FURTHER OBSERVATIONS ON THE PHENOMENA ENCOUNTERED IN ATTEMPTING TO TRANSMIT VARICELLA TO RABBITS.

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PLATE 38.

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The phenomena observed following the injection of blood from varicella patients into the testicles of rabbits and the macroscopic lesions resulting from the inoculation of testicular emulsions from these rabbits into the testicles, cornea, and skin of other rabbits in series have been described in a preceding paper.[1] At the time of the previous report, however, the results of inoculations of the skin with active testicular emulsions were so variable that it was impossible to predict regularly whether a visible reaction would occur in any individual rabbit even when a concentrated virus was smeared on the scarified skin. This irregularity in the occurrence of the skin reactions interfered with rapid progress in the study of the phenomena observed. Therefore a better method of inoculating the skin was sought, and the intradermal injections of small quantities of the active testicular emulsions were soon found to produce constant and specific reactions in the skin of rabbits. A description of the intradermal method of inoculation and the results of the experimental work facilitated by its use will be given in this paper.

Intradermal Inoculation.

Method.

Testicles removed[2] from rabbits 4 days following intratesticular injections of virus, were ground up with sterile, chemically clean sand and 10 cc. of Locke's solution. The testicular emulsion was centrifuged for 10 minutes at high speed. Por-

[1] Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1923, xxxviii, 673.

[2] All operations were performed under ether anesthesia.

tions of the decanted supernatant fluid were cultured on various media for ordinary anaerobic and aerobic bacteria. With other portions of the supernatant fluid and Locke's solution a series of dilutions from 1:1 to 1:1,000 was made and 0.2 cc. of each dilution was injected by means of a tuberculin syringe into the shaved skin of a rabbit. White elevated areas in the skin about 1 cm. in diameter appeared immediately at the sites of the intradermal injections and then rapidly disappeared. Daily examinations of the skin were made for 2 weeks.

Following the intradermal injections nothing except the effects of trauma was observed until the 3rd or 4th day, when it was noticed that the skin at the sites of the inoculations of concentrated material was red and raised (Fig. 1). The reactions at these points increased in size and intensity till the 5th to 7th day when the central portion of the inoculated areas was often very dark. Extending into the skin around the dark central area was a zone of less intense erythema 2 to 4 cm. in width. The lesions gradually subsided and in a fortnight disappeared without scar formation. At the sites of inoculation of the higher dilutions of the virus, visible reactions appeared more slowly,—5th or 6th day,—and they were smaller, less severe, and disappeared more rapidly.

In developing the method of intradermal inoculations of the virus, adequate controls were conducted by injecting fresh and heated emulsions of normal testicles into the skin of normal rabbits and fresh testicular emulsion containing active virus into the skin of immune rabbits. No reactions were seen in the controls. The absence of reactions following the control injections was so striking that it soon became evident that the skin lesions following the injections of testicular emulsions containing active virus were specific. While the method of smearing the testicular emulsions on the scarified skin was adequate for demonstrating the presence of a virus (Fig. 2); the results were so variable that it was impossible to titrate the virus accurately, to determine the effects of certain physical and chemical agents upon the virus, or to complete immunological studies with the virus. The results obtained following the introduction of the intradermal method, however, were so consistent and reliable as to facilitate the completion of the desired experiments in a satisfactory manner.

Virus III.

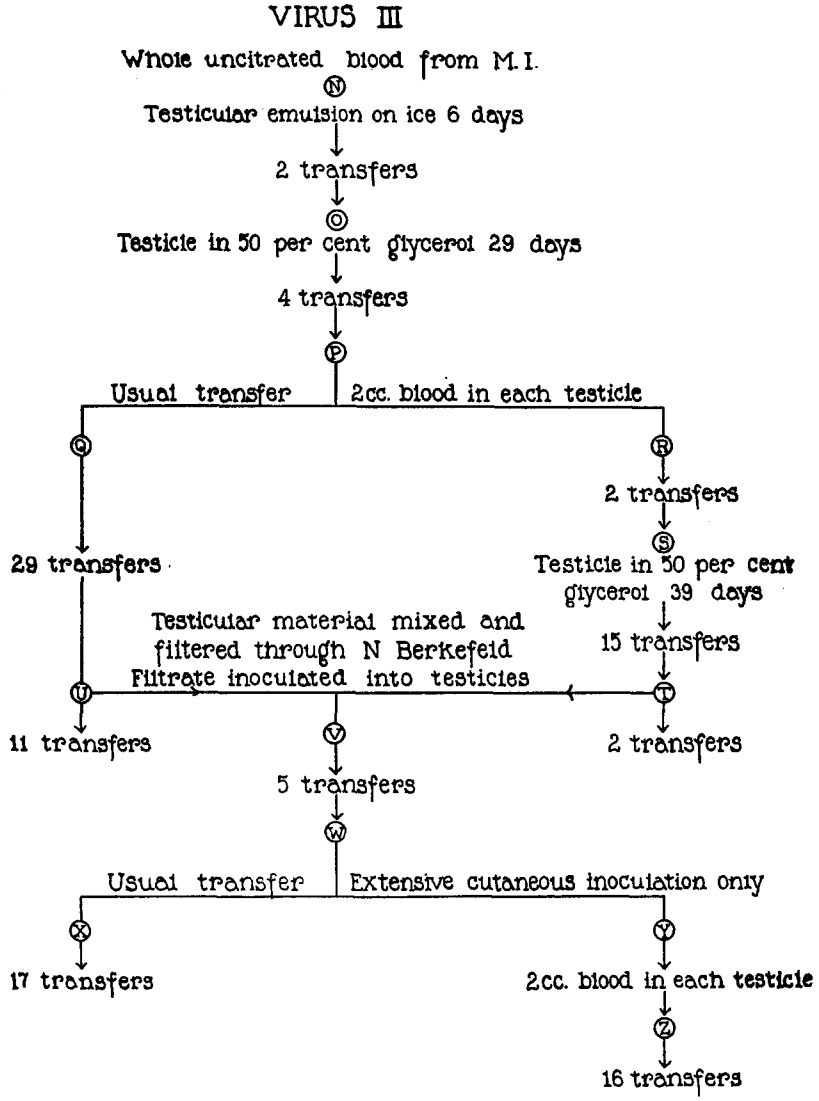
Before proceeding with a detailed description of the experiments, a brief statement concerning the virus used in most of them will be made. The lesions produced by Virus III were first recognized in the fourth testicular transfer following a primary inoculation of blood from a varicella patient into the testicles of a rabbit.[1] The virus has been transferred 65 times from rabbit to rabbit (Text-fig. 1). It now acts like a fixed virus in that a small amount of the virus when injected into the testicles of rabbits causes a sharp rise of temperature to 104–106°F. in the animals 3 or 4 days after the inoculation, and because intradermal injections of the virus in dilutions of 1:1,000 are regularly followed by visible skin reactions. Although the virus can be recovered from the heart's blood of rabbits (Text-fig. 1) following intratesticular or intradermal inoculations, it does not kill the animals even when injected intracerebrally. Portions of the testicular emulsions containing the virus have been shown repeatedly to be free of ordinary anaerobic and aerobic bacteria by means of cultures on blood agar, in broth, and in Smith-Noguchi tubes. Furthermore, ordinary bacteria have not been seen in stained films and dark-field preparations of the emulsions containing the virus, in stained sections of inoculated testicles, or in sections of inoculated testicles impregnated with silver nitrate.

