nodes; 2 rabbits were injected with 1.0 cc. doses of an unfiltered liver and spleen emulsion; and 2 rabbits received 1.0 cc. of whole blood. All 6 rabbits were successfully inoculated.

Rabbit P2.—Polish doe 14 months old. Fulminating infection of less than a week's duration. Marked ophthalmia with conjunctival serous discharge, watery nasal discharge, marked respiratory distress, pronounced edema of anogenital region, and enlarged, tense popliteal lymph nodes; muscular tremors, incoordination, a tendency toward convulsions, and almost complete paralysis of legs with twisting of body. Died. Unfiltered suspensions of the following organs were injected in both testicles of each of 2 rabbits: brain (0.5 and 0.2 cc.); spinal cord (0.5 and 0.1 cc.); and 1 rabbit each with liver (0.5 cc.) and spleen (0.5 cc.). All 6 rabbits were successfully inoculated. A rabbit injected with lung filtrate showed no clinical evidence of infection.

Rabbit X667-1.—Hybrid male, 5 weeks old. Early very severe case occurring toward the end of the epidemic. Extremely profuse papular skin eruption on the body, extremities, head and ears, serous nasal discharge with bloody crusts and papules about nares and lips. Killed. Liver filtrate in 0.1 and 0.03 cc. doses was injected in one testicle of each of 2 rabbits with negative clinical results. The injection of a skin filtrate (0.5 and 0.2 cc.) in one testicle of each of 2 rabbits gave positive results in both cases.

Rabbit HA46-1.—Havana male, 2 months old. Recent mild case developing at the end of the epidemic. There was a small cutaneous papule in one ear and two larger ones on the back; no constitutional symptoms. Killed. Unfiltered emul-

sions of the testicle, liver, and popliteal and right inguinal lymph nodes were injected. The results on the 2 rabbits injected with lymph node and testicle suspensions respectively were positive, but that on the rabbit injected with liver emulsion was negative.

Rabbit 8415.—Hybrid adult male, approximately 8 months old. A recovered case of about a week's duration. The condition which had included an orchitis had been mild. Killed. Negative post mortem findings. Unfiltered and filtered suspensions of the right testicle and of both popliteal lymph nodes were injected in 0.5 cc. doses in both testicles of 4 rabbits. A positive result was obtained in the rabbit injected with unfiltered testicular emulsion, but no clinical signs developed in the other animals.

Rabbit X213-1.—Hybrid male, 8 months old. Apparent complete recovery of approximately 3 weeks' duration; disease had been moderately severe with a diffuse orchitis, a few skin papules, marked right ophthalmia and keratitis, and moderate popliteal adenitis. The right testicle and right popliteal lymph node were removed under ether anesthesia; both organs appeared normal. Unfiltered and filtered testicular suspensions and an unfiltered emulsion of the popliteal lymph node were injected intratesticularly in 4 rabbits. The equivocal results which were obtained were regarded as negative.

Summary of Results of Original Inoculations from Spontaneous Pox Cases.—The intratesticular injection in rabbits of tissues from 6 cases of spontaneous pox of varying severity and duration produced a pronounced local and general reaction which, as will be described in the following section, could be duplicated by the serial passage of affected tissue. The marked acute hemorrhagic orchitis was especially striking. Positive results were obtained from the inoculation of Berkefeld V filtrates of tissues from 3 cases and with unfiltered tissue emulsions from 3 other cases.

In 5 of these cases the disease was well marked or pronounced while in the 6th it was very mild. Positive results were obtained in 23 of the 29 rabbits injected, or 79 per cent. The tissues and organs with which these findings were obtained were the following: testicle, ovary, liver, spleen, brain, spinal cord, popliteal lymph nodes, skin, whole and defibrinated blood. The failure to induce clinically positive results occurred in the case of certain materials obtained from 4 of the 6 rabbits, that is, in 2 rabbits injected with filtered defibrinated blood, in 2 injected with filtered liver emulsion, in 1 injected with unfiltered liver emulsion, and in 1 rabbit injected with filtered lung suspension. Among 14 rabbits injected with Berkefeld V filtrates, positive clinical results developed in 9 or 64 per cent, and among 15 rabbits injected with unfiltered materials in 14 or 93 per cent. The single failure encountered with the use of unfiltered material was with a suspension of liver obtained from a mild case of the disease.

