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Studies on the Binding of Staphylococcal ^{125}I-Labeled α-Toxin to Rabbit Erythrocytes†

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ABSTRACT: Staphylococcal α-toxin, a hemolytic exotoxin, can be iodinated using the lactoperoxidase method. ^{125}I-Labeled α-toxin binds to rabbit erythrocytes in an apparently irreversible and highly specific manner. The binding of ^{125}I-labeled α-toxin to erythrocytes of rabbit and human reflects the species specificity of native α-toxin. Binding of ^{125}I-labeled α-toxin is blocked by the presence of native α-toxin, ^{127}I-labeled α-toxin, or anti-α-toxin antibody. Simultaneous assays of ^{125}I-labeled α-toxin binding and leakage of intracellular $^{86}Rb^+$ suggest that toxin binding and membrane damage are separate, sequential functions. Both the rate and extent of binding are temperature dependent. Rabbit erythrocytes possess 5×10^3 binding sites/cell, while human erythrocytes possess no detectable binding sites. Treatment of rabbit erythrocytes with ^{125}I-labeled α-toxin appears to decrease the number of unoccupied binding sites. Chaotropic ions can inhibit ^{125}I-labeled α-toxin binding and cause bound ^{125}I-labeled α-toxin to dissociate from rabbit erythrocyte membranes. Treatment of intact rabbit erythrocytes with pronase reduces both the binding capacity of the cells for ^{125}I-labeled α-toxin, and the cells' sensitivity to hemolysis by native α-toxin. It is proposed that the primary binding site for α-toxin in biomembranes is a surface membrane protein.

A number of proposals have been made concerning the interaction of staphylococcal α-toxin with erythrocyte membranes and the mechanism of toxin-induced hemolysis. It has been proposed that α-toxin is a protease having activity for membrane proteins (Wiseman and Caird, 1972), and that the toxin is an esterase having specificity for cholesterol esters (Harvie, 1974). Weissman et al. (1966), Buckelew and Colacicco (1971), and Freer et al. (1973) have proposed that disruption of susceptible biomembranes may be explained solely by a direct interaction with membrane lipid. This proposal has been based on the interaction of α-toxin with artificial lipid dispersions (Weissman et al., 1966; Freer et al., 1973) and with lipid monolayers (Buckelew and Colacicco, 1971). That homogeneous α-toxin does release internal marker from liposomes has been confirmed recently (Cassidy et al., 1974). Although erythrocytes of different species vary in their sensitivity to the toxin, no such variation in sensitivity was found in liposomes derived from membrane lipid extracted from erythrocytes of different species. Also, the binding of radiolabeled α-toxin to erythrocytes has been found to reflect the species specificity of the native toxin (Cassidy and Harshman, 1973).

In a preliminary report (Cassidy and Harshman, 1973) we concluded that the binding of ^{125}I-labeled α-toxin to rabbit erythrocytes was complex in that an apparent decrease in

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^{125}I-labeled α-toxin binding occurred as the cells hemolysed. It is clear now that these results were a consequence of the assay method which failed to quantitatively recover all of the cell membranes after cell lysis (see Materials and Methods). The present work describes the binding characteristics of ^{125}I-labeled α-toxin to cell membranes, and characteristics of the α-toxin receptor in those membranes.

Materials and Methods

Staphylococcal α-toxin (form B) was prepared either by the method of Six and Harshman (1973), or by a new purification method involving adsorption chromatography on porous glass beads (Cassidy and Harshman 1976a). Tos-PheCH$_2$Cl[1]-trypsin was obtained from Worthington Biochemical Corp., pronase CB and lactoperoxidase grade B were from Calbiochem, bovine serum albumin, crystallized, simple sugars, and all other enzymes were from Sigma Chemical Co. Chaotropic salts were from Fisher Scientific Co. Na^{125}I, used to iodinate α-toxin, and ^{86}RbCl were from New England Nuclear. Rabbit erythrocytes were obtained from stock animals. Human erythrocytes were group O$^+$ cells obtained from outdated donor blood. Cells were washed in 0.15 NaCl, 0.02 M potassium phosphate, pH 7.4, and resuspended in the same buffer with 1 mg/ml bovine serum albumin (3×10^8 cells/ml). Occasionally 0.02 M Tris was substituted for phosphate in the buffer to avoid precipitation with other added salts.

Preparation of Iodinated Staphylococcal α-Toxin (^{125}I-Labeled α-Toxin). Staphylococcal α-toxin (B) prepared as described above was radiolabeled by the lactoperoxidase method of Morrison et al. (1971) using Na^{125}I. After the iodination reaction was complete, free ^{125}I was removed from the preparation by chromatography on Sephadex G-75 equilibrated with 0.15 M phosphate buffer of pH 7.4. A single symmetrical peak eluting just following the void volume generally contained 20–50% of the total radioactivity and retained hemolytic activity when assayed on rabbit erythrocytes by the method of Bernheimer and Schwartz (1963). Recovery of total protein from the column was generally 40–50%, presumably due to adsorption of the toxin to the column glass surfaces. After removal of an aliquot for protein assay of the pooled ^{125}I-labeled α-toxin, 1 mg/ml of bovine serum albumin was added and the solution frozen at −70°C. Under these storage conditions, ^{125}I-labeled α-toxin is stable over a period of 3–4 weeks. Specific radioactivities varied between 1.45 and 3.0 μCi/μg.

