# PARALLELISM IN THE LETHAL AND HEMOLYTIC ACTIVITY OF THE TOXIN OF CLOSTRIDIUM SEPTICUM*

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It has long been known that cultures of *Clostridium septicum* contain not only a toxin which is lethal for laboratory animals but a filtrable hemolysin as well. The relation between the toxin and the hemolysin, however, is not clear. The present report is concerned with attempts to determine whether *Cl. septicum* produces (1) two substances, namely a lethal toxin and a hemolytic agent, or (2) a single substance responsible for both the hemolytic and lethal properties. An answer to this question is of somewhat more than academic interest for a simple *in vitro* method of assaying unconcentrated toxin does not seem to be available and perhaps hemolysis tests will provide this.

It is the opinion of Menk (1), Robertson (2), Karube (3), and Weinberg, Nativelle, and Prévot (4), among others, that the hemolysin and lethal toxin are separate substances. The most carefully executed experiments are perhaps those of Menk (1) who found that although the hemolysin is as sensitive to thermal inactivation as the lethal toxin, the two could nevertheless be separated by specific adsorption of the former upon erythrocytes. Further evidence in support of Menk's contention is afforded by Robertson (2) in whose experience the hemolytic capacity and the lethal potency of cultures bore no constant relation to each other. Karube (3), on the other hand found that the hemolytic and lethal activities of cultures closely parallel each other, but adduced other evidence which led him to conclude that the two properties are not interdependent. In contrast to Menk's findings, Karube stated that the hemolysin is much more sensitive to thermal inactivation than is the lethal toxin.

In a recent report, Koerber and Alture-Werber (5) described a method of assaying *Cl. septicum* antitoxin based upon the capacity of immune serum to neutralize specifically the hemolytic activity of culture filtrate. The findings of Koerber and Alture-Werber suggest that hemolysin and lethal toxin may be a single substance, a conclusion which is not in agreement with those of the above cited authors.

## *Methods*

*Culture Technique.*—The smooth hemolytic variant of *Cl. septicum* strain 44 obtained from the National Institute of Health, was cultivated in a casein hydrolysate medium (6) according to the method for toxin production which is described in an accompanying paper (6). After

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removing the bulk of the bacteria by centrifugation, the supernatant fluid was stored in the refrigerator at 6–10°C. until used. The culture supernate is hereinafter referred to as such, or as "crude toxin."

*Estimation of Hemolytic Activity.*—Amounts of culture supernate were chosen to give the desired dilution when made to 1 ml. with the buffered saline containing M/12.9 NaCl and M/15 mono- and disodium phosphate, pH 7. To each dilution of culture supernate was added 1 ml. of 0.7 per cent suspension of washed human red cells in buffered saline, pH 7. After mixing the contents of the tubes by shaking, the tubes were placed at 36–38°C. for 30 minutes, and then centrifuged for 2 to 3 minutes in order to sediment the unhemolyzed cells. The degree of hemolysis was estimated by visual comparison of the color of the supernatant fluid in the test tubes with that of standard tubes. The latter were prepared by lysing a portion of the standard red cell suspension with saponin and diluting with suitable amounts of water to represent 10 per cent, 20 per cent, 30 per cent, etc., hemolysis. The hemolytic unit is the amount of hemolysin which liberates half of the total hemoglobin contained in 1 ml. of the standard red cell suspension, in a final volume of 2 ml. (1 ml. of hemolysin dilution plus 1 ml. of standard cell suspension). With cultures of unknown hemolytic activity, a preliminary titration employing twofold or fivefold dilutions was first done. After estimating the approximate hemolytic activity, the test was repeated using more closely spaced dilutions of culture. The method is reproducible to within 15 per cent and could doubtless be rendered more accurate should this prove desirable.

Owing to the presence, in cultures, of one or more substances of unknown nature which inhibit hemolysis, estimation of the hemolysin in cultures containing less than about 5 hemolytic units per ml. may be subject to appreciable error. In testing cultures whose activity is greater than 5 hemolytic units per ml., the hemolysin-inhibiting substances are "diluted out" so that their effect is probably negligible.

