TOXIN-ANTITOXIN REACTION WITHOUT NEUTRALIZATION

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It seems probable that the recent progress in the study of antigen and antibody reactions, particularly of agglutination and phagocytosis, is due to the circumstance that they can be studied on the surface of particulate matter (1). It seems similarly desirable to investigate the reactions between toxins and antitoxins on the surface of simple particles. The experiments to be reported are a study of how toxin and antitoxin react when they are in contact, the one adsorbed on collodion particles and the other in solution.

EXPERIMENTAL

Collodion particles were prepared according to Loeb's (2) method as modified by Kunitz (3). Collodion U.S.P. is poured in 2 l. of water while the mixture is being stirred. The collodion that is precipitated is washed with water several times, then pressed by hand and then between filter papers. The dry collodion is cut into small pieces and dissolved in a small amount of acetone at about 40°C. in a beaker. The beaker containing the collodion solution is placed in water at a temperature of about 40°C. Then while the acetone-collodion solution is stirred mechanically a mixture of water and acetone (three parts of water and one part of acetone) is added to it from a funnel through a capillary pipette, drop by drop, until a heavy gelatinous mass is formed. The supernatant cloudy fluid is poured into a flask and diluted with water. When the water is added to the collodion suspension more collodion particles are formed. The acetone can be removed from the suspension either by vacuum distillation at from 35° to 40°C. or by washing the particles with the aid of the centrifuge. The gelatinous mass of collodion from which the supernatant suspension was poured is dissolved in acetone and mixed with water-acetone mixture and treated as described above. The large particles are separated by centrifugalization and discarded. The final suspension contains particles of about the size of staphylococci.

Experiments with Diphtheria Toxin[1]

In previous papers (4) it was reported that collodion particles treated with diphtheria toxin are flocculated by diphtheria antitoxin and that

[1] The toxin and antitoxin preparations were obtained through the courtesy of Professor Karl F. Meyer and the H. K. Mulford Co.

when collodion particles treated with diphtheria toxin are injected into the skin of guinea pigs inflammation follows, which can be prevented by the simultaneous injection of diphtheria antitoxin.

In the present study the question was investigated whether toxin is neutralized when (1) collodion particles treated with toxin are suspended in antitoxin, and (2) when the order of treatment of particles is reversed, *i.e.*, when they are treated first with antitoxin and then with toxin.

The M.L.D. of the toxin was 0.003 cc. The unconcentrated antitoxic horse serum contained 500 units per cubic centimeter.

1. Treatment of Particles with Diphtheria Toxin.—1 cc. of a heavy suspension of collodion particles in saline solution was added drop by drop to 5 cc. of 1:10 dilution of diphtheria toxin. The mixture was gently shaken for 5 minutes and centrifugalized. The supernatant fluid was discarded, the wall of the tube wiped with filter paper, the particles suspended in 40 cc. of saline and washed three times. (The last washing fluid gave negative skin test in guinea pigs.) The particles were suspended in 0.5 cc. saline, and 0.25 cc. injected into the skin of a guinea pig or rabbit.

2. Treatment of Particles with Diphtheria Toxin and Antitoxin.—After the particles had been treated as described above, they were suspended in 5 cc. of a 1:10 dilution of diphtheria antitoxin, gently shaken for 5 minutes, washed three times, suspended in 0.5 cc. of saline; 0.25 cc. of the suspension was used for skin test.

3. Treatment of Particles with Diphtheria Toxin and Tetanus Antitoxin.—The same amount of the collodion suspension and the same amount of the diluted diphtheria toxin was used as in Experiment 1. After the particles were washed three times they were suspended in 5 cc. of a 1:10 dilution of tetanus antitoxin, shaken 5 minutes, centrifugalized and washed three times. The particles were then suspended in 0.5 cc. saline and 0.25 cc. injected into the skin of a rabbit or guinea pig.

4. Treatment of Particles with Diphtheria Antitoxin and Toxin.—Treatment of particles as described under 2 but in reversed order, first with antitoxin, then with toxin.

5. Treatment of Particles with Tetanus Antitoxin and Diphtheria Toxin.—The particles were treated as described under 3, but in reversed order.

