

STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

IV. STREPTOLYSIN.

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INTRODUCTION.

The preceding papers (1-3) of this series include reports on the oxidation-reduction of the "hemotoxins" of pneumococci and of Welch and tetanus bacilli. The present paper reports a study of the "spontaneous deterioration" of streptolysin.

Streptolysin may be considered as a substance possessing hemolytic properties, which is produced during the active growth of certain streptococci in a suitable culture medium. This hemolytic action of streptococci has been the subject of many investigations during the past 25 years (4-31). The facts of essential importance in the establishment of the nature of the lysin can be obtained from the studies of Ruediger (24, 25), von Hellens (31), and Meader and Robinson (21).

Although the fact has been disputed in the literature, the actual hemolytic powers of the free lysin are independent of the presence of streptococcus cells. The lytic substance is extremely labile and under the usual conditions rapidly loses its hemolytic property. The change is commonly termed a "spontaneous deterioration," since the loss in activity has been considered to be independent of the conditions imposed during the storage of streptococcus culture fluids.

The literature (5, 8, 19, 22, 16, 24, 25) furnishes little convincing evidence that streptolysin is an antigen. However, the similarity between the "spontaneous deterioration" of the streptococcus lysin and the oxidation of the antigenic lysins of *Pneumococcus* and tetanus justifies the inclusion of the present paper in this series of studies on the oxidation and reduction of immunological substances.

EXPERIMENTAL.

Methods.

Strains of Hemolytic Streptococci.—Two strains of hemolytic streptococci were used in most of the experiments. Strain M was isolated by Dr. William Moss from the tissues of the neck of a child suffering from cellulitis. Strain D is a "scarlet fever strain" which has been used in the preparation of "Dick toxin." Both of these strains produce wide zones of hemolysis in less than 24 hours when grown on meat infusion blood agar plates. Other strains from the collection of the Hospital of The Rockefeller Institute were included in a number of experiments.

Hemolysis Tests.—In the usual test for hemolytic properties of streptococci in liquid systems, the whole culture is added to a suspension of red blood cells. In the present investigation, however, it was necessary to make the actual hemolysis tests with bacteria-free fluids, in order to avoid the possibility of bacterial growth and actual elaboration of lysin during the hemolysis test itself.

In view of the marked instability of the lysin, the manipulation of the culture fluid preceding the measurements of the lysin activity was kept constant throughout all of the experiments. The cultures were grown in broth under vaseline seal. Representative samples were removed and put into narrow tubes, sealed with vaseline, then centrifuged for 45 minutes at high speed. The supernatant fluids used in the tests were added to the blood cell suspensions immediately after the centrifuging process.

The hemolysis tests were made by the same methods as in the preceding studies, and consisted in "titrations" of the minimum amount of the test fluid which suffices for hemolysis of a constant volume of red blood cells. Although there are theoretical objections to this method of measurement of lysin content, the data obtained seem sufficiently accurate for the purposes of the present investigation. In a number of protocols, the results of the lysin "titrations" are recorded in terms of lysin "units." In these instances, the lysin "unit" represents the amount of lysin required to cause 80 per cent complete hemolysis of a constant volume of red blood cells. It is understood that these "units" are not presented as absolute values.

The symbols introduced in the preceding paper (3) are used to distinguish between the different modifications of the lysin; *i.e.*, Ly_r = the "active" lysin present in fluids before treatment with the reducing agent; Ly_o = the oxidized lysin which, although itself "inactive," can be converted to the originally "active" substance by reduction treatment; Ly_x = irreversibly inactivated lysin.

The "Spontaneous Deterioration" of Streptolysin and "Reactivation" of the Inactive Lysin by Treatment with Reducing Agents.

Tests were made of the possibility of "reactivating" deteriorated streptolysin by the reduction treatment employed in the preceding

investigations. The results of a typical experiment are presented in Table I.

As shown in Table I, "spontaneously deteriorated" streptolysin is "reactivated" by the reduction treatment successfully employed in the "reactivation" of the hemolytically inactive oxidation products of the previously studied bacterial lysins. It seems, therefore, that these inactive products (as in the case of the pneumococcus, Welch, and tetanus lysins) represent reversible oxidation products which can be converted by reduction, to the original, hemolytically active lysin. Since inactivation can be brought about by aeration and the

TABLE I.
"Reactivation" of "Spontaneously Deteriorated" Streptolysin by Treatment with Reducing Agents.

Amount of fluid.	Hemolytic activity of "spontaneously deteriorated" fluids of streptococcus cultures.	
	Not treated with hydrosulfite.	Treated with hydrosulfite.
cc.		
1.0	—	++++
0.6	—	++++
0.4	—	++++
0.2	—	++++
0.1	—	++++
0.05	—	++

inactive products in turn can be "reactivated" by reduction, it appears that the long discussed "spontaneous deterioration" of streptolysin is simply a reversible oxidation-reduction process.