*Estimation of Lethal Toxin.*—Cultures were spun for 20 to 30 minutes in the Swedish angle centrifuge. The supernatant fluid was diluted in cold phosphate-buffered saline (M/12.9 NaCl and M/15 mono- and disodium phosphate, pH 7). In later experiments the diluent was modified by dissolving 1 gm. of gelatin in 100 ml. diluent. Although no striking difference accompanied the inclusion of gelatin, its use was continued on the ground that surface denaturation may occur in the absence of protective colloid.

0.1 ml. quantities of appropriate dilutions of culture supernate were injected intravenously into the tail veins of white mice. The $L.D._{50}$ is the volume of undiluted culture supernate which kills half the mice of a group within 72 hours after injection. Although mice weighing 16 to 20 gm. were used whenever possible, it was necessary at times to resort to the injection of mice which were somewhat overweight. This circumstance is unfortunate since the $L.D._{50}$ determinations are subject to large error under the best of conditions. Unexpectedly great variation among the individuals in groups of apparently similar mice receiving the same dose of toxin was observed.

*Estimation of Antihemolytic Potency of Antitoxin.*—*Decreasing quantities* of antitoxin were added to 0.5 ml. amounts of culture supernate, the latter having been previously diluted to contain 4 hemolytic units per ml. After adjusting the volume to 1 ml., with buffered saline at pH 7, the tubes containing culture supernate and antitoxin were mixed by shaking, and allowed to stand at room temperature. After 60 minutes, the hemolytic activity of the mixtures was determined in the manner previously described. The 50 per cent end-point was used, and the antihemolytic capacity of the antitoxin was estimated by comparison with that of National Institute of Health standard antitoxin.

*Estimation of Antilethal Potency of Antitoxin.*—The capacity of antitoxin to neutralize the lethal action of culture supernate was assayed according to the method of Bengston (7). The test dose of toxin used was the amount of toxin which when mixed with ½ unit of antitoxin

and injected intravenously into mice caused half the mice of a group to die within 72 hours. The potencies of unknown sera were found by comparing their capacity to neutralize toxin with that of the National Institute of Health standard antitoxin.

## EXPERIMENTAL

*The Lethal and Hemolytic Activity of Cultures.*—If the lethal and hemolytic properties of cultures are functions of a single substance, then parallelism in the degree to which different cultures are lethal and hemolytic should be demonstrable. In connection with studies on toxin production by strain 44, the lethal and hemolytic potencies of a large number of cultures, most of them grown in a medium of simplified composition (6), were determined. The conditions of incubation and the exact composition of the medium differed from one culture to the next, and were such as to allow considerable variation in the amounts of toxin and hemolysin formed. The results of determinations on 15 cultures are presented in Table I.

Table I shows that there is good parallelism between the lethal and hemolytic potencies. The most toxic cultures are the most hemolytic, while the least toxic cultures are least hemolytic. The mean ratio of the $L.D._{50}$ to the hemolytic unit, calculated from these and other data, is approximately 13.

*Effect of Hydrogen Peroxide on Hemolytic and Lethal Activity.*—If the lethal and hemolytic properties are functions of a single substance, then treatment of culture supernate with a substance which will partially inactivate the lethal factor might be expected to inactivate the hemolytic activity to the same extent. Hydrogen peroxide was chosen for this experiment.

Culture supernate having a hemolytic activity of 20 hemolytic units per ml. and a lethal activity of 300 to 400 $L.D._{50}$ per ml. was incubated at 37°C. with hydrogen peroxide in a final concentration of 0.1 per cent at pH 6.0. The titrations of hemolytic and lethal activities are set out in Table II. After 60 minutes, the hemolytic activity fell to 8 hemolytic units per ml., and the lethal activity to about 150 $L.D._{50}$ per ml. Thus, the reduction in hemolytic activity caused by hydrogen peroxide was proportional to the reduction in lethal activity. The initial hemolytic activity of the culture supernate could not be restored by the addition of sodium thioglycollate in a final concentration as high as 2 per cent.

