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PURE CULTIVATION IN VIVO OF VACCINE VIRUS FREE FROM BACTERIA.*

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PLATES 39 TO 50.

In spite of much effort no method has, up to the present time, been perfected by which vaccine virus can be propagated in a pure state, free from contaminating bacteria. The method of propagating vaccine virus universally practiced today consists in transmitting the virus from the skin of one calf to that of another. Although the inoculation of the virus is carried out under precautions as strictly aseptic as possible, the fresh product nevertheless yielded by the skin contains a not inconsiderable number of different bacteria derived from the skin surfaces, the air, etc.

The employment of glycerin as an elective germicide against the non-spore-bearing bacteria contained in fresh, or so called "green," vaccine pulp results in a great reduction both in number and variety of the contaminating microörganisms, without at the same time seriously impairing the activity of the vaccine virus (1). After contact with concentrated glycerin, in a refrigerator, ranging from one to three months, the virus usually is freed from most of the bacteria and becomes "ripe" for practical use in vaccination on human beings.

Different samples of the ripe vaccine preparations, as issued from various authorized sources, vary in their activities as well as in their germ contents. Among the organisms which may be encountered in such preparations are the following: streptococci, staphylococci, *Bacillus coli, Bacillus welchii, Bacillus xerosis, Bacillus subtilis,* and some other aerobic and anaerobic bacteria (2). The action of glycerin on bacterial spores, which may also be present in the pulp, is

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almost nil. Moreover, the prolonged action of glycerin tends gradually to reduce the activity of the vaccine virus itself, so that the fresh vaccine pulp forfeits some of its efficacy while undergoing the "ripening" process. At the end of the ripening process, which consumes from one to three months, the virus usually still contains a certain number of bacteria; bacteria-free virus is practically never produced by glycerination. But the residue of living bacteria, fortunately, is to all intents and purposes quite harmless and practically to be disregarded. And yet it is apparent that it is not only desirable to eliminate all bacteria from vaccine virus, but the saving of the time lost in "ripening" and the preservation unimpaired by glycerin of the full strength of the virus are also highly important considerations.

HISTORICAL.

The observations of Béraud (3), Weigert (4), Chiari (5, 6), Councilman, Magrath, and Brinkerhoff (7), and others (8) indicated that the variola virus localizes in different viscera and organs; the vaccine virus, on the other hand, according to Calmette and Guérin (9), and von Prowazek and Yamamoto (10), exhibits a high degree of affinity for pavement epithelium. When the vaccine virus is introduced directly into the blood or peritoneal cavity, it lodges in the epithelia of the skin and mucous surfaces, provided they are injured within a few hours of the inoculation, while in a few hours more it has completely disappeared from the circulation or the peritoneum. Should the epithelium not take it up, it is entirely lost.

In order to test the question whether still other epithelial cells take up the virus, Henseval and Convent (11) conducted in 1910 a series of experiments on the testes of rabbits. The virus that they employed contained staphylococci and *Bacillus subtilis*.[1] The effect was to produce some swelling, but no induration of the organ. Five to seven days after inoculation the testes were extirpated, emulsified in 66 per cent. glycerin, and tested on the shaved skin of rabbits. The activity was less than that of the original material. From the first testicular emulsion a second set of rabbits was injected; the swelling and induration produced was greater, but the activity of the emulsion was no stronger. No attempt was made either to free the vaccine of bacteria or to continue the testicular passages. Later, Paschen (12) injected vaccine (its bacterial content is not stated) into the testicle of a rabbit and noted the production of necrosis and cellular infiltration. As early as 1904 Tyzzer (13) attempted to obtain characteristic vaccinia lesions by inoculation of the testis of rabbits and a calf; the result was regarded as an acute inflammatory reaction. Von Prowazek and Miyaji (14) state that vaccine virus injected into the testicle of the rabbit can

[1] The number of contaminating bacteria in the three samples employed was 4, 10, and 25,000 per 0.01 c.c., respectively.

still be detected two days later, and that local necrosis of the tissue appears at the inoculation site. Nothing is said of the bacterial content.

That the testicle of the rabbit affords a favorable site for the multiplication of parasitic microörganisms can be inferred from the experiments of Parodi (15) and later of Uhlenhuth and Mulzer (16) with *Treponema pallidum,* of Nichols (17) with *Treponema pertenue,* and of a host of later workers with *Treponema pallidum* (18). I therefore turned my attention to the cultivation of pure strains of vaccine virus in the rabbit's testicle.

PURIFICATION OF VACCINE VIRUS PRIOR TO CULTIVATION.

At the outset and before describing the details of the experiments on the cultivation of vaccine virus it is necessary to point out a material difference between the employment of the rabbit's testicle for *Treponema pallidum* and for vaccine virus. In the case of the former the multiplication of the spirochætæ takes place slowly, so that the contaminating bacteria which are carried into the testicle with the inoculated fragments of syphilitic tissue may in time be suppressed by the bactericidal action of the vigorous tissues. On that account pure strains of the pallidum may be thus developed after a few passages. Vaccine virus, on the other hand, multiplies quickly, and the inflammatory reaction and necrosis of tissue which ensue quickly suppress the bactericidal process of the testicular tissues and place them in a favorable state for bacterial development. On that account the virus must first be freed from the bacterial contamination by other means.

Preliminary Purification.—Several antiseptic or disinfecting substances may be employed for reducing or removing contaminating bacteria. Glycerin is universally employed in practice to reduce the number, because of the small effect which it exerts on the virus itself. At low temperatures the antiseptic action is too weak to eliminate all bacteria, but at 37° C. in 60 per cent. strength all bacteria except spore-bearing species may be destroyed in a few days. Hence it is suitable for the purification when the virus is devoid of sporogenous bacteria. Addition of 1 per cent. phenol alone or 1 per cent. phenol and 60 per cent. glycerin both to the emulsion brings about a quicker elimination of bacteria, but this is accompanied by a more rapid deterioration of the virus itself. The same is true with the addition of one part per thousand of oil of cloves to 60 per cent. glycerin.

Recently Fornet (19), who has experimented with cultivation *in vitro* of vaccine virus, has recommended ether for removing the bacteria which do not possess spores. An emulsion of the virus can be freed of all bacteria except sporogenous ones by being shaken at room temperature for forty-eight hours with an excess of ether. The activity of the virus is, however, considerably diminished by ether treatment. By the ether method a vaccine pulp devoid of spore-bearing bacteria obtained as follows was secured free of all bacteria, and hence was suitable for cultivation in a pure state *in vivo*.

A sample[2] of glycerinated virus free from sporogenous bacteria was incubated at 37° C. for two days or longer, in order to destroy practically all the bacteria still present in it. The skin on the dorsal side of a rabbit was shaved and thoroughly cleansed with soap and rinsed with sterile distilled water. It was again shaved closely, after which the glycerinated virus was applied, in such strength as to produce separate eruptions. The vaccinated surface is protected from contamination by means of a sterile bandage. On the fourth or fifth day the bandage was removed and the surface washed first with absolute alcohol and then with ether. Several vesicles were selected and each was cleansed with 5 per cent. lysol solution and washed alternately with absolute alcohol and ether on sterile gauze. The eruptions were scraped with the edge of a sharp scalpel and the scrapings emulsified in several cubic centimeters of sterile saline solution. The emulsion was mixed with several volumes of ether and shaken in a sealed vessel for varying periods of time at room temperature. Samples were removed at the expiration of 1, 2, 4, 8, 12, 24, and 48 hours, from each of which cultures were prepared. When spores are absent sterility is usually obtained in twelve hours. The vaccinal activity falls at times to one-fifth of the original strength, but the characteristic properties remain unaltered.

TESTICULAR CULTIVATION IN RABBITS.

The vaccine emulsion so prepared is employed for the intratesticular inoculation of rabbits. Rabbits with well developed testicles should be chosen. After ether anesthesia an assistant holds the animal and fixes the testicles to prevent their withdrawal into the peritoneal cavity. The scrotal skin which is tightly stretched over the testicle is sterilized with 5 per cent. lysol solution and then painted with tincture of iodine. The operator next inserts the needle of a

[2] Several samples of the vaccine virus employed in the present investigation were furnished me by Dr. F. S. Fielder, Assistant Director of the Vaccine Laboratory of the Department of Health of the City of New York, to whom I wish to express my gratitude.

sterile syringe containing a 1 to 10 or 1 to 20 dilution of the emulsion into the testicular parenchyma along the long axis. The point of the needle is prevented from passing through the parenchyma to the tunica vaginalis. The contents are now gently forced out of the syringe into the organ at different regions by turning the direction of the needle. About one cubic centimeter of emulsion is injected into a testicle weighing from two to three grams. The organ is gently massaged to distribute the virus throughout the entire organ. The operation is practically painless.

The method just described is employed for the inoculation of the testicular strains of the virus, in which case an emulsion of testicle previously inoculated with the virus is used. The stock emulsion is prepared by grinding up the aseptically removed organs with sterile saline or 60 per cent. glycerin solution in the proportion of one gram of the tissue to two or three cubic centimeters of the fluid and any dilution of it; about 1 to 10 or 1 to 20 in saline is prepared for the purpose of the injection.

The testing for bacteria in the emulsion is an important point. Cultures are set up with plain and glucose bouillon, ascitic fluid with and without bouillon, and ascitic fluid with a piece of fresh sterile rabbit kidney with and without a layer of sterile paraffin oil. The cultures are incubated at 37° C. for three to four days, and then subcultures on plain and ascitic glucose slant agar, deep layer glucose agar, and ascitic tissue deep layer agar are made.[3] Film preparations stained by Gram or by Giemsa are also examined under the microscope, both from the cultures and from the testicles removed from the animal. In order to avoid occasional bacterial infection it has been found well to inoculate both testicles of each animal. The skin may be vaccinated at the same time. It is, moreover, advisable to use at least two rabbits for each transfer, because sometimes a rabbit reacts poorly to the vaccinal inoculation of both skin and testicle; but once the virulence of the virus has reached a certain height this precaution is no longer necessary.

[3] In the latter part of the present work initial culture in tissue bouillon and subcultures in glucose agar (deep) and plain agar (plate) were found to be sufficient.

RESULTS OF EXPERIMENTS WITH RABBITS.

As the accompanying table shows, the passage of the testicular virus from animal to animal offered some difficulty until it had been carried on for a number of generations. In this experiment success was not assured until the virus had been transferred about ten times. That this result was due to increase in virulence of the virus in the later generations may be deduced from the greater activity manifested when it was tested upon the skin, as well as from the greater severity of the reactions in the testicles themselves (table I).

TABLE I.

Date of transfer.	Generation.	Results.	Date of transfer.	Generation.	Results.
1914					
Jan. 29–Feb. 2	1	+	Sept. 28–Oct. 3	32	++
Feb. 2– 9	2	+	Oct. 3– 7	33	++
Feb. 9–14	3	+	Oct. 7–12	34	++
Feb. 14–22	4	+	Oct. 12–16	35	++
Feb. 23–27	5	+[4]	Oct. 16–20	36	++
Mar. 10–16	6	+[5]	Oct. 20–25	37	+++
Apr. 22–25	7	+[6]	Oct. 25–29	38	+++
May 1– 6	8	+	Oct. 29–Nov. 3	39	+++
May 25–29	9	+	Nov. 3– 7	40	+++
May 29–June 3	10	++	Nov. 7–12	41	+++
June 3– 6	11	++	Nov. 12–16	42	+++
June 8–12	12	++	Nov. 16–21	43	+++
June 12–15	13	++[7]	Nov. 21–26	44	+++
June 18–25	14	++	Nov. 26–30	45	+++
June 25–July 2	15	++	Nov. 30–Dec. 5	46	+++
July 2– 8	16	++	Dec. 5– 9	47	+++
July 8–13	17	++	Dec. 9–13	48	+++
July 13–16	18	++	Dec. 13–16	49	+++
July 17–20	19	++	Dec. 16–20	50	+++
July 20–23	20	++	Dec. 20–24	51	+++
July 26–30	21	++	Dec. 27–31	52	+++
July 30–Aug. 3	22	++	1915		
Aug. 4– 7	23	++	Jan. 4– 8	53	+++
Aug. 11–17	24	++	Jan. 8–12	54	+++
Aug. 17–22	25	++	Jan. 15–19	55	+++
Sept. 1– 4	26	++	Jan. 23–27	56	+++
Sept. 7–10	27	++	Jan. 31–Feb. 4	57	+++
Sept. 12–16	28	++	Feb. 8–12	58	+++
Sept. 16–20	29	++	Feb. 16–20	59	+++
Sept. 21–24	30	++	Feb. 24–28	60	+++
Sept. 24–28	31	++			

[4] From this material two further generations were carried on and then lost, so it was necessary to come back to this generation and to start again.