The Influence of Exposure to Air upon the "Deterioration" of Streptolysin.

In the experiments described below, a comparison is made of the relative rates of "deterioration" of streptolysin when the culture fluid is stored in sealed tubes and in shallow layers freely exposed to air.

Cultures of two strains of hemolytic streptococci were grown in infusion broth in tubes sealed with vaseline. After 12 hours incubation, uniform portions of the culture were removed; one series of each strain was placed in narrow tubes

and sealed with vaseline; a second series was placed in Erlenmeyer flasks which exposed a large surface of the fluid to the air.

Both series were then placed in the incubator at 38°C., the usual lysin "titrations" were made at the end of 6, 12, and 24 hours.

The results of one series of tests with Strain M are given in Table II.

In Table II, the results presented in the first three columns show that while the lysin gradually "deteriorates" to inactive products when protected from air by a heavy vaseline seal, free exposure to air causes a much more rapid inactivation.

The last three columns of Table II, which record the apparent lysin content of the fluid after treatment with the reducing agent, represent

TABLE II.

Influence of Exposure to Air upon the "Spontaneous Deterioration" of Streptolysin.

Amount of culture fluids.	Ly_r (Lysin activity before treatment with hydrosulfite).			$Ly_r + Ly_o$ (Lysin activity after treatment with hydrosulfite).		
	Original culture fluid (12 hr. culture).	Fluid exposed to air 6 hrs. at 38°C.	Fluid stored in sealed tube 6 hrs. at 38°C.	Original culture fluid.	Fluid exposed to air 6 hrs. at 38°C.	Fluid stored in sealed tube 6 hrs. at 38°C.
<i>cc.</i>						
1.0	++++	++	++++	++++	++++	++++
0.8	++++	±	++++	++++	++++	++++
0.6	++++	—	++++	++++	++++	++++
0.4	++++	—	++++	++++	++++	++++
0.2	+++	—	++	++++	++++	++++
0.1	+	—	—	++	++	++
0.05	—	—	—	—	—	—

the sum of the active lysin (Ly_r) plus its inactive, but reversible oxidation product (Ly_o). It is important to note that the inactive products formed from streptolysin either in sealed tubes or under conditions of free exposure to air can, in each instance, be converted to the original, active lysin by reduction treatment. Since the inactive products formed are apparently the same, it is fair to conclude that the inactivation processes consist of the same type of reaction—*i.e.*, oxidation processes are responsible for the "spontaneous deterioration" of streptolysin in sealed tubes as well as in aerated fluids. Whether the oxidizing agents involved in the actual oxidations of the lysin are

the same in each case, is, of course, not proved. The essential difference, however, in the "deterioration" of streptolysin under the two sets of conditions, seems to be a difference in the rate of the reaction.

A third point of interest revealed in Table II is that in spite of the greater "deterioration" of the lysin in the aerated series, treatment with the reducing agent restores the same original lysin activity to both the sealed and aerated fluids. Thus, while in the aerated fluids much more of the active lysin has been converted to its inactive, reversible oxidation product than in the fluids held in the sealed tubes, the sum of the active lysin plus its reversible oxidation product remains the same in each instance. Apparently, under the conditions of this experiment, none of the lysin was destroyed to non-reversible, inactive products.

The results of the tests with Strain D are not recorded in Table II. The difference in stability of streptolysin in cultures of the two strains (D and M) is worthy of note, as it was constantly evident throughout the entire investigation. For example, in the above experiment cultures of Strain M lost more of the original lysin activity after 6 hours storage at 38°C., than did Strain D after 20 hours. However, the same relation between the rates of inactivation of lysin in the fluids protected from and exposed to air were evident with the latter strain as that recorded above for the more labile cultures of Strain M.

Comparison of Lysin Stability and Methylene Blue Reduction in Culture Fluids Exposed to Different Conditions.

The object of the following experiment was to compare the stability of the active (or reduced) streptolysin in different systems with the methylene blue-reducing power of the same systems.

Samples of a 12 hour broth culture of Strain M were placed under the following conditions: (1) freely exposed to the air in a shallow layer in an Erlenmeyer flask; (2) a small surface exposed to the air (the height of the cylinder of fluid was 150 mm. while the surface exposed to air was only 15 mm. in diameter); (3) sealed from air by a heavy layer of vaseline; here, too, the surface of the broth was only 15 mm. in diameter while the depth was 150 mm. Samples (4) and (5) were, respectively, the same as (2) and (3), with the exception that a suspension of *B. coli* was added to each, to furnish additional reducing action.