Trace-labeled, ^{125}I-labeled α-toxin retained its full hemolytic activity, demonstrating that exposure to H_2O_2 does not inactivate α-toxin. However, when carrier iodide was present in the reaction and the protein was quantitatively iodinated, an incorporation of 1 g-atom of iodide per mol of α-toxin (assuming a molecular weight of 28 000 (Six and Harshman, 1973)) led to an inhibition of hemolytic activity of 90%. The specific modification of α-toxin by iodination is the subject of a separate study (Cassidy and Harshman, 1976).

Assay for Binding of ^{125}I-Labeled α-Toxin to Erythrocytes and Erythrocyte Membranes. In our previous method (Cassidy and Harshman, 1973), a centrifuge step to reduce the concentration of bovine serum albumin in the buffer was necessary since the Millipore HAWP filters being used tended to become clogged by the protein. Subsequently we found that the conditions of the centrifugation were inadequate to quantitatively pellet the cell membranes, thus giving rise to an apparent reduction in recovered bound toxin after hemolysis started. A survey of a number of filters led us to select the Millipore EAWP filter, which eliminates the centrifugation step, permits the quantitative recovery of the cell membranes, and allows completion of the wash and collection process in 10–15 s. Identical binding results can now be obtained starting either with intact cells or washed cell membranes. Maximum variation in duplicates does not exceed 15% with this procedure. The data reported are averages of duplicate and, in some cases, triplicate analysis.

The binding assay for ^{125}I-labeled α-toxin used in this study was modified from that used by Cuatrecasas (1973) to assay cholera toxin binding. One milliliter of erythrocyte suspension (3×10^8 cells/ml) was incubated with ^{125}I-labeled α-toxin in 0.15 M NaCl, 0.02 M phosphate buffer of pH 7.4 containing 1 mg/ml of bovine serum albumin, at 24 °C. At the end of the incubation period, the cells were diluted to 5 ml with the above buffer at 24 °C and filtered through Millipore filters, EAWP. The filters were then counted in a well-type γ counter. In each assay, controls were performed to determine nonspecific adsorption of the ^{125}I-labeled α-toxin to the Millipore filters and the erythrocytes. In all cases such nonspecific adsorption was less than 10% of the total bound radioactivity.

Assay for ^{86}Rb$^+$ and Hemoglobin Release. Rabbit erythrocytes were labeled intracellularly by incubation with ^{86}RbCl, and ^{86}Rb$^+$ and hemoglobin release were followed by the method of Hingson et al. (1969).

Results

Specificity of ^{125}I-Labeled α-Toxin Binding. Since, in this study, we followed the binding of α-toxin to rabbit erythrocytes by using ^{125}I-labeled α-toxin, it was necessary to establish that the iodine-labeled α-toxin was bound to the cell membranes at the same sites as was native toxin. Our recent observation that iodination of α-toxin decreases its hemolytic activity (Cassidy and Harshman, 1976) added particular emphasis to this requirement. Accordingly, we tested both native toxin and ^{127}I-labeled α-toxin for their ability to inhibit the binding of ^{125}I-labeled α-toxin to rabbit erythrocytes.

Native α-toxin inhibits ^{125}I-labeled α-toxin binding to rabbit erythrocytes when the two are added to the cells simultaneously (Figure 1). A preparation of ^{127}I-labeled α-toxin which had lost 90% of its hemolytic activity due to iodination (1.2 atoms of I/α-toxin molecule) retained its ability to inhibit ^{125}I-labeled α-toxin binding to rabbit erythrocytes and the inhibition curves were identical for both native and ^{127}I-labeled α-toxin. The iodotoxin preparation which retained only 10% of its original hemolytic activity retained completely its ability to bind to rabbit erythrocytes; thus, ^{125}I-labeled α-toxin can be used as a specific probe to detect α-toxin binding sites in cell membranes. Under these assay conditions, nonspecific binding is negligible.

Kinetics of ^{125}I-Labeled α-Toxin Binding and Membrane Injury. Figure 2 shows the relative kinetics of ^{125}I-labeled α-toxin binding, intracellular ^{86}Rb$^+$ release, and hemoglobin release at 20 and 0 °C. ^{86}Rb$^+$ has been shown to be a useful analogue of K$^+$ in determining K$^+$ release from erythrocytes (Hingson et al., 1969). At both 20 (Figure 2, top) and 0 °C (Figure 2, bottom), the binding of α-toxin precedes the release of ^{86}Rb$^+$, which we have taken as the first indicator of membrane damage. Hemoglobin release occurs later and at 0 °C is not even detectable over the 2 h of observation. A second-order rate constant for the binding of ^{125}I-labeled α-toxin may be calculated and from the data at 20 °C it was found to be $K_1 = 1.54 \times 10^6$ M^{-1} s^{-1}.

[1] Abbreviations used: Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl-chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