Effects of Certain Physical and Chemical Agents upon the Virus.

Filtration.—Virus III passes through Berkefeld filters N and V (Table I). Filters W were not used.

Method.

Fresh testicular emulsions were prepared in the usual manner except that at times larger amounts, 20 to 40 cc., of Locke's solution were added to the ground up testicles. After the emulsions had been centrifuged at high speed for 10 minutes, the supernatant fluid was decanted and filtered through paper. Influenza bacilli or colon bacilli, occasionally both, were added to the emulsions before filtration was started. This was done in each experiment to test the filters simultaneously with the filtration of the virus. Some of the filters were new, while others had been used two or three times previously. A water suction apparatus created the vacuum necessary for the filtration in all the experiments except one in which the filtration was accomplished under 50 pounds of pressure. The time



Transfers = consecutive transfers from the testicles of one rabbit to those of another.

Usual transfer = usual testicular transfers.

TEXT-FIG. 1. Outline of the transfer method pursued in the study of Virus III.

required to obtain enough filtrate for the experiments varied from 10 to 60 minutes. Portions of each filtrate were cultured aerobically and anaerobically in liquid and on solid media with and without blood. Other portions of the filtrate were injected into the skin and testicles of normal rabbits and into the skin of immune rabbits. In many of the experiments, testicles which had been inoculated with the filtrates were removed 4 days later and ground up in the usual manner. Then portions of these emulsions were injected into the skin and testicles of normal rabbits. In this way the virus in the filtrates was passed through several rabbits by means of testicular inoculations.

The cultures of the filtrates in the nine experiments summarized in Table I remained free of ordinary bacteria. In the first three experiments no visible reactions occurred in the rabbits' skin at the sites of inoculation of the filtrates. The animals in two of these experiments were immune, however, when they were inoculated intradermally with active virus 2 weeks later. In the other six experiments, intradermal inoculations of the filtrates produced visible reactions in the skin of normal rabbits but none in the skin of immune rabbits. Testicular emulsions from Rabbits 11 and 12 were mixed and filtered. Portions of the filtrate were injected into the skin and testicles of Rabbit 13. The virus from this filtrate has been passed through twenty-four rabbits by means of testicular inoculations and is now being carried as a stock strain. Rabbits 11, 12, and 13 in Table I are Rabbits T, U, and V in Text-fig. 1.

In the 8th and 9th experiments (Table I), the testicular emulsions before and after filtration were titrated in the skin of the same rabbit. In the 8th experiment the emulsion before it was filtered produced a visible reaction in the skin when diluted 1:1,000, yet the same emulsion after it was filtered produced such a reaction only when undiluted. In the 9th experiment the unfiltered emulsion diluted 1:200 produced a visible skin lesion, while the filtered emulsion produced no such lesion in dilutions higher than 1:10. Some of the virus passed through the filters, yet it is obvious that most of it was held back under the conditions of the experiments. No attempt will be made to discuss all the factors which may influence filtration. It seems, however, that filters which have been used several times are more impervious than unused ones, and that a relatively greater concentration of the virus passes through the filters when the testicular emulsions have been diluted. These two factors may account for the results of the titrations of the

TABLE I.

Summary of the Results of Nine Experiments Showing Conclusively that Virus III Passes through Berkefeld Filters N and V.

Testicular virus from rabbits	Berkefeld filter	Suction or pressure	Time of filtration	Test bacteria	Sterility of filtrate	Filtrate inoculated intradermally and intratesticularly into rabbits	Result of skin inoculations	Immune after 2 weeks
1	N	Water pump	10 min.	Influenza bacillus	Sterile	2 ↓ 3	—	Yes
							—	Yes
4	N	Water pump	15 min.	Influenza bacillus	Sterile	5 ↓ 6 ↓ 7	—	No
							—	No
							—	No
8	N	Water pump	30 min.	Influenza bacillus	Sterile	9	—	Yes
"	"	"	"	"	"	10	—	Yes
11 (T) and 12 (U)	N	Water pump	30 min.	Influenza bacillus	Sterile	13 (V) ↓ Still being passed from rabbit to rabbit by testicular inoculations	+	Yes
14	N	50 pounds pressure	30 min.	Influenza bacillus	Sterile	15 ↓ 16 ↓ 17 ↓ 18	+	Yes
							+	Yes
							+	Yes
							+	Yes
19	V	Water pump	10 min.	Influenza bacillus	Sterile	20 ↓ 21	+	No test
							+	No test
22	V	Water pump	60 min.	Influenza bacillus	Sterile	23	+	Yes
24	V	Water pump	60 min.	Colon bacillus	Sterile	25	+	Yes
26	V	Water pump	10 min.	Colon bacillus and Influenza bacillus	Sterile	27 ↓ 28	+	No test
							+	Yes

↓ = Passage of the virus from one rabbit to another by means of testicular inoculations.

Intradermal inoculations were used in testing for the presence of an immunity.

All intradermal inoculations were controlled with normal and with immune rabbits.

— = No visible skin reaction; + = visible skin reaction.

testicular emulsions before and after filtration in the 8th and 9th experiments. In the former the filter had been used twice previously and the testicular emulsion was concentrated, while in the latter the filter had not been used previously and the emulsion was diluted before filtration was attempted.

In the above experiments it has been shown conclusively that Virus III is filterable in spite of the fact that a great deal of it is held back by the filters.

TABLE II.

Summary of Results of Two Experiments Showing the Effect of Heat upon Virus III.

