Relation of In Vitro Inhibition by Chelates of Clostridium perfringens α-Toxin to Their Ability to Protect Against Experimental Toxemia

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The inhibition of Clostridium perfringens α-toxin by ethylenediaminetetraacetate (EDTA) and diethylenetriaminepentacetate (DTPA) was studied utilizing three different in vitro assay procedures: diffusion on egg yolk-agar, disintegration of muscle sections, and manometric assay with partially purified lecithin as substrate. DTPA was 10 to 20 times more efficient as an inhibitor than EDTA in systems containing relatively large amounts of calcium; these observations were similar to those observed in previous in vivo protection studies. A number of other chelating agents were tested for their ability to inhibit α-toxin in vitro and protect mice against it; the chelating agents which were the most efficient in vitro inhibitors had the greatest in vivo protective ability.

The α-toxin of Clostridium perfringens, a lecithinase (phospholipase C, EC 3.1.4.3), is important in initiating the lethal process of gas gangrene (11). Its in vitro activity is enhanced by certain metal ions and inhibited by metal-binding agents (10). Various metal-binding compounds have been investigated as possible in vivo therapeutic agents, and the chelating agent, ethylenediaminetetra-acetic acid (EDTA), protected animals against the toxin alone or experimental gas gangrene (3, 4, 13, 14).

Diethylenetriaminepentacetate acid (DTPA) also protects mice against α-toxin and is 10 to 20 times more efficient than EDTA (9). The greater efficiency of DTPA may be due to the differences in the in vivo behavior of the two compounds or to the differences in the effects of the two compounds on the reaction of α-toxin with its substrate. In this paper, studies on the inhibition of α-toxin by EDTA and DTPA with three different in vitro procedures are described; the results demonstrate that DTPA is superior to EDTA as an inhibitor of in vitro α-toxin activity.

The relationship of the degree of in vitro inhibition of lecithinase activity by these two chelates with their ability to protect animals against the toxin suggested that such a relationship may also exist with other chelating agents. Several other polycarboxylic amino acids were tested in vitro and in vivo, and a correlation between their behavior in both situations was found.

MATERIALS AND METHODS

Water. Glass distilled water was used.

Borate buffer. Borate buffer (pH 7.4) was prepared by dissolving 1.094 g of boric acid and 0.190 g of Na borate in a final volume of 100 ml of water.

Ringer solution for mammalian tissues. Ringer solution was prepared by dissolving 9.0 g of NaCl, 0.318 g of CaCl₂, 0.420 g of KCl, and 0.200 g of K₂CO₃ in 1 liter of water.

Chelating agents. EDTA, DTPA, and HEDTA (N-hydroxyethylenediaminetriacetic acid) were supplied by Dow Chemical Co., Midland, Mich. Nitriol-triacetic acid (NTA), ethyleneglycol-bis(β-amino-ethyl ether)-tetraacetic acid (EGTA), and cyclohexanediaminetetraacetic acid (CDTA) were supplied by Geigy Industrial Chemicals.

Lecithin. The substrate for manometric assay of α-toxin activity was commercially prepared egg yolk-lecithin (Nutritional Biochemicals Corp., Cleveland, Ohio) which was further purified in the following manner. Lecithin (50 g) was dissolved in 1,650 ml of 95% alcohol. The mixture was stirred for 1 hr at room temperature and filtered through Whatman no. 12 paper. The filtrate was stored at −20°C for 2 days. The precipitate that formed was removed by filtration through Whatman no. 1 paper. The filtrate was treated to remove metal ions with a chelating resin, Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.), by batch method. A 300-ml amount of the sodium form of the resin was suspended in distilled water. The original pH 11.6 was adjusted to 8.5 by the addition of 1 N HCl with constant stirring. The water was decanted, and the resin was washed several times with ethyl alcohol. A 1-liter amount of lecithin
solution was added to the resin and stirred continuously for 1 hr at room temperature. The lecithin was filtered through Whatman no. 42 filter paper on a Buchner funnel and stored at 4°C in glass bottles. Immediately before use, a volume of lecithin solution containing the desired dry weight was evaporated to dryness in a rotary evaporator at 40°C. Suspension of the lecithin in water was accomplished by swirling with glass beads. After suspension, the volume was adjusted to give 20 mg of lecithin in 1.6 ml of water.

Agar diffusion assay. Activity of the toxin on egg yolk-agar was determined by the method of Sheldon et al. (19) and was expressed as lecithinase units per milliliter.

