A STUDY OF THE COMPETITION OF LECITHIN AND ANTITOXIN FOR CL. WELCHII LECITHINASE*

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During recent years the similarity between the linkages of enzyme to substrate, and antigen to antibody has attracted considerable attention (2, 3). The recently observed identity between the toxic and enzymic action of the alpha toxin of *Cl. welchii* invited a more thorough analysis of such an analogy in this case. It had already been found by the discoverers of the enzymic nature of this toxin (4), that enzymatic action may be blocked by the antibody, a phenomenon previously recorded (5), but by no means general with respect to enzyme-antibodies (6–10). This inhibitory effect seemed a suggestion that antibody and lecithin might react with analogous regions of the lecithinase-toxin. The availability of a flexible manometric method to study this reaction (11) promised a more complete analysis of the triangular interrelationship between an enzyme, its substrate, and its antibody.

The assumption has been made (4) that other bacterial toxins may also be enzymic in nature. It is therefore possible that the competition found in these studies to exist between the antitoxin and the substrate for the enzyme-toxin may have a parallel in other bacterial toxins whose enzymic nature is as yet unrecognized.

Materials and Methods

The *Cl. welchii* (type A) toxic filtrates were obtained in several lots, either from Professor Milan A. Logan or from the Lederle Laboratories. Their known composition has been previously described (11). They were, in brief, glycerol-dialyzed filtrates consisting principally of alpha toxin. The toxic filtrate most used contained 1760 M.L.D. (mouse subcutaneous LD_{50}) per cc., and had an Lb of 43. One M.L.D. of toxic filtrate represented 6.1 γ of protein nitrogen. For the sake of convenience, the term toxin has been used hereafter rather than "toxic filtrate."

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A preliminary report of this method was given at Atlantic City, March 14, 1946, before the American Association of Immunologists (1).

The material presented in this paper and the preceding one (11) was awarded the Warren Triennial Prize for the year 1946.

The antitoxins were either commercially prepared polyvalent products labelled to contain on the average 500 units of *Cl. welchii* antitoxin per cc., or a monovalent *Cl. welchii* antitoxin containing 900 units per cc. In the latter preparation, 14 γ of protein nitrogen contained 1 unit of antitoxin. The activities of the toxin and antitoxin used were previously determined by Logan (12) and by the Lederle Laboratories, respectively, using the turbidimetric method of van Heyningen (13). In view of the difference in techniques used and choice of end point, the combining relationships of toxin-antitoxin mixtures as calculated from their data were in fair agreement with those found by the manometric method. A discussion of the interrelationships of the rather confusing terms M.L.D., Lb, and antitoxin unit has been given in the preceding paper (11).

The lecithin was prepared from purified total lipid extracts of hog liver (14).

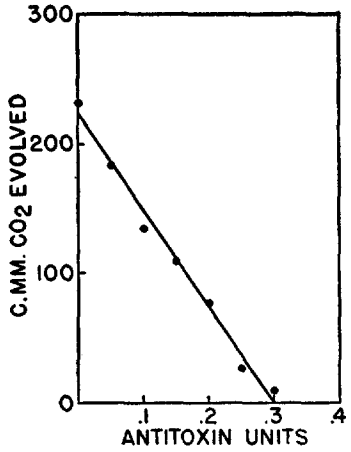

FIG. 1. Combining relationships of toxin and antitoxin. 36 M.L.D. of toxin and varying amounts of antitoxin were incubated together for 2 hours at 37°C. and then tipped into the lecithin-containing mixture. The carbon dioxide evolved between 5 and 15 minutes after tipping has been plotted.

Unless otherwise stated, the manometric conditions were as follows: flask contents: 0.2 cc. of 1 M sodium bicarbonate, 1.4 cc. of 2.5 per cent lecithin, 0.2 cc. of 0.04 M calcium chloride, 0.2 cc. of toxin containing 9 M.L.D. (diluted in 1 per cent albumin and 0.5 M sodium bicarbonate). Controls were run in all cases, using either untipped flasks or autoclaved toxin. The temperature was 37°C., the atmosphere 100 per cent carbon dioxide, and the calculated pH 6.7. The toxin and antitoxin were either mixed in advance and placed in the sidearms, or were mixed in the sidearms and then tipped into the center compartment. The addition of albumin was found desirable to stabilize the toxin and antitoxin in the dilutions used.

The calcium concentration used was an optimal concentration under our conditions (11). The importance of the calcium ion concentration in studies of toxin-antitoxin combination has been emphasized by Oakley and Warrack (21). The optimal concentration they recommend is 0.05 per cent, which is approximately the same (0.044 per cent) as that used here.

