63d CONGRESS : : 2d SESSION
DECEMBER 1, 1913-OCTOBER 24, 1914

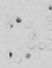
HOUSE DOCUMENTS

Vol. 10

WASHINGTON : : GOVERNMENT PRINTING OFFICE : : 1914

J66
copy 2

D. of D.
DEC 1 1915



CONTENTS

No.
1060. Hygienic Laboratory bulletin 94; 1, Collected studies on insect transmission of Trypanosoma evansi; 2, Summary of experiments in transmission of anthrax by biting flies.
1061. Same 95; Laboratory studies on tetanus.

63D CONGRESS } HOUSE OF REPRESENTATIVES { DOCUMENT
2d Session } { No. 1060

TREASURY DEPARTMENT
UNITED STATES PUBLIC HEALTH SERVICE

HYGIENIC LABORATORY—BULLETIN No. 94
JUNE, 1914

I. COLLECTED STUDIES ON THE INSECT TRANSMISSION OF *TRYPANOSOMA EVANSI*.

BY

M. BRUIN MITZMAIN

II. SUMMARY OF EXPERIMENTS IN THE TRANSMISSION OF ANTHRAX BY BITING FLIES.

BY

M. BRUIN MITZMAIN

WASHINGTON
GOVERNMENT PRINTING OFFICE
1914

ORGANIZATION OF HYGIENIC LABORATORY.

RUPERT BLUE, *Surgeon General, Public Health Service.*

ADVISORY BOARD.

Lieut. Col. Walter D. McCaw, United States Army; Medical Director Edward R. Stitt, United States Navy; Dr. A. D. Melvin, Chief of United States Bureau of Animal Industry; and Surg. John F. Anderson, United States Public Health Service, ex officio.

Prof. William H. Welch, Johns Hopkins University, Baltimore; Prof. Simon Flexner, Rockefeller Institute for Medical Research, New York; Prof. Victor C. Vaughan, University of Michigan, Ann Arbor; Prof. William T. Sedgwick, Massachusetts Institute of Technology, Boston.

LABORATORY CORPS.

Director.—Surg. John F. Anderson.
Assistant director.—Surg. Edward Francis.
Senior pharmacist.—C. O. Sterns, Ph. G.
Junior pharmacist.—Clyde Ritter, Ph. G.
Artist.—Leonard H. Wilder.
Acting librarian.—E. Foltz.

DIVISION OF PATHOLOGY AND BACTERIOLOGY.

In charge of division.—Surg. John F. Anderson.
Associates.—Surgs. W. J. Pettus, Hugh S. Cumming, Leslie L. Lumsden, Taliaferro Clark, Lunsford D. Fricks, Carroll Fox, Joseph W. Schereschewsky, Allan J. McLaughlin, Edward Francis; Passed Asst. Surgs. Arthur M. Stimson, Richard H. Creel, Friench Simpson, H. E. Hasseltine; Asst. Surgs. W. F. Draper, C. L. Williams.

DIVISION OF ZOOLOGY.

Professor of zoology.—Ch. Wardell Stiles, M. D., Sc. D., LL. D., Ph. D.
Associate.—Surg. Joseph Goldberger.
Assistant.—Walter D. Cannon, LL. B., A. B., M. D.

DIVISION OF PHARMACOLOGY.

Professor of pharmacology.—Carl Voegtlin, Ph. D.
Assistants.—Atherton Seidell, Ph. D.; Murray Galt Motter, A. M., M. D.; Martin I. Wilbert, Pharm. D.; George A. Menge, Ph. D.

DIVISION OF CHEMISTRY.

Professor of chemistry.—Earle B. Phelps, B. S.
Assistant.—Elias Elvove, M. S., Pharm. D.; A. F. Stevenson, B. S.

CONTENTS.

Page.

I. Collected studies on the insect transmission of *Trypanosoma evansi*........ 7
1. The relation of *Tabanus striatus* to surra dissemination............ 7
2. The relation of mosquitoes to surra............................ 20
3. Notes on the bionomics of *Lyperosia exigua* and the relation of this fly to experimental trypanosomiasis............................ 24
4. Mechanical transmission experiments with *Philaematomyia crassirostris*.. 31
5. Experiments with *Hippobosca maculata* in the transmission of surra. 33
6. Experiments with blood-sucking gnats............................ 35
7. The role of *Musca domestica* in surra conveyance................ 36

II. Summary of experiments on the transmission of anthrax by biting flies.... 41
The question of fecal contamination............................ 44
Transmission experiments.. 45

LIST OF ILLUSTRATIONS.

I.

Fig. 1. Stock sheds of Veterinary Research Laboratory showing conditions which aid in excluding tabanid fly infestation.................... 53
2. Spontaneous case of surra in a horse used for fly-biting experiments with *Tabanus striatus*.. 53
3. Experimental case of surra induced by the biting of horse flies infected by horse shown in figure 2.. 53
4. Experimental case of surra induced by the biting of horse flies infected by horse shown in figure 2.. 53
5. *Trypanosoma evansi* from smear of stomach contents of *Tabanus striatus* fed on surra-infected monkey.. 53
6. *Trypanosoma evansi* from smear of heart's blood of monkey dead as result of bites of horse flies.. 53
7. Showing method of exposing monkey to infestation with large numbers of stable flies and house flies.. 53

II.

Fig. 8. Showing method of applying biting flies on immobilized guinea pig... 53
9. *Tabanus striatus* 53
10. *Stomoxys calcitrans*.. 53

CONTENTS

PAGE

I. Collected studies on the insect transmission of *Trypanosoma evansi* ... [illegible]
 1. The relation of *Tabanus striatus* to surra dissemination ... [illegible]
 2. The relation of mosquitoes to surra ... 4
 3. Notes on the bionomics of *Lyperosia exigua* and the relation of this fly to experimental trypanosomiasis ... [illegible]
 4. Mechanical transmission experiments with [illegible] ... 31
 5. Experiments with *Hippobosca maculata* in the transmission of surra ... 33
 6. Experiments with blood-sucking ... [illegible]
 7. The role of *Musca domestica* in surra conveyance ... 36

II. Summary of experiments on the transmission of anthrax by biting flies ... 41
 The question of local contamination ... 44
 Transmission experiments ... 45

LIST OF ILLUSTRATIONS

I

FIG. 1. Stock sheds of Veterinary Research Laboratory showing conditions which aid in excluding stable fly infestation ... [illegible]
 2. Spontaneous case of surra in a horse used for fly-bite experiments with *Tabanus striatus* ... [illegible]
 3. Experimental case of surra induced by the biting of horse flies [illegible] shown in figure 2 ... [illegible]
 4. Experimental case of surra induced by the biting of horse flies infected by horse shown in figure 2 ... [illegible]
 5. Trypanosomes taken from smear of stomach contents of *Tabanus striatus* fed on surra-infected monkey ... [illegible]
 6. Trypanosomes from smear of heart's blood of monkey dead after bite of horse flies ... [illegible]
 7. Showing method of exposing monkeys to infestation with large numbers of stable flies and horse flies ... [illegible]

II

FIG. 8. Showing method of applying biting flies on immobilized guinea pigs ... [illegible]
 9. *Tabanus striatus* ... [illegible]
 10. *Stomoxys calcitrans* ... [illegible]

I. COLLECTED STUDIES ON THE INSECT TRANSMISSION OF TRYPANOSOMA EVANSI.

By M. Bruin Mitzmain, *Technical Assistant, United States Public Health Service.*

1. THE RELATION OF TABANUS STRIATUS TO SURRA DISSEMINATION.

Certain species of the *Tabanidae* have been associated with animal trypanosome transmission in the literature whenever the question of an invertebrate host has been considered. These flies have been connected with the direct method of conveyance in several experimental investigations and the latter will be reviewed as follows:

Rogers (1901) experimenting with surra in India used horseflies (species not given) which were caught and kept for varying periods of time after being allowed to bite and suck the blood of an animal which was suffering from surra, and whose blood at the time contained the *Trypanosoma evansi* in large numbers. They were subsequently allowed to bite healthy dogs and rabbits.

In every case in which the flies had been kept from one to four or more days after biting the infected animals, no disease ensued. When, however, flies which had just sucked infected blood were immediately allowed to bite another healthy animal positive results were obtained after an incubation corresponding with that of the disease produced when a minimum dose of infected blood is inoculated into an animal of the same species. The result was uncertain if only one or two flies were allowed to bite and especially if they were allowed to suck as much blood as they wished without being disturbed. If on the other hand several flies which just sucked an infected animal were induced to bite a healthy one and especially if they were disturbed and allowed to bite again several times infection was always readily produced in both rabbits and dogs.

In an experiment described as an example of the method employed, Rogers succeeded in transferring the disease from dog to dog through the biting of 12 horseflies.

Fraser and Symonds (1908) in a study of surra transmission in the Federated Malay States were able to transfer the infection from horses and bulls to the horse, dog, and rabbit in the mechanical method of biting. A single fly was found to be able to convey the disease. The species used in their experiments were *Tabanus minimus*, *T. partitus*, *T. vagus*, and *T. fumifer*.

Flies infected 24 hours produced surra by inoculation into susceptible animals, but all attempts from 1 to 10 days proved negative.

Ed. and Et. Sergent (1905) (1906) in two papers discuss their experiments in the transmission of nagana, dourine, and zousfana, trypanosomiases encountered in Algeria.

They succeeded in transmitting all three diseases by means of tabanids, *Atylotus tomentus* and *A. nemoralis.* They investigated especially the dromedary trypanosomiases, el-debab. and zousfana and concluded that tabanids could transmit these diseases by immediately successive bites. In their field observations they noticed that when several dromedaries were neighbors the flies bite first one and then another without interval of repose, precipitating themselves upon the animals in a swarm but rarely succeed in sucking blood at the first attempt until a fatigued or less attentive animal no longer repulses them—"So many successive bites so many lancet stabs." The experimental data presented demonstrated that it was possible to convey the trypanosomes from mouse to mouse with a single tabanid at a single thrust of its lancets when there was no interval between the bites, but when intervals of 15 minutes to 70 minutes were permitted to elapse infection by "stabbing" could not be carried. The Sergents also demonstrated mechanical transmission from rat to rat and from rat to mouse by means of the biting of two to eight flies. Also that six to eight tabanids could convey infection through their bites at an interval of 22 hours. After 24 hours no infection resulted and the flies did not harbor trypanosomes in their intestinal tracts.

Bruce and his collaborators (1910) working with *Tabanus secedens, T. fuscomarginatus,* and *T. thoracinus* and an African cattle trypanosomiasis (*Trypanosoma pecorum*) found that these tabanids appeared to be unable experimentally to transmit trypanosomes by the mechanical method of transmission. They could not state whether the flies could convey the disease after a period of development of the trypanosome. Direct application upon monkeys of flies from small cages proved unsatisfactory; therefore, in the hope that *Tabanidae* would live longer and feed more readily, a fly-proof kraal was constructed in the natural haunts of the fly.

In this experiment a calf was exposed to the bites of flies in a compartment shared by the infected calf. A healthy control calf was placed in another compartment. The wild flies used did not live more than three to four days in spite of the presence of running water, shrubs and foliage in the fly compartment. The experiment was declared negative.

Leese (1911) conducted a series of experiments with *Tabanidae* to demonstrate the rôle of biting diptera in the transmission of surra under natural conditions in India. He succeeded in convéying

Trypanosoma evansi with flies (*Tabanus* and *Haematopota*) removed from surra-infected camels in the open to a guinea pig and a white rat. Ten specimens of *Haematopota* and four specimens of two species of *Tabanus* were used in the direct mechanical method.

In another paper, Leese (1912), reviewing his experiments incriminating *Tabanidae*, concludes that in the case of surra the mechanical theory of transmission remains a perfectly satisfactory one, and that the theory of a life cycle of development for *Tr. evansi* in an invertebrate host is not tenable except by analogy.

Baldrey (1911), in an experimental investigation of the rôle of tabanids and other flies in surra conveyance, attempted to account for trypanosome development by feeding infected flies upon healthy guinea pigs and horses for 18 consecutive days. No infection resulted either in the direct method or otherwise. Dissected flies within 24 hours after infection when injected into a guinea pig and a pony produced the disease with fatal results. The author shows that *Tabanus* flies feed only once in five days, so that the only method of direct infection would be where a fly is disturbed in feeding and immediately attacks another animal.

Strong circumstantial evidence incriminating tabanids in surra dissemination is given by Mohler and Thompson (1909) in a study of the disease found in an importation of Indian cattle. They found that only 3 cases among the infected cattle originated in India and 15 additional cases developed after landing on Simonsons Island off New York. Three species of *Tabanus* and one of *Stomoxys* were the only flies found on the island. The authors held *Tabanus striatus* responsible for the outbreak.

In a series of experiments aimed to determine to what extent *Tabanus striatus* Fabricus, the common house fly of the Philippines, was involved as a mechanical carrier of trypanosomes the following conclusions were advanced. (P. 229, Phil. Jour. Science, Sec. B., vol. 8, No. 3, 1913.)

1. *Tabanus striatus* Fabricus for the first time recorded has been found to play a rôle in the transmission of surra. Bred horseflies have been employed for the first time in such experiments. Errors resulting from naturally infected wild flies have thus been eliminated.

2. Three experiments were successful in the direct or mechanical transmission by "interrupted" feeding when only a short interval was allowed between the bites on infected and healthy animals. In 16 experiments the minimum number of flies with which the infection could be transmitted was 2.

3. The maximum length of time that *Trypanosoma evansi* has been demonstrated microscopically in the gut of this species of fly after feeding on infected blood is 30 hours; the organisms were found in the fly's dejecta two and one-half hours after biting the infected animals;

and suspensions of flies, when injected subcutaneously, were found infective for animals for a period of 10 hours after the flies had fed on infected blood.

4. Trypanosomes of surra were not found to be transmitted hereditarily in *Tabanus striatus* Fabricus.

One monkey and two horses became infected as the result of the bites of horseflies used in this series. In analyzing the reason for such a small percentage of positive results (3 out of 16 attempts) with the use of animals whose blood was swarming with trypanosomes at the time of fly biting it was concluded that the feeding methods employed restricted the normal behavior of the parasites. In every instance the flies were induced to complete the infective meal upon the healthy host after a single insertion of its proboscis into the sick animal. The feeding is differently conducted by this fly in nature, as it was observed in every instance that a fly required several insertions of its proboscis at short intervals upon different hosts, and was never permitted by the host to become satiated at a single application of the skin-piercing apparatus.

The experiments in the purely mechanical method were therefore renewed with attempts to simulate more natural behavior of the blood-sucking gadfly. The two experiments bearing on this principle resulted successfully, both animals, a horse and a bull, showing undisputed evidence of the disease. The latter evidently recovered from the infection and later proved immune to inoculation of large doses of blood containing numerous trypanosomes which produced fatal results in a monkey. The horse proved infectious after an incubation period of five days, showing a moderate number of trypanosomes then and many times previous to its death which occurred on the fifty-second day after the bites were received.

TO DETERMINE IF A SINGLE FLY CAN TRANSMIT THE DISEASE.

In previous experiments nine trials with single flies resulted negatively. Here the fly was permitted to bite infected and healthy animals once only. Obviously not sufficient numbers of organisms were transferred on the probe of the fly to cause infection in this artificial way. In the present experiment a tabanid was induced to feed with the maximum number of interruptions upon two hosts alternately. Successive applications from a glass tube were made on surra infected horse and healthy horse. The interval during the transfer was only a few seconds, not more than 20 in any instance. In this manner each animal was bitten at short intervals a total of 26 times during 35 minutes by the same fly, which, however, did not appear abnormally engorged when removed for observation. The healthy horse, which was isolated in a screened stall, passed through an incubation period of nine days, when a few typical trypanosomes

were found in its blood. The animal died 67 days after the fly biting was performed. Heart's blood which contained numerous trypanosomes was injected into a monkey. The latter died upon the eleventh day with trypanosomes swarming in blood taken from its ear.

An additional trial with a single fly was made with monkeys as blood donors. Here an interval of 15 minutes was permitted between bites on infected and healthy hosts. The fly made three distinct stabs with its proboscis into the skin of both monkeys and could not be induced to continue.

Six days after the biting the healthy monkey showed a febrile condition and a moderate number of organisms in its circulation. Fifteen days later the animal died with swarms of *Trypanosoma evansi* in blood removed from his heart.

THE QUESTION OF THE LENGTH OF TIME TABANUS STRIATUS REMAINS INFECTIVE.

In all of the experiments in the direct mechanical method previously reported no provision was made for an interval longer than 1 minute. Consequently the limit of infection in these flies was not ascertained. This problem was taken up in the present investigation in an attempt to imitate as far as practicable the natural procedure of fly biting in the interrupted method. From the data presented in the table following, it is learned that 15 minutes is the maximum time that *Tabanas striatus* is able to infect through its biting.

TABLE I.—*Experiments in the direct method of transmission.*

Interval after biting infected host.	Trypanosome count of infected host when bitten.	Number of flies applied.	Total number of bites.	Healthy animal exposed.	Fate of animals exposed to fly bites.
3 minutes	Swarming	7	35	Horse 135	Positive on 9th day; monkey inoculated; died in 8 days; horse 135, died in 42 days. Disease also reproduced in bull No. 38.
5 minutes	Scanty to swarming.	26	133	Horse 344	Positive; 10 days incubation. Test monkey contracted surra, dying in 11 days; horse 344, died in 60 days.
Do	Swarming	2	6	Monkey X	Negative; succumbed later to blood inoculation.
7–8 minutes	Numerous	30	97	Horse 123	Positive; 6 days incubation; dead in 38 days. Reproduced surra with fatalities in monkey and guinea pig.
10 minutes	do	12	16	Horse 120	Positive; 5½ days incubation; dead in 35 days. Its blood killed 1 monkey and 2 guinea pigs.
15 minutes	Moderate to swarming.	18	18	Horse 300	Negative; tested later and found susceptible to surra.
Do	Swarming	1	3	Monkey 13	Positive; died with numerous trypanosomes in heart in 21 days.
20 minutes	Scanty to swarming	30	102	Bull 38	Negative; inoculated with blood of infected horse 30 days later. Showed trypanosomes and recovered.
1 hour	Swarming	12	12	Horse 339	Negative; 1 fly after feeding injected into a guinea pig produced surra.
2–3 hours	Numerous	10	10	Monkey 14	Negative.
3–4 hours	Swarming	39	39	Horse	Do.

EXPERIMENTS IN THE INDIRECT METHOD OF INFECTION.

Many attempts were made to keep alive large numbers of gadflies under artificial conditions in order to test the biological application of development to the experimental transmission of *Trypanosoma evansi*. This was finally achieved with a limited number of flies to the extent of 26 days in two experiments and 21 days in one experiment.

Methods.—In this series the flies were kept, when not applied on animals, in a large locally constructed refrigerator where the temperature was quite uniformly maintained at 23° to 25° C. Here flies were kept in a large breeding flask generously supplied with dry filter paper laid lengthwise in roughly rolled strips. When it was desired to feed the flies they were transferred to individual wide-mouthed test tubes plugged with cotton wool.

The parasites, after the initial engorgement on the surra-infected host, were applied in the various experiments once daily, although the individual fly bit on an average only once in three days. When the flies became disabled they were injected as soon as practicable in saline suspensions into guinea pigs. The following table presents a summary of the data in transmission experiments in the indirect method:

TABLE 2.—*Experiments in the indirect method of transmission.*

Interval after biting infected host.	Number of flies applied.	Healthy animal exposed.	Trypanosome count of infected host when bitten.	Fate of animals exposed to fly bites.
1–2 days	33	Horse 339	Swarming	Negative.
1–2 days	15	Horse 73	do	Do.
3–10 dyas	59	Horse 339	do	Do.
3–10 days	105	Horse 73	do	Do.
3–10 days	28	Horse B	Numerous	Do.
11–14 days	41	Horse 339	Swarming	Do.
11–14 days	87	Horse 73	do	Do.
11–14 days	25	Horse B	Numerous	Do.
15–19 days	15	Horse 339	Swarming	Do.
15–19 days	22	Horse 73	do	Do.
15–19 days	8	Horse B	Numerous	Do.
21 days	10	Horse 339	Swarming	Do.
21 days	13	Horse 73	do	Do.
21 days	2	Horse B	Numerous	Do.
23 days	6	Horse 339	Swarming	Do.
24–26 days	7	Horse 73	do	Do.
24–26 days	4	Horse 339	do	Do.

Results of experiments in the indirect method were strengthened by a number of instances of inoculations of flies into susceptible animals at various intervals after biting the infected host. Dissections and microscopical examinations for trypansomes were made prior to injecting.

Flies disabled by injuries or age were ground up in physiological salt solution and injected subcutaneously into guinea pigs and in one instance into a horse. Flies were inoculated after the following periods of possible infectivity:

Into guinea pigs: 2–3 days, 6 days, 9 days, 12 days, 14–15 days, 16 days, 17 days, 19 days, 21 days, 24 days.

Into a horse: 25 days and 26 days.

No suspicious protozoan organisms were encountered in the flies dissected and apparently no developmental forms were present as the animals inoculated remained normal one month following the experiments.

EVIDENCE OF INFECTION CONVEYED IN THE PROBOSCIS.

It often occurs in an outbreak that where large numbers of horses are corraled that only one animal of the lot may contract surra when it is obvious that the others are exposed to the same agencies. In two instances of this sort the writer collected tabanids, immediately after segregation of the sick horse, from animals which escaped infection and found in dissections in a few instances numerous surra-like organisms. A practical problem thus presented itself. How many horses could flies infect successively? Experimentally this would resolve itself into the determining if an infected fly in biting cleans its contaminated proboscis at a single insertion, or if infective organisms are retained so that when interrupted in the biting process infection may be carried to the next animal bitten. Or, in other words, is the conveyance of infection effected through the stab of the fly or assisted by the physiological action of regurgitation or activity of the salivary glands? To determine this point an experiment with horses and tabanids was conducted as follows: Three horses screened from each other were exposed to the successive bites of flies which had contaminated their labiums with blood of a horse whose blood was swarming with trypanosomes at the time of the experiment. A small trapdoor communicated between the screened stalls, permitting of the transfer of glass tube and fly. An attendant was stationed in each stall, so that the feeding could be better facilitated. Each fly was fed 30 seconds on the infected horse, and without interval 30 seconds on horse No. 58, followed immediately by 30 seconds on horse B, and after an interval of 5 minutes from the time of the infective bite, the fly was permitted to complete the meal on horse No. 73. In this manner 43 flies fed on the sick horse, 43 on the first contact horse No. 58, 39 flies bit the second contact, horse B, and 32 bites were completed upon horse No. 73.

After an incubation period of six days horse No. 58 showed a temperature of 40.3° C., followed by a morning temperature of 41.2° C. At this time blood from an ear vein revealed a moderate number of quite typical surra organisms. Sixty-two days following the fly applications horse No. 58 was down and struggling in the final stages of the disease. Numerous trypanosomes were seen in blood removed from the ear. One cubic centimeter of blood removed

from the jugular vein at this time was injected into monkey No. 75, which died with typical indications of surra infection 11 days later.

Horses Nos. 73 and B were held under observation for 40 days following the experiment, during which time no reacton was noted. Blood inoculated into monkeys and guinea pigs proved negative for trypanosomes.

It is concluded from this experiment that the biting of *Tabanus striatus* is innocuous in infecting more than one horse as a result of a previous contamination.

STUDY OF AN OUTBREAK OF SURRA IN WHICH TABANUS STRIATUS WAS THE PROBABLE AGENT OF INFECTION.

The writer was fortunate enough to have an opportunity to make an epidemological study of an outbreak of surra where intimate relations between host and carrier could be identified. This occurred from May to July, 1913, on the Government stock farm, 26 kilometers south of Manila, where a breeding station was maintained. Here the mares and colts were pastured while the stallions were quartered in darkened stables adjoining the pastures. The pastures for the brood mares were interspersed with groves of trees which afforded shade for the animals and incidentally very convenient resting places for the thousands of gad flies observed during the outbreak. During the heat of the day when tabanids and other flies were least active the herd was distributed among four stables which are designated in the table below as approximate centers for the dissemination of new cases of surra. During the hours of 10 a. m. to 4 p. m. the horses were pastured on land contiguous to the respective stables, the grazing areas being partitioned by wire fences and natural barriers of groves of trees. The outbreak of surra occurred when gad flies were the most prevalent day biting parasites. These flies originated from the shores of an 80-mile lake which was located 640 meters from the stock farm. A natural connection between lake and the reservation was afforded by an unbroken row of rain trees which attracted many hundred of resting flies at all times.

The source of the present outbreak is supposed to have originated in a mare which was brought to the stock farm for breeding the latter part of April, 1913. The disease in this animal was not recognized as surra, although it died with suspicious symptoms. The infection was identified May 9 in horse No. A–86, which was removed to the research laboratory for treatment of surra. This was followed one day later by three cases found in grazing animals stabled 400 meters from horse No. A–86. Three days later a horse kept in a stable 1,060 meters distant showed pronounced symptoms. From the last stable a total of 13 animals were found infected. In the nearest stable, a distance of 1,060 meters, seven cases developed. Two hundred and ten meters

from this stable two horses were removed and two more cases were found in the next stable about 200 meters distant from the preceding. It was observed that by far the greatest area (about 80 per cent) of woodland and stream was included in the tract from which the greatest number of cases was removed. Here the total number of horses present was about the same as that in the next pasture. Without doubt the greatest number of gad flies was observed and collected from the region where much water and woods abounded.

Systematic blood examinations of the horses were not made until the first five cases were identified, i. e., after the infection gained a foothold, so that it is not possible to state accurately the incubation period of the disease in the cases detected.

In collecting horseflies from the focus of infection several specimens of *Tabanus striatus* were removed from trees and also from horses showing engorged abdomens with swarms of trypanosomes. Surra was reproduced in a monkey and three guinea pigs by injections of suspensions of material of this sort.

The table following gives a list of cases of the infection with their respective dates of detection and a statement of the distances from the previous cases the animals were removed. These distances are approximate only, using as a basis the distance of one stable from another relative to the location of sick horses.

TABLE 3.—*Relation of cases to place of infection.*

No. of horse.	Date of detection.	Approximate distance from last case.	No. of horse.	Date of detection.	Approximate distance from last case.
		Meters.			*Meters.*
A-86	May 9, 1913	....	99	May 28, 1913	1,180
B-99	May 10, 1913	....	7	May 29, 1913	1,380
215	do	210	45	June 2, 1913	1,380
270	do	....	264	June 3, 1913	1,060
113	May 12, 1913	1,060	B-112	June 9, 1913	1,060
B-17	May 20, 1913	1,060	B-87	do	1,060
206	May 21, 1913	1,060	108 and B-53	June 13, 1913	Nil.
291	May 27, 1913	Nil.	179 and B-71	do	Nil.
182	do	210	A-9 and 115	June 16, 1913	Nil.
B-65	May 28, 1913	1,180	2	June 26, 1913	1,380
B-44	do	1,180			

Not any of the stallions which were quartered in darkened stables contracted the disease. Daily examinations of these quarters showed an absence of gadflies although the stable inhabiting species, *Hippobosca maculata* and *Stomoxys calcitrans* in addition to several species of mosquitoes were encountered.

In reference to the epidemiological study of the surra outbreak on the Government stock farm no evidence was presented to support any possible biological development of the surra organisms relative to an invertebrate host. Indeed the evidence is against the development of any latent infection. The horses and cattle under surveillance

were examined by microscope once weekly after the last case of horse surra was isolated. No clinical symptoms of the disease were manifested during four months following the blood examinations.

This study was further advanced by systematically collecting flies from this focus beginning two days following the last case of surra. These flies were applied during a period of two months to two healthy monkeys and the animals examined from time to time for indications of surra. One hundred and four flies were treated in this way without results. These monkeys reacted in the usual manner to injections of small does of virulent blood obtained from a horse which was spontaneously infected during the stock-farm outbreak.

THE OCCURRENCE OF SURRA INFECTION AND THE PREVALENCE OF TABANUS STRIATUS.

The locating of the breeding places of the gadfly, *Tabanus striatus*, occupied the writer's time during two years in studies of the epidemiology of surra in southern Luzon. This region has been notorious for all the time during American occupation on account of its annual loss due to epizootic surra. The disease has always been associated with the proximity of Bay Lake (Laguna de Bay) which gives rise to the Pasig River, at the mouth of which Manila is situated. The lake extends south of Manila for approximately 80 miles along the Provinces of Rizal and Laguna. It serves as an important waterway for native commerce between Manila and lake ports, communicating with interior barrios by means of carabaos and pack ponies. The latter relation is of interest epidemiologically, as the draft animals coming for many miles serve as ready vehicles for the flies which abound along the lake shore.

In a study of the Government archives embracing the American veterinary reports, it was ascertained that a few clearly demonstrated outbreaks of surra have occurred throughout the lake region. The military authorities have reported the necessity for abandoning certain strategic points as cavalry posts due to the tremendous loss of horses from surra. In 1902, as one example there were 113 cavalry horses destroyed in one outbreak of the disease at Santa Cruz and Pillar, towns on the upper end of the lake. Less severe outbreaks have occurred since in this region among officer's mounts.

The veterinary division of the insular government reports that there was a general widespread epizootic in 1908–1910, throughout Laguna Province, involving the loss of hundreds of mules, horses, and carabaos in some 12 towns on the lake shore. In 1909, 20 valuable horses of the Government stock farm at Alabang, Rizal Province, contracted the disease at a time when *Tabanus striatus* was markedly prevalent. In 1913, outbreaks of surra coming under the writer's personal notice occurred at several points along the lake

region and also in Manila. At the latter point enzootic conditions have prevailed from the time of American occupation to the present. The occurrence of epizootic surra in the lake Provinces in the latter part of 1913 has been of unusual interest on account of the coincidental faunal relations, which had been studied for several months prior to the cases observed. An accurate check was afforded to the incidence of surra on the strength of an exhaustive survey of faunal conditions pursued during April to September of the same year. This survey in antedating and correlating the epizootics is considered of tremendous value in supporting results of laboratory experiments. The biological relations were established before there arose the necessity for epidemiological studies. These consisted in a survey of the entire lake shore from a motor boat by water and in a walking tour by land. In all the regions where it was possible to traverse the sandy lake shore the breeding places of *Tabanus striatus* were located. In the detour of the infected region larvæ and pupæ of *Tabanus striatus* were found in abundance on the lake front embracing the following municipalities: Taguig, Balagbag, Alabang, Muntinlupa, San Pedro, Tunisan, Binan, Santa Rosa, Calamba, Los Banos, Bay, Santa Cruz, Jola Jola, Pelilla, Binangonan, and Morong. Flies from these localities were distributed to interior points by the following means: The adult flies were transported on the backs of slow-moving carabaos, bulls, and pack ponies; larvæ and pupæ were often transported in loads of shell and sand for road building and other purposes from the lake to interior points; and flies as they emerged from their habitats on the lake shore if no convenient host were available flew to the nearest rain tree and rested. In many lake towns there was afforded an unbroken communication from lake to the interior of the town by rows of rain trees. These served as the favorite native shade tree throughout these Provinces and were observed to be the most attractive resting place for thousands of both sexes of *Tabanus striatus*.

At every place visited native officials furnished stories of the devastation due to surra infection. Many of these were confirmed by official records and observed cases. Outbreaks were personally investigated at Los Banos, Calamba, Santa Rosa, Alabang, and Manila. In every instance sufficient evidence was obtained to incriminate *Tabanus striatus* as the carrier responsible for the dissemination of *Trypanosoma evansi*.

DARKENED STABLES RELATIVE TO INCIDENCE OF TABANUS STRIATUS.

Many investigators have observed that tabanids do not infest animals kept in a darkened stable. This is assumed by some as significant epidemiologically and others eliminate *Tabanidæ* on the

same grounds. For instance, Schat (1903) claims that cattle and horses in surra outbreaks in Java were not protected against surra when kept in stables because *Stomoxys calcitrans* and *Lyperosia exigua*, two stables inhabiting flies, had access to them. The horse-fly, *Tabanus* sp. of Java, is stated to play an insignificant part since it does not get inside the stables.

According to Laveran and Mesnil (1907) during the Mauritius surra epizootic the cattle belonging to the Indians were noticed to be immune on account of being boxed up in small, dark huts into which the the flies did not penetrate.

The influence of dark stables in India was commented on by Leese (1911), who states that much can be done in lessening the number of flies without interfering with the supply of fresh air by extending the eaves of stables as far downward on all sides as the height of the pony will admit. "I was not able to get the disease to spread in my controls by the few flies which penterated into the stable until they were tied up immediately outside of the protection afforded by the low roof. Only two or three *Tabanus* were seen biting in our stable during the whole surra season, although they were often seen just outside it."

Evidence of the protection against surra infection in the faunal conditions maintained in darkened stables is offered in the following example:

Horse No. 256 had been kept in an open corral and used for the saddle during November and December, at the end of which month it was placed in a partially darkened shed in company with other stock. On January 10, 1913, the attendant, noticing an unusual condition in the horse, reported the fact to the veterinary inspector, who found a temperature of 40.1° C. and ordered the removal of the animal. The native attendant neglected to isolate the animal until January 18, when an examination of its blood revealed numerous trypanosomes. Then the horse was removed to the screened stable and a careful examination made of the horse's companions. During the six weeks which followed not any of the 11 horses and 4 bulls showed symptoms of disease.

While the sick animal was quartered in the open shed many stable flies and hippoboscides were seen, but gadflies were not in evidence. One specimen of the latter species had been collected from the lighted portion of this shed 18 months before, but there were no recurrences, although the work bulls entered the shed with as many as 30 to 40 tabanids infesting them. These flies were seen to leave their hosts upon entering the interior of the shed.

Additional evidence of the protection afforded by darkened stables is presented in a practical experiment conducted in Manila during the time of maximum tabanid infestation. In this trial 3 horses and 2

carabaos were placed in a partially darkened shed, where 3 surra-infected carabaos in contiguous stalls were exposed to biting flies. The eight animals were not disturbed for a period of 2 months, during which time the infesting flies were collected and recorded.

Culicoides, *Phlebotomus*, and various species of mosquitoes were observed in addition to stable flies and *Lyperosia*.

During the entire period two specimens of *Tabanus striatus* were collected. The latter species was collected from carabaos in the pasture adjoining to the extent of 60 to 70 in a few minutes.

After exposure to the fly-biting experiment the animals were segregated in screened stalls and observed for 30 days with the usual precautions. No infection was found in the five healthy animals and the experiment was judged to be negative.

REFERENCES TO LITERATURE ON TABANIDAE.

ROGERS. The transmission of the *Trypanosoma evansi* by horseflies, and other experiments pointing to the probable identity of surra of India and Nagana or Tsetse-fly disease of Africa. Proc. Roy. Soc. (1901), vol. 48, pp. 163–170.

FRASER and SYMONDS. Surra, Studies from the Institute for Medical Research, Federated Malay States (1908), No. 9.

ED and ET. SERGENT. Etudes sur les trypanosomiases de berberie en 1905, Ann. Inst. Past. (1906), vol. 20, p. 665.

ED and ET. SERGENT. El-Debab, Trypanosomiase des dromadaires de l'Afrique du Nord. Ann. Inst. Past. (1905), vol. 19, p. 17.

BRUCE and others. Experiments to ascertain if Tabanidae act as the carrier of *Trypanosoma pecorum*. Proc. Roy. Soc. (1910), vol. 83, No. B–565, p. 349–357.

LEESE. Experiments regarding the natural transmission of surra carried out at Mohand in 1908. Indian Civil Veterinary Department Memoirs (1911), No. 2.

———. Biting flies and surra. The Journal of Tropical Veterinary Science (1912), vol. 7, No. 1.

BALDREY. The evolution of *Tr. evansi* through the fly: *Tabanus* and *Stomoxys*. Journal of Tropical Veterinary Science (1911), vol. 6, No. 3, p. 271–282.

MOHLER and THOMPSON. A study of surra found in an importation of cattle followed by prompt eradication. Twenty-sixth Ann. Rep. Bur. Animal Ind. (1909), 81–98.

2. THE RELATION OF MOSQUITOES TO SURRA.

Mosquitoes have been suspected of disseminating surra in the Philippines since the earliest investigations. However, equal stress has been placed on the incrimination of all of the blood-sucking forms. Concerning the relation of mosquitoes, the native people of the Philippines believe strongly in the guilt of these insects as trypanosome transmitters. Many believe that protection can be afforded domestic animals by grazing at distances from lowlands which breed mosquitoes.

Working in Manila, Curry (1902) summarizes his studies on surra conveyance by emphasizing stable flies rather than mosquitoes, which latter, he states, Howard wrote him should be looked upon with suspicion.

Leese (1911) considered that he had satisfactorily eliminated *Anopheles* and *Culex* and *Stegomyia* in several surra transmission experiments performed in India. His experiments gave no support to the factor of a developmental cycle of *Tr. evansi* in the invertebrate host. His conclusions are based on experiments at intervals after infective biting of 2 days with 58 mosquitoes to 11 to 16 days with 4 mosquitoes.

Dutton, Todd, and Hannington (1907) failed to transmit *Tr. gambiense* in their experiments with Anopheline mosquitoes *Pyretophorus costalis*. Dissections were made 12 hours to 11 days after infection. The insects were kept alive by feeding every two days on uninfected animals. *Trypanosoma gambiense* was seen in periods ranging from 12 to 42 hours following the infective meal. They quote some authors who show that *Tr. gambiense* survives ingestion by mosquitoes for only 36 hours, and remains virulent to mice for a period of 14 hours.

The possibility of the transmission of trypanosomes by mosquitoes has been shown by Roubaud and Lafont (1914) in an investigation conducted in French West Africa. *Stegomyia fasciata* was used in experiments with *Trypanosome gambiense* in rodents and monkeys. Only the direct method of transmission was attempted. Mosquitoes in these experiments harbored viable trypanosomes for more than 24 hours.

In a large series of experiments with surra in the Philippines I was not able to transmit the infection with several species of mosquitoes with animals kept in screened stables, in screened cages, or by applying possibly infected mosquitoes from glass tubes and bottles upon many healthy animals.

In the experiments with monkeys the mosquitoes, after feeding on infected horses were applied from a large bottle, into which the monkey's tail was thrust and held from 1 to 2 hours, until all of the insects had fed. In several experiments the monkey was placed in a small cage after it was denuded of much of its hair and immobilized for 3 hours daily, during which time the mosquitoes were observed to feed.

The character of the infection induced by these insects in the latter experiment would rest almost wholly in transmission by the indirect method or infection after a considerable interval. These nematocerous insects are not involved in interrupted feeding in nature, since the biting to the point of engorgement is completed almost wholly upon the same host. However, in experiments mosquitoes easily adapt themselves to interruption in the feeding process. The mouth is immediately withdrawn when forcibly interrupted and feeding reassumed in an adjoining area upon the skin of the host's body.