From 1 of 2 rabbits which had recovered for about a week from a moderately severe attack, the virus was demonstrated in the unfiltered but not in the filtered

testicular emulsion; it was not demonstrated in either the filtered or the unfiltered suspension of the popliteal lymph nodes. From the 2nd rabbit with a recovery period of approximately 3 weeks after a comparatively severe attack, the right testicle and right popliteal lymph node were used; the injection of 4 rabbits with filtered and unfiltered emulsions gave negative clinical results.

Serial Passage of Virus. Acute Fulminating Infection

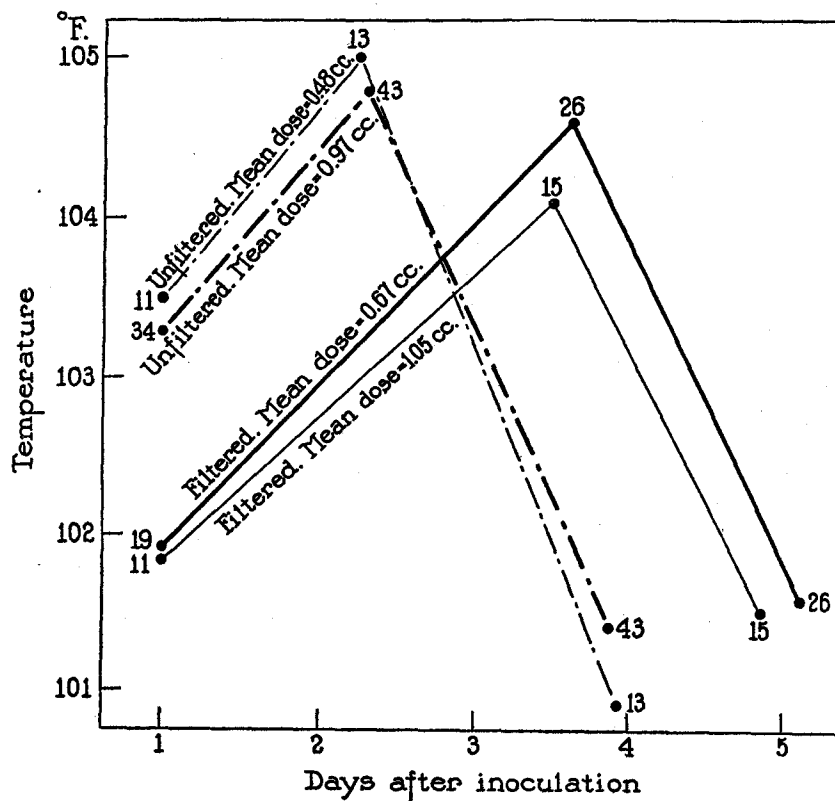
Two clinical types of the experimental disease predominated. Under conditions of the routine serial passage of virus in which filtered or unfiltered testicular tissue suspensions were injected by the intratesticular route, the infection was of an acute fulminating nature and with few exceptions resulted fatally within a week and usually within 3 to 5 days. In these cases the clinical manifestations were largely confined to the testicular lesion and to fever. In circumstances which permitted a longer survival or recovery, a wide variety of symptoms characteristic of spontaneous pox developed (1). These circumstances included the use of a very small dosage in the case of testicular tissue-virus injected intratesticularly, the employment of routes of injection other than the intratesticular, and finally, the use of tissues other than the testicle as a source of virus.

The Xy171 strain of virus which was used for most experiments was carried for 15 consecutive passages by means of bacteriologically sterile Berkefeld V filtrates of fresh or 24 hour old testicular emulsions kept at ice box temperature. Injections were made in one or both testicles of rabbits, usually in 0.5 cc. amounts. Beginning with the 9th generation and continuing for 10 serial passages, unfiltered testicular emulsions were also used. The results of the first passage from the spontaneous case (rabbit Xy171²) did not differ from those observed in subsequent generations, and with two exceptions to be discussed later, a level of high virulence was continuously maintained in both the filtered and the unfiltered series. The only essential difference in the reaction to the filtered as compared with the unfiltered inoculum was that of time, that is, the initiation and duration of the reaction and the fatal outcome occurred 1 to 3 days later in the case of rabbits inoculated with filtrates. The explanation of this difference is presumably that of a lowered virus content brought about by the process of filtration.