α-Toxin. Toxin was prepared by methanol precipitation according to "procedure A" described by Lynch and Moskowitz (8). After resuspension in cold borate buffer, the toxin was centrifuged to remove insoluble material. To remove metal ions from the toxin, it was made 0.5M with EDTA, held overnight at 4°C, and then exhaustively dialyzed at 4°C against changes of borate buffer. The dialyzed toxin was stored in small samples (0.5 to 1.5 ml) at -20°C. A typical preparation had 88 lecithinase units (L units) per ml and 1.3 × 10^-4 g of N per L unit.

Bovine serum albumin (BSA) was previously shown to stabilize the toxin (17), and all enzyme dilutions for use in the various assay systems had a final concentration of 1% BSA (fraction V, Armour Pharmaceutical Co., Kankakee, Ill.). The enzyme dilution included this addition of BSA. Thus, for a dilution of 1:6, equal volumes of 2% (w/v) BSA in water and a 1:3 enzyme dilution in borate buffer were mixed together.

Manometric assay. A Warburg apparatus was used to measure α-toxin action against lecithin according to the procedure developed by Zamecnik et al. (21). Warburg flasks were set up for assay with 20 mg of lecithin in 1.6 ml of water, 0.4 ml of 1.19 M NaHCO₃, 0.1 to 0.5 ml of metal solution (CaCl₂ or ZnCl₂), 0.6 ml to 0.5 ml of chelating agent, 0.2 ml of 1:6 enzyme dilution, and enough water to give a total reaction volume of 3.2 ml. The assembled manometer and flask were gassed with 100% CO₂ before equilibration at 37°C. The final pH of the reaction mixture was 7.1. Unless otherwise specified, the reaction was initiated by addition of enzyme from a side arm to the other reaction components in the main compartment of the flask.

Animals. Rockland Swiss albino mice (25 to 30 g) were used for the muscle section assays and for the in vivo protection studies.

RESULTS

Agar diffusion studies. Table 1 shows the results of experiments to compare the inhibitory effects of the calcium chelate of EDTA (CaEDTA) and the calcium chelate of DTPA (CaDTPA) on the activity of α-toxin as measured by agar diffusion (19). The various concentrations of chelating agent and toxin were mixed and the mixture was added to the assay disc. Maximal inhibition occurred with 10 μmoles of CaEDTA/ml. A low amount of activity remained after this concentration of chelate, and doubling the amount of chelate did not cause a further decrease in activity. With CaDTPA, maximal inhibition occurred with only 0.5 μmole of chelate/ml, and with this compound there was complete inhibition.

Muscle section studies. Gas gangrene is a disease involving destruction of muscle tissue. Several workers have performed histological investigations of α-toxin activity (1, 16, 20), and the results of these studies show that the toxin is responsible for muscle damage. The advantage of such in vitro systems is that conditions approximating those found in vivo are present. A muscle section assay was developed to determine the relationship between inhibition by CaEDTA and CaDTPA when the α toxin is acting directly on muscle. Emphasis was placed on the development of a relatively simple, rapid estimate of the histological effects of α-toxin.

A white mouse was sacrificed and the gastrocnemius muscle was removed from one of the hind legs. The muscle was rinsed in Ringer solution, frozen, and sectioned with a microtome cryostat (model CT, International Equipment, Needham Heights, Mass.). Sections were placed in Ringer solution at room temperature and each one was treated in the following manner.

The section was transferred to a small glass beaker (diameter, 19 mm) containing 2.0 ml of Ringer solution. The Ringer solution was drawn off with a capillary pipette fitted with a rubber bulb, thus allowing the tissue to rest flat on the bottom of the beaker. Then 0.5 ml of incubation solution containing Ringer solution and either toxin alone or toxin mixed with the chelates was added. During this addition, the tip of the pipette was placed against the inside edge of the beaker so that the liquid flowed slowly down the side.

| Table 1. Inhibition of α-toxin by CaEDTA and CaDTPA as measured by agar diffusion |
|-------------------------------|-----------------|----------------|----------------|
| CaEDTA | Activity | CaDTPA | Activity |
| μmole/ml | % | μmole/ml | % |
| 0.0 | 100 | 0.0 | 100 |
| 0.5 | 25 | 0.01 | 80 |
| 1.0 | 20 | 0.05 | 36 |
| 2.0 | 11 | 0.1 | 11 |
| 5.0 | 3 | 0.2 | 2 |
| 10.0 | 2 | 0.5 | 0 |
| | | 1.0 | 0 |

* At 100 lecithinase units per ml.
With this procedure, the tissue floated up off the bottom of the beaker. Incubation was carried out for 60 min at 37 °C.