The experiments in the direct method of infection are included in the table following. Two species, namely, *Aedes* (*Stegomyia*) *calopus* and *Culex fatigans* are considered in relation to the various hosts.

TABLE IV.—*Experiments with mosquitoes.*

AEDES (STEGOMYIA) CALOPUS.

Period during which mosquitoes were transferred.	Infected animal used as donor.	Number of days trypanosomes were present.	Healthy host exposed to mosquito bites.	Number of mosquitoes transferred from sick host seen biting healthy animal.	Interval between feedings.	Date of conclusion of experiment.
May 17-27	Horse 40	5	Horse 44.	134	20 minutes to 40 hours	June 23
May 11-29	....do	12	Monkey 5	113	30 minutes to 48 hours	June 15
June 23-29	Horse 42	7	Mule 3	621	20 minutes to 4 hours	Aug. 2
Aug. 2-19	Horse C	18	Monkey 7	654	10 minutes to 1 hour	Sept. 5
June 5-22	Horses 37 and 42.	14	Horse 43.	884	15 minutes to 1½ hours	June 22

CULEX FATIGANS.

Period during which mosquitoes were transferred.	Infected animal used as donor.	Number of days trypanosomes were present.	Healthy host exposed to mosquito bites.	Number of mosquitoes transferred from sick host seen biting healthy animal.	Interval between feedings.	Date of conclusion of experiment.
July 3-12	Horse C	10	Horse 44.	984	15 minutes to 1½ hours	July 15
July 14-Aug. 20	....do	38	Horse B.	979	5 minutes to 8 days	Sept. 1

The matter of hereditary transmission was likewise disposed of in an experimental way in conjunction with the indirect method. Three hundred specimens of *Aedes* (*Stegomyia*) *calopus*, collected during the act of biting a horse which had numerous surra trypanosomes in its blood, were placed in a screened stable with a healthy horse. Here the insects were encouraged to lay their eggs in vessels provided for the purpose. The larvæ which developed were removed from time to time and placed in suitable containers in a separate stall where another healthy horse was provided for the mosquitoes emerging.

No mosquitoes remained after 36 days, when the experiment was concluded, and the horses were further observed during 30 days. No infection resulted.

Mosquitoes proved to harbor surra trypanosomes a greater length of time than any other blood-sucking form. Several specimens of *Aedes (Stegomyia) calopus* were found with active trypanosomes resembling *evansi*, for a period of 42 hours after an infective bite. These were located in the proventriculus. None was encountered in the salivary glands or in dissections of the mouth parts in various periods up to 36 days after biting sick animals. In this series 30 hours was the longest period the trypanosomes in these mosquitoes (*Aedes (Stegomyia) calopus*, *Culex fatigans* and *C. ludlowi*) were found virulent by inoculation into a monkey. The species infected the maximum period was found to be *Aedes (Stegomyia) calopus.*

MOSQUITO EXPERIMENTATION IN THE FIELD.

The question of the possibility of dissemination of surra through mosquitoes was put to a practical test during an outbreak of the disease at the Government experiment station, 26 kilometers south of Manila. At this location, from May to July, 1913, a total of 24 horses contracted surra. During this period the region was under personal surveillance, in which a careful epidemilogical study was conducted. It was concluded that the Philippine horsefly, *Tabanus striatus* was responsible for the distribution of the infection. The prevalence of this fly was marked a few weeks preceding the first cases diagnosed and a short time following the last cases isolated. The field experiments with mosquitoes were performed during the occurrence of the spontaneous cases toward the end of the outbreak.

Four healthy ponies were placed in an improvised corral immediately adjoining the pasture where the majority of the surra cases occurred. To make the test more severe a pony suffering from the disease was quartered in the same corral and permitted to mingle with the four healthy ponies. The exposure to nocturnal parasites continued during eight nights, when systematic collections were made, by the aid of a hunter's lamp, of the blood-sucking forms molesting the five ponies. Similar collections were made from a few of the horses in the adjoining pasture. Two species of *Anopheles*, one species of *Aedes*, and four species of *Culex* were recognized. Mosquitoes were the predominant forms present; a species of sand fly, *Phlebotomus*, was recognized and found to be quite abundant.

The occurrence of *Tabanus striatus* in the pastures was carefully noted during this test. Their activities were timed to be within the limits of 5 a. m. and 7 p. m. Therefore it was necessary to avoid these hours in order to eliminate the horsefly as a transmitting agent. To be sure, a few diurnal mosquitoes (principally *Aedes (Stegomyia)*

calopus) infested horses in this locality, but with the experimental evidence at hand these can be safely ignored for the present.

The five ponies were removed from their screened stalls as immediately after 7 o'clock as was practicable and placed in the open corral until 4.45 a. m. The animals were kept in individual fly-screened stalls during daylight.

After the eighth night the animals were no longer exposed but kept under observation for a long period in the screened stable. A fresh animal was then placed in the corral, where it was exposed during 35 nights in order to attract mosquitoes in an attempt to provide for the possibility of trypanosome infection carried either hereditarily or through a developmental cycle in the mosquito.

After the experiments the five healthy ponies were not removed from the screened stable for a period of 42 days during which time their temperatures were observed and blood examinations made. No reaction was noted, so the experiment was declared negative.

REFERENCES ON MOSQUITOES AND TRYPANOSOMIASES.

CURRY. A report on surra with studies on the mode of transmission. American Medicine (1902), vol. 4, 95–99.

LEESE. Experiments regarding the natural transmission of surra carried out at Mohand in 1908. Indian Civil Veterinary Memoirs (1911), No. 2.

DUTTON, TODD, and HARRINGTON. Trypanosome transmission experiments. Ann. Trop. Med. and Parasit. (1907), vol. 1, 201–229.

ROUBAUD and LAFONT. Experiments on the transmission of human Trypanosomes of Africa by means of the *Stegomyia fasciata*. Bull. de le Soc. de Path. Exotique (1914), No. 1. Borrowed from translation in The Amer. Jour. of Trop. Dis. and Prev. Med. (1914), vol. 1, No. 9658.

3. NOTES ON THE BIONOMICS OF LYPEROSIA EXIGUA AND THE RELATION OF THIS FLY TO EXPERIMENTAL TRYPANOSOMIASIS.

In relation to draft animals in the Philippine Islands, *Lyperosia exigua* de Meij is the most predominant of all species of blood-sucking flies. It is prevalent at all seasons and at all hours; found at night usually at rest on bovines, accompanying mosquitoes and sand flies. On horses this fly is relatively an accidental parasite, preferring the hosts upon whose excreta it lays its eggs; carabao and cattle are selected in this regard.

The egg stage.—Under artificial conditions the captive fly begins egg laying within a few minutes. They are laid on the glass or in a suitable medium either singly or in clusters; when the latter is the case the eggs number 10 usually, but two clusters of 18 and 25 have been observed. The eggs are quite viscid when laid, adhering to the surface of the vessel by their convex sides. The color of the egg is ordinarily white, sometimes white with brown periphery, yellow, and light brown. At all events, the egg darkens perceptibly after incubation has fairly commenced. The white egg when exposed to the air changes in a few minutes to yellow and rapidly to brown.

The size of the egg is, with little variation, 1.30 millimeters by 0.35 millimeters. It is considerably smaller than the usual muscid type of egg, though it has the same general form. The egg shell is relatively thick and tough, due probably to the fact that the eggs are laid normally on the surface of the manure, and is thus constructed to resist exposure.

Although the *Lyperosia* will deposit eggs in empty glass vessels, repeated trials give convincing evidence that these flies will not oviposit on horse manure placed in glass jars. This manure mixed with corn meal or bran is not more attractive. But when eggs and newly hatched larvæ of this species are placed in jars with moist horse manure, development follows and the young forms reach maturity.

Hatching.—In glass tubes eggs fail to hatch unless moisture is provided; they dry up within a short time. About 10 hours after deposition the egg shows signs of embryonic movement, and 4 or 5 hours later the larva emerges. With the aid of the microscope the first sign of hatching can be discerned through the exochorion, consisting of a squirming action. By a pressure of the anal portion and an alternate sliding of the head end a decided piston-like movement

results. The head capsule presses against the cephalic end of the egg as the processes upon which the anal stigmata are set force the body in that direction. Then a backward glide is effected in order, apparently, to bring into play the two cutting spines of the pharyngeal apophyses. These working alternately against the two margins of the micropyle canal, slit ajar the upper portion through which the young larva effects its escape. The latter movement is extremely rapid; it is virtually a glide through the forced open micropyle canal.

The larva.—In appearance the larva is not unlike other muscid larvæ, but is probably whiter and more delicately marked. When hatched it is a footless maggot, 1.5 millimeters long and rather chunky. The white cuticle is contrasted by the black pharyngeal apophyses which terminate in the pair of acute hatching spines directed caudally, and the black anal stigmata which, in this species, are borne on two minute pedunculated processes.

The young larva is extremely active in procuring its food. Unlike the larva of *Stomoxys calcitrans*, it can not survive overnight without food and dies in a few hours when left in a test tube with a decoction of manure or in salt solution. Development in this species is quite rapid when the optimum food conditions are present. When placed in horse manure, growth is retarded. In nature the eggs are laid on the surface of cow and carabao manure, but the larvæ do not remain on the surface, but seek the liquid portions of the food medium. Three days suffice for full larval development, when the puparium is formed. At this stage it is not quite 7 millimeters in length. Preparatory to forming the pupal case the larva imbibes a great amount of the surrounding moisture. In so doing the head is kept constantly quivering from side to side. Almost imperceptibly there ensues a clearing action of the black contents of the intestinal tract, and soon this appears yellowish in conformity with the external cuticle. The body segments contract from the oval end cephalically, and with the invagination of the head capsule the barrel-formed puparium is completed. The color of this rapidly changes from a yellow to a bright terra cotta. The dimensions of the puparium are rather constantly 4 millimeters by 2 millimeters.

The pupa.—The pupal stage requires at least four days, the fly emerging in four to five days ordinarily. Unlike *Stomoxys*, *Philæmatomyia*, and other muscid flies, the postpupal stage in this species is a condensed period, the adult fly bursting through the puparium in a few seconds and within three minutes is apparently fully developed. With wings spread it is seen walking to the lighted portion of the jar, and at once assumes flight if permitted to escape.

Life cycle.—The entire life cycle of *Lyperosia exigua* as observed at various times under laboratory conditions is summarized in Table I.

TABLE I.—*Showing the length of time required for the life stages of Lyperosia exigua.*

Date.	Material used.	Egg.	Larva.	Pupa.	Cycle.
		Hours.	*Days.*	*Days.*	*Days.*
Sept. 6, 1911	Carabao manure	18	5	5	11
Sept. 19, 1911	do	14	4	5	10
Feb. 14, 1912	Cow manure	16	4	4	9
Feb. 16, 1912	do	16	4	5	10
July 26, 1912	Guinea-pig manure	16	5	5	11
July 27, 1912	Carabao manure	14	4	5	10
Apr. 11, 1913	Cow manure	16	4	4	9
May 26, 1913	do	12	4	4	9

Average life cycle, 9 to 11 days.

The adult fly is prepared to feed within an hour. Both sexes draw blood. The action of biting is not unlike that of the stable fly, but the *Lyperosia* usually requires shorter and more frequent bites for complete satiation.

The fly in nature, like its relative, the horn fly of the United States, usually feeds in a group, but is never found resting upon the base of the horns. When found feeding on the horse, it is never in a swarm which occurs on carabaos and cattle. The parasitism of the horse occurs usually in individual instances.

This species, unlike *Stomoxys calcitrans,* is difficult to keep for laboratory purposes, rarely surviving beyond ten days when fed daily. Therefore, its use in transmission experiments was limited to the direct method.

TRANSMISSION EXPERIMENTS WITH TRYPANOSOMA EVANSI.

In several epizootics in widely separated regions in the Orient and in Africa, species of *Lyperosia* have been suspected to be the cause of the dissemination of trypanosomiases. Schat (1903) in his dissertation on the study of the spread of surra in Java is quite decided in his incrimination of *Lyperosia exigua* in addition to the stable fly. His observations were almost entirely epidemiological and meager experimental evidence is presented. Leese (1912) in a careful survey of epidemiological conditions on surra in India found *Lyperosia* prevalent in enzootic areas where horseflies and other blood-sucking forms were absent, and suggests the probability of *Lyperosia minuta* acting as a mechanical carrier. In his experiments reported in 1911 the results obtained led him to state that this species of fly plays an insignificant rôle as a carrier. Austen (1909) mentions in a footnote that a medical officer in British Africa reported the occurrence of *Lyperosia* in an important rôle as a transmitter of trypanosomiasis.

Lyperosia exigua is found to infest the draft animals of the Philippines in tremendous numbers at a time of the year when surra is present. In this connection it is often associated with the gadfly *Tabanus striatus* and usually with *Stomoxys calcitrans,* the stable fly. It therefore devolves upon the investigator to determine to what

extent *Lyperosia* may play a rôle in the absence of other blood-sucking flies.

In the experimental data presented three methods of procedure are included:

First. Exposing healthy animals and infected animals to the bites of flies under natural conditions. Here counts were made of flies taken in the act of biting the hosts kept in the open.

Second. The experimental animals were exposed in a screened stable to the biting of a specified number of flies collected from known sources.

Third. Bred flies were applied from test tubes upon infected and healthy animals at stated intervals.

First method.—Experiments under natural conditions were pursued during 1911 from the latter part of April to the latter part of October. At the time of the year selected this species of fly abounds in swarms, being the most prevalent in numbers of the blood-sucking flies. The animals used were carabaos and horses. Surra-infected carabaos were used to supply the infection, they being preferred as the primary host on account of being the most susceptible of the draft animals to *Lyperosia* infestation.

The three experiments were conducted with the animals in the open under well-lighted sheds and kept together as closely as practicable. The flies were collected twice daily and only the number of specimens of *Lyperosia* was recorded; other species of parasitic flies were observed but not recorded. The hosts were examined for blood parasites, in addition the temperatures were recorded and the animals observed for clinical symptoms.

The table following gives a résumé of the three experiments conducted:

TABLE II.—*Compilation of experiments in which healthy horses and sick carabaos were exposed to Lyperosia flies in the open.*

	Period of experiment.		
	Apr. 27–June 8.	Aug. 19–Sept. 8.	Sept. 9–Oct. 24.
Sick carabaos used	1	1	4
Horses exposed to flies	1	1	2
Flies taken from carabaos	1,811	487	5,802
Flies taken from horses	23	138	432
Infected carabaos showed trypanosomes	17	0	22

After each experiment the horses were placed in screened stalls and blood examinations and temperatures taken daily for 30 days. No reactions were noted.

Second method.—The next two experiments were attempted with carabaos in a fly-screened cage and the insects from known sources introduced at stated intervals.

A fly-proof cage with a substantial fly-proof entrance, 7.3 meters by 9.1 meters, with a height of 4.5 meters graduated to 3.6 meters

was so partitioned that two surra-infected carabaos and a healthy carabao could occupy adjoining stalls without bodily contact and still be exposed to the bites of flies placed in the inclosure. In the first trial two surra-infected carabaos, Nos. 3228 and 3252, which were in advanced stages of the disease, were placed on one side of the coarse-screen partition and the healthy carabao, No. 16, was selected to be exposed.

The parasites used were *Lyperosia* flies collected in open sheds from healthy work carabaos. These were transferred twice daily to the caged animals and were placed promiscuously in the screened inclosure. The parasites attached themselves to the new hosts quite readily. In all over 5,000 flies were employed during the course of the experiment lasting about one month, from January 12 to February 13, 1912. The healthy animal, carabao No. 16, was not removed to the adjoining inclosure until two weeks later, February 28, when all of the flies had disappeared.

As shown in Table III following, blood examinations of the three carabaos were made daily. Carabao No. 3228 was positive for trypanosomes upon 11 days and carabao No. 3252 was positive for an equal number of days, and between the two carabaos the disease was present in a fly-communicable form during 18 days of the experiment.

TABLE III.—*Résumé of experiment in which sick carabaos and a healthy carabao were exposed to Lyperosia flies.*

Date.	Number of flies placed in the inclosure.		Trypanosome examination in carabaos.	
	a. m.	p. m.	No. 3228.	No. 3252.
1912				
Jan. 12	11	....	Positive...	Positive.
Jan. 13	170	110	...do.......	Do.
Jan. 15	122	115	...do.......	Negative.
Jan. 16	183	87	...do.......	Do.
Jan. 17	153	106	Negative..	Positive.
Jan. 19	70	....	Positive...	Do.
Jan. 22	75	....	Negative..	Do.
Jan. 23	88	....	...do.......	Do.
Jan. 24	....	20	Positive...	Do.
Jan. 25	40	48	Negative..	Do.
Jan. 26	103	230	Positive...	Negative.
Jan. 27	....	112	Negative..	Do.
Jan. 28	75	40	...do.......	Do.
Jan. 29	290	....	...do.......	Do.
Jan. 30	....	120	...do.......	Do.
Jan. 31	102	....	Positive...	Do.
Feb. 2	....	93	Negative..	Do.
Feb. 3	127	445	...do......	Positive.
Feb. 4	....	339	...do......	Do.
Feb. 5	593	....	...do.......	Do.
Feb. 6	432	114	...do.......	Negative.
Feb. 7	131	262	Positive...	Do.
Feb. 8	138	70	...do......	Do.
Feb. 13	131	....	...do......	Do.

Carabao No. 16, the healthy contact, showed no evidence of the disease either by temperature reaction or blood inoculation into guinea pigs when released February 28. Subsequent observation

of this animal during its two months' quarantine convinced the observer that the experiment terminated negatively.

A similar experiment was attempted with two infected carabaos as blood donors but with the normal carabao in an isolated screened inclosure. During the course of one month beginning February 28, 1912, more than 3,000 flies were placed on the sick carabaos and transferred at intervals of five minutes to three hours to the stall occupied by healthy carabao No. 7. Many of these flies were observed to settle on the second host and caught while biting. Three months following the last experiment the blood of carabao No. 7 was devoid of trypanosomes and the two guinea pigs inoculated remained negative.

Third method.—Finally a series of experiments was conducted under more strictly laboratory conditions. The direct method of applying the flies was used in six experiments with no appreciable interval between the bites of flies directly removed from the infected host, and in seven experiments with intervals varying from 5 hours to 10 days. The table below gives a summary of these experiments.

TABLE IV.—*Experiments in mechanical transmission with Lyperosia and Trypanosoma evansi.*

Interval between feedings.	Number of flies fed.	Condition of blood on infected host.	Healthy animal used.
30 seconds	2	Swarming	Horse 339.
20 seconds	2	do	Monkey 27; died Apr. 1. Neg.
5 seconds to 5 minutes	23	do	Monkey 26; died Apr. 3. Neg.
50 seconds average	40	do	Horse 69.
5 seconds to 2 minutes	61	do	Horse 277.
10 seconds to 1 minute	40	Numerous	Monkey 30.
14 to 20 hours	30	Swarming; animal dying	Horse 66.
5 to 7 hours	39	do	Horse B-120.
24 to 48 hours	29	do	Horse 982.
1 to 4 days	28	Numerous	Monkey 28; dead Apr. 4 (peritonitis).
5 to 7 days	14	do	Monkey 29; dead Apr. 3. Neg.
1 to 6 days	51	Moderate	Guinea pig K; reacted subsequently to surra by blood inoculation.
1 to 10 days	59	do	Carabao 9.

All of the healthy animals used in the 13 experiments above have proved negative for surra infection. Monkeys Nos. 26, 27, 28, and 29 either died from injuries, naturally, or were killed for purposes of necropsy. These animals showed at post-mortem no lesions indicative of surra.

The blood examinations were also negative. Guinea pigs inoculated from the animals exposed to the fly bites have proved negative after one month to six weeks of frequent examinations.

Carabao No. 9, which received the bites of 59 flies, was six months later inoculated with a suspension of 30 *Lyperosia* flies removed from a surra-infected horse. The carabao's blood yielded trypanosomes after an incubation period of 12 days. This animal died 8 months later and though trypanosomes were present in several organs its

untimely death was due more probably to the cramped quarters in which it was confined. However, two guinea pigs inoculated with the blood of this carabao reacted with surra infection and died as a result.

An attempt was made to eliminate the possibility of transmission of surra organisms through heredity in the fly. Several thousand of flies were liberated in the screened inclosure with two surra-infected carabaos, and in 12 days or less not any of the flies remained. In the meanwhile a new generation of flies was produced from the accumulated manure of the two animals. The new flies in turn fed on the sick carabaos, laying their eggs on fresh depositions of manure. Daily this manure was carefully transferred to a separate compartment where a healthy donkey was quartered. In due time the second generation of flies made their appearance and infested the new host.

The experiment was continued for 5 weeks, and 10 days later blood of the donkey was inoculated into one monkey and used a month later for an experiment in which it was inoculated with blood from one of the two surra-infected carabaos. The animal responded to the inoculation with a high temperature and a moderate number of typical blood organisms on the seventh day and died 18 days later with quite characteristic post-mortem lesions of surra.

SUMMARY OF EXPERIMENTS.

Experiments in the direct method with *Lyperosia exigua* and *Trypanosoma evansi* gave negative results.

Horses, which were exposed under natural conditions to the biting of flies infesting sick carabaos, did not become infected.

When thousands of flies infested sick and healthy carabaos placed in a large screened inclosure no infection was transferred.

Only negative results were obtained in the interrupted method of feeding flies in 13 experiments with various animals. In six trials there was no appreciable interval between the bites of flies removed from the infected hosts, and in seven experiments intervals occurred varying from 5 hours to 10 days. The greatest number of flies employed was 61.

In one experiment with several thousands of flies the possibility of transmission of *Tr. evansi* hereditarily was eliminated.

REFERENCES CITED.

SCHAT. Verdere mededeelingen over surra. Mededeel Proefstation Oost—Java (1903), vol. 3, No. 44.

LEESE. Biting flies and surra. The Journal of Tropical Veterinary Science (1912), vol. 7, No. 1.

——— Experiments regarding the natural transmission of surra carried out at Mohand in 1908. Indian Civil Veterinary Memoirs (1911), No. 2.

AUSTEN. African blood-sucking flies. British Museum of Natural History, London (1909).

4. MECHANICAL TRANSMISSION EXPERIMENTS WITH PHILÆMATOMYIA CRASSIROSTRIS.

It was aimed to investigate, if practicable, every blood-sucking species of fly of the Philippines which appear in sufficient numbers to warrant experimentation. During certain months of the year, principally April and May, *Philæmatomyia crassirostris* Stein appears to equal in numbers the ubiquitous stable fly. This fact seemed sufficient justification to attempt to use this fly in surra transmission experiments.

This species, which has been previously reported from British India and Africa, is noted for its very peculiar mouth apparatus, which at first glance would appear to belong to a nonblood-sucking form. It is, however, a very efficient blood sucker, as can be observed from its engorged abdomen and the wound it makes on the host's cuticle. It is very much like *Musca domestica* in general appearance, but it has the general habits of the stable fly. It prefers cattle for its infestation and for the egg deposition on the manure of these animals.

Eight experiments were tried with guinea pigs in transferring infection at an interval less than one minute. From 6 to 36 flies were applied, when the infected animal's blood contained trypanosomes from moderate to numerous in numbers. In two trials 36 and 29 flies, respectively, were applied on two successive days.

One experiment at an interval of one hour between the bites was attempted with 18 flies from monkey to monkey, and three experiments with 2, 11, and 19 flies were attempted with a 24-hour interval.

Only one experiment in the direct method of transmission proved successful. In this trial monkeys were used during six successive daily applications. The blood of the surra monkey employed during the six days contained trypanosomes moderate in numbers upon four days and numerous to swarming upon two days. Fresh flies were applied daily in the following numbers: 2, 25, 8, 17, 33, 19, a total of 104 for the experiment. Only laboratory-bred flies were employed in this experiment.

Six days following the last application of flies upon the healthy host the latter suffered with fever and had a moderate number of surra trypanosomes in its blood. Beginning three days later the trypanosomes were found swarming in numbers until death, which occurred 14 days after the onset of symptoms. Blood from the

heart of this monkey when injected subcutaneously reproduced the disease in a guinea pig, which died after three months, and in a monkey, which showed an incubation period of only three days. The latter died ten days later in a greatly debilitated condition. Death occurred during the night and a great number of dead trypanosomes were seen on the following morning in blood from the heart. A moderate number of "round bodies" were found in stained preparations from the spleen of this monkey.

5. EXPERIMENTS WITH HIPPOBOSCA MACULATA IN THE TRANSMISSION OF SURRA.

The genus *Hippobosca* has been generally overlooked by investigators who have attempted to determine the rôle of the important blood-sucking flies in the conveyance of surra and other trypanosomiases. However, species of *Hippobosca* have been suspected upon several occasions of transmitting pathogenic trypanosomes; and one, *H. rufipes*, has been proved experimentally to transmit Tr. theileri, the cause of gallsickness in cattle of South Africa. (Laveran and Mesnil, 1907.)

Two species of this genus occur in the Philippines, namely, *equina* and *maculata*, but only the latter was investigated. *Hippobosca maculata* Leach is found upon all draft animals, though rarely infesting the carabao, in all months of the year. The habits of the fly are in most respects dissimilar to all other species. It is often found to be the only parasite present particularly on horses. It appears to be uninfluenced by light reactions, seeming to attack equally well in darkened stables and in open pastures. The feeding peculiarities of *maculata* makes it unpromising as a direct contaminator, as it prefers to finish its protracted meal upon the same host. Experimentally it is difficult to interrupt its biting and requires several minutes to recommence feeding on a second host when an unusual length of time is required before it becomes satiated.

Due to the difficulty of rearing a large number of flies at one time, since each female deposits but an individual progeny, the feeding experiments were conducted except in one instance with wild flies captured from sources known to be surra free.

Twelve experiments in the direct method of feeding were pursued, as indicated in the following table:

TABLE V.—*Experiments in the direct method of feeding with hippoboscid flies.*

Date.	Infected animal used.	Condition of blood relative to trypanosomes present.	Healthy animal used.	Number of flies applied.	Length of time fed on surra host (average per fly).		Interval during feeding (average per fly).		Time required to complete meal on healthy host (average per fly).	
1912.					*Min.*	*sec.*	*Min.*	*sec.*	*Min.*	*sec.*
June 13	Guinea pig 127	Scanty	Guinea pig 92	5	3	36	3	0	7	24
June 14	do	Numerous	Guinea pig 94	9	3	6	2	26	9	6
June 15	do	Swarming	Guinea pig G	6	2	0	2	5	4	50
June 16	do	do	Guinea pig I	6	2	10	3	50	8	0
June 17	do	do	Guinea pig L	9	2	12	2	20	4	40
June 17	do	do	Guinea pig M	6	2	0	3	30	7	0
June 18	do	do	Guinea pig O	5	1	48	1	0	7	24
June 18	do	do	Guinea pig P	8	2	0	1	30	7	7
June 18	do	do	Guinea pig Q	4	2	0	4	0	4	15
June 19	do	Numerous	Guinea pig 89	10	1	40	3	50	9	12
June 19	do	do	Guinea pig 112	5	1	12		24	5	24
June 20	Monkey B	do	Monkey, M	10	4	0	2	30	8	0

NOTE.—The flies used were discarded after each experiment.

The experiments in the direct method of application were augmented by a series of feedings on clean animals after the flies were induced to feed on a surra animal whose blood proved by animal inoculation to be pathogenic. Thirty-three flies were fed three days upon monkey B whose blood was heavily infected with trypanosomes during each day of this period. On the fourth day and for 26 successive days the flies were applied to clean animals. The two surviving flies died on the twenty-seventh day of the experiment. The table following outlines the results of the fly feeding tests:

TABLE VI.—*Experiments representing the indirect method of applying hippoboscid flies.*

Times since last feeding on surra host.	Healthy animal used.	Number of flies applied.	Average length of time each fly fed.
			Minutes.
1 day	Guinea pig 93	19	10
2 days	Monkey C	24	10
3 days	Monkey D	19	11
4 days	Monkey G	17	11
5 days	Monkey H	23	11
6 days	Monkey I	22	11
7 days	Monkey J	21	12
8 days	Monkey K	17	10
9 days	Monkey L	14	13
10 days	Monkey M	13	11
11 days	Monkey N	13	10
12 days	Monkey P	11	13
13 days	Monkey Q	10	10
14 days	Monkey S	7	8
15 days	Monkey 1	6	12
16 days	Monkey 2	6	14
17 days	Monkey 3	5	9
18 days	Monkey 4	5	13
19 days	Monkey 5	5	16
20 days	Monkey 6	3	15
21 days	Monkey 7	3	10
22 days	Monkey 8	3	10
23 days	Monkey 9	2	11
24 days	Monkey 10	2	7
25 days	Guinea pig 50	2	10
26 days	do	2	7

With two bred flies a final experiment was attempted and no infection resulted. As in the above experiment the flies were fed three consecutive days on a sick monkey then applied once daily to a clean monkey. Thirty-three days after feeding upon the infected animal both flies were dead. Forty days after the experiment it was apparent, by blood examination and temperatures, that all of the animals bitten by the hippoboscid flies were free from infection.

REFERENCE CITED.

LAVERAN and MESNIL. Trypanosomes and Trypanosomiases. Nabarro English Edition. Bailliere, Tindall and Cox, London (1907), p. 351.

6. EXPERIMENTS WITH BLOOD-SUCKING GNATS.

Experiments in direct transmission with *Culicoides judicaudus* Bezzi were conducted with captive flies. Ten experiments with monkeys and guinea pigs proved negative. In one trial from monkey to monkey 326 gnats were used to bite both hosts on two successive days, 200 upon the first day and 126 were applied upon the second day. In all of these experiments the gnats were applied individually from small test tubes. The insects were used once only, then discarded.

In one trial during six days the indirect method was attempted. This likewise proved negative.

In regard to other similar blood-sucking forms one species which is indeed ubiquitous during probably five to six months may be considered here. A *Phlebotomus* was observed upon many occasions to enter the fine meshes of brass screening surrounding the stalls housing experimental animals. Infected and healthy horses kept in these stables were attacked in common and several instances were noted of transfer from surra infected to healthy animals. No instance of infection brought about through this means was observed during three years of experience.

In the field experiments with carabaos and horses, and with horses and horses, ample opportunity was afforded for the transmission due to bites of thousands of *Phlebotomus* flies. Conveyance of the disease in any instance did not result on this account.

7. THE RÔLE OF MUSCA DOMESTICA IN SURRA CONVEYANCE.

The possibility of infection conveyed by the house fly in a passive rôle has long been recognized. In reference to trypanosome transmission, it is presupposed that the method is correlated with a previous skin abrasion affording the channel of entrance for the pathogenic organisms. Consideration of skin abrasions occurring through injury or disease must include the mechanical openings effected by various ectoparasites. The latter include such forms as the ticks, gadflies, and stable flies. These parasites are important factors commensurate to the size of the openings punctured in the host's cuticle and which remain after withdrawing the piercing mouth parts in satisfying the desire for blood. It is obvious that in the presence of muscids with mouths constructed for lapping contaminated products unlimited possibilities for conveyance of surra from host to host present themselves.

The conveyance of trypanosome-infected blood by the house fly in the presence of abrasions produced through sores and wounds was taken into account by early workers in trypanosomiasis of the Philippines.

Musgrave and Clegg and Musgrave and Williamson (1903) considered this as an important factor in the prevalence of surra.

These writers state that they transmitted surra infection to healthy animals through the agency of house flies, and more recently Darling (1911) conveyed *Tr. hippicum* to the mule experimentally by means of house flies. Darling concluded one positive and two negative experiments with house flies fed artificially on the blood of an infected guinea pig. In this investigation a few drops of blood were placed on a glass plate, covered with a jar, and 18, 9, and 6 flies in three experiments fed about 5 minutes, and after an interval of 30 seconds were applied for 5 minutes to the shaved scratched skin of three mules. The positive result was obtained in the case of the mule infested with 18 house flies.

In the present study the investigation was pursued from various angles. The purely mechanical method of contamination was not considered until the more important biological relations were exhausted. Only *Stomoxys calcitrans* was considered as a correlative factor in reference to the house fly as a carrier of infection. In a previous report (1912) I have described the intimate relations existing in the feeding habits of the two species of flies. It was pointed out that the coprophagous flies, and especially the house fly, acted in a sense as secondary passive parasites by lapping to a point

of engorgement the blood brought to the host's epidermis by the probing of the labium of the stable fly. It was observed under natural conditions that an unusual percentage of nonbiting flies were attracted to domestic animals not alone to act their rôle as scavengers, but were found to accompany stable flies and gadflies to avail themselves of the food provided through the wounds made by the latter.

With this peculiar phase of parasitism in mind, experiments were conducted to determine the relationship of *Musca domestica* as a carrier of trypanosomes. It was aimed to prove first that this fly could harbor infective organisms, and was determined satisfactorily by numerous dissections and injections of saline suspensions of abdominal contents of flies fed on the abraded tail of the surra-infected monkey. Two guinea pigs and one monkey inoculated with material of this sort sickened after an average incubation period and died giving indications of the nature of the disease at autopsy. Preparations of the blood of the three animals showed *Trypanosoma evansi* in large numbers.

Attempts were made to simulate the normal relationship of parasitism in *Musca domestica* and *Stomoxys calcitrans* by placing many flies of the two species in a common bottle and permitting them to attack the inclosed tail of a surra-infected monkey. Only laboratory-bred flies were employed. Two hundred and fifty selected house flies were allowed to lap the blood from the tail of an infected monkey, upon which 200 stable flies had previously fed. The monkey's tail was withdrawn from the bottle when the majority of the house flies appeared fairly well engorged. Then 200 *clean Stomoxys* were introduced into the bottle with the fed *Musca* and the tail of a fresh monkey placed within. Here the attempts to imitate natural conditions were quite successful, the house flies applying their mouth parts to punctures made by the stable flies. Similar experiments were conducted with the two species of flies in one trial from monkey to monkey, and three trials, slightly modified, from guinea pig to guinea pig. Large numbers of laboratory-bred flies were used, and the infected blood donors in each instance were positive microscopically.

The five experiments were followed by negative results demonstrating under these conditions that *Musca domestica* does not transport infection through the channel afforded by the wound caused by the skin-piercing apparatus of *Stomoxys calcitrans.*

The possibility of surra infection being carried by the fly's feet was tested also. This was performed in the same manner with both species as in the preceding experiments, but with this difference—the infected *Musca* were introduced from a separate bottle, and by careful manipulation of the wire support attached to the monkey's tail the flies were permitted to alight on the appendage, but were prevented from feeding. Two experiments with monkeys involved the application of

30 stable flies and 50 house flies in one instance, and 200 stable flies and 250 house flies in the other instance.

Guinea pigs were also used in three experiments with fewer flies.

The five experiments failed to demonstrate that the wound made by the labium of the stable fly was a suitable channel for the introduction of trypanosomes carried on the pulvilli of *Musca domestica.*

Finally to serve as controls to these experiments four tests were made to decide the question of the possibility of the punctures caused by the bite of the stable fly serving as sites for the introduction of trypanosomes in virulent blood. In these four experiments the bites of 7, 8, 13, and 20 laboratory-bred *Stomoxys* were followed immediately by the rubbing in of freshly drawn virulent blood conveyed on a plantinum loop.

The guinea pig inoculated subcutaneously with similar material contracted surra. The four guinea pigs used for the fly biting escaped infection. One of the latter reacted six weeks later to an injection of the blood of the original blood donor, which died at this time.

The practical significance of the conveyance of trypanosomes obtained by *Musca domestica* from the site of the probing of the stable fly to exposed wounds was finally investigated. Monkeys and horses were employed in this series. The house flies after apparent engorgement of blood derived from the probes of stable flies were transferred to clean bottles and the abraded surface of the monkey's tail presented for the completion of the meal. The two horses exposed were scarified by scratching the haunch with a sharp scalpel.

In transferring bottles and tubes applied to the infected host the mouth of the vessel was carefully wiped with a cloth saturated with strong alcohol. A few minutes were permitted to elapse before the vessel was then applied to the broken skin of the healthy host. Four of the five experiments attempted resulted in positive transmission. One monkey which escaped infection was later proved to be susceptible to subcutaneous inoculation of virulent blood.

The table following shows how the animals and flies were treated in these experiments:

Hosts employed.		Flies employed.		Fate of healthy hosts exposed to infestation of Musca.
Infected.	Healthy.	Stomoxys.	Musca.	
Monkey B...	Monkey L..	70	22	Positive; incubation period 4 days; died 10 days later. Trypanosomes seen in blood from ear and from heart.
Do.......	Monkey T..	92	48	Negative; died later when injected with 2 c. c. of blood of horse 30.
Monkey C....	Monkey R..	100	29	Positive; incubation period 3½ days; blood from ear reproduced the disease in 2 Guinea pigs. Trypanosomes numerous at death in 11 days.
Do.......	Horse 28....	112	40	Positive; incubation period 5 days. Trypanosomes moderate—disease reproduced by injection of blood into horse 27 and 2 guinea pigs. Horse 28 died in 47 days.
Horse 27.....	Horse 30....	98	32	Positive; incubation period 6 days. Trypanosomes numerous—reproduced diseaseby injection of blood into Monkey T and one guinea pig. Horse 30 died after 35 days.

ACKNOWLEDGMENTS.

I wish to acknowledge my indebtedness to Prof. Dr. M. Bezzi, of Torino, Italy, and Mr. Austen, of the British Museum, for the identification of the species of flies discussed in these papers.

I am also grateful to the Philippine Bureau of Agriculture for their courtesies in permitting me to use the photographs shown in these papers.

REFERENCES CITED.

MUSGRAVE and CLEGG. Trypanosoma and trypanosomiasis with especial reference to surra in the Philippines. Publ. Bur. Gov. Labs. (1903), No. 5, pp. 85-87.

DARLING. Experimental infection of the mule with Trypanosoma hippicum by means of *Musca domestica*. Jour. Exp. Med. (1911) vol. 15, No. 4, pp. 365-366.

MITZMAIN. The rôle of *Stomoxys calcitrans* in the transmission of *Trypanosoma evansi*. Philippine Journal of Science (1912), Sec. B. vol. 7, p. 475.

II. SUMMARY OF EXPERIMENTS IN THE TRANSMISSION OF ANTHRAX BY BITING FLIES.

By M. Bruin Mitzmain, *Technical Assistant, United States Public Health Service.*

In an exhaustive and critical review of the subject of anthrax and flies, Nuttall introduces the subject in his treatise in the Johns Hopkins Hospital reports as follows:

> After having read the rather extensive literature on the subject I have been struck by the very few positive cases recorded on anthrax arising from the bites of flies. Many authors present very positive opinions, but no evidence. In many cases the sharp pain which draws attention to the part affected, as well as the local appearances, have undoubtedly led to false statements. This was already stated by older authors, and a number of these in consequence go so far as to deny that malignant pustule may arise from the bite of a fly.

