STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

III. TETANOLYSIN.

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In the preceding papers, certain principles previously established in studies of the oxidation-reduction of blood pigments have been applied to the oxidation-reduction of pneumococcus hemotoxin (1) and the lysin of the Welch bacillus (2). In the present paper, these studies are extended to tetanolysin, the hemotoxin produced by the tetanus bacillus.

The hemotoxin, or lysin, of the tetanus bacillus was first recognized by Ehrlich (3, 4) who clearly differentiated it from the true killing toxin. Much of the lysin-inhibiting or lysin-neutralizing effect of normal sera (3–7) can be referred to the lipoid constituents (8, 9, 10, 13) although several investigators (11, 12) have reported that certain protein constituents are also concerned.

The antibodies of tetanolysin and tetanospasmin have been differentiated by later workers on much the same grounds as those of Ehrlich. Further evidence of the specificity of the tetanus hemotoxin is furnished by the fact that it is not neutralized by the antihemotoxin produced by immunization with the lysins of Pneumococcus,[1] staphylococcus (14), and of other, anaerobic bacteria (15).

Tetanolysin has been used to illustrate the rate of combination of toxins and antitoxins, in so called *"Heilversuche"* experiments (16, 17); to demonstrate the effect of fractional addition of toxins in toxin-antitoxin mixtures (19); and in a number of other fundamental investigations. The physical-chemical relations involved in the reactions by which the toxin is destroyed, and the effect of temperature upon the rate of hemolysis have also been studied (4, 19, 20, 21, 10). As shown in the preceding paper (2) the activity of tetanolysin is influenced to a much less marked degree by differences in the pH of the hemolysis test system, than is the Welch lysin.

[1] Unpublished experiments of our own.

Tetanolysin has been chosen for the purposes of this investigation, as an example of an antigen of bacterial origin. This lysin has more than ordinary interest, since it has been used in classical experiments, the results of which have been applied in the establishment of many of the fundamental principles of immunology. The experiments reported here are concerned with the conditions governing the oxidation-reduction of tetanus hemotoxin (tetanolysin), and with the nature of the hemolytically inactive products formed during the oxidation of the active lysin.

EXPERIMENTAL.

Methods.

The methods used in this investigation were essentially the same employed in the preceding studies (1, 2). A typical strain of tetanus bacillus, which was furnished by Dr. Charles Krumwiede of the Bureau of Laboratories of the New York City Department of Health, was used in all of the experiments.

In measuring the lysin content of culture fluids treated with hydrosulfite, it is important to avoid exposure of the "reduction mixture" to air before its addition to the red blood cell. The possibility of the destruction of the lysin by neglect of this factor was pointed out in the first paper (1).

In later experiments, the following procedure has proved satisfactory. The mixtures of lysin fluid plus reducing agent were sealed with vaseline as previously described. After a reduction period of 10 to 15 minutes, measured amounts of the reduced fluids are added immediately to the blood cell solution used in the final hemolysis tests. The blood cells are held in a cold water bath at 8–10°C. for 15 to 20 minutes before being placed at 38°C. The test mixtures are shaken several times during the preliminary incubation period at low temperature. This procedure has proved more satisfactory than incubating them immediately at 38°C. Comparative lysin titrations of unoxidized fluids not treated with hydrosulfite with the same fluids treated with the reducing agent have emphasized the importance both of rapid addition of the test samples to the blood cells, and of the preliminary period at low temperature.

If the hydrosulfite-treated fluids are exposed to air for even a few minutes, some of the lysin is destroyed; if the aeration is prolonged for an hour and a half, practically all of the lysin may be destroyed. This rapid destruction of the lysin is undoubtedly due to agents formed from the hydrosulfite in presence of air, since the mixtures of lysin fluid plus phosphate solution are not rapidly destroyed when exposed to air.