*Removal of Lethal and Hemolytic Activity by Adsorption.*—The purpose of this experiment was to find out whether the agent responsible for hemolysis could be separated from the lethal toxin by adsorption upon charcoal or kaolin. Treatment of the supernatant fluid of a culture with charcoal or kaolin completely removes both lethal and hemolytic activities. By controlling the temperature, hydrogen ion concentration, and the time interval during which the adsorbent is in contact with the culture supernate, the weight of adsorbent which will remove only a portion of the hemolytic activity can be found. Both lethal and

hemolytic activities of culture supernate can be measured before and after adsorption, and the fraction of each adsorbed determined by difference. The results of such experiments are given in Table III, in which it is seen that the portion of lethal activity removed by adsorption with either charcoal or kaolin is approximately equivalent to the portion of hemolytic activity removed.

Inasmuch as charcoal and kaolin are relatively non-specific adsorbents, attempts were made to separate the hemolytic from the lethal activity by adsorbing upon erythrocytes the agent responsible for hemolysis. Menk (1) has reported that hemolysin can be separated from lethal toxin by this procedure.

TABLE I

*Comparison of the Hemolytic and Lethal Activities of 15 Cultures of Strain 44 of Cl. septicum*

| Culture No. | Hemolysin | Lethal toxin | Ratio of L.D.$_{50}$ to hemolytic unit |
|---|---|---|---|
| | *H.U./ml.* | *L.D.$_{50}$/ml.* | |
| 1 | 15 | 175 | 12 |
| 2 | 8 | 100 | 13 |
| 3 | 0 | 10 | — |
| 4 | 27 | 350 | 13 |
| 5 | 18 | 200 | 11 |
| 6 | 20 | 330 | 17 |
| 7 | 12 | 75 | 6 |
| 8 | 43 | 500 | 12 |
| 9 | 5 | 70 | 14 |
| 10 | 18 | 275 | 15 |
| 11 | 40 | 600 | 15 |
| 12 | 17 | 125 | 7 |
| 13 | 5 | 100 | 20 |
| 14 | 3 | 70 | 23 |
| 15 | 20 | 350 | 18 |

0.2 ml. of culture supernate diluted to contain 5.5 hemolytic units and 100 L.D.$_{50}$ of toxin, was mixed in the cold with 10 ml. of a 5 per cent suspension of sheep erythrocytes in 6 per cent glucose. The mixture was maintained at 0–3°C. for 60 minutes and then centrifuged in the cold. When the sedimented erythrocytes were resuspended in buffered saline, they hemolyzed completely in 5 minutes at 37°C. The fluid in which the sheep erythrocytes had been suspended was slightly tinged with hemoglobin. It contained no demonstrable hemolytic activity, 1 ml. failing to produce detectable hemolysis of 1 ml. of standard human erythrocyte suspension at 37°C. No lethal activity could be found in this fluid as judged by the survival of 3 mice each injected intravenously with 1 ml.

In this experiment the whole, or the greater part, of both the hemolytic and lethal activities was removed by the sheep erythrocytes. Since the toxin initially contained 10 L.D.$_{50}$ per ml., and since after treatment with sheep erythrocytes the supernatant fluid contained less than 1 L.D.$_{50}$ per ml., it is clear that at least 90 per cent of the lethal activity had been removed by the sheep

erythrocytes. The significance of these results, however, is diminished by the possibility that the lethal toxin may have been non-specifically adsorbed on the red cells. Moreover, the quantitative estimation of the low hemolysin concentration employed is difficult. Attempts to carry out this experiment using greater concentrations of hemolysin failed because considerable hemolysis occurred during the period of adsorption even though this was done at 0°.