[5] From this two generations were carried on and lost, necessitating a return.

[6] Only one out of several rabbits gave good results.

[7] Two rabbits were inoculated with this material, but only one gave a satisfactory result.

The testicular strain indicated in the table has passed through sixty successive generations within twelve months. It is interesting to find that considerable resistance to testicular adaptation was exhibited at the seventh transfer, after which no serious obstacle was met in carrying on the passages.

TESTICULAR VACCINAL PROCESSES IN RABBITS.

In order to follow the course of the vaccinal processes in the testicular tissue the following experiments were carried out.

Thirteen male rabbits were inoculated into each testicle with 1 c.c. of a 1:20 dilution of the saline testicular emulsion derived from the rabbit inoculated with the thirtieth generation of the testicular strain and castrated under ether anesthesia on the fourth day.

The testicles of these animals were removed one after the other and tested successively for activity on the skin, cornea, and testicles of normal rabbits every twenty-four hours, over a period of eighteen days, and then after 3, 4, 5, and 8 weeks.

Table II shows the results obtained.

During the first twenty-four hours the testicle presents little change, except that microscopic foci of infiltration of polynuclear leucocytes and exudate are observed in the interstitial spaces (figure 32, compare with normal, figures 30 and 31). At the end of forty-eight hours the swelling and induration of the organ begin to increase rapidly and the testicle becomes congested and edematous (figure 2). The content of the virus, which was almost zero after twenty-four hours, now reaches about 100 times that found at the end of the first day. When examined in sections an enormous leucocytic infiltration is seen in the interstitial tissues, and some leucocytes are contained in tubules (figure 33). The testicular cells are hydropic and fill up the tubular lumen. At the end of three days the infiltration has increased in intensity and extent (figures 3 and 18, compare with normal, figure 17), and the vaccinal activity has risen to at least 300 times that present twenty-four hours and about three times that present forty-eight hours after the inoculation.

The external changes present in the four day specimen (figures 4 and 34) resemble those occurring in the three day specimen, except that the testicle is more compact and less elastic and the amount of

TABLE II.

Testicles removed after	Gross appearance of specimens.	Tests for activity of emulsions on rabbits.			
		Dilution of emulsion.	Skin.	Cornea.	Testicle.
24 hrs.	Almost no swelling; traumatic hemorrhages	1 : 10	1 eruption	−	+
		1 : 100	−	.....	.....
		1 : 1,000	−	.....	−
48 hrs.	Vascular injection, edema, and moderate induration (figure 2)	1 : 10	++	++	++
		1 : 100	+	−	.....
		1 : 1,000	<+	−	.....
		1 : 10,000	−	−	−
3 dys.	Marked induration, great congestion, greyish mottling, edema (figure 3)	1 : 10	++	++	++
		1 : 100	++	+	.....
		1 : 1,000	+	−	.....
		1 : 10,000	−	−	+
4 dys.	Severe congestion, edema, induration and swelling; numerous yellowish grey spots on the organ (figure 4)	1 : 10	Confluent	++	++
		1 : 100	+++	+	.....
		1 : 1,000	++	+	.....
		1 : 10,000	<+	−	++
5 dys.	Similar to the last (figure 1)	1 : 10	Confluent	++	++
		1 : 100	Confluent	++	.....
		1 : 1,000	++	+	.....
		1 : 10,000	+	?	++
5 dys.	Similar to the last (figure 5)	1 : 10	Confluent	++	++
		1 : 100	+++	+	.....
		1 : 1,000	++	−	.....
		1 : 10,000	−	?	++
5 dys.	Extensive hemorrhages; otherwise similar to the last	1 : 10	Confluent	++	++
		1 : 100	++	+	.....
		1 : 1,000	+	−	.....
		1 : 10,000	−	−	+
6 dys.	Marked induration, but somewhat less than on the previous day. Less edema (figure 6)	1 : 10	Confluent	++	++
		1 : 100	++	+	.....
		1 : 1,000	+	−	.....
		1 : 10,000	−	−	+
7 dys.	Induration somewhat less than on the preceding day. Yellowish grey specks prominent on the surface (figure 7)	1 : 10	++	++	++
		1 : 100	++	+	.....
		1 : 1,000	+	−	.....
		1 : 10,000	−	−	+
8 dys.	General induration disappearing; organ flabby; disseminated yellowish grey spots (figure 8)	1 : 10	+	++	++
		1 : 100	<+	−	.....
		1 : 1,000	−	−	.....
		1 : 10,000	−	−	−
9 dys.	Induration gone; soft and pale, slightly below original volume; some focal infiltration (figure 9)	1 : 10	+	+	++
		1 : 100	<+	−	.....
		1 : 1,000	<+	.....	.....
		1 : 10,000	−	.....	−
10 dys.	Induration gone; general atrophy; some focal induration (figure 10)	1 : 10	+	+	++
		1 : 100	<+	−	.....
		1 : 1,000	<+	.....	.....
		1 : 10,000	−	.....	−
11 dys.	Marked reduction in size; no induration (figure 11)	1 : 10	+	+	++
		1 : 100	<+	−	.....
		1 : 1,000	−	.....	.....
		1 : 10,000	−	.....	−
12 dys.	No induration, soft and pale, much smaller than original (figure 12)	1 : 10	+	+	+
		1 : 100	−	−	.....
		1 : 1,000	−	.....	.....
		1 : 10,000	−	.....	−

TABLE II.—*Concluded.*

Testicles removed after	Gross appearance of specimens.	Tests for activity of emulsions on rabbits.			
		Dilution of emulsion.	Skin.	Cornea.	Testicle.
13 dys.	Normal in size, pale and soft; some greyish spots	1 : 2	+	+	.....
		1 : 10	<+	.....	+
		1 : 20	−	−	.....
		1 : 100	−	.....	.....
14 dys.	Similar to the last	1 : 2	−	−	.....
		1 : 10	−	.....	+
		1 : 20	−	.....	.....
15 dys.	Small, pale, flabby; few greyish foci	1 : 2	+	+	.....
		1 : 10	<+	−	+
		1 : 20	<+	−	.....
16 dys.	Similar to the last	1 : 2	+	+	.....
		1 : 10	<+	−	+
		1 : 20	<+	−	.....
17 dys.	Marked atrophy	1 : 2	−	−	+
		1 : 10	−	−	.....
		1 : 20	−	−	.....
18 dys.	Similar to the last	1 : 2	−	−	−
		1 : 10	−	−	−
21 dys.	Small, fibrous; few minute foci of infiltration	1 : 2	<+	+	+
		1 : 10	−	−	.....
28 dys.	Similar to the last	1 : 2	1 eruption	−	+
		1 : 10	−	−	.....
35 dys.	Marked atrophy; small area of necrotic tissue present	1 : 2	−	−	−
		1 : 10	−	−	−
56 dys.	One testicle reduced to a small fibrous mass; the other less atrophied	1 : 2	−	−	−
		1 : 10	−	−	−

exudate in the tunica vaginalis and in the testicular tissue itself is more copious. The color of the testis has become purplish red, spotted here and there with irregular yellowish areas of different dimensions (figures 13 to 16). The organ is easily torn. The sections show numerous groups of several tubules each, which have lost the property of taking up the basic stain, indicating a total necrosis of the structures (figures 19 and 36). These changes are not universal, since areas exist in which no apparent serious alteration has taken place. At this stage the vaccinal power is nearly three times as great as it was at the end of three days, and at least 1,000 times as great as at the end of the twenty-four hour period. The changes in the five day specimen are about the same as those of the four day specimen. The vaccinal activity seems now to have attained its maximum height, since almost confluent eruptions on the skin of rabbits are yielded in dilutions of 1 to 1,000. The sections indicate beginning disintegration of the leucocytic elements and of

affected tubular cells (figures 5, 29, 29 a, and 35; compare with normal, figure 28).

At the expiration of six days the testicle has become of softer consistency (figure 6), and the edema and cellular infiltration have begun to recede. Microscopically foci of small round cells in the infiltrated areas and fibrin masses are distinguished (figures 37 and 38). The vaccinal activity is similar to that of the five day specimen. After seven days the resolution of the infiltration proceeds more rapidly (figures 7 and 20), while the vaccinal activity is less than that of the preceding day. From now on the testicles diminish rapidly in volume, so that the ten day specimen is below normal in size (figures 8, 9, 10, 21, and 22). The organs are now pale and of soft texture. The activity of the virus is much less than it was at the end of seven days. At the end of eleven days it is still weaker. From twelve to eighteen days the shrinkage of the organ continues and the sections show loss of testicular cells (figures 11, 12, 23 to 27, and 39 to 44) and collapse of the tubules. The virus has now disappeared wholly or almost wholly, and in some instances no vaccinal effect could be obtained in any concentration. Hence this period may be regarded as that of elimination of the virus, while the exact moment of disappearance probably depends upon the quantity of the virus originally developed and the degree of immunity displayed. At later periods, namely after five weeks, no virus could be detected, while the atrophy of the testicular parenchyma may be complete, so that a fibrous mass containing a few unusually thin tubules, lined with a single layer of epithelial cells may alone remain (figures 27 and 45).

The tunica vaginalis is often intensely infiltrated with polynuclear leucocytes (figure 46) and contains a considerable amount of the virus; apparently the virus multiplies here as it does in other epithelial cells. The epididymis shows a slight infiltration only during the early period.

Spermatogenesis ceases quickly and the various sperm cells quickly degenerate under the influence of the vaccinal process.

EFFECTS OF THE TESTICULAR VACCINAL STRAIN ON THE SKIN AND CORNEA.

The testicular strains of the virus were employed to inoculate the shaved skin and scarified corneal surfaces of rabbits. The effects of the vaccinations so carried out may be followed in table III. They will be recognized as characteristic of the effects produced by active vaccine virus as usually prepared in the calf. The microscopical features of the process are also typical, including the presence of the Guarnieri, or vaccine, bodies so called (figures 49 and 50).

TABLE III.

At the end of	Average course in rabbits.		
	Skin.	Cornea.	Temperature.[8]
24 hrs.	Diffuse reddening	Slight swelling	38.9°
48 hrs.	Mottled reddish areas	Slight turbidity	39.5°
3 dys.	Fairly defined flat erythema, in part confluent	Distinct turbidity	40.0°
4 dys.	In part confluent, mostly discrete raised papules with induration and areola	Advancing in degree and extent	40.8°
5 dys.	Distinct raised round vesicles with areola	Sometimes ulceration	40.2°
6 dys.	Development into pustular eruptions	Ulceration	39.8°
7 dys.	Pustules with scanty content; tendency to dry up	No change	38.8°
8 dys.	Inflammatory processes disappearing	Ulcer persists	38.9°
9 dys.	Stage of crust formation and desquamation	Ulcer persists	38.7°
10 dys.	Stage of crust formation and desquamation	Ulcer persists	38.8°

EFFECTS OF VARYING CONCENTRATION OF TESTICULAR VIRUS UPON THE SKIN, CORNEA, AND TESTICLE.

In order to determine the sensitiveness of the skin, cornea, and testicle to vaccine virus, emulsions of the testicular virus of different concentrations were prepared and inoculated into the several parts mentioned.

For this purpose a specimen of testicle representing the thirty-first passage or generation was employed. The dilutions in sterile saline were as follows: 1 to 10, 1 to 100, 1 to 300, 1 to 1,000, 1 to 3,000, 1 to 10,000, 1 to 30,000, 1 to 100,000. The skin and cornea were

[8] Average from fifteen rabbits.

inoculated in the usual manner, and 0.5 of a cubic centimeter was injected into the testicle.

The result is summarized in table IV and may be stated as follows: Up to a dilution of 1 to 300 the testicular emulsion causes marked vaccinal reactions in the skin, cornea, and testis. The skin surface still reacts slightly at the 1 to 1,000 dilution, but the cornea does not. Beyond the 1 to 1,000 dilution the skin reacts no longer, while the testicle continues to react even to dilutions of 1 to 100,000, the limit of the experiment. However, dilutions of 1 to 3,000 and higher retarded somewhat the testicular reaction, which in the end

TABLE IV.

