

THE EFFECT OF GENTIAN VIOLET ON THE BACILLUS TETANI, TETANUS TOXIN AND CERTAIN LABORATORY ANIMALS *

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We were led through the work of Churchman¹ on the selective action of gentian violet to investigate the devitalization by this dye of toxic cultures of the bacillus tetani intended for immunization of horses.

The use of phenol, or tricresol, or of toluene fails when applied to tetanus cultures, because of the resistance of the spores and the lability of the toxin. Sedimentation and centrifugalization are uncertain and unsafe even when a preservative is added. Berkefeld filtration with or without antiseptic is effective and safe only if extreme care is taken to prevent the spilling of the culture. One of us² has devised an apparatus to facilitate safe separation of the culture from the oil used to exclude air from the medium.

HISTORICAL

As early as 1886 Pfeffer³ showed that certain anilin dyes are harmful to higher plants. The following year Spina,⁴ Roszahegyi,⁵ and Noegerath⁶ demonstrated the reduction of sodium indigo-sulphonate and methylene blue by certain bacteria.

Bekh working with Penzoldt⁷ early proved the truly selective action of certain dyes as did Cornil and Babes,⁸ while Drigalski and Conradi⁹ later emphasized the selective action of crystal violet upon cocci in their well known medium for the isolation of the bacillus typhosus.

Walker and Murray¹⁰ and Vay¹¹ noted morphological changes in organisms grown on media containing dyes and Dreyer, Krieglér and Walker¹² claimed the lethal time to vary in the ratio of the inverse square of the dye concentration.

* Received for publication September 2, 1914.

1. Jour. Exper. Med., 1912, 16, p. 221.

2. Univ. Cal. Pub. in Path., 1913, 11, p. 97.

3. Quoted by Simon and Wood, Am. Jour. Med. Sc., 1914, 147, p. 247.

4. Centralbl. f. Bakteriöl., Abt. I, Orig., 1887, 11, p. 71.

5. Ibid., p. 418.

6. Fortschr. d. Med., 1888, 6, p. 1; quoted by Churchman, Jour. Exper. Med., 1912, 16, p. 221.

7. Arch. f. exper. Path. u. Pharmacol., 1890, 26, p. 310.

8. Les Bacteries, Paris, Third edition, 1890, p. 76.

9. Ztschr. f. Hyg. u. Infektionskrankh., 1902, 39, p. 283.

10. Brit. Med. Jour., 1904, 11, p. 16.

11. Centralbl. f. Bakteriöl., Abt. I, Orig., 1910, 55, p. 193.

12. Jour. Path. and Bacteriol., 1911, 15, p. 134.

As to the mechanism of the germicidal action of dyes, Kriegler¹³ anticipated the conclusions of Simon and Wood¹⁴ in finding diminished lethal action among certain members of the rosanilin and thiazin groups associated with decreasing basicity. May¹⁵ confirmed this for fuchsin and proved the importance of temperature.

Color is an insignificant element in the germicidal behavior of dyes, Simon and Wood having further shown the activity to rest in the basic auxochromic groups of the triamino triphenyl methanes and certain other dyes.

That dyes other than gentian violet possess selective power for bacteria was shown by Churchman,¹⁶ Signorelli,¹⁷ Krumwiede and Pratt,¹⁸ Smythe,¹⁹ Torrey,²⁰ and DeWitt.²¹ Unna's²² success in Gram's staining method with dyes of the para-rosanilin class may be recalled with interest in this regard.

Dye susceptibility may be overcome through adaptation as shown by Shiga,²³ Fitzgerald and Mackintosh,²⁴ while a closely related point is the discovery of susceptible strains by Churchman and Michael²⁵ in a group usually resistant.

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Throughout these experiments, except where specifically noted to the contrary, a filtered saturated solution of Grüber's gentian violet in distilled water was used. This was prepared by the addition of 2 gm. per 100 c.c. distilled water, which is slightly in excess of the amount, 1.75 gm., stated as necessary for saturation by Stitt,²⁶ or 1.5 gm. by Wood.²⁷ Just previous to each test, the portion used was sterilized by placing the test tube container in boiling water several minutes. For use, dilutions such that 1 c.c. contained 0.1 c.c., 0.01 c.c., 0.001 c.c., etc., of saturated solutions were made in sterile 0.85 percent NaCl. These were boiled a few minutes and then cooled. The volume of medium or culture exposed to the action of the dye was 9 c.c. in each case, the addition of 1 c.c. diluted dye yielding the required dilution as noted in the protocols. Controls having no gentian violet were brought to equal volumes with 0.85 percent NaCl.