TABLE II

*Reduction in Hemolytic and Lethal Activity of Culture Supernate Treated with Hydrogen Peroxide*

| | Titrations of hemolytic activity | | | | | | | Titrations of lethal activity | | | Ratio of lethal to hemolytic activity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dilution of culture supernate: 1:2 | 1:5 | 1:10 | 1:15 | 1:20 | 1:25 | Hemolytic activity | Culture supernate injected | Proportion of mice killed | L.D.50 per ml. culture supernate | |
| | Hemolysis | | | | | | | | | | |
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *H.U./ml.* | *ml.* | | | |
| Culture supernate at 4°C. for 60 min. | 100 | 100 | 100 | 80 | 50 | tr | 20 | 1/100<br>1/200<br>1/300<br>1/400 | 3/3<br>3/3<br>4/6<br>1/3 | 300 to 400 | 15 to 20 |
| Culture supernate at 37°C. for 60 min. | 100 | 100 | 95 | 70 | 45 | tr | 19 | — | — | — | — |
| Culture supernate containing 0.1 per cent $H_2O_2$ at 37°C. for 60 min. | 100 | 70 | 35 | 0 | 0 | 0 | 8 | 1/20<br>1/50<br>1/100<br>1/150<br>1/200 | 2/2<br>4/4<br>4/4<br>2/4<br>0/2 | 150 | 19 |
| 0.1 per cent $H_2O_2$ in phosphate-buffered saline | 0<br>0 | 0<br>0 | 0<br>0 | <br>0 | <br>— | <br>— | — | 1/20<br>1/50<br>1/100 | 0/1<br>0/2<br>1/2 | These are controls for toxicity and hemolytic activity of $H_2O_2$ | |

tr, trace of hemolysis; 0, no hemolysis.

*Partial Loss of Lethal and Hemolytic Activity on Standing at 37°C.*—Neither the lethal toxin nor the hemolysin present in unconcentrated culture supernates is stable at 37°C. at pH 6.2. It was of interest to know whether or not, under these conditions, the lethal toxin and the hemolysin are inactivated to the same extent. As shown in Table III, lines *a*, *i*, and *j*, exposure of culture supernate to a temperature of 37°C. at pH 6.2 for 3 hours caused a loss of 30 to 40 per cent of both the lethal and hemolytic activities.

*Loss of Hemolytic and Lethal Activity During Attempts to Purify the Hemolysin.*

—While attempting to purify the hemolysin, both the hemolytic and lethal activities were followed. Purification was carried out chiefly by fractional precipitation with ammonium sulfate, during the course of which procedure most of the hemolytic and lethal activities were lost. A culture of *Cl. septicum* was treated in the following manner.

TABLE III

*Decrease in Lethal and Hemolytic Activity of Crude Toxin by Adsorption on Charcoal and Kaolin and after Standing at 37°C. for 180 Minutes*

| | Hemolytic activity | | Lethal activity | |
|---|---|---|---|---|
| | *H.U./ml.* | | L.D.50/ml. | |
| (*a*) Before adsorption on charcoal | 20 | | 330 | |
| (*b*) Supernate after adsorption on charcoal (5 ml. crude toxin plus 15 mg. activated charcoal for 5 min. at 27°C. at pH 6.2) | 4 | | 80 | |
| (*c*) Decrease in activity $(a - b)/a$ | | *0.80* | ..... | *0.76* |
| (*a*) Before adsorption on charcoal | 20 | | 330 | |
| (*d*) Supernate after adsorption on charcoal (5 ml. crude toxin plus 15 mg. activated charcoal for 5 min. at room temperature at pH 6.2) | 5 | | 76 | |
| (*k*) Decrease in activity $(a - d)/a$ | | *0.75* | ..... | *0.76* |
| (*a*) Before adsorption on kaolin | 20 | | 330 | |
| (*e*) Supernate after adsorption on kaolin (5 ml. crude toxin plus 25 mg. kaolin for 5 min. at 27°C. at pH 6.2) | 1 | | 52 | |
| (*f*) Decrease in activity $(a - e)/a$ | | *0.95* | ..... | *0.85* |
| (*a*) Before adsorption on kaolin | 20 | | 330 | |
| (*g*) Supernate after adsorption on kaolin (4 ml. crude toxin plus 10 mg. kaolin for 5 min. at room temperature at pH 6.2) | 6 | | 142 | |
| (*h*) Decrease in activity $(a-g)/a$ | | *0.70* | ..... | *0.57* |
| (*a*) Crude toxin at 37°C. for 0 min., pH 6.2 | 20 | | 330 | |
| (*i*) " " " 37 " " 180 " " 6.2 | 12 | | 225 | |
| (*j*) Decrease in activity $(a - i)/a$ | | *0.40* | ..... | *0.32* |