The three outstanding features of the acute fulminating type of reaction were a hemorrhagic orchitis with an accompanying scrotal edema, fever, and death.

² The positive results obtained with tissues from other spontaneous cases listed in the previous section were entirely similar to those obtained from rabbit Xy171. These other strains, however, were not carried for as many consecutive animal passages.

Orchitis.—The local reaction in the inoculated testicle was extremely marked. Usually within 24 hours small areas of induration or a diffuse resistance were palpable. By the 2nd or 3rd day, the testicle was greatly swollen and diffusely indurated and marked congestion, hemorrhage, and extensive necrosis were conspicuous features. The condition was accompanied by massive scrotal edema and frequently, the scrotal tissues were congested or hemorrhagic (Figs. 1 and 2). An edematous swelling of the prepuce was not uncommon (Fig. 3).



TEXT-FIG. 1. Analysis of rectal temperatures of acute rabbit pox animals, excluding those which were killed. Mean temperature 24 hours after inoculation, highest temperature observed, and last temperature taken before death. Intra-testicular inoculations of unfiltered and Berkefeld V filtered testicular tissue-virus emulsions.

Fever.—Fever regularly developed and was usually present on the 2nd or 3rd day; rectal temperatures of 105° and 106°F. were frequent. Some time before death, usually within 24 or 48 hours, an abrupt fall in the temperature to sub-normal levels was often observed. In other instances, the last temperature recorded was abnormally high.

The curves in Text-fig. 1 illustrate the character of the febrile reaction. The curves were drawn from three mean temperature values, namely, 24 hours after inoculation, the highest temperature recorded and the last temperature taken within 24 hours of death. Both filtered and unfiltered inocula are represented, but the values for all animals which were killed were excluded. It will be noted that in the case of the group injected with unfiltered emulsions the temperature reached febrile levels within the first day after inoculation, whereas the mean value for the filtrate series was still within normal limits. The highest values recorded occurred between the 2nd and 3rd day after inoculation in the case of the unfiltered group and between the 3rd and 4th day in the filtrate group. A similar time difference is seen in the values for the last temperature within 24 hours of death. Furthermore, the mean value of the highest temperatures recorded for the unfiltered series exceeded that for the animals injected with filtrates.

The mean amount of filtrate used for inoculation was 0.67 cc. and of unfiltered emulsion 0.97 cc. That the difference in the febrile reaction of the two groups was not attributable to the differences in volume of inocula but was a matter of virus content is shown by the second set of curves, the values for which were obtained by omitting the temperature records on all animals inoculated with doses of more than 2.0 and less than 0.6 cc. in the case of filtrates and of more than 0.9 cc. in the case of unfiltered material. In this calculation the mean dosage for the filtrate group, 1.05 cc., was more than twice the size of that for the unfiltered group, 0.48 cc. The second curves differ in no essential respect from the first ones.

Fatal Outcome.—The third feature of the reaction in the acute fulminating type of experimental pox was a fatal outcome. This result is shown in the following tabulation which summarizes the observations on 70 rabbits inoculated with the Xy171 strain in which the disease was allowed to progress uninterruptedly. Of these animals 49 belonged to the routine passage series and 21 were inoculated for other purposes. 5 passage animals have been omitted because of recovery or an unusually long survival; these exceptional findings are discussed in the following section.

Inoculum	No. of rabbits	No. of deaths	Mean time of death						
		per cent	days						
Filtered testicular tissue emulsion	31	100	5.84						
Unfiltered " " "	39	100	4.1						
		Day of death							
		1	2	3	4	5	6	7	8
Filtered testicular tissue emulsion	31	—	—	—	3	8	13	5	2
Unfiltered " " "	39	—	—	6	23	10	—	—	—

These observations show that in the case of intratesticular injection of Berkefeld V filtrates of testicular tissue-virus, the majority of deaths,

26 or 84 per cent, occurred on the 5th, 6th, and 7th days after inoculation, and that none occurred as early as the 3rd day. In contrast to this finding 6 or 8 per cent of the rabbits injected with unfiltered emulsions died on the 3rd day and the remainder on the 4th and 5th days.