Nuttall finally concludes after presenting an imposing array of literature—

> It does seem high time, though, after nearly a century and a half of discussion, to see what would be the result of properly carried out experiments.

There has come to the notice of the writer only one piece of experimental investigation concerning the transmission of anthrax by biting of flies. Schuberg and Kuhn in 1912 succeeded in transferring, with *Stomoxys calcitrans* as a porter, anthrax infection from cadaver to living animal. An interval of 10 minutes or more was recorded in this experiment. They also demonstrated that anthrax organisms remained viable in the stomachs of the flies for considerable periods. The fecal deposits of these flies were found to produce pure cultures of anthrax.

That the anthrax organisms remain unchanged when passed through the insects' bodies has been shown by the above-mentioned authors and also in the researches of Graham-Smith who recovered cultures of anthrax from the feces of house flies three years after deposition.

Schuberg and Kuhn in their experiments infected their flies by inducing them to feed on exposed organs of anthrax cadavers. Transmission was affected through the biting of a healthy animal. Attention may be drawn to the question of practicability of experiments of this sort, for it has been observed in the Philippines that the stable

fly, *Stomoxys calcitrans*, does not, except under artificial conditions, attack the carcass of an animal whether recently dead or opened and exposed for some time.

Stomoxys calcitrans, as well as *Tabanus striatus*, the horsefly, can be induced to feed on animals dying of anthrax and also upon their bodies a short time after death. The results of this precedure are outlined in the following experiments:

Experiments with Tabanus striatus.—A guinea pig dying of anthrax, i. e., 30 minutes prior to death, was used. One tabanid fly in one experiment and three flies in another experiment were used to bite the sick animal and transferred directly to a healthy animal. When the sick animal died, flies of the same species, *T. striatus*, were applied to the unbroken skin of the dead animal and after contaminating their proboscides were transferred immediately to the healthy animals. Two experiments were tried with this method up to 35 minutes after death. One tabanid was used in one experiment and five flies in the other experiment.

Checks.—The first series was controlled by microscopic examination of blooddrops taken from the ear. A moderate number of anthrax-like rods were present in blood drawn prior to the two experiments.

The second series, in which flies were permitted to bite the dead animal, was controlled by the obtaining of pure cultures of *B. anthrax* from the mucous layer of the skin beneath the site of application of the fly feeding.

Results of the fly biting.—Anthrax infection resulted in the two animals bitten by flies which had previously bitten the dying animal. One of the two newly infected animals recovered, after a pure culture of anthrax had been obtained from blood from its ear, three days following the bite of a single tabanid fly. The other guinea pig died of typical anthrax in three and one-half days after three flies had bitten it. The organism was reproduced in cultures and animal inoculation from the spleen pulp of the dead animal. In neither of the experiments, wherein the initial biting was performed on the cadaver, did infection result. Later the two guinea pigs were proved susceptible by injections of pure cultures of anthrax.

Three experiments were conducted with *Stomoxys calcitrans*, by the interrupted method of feeding, in similar attempts to carry infection from dying or dead guinea pigs to healthy guinea pigs. There were employed 10, 18, and 35 flies, respectively, in three experiments controlled in two instances by attempts to transmit the disease from the sick animal 30 minutes to 5 minutes before death. The latter experiments resulted in anthrax transmission, both guinea pigs dying three days after 12 and 15 flies, respectively, had been allowed to bite them. The experiments in which a dead animal had been used for the initial bite resulted negatively, and the three guinea pigs were

found 20 to 24 days later to be susceptible to injections of pure culture of anthrax bacilli.

As many as 18 experiments were tried in efforts to transmit the disease through the biting of large numbers of flies of the two species, *Stomoxys calcitrans* and *Tabanus striatus*, with animals 48 to 7 hours preceding death. Only negative results were obtained. One horse, two bulls, and several guinea pigs inoculated with fatal doses of *B. anthrax* were used in these experiments. The exposed animals utilized were 4 horses, 6 bulls, and 8 guinea pigs. Only the guinea pigs and 1 horse were tested for susceptibility, and of these 1 horse and 6 of the 8 guinea pigs employed died as a result of injections of pure cultures. In every instance either a pure or a mixed culture of *B. anthrax* was obtained from the blood of the animals used in attempts to infect the flies.. Three of the pure cultures thus obtained were used to reproduce the disease in the horse and 6 guinea pigs just mentioned.

The foregoing experience convinced the investigator that it was justifiable to exact a more definite criterion of the presence of infection. The time element in the matter of duration of the disease and even that of clinical symptoms proved to be quite unsatisfactory tests for the availability of an animal as a blood donor. An infallible test in this regard was afforded by a microscopical examination of the peripheral blood (that from the ear) to insure more positive results in the experiments following. The results were the same whether the antrax-like organisms were scanty in numbers or abundant or whether the rods were present singly or formed in chains. Only the direct method of transmission in which the fly's biting was interrupted was attempted in this series of experiments. Guinea pigs were used and the flies applied were *S. calcitrans* and *T. striatus*. Five experiments with stable flies and three experiments with gadflies gave positive results. None in this series resulted negatively. In the experiments with gadflies and three of the experiments with stable flies no appreciable interval (45 seconds or less) was allowed between the biting of diseased and healthy hosts. In two experiments with *Stomoxys calcitrans* intervals of 10 minutes and 20 minutes, respectively, were given the flies prior to completing the feeding upon the healthy animals. There were used 10 to 43 flies in the experiments with *Stomoxys* and 1 to 5 flies were used in the experiments with *Tabanus*. In the latter series, where a single gadfly was used, it was transferred from sick to healthy animal in one trial, twice successively, and in another trial a fly was employed three times in succession.

Methods.—At first a gauze covering for the experimental guinea pigs was employed. Later the animals were merely immobilized with gauze straps fastened to a board. Care was exercised to avoid abrasions where insects were applied on skin of the host. Flies

were, without exception, applied from individual tubes. It was thought necessary at first to clean the mouth of the tube with an antiseptic fluid at each application of the fly at the time of its transfer. but this procedure was discontinued later, as it was deemed unessential.

The following criteria were adopted in declaring a transmission experiment positive. If the exposed animal did not die within five days after being bitten by flies removed from an infected host, a culture was made from blood taken from an ear vein. If a mixed culture resulted it was further injected into a clean animal and the death of the animal with the positive microscopical findings were considered sufficient verification. When an animal died as a result of fly bites, blood drops and stained smears were prepared from the heart and spleen. It was assumed that death was due to anthrax when the organisms seen were large square-ended nonmotile rods occurring singly and in segments of two or more. These organisms were gram positive and reacted typically to McFadyean's staining test. Two guinea pigs were vaccinated with spleen pulp of the dead animal, and cultures were made from the spleens of the test animals. Cultures on agar were deemed sufficient. These produced, after 48 hours incubation, very definite, wavy-bordered colonies of a dull ground-glass appearance. The individual filaments of the colony could be readily discerned with the low-power microscope. Hanging drop preparations from the cultures revealed innumerable numbers of large nonmotile filamentous rods containing very distinct oval spores. Stained preparatons emphasized the square-ended structure of the individual bacterium, the spores staining uniformly red in contrast to the tinctorial reaction of the vegetative forms. All of these cultures were indistinguishable from the cultures used primarily to infect the original blood donors.

THE QUESTION OF FECAL CONTAMINATION.

Methods.—Twelve laboratory-bred *Stomoxys calcitrans* and an equal number of *Tabanus striatus* were selected for this study. The flies were fed daily, or, in the case of the gadflies, as often as they desired (never more than once in three days). The first meal of blood was derived from an animal which proved susceptible to an inoculation of an agar culture of anthrax, and in whose blood the organisms were demonstrated microscopically. Upon the second day and after, the subsequent feedings were made upon two healthy guinea pigs, one for each species of fly, until the twentieth day when the two remaining stable flies were killed for cultural purposes. Incidentally the healthy animals bitten did not suffer from the biting of the infected flies. After each daily feeding, the flies were transferred individually to sterile test tubes and permitted to deposit upon the glass of the

tubes. Two cultures from each day's fresh fecal deposits were made upon slant agar and incubated in the usual way. The mixed cultures, resulting after 48 hours, were suspended in physiological saline and injected into either two guinea pigs or two white mice. After the eighth day, when growth was less vigorous, pure cultures were made by subinoculations on Petri dishes. These were continued up to the twentieth day, when the experiments terminated.

Results obtained.—Only the vegetative form of the anthrax bacterium could be demonstrated throughout the entire series, either in the body of the insect or in the fecal deposits. Of the latter, both the watery liquid and the heavier light-brown droppings were examined and cultivated. The virulence of the organisms present was demonstrated by the results of the animals inoculated from suspensions of the agar cultures.

There was a uniform fatality from the material used beginning with three hours up to and including 9 days. The following cultures, including those made 9 days after fly infection, proved virulent: Three hours, 20 hours, 46 hours, 76 hours, 98 hours, 117 hours, 6 days, 7 days, 8 days, and 9 days. Beginning with the cultures of feces made after ninth day following the infective meal, all proved avirulent as far as animal inoculation was concerned. All of the ordinary cultural and morphological tests were positive from the ninth day to the twentieth day with the material obtained from *Stomoxys calcitrans*, and from the sixth to the tenth day with material obtained from *Tabanus striatus*. Spores were not demonstrated either in the body of the flies or in the fresh fecal deposits at any time following the bite upon the infected animal. In these experiments no attempt was made to test the viability of anthrax spores possibly developing from the fly's feces after protracted periods following deposition.

The tables following summarize the data compiled from the various experiments included in the foregoing discussion:

TABLE I.—*Transmission experiments with the host dying and later dead of anthrax.*

WITH STOMOXYS CALCITRANS.

Infected animal used.	Healthy animal used.	Number of flies applied.	Time flies were applied.		Results in animals exposed to fly bites.
			Before death of infected host.	After death of infected. host.	
			Minutes.	*Minutes.*	
Guinea pig 21	Guinea pig 92	10	....	5	Negative; 3 guinea pigs later succumbed to injections of pure culture.
	Guinea pig 48	18	....	8–10	
	Guinea pig 37	35	....	20–45	
	Guinea pig 22	12	30–20	....	Dead in 3 days; typical anthrax infection.
	Guinea pig 23	15	15–5	....	Dead in 3 days; anthrax cultures from spleen. Injection of spleen pulp into healthy guinea pig produced death in 56 hours.

Table I.—*Transmission experiments with the host dying and later dead of anthrax*—Continued.

WITH TABANUS STRIATUS.

Infected animal used.	Healthy animal used.	Number of flies applied.	Time flies were applied.		Results in animals exposed to fly flites.
			Before death of infected host.	After death of infected host.	
			Minutes.	*Minutes.*	
Guinea pig 24	Guinea pig 93	1	....	10	Negative; both guinea pigs used later found susceptible to injection of pure anthrax culture.
	Guinea pig 112	5	....	20–35	
	Guinea pig 40	1	30	....	Positive; recovered after culture obtained from ear on third day.
	Guinea pig 118	3	20–3	....	Dead in 3½ days; culture and test animal reproduced anthrax organisms.

Table II.—*Experiments in anthrax transmission with infected animals some time prior to death.*

Kind of animal used.		Time prior to death of infected host flies were applied.	Flies applied.		Fate of infected host employed.	Result of fly biting of healthy host.
Infected.	Healthy.		Number.	Species.		
		Hours.				
Horse 37	Horse 70	48	6	T. striatus	Died 5 days after injection of pure culture.	Negative.
Do	Horse 68	45	10	do		
Do	Horse 94	37	32	S. calcitrans		
Do	Horse 138	17	40	do	Pure culture recovered from jugular vein immediately after death.	Negative; died in 6 days as result of injection of culture.
Bull 35	Bull 40	48	9	T. striatus	Dead in 4 days after injection of spleen of guinea pig 111.	Negative.
Do	Bull 41	40	12	do		
Bull 39	Bull 42	37	38	S. calcitrans	Killed on third day.	Negative.
Do	Bull 43	12	44	do	Mixed culture of anthrax obtained from, ear vein.	Negative.
Do	Bull 44	14	28	do		
Do	Bull 46	8	42	do		
Guinea pig 111	Guinea pig 110	43	30	do	Died on third day. Spleen pulp injected into bull 38 produced typical symptoms.	Negative proved susceptible to a later injection of pure culture.
Do	Guinea pig 129	7	3	T. striatus		
Guinea pig 109	Guinea pig 123	12	5	do	Dead within 39 hours. Heart's blood injected into guinea pigs 114 and 119.	Do.
Guinea pig 113	Guinea pig 201	8	21	S. calcitrans	Dead on second day.	Negative proved susceptible to injection of mixed culture.
Guinea pig 114	Guinea pig 207	24	2	T. striatus	Dead on fourth day.	
Do	Guinea pig 221	16	29	S. calcitrans	do	
Guinea pig 119	Guinea pig 222	8	39	do	Dead on 3d day	Negative.
Do	Guinea pig 187	7	1	T. striatus	do	Do.

TABLE III.—*Experiments in anthrax transmission with the initial feeding on animals in whose blood organisms were found microscopically.*

Animals serving as blood donors.		Interval during application of infected flies.	Flies applied.		Results of fly biting in the hosts exposed.
Infected guinea pig.	Healthy guinea pig.		Number.	Species.	
A-13	300	40 seconds	10	S. calcitrans	Positive; pure culture obtained from ear vein on fourth day killed a test guinea pig.
A-13	301	35 seconds	30	do	Positive; dead on third day; spleen pulp produced the disease in guinea pig A-19.
A-7	308	45 seconds	43	do	Positive; dead on fourth day.
A-19	314	10 minutes	20	do	Positive; dead in 3½ days.
A-19	321	20 minutes	18	do	Positive; dead in 2½ days. Pure culture obtained; heart's blood produced disease with death in test guinea pig in 3 days.
A-7	309	40 seconds	5	T. striatus	Positive; guinea pig dead on third day.
	310	45 seconds	1	do	Positive; guinea pig dead on third day; spleen pulp produced disease and death in test guinea pig in 2 days.
	311	40 seconds	1	do	Positive; mixed culture obtained from ear vein in 4½ days produced death in test animal in 3 days.

TABLE IV.—*Experiments in the viability of cultures of feces from flies fed on anthrax-infected animals.*

STOMOXYS CALCITRANS.

Length of time following biting infected host.	Nature of culture made.	Result of animal inoculation.
3 hours	Typical in every essential feature; colonies dull grayish composed of bundles of filaments. Borders wavy-ground glass appearance. Bacterium nonmotile square ended rods occurring singly and in chains. Spores numerous after 48 hours' growth. Rather large ovoid staining brilliantly.	Culture produced death in 2 guinea pigs.
20 hours		Do.
46 hours		Do.
76 hours		Culture produced death in 1 guinea pig.
98 hours		Feces produced death in guinea pig injected with saline suspension.
117 hours		Culture produced death in 2 guinea pigs.
6 days		
7 days		
8 days		
9 days		
10-20 days	Similar in character except nonuniformity of motility of individual bacterium and morphology. Strains of 14, 15, and 19 days with slightly round ended rods.	Culture produced death in 2 mice. Guinea pigs and mice injected with from 4 loops to entire culture escaped infection.

TABANUS STRIATUS.

Length of time following biting infected host.	Nature of culture made.	Result of animal inoculation.
1 day	Characteristic cultures produced on agar. Streak subcultures of mixed material gave typical colonies when isolated on plates.	Culture killed 2 guinea pigs.
2 days		Do.
3 days		Do.
4 days		Feces of fourth day produced death when injected into a guinea pig; cultures also pathogenic.
5 days		Culture killed 2 guinea pigs.
6 days		Culture killed 1 guinea pig.
7 days	Cultures similar in general features to preceding. Organisms indistinguishable as to morphology and staining.	4 loops of culture and also entire cultures failed to affect guinea pigs and mice.
8 days		
9 days		
10 days		

REFERENCES CITED.

NUTTALL. The rôle of the insects, arachnids and myriapods as carriers in the spread of bacterial and parasitic diseases of man and animals. Johns Hopkins Hospital Reports (1899), vol. VIII, pp. 2–15.

SCHUBERG and KUHN. Ueber die Uebertragung von Krankheiten durch einheimische stechende Insekten. Arb.a. d. kais. Gesundheitsamte (1912), Heft 2. Bd. XL, 209–234.

GRAHAM-SMITH. Flies in relation to disease: Nonblood-sucking flies. Cambridge University Press. London (1913).

HYGIENIC LABORATORY BULLETINS OF THE PUBLIC HEALTH SERVICE.

The Hygienic Laboratory was established in New York, at the Marine Hospital on Staten Island, August, 1887. It was transferred to Washington, with quarters in the Butler Building, June 11, 1891, and a new laboratory building, located in Washington, was authorized by act of Congress March 3, 1901.

The following *bulletins* [Bulls. Nos. 1–7, 1900 to 1902, Hyg. Lab., U. S. Mar.-Hosp. Serv., Wash.] have been issued:

*No. 1.—Preliminary note on the viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 2.—Formalin disinfection of baggage without apparatus. By M. J. Rosenau.

*No. 3.—Sulphur dioxid as a germicidal agent. By H. D. Geddings.

*No. 4.—Viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 5.—An investigation of a pathogenic microbe (*B. typhi murium* Danyz) applied to the destruction of rats. By M. J. Rosenau.

*No. 6.—Disinfection against mosquitoes with formaldehyde and sulphur dioxid. By M. J. Rosenau.

†No. 7.—Laboratory technique: Ring test for indol, by S. B. Grubbs and Edward Francis; Collodium sacs, by S. B. Grubbs and Edward Francis; Microphotography with simple apparatus, by H. B. Parker.

By act of Congress approved July 1, 1902, the name of the "United States Marine-Hospital Service" was changed to the "Public Health and Marine-Hospital Service of the United States," and three new divisions were added to the Hygienic Laboratory.

Since the change of name of the service the bulletins of the Hygienic Laboratory have been continued in the same numerical order, as follows:

*No. 8.—Laboratory course in pathology and bacteriology. By M. J. Rosenau. (Revised edition, March, 1904.)

†No. 9.—Presence of tetanus in commercial gelatin. By John F. Anderson.

*No. 10.—Report upon the prevalence and geographic distribution of hookworm disease (uncinariasis or anchylostomiasis) in the United States. By Ch. Wardell Stiles.

*No. 11.—An experimental investigation of *Trypanosoma lewisi*. By Edward Francis.

*No. 12.—The bacteriological impurities of vaccine virus; an experimental study. By M. J. Rosenau.

*No. 13.—A statistical study of the intestinal parasites of 500 white male patients at the United States Government Hospital for the Insane; by Philip E. Garrison, Brayton H. Ransom, and Earle C. Stevenson. A parasitic roundworm (*Agamomermis culicis* n. g., n. sp.) in American mosquitoes (*Culex sollicitans*); by Ch. Wardell Stiles. The type species of the cestode genus *Hymenolepis*, by Ch. Wardell Stiles.

*No. 14.—Spotted fever (tick fever) of the Rocky Mountains; a new disease. By John F. Anderson.

*No. 15.—Inefficiency of ferrous sulphate as an antiseptic and germicide. By Allan J. McLaughlin.

*No. 16.—The antiseptic and germicidal properties of glycerin. By M. J. Rosenau.

*No. 17.—Illustrated key to the trematode parasites of man. By Ch. Wardell Stiles.

*No. 18.—An account of the tapeworms of the genus *Hymenolepis* parasitic in man, including reports of several new cases of the dwarf tapeworm (*H. nana*) in the United States. By Brayton H. Ransom.

*No. 19.—A method for inoculating animals with precise amounts. By M. J. Rosenau.

*No. 20.—A zoological investigation into the cause, transmission, and source of Rocky Mountain "spotted fever." By Ch. Wardell Stiles.

*No. 21.—The immunity unit for standardizing diphtheria antitoxin (based on Ehrlich's normal serum). Official standard prepared under the act approved July 1, 1902. By M. J. Rosenau.

*No. 22.—Chloride of zinc as a deodorant, antiseptic, and germicide. By T. B. McClintic.

*No. 23.—Changes in the Pharmacopœia of the United States of America. Eighth Decennial Revision. By Reid Hunt and Murray Galt Motter.

No. 24.—The International Code of Zoological Nomenclature as applied to medicine. By Ch. Wardell Stiles.

*No. 25.—Illustrated key to the cestode parasites of man. By Ch. Wardell Stiles.

*No. 26.—On the stability of the oxidases and their conduct toward various reagents. The conduct of phenolphthalein in the animal organism. A test for saccharin, and a simple method of distinguishing between cumarin and vanillin. The toxicity of ozone and other oxidizing agents to lipase. The influence of chemical constitution on the lipolytic hydrolysis of ethereal salts. By J. H. Kastle.

*No. 27.—The limitations of formaldehyde gas as a disinfectant, with special reference to car sanitation. By Thomas B. McClintic.

*No. 28.—A statistical study of the prevalence of intestinal worms in man. By Ch. Wardell Stiles and Philip E. Garrison.

*No. 29.—A study of the cause of sudden death following the injection of horse serum. By M. J. Rosenau and John F. Anderson.

†No. 30.—I. Maternal transmission of immunity to diphtheria toxine. II. Maternal transmission of immunity to diphtheria toxine and hypersusceptibility to horse serum in the same animal. By John F. Anderson.

†No. 31.—Variations in the peroxidase activity of the blood in health and disease. By Joseph H. Kastle and Harold L. Amoss.

†No. 32.—A stomach lesion in guinea pigs caused by diphtheria toxine and its bearing upon experimental gastric ulcer. By M. J. Rosenau and John F. Anderson.

*No. 33.—Studies in experimental alcoholism. By Reid Hunt.

†No. 34.—I. *Agamofilaria georgiana* n. sp., an apparently new roundworm parasite from the ankle of a negress. II. The zoological characters of the roundworm genus *Filaria* Mueller, 1787. III. Three new American cases of infection of man with horsehair worms (species *Paragordius varius*), with summary of all cases reported to date. By Ch. Wardell Stiles.

†No. 35.—Report on the origin and prevalence of typhoid fever in the District of Columbia. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle. (Including articles contributed by Ch. Wardell Stiles, Joseph Goldberger, and A. M. Stimson.)

†No. 36.—Further studies upon hypersusceptibility and immunity. M. J. Rosenau and John F. Anderson.

†No. 37.—Index-catalogue of medical and veterinary zoology. Subjects: Trematoda and trematode diseases. By Ch. Wardell Stiles and Albert Hassall.

No. 38.—The influence of antitoxin upon post-diphtheritic paralysis. By M. J. Rosenau and John F. Anderson.

†No. 39.—The antiseptic and germicidal properties of solutions of formaldehyde and their action upon toxines. By John F. Anderson.

†No. 40.—1. The occurrence of a proliferating cestode larva (*Sparganum proliferum*) in man in Florida, by Ch. Wardell Stiles. 2. A reexamination of the type specimen of *Filaria restiformis* Leidy, 1880=*Agamomermis restiformis*, by Ch. Wardell Stiles. 3. Observations of two new parasitic trematode worms: *Homalogaster philippinensis* n. sp., *Agamodistomum nanus* n. sp., by Ch. Wardell Stiles and Joseph Goldberger. 4. A

reexamination of the original specimen of *Tænia saginata abietina* (Weinland, 1858), by Ch. Wardell Stiles and Joseph Goldberger.

†No. 41. Milk and its relation to the public health. By various authors.

†No. 42.—The thermal death points of pathogenic micro-organisms in milk. By M. J. Rosenau.

†No. 43.—The standardization of tetanus antitoxin (an American unit established under authority of the act of July 1, 1902). By M. J. Rosenau and John F. Anderson.

No. 44. Report No. 2 on the origin and prevalence of typhoid fever in the District of Columbia, 1907. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle.

†No. 45.—Further studies upon anaphylaxis. By M. J. Rosenau and John F. Anderson.

†No. 46.—*Hepatozoon perniciosum* (n. g., n. sp.); a hæmogregarine pathogenic for white rats; with a description of the sexual cycle in the intermediate host, a mite (*lelaps echidnimus*). By W. W. Miller.

No. 47.—Studies on Thyroid: I. The relation of iodine to the physiological activity of thyroid preparations. By Reid Hunt and Atherton Seidell.

†No. 48.—The physiological standardization of digitalis. By Charles Wallis Edmunds and Worth Hale.

No. 49.—Digest of comments on the United States Pharmacopœia. Eighth decennial revision for the period ending December 31, 1905. By Murray Galt Motter and Martin I. Wilbert.

No. 50.—Further studies upon the phenomenon of anaphylaxis. By M. J. Rosenau and John F. Anderson.

No. 51.—Chemical tests for blood. By Joseph H. Kastle.

No. 52.—Report No. 3 on the origin and prevalence of typhoid fever in the District of Columbia (1908). By M. J. Rosenau, Leslie L. Lumsden, and Joseph H. Kastle.

No. 53.—The influence of certain drugs upon the toxicity of acetanilide and antipyrine. By Worth Hale.

No. 54.—The fixing power of alkaloids on volatile acids and its application to the estimation of alkaloids with the aid of phenolphthalein or by the Volhard method. By Elias Elvolve.

No. 55.—Quantitative pharmacological studies; adrenalin and adrenalin-like bodies. By W. H. Schultz.

No. 56.—Milk and its relation to the public health. (Revised edition of Bulletin No. 41.) By various authors.

No. 57.—I. The presence of tubercle bacilli in the circulating blood in clinical and experimental tuberculosis. By John F. Anderson. II. The viability of the tubercle bacillus. By M. J. Rosenau.

No. 58.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary for the period ending December 31, 1906. By Murray Galt Motter and Martin I. Wilbert.

No. 59.—The oxidases and other oxygen catalysts concerned in biological oxidations. By Joseph Hoeing Kastle.

No. 60.—A study of the anatom of Watsonius (n. g.), Watsoni of man, and of 19 allied species of mammalian trematode worms of the superfamily Paramphistomoidea. By Ch. Wardell Stiles and Joseph Goldberger.

No. 61.—Quantitative pharmacological studies: Relative physiological activity of some commercial solutions of epinephrin. By W. H. Schultz.

No. 62.—The taxonomic value of the microscopic structure of the stigmal plates in the tick genus Dermacentor. By Ch. Wardell Stiles.

†No. 63.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary (third edition) for the calendar year ending December 31, 1907. By Murray Galt Motter and Martin I. Wilbert.

†No. 64.—Studies upon anaphylaxis with special reference to the antibodies concerned. By John F. Anderson and W. H. Frost.

No. 65.—Facts and problems of rabies. By A. M. Stimson.

†No. 66.—I. The influence of age and temperature on the potency of diphtheria antitoxin. By John F. Anderson. II. An organism (*Pseudomonas protea*) isolated from water, agglutinated by the serum of typhoid fever patients. By W. H. Frost. III. Some considerations on colorimetry, and a new colorimeter. By Norman Roberts. IV. A gas generator, in four forms, for laboratory and technical use. By Norman Roberts.

†No. 67.—The solubilities of the pharmacopœial organic acids and their salts. By Atherton Seidell.

†No. 68.—The bleaching of flour and the effect of nitrites on certain medicinal substances. By Worth Hale.

No. 69.—The effects of restricted diet and of various diets upon the resistance of animals to certain poisons. By Reid Hunt.

No. 70.—A study of melting-point determinations with special reference to the melting-point requirements of the United States Pharmacopœia. By George A. Menge.

No. 71.—1. Some known and three new Endoparasitic Trematodes from American fresh-water fish. By Joseph Goldberger. 2. On some new parasitic Trematode worms of the genus *Telorchis*. By Joseph Goldberger. 3. A new species of Athesmia from a monkey. By Joseph Goldberger and Charles G. Crane.

†No. 72.—I. Report on an Outbreak of Typhoid Fever at Omaha, Nebr. (1909–1910), by L. L. Lumsden. II. The Water Supply of Williamson, W. Va., and its Relation to an Epidemic of Typhoid Fever. By W. H. Frost.

No. 73.—The effect of a Number of Derivatives of Choline and Analogous Compounds on the Blood Pressure. By Reid Hunt and R. de M. Taveau.

†No. 74.—Digitalis standardization and the variability of crude and of medicinal preparations. By Worth Hale.

No. 75.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary (third edition) for the calendar year ending December 31, 1908. By Murray Galt Motter and Martin I. Wilbert.

No. 76.—The physiological standardization of ergot. By Charles Wallis Edmunds and Worth Hale.

No. 77.—Sewage pollution of interstate and international waters, with special reference to the spread of typhoid fever. By Allan J. McLaughlin.

No. 78.—Report No. 4 on the origin and prevalence of typhoid fever in the District of Columbia (1909). By L. L. Lumsden and John F. Anderson. (Including articles contributed by Thomas B. McClintic and Wade H. Frost.)

†No. 79.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary (third edition) for the calendar year ending December 31, 1909. By Murray Galt Motter and Martin I. Wilbert.

†No. 80.—Physiological studies in anaphylaxis. Reaction of smooth muscle from various organs of different animals to proteins. (Including reaction of muscle from nonsensitized, sensitized tolerant, and immunized guinea pigs.) By William H. Schultz.

No. 81.—Tissue proliferation in plasma medium. By John Sundwall.

No. 82.—I. Method of standardizing disinfectants with and without organic matter. By John F. Anderson and Thomas B. McClintic. II. The determination of the phenol coefficient of some commercial disinfectants. By Thomas B. McClintic.

†No. 83.—Sewage pollution of interstate and international waters with special reference to the spread of typhoid fever. II. Lake Superior and St. Marys River. III. Lake Michigan and the Straits of Mackinac. IV. Lake Huron, St. Clair River, Lake St. Clair, and the Detroit River. V. Lake Ontario and St. Lawrence River. By Allan J. McLaughlin.

†No. 84.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary (third edition) for the calendar year ending December 31, 1910. By Murray Galt Motter and Martin I. Wilbert.

†No. 85.—Index catalogue of medical and veterinary zoology. Subjects: Cestoda and cestodaria. By Ch. Wardell Stiles and Albert Hassall.

By act of Congress approved August 14, 1912, the name of the "Public Health and Marine-Hospital Service of the United States" was changed to the "Public Health Service." Since the change in name the bulletins of the Hygienic Laboratory have been issued without break in their numerical order.

†No. 86.—Studies on typhus. By John F. Anderson and Joseph Goldberger.

No. 87.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and on the National Formulary (third edition) for the calendar year ending December 31, 1911. By Murray Galt Motter and Martin I. Wilbert.

No. 88.—Method for determining the toxicity of coal-tar disinfectants, together with a report on the relative toxicity of some commercial disinfectants. By Worth Hale.

No. 89.—Sewage pollution of interstate and international waters with special reference to the spread of typhoid fever. VI. The Missouri River from Sioux City to its mouth. By Allan J. McLaughlin.

No. 90.—Epidemiologic studies of acute anterior poliomyelitis. I. Poliomyelitis in Iowa, 1910. II. Poliomyelitis in Cincinnati, 1911. III. Poliomyelitis in Buffalo and Batavia, N. Y., 1912. By Wade H. Frost.

No. 91.—I. The cause of death from subdural injections of antimeningitis serum. By Worth Hale. II. Some noncholera selective media. By Joseph Goldberger.

No. 92.—Gaseous impurities in the air of railway tunnels. By Atherton Seidell and Philip W. Meserve.

No. 93.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and on the National Formulary (third edition) for the calendar year ending December 31, 1912. By Murray Galt Motter and Martin I. Wilbert.

No. 94.—I. Collected studies on the insect transmission of *Trypanosoma evansi*. By M. Bruin Mitzmain. II. Summary of experiments in the transmission of anthrax by biting flies. By M. Bruin Mitzmain.

MAILING LIST.

The Service will enter into exchange of publications with medical and scientific organizations, societies, laboratories, journals, and authors. ALL APPLICATIONS FOR THESE PUBLICATIONS SHOULD BE ADDRESSED TO THE **Surgeon General, Public Health Service, Washington, D. C.,** EXCEPT THOSE MARKED (*) AND (†).

The editions of the publications marked (*) are no longer available for distribution by the Surgeon General of the Public Health Service. Copies of those marked (†) may, however, be obtained from the Superintendent of Documents, Government Printing Office, Washington, D. C., who sells publications at cost, and to whom requests for publications thus marked should be made.

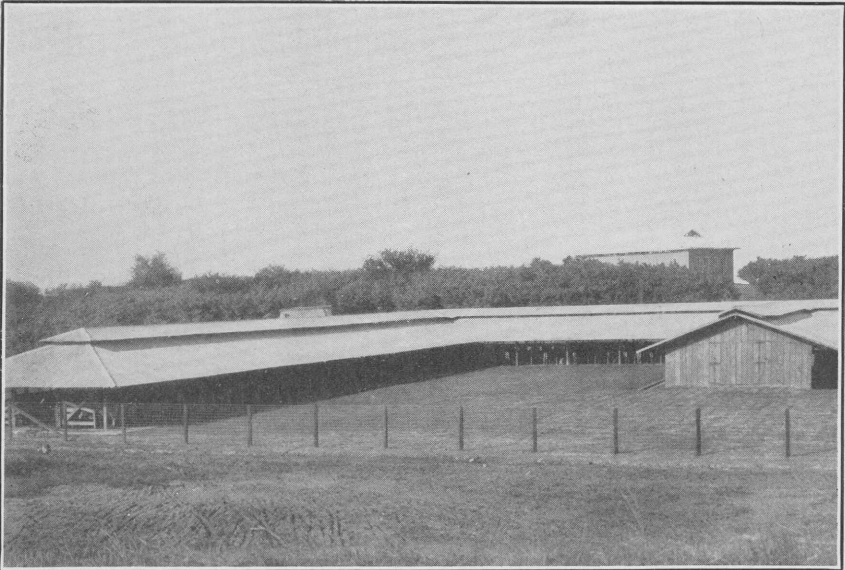


FIG. 1.—STOCK SHEDS OF VETERINARY RESEARCH LABORATORY, SHOWING CONDITIONS WHICH AID IN EXCLUDING TABANID FLY INFESTATION.

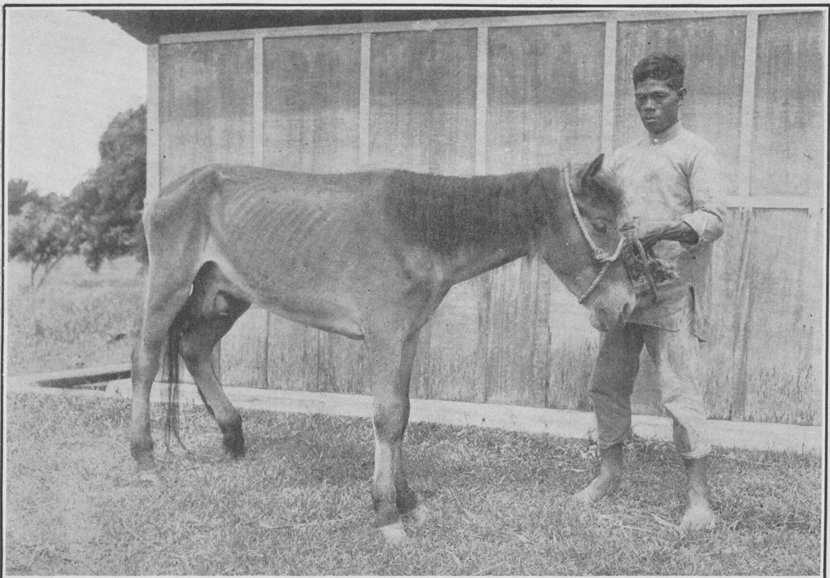


FIG. 2.—SPONTANEOUS CASE OF SURRA IN A HORSE USED FOR FLY-BITING EXPERIMENTS WITH TABANUS STRIATUS.

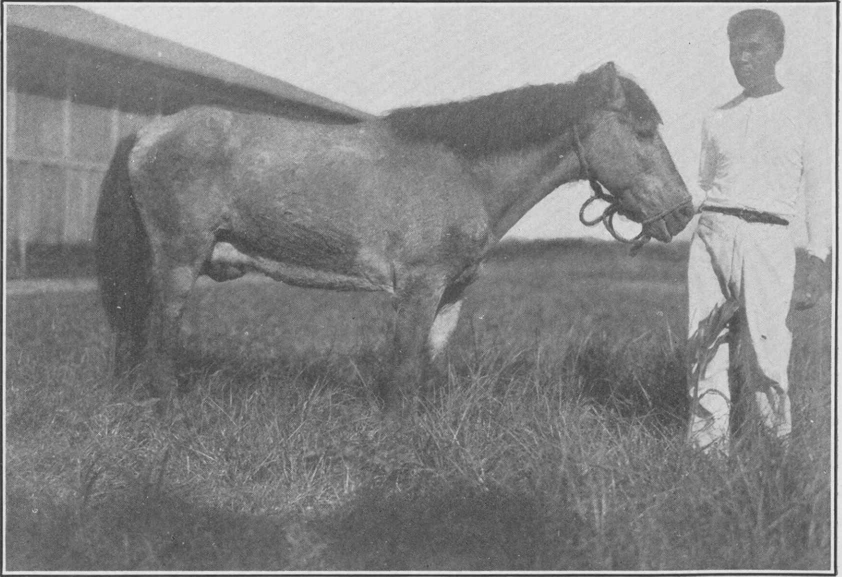


FIG. 3.—EXPERIMENTAL CASE OF SURRA INDUCED BY THE BITING OF HORSE FLIES INFECTED BY HORSE SHOWN IN FIG. 2.

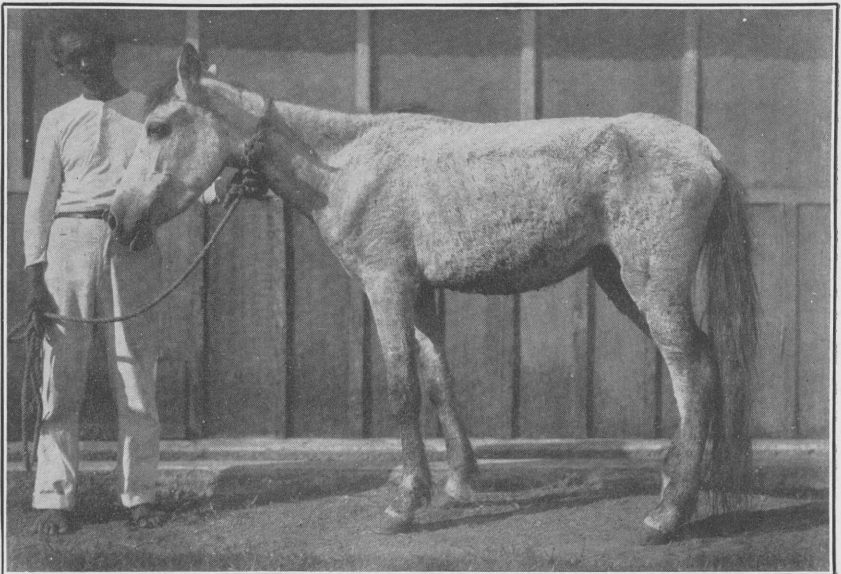


FIG. 4.—EXPERIMENTAL CASE OF SURRA INDUCED BY THE BITING OF HORSE FLIES INFECTED BY HORSE SHOWN IN FIG. 2.