Testicular virus from rabbit	Test rabbit	Unheated virus control	Heated virus						
			30 min. 45°C.	30 min. 55°C.	30 min. 65°C.	10 min. 55°C.	20 min. 55°C.	40 min. 55°C.	50 min. 55°C.
1	2 normal	+	+	−	−	−	−	−	−
1	3 immune	−	−	−	−	−	−	−	−
4	5 normal	+	+	−	−	−	−	−	−
4	6 immune	−	−	−	−	−	−	−	−

Intradermal inoculations were used in the experiments.
+ indicates a visible reaction at the site of inoculation.
− indicates the absence of a visible reaction at the site of inoculation.

Heat.—Virus III is very sensitive to heat. The results of two experiments which were performed to determine the effect of heat upon the virus are summarized in Table II. Portions of fresh testicular emulsions containing active virus which produced visible skin lesions in dilutions of 1:1,000 were heated various lengths of time at different temperatures. Then a small quantity of each portion was injected into the skin of the same normal rabbit and also into the skin of the same immune rabbit. No visible reactions were obtained in the immune rabbit. Definite reactions occurred, however, in the skin of the normal rabbit at the sites of inoculation of the unheated virus and the virus heated at 45°C. for 30 minutes. The portions of virus heated 10, 20, 30, 40, and 50 minutes at 55°C. and 30 minutes at 65°C. respectively produced no visible reaction in the skin of the normal rabbit.

TABLE III.

Summary of the Results of the Experiments Showing the Effect of Physiological Salt Solution, Locke's Solution, and Glycerol upon the Viability and Activity of Virus III.

Rabbit	Unfiltered testicular virus, 1, preserved in physiological salt solution unsealed on ice.		Unfiltered testicular virus, 2, preserved in Locke solution on ice. Sealed with vaseline.	
	2 days	5 days	23 days	
Immune	—	—	—	
Normal	+	—	—	
	Unfiltered testicular virus, 3, preserved in Locke solution unsealed on ice.		Unfiltered testicular virus, 3, preserved in Locke solution on ice. Sealed with vaseline.	
	30 days		30 days	
Immune	—		—	
Normal	—		—	
	Unfiltered testicular virus, 4, preserved in Locke solution on ice. Sealed with vaseline.		Unfiltered testicular virus, 4, preserved in Locke solution and 40 per cent glycerol on ice. Sealed with vaseline.	
	54 days		54 days	
Immune	—		—	
Normal	—		—	
	Berkefeld filtrate of testicular virus, 5, preserved in Locke solution unsealed on ice.		Berkefeld filtrate of testicular virus, 5, preserved in Locke solution on ice. Sealed with vaseline.	
	28 days	61 days	28 days	61 days
Immune	—	—	—	—
Normal	—	—	+	—
	Berkefeld filtrate of testicular virus, 6, preserved in Locke solution unsealed on ice.		Berkefeld filtrate of testicular virus, 6, preserved in Locke solution on ice. Sealed with vaseline.	
	17 days	52 days	17 days	52 days
Immune	—	—	—	—
Normal	—	—	+	—
	Berkefeld filtrate of testicular virus, 7, preserved in Locke solution on ice. Sealed with vaseline.		Berkefeld filtrate of testicular virus, 7, preserved in Locke solution and 40 per cent glycerol on ice. Sealed with vaseline.	
	24 days		24 days	
Immune	—		—	
Normal	—		+	

Intradermal inoculations were used in these experiments.
+ indicates a visible reaction at the site of inoculation.
— indicates absence of a visible reaction at the site of inoculation.

Physiological Salt Solution, Locke's Solution, and Glycerol.—It seemed of interest to ascertain under what conditions the virus can be best preserved in an active state *in vitro*. Therefore the effects of physiological salt solution, Locke's solution, and glycerol upon the viability and activity of the virus were observed. These observations are summarized in Table III. Under certain conditions the virus evidently deteriorates rapidly when removed from the body and quickly ceases to produce visible lesions in the skin. Even though the virus deteriorates rapidly *in vitro*, some of it remains viable in physiological salt solution and in 40 to 50 per cent glycerol various lengths of time (Text-fig. 1) and is recoverable in a highly active state after several passages through rabbits.

The virus (Table III) in the unfiltered testicular emulsions, with or without the addition of glycerol, sealed or unsealed, after a short time ceased to produce visible skin reactions. The virus in the Berkefeld filtrates of the testicular emulsions sealed with vaseline, however, continued to produce definite skin reactions longer than did the virus in the unfiltered emulsions preserved under similar conditions. The difference in the preservation of the virus under these conditions becomes more evident when the difference in its concentration in the filtered and unfiltered fresh emulsions is considered. The fresh unfiltered material was active in most instances in dilutions of 1:1,000 while the filtrates were active only in the undiluted portion or in dilutions of 1:10. The addition of glycerol to the filtrates before they were sealed with vaseline also seemed to aid in the preservation of the virus. The data obtained so far indicate that the best method of preserving the virus in an active state is to filter the testicular emulsions, add glycerol to the filtrates up to 40 per cent of the total volume, seal with vaseline, and store on ice.

Study of the Immunity Produced by the Virus.

Active Immunity.[3]

One of the striking characteristics of most of the so called filterable viruses is that an infection caused by them leads to the appear-

[3] In the experiments reported under Active Immunity, a viable virus was always used. This was carefully controlled even when no note of it appears in the report of the individual experiments.

TABLE IV.

Summary of the Results of Studies upon the Active Immunity Produced by Virus III.

Rabbit	Material used for immunizing dose	Site of inoculation of immunizing dose	Reaction to immunizing dose	Time between immunizing & testing doses	Material used for testing dose	Site of inoculation of testing dose	Result
1	Fresh testicular virus	Testicles & skin	Visible reaction testicles & skin	4 days	Fresh testicular virus	Skin intradermally	Partially immune
2	"	"	"	5 "	"	"	Immune
3	"	Skin intradermally	Visible reaction	10 "	"	"	"
4	"	"	"	14 "	"	"	"
5	"	"	"	60 "	"	"	"
6	Testicular virus preserved on ice 42 days in 40 per cent glycerol	"	No visible reaction	18 "	"	"	"
7		"	"	18 "	"	"	"
8		"	"	18 "	"	"	"
9	Normal testicle preserved in 40 per cent glycerol on ice 31 days	"	"	18 "	"	"	Not immune
10		"	"	18 "	"	"	"
11		"	"	18 "	"	"	"
12		"	"	18 "	"	"	"
13		"	"	18 "	"	"	"
14	Fresh testicular virus	Brain	Fever	25 "	"	"	Immune
15	"	"	"	29 "	"	"	"
16	"	"	"	29 "	"	"	"
17	Blood containing active virus	Intravenously	No visible reaction	16 "	"	"	"
18	"	"	"	16 "	"	"	"
19	"	"	"	14 "	"	"	"
20	"	"	"	14 "	"	"	"
21	"	Testicles	"	16 "	"	"	"
22	"	"	"	16 "	"	"	"
23	"	"	"	14 "	"	"	"
24	"	"	"	14 "	"	"	"
25	Fresh testicular virus	Intravenously	"	17 "	"	"	"
26	"	Nose	"	20 "	"	"	"
27	"	"	"	20 "	"	"	"
28	"	"	"	28 "	"	"	"
29	"	"	"	28 "	"	"	"
30	"	"	"	28 "	"	"	"
31	"	Smeared on unshaved skin of ears	"	23 "	"	"	Not immune
32	"		"	23 "	"	"	"
33	"		"	23 "	"	"	"
34	"	Testicles & skin	Visible reaction	6 months	"	"	Immune