Relations very similar to the above were encountered by Conant and Fieser (25) in the determination of hemoglobin in solutions previously reduced by hydro-

sulfite. They were also evident in earlier experiments of our own (26) on the determination of hemoglobin in mixtures previously reduced by bacteria in the absence of air. The advantage of low temperature in the preliminary incubation of the hemolysis tests probably depends upon the same principles that made it more desirable to saturate the hemoglobin at low temperature. The lysin can combine with the blood cell at a low temperature just as hemoglobin can combine with oxygen, although the destruction of both the lysin and the hemoglobin by the products of hydrosulfite is retarded to a greater extent by decreased temperature. Possibly also, the lysin after it once combines with the blood cell is less easily oxidized or destroyed, just as ferrous hemoglobin proves more stable after combination with oxygen or carbon monoxide (26).

TABLE I.

Tetanolysin Accumulation in Plain Broth and Glucose Broth at Different Ages of the Culture.

Amount of culture fluid.	Plain broth culture.			Glucose broth culture.		
	Age of culture.			Age of culture.		
	16 hrs.	36 hrs.	12 days.	16 hrs.	36 hrs.	12 days.
cc.						
0.03	++++	++++	++++	++++	++++	++++
0.01	++++	++++	++++	++++	++++	++++
0.004	++	++	+++	+++	++++	++++
0.002	−	−	+	+	+	+
0.001	−	−	−	±	±	±

Tetanolysin Accumulation in Plain Broth and Glucose Broth at Different Ages of the Culture.

The primary object of the following experiment was to determine the period of growth of the culture at which the lysin is produced. It was also desired to determine the stability of the lysin when cultures were held at 38°C. under the usual conditions.

The cultures were grown in infusion broth containing no added glucose and in the same broth containing 1 per cent glucose. The lysin was added to 3 cc. of 2 per cent sheep cells suspended in salt solution; the hemolytic test mixtures were incubated 1 hour at 38°C. and then centrifuged. The results are given in Table I.

The results of these experiments (Table I) show that tetanolysin, like the Welch lysin, is produced early in the growth of the culture. This fact distinguishes both of these lysins from the endocellular hemotoxin of Pneumococcus

which is liberated in the medium only upon disintegration of the bacterial cells.

The lysin content of both the plain broth and the glucose broth cultures remained surprisingly constant in cultures varying in age from 16 hours to 12 days. (The pH of the cultures, if measured with a minimum exposure to air, was approximately 6.5.) The presence of glucose influenced the lysin production to a very slight degree; though when any difference existed, it was in favor of the glucose broth culture. In similar experiments with the Welch bacillus a greater lysin content was obtained in the absence of glucose. One would naturally expect the presence of glucose to alter the products of growth of the gas bacillus to a greater extent than it would in the case of the tetanus organism. However, the lower yield of lysin in glucose cultures of the gas bacillus cannot be referred entirely to the influence of the presence of the fermentable sugar upon the direction of the metabolism of the bacteria. These cultures become quite acid and the precipitation of the lysin or other effects of acidity must also be considered as factors.

TABLE II.

Heat Lability of Tetanolysin.

Amount of lysin.	Original lysin (titre).	Hemolysis by lysin heated for			
		10 min. at 55°C.	10 min. at 60°C.	2½ min. at 65°C.	10 min. at 65°C.
cc.					
0.5	++++	++++	++++	++++	−
0.2	++++	++++	++++	++++	−
0.1	++++	++++	+++	++++	−
0.05	++++	++++	+++	++++	−
0.03	++++	++++	±	+	−
0.02	++++	++++	−	−	−
0.01	++++	++++	−	−	−
0.002	++	±	−	−	−
0.001	±	−	−	−	−

Heat Lability of Tetanolysin.

Experiments were made to compare the lability of the tetanus hemotoxin with the lability of the other hemotoxins studied in this series of papers. The lysin was heated in narrow thin walled tubes protected from air. The reaction of the fluids tested, if not exposed to air, was pH 6.6. The tests for active lysin were made immediately after the heating test. The results are given in Table II.

The results of these experiments (Table II) show that tetanolysin is completely inactivated by 10 minutes exposure to 65°C. At this temperature the inactiva-

tion processes proceed rapidly, as about the same amount of lysin is destroyed by 2½ minutes exposure to 65°C. as is destroyed in 10 minutes at 60°C. This degree of heat resistance is considerably higher than that found in tests made under similar conditions with the hemotoxins of the Pneumococcus, the Welch bacillus, and El Tor strains of the cholera vibrio. The order of heat lability of the hemotoxins of these different bacteria is as follows: Pneumococcus, cholera vibrio, Welch bacillus, and tetanus bacillus.

