Characterization of Detergent-Solubilized Iodine-125-Labeled α-Toxin Bound to Rabbit Erythrocytes and Mouse Diaphragm Muscle†

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ABSTRACT: Triton X-100 extracts of staphylococcal ^{125}I-labeled α-toxin-treated rabbit erythrocytes are resolved into two bands of radioactivity by sucrose density centrifugation and also by polyacrylamide disc gel analysis of sodium dodecyl sulfate extracts. The smaller band has an apparent molecular size of 2.8×10^4 daltons and comigrates with native α-toxin. The larger band has a molecular size of about 10^6 daltons and is remarkably stable to proteolytic digestion unless pretreated for 1 min at 100 °C in 2 M urea. Analysis by agarose–polyacrylamide slab gel electrophoresis of the sodium dodecyl sulfate extract permits resolution of the high molecular size band into two components of 1.5×10^6 and 1.1×10^6 apparent molecular weight. These forms are temperature sensitive and pretreatment for 1 min at 75 °C in sodium dodecyl sulfate causes disappearance of the 1.5×10^6 band and formation of a new band of 1.6×10^5 apparent molecular weight. Pretreatment at 100 °C in sodium dodecyl sulfate causes all the radioactivity to migrate with native α-toxin. A model explaining these results in terms of formation of monomer and multimer complexes is reported. Antisera prepared against either α-toxin or rabbit erythrocyte membranes cause precipitation of the complex when assayed by the indirect precipitin method. That antisera to human erythrocytes, which do not specifically bind α-toxin, can also cause precipitation of ^{125}I-labeled α-toxin–rabbit erythrocyte complexes remains unexplained. Extension of these observations with rabbit erythrocytes to other tissues in the mouse is reported. To date, only muscle tissue appears to bind α-toxin in a high molecular weight form analogous to that seen with rabbit erythrocytes. However, α-toxin bound to muscle appears to be "nonspecifically" bound.

Staphylococcal α-toxin, a hemolytic exotoxin, has been shown to bind to rabbit erythrocytes in an apparently irreversible and highly specific manner (Cassidy & Harshman, 1976a). Previous workers have proposed that disruption of susceptible membranes may be explained solely by a direct interaction of α-toxin with membrane lipid (Weissman et al., 1966; Buckelew & Colacicco, 1971; Freer et al., 1973). Their proposal was based on the interaction of α-toxin with lipid monolayers (Buckelew & Colacicco, 1971) and with artificial lipid dispersions (Weissman et al., 1966; Freer et al., 1973). Our own studies (Cassidy et al., 1974) confirmed that homogeneous α-toxin does release internal markers from liposomes. However, release of markers from liposomes, prepared from membrane lipid extracted from erythrocytes of different species, failed to show the selective species sensitivity to α-toxin.

In studies using ^{125}I-labeled α-toxin, we demonstrated that the sensitivity of erythrocytes to α-toxin from various species is reflected directly in the binding of α-toxin to the membranes (Cassidy & Harshman, 1973). This observation, combined with our studies, citing earlier that α-toxin is specifically bound to rabbit erythrocytes (Cassidy & Harshman, 1976a), led us to conclude that the primary binding site for α-toxin in biomembranes is a surface membrane protein. The present work describes our efforts to characterize the nature of the complex formed between α-toxin and the surface membrane protein receptor of rabbit erythrocytes. Also, we report our progress in seeking such a membrane receptor in other tissues of the mouse.

Materials and Methods

Staphylococcal α-toxin was prepared by use of the method of absorption chromatography on porous glass beads (Cassidy & Harshman, 1976b). Lactoperoxidase grade B and pronase CB were obtained from Calbiochem; bovine serum albumin, crystallized, from Sigma; Triton X-100, from Beckman; sodium dodecyl sulfate ($NaDodSO_4$) and *N*-lauroylsarcosine sodium salt (sarkosyl), from Sigma; and gel electrophoresis reagents, from Bio-Rad. $Na^{125}I$, used to iodinate α-toxin, was from New England Nuclear and *N*-hydroxysuccinamide (Bolten–Hunter reagent), from Eastman Kodak. Rabbit erythrocytes were obtained from stock animals. Human erythrocytes were group O^+ cells obtained from outdated donor blood. Cells were washed in 0.15 M NaCl, 0.02 M potassium phosphate, pH 7.4, and resuspended in the same buffer with 1 mg/mL of bovine serum albumin (3×10^8 cells/mL). Mouse neuroblastoma NB41AD and Neuro 2a were obtained from the American Type Culture collection.