Dilution of emulsion.	Reactions following the inoculation of different dilutions of the emulsion as indicated in the first column.			Tests of activity of emulsions prepared from testicles[9] which had been injected with different dilutions.			
	Skin.	Cornea.	Testicle.	Dilution of emulsion of each testicle.	Skin.	Cornea.	Testicle.
1 : 10	+++	+	3 dys. already marked induration and edema	1 : 100	+	+	++
				1 : 1,000	+	.....	++
1 : 100	++	+	3 dys. already marked induration and edema	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 300	++	+	3 dys. already marked induration and edema	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 1,000	+	−	3 dys. already marked induration and edema	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 3,000	−	−	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 10,000	−	−	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 30,000	−	−	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 100,000	−	−	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
				1 : 1,000	+	.....	++

was quite identical with that produced by the stronger concentration. In an experiment made somewhat later and after the testicular strain of the virus had been intensified by repeated passages, a skin reaction of small degree but of characteristic kind could be elicited in the 1 to 100,000 dilution.

This experiment serves also to bring out distinctly the fact of

[9] The testicles were removed from the animals on the sixth day after the inoculation.

the rapid multiplication of the virus in the testicle of rabbits, and is shown in the second half of table IV. The vaccinal effect upon the skin, cornea, and testicle became essentially the same in kind and degree, irrespective of the concentration of the virus emulsion injected originally into the testicle. At the expiration of six days the testicles inoculated with virus in the 1 to 100,000 dilution yielded a virus emulsion as active as the original testicular material employed for preparing the stock emulsion. The rapidity and ease of multiplication of the virus within the testicle is well illustrated by this experiment. The causes of the small variations in effect as indicated in the table as occurring between emulsions of testicles receiving the higher and lower dilutions of virus are not at once apparent. Possibly certain rabbits restrain the multiplication of the virus more than others; possibly concomitant immunity reactions come into play.

TESTICULAR CULTIVATION IN BULLS.

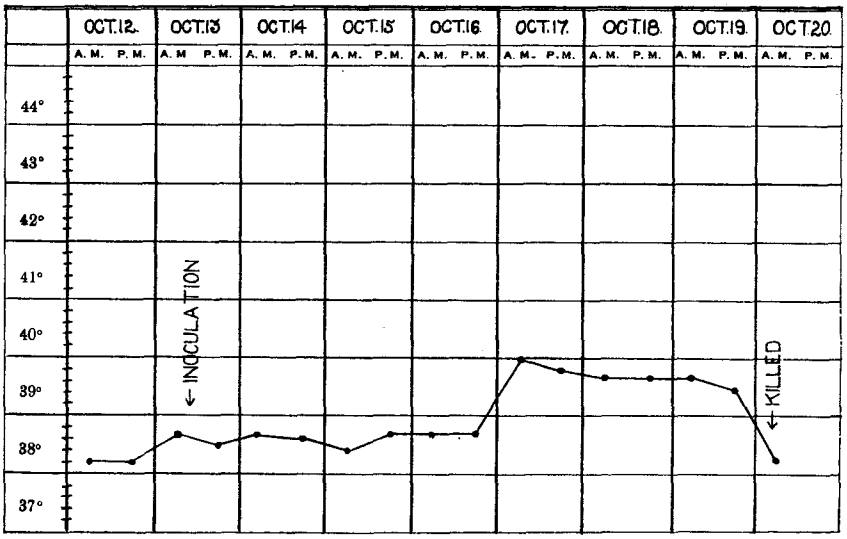
Experiments were next performed on the testicles of young bulls, with the view of ascertaining whether this species of animal would be suitable for use, both from the quantitative and qualitative relation of the virus, since it has long been customary to produce vaccine in the skin of this species.

Bull 1.—Weight, 750 pounds. The scrotal skin was shaved, washed with soap, and sterilized by sublimate alcohol. The vaccine virus employed for the injection consisted of testicular emulsion derived from the testicles of two rabbits which had been inoculated with the 30th generation of the testicular strain and castrated at the end of seven days. This emulsion produced a fairly thick eruption on the skin of rabbits in the dilution of 1 : 100. On Oct. 13, 1914, 10 c.c. of the emulsion diluted 1 : 100 were injected into the right, and 15 c.c. into the left testicle under aseptic precautions.

The testicles showed signs of swelling on the third day (forty-eight hours), and increased further in size and density up to the end of six days. The left testicle was much more swollen than the right. The temperature remained normal (38.7° to 38.9°) until the end of the fifth day, when it rose to 40.6°, 40.1° (6th day), and remained at 40.4° as late as the end of the sixth day. On the morning of the eighth day the temperature was 38.7° (text-figure 1).

The animal was slaughtered and the testicles were removed under aseptic precautions on the eighth day. The subcutaneous tissue and the tunica vaginalis were edematous and hemorrhagic, especially along the needle track. The testicles themselves were also edematous and showed a mass of coagulated blood at the site where a blood vessel must have been injured by the needle puncture.

Compared with the normal the vaccinated testicles were firmer and more pinkish in color, besides showing numerous ecchymoses. The left testicle was more altered than the right; the former weighed 310 and the latter 250 gm.



TEXT-FIG. 1. Bull 1.

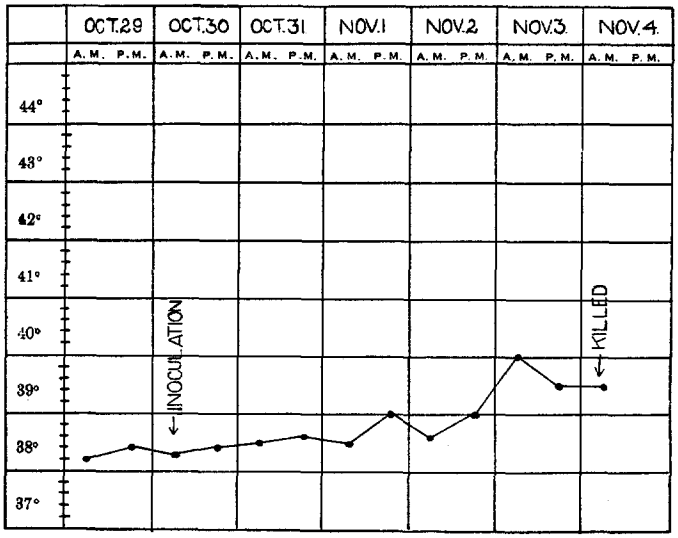
Emulsions in sterile saline solution were made with the tissues immediately about the point of insertion of the needle and also of portions remote from the site of injection. The tests for activity were made upon the shaved skin, cornea, and testicles of rabbits.

TABLE V.

Bull 1.	Tests on rabbits.			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of tissue around site of injection.....	1 : 10	+++	++	++
	1 : 100	+	+	++
	1 : 1,000	<+	−	++
	1 : 10,000	−	.....	++
Emulsion of tissue remote from site of injection.	1 : 10	++	++	++
	1 : 100	+	−	++
	1 : 1,000	−	.....	−
	1 : 10,000	−	.....	−
Exudate in tunica vaginalis.....	Undiluted	−	−	−
	1 : 10	−	−	−

The exudate contained in the tunica vaginalis was likewise tested. Both exudate and emulsions were found sterile for bacteria. Table V summarizes the results, which show that the virus multiplied to a much smaller extent in the testicular tissues of the bull than is the rule in the testicle of the rabbit. That the virus multiplied in some degree is highly probable; and that the edematous fluid in the tunica vaginalis was devoid of virus is an interesting point.

Bull 2.—Weight, 680 pounds. Oct. 30, 1914. The left testicle was injected at four different sites in order to distribute the virus widely with a 1 : 100 dilution of rabbit testicular virus in the thirty-third generation, the total quantity inoculated amounting to 20 c.c. At the same time the skin of the ventral surface below the navel was shaved, scarified, and inoculated with some of the same emulsion. The inoculated testicle after forty-eight hours was tense on palpation, distinctly swollen, and indurated. The swelling and induration increased during the next three days. The skin showed distinct papules three days after the inoculation, which became typical pustules two days later (figure 53). The temperature rose to 40° to 40.7° on the fourth day, and was 40° on the sixth day. The bull was slaughtered on the sixth day and the testicles were aseptically removed (text-figure 2).



TEXT-FIG. 2. Bull 2.

The scrotum was found to be edematous and infiltrated, but no adhesion was present between the tunica and the testicular surface. The moderate amount of serous and hemorrhagic exudate proved to be sterile for bacteria. Upon section

serous exudate oozed freely from the testicle. The points of injection could be detected by the presence of numerous minute hemorrhages within areas which were distinctly firmer than the surrounding parts. Coagula of blood occurred here and there (figures 54 and 55). The uninoculated right testicle showed no change. The weight of the left testicle was 229 and that of the right 199 gm.

Emulsions were prepared both from the tissues immediately about the sites of injection and from parts remote from them. The tests were made on the skin, cornea, and testicles of rabbits.

TABLE VI.

Bull 2.	Tests on rabbits.			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of tissue around zone of injection.	1 : 10	Confluent	++	++
	1 : 100	++	+	++
	1 : 1,000	+	.....	++
	1 : 10,000	<+	.....	++
Emulsion of tissue remote from site of injection.....	1 : 10	++	++	++
	1 : 100	+	+	++
	1 : 1,000	<+	.....	+
	1 : 10,000	−	.....	−
Exudate in tunica vaginalis	Undiluted	<+	+	+
	1 : 30	−	−	−

Table VI, which summarizes the results, indicates that the tissues about the sites of inoculation contain an amount of the virus about equal to that of rabbit's testicles inoculated with similar material, and that the degree of activity possessed by the two testicular strains is about the same. On the other hand, the emulsion made from the remote portions of the testicle produced far less effect, although it was still active up to the dilution of at least 1 to 1,000. The exudate in the tunica contained a trace of the virus, but this may have been due to a contamination of this exudate in the opening of the testicle.

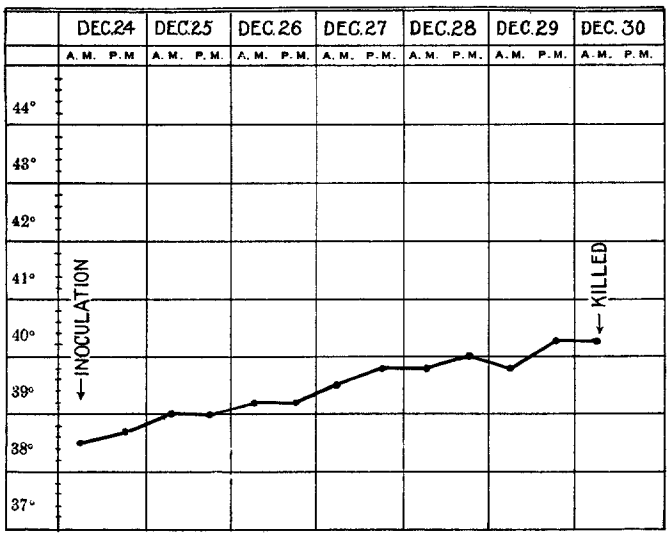
Bull 3.—Weight, 720 pounds. Nov. 11, 1914. Both testicles were inoculated with an emulsion of a testicular strain obtained from a rabbit inoculated with the testicular strain of bull 2. The emulsion was, however, contaminated with a *xerosis* bacillus. The local and general reactions were similar to those described in the previous two bulls. In spite of the admixture of *xerosis* bacilli with the virus the local reaction was not different from those following the injection of a pure virus. The animal was slaughtered on the ninth day after injection.

The testicular tissues were edematous, showed several well localized areas of

a greyish white color, and some hemorrhages caused by the trauma of the needle. The tissues were not softened. Cultures made from the emulsions of the testicles showed the presence of a few *xerosis* bacilli, but no other bacteria.

The vaccinal activity as tested on the rabbit showed that it was much weaker than that of bull 2.

Bull 4.—Weight, 140 pounds. Dec. 24, 1914. 4 c.c. of a 1:40 dilution of an emulsion of the fiftieth generation of testicular rabbit virus were injected into the left, and 3 c.c. of a 1:10 dilution of the testicular virus from bull 2 were injected into the right testicle. The organs were examined every twenty-four hours, but no particular difference was noticed on the two sides. Both showed marked induration within thirty hours. On the fifth day the swelling appeared to have reached the maximal stage. The temperature rose after three days and reached 40.8° on the sixth day. The animal was slaughtered at the end of six days (text-figure 3).



TEXT-FIG. 3. Bull 4.