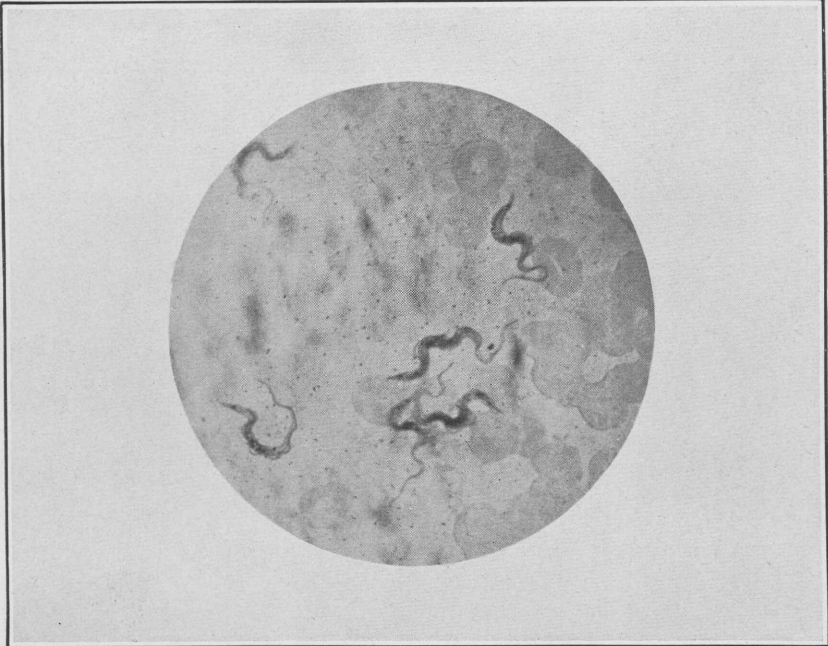


FIG. 5.—TRYPANOSOMA EVANSI FROM SMEAR OF STOMACH CONTENTS OF TABANUS STRIATUS FED ON SURRA-INFECTED MONKEY.

FIG. 6.—TRYPANOSOMA EVANSI FROM SMEAR OF HEART'S BLOOD OF MONKEY DEAD AS A RESULT OF BITES OF HORSE FLIES.

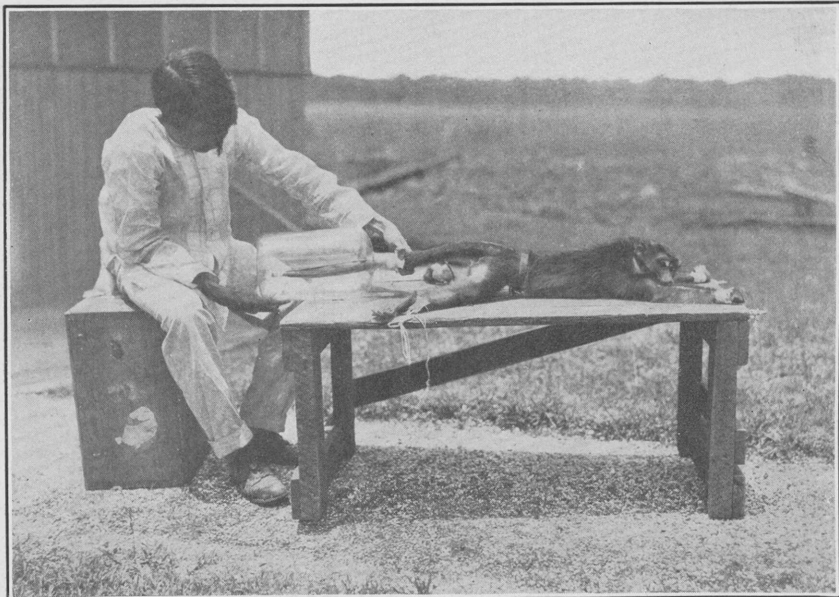


FIG. 7.—SHOWING METHOD OF EXPOSING MONKEY TO INFESTATION WITH LARGE NUMBERS OF STABLE FLIES AND HOUSE FLIES.

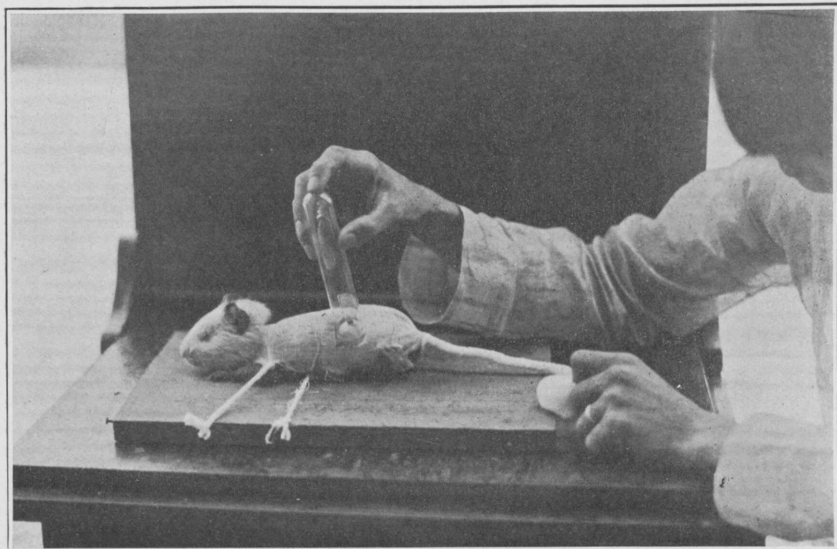


FIG. 8.—SHOWING METHOD OF APPLYING BITING FLIES ON IMMOBILIZED GUINEA PIG.

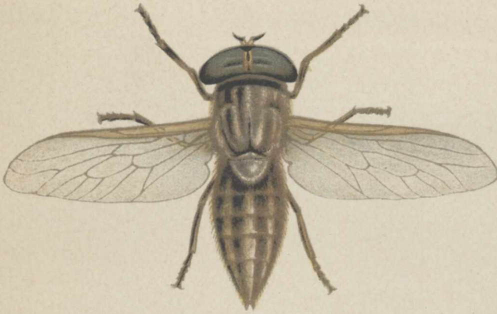


Fig. 9. Tabanus striatus (x2 .5)

L. H. Wilder

Fig. 10. Stomoxys calcitrans x

A Hoen & Co. Baltimore.

63D CONGRESS 2d Session } HOUSE OF REPRESENTATIVES { DOCUMENT No. 1061

TREASURY DEPARTMENT
UNITED STATES PUBLIC HEALTH SERVICE

HYGIENIC LABORATORY.—BULLETIN No. 95
AUGUST, 1914

LABORATORY STUDIES ON TETANUS

I. Conditions Surrounding Tetanus Spores Artificially Implanted Into Vaccine Virus

II. The Behavior of Tetanus Spores Injected Subcutaneously Into Guinea Pigs and White Mice

III. Miscellaneous Observations Upon Tetanus

BY

EDWARD FRANCIS

WASHINGTON
GOVERNMENT PRINTING OFFICE
1914

ORGANIZATION OF HYGIENIC LABORATORY.

RUPERT BLUE, *Surgeon General, Public Health Service.*

ADVISORY BOARD.

Major E. R. Whitmore, United States Army; Medical Director Edward R. Stitt, United States Navy; Dr. A. D. Melvin, Chief of United States Bureau of Animal Industry; and Surg. John F. Anderson, United States Public Health Service, *ex officio.*

Prof. William H. Welch, Johns Hopkins University, Baltimore; Prof. Simon Flexner, Rockefeller Institute for Medical Research, New York; Prof. Victor C. Vaughan, University of Michigan, Ann Arbor; Prof. William T. Sedgwick, Massachusetts Institute of Technology, Boston; Prof. Mazyck P. Ravenel, University of Wisconsin, Madison, Wis.

LABORATORY CORPS.

Director.—Surg. John F. Anderson.
Assistant Director.—Surg. A. M. Stimson.
Senior Pharmacist.—C. O. Sterns, Ph. G.
Junior Pharmacist.—Clyde Ritter, Ph. G.
Artist.—Leonard H. Wilder.
Acting Librarian.—E. Foltz.

DIVISION OF PATHOLOGY AND BACTERIOLOGY.

In charge of division.—Surg. John F. Anderson.

Assistants.—Surgs. Hugh S. Cumming, Leslie L. Lumsden, Taliaferro Clark, Lunsford D. Fricks, Carroll Fox, Joseph W. Schereschewsky, Edward Francis, Arthur M. Stimson, Richard H. Creel; Passed Asst. Surgs. R. M. Grimm, W. M. Bryan, H. E. Hasseltine, J. P. Leake; Asst. Surgs. W. F. Draper, C. L. Williams.

DIVISION OF ZOOLOGY.

Professor of zoology.—Ch. Wardell Stiles, M. D., Sc. D., LL. D., Ph. D.
Assistants.—Surg. Joseph Goldberger; Walter D. Cannon, LL. B., A. B., M. D.

DIVISION OF PHARMACOLOGY.

Professor of pharmacology.—Carl Voegtlin, Ph. D.
Assistants.—Atherton Seidell, Ph. D.; Murray Galt Motter, A. M., M. D.; Martin I. Wilbert, Pharm. D.; George B. Roth, A. B., M. D.

DIVISION OF CHEMISTRY.

Professor of chemistry.—Earle B. Phelps, B. S.
Assistants.—Elias Elvove, M. S., Pharm. D.; Albert Stevenson, B. S.

CONTENTS.

Page.

Introduction and summary 7

I. Conditions surrounding tetanus spores artificially implanted into vaccine virus 11
   - Heat as an agent in preventing acid-production in glucose bouillon inoculated with vaccine virus 18
   - Glycerination as a factor in eliminating nonspore-bearing and acid-producing organisms 19
   - Excessive acid production prevented by omitting glucose from culture medium 21
   - *Bacillus Welchii* resists glycerination and heat, but produces little acidity in ordinary bouillon 22
   - The animal test for tetanus spores 24
   - Summary 30

II. The behavior of tetanus spores injected subcutaneously into guinea pigs and white mice 35
   - Effect in guinea pigs of the injection of quinine or staphylococci at the site of injection of tetanus spores 35
   - Effect in white mice of the injection of quinine or staphylococci at the site of injection of tetanus spores 37
   - Effect in guinea pigs of the injection of quinine or staphylococci elsewhere than at the site of injection of tetanus spores 42

III. Miscellaneous observations upon tetanus 57
   - Inhibition of germination of tetanus spores by carbolic acid 57
   - Culture media for tetanus 58
   - Inhibition of germination of tetanus spores in glucose bouillon by anaerobes found in vaccine virus 59
   - Activators of tetanus spores 61
   - Tetanus toxin heated to 80° C. is not rendered lethal by quinine 61
   - Symbiosis versus antagonism 62
   - Heated tetanus spores 63
   - Washed tetanus spores 64
   - Isolation of tetanus organism from a wound 66
   - Differences in reactions of guinea pigs and white mice toward tetanus spores injected subcutaneously 67
   - Tetanus and vaccination in monkeys 68
   - Cleanliness in collecting vaccine virus from the calf 70
   - Vaccination favors the ejection rather than the absorption of foreign matters 72
   - The amount of tetanus toxin at the site of inoculation of tetanus spores and the amount in the blood 73

INTRODUCTION AND SUMMARY.[1]

In a period of 12 years, during which the Hygienic Laboratory of the Public Health Service has examined vaccine virus obtained under the greatest variety of circumstances and from many sources, no instance has been found of contamination of the virus with tetanus; *artificial contamination* of vaccine virus with tetanus spores was therefore necessarily resorted to in every instance, in order to obtain material for the tests and experiments reported upon in this bulletin.

Cases of tetanus reported as following vaccination direct our attention to the examination of vaccine virus for the possible presence of the tetanus organism. On account of the anaerobic nature of *Bacillus tetani,* anaërobiosis and apparatus and technique designed to produce oxygen-free conditions have heretofore overshadowed other considerations just as essential to the growth of the tetanus organism as is anaërobiosis. A thorough knowledge of all the biologic characteristics of the tetanus bacillus is a prerequisite to any intelligent attempt to recover that organism from vaccine virus supposedly contaminated with the organism.

Symbiosis and the oxygen-absorbing property of cocci, bacillus subtilis, and other organisms have been generally considered to be helpful accessories to the production of a strong tetanus toxin not only in wounds but in culture media as well.

In the subcutaneous tissue the germination of tetanus spores is favored by staphylococci but in glucose bouillon, their germination is inhibited by staphylococci. Striking examples of the opposite phenomena of symbiosis and antagonism are thus furnished by two and the same organisms when brought together under different conditions.

Conditions conducive to the growth of tetanus in pure culture may be inhibitive to its growth in mixed culture.

An attempt to recover tetanus from vaccine virus is merely an attempt to grow tetanus in mixed culture. Among the organisms which may contribute to the mixed culture are *streptococci, staphylococci, Bacillus coli, Bacillus Welchii,* and other aerobic and anaerobic bacteria.

If one attempts to germinate tetanus spores in fermentation tubes of glucose bouillon liberally inoculated also with any of the organisms just mentioned, he will almost certainly get no production of tetanus toxin.

[1] Manuscript submitted May 8, 1914.

If one contaminates with tetanus spores a "green" vaccine virus, or a virus glycerinated for months but containing glycerin-resisting anaerobes, and plants these contaminated viruses into fermentation tubes of glucose bouillon, he will almost certainly get no production of tetanus toxin.

While consortism of the tetanus bacillus with some other organism may be mutually helpful under certain conditions, antagonism or inhibition prevails when mixed cultures of the nature just cited are planted into fermentation tubes of glucose bouillon and immediately incubated. The reason for the nongermination of tetanus spores under these conditions is the early production of a high degree of acidity in the culture medium.

The Public Health Service tests vaccine virus for the persence of tetanus by four methods:

(*a*) By planting the virus into fermentation tubes of glucose bouillon and immediately incubating.

(*b*) By planting it into fermentation tubes of glucose bouillon and heating at 80° C. for an hour before incubation.

(*c*) By planting it into fermentation tubes of ordinary bouillon.

(*d*) By inoculating it subcutaneously into guniea pigs.

Tetanus spores contaminating a virus which contains many nonspore-bearing, acid-producing bacteria will almost certainly fail of detection by method (*a*) but are almost certain of detection by method (*b*).

Tetanus spores contaminating a virus which contains many spore-bearing, heat-resisting, acid-producing anaerobes will usually fail of detection by method (*b*) and usually produce toxin by method (*c*).

No one of the four methods of testing is suitable for determining the presence of tetanus in all samples of vaccine virus on account of the varied flora of the virus; two methods are, however, usually sufficient for any single sample of virus.

Failures to detect tetanus do occasionally occur in virus artificially contaminated with tetanus spores when planted into culture media under most favorable conditions, or when injected into susceptible animals; the reasons for the failures are not always apparent.

Bouillon inoculated with vaccine virus contaminated with tetanus spores and showing an acidity of less than +4.5 at the end of 24 hours of incubation will, with few exceptions, contain tetanus toxin; an acidity higher than +4.5 will prevent toxin production; the exceptions to the rule will be found in samples showing early titrations between +4.0 and +5.0.

Agents contributing to a high acidity of culture medium within the first 24 hours of growth are nonspore-bearing organisms, heat-resisting anaerobes and glucose.

Prolonged glycerination of vaccine virus or treatment of the virus for a time with a small amount of carbolic acid reduces the nonspore-bearing organisms and thereby prevents the development of high acidity of glucose bouillon into which the virus is planted for the purpose of determining whether it contains tetanus spores.

Heating of vaccine virus at 80° for an hour before incubation in glucose bouillon kills nonspore-bearing, acid-producing organisms and thus allows tetanus spores, if present, to germinate in a favorable medium of low acidity.

Withholding the glucose from the culture medium removes a source of acid production and thereby contributes to the germination of tetanus spores in a virus which contains at the same time heat-resisting anaerobes.

Tetanus spores in pure culture and free from tetanus toxin when injected subcutaneously into guinea pigs and white mice do not give rise to tetanus, but lie dormant as an inert body at the point of injection, awaiting either destruction by the phagocytes or an awakening into activity and toxin production by the irritation of some foreign body—particularly quinine or staphylococci.

The length of time during which spores will remain locally at the point of injection and susceptible of activation by quinine or staphylococci injected at the site of spore injection is approximately one month in the guinea pig and four months in the mouse.

Quinine and straphylococci are equally capable of activating tetanus spores when injected into the site of a spore injection in a guinea pig.

Quinine is incapable of activating tetanus spores in a mouse, but staphylococci injected into the site of a spore injection will activate the spores in a mouse with regularity up to four months.

If at the time of injection of tetanus spores into one part of the body of a guinea pig, there be injected into another part of his body some quinine or staphylococci, the animal will usually contract tetanus and cultures made from the quinine lesion or the staphylococcus lesion may be positive for tetanus; this shows that tetanus spores have been transported from the original site of injection and have been implanted into the quinine lesion or staphylococcus lesion; whether tetanus spores thus transplanted germinate and produce tetanus toxin in their new location is an open question.

Cases of tetanus occasionally occur in man following a hypodermic injection of quinine; based upon studies in guinea pigs, it has been suggested in a case of this kind that tetanus toxin is produced in the quinine lesion by tetanus spores picked up in another part of the body and transported by phagocytes to the site of the quinine injection.

From the results of studies in guinea pigs here reported in which tetanus spores injected on the abdomen were found in the staphylococcus lesions between the shoulders, one might expect to find in man that abscesses and other staphylococcus-infected lesions of closed anaerobic spaces would harbor stranded tetanus spores transported by phagocytes from some other part of the body.

The germination of tetanus spores was inhibited or prevented in glucose bouillon containing one-tenth per cent carbolic acid.

"Washing" of tetanus spores by successive suspensions and centrifugalizations in large quantities of saline solution did not rid them of their toxin.

Heating of tetanus spores at 80° C. for an hour was found to render them uniformly free from toxin without impairing their viability. Heating of the spores at 100° C. for 15 minutes killed them.

The subcutaneous tissue of a guinea pig seems both more susceptible and more destructive to tetanus spores than is the subcutaneous tissue of a white mouse.

Tetanus toxin appearing on media inoculated with tissue from a tetanus wound may have been carried over into the media with the tissue; it need not necessarily have been produced by the growth of tetanus in the media.

Eleven samples of glycerinated vaccine virus, naturally contaminated with *Bacillus Welchii*, artificially contaminated with tetanus spores, planted into glucose bouillon and heated at 80° C. for an hour before incubation, produced no tetanus toxin on account of the development of a high acidity of the media within the first 24 hours of incubation.

Gentle scrubbing with a soft brush and sterile water removed nearly all tetanus spores from the vaccinated area of a calf grossly contaminated with tetanus spores.

Eight rhesus monkeys vaccinated in five places with vaccine virus heavily contaminated with tetanus spores all developed good "takes" but none contracted tetanus; one rhesus monkey remained well after tetanus spores were thoroughly rubbed into his "take."

A vaccination sore seems to favor ejection of foreign matter such as tetanus spores and *Bacillus Welchii* rather than the absorption of the same.

I. CONDITIONS SURROUNDING TETANUS SPORES ARTIFICIALLY IMPLANTED INTO VACCINE VIRUS.

In accordance with the law of July 1, 1902, and regulations made in conformity thereto, the Treasury Department through the Public Health Service is charged with the inspection and licensing of manufacturers of "viruses, serums, toxins, and analogus products" sold in interstate traffic. These products are required to be labeled with the name of the manufacturer and his place of business; the name of the product and the date beyond which it can not reasonably be expected to retain its potency; the laboratory number of the product by which on reference to the firm's records, all steps in the preparation of the product can be traced.

Cases of tetanus occasionally occur following vaccination against smallpox and on investigation of these cases by officers of the Public Health Service, the question immediately arises as to whether the tetanus organism was present in the virus with which the vaccination was performed or whether it gained entrance into the vaccination sore from unclean skin, dressing, fingers, or surroundings.

Contamination of vaccine virus with tetanus or its freedom from this contamination can be determined by laboratory procedure, but a definite statement as to whether the vaccination sore became infected through unclean surroundings can not be made on account of the great number of avenues by which the infection might have gained entrance.

During the past two years the Hygienic Laboratory of the Public Health Service has made an extensive study of vaccine viruses with the view of determining whether they were free from tetanus organisms. Samples were bought at random on the market and were also obtained for examination direct from the manufacturers. The latter samples were in 5 cubic centimeter amounts and each represented a portion of the total yield of virus collected from one calf. The examination of the bulk output of a calf rendered unnecessary the examination of thousands of capillary tubes or points through which the bulk was retailed. One hundred and sixty-one bulk lots of virus collected from an equal number of calves were examined. These lots represented an output of a million and a half vaccinations.

Cases of tetanus reported as following vaccination were visited and on investigation the original package which contained the

virus used on the patient could usually be found. Usually the package still contained unused virus. If the package was empty its labels furnished all data necessary for obtaining on the open market or from the manufacturer duplicate samples of the particular virus used on the patient. Examination of this material obtained under the greatest variety of circumstances and from many sources all proved negative for tetanus.

In testing vaccine virus for the presence of tetanus or its products, the cultural methods employed involve a knowledge of the conditions which surround the tetanus organism after it comes to constitute a contaminating element in vaccine virus. "Green" pulp freshly collected from the calf constantly contains particles of epidermis and staphylococci in addition to the vaccine organism; it often contains also some streptococci, colon bacilli and hay bacilli; it sometimes contains *Bacillus pyocyaneus*, *Bacillus fecalis alkaligines*, *Bacillus Welchii*, and spore-bearing anaerobes. Should tetanus spores also by chance have reached the calf's abdomen they too might be collected with the pulp.

Given a "green" pulp contaminated with tetanus spores, how should one proceed to detect the presence of those spores? We are presented with tetanus in mixed culture—mixed with one or more of the organisms which have been found to contaminate vaccine virus. Heretofore it has been thought sufficient to plant the suspected pulp or virus into a fermentation tube of glucose bouillon and let it incubate at 37° C. for two weeks. By the end of this time it was thought that tetanus spores should have indicated their presence by the formation of a toxin which causes characteristic fatal symptoms in a mouse or guinea pig when injected subcutaneously in very small amount. If tetanus spores were the only kind of bacteria in the sample submitted for examination, they undoubtedly would grow and produce toxin when planted as above indicated. But in testing vaccine virus for tatanus, we are compelled to give just as careful attention to the inhibiting powers of the other organisms mixed with the virus as we give to the peculiar conditions needed for the maximum growth of the tetanus bacillus. Cocci, colon bacilli, and certain anaerobes when mixed with tetanus spores and planted into glucose bouillon will often so master the situation as to completely prevent any germination of the tetanus spores.

The inhibition which these organisms exercise is accomplished by the acidity which accompanies their growth in glucose bouillon. *Staphylococci*, *Bacillus coli* or *Bacillus welchii* will by the end of 24 hours of growth in glucose bouillon raise the acidity of the media from its initial +1.0 to +5.0 or +7.0. At this time tetanus spores, under the most favorable circumstances, are usually just beginning to show growth, whereas the organisms mentioned have already reached

almost their maximum growth and have so altered the medium as to render it unfavorable for the growth of tetanus.

If, then, a mixture of tetanus spores and acid-forming organisms be planted into glucose bouillon, the condition which surrounds the tetanus spore just at the time of expected germination is one of high acidity, the effect of which is to prevent its germination. The tetanus spore therefore lies completely dormant in an acid environment unfavorable to its growth. The nongerminating spore produces no toxin, and, giving no index of its presence, it may be entirely overlooked. The relation between reaction of media and germination of tetanus spores has special application in the problem of detecting tetanus in vaccine virus. The relation is so definite that one can predict at the end of 24 hours of growth almost to a certainty whether or not tetanus will be recovered from a known infected virus.

In order to obtain materials for studying the conditions surrounding tetanus spores in vaccine virus, it became necessary to artificially infect a large series of viruses with tetanus. Glycerinated viruses from 161 calves and unground vaccine pulps from 29 calves were artificially contaminated with tetanus spores and planted into fermentation tubes of nutrient bouillon. The data obtained from that study showing the relation of acidity of media to germination of tetanus spores will now be submitted.

TABLE No. 1.—*Relative efficiency of different cultural conditions for recovering tetanus from vaccine materials which have been artificially contaminated with tetanus spores.*

Number of pulp and its corresponding vaccine virus.	Number of days between collection from calf and testing.	Pulp nonglycerinated artificially contaminated with tetanus spores.								Glycerinated virus artificially contaminated with tetanus spores.					
		Glucose bouillon, unheated.			Glucose bouillon, 80° 1 hour.		Ordinary bouillon, unheated.			Glucose bouillon, unheated.			Ordinary bouillon, unheated.		
		Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Reaction of open arm at end of 48 hours.	Mice inoculated with growth at end of 9 days.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.
1	143	6.0	9.5	1 c. c. neg.											
2	139	6.0	11.3	1 c. c. neg.						3.3		0.001 tet.			
3	120	5.5	12.5	1 c. c. neg.	3.5	0.001 tet.	2.4	3.0	0.001 tet.	1.8		0.001 tet.			
4	108	6.5	9.5	1 c. c. neg.	2.5	0.001 tet.	2.7	3.0	0.001 tet.	1.6		0.001 tet.			
5	89	5.0	11.5	1 c. c. neg.	3.5	0.001 tet.	2.5	3.0	0.001 tet.	1.5		0.001 tet.			
6	69	6.0	9.5	1 c. c. neg.			4.3	3.7	0.001 tet.	3.3		0.001 tet.			
7	34	5.8	10.0	1 c. c. neg.			2.3	2.6	0.001 tet.	4.5		0.001 tet.			
8	17	5.7	8.7	1 c. c. neg.	3.7	0.001 tet.	3.6	2.3	0.001 tet.	5.5		1 c. c. neg.			
9	18	5.0	7.0	1 c. c. neg.	2.2	0.001 tet.	2.5	2.7	0.001 tet.	5.6		1 c. c. neg.			
10	5	5.4	10.0	1 c. c. neg.	4.2	0.001 tet.	2.0	2.1	0.001 tet.	6.2		1 c. c. neg.			
11	5	5.5	11.0	1 c. c. neg.	2.5	0.001 tet.	2.3	3.5	0.001 tet.	5.8		1 c. c. neg.			
12		7.0	10.5	1 c. c. neg.	3.3	0.001 tet.	2.5	4.0	0.001 tet.	5.7		1 c. c. neg.			
13	37	6.0	9.0	1 c. c. neg.			2.0	4.0	0.0001 tet.	5.5	9.2	1 c. c. neg.	1.5	2.4	0.0001 tet.
14	36	7.0	9.6	1 c. c. neg.			2.3	4.1	0.0001 tet.	6.0	9.0	1 c. c. neg.	5.0	2.0	0.0001 tet.
15	15	8.0	11.3	1 c. c. neg.			2.3	4.6	0.0001 tet.	6.7	8.7	1 c. c. neg.	2.5	4.0	0.0001 tet.
16	12	7.7	10.1	1 c. c. neg.			2.9	5.1	0.0001 tet.	6.4	9.2	1 c. c. neg.	3.0	4.5	0.0001 tet.
17	7	7.2	10.7	1 c. c. neg.			2.3	4.5	0.0001 tet.	5.5	7.4	1 c. c. neg.	2.0	3.4	0.0001 tet.
18	1	6.1	10.1	1 c. c. neg.			2.1	3.9	0.0001 tet.	6.1	8.8	1 c. c. neg.	4.7	3.8	0.0001 tet.
19	6	5.5	6.6	1 c. c. neg.			2.4	3.5	0.0001 tet.	6.6	8.0	1 c. c. neg.	2.8	3.0	0.0001 tet.
20	5	6.4	9.0	1 c. c. neg.			2.4	4.5	0.0001 tet.	5.7	9.2	1 c. c. neg.	3.7	4.6	
21	285				1.8	0.001 tet.	2.0	2.5	0.0001 tet.						
22	280				2.2	0.001 tet.									
23	278						1.7	3.0	0.0001 tet.						
24	242						2.4	3.0	0.0001 tet.						
25	222						2.8	3.5	0.0001 tet.						
26	220						1.8	2.5	0.0001 tet.						
27	216				4.0	0.001 tet.	4.0	3.2	0.0001 tet.						
28	213						2.5	3.6	0.0001 tet.						
29	213				2.6	0.001 tet.	2.5	5.2	0.0001 tet.						
													Glucose bouillon, 80° 1 hour.		
30	46										10.0	1 c. c. neg.	3.0		0.0001 tet.
31	41										6.7	1 c. c. neg.	2.0		0.0001 tet.
32	25										8.5	1 c. c. neg.	2.3		0.0001 tet.
33	30										9.5	1 c. c. neg.	3.0		0.0001 tet.

The viruses studied in this table represent samples collected from 33 calves. The first 20 calves supplied samples not only of the ordinary commercial glycerinated virus, but also the green unground pulp just as it is taken from the calf.

All samples were artificially contaminated with tetanus spores and planted into fermentation tubes of either glucose bouillon or ordinary bouillon, in order to determine under what conditions tetanus could best be recovered. Certain of the tubes were kept for an hour in an Arnold steam sterilizer regulated at 80° C. and then incubated. The others were incubated without heating.

Twenty samples of vaccine pulp incubated without previous heating developed, at the end of 24 hours, acidities between +5.0 and +8.0 but none developed tetanus toxin, as shown by the results of the injection of 1 c. c. amounts into mice at the end of 9 days' incubation. It will be noted in the same table that 18 of these samples, and also nine other pulps when planted into either glucose bouillon heated to 80° C. for an hour before incubation or into ordinary bouillon not heated, all had early reactions between +1.7 and +4.3 and that all developed strong tetanus toxins.

In tests made upon vaccine pulp, the efficiency of glucose bouillon heated and of ordinary bouillon, stands in marked contrast with the inefficiency of glucose bouillon unheated. In the tests with glycerinated virus the same thing is true with the qualification that glucose bouillon unheated was efficient in recovering tetanus from viruses that had been glycerinated longer than 46 days.

Samples of glycerinated virus which had been glycerinated less than 46 days and in which the glycerin had not had time to sufficiently reduce the cocci, produced high acidities in glucose bouillon unheated and consequently no tetanus toxin was formed, but the same samples planted into glucose bouillon and heated or planted into ordinary bouillon, produced low acidities and strong tetanus toxin.

Samples 1 to 6 of glycerinated virus in which the glycerin had acted for long periods of time showed low acidities at the end of 48 hours of growth and a consequent production of strong tetanus toxin after nine days.

Titration of media was made as follows: With a 5 c. c. pipette introduced into the open arm of a fermentation tube, 5 c. c. of the growth were removed and placed in a porcelain dish to which phenolphthalein was also added; without dilution, the medium was placed over a flame and at the moment of reaching the boiling point, the flame was withdrawn and one-twentieth normal sodium hydrate solution was allowed to run in from a burette until a pink color appeared. The number of cubic centimeters of sodium hydrate used indicated also the percentage of acidity of the medium.

Sixteen tests, extending over 16 months, were made of a vaccine virus obtained from a calf which was vaccinated on the abdomen and inner sides of the thighs with vaccine virus artificially contaminated with tetanus spores. One week later the virus was collected from the calf and glycerinated, and then placed in the icebox at 5° C. where it was kept throughout the tests. The virus was planted each time in glucose bouillon and incubated without heating. Titrations at the end of 24 hours incubation showed reactions between +5.0 and +7.0. (See Table 2.) Tetanus toxin developed in none, on account of the high acidity developing in the tubes during the first 24 hours.

TABLE No. 2.—*Relative efficiency of glucose bouillon unheated, the same medium when heated to 80° C. before incubation, and ordinary bouillon, in recovery of tetanus from vaccine virus.*

Date of collection of virus from calf.	Date of testing of virus for tetanus.	Glucose-bouillon, unheated.			Glucose-bouillon, 80°, 1 hour.			Ordinary bouillon, unheated.		
		Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.
1912.	1912.									
July 16	July 20	5.0	8.7	1 c. c. neg.	4.0	5.0	1 c. c. neg.	......	......	......
	...do....	5.1	7.1	1 c. c. neg.	3.0	3.3	0.1 c. c. tet.	......	......	......
	July 30	6.0	9.1	1 c. c. neg.	3.0	5.7	0.001 c. c. tet.	......	......	......
	...do....	6.0	9.3	1 c. c. neg.	2.4	2.8	0.1 c. c. tet.	......	......	......
	Oct. 15	6.0	9.5	1 c. c. neg.	4.5	5.8	1 c. c. neg.	......	......	......
		5.5	7.6	1 c. c. neg.	4.0	5.0	1 c. c. neg.	......	......	......
		6.0	7.7	1 c. c. neg.	1.6	5.5	0.001 c. c. tet.	......	......	......
	1913									
	Jan. 13	5.2	8.8	1 c. c. neg.	1.2	4.1	0.001 c. c. tet.	......	......	......
		5.4	12.0	1 c. c. neg.	3.3	4.2	1 c. c. tet.	......	......	......
		5.7	10.0	1 c. c. neg.	4.0	1.8	0.00001 c. c. tet.	......	......	......
	Dec. 2	......	8.5	1 c. c. neg.	......	......	......	3.2	2.1	0.01 c. c. tet.
			9.0	1 c. c. neg.	......	......	......	......	2.7	0.001 c. c. tet.
		6.0	6.5	1 c. c. neg.	......	......	......	......	4.5	1 c. c. neg.
			7.8	1 c. c. neg.	......	......	......	3.1	2.2	0.001 c. c. tet.
		6.0	8.5	1 c. c. neg.	......	......	......	4.5	3.2	0.001 c. c. tet.
		7.0	5.8	1 c. c. neg.	......	......	......	4.0	5.0	1 c. c. tet.

Ten parallel tests of the same virus were made in glucose bouillon in which the virus was exposed to 80° C. for one hour before incubation. The reaction of all at the end of 48 hours' growth were between +1.2 and +4.5, and all developed tetanus toxin except three, and these were the three of highest early acidity. Six parallel tests were made in ordinary bouillon unheated and the growth of tetanus showed in all except one, in which the acidity ran rather high.

Table 2 contrasts the inefficiency of glucose bouillon unheated with the efficiency of the same medium heated for an hour at 80° C. before incubation, and with the efficiency of ordinary bouillon in recovering tetanus from vaccine virus.

Eighteen tests extending over eight months were made of a vaccine virus artificially contaminated with tetanus spores. The virus was planted each time into glucose bouillon and incubated without pre-

vious heating. In 14 of the tests, tetanus toxin did not develop owing to the acidities which ranged at the end of 24 hours of growth between +4.7 and +9.0. In four tests strong tetanus toxin did develop, but in these the reactions at the end of 24 hours were between +1.2 and +3.0. (See Table No. 18 under "glucose bouillon, unheated, virus collected from unwashed side of calf".) In the same table note that in nine parallel tests made of the same virus, but in which the material was exposed to 80° for an hour before incubation, the reactions at the end of nine days were all between +1.3 and +2.6 and all developed strong tetanus toxins.

Eight samples of vaccine virus artificially contaminated with tetanus spores and staphylococci, and planted and incubated without previous heating, all developed acidities of +4.5 to +6.7 by the end of 24 hours' growth, and none produced any tetanus toxin (see Table No. 3).

The same table shows that in 6 tubes of glucose bouillon planted from the sediment of 6 of the above tubes and heated to 80° C. for one hour before incubation, the reactions remained between +2.0 and +4.2 and all developed tetanus toxin except one, the exception being the one showing the highest early acidity. The sediment from the other two tubes of glucose bouillon unheated which failed to produce tetanus toxin was injected subcutaneously into animals which contracted tetanus and died.

TABLE No. 3.—*The inhibition of germination, but not destruction, of tetanus spores by acidity of culture medium.*

Number of vaccine. virus.	Column 1. Glycerinated vaccine virus artificially contaminated with tetanus spores and staphylococcus and planted into glucose bouillon, unheated.			Column 2. Sediments from fermentation tubes of Nos. 3, 4, 5, 6, 8, and 11 were planted into glucose bouillon heated 80°, 1 hour.		
	Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 14 days.	Mice inoculated with growth at end of 14 days.	Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 14 days.	Mice inoculated with growth at end of 14 days.
3	6.7	11.0	1 c. c. neg.	4.2	5.0	1 c. c. neg.
4	6.4	13.0	1 c. c. neg.	3.7	5.5	1 c. c. tet.
5	6.3	9.0	1 c. c. neg.	2.3	1.2	0.001 tet.
6	6.6	9.0	1 c. c. neg.	2.0	5.6	0.001 tet.
8	6.4	8.0	1 c. c. neg.	2.4	5.7	0.1 c. c. tet.
11	6.6	8.2	1 c. c. neg.	4.0	6.0	0.001 tet.
12	4.5	7.2	1 c. c. neg.	....	....	....
13	4.5	8.5	1 c. c. neg.	....	....	....
	Four tetanus controls all showed growth. Sediments from fermentation tubes of Nos. 12 and 13 were injected subcutaneously into guinea pigs, which developed tetanus and died. Scrapings from sites of inoculation of guinea pigs contained, respectively, 10,000 and 100 Mlds. of tetanus toxin for a mouse.					

Column 1 of Table No. 3 illustrates the absence of tetanus toxin in fermentation tubes showing early high acidities, and column 2 shows the production of toxin by those same spores after removal from their acid environment and implantation in a medium in which the production of acid is prevented by exposure to 80° C. for one hour before incubation. The spores germinated in all instances, except one, which was the one of greatest early acidity.

The viability of the spores was tested by the inoculation of the sediment of two tubes (Nos. 12 and 13) into animals. Tetanus developed in both animals.

From these tests it is apparent that high early acidity inhibits the germination of tetanus spores but does not kill them.

HEAT AS AN AGENT IN PREVENTING ACID PRODUCTION IN GLUCOSE BOUILLON INOCULATED WITH VACCINE VIRUS.