Sample (6) consisted of a sealed tube of the sterile culture fluid. A second series exactly the same as that described above was prepared with the exception that methylene blue was added to each of the tubes.

Series I and Series II were now placed at 38°C. At the end of 6, 12, 24, 48, and 72 hours, lysin titrations were made on Series I, and observations of the degree of reduction of methylene blue were made on Series II.

In all experiments of the type described above the lysin proved most stable in the fluids in which the reduction of the dye was most complete and most persistent. Thus, as indeed might be expected, conditions least favorable for the conversion of the colorless dye to its colored oxidation product likewise proved to be least favorable for the conversion of streptolysin to its hemolytically inactive oxidation product.

In the unsealed tubes, the reduction of methylene blue was transient; upon the cessation of growth activity of the bacteria, the dye was more or less rapidly converted to its colored oxidation product. In these tubes, in which both dye and lysin were finally oxidized, the time at which the maximum amount of lysin was in the active or reduced state, coincided with the time at which the maximum amount of the dye was in the colorless or reduced condition. Methylene blue, however, is much more readily maintained in the reduced condition than is the active streptolysin.

In the fluids which were sealed from air, the greater tendency of the lysin toward oxidation limited the application of the comparison of the persistence of methylene blue reduction with the persistence of active lysin. Even the sterile culture fluid containing no bacteria possessed sufficient reducing power to maintain the dye in the reduced state, if protected from air by a vaseline seal. Thus, it is evident that streptolysin is a substance much more readily oxidized than is methylene white, since the conversion of the colorless dye to its oxidation product was prevented in systems in which the active (or reduced) lysin was more or less rapidly converted to the inactive oxidation product.

Heat Lability of Streptolysin.

That streptolysin is a heat-labile substance has been well established in the literature (15, 14, 31, 27, 18, 20, 5, 31), but the relative degree of heat lability varied in the results of different investigators. Experiments on the heat lability

of the streptolysin in the fluids used in the present studies were made to furnish data required in the next experiment. The fluids were heated in sealed tubes in the absence of air; the reaction of the fluids was approximately pH 6.8. The results of these experiments showed that the lysin, when heated under these conditions, was completely inactivated by 5 minutes exposure to 55°C., while over half of the lysin was destroyed by 10 minutes exposure to 50°C. Thus, as shown by comparison with the results in the preceding studies (1-3) streptolysin possesses about the same degree of heat lability as the lysins of the Welch bacillus and the El Tor cholera vibrio; it is somewhat less heat-labile than pneumococcus hemotoxin, and somewhat more thermolabile than the lysin of the tetanus bacillus.

Attempt to "Reactivate" Heat-Inactivated Streptolysin by Treatment with Reducing Agents.

The heated fluids used in the experiments were exposed to 55°C. for 5 minutes (the minimum heating treatment found in the preceding experiment to effect the complete inactivation of the lysin). The "oxidized" or "deteriorated" lysin consisted of the centrifuged supernatant of a 5 day culture of the same strain. In both cases, the cultures had been grown and stored in vaseline-sealed tubes. The heating tests were likewise made in vaseline-sealed tubes.

The results of a number of experiments proved that heat-inactivated streptolysin cannot be "reactivated" by treatment with the reducing agent, sodium hydrosulfite. Thus, the heat inactivation of streptolysin is due to the formation of inactive products distinctly different from the reversible oxidation products formed during the "spontaneous deterioration" of the lysin.

Relative Amounts of Active and "Deteriorated" Lysin in Different Stages of Growth of Streptococcus Cultures.

Previous studies of streptolysin have furnished measurements not of actual lysin production, but of its relative stability at the time of the tests. In the experiment described below, streptolysin production and "deterioration" during different periods of growth of the culture were followed in a number of experiments by means of methods whereby the inactive reversible oxidation product of the lysin is detected.

Tubes containing 100 cc. of infusion broth sealed with vaseline were inoculated with 0.4 cc. of a 6 hour culture of Strain M. "Titrations" of the lysin

were made at the end of 6, 12, 24, and 48 hours. A constant volume of 4.0 cc. was maintained in the hemolysis systems; and the following increments of diluted culture fluid were tested: 0.80, 0.60, 0.50, 0.40, 0.35, 0.30, 0.250, 0.225, 0.175, 0.150, 0.125, and 0.100 cc. The hemolysis tests were incubated 45 minutes and then centrifuged.