13. Centralbl. f. Bacteriol., Abt. I, Orig., 1911, 59, p. 481.

14. Am. Jour. Med. Sc., 1914, 147, pp. 247, 524.

15. Jour. Am. Med. Assn., 1912, 58, p. 1174.

16. Jour. Exper. Med., 1913, 17, p. 373; Ibid., 18, p. 187.

17. Centralbl. f. Bakteriologie, Orig., 1912, 64, p. 496.

18. Proc. New York Path. Soc., 1913, N. S. 13, p. 43; Centralbl. f. Bakteriologie, Abt. I, Orig., 1913, 68, p. 562; Jour. Exper. Med., 1914, 19, p. 20; Ibid., p. 501.

19. Centralbl. f. Bakteriologie, Abt. I, Orig., 1913, 71, p. 319.

20. Jour. Infect. Dis., 1913, 13, p. 263.

21. Ibid., 12, p. 68.

22. Quoted by Benians, Jour. Path. and Bacteriol., 1912, 7, p. 199.

23. Ztschr. f. Immunitätsf., Orig., 1913, 18, p. 65.

24. Proc. Soc. for Exper. Biol. and Med., 1913, 10, p. 149.

25. Jour. Exper. Med., 1912, 16, p. 822.

26. Practical Bacteriology, Blood Work, Parasitology, Appendix, Philadelphia, 1911.

27. Chemical and Microscopical Diagnosis, New York, 1909, p. 683.

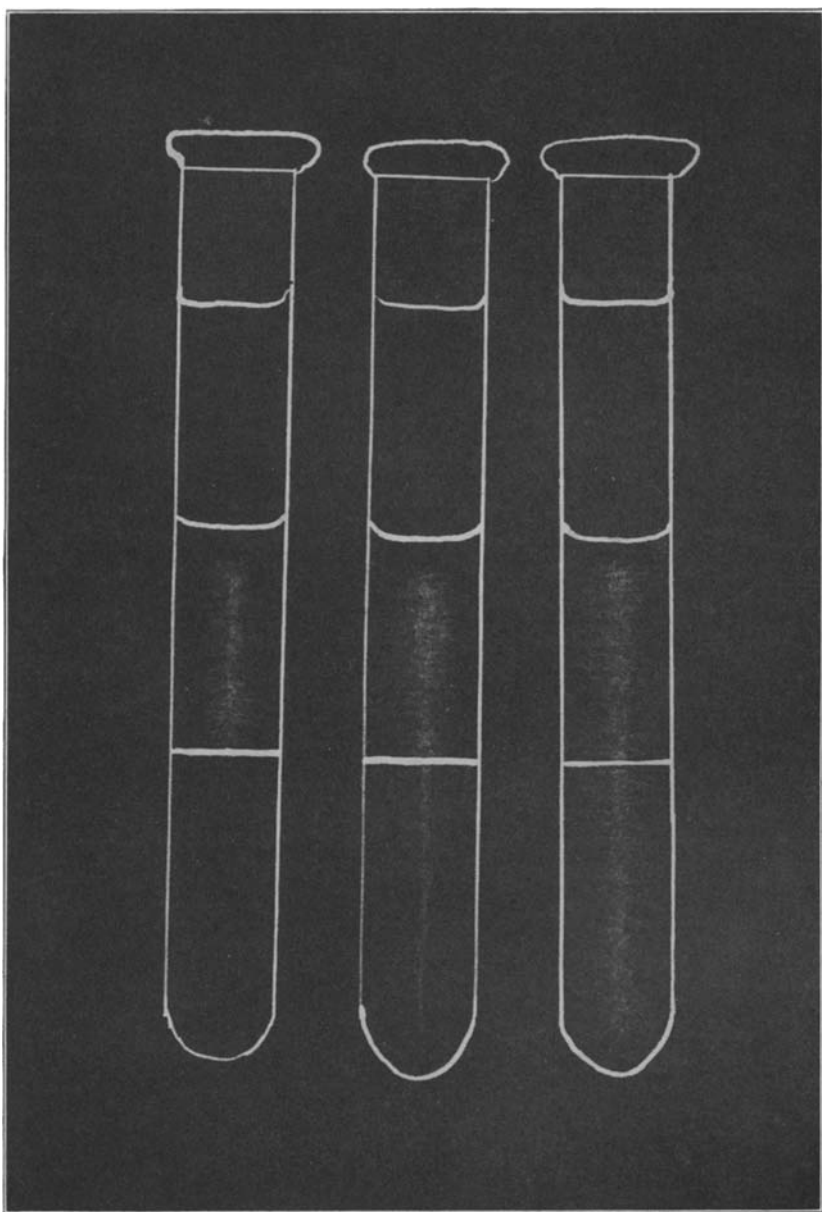


Fig. 1.—Inhibitory effect of gentian violet on the growth of the bacillus tetani in dextrose agar cultures.