Untreated collodion particles cause a nodule when injected into the skin of guinea pigs or rabbits; the skin over the nodule is not discolored. In the presence of the adsorbed toxin the nodule is usually larger, the skin becomes red or purple and it may undergo superficial necrosis. The discoloration of the skin usually appears 2 days after inoculation and it may increase for 2 days. Positive skin reactions

were observed only in guinea pigs weighing more than 500 gm. and not all guinea pigs used for testing reacted. Rabbits reacted more

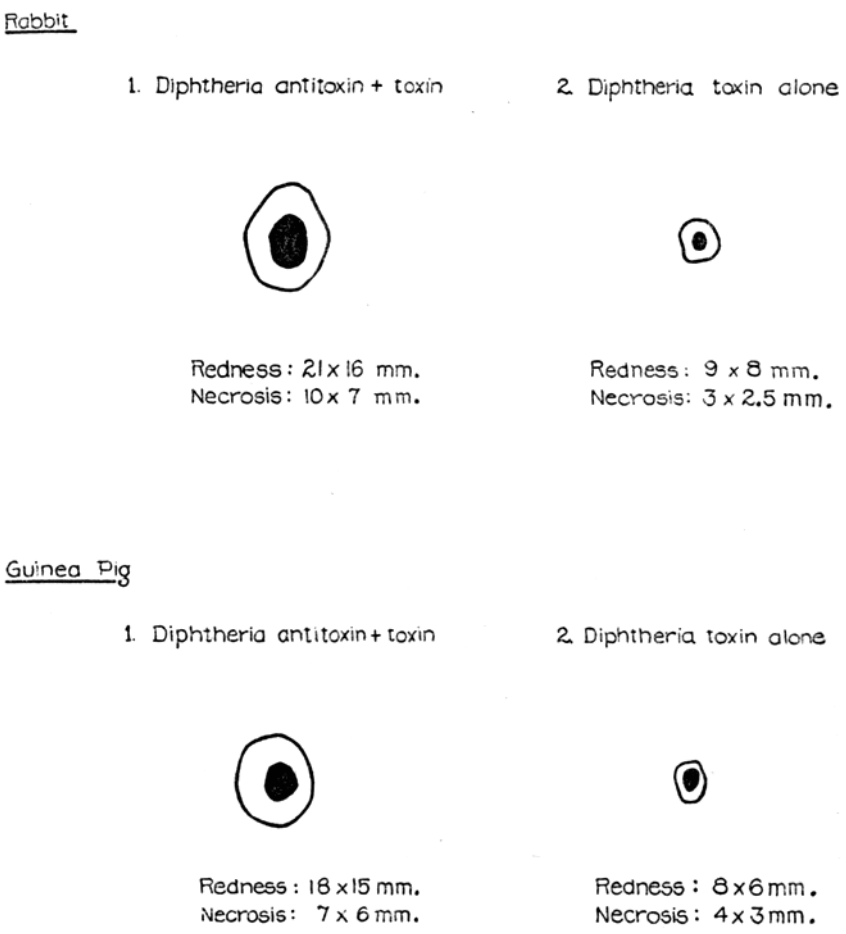

DIAGRAM 1. Area of redness and necrosis at site of injection of treated collodion particles.

uniformly. It was found that collodion particles treated with toxin and then with antitoxin produced a nodule over which the skin remained normal. With toxin and normal serum the reaction was either the same as with toxin alone or weaker. Particles treated first with

normal serum or tetanus antitoxin and then with toxin caused no reaction. When, however, collodion particles treated first with diphtheria antitoxin and then with toxin were injected the skin over the nodule became inflamed and necrotic; the inflammation was strikingly more intense than with particles treated with toxin alone.

Diagram 1 illustrates the areas of redness and necrosis in rabbits and guinea pigs caused by injections of treated collodion particles.

These experiments may be summarized as follows:

	On the collodion particles		*In solution*		*Collodion particles*
I	Diphtheria toxin	+	Diphtheria antitoxin	:	Not toxic
II	Diphtheria toxin	+	Tetanus antitoxin	:	Toxic
III	Diphtheria antitoxin	+	Diphtheria toxin	:	Toxic
IV	Tetanus antitoxin	+	Diphtheria toxin	:	Not toxic

Experiment with Tetanus Toxin

The M.L.D. of the toxin was 0.0001 cc. The unconcentrated antitoxic horse serum contained 700 units per cubic centimeter.