The results of this experiment are presented in Fig. 1. Each "unit" of lysin represents the amount of lysin which causes approximately 80 per cent hemolysis of 4 cc. of a 2 per cent suspension of sheep cells. These "units" are not presented

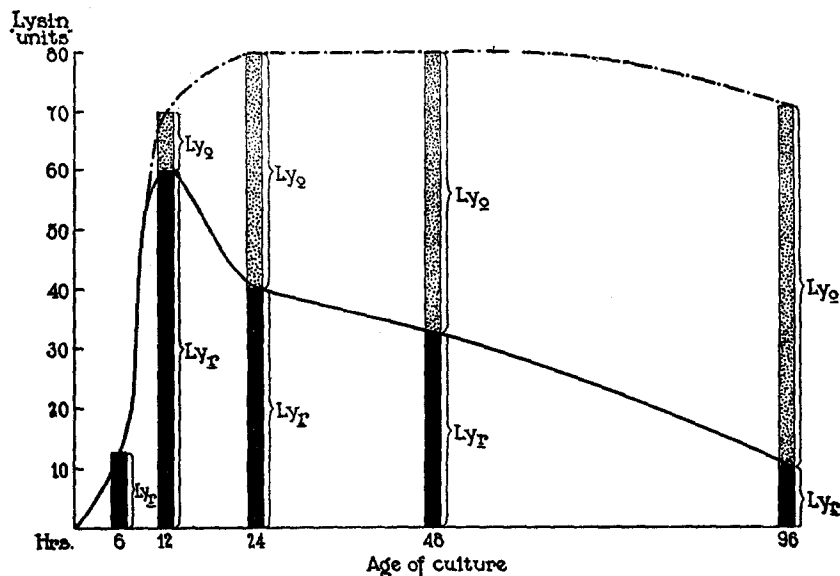


FIG. 1. Relative amounts of active and deteriorated lysin in streptococcus cultures at different ages of the culture.

The black unbroken line represents the active, reduced lysin (Ly_r); the broken line represents the sum of the active lysin plus its hemolytically inactive, but reversible, oxidation product ($Ly_r + Ly_o$). The black shaded areas represent the amount of active lysin (Ly_r) in the fluid at the time of the tests; the stippled areas represent the amount of the reversible oxidation product (Ly_o).

as absolute values, but are used merely as a convenient index for the approximate comparison of the relative lysin content of the fluid at different stages of growth of the culture.

In Fig. 1, the continuous line and the shaded areas represent the amount of lysin in the active state at the time of the test, the results having the character of those which have been reported for years in

the literature. The broken lines and stippled areas, however, represent the hemolytically inactive oxidation product of the original lysin and contribute data impossible to obtain by measurements of active lysin alone. Hence, the broken line in Fig. 1 presents a picture which more closely approaches the actual progress of streptolysin production than do any of the previous "curves of lysin production."

While there is little or no "deteriorated" lysin present during the period of maximum growth of the streptococci, the "deterioration" has begun, in this particular culture, by the time of the 12 hour test. Moreover, it is evident in Fig. 1 that the amount of active lysin may begin to diminish before the maximum amount of "total lysin" ($Ly_r + Ly_o$) has been liberated into the medium. This is an important fact and demonstrates that, at least with certain strains of streptococci, the measurement of lysin by the usual method never furnishes the value of the total amount of lysin actually elaborated by the culture. This has been especially true with our Strain M, with which it has never been possible to obtain samples of culture fluid in which there was no "deteriorated" lysin—*i.e.*, fluids in which Ly_r was not less than $(Ly_r + Ly_o)$. We have conducted a number of experiments of the type described above with different strains of streptococci. The fact that with some strains the lysin "deterioration" begins before the liberation of the maximum amount of "total lysin" while with other strains the lysin proves more stable, is evidence that the usual measurements of active lysin cannot furnish a proper basis of comparison of actual lysin production by different streptococci.¹

¹ If practically all of the lysin had "deteriorated" in the fluid tested, the usual method of lysin measurement might lead one to believe that the culture tested was totally devoid of lysin-producing powers, or at least that no lysin had been produced in the tested culture medium.

It would be a mistake however, to emphasize this possibility as a likely source of error in determining whether or not a particular strain of streptococcus is a "hemolytic streptococcus." As a matter of practice, the criterion used in the separation of "hemolytic" streptococci from "non-hemolytic" strains is the production of distinct wide zones of hemolysis during the early growth of the colonies on a blood agar plate (the "beta hemolysis" of Smith and Brown). Where hemolysis in liquid systems is used as a descriptive or systematic criterion

In Fig. 1, it is also significant that while the amount of active lysin rapidly diminishes, the sum of the active lysin and its inactive, reversible "deterioration" product ($Ly_r + Ly_o$) remains constant for several days. In most of our experiments, the sum of these two products remained the same for 5 or 6 days. The fact that still older culture fluids were found not to regain all of their original active lysin content after the reduction treatment can be explained by the formation of irreversible degradation products (Ly_x) which cannot be converted by reduction to the originally active lysin.