The testicles had increased in volume, especially the one on the left side injected with the bull-rabbit strain; they were markedly edematous and congested. On section numerous greyish yellow specks were seen to be scattered throughout the organ, and were especially numerous along the sites of injection. Similar greyish yellow areas, it will be recalled, occur almost constantly in the inoculated rabbit's testicles.

The emulsions were made from both testicles separately and tested for their vaccinal activity, together with the controls of the standard glycerinated vaccine issued by the Department of Health of New

York City. The testicular emulsions were free of bacteria; the Health Department vaccine, of course, was not.

TABLE VII.

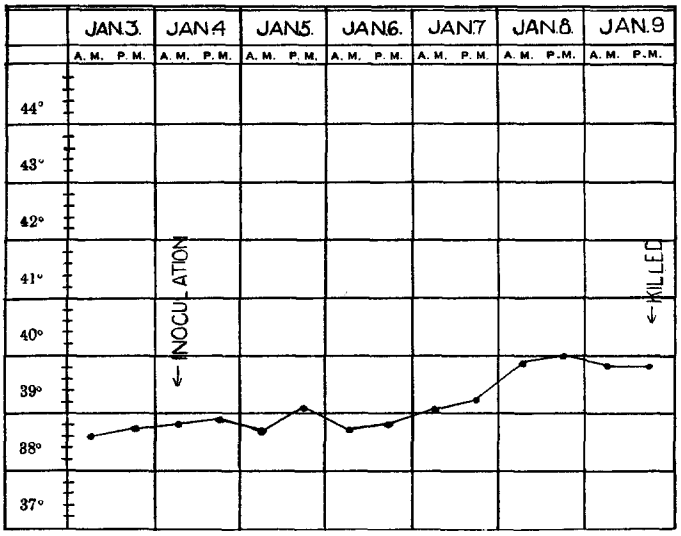
	Bull 4.	Dilution of emulsion.	Tests on rabbits.			Tests on calves.
			Skin.	Cornea.	Testicle.	Skin.
	Emulsion of testicle inoculated with bull-rabbit testicular strain	1 : 10	Confluent	++	++	Confluent.
		1 : 100	Confluent	+	++	++
		1 : 1,000	+	±	++	+
		1 : 10,000	<+	−	++	<+
	Emulsion of testicle inoculated with bull-rabbit testicular strain no. 2	1 : 10	Confluent	++	++	Confluent.
		1 : 100	Confluent	++	++	Almost confluent.
		1 : 1,000	++	+	++	++
		1 : 10,000	+	−	++	<+
Regular vaccine as controls	N. Y. D. H. standard vaccine 1st sample	1 : 10	Confluent	++	++	Confluent.
		1 : 100	Confluent	++	++	Almost confluent.
		1 : 1,000	++	+	+	++
		1 : 10,000	<+	−	<<+	<+
	N. Y. D. H. standard vaccine 2d sample	1 : 10	Confluent	++	++	Confluent.
		1 : 100	++	+	+	++
		1 : 1,000	+	−	−	+
		1 : 10,000	−	−	−	−

The above experiments (table VII) demonstrate that a strong bacteria-free vaccine virus can be produced in the testicular tissues of the young bull by injecting the testicular strain derived from a bull or from a rabbit, and they also show that the vaccinal activity reaches the same strength as that possessed by a standard virus propagated on the skin of a calf. Moreover, no differences were noted in the type and course of the vaccinal processes as produced in the skin of rabbits and calves by the vaccines from the several sources. The only distinctions are quantitative ones, since the skin virus is more quickly diluted beyond effective strength than the testicular virus.

The next experiment was devised to compare the effect of the testicular inoculation of a testicular and a skin strain of vaccine, respectively, into the same calf.

Bull 5.—Weight, 160 pounds. Jan. 4, 1915. 3 c.c. of a 1 : 10 dilution of an emulsion of the testicular strain derived from bull 4 were injected into the right, and 3 c.c. of a 1 : 10 dilution of the New York City Department of Health standard vaccine into the left testicle. The next day the swelling and injection of the right testicle were marked, while the left was far less altered. This difference

became more pronounced, and on the sixth day only the right side presented the typical reaction, swelling, and induration previously noted, while the left was but slightly enlarged. The animal was slaughtered at the end of five and one-half days (text-figure 4).



TEXT-FIG. 4. Bull 5.

Upon exposing the testicles it was found that the left showed infiltration in small amount and only about the site of the injection, while the right was edematous throughout, greatly enlarged, and dotted with numerous minute foci of greyish yellow appearance. While the consistency of the left testicle which weighed 6.5 gm. was about normal, that of the right, which weighed 8 gm., was diminished.

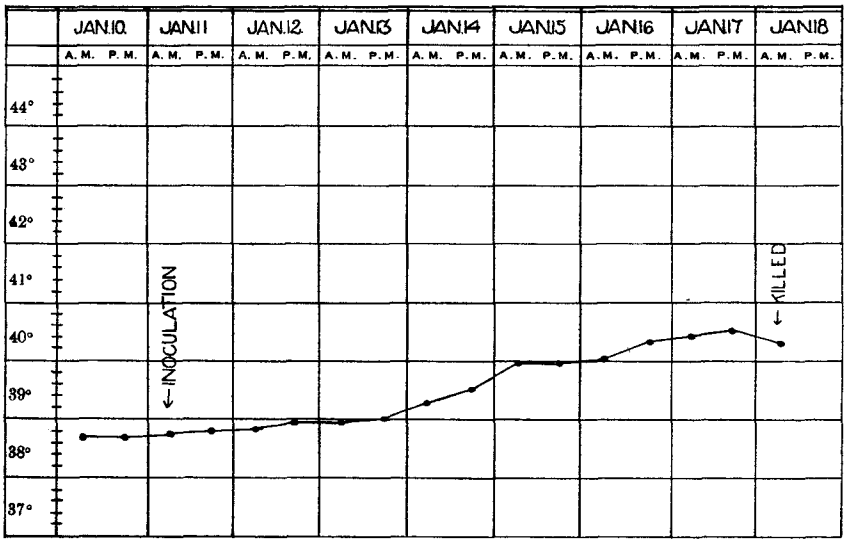
The vaccinal strength of both testicles was tested on the rabbit.

TABLE VIII.

Bull 5.	Tests on rabbits.			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of testicle inoculated with calf testicular strain no. 4	1 : 10	Confluent	++	++
	1 : 100	++	++	++
	1 : 1,000	+	−	++
	1 : 10,000	−	−	++
Emulsion of testicle inoculated with skin strain (N. Y. D. H.)	1 : 10	+	+	+
	1 : 100	<+	−	−
	1 : 1,000	−	.....	−
	1 : 10,000	−	.....	−

As table VIII indicates, the local reaction and the vaccinal activity were more marked in the case of the testicular than of the skin strain, from which it may be inferred that adaptation of the strain to the testicle is necessary as a prerequisite to the production by this means of strong vaccine.

Bull 6.—Weight, 225 pounds. Jan. 11, 1915. The left testicle was injected with an emulsion of the testicle that had been inoculated with the standard skin strain of vaccine, as mentioned above, and the right testicle with an emulsion of the testicle previously inoculated with a testicular strain. The local and general reactions were similar to those observed and described, except that the left testicle, weighing 9 gm., showed far less change than the right, which weighed 12.5 gm.



TEXT-FIG. 5. Bull 6.

(figures 56 and 57). The bull was slaughtered at the end of five and one-half days, and the vaccinal activity of each testicle was tested on the rabbit (table IX and text-figure 5).

Histological Changes.—The histological changes observed in the testicles of bulls inoculated with testicular strains of vaccine virus are similar to those enumerated in these organs of the rabbit. An acute inflammatory reaction arises, in which many polymorphonuclear leucocytes invade the interstitial tissues together with a serous exudate; the adjacent tubules are compressed by pressure of the exu-

TABLE IX.

Bull 6.	Tests on rabbits.		
	Dilution of emulsion.	Skin.	Cornea.
Emulsion of left testicle inoculated with skin strain.	1 : 10	+	+
	1 : 100	<+	−
	1 : 1,000	−	.....
	1 : 10,000	−	.....
Emulsion of right testicle inoculated with testicular strain	1 : 10	+++	++
	1 : 100	++	+
	1 : 1,000	+	.....
	1 : 10,000	−	.....

date, which also invades them, and the epithelial cells degenerate. The reaction is multifocal and affects parts remote from the site of inoculation, although the severer effects occur adjacent to the inoculation. Hemorrhages occur also (figures 57 to 66).

IDENTITY OF THE SKIN AND TESTICULAR STRAINS OF VACCINE VIRUS.

It may now be considered as established that vaccine virus derived from the specific skin lesions is capable of multiplying indefinitely in a pure state within the testis of the rabbit and the bull. There would appear to be no necessity for restoring the virus to skin surfaces in order to continue the propagation in the testicles. The testicular epithelium, once the virus is adapted to it, seems to provide as suitable a medium for its multiplication as the skin epithelium. Moreover, in order that the virus should propagate in the testis no previous injury of the organ is necessary, save that caused by the injection, so that in this respect the testicle may be regarded as exceeding the skin surface in susceptibility to its presence. Possibly anatomical structure accounts for this difference; unless scarified the virus has no opportunity of adhering to and entering intimately into relation with the skin surface, while injection into the testicle brings the virus at once into immediate proximity to the tissues composing that organ.

The question which arises is whether the skin and testicular strain of virus remain identical in physiological properties. One has, of course, been derived from the other; but it remains to be considered whether in the course of adaptation the testicular strain has undergone modification.

Several criteria can be employed to decide this point: (a) type and course of the eruption of the skin of susceptible hosts (rabbit, calf, man); (b) characteristic lesion of the cornea of the rabbit; (c) histological processes within the lesions caused by the virus; (d) phenomena of immunity arising after vaccination; (e) resistance to the action of certain agencies, such as glycerin, ether, chloroform, phenol, drying, and heating.

It may be assumed that merely completely freeing vaccine virus from the usual bacteria which contaminate it in practice will not change its physiological properties. This probability is indicated by the effect of glycerination which reduces the bacterial content. The practical advantage of this reduction is obtained in milder vaccinal effects in man, which are equally protective with the severer effects resulting from bacterial coöperation.

Hence it may be hoped that the complete removal of the contaminating bacteria should still further improve the results of vaccination in that the secondary operation of bacteria contained within the virus will be entirely eliminated. The employment of such a pure strain virus will, at the same time, do away entirely with the potential danger of contamination of the virus with tetanus bacilli or spores, a subject which has always engaged the attention of producers, and respecting which special precautions are taken.

Skin Reactions.—Both skin and testicular strains of virus were inoculated simultaneously on different parts of the shaved skin surfaces of rabbits and calves, after which the course of the vaccination was followed. No differences were noted (figures 67, 68, and 69).

Cornea.—The rabbit's cornea inoculated with skin and testicular virus, respectively, undergoes the same series of changes, and in each case Guarnieri bodies are produced. The results are indistinguishable (figures 49 and 51).

Histological Lesions.—When the testicular strains of virus are reimplanted upon the skin of the rabbit and calf, and the minute histological changes produced are compared with those caused by the skin strains of virus, no essential differences have been detected (figures 47, 48, 50, and 52). Moreover, the microscopical changes in the testicles produced by skin or adapted testicular strains of virus are essentially identical.

IMMUNITY PHENOMENA.

Considered from the practical standpoint the most important effect of vaccine virus is the immunity to reinoculation that it confers. Hence, experiments were conducted with the testicular strains in order to determine the immunity effects which it produces.

Experiment I.—Rabbit. First vaccination, Oct. 5, 1914, at three separate places on the shaved skin, with regular, glycerinated stock virus. Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 12, 1915. The three places previously vaccinated were now revaccinated as follows: (a) an area with some of the virus used previously; (b) an area with rabbit testicular strain; (c) an area with bull testicular strain. The three areas reacted in identical manner: each area became congested, but neither vesicles nor papules appeared. The reddening of the skin was probably allergic in origin (20).

The control rabbit developed confluent eruptions from each of the three samples of virus.

Experiment II.—Rabbit. First vaccination, Oct. 5, 1914, at three separate places on the shaved skin, with a testicular strain of vaccine virus from a rabbit. Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 12, 1915. The three places previously vaccinated were revaccinated as follows: (a) an area with some of the virus used previously; (b) an area with bull testicular strain; (c) an area with New York City Department of Health vaccine. On the following day slight transient scattered erythema was noted, but neither papules nor pustules.

The control rabbit developed typical confluent eruptions from each of the three samples of virus.