RESULTS

Toxin-Antitoxin Combining Relationships.—Fig. 1 demonstrates a practically straight line relationship for the toxin-antitoxin combining reaction. Working

under different conditions and using a different method of assay, Macfarlane and Knight (4) have reported a deviation from a straight line relationship at higher antitoxin concentrations. It has occurred to us that the difference between these two sets of data may be explained by the fact that in our experiments a considerably longer incubation period of toxin-antitoxin was used. Macfarlane and Knight incubated toxin-antitoxin for 15 minutes at room

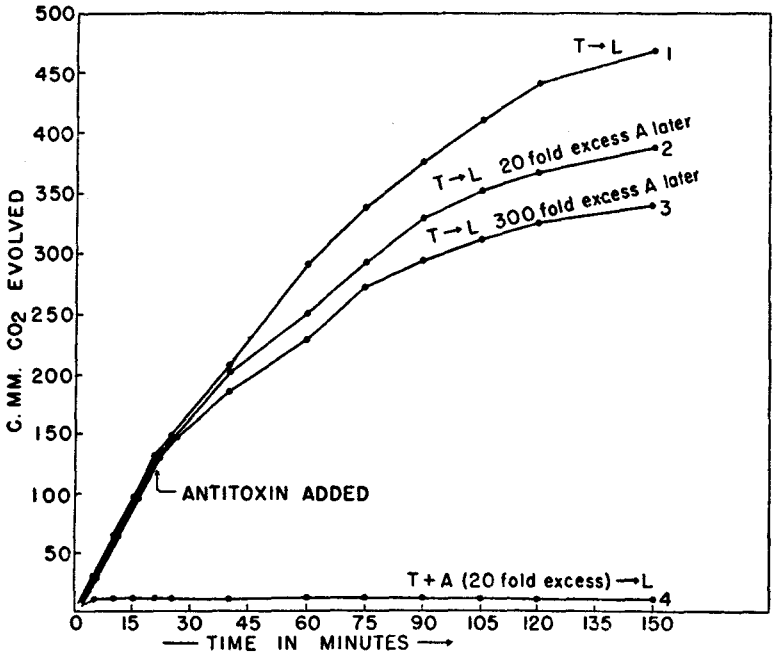

FIG. 2. Effect of adding antitoxin after lecithinase reaction has started. 1.6 units of antitoxin provide approximately a 20-fold excess of antitoxin over the 9 M.L.D. of toxin used (*cf.* Fig. 1). In curves 2 and 3, antitoxin was tipped 20 minutes after the toxin and lecithin had begun to react. In curve 4, the toxin and antitoxin (T + A) were mixed in a sidearm for 30 seconds, then tipped into the center compartment.

temperature, a condition which we have found to be not quite sufficient to ensure completion of the combining reaction (*cf.* Fig. 4).

At the point in Fig. 1 where the toxin is approximately neutralized by the least amount of antitoxin, the ratio of the toxin nitrogen to antitoxin nitrogen is approximately 30 to 1. Since neither the toxin nor antitoxin preparation is free from impurities, however, this figure remains a rough approximation.

Effect of Addition of Antitoxin after the Toxin Is in Contact with Lecithin.— The most important finding in our study is the observation that the combination of antitoxin with toxin is enormously inhibited by the presence of lecithin. Fig. 2 illustrates the effect of adding antitoxin 20 minutes after lecithin has

been added to the toxin. Curves 2 and 4 bring out most clearly the difference which occurs in the toxin-antitixoin combination depending on whether the antitoxin is added before or after the toxin comes in contact with lecithin. Even with such a large excess of antitoxin as that used in curve 3, there is activity left over when the enzyme has been incubated with lecithin for 20 minutes previously.

The above finding suggested a study of the influence of time of incubation, of lecithin with toxin, on the availability of the latter for antitoxin. Such experiments are reproduced in Fig. 3. In curve 4 of that figure lecithin was in contact with toxin for only 5 seconds before addition of antitoxin. The slope of this curve is less than that of curves 2 and 3, suggesting that a very brief contact of lecithin with toxin, prior to addition of antitoxin, does not interfere with the toxin-antitoxin combination as effectively as does a longer contact. There appears to be a definite time factor involved in the combination of toxin-enzyme and lecithin. The reservation must be made, however, that mechanical mixing of reactants may be an important consideration where such a short time period is involved.