All testicular virus used for immunizing and testing doses was controlled with animals known to be normal and immune.

The virus was demonstrated in the blood said to contain active virus by passage through rabbits' testicles.

The testicular virus preserved in glycerol was still viable as an active virus was recovered by passage through rabbits' testicles.

ance in recovered animals of a marked lasting immunity, or refractory state. Therefore it seemed of interest to determine if the animals which had recovered from a primary inoculation of the virus under investigation were refractory to subsequent inoculations of the same virus. It was soon found that testicular emulsions containing active virus when injected into rabbits regularly produced an immunity which did not follow inoculations with emulsions of normal testicles.

Time of Appearance of the Immune or Refractory State.—The time of appearance of the refractory state in animals probably depends on a number of factors; for example, the amount, condition, and mode of administration of the virus. It seemed of interest to determine how soon a refractory state can be established in rabbits following injections of large amounts of Virus III. 5 days were found to be the shortest length of time within which a rabbit becomes immune to the virus.

Rabbit 1 (Table IV) received in each testicle 1 cc. of a fresh testicular emulsion containing Virus III and also 0.2 cc. of the same emulsion intradermally. Typical reactions occurred in the testicles and skin. 4 days after the primary inoculations the rabbit was reinoculated intradermally with active Virus III. A mild accelerated reaction appeared at the site of reinoculation. Rabbit 2 was treated in a similar manner with the exception that the reinoculation of the skin was made 5 days after the primary injection. While definite reactions occurred after the primary injections, none was observed following the second intradermal inoculation.[4]

Immunity Following Intradermal Inoculations of Active Virus III.—The skin of rabbits became immune within a short time after intradermal injections of small amounts of active virus.

Rabbits 3, 4, and 5 (Table IV) received intradermally 0.2 cc. of a fresh testicular emulsion containing active Virus III. Typical reactions followed the primary inoculations. The skin of these animals, however, was immune to active virus after 10, 14, and 60 days, respectively.

Immunity Following Intradermal Inoculations of Virus III Preserved in Glycerol.—A glycerolated virus, which was still viable, im-

[4] The virus used in each of the immunity experiments was controlled on normal and on immune rabbits.

munized rabbits when injected intradermally. The results of this experiment are summarized in Table IV.

Immunity Following Intracerebral Inoculations of Virus III.— Small amounts of fresh testicular emulsions containing active virus when injected into the brains of young rabbits (1,000 gm.) caused febrile reactions more severe than those produced in control animals by intracerebral inoculations of emulsions of normal testicles. The rabbits did not die after the intracerebral inoculations and the animals which received the virus developed an immunity.

Rabbits 14, 15, and 16 (Table IV) were inoculated intracerebrally with 0.2 cc. of a fresh testicular emulsion containing active Virus III. Sharp febrile reactions occurred 3 to 5 days after the injections. The animals were reinoculated intradermally with active Virus III 25 to 29 days later. No reactions followed the second inoculations.

Immunity Following Intravenous Inoculations of Active Virus III.— A refractory state was established in animals following intravenous injections of the virus.

Rabbits 17, 18, 19, 20, and 25 (Table IV) were inoculated intravenously either with fresh infective rabbit blood or with fresh testicular emulsion containing active virus. 14 to 17 days later the animals were reinoculated intradermally with fresh active Virus III without the development of any visible reactions.

Immunity Following Intranasal Instillations of Active Virus III.— The instillation of active Virus III into the nostrils of rabbits was followed by the appearance of an immunity in the skin of the animals against the virus. No lesions were observed around the external nasal orifices following the intranasal inoculations of the virus. Lesions might have occurred in the mucous membranes inaccessible for examination in living animals. This could not be determined, however, as it was impossible to sacrifice a rabbit for an examination of the nasal mucous membranes and at the same time save the animal for future immunity work. That the virus instilled into the nose had produced a reaction somewhere in the animals was evidenced by the immunity that subsequently developed.

0.5 cc. of a fresh testicular emulsion containing active Virus III was instilled into each nostril of Rabbits 26, 27, 28, 29, and 30 (Table IV). The nasal mucous membranes were not injured at the time of the inoculations and no reactions were noticed in the animals during the 2 weeks of observation. The skin of the rabbits was refractory to active virus 20 to 28 days later. The experiment was controlled by rubbing active virus on the unshaved skin of the ears of Rabbits 31, 32, and 33 (Table IV). The skin was not shaved because shaving alone often injures the skin enough to permit the entrance of the virus into the body. The skin of the control animals, which received applications of the virus on the unshaved skin of the ears, was not refractory to the virus 23 days later.

Duration of the Immunity.—At present it is impossible to state how long the immunity persists in animals. It is known, however, that the immunity lasts for at least 6 months, as all the rabbits, when tested 6 months after the primary inoculations, were still immune to the virus.

Immunological Relationship of Three Strains of the Virus under Investigation.—Three strains of the virus under investigation have been shown experimentally to be immunologically identical. These strains are designated Virus III, Virus IV, and Virus V.

Three rabbits each were inoculated intradermally with Virus III, Virus IV, and Virus V. A typical reaction followed the primary inoculations in all the animals. 2 weeks later these rabbits were reinoculated intradermally with active Virus III, the most vigorous strain, and in no instance was there a reaction at the site of the second inoculation.

Immunological Relationship between Vaccine Virus and Virus III.—Experiments have failed to demonstrate an immunological relationship between vaccine virus and Virus III.