A contains 1-1,000 saturated gentian violet, below the paraffin.

B contains 1-10,000 saturated gentian violet, below the paraffin.

C contains no gentian violet.

The strain of the tetanus bacillus used was obtained in April, 1912, from Dr. J. F. Anderson of the Hygienic Laboratory at Washington, D. C.

Experiment 1.—Nine cubic centimeters of sterile neutral 1 percent dextrose agar were placed in each of 3 tubes and the requisite gentian violet solution added, together with a small piece of paraffin. These were boiled 10 to 15 minutes to expel oxygen and melt the paraffin, and were then allowed to cool and harden. Seven to eight cubic centimeters of sterile neutral 1 percent dextrose agar were finally poured into each, leaving the relations within the tubes as in Figure 1. After inoculation from a pure six-day culture of the tetanus bacillus in MgCO_3 broth² by deep agar stab through the paraffin, the cultures were incubated at 37 C. for 3 days, with the results apparent in Figure 1. Thus it appeared that the presence of 1 part saturated gentian violet in 10,000 parts neutral dextrose agar is markedly inhibitory while 1 part in 1,000 prevents visible growth.

The next step was to determine if a similar amount of gentian violet in a broth culture of the tetanus bacillus would destroy the organism or inhibit its growth on subculture. Preliminary experiments had indicated that a larger quantity of gentian violet was necessary to prevent positive subcultures from tetanus emulsions than to inhibit growth when incorporated in the medium. It is not surprising that such is the case in view of the larger number of organisms to be acted upon in the latter instance. Churchman¹ has mentioned that inhibition is more constant when the dye is incorporated in media than when applied directly to the bacterial bodies.

Krumwiede and Pratt¹⁸ have shown that the reaction is quantitative for certain organisms, an observation in accord with our own on the tetanus bacillus as well as on the anthrax bacillus, staphylococcus albus and aureus, and the gonococcus. With these, and Fitzgerald and Mackintosh,²⁴ we have found the presence of serum albumin to partially inhibit the bacteriostatic effect of the dye.

The following experiment was made on the same day with the tetanus suspension, gentian violet dilutions, and dextrose agar used in Experiment 1. The results are therefore comparable:

Experiment 2.—Nine cubic centimeters of suspension were placed in each of 4 test tubes and diluted gentian violet added in each instance to produce a total volume of 10 c.c. with a gentian violet content as noted in the protocol. The mixtures were then incubated at 37 C. for one and one-half hours and shake cultures in neutral 1 percent dextrose agar prepared. A second series of cultures was made after twenty-four hours' incubation, followed in each instance by seventy-two hours' residence in the thermostat at 37 C. Readings were made after seventy-two hours' incubation of the cultures.

Ratio of Saturated Gentian Violet to Mixture	Plants Made After One and One-Half Hours	Plants Made After 24 Hours' Incubation
1-100	Few distinct punctiform colonies	None
1-1,000	Good, diffused slightly	Fair
1-10,000	Marked, very diffuse	Good
None	Marked, very diffuse	Marked

No aerobic growth was observed in any tube, thus indicating freedom from aerobic contamination. The filtrate of a portion of the culture produced severe tetanus in a guinea-pig, weighing 312 gm., in 4 days after the inoculation of 0.00005 c.c.

These experiments justify the conclusion that whereas 1 part saturated gentian violet solution in 1,000 parts of neutral 1 percent dextrose agar prevents visible growth of the tetanus bacillus and 1 part in 10,000 is almost completely inhibitive, 1 part saturated gentian violet in 1,000 parts of 1 percent dextrose broth culture of the tetanus bacillus during contact at 37 C. for twenty-four hours does not preclude a positive subculture on 1 percent dextrose agar, while 1 part in 10,000 under similar conditions yields a subculture as luxuriant as in the case of the control test with no gentian violet.