430 ml. of culture having an activity of 23 hemolytic units (H.U.) per ml. were centrifuged without loss of activity. The supernatant fluid (*a*), of which the pH was 5.7, was passed through a Seitz filter. The filtrate (*b*), containing 17.5 H.U. per ml., was transferred to a cellophane sac, a few drops of toluene added, and evaporated in a current of air for 20 hours, by the end of which time the volume was reduced to 1/6 the initial volume. A viscous layer having a volume of 7 ml. separated at the bottom of the sac. This bottom layer which contained 60 H.U. per ml. was discarded. The top layer (*c*) had a volume of 72.5 ml., and contained 80 H.U. per ml., or about 60 per cent of the initial activity.

The next step resulted in the loss of most of the activity. To the top layer (*c*) of the cellophane sac concentrate was added 2.33 volumes of saturated ammonium sulfate, pH 5.5

(abbreviated S.A.S.), at room temperature. The precipitate which formed was separated from the inactive supernatant fluid and was washed once in 20 ml. of 0.7 S.A.S. Most of the washed precipitate went into solution upon the addition of 10 ml. of distilled water, to form a slightly cloudy, brown fluid. This was centrifuged, and the small amount of insoluble precipitate discarded; the supernatant fluid (*d*) contained 108 H.U. per ml., or about 10 per cent of the initial activity.

To fraction *d* was added 1.5 volumes of S.A.S. in the cold. The inactive supernatant fluid was separated by centrifugation from the precipitate which had formed and was discarded. After washing in 20 ml. 0.6 S.A.S., the precipitate was dissolved in 10 ml. distilled water (*e*). This fraction had an activity of 75 H.U. per ml.

To fraction *e* was added 1.23 volumes of S.A.S. in the cold. The inactive supernatant fluid was separated by centrifugation from the precipitate which had formed and was discarded. After washing with 20 ml. 0.55 S.A.S., the precipitate was dissolved in 5.0 ml. distilled water (*f*). This fraction had an activity of 130 H.U. per ml.

Fraction *f* was dialyzed against 800 ml. distilled water in the refrigerator for 60 hours with occasional agitation, during which time a precipitate formed in the dialyzing sac. The precipitate, after separation by centrifugation, was found to be slightly more active than the supernatant fluid (*h*), and was taken up in 5.0 ml. 0.9 per cent NaCl solution (fraction *g*). This fraction had an activity of 30 H.U. per ml. and represented therefore, about 1.5 per cent of the initial activity.

The hemolytic and lethal potencies of the active fractions are presented in Table IV. It would appear that it is not possible to separate to a significant extent the hemolytic from the lethal activity by the methods employed. Moreover, the loss of a large portion of the hemolytic activity was accompanied by a correspondingly large loss in lethal activity. Although the portion of the initial lethal activity recovered in the various active fractions was somewhat less than that of the hemolytic activity, the difference in the two, in view of the probable error in estimation of lethal activity (see Discussion), is not considered to be significant.

*The Capacity of Cl. septicum Antitoxin to Neutralize the Lethal and Hemolytic Properties of Toxin.*—The purpose of this experiment was to find out whether or not parallelism exists between the antihemolytic and antilethal capacity of antitoxin.