Fifty-eight failures to detect tetanus in known infected vaccine material have just been cited above. In each instance the material was planted into a fermentation tube of glucose bouillon and incubated without exposure to 80° C. In each instance an acidity of +4.5 or more developed by the end of 24 hours of growth. Staphylococci and colon bacilli through their acid production were probably responsible for all the failures. The elimination of those organisms from the samples ought therefore to be accompanied by low acidity and production of tetanus toxin. If two tubes of glucose bouillon are inoculated with a freshly collected virus and one tube is incubated without further treatment while the other is exposed to 80° C. for an hour before incubation, the reaction of the media at the end of 24 hours will be decidedly higher in the unheated than in the heated tube. In attempting to recover tetanus from vaccine material by planting it into glucose bouillon, the interference which the nonspore-bearing organisms exercise upon the germination of the tetanus spore by their acid production must be reckoned with and prevented. This can be accomplished by an exposure of the contaminated pulp or virus to a temperature of 80° C. for an hour before incubation. The heat kills the nonspore-bearing organisms, but leaves the tetanus spores unaffected and free to germinate and produce their toxin.

As the 58 failures to recover tetanus in glucose bouillon unheated were being cited above, there was also cited a total of 37 parallel tests of the same material planted into glucose bouillon but exposed to 80° C. for an hour before incubations. The latter tests were all accompanied by reactions of media at the end of 24 hours no higher than +4.5 and all developed tetanus toxin except the four of highest early acidity.

Contrasting the efficiency of glucose bouillon heated at 80° C. for an hour before incubation with that of glucose bouillon incubated

without such heating, we find that in 55 tests of material artificially contaminated with tetanus spores and incubated after heating to 80° C., there were four failures to recover tetanus, whereas in 55 parallel tests of the same material incubated without previous heating there were 43 failures. (See tables 1, 2, 3, 4, 5, and 18.)

GLYCERINATION AS A FACTOR IN ELIMINATING NONSPORE-BEARING ACID-PRODUCING ORGANISMS FROM VACCINE VIRUS.

The pulp as it is collected from the calf must be freed as far as possible from the bacteria which unavoidably are collected with it in spite of most careful attention to the surroundings of the calf. In commercial practice glycerin is the agent most relied upon for removing the bacterial contamination of fresh pulp. It is generally used as a 50 per cent solution in water; 25 grams of pulp are added to 75 c. c. of 50 per cent glycerin; this mixture is ground and then emulsified and set aside in order to allow the glycerin to reduce the number of bacteria. Spore-bearing organisms are practically unaffected by glycerin, but the nonspore-bearing ones are greatly reduced, depending upon their number and the length of time during which the glycerin is allowed to act. If the greatest care has been taken in protecting the vaccinated area of the calf, the glycerin will usually have quite satisfactorily reduced the nonspore-bearing organisms by the end of 60 days. Low acidity in glucose bouillon inoculated with vaccine virus is an index of great reduction of colon bacilli and cocci in the virus; a reaction between +1.5 and +4.5 is significant of fair absence of those organisms.

Data will now be submitted on 144 attempts to recover tetanus organisms from an equal number of glycerinated commercial vaccine viruses artificially contaminated with tetanus spores, planted into glucose bouillon and incubated without previous heating. A relation is clearly brought out between the number of days of glycerination, the early reaction of the media, and the success or failure in recovering tetanus. We should expect a long period of glycerination to be accompanied by a low reaction and strong toxin production in glucose bouillon.

Six glycerinated viruses which had glycerinated between 69 and 143 days were artificially contaminated with tetanus spores and planted into glucose bouillon and incubated without previous heating. All developed early acidities between +1.5 and +4.5, and all produced strong tetanus toxins. (See Table No. 1 under "glycerinated virus, glucose bouillon, unheated.") In the same column note that 17 viruses similarly planted and tested, but which had glycerinated from 1 to 46 days, all developed acidities between +5.5 and +6.7 by the end of 24 hours, and consequently there was no production of tetanus toxin.

Eighteen samples of vaccine virus which had glycerinated from 46 to 330 days were contaminated with tetanus spores, planted into fermentation tubes of glucose bouillon and incubated without exposure to 80° C. Fourteen samples developed early acidities between +1.5 and +4.5 and strong tetanus toxins. Four samples contained *Bacillus welchii,* which caused acidities of +5.5 by the end of 24 hours and prevented the growth of tetanus. (See Table No. 4 under "glucose bouillon, unheated.")

Fifty-four viruses which had glycerinated between 55 and 90 days were planted, after being artificially contaminated with tetanus spores, into fermentation tubes of glucose bouillon and incubated without previous heating. Titrations of the open arm of the tubes at the end of 24 hours showed that the acidity of none had exceeded +4.5, and that only 15 had exceeded +4. Tetanus grew in all 54 tubes. (See Table No. 6 under "glucose bouillon, unheated.") Twelve other viruses included in Table 6 had glycerinated for only 11 days or less. The two tubes showing the highest early acidity developed no tetanus toxin. The other 10 tubes ranged between +4.5 and +5.5, but all produced toxin in spite of their high acidities.

Twenty-five samples of vaccine virus which had glycerinated between 280 and 575 days were artificially contaminated with tetanus spores, planted into fermentation tubes of glucose bouillon and incubated without previous heating. Titrations at the end of 24 hours of growth ranged between +2 and +4.5 in 20 of the number, and in all of these tetanus toxin developed; the five remaining tubes titrated between +5.8 and +7 and were negative for tetanus toxin. (See Table No. 5 under "glucose bouillon unheated.") Twelve other samples included in this table had glycerinated between 6 and 16 days. They titrated at the end of 24 hours of growth between +5.6 and +7.2. In spite of these high acidities there did develop in all tubes tetanus toxin which was of very low strength in those of highest acidity and of moderate strength in those of lowest acidity.

Reduced bacterial contamination of vaccine virus brought about by glycerin shows itself in low acidity when the virus is planted into glucose bouillon. The effect of glycerin acting for variable periods on 144 commercial viruses has been given above in detail and may be summarized as follows: One hundred and three of the 144 samples were glycerinated 46 days or longer; 94 of the 103 were accompanied by early reaction in glucose bouillon no higher than +4.5 and by toxin production. In the remaining 9 samples the early reactions exceeded +5.4 and in them no tetanus toxin developed. In 41 of the 144 samples, glycerination for only 46 days or less was accompanied in all instances by reactions in glucose bouillon of +4.5 or

higher; there was absence of tetanus toxin in 19 of the 41 and presence of tetanus toxin in 22.

The efficiency of 46 or more days of glycerination in reducing bacterial contamination, and therefore in preventing acidity and in permitting tetanus spores to germinate, is shown by the recovery of tetanus from all but 9 of 103 vaccine viruses exposed to glycerination for 46 days or longer (91.26 per cent). The inefficiency of less than 46 days of glycerination is shown by the absence of tetanus toxin in 19 out of 41 samples which had glycerinated for only 46 days or less (46.34 per cent). An absence of any glycerination in 20 samples of pulp was accompanied by an absence of tetanus toxin in all.

EXCESSIVE ACID PRODUCTION PREVENTED BY OMITTING GLUCOSE FROM CULTURE MEDIUM.

While high acidity in glucose bouillon due to nonspore-bearing organisms has been shown to be successfully prevented by exposure to a temperature of 80° or by prolonged glycerination, it is also true that these organisms when planted into ordinary bouillon usually fail to produce sufficient acid to prevent the germination of tetanus spores. Forty-two negative tests for tetanus in glucose bouillon unheated, in all of which the early acidities ranged between +4.7 and +9, *were repeated* with the same vaccine material, but using ordinary bouillon and incubating without previous heating. The reaction by the end of 48 hours now remained between +1 and +4 in all except five and tetanus grew in all except one. (See Tables 1, 2, 18, 4, and 6 under "ordinary bouillon, unheated.")

Forty-two samples of glycerinated vaccine virus which had proven positive for tetanus toxin in glucose bouillon were planted into fermentation tubes of ordinary bouillon and incubated without previous heating. The reactions at the end of 48 hours were between +1 and +3.2 in 27 samples which developed strong tetanus toxins. (See tables Nos. 4, 5, and 6 under "Ordinary bouillon, unheated.") The remaining 15 samples developed early reactions between +4 and +5. Eleven of these developed no tetanus toxin, and 4 developed toxin of very low strength.

Contrasting the efficiency of unheated ordinary bouillon with that of unheated glucose bouillon in recovering tetanus from vaccine material artificially contaminated with tetanus spores, we find that in 86 tests of material planted into ordinary bouillon and incubated without exposure to 80°, there were only 11 failures to recover tetanus, whereas in 86 parallel tests of the same material planted into glucose bouillon without exposure to 80° there were 41 failures. (See Tables 1, 2, 18, 4, 5, and 6.)

BACILLUS WELCHII RESISTS GLYCERINATION AND HEAT BUT PRODUCES LITTLE ACIDITY IN ORDINARY BOUILLON.

In treating the subject of acid production thus far, consideration has been given to only the nonspore-bearing organisms, i. e., those which are killed by an exposure to 80° C. *Bacillus welchii* was found in 15 samples of glycerinated vaccine virus out of 30 samples obtained from as many different calves. It resisted a temperature of 80° C. and is an anaërobe. It grows under exactly the same conditions as are most favorable to the growth of the tetanus bacillus, but it is inimical to the germination of tetanus spores. When planted into fermentation tubes of glucose bouillon and heated to 80° C. for an hour before incubation, it caused a high degree of acidity by the end of 24 hours.

The behavior in vaccine virus of *B. Welchii* toward *B. tetani* is brought out in the results tabulated in Table No. 4.

TABLE No. 4.—*Relative efficiency of ordinary bouillon and glucose bouillon in recovering tetanus from vaccine virus naturally contaminated with B. welchii and artificially contaminated with tetanus spores.*

Number of vaccine virus.	Number of days between collection of virus from calf and testing for tetanus.	Glucose bouillon, unheated.			Glucose bouillon, heated 1 hour at 80°.				Ordinary bouillon, heated 1 hour at 80°.			
		Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Tetanus controls.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Tetanus controls.
2	324	....	....	....	6.2	8.2	1 c. c. neg.	In each of 4 fermentation tubes heated to 80° 1 hour, 0.0001 c. c. was the MLD for mice; 0.00001 c. c. caused no symptoms; the reactions were 2, 2, 2.1, 2.2.	1.2	1.8	0.0001 tet.	In each of 3 fermentation tubes heated to 80° 1 hour, 0.0001 c. c. was the MLD for mice; 0.00001 c. c. caused no symptoms; the reactions were 1.7, 1.8, 2.
4	308	....	....	....	6.5	7.9	1 c. c. neg.		1.6	1.9	.0001 tet.	
6	285	....	....	....	5.8	8.3	1 c. c. neg.		3.9	4.3	.0001 tet.	
7	274	....	....	....	6.5	8.4	1 c. c. neg.		1.3	1.5	.0001 tet.	
9	261	....	....	....	6.1	8.1	1 c. c. neg.		1.5	2.0	.0001 tet.	
12	245	....	....	....	6.7	8.5	1 c. c. neg.		1.3	2.3	.0001 tet.	
13	244	....	....	....	6.8	8.0	1 c. c. neg.		2.5	2.7	.0001 tet.	
14	247	....	....	....	5.9	8.0	1 c. c. neg.		1.2	1.7	.0001 tet.	
15	247	....	....	....	6.6	8.0	1 c. c. neg.		3.7	2.9	.0001 tet.	
16	252	....	....	....	6.4	7.4	1 c. c. neg.		2.6	3.4	.0001 tet.	
17	208	....	....	....	6.2	7.5	1 c. c. neg.		3.3	3.0	.0001 tet.	
					Reaction at end of 48 hours.				Ordinary bouillon, unheated.			
1	330	1.7	....	Tetanus.	2.5	....	....	....	3.0	1.3	0.0001 tet.	
3	316	5.5	....	1 c. c. neg.	3.2	....	....	....	2.0	1.5	.0001 tet.	
5	305	5.5	....	1 c. c. neg.	1.7	....	....	....	1.7	1.5	.0001 tet.	
8	284	4.5	....	Tetanus.	4.0	....	....	....	1.6	1.6	.0001 tet.	
10	276	2.2	....	Tetanus.	4.0	....	....	....				
11	276	4.5	....	Tetanus.	1.7	....	....	....	1.0	1.6	.0001 tet.	
19	207	....	....	Tetanus.	....	2.4	0.0001 tet.	....	1.1	1.2	.0001 tet.	
18	227	3.6	4.6	0.0001 tet.	....	6.0	.0001 tet.	....	2.5	3.0	.0001 tet.	
20	193	1.5	1.2	.0001 tet.	....	3.0	.0001 tet.	....	1.1	1.1	.0001 tet.	
21	123	4.4	....	....	....	2.0	.0001 tet.	....				
22	115	1.6	3.2	.0001 tet.	....	2.2	.0001 tet.	....	1.1	1.5	.0001 tet.	
23	102	1.5	1.3	.0001 tet.	....	2.2	.0001 tet.	....	1.0	1.2	.0001 tet.	
24	87	1.5	1.1	.0001 tet.	....	2.2	.0001 tet.	....	....	2.3	.0001 tet.	
25	82	4.5	4.9	.0001 tet.	....	3.0	.0001 tet.	....	....	1.0	.0001 tet.	
26	82	1.5	1.8	.0001 tet.	....	2.0	.0001 tet.	....	1.0	1.0	.0001 tet.	
27	72	2.7	2.0	.0001 tet.	....	4.0	.0001 tet.	....	1.2	2.3	.0001 tet.	
30	46	1.7	6.9	0.01 tet.	....	2.2	.0001 tet.	....	....	2.0	.0001 tet.	
28	66	5.5	6.2	1 c. c. neg.	....	5.0	.0001 tet.	....	3.1	2.8	.0001 tet.	
29	66	5.5	5.1	1 c. c. neg.	....	2.0	.0001 tet.	....	3.0	3.1	.0001 tet.	

These 30 samples were artificially contaminated with tetanus spores and planted into fermentation tubes of different media. Of these 30 samples, 11 in which *B. Welchii* occurred, were planted into fermentation tubes of glucose bouillon and heated for an hour in an Arnold steam sterilizer regulated at 80° C. and each showed a high acidity (+5.5 to +6.8) in the open arm of the tube at the end of 24 hours incubation and there was a consequent failure of germination of the tetanus spores, as shown by the absence of tetanus toxin in 1 c. c. of growth at the end of 9 days, when injected into mice.

The high acidity in each instance was due to *B. Welchii*, the presence of which was due to natural contamination.

The same 11 samples were also planted into ordinary bouillon and, after being heated for an hour at 80° C., were incubated. The reaction of the media in the open arms at the end of 48 hours remained low (+1.2 to +3.9) and this permitted the tetanus spores to germinate and produce a strong toxin in each tube by the ninth day, the M. L. D. of each being 0.0001 c. c.

The other 19 samples were planted (*a*) into fermentation tubes of glucose bouillon and immediately incubated, or (*b*) into glucose bouillon and heated for 1 hour in an Arnold sterilizer at 80° C., or (*c*) planted into ordinary bouillon unheated. All produced low acidities in the media and permitted the germination of tetanus spores and the production of strong toxin, with the exception of four (Nos. 3, 5, 28, and 29, Table 4) in glucose bouillon unheated. In these four tubes *B. Welchii* again appeared and produced an early acidity sufficiently high within the first 24 hours to prevent any germination of the tetanus spores. These 19 viruses had all been glycerinated for 46 days or longer and were therefore comparatively free from nonspore-bearing organisms and produced low reactions in glucose bouillon unheated.

Contrasting the efficiency of ordinary bouillon incubated after exposure to 80° C. with glucose bouillon incubated after exposure to 80° C., we find that 11 samples of vaccine virus artificially contaminated with tetanus spores and naturally contaminated with *Bacillus Welchii*, when planted into ordinary bouillon, all developed early reactions between +1.2 and +3.9 and all produced strong tetanus toxins, while the same samples planted into glucose bouillon developed early reactions between +5.8 and +6.8 and none developed tetanus toxin. (See Table 4.)

THE ANIMAL TEST FOR TETANUS SPORES IN GLYCERINATED VACCINE VIRUS.

Table No. 5 shows the results of the tests made on 41 samples of vaccine virus, which was obtained from 41 calves and which was artificially contaminated with tetanus spores and either planted into

glucose bouillon and immediately incubated, or planted into glucose bouillon and kept for 1 hour at 80° C., or inoculated subcutaneously into guinea pigs on the abdomen. These tests, and a subsequent series shown in Table No. 6, were made to determine whether tetanus could be more constantly detected in vaccine virus by cultural methods or by inoculation of the virus directly into guinea pigs.

TABLE No. 5.—*Comparative efficiency of cultural tests under various conditions and animal tests in recovering tetanus from vaccine virus artificially contaminated with tetanus spores.*

Number of vaccine virus.	Number of days between collection of virus from calf and testing.	Glucose bouillon, unheated.			Glucose bouillon, heated 80°, 1 hour.			Incubation of tetanus, in days, in inoculated guinea pigs.	Tetanus controls.
		Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 14 days.	Mice inoculated with growth at end of 14 days.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.		
1	575	2.0	2.7	Tetanus.	.....	.....	.....	4 days, tet.	Two guinea pigs inoculated subcutaneously on the abdomen with the same dose of tetanus spores as was injected into guinea pigs 1 to 29 remained well. No. 9 in Table No. 10 was one of them.
2	575	2.2	2.9	Tetanus.	.....	.....	.....	6 days, tet.	
3	310	3.1	3.5	Tetanus.	.....	.....	.....	Remained well.	
4	309	5.8	7.5	1 c. c. neg.	3.3	2.9	0.001 tet.	Remained well.	
5	309	7.0	8.2	1 c. c. neg.	3.1	2.5	.0002 tet.	3 days, tet.	
6	308	4.3	3.2	.....	.....	.....	.....	.....	
7	308	6.1	7.6	1 c. c. neg.	3.0	3.3	0.0002 tet.	6 days, tet.	
8	303	6.5	7.5	1 c. c. neg.	3.1	2.8	0.0002 tet.	3 days, tet.	
9	290	6.7	7.8	1 c. c. neg.	3.0	3.3	0.00001 tet.	4 days, tet.	
10	290	3.5	3.1	0.02 c. c. tet.	3.2	3.0	0.002 tet.	.....	
11	290	2.6	4.0	.....	.....	.....	.....	Remained well.	
12	284	3.4	3.4	.....	.....	.....	.....	6 days, tet.	
13	330	2.4	2.2	Tetanus.	.....	.....	.....	6 days, tet.	
14	330	2.6	2.3	Tetanus.	.....	.....	.....	6 days, recovered.	
15	338	2.9	4.0	Tetanus.	.....	.....	.....	Remained well.	
16	330	2.5	1.5	Tetanus.	.....	.....	.....	8 days, tet.	
17	316	2.7	1.3	Tetanus.	.....	.....	.....	Remained well.	
18	316	2.9	4.5	Tetanus.	.....	.....	.....	5 days, tet.	
19	316	2.4	3.5	Tetanus.	.....	.....	.....	6 days, tet.	
20	317	4.0	4.1	.....	.....	.....	.....	5 days, tet.	
21	310	2.3	2.0	Tetanus.	.....	.....	.....	4 days, tet.	
22	311	2.0	2.8	Tetanus.	.....	.....	.....	4 days, tet.	
23	291	4.5	6.0	Tetanus.	.....	.....	.....	6 days, tet.	
					Ordinary bouillon unheated.				
24	289	4.0	4.4	Tetanus.	2.4	2.3	0.0001 tet.	5 days, tet.	
25	289	3.0	2.6	Tetanus.	1.6	3.2	0.0001 tet.	6 days, tet.	
26	288	2.0	2.1	Tetanus.	2.0	3.2	0.0001 tet.	5 days, tet.	
27	284	4.5	4.5	Tetanus.	1.6	3.1	0.0001 tet.	6 days, tet.	
28	282	3.8	4.0	Tetanus.	4.5	4.0	1 c. c. neg.	4 days, tet.	
29	280	2.0	1.6	Tetanus.	1.7	2.3	0.0001 tet.	5 days, tet.	
00	16	4.5	7.0	0.001 tet	4.0	2.5	0.01 c. c. tet.	.....	
02	16	4.2	7.5	0.001 tet.	5.0	.....	1 c. c. neg.	.....	
03	9	5.2	7.0	1 c. c. tet.	2.2	3.0	0.0001 tet.	.....	
08	16	2.0	7.4	0.001 tet.	1.8	2.4	0.0001 tet.	.....	
018	11	5.0	7.5	0.001 tet.	3.2	3.0	0.0001 tet.	.....	
019	11	5.0	8.5	0.001 tet.	4.1	.....	1 c. c. neg.	.....	
024	20	4.0	.....	0.1 c. c. tet.	2.9	2.3	0.0001 tet.	.....	
32	7	4.0	.....	0.01 c. c. tet.	1.5	1.7	0.0001 tet.		
37	7	4.0	.....	0.01 c. c. tet.	5.0	5.7	0.1 c. c. tet.	.....	
38	6	4.0	.....	0.01 c. c. tet.	4.4	5.5	0.1 c. c. tet.	.....	
43	6	4.5	.....	0.01 c. c. tet.	4.6	5.5	1 c. c. tet.	.....	
44	6	4.2	.....	0.1 c. c. tet.	1.8	2.3	0.0001 tet.	.....	

Of 41 samples planted into glucose bouillon and immediately incubated, 36 developed tetanus toxin of widely varying strengths. In five of the samples (Nos. 4, 5, 7, 8, and 9) the tetanus spores did not germinate and the acidities of these tubes at the end of 48 hours

were among the highest. These five numbers, however, when planted into glucose bouillon and heated to 80° C. for an hour before incubating all produced low acidities and very strong tetanus toxin.

Nos. 24 to 44, planted into ordinary bouillon, developed very strong tetanus toxin, with the exception of six tubes having the highest acidities, three of which developed no tetanus toxin and the other three developed extremely weak toxin.

Twenty-seven samples of virus, artificially contaminated with tetanus spores, when inoculated subcutaneously into guinea pigs on the abdomen, failed to produce any symptoms of tetanus in five of the animals, and a sixth recovered from an attack of tetanus. The other 21 animals all developed fatal tetanus.

A second series of tests similar to the one just described was carried out, the results of which are tabulated in Table No. 6. This series covered 67 samples of vaccine virus from as many calves and was planted or inoculated in the same manner as in the preceding series, except that no glucose bouillon heated to 80° C. for one hour before incubating was employed.

TABLE No. 6.—*Relative efficiency of cultural tests under various conditions and animal tests in recovering tetanus from vaccine virus artificially contaminated by tetanus spores.*

Number of vaccine virus.	Number of days between collection of virus from calf and testing.	Glucose bouillon, unheated.			Ordinary bouillon, unheated.			Incubation of tetanus, in days, in inoculated guinea pigs.	Tetanus controls.
		Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 14 days.	Mice inoculated with growth at end of 14 days.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.		
3	90	1.7	1.0	0.0001 tet.	......	......	......	3 days, tet.	Five guinea pigs inoculated subcutataneously on hind leg with same dose of tetanus spores as was injected into guinea pigs 3 to 48 of this table, remained well. They are represented by numbers 4, 5, 6, 7, 8, in table No. 10.
4	90	3.1	3.8	0.0001 tet.	......	......	......	3 days, tet.	
5	89	2.5	3.7	0.0001 tet.	......	......	......	3 days, tet.	
7	89	3.3	4.0	0.0001 tet.	......	......	......	6 days, tet.	
8	89	1.5	1.0	0.0001 tet.	......	......	......	3 days, tet.	
10	89	3.7	3.5	0.0001 tet.	......	......	......	9 days, tet.	
11	90	1.7	4.0	0.0001 tet.	......	......	......	6 days, tet.	
12	90	3.8	3.7	0.0001 tet.	......	......	......	5 days, tet.	
16	87	2.7	1.2	0.0001 tet.	......	......	......	4 days, tet.	
17	86	1.2	3.1	0.0001 tet.	......	......	......	4 days, tet.	
19	87	3.5	4.2	0.0001 tet.	......	......	......	3 days, tet.	
22	87	1.3	2.6	0.0001 tet.	......	......	......	4 days, tet.	
24	82	4.0	4.0	0.0001 tet.	......	......	......	5 days, tet.	
27	83	3.5	7.5	0.0001 tet.	......	......	......	7 days, tet.	
28	......	1.7	3.0	0.0001 tet.	......	......	......	4 days, tet.	
40	80	1.7	1.4	0.0001 tet.	......	......	......	3 days, tet.	
43	74	4.0	5.0	0.0001 tet.	......	......	......	5 days, tet.	
48	75	1.3	3.7	0.0001 tet.	......	......	......	6 days, tet.	
64	72	......	......	......	......	......	......	Remained well.	
88	65	4.3	4.4	0.0001 tet.	......	......	......	8 days, tet.	
89	65	2.2	1.8	0.0001 tet.	......	......	......	Remained well.	
90	65	4.3	7.2	0.0001 tet.	......	......	......	5 days, tet.	
91	66	4.5	9.0	0.0001 tet.	......	......	......	7 days, tet.	
92	66	2.6	5.5	0.0001 tet.	......	......	......	4 days, tet.	
93	66	4.2	6.2	0.0001 tet.	......	......	......	Remained well.	
94	66	1.5	2.0	0.0001 tet.	......	......	......	12 days, tet.	
95	65	4.5	7.0	0.0001 tet.	......	......	......	Remained well.	
96	65	2.5	7.2	0.0001 tet.	......	......	......	9 days, tet.	
97	62	3.0	2.0	0.0001 tet.	......	......	......	9 days, tet.	
99	61	3.9	7.6	0.0001 tet.	......	......	......	5 days, tet.	

Table No. 6.—*Relative efficiency of cultural tests under various conditions and animal tests in recovering tetanus from vaccine virus artificially contaminated by tetanus spores*—Continued.

Number of vaccine virus.	Number of days between collection of virus from calf and testing.	Glucose bouillon, unheated.			Ordinary bouillon, unheated.			Incubation of tetanus, in days, in inoculated guinea pigs.	Tetanus controls.
		Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 14 days.	Mice inoculated with growth at end of 14 days.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.		
100	61	4.0	6.3	0.0001 tet.	......	......	......	6 days, tet......	
101	60	4.0	6.3	0.0001 tet.	......	......	......	4 days, tet......	
102	62	1.5	1.2	0.0001 tet.	......	......	......	10 recovered......	
105	62	4.0	5.0	0.0001 tet.	......	......	......	4 days, tet......	
106	62	4.5	4.5	0.0001 tet.	......	......	......	3 days, tet......	
107	60	3.5	4.0	0.0001 tet.	......	......	......	9 days, tet......	
108	62	4.2	9.5	0.0001 tet.	......	......	......	3 days, tet......	
109	60	2.1	1.8	0.0001 tet.	......	......	......	7 days, tet......	
110	61	2.4	2.7	0.0001 tet.	......	......	......	3 days, tet......	
112	59	1.2	6.1	0.0001 tet.	......	......	......	4 days, tet......	
113	59	3.7	4.8	0.0001 tet.	......	......	......	8 days, tet......	
114	59	4.5	8.8	0.0001 tet.	......	......	......	5 days, tet......	
115	59	4.0	5.1	0.0001 tet.	......	......	......	4 days, tet......	
116	59	1.7	4.6	0.0001 tet.	......	......	......	Remained well....	
118	59	4.2	4.8	0.0001 tet.	......	......	......	Remained well....	
119	59	4.2	4.5	0.0001 tet.	......	......	......	Remained well....	
120	59	4.5	4.1	0.01 tet.	......	......	......	4 days, tet......	
121	59	4.5	6.5	0.001 tet.	......	......	......	5 days, tet......	
122	56	4.2	4.0	0.01 tet.	4.3	4.6	1 c. c. neg.	6 days, tet......	
123	56	4.0	7.5	0.01 tet.	4.0	4.5	1 c. c. neg.	5 days, tet......	
124	56	4.5	8.4	0.01 tet.	4.6	4.8	1 c. c. neg.	5 days, tet......	
126	56	4.0	5.4	0.001 tet.	4.3	4.6	1 c. c. neg.	6 recovered......	
127	55	4.4	8.1	0.001 tet.	4.0	4.1	1 c. c. neg.	6 days, tet......	
128	55	4.0	10.0	0.001 tet.	4.0	4.6	1 c. c. neg.	5 days, tet......	
129	55	3.2	5.5	0.001 tet.	4.0	4.2	1 c. c. neg.	10 recovered......	
02	11	6.0	10.2	1 c. c. neg.	1.8	3.0	0.001 tet.	......	
03	11	5.5	10.3	0.001 tet.	......	......	......	......	
04	10	5.6	9.2	1 c. c. neg.	......	......	......	......	
05	11	5.0	7.5	0.001 tet.	......	......	......	......	
06	10	5.5	12.5	0.001 tet.	......	......	......	......	
08	7	5.4	8.5	0.001 tet.	4.0	4.3	1 c. c. neg.		
011	6	4.6	7.6	0.001 tet.	......	......	......	......	
012	6	5.1	8.0	0.001 tet.	2.5	3.6	0.001 tet.	......	
014	6	4.5	8.4	0.001 tet.	1.4	3.2	0.001 tet.	......	
015	6	4.5	7.0	0.001 tet.	1.6	2.3	0.001 tet.	......	
018	7	5.0	7.5	0.001 tet.	......	......	......	......	
021	7	5.0	11.5	0.001 tet.	......	......	......	......	

Of the 67 samples planted into fermentation tubes of glucose bouillon, unheated, all developed tetanus toxin except two (Nos. 02 and 04), and the acidities of these two samples at the end of 48 hours were higher than the acidities of any of the other 65 samples. Sample No. 02, when planted into ordinary bouillon, developed a low acidity and a strong tetanus toxin.

Fifty-five samples of virus artificially contaminated with tetanus spores, when inoculated subcutaneously into guinea pigs on the abdomen, failed to produce any symptoms in seven of the animals. In three the incubation period was long, the symptoms mild, and the animals recovered; while the other 45 animals all developed fatal tetanus.

Table 7 shows the results of the injection of 11 samples of commercial vaccine virus artificially contaminated with tetanus

spores into 26 guinea pigs subcutaneously on the abdomen and into 26 mice subcutaneously on the back.

Table No. 7.—*Showing that the susceptibility of guinea pigs to tetanus infection is greater than that of white mice.*

Number of vaccine virus.	Inoculated Apr. 8, 1913, with 4 drops of vaccine virus artificially contaminated with tetanus spores.	Incubation of tetanus in days.	May 23.	May 24.	May 25.	Remarks.
2	Mouse	Remained well	Staphylococcus injected.	Neg.	Dead.	Scrapings from site of inoculation contained over 500 M. L. D.'s of tetanus toxin for a mouse.
	do	11 days				
	2 guinea pigs	4 and 4 days				
4	Mouse	Remained well	Staphylococcus injected.	Neg.	Tet.	Do.
	do	do	do	Neg.	Tet.	Do.
	2 guinea pigs	4 and 4 days				
6	2 mice	8 and 9 days				
	2 guinea pigs	4 and 4 days				
7	2 mice	9 and 10 days				
	2 guinea pigs	4 and 4 days				
9	2 mice	2 and 3 days				
	2 guinea pigs	2 and 5 days				
12	Mouse	Remained well	Staphylococcus injected.	Neg.	Dead.	Do.
	do	3 days				
	2 guinea pigs	3 and 3 days				
13	2 mice	7 and 11 days				
	2 guinea pigs	5 and 5 days				
14	Mouse	Remained well	Staphylococcus injected.	Neg.	Tet.	Do.
	do	12 days				
	2 guinea pigs	4 and 5 days				
15	Mouse	Remained well	Staphylococcus injected.	Neg.	Tet.	Do.
	do	6 days				
	2 guinea pigs	4 and 4 days				
16	2 mice	11 and 11 days				
	2 guinea pigs	3 and 3 days				
	Inoculated Mar. 18, 1913.					
912	Mouse	Remained well	Staphylococcus injected.	Neg.	Tet.	Do.
	do	do	do	Neg.	Tet.	Do.
	do	do	do	Neg.	Tet.	Do.
	3 mice	6, 6, and 11 days.				
	6 guinea pigs	4, 4, 5, 5, 5, and 5 days.				

All these viruses, except No. 912, were the same as those employed in Table No. 4. No. 912 contained no anaerobes and one drop smeared over three agar plates gave a growth of only five colonies. The others all contained *B. welchii*, but produced no growth on agar slants. All were artificially contaminated with tetanus spores and injected in 4-drop amounts subcutaneously on the back into 26 mice and subcutaneously on the abdomen into 26 guinea pigs.

All the guinea pigs promptly contracted fatal tetanus after an average incubation period of four days. Seventeen of the mice contracted fatal tetanus after an average incubation period of eight

days. Nine mice showed no symptoms of tetanus. However, each of the nine mice that remained well had a mate which was similarly injected with the same amount of the same material and each mate, with one exception, contracted typical tetanus from which it died.

After a lapse of 45 days in the case of six of these nine mice and 66 days in the other three it was desired to determine whether the mice were still harboring viable tetanus spores. They were therefore injected subcutaneously on the back with a 24-hour agar culture of staphylococcus and all nine promptly contracted fatal tetanus. Scrapings from the site of inoculation in each instance contained over 500 M. L. Ds. of tetanus toxin for a mouse.

The point of special interest in this table is that mice were apparently less susceptible to certain vaccine viruses contaminated with tetanus spores than were guinea pigs. The viruses all contained *Bacillus welchii* but showed no growth of aerobes when planted upon agar slants, except virus 912 which contained no anaerobes and one drop smeared over three agar plates gave a growth of five colonies.

Combining the results of animal tests tabulated in Tables Nos. 5, 6, and 7, we find that samples of 93 glycerinated vaccine viruses artificially contaminated with tetanus spores were inoculated in 1 c. c. amounts subcutaneously on the abdomen into an equal number of guinea pigs each weighing 250 grams. The local reactions in the guinea pigs were marked. By the end of four to six days the site of inoculation in each was broken down into pus which was surrounded by intensely inflamed and injected tissue. Seventy-seven animals showed marked characteristic symptoms of tetanus and died, 12 manifested no symptoms of tetanus, 4 developed tetanus but recovered. No satisfactory explanation is offered for the nondevelopment of tetanus in the 12 guinea pigs. Their lesions were as severe as were those of the animals which succumbed. Ulceration and scar formation took place at the site of inoculation. (See Tables 5, 6, and 7.)

One cubic centimeter amounts of the same 93 viruses, similarly contaminated, were, at the same time, planted into fermentation tubes of glucose bouillon. Seventy-seven tubes showed early reactions no higher than +4.5 and all of these developed tetanus toxin. Sixteen tubes showed reactions at the end of 24 hours between +5.8 and +6.8 and none of these developed any tetanus toxin. (See Tables 4, 5, and 6.)

In contrasting the efficiency of the guinea-pig test with that of glucose bouillon, we find that 93 samples of glycerinated vaccine virus artificially contaminated with tetanus spores and injected subcutaneously into 93 guinea pigs failed to produce tetanus in 12 animals, whereas the 93 tubes of glucose bouillon failed in 16 instances to produce tetanus toxin. (See Tables 4, 5, 6, and 7.)

SUMMARY OF ATTEMPTS BY FOUR METHODS TO RECOVER TETANUS FROM ARTIFICIALLY CONTAMINATED GLYCERINATED VACCINE VIRUS.

Glycerinated virus collected from 161 calves was artificially contaminated with tetanus spores and was either (*a*) planted into fermentation tubes of glucose bouillon and incubated without previous heating, or (*b*) planted into fermentation tubes of glucose bouillon and heated to 80° for an hour before incubation, or (*c*) planted into fermentation tubes of ordinary bouillon, or (*d*) inoculated subcutaneously into guinea pigs.

(*a*) One hundred and forty-four samples were planted into glucose bouillon and incubated without previous heating. In 94 of these the reactions of the media in the open arm of the tubes at the end of 24 hours of growth were between +1.2 and +4.5, and tetanus toxin developed in all. In 10 samples the early reactions were between +4.5 and +5.5, and all of these developed strong tetanus toxin. In 12 samples the early reactions were between +5.6 and +7.2, and all developed tetanus toxin of widely varying strength. In 28 samples the early reactions were between +5.5 and +7, and in none of these was any tetanus toxin formed.

(*b*) Thirty-four samples were planted into glucose bouillon and heated to 80° C. for one hour before incubation. In 23 of these the early reactions were low, and each developed strong tetanus toxin. In the other 11 samples the early reactions were between +5.8 and +6.8, due to the presence of Bacillus Welchii, and no tetanus toxin was found.

(*c*) Sixty-five samples were planted into ordinary bouillon. In 49 of the number the early reactions ranged between +1 and +4, and all of these developed strong tetanus toxin. In 16 samples the early reactions were between +4 and +5; 5 of these developed tetanus toxin, but the other 11 developed none.

(*d*) Ninety-three samples inoculated subcutaneously into 93 guinea pigs produced tetanus in all except 12.

The summary brings out the following facts: Four kinds of tests were used in endeavoring to recover tetanus from 161 artificially contaminated glycerinated vaccine viruses. No one method of testing was found to be uniformly successful. In no instance was it necessary, however, to apply all four kinds of tests to the same virus; in only one virus was it necessary to apply three kinds of tests before detecting the tetanus; two kinds of tests were sufficient to detect tetanus in any of the viruses except one. A single cultural test failed to recover tetanus in 50 viruses and a single guinea pig test failed in 12 out of 93 viruses and a single mouse test failed in 6 out of 11 viruses so tested. There was only one instance in which one cultural test and one guinea pig test applied to the same virus

both failed. In all instances where a single cultural test failed, another kind of cultural test made on that same virus or a single guinea pig test made upon it did detect tetanus. In all instances except one, where a single guinea pig test failed, a single cultural test made upon the same virus was successful.

TABLE No. 8.—*Summary of Tables 1, 2, 18, 3, 4, 5, 6, and 7, showing successes and failures in recovering tetanus from glycerinated commercial vaccine virus and other vaccine materials, all samples having been artificially contaminated with tetanus spores and either planted into fermentation tubes of nutrient bouillon or injected subcutaneously into guinea pigs.*

GLYCERINATED COMMERCIAL VACCINE VIRUSES.