*The Influence of Yeast Extract in the Culture Medium upon the
Production and Stability of Streptolysin.*

As suggested by Meader and Robinson's (21) work, one of the constituent which is required for streptolysin production bears certain resemblances to the so called "accessory substances." The work of Avery and his associates (32), as well as that of other investigators, has demonstrated the marked effect which yeast extract may have upon microbial activity. Experiments on the influence of yeast extract upon the formation and stability of streptolysin seemed especially pertinent to the present investigation, since certain constituents of yeast extract had been found in previous studies (33) to have an intimate relation to the oxidation-reduction activities of different bacteria.

In experiments of the type described below, comparisons were made of the lysin produced in cultures grown in "unfiltered" muscle infusion broth with that produced in the same broth enriched by the addition of yeast extract. In the comparison of lysin production in different culture media, the methods developed in the preceding papers offer certain definite and obvious advantages since they furnish values more closely approaching the "total lysin" actually produced in the tested medium.

"Unfiltered" Muscle Infusion Broth.—Finely minced, fat-free, heart muscle was infused in slightly acidified tap water for several hours and then gradually

the whole culture is added to a suspension of blood cells, in place of the bacteria-free fluids used in the hemolysis tests in this study. Under these conditions, with a reasonably young, whole culture the danger of failure to detect lytic capacity is much more remote than would appear from the results of the preceding experiments, in which all tests were made with the bacteria-free culture fluids.

brought to a boil. Peptone and salt were added to the decanted infusion, and the broth, still slightly acid, was autoclaved for 10 minutes. The broth was adjusted to pH 7.8, let stand for several hours, and the clear supernatant siphoned off. This medium was finally sterilized in the autoclave for 8 minutes in tubes containing 100 cc. of the medium. After sterilization, a layer of sterile vaseline was added to the hot broth.

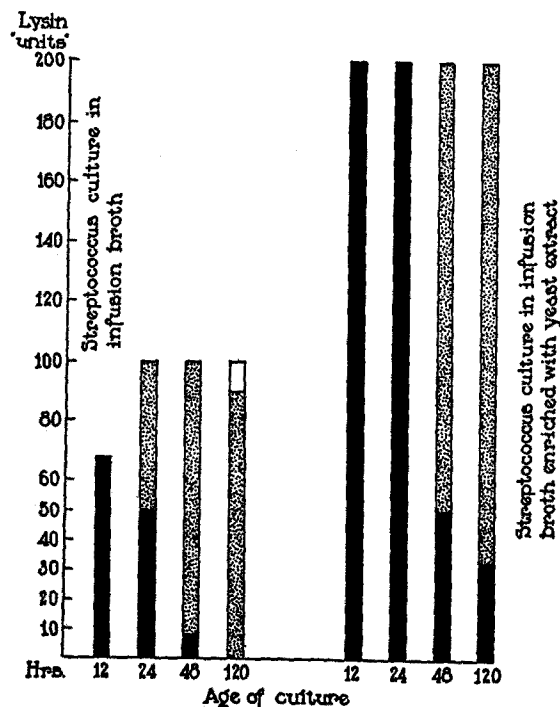


FIG. 2. The influence of yeast extract in the culture medium upon the production and stability of streptolysin.

The black shaded areas represent active (or reduced) lysin (Ly_r). The stippled areas represent the hemolytically inactive, reversible oxidation product (Ly_o) of the original or reduced lysin. The white area represents hemolytically inactive degradation products (Ly_x) of the lysin which cannot be "reactivated" by reduction treatment.

Broth Enriched with Yeast Extract.—Yeast extract broth was prepared by adding 7.5 cc. of yeast extract to each of several tubes of the above described meat infusion broth. The yeast extract was furnished by Dr. O. T. Avery of the Hospital of The Rockefeller Institute. The method of preparation of the yeast extract has been described in previous papers (34).

The lysin measurements were made by the methods used in the preceding experiments. Human blood cells were used in these experiments.

The results of an experiment performed as described above are presented in graphic form in Fig. 2. The same lysin "units" are employed as those used in the presentation of the experiment illustrated in Fig. 1.

The results of these experiments (Fig. 2) show that the addition of yeast extract to the medium enhances lysin formation, since it causes an increase in both the rate of lysin elaboration and the "total lysin" production. However, it is more pertinent to the present study to observe that the lysin after its elaboration "deteriorates" much more slowly in the enriched medium. For example, although much of the lysin formed in the unenriched culture medium "deteriorated" between the 12 and 24 hour tests, during the same period in the yeast extract broth, none of the lysin was converted to inactive oxidation products.

