

STUDIES ON THE OXIDATION AND REDUCTION OF  
IMMUNOLOGICAL SUBSTANCES.

VII. THE DIFFERENTIATION OF TETANOLYSIN AND  
TETANOSPASMIN.

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INTRODUCTION.

It is well known that the tetanus bacillus produces both a hemotoxin and a true, killing toxin ("tetanolysin" and "tetanospasmin"). Historically, the existence of the true toxin was proved (1) about 10 years before recognition of the hemotoxin by Ehrlich in 1898 (2, 3). The properties of tetanospasmin are described in detail in the larger handbooks of immunology, and the literature on the properties of tetanolysin has been reviewed in a previous paper in this series (4). Both of these products of the tetanus bacillus belong to the group of antitoxinogens and possess two fundamental properties in common: (1) they are primarily toxic substances which possess an affinity for particular cells (tetanolysin for erythrocytes and tetanospasmin for nerve cells, especially those of the gray matter of the brain); (2) they are antigenic, invoking the production of a specific neutralizing antibody (antihemotoxin and antitoxin) when systematically injected into an appropriate animal.

Tetanolysin and tetanospasmin have been used as representative "antitoxinogens" in classical experiments, the results of which have been used in the establishment of many of the fundamental principles of immunology. The theoretical importance of these principles, as well as the practical importance of tetanus itself, makes it desirable to obtain a definite differentiation of these two antigenic products of the tetanus bacillus. Although it is generally recognized that they are

distinct substances, there is little experimental evidence available in the literature to show the distinction between them. The most satisfactory differentiation is found in a paper by Madsen (3) (written in Ehrlich's laboratory), who cites, from apparently unpublished experiments of Ehrlich, the following points of difference: (1) the lysin and toxin appear in different proportions in cultures of different strains; that is, some strains are good lysin producers and poor toxin producers and *vice versa*; (2) the lysin is much more labile than the toxin both to "spontaneous deterioration" and to heat; (3) they have different binding relations; when red blood cells are added to tetanus culture fluids the lysin combines with red blood cells while the true toxin or tetanospasmin remains in solution; (4) the lysin and the toxin have separate or specific antitoxins. None of Ehrlich's experimental data is given in support of these observations. (The reference quoted in all the large German source books or handbooks for this differentiation is a short statement by Ehrlich (2) of the discovery of tetanolysin, which mentions only the fourth of the above points of distinction.)

The present paper deals with the differentiation of tetanolysin and tetanospasmin. Both of these substances are antitoxinogens and both are contained in culture fluids of tetanus bacilli. Since most of the differences in properties proposed for their differentiation are quantitative and by no means absolute, the absence of any experimental data in the literature makes it desirable to show the degree of separation of the two substances obtainable on the basis of previously reported (2,3) properties as well as to present new and additional points of distinction.

This report, therefore, includes the results of experiments showing differences in the following properties of these two antigenic products of the tetanus bacillus: (1) time of liberation in broth cultures; (2) effect of absorption with red blood cells; (3) heat lability; (4) susceptibility to oxidation by air.

#### EXPERIMENTAL.

*Methods.*—The tetanolysin and tetanospasmin used in these experiments were contained in broth culture fluids freed from bacteria by filtration through Berkefeld candles. All cultures before filtration were centrifuged under vaseline seal at high speed to remove most of the bacteria and thus insure rapid filtration.

The lysin "titrations" were carried out by the methods described in a previous paper (4) in this series. In some of the experiments, titrations were made with fluids treated with  $\text{Na}_2\text{S}_2\text{O}_4$  so that the measurements would include the hemolytically inactive but reversible oxidation products of the lysin in the culture fluids under examination.

The toxin "titrations" were made by subcutaneous injection of different dilutions of the culture fluids in order to determine the minimum dose causing death and also the minimum dose causing definite paralysis. White mice of approximately the same weight were used; all injections were made at the base of the tail. In spite of the frequent reports of the unreliability of mice for toxin measurements, our results were always regular when mice of uniform age and weight were selected for the experiments.