Column 1.	Column 2. Glucose bouillon, unheated.	Column 3. Glucose bouillon, heated 80°, 1 hour.	Column 4. Ordinary bouillon, unheated.	Column 5. Guinea pigs, injected subcutaneously.
Table 1 (23 viruses)	4 neg.	4 pos., 2.0 to 3.0		
	7 neg., 5.5 to 6.7		7 pos., 1.5 to 3.0, except 4.7 and 5.0.	
	6 neg., 5.5 to 6.2			
	6 pos., 1.5 to 4.5			
Table 4 (30 viruses)	2 neg., 5.5, 5.5	2 pos.	2 pos., 3.0 and 3.1	
	2 neg., 5.5, 5.5		2 pos., 1.7 and 2.0	
	10 pos., 1.5 to 4.5	10 pos.	10 pos., 1.0 to 2.5	
	3 pos., 1.7, 4.5, 4.5		3 pos., 1.0, 1.6, 3.0	
	1 pos., 2.2			
		1 pos., 2.0		
		11 neg., 5.8 to 6.8 (B. Welchii).	11 pos., 1.2 to 3.9 (80°, 1 hour).	11 pos. (Table 7).
Table 5 (41 viruses)	4 neg., 6.1 to 7.0	4 pos., 3.0 to 3.1		4 pos.
	1 neg., 5.8	1 pos., 3.3		1 neg.
	1 pos., 3.5	1 pos., 3.2		
	10 pos., 4.0 to 4.5, except 5.0 and 5.2.		10 pos., 1.5 to 4.0, except 4.4, 4.6, 5.0.	
	2 pos., 4.2 and 5.0		2 neg., 4.1 and 5.0	
	1 pos., 3.8		1 neg., 4.5	1 pos.
	5 pos., 2.0 to 4.5		5 pos., 1.6 to 2.4	5 pos.
	10 pos., 2.0 to 4.5			10 pos.
	3 pos., 2.7, 2.9, 3.1			3 neg.
				1 neg.
				2 pos.
Table 6 (67 viruses)	1 neg., 5.6			
	6 pos., 5.0 to 5.5			
	1 neg., 6.0		1 pos., 1.8	
	1 pos., 5.4		1 neg., 4.0	
	3 pos., 4.5, 4.5, 5.1		3 pos., 1.4, 1.6, 2.5	
	7 pos., 3.2 to 4.5		7 neg., 4.0 to 4.6	7 pos.
	6 pos., 1.5 to 4.5			6 neg.
	41 pos., 1.2 to 4.5			41 pos.
				1 neg.
Total	116 pos., 28 neg.	23 pos., 11 neg.	54 pos., 11 neg.	81 pos., 12 neg.
VACCINE MATERIALS NOT INTENDED AS SUCH FOR COMMERCIAL USE.				
Table 1 (29 pulps)	2 neg., 6.0 and 6.0			
	8 neg., 5.0 to 7.0	8 pos., 2.2 to 3.7, except 4.2.	8 pos., 2.0 to 3.6, except 4.3.	
	10 neg., 5.5 to 8.0		10 pos., 2.0 to 2.9	
		3 pos., 1.8, 2.6, 4.0	3 pos., 2.0, 2.5, 4.0	
		1 pos., 2.2		
			5 pos., 1.7 to 2.5	
Table 2, experimental calf virus.	7 neg., 5.1 to 6.0	7 pos., 1.2 to 4.0		
	3 neg., 5.0, 5.5, 6.0	3 neg., 4.0, 4.0, 4.5		
	5 neg., 6.0 to 7.0		5 pos., 3.1 to 4.0, except 4.5.	
	1 neg., 6.0			
Table 18, experimental calf virus.	8 neg., 4.9 to 6.6	8 pos., 1.3 to 2.6		
	1 pos.	1 pos., 2.0		
	6 neg., 4.7 to 9.0		6 pos., 2.8 to 3.5, except 4.4.	
	3 pos., 1.2, 1.6, 3.0		3 pos., 1.0, 2.4, 3.0	

TABLE NO. 8.—*Summary of Tables 1, 2, 18, 3, 4, 5, 6, and 7, etc.*—Continued.

VACCINE MATERIALS NOT INTENDED AS SUCH FOR COMMERCIAL USE—Continued.

Column 1.	Column 2. Glucose bouillon, unheated.	Column 3. Glucose bouillon, heated 80°, 1 hour.	Column 4. Ordinary bouillon, unheated.	Column 5. Guinea pigs, injected subcutaneously.
Table 3, 8 viruses + staphylococcus).	5 neg., 6.3 to 6.6... 1 neg., 6.7........ 2 neg., 4.5 and 4.5.	5 pos., 2.0 to 4.0... 1 neg., 4.2........	....	
Total............	4 pos., 58 neg.....	33 pos., 4 neg.....	40 pos.............	
Grand totals..... Percentages of failures..	120 pos., 86 neg.... 41.7 per cent.......	56 pos., 15 neg..... 21.1 per cent.......	94 pos., 11 neg..... 10.5 per cent.......	81 pos., 12 neg. 12.9 per cent.

EXPLANATION OF TABLE NO. 8.

Positive means success in recovering tetanus.

Negative means failure to recover tetanus.

The figures indicate percentage of acidity of the media determined by titration at the end of 24 hours of growth.

Viruses reacting alike or subjected to the same tests are placed in groups.

Data in transverse lines relate to the same virus.

Data in vertical columns relate to different viruses.

Column 1 gives the materials that were used; 23 viruses from 23 calves; 30 viruses from 30 calves; 41 viruses from 41 calves; 67 viruses from 67 calves; 29 pulps from 29 calves; experimental virus from 1 calf; 8 viruses to each of which a staphylococcus was added. All material used was artificially contaminated with tetanus spores.

Column 2 indicates whether or not tetanus was recovered from the various artificially contaminated viruses, pulps, etc., when planted into fermentation tubes of glucose bouillon and incubated without previous exposure to 80° and what the titrations were at the end of 24 hours of growth. No virus or pulp is treated twice in the same column excepting the two experimental calf viruses, on each of which numerous tests are reported in each column.

Column 3 shows the results obtained when the samples were planted into fermentation tubes of glucose bouillon and heated at 80° for an hour before incubation.

Column 4 gives results after planting into fermentation tubes of ordinary media and incubating without previous exposure to 80°.

Column 5 shows whether or not a guinea pig contracted tetanus after being injected subcutaneously on the abdomen with the same material and in the same amount as was planted into the fermentation tubes.

The first half of the table deals with glycerinated commercial vaccine viruses which were being supplied to the trade or which were later placed on the market after longer glycerination.

The second half relates to the unground pulps and experimental viruses, none of which were intended as such for the market.

Table 8 brings together the results of 475 attempts to recover in the laboratory tetanus spores which had been intentionally put into the vaccine materials for the purpose of studying conditions which surround them there and to determine upon methods of proving their presence therein. Out of the entire 475 attempts to recover tetanus there were 124 failures, or 26.1 per cent of failures.

There were 41.7 per cent of failures in endeavoring to recover tetanus from 206 artificially contaminated vaccine materials by planting them into fermentation tubes of glucose bouillon and incubating without previous exposure to 80°. There were 21.1 per cent of failures in attempting to recover tetanus from 71 artificially contaminated samples planted into fermentation tubes of glucose bouillon and heated to 80° for an hour before incubation. There were

10.5 per cent of failures in 105 attempts in fermentation tubes of ordinary bouillon incubated without previous heating.

Ninety-three guinea pigs injected subcutaneously on the abdomen with the same materials and in the same amounts as were planted into fermentation tubes gave 12.9 per cent of failures to show symptoms of tetanus.

The cause of the failures in culture media can be deduced from the data furnished by the table. That data is arranged to show the relation between the reaction of the media at the end of 24 hours of growth and the germination of tetanus spores contained in that media.

The failures to recover tetanus from known infected material planted into nutrient media were found to be due to the development within the first 24 hours of a high acidity of the media which prevented the germination of the tetanus spores. Out of a total of 112 tubes which proved negative for tetanus 102 titrated +4.5 or more at the end of 24 hours of growth. The other 10 titrated between +4 and +4.5.

Out of a total of 270 tests which proved positive for tetanus 255 titrated between +1 and +4.5 at the end of 24 hours of growth. Ten did not go over +5 and the other five did not titrate higher than +5.5 at the end of 24 hours. Retests in media calculated to restrict acid production were made in a total of 96 of the 112 samples in which early acidities of +4.5 or more had prevented toxin production; 86 now titrated at the end of 24 hours no higher than +4 and each one of these produced tetanus toxin; 10 titrated between +4 and +4.5, and of these 5 produced toxin and 5 did not.

Based on the data furnished, the generalization might be made that bouillon inoculated with vaccine virus contaminated with tetanus spores and showing an acidity of less than +4.5 at the end of 24 hours of incubation will contain tetanus toxin; an acidity higher than +4.5 will prevent toxin production; exceptions will be found where the early titrations are between +4 and +5.

47026°—14——3

II. THE BEHAVIOR OF TETANUS SPORES INJECTED SUBCUTANEOUSLY INTO GUINEA PIGS AND WHITE MICE.

Since the subcutaneous injection of guinea pigs and white mice with vaccine virus constitutes one of the methods of testing the virus for contamination, a thorough knowledge of the resistance and susceptibility of these animals to tetanus is therefore highly desirable.

THE EFFECT IN GUINEA PIGS OF THE INJECTION OF QUININE OR STAPHYLOCOCCI AT THE SITE OF INJECTION OF TETANUS SPORES.

Tetanus spores in pure culture and free from tetanus toxin, when injected subcutaneously into a guinea pig, do not give rise to tetanus. The animal remains well. (See Table No. 9, guinea pigs 5, 6, 11, and 12.) The spores lie dormant as an inert body at the point of injection awaiting either destruction by the phagocytes or an awakening into activity and toxin production by the irritation of some foreign body, particularly quinine or staphylococci. If quinine or staphylococci be combined with the spores before injection and both are injected at the same time into the same site, tetanus and death of the animal follow promptly and regularly within 3 to 6 days. (See Table No. 9, guinea pigs 1, 2, 3, 4, 7, 8, 9, and 10.)

TABLE NO. 9.—*Activation in guinea pigs of tetanus spores by quinine or staphylococcus.*

No. of guinea pig.	Spores injected.	Quinine injected.	Remarks.
1	On abdomen May 22, 1912	May 22, 1 gr. on abdomen	May 25, tetanus, died.
2	do	do	May 27, tetanus. May 28, dead.
3	do	do	May 25, tetanus, died.
4	do	do	May 25, tetanus. May 26, died.
5 control	do		June 30, still well.
6 control	do		Do.
	Spores injected.	*Staphylococcus injected.*	
7	May 25, 1912, on abdomen	May 25, 1912, on abdomen	May 27, tetanus, died.
8	do	do	Do.
9	do	do	May 27, tetanus, May 28, died.
10	do	do	May 27, tetanus. May 28, dead.
11 control	do		June 22, still well.
12 control	do		July 30, still well.

If a length of time be allowed to elapse after the injection of the spores, before injecting quinine or staphylococci into the site of the spore injection, tetanus will or will not follow in a guinea pig depending upon whether the interval has been sufficient to allow the devour-

ing process of the phagocytes to effect only a partial or a complete destruction of the spores. An effort was made to determine how long tetanus spores will be harbored by a guinea pig in its subcutaneous tissues before they become destroyed by the phagocytes. Quinine or staphylococci injected at the site of the spore injection was depended upon to activate any spores which had escaped phagocytosis.

Forty-five tests in guinea pigs were made in which various spaces of time elapsed between the subcutaneous injection of tetanus spores and the injection of quinine or staphylococci into the site of the spore injection. (See Table No. 10, guinea pigs Nos. 1 to 25; see Table No. 13, guinea pigs Nos. 1 to 15, except 7; guinea pigs 23, 24, 25, 32, 33, and 34; see summary, Table No. 12.) The intervals between the two injections varied from 9 to 120 days and the result was indicated either as a success in producing tetanus or as a failure to do so.

TABLE NO. 10.—*Effect of lapse of time between injection of tetanus spores and injection of activator, both at same site, in guinea pigs.*

No. of guinea pig.	Spores injected.	Quinine injected.	Staphylococcus injected.	Remarks.
1	Nov. 29, 1912, on abdomen.	Feb. 17, 18, 20; 1 grain each.	....	Ulcer 1 inch in diameter; Apr. 9, still well.
2	.....do................	Feb. 25; 2 grains........	....	Do.
3	.....do................	....	Mar. 21	Abscess followed by scar; Apr. 9, still well.
4	Dec. 30, 1912, on hind leg.	....	...do....	Apr. 11, still well.
5	.....do................	....	...do....	Do.
6	.....do................	Feb. 17, 18, 20; 1 grain each.	....	Mar. 7, ulcer 1 inch in diameter; Mar. 21, died; no tetanus.
7	.....do................	.....do..................	....	Mar. 7, ulcer 1 inch in diameter; Apr. 11, still well.
8	.....do................	Feb. 25; 2 grains........	....	Do.
9	Dec. 31, 1912, on abdomen.	Mar. 28; 1½ grains.......	....	Marked local reaction. No ulcer; Apr. 5, dead; scrapings from site contained 10 MLDs for mouse.
10	Jan. 1, 1913, on abdomen.	....	Mar. 21	Apr. 11, abscess.
11	.....do................	....	Mar. 27	Do.
12	Jan. 2, 1913, on abdomen.	....	...do....	Apr. 7, open abscess; Apr. 11, still well.
13	Jan. 11, 1913, on abdomen.	Feb. 25; 2 grains........	....	Mar. 7, ulcer 1 inch diameter; June 4, still well.
14	Jan. 13, 1913, on abdomen.	Feb. 28; 1½ grains.......	....	Apr. 5, ulcer 1 inch diameter; Apr. 9, still well.
15	Jan. 25, 1913, on abdomen.	....	Mar. 27	Apr. 7, abscess ruptured; June 4, still well.
16	.....do................	....	Mar. 21	Mar. 26, marked redness; Apr. 7, abscess ruptured; June 4, still well.
17	Jan. 31, 1913, on abdomen.	....	...do....	Apr. 7, abscess; June 4, still well.
18	Mar. 22, 1913, on abdomen.	Mar. 31, Apr. 11; 1 grain each.	....	Apr. 7, ulcer 1 inch diameter; Apr. 20, still well.
19	.....do................	Mar. 31, Apr. 1; 1 grain each.	....	Apr. 7, induration; Apr. 8, tetanus; died; no ulcer.
20	.....do................	Apr. 7; 1 grain..........	....	Apr. 11, induration; Apr. 13, tetanus; Apr. 15, dead; no ulcer.
21	..... o................	.....do..................	....	Apr. 13, ulcer 1 inch diameter; Apr. 14, tetanus; Apr. 15, death.
22	.....do................	....	Apr. 7	Apr. 11, general induration; tetanus; Apr. 13, dead; no ulcer.
23	.....do................	....	...do....	Apr. 12, abscess ruptured; Apr. 20, still well.
24	May 22, 1913, on abdomen.	June 5; 1 grain..........	....	June 14, tetanus; died.
25	.....do................	.....do..................	....	July 31, still well.

An interval of 9 days was followed by 1 success and 1 failure.

An interval of 14 days was followed by 1 success and 1 failure.

An interval of 16 days was followed by 3 successes and 1 failure.

An interval of 30 days was followed by 6 successes and 10 failures.

An interval between 30 and 60 days was followed by 7 failures.

An interval between 60 and 90 days was followed by 2 successes and 11 failures.

An interval between 90 and 120 days was followed by 1 failure.

The shorter the interval, the more constant is the activating power of quinine or staphylococcus. The longer the interval, the more complete is the destruction of spores by the phagocytes.

When the injections were simultaneous, 8 guinea pigs all contracted tetanus; when the interval was between 9 and 30 days, 11 contracted tetanus and 13 did not; when the interval was between 30 and 90 days, 2 contracted tetanus and 18 did not; when the interval was 120 days, 1 did not contract tetanus—only 1, however, was tested.

While a simultaneous injection of tetanus spores and quinine or staphylococci is certain to produce tetanus in a guinea pig, an interval of 9 to 30 days between the injections gives the phagocytes and spores about an equal chance, and half of the animals will contract tetanus and half will not; any lengthening of the interval beyond 30 days very rapidly shifts the advantage to the phagocytes and no tetanus follows.

The data indicates that after an interval of 30 days, tetanus spores are practically all destroyed in the subcutaneous tissue of a guinea pig, and the chances of any spores remaining dormant and susceptible of activation longer than 30 days are very remote.

THE EFFECT IN WHITE MICE OF THE INJECTION OF QUININE OR STAPHYLOCOCCI AT THE SITE OF INJECTION OF TETANUS SPORES.

White mice injected subcutaneously on the back with a pure culture of tetanus spores free from tetanus toxin do not contract tetanus in the absence of a suitable activator of the spores. (See Table No. 11, Mice 1 to 20.)

TABLE No. 11.—*Relative efficiency of quinine and staphylococci as activators of tetanus spores in white mice.*

Mouse No.	Spores injected.	Mar. 15.	Mar. 16.	Mar. 17.	Mar. 18.	Mar. 19 and 24.	April.	May 23.	May 24.	May 25.	May 26.	Remarks.
1	Feb. 4, 1913	Staphylococcus injected.	Neg.	Tet.	Dead.							Scrapings from site of inoculation contained over 1,000 MLDs of tetanus toxin for a mouse.
2	do	Quinine injected	Neg.	Neg.	Neg.	Quinine injected.	Still well.					
3	Feb. 16, 1913	Staphylococcus injected.	Neg.	Tet.	Dead.							Do.
4	do	Quinine injected	Neg.	Neg.	Neg.	Quinine injected.						Died within 2 hours after last injection of quinine.
5	Feb. 17, 1913	Staphylococcus injected.	Neg.	Tet.	Dead.							Scrapings from site of inoculation contained over 1,000 MLDs of tetanus toxin for a mouse.
6	do	Quinine injected	Neg.	Neg.	Neg.	Quinine injected.	Still well.	Staphylococcus injected.	Neg.	Tet.	Died.	Scrapings from site of inoculation contained over 500 MLDs of tetanus toxin for a mouse.
7	Feb. 8, 1913	do	Neg.	Neg.	Neg.	do	do	do	Neg.	Tet.	Dead.	Do.
8	Nov. 26, 1912	Staphylococcus injected.	Neg.	Neg.	Neg.		do	do				Remained well.
9	do	Quinine injected	Neg.	Neg.	Neg.	Quinine injected.	do					
10	Nov. 24, 1912	Staphylococcus injected.	Neg.	Neg.	Neg.		do					
								July 18.	July 19.	July 20.	July 21.	
11	Mar. 17, 1913	Apr. 11, quinine injected.	Remained well					Staphylococcus injected.	Neg.	Tet.	Dead.	Scrapings from site of inoculation contained over 10,000 MLDs of tetanus toxin for a mouse.
12	do	do	do					do	Neg.	Tet.	Dead.	Do.
13	Mar. 25, 1913	do	do					do	Neg.	Tet.	Dead.	
14	Mar. 18, 1913	do	Died within 1 hour after injection of quinine.									
15	Apr. 26, 1913	June 20, staphylococcus injected.	June 25, tetanus; June 26, death									Scrapings from site of inoculation contained over 500 MLDs of tetanus toxin for a guinea pig.
16	do	do	June 27, tetanus; June 28, tetanus; June 29, death.									Scrapings from site of inoculation contained over 100 MLDs of tetanus toxin for a guinea pig.
17	May 28, 1913	May 28, 29, 30, quinine injected.	July 18, still well					Staphylococcus injected.	Tet.	Death.		
18	do	May 29, 30, 31, quinine injected.	do									
19	do	May 30, 31, June 1, quinine injected.	do									
20	do	May 31, June 1, 2, quinine injected.	do									

All mice in Table No. 11 were injected subcutaneously on the back with tetanus spores. Some of the mice were injected with quinine at the site of the spore injection at the time of the spore injection or later, and others were injected with staphylococci at the site of spore injection at intervals as long as four months after the spore injection. There should also be included in this table nine mice from Table No. 7, inoculated with viruses Nos. 2, 4, 12, 14, 15, and 912, in which staphylococcus activated tetanus spores in all instances after lying dormant in the mice for 45 and 66 days.

Quinine, as shown in Table No. 11, is incapable of activating tetanus spores injected in the manner indicated above even if the quinine be combined with the spores at the time of injection. Mice 17, 18, 19, and 20 of Table No. 11 were each given a single injection of tetanus spores subcutaneously on the back. Each mouse also received on each of the three successive days an injection of one-tenth grain of quinine into the site of his spore injection. The interval in the four mice between his spore injection and his first quinine injection was 0, 1 day, 2 days, and 3 days, respectively. Mice 13, 11, and 12 each received one-tenth grain of quinine into the site of his spore injection after a lapse of 17, 25, and 25 days, respectively. Mice 2, 4, 6, and 7 each received three injections of quinine of one-tenth grain each into the site of his spore injection after intervals between 26 and 48 days.

In all instances just cited, quinine failed to activate the spores. The amount of quinine injected could not have been increased on account of the danger of its killing the animals. One-tenth grain causes staggering in a mouse weighing fifteen grams and not infrequently kills, as noted in mice 4 and 14.

In marked contrast to quinine's total absence of activating power in 12 mice, we find that a single injection of staphylococci into the site of a previous injection of tetanus spores caused tetanus in 20 out of 22 mice. (See tables 7, 11, and 12.) A considerable interval of time elapsed betweeen the injection of spores and the injection of staphylococci. The length of interval and the success or failure in causing tetanus were as follows:

An interval of 30 days was followed by 2 successes.

An interval between 40 and 66 days was followed by 13 successes.

An interval between 90 and 123 days was followed by 5 successes and 2 failures.

The data shows that white mice will harbor tetanus spores in their subcutaneous tissue for at least four months, at the end of which time an injection of staphylococci into the site of the spore injection will quite constantly activate the spores to a production of tetanus toxin.

We have shown that quinine does not activate tetanus spores in a mouse, and that staphylococci do. We will now show that tetanus spores which failed of activation by quinine were later activated by staphylococci.

Mice 11, 12, and 13 in Table No. 11 failed to contract tetanus when quinine was injected 25, 25, and 17 days, respectively, after spore injection; and yet a single injection of staphylococci given 123, 123, and 115 days, respectively, after spore injection did cause fatal tetanus in those same mice within 3 days.

Mice 6 and 7 in Table No. 11 failed to contract tetanus when three injections of quinine were given between 26 and 44 days after spore injection; yet a single injection of staphylococci given 79 and 88 days, respectively, after spore injection caused fatal tetanus in both within 3 days.

Mouse 17 of table 11 failed to contract tetanus when given an injection of quinine at the site of the spore injection at the time of the spore injection and on each of the 2 succeeding days; yet a single injection of staphylococci given into the site of the spore injection 51 days after the spore injection caused fatal tetanus within 2 days.

That the six mice just cited died of tetanus was shown not only by the typical symptoms which they presented but also by the results of inoculations made into other mice of the scrapings collected from their sites of injection. Subinoculations showed that as high as 10,000 minimal lethal doses of tetanus toxin for a mouse had been produced at the site of injection of staphylococci by tetanus spores, which had failed of activation by repeated injections of quinine in almost fatal amounts.

The above experiments show that mice injected on the same day and with the same amounts of tetanus spores usually contracted tetanus when injected later with staphylococci, but failed in all instances to contract tetanus when injected with quinine. An injection of staphylococcus following the unsuccessful use of quinine did, moreover, uniformly produce tetanus.

Table 12 gives a summary of the results tabulated in tables Nos. 7, 9, 10, and 11, howing t he comparative efficiency of quinine and staphylococci in activating tetanus spores lying dormant in the subcutaneous tissue of guinea pigs and mice. Spores and activator were both injected at the same site.

TABLE NO. 12.—*Summary of Tables Nos. 7, 9, 10, and 11, showing comparative efficiency of quinine and staphylococci in activating tetanus spores lying dormant in the subcutaneous tissue of guinea pigs and mice, spores and activator being injected at the same site.*

Interval between injection of tetanus spores and injection of quinine or staphylococci into the site of the spore injection.	Success or failure in producing tetanus in guinea pigs and mice.			
	Quinine.		Staphylococcus.	
	Success.	Failure.	Success.	Failure.
(*a*) Guinea pigs injected (on abdomen):				
Simultaneous injection	4		4	
9 days' interval	1	1		
14 days' interval	1	1		
16 days' interval	2		1	1
30 days' interval	5	9	1	1
Between 30 and 60 days' interval		5		2
Between 60 and 90 days' interval	1	2	1	9
Between 90 and 120 days' interval				1
(*b*) Mice injected (on back):				
Simultaneous injection		1		
1 day's interval		1		
2 days' interval		1		
3 days' interval		1		
17 days' interval		1		
25 days' interval		2		
30 days' interval		2	2	
Between 40 and 66 days' interval		2	13	
Between 90 and 120 days' interval		1	5	2
Total	14	30	27	16

Under heading (*a*), guinea pigs were found to harbor tetanus spores quite constantly for about two weeks. At the end of 30 days the failures in activating the spores were about twice as numerous as the successes. After 30 days only two successes followed 21 attempts at activation.

Under the heading (*b*), mice were found to harbor tetanus spores quite uniformly for four months. Quinine failed absolutely in all attempts to activate tetanus spores in mice, although the injections of spores and quinine were made into the same site simultaneously or separated by intervals as long as four months. Staphylococci activated tetanus spores in mice in all instances except two; two failures occurred when the injections of staphylococci were made four months after the injections of the spores.

The grand totals give quinine 14 successes as against 30 failures and give staphylococcus 27 successes and 16 failures in attempts to activate tetanus spores in the subcutaneous tissues of guinea pigs and mice when spores and activator were injected at the same site.

THE EFFECT IN GUINEA PIGS OF THE INJECTION OF QUININE OR STAPHYLOCOCCI ELSEWHERE THAN AT THE SITE OF INJECTION OF TETANUS SPORES.

Any activation or nonactivation of tetanus spores by quinine or staphylococci which has been cited thus far in guinea pigs or mice has followed the injection of spores and activator at the same time or at different times into the same site of the body; in mice both injections were made into the subcutaneaus tissue of the back; in guinea pigs both injections were made into the subcutaneous tissue of the abdomen or the hind leg. Data will now be submitted upon results obtained in guinea pigs after simultaneous injection of tetanus spores either on the hind leg or on the abdomen and quinine or staphylococcus either on the chest or between the shoulders. All references will be to Table No. 13.

TABLE NO. 13.—*Effect of simultaneous injection of tetanus spores and activator at different sites in guinea pigs.*

1	Spores injected on hind leg, May 25, 1912.	Quinine injected on chest May 25, 1 grain.	Staphylococci injected on hind leg June 25, 26, 27, 28, 29.	July 2, tetanus.	July 3, died ...	The entire lesion on chest or shoulders was excised and planted into six fermentation tubes of ordinary bouillon. The M. L. D. for mice of tetanus toxin appearing in each tube is indicated below.						Mice inoculated with growth from culture taken from site of injection of spores on leg.
2	.....do.........	.....do.........	Staphylococci injected on leg July 30, 31.	Aug. 3, tetanus.	Aug. 4, died...							
3	.....do.........	.....do.........	.....do.........	Aug. 26, still well.	....							
4	.....do.........	.....do.........	.....do.........	.....do......	....							
5	.....do.........	....	.....do.........	.....do......	....							
6	.....do.........	....	Staphylococci injected on leg, June 24, 25, 26, 27, 28, 29.	.....do......	....							
						Cultures taken from chest lesion.						
7	Spores injected on hind leg, Apr. 29, 1913.	Quinine injected 1 grain on chest each day, Apr. 28, 29, 30.	....	May 2, tetanus.	May 9, killed ..	0.0001 tet.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	0.0001 tet.
8	.....do.........	.....do.........	Quinine injected 1 grain on leg each day, May 29, 30, 31.	June 6, tetanus.	Died.........	....	....	....	....	....	....	
9	.....do.........	.....do.........	.....do.........	June 5, tetanus.	.....do......	....	....	....	....	....	....	
10	.....do.........	.....do.........	.....do.........	Remained well.	July 20, O. K..	....	....	....	....	....	....	
11	.....do.........	.....do.........	.....do.........	.....do......	.....do......	....	....	....	....	....	....	Mice inoculated with growth from cultures taken from site of injection of spores on abdomen.
12	.....do.........	.....do.........	.....do.........	.....do......	.....do......	....	....	....	....	....	....	
13	.....do.........	....	.....do.........	.....do......	.....do......	....	....	....	....	....	....	
14	.....do.........	....	.....do.........	.....do......	.....do......	....	....	....	....	....	....	
15	.....do.........	....	.....do.........	June 5, tetanus.	June 7, dead..	....	....	....	....	....	....	
						Cultures taken from shoulder lesion.						
16	Spores injected on abdomen, May 6, 1913.	Quinine injected between shoulders May 5, 1 grain; May 6, 1 grain; May 7, 1 grain; May 8, 1 grain.	....	May 11, tetanus.	May 12, killed.	Growth	Growth	0.01 tet.	0.01 tet.	0.0001 tet.	0.0001 tet.	Growth.
17	.....do.........	.....do.........	....	.....do......	.....do......	Growth	Growth	.01 tet.	.01 tet.	.01 tet.	.0001 tet.	Growth.
18	.....do.........	.....do.........	....	May 12, tetanus.	May 14, dead..	Growth	Growth	.01 tet.	.01 tet.	.01 tte.	.0001 tet.	0.001 tet.
19	.....do.........	.....do.........	....	May 13, tetanus.	May 16, died ..	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	.001 tet.	.001 tet.	.001 tet.	.0001 tet.

TABLE NO. 13.—*Effect of simultaneous injection of tetanus spores and activator at different sites in guinea pigs*—Continued.

20	Spores injected on abdomen, May 6, 1913.	Quinine injected between shoulders May 5, 1 grain; May 6, 1 grain; May 7, 1 grain; May 8, 1 grain.		May 17, tetanus.	May 17, died	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	.001 tet.
21	do	do		Remained well.	May 23, killed.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	.0001 tet.
22	do	do		do	do	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	1 c. c. neg.	.0001 tet.
23	do		Quinine injected June 6, 1 grain on abdomen; June 7, 1 grain on abdomen; June 8, 1 grain on abdomen.	do	July 20, O. K.							
24	do		do	do	do							
25	do		do	June 14, tetanus.	June 15, dead							
						Cultures taken from shoulder lesion.						
26	Spores injected, on abdomen, May 17, 1913.	Staphylococci injected May 16, between shoulders; May 17, between shoulders; May 18, between shoulders.		May 25, rigidity.	May 26, killed.	0.01 tet.	0.001 tet.	1 c. c. tet.	0.0001 tet.	0.01 c. c. tet.	0.01 c. c. tet	.01 c. c. tet.
27	do	do		May 25, curvature.	do							.01 c. c. tet.
28	do	do		May 25, rigidity.	May 27, killed.	.1 c. c. tet.	.1 c. c. tet.	.1 c. c. tet.	.01 c. c. tet.	.01 c. c. tet.	.01 c. c. tet.	.01 c. c. tet.
29	do	do		do	do	0.001 tet.	.001 tet.	.1 c. c. tet.	.01 c. c. tet.	.01 c. c. tet.	.001 tet.	.01 c. c. tet.
30	do	do		May 26, sick.	do	.1 c. c. tet.	.001 tet.	.01 c. c. tet.	.1 c. c. tet.	.01 c. c. tet.	.1 c. c. tet.	.01 c. c. tet.
31	do	do		No tetanus.	do	.1 c. c. tet.	.04 c. c. tet.	.1 c. c. tet.	.04 c. c. tet.	.3 c. c. tet.	.001 tet.	.01 c. c. tet.
32	do		Quinine injected June 17, 1 grain on abdomen; June 18, 1 grain on abdomen; June 19, 1 grain on abdomen.	Remained well.	July 20, O. K.							
33	do		do	June 27, tetanus.	Died							
34	do		do	June 25, dead; no tetanus.								

Guinea pigs 1, 2, 3, and 4 were injected subcutaneously with tetanus spores on the hind leg and at the same time with 1 grain of quinine subcutaneously on the chest. None of the animals showed symptoms of tetanus by the end of a month. Evidently a single injection of 1 grain of quinine on the chest did not activate tetanus spores injected at the same time on the hind leg. That the spores in the leg were viable and susceptible of activation was shown by the tetanus and death which occurred in guinea pig 1 following the injections of staphylococci directly into the site of the spore injection on June 25, 26, 27, and 28. The viability of spores in guinea pig 2 was shown by the tetanus and death which took place following the injections on July 30 and 31 of staphylococci directly into the site of the spore injection.

Guinea pigs 7 to 12 were injected subcutaneously on the left hind leg with tetanus spores. On the day preceding the spore injection, on the same day as the spore injection, and on the succeeding day each animal was injected with 1 grain of quinine subcutaneously on the chest.

On May 2 guinea pig 7 showed a slight stiffness of the left hind leg, which by the 5th stuck straight backward and was accompanied by a lateral curvature of the body. On the 8th all four legs were stiff and projected backward, but the left hind leg stuck backward rigidly and persistently, while the other three were relaxed a little at times. The animal was killed on the 9th. At the autopsy the entire site of the injection of quinine on the chest was excised and divided into six portions, each of which was planted into a fermentation tube of ordinary bouillon and incubated for 10 days, at the end of which time the growths were tested for tetanus toxin by injection into white mice. Strong tetanus toxin appeared in one tube but none had developed in the other five. The site of inoculation of spores in the leg was excised, planted into ordinary bouillon and incubated; the growth showed strong tetanus toxin. The reaction of the media in none of the tubes exceeded +3.3.

Guinea pig 8 carried his left hind leg backward at times on May 3 and by the 8th the leg was held persistently backward. On the 20th it stuck backward at times and a week later was apparently normal.

Guinea pig 9 carried his left hind leg backward from the 3d to the 10th.

Guinea pig 10 stuck his leg backward at times on the 5th and 6th.

Guinea pig 11 showed some local symptoms in his left hind leg on the 7th and 8th.

Guinea pig 12 showed a backward projection of his left hind leg from the 2d to the 8th.

Control guinea pigs 13, 14, and 15 which had been injected with spores only, but with no quinine, showed no stiffness of the left hind

leg. Guinea pigs 8 to 12 having recovered from local leg tetanus following the injections of quinine on the chest, were later injected with quinine directly into the site of their spore injection; two promptly contracted tetanus and died; three remained well. Guinea pigs 7 to 12 showed the effects of three injections of quinine on the chest in activating tetanus spores injected at the same time on the hind leg. In all animals local tetanus developed; in the case of one animal the symptoms became general and caused death, which was followed by the demonstration of tetanus organisms in a culture from the site of the quinine injection.

The question naturally arises as to whether all the symptoms of tetanus displayed by these six animals were caused by the spores located in the leg or whether some symptoms were referable to spores transported to the seat of the quinine injection on the chest. Guinea pig 7 is the only one in which we are positive that spores actually lodged in the chest lesion. The characters of the chest lesions in the five which did not contract general tetanus were no different from those of the one which did develop general tetanus. If tetanus spores lodged in the necrotic tissue of the chest wall of one animal, is it not probable that they did so in the other five as well, and if they did, why did general tetanus not follow in their cases also?

Guinea pig 7 first developed local tetanus, which was confined to the left hind leg for three days and then extended to the left side of his body and later became general. While it is possible that some of his general symptoms were referable to spores lodged at the site of his quinine injection on the chest, it is equally tenable that all of his tetanus symptoms were referable to the spores in his leg. Why refer any of his symptoms to the spores on his chest?

Guinea pigs 16 to 22 were injected with tetanus spores subcutaneously on the lower abdomen. On the day preceding the spore injection, on the same day, and on each of the two succeeding days, one grain of quinine was injected subcutaneously between the shoulders of each animal. Five animals contracted tetanus and either died or were killed; the other two remained well, but were killed on the seventeenth day. At autopsy the entire shoulder lesion of each was excised, divided into six parts, and each part was planted into a fermentation tube of ordinary bouillon and incubated for nine days. The reaction of the media in none of the 42 tubes exceeded +3.7. The cultures from the three animals in which the incubation periods of tetanus had been 5, 5, and 6 days, all showed production of tetanus toxin. Cultures from the animal in which the incubation was 7 days were half positive and half negative for tetanus toxin. Cultures from the animal in which the incubation period was the longest, or 11 days, were all negative for tetanus toxin. Cultures from the two animals which

had remained well and were killed on the seventeenth day were all negative. Cultures from the site of inoculation of spores on the abdomen contained strong tetanus toxin in the case of each animal. Control guinea pigs 23, 24, and 25, which received spores but no quinine, were still well at the end of a month, when an attempt was made to activate the spores in their legs by injections of quinine directly into the site, with the result that one contracted tetanus and two did not.

Guinea pigs 16 to 20 contracted tetanus following the injection of tetanus spores on the lower abdomen and quinine between the shoulders. What was the source of the tetanus toxin which killed these five animals? Was it all derived from the spores on the abdomen or was a part of it produced by the spores which were proven to have lodged in the necrotic tissue between the shoulders of four of the five animals? The lower abdomen was selected as the site of the spore injection because the symptoms which follow in a guinea pig after the injection of tetanus toxin at this site constitute a familiar picture in this laboratory, and if toxin were now to be derived from spores located between the shoulders in addition to that produced on the abdomen some deviation from the common picture was to be expected. Although the animals were closely watched there was nothing in their symptoms to indicate that there was an additional source of toxin production between the shoulders. Guinea pig 20 was the last of the five to contract tetanus and was therefore the one in which to have most reasonably expected a focus of infection in the shoulder lesion, yet this is the one in which all cultures from that lesion were negative for tetanus. The two animals which remained well and were killed had shoulder lesions apparently the same as those of the five which contracted tetanus, and yet all cultures from their shoulder lesions were negative.

Guinea pigs 26 to 31 were injected subcutaneously on the lower abdomen with tetanus spores. On the day preceding the spore injection, on the same day, and on the succeeding day, staphylococci were injected subcutaneously between the shoulders of each animal. On the eighth day, four animals showed mild symptoms of tetanus, such as rigidity and curvature; one seemed sick, and one was still well. On the ninth and tenth days all were killed, and at autopsy the entire shoulder lesion of each was excised and divided into six portions, each one of which was planted into a fermentation tube of ordinary bouillon and incubated for seven or eight days. Through error the tissue from guinea pig 27 was planted into glucose bouillon instead of ordinary bouillon, with the result that the reactions of all six tubes at the end of 24 hours were +7 or higher, and consequently no tetanus toxin was formed from the tissues of No 27. The reaction of the 30 tubes of ordinary bouillon inoculated with tissue from five animals all remained under +3.5 and tetanus toxin appeared in all. Cul-

tures from the site of inoculation of spores on the abdomen were made in the case of each animal; in each instance 0.01 c. c. of the growth promptly produced tetanus and death in mice, but the M.L.D. was not further determined. Control guinea pigs 32, 33, and 34, which received spores only, were still well at the end of a month, when they were used for another experiment.