Inactivation of Tetanolysin by Exposure to Air, and "Reactivation" of the Oxidized Lysin by Treatment with Reducing Agents.

Experiments were made to determine whether the inactive substances formed in aerated tetanus culture fluids included a reversible oxidation product. The methods employed were the same as those

TABLE III.

"Reactivation" of Oxidized Tetanolysin by Reduction.

Tetanolysin.	Hemolytic titre.			
	Amount of lysin.			
	0.10 cc.	0.03 cc.	0.01 cc.	0.005 cc.
Oxidized lysin not treated with $Na_2S_2O_4$.	−	−	−	−
Oxidized lysin after reduction by $Na_2S_2O_4$	++++	++++	++++	++

used in the preceding investigations. A protocol of a typical experiment is given in Table III to illustrate the results.

The "reactivation" of the hemolytically inactive fluid by treatment with the reducing agent (Table III) can be interpreted as the conversion of a non-hemolytic, reversible oxidation product to the originally "active," reduced lysin. Hence, the inactivation of tetanolysin upon aeration of culture fluids is an oxidation process which yields a reversible product.

The Influence of Temperature upon the Oxidation of Tetanolysin.

The following experiments were made to determine the effect of temperature upon the oxidation of the tetanolysin.

The bacteria-free supernatant fluid of a 12 day glucose broth culture was used in the experiments. 10 cc. portions were exposed to air at 5°, 22°, and 38°C. Samples were taken at the times indicated in the protocol. The pH of the cul-

TABLE IV.

Influence of Temperature upon the Oxidation of Tetanolysin.

Amount of culture.	Original (before exposure).	Exposed to air. 5°				Exposed to air. 22°				Exposed to air. 37°			
		2 hrs.	8 hrs.	11 hrs.	24 hrs.	2 hrs.	8 hrs.	11 hrs.	24 hrs.	2 hrs.	8 hrs.	11 hrs.	24 hrs.
cc.													
0.4	++++	++++	++++	++++		++++	++++	++++	++++	++++	++++	+	−
0.2	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	−	−
0.1	++++	++++	++++	++++	++++	++++	++++	++++	++	++++	±	−	−
0.05	++++	++++	++++	++++	++++	++++	++++	+++	+	++++	−	−	−
0.03	++++	++++	++++	++++	++++	++++	++++	++	−	++++	−	−	−
0.02	++++	++++	++++	++++	++	++++	+	+	−	++++	−		−
0.01	++++	++++	++++	+++	±	++++	−	−	−	++++	−		−
0.004	+++	++	+	++	−	++	−	−	−	++	−		−
0.002	+	+	−	±	−	+	−	−	−	±	−		−
0.001	±	−		−	−								−

ture after exposure to air was 7.2. The hemolysis tests were made with 3 cc. of blood cells suspended in salt solution. The results are given in Table IV.

The data in Table IV illustrate the relative rates of oxidation of tetanolysin at different temperatures. As might be expected, the lysin is oxidized much more rapidly at the temperature of the body than at room temperature, being almost entirely destroyed after 11 hours exposure to air at 37°C. The oxidation proceeds much more slowly at the temperature of the ice box.

Attempt to "Reactivate" Heated Tetanolysin by Treatment with Reducing Agents.

In the preceding papers (1, 2) it has been shown that the heat-inactivated products of the hemotoxins of Pneumococcus and of the Welch bacillus cannot be "reactivated" by reducing agents. This obtained in tests made with lysins which had been heated the minimum amount required for their complete inactivation. Tests were made to determine if the same relation holds true in the case of tetanolysin.

The results of these experiments were the same as those obtained in analogous experiments with the other lysins. If inactivated by heat, neither the "reduced" active lysin nor the "oxidized" lysin can be "reactivated" subsequently by treatment with sodium hydrosulfite. Hence, it would seem that the inactivation of tetanolysin by heat is due to the formation of products distinctly different from the oxidation product.