Eight rabbits which had been previously shown by test to be immune to Virus III, were inoculated on the scarified skin with vaccine virus. An eruption typical of vaccinia occurred in each rabbit. Five rabbits which had been previously shown by test to be immune to vaccine virus were inoculated intradermally with active Virus III. A typical reaction occurred in each rabbit at the site of the inoculation. The vaccine virus and Virus III used in this experiment were controlled in normal and immune rabbits.

Relationship between Virus III and the Virus of Symptomatic Herpes.[5]—Virus III seems to be more closely allied to the virus of symptomatic herpes than to vaccine virus. The intranuclear changes seen

[5] Two strains of the virus of symptomatic herpes were supplied by Dr. Flexner.

in cells injured by Virus III and the virus of herpes are almost identical. In spite of this similarity, definite differences in the behavior of the two viruses have been observed. Virus III never kills young rabbits when injected intracerebrally, does not produce vesicles following skin inoculations, gives more constant results when injected intradermally than does the herpes virus, and produces only a mild reaction when inoculated upon a scarified cornea.

Furthermore, the two viruses are immunologically distinct, as shown by the following experiments.

Rabbit A, immune to Virus III for 30 days, received an intracerebral inoculation of 0.25 cc. of a strong herpes virus. The rabbit showed the usual symptoms of encephalitis and died on the 3rd day after the inoculation.

Rabbit B, immune to Virus III for 70 days, received an intracerebral inoculation of 0.25 cc. of a weak herpes virus. The animal showed the usual symptoms of encephalitis, had a temperature of 108°F. on the 7th day, and died on the 8th day following the inoculation.

Rabbit C (1,000 gm.) received intracerebrally 0.25 cc. of a weak herpes virus which had been preserved in glycerol on ice for 3 months. On the 4th day after inoculation the animal's temperature rose to 104°F., remained high for 4 days, during which time the rabbit was sick and ataxic. The animal recovered and 10 days later proved equally susceptible as control animals to Virus III injected intradermally.

Rabbit D (1,800 gm.) showed the usual reaction following the intradermal inoculation of Virus III. 14 days later the animal was inoculated intradermally with active Virus III on one side of the body and with active herpes virus on the other side. No reaction was observed at the site of the reinoculation of Virus III. Typical lesions, however, occurred at the site of the inoculation of the herpes virus.

Absence of Immunity in Rabbits Following a Single Intradermal Injection of Virus III Which Had Been Heated 10 Minutes at 55° C.—A single intradermal injection of Virus III, which had been heated 10 minutes at 55° C., was not followed by any appreciable immunity in rabbits, while one of the unheated virus was regularly followed by a refractory state.

Passive Immunity.

Failure of Intravenous Injections of Immune Rabbit Serum to Protect Normal Rabbits against Virus III.—After it was established that rabbits can be immunized actively by injections of viable virus, it was considered desirable to determine if animals can also be protected

passively against the virus by injections of serum from immune animals. The serum was collected from immune rabbits, 2 or 3 weeks after the immunizing dose of virus, and contained virucidal properties as will be shown further on. A number of rabbits received intravenous injections of 5 to 10 cc. of the immune serum and 24 hours later were inoculated intradermally with active Virus III. The rabbits which had received the serum, and normal control animals, proved equally susceptible to the virus, while actively immunized rabbits were refractory. It has not been determined whether the negative outcome of these experiments was due to the injection of too small an amount of serum, to the length of time allowed between the injection of the serum and the inoculation of the virus, or to an impossibility of demonstrating passive immunity in rabbits to Virus III following the intravenous injection of immune serum. Even though a definite explanation of the negative results is lacking, it is, nevertheless, very evident that rabbits were not protected by the intravenous injection of immune serum under the conditions outlined in the experiments.

Virucidal Action of Immune Rabbit Serum.

Neutralization of Virus III by Immune Rabbit Serum in Vitro.— The immune rabbit serum, which did not protect normal rabbits when 5 to 10 cc. of it were given intravenously, was found to be virucidal when mixed with the virus *in vitro*.

Various mixtures of the virus and sera with controls were set up and incubated at 37° C. for 2 hours as indicated in Table V. Then a small quantity of each mixture was injected intradermally into the same normal rabbit. The rabbit was examined daily for 2 weeks for the appearance of reactions at the sites of the injections.

The tests showed that immune rabbit sera neutralized the virus, while normal rabbit sera, Locke's solution, and physiological salt solution did not. The results were striking, as witness the skin of the rabbit (Fig. 3) and the summary of three experiments in Table V. The results were the same when complement was included or excluded from the mixtures *in vitro*. At present it is not known whether complement is essential for the neutralization of the virus, as the

results of the various mixtures after incubation must be determined in the skin of living animals where complement may be supplied

TABLE V.

Summary of the Results of Three Experiments Showing that Immune Rabbit Serum Neutralizes Virus III in Vitro.

Rabbit	Mixtures incubated 2 hrs. at 37°C. and then small quantities of each were injected intradermally into rabbits	Dilutions of the virus								
		1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256	1-512	1-1024
3	.5cc. (salt solution and complement) + .5cc. various dilutions of virus	+	+	+	+	+					
	.5cc. (normal rabbit serum, 1, and complement) + .5cc. various dilutions of virus	+	+	+	+	+					
	.5cc. (immune rabbit serum, 1, and complement) + .5cc. various dilutions of virus	−	−	−	−	−					
4	.5cc (salt solution and complement) + .5cc. various dilutions of virus			+	+	+	+	+	+	+	+
	.5cc. (normal rabbit serum, 2, and complement) + .5cc. various dilutions of virus			+	+	+	+	+	+	+	+
	.5cc. (immune rabbit serum, 5, and complement) + .5cc. various dilutions of virus			−	−	−	−	−	−	−	−
6	.5cc. fresh immune rabbit serum, 7, + .5cc. various dilutions of virus	−			−			−			−
	.5cc. inactivated immune rabbit serum, 7, + .5cc. various dilutions of virus	−			−			−			−
	.5cc. Locke solution + .5cc. various dilutions of virus	+			+			+			+

Normal rabbit serum, 1, and immune rabbit serum, 1, came from the same animal, the former before immunization, the latter 14 days after an intradermal injection of the virus.

+ indicates a visible lesion at the site of inoculation.

− indicates absence of a visible lesion at the site of inoculation.

for the completion of the reaction if this has not already been accomplished *in vitro*.