Effect of Time on Degree of Completion of Toxin-Antitoxin Combination.—Another approach to the competition problem was made by adding antitoxin to toxin, and allowing them to react for varying intervals before addition of lecithin. Experiments of this type show, as illustrated in Fig. 4, that the early addition of lecithin interferes with the completion of the toxin-antitoxin combination. In this way, the addition of lecithin makes it possible to measure the time involved in carrying the toxin-antitoxin combination to completion. From the moment of addition of lecithin, the enzymatic reaction proceeds almost linearly for a time, although eventually the antitoxin slowly reacts through the lecithin barrier as shown in Fig. 2.

Effect of Adding Toxin to a Lecithin-Antitoxin Mixture.—In the two previous sets of experiments shown in Figs. 3 and 4 respectively, the conditions were such that first, the effect of previous combination of toxin with lecithin on the final reaction between toxin and antitoxin was studied. Second, the effect of time of interaction of toxin and antitoxin prior to addition of lecithin on the remaining enzymatic activity was investigated. The present series of curves represented in Fig. 5 shows the result of a simultaneous attack on the toxin-enzyme by the lecithin and the antitoxin. The outcome of such a competition between substrate and antitoxin is most clearly illustrated by comparison of curves 2 and 5. In curve 2, where the lecithin and antitoxin simultaneously come into contact with the toxin, much of the enzyme is diverted to combination with the lecithin. In curve 5, through a previous combination of the same amount of antitoxin with the enzyme, a subsequent activity toward lecithin is completely blocked. Curves 3 and 4 illustrate an additional point, that where lecithin and a considerable excess of antitoxin come into simultaneous contact

with toxin, the competitive activity of lecithin is not strong enough to prevent a more complete toxin-antitoxin combination.

Effect of Other Lipids[1] *on the Toxin-Antitoxin Combining Reaction.*—Experiments reported in the preceding paper (11) show that the *Cl. welchii* lecithinase splits lecithin most specifically, being without activity toward the other phospholipids tested. An effort has been made here to determine whether lecithin is unique in its ability to interfere with the toxin-antitoxin combination, or

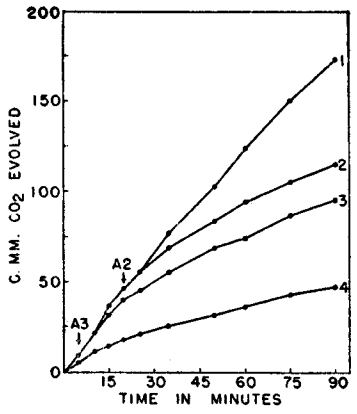

FIG. 3

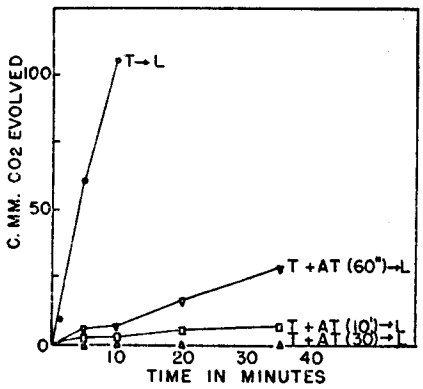

FIG. 4

FIG. 3. Effect of addition of antitoxin at various times after lecithin was added to toxin. A 480-fold excess of antitoxin is present (24 units of antitoxin *versus* 6 M.L.D. toxin). Interval between addition of lecithin and antitoxin to the toxin:

1. No antitoxin added.
2. 20 minutes. The arrow at A2 indicates the point at which antitoxin was added.
3. 5 minutes. The arrow at A3 indicates the point at which antitoxin was added.
4. 5 seconds.

FIG. 4. Effect of time of incubation of antitoxin with toxin on completeness of toxin-antitoxin combination. Antitoxin (AT) tipped into toxin (T) in sidearm, and mixture incubated for times indicated before tipping into lecithin-containing mixture (L). Vessel contents: 0.4 cc. of 1 M sodium bicarbonate, 1.4 cc. of 4 per cent lecithin, 0.2 cc. of 0.04 M calcium chloride, 0.1 cc. of toxin (containing 9 M.L.D.), and 0.1 cc. of antitoxin (containing 0.2 units).

whether this property is shared by other lipids. For example, toxin was mixed with sphingomyelin, then antitoxin was added. This mixture was next tipped into lecithin. There was no hydrolysis of lecithin. Similar results were obtained with phosphatidyl ethanolamine and ox brain cerebrosides. It thus appears that the interference of lecithin with the toxin-antitoxin combination is not due to a non-specific coating of the antitoxin or toxin by the lecithin, but rather suggests competition for combination with the same region on the toxin-

[1] The authors are indebted to Dr. Jordi Folch for supplies of these substrates.

enzyme molecule. Phosphatidyl serine, phosphatidyl ethanolamine, sphingomyelin, and ox brain cerebrosides have been found (11) to exercise a partial inhibition on the action of toxin on lecithin, which adds a complication to the type of experiment described above.