#### *The Effect of Berkefeld Filtration upon Tetanolysin and Tetanospasmin.*

The literature (5) is practically unanimous in stating that tetanus culture fluids cannot be filtered without great losses in the content of both the lysin and the toxin. The loss is commonly ascribed to absorption or combination of the lysin and toxin to the constituents of the filter. Zunz (6) included kaolin, diatomaceous earth, talc, and clay in a study of the absorption of tetanus lysin and toxin. Although he found that absorption of tetanus fluids with some of these substances removed different proportions of the lysin and toxin, none of his absorptions removed all of either the lysin or toxin without also removing a large proportion of the other.

While it did not seem probable that Berkefeld filtration would effect any specific separation of the lysin and toxin, it was desired to use the sterile, filtered fluids for our experiments if the filtration did not cause too great a decrease in the active substances. Hence, comparisons were made of the lysin and toxin contents of a number of different tetanus fluids before and after Berkefeld filtration. A protocol of a typical experiment is given in Table I.

The results in Table I present a comparison of the lysin and toxin contents of the centrifuged supernatant of a 7 day old glucose broth culture, before and after Berkefeld filtration. In the case of the lysin, the slight differences in the hemolytic activity of the filtered and unfiltered fluids are detectable only in the two smallest increments of fluid tested. In the case of the toxin, a slight diminution is indicated by the smaller amount of the unfiltered fluid which was able to cause paralysis. However, with the test increments employed in the titration, no difference could be shown in the minimum lethal dose. Thus, although some loss in both lysin and toxin did occur incident to the filtration, the loss was small and not of the order of magnitude reported in the literature. Similar comparisons made with the fluids of 20 day and 30 day cultures showed no greater losses in either toxin or lysin. (Since no disinfectants were added to the supernatant fluids of the tetanus cultures, it is obvious that they contained a few tetanus spores. It is interesting to note that although a few live spores were

necessarily injected in the tests, their presence in the unfiltered fluids apparently did not affect the toxin titrations.)

It is evident that the loss of lysin and toxin in our experiments was extremely slight in comparison with the great losses attributed to filtration in the literature. Our precautions to prevent loss of the active substances consisted chiefly in attempts to carry out the filtration as rapidly as possible and at a low temperature. (Since pH is frequently an important factor in determining successful filtration, it is desirable to state that the reaction of the fluids at time of filtra-

TABLE I.

*Effect of Berkefeld Filtration upon the Lysin and Toxin Contained in the Centrifuged Supernatants of Tetanus Culture Fluids.*

Lysin titration*			Toxin titration**		
Amount of culture fluid	Hemolytic activity		Amount of culture fluid	Time of death or condition of mice 4 days after injection	
	Before filtration	After filtration		Before filtration	After filtration
cc.			cc.		
0.005	++++	++++	0.003	1 to 2	1 to 2
0.003	+++	+++	0.002	1 to 2	1 to 2
0.002	++	++	0.001	PP	0
0.001	+	+	0.0008	P	0
0.0005	±	±	0.0006	P	0

\* +++++ = complete hemolysis.

+++ = hemolysis approximately 3/4 complete.

++ = hemolysis approximately 1/2 complete.

+ = hemolysis approximately 1/4 complete.

± = hemolysis approximately 1/20 complete.

0 = no hemolysis.

\*\* Numerals indicate time of death in days.

PP = marked paralysis within 4 days.

P = slight paralysis within 4 days.

0 = no paralysis.

tion was approximately pH 6.7 before exposure to air.) The culture fluids were chilled and then centrifuged at high speed, under vaseline seal. The clear supernatant fluid was again chilled to about 5°C. and finally filtered through a dry (V type) Berkefeld filter into a flask packed in ice; the filtrate was sealed with vaseline immediately after filtration. The loss in filtration under these conditions was so slight that it seemed unnecessary to carry out the process in a nitrogen atmosphere as previously employed in the filtration of pneumococcus extracts (7).