The question of dosage in these two groups of rabbits is not considered in the above tabulation because it was found that within wide limits of dosage, comparable results were obtained. Thus, of the 31 rabbits injected with filtrates, 20 which received doses of 0.1 to 1.0 cc. were found dead in 4 to 8 days or on an average of 5.8 days. 6 rabbits given 2.0 or 3.0 cc. were found dead in 5, 6, or 7 days, or on an average of 6 days. And 5 rabbits injected with amounts of 0.0016 to 0.05 cc. were found dead in 4 to 8 days, or on an average of 6 days. The dosage of filtrates injected was more variable than that of unfiltered emulsions because most of the work with filtrates was done early in the course of the investigation when the question of dosage was being studied. Of the 39 rabbits injected with unfiltered inocula, 27 which received doses of 1.0 cc. were found dead in 3 to 5 days, or on an average of 4.1 days. 3 rabbits given doses of 2.0 cc. were found dead in 2, 3, and 5 days, or on an average of 3.3 days. 6 animals injected with 0.6 cc. doses were found dead in 3 to 5 days, or on an average of 4.2 days. 2 others given 0.4 cc. doses and 1 given 0.2 cc. were found dead in 4 and 5 days.

General Symptomatology.—In addition to the three principal features of the acute fulminating condition, there were others of a more general nature. The animals became obviously ill. Apathy and a disinclination to move were commonly seen, the fur was dull and rough, the appetite was diminished, the animal often became thin and weak, and frequently a mucous diarrhea developed (Fig. 3). Prostration of varying degree was usually seen a day or two before death. In other cases, however, there was a remarkable absence of general symptoms of illness, and the rabbit would appear to be in good condition as late as the day before death.

In animals surviving for as long as a week, a maculopapular eruption of the skin and mucocutaneous borders, a blepharitis and conjunctivitis, and a watery nasal discharge were sometimes observed. These manifestations were characteristic features of the less acute experimental infection which is discussed in the following paper (1), and consequently need only to be mentioned here.

Variations of the Acute Fulminating Infection

A fatal outcome occurred so regularly in rabbits injected intratesticularly with testicular tissue-virus emulsion even under conditions

of a small dosage that the few exceptions to the general rule merit special mention.

The true incidence of recovery cannot be determined because the disease was not allowed to progress to an uninterrupted conclusion in all cases. Stock animals were not infrequently sacrificed to furnish material for the next passage of virus and for other experiments in order to avoid bacterial contamination of tissues. They were killed on an average of a day before the mean time of death of the other animals. For example, in the routine filtrate series of the Xy171 strain with which most of the experimental work was done, 10 of the rabbits were killed at a mean time of 5 days after inoculation. It will be recalled that the mean time of death of 31 rabbits with an uninterrupted disease was 5.8 days. In the case of unfiltered inocula, 6 rabbits were killed on an average of 3.3 days after inoculation and the mean time of death of 39 animals was 4.1 days. While it cannot be said that none of these 16 rabbits might not have recovered, there is a reasonable certainty that at least the majority would have died. But among the serial passage rabbits of the Xy171 strain of virus which were not sacrificed, there were 2 instances of recovery, the explanation of which is not apparent.