While certain constituents of yeast extract may have a specific effect upon the formation of streptolysin, the differences observed between the media used in the above experiment could also be explained by known effects of yeast extract upon bacterial growth activity. The increased rate of lysin formation as well as the greater total production of lysin may be due to a more rapid rate of growth of the streptococci in the enriched medium. It is probable that the deterioration of the lysin in any medium depends upon the loss in the reducing activity which was maintained during the period of active bacterial growth. If the yeast extract enables the streptococci to retain their life or growth activity for a longer period, the increased stability of the lysin is easily explained by the more persistent reducing conditions maintained in the yeast broth.

The difference in stability of the lysin in the two culture media offers another example of the advantages of the method of lysin measurement described. In the case of the unenriched broth, with certain strains of streptococci, a significant amount of active lysin is always converted to inactive products before the total amount of lysin actually elaborated has been liberated into the medium. Since in the yeast extract cultures the lysin proved more stable, it is obvious that a fair comparison of the actual production of lysin in the two media must take into account the inactive products formed by the deterioration of originally active lysin.

The Inhibition or Prevention of the Usual "Spontaneous Deterioration" of Streptolysin by Storing the Culture Fluids in a Sealed System Containing Sodium Hydrosulfite.

From analogous studies on hemoglobin (35), it seemed possible that the "spontaneous deterioration" of the bacterial lysin might be prevented if reducing conditions were imposed during its storage. To test the validity of this assumption, sodium hydrosulfite was added to streptococcus culture fluids in sealed tubes; and the stability of the lysin under these conditions was compared with the stability of the lysin in the same culture fluids to which no reducing agents had been added.

Cultures of Strain M grown in unenriched muscle infusion broth were chosen, as the lysin of this strain had proved most labile throughout all of the preceding experiments.

Portions of the streptococcus culture fluid, taken after stirring the culture, were assumed to be representative samples containing equal amounts of the lysin. The following series of tubes were then prepared:

1. *Culture Fluid Alone.*—4 to 5 cc. of the culture fluid were placed in narrow agglutination tubes so that the tubes were filled to within $\frac{1}{2}$ to $\frac{3}{4}$ of an inch from the top.

2. *Culture Fluid Plus Hydrosulfite.*—Tubes containing 25 mg. of $\text{Na}_2\text{S}_2\text{O}_4$ had previously been sterilized in hot air at 140°C . To these tubes, measured 5 cc. portions of the culture were added, which gave a concentration of 0.5 per cent of hydrosulfite.

pH of the Fluids.—The reaction of the culture fluid alone was approximately pH 6.6. The addition of the hydrosulfite resulted in a drop in pH, so that the culture plus hydrosulfite was about pH 5.9.

In an attempt to obtain a series containing the reducing agent but at the pH of the culture fluid alone, sterile NaOH was added to six of the hydrosulfite series. Unfortunately, through an error, too much alkali was added, and the final reaction at which these tubes of fluid were stored was about pH 9.0.

Sealing of the Tubes.—The tubes were sealed with care to make the exclusion of air as absolute as possible. The narrow tubes were completely filled with melted sterile vaseline immediately after the addition of the culture fluid. The excess vaseline escaped through the groove when a sterile, grooved cork stopper was carefully forced into the tube. The cork was finally completely sealed with sealing wax.

Storage of the Fluids.—A series of tubes containing culture fluid alone and tubes containing culture fluid plus $\text{Na}_2\text{S}_2\text{O}_4$ were stored at 38°C .; a second series were stored at room temperature. The temperature of the room varied con-

siderably; during the first 5 days it was approximately 25°C.; between the 5th and 21st days, it reached 32°C. on several occasions. The 140 day sample unfortunately was exposed to several prolonged periods of hot weather in which the temperature was frequently 35°C.

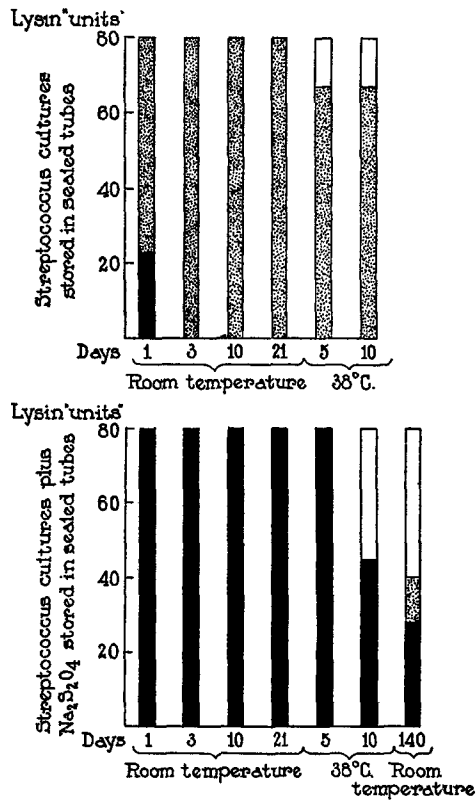


FIG. 3. Inhibition of the "spontaneous deterioration" of streptolysin by storing the culture fluids in a sealed system containing sodium hydrosulfite.