*Comparison of the Time of Liberation of the Lysin and Toxin in Tetanus Culture Fluids.*

Both the lysin and the toxin of the tetanus bacillus are commonly considered as "exocellular" bacterial products, since both of them are liberated into the culture fluid and are obtained in filtrates devoid of the bacterial cells. Although both of them are exocellular substances, it seemed desirable to determine whether or not the two products might not be differentiated by the stage of growth during which they are liberated into the culture fluid. The experiments consisted of titrations of the lysin and toxin on the filtrates of cultures of different ages. A condensed protocol of a typical experiment is presented in Table II.

TABLE II.  
*Time of Liberation of Lysin and Toxin in Tetanus Culture Fluids.*

Lysin titration				Toxin titration			
Amount of culture fluid	Hemolytic activity			Amount of culture fluid	Time of death or condition of mice 4 days after injection		
	19 hr. culture filtrate	7 day culture filtrate	30 day culture filtrate		19 hr. culture filtrate	7 day culture filtrate	30 day culture filtrate
cc.				cc.			
0.01	++++	++++	++++	0.02	3	1	
0.008	++++	++++	++++	0.01	4 to 5	1	
0.005	++++	++++	+++	0.005	PP	1 to 2	1 to 2
0.003	+++	+++	++	0.004	P	1 to 2	1 to 2
0.002	++	++	+	0.003	P	1 to 2	1 to 2
0.001	+	+	±	0.002	0	1 to 2	1 to 2
0.0005	0	0	0	0.001	0	0	0

The results (Table II) show that tetanolysin and tetanospasmin may be differentiated to a certain extent by the time of their liberation into the culture fluid. The 19 hour culture, with the relatively large inoculum used in this experiment, had already attained its maximum growth; while the 7 day and 30 day cultures must be considered to represent cultures which had passed far beyond the period of active bacterial growth. It is evident from the titrations of the filtrates of these cultures, that the lysin had reached its maximum in the 19 hour culture, while the toxin attained its maximum only in the older cultures. Hence, tetanolysin can be considered as a substance which is elaborated and liberated into the culture fluid during the period of active growth of tetanus bacilli; tetanospasmin, on the other hand, is either elabo-

rated much more slowly or is retained longer within the bacterial cell for it is not set free in the culture fluid until long after the period of bacterial multiplication. From a physiological point of view, it is interesting to observe that these two antigenic products of tetanus bacilli (lysin and toxin) differ from each other in respect to the time of their elaboration or liberation, in much the same way as do the two principal end-products of bacterial nitrogen metabolism; *i.e.*, the lysin, like ammonia, accumulates in the culture fluid during the period of bacterial multiplication; the toxin, like amino acids, accumulates

TABLE III.

*Separation of Tetanolysin and Tetanospasmin by Absorbing Tetanus Culture Fluids with Red Blood Cells.*

Lysin titration			Toxin titration		
Amount of culture fluid	Hemolytic activity		Amount of culture fluid	Time of death or condition of mice 4 days after injection	
	Absorbed with red blood cells	Unabsorbed control		Absorbed with red blood cells	Unabsorbed control
<i>cc.</i>			<i>cc.</i>		
0.06	±	++++	0.0010	1 to 2	1 to 2
0.03	0	++++	0.0007	1 to 2	2 to 3
0.010	0	+	0.0005	2 to 3	2 to 3
0.005	0	±	0.0002	3 to 4	3 to 4
0.003	0	0	0.0001	PP	PP

during later periods when growth has ceased and when the bacterial enzymes alone remain operative.