Preparation of Iodinated Staphylococcal α-Toxin. Staphylococcal α-toxin was radiolabeled by the lactoperoxidase method of Morrison et al. (1971) using $Na^{125}I$ and further purified as described by Cassidy & Harshman (1976a). The properties of ^{125}I-labeled α-toxin have been described (Cassidy & Harshman, 1976c). The biological activity was measured on rabbit erythrocytes by use of the hemolytic assay described by Bernheimer & Schwartz (1963). Radiolabeling with ^{131}I-labeled *N*-hydroxysuccinamide was done according to the procedure described by Bolton & Hunter (1973).

Preparation of Disc and Slab Gels. Stock acrylamide contained 20 g of acrylamide and 1.48 g of bis(acrylamide) in 100 mL of H_2O. The 3E buffer consisted of 29.1 g of Tris base, 16.3 g of sodium acetate, and 2.23 g of Na_2EDTA, pH 7.2, in a volume of 2 L. Gels were prepared by mixing together at 45 °C the following: 5 mL of stock acrylamide; 16.67 mL of 3E buffer, with or without 0.3% $NaDodSO_4$ added; 2.33 mL of water; 25 mL of 2% melted agarose; 0.5 mL of 10% persulfate; and 0.5 mL of 10% TEMED. Gels were allowed to polymerize overnight.

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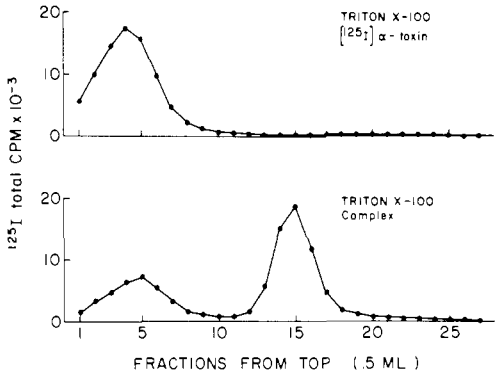

FIGURE 1: Sucrose density centrifugation of Triton X-100 solubilized rabbit erythrocyte membranes treated with ^{125}I-labeled α-toxin. Upper: ^{125}I-labeled α-toxin standard gradient. Lower: ^{125}I-labeled α-toxin-treated rabbit erythrocyte membranes. The radioactive peaks in the lower gradient were calculated to have sedimentation constants of 3 and 12 S. Twenty milliliters of 1% v/v washed rabbit erythrocytes was incubated for 1 h at 25 °C with 0.7 μg/mL of ^{125}I-labeled α-toxin in phosphate-buffered saline (1 mg/mL of bovine serum albumin) (PBSA). The erythrocyte membranes were washed twice in 20 mL of PBSA and three times in 20 mL of 5 mM sodium phosphate buffer, pH 7.2. The membranes were dissolved in 2 mL of 10% w/v Triton X-100 in PBS, and after a low-speed centrifugation, the supernatant (0.2 mL) was layered onto a 5–25% w/v sucrose gradient in 1% w/v Triton X-100. The gradient was centrifuged for 16 h at 20 °C and 38 000 rpm in a Beckman SW 40.1 rotor. Samples were collected from the top.

Results

Sucrose Density Gradient Centrifugation. Specifically bound ^{125}I-labeled α-toxin retained on toxin-treated rabbit erythrocytes is present as a complex of very high S value when solubilized with Triton X-100 detergent and centrifuged through sucrose density gradients. The results of a duplicate experiment are presented in Figure 1. The proportion of bound ^{125}I-labeled α-toxin in the low (3 S) and high (12 S) value peaks is usually about 40–60%, respectively, of the total bound radioactivity when trace-labeled preparations of toxin are used. In previous work we have shown that all of the radioactivity can be competed with native α-toxin and is, thus, specifically bound (Cassidy & Harshman, 1976a).