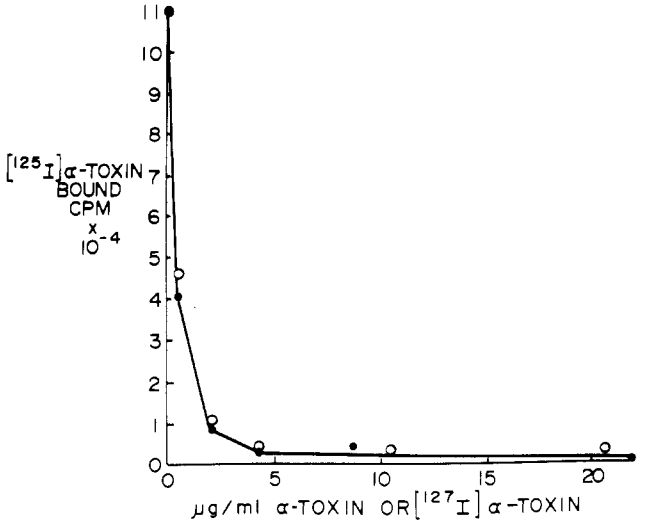

FIGURE 1: Inhibition of ^{125}I-labeled α-toxin binding by native α-toxin or ^{127}I-labeled α-toxin. ^{125}I-Labeled α-toxin (0.28 μg) was added simultaneously with either native α-toxin or ^{127}I-labeled α-toxin (1.2 atoms of I/α-toxin molecule) to 1 ml of a suspension of rabbit erythrocytes (3×10^8 cells per ml). After 30 min at 24 °C, ^{125}I-labeled α-toxin binding was determined. (●) Native α-toxin, 27 800 hemolytic units/mg; (○) ^{127}I-labeled α-toxin, 2040 hemolytic units/mg. ^{125}I-Labeled α-toxin specific activity was 4.15×10^9 cpm/mg.

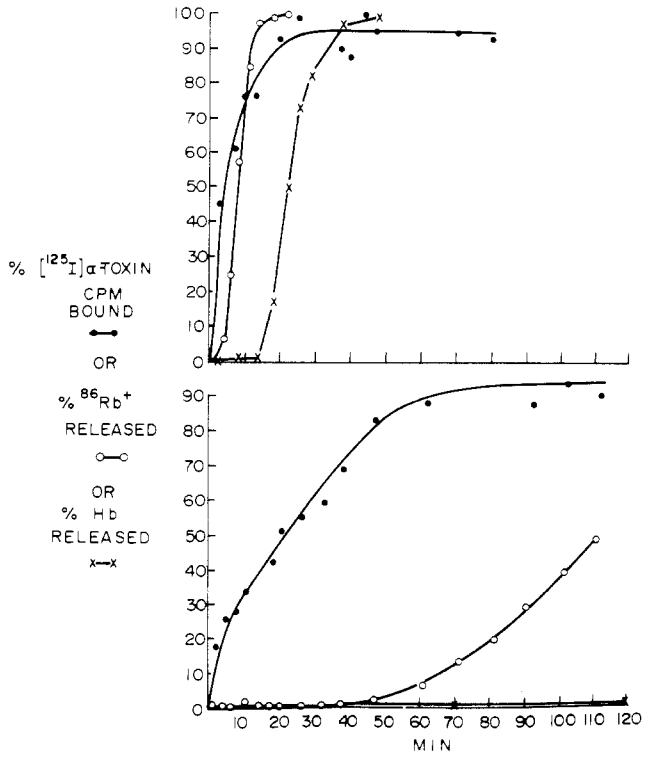

FIGURE 2: Kinetics of ^{125}I-labeled α-toxin binding and membrane injury. ^{125}I-Labeled α-toxin (6.9 μg) was added to 40 ml of a suspension of rabbit erythrocytes (3×10^8 cells/ml); final ^{125}I-labeled α-toxin concentration was 0.17 μg/ml. The suspensions were incubated at either 20 (top) or 0 °C (bottom). At times indicated samples were withdrawn and assayed for ^{125}I-labeled α-toxin bindings, intracellular ^{86}Rb$^+$ release, and hemoglobin release (Hingson et al., 1969). ^{125}I-Labeled α-toxin specific activity was 2.46×10^9 cpm/mg.

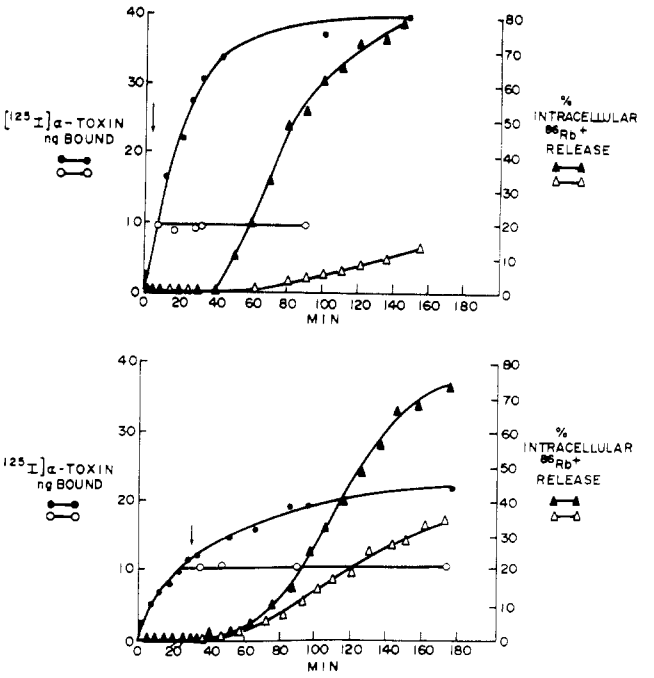

FIGURE 3: Effect of anti-α-toxin on ^{125}I-labeled α-toxin binding and intracellular ^{86}Rb$^+$ release. The kinetics of ^{125}I-labeled α-toxin binding and ^{86}Rb$^+$ release were followed as in Figure 2, at a final ^{125}I-labeled α-toxin concentration of either 0.60 (top) or 0.15 μg/ml (bottom). Specific anti-α-toxin antibody has been calculated to have been in 2.5- and 6-fold molar excess respecvely at the two ^{125}I-labeled α-toxin concentrations. Arrow indicates time of anti-α-toxin addition. (Open symbols) After the addition of anti-α-toxin serum. ^{125}I-Labeled α-toxin specific activity was 2.46×10^9 cpm/mg.