Experiment 3.—A week old culture of the tetanus bacillus in dextrose broth was divided between two tubes in equal quantities of 9 c.c. each. To Tube A was added 1 c.c. of 1-10 saturated solution of gentian violet in 0.85 percent NaCl. To Tube B was added 1 c.c. 0.85 percent NaCl. Both were incubated 24 hours at 37 C. and were then washed free from toxin by five successive centrifugalizations in 0.85 percent NaCl. The toxic filtrate from the unused portion of culture being proved by test to contain about 10,000 M. L. D. per c.c., five repeated washings may be considered sufficient to remove all but less than 1 M. L. D. of toxin from the bacteria. The sediment from each tube was re-emulsified in 0.85 percent NaCl and injected subcutaneously into a guinea-pig. Aerobic cultures from each sediment showed the presence of a contaminating gram-positive coccus.

As a result of this test both guinea-pigs died from tetanus in 27 and 20 hours, respectively.

Experiment 4.—A new, week-old culture of the tetanus bacillus in dextrose broth was tested for purity by microscopic examination and successfully barren aerobic subculture on an agar slant. It was distributed in quantities of 9 c.c. each in sterile test tubes and 1 c.c. of saturated gentian violet dilutions in 0.85 percent salt solution added so that the final volume of 10 c.c. in each tube contained the proportion of saturated gentian violet solution indicated in Table 1. The mixtures were then incubated for 24 hours at 37 C. and subcultures made by stab to deep 1 percent dextrose agar.

The mixtures, containing respectively 1-10, 1-100, and 1-200, were centrifugalized, the supernatant fluid decanted, and the sediment washed by centrifugalization seven successive times in 0.85 percent NaCl. Each was then injected subcutaneously into a guinea-pig. The result shows that the bacilli may be deemed dead after treatment with 1-10 saturated gentian violet at 37 C. for

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24 hours. But tho they were unable to grow in deep dextrose agar after treatment with 1-600 (and even less) saturated gentian violet for a similar period, the injection reproduced the disease in the guinea-pig after exposure to a dilution of 1-100 parts saturated gentian violet solution.

Exposure in the tube containing 1 part in 10, however, resulted in the destruction of the organisms.

TABLE 1
THE BACTERIO-STATIC ACTION OF GENTIAN VIOLET ON THE BACILLUS TETANI

Experiment	Tube	Final Proportion of Saturated Gentian Violet in Mixture*	Result After Incubation of Sub-culture to Deep Dextrose Agar	Symptoms Following Injection of Mixture into Guinea-Pig†									
				Days									
				1	2	3	4	5	6	7	8	9	10
3	A	1-100	d	+								
	B	None	+									
4	A	1-10	Sterile	—	—	—	—	—	—	—	—	—	—
	B	1-100	Sterile	—	—	a	c	d	d	d	d	d	c
	C	1-200	Sterile	—	—								
	D	1-400	B. tetani	—	—	d	+						
	E	1-600	Sterile										
	F	1-800	Sterile										
	G	1-1000	Sterile										
	H	1-2000	Sterile										
	I	None	B. tetani plus contamination										

* Incubated 24 hours at 37 C.

† — = none; a = slight—i. e. just perceptible; b = marked—pig regains feet when placed on back; c = severe—pig regains feet with difficulty only; d = very severe—pig cannot regain feet; + = death.

The weight of the guinea-pigs ranged from 235-279 gm.

It must be emphasized here that inoculations of media were made with a platinum loop carrying an extremely small number of bacterial bodies as compared to a 5 c.c. syringe. It is not conclusive from this, then, that the organisms successfully propagating in the animal body might not have grown successfully also in the culture tube. In fact, the irregular appearance of a positive culture at 1-400 indicates that the amount of material used to inoculate the culture media was scarcely sufficient to be representative. Possible error then would consist in assuming negative cultures as proof of the death of the organisms while the result of the animal test shows that more than 1 part gentian violet solution to 100 parts culture is necessary to destroy the virulence of such emulsions. On the other hand, we are not justified from these experiments in assuming the presence of living organisms in even washed cultures which produce tetanus, since there may be sufficient toxin adherent to the possibly dead bodies to cause symptoms and death from tetanus.

THE EFFECT OF GENTIAN VIOLET ON TETANUS TOXIN

The determination of the effect of gentian violet on the toxin of tetanus presents less difficulties than the preceding phase of the subject since the bacillus and its spores may be eliminated by Berkefeld filtration of a toxic culture.