The high S value toxin appears to be rather resistant to denaturation by urea and proteolytic attack by pronase. As Figure 2 shows, incubation in 2 M urea either with or without a 1-min treatment at 100 °C does not lead to a total destruction of the high S value material. Incubation with 25 μg/mL of pronase for 2 h also does not completely degrade the material. Only when the Triton-solubilized material is first treated at 100 °C in 2 M urea, cooled to 37 °C, and digested with pronase does all the labeled ^{125}I-containing material appear at the top of the sucrose gradient. A comparable experiment using human erythrocytes which do not specifically bind ^{125}I-labeled α-toxin shows that the small amount of radioactivity retained by human erythrocytes is all in a low S value form (Figure 3). A comparison between the appearance of the rabbit and human erythrocyte sucrose gradients also shows that both the high and low S value forms of the toxin are specifically bound to rabbit erythrocytes.

$NaDodSO_4$ Agarose–Polyacrylamide Gel Electrophoresis. When rabbit erythrocyte membranes exposed to ^{125}I-labeled α-toxin are dissolved directly in sodium dodecyl sulfate containing buffer, apparent high and low molecular forms of the ^{125}I-labeled α-toxin are observed on 2% polyacrylamide–1% agarose gel electrophoresis. On disc gels, the high molecular weight peak has an apparent molecular mass of about 10^6 daltons and the low peak comigrates with ^{125}I-labeled α-toxin

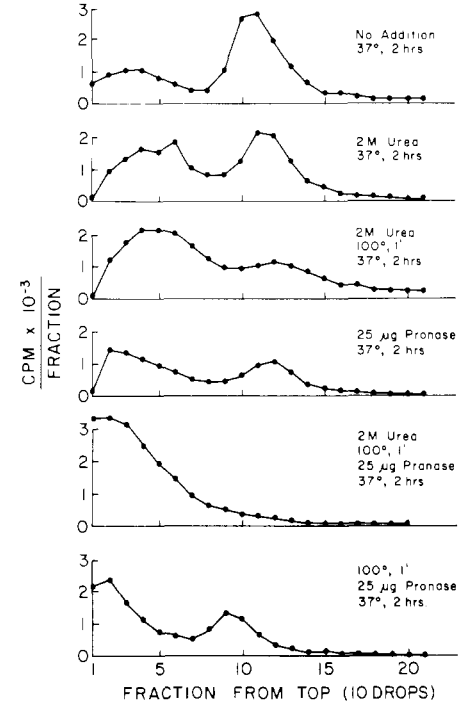

FIGURE 2: Effect of denaturing conditions and pronase digestion on solubilized ^{125}I-labeled α-toxin from treated rabbit erythrocyte membranes. Initial extraction of membranes as in Figure 1. Pretreatment of extracts as indicated. For further details, see text.

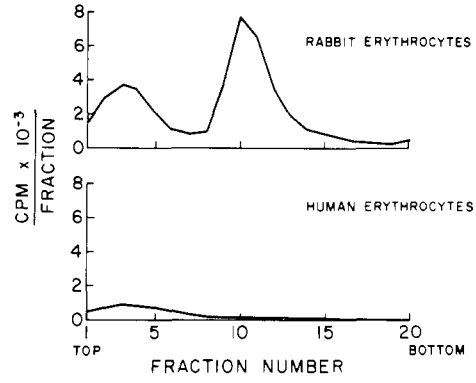

FIGURE 3: Sucrose density gradient centrifugation of rabbit and human erythrocyte membranes treated with ^{125}I-labeled α-toxin and solubilized in Triton X-100 buffer. Upper: rabbit erythrocyte membranes. Lower: human erythrocyte membranes. Sample prepared and run as in Figure 1 except that a 1/50 dilution into H_2O at 4 °C was included to osmotically lyse the human cells which do not lyse on exposure to α-toxin. Similar treatment of the rabbit erythrocyte has no effect on the results.

(2.8×10^4 daltons). The same results are found if the membranes are first dispersed in Triton X-100 containing buffer and transferred to $NaDodSO_4$-containing buffer prior to electrophoresis, whether or not mercaptoethanol or dithiothreitol is present. If the sample is prepared by raising the temperature to 100 °C for 1 min with $NaDodSO_4$ present, all the ^{125}I counts comigrate with ^{125}I-labeled α-toxin (Figure 4).