Although, as expected, both the rate of α-toxin binding and the rate of ^{86}Rb$^+$ released were slowed at 0 °C, the time required for the initiation of ^{86}Rb$^+$ release appeared inordinately prolonged, 3 min at 20 °C vs. 50 min at 0 °C. This observation suggested to us that the induction of membrane injury may be not only a sequential but also a separate event from the event of α-toxin binding. To follow this possibility we next examined, at 0 °C, the kinetics of toxin binding and ^{86}Rb$^+$ release at two different initial ^{125}I-labeled α-toxin concentrations. The lag time at 0 °C for the initiation of ^{86}Rb$^+$ release with either 0.15 μg of ^{125}I-labeled α-toxin/ml or 0.60 μg is approximately 50 min (Figure 3). The rate of ^{86}Rb$^+$ release, on the other hand, is slower when less toxin is used. This result suggests that some time-dependent event(s) is required to produce membrane damage but that the extent of damage, i.e., the number of damage foci, and hence the rate of ^{86}Rb$^+$ release, is dependent on the number of α-toxin molecules bound to the membrane, which under these conditions is a function of the initial α-toxin concentration.

To develop this concept further, we employed anti-α-toxin serum to inhibit α-toxin binding and to block further membrane damage (Arbuthnott, 1970). The conditions were selected such that antitoxin was added to both the high and low initial concentrations of α-toxin at such times as to permit the same total amount of α-toxin (10 ng) to bind to the membranes (Figure 3, open circles). When the antiserum was added early, after only 4 min of exposure, the rate of ^{86}Rb$^+$ release is considerably reduced as compared with the rate obtained when the antiserum is added late, at 30 min (Figure 3, bottom). Thus, anti-α-toxin serum not only blocks further binding by free α-toxin but, up to a certain point in time, also blocks the ability of bound toxin to induce membrane damage, else the rate of ^{86}RB$^+$ release in both experiments would have been the same.

To more clearly demonstrate that the rate of ^{86}Rb$^+$ release is a function of the amount of toxin bound, provided that the

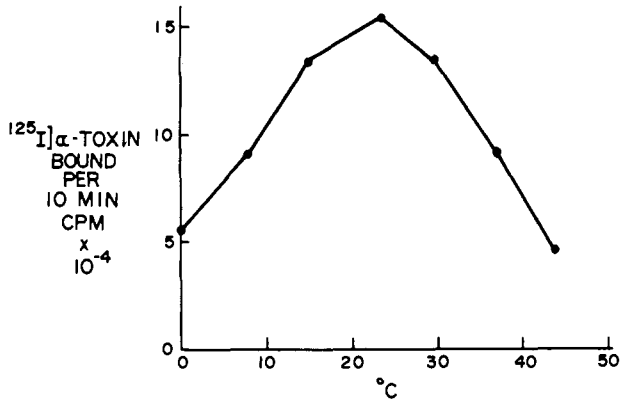

FIGURE 4: Effect of temperature on the rate of ^{125}I-labeled α-toxin binding. ^{125}I-Labeled α-toxin (0.15 μg/ml) was incubated with rabbit erythrocyte suspensions (3×10^8 cells/ml) at the indicated temperature 10 min. ^{125}I-Labeled α-toxin binding was determined using wash buffer which was equilibrated at the indicated temperature. ^{125}I-Labeled α-toxin specific activity was 2.3×10^9 cpm/mg.

Table I: Correlation of Amount of ^{125}I-Labeled α-Toxin Bound with the Maximum Rate of $^{86}Rb^+$ Leakage from Rabbit Erythrocytes.[a]

^{127}I-Labeled α-Toxin Concn (μg/ml)	Time of Anti-α-toxin Addition (min)	^{125}I-Labeled α-Toxin Bound (ng/Tube)	Rate of $^{86}Rb^+$ Leak (%/h)	
			Calcd	Obsd
0.15		22	41	45
0.60		40	75[b]	75
0.15	30	10.5	20	20
0.60	4	10.0	19	8

[a] Data derived from those shown in Figure 3. [b] Rate used for calculation of expected maximal rates.

membrane injury step is permitted to proceed for a period of time after α-toxin binding, we tabulated the maximum observed rates of $^{86}Rb^+$ release with the amounts of α-toxin bound (Table I). The calculated values were obtained using the rate of 75% $^{86}Rb^+$ release per h at 40 ng of α-toxin bound as a base and assuming a simple linear relationship between rate of release and amount of toxin bound. The data show close correspondence between the calculated and observed rates of $^{86}Rb^+$ release at 10, 22, and 40 ng of α-toxin bound. The exception occurs when the bound α-toxin is exposed to specific antisera early (4 min) after binding, thus retarding the development of foci of membrane injury.

Temperature Effects on ^{125}I-Labeled α-Toxin Binding. The rate of ^{125}I-labeled α-toxin binding to rabbit erythrocytes is temperature dependent, displaying an optimum rate of binding at approximately 24 °C (Figure 4). Incubation of ^{125}I-labeled α-toxin at different temperatures for 30 min does not affect the toxin's subsequent rate of binding to cells when incubated at 24 °C.