Effect of Changes in Temperature and pH on the Lecithin-Antitoxin Competition.—There was no appreciable change in the relative distribution of toxin between lecithin and antitoxin when the experiment was run at 22°C. and compared with a similar experiment at 37°C. Likewise, changing the pH of the reaction from 6.6 to 8.2 had no noticeable effect on this competition.

Precipitation of Toxin-Antitoxin Complex.—In a series of ten micro test tubes,

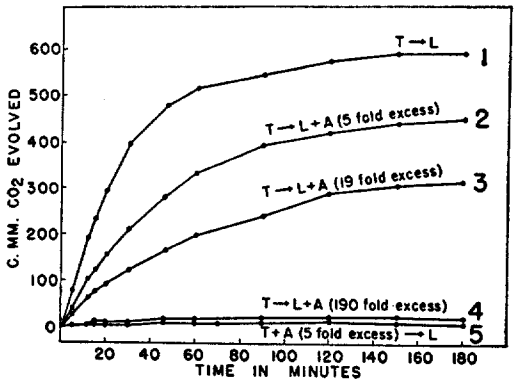

FIG. 5. Effect of adding toxin to a mixture of lecithin and antitoxin. 18 M.L.D. of toxin used.

1. Toxin-lecithin without antitoxin.
2. Toxin tipped into lecithin and a fivefold excess of antitoxin.
3. Toxin tipped into lecithin and a 19-fold excess of antitoxin.
4. Toxin tipped into lecithin and a 190-fold excess of antitoxin.
5. Toxin and a fivefold excess of antitoxin mixed before tipping into lecithin.

0.1 cc. of monovalent antitoxin containing 0.4 unit was mixed with 0.1 cc. of toxin containing from 0.28 to 140 M.L.D. In a second series of thirteen test tubes, 0.1 cc. of toxin, containing 18 M.L.D., was mixed with 0.1 cc. of antitoxin containing from 2.56 to 0.0062 units. After 2 hours' incubation at 25°C. no precipitation was observed. After 18 hours' incubation at 37°C. there was faint cloudiness in the tubes where approximately equivalent amounts of toxin and antitoxin were present. The precipitation reaction of *Cl. welchii* toxin-antitoxin mixtures therefore appears to be a slow one, and where either component is present in great excess, precipitation is not observed. It is concluded from these experiments that under the conditions of the manometric experiments no appreciable, if any, precipitation did occur.

Experiments with Rattlesnake Venom.—After this experience with the *Cl. welchii* lecithinase, we were tempted to test the different lecithinase of the

venom of the rattlesnake (*Crotalus terrificus*)[2] for the possibility of a similar competitive relationship between its antitoxin and lecithin. In the preceding paper (11) it was mentioned that the manometric method was found applicable to the hydrolysis of lecithin catalyzed by the snake venom, a reaction which results in a liberation of lysolecithin and a fatty acid (15). 10 cc. of antiserum were found just sufficient to inhibit the lecithinase activity of 9 mg. of dried venom. The experiments shown in Fig. 6 seem to bear out our suspicion that a similar relationship between lecithin and antitoxin exists in this case likewise. Even though our antitoxin preparations were rather crude, it appears permis-

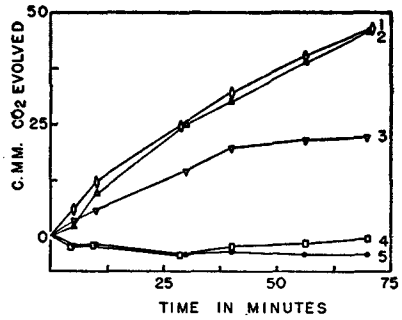

FIG. 6. Rattlesnake venom—substrate-antiserum competition. Vessel contents: 0.1 cc. of 1 M sodium bicarbonate, 1.4 cc. of 8 per cent lecithin, 0.2 cc. of 0.04 M calcium chloride, 0.1 cc. of venom (containing 0.2 mg. dried venom), 0.4 cc. of diluted antiserum (in sidearm) in certain flasks. Temperature 37°C., 100 per cent carbon dioxide atmosphere, calculated pH 7.7.