Neutralization of Virus III Locally in Vivo by Immune Rabbit Serum.—Doubt arose as to whether the neutralization of the virus

by immune serum was completed *in vitro* or whether a part or all of the reaction occurred locally in the skin of the normal rabbit following the injections of the various mixtures described in the preceding experiments. It was early found that mixtures of virus and sera which were injected immediately into the skin of rabbits gave results identical with those obtained following injections of similar mixtures which had been incubated 2 hours at 37° C. (Tables

TABLE VI.

Summary of the Results of One Experiment Showing that Immune Rabbit Serum Neutralizes Virus III in Vivo When the Serum and the Virus Are Injected into the Same Part of the Skin at or about the Same Time.

RABBIT A

Right side		Left side	
Front	Result	Front	Result
Small amount of fresh virus injected intradermally.	+	.5cc. fresh virus + .5cc. Locke solution mixed and a small amount of the mixture injected immediately intradermally.	+
Rear		Rear	
An area of skin 3 cm. in diameter was infiltrated with 1.5cc. of inactivated immune rabbit serum. 10 min. later a small amount of fresh virus was injected intradermally in the center of the infiltrated area.	−	.5cc. fresh virus + .5cc. inactivated immune rabbit serum mixed and a small amount of the mixture injected immediately intradermally.	−

+ indicates the usual visible reaction following an intradermal injection of fresh active virus.

− indicates the absence of a visible reaction at the site of injection after 48 hours.

V and VI). Furthermore, it was found that, if a small area in the skin of a normal rabbit was infiltrated with immune serum and then 10 minutes later active virus was injected in the center of the infiltrated area, no lesion occurred. Although no lesion occurred in the area of skin protected by the immune serum, a typical reaction appeared at the site of the injection of virus into a control area of the skin only a short distance away (Table IV).

Identification of Virus III.

Virus III was recovered by injecting blood of varicella patients into the testicles of rabbits and then making repeated transfers at 4 day intervals from rabbit to rabbit by means of testicular inoculations. The method employed in recovering the virus, the fact that more than half of the experiments which were performed in attempting to recover the virus resulted negatively, the macroscopic and microscopic lesions produced in rabbits by the virus, led us to infer that we were not improbably working with the etiological agent of chicken-pox. It was realized that the final proof that the virus is the etiological agent of varicella was lacking and in a previous paper[1] we expressly stated that: "Further evidence must be obtained, however, before one can think and speak definitely of this virus as the etiological agent of varicella." A better understanding of the behavior of the virus in animals and better methods of working with the virus were necessary before we could rely on the results of experiments performed to identify the virus. A description of the necessary methods and the behavior of the virus in normal and immune animals have been described above. With this information we were then prepared to undertake the identification of Virus III. The results of this work follow.

Failure of Intravenous Injections of Serum and Whole Blood from Convalescent Chicken-Pox Patients to Protect Normal Rabbits against Virus III.—Neither 5 to 10 cc. of whole blood nor 5 to 10 cc. of serum from patients convalescent from chicken-pox, when injected intravenously, protected normal rabbits against Virus III inoculated intradermally 24 hours after the administration of the serum or blood. This was considered to be in accord with experiments reported above in which it was shown that intravenous injection of immune rabbit serum which neutralized the virus *in vitro* would not protect normal animals against Virus III inoculated intradermally.

Failure of Serum from Patients Convalescent from Chicken-Pox to Neutralize Virus III in Vitro.—Since immune rabbit serum neutralizes Virus III *in vitro* and since a reliable technique has been devised by which this neutralization can be demonstrated, it seemed wise to determine if the serum of patients convalescent from vari-

cella possesses any demonstrable virucidal properties for Virus III or if the serum collected during convalescence neutralizes more virus than does the serum collected during the first 2 days of the disease. The results of these experiments showed that sera from two normal adults and from fourteen patients convalescent from varicella have no demonstrable neutralizing effect upon Virus III *in vitro*. Furthermore, no difference could be detected in the sera collected from four patients during the disease and during convalescence. We felt that the serum from a patient convalescent from varicella should neutralize Virus III *in vitro*. When it was found, however, that the serum did not neutralize the virus, it occurred to us that the virus might be too concentrated or too active to be affected by a small amount of immune bodies that might appear in the serum of convalescents.

Failure of Rabbits to Be Actively Immunized against Virus III by Injections of Whole Blood, Vesicle Fluid, or Nasal Washings from Varicella Patients.—Since it was known that the virus is present in small amounts of blood taken from a rabbit 4 days after intratesticular inoculation with Virus III, since an active immunity promptly appears in rabbits inoculated with small amounts of this blood, and since there is evidence that the blood of varicella patients contains the etiological agent of chicken-pox, we decided to see if animals could be actively immunized against Virus III by injections of fresh blood from patients with chicken-pox early in the disease. In this manner we hoped to establish some relationship between Virus III and the etiological agent of varicella. Vesicle fluid and nasal washings were also injected into a few animals. Control animals received similar amounts of fresh blood from normal humans. After the inoculations the animals were kept 21 to 67 days and then the presence of an immunity in them was determined by means of intradermal inoculations of various dilutions of active Virus III. In each experiment the virus was controlled in normal and immune animals. Eleven experiments were performed in which 39 test and 23 control animals were employed. The results of these experiments are summarized in Table VII. The percentage of immune rabbits was the same in the control as in the experimental animals, 26 per cent. Experiments 3, 10, and 11 taken alone might be in-

TABLE VII.

Summary of Eleven Experiments Showing that Rabbits Could Not Be Actively Immunized against Virus III by Injections of Blood, Nasal Washings, or Vesicle Fluid from Varicella Patients. These Experiments Also Show that a Certain Number of Rabbits Are Refractory to Virus III Regardless of the Materials Previously Inoculated.