In the 8th generation of the filtrate series, 3 rabbits inoculated in one testicle with 0.5, 0.25, and 0.1 cc. respectively developed typical local and febrile reactions. The 2 rabbits given the largest doses were killed on the 5th day and material from them was used to inoculate the 9th generation rabbits. The 3rd animal which was castrated on the 9th day developed a scanty papular cutaneous eruption and eventually recovered. Meanwhile, of the 9th generation 2 animals inoculated with 1.0 and 0.5 cc. of filtered virus emulsion developed a less pronounced testicular reaction than usual, the development of fever was delayed, and death did not occur until the 10th day. The 3rd rabbit of the 9th generation was inoculated with bilateral testicular injections of 0.2 cc. and 5 intracutaneous injections of dilutions of 1:10 to 1:100,000. No generalized clinical manifestations were observed except a diarrhea and death was delayed to the 18th day. In both the 8th and 9th rabbit passages, the disease was thus definitely less severe than in the previous generations. In the next 4 serial passages, the larger doses of 2 and 3 cc. were injected; these rabbits died on the 5th, 6th, and 7th days. The 15th generation comprised 2 rabbits inoculated with 2.0 and 1.0 cc. doses respectively. These animals were found dead on the 5th and 6th days. Although the numbers of animals are small and the doses employed were larger than those ordinarily used, the resumption of a fatal outcome within the time previously experienced indicates that the severity of the infection had approached or had resumed its former high level.

The only other instance of recovery after intratesticular injection of the Xy171 strain of virus in testicular tissue occurred after the inoculation of unfiltered material. 2 rabbits injected in both testicles with 0.5 cc. doses developed a typical local and febrile reaction. 1 animal died on the 5th day; the other developed a generalized cutaneous papular eruption and recovered. 2 other rabbits inocu-

lated intradermally with the same specimen of virus died on the 8th and 10th days respectively.

With the AB18 strain of virus³ there were 4 instances of recovery after the intratesticular injection of testicular tissue emulsions which had been filtered twice through Berkefeld V candles before a sterile filtrate was obtained. 3 of the cases were observed in a group of 5 rabbits which were inoculated in one testicle with 0.1 cc. doses. The first rabbit injected with full strength filtrate recovered after the development of a typical local and febrile reaction, a metastatic orchitis, typical papular lesions of the lips and nose, and involvement of the eyes. The other rabbits were injected with dilutions of 1:10, 1:100, 1:1,000, and 1:10,000 respectively. All developed well marked testicular and febrile reactions; the 2 injected with the lower dilutions died on the 7th and 13th days while those which received the higher dilutions recovered. The filtrate was kept in the refrigerator and 7 days after the first inoculation, a rabbit was injected in one testicle with 0.1 cc. and 6 days later, or 13 days after the first inoculation, 2 rabbits were injected in one testicle with 0.5 and 0.1 cc. respectively. Full strength filtrate was used for these injections. All 3 rabbits developed fever and a typical orchitis. The first was ill and was killed on the 8th day; the others were found dead on the 9th day. The 4th instance of recovery was a rabbit inoculated in one testicle with 0.1 cc. of a testicular tissue-virus emulsion which had been filtered through 2 Berkefeld V candles. This animal developed typical local and generalized manifestations of pox and was severely ill, but recovery eventually took place.

As far as the results on these 4 animals inoculated with the AB18 strain of virus are concerned, it appears that a sufficiently small dosage was obtained by means of a double filtration and in 2 cases with the added factor of virus dilution, so that the usual fatal outcome was avoided although it was potent enough to produce unmistakable clinical symptoms. But this explanation cannot be applied to the 2 instances of recovery in the Xy171 strain for dilution of virus was not made. No reason for this unusual result could be found at the time. An explanation based on individual animal variation with increased resistance does not seem applicable to the results observed in connection with the first of these cases. It is possible that elimination of virus brought about by castration on the 9th day was sufficient to turn the tide toward recovery in an animal that had already survived

³ There was no indication that the AB18 strain of virus differed from the Xy171 strain unless the present instances of recovery be so considered. Fewer experiments were done with the strain so that the incidence of recoveries was relatively much higher. In the absence of a sufficient number of comparable tests, however, the difference in results cannot be ascribed to an essential difference in the strains.

longer than the average time. On the other hand, the decreased severity of the infection was continued over to the next passage animals which were not inoculated with material from the rabbit which eventually recovered, and this finding was in turn followed by an apparent resumption of disease severity. That these results may have been brought about by changes in virulence of the virus should be considered, but no evidence for such an explanation is apparent.

Retention of Virulence of Virus in Stored Tissue

The virulence of the virus was retained under conditions of the storage of tissues in the refrigerator, either with or without the addition of 50 per cent glycerol for as long as 127 days, as shown by the results obtained in 14 experiments, 12 with the Xy171 strain and 1 each with the AB18 and X667 strains. A total of 28 rabbits were injected with these tissues.