Immune Precipitation by Antierythrocyte Antibodies. Both the ^{125}I counts solubilized with either Triton X-100 or sodium sarkosyl (used instead of $NaDodSO_4$ because of low-temperature incubation) precipitated with antibodies raised against whole rabbit or human erythrocytes (Table I). This unexpected finding may indicate that the component in rabbit erythrocyte membranes which binds ^{125}I-labeled α-toxin is also present in human erythrocytes but in an altered form, able to

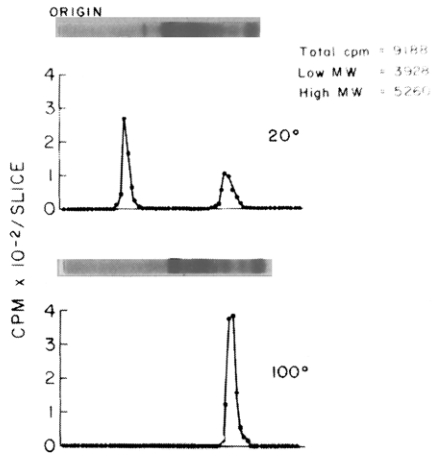

FIGURE 4: 2% polyacrylamide–1% agarose gel electrophoresis of ^{125}I-labeled α-toxin-treated rabbit erythrocyte membranes dissolved directly in $NaDodSO_4$ buffer. Upper: sample preparation temperature, 20 °C. Lower: sample preparation temperature, 100 °C. Membranes were prepared as in Figure 1 but the Triton X-100 step was omitted and membranes were directly dissolved in buffer containing 1% w/v $NaDodSO_4$. Samples were incubated either at 20 or at 100 °C for 1 min and electrophoresed.

Table I: Precipitation of Detergent-Solubilized Radioactivity from Rabbit Erythrocytes Treated with ^{125}I-Labeled α-Toxin

	% radioactivity precipitated	
antiserum[a]	Triton X-100 solubilized membranes	sodium sarkosyl solubilized membranes
	2	0
anti-rabbit erythrocyte	16	20
anti-human erythrocyte	14	24
anti-α-toxin	54	2

[a] First antibody in double antibody immune precipitation. Second antibody was either anti-chicken or anti-rabbit IgG. Antierythrocyte antiserums produced in chicken; anti-α-toxin produced in rabbit.

cross-react with antibodies prepared against rabbit erythrocytes but unable to interact with the toxin. An incidental finding is that α-toxin solubilized in sodium sarkosyl does not precipitate with anti-α-toxin antiserum.

A large preparation of the immune precipitate was prepared by reaction of sodium sarkosyl solubilized membranes treated with ^{125}I-labeled α-toxin with anti-rabbit erythrocyte antiserum. Electrophoresis on 2% polyacrylamide gels showed that both high and low molecular weight forms of the ^{125}I-labeled α-toxin were present, thus indicating that both forms are specifically associated with membrane components.

Heat Stability of High Molecular Weight Forms of ^{125}I-Labeled α-Toxin. The solubilized ^{125}I-labeled α-toxin from treated rabbit erythrocyte membranes has also been examined on 2% polyacrylamide–1% agarose slab gel electrophoresis which provides better resolution of radioactive components through autoradiography. In this electrophoresis system, the solubilized ^{125}I-labeled α-toxin, which appeared to be about 10^6 apparent molecular weight on disc gels, was resolved into two narrowly resolved bands of 1.5×10^6 and 1.1×10^6 apparent molecular weight (Figure 5). At a sample preparation temperature of 25 °C in $NaDodSO_4$ buffer, no intermediate labeled ^{125}I-containing bands were seen between these high molecular weight bands and the low molecular