Experiment III.—Rabbit. First vaccination, Feb. 2, 1915, at three places on the shaved skin, with testicular strain from a bull calf (No. 4). Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 17, 1915. The three places previously vaccinated were revaccinated as follows: (a) an area with some of the virus used in the first vaccination; (b) an area with a rabbit testicular strain; (c) an area with the regular New York City Department of Health vaccine. Only moderate allergic reactions were observed.

Two other rabbits similarly tested gave identical results with the above experiment, except for the presence in one of them of a slight erythema along the scarified lines, which lasted three days.

Controls on a normal rabbit showed that the strength of the vaccine strains employed for the foregoing experiments was such as to produce confluent eruptions.

The experiments described are conclusive in demonstrating that the skin and testicular strains of vaccine virus yield identical immunity reactions both as regards kind and degree.

TESTICULAR CULTIVATION AS A MEANS OF PROPAGATION OF PURE VACCINE VIRUS.

It is obvious that a method of propagating the vaccine virus free from bacterial contamination would remove all danger of accidental infection through the introduction at the time of vaccination of an impure preparation containing certain bacteria. Without stopping to consider how often severe effects have resulted from bacterial contamination of the virus it may be urged that the use of a pure preparation will altogether preclude the possibility of a mixed infection from this source. The indefinite propagation of a highly potent vaccine virus in the testicular tissues of animals is comparatively a simple procedure, so that by means of a strictly aseptic technique combined with careful bacteriological control of the product, it will now become a simple matter to supply the medical profession with an absolutely pure virus, for the purpose of vaccination. As is well known, the method hitherto practised for propagating vaccine consists in utilizing the emulsion of the skin pulp from vaccinated calves. The usual fresh pulp contains many and various bacteria, so that to free the pulp of them it must be left in contact for a period with 60 per cent. glycerin or 1 per cent. phenol, before it is regarded as ready for use on human subjects. Since the strength of the virus also deteriorates with time the process of vaccine preparation has not been perfected from the practical point of view. On the other hand, a pure virus should be ready for use as soon as the usual bacteriological and potency tests are completed, that is, within about one week after the preparation of the emulsion of the testicle containing it.

The introduction of a pure vaccine into the public health service would be welcome, even if it were somewhat more expensive to produce than the usual vaccine. As a matter of fact it appears to be even more economical than the latter, as the following calculation shows.

A male rabbit weighing about 3 kg. yields from 7 to 10 gm. of testicular material containing the pure culture of vaccine. When this amount of tissue is emulsified in from 15 to 20 c.c. of 50 per cent. sterile glycerin, it will yield from 20 to 28 c.c. of a finished product of high potency. A sample of this product is diluted 1 : 1,000 and tested on the skin of rabbits. If a confluent eruption results,

which may reasonably be expected, the stock suspension may be further diluted with four times its volume of sterile 50 per cent. glycerin, since a sample of glycerinated virus which produces a confluent eruption in a dilution of 1:200 is sufficiently strong for use in man. Hence a single rabbit may yield 125 c.c. of finished glycerinated virus, or even more.

The average yield from the calf, according to Fielder,[10] is put at 40 to 100 gm. of fresh pulp, which emulsified with four parts of 50 per cent. glycerin gives 250 to 300 c.c. of vaccine virus ready for use within three months.

A comparison of the cost is in favor of the rabbit vaccine. For the preparation of 1,000 cubic centimeters of the finished vaccine product about 40 rabbits or 4 calves would be required. The initial cost of the former would be considerably smaller.

Moreover, the employment of rabbits instead of calves has advantages of another kind from those which have just been emphasized. The prevalence of foot-and-mouth disease among cattle has closed the market in many states to calves, so that the propagation of vaccine virus in the ordinary way has recently suffered. No such interruption is to be feared with respect to the use of rabbits. During the prevalence of such an epidemic it becomes essential also to keep the calves intended for vaccination under observation before use for a sufficient period to insure their freedom from foot-and-mouth disease infection.

Finally, it may be added that the bull may also come to be used for the testicular cultivation of the virus. However, the method has not yet been worked out for this animal as completely as it has for the rabbit. Hence it must be left to the future to determine whether the bull is strictly suitable for the purpose. This part of my work suffered interruption because of the prevailing epidemic of foot-and-mouth disease and the resulting quarantine upon cattle.

STANDARDIZATION OF VACCINE VIRUS.

Several methods are employed for determining the efficacy and potency of vaccine virus before distributing it for general use. The accepted methods agree in using animals before permitting the virus to be used on man. Theobald Smith recommends the skin of the calf, and requires that the eruptions produced there must correspond with those of the skin of a child. Calmette and Guérin (9, 21) and

[10] On the other hand, Rosenau (1) gives the amount of fresh pulp from a calf as 20 to 40 gm., which will yield 50 to 120 c.c. of the glycerinated emulsion.

Camus (22) employ the skin of the rabbit; while Chaumier (23) and others[11] first employ animals, and then themselves observe the vaccination effects on a few children before issuing the virus. There is agreement among the authorities that the skin of the rabbit and calf are reliable indications of the effects produced on human subjects.

Henseval and Convent (24) consider that a preparation which produces on the skin of the rabbit almost confluent eruptions in a dilution of 1 to 500 should be considered a suitable vaccine. Such a sample always causes an uninterrupted eruption along a line (linear eruption) on the skin of a child. They recommend as still usable even a weaker specimen provided it produces a continuous eruption in a 1 to 100 dilution.

The testicular vaccine strains which the author prepared in rabbits either fulfill or exceed the above requirement, since a few samples produced confluent eruptions in dilutions of 1 to 10,000. On the other hand, the products from the testicles of the bull were less active, and a few only of the preparations reached the lower standard.

PURE VACCINATION IN MAN.

The tests which were carried out in detail and with minute care in animals with the pure testicular strains of vaccine virus all indicated that the virus would produce typical vaccinal effects in the human subject.

Several adults, chiefly physicians familiar with the cultivation experiments, immediately volunteered for vaccination, which was carried out by the linear and scarification methods. Among the tests, which were all revaccinations, some were positive and others negative. In the positive results the vaccinal eruption was typical and no secondary infection arose to complicate or intensify the vaccination.

Among those in whom the vaccination was positive was a physician. After his experience he vaccinated his own child, three years and two months old, who had not previously been successfully

[11] The Department of Health of the City of New York issues only the preparations which have produced on primary vaccination cases fifteen typical takes on fifteen insertions made on five children, thus requiring 100 per cent. of positive takes for each sample of vaccine.

vaccinated. The linear eruption appeared on the fifth day, increased in intensity during the three succeeding days when it reached the maximum, and then receded. No pain or itching was complained of, and the highest temperature was 37.5° C., which was noted on the eighth day. On the fourteenth day scab formation was well advanced. I am indebted to the father of the child for the two photographs (figures 70 and 71) which illustrate the vaccinal effects on the fifth and sixth days.

SUMMARY.

Vaccine virus freed from all associated bacteria by means of suitable disinfecting agents can be propagated in a pure state in the testicles of rabbits and bulls. The virus cultivated in this manner is not only devoid of all bacteria, but appears capable of indefinite transfer from one animal to another. Sixty passages in rabbits of a pure strain have been made within one year.

Several transfers from testicle to testicle are required to bring about accurate adaptation of the virus to the testicular parenchyma, so that continued propagation in this way can be certainly secured. During the first transfers from testicle to testicle the activity of the virus may be less than the original skin specimen from which the pure strain was derived; but as the transfers proceed the activity rises until, when the adaptation is complete, the activity of the testicular equals that of the skin strain.

The multiplication of the virus within the testicle is maximum on the fourth or fifth day after inoculation; the quantity of virus remains about stationary until the eighth day, when diminution begins. At the expiration of five weeks no more virus could be detected in the testicle.

The vaccinal processes in the skin, cornea, and testicle of rabbits are practically identical whether the virus employed for the inoculation has been the original skin strain or the pure testicular strain; and the skin lesions produced in the calf with the two strains are also identical.

In conformity with the finding mentioned in the last paragraph it has been found that human beings react to the pure testicular strain of vaccine virus in an entirely typical manner. In the case

both of original vaccination and revaccination the vaccinal effects cannot be distinguished from those arising from uncomplicated skin virus.

Pure strains of testicular virus are readily produced, and once secured they may be propagated in a pure state by the method described in rabbits or bulls without difficulty and with economy. The pure strains thus obtained should supply an ideal form of virus for employment in the vaccination of human beings.

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EXPLANATION OF PLATES.

PLATE 39.

FIG. 1. The right testicle shows swelling due to the injection of pure vaccine virus made five days previously; the left side is normal.

FIGS. 2 to 12. The conditions of the testicles at various stages in the vaccinal processes produced by means of a pure vaccine virus, as follows: 2, 48 hours; 3, 3 days; 4, 4 days; 5, 5 days; 6, 6 days; 7, 7 days; 8, 8 days; 9, 9 days; 10, 10 days; 11, 11 days; and 12, 12 days after the inoculation. The yellowish grey mottles of irregular dimension can be seen in the specimens shown in figures 4, 5, and 6. The marked atrophy of the organ is easily noticeable in the specimens shown in figures 9, 11, and 12. All natural size.

PLATE 40.

FIG. 13. A testicle four days after inoculation with pure vaccine virus. It shows marked vascular injection, edema, and several small yellowish grey spots visible on the surface.

FIG. 14. The organ on section, in which several lobules can be seen undergoing necrosis, as indicated by the greyish yellow, somewhat indurated areas. The parenchyma has bulged out of the tunica albuginea on account of the edema.

FIGS. 15 and 16. Two specimens of testicles at the height of the vaccinal processes, one (figure 16) with hemorrhagic reactions. In the latter instance the necrotic changes are much more pronounced, as is indicated by the general yellowish grey color of the lobules on section.

PLATES 41 AND 42.

FIG. 17. The structure of the testicle of a normal rabbit under a low power (magnified 13 diameters).

FIG. 18. Diffuse interstitial infiltration of the vaccinated testis after three days. The compression of the tubules through intertubular edema and infiltration is well shown.

FIG. 19. A four day specimen which presents a large, irregularly defined infiltration and the destruction of the tubules.

FIGS. 20 to 24. Gradual absorption of the inflammatory products with distinct diminution of tubules. There are still many foci of infiltration.

FIGS. 25 and 26. The structure of testicles which had been vaccinated three and seven weeks previously.

FIG. 27. A longitudinal section of a testicle which shrank as the result of vaccination fifty-six days previously. There are only a few tubules left, all without any spermatogenetic activities. Magnification: ×13 throughout.

PLATE 43.

FIG. 28. A section of normal rabbit's testicle stained by the Borrel-Calkin method (25).

FIGS. 29 and 29 a. The peculiar round or oval bodies of varying size within the affected cellular elements in the vaccinated testicles of rabbits. These bodies appear brilliantly red when stained by the Borrel-Calkin method, and they resemble the vaccine bodies so called. These bodies may have been derived from the nuclei of the cells under the influence of the vaccine virus. Magnification: × 340 throughout.

PLATE 44.

FIGS. 30 and 31. The structure of a normal testicle of the rabbit.

FIG. 32. An early polynuclear infiltration of the interstitial spaces of a vaccinated testicle of the rabbit after twenty-four hours.

FIG. 33. A similar but more advanced stage after three days.

FIG. 34. Extensive involvement of the testicular tubules as well as the interstitial tissues after four days.

FIG. 35. A focus where polynuclears are seen to have begun to break up after five days.

FIG. 36. An area of extensive necrosis occurring in a specimen removed after four days.

FIG. 37. The cellular infiltration and fibrin in a specimen after five days (Weigert stain).

FIG. 38. An intratubular infiltration occurring in a specimen seven days after inoculation. The cells are basophilic leucocytes. Magnification: × 170 throughout.

PLATE 45.

FIG. 39. A tubule completely filled with leucocytes in a specimen eight days after inoculation.

FIGS. 40 and 41. Enormous cellular infiltration along the tunica albuginea in nine day specimens.

FIG. 42. The detachment of the degenerated testicular cells from the wall of affected tubules in a twelve day specimen.

FIG. 43. A focus of infiltration in a twenty-one day specimen. Such a focus is rather rare in old specimens.

FIG. 44. A focus of infiltration which was found in a fifty-six day specimen.

FIG. 45. The general changes of the structure of the vaccinated testicle after fifty-six days.

FIG. 46. The infiltration of the tunica vaginalis in a rabbit with polynuclear leucocytes in a four day specimen.