The black areas represent the reduced or active lysin (Ly_r); the dotted or stippled areas represent the hemolytically inactive reversible oxidation product of the lysin (Ly_o); the white areas represent further, inactive degradation products (Ly_z) which cannot be "reactivated" by reduction treatment.

Lysin Measurements.—The tubes of culture fluid were centrifuged and the bacteria-free supernatants used in the tests. 2 cc. of the test fluid were placed in each of two tubes; one of the tubes already contained 2.0 cc. of 0.1 M phosphate solution; 2.0 cc. of 1.0 per cent $Na_2S_2O_4$ in 0.1 M phosphate solution were

added to the other tube. After allowing time for reduction of the second tube, aliquots of these mixtures were used in the lysin titrations. Tests of the first of these mixtures represent measurements of the active lysin alone; the tests of the second of the above mixtures include not only the active lysin but the inactive, reversible oxidation product.

Controls in which uninoculated broth was stored in sealed tubes containing $\text{Na}_2\text{S}_2\text{O}_4$ were included to eliminate the possible formation of hemolytic substances by action of hydrosulfite upon the culture medium itself.

The results of this experiment are presented graphically in Fig. 3. In this figure, the measurements of lysin content of the fluids are recorded in terms of "lysin units," one "unit" representing the amount of lysin required to cause approximately 80 per cent hemolysis of 3 cc. of a 2 per cent suspension of blood cells.

In streptococcus cultures stored in sealed tubes under the usual conditions (*i.e.*, containing no added reducing agent), the active lysin is always soon converted to inactive products. In the experiment presented (Fig. 3) the fluid had become entirely devoid of lytic action after 3 days storage at room temperature. In fact, the "spontaneous deterioration," as might be expected from previous results (Fig. 1) with this especially labile strain, had already begun at the beginning of the storage experiment.

In contrast to the usual rapid "deterioration" in streptococcus cultures, the series in which hydrosulfite had been present during storage retained their entire original lysin activity for at least 21 days storage at room temperature, and for over 5 days at 38°C. Upon addition of the hydrosulfite at the beginning of the storage period, the hemolytically inactive oxidation product already formed in the fluid was reduced back to the original, active lysin. Then after the system was sealed the excess reducing agent maintained conditions which made impossible the usual "spontaneous" deterioration of lysin during the subsequent storage. It is evident that under these conditions (*i.e.*, stored in a sealed system in the presence of an active reducing agent), streptolysin, a classically labile bacterial product, would appear to be a relatively stable substance.

The results obtained with the hydrosulfite fluids after storage for 10 days at 38°C. and 140 days at "room temperature" introduce certain complications which, however, do not detract from the important relation evident in the results already discussed. Although some active lysin still persisted in these fluids, a

considerable portion has become inactive. It is possible that the failure of these fluids to retain their original lysin activity is due to some effect of prolonged exposure to high temperatures upon the hydrosulfite, as well as upon the lysin. (During the later part of the storage of the 140 day "room temperature" tube, it was subjected for several days to a temperature of 35°C.) It is significant that the lysin in these instances was converted to irreversible products rather than to the usual reversible oxidation product.

DISCUSSION.

Streptolysin is an extremely labile bacterial product. Under ordinary conditions, its loss of activity is so rapid and so pronounced that the hemolytic property of streptococcus culture fluids is recognized throughout the literature as "*une propriété éphémère*" (13).

In the present paper, it is shown that the hemolytically inactive product formed by this "spontaneous deterioration" can be "reactivated" by treatment with a chemical reducing agent. If the "reactivation" represents the reduction of an inactive, reversible oxidation product to the original active substance (reduced streptolysin), the process involved in the usual "spontaneous deterioration" of streptolysin can be regarded as an oxidation reaction which yields a reversible product.