		Test animals						Control animals						
Experiment	Patient	Number rabbits	Material injected	Site of injection	Time before testing immunity	Number immune	Number not immune	Normal man	Number rabbits	Material injected	Site of injection	Time before testing immunity	Number immune	Number not immune
1	Rev.	3	Blood	Testicles	21 days	2	1	F	3	Blood	Testicles	21 days	1	2
2	Rec.	3	"	"	21 "		3	T.	2	"	"	21 "		2
		2	Nasal washings	Skin	21 "		2							
		1	Vesicle fluid	"	21 "	1								
3	Bo.	3	"	"	25 "		3	R.	2	"	"	25 "		2
		1	Nasal washings	"	25 "		1							
		3	Blood	Testicles	25 "	3								
4	Fer.	5	"	"	26 "		5	T	5	"	"	26 "		5
		2	Vesicle fluid	Skin	32 "		2							
5	Ric.	3	Blood	Testicles	41 "		3	T.	3	"	"	41 "	1	2
6	Ka.	1	"	Vein	30 "	1		T.	1	"	Vein	30 "		1
7	Da.	1	Vesicle fluid	Skin	48 "		1							
8	Co.	2	"	"	45 "		2							
9	Pe.	2	"	"	41 "		2	T.	2	"	"	54 "	2	
		2	Blood	Testicles	54 "		2		2	"	Testicles	54 "	2	
		2	"	Vein	54 "	1	1							
10	He.	1	"	Testicles and vein	67 "	1		T.	1	"	Testicles and vein	67 "		1
11	A&B	2	"	"	67 "	1	1	F.	2	"	"	67 "		2
Total						10	29	Total					6	17
Per cent immune							25.6	Per cent immune						26.08

Immunity determined by intradermal inoculations of 0.2 cc. various dilutions of fresh active Virus III.
The percentage of animals immune was the same in the test and the control animals.
In Experiment 3 the test animals which received blood were caged together twenty-five days.
In Experiment 9 the control animals were caged together fifty-four days.

terpreted as evidence in favor of Virus III being the etiological agent of chicken-pox, but when all eleven of the experiments are considered together, no evidence can be obtained from them in support of such a deduction.

Further study of the experiments reveals that the failure of 26 per cent of the rabbits to react to Virus III was not dependent upon

TABLE VIII.

Summary of an Experiment Showing that the Serum of One of Six Stock Rabbits Neutralized Virus III in Vitro, and that the Virucidal Properties of the Sera Usually Parallel the Intradermal Tests with the Virus.

Rabbit	Neutralization of Virus III by the serum of the rabbit before inoculation with blood	Inoculation of blood into testicles and vein of rabbit	Time allowed between inoculation of blood and second neutralization tests	Neutralization of Virus III by serum of the rabbit after inoculation with blood	Immunity of rabbit as determined by intradermal inoculation of Virus III
A	Yes	From patient with varicella	67 days	Yes	Yes
B	No	"	"	"	"
C	"	"	"	No	No
D	"	From normal adult	"	"	"
E	"	"	"	"	"
F	"	"	"	"	"

The serum from one of six rabbits, A, taken from stock neutralized Virus III.
Sixty-seven days later two sera, A and B, neutralized Virus III.
The results of the second neutralization experiment were identical with those obtained by intradermal injection of Virus III.

the previous inoculations of blood, vesicle fluid, or nasal washings. The control animals in Experiments 2, 4, 5, 6, 9, and 10 received blood from the same normal man. In spite of this, the four control animals in Experiment 9 were immune and only one of the twelve rabbits in Experiments 2, 4, 5, 6, and 10 which received blood from the same subject was found to be immune. It is interesting to note that the four control rabbits in Experiment 9 were caged together for 54 days and that the three test animals which received blood in Experiment 3 and which were later found to be immune were also caged together for 25 days. In our previous work of making routine transfers we had found that only about 15 per cent of 200 young stock

rabbits (1,800 gm.) failed to react to intradermal inoculations of Virus III and were surprised to find so many refractory rabbits in these experiments.

As the work progressed and many refractory animals were found among the controls, it became necessary to ascertain in some way if normal stock rabbits were refractory before the experiments were begun. The only way to accomplish this was to determine the virucidal properties of each rabbit's serum for Virus III *in vitro* before inoculating the animal with blood, determine it again before testing the skin of the animal with Virus III, and then compare the results obtained by the two methods. The results of one such experiment are summarized in Table VIII. The animals in this experiment are identical with those in Experiments 10 and 11 in Table VII. From these results it can be seen that the sera of certain stock rabbits neutralize Virus III *in vitro*, that the presence of an immunity in a rabbit as evidenced by the failure of a visible reaction to follow the intradermal inoculation of Virus III closely parallels that evidenced by the virucidal properties of the rabbit's serum for Virus III *in vitro*, and that the immunity shown to exist in the test and control animals in Table VII was not dependent upon the blood, nasal washings, or vesicle fluid injected into the rabbits.

The power of a rabbit's serum to neutralize Virus III *in vitro* does not necessarily parallel the failure of a reaction to follow an intradermal inoculation of Virus III in the same animal. The virucidal properties of a rabbit's serum may disappear in 3 to 6 months. The animal remains refractory, however, to the virus. This phenomenon is known to exist in rabbits immune to vaccine virus. If young rabbits are chosen, few refractory ones are encountered and the skin reactions and the power of the serum to neutralize the virus *in vitro* usually parallel each other.

Twenty sera from stock rabbits of different ages have been examined for virucidal properties for Virus III. Four of these sera, 20 per cent, neutralized the virus *in vitro*. The animals whose sera neutralized the virus *in vitro* were subsequently found to be refractory when the skin was tested with active Virus III.

Inoculation of Active Virus III into Animals Other than Rabbits.—Guinea Pigs: Two guinea pigs received intradermal inoculations of

active Virus III. No visible reaction appeared in either pig during 3 weeks of observation.

White Mice: Six white mice received intraperitoneal injections of 0.25 to 1.25 cc. of active Virus III. During 10 days of observation all the mice remained well and active.

Monkeys: Each of two monkeys (*Macacus rhesus*) received an intravenous injection of 2 cc. of active Virus III in 8 cc. of physiological salt solution, and an intradermal inoculation of 0.2 cc. of the same virus. No visible skin reactions, no significant changes in the temperature or in the blood count occurred in either monkey during 3 weeks of observation.

Humans: Two men, physicians, volunteered for intradermal inoculations of active Virus III. One of the men had had varicella in childhood, the other had never suffered from varicella. Each volunteer received 0.2 cc. of active virus intradermally in two places on the left upper arm. The volunteer who had not suffered from varicella experienced no general reaction and only a mild local one consisting of redness and tenderness in the immediate vicinity of the inoculation which disappeared entirely in 3 days. The man who had had varicella in childhood experienced a more severe reaction. 8 hours after the inoculation a chilly sensation, headache, backache, and general malaise were noticed. The arm at the site of the inoculation became red, swollen, tender, and painful. The general reaction disappeared after 48 hours. The local reaction increased in intensity for 36 hours; the redness, swelling, and tenderness extended half way down the forearm. The axillary glands were swollen and tender. The local reaction gradually subsided and disappeared in 5 days. In neither of the subjects did a vesicle or an open lesion appear at the site of the inoculation. No generalized eruption appeared in either man. Both individuals were still well a month after the injections. The blood serum of neither man neutralized Virus III *in vitro* before the inoculations and is now being studied at weekly intervals for the occurrence of virucidal properties.