The Distinction between the Reversible and Irreversible Inactivation of Tetanolysin.

The preceding experiments have dealt with two types of inactivation: that due to oxidation, which yields a reversible inactive product, and that brought about by heat, which is irreversible (protein denaturization). It is possible that when culture fluids are aerated, a part of the lysin may also be converted to irreversible products, just as in mixtures of "ferrous" (24), and "ferric" hemoglobin part of the blood pigment may be changed to irreversible products (globin, hematin, etc.). The detection of the irreversibly inactivated lysin corresponds to that of irreversibly inactivated products of hemoglobin.

In any mixture containing hemoglobin and its degradation products, the amount of "active" hemoglobin can be estimated by determining its oxygen- or carbon monoxide-combining capacity. In the same mixtures, the amount of methemoglobin, the reversible oxidation product, may be estimated by reducing the mixture and then determining the increase in oxygen or carbon monoxide capacity. This increase in "activity" can be referred to the reduction of "inactive" methemoglobin to "active" hemoglobin. The detection of other degradation products, such as globin and hematin, requires data on the initial content of hemoglobin in the original fluid before any deterioration has occurred. With these data obtained (the initial "total hemoglobin"), the further degradation products (such as globin and hematin) are estimated by subtracting the sum of

TABLE V.

Mixtures of "Active" Tetanolysin and Its "Inactive," Reversible Oxidation Product in Culture Fluids Exposed to Air at Different Temperatures.

Amount of culture fluid.	Lysin in untreated fluids (before treatment with reducing agent).				Lysin in fluids after treatment with reducing agents.			
	Unexposed culture fluid.	Culture fluid exposed to air for 24 hrs. at			Unexposed culture fluid.	Culture fluid exposed to air for 24 hrs. at		
		5°C.	22°C.	37°C.		5°C.	22°C.	37°C.
cc.								
0.2	++++	++++	++++	−	++++	++++	++++	++++
0.1	++++	++++	++++	−	++++	++++	++++	++++
0.05	++++	++++	++	−	++++	++++	++++	++++
0.04	++++	++++	−	−	++++	++++	++++	++++
0.03	++++	++++	−	−	++++	++++	++++	+++
0.02	++++	+++	−	−	++++	++++	+++	++
0.01	++++	+	−	−	++++	++++	+++	+
0.005	+++	−	−	−	+++	+++	+	−
0.003	+	−	−	−	++	−	−	−

the hemoglobin and methemoglobin which are present in the deteriorated mixture, from the initial amount of hemoglobin in the undeteriorated fluid. In the case of mixtures of the different modifications of active hemotoxins, the principles involved are exactly the same.

In examination of the lysins, the first data required are measurements of "total lysin." These are obtained by determining the "active lysin" content in undeteriorated culture fluids. The fluid of a young culture, or a fluid which has been protected from deterioration, is titrated for "active lysin" both before and after treatment with a reducing agent. If the lysin content is not increased by the reduction treatment, it is assumed that all of it is present in the "active" form, and that the values obtained represent the "total lysin" produced by the

culture. With the preliminary data at hand, the following measurements are made of the lysin in the deteriorated culture fluids. The "active lysin" is estimated by a titration of the lytic activity of the fluid. The fluid is then treated with the reducing agent and the titration is repeated upon the reduced mixture. As in the case of the blood pigment derivatives, the increase in the "active" substance upon reduction represents the reversible oxidation product. The other degradation products are estimated as the difference between the "total lysin" and the sum of the reduced "active" lysin and the reversible oxidation product.

A number of different mixtures in deteriorated culture fluids have been examined by these methods. The experiment given in Table V represents an examination of the cell-free supernatant of a 12 day culture, portions of which had been exposed to air at 5°, 22°, and 37°C.

The lysin "titrations" of the unexposed culture fluid and of the fluids exposed to air at different temperatures are presented in Table V. The first four columns include the titrations of the fluids prior to treatment with a reducing agent. The results recorded in these columns, therefore, represent the lysin which is in the active or reduced state. The last four columns of the table include the titrations of the same fluids after the reduction treatment. The results of these titrations represent the sum of the active lysin plus the inactive but reversible oxidation product.