*Separation of Tetanolysin and Tetanospasmin by Absorbing Tetanus Culture Fluids with Red Blood Cells.*

The technique employed in the absorption of tetanolysin was essentially the same as that utilized in the preceding paper (8) for the absorption of pneumococcus hemotoxin. The same temperature precautions were observed to reduce the possibilities for hemolysis during the absorption. The red blood cells used in the absorption were washed with especial care in order to avoid any possibility of combination of the toxin with lipoid or other serum constituents.

The filtrate of a 7 day culture (which contained both the lysin and the toxin) was diluted with 33 volumes of sterile salt solution. The diluted fluid was divided into two portions and put into separate tubes; 1 cc. of washed red blood

cells (rabbit) was added to one tube; no blood cells were added to the second tube, which served as the "unabsorbed control." After a 12 hour period allowed for combination of the lysin with the blood cells, both tubes were centrifuged, the supernatant fluids pipetted off and titrated for lysin and toxin. A condensed protocol of a typical experiment is presented in Table III.

The results of experiments of this type (Table III) furnish the most satisfactory proof that tetanolysin and tetanospasmin are two distinct and separate substances. Although the culture fluid contains both the lysin and the toxin, the lysin can be completely removed by absorption with red blood cells without diminishing the toxin content at all. (The very slight amount of lysin (Table III) left in the absorbed fluid is probably due to the equilibrium established between the erythrocyte-lysin combination and the free lysin. Even this small amount of residual lysin can be removed by a second absorption without decreasing the toxin content.) That the lysin had been removed by true combination with the erythrocytes was proved by the prompt hemolysis of the "absorbed" cells as soon as they were resuspended in warm salt solution; the "absorbed" cells could be washed several times at a low temperature without removing the combined lysin.

The results of this experiment which confirm Ehrlich's observation (3) furnish an interesting example of the specificity of bacterial "antitoxinogens" for particular cells: both of these primarily toxic antigenic products are produced by the same bacillus, but one of them, tetanolysin, possesses a specific affinity for red blood cells, while the other, tetanospasmin, possesses a like affinity for the cells of nerve tissue.

#### *Comparison of the Heat Labilities of Tetanus Lysin and Toxin.*

One of Ehrlich's points of differentiation between tetanus lysin and toxin is their difference in heat lability. Our experiments to determine the difference in the degree of heat lability of the two substances included the following heating treatments: 10 minutes at 55°C.; 5 minutes at 60°C.; and 10 minutes at 60°C. The fluids were protected from air during the heating tests. The results are presented in Table IV.

The results (Table IV) as a whole show that the distinction between the two substances on the basis of differences in heat lability is only quantitative, since it proved impossible to destroy all the lysin without

at the same time inactivating a considerable portion of the toxin. Indeed, when the heating tests were made at 55°C. the toxin inactivation was not much less than the lysin inactivation. While the rates of the inactivation of the two substances seemed approximately the same at 55°C., an increase of 5°C. apparently accelerates the lysin inactivation more than it does the toxin inactivation; and at 60°C. the distinction between the heat labilities of the two substances became more pronounced. 5 minutes exposure of the fluid to 60°C. destroyed practically all the lysin, while it only reduced the toxin to about one-

TABLE IV.  
*Comparison of the Heat Labilities of Tetanus Lysin and Toxin.*

Lysin titration					Toxin titration				
Amount of culture fluid	Hemolytic activity				Amount of culture fluid	Time of death or condition of mice 96 hrs. after injection			
	Unheated	10 min. at 55°C.	5 min. at 60°C.	10 min. at 60°C.		Unheated	10 min. at 55°C.	5 min. at 60°C.	10 min. at 60°C.
cc.					cc.				
0.5	++++	++++	±	0	0.02		1	1 to 2	2 to 3
0.2	++++	++++	0	0	0.01		1 to 2	2 to 3	3 to 4
0.1	++++	++++	0	0	0.006		1 to 2	3 to 4	4 to 5
0.05	++++	++++	0	0	0.003	1 to 2	1 to 2	PP	PP
0.03	++++	++++	0	0	0.002	1 to 2	2 to 3	P	0
0.02	++++	+++	0	0	0.001	1 to 2	3 to 4	0	0
0.01	++++	+++	0	0	0.0007	2 to 3	PP	0	0
0.002	++	0	0	0	0.0005	2 to 3	PP	0	0
0.001	+	0	0	0	0.0002	PP	P	0	0