Free exposure to air is not required for the oxidation of streptolysin. Although aeration of the culture fluid in shallow layers results in a much more rapid inactivation of the lysin, culture fluids held in tubes sealed from air gradually lose their hemolytic activity. The "deterioration" of streptolysin in tubes protected from air is in marked contrast to the stability of the lysins of Welch and tetanus bacilli, which, at least in culture fluids more acid than pH 7.0, remain active for months if the culture tubes are sealed with vaseline. The inactivation of the lysin in sealed tubes is due to the formation of a reversible oxidation product and hence, must be accepted as an oxidation process which yields a product identical or similar to that formed in aerated culture fluids. It is quite possible that the exclusion of air was not absolute in any of our experiments, and in the absence of exact controls of the gas content of the culture fluids, one cannot meet the question of whether or not traces at least of molecular oxygen are required for the oxidation of streptolysin.

The usual method for measuring bacterial lysins in culture fluids detect only the lysin which is in the active state at the time of the test. The methods previously employed in the studies (2, 3) of the Welch and tetanus lysins furnish in addition to measurements of active lysin, measurements of total lysin production and of the ratio of active to total lysin.

Applications of these methods have furnished a picture more complete than those existing heretofore of the actual course of lysin production and of lysin "deterioration" at various stages of growth of different strains of streptococci. The most important facts revealed were the following: (1) the content of active lysin (Ly_r) frequently decreases before the maximum amount of total lysin ($Ly_r + Ly_o$) is liberated into the medium; (2) the ratio $\left(\frac{Ly_r}{Ly_r + Ly_o}\right)$ varies with different strains and with different conditions to which the culture is subjected. These two facts in themselves are sufficient evidence that methods detecting only active lysin (Ly_r) cannot furnish a proper basis for comparisons of actual lysin production, either in experiments with different strains under the same conditions or in experiments with the same strain under different conditions.

The "spontaneous deterioration" of streptolysin, like the usual "spontaneous deterioration" of hemoglobin, consists in the conversion of an active substance to its inactive reversible oxidation product. As previously shown for hemoglobin (35), the formation of the inactive oxidation product is inhibited or wholly prevented in sealed systems containing an active reducing agent. For example, with one of the more labile strains studied, all of the active lysin "deteriorates" to inactive products in 2 or 3 days when stored at room temperature in sealed tubes containing no added reducing agent; on the other hand, in sealed tubes to which hydrosulfite had been added, the same culture fluids were stored for more than 21 days without loss in the apparent content of active lysin and without formation of a detectable trace of the inactive "deterioration" product. Apparently, therefore, if reducing conditions of sufficient intensity be maintained in the culture fluid, the ratio of active lysin to total lysin (*i.e.*, $\frac{Ly_r}{Ly_r + Ly_o}$) remains constant for a considerable

time at a value approximating unity. It is probable that the reducing conditions established by the streptococcus cells during the period of maximum growth diminish in intensity upon the cessation of the bacterial growth activity and this failure of the bacterial culture to maintain the strong reducing conditions which obtained during the period of active lysin elaboration is the cause of the conversion of active lysin to its inactive oxidation product (*i.e.*, a decrease in the ratio $\left(\frac{Ly_r}{Ly_r + Ly_o}\right)$). From this point of view, one would expect the conversion of the active lysin to its oxidation product to be inhibited in any system in which reducing conditions are maintained for a longer period. If the ratio of active lysin to total lysin $\left(\frac{Ly_r}{Ly_r + Ly_o}\right)$ be accepted as an index of reducing conditions in the culture fluid, it is interesting to observe that observations of an unusual persistence of active streptolysin in certain cultures (as for example, the yeast extract cultures of our Strain D) indicate that reducing conditions of sufficient intensity are maintained for a longer period in these cultures.

It has been mentioned before that the lysin of streptococcus "deteriorates" in vaseline-sealed culture fluids, while the lysin in culture fluids of pneumococci (36), tetanus (3), and Welch bacilli (2) are relatively stable if similarly protected from air. An explanation of the greater tendency of streptolysin toward "deterioration" involves two factors: either reducing conditions of sufficient intensity are maintained for a longer period in the cultures of the other bacteria, or streptolysin itself is a substance intrinsically more easily oxidized than the other lysins. The importance of the first factor has already been illustrated by the relative stability of the usually labile streptolysin when stored in a sealed system containing an added chemical reducing agent. A true evaluation of the second factor must depend upon measurements of the reducing intensities obtaining in the respective culture fluids. However, the fact that streptolysin is converted to its oxidation product in systems of sufficient reducing power to prevent a similar conversion of methylene white indicates that the lysin itself is a relatively easily oxidized substance.

SUMMARY.

The "spontaneous deterioration" of streptolysin has been studied by the methods already used in investigations of the oxidation-reduction of blood pigments and of the lysins of other bacteria. From the results of this study, it may be concluded that the commonly observed "spontaneous deterioration" of streptolysin consists in the conversion of the originally active lysin to a hemolytically inactive oxidation product. This process is a reversible one, and the activity of the lysin is restored by reduction.

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