DISCUSSION.

From the work reported previously[1] and that presented in this paper it is evident that an active, transmissible agent is being studied which partakes of the characters of the so called filterable viruses. By immunological tests in humans and rabbits it has been impossible to obtain any evidence that this virus is associated in patients with the manifestations of varicella. On the other hand, the studies do bring evidence that we are dealing with a specific previously unknown virus which is quite distinct from vaccine virus and the virus of symptomatic herpes. There is no evidence that Virus III is the etiological agent of rabbit snuffles. The virus of infectious myxomatosis is the only filterable virus indigenous to rabbits concerning which we have been able to find reports in the literature. This virus was first reported by Sanarelli[6] and further described by Splendore[7] and Moses.[8] It produces fatal myxomatous tumors in rabbits, is filterable, and can be transmitted indefinitely from rabbit to rabbit. The exact source and nature of Virus III and its relationship to human or rabbit diseases remain to be determined.

CONCLUSIONS.

1. The intradermal method of inoculating Virus III, a hitherto unknown filterable virus producing lesions in rabbits, gives more reliable results than those obtained by smearing the virus on the scarified skin.

2. Virus III, heated 10 minutes at 55°C., will not produce visible reactions in the skin of rabbits.

3. Virus III passes through Berkefeld N and V filters.

4. The data obtained so far indicate that the best method of preserving Virus III in an active state is to filter the testicular emulsions containing the virus, add glycerol to the filtrate up to 40 per cent of the total volume, seal with vaseline, and store on ice.

5. Viable Virus III produces a definite immunity in rabbits which persists for at least 6 months. The immunity follows intradermal,

[6] Sanarelli, G., *Centr. Bakt., 1. Abt.*, 1898, xxiii, 865.

[7] Splendore, A., *Centr. Bakt., 1. Abt., Orig.*, 1909, xlviii, 300.

[8] Moses, A., *Mem. Inst. Oswaldo Cruz*, 1911, iii, 46.

intratesticular, intravenous, intracerebral, or intranasal inoculations of the virus.

6. A single intradermal injection of Virus III, which has been killed by heat, will not produce a demonstrable immunity in rabbits.

7. No passive immunity to Virus III could be demonstrated in rabbits which had received intravenous injections of 5 to 10 cc. of immune rabbit serum 24 hours previously.

8. Immune rabbit serum neutralizes Virus III either *in vitro*, or locally in a rabbit's skin when the immune serum and the virus are injected into the same part of the skin at or about the same time.

9. Three strains of the virus under investigation are immunologically identical.

10. Virus III and vaccine virus are immunologically distinct.

11. Virus III and the virus of symptomatic herpes are immunologically distinct.

12. No passive immunity to Virus III could be demonstrated in rabbits which had received intravenous injections of 5 to 10 cc. of serum or whole blood from patients convalescent from varicella.

13. Sera from two normal adults and from fourteen patients convalescent from varicella did not neutralize Virus III *in vitro*.

14. Rabbits could not be actively immunized against Virus III by injections of whole blood, vesicle fluid, or nasal washings from patients with varicella.

15. Four of twenty sera collected from stock rabbits of different ages, 20 per cent, neutralized Virus III *in vitro*. The animals whose sera neutralized Virus III failed to show a reaction at the site of intradermal inoculations with the same virus. About 15 per cent of 200 young stock rabbits (1,800 gm.) used in routine transfers were found to be refractory to Virus III, as evidenced by a failure to react to intradermal inoculations of the virus.

16. No susceptibility to Virus III was observed in guinea pigs, mice, or monkeys.

17. A volunteer who had never suffered from varicella experienced no general reaction and only a mild local one following an intradermal inoculation of Virus III. A volunteer who had had chicken-pox in childhood experienced a moderate general action, *viz.*, fever, headache,

backache, and general malaise, and also a moderate local reaction, *viz.*, redness, swelling, tenderness, and pain, following an intradermal inoculation of Virus III.

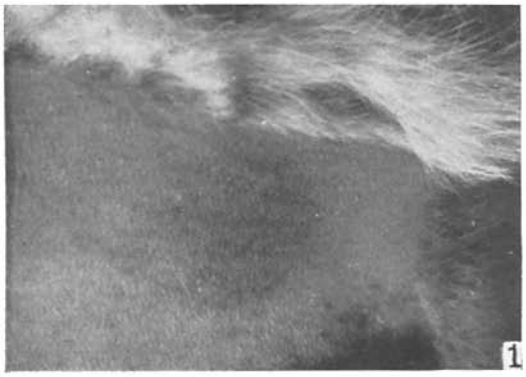
18. The study of the immunological reactions has failed to bring any evidence that the virus under investigation bears an etiologic relationship to varicella.

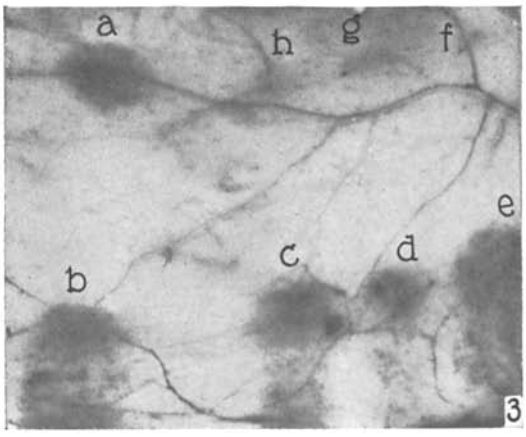
EXPLANATION OF PLATE 38.

FIG. 1. Usual reaction in the skin of a rabbit following the intradermal inoculation of active Virus III. Photograph made 5 days after the inoculation.

FIG. 2. Usual reaction in the skin of a rabbit when active Virus III is smeared along the lines of scarification. Five lines were made on the skin. Lines 2 and 4 were inoculated. Lines 1, 3, and 5 were not inoculated. Photograph made 5 days after inoculation.

FIG. 3. Photograph of rabbit's skin in which the mixtures of Experiment 1 in Table V were tested. *a* and *b* are sites of injection of mixtures of salt solution and dilutions of the virus; *c*, *d*, and *e* are sites of injection of mixtures of normal rabbit serum and dilutions of the virus; *f*, *g*, and *h* are sites of injection of mixtures of immune rabbit serum and dilutions of the virus. The immune serum neutralized the virus as shown by the failure of lesions to appear at *f*, *g*, and *h*.





(Rivers and Tillett: Varicella.)