FIGS. 47 and 48. The vaccinal reactions on the skin of rabbits. Magnification: × 170 throughout.

PLATE 46.

FIG. 49. The Guarnieri, or vaccine bodies, so called, in the corneal epithelium of a rabbit inoculated with pure testicular vaccine virus. Three day specimen.

FIG. 50. The vaccinal reaction in the skin of a rabbit inoculated with the testicular strain of vaccine virus. Stained with Calkin's modification of Borrel's method.

FIG. 51. The vaccine bodies in a rabbit's cornea inoculated with regular vaccine strain.

FIG. 52. Normal rabbit skin stained with the Borrel-Calkin method.

PLATE 47.

FIG. 53. The inoculated (left) and normal (right) testicles of bull 2 on the sixth day after the injection of pure testicular vaccine from a rabbit. The left side is seen to be somewhat larger than the right. The skin shows typical vaccinal pustules also produced with the same material.

FIG. 54. The inoculated testicle and the tunica vaginalis of the same animal, the latter showing much hemorrhage.

FIG. 55. The same organ on section. One notices the hemorrhagic foci and necrotic area.

FIGS. 56 and 57. The testicles of bull 6. The larger one was inoculated with the testicular, and the smaller with a skin strain of vaccine virus, and both were removed on the sixth day.

PLATE 48.

FIG. 58. The structure of a normal testicle of a young bull. One recognizes at once that the spermatogenesis is in full display.

FIGS. 59 to 61. The reactions following the injection of pure testicular vaccine strain into the organ. They represent changes found on the sixth day.

FIGS. 62 to 66. The vaccinal processes in the testicles of bull calves. The changes are similar to those found with young bulls and rabbits, except that the animals were too young to have any spermatogenesis, even in the unaffected areas of the organ. Both in the young bulls and in the bull calves the interstitial infiltration with polynuclear leucocytes is marked. Magnification: × 170 throughout.

PLATE 49.

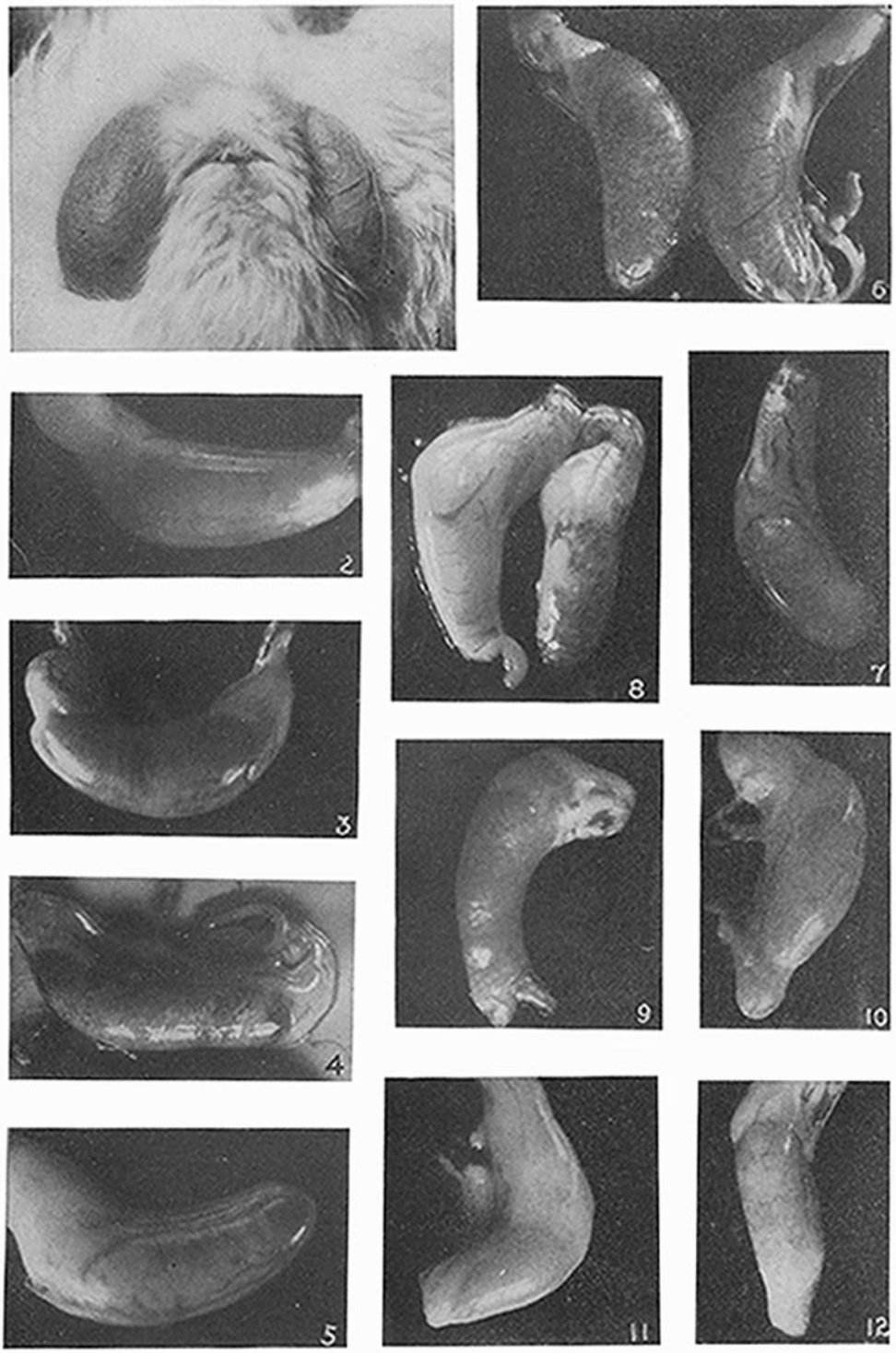
FIG. 67. The typical vaccinal effect on the skin of a rabbit inoculated with a pure testicular strain of vaccine virus on the sixth day.

FIG. 68. A confluent eruption on the skin of a rabbit vaccinated with a pure testicular strain.

FIG. 69. The typical vaccinal effect on the skin of a calf inoculated with a pure testicular strain of vaccine virus obtained from a rabbit, on the sixth day. Natural size.

PLATE 50.

FIGS. 70 and 71. The fifth and the sixth day phases of the first vaccination in a child three years and two months old. The virus was prepared in the rabbit according to the method described in this work, and was absolutely free from bacteria. Notice the uninterrupted linear eruption on both sides. In figure 71 the mottled appearance around the site of vaccination is due to the adhesion of the vaccine shield.



(Noguchi: Cultivation of Vaccine Virus.)

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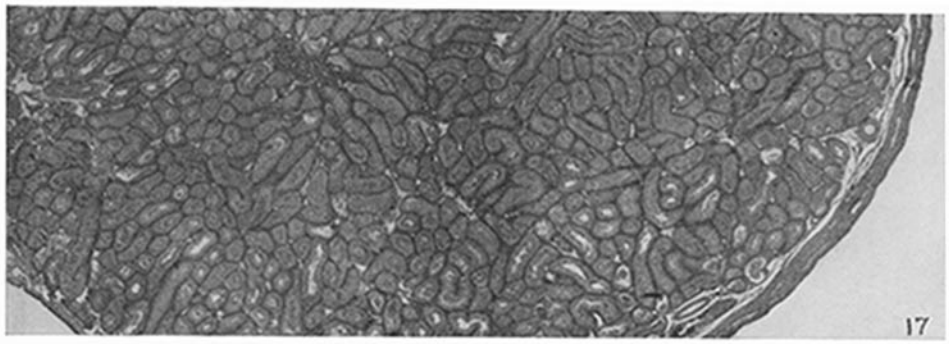
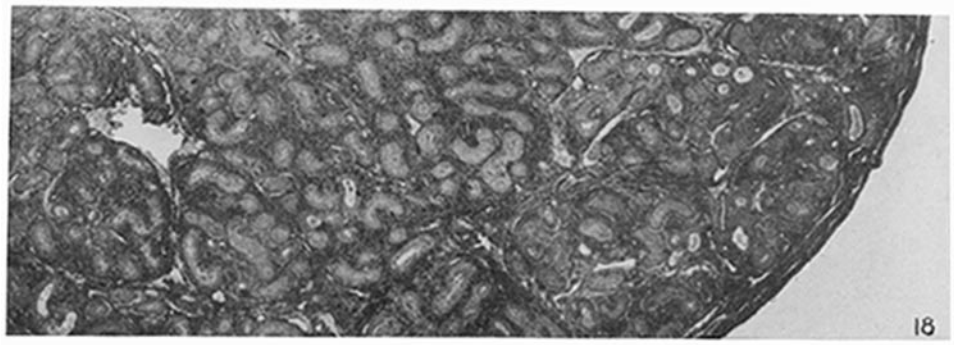
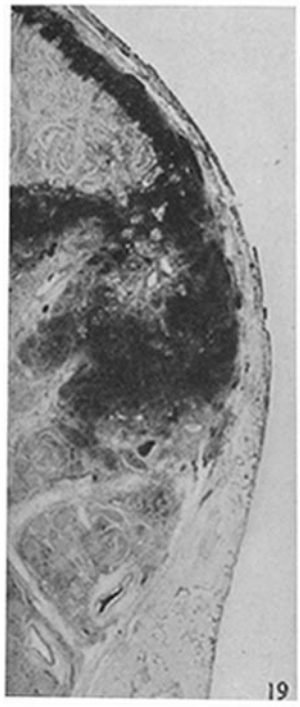
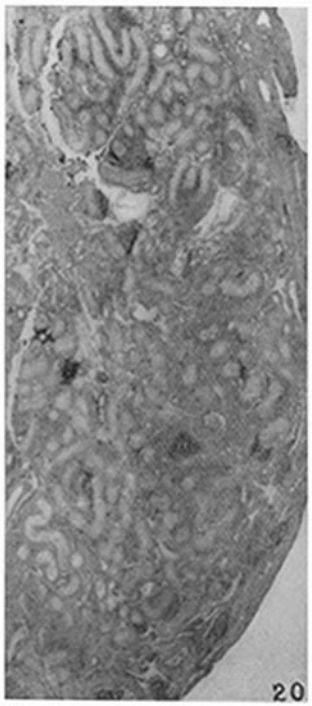
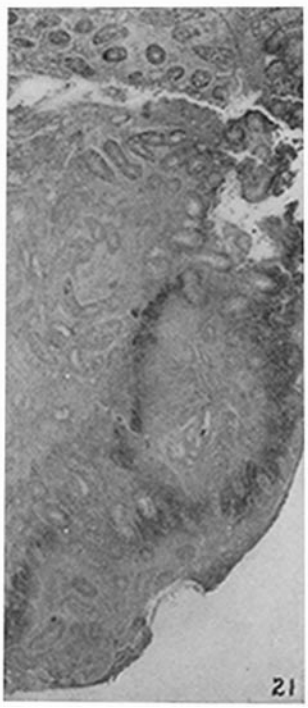
(Noguchi: Cultivation of Vaccine Virus.)



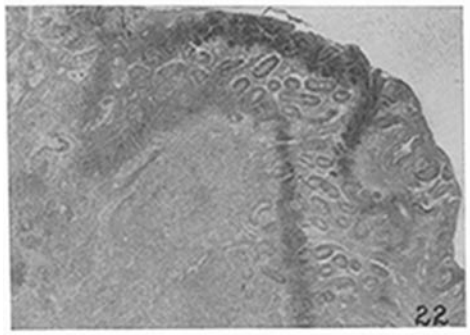


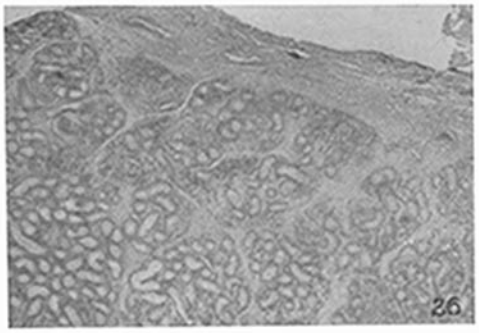


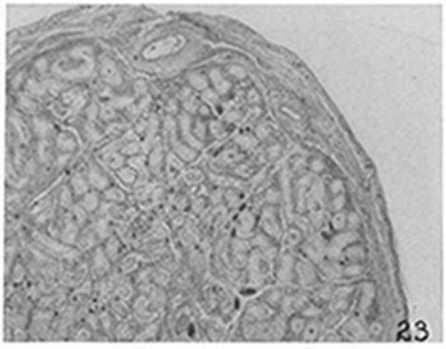


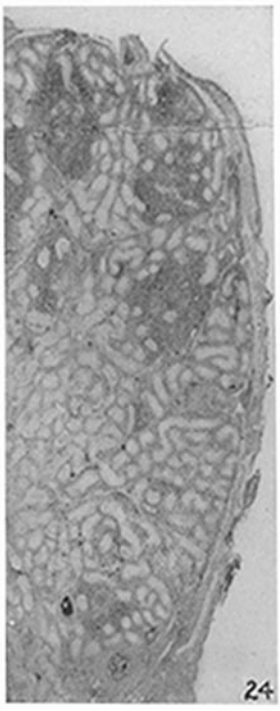


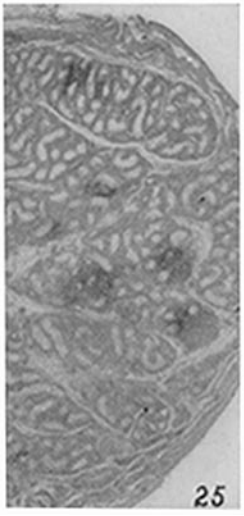
(Noguchi: Cultivation of Vaccine Virus.)