Experiment 5.—In this test, use was made of a solution of the Hygienic Laboratory test toxin "D" in 0.85 percent NaCl containing 0.0006 gm. dried tetanus toxin per c.c. To 9.9 c.c. in test tube "A" was added 0.1 c.c. saturated aqueous gentian violet, thus making a dilution of 1-100. To a similar amount in Tube "B" was added 0.1 c.c. distilled water. Both were incubated one-half hour at 37 C. Dilutions from each were made by adding 1 c.c. to 99 c.c. 0.85 percent NaCl solution. One cubic centimeter of this dilution should contain approximately 1 M. L. D. and this amount was injected from each into separate guinea-pigs. There was no perceptible difference between the results in these animals, both of which developed mild tetanus on the second day, very severe tetanus on the third day, and died within the same hour late on the third day after injection.

Experiment 6.—To 9 c.c. tetanus culture filtrate was added 1 c.c. saturated aqueous gentian violet in Tube "A." Similarly to 9 c.c. in Tube "B" was added 1 c.c. 0.85 percent NaCl. Both mixtures were incubated at 37 C. for 24 hours and then each diluted to 1-1,000. One cubic centimeter of each dilution was injected subcutaneously into a guinea-pig with the result that the guinea-pig receiving the gentian violet treated toxin did not show symptoms of tetanus in 11 days, while the control developed tetanus on the second day, and died of the disease on the sixth day after injection.

It may be noticed in these preliminary experiments that there are three important factors at variance, namely, the strength of gentian violet, the strength of toxin, and the time of exposure. Another experiment was therefore done in order to ascertain the effect of variation in the strength of gentian violet alone.

Experiment 7.—To each of 4 test tubes containing 9 c.c. fresh tetanus toxin was added 1 c.c. gentian violet dilution in 0.85 percent NaCl such that the mixtures contained respectively 1-10, 1-100, 1-1,000, and no gentian violet in solution. These were incubated at 37 C. for 24 hours. Dilutions were then made in 0.85 percent NaCl such that 1 c.c. contained approximately 1 M. L. D., i. e., 0.00012 c.c. tetanus toxin, and this amount was injected subcutaneously into a normal guinea-pig. All of these guinea-pigs died of tetanus, excepting the one receiving toxin treated with gentian violet 1-10, this animal surviving without symptoms of any sort. Table 2 is a summary of Experiments 5, 6, and 7.

Since a dilution of 1-10 is the least amount, within the limits tested, of gentian violet which suffices to kill tetanus spores, it is obviously impossible to devitalize tetanus cultures by this method and at the same time retain the full toxicity of the filtrate. We are now interested in determining how complete the destruction of toxin might be in the presence of this strength of the dye.

TABLE 2
THE EFFECT OF GENTIAN VIOLET ON TETANUS TOXIN

Mixture of Gentian Violet with Tetanus Toxin					Symptoms Following Subcutaneous Injection of 1 M. L. D.*											
Experi- ment	Tube	Proportion of Saturated Aqueous Gentian Violet Solution	Potency of Tetanus Toxin per c.c.	Time of Incuba- tion of Mixture in Hours	Guinea-Pig Weight in Grams	Days										
						1	2	3	4	5	6	7	8	9	10	
5	A	1-100 None	100 M. L. D. 100 M. L. D.	0.5 0.5	410 455	..	a a	++								
	B															
6	A	1-10 None	1000 M. L. D. 1000 M. L. D.	24 24	398 392	—	a —	b —	c —	d —	— +	— —	— —	— —	— —	— —
	B					—	—	—	—	—	—	—	—	—	—	—
7	A	1-10	8000 M. L. D.	24	330	—	—	—	d	+	d	—	—	—	—	—
	B	1-100	8000 M. L. D.	24	335	—	—	c	d	d	d	+	—	—	—	—
	C	1-1000	8000 M. L. D.	24	340	—	—	c	d	d	d	d	+	—	—	—
	D	None	8000 M. L. D.	24	345	—	a	a	a	b	d	d	+	—	—	—

* — = none; a = slight—i. e., just perceptible; b = marked—pig regains feet when placed on back; c = severe—pig regains feet with difficulty only; d = very severe—pig cannot regain feet; + = death.

Experiment 8.—A fresh saturated solution of gentian violet was made in 0.85 percent NaCl. One cubic centimeter of this solution was added to 9 c.c. of a potent tetanus toxin, and at the same time, 1 c.c. of 0.85 percent NaCl was added to another tube containing 9 c.c. of this toxin. These mixtures were placed in the thermostat room at 37 C. for 24 hours. Dilutions in 0.85 percent NaCl, ranging from 0.2 to 0.0002 c.c. toxin per c.c. were then made, as outlined in the protocol below, and guinea-pigs injected subcutaneously with 1 c.c. of each dilution. Similarly, a guinea-pig received 0.0002 c.c. of the toxin to which only the salt solution had been added in 1 c.c. of 0.85 percent NaCl.