7 specimens of antitoxic horse sera[1] were tested. 5 of these were commercially available "gas gangrene antitoxin" containing antitoxin against *Cl. welchii* as well as *Cl. septicum*. Of the remaining 2, one (No. 1) was monovalent, unconcentrated, antitoxic horse serum, while the other (NIH) was the standard glycerinated antitoxin of the National Institute of Health. All the sera were titrated against a constant quantity of test toxin in the manner described under Methods. The results of the estimations of antihemolytic and antilethal potencies comprise Table V.

It is seen that, with the exception of antitoxin 5, bottle 1, there is a good agreement in the antihemolytic and antilethal potencies of the antitoxins tested.

[1] Kindly supplied by the National Institute of Health, Lederle Laboratories, Inc., National Drug Company, Sharp and Dohme, Eli Lilly and Company, and Parke, Davis and Company.

TABLE IV

*Hemolytic and Lethal Activities of Culture Supernate Fractionated Chiefly by Means of Ammonium Sulfate*

| Fraction | Titration of lethal activity | | | | | | | | Hemolytic activity | Ratio of lethal to hemolytic activity | Per cent initial hemolytic activity | Per cent initial lethal activity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ml. injected intravenously | | | | | | | Lethal activity | | | | |
| | 1/50 | 1/100 | 1/200 | 1/400 | 1/800 | 1/1600 | 1/3200 | | | | | |
| | | | | | | | | L.D.50/ml.* | H.U./ml. | | | |
| (*a*) Culture supernate 430 ml.‡ | | 4/6§<br>6/6 | 5/5<br>6/6 | 5/6<br>2/6 | 0/6<br>2/6 | | | 460<br>400 | 23 | 20<br>17.4 | 100 | 100 |
| (*b*) Seitz filtrate 425 ml. | | | | | | | | — | 17.5 | — | 75 | — |
| (*c*) Cellophane sac concentrate (top layer) 72.5 ml.‡ | | | | 6/6<br>6/6 | 3/6<br>5/6 | 0/6<br>0/6 | 0/6<br>0/6 | 800<br>1060 | 80 | 10.0<br>13.3 | 59 | 34<br>45 |
| (*d*) Fraction precipitated by 0.7 S.A.S.‖ 8.3 ml. | | | | 6/6 | 5/6 | 2/6 | 0/6 | 1200 | 108 | 11.1 | 9 | 5.8 |
| (*e*) Fraction precipitated by 0.6 S.A.S. 10 ml. | | | | | | | | — | 75 | — | 7.6 | — |
| (*f*) Fraction precipitated by 0.55 S.A.S. 5 ml. | | | | 6/6 | 6/6 | 0/6 | 0/6 | 1130 | 130 | 8.7 | 6.6 | 3.3 |
| (*g*) Water-insoluble precipitate obtained upon dialysis of fraction *f* 5 ml. | | | 5/6 | 1/6 | 0/6 | 0/6 | | 280 | 30 | 9.3 | 1.5 | 0.8 |
| (*h*) Water-soluble fraction obtained upon dialysis of fraction *f* 9.5 ml. | 6/6 | 2/6 | 1/6 | 0/6 | | | | 93 | 13 | 7.2 | 1.2 | 0.5 |

* L.D.50 per ml. is calculated by the method of Reed and Muench (8).

‡ Duplicate titrations of lethal activity were done on fractions *a* and *c*.

§ 6/6, 3/6, etc., are the number of mice dead in 72 hours over the number of mice injected.

‖ S.A.S. indicates saturated ammonium sulfate.

Antitoxin 5, bottle 1, appears, according to the antihemolytic test, to contain only about 1/3 as much antitoxin as it does by the antilethal test. The peculiar behavior of this specimen of antitoxin was confirmed by the observation that a neutral mixture with toxin (*i.e.* a mixture which is not lethal for mice) hemolyzed a suspension of red blood cells, while neutral mixtures of other antitoxins were completely non-hemolytic. The antitoxin contained in a second bottle, obtained at the same time from the same source, and bearing the same lot number, yielded antihemolytic and antilethal values which were in good agreement with each other. 4 additional samples of lot 271043 obtained subsequently and stated to contain approximately 800 units of antitoxin per ml., were found by the antihemolytic test to contain 970, 970, 800, and 970 units of