In the first experiment 1 cc. of the suspension of collodion particles was treated with 5 cc. of a 1:10 dilution of tetanus toxin. After the particles had been washed and suspended in 0.5 cc. of saline 0.1 cc. of the suspension was injected into the right hind leg of a mouse. The mouse had local tetanus in 2 days and died in 4 days after the injection. Mice injected with the last washing fluid remained free from tetanus. When 0.1 cc. of the suspension of collodion particles treated with toxin was suspended in 5 cc. of tetanus antitoxin diluted from 1:10 to 1:50,000 and washed no symptoms were produced in mice. Diphtheria antitoxin had no effect on the toxicity of the particles.

After it had been found that tetanus toxin is adsorbed on collodion particles and is neutralized by tetanus antitoxin, but not affected by normal horse serum or diphtheria antitoxin, experiments were performed to ascertain whether the order of treatment would influence the result, *i.e.*, whether antitoxin *adsorbed* on the particles would neutralize the toxin. Therefore, particles were treated: (*a*) first with tetanus antitoxin and then with tetanus toxin; (*b*) first with diphtheria antitoxin and then with tetanus toxin.

In these experiments the dilutions of serums and toxin were varied. With antitoxin dilution 1:1 and toxin dilution 1:1 or 1:10, with antitoxin dilution 1:10 and toxin dilution 1:1 or 1:10 the mice in the experiments with diphtheria antitoxin and tetanus toxin showed no symptoms at all; in the experiments with tetanus antitoxin and tetanus toxin they either died or had severe symptoms of local

tetanus. When antitoxin was used in dilution 1:10 and toxin 1:100 the mice in both types of experiment remained free from tetanus.

When these results are summarized they are seen to parallel the results of the similar experiment with diphtheria toxin.

	On the collodion particles		*In solution*		*Collodion particles*
I	Tetanus toxin	+	Tetanus antitoxin	:	Not toxic
II	Tetanus toxin	+	Diphtheria antitoxin	:	Toxic
III	Tetanus antitoxin	+	Tetanus toxin	:	Toxic
IV	Diphtheria antitoxin	+	Tetanus toxin	:	Not toxic

When the tetanus toxin was fresh (1 month old) the collodion particles treated with the antitoxin and then the toxin were either as toxic or even more toxic than particles treated with toxin alone. When the toxin was 8 months old the particles treated with antitoxin were not so toxic as particles treated with toxin alone. In several experiments the particles were treated with normal horse or rabbit serum, and in one experiment with egg white diluted 1:10 before they were mixed with tetanus toxin. Such particles remained non-toxic like those treated first with diphtheria antitoxin and then with tetanus toxin.

The experiments with tetanus toxin showed that:

1. Collodion particles adsorbed tetanus toxin and retained it in salt solution, but the adsorbed toxin was at least in part released in the animal.

2. The adsorbed toxin was neutralized by suspending the particles coated with toxin in a dilution of antitoxin. It is not clear from this experiment whether the neutralization occurs on the collodion particles before injection, for it is possible that the toxin is neutralized after both toxin and antitoxin have been released in the animal.

3. The neutralization is specific.

4. If collodion particles are treated first with diphtheria antitoxin or normal horse serum or egg white and then with tetanus toxin, the particles are not toxic for mice.

5. If collodion particles are treated first with tetanus antitoxin then with tetanus toxin the particles become toxic for the animal. Since particles first treated with diphtheria antitoxin and then with tetanus toxin are not toxic, the toxicity must be due to the action of tetanus antitoxin upon the tetanus toxin.

These results can be explained by assuming that the antitoxin adsorbed on the particles is able to adsorb toxin without being able to neutralize it. Since the toxin adsorbed on particles that had been previously treated with antitoxin remains unneutralized, the question arises whether or not the adsorbed toxin is susceptible to neutralization by antitoxin. It was found that when particles treated first with antitoxin and then with toxin were treated again with antitoxin they produced no symptoms in mice.