At the end of the incubation time, 1.5 ml of Ringer solution was added carefully to the beaker. The section was gently lifted out of the beaker on a glass rod. This same rod was used for all subsequent transfers of the section during the staining procedure. The section was placed in 0.1% (w/v) aqueous methylene blue for 15 sec and dipped in physiological saline twice; it was then floated out in a 50-ml beaker containing saline. Finally, the tissue was floated onto a clean microscope slide and allowed to drain dry.

Slides were examined macroscopically for the loss of integrity of the tissue section as a result of toxin action. An arbitrary 4+ scale was used for scoring the sections, with 1+ representing minimal damage as seen in control sections incubated in Ringer solution (Fig. 1) and 4+ representing greatest damage as seen in control sections incubated in toxin (Fig. 2). Scoring was done independently by more than one individual.

A 60-min incubation of tissue sections of various thicknesses in Ringer solution revealed that 64-μm sections remained relatively intact (Fig. 1); this thickness was utilized in the experiments.

Different regions of muscle varied in their ability to remain intact; thus, all sections used in an experiment were obtained from the same region.

Incubation of sections in various toxin dilutions showed that a 1:16 dilution of toxin gave easily detectable loss of section integrity (Fig. 2). A series of dilutions of CaEDTA and of CaDTPA were also tested for effects on section integrity. Concentrations as high as 1.0 μmole of CaEDTA and 0.5 μmole of CaDTPA produced no visible tissue damage.

Experiments were performed to determine whether CaEDTA and CaDTPA could protect the muscle from the effects of α-toxin. Incubation solutions were prepared by mixing one volume of 1:4 toxin dilution with two volumes of double-strength Ringer solution and one volume of the desired chelating agent dilution. Toxin controls were made by substituting water for the chelating agent; Ringer controls were made by substituting water for both the chelating agent and the toxin. The results of these experiments are shown in Table 2. As little as 0.005 M CaDTPA provided complete protection against the toxin, whereas 0.1 M CaEDTA was required to give the same degree of protection.

**Manometric studies.** The results of the agar-diffusion and muscle-section assays demonstrated that DTPA is a better inhibitor of α-toxin than is EDTA. However, these systems are relatively

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**Fig. 1.** Tissue section incubated 60 min in Ringer's solution. Rated 1+.

**Fig. 2.** Tissue section incubated 60 min in Ringer's solution plus α-toxin. Rated 4+. 

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TABLE 2. CaEDTA and CaDTPA protection of muscle sections against action of α-toxin

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Section integrity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ringer's solution</td>
<td>1+</td>
</tr>
<tr>
<td>Toxin</td>
<td>4+</td>
</tr>
<tr>
<td>Toxin plus CaEDTA (µmole)</td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>4+</td>
</tr>
<tr>
<td>0.005</td>
<td>4+</td>
</tr>
<tr>
<td>0.01</td>
<td>4+</td>
</tr>
<tr>
<td>0.1</td>
<td>1+ to 2+</td>
</tr>
<tr>
<td>Toxin plus CaDTPA (µmole)</td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>4+</td>
</tr>
<tr>
<td>0.005</td>
<td>2+</td>
</tr>
<tr>
<td>0.005</td>
<td>1+</td>
</tr>
</tbody>
</table>

* Scored on a 4+ scale: 1+ = minimal damage, 4+ = greatest damage.

...crude and a more quantitative method assay of lecinthinase activity, the manometric assay developed by Zamecnik (21) and modified by D. R. Sheldon (Ph.D. Thesis, Purdue University, 1960), was also utilized to determine the inhibitory activity of the two chelates.

Utilizing the Warburg apparatus, Sheldon found that much less than equimolar amounts of EDTA were required to inhibit the α-toxin in the presence of calcium, but equimolar amounts were required when zinc was present as the activating metal. The present experiments were carried out to determine the relative effectiveness of EDTA and DTPA in calcium- and zinc-activating systems. Reaction components were treated to remove extraneous metal ions, and no activity was evident unless calcium or zinc was added to the reaction mixture. The amount of metal added to the system (8 µmoles of calcium; 9 µmoles of zinc) was the minimal amount that activated the reaction at a maximal linear rate throughout the first 30 min.