If the kinetics of ^{125}I-labeled α-toxin binding are followed at 8, 24, and 44 °C, two separate effects of temperature are revealed (Figure 5). At 8 °C only the rate of binding is decreased while the total amount of ^{125}I-labeled α-toxin bound is identical with that bound at 24 °C. Increasing the temperature to 44 °C results in both a decrease in the rate of binding and a decrease in the amount of toxin bound. This effect could

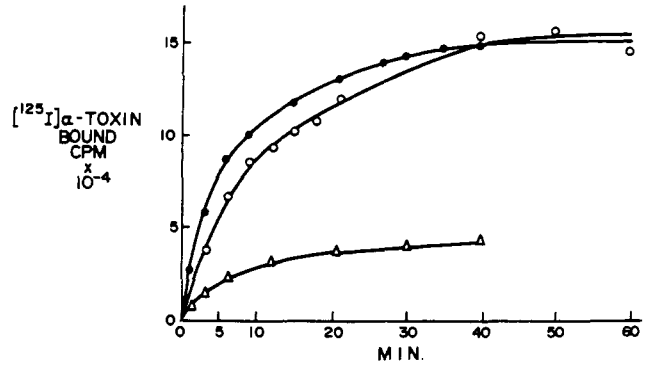

FIGURE 5: Effect of temperature on the kinetics of ^{125}I-labeled α-toxin binding. Kinetics of ^{125}I-labeled α-toxin binding was determined at 24 (●), 8 (○), and 44 °C (△), as in Figure 4 (final ^{125}I-labeled α-toxin concentration was 0.15 μg/ml). ^{125}I-Labeled α-toxin specific activity was 2.3×10^9 cpm/mg.

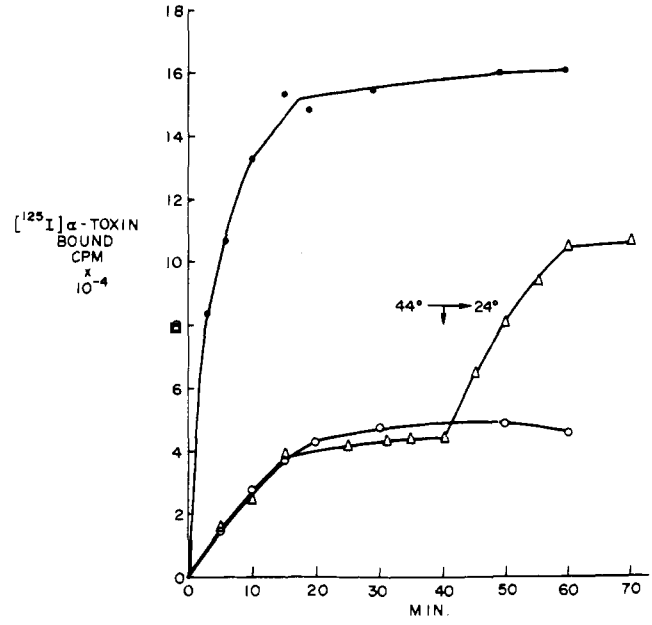

FIGURE 6: Effect of temperature shift from 44 to 24 °C on ^{125}I-labeled α-toxin binding. ^{125}I-Labeled α-toxin (0.15 μg/ml) was incubated either at 24 or 44 °C and ^{125}I-labeled α-toxin binding determined. A third rabbit erythrocyte suspension was incubated with ^{125}I-labeled α-toxin at 44 °C for 40 min. The temperature was then rapidly shifted to 24 °C by equilibrating the incubation flask with a large volume of water at 24 °C. ^{125}I-Labeled α-toxin specific activity was 2.3×10^9 cpm/mg.

either be due to a temperature-dependent change in the association constant of the toxin membrane complex or to a reduction in the total number of toxin binding sites available with increasing temperature. The temperature-dependent change in the extent of ^{125}I-labeled α-toxin binding can be reversed by a temperature shift from 44 to 24 °C (Figure 6). This observation makes unlikely the possibility that incubation of the cells at 44 °C irreversibly denatures the ^{125}I-labeled α-toxin binding sites.

Effect of Chaotropic Ions on ^{125}I-Labeled α-Toxin Binding. Chaotropic ions are thought to disrupt the structure of water and cause the solvent environment to become relatively less polar, which favors the disruption of hydrophobic interactions (Hatefi and Hanstein, 1974). These ions have been arranged in the so-called chaotropic series according to decreasing potency: $SCN^- > I^- > Br^- > Cl^- > CH_3COO^- > SO_4^{2-} > HPO_4^{2-}$. The rapid reversal of the binding of ^{125}I-labeled

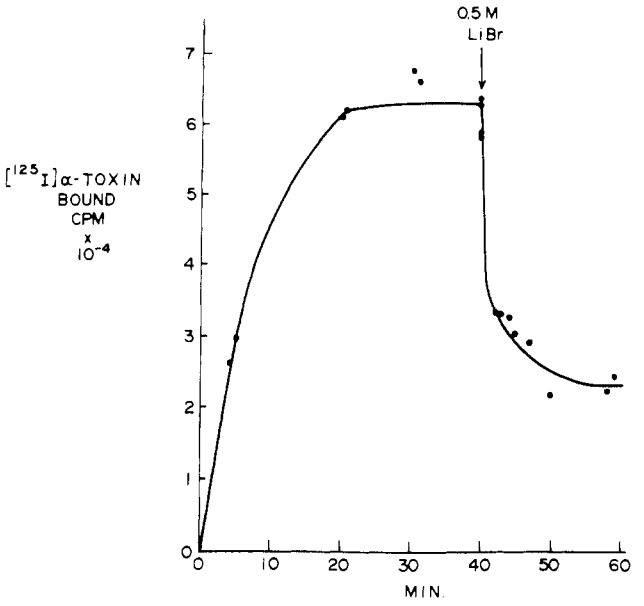

FIGURE 7: Effect of LiBr on ^{125}I-labeled α-toxin binding. ^{125}I-Labeled α-toxin (0.15 μg/ml) was incubated for 40 min with 20 ml of a suspension of rabbit erythrocytes (3×10^8 cells/ml in 0.15 M NaCl, 0.02 M tris(hydroxymethyl)aminomethane (pH 7.5), 1 mg/ml bovine serum albumin); 5 M LiBr was added to the remaining cell suspension at 40 min to bring the final concentration of LiBr to 0.5 M. ^{125}I-Labeled α-toxin binding was determined at the indicated times. ^{125}I-Labeled α-toxin specific activity was 2.46×10^9 cpm/mg.