Six experiments were carried out with Berkefeld V filtrates of tissues stored in the ice box for 13 to 67 days (Table I). In Experiments 2, 3, 4, and 5 the emulsions for injection were prepared from testicular tissue preserved in 50 per cent glycerol; in Experiment 1 the testicular tissue filtrate itself without the addition of glycerol was kept in the ice box and a similar procedure with a skin filtrate was followed in Experiment 6. The tissues were obtained from 2 spontaneous cases, rabbits Xy171 and X667, from a rabbit of the 2nd generation of the Xy171 strain, and from a rabbit of the 3rd generation of the AB18 strain. The 6 specimens of virus filtrates were injected intratesticularly in 11 rabbits in amounts ranging from 0.3 to 3.0 cc.

The results showed clinical evidence of infection in 9 rabbits; there were 5 deaths and 1 animal with outspoken symptoms was killed. The average time of death was 9.5 days after inoculation. There were 3 recoveries. In 2 rabbits, 1 each in Experiments 4 and 6, no clinical signs of infection were observed although in both cases another rabbit inoculated with the same filtrate developed pox. In Experiment 4 an animal injected with 0.4 cc. was clinically negative; the other injected with 0.8 cc. was clinically positive and was found dead on the 12th day.

Of particular interest was the result obtained with a skin filtrate derived from a spontaneous case of pox (Experiment 6). 2 rabbits inoculated intratesticularly

TABLE I
Retention of Virulence in Stored Virus
Testicular Tissue Preserved in 50 Per Cent Glycerol in Ice Box. Uni- and Bilateral
Intratesticular Inoculation

Experiment	Strain	Generation	Time after inocu-	Length of storage	Inoculum	Rabbits inocu-	Total dosage		Results
			lation	days			days	cc.	
1	AB18	III	7	13*	Filtered 2×	A	0.5	9	Found dead
							B	0.1	9
2	Xy171	Spontaneous case	—	14	"	A	1.0	8	" "
3	"	II	4	25	"	A	2.0		Recovered
							B	1.0	
4	"	Spontaneous case	—	28	"	A	0.8	12	Found dead
							B	0.4	
5	"	" "	—	32	" 2×	A	1.0		Recovered
							B	0.3	19
6	X667	" "	—	67†	"	A	3.0		No clinical evidence of infection
							B	2.0	9
7	Xy171	VI	4	18	Not filtered	A	2.0	4	Found dead
							B	1.0	3
8	"	IV	8	26	" "	A	2.0	4	Found dead
							B	1.0	5
9	"	XV	5	65	" "	A	1.0	4	Ill. Killed
							B	1.0	4
10	"	III	6	72	" "	A	3.0	3	" "
							B	2.0	4

* Filtrate stored in ice box without glycerol.

† Skin filtrate stored without glycerol in ice box.

TABLE I—*Concluded*

Experiment	Strain	Generation	Time after inoculation	Length of storage	Inoculum	Rabbits inoculated	Total dosage		Results
			days	days			cc.	days	
11	Xy171	II	8	78†	Not filtered	A	3.0	7	Moribund. Killed
						B	2.0	6	Found dead
12	"	I	7	86	" "	A	3.0	9	Critically ill. Killed
						B	2.0	6	Found dead
13	"	Spontaneous case	—	93	" "	A	3.0	6	" "
						B	2.0	5	" "
14	"	I	5	127	" "	A	2.0	4	Died
						B	1.6	4	Ill. Killed
						C	1.0	4	" "

† Tissue stored in taped Petri dish without glycerol.

with the fresh filtrate in doses of 0.5 and 0.2 cc. respectively developed a typical infection and died on the 7th and 14th days. The filtrate was kept in a taped flask in the ice box without the addition of glycerol or other preservative for 67 days and was then used for the inoculation of 2 rabbits in 2.0 and 3.0 cc. amounts injected (Experiment 6). In the animal injected with the larger dose no clinical evidence of infection was observed but the other developed a marked orchitis, a pronounced cutaneous eruption, and various constitutional symptoms and was killed on the 9th day. It is probable that the reason for the negative result in the former case was that the animal was an immune since it was one of the few rabbits used in the experimental work which came from the colony in which pox had occurred.