Two sets of guinea pigs have just been reported upon. In one set quinine injected between the shoulders activated tetanus spores injected on the lower abdomen; in the other set, staphylococcus injected between the shoulders activated tetanus spores injected on the lower abdomen.

In the quinine-injected set, five animals promptly developed fatal tetanus and two remained well; cultures from the shoulder lesions of all seven animals were taken and were found positive for tetanus in four animals and negative in three.

In the staphylococcus-injected set, four animals developed mild symptoms of tetanus, one remained well, and one became sick. Cultures were taken from the shoulder lesions of five of the six animals and were all positive for tetanus.

If the transportation of tetanus spores to the quinine lesions was accomplished by phagocytes, it is presumed that spores reached the staphylococcus lesions in the same way.

Fifty per cent of the cultures from the quinine lesions were positive for tetanus, while 100 per cent of those from the staphylococcus lesions were positive.

Inasmuch as the animals which harbored tetanus spores most constantly (100 per cent) in the necrotic tissue between the shoulders developed only mild tetanus or none at all, it hardly seems reasonable to ascribe symptoms of tetanus to spores transported by phagocytes and lodged in necrotic tissue; the effect of quinine or staphylococci in activating tetanus spores is most probably exercised at the original site of injection of the spores.

Before commenting upon the significance of activation or non-activation by quinine and staphylococcus of dormant tetanus spores in guinea pigs and white mice, and before drawing inferences and deductions as to the relation in man between an attack of tetanus and a previous injection of quinine or a previous staphylococcus infection, it might be well to give some quotations from Semple.[1]

Every now and again we are confronted with the observation that cases of tetanus occur after the hypodermic or intramuscular administration of quinine, even in cases where every possible care has been taken to insure sterility of the syringe and fluid injected.

* * * * * * *

[1] The relation of Tetanus to the Hypodermic or Intramuscular Injection of Quinine by Lieut. Col. Sir D. Semple, Kt., M. D. Scientific Memoirs by Officers of the Medical and Sanitary Departments of the Government of India. Calcutta, India, 1911. No. 43; new series.

At first sight it would seem impossible when given a sterile syringe, sterile quinine solution, and sterile skin at the site of inoculation, to produce tetanus, and so it would be except for the fact that there are people who harbor in their bodies tetanus spores, which may lie dormant in a recently healed up wound or abrasion, or possibly in an old healed up injury long since forgotten. There are also many healthy individuals who harbor tetanus germs in their intestinal tracts.

* * * * * * *

On numerous occasions I have isolated tetanus bacilli from garden earth, stable floors, and stable manure, and in four cases out of ten examined from human feces.

* * * * * * *

It is conceivable that a few tetanus spores absorbed from the intestinal tract, and carried by phagocytes to a part of the body where there was dead tissue (e. g. the site of quinine injection), would give rise to tetanus. Would this method of infection account for some of the cases in which quinine has been given?

* * * * * * *

Granted that it is possible for tetanus spores to enter the circulation from the intestinal tract where the mucous membrane is injured, ulcerated, or in any way damaged to such an extent as to be incapable of keeping back infection, what is to prevent such spores getting stranded in an injury somewhere else in the body where there is dead anaerobic tissue? The hypodermic or intramuscular injection of quinine would prepare a suitable focus in a case of this kind, and so would injuries of various sorts which are constantly occurring. It seems to me that this is a reasonable possibility of infection in cases of so-called idiopathic tetanus, and in many other injuries including quinine injections, but to say more on the subject at present would savor of conclusions arrived at in the absence of proof.

* * * * * * *

In his Table XIII Semple reports experiments with eight guinea pigs injected subcutaneously on the hind legs with pure tetanus spores free from toxin or other irritants; at the end of 30 days six of the animals were chloroformed and the tissues at the sites of their spore injections were excised for anaerobic cultures in broth, all of which gave growth of tetanus bacilli; just at the end of six months similar cultures were taken from the two other animals and these likewise gave growth of true tetanus bacilli virulent to other guinea pigs.

It is evident from these results that tetanus spores can remain localized in the tissues for months. We know from experiments carried out years ago by Metchnikoff, Vincent, and others that when washed tetanus spores are injected hypodermically into animals the phagocytes pick them up and kill them off, and that the spores can be demonstrated inside phagocytes taken from the sites soon after injection. Some of the spores must escape being efficiently disposed of by the phagocytes, and those which escape get fixed in a safe place in the tissues, and remain there without giving rise to any symptoms.

When a person contracts tetanus through a wound, it means that tetanus spores have entered the wound and found suitable conditions in which to germinate and give rise to the disease.

On the other hand should the local conditions be unfavorable for the tetanus spores to germinate, and should they escape phagocytosis, what is to prevent them remaining stowed away and locked up in the tissues for months after the wound has healed? A condition of this kind would correspond to what is found in guinea pigs when washed

tetanus spores are injected locally, except that in a tetanus-infected wound the chances are more in favor of the spores finding suitable conditions for germination on account of other wound-infecting organisms accompanying them. There is still another condition necessary when a wound is infected with tetanus spores, viz, anaerobic conditions. I have no doubt that in many tetanus-infected wounds, owing to the absence of anaerobic conditions, tetanus fails to set in, and that the spores which escaped phagocytosis get hidden away in the tissues locally.

Semple reports 21 guinea pigs and 3 monkeys which were each given a single injection of tetanus spores on the hind leg and an injection of quinine on the chest on each of three successive days, except that in the monkeys the third injection of quinine was delayed three or four days. In 14 animals the quinine injections were begun on the day preceding the spore injection; in 7 they were begun on the day of the spore injection; in 3 they were started on the day following that of the spore injection. Seven animals contracted tetanus and died, 10 contracted tetanus and recovered, and 7 remained well. Cultures were made from the site of the quinine injections in 5 of the 7 animals which died. In all of these tetanus organisms were recovered. In cultures from the liver, the spleen, or blood of these animals no tetanus organisms were found.

On referring to the above-mentioned tables it will be seen that in every instance except one, in which cultivations were made from the site of the quinine injections in the circumstances mentioned, tetanus bacilli were recovered. Vincent in 1904 recorded similar results in some cases of experimental tetanus in guinea pigs in which quinine had been given hypodermically. It is therefore obvious from the results recorded in this paper, and from those recorded by Vincent, that the transfer of tetanus micro-organisms from the original site of infection to the site of quinine infection is one of the results to be expected when quinine is given hypodermically in tetanus infections; it is also a possible result to be expected in latent spore infections. We know that tetanus spores are to be found in the intestinal tracts of a large percentage of mankind and in some animals; would a similar transference of infection take place from the intestinal tract of some of these cases when quinine is given hypodermically?

* * * * * * *

Quinine when given hypodermically to a spore-infected person would produce favoring conditions for the production of tetanus in two ways—

(1) By a paralyzing effect on the phagocytes when given in large doses and for some time.

(2) By destroying tissues at the seat of injection, and by this means producing a suitable local anaerobic focus where a stray phagocyte carrying tetanus spores might get stranded.

It will be seen from the experiments in this paper that a hypodermic injection of quinine in animals invariably destroys tissue at the site of injection, and dead subcutaneous tissue would act in every way as a suitable medium for the germination of tetanus spores irrespective of how they found their way there.

In 1904 Vincent brought forward observations that proved that the physiological action of quinine can prevent phagocytes from successfully coping with tetanus spores; and that quinine, heat and other depressing influences, diminish the resistance to tetanus in animals inoculated with spores.

Small doses of quinine would increase the leucocytes and probably also increase phagocytosis, but when large doses are given the paralyzing effect of the drug on

leucocytes has to be taken into account. Further, there is another condition to be taken into account when quinine is given hypodermically, viz, the malarial infection for which it is given; this would lower resistance to most infections, tetanus included.

* * * * * * *

When tetanus spores are carefully prepared and freed from any traces of toxine or contaminations of any kind they may be injected into susceptible animals without producing tetanus. This is an old and well known experiment and never fails when properly carried out. Its success depends upon the fact that the phagocytes can pick up and digest tetanus spores in the absence of any irritant or other material likely to distract them; but when virulent spores mixed with toxine, other bacteria or other spores, sterile powdered charcoal, sterile sand, quinine, lactic acid, or anything which keeps away phagocytes, or occupies their attention, are injected hypodermically into susceptible animals, such as guinea pigs, or monkeys, tetanus is the result.

* * * * * * *

1. *Quinine.*—When quinine is injected hypodermically into guinea pigs, it soon becomes absorbed, but immediately upon injection, or before absorption has taken place, the tissues at the site of the injection are severely damaged. The local destruction of tissues in most cases is so complete that a slough forms, which involves the skin and comes to the surface, leaving an open sore within a few days from the time the quinine was given. The acidity of the solution may to a certain extent account for this local action, but it happens also when a preparation absolutely neutral in reaction is given.

The effect of a localized necrotic subcutaneous focus would be to establish a suitable anaerobic medium for the growth of the tetanus spores, should they by any chance become lodged there. At the same time one of the immediate effects of a large dose of quinine would be to produce a more or less paralyzing effect on the phagocytes generally, and a negative chemiotaxis on the phagocytes locally, conditions which would still further the growth of any tetanus spores which happened to get lodged at the spot.

2. *Washed tetanus spores.*—Vaillard and Vincent have proved that when "washed tetanus spores" are injected hypodermically into guinea pigs, they are picked up and digested by the phagocytes which crowd to the seat of injection, provided there is nothing introduced with the spores to destroy tissue, produce a negative chemiotaxis, or in any way to divert the phagocytes from efficiently performing their function of bactericidal agents.

It may happen even with active phagocytes that some of the spores escape destruction and become stowed away locally, to grow and produce infection at some future time when conditions become favorable. It may also happen that some of the phagocytes require a considerable time to kill off and digest the toughest of the spores, and that phagocytes in this condition on reentering the circulation may get stranded or lodged in a suitable site where the spores can grow and give rise to tetanus. The subcutaneous or intramuscular injections of quinine would produce such foci provided spore-laden phagocytes become lodged there.

On the assumption that this is what happens when quinine and tetanus spores are injected into separate parts of the body, one would expect more infections in susceptible animals when the quinine is injected on the day before, or at the same time as the spores, and fewer infections when the spores are injected a day or more before the quinine. Also one would expect never to fail in producing tetanus when solutions of quinine or other irritants, such as lactic acid, are injected mixed with the spores.

On the other hand one would expect to fail in producing tetanus when nonirritants (which become rapidly absorbed), such as morphia or normal sterile saline solution, are injected mixed with tetanus spores. As a matter of fact, the accuracy of these conjectures is verified by experimental tests as will be seen by referring to the numerous experiments detailed in the succeeding part of this paper.

IN INSTANCES WHERE SYMPTOMS OF TETANUS FOLLOW THE INJECTION OF SPORES INTO THE HIND LEG AND QUININE UPON THE CHEST, IS THE TETANUS TOXIN PRODUCED AT THE SITE OF THE QUININE INJECTION OR AT THE SITE OF THE SPORE INJECTION?

Semple reports 21 guinea pigs which were injected with tetanus spores on the hind leg and with quinine on the chest. A quinine injection was made on each of three or more succeeding days, the first quinine injection having been made either on the day preceding the spore injection or on the same day as the spore injection or on the day following the spore injection. Ten of the animals contracted tetanus and recovered, 4 contracted tetanus and died, and 7 remained well. The symptoms of the 10 which contracted tetanus and recovered were as follows:

On the tenth day from the time the spores were given, one animal developed tetanus in the leg into which the spores had been injected, and the next day the tetanus symptoms had extended to the side on which the spores were injected and the disease became chronic and lasted for a month, when recovery took place.

* * * * * * *

Symptoms localized to right leg and right side of body. (Guinea pigs were inoculated in right hind leg.)

* * * * * * *

Disease localized to right leg and right side of body; very severe for a week, when daily improvement set in.

* * * * * * *

In all these six animals, the disease ran a chronic course and was confined to the left hind leg (seat of inoculation of spores) and the left side of body; and for over a fortnight they were bent to the infected side, and the infected leg stuck out stiff when they crawled along on being taken out of their cages. After a month they all eventually recovered.

All of the symptoms of tetanus which any of the above 10 animals presented seem to be clearly referable to spores located at the original site of their injection in the hind leg and not to any spores which may have become transported to the site of the quinine injection on the chest wall.

The symptoms of the four animals which contracted tetanus and died were the following:

Symptoms local at outset, then became general.

* * * * * * *

Symptoms local at outset, then became general.

* * * * * * *

Disease local at first, then became general.

* * * * * * *

Symptoms local at first, advanced slowly, then became severe and general the day before death in case of Guinea pig No. 2.

* * * * * * *

In the No. 2 guinea pig of this series, local tetanus developed on the fifth day after spores were given, and the disease remained localized to the leg inoculated with spores

for nine days, after which acute, generalized tetanus set in, and the animal died two days later. Immediately after death anaerobic cultivations in broth tubes were made from the seat of injection of spores (right hind leg); seat of injection of quinine (chest); spleen, liver, lungs, kidneys, heart's blood. When these cultivations had been incubated for a month at 37° C. they were opened and examined with the following results:

A rich growth of tetanus bacilli with well formed spores from the seat of injection of quinine on the chest, and also from seat of injection of spores on the leg. A few micrococci in the cultivation from the lungs, and the remaining four cultivations from the liver, spleen, kidneys, and heart's blood were sterile.

After this result it was deemed advisable to test further whether tetanus germs can be conveyed from a remote site of infection, such as the leg, to another part of the body, such as the chest, where quinine had been injected. The results of the experiments detailed in Tables VIII, IX, and XII prove that this is what happens in probably every case, and that a hypodermic injection of quinine establishes a very favorable local site for the growth of tetanus germs should any ever reach the spot. A phagocyte carrying a few tetanus spores which happened to get stranded in such a site would prove the starting point of a fresh focus of infection.

Is this what happened in No. 2 guinea pig of this series when it had chronic and localized tetanus for nine days, and then acute and fatal symptoms suddenly set in? In 1904 Vincent, when experimenting with quinine and tetanus infections, also obtained results which favor the view that quinine injections produce foci favorable for the growth of tetanus bacilli, and that infections may be conveyed from the original site of the disease to these foci.

The four animals which died presented early symptoms referable to the spores in the leg. Their late symptoms were general, due to toxin, the source of which may just as reasonably have been the spores in the leg as those transported to the site of injection of quinine on the chest.

In Table XII three monkeys were injected on the hind leg with tetanus spores and with quinine on the chest. All three developed tetanus and died. Cultures from the lesions on the leg and chest were positive for tetanus bacilli, but cultures from the liver and spleen were all negative. Cultures were made from the blood of one monkey; these remained negative.

The incubation period was more prolonged in the case of the monkey, and when symptoms set in they resembled those found in the cases of tetanus in the human subject, lockjaw being an early and prominent symptom, and local tetanus at the site of inoculation being absent (as an early symptom at any rate) in all three cases, whereas it is always the first symptom in a guinea pig, and when not lethal is often the only symptom.

Semple suggests as possibilities that cases of tetanus following the subcutaneous or intramuscular injection of quinine may be due to the germination of tetanus spores in the necrotic tissue at the site of the quinine injection; that the human intestinal tract is a source from which the tetanus spores may be absorbed through an injured or ulcerated mucous membrane; that a recently healed up wound or abrasion or an old healed up injury long since forgotten may harbor dormant or latent tetanus spores; that tetanus spores can remain

stowed away or locked up in the tissue locally for months and very probably for years; that phagocytes may carry tetanus spores from some spore infected locality of the body to a different part of the body where there is dead anaërobic tissue produced by quinine or injuries of various sorts; that stray spore-laden phagocytes getting stranded in dead tissue, germinate there and produce tetanus toxin.

Many of the possibilities suggested by Semple should be susceptible of demonstration and it may be well to inquire how far they are borne out by laboratory data. (1) Have tetanus organisms or their toxins been found in the necrotic tissue at the site of a quinine injection in man? (2) To what extent have tetanus spores been found in the feces of human cases of so-called idiopathic tetanus? (3) On account of the high mortality in tetanus, opportunity is offered to examine not only the intestinal contents throughout, but to determine the condition of the mucous membrane as well; have such examinations disclosed injuries or ulcers of the bowels or the presence of tetanus spores in the bowel contents? (4) Can laboratory animals whose gastro-intestinal tracts have been artificially contaminated with tetanus spores be made to contract tetanus by injections of quinine, or can tetanus spores be demonstrated in parts of their bodies other than the lumen of the gut? (5) Have tetanus spores been found in healed wounds of patients who had exhibited no symptoms of tetanus? (6) Have latent tetanus spores been found lying dormant in the healed tetanus wound of a patient after recovery from his attack of tetanus? (7) Has tetanus been produced in animals when the injection of tetanus spores and quinine were separated, not only in place, but by a considerable length of time as well?

On questions 1, 2, 3, 5, and 6, I am unable to submit data, since their elucidation requires the presence of clinical facilities and cases of human tetanus which are not available at this laboratory at the present time.

In regard to question 4, the demonstration of the problem has not yet been attempted in this laboratory.

The hypothesis is made that tetanus in man following an injection of quinine is due to the tetanus spores which have become transported by phagocytes into the site of the quinine injection from a site elsewhere in the body where they had been implanted at some time previous to that of the quinine injection. If this hypothesis be true, one should expect to find it corroborated by a laboratory experiment in which not only the spore site and the quinine site were located in different parts of the body, but in which a considerable interval of time had separated the injections of spores and quinine. The hypothesis does not provide for a simultaneous implantation of spores and injection of quinine; it is assumed that the spores at some time previous gained entrance to the body tissues through external

wounds now healed or through an injured intestinal mucous membrane. In the experiments in animals a separation in respect to time does not seem to have been made between the spore injection and the quinine injection and yet such a separation is assumed to occur in man. Out of Semple's 24 animals in which the site of injection of spores and quinine were the hind leg and the chest respectively, the time of the first quinine injection was one day previous to that of the spore injection in 14 instances, it was the same day as the spore injection in 7, and on the day following the spore injection in 3. The results might have been very different had the injection of quinine been delayed for weeks or months after the spore injection. Such a separation in time of injections is called for by the hypothesis.

III. MISCELLANEOUS OBSERVATIONS UPON TETANUS.

In the preparation of the data for this bulletin, considerable quantities of media, material, and animals were used; some general observations upon the properties of the tetanus bacillus were made which will now be presented.

INHIBITION OF GERMINATION OF TETANUS SPORES BY CARBOLIC ACID.

Some manufacturers of vaccine virus do not rely upon glycerin alone to reduce the nonspore-bearing organisms but use carbolic acid in addition to the glycerin. The solution used for grinding the vaccine is made as follows:

50 c. c. glycerin.
$\frac{9}{10}$ c. c. carbolic acid.
49 c. c. water.

To every gram of the vaccine pulp, 4 c. c. of the carbolized glycerin solution is added. The amount of carbolic acid in each cubic centimeter of the finished virus would therefore be approximately 0.007 c. c. or $\frac{7}{10}$ per cent.

If 1 c. c. of such a virus should contain tetanus spores and should be tested for tetanus by planting the entire cubic centimeter into 6 c. c. of bouillon, the spores if they germinated would be compelled to do so in $\frac{1}{10}$ per cent solution of carbolic acid; if planted into 13 c. c. of bouillon, the strength of the carbolic acid would be reduced to $\frac{1}{20}$ per cent, etc. These percentages of dilution of carbolic acid are based on a thorough mixing of vaccine virus with the entire culture medium.

If instead of thoroughly mixing virus and culture medium, the heavy virus is allowed to settle to the bottom of the medium, it is conceivable that the dilution of the carbolic acid might not be greater than $\frac{1}{10}$ per cent. The question arises as to what percentage of carbolic acid will prevent the germination of the tetanus spores.

The attempt was made to germinate tetanus spores in glucose bouillon containing $\frac{1}{10}$ per cent carbolic acid with the result that two out of five tubes thus inoculated showed no growth of tetanus while growth in the other three was delayed 36 and 60 hours. (See Table No. 14.)

Table No. 14.—*Inhibiting effect of one-tenth per cent carbolic acid on the germination of tetanus spores.*

	Glucose bouillon.			Glucose bouillon, $\frac{1}{10}$ per cent carbolic acid.			
	Straight tubes.		Fermentation tubes, tetanus spores.	Fermentation tubes.			
	Tetanus spores.	Tetanus culture 48 hours old.		Tetanus spores.	Tetanus culture 48 hours old.	Bacillus coli.	Staphylococcus.
EXPERIMENT 1.							
Feb. 21, 5 p. m.	3 tubes.	3 tubes.	6 tubes.	2 tubes.	2 tubes.	2 tubes.	2 tubes.
Feb. 22, 9 a. m.	Growth.	Growth.	Growth.	No growth.	Growth.	Heavy growth.	Heavy growth.
Feb. 23				do.			
Feb. 24				1 growth Other No growth			
Feb. 27				do.			
EXPERIMENT 2.							
Feb. 24, 3 p. m.		1 tube.	1 tube.	3 tubes.	3 tubes.		
Feb. 25, 9 a. m.		Growth.	No growth	No growth.	Growth.		
Feb. 25, 3 p. m.			Growth.	do.			
Feb. 26				2 growth Other No growth			
Mar. 2				do.			

Ten control tests in which tetanus spores were inoculated into glucose bouillon to which no carbolic acid had been added showed growth of tetanus within 18 hours in all except one, in which growth appeared within 24 hours. *Bacillus coli* and staphylococcus grew readily in $\frac{1}{10}$ per cent carbolic acid; in 18 hours their nonrestraint would therefore bring about a lengthened period of high acidity preceding the delayed germination of the tetanus spores.

A young culture of tetanus showed growth in glucose bouillon containing 0.1 per cent of carbolic acid.

CULTURE MEDIA FOR TETANUS.

Glucose bouillon, freshly prepared, made from lean beef, not fermented with colon bacilli, containing 1 per cent peptone, and 1 per cent glucose, titrated 1 per cent acid to phenolphthalein, tubed into Smith fermentation tubes and raised to the boiling point just before inoculation in order to expel air, is the medium in most common use in laboratories for testing for tetanus.

Ordinary bouillon prepared in every respect like the above, except that the glucose is omitted, is not generally considered to be the best medium for growing tetanus on account of the nonaddition of glucose.

While the presence of glucose in the medium favors the growth of tetanus in pure culture, it hinders the germination of tetanus in mixed culture almost to the degree of absolute prevention provided the contamination consists of many cocci, colon bacilli, *Bacillus*

welchii or other acid-forming organisms. For a mixed culture of the character stated, ordinary bouillon is far superior to glucose bouillon, because the acidity of the medium remains sufficiently low to allow tetanus to germinate.

An attempt was made to determine how much superior glucose bouillon is to ordinary bouillon in growing tetanus in pure culture. In the course of the work many fermentation tubes of the media were inoculated with tetanus spores for the purpose of controls; 229 tubes of glucose bouillon inoculated with pure tetanus spores all developed growth of tetanus except 8, in which the medium remained perfectly clear; 65 tubes of ordinary bouillon similarly inoculated all showed growth of tetanus except 4. Neither medium was found to be perfect. The percentage of failures to grow tetanus in glucose bouillon was 3.5; the percentage of failures in ordinary bouillon was 6.1. The same strain of organism was used throughout the work; no difference could be noticed in the strength of toxin produced in the two media; the M.L.D. of growth in either bouillon was 0.00001 c. c for a white mouse; growth appeared no sooner in one medium than in the other.

No special apparatus or methods designed to exclude oxygen were employed other than those of the Smith fermentation tube shown in figure (No. 2). In the open arm of the tube one can with great convenience make inoculations and titrations without disturbing the growth of tetanus in the long arm.

Theobald Smith first suggested the addition of a piece of sterile tissue to the media in order to further the growth and sporulation of tetanus organisms. If in testing vaccine virus one adds 1 c. c. of the virus to a fermentation tube, there is thereby added to the media a considerable amount of tissue which may favor the growth of tetanus; the tissue added is, however, far from sterile.

INHIBITION OF GERMINATION OF TETANUS SPORES IN GLUCOSE BOUILLON BY ANAEROBES FOUND IN VACCINE VIRUS.

When testing a vaccine virus for the presence of tetanus spores, we subject the virus to conditions of growth most favorable, not only to the tetanus bacilli but to other strict anaerobes as well.

Bacillus Welchii (*bacillus aerogenes capsulatus*) was found in 15 samples of glycerinated vaccine virus. Like tetanus, it resisted exposure to a temperature of 80° C. for an hour, but unlike tetanus, it produces a high acidity in glucose bouillon within the first few hours of growth; the acidity is sufficient to prevent the germination of tetanus spores. (See Table No. 4.) The organism was isolated from viruses which had glycerinated from 7 to 10 months and were apparently sterile when planted on agar slants. One to four drops, however, planted into fermentation tubes and heated to 80° C. for an

hour before incubation, quickly produced fermentation and yielded the organism in pure culture. The organism isolated was a plump bacillus often in pairs, nonmotile, gram-positive, strictly anaerobic, apparently without spores and yet in vaccine virus it resisted 80° C. for an hour; in fermentation tubes of glucose bouillon and milk there was fermentation and production of acidity between +5.0 and +7.0; the latter medium presented the typical appearance of "stormy fermentation." A rabbit killed within a few minutes after intravenous injection with a young culture and incubated for 24 hours presented an emphysematous condition of the subcutaneous tissue of his body, extending to the tips of his extremities and ears and showed the effects of a very destructive process in his blood, kidneys, spleen, and liver; smears from the latter organ showed a plump bacillus which stained gram-positive.

An unidentified strict anaerobe, forming oval spores, was isolated from vaccine virus. This organism constantly produced a maximum acidity of about +5.0 in glucose bouillon by the end of 24 hours and this acidity always inhibited the germination of tetanus spores. (See Table No. 15.)

TABLE NO. 15.—*Inhibition of germination of tetanus spores in glucose bouillon by the presence of an unidentified spore-forming, acid-producing anaerobe obtained from vaccine virus.*

	Column 1. An unidentified strictly anaerobic fine rod with oval spores was planted into 6 fermentation tubes, glucose bouillon, heated 65°, ½ hour.	Column 2. Sediments from 6 fermentation tubes of column 1 were artificially contaminated with tetanus spores and planted over into 6 fermentation tubes, glucose bouillon, unheated.			Sediments from 6 fermentation tubes of column 2 were artificially contaminated with tetanus spores and planted over into 6 fermentation tubes, glucose bouillon, heated 70°, 1 hour.		
	Reaction of open arm at end of 24 hours.	Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 8 days.	Mice inoculated with growth at end of 8 days.	Reaction of open arm at end of 24 hours	Reaction of entire contents at end of 12 days.	Mice inoculated with growth at end of 12 days.
1	5.0	5.3	5.6	1 c. c. neg.	4.5	5.5	1 c. c. neg.
2	5.0	5.5	5.0	1 c. c. neg.	4.5	5.6	1 c. c. neg.
3	4.8	5.6	5.3	1 c. c. neg.	4.3	5.2	1 c. c. neg.
4	5.0	5.0	5.5	1 c. c. neg.	4.7	5.2	1 c. c. neg.
5	5.0	5.7	....	1 c. c. neg.	5.0	6.4	1 c. c. neg.
6	4.8	5.0	5.2	1 c. c. neg.	5.5	5.8	1 c. c. neg.
					Six tetanus controls all showed growth.		

The organism was obtained in pure culture and twelve attempts were made to grow tetanus in combination with it, but without success. The control tubes of tetanus spores all showed growth. Unfortunately the culture was thrown out before determining whether its acid production would be sufficiently limited in ordinary bouillon to allow tetanus spores to germinate.

ACTIVATORS OF TETANUS SPORES.

Quinine obtained from a quarter-pound can of quinine hydrochloride (Merck) was used in these experiments. A gram was usually weighed out and added to a sterile glass-stoppered cylinder containing 15 c. c., of sterile water. With the application of a little heat and shaking, the quinine went into solution. One c. c. of this solution was taken to contain 1 grain of quinine.

Guinea pigs weighing between 200 and 300 grams, after subcutaneous injection of 1 grain of quinine immeditaely develop incoordination, staggering, and an indifference to being handled; the local lesion shows intense injection within an hour, followed in 24 hours by induration and later by ulceration and scar formation. One grain is considered the maximum amount which can with safety be injected at one time into guinea pigs of the weight indicated; that amount occasionally proved fatal within an hour.

White mice weighing about 15 grams were injected with one-tenth grain of quinine subcutaneously on the back. This amount usually caused staggering and occasionally killed. It was, however, injected daily for four successive days in several instances.

Staphylococcus albus obtained from a chronic ear discharge and staphylococcus aureus taken from the stock cultures of the laboratory were used with equal success. The entire growth from an agar slant was often injected subcutaneously into a guinea pig and one-tenth of that amount into a white mouse. By injecting different amounts into healthy animals, one can ascertain, after a few days, the maximum amount of a particular strain which can with safety be injected.

TETANUS TOXIN HEATED TO 80° C. IS NOT RENDERED LETHAL BY QUININE.

Glucose bouillon was inoculated with tetanus and incubated for 14 days, at the end of which time it was filtered through a Berkefeld filter. The M.L.D. of the filtrate for mice was 0.0001 c. c. The filtrate was heated to 80° C. for one hour and then injected subcutaneously on the abdomen into six guinea pigs weighing 300 grams. Each animal received 4 c. c. of the heated toxin daily for three successive days. Each animal also received an injection between the shoulders of one grain of quinine daily for four successive days, the first of which was given on the day preceding the first toxin injection. The injection of quinine resulted in each instance in the formation of a large ulcer, but there were no symptoms of tetanus at any time in the animals. Two guinea pigs which received 1 c. c. and 3 c. c., respectively, of the heated toxin but no quinine, likewise remained well. Two mice which received 2 c.c. and 1 c. c. respectively, of the heated filtrate also remained well. Cultures of the filtrate before and after heating were negative in fermentation tubes of glucose bouillon.

SYMBIOSIS VERSUS ANTAGONISM.

Symbiosis is strikingly illustrated by the germination of tetanus spores in the subcutaneous tissue brought about by the presence of cocci. Pure tetanus spores injected subcutaneously do not produce tetanus, but admixture with cocci at the time of injection or sometime after injection does produce tetanus.

Antagonism or inhibition is equally well illustrated by the prevention of germination of tetanus spores in glucose bouillon brought about by the same cocci upon which in the subcutaneous tissue germination was dependent. If tetanus spores and cocci are inoculated into glucose bouillon and if these cocci are present in sufficient number to raise the acidity of the media to + 4.5 during the first 24 hours of growth, the cocci will thereby almost certainly prevent germination of the tetanus spores.

Clear-cut illustrations of the two opposite phenomena are thus furnished by the same organisms when brought together under different conditions.

Bacillus subtilis is governed by cocci in glucose bouillon in very much the same way as tetanus spores are. If two tubes are each inoculated with subtilis spores and cocci and if before incubation one tube is heated to 80°C, the surface of that tube will develop a scum of subtilis while the unheated one will generally show no such scum. The cocci in the unheated tube exercise a decisive inhibition upon a luxuriant aerobe, probably through their acid production. Vaccine virus inoculated into two fermentation tubes of glucose bouillon, one of which was later exposed to 80° C. frequently produced a scum of subtilis on the heated tube and no scum on the unheated one.

Considerable importance has been ascribed to the oxygen-absorbing property of cocci and *Bacilus subtilis* in rendering anaerobic and conquently more favorable, the media in which a growth of *Bacillus tetani* is desired; throughout this study very numerous instances occurred of coexistent growth of tetanus and cocci and of tetanus and *Bacilus subtilis,* but no instance was found in which such a mixed infection resulted in greater strength of tetanus toxin than appeared in control pure cultures of the tetanus bacillus.

The strain of organism used was a strong toxin producer. It gave toxin after 14 days growth in fermentation tubes of glucose bouillon, the M.L.D. of which for white mice was 0.00001 c. c.

A very noticeable effect of the coincident growth of tetanus and subtilis in a Smith fermentation tube of glucose bouillon was the production in the closed arm of three or four times as much gas as was formed by a pure culture of tetanus. Tetanus alone usually produced about an inch of gas in the closed arm of the tube, but a combination with subtilis gave a scum on the surface of the open

arm and usually 3 to 5 inches of gas in the closed arm. *Bacillus subtilis* does not ferment glucose bouillon. Increased gas production did not, however, indicate any increase in virulence of toxin. Tetanus and subtilis grew well together. One reason for their harmonious growth is found in the low acidity produced by subtilis in glucose bouillon. The time of highest acidity in a culture of subtilis is at the end of 48 hours; the average of 31 titrations in the open arm of a fermentation tube after 48 hours of growth was +3.1. The reaction then gradually falls until at the end of 9 to 12 days it is sometimes neutral. The average of 31 titrations in the open arm at the end of 9 to 12 days was +1.1.

HEATED TETANUS SPORES.*

Heating the spores at 80° C. for one hour was found to render them uniformly free from toxin without impairing their viability. Heating the spores at 100° C. for 15 minutes killed them. The method of preparing all the spores used in this work was as follows: Smith fermentation tubes containing about 25 c. c. of glucose bouillon or ordinary bouillon were inoculated with tetanus spores and incubated for two weeks at 37° C. During the first few days of growth the media was cloudy throughout but by the end of two weeks the organism had developed into spores and had settled to the bottom and formed a sediment which was readily pipetted off with a capillary pipette introduced through the opening in the short arm of the tube. In drawing up the sediment, about 1 c. c. of spores and bouillon would be removed from each tube. Usually the sediment was collected from about 30 tubes at a time and after being mixed was drawn up into a bulb pipette of about 40 c. c. capacity such as is illustrated in figure No. 1. The bulb after being sealed in the flame was immersed in a water bath and kept at 80° C. for one hour, after which it was kept in the cold room at 15° C. A bulb containing 30 c. c. of such a suspension afforded a very convenient means of keeping constantly on hand in sterile condition a large bulk of tetanus spores, sufficient in amount to do many experiments and of even quality so that results were reliably comparable. When spores were wanted for an experiment, the bulb was shaken and the stem of the bulb was filed near the end; the end was then passed through a flame, and with sterile forceps the end was broken at the file mark or a white-hot thick platinum wire was held on the file mark to crack the glass. A fine capillary pipette was introduced and the desired amount was drawn off. The stem was then sealed in the flame and the bulb was replaced in the cold room. When a bulb is being opened very often an infection of the contents of the bulb with a coccus sometimes occurs. A reheating of the sealed bulb in a water bath at 80° for an hour renders it sterile again. As the spores must necessarily be

free from contamination, it is always well to test for sterility by planting a drop on an agar slant every time a capillary tube is introduced into the bulb. The size of the capillary tube was usually such as would drop 40 to 70 drops per c. c. and one or two drops of the stock suspension was always found to contain sufficient tetanus spores to contaminate the material which was about to be inoculated into a fermentation tube or injected into a guinea pig or a white mouse.

Tetanus spores prepared in the manner described above and stored in bulbs in the cold room at 15° C. were tested for viability after various periods of storage. Nine bulbs which had been kept a year and a half were opened and one drop from each was planted into a fermentation tube of glucose bouillon and incubated for 10 days, at the end of which time 0.0001 c. c. of the growth from each tube was injected into a mouse and produced tetanus and death within three days. The bulbs which had been stored for a year were similarly tested and gave the same results. It is suggested that pure cultures of tetanus could be preserved in laboratories in bulbs rather than in culture media and thus not only save the work incident to carrying over the cultures but avoid the risk of contamination incident to the manipulation.

WASHED TETANUS SPORES.

How can tetanus spores be rendered free from toxin? At first thought it might seem possible to rid them of their toxin by successive suspensions and centrifugalizations in large quantities of saline solution. Mechanically speaking, the amount of tetanus toxin which would remain in 1 c. c. of tetanus spores after seven successive suspensions and precipitations in 200 c. c. of saline solution would be inappreciable, and yet on reference to Table 16 we find that the saline solution from the seventh such washing caused tetanus in mice and gave a growth of tetanus in media.

TABLE No. 16.—*Tetanus spores after 7 washings with saline solution by centrifugalization were not freed from toxin.*

EXPERIMENT 1.

	Mice inoculated.	Result.	Fermentation tube inoculated.	Smear of sediment.
	C. c.			
Saline solution (3)	2.0	Tetanus, death in 18 hours	Growth	
	2.0	Tetanus, death in 36 hours		
	1.0	Tetanus, death in 24 hours		
	1.0	Tetanus, death in 36 hours		
Saline solution (4)			Growth	
Saline solution (5)	2.0	Tetanus, death in 36 hours	do	
	1.0	do	do	
Sediment (5)	.02	Tetanus, death in 24 hours	do	Tetanus spores.
	.004	Tetanus, death in 36 hours		
	.002	do		
	.0004	do		
	.0002	Tetanus, death in 60 hours		

TABLE NO. 16.—*Tetanus spores after 7 washings with saline solution by centrifugalization were not freed from toxin*—Continued.

EXPERIMENT 2.

	Mice inoculated.	Result.	Fermentation tube inoculated.	Smear of sediment.
	C. c.			
Saline solution (3)	2.0	Tetanus, death in 18 hours		
	1.0	do		
Saline solution (4)	2.0	Tetanus, death in 36 hours	Growth	
	1.0	do	do	
Saline solution (5)	2.0	Tetanus, death in 24 hours	do	
	1.0	Tetanus, death in 42 hours	do	
Saline solution (6)	2.0	do	do	
	1.0	do	do	
Saline solution (7)	2.0	Tetanus, death in 36 hours	do	
	1.0	Tetanus, death in 84 hours	do	
Sediment (7)	.02	Tetanus, death in 36 hours	do	Tetanus spores.
	.004	do		
	.002	Tetanus, death in 60 hours		
	.0004	Remained well		
	.0002	do		

In experiment 1, 800 c. c. of a tetanus bouillon culture planted April 12, 1912, was on May 1 poured into four centrifugalizing bottles of 200 c. c. capacity each, and these were centrifuged for one and one-quarter hours. At the end of this time the bouillon was pipetted off and found to have an M.L.D. for mice of 0.0001 c. c. The sediment from the four bottles aggregating 4 c. c. was collected into one bottle and suspended in 200 c. c. of saline solution (1). This was centrifuged for an hour and a quarter, at the end of which time the saline solution was pipetted off, leaving approximately 1 c. c. of sediment. This sediment was suspended in 200 c. c. of saline solution (2) and centrifuged again for an hour and a quarter. This process was repeated until the fifth saline solution had been used. The third, fourth, and fifth saline solutions were found to kill mice when injected subcutaneously in 1 c. c. amounts, and this same amount planted into fermentation tubes of glucose buoillon gave a growth in each instance. A smear made from the sediment after the fifth washing showed an abundance of tetanus spores.