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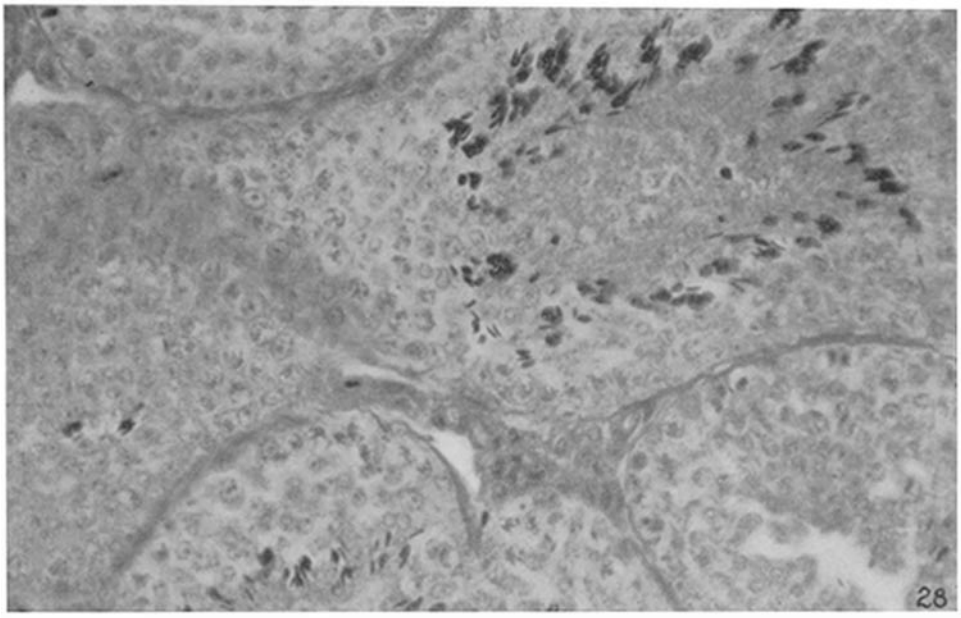

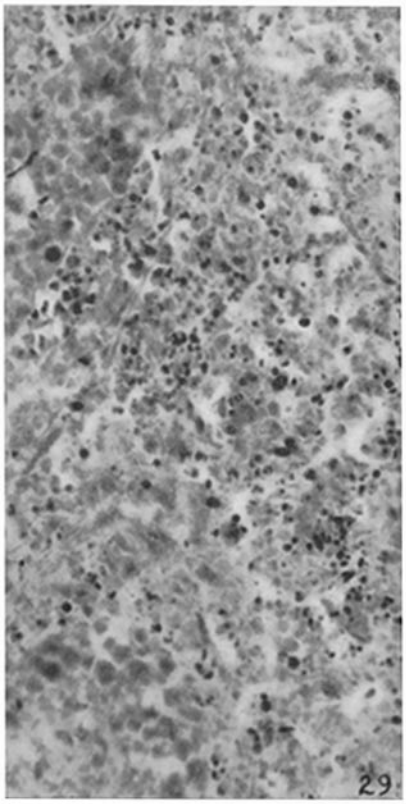

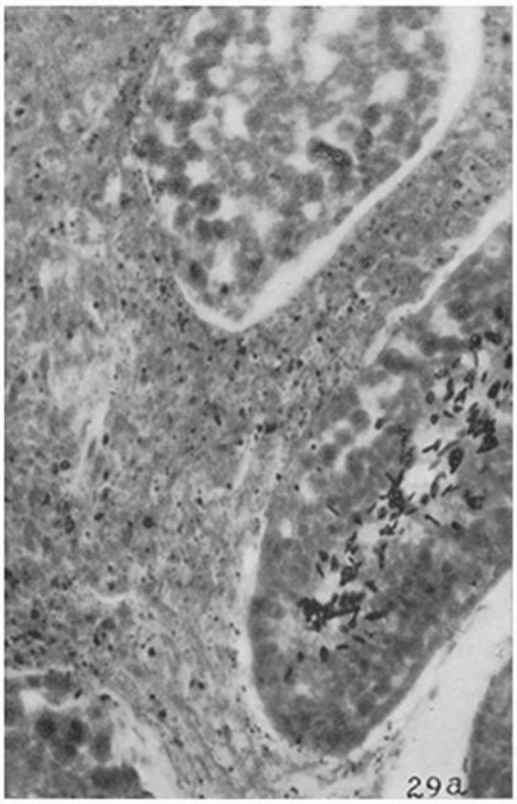

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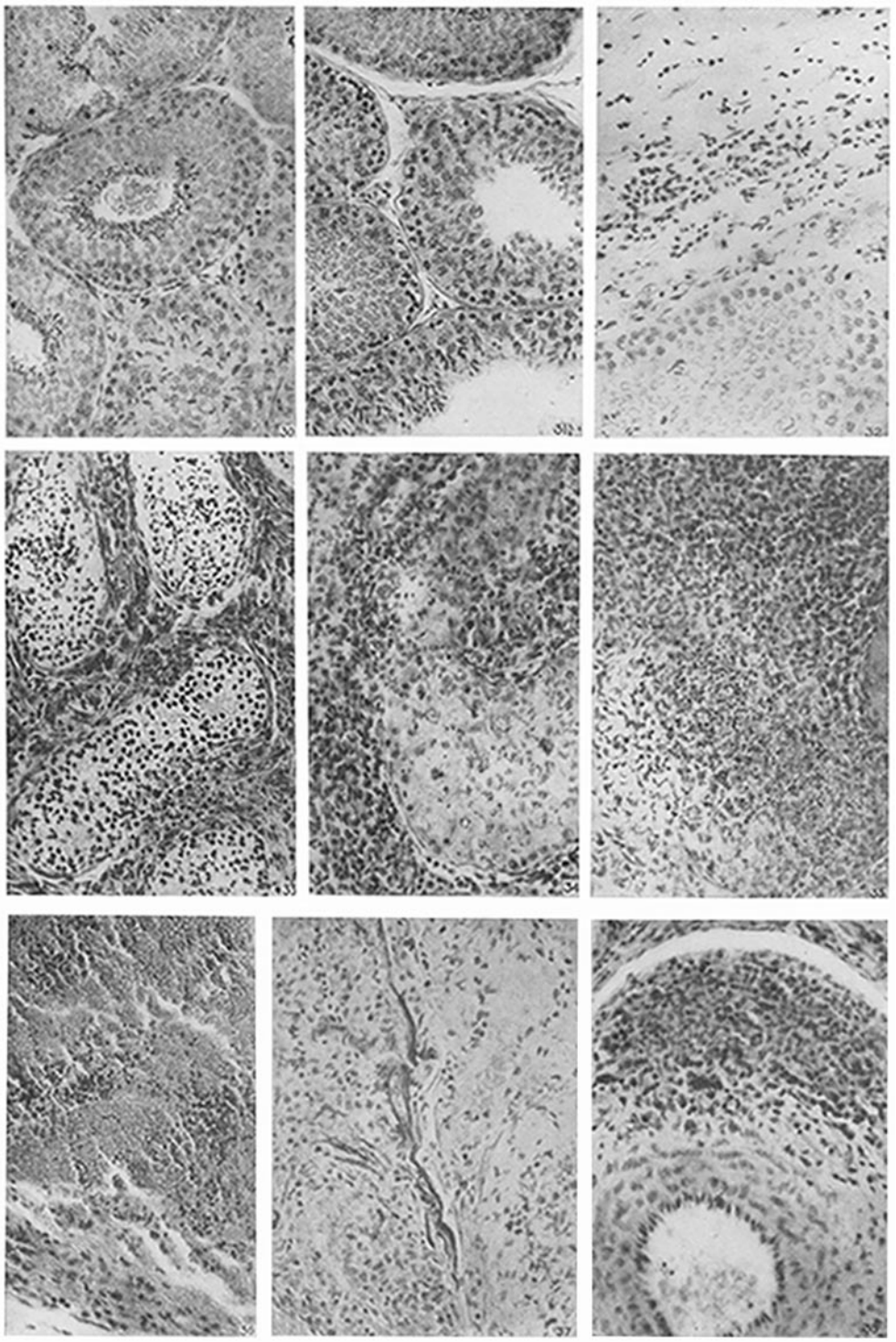

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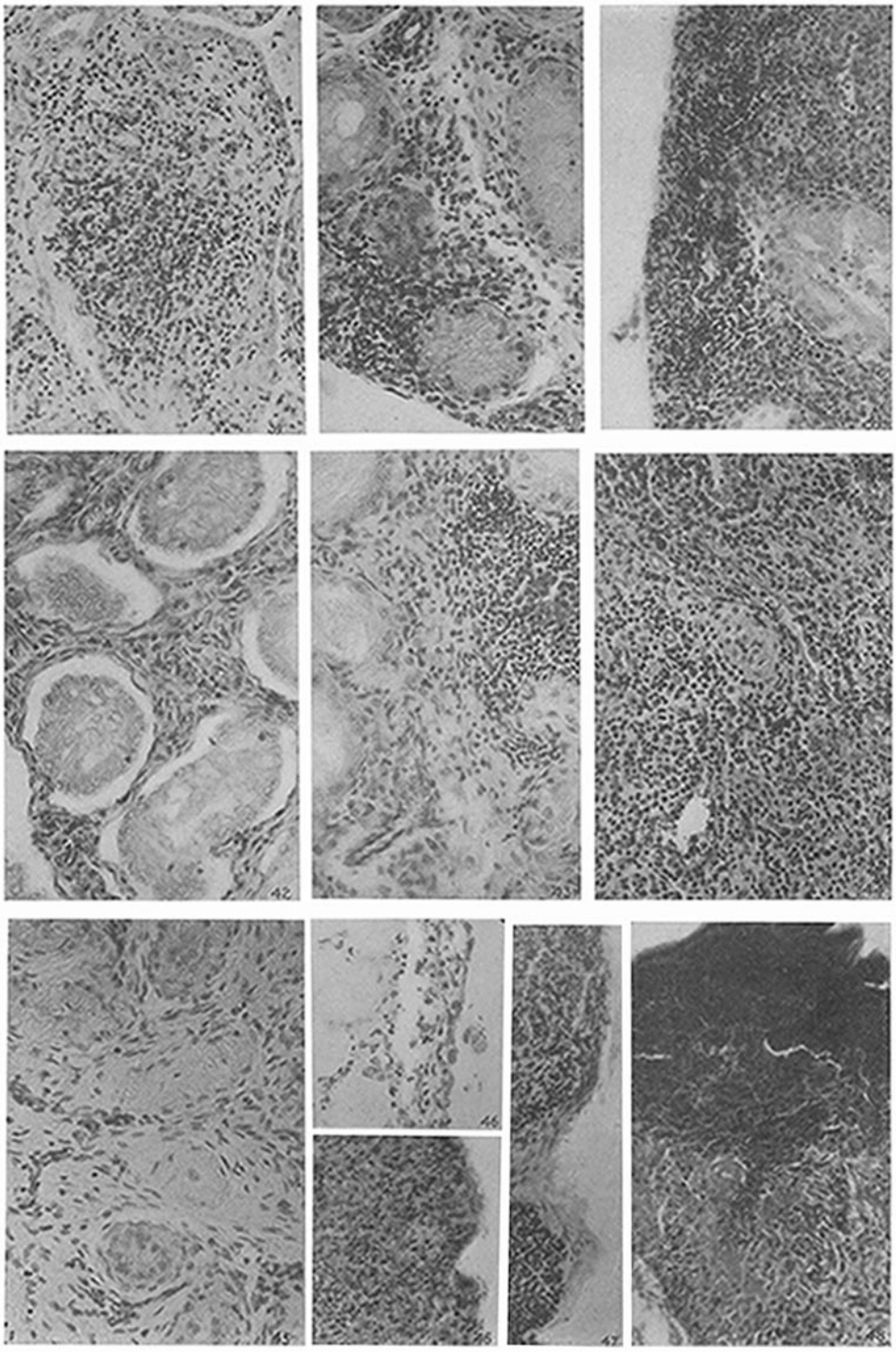