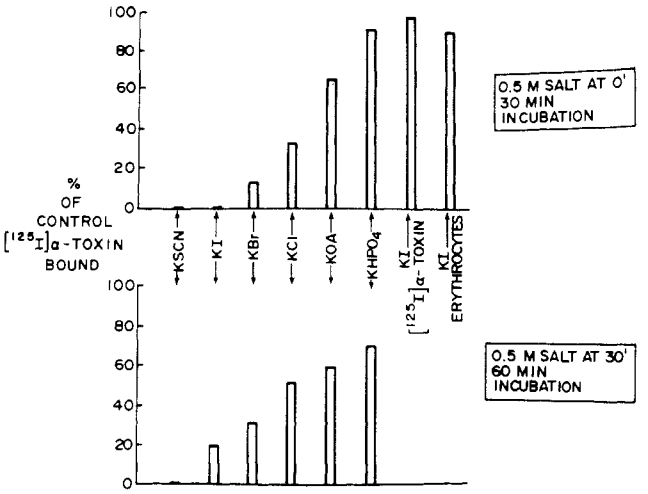

FIGURE 8: Effect of chaotropic ions on ^{125}I-labeled α-toxin binding. ^{125}I-Labeled α-toxin (0.15 μg/ml) was incubated with a rabbit erythrocyte suspension as in Figure 7. In the top panel, 0.5 M salt was added at zero time and ^{125}I-labeled α-toxin binding was determined after 30 min at 24 °C. In two control experiments, 0.15 μg of ^{125}I-labeled α-toxin was incubated for 30 min at 24 °C in 0.05 ml of 0.5 M KI, 0.02 M tris(hydroxymethyl)aminomethane (pH 7.4) solution, 1 mg/ml bovine serum albumin. One milliliter of rabbit erythrocyte suspension was then added and ^{125}I-labeled α-toxin binding was determined after an additional 30 min. Alternately, 1 ml of the cell suspension was incubated for 30 min in 0.15 M NaCl, 0.02 M tris(hydroxymethyl)aminomethane (pH 7.4), 1 mg/ml bovine serum albumin, 0.5 M KI. The cells were then centrifuged and the cell pellet was resuspended in the same buffer without KI. ^{125}I-Labeled α-toxin (0.15 g) was added and ^{125}I-labeled α-toxin binding determined after an additional 30 min incubation at 24 °C. In the bottom panel, ^{125}I-labeled α-toxin was incubated with the rabbit erythrocyte suspension for 30 min and 0.5 M salt was added. At 60 min, ^{125}I-labeled α-toxin binding to the erythrocytes was determined. ^{125}I-Labeled α-toxin specific activity was 2.46×10^9 cpm/mg.

α-toxin to rabbit erythrocytes by the chaotropic ion Br^- is illustrated in Figure 7. Moreover, chaotropic ions can both prevent initial toxin binding (Figure 8, top) and reverse toxin binding (Figure 8, bottom); the potency with which they affect these processes follows the chaotropic series. When the strong chaotropic ion I^- is incubated with either ^{125}I-labeled α-toxin or rabbit erythrocytes and is subsequently diluted or washed out of the binding assay system, no inhibitory effect on subsequent ^{125}I-labeled α-toxin binding is observed (Figure 8, top last two columns).

Effect of Sugars on ^{125}I-Labeled α-Toxin Binding. In order to determine if a carbohydrate moiety is an important part of the binding site, ^{125}I-labeled α-toxin was incubated in 0.2 ml of a 50 mM solution of various sugars for 60 min at 24 °C, prior to performing the binding assay. The following sugars were tested: L-fucose, D-galactose, D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, sucrose, α-methyl D-glucoside, and *N*-acetylneuraminic acid. All sugars tested failed to significantly affect ^{125}I-labeled α-toxin binding to rabbit erythrocytes. In addition, the glycoproteins fetuin and rat serum mucoid failed to affect toxin binding at an incubation concentration of 0.5 mg/ml.

Effect of Enzymatic Digestion of Erythrocytes on ^{125}I-Labeled α-Toxin Binding. When suspensions of rabbit erythrocytes were digested for extended periods with pronase (1 mg/ml), both ^{125}I-labeled α-toxin binding and the cells' sensitivity to α-toxin-induced hemolysis decreased and in 7 h had decreased to 6 and 10% of the control values, respectively (Figure 9). Over the 7-h incubation period, the osmotic fragility of the cells remained unchanged. Equivalent treatment of the cells with trypsin (1 mg/ml) produced a decrease in ^{125}I-labeled α-toxin binding of 12% in 7 h and had no effect on the hemolytic sensitivity of the cells. Digestion of rabbit erythrocytes with α-chymotrypsin, β-glucosidase, μ-galactosidase, hyaluronidase, phospholipase A, and phospholipase D had no significant effect on either the toxin-binding capacity or the hemolytic sensitivity of the cells. Digestion of human erythrocytes with the above enzymes including pronase had no effect on toxin binding or sensitivity to hemolysis.