Figure 3 shows inhibition by EDTA and DTPA in the presence of zinc. With EDTA, complete inhibition occurred when the amount of chelating agent is equal to that of the metal (Fig. 3a). With DTPA, relatively small amounts of chelating agent produced complete inhibition at early time periods (Fig. 3b); however, only those flasks containing equimolar amounts of zinc and DTPA remained without activity throughout the entire reaction time.

Figure 4 shows the inhibition produced by the two chelating agents in the presence of calcium. With this metal as activator, far less than equimolar amounts of chelating agent are necessary; maximal inhibition occurred with 0.1 µmole of EDTA (Fig. 4a) and with 0.005 µmole of DTPA (Fig. 4b).

The results of the manometric assays are in agreement with those of the agar-diffusion and muscle-section assays. In all systems, DTPA was a better inhibitor of α-toxin activity than is EDTA. Furthermore, when calcium was the predominant metal in the manometric assay, DTPA was approximately 20 times more effective than EDTA, a relationship similar to that found in the in vivo systems (9). This finding suggested that in vitro assay systems in which calcium...
TABLE 3. In vivo and in vitro action of polycarboxylic amino acid chelating agents on α-toxin activity

<table>
<thead>
<tr>
<th>Chelating agent</th>
<th>In vivo protectiona</th>
<th>In vitro inhibitionb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg survival time  hr</td>
<td>Std deviation</td>
</tr>
<tr>
<td>EDTA</td>
<td>212 ± 50</td>
<td>9.0 ± 0</td>
</tr>
<tr>
<td>DTPA</td>
<td>429* ± 47</td>
<td>9.0 ± 0</td>
</tr>
<tr>
<td>HEDTA</td>
<td>194 ± 33</td>
<td>9.0 ± 0</td>
</tr>
<tr>
<td>CDTA</td>
<td>156 ± 14</td>
<td>9.0 ± 0</td>
</tr>
<tr>
<td>EGTA</td>
<td>118 ± 24</td>
<td>9.0 ± 0</td>
</tr>
<tr>
<td>NTA</td>
<td>116 ± 37</td>
<td>9.0 ± 0</td>
</tr>
<tr>
<td>Control (no chelate)</td>
<td>94 ± 25</td>
<td></td>
</tr>
</tbody>
</table>

a Survival time after injections of eight minimal lethal doses of toxin and 10 μmoles of Ca chelate.

b Amount of chelating agent necessary for maximal inhibition at 30 min.

* Zn, 9 μmoles.

* Ca, 8 μmoles.

* Three of eight mice injected did not die.

The results presented may give information about the ability of a chelating agent to protect animals against α-toxin. This possibility was tested by examination of several different polycarboxylic amino acids.

In vivo and in vitro comparisons of polycarboxylic amino acids. HEDTA, CDTA, EGTA, and NTA have structures similar to EDTA, and their ability to inhibit α-toxin activity in the Warburg apparatus was tested in the manner described for EDTA and DTPA. The results of these experiments are shown in Table 3. In zinc-activated systems, the amount of chelating agent necessary for complete inhibition was equivocally as to that of the metal; however, in calcium-activated systems, the amount of the chelating agent necessary for maximal inhibition was less than the amount of metal present. The inhibitory activity of HEDTA and CDTA was similar to EDTA, but EGTA and NTA were much less effective.

The compounds were tested for their ability to protect white mice against α-toxin by using the procedure of Lynch and Moskowitz (9). Mice were injected intraperitoneally with toxin at a minimal lethal dose of 8 and with 10 μmoles of the calcium chelate of each polycarboxylic amino acid. Survival times were recorded and results expressed (Table 3) as average survival times.

In general, the more chelating agent necessary for complete inhibition of calcium-activated toxin in vitro, the shorter the protection time afforded by the calcium chelate in vivo. Under these conditions, the calcium chelate of CaEDTA and the calcium chelate of NTA (CaNTA), those compounds least effective in vitro, provided little or no protection; the calcium chelate of CaHEDTA and the calcium chelate of CDTA (CaCDTA), those compounds which were effective in vitro inhibitors, protected the mice to a much greater extent. Application of the t test to the data for average survival times indicated that at the 10% level, there was no significant difference between values for CaEGTA and CaNTA and the control value (p 1.43 < 1.81 for CaEGTA; p 1.24 < 1.77 for CaNTA). However, t values for both CaHEDTA and CaCDTA data were significant at the 0.1% level (p 4.63 > 4.59 for CaHEDTA; p 5.00 > 4.59 for 1 + CaCDTA).