TABLE 3
PROPORTION OF TETANUS TOXIN DESTROYED BY CONTACT WITH 10 PERCENT SATURATED GENTIAN VIOLET

Toxin Content of 1 c.c. Diluted Mixture	Weight of Guinea-Pig	Symptoms after Subcutaneous Injection of 1 c.c.*									
		Days									
		1	2	3	4	5	6	7	8	9	10
0.2	480	—	b	d	+	—	—	—	—	—	—
0.02	495	—	a	b	b	b	c	b	?	b	b
0.002	485	—	—	?	a	a	—	b	?	a	a
0.0002	475†	—	—	—	—	—	—	—	+	—	—
0.0002 (Control)	475	+	—	—	—	—	—	—	—	—	—

* — = none; a = slight—i. e., just perceptible; b = marked—pig regains feet when placed on back; c = severe—pig regains feet with difficulty only; d = very severe—pig cannot regain feet; + = death.

† Death not due to tetanus.

The results, as shown in Table 3, indicate that there is almost total, tho not complete, destruction of toxicity under the influence of 1 part saturated gentian violet solution to 9 parts tetanus toxin, when incubated twenty-four hours at 37 C. It may be noted here that the toxin was very powerful; there must have been many times the M.L.D. given to the control guinea-pig in 0.0002 c.c. untreated toxin. Yet a dose ten times as large of treated toxin failed to cause more than slight symptoms of tetanus during a period of ten days of observation, a dose one hundred times as large gave only moderate symptoms, and a thousand times as much was required to kill the injected animal with tetanus, which it did upon the fourth day.

Assuming that 0.0002 c.c. of the toxin contained only 1 M.L.D. for the test animal, 0.02 c.c. of the toxin would contain 100 M.L.D., and death should occur in a few hours after injection. The symptoms of the animal receiving this amount of treated toxin, however, indicate the presence of less than 1 M.L.D. of potent toxin, which signifies

the destruction of over 99 percent of the toxin by the action of 0.1 saturated solution of gentian violet. The guinea-pig which received 0.0002 c.c. treated toxin died upon the eighth day, but not of tetanus. The cause of death was not ascertained.

These experiments on tetanus toxin yielded such regular results that we were surprised that Churchman²⁸ found gentian violet to give inconstant results with tetanus toxin. Dr. Meyer²⁹ considers adsorption the basis of the destructive action of the dye, a view which finds affirmation in our experiments.

THE TOLERANCE OF RABBITS AND GUINEA-PIGS TO GENTIAN VIOLET

The purpose of recording the tests below is to demonstrate the surprisingly slight toxicity of injections of gentian violet for small animals.

Subcutaneous injections.—Following are the details of a test made upon three guinea-pigs by a single injection each of the dye subcutaneously. A saturated solution of Grüber's gentian violet in distilled water was used. Immediately previous to use, it and the dilutions made therefrom, in 0.85 percent NaCl, were sterilized by boiling. On cooling, the injections were made in each instance with the usual aseptic precautions.

Guinea-pig A, weight 592 gm., was injected subcutaneously with 1 c.c. saturated aqueous gentian violet. In 24 hours a marked induration appeared. In four days there was an ulcerated phlegmon involving necrosis of the inguinal glands. This animal recovered fully after daily treatment for two weeks with 2 percent tricresol solution to prevent infection. During this period the odor from the wound was very disagreeable. The animal was in splendid condition two and one-half months later.

Guinea-pig B, weight 440 gm., was injected subcutaneously with 1 c.c. of 10 percent saturated gentian violet in 0.85 percent NaCl. In five days a slight induration was noticed. In nine days there was a small open ulcer. This pig recovered fully in two weeks without treatment, and remained in good condition for at least two and one-half months.

Guinea-pig C, weight 250 gm., was injected subcutaneously with 0.5 c.c. of 10 percent saturated gentian violet in 0.85 percent NaCl. There were no visible effects or lesions and the animal was released from observation after three weeks.