Seven specimens of unfiltered emulsions prepared from testicular tissues stored from 18 to 127 days in the ice box were injected intratesticularly in 17 rabbits. The dosage varied from 1.0 to 3.0 cc. A typical marked hemorrhagic orchitis and fever developed in each instance, and 10 of the animals died in 3 to 6 days. 5 rabbits were critically ill on the 3rd, 4th, and 9th days and were killed. The testicular tissue employed in these experiments was obtained from the original Xy171 case and from rabbits of the 1st, 3rd, 4th, 6th, and 15th generations of the serial passage of this strain. In Experiment 11 the intact testicle obtained from a 2nd generation rabbit 8 days after

inoculation was kept in a taped Petri dish without glycerol for 78 days at ice box temperature. 2 rabbits were injected with the unfiltered emulsion prepared from this tissue; one animal was found dead 6 days later and the other was moribund on the 7th day and killed.

The clinical findings of these experiments were indistinguishable from those in which fresh tissue-virus was employed, and it thus appeared that storage of tissues in glycerol at refrigerator temperature for as long as 127 days was not associated with a lowered activity of the virus. In 2 specimens stored without glycerol for 67 and 78 days, one a skin filtrate and the other testicular tissue, a potent virus was demonstrated. The findings indicate, as was also found with fresh tissue-virus inocula, that the potency of Berkefeld V filtrates was not as great as was that of unfiltered emulsions. The results on the rabbits injected with filtrates prepared from stored tissues were more irregular than were those of the unfiltered group and hence it is possible that conditions of storage lead to diminution of virus, either quantitatively or qualitatively, which is not apparent in stored unfiltered tissue emulsions.

SUMMARY AND CONCLUSIONS

A filterable agent was isolated from 7 cases of spontaneous rabbit pox by the intratesticular injection in rabbits of a variety of tissues. The virus was transmitted for 15 consecutive testicle to testicle passages in rabbits by Berkefeld V filtrates of testicular tissue emulsions. Unfiltered emulsions were more potent than filtrates. The virulence of the virus was maintained by ice box storage of infected tissues for as long as 127 days. The pathogenic properties of the virus persisted under conditions of animal passage for the 9 months from January to October, 1933, covered by these studies.

The reaction produced in rabbits by the inoculation of tissue-virus emulsions was of two principal types, the first of which has been described and analyzed in the present paper. In the case of intratesticular injections, particularly of testicular tissue inocula, an acute fulminating and rapidly fatal condition regularly developed. The outstanding features were a massive hemorrhagic orchitis with marked scrotal edema, fever, and death within a week. The second type of reaction which is taken up in the next paper of this series (1) was

observed under conditions in which the animal survived a week or longer. Occasional examples occurred in rabbits of the so called intra-testicular series. The reaction was characterized by the development of a disease syndrome with a diversity of clinical manifestations which, it may be stated here for the sake of continuity, was indistinguishable from spontaneous rabbit pox.

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EXPLANATION OF PLATE 20

FIG. 1. Marked orchitis and beginning scrotal edema 3 days after bilateral testicular inoculation of 0.2 cc. Berkefeld V filtrate of testicular tissue emulsion; 3rd generation of filtrate series of Xy171 strain of pox virus. Rabbit found dead on the 6th day.

FIG. 2. Marked hemorrhagic orchitis and scrotal edema 4 days after bilateral testicular inoculation of 0.5 cc. Berkefeld V filtrate of testicular tissue emulsion; 2nd generation of filtrate series of Xy171 strain of pox virus. Rabbit found dead on the 8th day.

FIG. 3. Marked orchitis, edema of scrota and prepuce; mucous rectal cast. 8 days after bilateral testicular inoculation of 0.04 cc. Berkefeld V filtrate of testicular tissue emulsion; 2nd generation of filtrate series of Xy171 strain of pox virus. Rabbit died on the 8th day.



Photographed by Joseph B. Haulenbeck

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