Saturability of ^{125}I-Labeled α-Toxin Binding. Increasing the ^{125}I-labeled α-toxin concentration in the binding assay leads to an apparent saturation of binding sites (Figure 10). In contrast to the results with rabbit erythrocytes, no binding to human erythrocytes is detectable at any concentration of α-toxin tested. A Scatchard plot of the data (Figure 10, insert) shows that a linear relationship is obtained with an excellent regression figure, $r = -0.998$. From these data an apparent dissociation constant can be calculated, $K_d = 6 \times 10^{-9}$ M at 20 °C, and the number of binding sites per rabbit erythrocyte estimated, 5×10^3/cell. Assuming random distribution over a cell surface of 145 μm^2, an average of 30 binding sites per μm^2 of surface is obtained.

Using the apparent dissociation constant and the forward rate constant obtained from data in Figure 2, the dissociation rate constant can be calculated, $K_{-1} = K_1 K_d = 9.25 \times 10^{-3}$ s^{-1}. Thus, at 20 °C approximately 50% of the bound α-toxin should dissociate per min. We elected to measure the dissociation rate constant directly. To this end, a 260-fold excess of unlabeled α-toxin was added to a suspension of rabbit erythrocytes that already had been equilibrated with 0.34 μg/ml of ^{125}I-labeled α-toxin for 60 min (Figure 11). Unexpectedly, no significant dissociation of the bound ^{125}I-labeled α-toxin was detected over a period of 540 min.

To pursue the basis for this extraordinary discrepancy between the calculated and observed dissociation rates of α-toxin, we examined two possibilities: (a) that the added ^{125}I-labeled α-toxin was being inactivated and (b) that a decrease in the

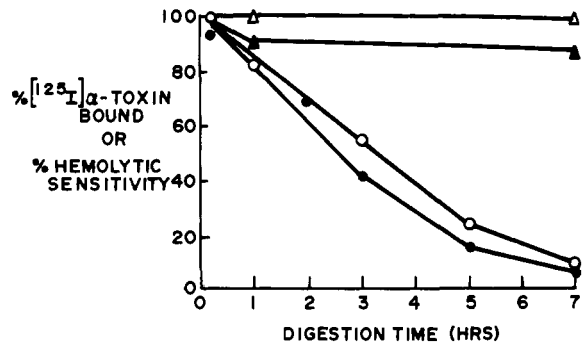

FIGURE 9: Effect of proteolytic digestion of rabbit erythrocytes on ^{125}I-labeled α-toxin binding capacity or hemolytic sensitivity. Rabbit erythrocyte cell suspensions were incubated at 24 °C in phosphate-buffered saline with either no addition, Tos-PheCH_2Cl trypsin (1 mg/ml), or pronase CB (1 mg/ml) for 7 h. At various times, aliquots were removed, washed extensively by centrifugation in 0.15 M NaCl, 0.02 M tris(hydroxymethyl)aminomethane (pH 7.4), 1 mg/ml bovine serum albumin and resuspended in the same buffer to 3×10^8 cells/ml. The cells were then chilled to 0 °C and held at that temperature until ^{125}I-labeled α-toxin binding and hemolytic assays were performed. ^{125}I-Labeled α-toxin binding assay was performed at 24 °C using a final concentration of ^{125}I-labeled α-toxin of 0.15 μg/ml. Hemolytic assays using native α-toxin were performed by the method of Bernheimer and Schwartz (1963) and the results expressed as the percentage of hemolytic units obtained by assaying native α-toxin on digested cells relative to the hemolytic units obtained by assay on undigested cells. (△) Trypsin-treated cells, ^{125}I-labeled α-toxin bound; (▲) trypsin-treated cells, hemolytic sensitivity; (○) pronase-treated cells, ^{125}I-labeled α-toxin bound; (●) pronase-treated cells, hemolytic sensitivity. ^{125}I-Labeled α-toxin specific activity was 2.17×10^9 cpm/mg.

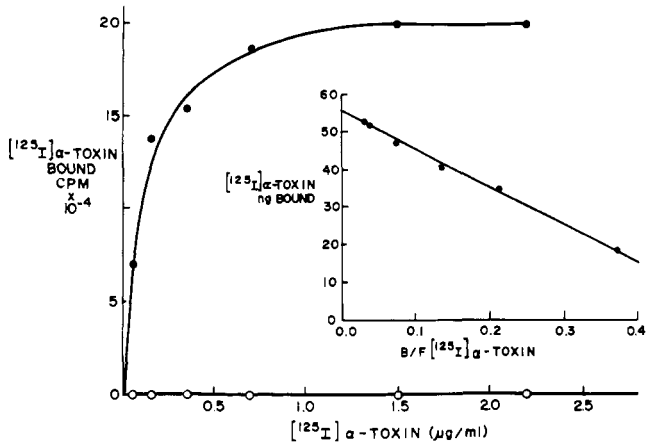

FIGURE 10: Saturability of ^{125}I-labeled α-toxin binding. ^{125}I-Labeled α-toxin was incubated, at increasing concentrations, with 1 ml of a suspension of either rabbit or human erythrocytes (3×10^8 cells/ml) for 30 min at 24 °C. ^{125}I-Labeled α-toxin binding was then determined. RRBC, rabbit erythrocytes (●); HRBC, human erythrocytes (○). ^{125}I-Labeled α-toxin specific activity was 4.15×10^9 cpm/mg.

number of toxin-binding sites on the cells was occurring. Either or both of these events would give an erroneous apparent equilibrium.

It was found, in experiments not shown, that ^{125}I-labeled α-toxin remaining unbound after a period of 60 min incubation with a rabbit erythrocyte suspension was fully active and capable of binding to a second addition of cells. Thus, the explanation that ^{125}I-labeled α-toxin was being inactivated during incubation with the erythrocytes was not applicable.