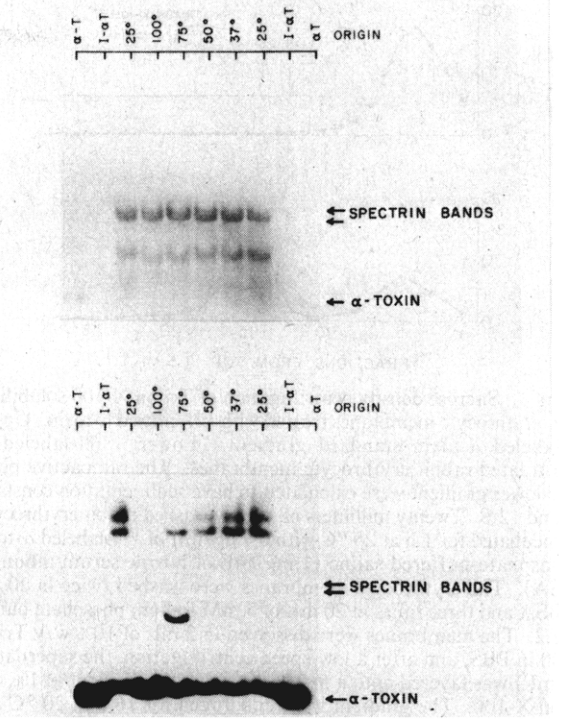

FIGURE 5: 2% polyacrylamide–1% agarose slab gel electrophoresis of ^{125}I-labeled α-toxin-treated rabbit erythrocyte membranes. Samples from right to left are native α-toxin, ^{125}I-labeled α-toxin, toxin-treated membranes, sample preparation temperature 25, 37, 50, 75, 100, and 25 °C ^{125}I-labeled α-toxin, and native α-toxin.

weight band which comigrated with ^{125}I-labeled α-toxin and native α-toxin. However, as shown in Figure 5, as the sample preparation temperature is raised, a sharp transition temperature is seen at about 75 °C. If the sample is incubated for 1 min at 75 °C before electrophoresis, the 1.5×10^6 dalton band disappears and a band of intermediate molecular weight, 1.6×10^5 daltons, is formed. If the temperature is increased to 100 °C, all high molecular weight bands disappear, and all radioactivity comigrates with ^{125}I-labeled α-toxin.

A very interesting, and so far unexplained, finding is that if rabbit erythrocyte membranes exposed to ^{125}I-labeled α-toxin are subjected to $NaDodSO_4$ electrophoresis in a 7.5% polyacrylamide slab system, only the 1.6×10^5 dalton high molecular weight radioactive band is observed in addition to the low molecular weight ^{125}I-labeled α-toxin band. We feel that this observation may be explained by the different pH buffers in which the two electrophoresis systems are run. The agarose system is run at pH 7.2 while the 7.5% acrylamide system is run at pH 8.3. We have indication from other experiments that the high molecular weight, ^{125}I-labeled α-toxin components, which are extremely stable in $NaDodSO_4$ buffer at neutral pH, are much more labile at high pH. Naturally, we have attempted to identify the component(s) which may be present in the high molecular weight forms of ^{125}I-labeled α-toxin seen on sucrose density gradients and $NaDodSO_4$ gel electrophoresis. The extensive series of experiments concerning this problem have failed. In an effort to radiolabel the membrane components of the complex, we have purified the 1.5 plus 1.1×10^6 dalton bands from agarose disc gel electrophoresis and have attempted to label the material with ^{131}I using [^{131}I]-*N*-hydroxysuccinamide (Bolton-Hunter reagent). No labeling was observed. We believe the result derives from the high concentration dependence of the labeling reactions and the extremely low concentration of the purified material.

^{125}I-Labeled α-Toxin Binding to Mouse Tissues and Mouse

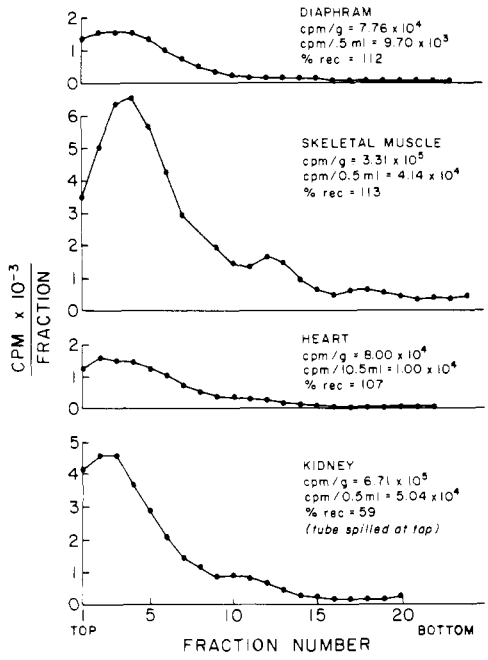

FIGURE 6: Sucrose density centrifugation of mouse tissues treated in vivo with ^{125}I-labeled α-toxin. C3H mice (20 g) were injected interperitoneally with 12 μg of ^{125}I-labeled α-toxin. One hour later, the animals were sacrificed and the organs removed on ice. Centrifugations were performed as in Figure 1 using supernatants from homogenates of the organs in PBS containing 10% w/v Triton X-100.