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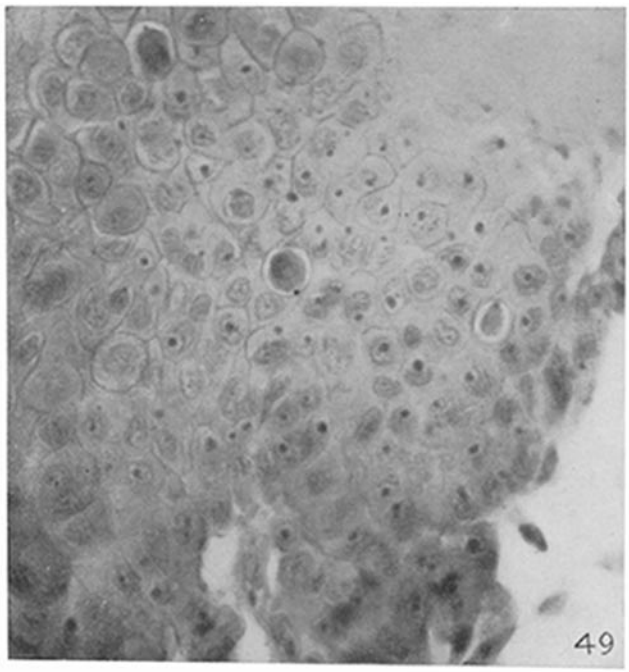

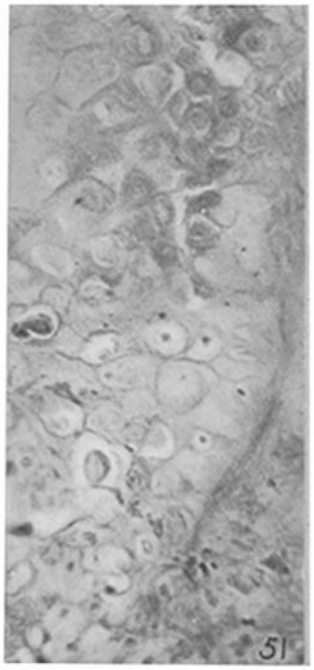

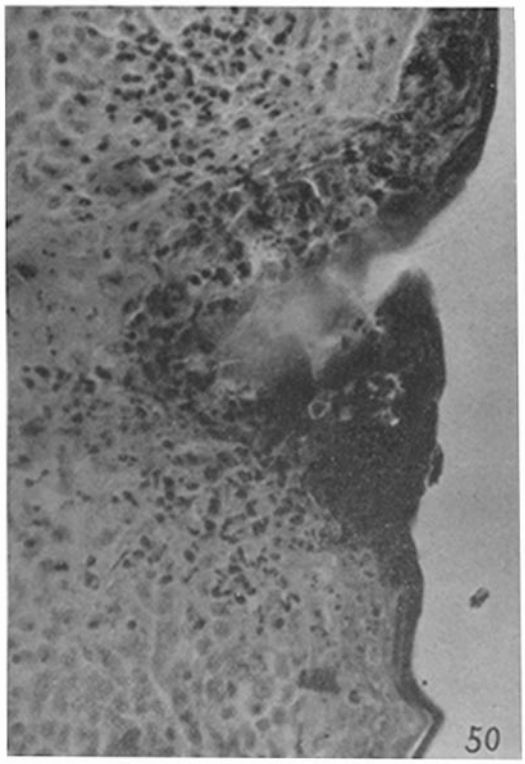

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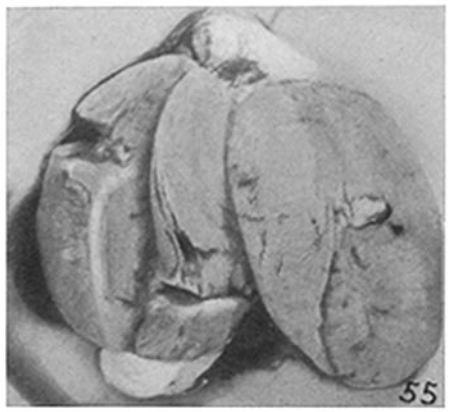

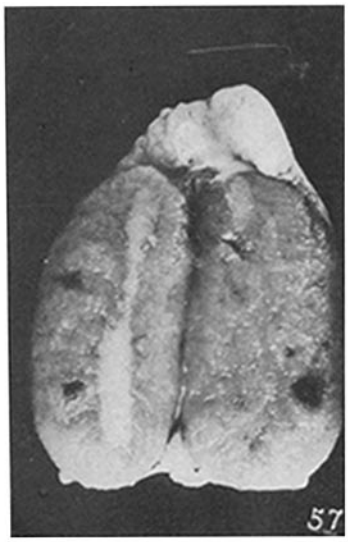

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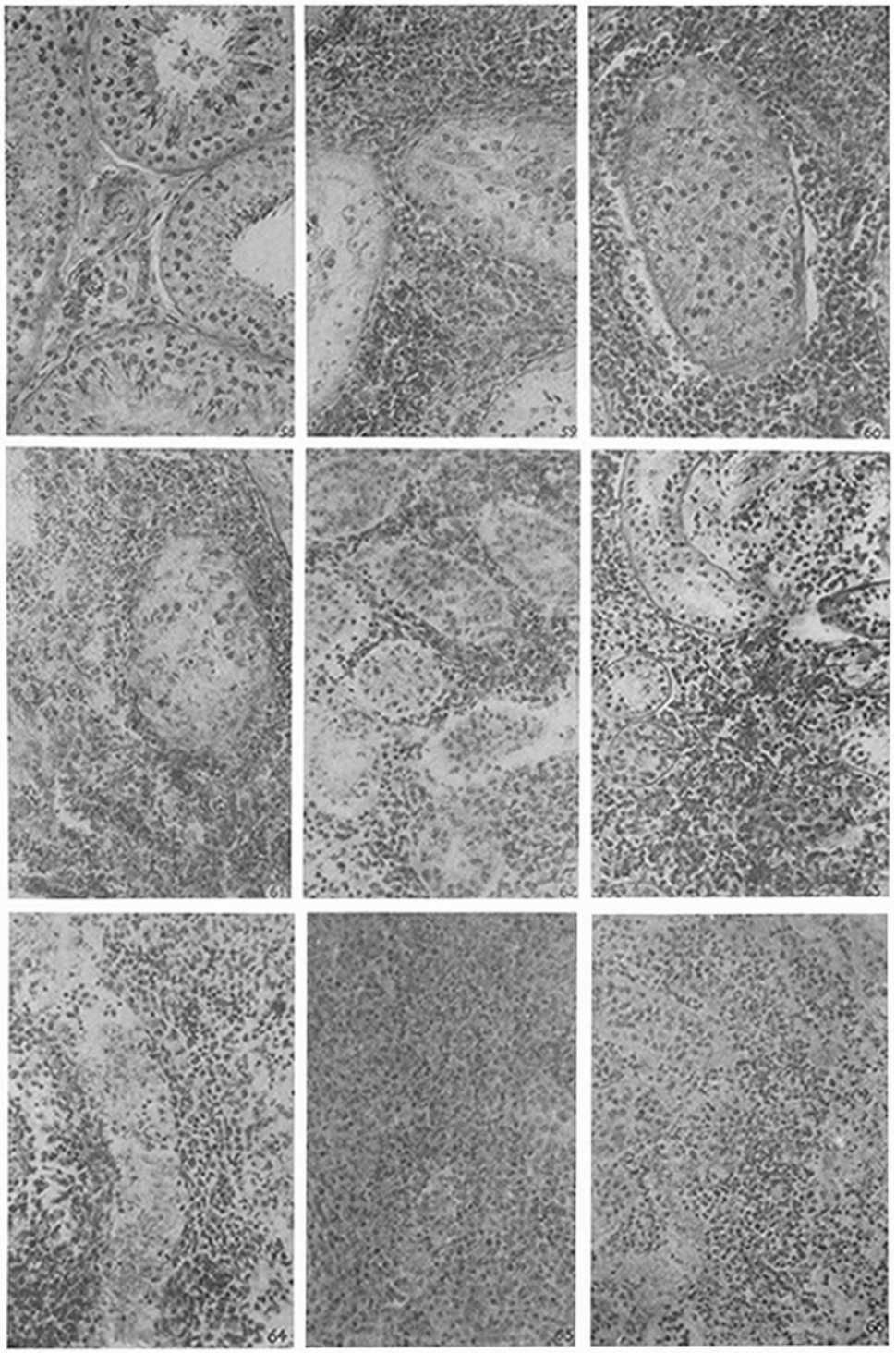

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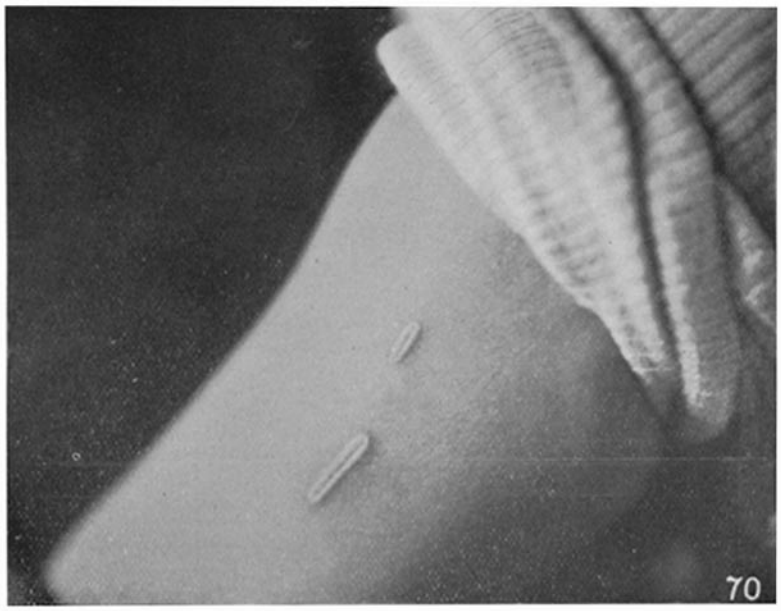

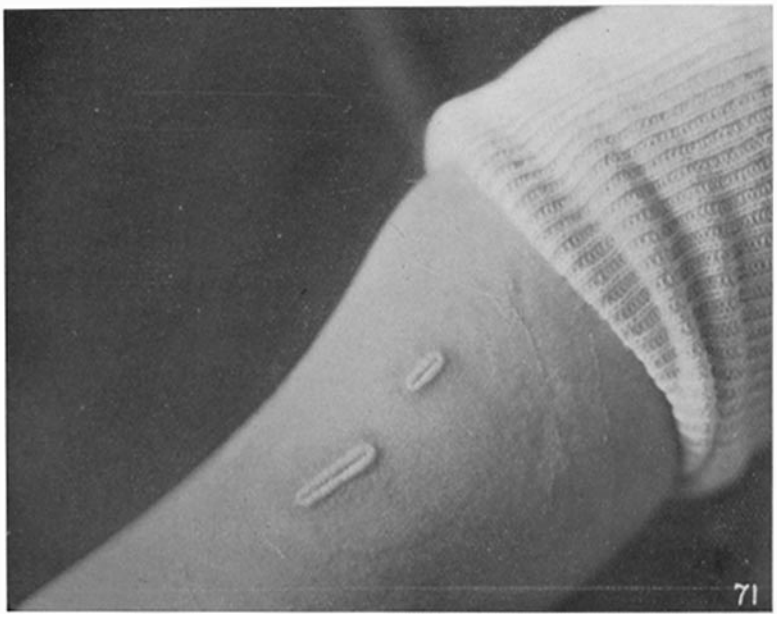

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