A comparison of the lysin activity of the unexposed fluid and of the exposed fluids, before and after treatment with a reducing agent, reveals the following relations. The unexposed fluid exhibits no increase in apparent lysin content upon treatment with a reducing agent, which proves the absence of the reversible oxidation product of the lysin. The value of the lysin titration of the unexposed fluid after the reduction treatment may be taken as "total lysin" present in the culture fluid. In contrast to the unexposed fluid which showed no increase in activity upon reduction treatment, all of the exposed fluids exhibited an unmistakable increase in activity when reduced. The difference between the apparent lysin content of each of the fluids before and after reduction, may be assumed to represent the degree of "reactivation" of the inactive, but reversible oxidation product.

It is evident, however, that only one of the exposed fluids regained the original "total lysin" value after reduction treatment. The failure to regain the original lysin activity, as shown by the dif-

ference between the original or "total lysin" and the sum of the active lysin plus its reversible oxidation product in the deteriorated fluid may most easily be explained by the formation of other irreversible degradation products which probably arise from the deterioration of the first formed reversible oxidation product. The irreversibly inactivated products of the lysin can be compared to degradation products of hemoglobin, such as globin and hematin, which, unlike methemoglobin, cannot be reduced to the original or "active" blood pigment. While this explanation seems the most likely one, it is necessary to admit the possibility of hydrosulfite failing to reduce completely all of the reversible product.

TABLE VI.

Mixtures of Different Products of Tetanolysin in Culture Fluids Exposed to Different Conditions.

Previous treatment of culture fluid.	Ly_r (original, active reduced lysin).	Ly_o (inactive reversible oxidation product).	Ly_x (other inactive degradation products).
Not exposed to air	200	0	0
Exposed to air for 24 hrs. at 5°C.	50	150	0
" " " " 24 " " 22°"	7	143	50
" " " " 24 " " 37°"	0	33	167

It is evident from the conditions of the above experiment that each of the fluids examined (Table V) originally contained the same amount of the active, or reduced, lysin. The various proportions of the different modifications of tetanolysin that may be present in the same culture fluids after exposure under the different conditions can be graphically illustrated by collecting the data of Table V in the form given in Table VI.

The "units" of lysin represent the amount of lysin which suffices to give approximately 80 per cent hemolysis of 4 cc. of a 2 per cent suspension of blood cells. These "units" at best are only approximate and are not presented as representing absolute values. They serve, however, to illustrate the principles involved.

The amounts of the different products of the lysin (Table VI) are calculated in exactly the same manner as one would calculate the analogous products of

hemoglobin from data obtained by the same methods as those used in the above lysin measurements. The analogy is made clear by the following relationships.

Lysins:

1(*a*) "Total Ly" = (Ly_r + Ly_o + Ly_x)
where Ly_r = reduced or active lysin; Ly_o = the reversible oxidation product of Ly_r; Ly_x = the irreversibly inactivated products of the lysin.

(*b*) Ly_o = (Ly_r + Ly_o) − Ly_r

(*c*) Ly_x = "Total Ly" − (Ly_r + Ly_o)

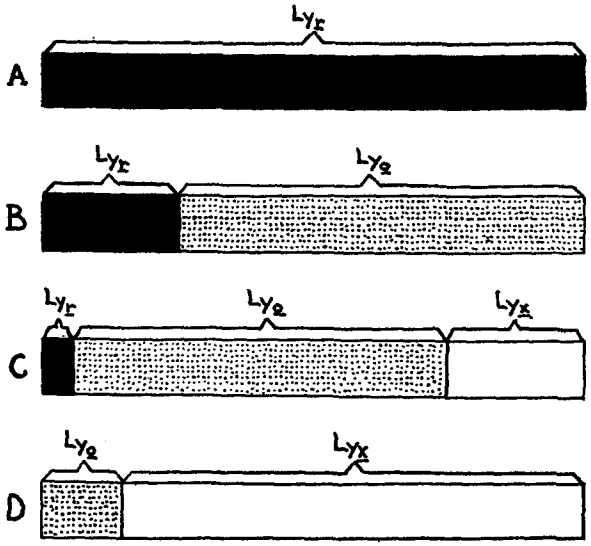

FIG. 1. Examples of the variety of mixtures of active lysin and its inactive modifications that may be present in tetanus culture fluids after exposure to different conditions. *A*, original culture fluid (not exposed to air). *B*, culture fluid aerated for 24 hours at 5°C. *C*, culture fluid aerated for 24 hours at 22°C. *D*, culture fluid aerated for 24 hours at 37°C.