Neuroblastoma Tissue Culture Cells. Two methods of approach were taken to search for ^{125}I-labeled α-toxin binding sites in tissue other than blood cells. An in vivo approach involved injecting mice with sublethal doses (over the time of the test) of ^{125}I-labeled α-toxin, sacrificing the animals, and removing tissues. The tissues were minced and stirred in buffer in an attempt to remove as many blood cells as possible and any unbound ^{125}I-labeled α-toxin. The minced tissues were then homogenized in a Triton X-100 containing buffer, and after a low-speed centrifugation step to remove insoluble material, samples were applied to sucrose gradients similar to those used for the erythrocyte work. In a survey of mouse diaphragm, leg skeletal muscle, heart, kidney, and liver, only the skeletal muscle tissue showed a radioactive peak in the 12S region of the sucrose gradients (Figure 6).

The second approach used was an in vitro incubation of isolated mouse diaphragms with ^{125}I-labeled α-toxin. This approach allowed a higher concentration of toxin to be used and for the thin muscle tissue to be washed extensively after binding the toxin and before homogenization. The results of the sucrose gradient centrifugation of diaphragm supernatants are shown in Figure 7. About one-third of the total bound radioactivity was in a form having a high (about 12 S) S value. However, neither the high nor the low S value ^{125}I-labeled α-toxin-containing peaks were reduced by diluting the ^{125}I-labeled α-toxin in the diaphragm incubation medium with native α-toxin (250-fold isotope dilution). Convention usually dictates that ^{125}I-labeled α-toxin bound under these conditions is "nonspecifically" bound. However, we do not understand why all such nonspecific, soluble complexes of the toxin with components of the diaphragm should appear as a high S value peak rather than being polydispersed across the sucrose gradient.

Similar experiments using two permanent tissue culture cell lines, mouse neuroblastoma NB41AD and Neuro 2a, failed to show specific binding of ^{125}I-labeled α-toxin to cell monolayers, a high S value radioactive peak on sucrose gradients,

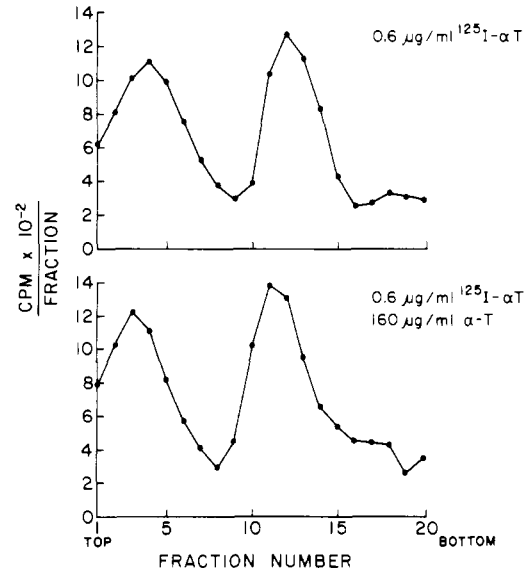

FIGURE 7: Sucrose density centrifugation of mouse diaphragm treated with ^{125}I-labeled α-toxin and solubilized in Triton X-100 buffer. Two diaphragms were used for each experiment: total cpm bound = 1.28 × 10⁴; total cpm in high molecular weight peak = 4.68 × 10³; total picomoles of α-toxin bound/mg of tissue = 16.0; total picomoles of α-toxin bound/mg of tissue associated with the high molecular weight peak = 5.8.

or any cytotoxic effect of native toxin even at high toxin concentrations.