The minimal lethal dose of tetanus toxin that had been in contact with particles treated either with tetanus or diphtheria antitoxin was found to be the same and hardly different from that of the toxin before adsorption. Apparently the particles adsorb so little toxin that the difference might escape detection.

Experiments with Botulinus Toxin

These experiments did not produce consistent results. The toxin was adsorbed and retained on collodion particles; however in some experiments the collodion particles treated with toxin and antitoxin proved to be toxic. When collodion particles were treated first with tetanus or *botulinus* antitoxin and then with toxin, and injected into mice, the results varied so much that no conclusions could be drawn.

DISCUSSION

Toxins are adsorbed by various colloids: charcoal, colloidal iron hydroxide, kaolin (5). In the present study a negatively charged colloid, a suspension of collodion particles, was used for the adsorption of toxins and antitoxins. Collodion particles seem to have the advantage over other adsorbents that collodion is insoluble in water, is changed in the animal only with difficulty, if at all, is suitable for studying flocculation reactions and can be used in cataphoretic studies. Loeb, Northrop, Hitchcock and Kunitz (6) have used collodion as adsorbent in studying properties of protein that cannot be observed in solution. In connection with a study on the surface properties of tubercle bacilli, I found that collodion particles coated with egg white are flocculated by anti-egg white serum (7). Jones reported that collodion particles can be coated with various proteins and flocculated with precipitin serums (8). Mudd, Lucké, McCutcheon and Strumia have studied the mechanism and have also demonstrated

that collodion particles treated with proteins and specific antiserum are phagocytosed like bacteria treated with bacteriotropins (9). I have reported (4) that collodion particles treated with tetanus toxin are *not* flocculated by tetanus antitoxin, whereas collodion particles coated with diphtheria toxin are flocculated by the homologous antitoxin. Bedson (10) found that herpes virus can be adsorbed and neutralized on the surface of collodion particles.

In the present study it was found that tetanus, diphtheria and *botulinus* toxins and antitoxins can be adsorbed upon collodion particles, and that the adsorbed tetanus and diphtheria toxins can be neutralized by their corresponding antitoxins. When the order of treatment was reversed an interesting phenomenon was observed: although treatment with a heterologous toxin of collodion particles coated first with antitoxin resulted in non-toxic particles, treatment with the hemologous toxin resulted in toxic particles. The results of the experiments showing this cross-specificity may be summarized as follows:

	On collodion particles		*In solution*		*Collodion particles*
A	Tetanus antitoxin	+	Tetanus toxin	:	Toxic
B	Diphtheria antitoxin	+	Tetanus toxin	:	Not toxic
A′	Diphtheria antitoxin	+	Diphtheria toxin	:	Toxic
B′	Tetanus antitoxin	+	Diphtheria toxin	:	Not toxic

It is very probable that the particles treated with non-specific serum are not able to adsorb toxin because the protein on the particles does not adsorb toxins, whereas in the specific combination the antitoxin antibody is able to adsorb more toxin than it can neutralize. This observation can be compared with that of Jones, who found that "collodion particles exposed to immune serum and subsequently washed fail to agglutinate in the presence of antigen."

There is a certain—perhaps superficial—similarity between this apparently paradoxical phenomenon and the Bordet-Danysz reaction in regard to the importance of the order of mixing the reagents. Danysz found that when to a definite amount of antitoxin a definite quantity of toxin is added the mixture is more toxic if the latter (diphtheria toxin or ricin) is added fractionally than when the whole amount is added at once. However, if the antitoxin is added fractionally to the

toxin the resulting mixture is not more toxic than when the entire amount of antitoxin is added at once. In the reaction on collodion particles as well the anomalous excess of toxicity results only if toxin is added to antitoxin but not with the reversed order.