A number of tests not noted herein had indicated that 10 c.c. of 1 percent saturated gentian violet in 0.85 percent NaCl has no apparent effect in subcutaneous injections of rabbits. In order to determine the result of repeated injections two animals were placed in a cage, the intention being to inject the heavier one, designated Rabbit A, weight 2,400 gm., subcutaneously every few days with 10 c.c. of a sterile 1 percent saturated solution of gentian violet in 0.85 percent NaCl. Rabbit B, weight 2,150 gm., was to be observed without

28. Jour. Am. Med. Assn., 1913, 61, p. 302; Proc. Soc. for Exper. Biol. and Med., 1914, 11, p. 54.

29. Personal communication.

injection as a control upon Rabbit A. After four days, it was found that both rabbits had lost approximately 15 and 10 percent in weight, respectively. Inquiry indicated fighting as a probable cause and they were therefore separated, with a rapid increase in weight to about normal. At this time the plan of experiment was changed so that Rabbit B received 10 c.c. of sterile 0.85 percent NaCl subcutaneously, at the same time that A was treated with 10 c.c. of 1 percent gentian violet solution. In all, A received five 10 c.c. doses of 1 percent saturated gentian violet solution; B received three 10 c.c. doses of 0.85 percent NaCl. Table 4 shows that the relative weights of these animals had interchanged at the end of one month altho there was no sign of disturbance of function in either, except slight swellings at the site of inoculation in Rabbit A after the second and third injections. These disappeared rapidly in about thirty-six hours.

TABLE 4

COMPARATIVE WEIGHTS OF RABBITS RECEIVING SUBCUTANEOUS INJECTIONS OF GENTIAN VIOLET AND 0.85 PERCENT SALT SOLUTION

Day of Observation	Rabbit A		Rabbit B	
	Treatment	Weight in Grams	Treatment	Weight in Grams
1	10 c.c. 1 percent satur. gentian violet s. c.	<i>2400</i>	None	2150
3	10 c.c. 1 percent satur. gentian violet s. c.	<i>2260</i>	None	2120
5*	10 c.c. 1 percent satur. gentian violet s. c.	2025	10 c.c. 0.85 percent NaCl	1940
8	10 c.c. 1 percent satur. gentian violet s. c.	<i>2360</i>	10 c.c. 0.85 percent NaCl	2260
10	10 c.c. 1 percent satur. gentian violet s. c.	<i>2350</i>	10 c.c. 0.85 percent NaCl	2200
14	10 c.c. 1 percent satur. gentian violet s. c.	<i>2280</i>	10 c.c. 0.85 percent NaCl	2180
16	10 c.c. 1 percent satur. gentian violet s. c.	<i>2200</i>	10 c.c. 0.85 percent NaCl	2150
18	10 c.c. 1 percent satur. gentian violet s. c.	<i>2325</i>	10 c.c. 0.85 percent NaCl	2270
21	10 c.c. 1 percent satur. gentian violet s. c.	<i>2140</i>	10 c.c. 0.85 percent NaCl	<i>2290</i>
23	10 c.c. 1 percent satur. gentian violet s. c.	<i>2160</i>	10 c.c. 0.85 percent NaCl	2275
25	10 c.c. 1 percent satur. gentian violet s. c.	<i>2200</i>	10 c.c. 0.85 percent NaCl	<i>2345</i>
29	10 c.c. 1 percent satur. gentian violet s. c.	<i>2240</i>	10 c.c. 0.85 percent NaCl	<i>2350</i>

* Animals previously in one cage; now separated.

The weight of the heavier animal at each observation is printed in *italics* to indicate the fact more clearly.

These tests confirm the observations of Churchman and Herz³⁰ as to the possibility of treating animals with considerable amounts of dye without material harm. We have gained the idea that subcutaneous injections are more or less irritating locally, but not fatal in comparatively large repeated doses. In some instances certain glands, near the site of injection, appeared particularly susceptible.

Intravenous injections.—Rabbit C, half-grown, was subjected to the intravenous inoculation of 1 c.c. saturated gentian violet in distilled water without visible symptoms of any sort up to the eighth day. At that time, a second intravenous injection was attempted but most of the stain was injected into the subcutaneous tissues near the base of the ear. The following day the jaw and ear were badly swollen and five days following the injection the animal was killed because of its obvious suffering. On necropsy the viscera were normal with the exception of the intestines, which were cyanotic. The ear and face were much swollen on the side of the injection and one section displayed the parotid gland neatly outlined in pale blue with the connective tissues surrounding it presenting a jelly-like appearance through the presence of edema fluid. The ear was edematous and thickened, but not markedly stained.