tenth its original potency. A phenomenon frequently observed in the heat inactivation of toxins is evidenced in the results of the heating at 60°C.: while the greater part of the toxin was inactivated by 5 minutes exposure to 60°C., exposure for twice this period caused no further increase in the M.L.D. of the toxin and only a slight increase in the time of death.

Hence, the results of this experiment show that a more satisfactory distinction between tetanolysin and tetanospasmin can be obtained at 60°C. than at lower temperatures; but that any distinction made between them from the standpoint of heat lability must be quantitative in nature.



*Differences in the Susceptibilities of Tetanolysin and Tetanospasmin to Oxidation by Air.*

Ehrlich's differentiation of tetanolysin and tetanospasmin includes the statement that the "spontaneous deterioration" of the lysin is more rapid than that of the toxin. The following experiment was made to furnish data showing the differences in the susceptibility of the two substances to oxidation when filtrates containing both of them are exposed to air. In these experiments, the lysin titrations included measurements of fluids treated with  $\text{Na}_2\text{S}_2\text{O}_4$  which serves to convert any hemolytically inactive reversible oxidation products of the lysin

TABLE V.

*Differences in the Susceptibilities of Tetanolysin and Tetanospasmin to Oxidation by Air.*

Amount of culture fluid	Lysin titration				Toxin titration		
	Hemolytic activity				Amount of culture fluid	Time of death or condition of mice 96 hrs. after injection	
	Not exposed to air		Exposed to air			Not exposed to air	Exposed to air
	Not treated with $\text{Na}_2\text{S}_2\text{O}_4$	Treated with $\text{Na}_2\text{S}_2\text{O}_4$	Not treated with $\text{Na}_2\text{S}_2\text{O}_4$	Treated with $\text{Na}_2\text{S}_2\text{O}_4$			
cc.				cc.			
0.05	++++	++++	++	++++	0.004	3 to 4	3 to 4
0.03	++++	++++	+	++++	0.003	PP	PP
0.016	++++	++++	0	++++	0.0024	PP	PP
0.008	++++	++++	0	+++	0.0016	PP	PP
0.004	++	+	0	+	0.0008	0	0

to the active form. These measurements furnished a means of determining whether the loss in hemolytic activity previously termed "spontaneous deterioration" was in fact an oxidation, since only oxidation products could be "reactivated" by the reducing agent.

Tetanus culture filtrate containing both the lysin and the toxin was divided into two portions. One portion was placed in a long narrow tube and sealed with vaseline; the other portion was freely exposed to air in a shallow layer in an Erlenmeyer flask. Both the sealed and the exposed fluids were kept at  $35^\circ\text{C}$ . for 24 hours, and then titrated for lysin and toxin by the methods used in the previous experiments. The results are presented in Table V.

The results of this experiment (Table V) show a definite difference in the susceptibility of tetanolysin and tetanospasmin to inactivation by oxidation with air. The exposure to air caused a much greater diminution of the content of active lysin than occurred with the toxin. That the marked difference between the hemolytic activities of the sealed and aerated fluids was due to an actual oxidation was proved by the fact that when the aerated fluid was treated with a reducing agent it regained an activity approximately identical to that of the unexposed fluid. Since only oxidation products of the lysin could be reactivated by the reducing agent, this evidence contributes information not furnished in the literature where the loss in activity is indefinitely referred to as "spontaneous deterioration." In contrast to the marked decrease in active lysin, exposure of the same fluid to air caused no significant loss in the active killing toxin; in fact no loss at all could be detected with the test doses employed in the experiment.