1. Venom and lecithin.
2. Venom and lecithin, albumin tipped in 10 minutes later (instead of antiserum).
3. Venom and lecithin, antiserum tipped in 10 minutes later.
4. Venom tipped into a mixture of antiserum and lecithin.
5. Venom and antiserum mixed, then tipped into lecithin 10 minutes later.

The antiserum was a rather crude preparation and a "bump" occurred in tipping antiserum into the venom-lecithin-containing main compartment. Readings have therefore been recorded beginning 10 minutes after tipping of the antiserum.

sible to conclude that the combination of lecithin with the venom lecithinase interferes with the combining of antitoxin with the venom, as illustrated in curve 3. When the venom lecithinase was tipped into a mixture of lecithin and antiserum, however, there was no residual lecithinase activity, a result which indicates a difference in relative affinities as compared with the *Cl. welchii* lecithinase (*cf.* Fig. 5).

DISCUSSION

The experiments just described have shown that lecithin may prevent the antitoxin from combining with the toxin-lecithinase. In the description of

[2] The authors are indebted to Dr. O. Bier, Institute Butantan, São Paulo, Brazil, for supplies of venom and antiserum.

the phenomenon the term competition has been used here rather freely. This competition is, however, of a somewhat unusual kind and seems to require a further comment. Most frequently the term competitive inhibition is used in enzyme chemistry when both the substrate as well as the inhibitor form dissociable compounds with the enzyme (16). In the present case only the enzyme-substrate complex is easily dissociable, as shown by Fig. 6 of the previous paper (11). On the inhibitor side, however, the linear proportionality between inhibition and antibody concentration (*cf.* Fig. 1) indicates a practically irreversible combination, provided time has been allowed for completion.

If, on the other hand, the lecithin is present as a competitor before reaction between antibody and toxin has occurred or while it is still incomplete, then the time required for the combination may be lengthened considerably. Eventually the antibody tends to compete successfully, particularly if added in large excess. This situation may best be described as a *kinetic competition*. When thermodynamic equilibrium is reached eventually, the antibody should and does prevail.

The situation may be summarized as follows: The forces binding lecithin to the toxin are less strong than those attaching antibody to enzyme. They do, however, both attach themselves to the same portion of the enzyme protein. When now the protein is charged with lecithin and then approached by the antibody the probability for a successful linking is considerably diminished. Once linkage is completed, the lecithin appears unable to replace the antitoxin.

Experiments on antigen-antibody–combining relationships (17–20) usually depend on measurement of the weight of the precipitated complex. Thus, in addition to the combination of antigen with antibody, an aggregation of combined antigen-antibody molecules is involved in the precipitation mechanism. The present enzymatic technique offers the theoretical advantage of separating the antigen-antibody–combining reaction from this secondary aggregation process.

There is a clinical implication in the finding that the presence of lecithin in combination with toxin interferes with toxin-antitoxin combination. It has been recognized that *Cl. welchii* antitoxin is less effective late in the therapy of *Cl. welchii* infection than early in the course of the disease. While there are, of course, other important reasons for this circumstance, (such as impairment of blood supply to affected regions), the present experiments indicate that once the toxin is in contact with its substrate, large concentrations of antitoxin are unable to prevent the enzymatic reaction from continuing. A similar situation may apply in the case of other bacterial toxins as well.

SUMMARY

Lecithin has been found to interfere with the combining reaction of *Cl. welchii* alpha toxin (lecithinase) and its antitoxin. If the lecithinase is first brought into contact with lecithin, and the antitoxin is then added, the antitoxin

fails to stop the enzymatic reaction, but gradually decelerates it. If the lecithinase is brought into contact with both lecithin and antitoxin at the same instant, it appears to combine in part with each, and the enzymatic process takes place at a reduced rate, which gradually declines further. If the lecithinase is first brought into contact with antitoxin, before the lecithin is added, the enzymatic reaction is completely inhibited.

This ability of lecithin to inhibit the antitoxin-toxin combination cannot be explained adequately as a non-specific coating of the toxin-enzyme by the lecithin. It is rather suggested that lecithin and antitoxin compete specifically for combination with the same regions on the enzyme molecule.

Lecithin has similarly been found to interfere with the combination of *Crotalus terrificus* venom and its antiserum.

The above findings provide a partial explanation for the lack of effectiveness of antitoxin when given late in the course of *Cl. welchii* infection.

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