TABLE V

*Capacity of Antitoxin to Neutralize the Hemolytic and Lethal Actions of Culture Supernate*

| Antitoxin No. | Units of antitoxin* per ml. | |
|---|---|---|
| | Determined by comparison of antihemolytic capacity with that of antitoxin NIH | Determined by comparison of antilethal capacity with that of antitoxin NIH |
| NIH | 100 | 100 |
| 1 | 230 | 180 |
| 2 | 1700 | 2000 |
| 3 | 515 | 460 |
| 4 | 3200 | 3100 |
| 5 (lot 271043, bottle 1) | 320 | 1000 |
| 5 (" 271043, " 2) | 850 | 900 |
| 6 (" 326995) | 400 | 500 |

* The unit of antitoxin is that used by the National Institute of Health.

antitoxin per ml. It is thought that the anomalous antihemolytic value of the first bottle of antitoxin 5 could possibly be explained by the presence of a contaminating hemolytic substance (as for example, a detergent used in cleansing the bottle before filling), but since the contents of this bottle became exhausted, unfortunately this hypothesis could not be tested. The antihemolytic and antilethal capacities of another sample of antitoxin (No. 6 of Table V) obtained from the same source at a later date, and bearing a different lot number, were in essential agreement.

## DISCUSSION

It is not easy to reconcile the results of the foregoing experiments with those of a number of other authors, especially with those who consider that the lethal and hemolytic properties are attributable to distinct and separate components of culture filtrate or supernate. In particular, Menk (1) has presented evidence for the non-identity of toxin and hemolysin by specifically adsorbing the latter

upon erythrocytes. Using methods similar to those employed by Menk, separation of lethal toxin from hemolysin could not be effected in the present study.

There exists the possibility that the hemolysin which has been studied in the present investigation may possess different properties from the hemolysin studied by Menk. Measurement of the lethal and hemolytic activities of cultures of strains other than strain 44 have not been sufficiently extensive to permit the conclusion that the findings with strain 44 are of general application. As will be shown in a separate report (9), the hemolysin we have studied does not exhibit optimum activity in the region of pH 6.8–7.6, nor is the hemolytic action inhibited at pH 5.8. Menk found that *Cl. septicum* hemolysin was inhibited at pH 8.0 as well as at pH 5.8, but not at 6.8 nor 7.6. The significance of these differences, however, is difficult to assess because, according to Walbum (10), the dependence of hemolytic activity upon hydrogen ion concentration is influenced to a great extent by the nature of the buffer system. Other investigators working with other strains of *Cl. septicum* and under a different set of experimental conditions may not find the parallelism in lethal and hemolytic activity which has been encountered in the present study. It is possible that not one, but two hemolysins may be produced by *Cl. septicum*, as is known to be the case with group A hemolytic streptococci (11) and with *Cl. welchii* (12). The latter species, moreover, may produce one or both hemolysins depending upon the strain and method of cultivation (13). The question of the possible production of two hemolysins by *Cl. septicum* clearly requires investigation, and can probably best be answered by studying freshly isolated strains. In the strain used in the present investigation, there is no evidence that more than one hemolysin is produced.

It is of importance that one have some idea of the magnitude of error involved in the methods of estimation of lethal and hemolytic activity. The significance of experiments designed to separate lethal from hemolytic activity obviously depends upon the precision with which these properties are assayed. The least satisfactory measurements in the experiments herein described are those of the lethal principle. Although there is fair agreement between the duplicate titrations of lethal toxin given in parts *a* and *c* of Table IV, other duplicate titrations which have been carried out suggest that the error in the *in vivo* determinations may be as large as 50 to 100 per cent. The error in the estimation of hemolytic activity, compared to that of the lethal activity, is probably negligible, except in preparations which are weakly hemolytic. In such preparations, the value of the hemolytic activity tends to be too low, owing to interference with hemolysis by inhibitor. The existence in cultures of a thermostable inhibitor to hemolysis can be demonstrated by diluting a preparation of hemolysin either in buffered saline, or in culture supernate previously heated to 56°C. for 3 hours. In the first instance, the hemolysin has an activity of 35

hemolytic units per ml.; in the second, it has an apparent activity of only 15 hemolytic units per ml. Similar observations have been made by Menk (1).