The question naturally arises why the particles treated with antitoxin and then with toxin are toxic, when the opposite order of treatment yields non-toxic particles. In the experiments with tetanus toxin the particles treated with antitoxin and toxin are not more toxic than those treated with toxin alone. Therefore it might be possible that while the particles treated with antitoxin are in contact with toxin they lose their previously adsorbed antitoxin film and adsorb toxin. However, when particles treated with antitoxin and toxin are heated at 55°C. for $\frac{1}{2}$ hour (to destroy the toxin) antitoxin is released when injected into mice, showing that the antitoxin was retained during the contact with toxin. 1 cc. of a suspension of such particles protected mice against one lethal dose of toxin. In the diphtheria toxin experiments particles treated with antitoxin and toxin are even more toxic than those treated with toxin alone. Apparently a specific binding between the toxins and antitoxins is not sufficient for neutralization. Three possibilities may be considered:

1. It is possible that in the toxin and antitoxin molecules there are two groups participating in the neutralization reaction as was assumed by Ehrlich. The combination of collodion, antitoxin and toxin may be toxic because when the antitoxin is adsorbed on collodion it has only one free group, which combines with the toxin, the haptophor group, but the neutralizing ergophor group is fixed to the particle and not free to act upon, to neutralize, the toxin.

2. The second, perhaps the more likely, explanation is the following: It is well established that a definite number of bacteria or red blood cells can adsorb various amounts of agglutinins from a solution containing agglutinins depending upon the concentration (titer) of the solution, temperature, etc., and that they can adsorb a considerably greater number of units of agglutinins than is necessary for agglutination (Bordet). It is possible that toxins adsorbed on collodion particles adsorb more units of antitoxins that is necessary for neutralization. Similarly it is possible that antitoxins adsorbed on collodion particles adsorb more units of toxins than they are able to neutralize.

When collodion particles are treated first with toxin then with antitoxin the antitoxin is in excess and with the reversed order of treatment the toxin is in excess.

3. Another possibility is that neutralization occurs only if the antitoxin is in the surface layer of the collodion-toxin-antitoxin complex so that the surface of the complex has the properties of the antitoxin. At present it is thought that in agglutination of bacteria or red blood cells by immune serum the surface of the cell is coated entirely or partially with the antibody and the complex has the properties of the antibody (denatured globulin, insoluble in salt solution) (11). The suggestion that neutralization depends upon establishing the surface properties of the antitoxin for the toxin-antitoxin complex is also supported by the flocculation reaction, which occurs in neutral or almost neutral mixture of diphtheria and tetanus toxins and antitoxins.

The second and third possibilities are not mutually exclusive but may both operate at the same time. Thus on particles treated first with antitoxin and then with toxin the excess of toxin present may be more effective because it is situated largely at the outer surface of the collodion-antitoxin-toxin aggregate and is then able to come into contact with the tissue cells for which it is toxic. Similarly on particles treated first with toxin and then with antitoxin the excess of antitoxin may act in part by coating the outer surface of the collodion-toxin aggregate and thus preventing contact between toxin and tissue cells.

It is also possible that neutralization of toxin by antitoxin is conditioned by specific adsorption. The process of the essential neutralization and the specific adsorption of toxin and antitoxin may follow different rules; the former may occur according to definite, and the latter, according to variable proportions.

The observations reported here showing the significance of the order of treatment of collodion particles in neutralization, together with the similar observations made by Jones with precipitinogen and precipitin establish an analogy between the toxin-antitoxin reaction and other antigen-antibody reactions, particularly agglutination and precipitation. This similarity is strengthened by experiments showing that tannin adsorbed on red blood cells mediates agglutination, promotes phagocytosis, prepares red blood cells for lysis by complement

and that it combines with and detoxifies toxins adsorbed on collodion particles (12).

CONCLUSIONS

1. Collodion particles adsorb diphtheria or tetanus or *botulinus* toxins. These toxins are retained on the particles when washed but are at least in part released in the animal.

2. The adsorbed toxins are neutralized by adsorption of the corresponding antitoxins but are unaffected by other serums.

3. When collodion particles are treated first with tetanus antitoxin, then with diphtheria toxin, they are not toxic, but they become toxic when they are treated first with diphtheria antitoxin, then with the diphtheria toxin. Similarly when collodion particles are treated first with diphtheria antitoxin and then with tetanus toxin, they do not become toxic, but they become toxic when they are treated with tetanus antitoxin and tetanus toxin.

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