Comparison of the above differences in the ease of oxidation of the lysin and toxin with the previous results on differences in heat lability (Table IV) indicates that one can obtain a more satisfactory separation of these two products by taking advantage of differences in their susceptibility to oxidation than can be obtained by differences in their heat labilities. However, it must not be deduced from the results in Table V that tetanospasmin is not affected at all by exposure to air for we have frequently observed marked losses in tetanus toxin content of fluids which have been exposed to air for several days (9).

#### DISCUSSION.

The preceding experiments have shown the following points of differentiation between tetanolysin and tetanospasmin: (1) the lysin is set free during the period of active growth of the culture, while most of the toxin liberation occurs after bacterial multiplication has ceased; (2) the lysin possesses specific combining affinity toward red blood cells at 0°C., and the toxin does not possess this property at all; (3) the lysin is more heat-labile than the toxin, but the difference in this property is only relative; (4) the lysin is also more susceptible to oxidative inactivation than is the toxin.

Although much of this information has been reported as statements of fact in the literature, an extensive search has revealed no experi-

mental data for the differentiation of tetanolysin and tetanospasmin. The importance of the two antitoxinogens of the tetanus bacillus makes it desirable to present evidence showing the degree of separation of the two products (lysin and toxin) which can be made upon the basis of differences in the above properties. The most satisfactory separation of the lysin and toxin is obtained by absorption with red blood cells which specifically removes tetanolysin without decreasing the tetanospasmin content of the culture fluid. The separation effected on the basis of the other properties is only a relative one. Heating tetanus culture filtrate at 60°C. for short periods, destroys practically all the lysin, but a significant amount of the toxin is also inactivated by the same treatment. The separation of the two substances by means of differences in their susceptibility to oxidation is likewise only a quantitative separation; but if the oxidation treatment is properly chosen, most of the lysin can be inactivated without causing significant losses in toxin.

The separations of the lysin and the toxin by absorption with red blood cells, or by fractional inactivation by heat, or by oxidation yield a fluid containing an increased proportion of the toxin and a decreased proportion of the lysin. The opposite result (*i.e.* a culture fluid containing the maximum amount of tetanus lysin and the minimum amount of tetanospasmin) can be obtained by taking advantage of differences in the period of growth in which the two products are liberated. Tetanus culture fluids if filtered shortly after the end of the period of active bacterial cell growth, contain the maximum amount of lysin, and only minimum amounts of the true toxin; due to the delayed liberation of toxin and the cessation of lysin elaboration, older cultures contain an increased proportion of the true toxin.

The differentiation of the hemotoxin (tetanolysin) and the true toxin (tetanospasmin) of the tetanus bacillus is of particular importance from an immunological point of view, since both of them are antitoxinogens which have been utilized in the illustration of important fundamental principles. While it has become increasingly more evident that bacterial cells and bacterial filtrates represent complex systems containing a number of different antigenic constituents, only a few of these antigens would be included in the antitoxinogen group since the majority of them give rise to sensitizing antibodies rather than the

neutralizing type of antibody. From this aspect, the recognition of tetanolysin and tetanospasmin as distinct and separate substances acquires interest as an example of two primarily and specifically toxic antigens (antitoxinogens) which are produced and liberated in the same culture fluid by one species of bacteria. There are few, if any, other known examples of the production by the same bacteria of two distinct and different antigens of the toxin type in which the true antigenic nature of each of the products is as definitely established as in the case of the hemotoxin and true toxin of the tetanus bacillus.

#### SUMMARY.

Tetanolysin and tetanospasmin possess in common the immunological properties of the group of "antitoxinogens." Both of them are contained in undeteriorated culture fluids of the tetanus bacillus, but each of them represents a distinct and separate antigenic substance. Experimental data illustrating points of difference in their properties are presented in this paper.

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