Reversal studies. The in vivo protective effects of EDTA can be reversed by injections of zinc but not by calcium (15), and the effects of these metals on DTPA protection are the same. The reversal of in vitro inhibition by EDTA and DTPA was studied by utilizing the manometric assay. Chelating agent was added to a maximally activated system in a concentration just sufficient to give complete inhibition; then various amounts of calcium or zinc were added to determine whether they restored activity to the system.

Substrate, buffer, activating metal, and chelating agent were placed in the flask. Enzyme was placed in one side arm and metal in the other side arm. At zero-time, enzyme was tipped into the flask and observations were made for 15 min to confirm the inhibition of activity. Metal was then added to the flask from the second side arm and the reaction was followed for 2 min.

An 8-μmole amount of calcium was required for maximal activation, and 0.1 μmole of EDTA or 0.005 μmole of DTPA completely inhibited the activity. The addition of calcium in concentrations up to 16 μmoles did not restore any activity.

A 9-μmole amount of zinc was required for maximal activation, and 9 μmoles of EDTA or DTPA was required to completely inhibit the system. The addition of 9 μmoles of zinc completely restored activity to the EDTA-inhibited system, but complete restoration of activity in the DTPA-inhibited system did not occur until 12 μmoles of zinc was added.

**DISCUSSION**

It has been demonstrated that DTPA is 10 to 20 times more effective than EDTA in protecting animals against α-toxin (9). The difference in the in vivo effects of these two compounds may be
related to the greater retention of DTPA in the body and its ability to gain entrance into cells (9). However, the results of the studies reported here indicate that the most important factor in the superior protective ability of DTPA can undoubtedly be ascribed to its greater ability to inhibit the α-toxin.

The state of the substrate (degree of dispersion) has a marked influence on the activity of the α-toxin (Senff and Moskowitz, unpublished data) as well as that of other phospholipases (2, 5, 8), and variation in the state of the substrate may influence the behavior of inhibitors of α-toxin. However, despite the fact that the three systems investigated differ with regard to the condition of substrate, DTPA is a more efficient inhibitor than EDTA in all cases. This suggests that the state of the substrate in vivo is not a factor in the difference of the behavior of the compounds in vivo.

The metal ion content of the assay medium has an effect on the relative effectiveness of DTPA and EDTA. In the agar-diffusion and muscle-section assays, where more calcium than zinc is undoubtedly present, DTPA was approximately 10 times as effective as EDTA. In the manometric studies with calcium as activating metal, DTPA was approximately 20 times better than EDTA, but with zinc the difference between the two compounds was much reduced. Thus, when calcium is the metal present in greatest amounts, the in vitro difference between DTPA and EDTA is similar to that found in vivo. This relationship was also found with HEDTA, CDTA, EGTA, and NTA.

Calcium is present in the body in far greater amounts than zinc. Thus, one of the essential properties of a chelating agent that may have potential in vivo protective ability would be the ability to inhibit the α-toxin in the presence of a marked excess of calcium. This property was shared by the four chelating agents that conferred protection against the toxin in vivo.

Although excess calcium did not reverse the inhibition of the effective chelating agents, zinc did effect reversal. More zinc was necessary to reverse the inhibition by DTPA than by EDTA and this may be related to the fact that DTPA binds zinc to a greater extent than EDTA (stability constants of 18.14 and 16.58, respectively). The fact that relatively small amounts of zinc are present in the body and that this zinc is not free is complexed with proteins is probably a factor in the ability of the chelating agents to act in vivo.

The results of these inhibition and reversal studies suggest an explanation for the finding that citrate, although it is an in vitro inhibitor of α-toxin, is ineffective in providing in vivo protection (12). M. W. Deverell (Ph.D. Thesis, Purdue University, 1957) showed that relatively large amounts of citrate are necessary to bring about in vitro inhibition and that calcium could reverse this inhibition. Thus, this inhibitor is much less effective than the polycarboxylic amino acids in the presence of calcium, and it would be expected that it would be less effective in vivo, where this ion is present in large amounts.

The results of these studies indicate that there are several polycarboxylic amino acids which could be useful in treatment of gas gangrene, and that DTPA would probably be the most effective of those tested.

LITERATURE CITED
In M. J. Seven (ed.), Metal-binding in medicine. J. B. Lippincott Co., Philadelphia.