Discussion

The bulk of the experiments and results reported here concerned the characterization of the forms of ^{125}I-labeled α-toxin specifically bound to rabbit erythrocyte membranes. The results give strong circumstantial evidence that the toxin interacts with a membrane protein forming very high molecular weight complexes. These complexes are extremely stable to both proteolytic digestion and denaturation in urea or $NaDodSO_4$ solutions. The fact that the radioactive complexes form discrete bands on $NaDodSO_4$ electrophoresis gels rather than being polydispersed also argues the fact that the complexes do not derive from some nonspecific interaction of the toxin with solubilized erythrocyte membrane proteins. Under all conditions so far studied with erythrocytes, the formation of the high molecular weight complexes is correlated with specific binding of the ^{125}I-labeled α-toxin to membranes. A model, suggested to us by Professor Sidney Colowick, which explains these results in terms of formation of monomer and multimer complexes is presented in Figure 8. α-Toxin (T) is shown to combine with the membrane receptor (O) to form the monomer O–T, with the apparent molecular weight of 1.6 × 10⁵. This initial binding to form a monomer complex would be easily reversed on $NaDodSO_4$ solution at 25 °C and would account for the 30–40% of bound counts solubilized from toxin-treated rabbit erythrocyte membranes, which comigrate with ^{125}I-labeled α-toxin. The monomers associate to form the hexamer, which has an apparent molecular weight of 1.1 × 10⁶. The hexamers, in turn, either condense to form the dodecamer or, more likely, each α-toxin molecule (T) associates with a second binding site (O) to form a hexamer with an apparent molecular weight of 1.5 × 10⁶. Both the dodecamer form and the hexamer with two binding sites per α-toxin molecule could explain the observed 12S form. However the latter model has the additional virtue that it nicely explains the apparent loss of available binding sites, when the rabbit erythrocytes are first exposed to subsaturating con-

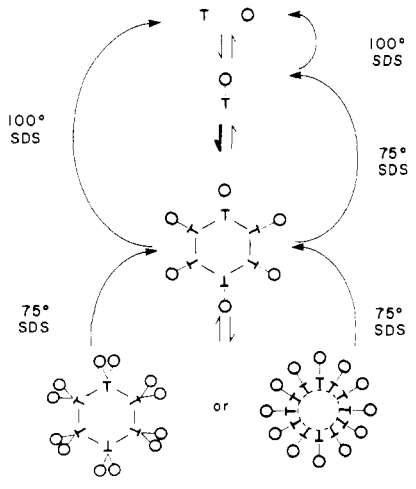

FIGURE 8: A possible model of the interaction of α-toxin with membrane protein of the rabbit erythrocyte. The relative positions of the membrane protein of the rabbit erythrocyte (O) and the α-toxin (T) in the complexes are drawn for illustration. A model in which the α-toxin moiety is on the outside is equally possible. The estimated molecular weight of α-toxin is 28 000 and of the monomer complex, 160 000, thus giving a calculated value for the membrane proton receptor of 130 000. The hexamer form would have a calculated molecular weight of 0.9×10^6 and the dodecamer form, 1.8×10^6. The measured values were 1.1×10^6 and 1.5×10^6, respectively. For further details, see text.

centrations of α-toxin, a topic we have already discussed above.

Thus far, specific binding of ^{125}I-labeled α-toxin has been demonstrated only by use of erythrocytes. At present, both the in vivo and in vitro binding studies involving mouse tissue have not shown classical specific binding to any of the tissues examined, although there are indications that muscle tissue may be involved. The recent report that rabbit muscle fibers can be rendered permeable to Ca^{2+} by exposure to α-toxin (Cassidy et al., 1978) lends support to this conclusion. In a separate paper we reported our observations with rabbit vagus nerves that suggest that myelin membranes may also be a sensitive target tissue for staphylococcal α-toxin (Szmigielski et al., 1978). Of particular interest is the observation that α-toxin binding to rabbit vagus nerves appears to be specific (Szmigielski & Harshman, 1978).

The observation that antisera to rabbit erythrocytes as well as antisera to human erythrocytes (which do not specifically bind α-toxin) both effectively react with the α-toxin rabbit membrane complex is unexpected and currently unexplained. The result would suggest that a serologically cross-reacting material (CRM) but biologically modified material may be a common component on the membrane of cells. Such CRM material, whose normal physiological function remains unknown, could be either topologically unavailable for reaction with α-toxin or genetically modified so as to have lost the α-toxin binding region, while retaining the CRM characteristic. These possibilities remain unresolved at present.

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