Reference to part *e* of Table IV shows that a particular culture supernate having a lethal-hemolytic ratio of 17, after treatment with kaolin had a lethal-hemolytic ratio of 52. The ratio of 52, however, is calculated from an apparent hemolytic activity of only 1 hemolytic unit per ml. The true hemolytic activity, *i.e.* the hemolytic activity in the absence of inhibitor, is probably 2 or 3 hemolytic units, so that the true lethal-hemolytic ratio is probably closer to 17 to 26.

In view of the possible error in the estimation of lethal activity, to what extent may one expect variation in the experimental value of the lethal-hemolytic ratio? The average value of the lethal-hemolytic ratio of a large number of cultures is approximately 13. If the error in the estimation of lethal activity is as large as 50 or 100 per cent, as it probably is in some instances, then one would expect the lethal-hemolytic ratio to vary from about 6 to 26. This range of variation is very close to that which has been found experimentally, as can be seen for example, in Table I in which the lethal-hemolytic ratio has apparent values of from 6 to 23.

It has not been found possible to separate, by the various methods employed, the lethal from the hemolytic principle: lethal and hemolytic activities have paralleled each other in all instances. These findings suggest that the lethal toxin and the hemolysin may be one substance. More cogent evidence for this concept, however, is the establishment by Koerber and Alture-Werber (5) and its confirmation in the present study, that the antilethal and antihemolytic potencies of horse sera containing *Cl. septicum* antitoxin are parallel. This finding indicates that both effects are due either to a single antibody, or to two antibodies which are always or nearly always present in the same ratio. If the second possibility be excluded on the ground of improbability, one must then conclude that one is dealing with a single kind of antibody formed in response to a single antigen possessing both lethal and hemolytic properties. Further evidence in favor of the identity of hemolysin and lethal toxin is presented in an accompanying paper (6) in which it is shown that the loss of the capacity of a strain to produce hemolysin is accompanied by a loss of capacity to produce lethal toxin.

The experimental findings may, however, be interpreted in a different way. It is possible that the hemolysin and lethal toxin are two distinct substances possessing physical and chemical properties which are so similar that separation of the two cannot be achieved readily. In addition, their parallel production in cultures might lead to the formation of equivalent amounts of antihemolytic and antilethal antibodies in horses which have been hyperimmunized with culture filtrate. The relatively limited data which have been presented in this paper are insufficient to rule out these possibilities.

SUMMARY

The relation of the lethal toxin to the hemolysin produced by *Clostridium septicum*, strain 44, has been investigated. The following results suggest that the hemolytic and lethal actions of crude toxin are functions of a single substance or that they are functions of two substances which have similar physical, chemical, and antigenic properties. (1) Within the limits of experimental error, the lethal activity of cultures is directly proportional to their hemolytic activity. (2) Treatment of a culture supernate with hydrogen peroxide diminishes to the same extent its hemolytic and lethal activities. (3) The hemolytic principle and the lethal toxin are adsorbed to approximately the same extent by charcoal. (4) The hemolytic principle and the lethal toxin are adsorbed to approximately the same extent by kaolin. (5) Treatment of culture supernate with erythrocytes results in the removal of most of the lethal activity as well as of most of the hemolytic activity. (6) Both lethal toxin and hemolytic principle are partially destroyed in dilute solution at 36°C. The loss in lethal and hemolytic activities occurs in parallel. (7) The lethal and hemolytic activities of cultures are found to be inseparable by fractional precipitation with ammonium sulfate. (8) The antihemolytic capacity of antitoxic horse serum is found to be directly proportional to the antilethal capacity.

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