Hemoglobin:

2(*a*) "Total Hb" = (Hb_{ous} + Hb_{ic} + Hb_x)
where Hb_{ous} = "active" Hb (Hastings' (24) "ferrous Hb"); Hb_{ic} = "inactive" Hb (MetHb or Hastings' (24) "ferric Hb"), the reversible oxidation product of Hb_{ous}; Hb_x = the irreversible inactive products of Hb (globin, hematin, etc.).

(*b*) Hb_{ic} = (Hb_{ous} + Hb_{ic}) − Hb_{ous}

(*c*) Hb_x = "Total Hb" − (Hb_{ous} + Hb_{ic})

The value of (Ly_r + Ly_o) is obtained by estimation of the active lysin after reduction treatment of the culture fluid, just as the value of (Hb_{ous} + Hb_{ic}) is obtained after reduction treatment of the hemoglobin solution.

Using the above approximate data of Table VI, it is interesting to present the graphic example shown in Fig. 1 of the variety of mixtures of active lysin and inactive lysin derivatives that may occur in deteriorated tetanus culture fluids.

Fig. 1 illustrates the fact that a series of cultures fluids with the same initial content of lysin may contain at the time of analysis, a variety of mixtures of active lysin and its inactive degradation products. The obvious conclusion, hitherto often overlooked, is that the lysin detected at any one time by the ordinary methods is determined not only by the actual lysin-producing capacity of the culture, but is equally dependent upon the conditions to which the culture fluid has been exposed previous to the time of analysis.

DISCUSSION.

Tetanolysin is inactivated when the culture fluids are exposed to air. The inactive product thus formed is the reversible oxidation product of the "active" reduced lysin since it is converted to the "active" lysin by treatment with the proper reducing agent. In addition to the reversible oxidation product, other inactive degradation products of the lysin are formed upon the aeration of tetanus culture fluids. These substances cannot be "reactivated" by reduction treatment and they probably represent degradation products of the first formed reversible oxidation product.

The methods used in the analysis of mixtures of hemoglobin and its usual degradation products are applicable to the examination of mixtures of "active" lysin and its inactive products. A complete analysis of the lysin products present in a culture fluid requires the following data: (1) the "total lysin" produced by the culture as revealed by the examination of the culture fluid before any of the lysin has deteriorated; (2) the "active" or reduced lysin present in the fluid at the time of examination; and (3) the sum of the "active" reduced lysin plus its inactive reversible oxidation product as revealed by lysin titrations of the fluid after treatment with a proper

reducing agent. If these data are at hand, it is possible to estimate the relative amounts of "active" reduced hemotoxin (Ly_r), of its inactive, reversible oxidation product (Ly_o), and of the irreversible degradation products (Ly_x) which cannot be "reactivated" by reduction treatment. It is possible that the irreversible degradation products may be formed by the deterioration of the reversible oxidation product (*i.e.*, $Ly_r \rightarrow Ly_o \rightarrow Ly_x$) in a manner analogous to the formation of globin and hematin by the splitting of methemoglobin (*i.e.*, Hb→MetHb→globin and hematin) which is the most usual course of blood pigment destruction.

SUMMARY.

A preliminary study was made of the rate of formation of tetanolysis and of the effect of glucose upon the lysin production. The heat lability of tetanolysin was next compared with that of the lysins of other bacteria. Finally the methods used to estimate the relative amounts of "active" hemoglobin and its "inactive" derivatives in deteriorated solutions of blood pigments were applied to the differentiation of the various derivatives of "active" tetanolysin present in deteriorated culture fluids.

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