Rabbit D, an adult, weight 2,590 gm., a month previous subjected to a non-virulent subcutaneous injection of the staphylococcus aureus, was inoculated intravenously with 1 c.c. saturated aqueous solution of gentian violet on three successive days. These injections were successful excepting the last, of which approximately 0.1 c.c. escaped into the subcutaneous tissue of the ear. There were no signs of discomfort of the animal nor changes in the tissues until the following morning when this ear was found slightly edematous and paralyzed. The rabbit was dead the next day.

Necropsy revealed a marked subcutaneous edema of the ear and a markedly hemorrhagic condition of the lungs; all of the other organs appeared normal, particularly the heart, liver, stomach, spleen, kidneys, intestines, mesentery, and peritoneum; heart blood cultures gave a gram-negative coliform bacillus.

Rabbit E, an adult, which had been used before, was injected intravenously with 1 c.c. saturated aqueous gentian violet. There was a slight swelling and paralysis of the ear, due, no doubt, to faulty technic of injection, but the animal survived until the sixteenth day, when it was found dead. Necropsy proved death due to a staphylococcal abscess of the pleural cavity.

At the same time a similar animal, Rabbit F, was injected intravenously with 5 c.c. of a saturated aqueous solution of gentian violet. The lips, ears, tongue, and eyelids became intensely purple in a few minutes followed immediately by stupor, loss of motor power, and motor reflexes. The animal quickly became unconscious and during a paroxysm of choking, in which the urine was voided, it died, a period of about fifteen minutes having elapsed since the injection. Necropsy showed the stain distributed throughout the body and especially notable in visceral organs.

Of interest in this connection is a series of observations upon another rabbit. This animal, Rabbit G, had previously been injected intravenously, without observable effect, with 2 c.c. saturated gentian violet in 0.85 per cent NaCl. After the outcome of the injections of Rabbits E and F, 5 c.c. of a sterile saturated solution of gentian violet in 0.85 percent NaCl, were injected intra-

30. Jour. Exper. Med., 1913, 18, p. 579.

venously, a period of ten weeks having elapsed since the first injection. No observable effects followed this treatment. A week later, 10 c.c. were injected intravenously, using, in all intravenous injections, the marginal vein of the ear. Again there were no indications of discomfort or illness.

We desire to note here that the solution used for the last two injections was several months old, but if the age of the solution is a factor in the toxicity of gentian violet, it did not operate in the case of the saturated solution of gentian violet in 0.85 percent NaCl which was made at about the same time as the aqueous solution and kept under the same conditions of room temperature and diffuse light. For when 5 c.c. of the latter, i e., the old aqueous solution, were injected intravenously two weeks later, the rabbit, now very large, weighing 2,700 gm., was dead in three minutes after a succession of symptoms essentially like those described for Rabbit F. Immediate necropsy showed the mucosa and viscera deeply stained, but, altho the animal was very fat, there was no adsorption of the dye by the fat, which stood out prominently upon the reddish purple background of the stained tissues.

That 5 c.c., or even 10 c.c., distilled water injected intravenously does not cause sudden death was easily demonstrated in Rabbit H, weight 1,240 gm., which was so treated. There was no evident discomfort, altho the weight of this rabbit was less than half that of Rabbit G.

Further comparison between aqueous solutions and salt solutions of gentian violet should be made. Our experience indicates a change in technic from saturated solutions to solutions containing definite proportions, less than saturation, of solid dye.

Intravenous injections of gentian violet are thus more toxic than subcutaneous inoculations, the dye being quickly diffused and sudden death occurring when 5 c.c. or more of aqueous solution were injected in the ear vein.

SUMMARY

Gentian violet distinctly inhibits the growth of the tetanus bacillus when 1 part saturated solution is added to 10,000 of dextrose agar. In dextrose broth, however, exposure for twenty-four hours at 37 C., at this concentration, has no perceptible effect on subcultures, tho cultures failed from broth containing 1 part in 100. The organisms are not destroyed, however, as can be shown by animal inoculations. One part saturated solution in ten of broth destroys the virulence of a toxic culture.

At this concentration more than 99 percent of the toxin is destroyed by contact at 37 C. for twenty-four hours. One part in one hundred has no perceptible effect.

The necessity of using more than a 1 percent saturated solution for devitalization precludes the employment of gentian violet for the practical purpose attempted through excessive soiling of laboratory ware. Within the limits tested, the toxin appears to be destroyed under practically the same conditions as the spores.

Guinea-pigs and rabbits withstand considerable injections of gentian violet in 0.85 percent NaCl. Distilled water solutions appear to be more toxic than those in salt solution, and subcutaneous injections less than intravenous injections.