In experiment 2, 800 c. c. of the same bouillon culture as was used in experiment 1, was on April 29, centrifuged and the sediment was washed twice with saline solution as described above. On April 30, 800 c. c. of the same culture was treated in the same way. On May 1, the washed sediment from both lots was mixed and washed five more times in the manner already described. The saline solution after each centrifugalization was found to produce tetanus in mice and to grow when inoculated into fermentation tubes. The sediment after the seventh washing showed tetanus spores in a stained smear.

No attempts were made to rid tetanus spores of their toxin by successive suspension in saline solution and filtration through a Berkefeld filter. Presumably the result would be the same as in centrifugalization.

Semple applies the expression "washed spores" to spores which have been heated instead of washed in the ordinary sense. His "washed spores" were prepared as follows:

Ten c. c. of anaerobic-broth cultures which had been grown for a month at a temperature of 37° C. were used; 2 c. c. were pipetted off from the bottom of a culture and transferred to a sterile test tube and then heated in a water bath at a temperature of 75° C. for 5 minutes.

ISOLATION OF THE TETANUS ORGANISM FROM A WOUND.

If the scrapings from a wound are heated at 80° C. for an hour and then planted into media, any toxin which appears in the media must be due to a growth of the tetanus organism in the media. If scrapings are planted without heating, the toxin which appears in the media may be due either to a multiplication of the tetanus organism in the media or to tetanus toxin carried over into the media with the tissues, or to a combination of both.

If the tissues from a suppurating wound, even though they contain tetanus organisms are planted into glucose bouillon and incubated without exposure to 80° C., there will probably be no multiplication of the tetanus organisms on account of the early acidity of the medium produced by staphylococci; tissues from such a wound should be planted into ordinary bouillon.

A tube of bouillon into which has been planted the tissues from a tetanus wound may after two weeks' incubation be found to contain tetanus toxin when inoculated into mice. The tetanus toxin in that case need not necessarily have been derived from a growth of tetanus bacilli in the bouillon; there may have been no multiplication whatever of tetanus organisms in the media; the tissues may have carried over into the media the identical toxin which, after two weeks' incubation, is inoculated into mice and causes tetanus in them.

If in Table 17 the scrapings from the tetanus wounds of the guinea pigs had been planted into media and incubated without exposure to 80° C., 10,000 M.L.Ds. of tetanus toxin would have been carried over with the tissues into the media; after incubation, the media on inoculation into mice would have caused tetanus. In that case the impression might have been gained that the tissues contained tetanus bacilli which had multiplied in the media and produced toxin. On the other hand it appears that the tissues contained no tetanus bacilli. If the tissues on removal from the guinea pigs had contained even vegetating tetanus bacilli, they should have formed spores in two weeks at 5° C. and should then have resisted 80° C. and germinated in the bouillon with the production of toxin. Only one produced toxin. Why should the tetanus organisms have been so easy of recovery from the mouse wounds and almost nonrecoverable from the guinea pig wounds?

TABLE NO. 17.—*Differences in reactions of guinea pigs and white mice to injection with equal amounts of tetanus spores combined with staphylococcus or quinine.*

Date.		Glucose bouillon.			
		80°, 1 hour.		Unheated.	
1912.					
July 9	....	2 gtts.	2 gtts.	2 gtts.	2 gtts.
July 11	Reaction at end of 48 hours..........	2.2	2.4	6.3	4.4
July 18	Reaction at end of 9 days..........	3.3	2.5	6.1	5.6
Do....	M.L.D. of toxin for mouse at end of 9 days...	0.00002 c.c.	0.0001 c.c.	0.001 c.c.	0.001 c.c.

Date.		Guinea pigs inoculated subcutaneously on abdomen.					
1912.							
July 9	....	2 gtts.+ staph-coc.	2 gtts.+ staph-coc.	2 gtts.+ quinine.	2 gtts.+ quinine.	2 gtts.	2 gtts.
July 11	Reaction at end of 48 hours	Tetanus †	Tetanus †	Local reaction.	Local reaction.	No reaction.	No reaction.
July 12	....	....	....	Tetanus †	Local reaction.	No reaction.	No reaction.
July 13	....	....	....	....	Tetanus †	Tetanus †	Tetanus †
July 15	M.L.Ds of tetanus toxin for a mouse in scrapings from site of inoculation of guinea pig	10,000	10,000	10,000	10,000	1,000	1,000
Aug. 14	Reaction of growth at end of 14 days of incubation.	4.2	5.0	3.1	2.8	3.8	3.0
Do....	M.L.D. of tetanus toxin for mouse at end of 14 days' incubation........	0	0.01 c.c.	0	0	0	0

Date.		Mice inoculated subcutaneously on back.					
1912.							
July 9	....	2 gtts.+ staph-coc.	2 gtts.+ staph-coc.	2 gtts.+ quinine.	2 gtts.+ quinine.	2 gtts.	2 gtts.
July 11	Reaction at end of 48 hours	Tetanus †	Tetanus †	Tetanus †	Negative.	Tetanus.	Tetanus.
July 12	....	....	....	....	Slight tetanus.	Death.	Tetanus.
July 13	....	....	....	....	Slight tetanus.	....	Tetanus.
July 14	....	....	....	....	....	....	Death.
July 15	M.L.Ds. of tetanus toxin for amouse in scrapings from site of inoculation of mouse	500	5,000	5,000	Recovered	5	5
Aug. 14	Reaction of growth at end of 14 days of incubation	1.1	3.4	8.7	....	1.0	1.7
Do....	M.L.D. of tetanus toxin for mouse at end of 14 days' incubation.......	0.0001 c.c.	0.001 c.c.	0.0001 c.c.	....	0.001 c.c.	0.01 c.c.

On the death of each guinea pig and mouse, scrapings were made from the entire site of inoculation and placed in the ice box at 5° C., until July 31, when they were planted into glucose bouillon and heated to 80° for one hour in the Arnold steam sterilizer and then incubated.

DIFFERENCES IN REACTIONS OF GUINEA PIGS AND WHITE MICE TOWARD TETANUS SPORES INJECTED SUBCUTANEOUSLY.

Table No. 17 illustrates the difference in the reactions of the inflamed subcutaneous tissue of guinea pigs and white mice toward tetanus spores. In this experiment vaccine virus, artificially contaminated with tetanus spores, was tested as follows:

Two drops were planted into each of four fermentation tubes of glucose bouillon, two of which were heated to 80° C. for one hour before incubation, the other two being unheated. Two drops, with the addition of staphylococci, were inoculated into each of two

guinea pigs and two mice. Two drops, containing quinine, were injected into each of two guinea pigs and two mice. Two drops, containing no activator, were inoculated into each of two guinea pigs and two mice. Strong toxin developed in all the fermentation tubes. All the animals contracted tetanus, but greater promptness marked the development of tetanus in the animals in which staphylococci activated the spores than in those in which quinine was the activator. The six guinea pigs and five mice promptly died. One mouse developed slight tetanus and recovered.

On the death of each animal, the entire site of inoculation was scraped up and placed in the ice box at 5° C. for a period of two weeks, at the end of which time the material was planted into fermentation tubes of glucose bouillon and heated for one hour in an Arnold steam sterilizer at 80° C. and then incubated for two weeks. At the end of this time inoculations of the bouillon growth were made into white mice. The cultures from the scrapings from the guinea pigs were all negative for tetanus toxin except one; cultures from the scrapings from the white mice all showed strong tetanus toxin.

Tetanus spores which were thus rendered active immediately upon injection into the subcutaneous tissue of guinea pigs and mice were recovered in cultures from the sites of inoculation in five out of five white mice but in only one out of six guinea pigs. (See Table No. 17.)

Pure tetanus spores inoculated subcutaneously into guinea pigs and white mice remained dormant locally and susceptible of activation by quinine or staphylococcus for four months in the mouse and one month in the guinea pig, generally speaking. (See Table No. 12.)

A more destructive phagocytic action in guinea pigs than in mice would seem to explain the differences indicated above in the behavior of guinea pigs and white mice toward tetanus spores injected into the subcutaneous tissues.

Tetanus spores are more easily activated in guinea pigs than in white mice. See Table No. 7, in which 26 guinea pigs all contracted fatal tetanus after an average incubation period of four days, whereas out of 26 mice which received like amounts of the same material, only 17 contracted tetanus after an average incubation period of eight days; the other nine remained well for 45 or 66 days, when their spores were then activated with staphylococci.

Quinine fails absolutely to activate pure tetanus spores in white mice, whereas it activates spores readily in guinea pigs. (See Table No. 12.)

TETANUS AND VACCINATION IN MONKEYS.

If the occasional case of tetanus which follows vaccination in children is dependent upon the production of toxin by a few stray spores in the vaccination sore, it seems reasonable that an animal

susceptible to both tetanus and vaccinia should contract tetanus when vaccinated in multiple places on his body with vaccine virus heavily contaminated with tetanus spores.

While the calf is very susceptible to vaccination, it is only weakly susceptible to tetanus; two calves vaccinated in the ordinary way on the abdomen and insides of the thighs with virus heavily contaminated with tetanus spores did not contract tetanus during a period of two months following vaccination with contaminated virus. Tests made on the virus collected from the calves are presented in Tables No. 2 and 18.

The rhesus monkey is highly susceptible to vaccinia and is about half as susceptible to tetanus toxin as a guinea pig is, weight for weight. This animal was therefore selected for experiment.

In experiment I, material consisting of a mixture of one-half cubic centimeter of glycerinated vaccine virus and one-half cubic centimeter of a rich suspension of tetanus spores was used to vaccinate five monkeys, each in five places on the back; two drops of the mixture when planted into a glucose fermentation tube developed a toxin, the M.L.D. of which for a white mouse was 0.001 cubic centimeter. By the end of five days each monkey had five pronounced "takes," each of which developed into a raised rough crust which fell off at the end of about two weeks. In the case of one monkey a portion of the crust was removed on the fourteenth day and injected subcutaneously into two guinea pigs, both of which developed tetanus; this same monkey was injected with staphylococci in and around his vaccination sores on the ninth, twenty-second, twenty-third, and twenty-fourth days of vaccination. Another monkey was similarly injected with staphylococci. None of the five contracted tetanus.

In experiment II, vaccine virus plus a suspension of tetanus spores was used to vaccinate each of three monkeys in five places on the back. All had good "takes" but none contracted tetanus. Two drops of the material with which the three monkeys were vaccinated was heated in an ampule at 65° C. for an hour and then planted into each of two fermentation tubes of glucose boullion; tetanus toxin developed in each, the M.L.D. of which for a white mouse was 0.00001 cubic centimeter. One drop of the material with which the monkeys were vaccinated was injected subcutaneously into the hind leg of a monkey. On the third day his leg was rigidly extended but he could open his jaws 1½ inches and close them tightly. The symptoms became more marked and the animal was chloroformed two days later. The site of inoculation was excised, divided into five parts, heated for one hour at 70° C. and planted into five tubes of glucose bouillon, three of which developed toxin, the M.L.D. of which was 0.00001 cubic centimeter; the other two tubes developed no tetanus toxin.

In experiment III, a monkey was vaccinated with plain virus and on the fifth day, when a good "take" had developed, tetanus spores were rubbed into the "take." The animal remained free from symptoms of tetanus.

CLEANLINESS IN COLLECTING VACCINE VIRUS FROM THE CALF.

The effect of mechanical cleansing of the vaccinated area of the calf is shown in the tests presented in Table No. 18. The entire abdomen and insides of the thighs of a calf had been vaccinated with virus to which a rich suspension of spores had been added.

TABLE NO. 18.—*Effect of mechanical cleansing of the vaccinated area of calf before gathering the pulp in reducing the contamination of the product by tetanus spores.*

Date of collection of virus from calf.	Date of testing of virus for tetanus.	Glucose bouillon, unheated.			Glucose bouillon, 80°, 1 hour.			Ordinary bouillon, unheated.		
		Reaction of open arm at end of 24 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.	Reaction of open arm at end of 48 hours.	Reaction of entire contents at end of 9 days.	Mice inoculated with growth at end of 9 days.
VIRUS COLLECTED FROM UNWASHED SIDE OF CALF.										
1913. Mar. 25	1913. Apr. 15	6.6	9.3	1 c. c. neg.	......	2.1	0.0001 tet.	......	......	......
		6.6	......	1 c. c. neg.	......	2.0	.0001 tet.	......	......	......
		4.9	8.9	1 c. c. neg.	......	2.6	.001 tet.	......	......	......
	Apr. 21	......	9.7	1 c. c. neg.	......	1.7	.001 tet.	......	......	......
		......	6.5	1 c. c. neg.	......	1.5	.001 tet.	......	......	......
		......	7.9	1 c. c. neg.	......	1.3	.001 tet.	......	......	......
	Apr. 23	......	10.0	0.01 c. c. tet.	......	2.0	.001 tet.	......	......	......
		......	6.2	1 c. c. neg.	......	1.7	.001 tet.	......	......	......
		......	6.4	1 c. c. neg.	......	2.4	.001 tet.	......	......	......
	Apr. 29	4.8	6.5	1 c. c. neg.	......	......	......	......	2.1	.0001 tet.
		5.2	7.0	1 c. c. neg.	......	......	......	3.2	3.7	.0001 tet.
		5.0	5.5	1 c. c. neg.	......	......	......	3.5	2.8	.0001 tet.
	Dec. 2	1.2	1.5	.0001 tet.	......	......	......	1.0	1.3	.0001 tet.
		3.0	1.5	.0001 tet.	......	......	......	3.0	3.1	.001 tet.
		8.5	5.5	1 c. c. neg.	......	......	......	2.8	3.2	.001 tet.
		1.6	1.8	.0001 tet.	......	......	......	2.4	1.3	.0001 tet.
		9.0	5.2	1 c. c. neg.	......	......	......	4 4	3.3	.0001 tet.
		4.7	4.8	1 c. c. neg.	......	......	......	3.4	4.0	.01 tet.
VIRUS COLLECTED FROM WASHED SIDE OF CALF.										
1913. Mar. 25	1913. Apr. 15	......	7.7	1 c. c. neg.	......	1.5	1 c. c. neg.	......	......	......
		......	6.1	1 c. c. neg.	......	1.8	1 c. c. neg.	......	......	......
		......	7.0	.001 tet.	......	3.0	1 c. c. neg.	......	......	......
	Apr. 21	......	5.5	1 c. c. neg.	......	1.6	1 c. c. neg.	......	......	......
		......	6.7	1 c. c. neg.	......	4.0	1 c. c. neg.	......	......	......
		......	6.2	.001 tet.	......	3.2	1 c. c. neg.	......	......	......
	Apr. 23	......	6.9	1 c. c. tet.	......	1.9	1 c. c. neg.	......	......	......
		......	6.4	1 c. c. neg.	......	1.7	1 c. c. neg.	......	......	......
		......	6.7	1 c. c. neg.	......	3.6	1 c. c. neg.	......	......	......
	Apr. 29	......	6.0	.001 tet.	......	......	......	......	2.3	.001 tet.
		......	8.2	.0001 tet.	......	......	......	......	2.0	.001 tet.
		4.8	5.3	1 c. c. neg.	......	......	......	3.5	3.7	1 c. c. neg.
	Dec. 2	......	3.5	1 c. c. tet.	......	......	......	3.0	3.2	1 c. c. neg.
		4.5	5.5	1 c. c. neg.	......	......	......	......	1.5	.0001 tet.
		4.0	4.3	1 c. c. neg.	......	......	......	4.0	4.0	1 c. c. neg.
		......	1.7	1 c. c. neg.	......	......	......	2.0	1.5	1 c. c. neg.
		......	1.7	1 c. c. neg.	......	......	......	......	3.0	1 c. c. neg.
		......	3.3	1 c. c. neg.	......	......	......	1.7	2.0	.0001 tet.

On March 18, 1913, a calf was vaccinated by linear incisions on the abdomen and insides of the thighs with vaccine virus heavily

contaminated with tetanus spores. Vesicles appeared on the 22d; on the 25th the virus was collected. One longitudinal half of the abdomen and the inside of the corresponding thigh were gently scrubbed with sterile brush and water before collection of the virus. Virus was collected from the other side without any preliminary treatment. The two viruses were ground separately and kept in separate bottles at room temperature throughout the experiment. The emulsion consisted of 20 per cent pulp plus 80 per cent of 50 per cent glycerin in water.

(*a*). *Tests on virus from the unwashed side.*—Within the first two weeks of glycerination, 12 fermentation tubes of glucose bouillon were inoculated with virus from the unwashed side and immediately incubated. With a single exception all 12 tubes at the end of nine days growth were negative for tetanus toxin because of overgrowth of cocci and consequent high acidity. Twelve tubes similarly inoculated but containing either glucose bouillon followed by heating to 80° for one hour or containing ordinary bouillon, all developed low acidities and all produced strong tetanus toxins. Tests made on December 2 after eight months of glycerination indicated strongly that the cocci in the virus had died out considerably because out of six inoculations into glucose fermentation tubes unheated, three showed low acidity and strong toxin production while in the other three the acidity was only moderately high. Six inoculations made December 2 into ordinary bouillon were all productive of low acidities and strong tetanus toxins.

(*b*). *Tests on virus from the washed side.*—The growths in glucose bouillon unheated showed high acidities in April and low acidities in December after eight months of glycerination. We would naturally look for growth of tetanus in the glucose bouillon heated and in ordinary bouillon in both of which low acidities were constant; yet out of 18 such tubes only four showed tetanus.

In all, 18 tests for tetanus upon the virus collected from the unwashed side made in media calculated to restrict acid production were all positive for tetanus, while 18 tests upon virus collected from the washed side made in media favorable to the germination of tetanus spores were all negative for tetanus except four.

Gentle scrubbing with a soft brush and sterile water had produced the effect of nearly removing from half of the vaccinated area a heavy contamination of tetanus spores.

The fact of being able to so completely remove tetanus spores from a vaccination site by a gentle, mechanical superficial cleansing indicates that the vaccination process had very little tendency to carry the spores into the deep tissues of the skin; the spores seemed to have remained on the outside rather than to have followed the vaccine virus into the tissue.

VACCINATION FAVORS THE EJECTION RATHER THAN THE ABSORPTION OF FOREIGN MATTER.

So far as tetanus spores are concerned, the process of vaccination does not seem to offer any specially favorable conditions for their germination; the spores seem to remain extracorporeally within a crust which the body is endeavoring to throw off rather than to absorb. The vaccinated area of a calf was rendered almost free of a heavy contamination of tetanus spores by gentle scrubbing. Nine monkeys with marked vaccination "takes" all failed to contract tetanus in spite of the fact that the crusts from their sores produced tetanus in guinea pigs and subcutaneous injection into a monkey of material with which the monkeys had been vaccinated produced fatal tetanus.

THE AMOUNT OF TETANUS TOXIN AT THE SITE OF INOCULATION OF TETANUS SPORES AND THE AMOUNT IN THE BLOOD.

Scrapings made from the site of inoculation of tetanus spores in guinea pigs and mice contain not only tetanus organisms but usually large amounts of tetanus toxin. If the scrapings from a wound are diluted to the one ten-thousandth part and that part promptly kills a mouse with symptoms of tetanus, there were then at least 10,000 M.L.Ds. of tetanus toxin for a mouse in that wound. Scrapings from 23 mice contained an average for each wound of over 1,700 M.L.Ds. of toxin for a mouse; scrapings from 11 guinea pigs contained an average for each wound of 4,800 M.L.Ds. for a mouse.

In marked contrast to the large amount of toxin which we find in the tissues at site of inoculation of tetanus spores, we find a very small amount in the blood serum.

TABLE No. 19.—*Tetanus toxin in the blood serum of guinea pigs dying from tetanus after subcutaneous injection with tetanus spores.*

No. of guinea pig.	Date of inoculation of guinea pig with tetanus spores.	Date of onset of symptoms in guinea pig.	Blood serum collected from dead guinea pig and injected into fresh animals.	Result.
1	May 24	May 31, tet.†	May 31, 1½ c. c. serum into guinea pig	Remained well.
2	...do	May 28, tet.†	May 28, 1 c. c. serum into guinea pig	May 30, tet. May 31, tet.†
3	May 25	May 27, tet.†	May 28, 4 c. c. serum into guinea pig	May 29, tet.
4	...do	...do	May 28, 2 c. c. serum into guinea pig	May 29, neg.
5	...do	May 27, tet.		
		May 28, tet.†	May 28, 3½ c. c. serum into guinea pig	May 29, neg. May 30, tet.†
6	June 1	June 7, tet.†	June 7, 2 c. c. serum into mouse	June 11, tet. June 14, tet.†
			June 7, 2½ c. c. serum into guinea pig	Remained well.
7	...do	July 3, tet.	July 4, 2 c. c. serum into mouse	
		July 4, tet.†	July 5, 2 c. c. serum into same mouse	July 6, neg. July 7, tet.†
8	June 18	June 22, tet.	June 24, 2 c. c. serum into mouse	
		June 24, tet.†	June 25, 1 c. c. serum into same mouse	Remained well.
9	...do	June 21, tet.†	June 22, 2 c. c. serum into mouse	
			June 23, 1 c. c. serum into same mouse	June 24, tet. June 25, tet.†

† The animal died.

By reference to Table 19 we see that the amount of uncombined toxin in the blood serum of guinea pig dying acutely with tetanus was inappreciable in some instances.

The guinea pigs represented in this table were selected at random. They all died acutely or were chlorformed and were autopsied at the moment of death. The blood was drawn from the heart. After clotting had taken place, the serum was drawn off and injected. The actual number of M.L.Ds. of toxin in the blood serum was not determined, but the indications pointed to a very small number in the most toxic serum.

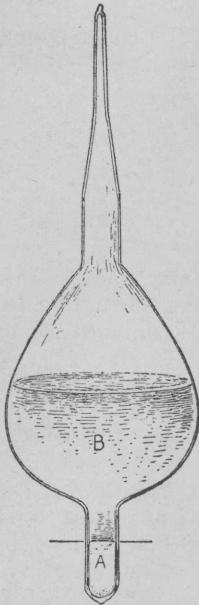


FIG.1. – AMPUL CONTAINING SUSPENSION OF TETANUS SPORES.
A. – SEDIMENT OF TETANUS SPORES.
B. – BOUILLON.

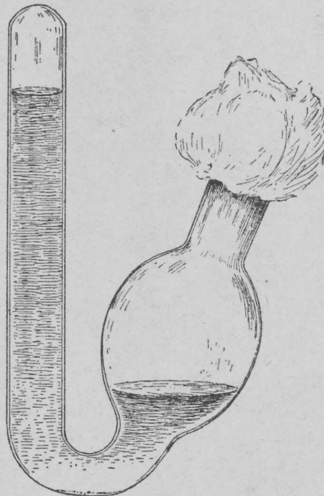


FIG.2 – SMITH FERMENTATION TUBE.

HYGIENIC LABORATORY BULLETINS OF THE PUBLIC HEALTH SERVICE.

The Hygienic Laboratory was established in New York, at the Marine Hospital on Staten Island, August, 1887. It was transferred to Washington, with quarters in the Butler Building, June 11, 1891, and a new laboratory building, located in Washington, was authorized by act of Congress March 3, 1901.

The following *bulletins* [Bulls. Nos. 1-7, 1900 to 1902, Hyg. Lab., U. S. Mar.-Hosp Serv., Wash.] have been issued:

*No. 1.—Preliminary note on the viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 2.—Formalin disinfection of baggage without apparatus. By M. J. Rosenau.

*No. 3.—Sulphur dioxid as a germicidal agent. By H. D. Geddings.

*No. 4.—Viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 5.—An investigation of a pathogenic microbe (*B. typhi murium* Danyz) applied to the destruction of rats. By M. J. Rosenau.

*No. 6.—Disinfection against mosquitoes with formaldehyde and sulphur dioxid. By M. J. Rosenau.

†No. 7.—Laboratory technique: Ring test for indol, by S. B. Grubbs and Edward Francis; Collodium sacs, by S. B. Grubbs and Edward Francis; Microphotography with simple apparatus, by H. B. Parker.

By act of Congress approved July 1, 1902, the name of the "United States Marine-Hospital Service" was changed to the "Public Health and Marine-Hospital Service of the United States," and three new divisions were added to the Hygienic Laboratory.

Since the change of name of the service the bulletins of the Hygienic Laboratory have been continued in the same numerical order, as follows:

*No. 8.—Laboratory course in pathology and bacteriology. By M. J. Rosenau. (Revised edition, March, 1904.)

†No. 9.—Presence of tetanus in commercial gelatin. By John F. Anderson.

*No. 10.—Report upon the prevalence and geographic distribution of hookworm disease (uncinariasis or anchylostomiasis) in the United States. By Ch. Wardell Stiles.

*No. 11.—An experimental investigation of *Trypanosoma lewisi*. By Edward Francis.

*No. 12.—The bacteriological impurities of vaccine virus; an experimental study. By M. J. Rosenau.

*No. 13.—A statistical study of the intestinal parasites of 500 white male patients at the United States Government Hospital for the Insane; by Philip E. Garrison, Brayton H. Ransom, and Earle C. Stevenson. A parasitic roundworm (*Agamomermis culicis* n. g., n. sp.) in American mosquitoes (*Culex sollicitans*); by Ch. Wardell Stiles. The type species of the cestode genus *Hymenolepis*, by Ch. Wardell Stiles.

*No. 14.—Spotted fever (tick fever) of the Rocky Mountains; a new disease. By John F. Anderson.

*No. 15.—Inefficiency of ferrous sulphate as an antiseptic and germicide. By Allan J. McLaughlin.

*No. 16.—The antiseptic and germicidal properties of glycerin. By M. J. Rosenau.

*No. 17.—Illustrated key to the trematode parasites of man. By Ch. Wardell Stiles.

*No. 18.—An account of the tapeworms of the genus *Hymenolepis* parasitic in man, including reports of several new cases of the dwarf tapeworm (*H. nana*) in the United States. By Brayton H. Ransom.

*No. 19.—A method for inoculating animals with precise amounts. By M. J. Rosenau.

*No. 20.—A zoological investigation into the cause, transmission, and source of Rocky Mountain "spotted fever." By Ch. Wardell Stiles.

*No. 21.—The immunity unit for standardizing diphtheria antitoxin (based on Ehrlich's normal serum). Official standard prepared under the act approved July 1, 1902. By M. J. Rosenau.

*No. 22.—Chloride of zinc as a deodorant, antiseptic, and germicide. By T. B. McClintic.

*No. 23.—Changes in the Pharmacopœia of the United States of America. Eighth Decennial Revision. By Reid Hunt and Murray Galt Motter.

No. 24.—The International Code of Zoological Nomenclature as applied to medicine. By Ch. Wardell Stiles.

*No. 25.—Illustrated key to the cestode parasites of man. By Ch. Wardell Stiles.

*No. 26.—On the stability of the oxidases and their conduct toward various reagents. The conduct of phenolphthalein in the animal organism. A test for saccharin, and a simple method of distinguishing between cumarin and vanillin. The toxicity of ozone and other oxidizing agents to lipase. The influence of chemical constitution on the lipolytic hydrolysis of ethereal salts. By J. H. Kastle.

*No. 27.—The limitations of formaldehyde gas as a disinfectant with special reference to car sanitation. By Thomas B. McClintic.

*No. 28.—A statistical study of the prevalence of intestinal worms in man. By Ch. Wardell Stiles and Philip E. Garrison.

*No. 29.—A study of the cause of sudden death following the injection of horse serum. By M. J. Rosenau and John F. Anderson.

†No. 30.—I. Maternal transmission of immunity to diphtheria toxine. II. Maternal transmission of immunity to diphtheria toxine and hypersusceptibility to horse serum in the same animal. By John F. Anderson.

†No. 31.—Variations in the peroxidase activity of the blood in health and disease. By Joseph H. Castle and Harold L. Amoss.

†No. 32.—A stomach lesion in guinea pigs caused by diphtheria toxine and its bearing upon experimental gastric ulcer. By M. J. Rosenau and John F. Anderson.

*No. 33.—Studies in experimental alcoholism. By Reid Hunt.

†No. 34.—I. *Agamofilaria georgiana* n. sp., an apparently new roundworm parasite from the ankle of a negress. II. The zoological characters of the roundworm genus *Filaria* Mueller, 1787. III. Three new American cases of infection of man with horsehair worms (species *Paragordius varius*), with summary of all cases reported to date. By Ch. Wardell Stiles.

†No. 35.—Report on the origin and prevalence of typhoid fever in the District of Columbia. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle. (Including articles contributed by Ch. Wardell Stiles, Joseph Goldberger, and A. M. Stimson.)

†No. 36.—Further studies upon hypersusceptibility and immunity. B. J. Rosenau and John F. Anderson.

†No. 37.—Index-catalogue of medical and veterinary zoology. Subjects: Trematoda and trematode diseases. By Ch. Wardell Stiles and Albert Hassall.

No. 38.—The influence of antitoxin upon post-diphtheritic paralysis. By M. J. Rosenau and John F. Anderson.

†No. 39.—The antiseptic and germicidal properties of solutions of formaldehyde and their action upon toxines. By John F. Anderson.

†No. 40.—1. The occurrence of a proliferating cestode larva (*Sparganum proliferum*) in man in Florida, by Ch. Wardell Stiles. 2. A reexamination of the type specimen of *Filaria restiformis* Leidy, 1880=*Agamomermis restiformis*, by Ch. Wardell Stiles. 3. Observations of two new parasitic trematode worms: *Homalogaster philippinensis*

n. sp., *Agamodistomum nanus* n. sp., by Ch. Wardell Stiles and Joseph Goldberger. 4. A reexamination of the original specimen of *Tænia saginata abietina* (Weinland, 1858), by Ch. Wardell Stiles and Joseph Goldberger.

†No. 41.—Milk and its relation to the public health. By various authors.

†No. 42.—The thermal death points of pathogenic micro-organisms in milk. By M. J. Rosenau.

†No. 43.—The standardization of tetanus antitoxin (an American unit established under authority of the act of July 1, 1902). By M. J. Rosenau and John F. Anderson.

No. 44.—Report No. 2 on the origin and prevalence of typhoid fever in the District of Columbia, 1907. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle.

†No. 45.—Further studies upon anaphylaxis. By M. J. Rosenau and John F. Anderson.

†No. 46.—*Hepatozoon perniciosum* (n. g., n. sp.); a hæmogregarine pathogenic for white rats; with a description of the sexual cycle in the intermediate host, a mite (*lelaps echidnimus*). By W. W. Miller.

No. 47.—Studies on Thyroid: I. The relation of iodine to the physiological activity of thyroid preparations. By Reid Hunt and Atherton Seidell.

†No. 48.—The physiological standardization of digitalis. By Charles Wallis Edmunds and Worth Hale.

No. 49.—Digest of comments on the United States Pharmacopœia. Eighth decennial revision for the period ending December 31, 1905. By Murray Galt Motter and Martin I. Wilbert.

No. 50.—Further studies upon the phenomenon of anaphylaxis. By M. J. Rosenau and John F. Anderson.

No. 51.—Chemical tests for blood. By Joseph H. Kastle.

No. 52.—Report No. 3 on the origin and prevalence of typhoid fever in the District of Columbia (1908). By M. J. Rosenau, Leslie L. Lumsden, and Joseph H. Kastle.

No. 53.—The influence of certain drugs upon the toxicity of acetanilide and antipyrine. By Worth Hale.

No. 54.—The fixing power of alkaloids on volatile acids and its application to the estimation of alkaloids with the aid of phenolphthalein or by the Volhard method. By Elias Elvolve.

No. 55.—Quantitative pharmacological studies; adrenalin and adrenalin-like bodies. By W. H. Schultz.

No. 56.—Milk and its relation to the public health. (Revised edition of Bulletin No. 41.) By various authors.

No. 57.—I. The presence of tubercle bacilli in the circulating blood in clinical and experimental tuberculosis. By John F. Anderson. II. The viability of the tubercle bacillus. By M. J. Rosenau.

No. 58.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary for the period ending December 31, 1906. By Murray Galt Motter and Martin I. Wilbert.

No. 59.—The oxidases and other oxygen catalysts concerned in biological oxidations. By Joseph Hoeing Kastle.

No. 60.—A study of the anatom of Watsonius (n. g.), Watsoni of man and of 19 allied species of mamalian trematode worms of the superfamily Paramphistomoidea. By Ch. Wardell Stiles and Joseph Goldberger.

No. 61.—Quantitative pharmacological studies; Relative physiological activity of some commercial solutions of epinephrin. By W. H. Schultz.

No. 62.—The taxonomic value of the microscopic structure of the stigmal plates in the tick genus Dermacentor. By Ch. Wardell Stiles.

†No. 63.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary (third edition) for the calendar year ending December 31, 1907. By Murray Galt Motter and Martin I. Wilbert.

†No. 64.—Studies upon anaphylaxis with special reference to the antibodies concerned. By John F. Anderson and W. H. Frost.

No. 65.—Facts and problems of rabies. By A. M. Stimson.

†No. 66.—I. The influence of age and temperature on the potency of diphtheria antitoxin. By John F. Anderson. II. An organism (Pseudomonas protea) isolated from water, agglutinated by the serum of typhoid fever patients. By W. H. Frost. III. Some considerations on colorimetry, and a new colorimeter. By Norman Roberts. IV. A gas generator, in four forms, for laboratory and technical use. By Norman Roberts.

†No. 67.—The solubilities of the pharmacopœial organic acids and their salts. By Atherton Seidell.

†No. 68.—The bleaching of flour and the effect of nitrites on certain medicinal substances. By Worth Hale.

No. 69.—The effects of restricted diet and of various diets upon the resistance of animals to certain poisons. By Reid Hunt.

No. 70.—A study of melting-point determinations with special reference to the melting-point requirements of the United States Pharmacopœia. By George A. Menge.

No. 71.—1. Some known and three new endoparasitic trematodes from American fresh-water fish. By Joseph Goldberger. 2. On some new parasitic trematode worms of the genus *Telorchis*. By Joseph Goldberger. 3. A new species of Athesmia from a monkey. By Joseph Goldberger and Charles G. Crane.

†No. 72.—I. Report on an outbreak of typhoid fever at Omaha, Nebr. (1909–1910), by L. L. Lumsden. II. The water supply of Williamson, W. Va., and its relation to an epidemic of typhoid fever. By W. H. Frost.

No. 73.—The effect of a number of Derivatives of Choline and Analogous Compounds on the Blood Pressure. By Reid Hunt and R. de M. Taveau.

†No. 74.—Digitalis standardization and the variability of crude and of medicinal preparations. By Worth Hale.

No. 75.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary (third edition) for the calendar year ending December 31, 1908. By Murray Galt Motter and Martin I. Wilbert.

No. 76.—The physiological standardization of ergot. By Charles Wallis Edmunds, and Worth Hale.

No. 77.—Sewage pollution of interstate and international waters, with special reference to the spread of typhoid fever. By Allan J. McLaughlin.

No. 78.—Report No. 4 on the origin and prevalence of typhoid fever in the District of Columbia (1909). By L. L. Lumsden and John F. Anderson. (Including articles contributed by Thomas B. McClintic and Wade H. Frost.)

†No. 79.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary (third edition) for the calendar year ending December 31, 1909. By Murray Galt Motter and Martin I. Wilbert.

†No. 80.—Physiological studies in anaphylaxis. Reaction of smooth muscle from various organs of different animals to proteins. (Including reaction of muscle from nonsensitized, sensitized, tolerant, and immunized guinea pigs.) By William H. Schultz.

No. 81.—Tissue proliferation in plasma medium. By John Sundwall.

No. 82.—I. Method of standardizing disinfectants with and without organic matter. By John F. Anderson and Thomas B. McClintic. II. The determination of the phenol coefficient of some commercial disinfectants. By Thomas B. McClintic.

†No. 83.—I. Sewage pollution of interstate and international waters with special reference to the spread of typhoid fever. II. Lake Superior and St. Marys River. III. Lake Michigan and the Straits of Mackinac. IV. Lake Huron, St. Clair River,

Lake St. Clair, and the Detroit River. V. Lake Ontario and St. Lawrence River. By Allan J. McLaughlin.

†No. 84. Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary (third edition) for the calendar year ending December 31, 1910. By Murray Galt Motter and Martin I. Wilbert.

†No. 85.—Index catalogue of medical and veterinary zoology. Subjects: Cestoda and cestodaria. By Ch. Wardell Stiles and Albert Hassall.

By act of Congress approved August 14, 1912, the name of the "Public Health and Marine-Hospital Service of the United States" was changed to the "Public Health Service." Since the change in name the bulletins of the Hygienic Laboratory have been issued without break in their numerical order.

†No. 86.—Studies on typhus. By John F. Anderson and Joseph Goldberger.

No. 87.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and on the National Formulary (third edition) for the calendar year ending December 31, 1911. By Murray Galt Motter and Martin I. Wilbert.

No. 88.—Method for determining the toxicity of coal-tar disinfectants, together with a report on the relative toxicity of some commercial disinfectants. By Worth Hale.

No. 89.—Sewage pollution of interstate and international waters with special reference to the spread of typhoid fever. VI. The Missouri River from Sioux City to its mouth. By Allan J. McLaughlin.

No. 90.—Epidemiologic studies of acute anterior poliomyelitis. I. Poliomyelitis in Iowa, 1910. II. Poliomyelitis in Cincinnati, 1911. III. Poliomyelitis in Buffalo and Batavia, N. Y., 1912. By Wade H. Frost.

No. 91.—I. The cause of death from subdural injections of antimeningitis serum. By Worth Hale. II. Some new cholera selective media. By Joseph Goldberger.

No. 92.—Gaseous impurities in the air of railway tunnels. By Atherton Seidell and Philip W. Meserve.

No. 93. Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and on the National Formulary (third edition) for the calendar year ending December 31, 1912. By Murray Galt Motter and Martin I. Wilbert.

No. 94.—I. Collected studies on the insect transmission of *Trypanosoma evansi*. By M. Bruin Mitzmain. II. Summary of experiments in the transmission of anthrax by biting flies. By M. Bruin Mitzmain.

No. 95. Laboratory studies on tetanus. By Edward Francis.

MAILING LIST.

The Service will enter into exchange of publications with medical and scientific organizations, societies, laboratories, journals, and authors. ALL APPLICATIONS FOR THESE PUBLICATIONS SHOULD BE ADDRESSED TO THE **"Surgeon General, Public Health Service, Washington, D. C.,"** EXCEPT THOSE MARKED (*) AND (†).

The editions of the publications marked (*) are no longer available for distribution by the Surgeon General of the Public Health Service. Copies of those marked (†) may, however, be obtained from the Superintendent of Documents, Government Printing Office, Washington, D. C., who sells publications at cost, and to whom requests for publications thus marked should be made.

○