To test the possibility that incubation with ^{125}I-labeled α-toxin reduces the number of available unbound sites, rabbit erythrocytes were first equilibrated with low levels of α-toxin and then exposed to a constant high amount of α-toxin by ap-

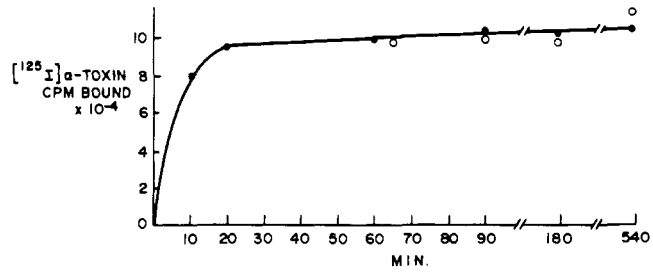

FIGURE 11: Reversibility of ^{125}I-labeled α-toxin binding. ^{125}I-Labeled α-toxin (final concentration 0.34 g/ml) was incubated at 24 °C with 20 ml of a suspension of rabbit erythrocytes (3×10^8 cells/ml). One-milliliter samples were withdrawn to measure ^{125}I-labeled α-toxin binding (●). At 60 min, 10 ml of the suspension was withdrawn and mixed with native α-toxin (final concentration was 88 μg/ml) (○). ^{125}I-Labeled α-toxin specific activity was 2.40×10^9 cpm/mg.

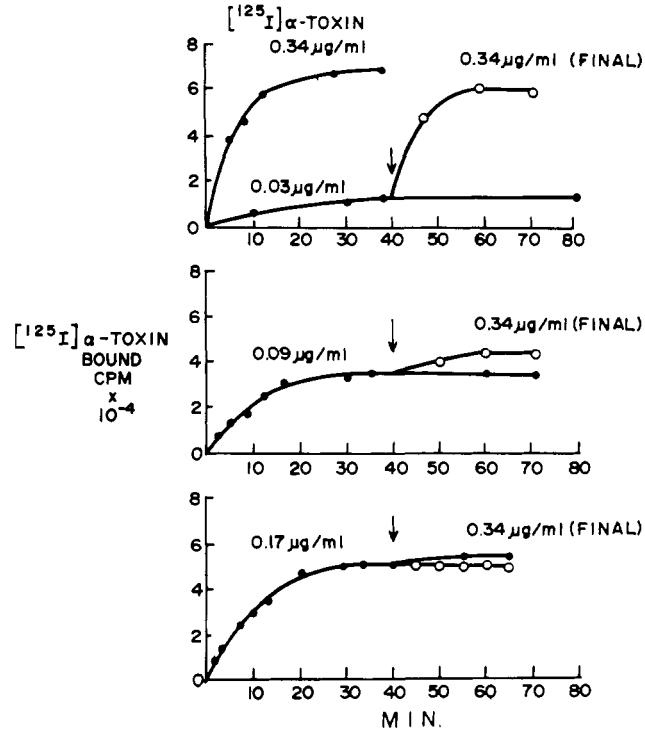

FIGURE 12: Loss of unoccupied binding sites. ^{125}I-Labeled α-toxin at the indicated initial concentrations was incubated at 24 °C with a suspension of rabbit erythrocytes (3×10^8 cells/ml). At 40 min, the final ^{125}I-labeled α-toxin concentration was raised to 0.34 μg/ml (indicated by arrow). ^{125}I-Labeled α-toxin specific activity was 2.4×10^9 cpm/mg.

propriate additions of toxin (Figure 12). The results show that true equilibrium binding of ^{125}I-labeled α-toxin to rabbit erythrocytes does not occur. For example, if the cells are incubated with an initial toxin concentration of 0.17 μg/ml (Figure 12, bottom) and binding is allowed to attain an apparent equilibrium, no further binding of ^{125}I-labeled α-toxin is observed if the toxin concentration is then raised to 0.34 μg/ml. If the initial concentration of toxin is such that a small number of sites are occupied (Figure 12, top), the cells retain a significant, but not complete, capacity to bind further toxin. The decrease in apparent number of ^{125}I-labeled α-toxin binding sites is related to the initial toxin concentration and therefore to the number of toxin binding sites initially occupied. This finding suggests that α-toxin-occupied binding sites affect neighboring unoccupied binding sites making them unavailable for reaction with free α-toxin, thus affecting the apparent equilibrium.

Discussion

As Madoff et al. (1964) previously showed, α-toxin causes a rapid release of intracellular potassium ion, and later causes the release of hemoglobin from the damaged erythrocyte. Using $^{86}Rb^+$ as an analogue of K^+, we have demonstrated that $^{86}Rb^+$ release by the rabbit erythrocyte closely follows the binding of ^{125}I-labeled α-toxin, and may reflect a very rapid process carried out by the toxin after binding which causes irreversible damage to the erythrocyte membrane. When both the rate of toxin binding and the rate of hemolysis were slowed by incubation at 0 °C, several steps could be resolved in the hemolytic sequence (Table I, Figures 2 and 3). Taken together these results indicate that cell damage by α-toxin can be dissected into a series of sequential events: (a) a relatively rapid, time-dependent binding of free α-toxin; (b) a slower, time-dependent induction of foci of membrane injury that leads to $^{86}Rb^+$ release; and (c) the eventual colloidal osmotic lysis of the cells (Seeman, 1974) as indicated by hemoglobin release. Further, the data indicate that specific anti-α-toxin antibody, when added early to the reaction, not only blocks additional binding of free toxin, but also prevents membrane injury by already bound α-toxin. During the course of our characterization of iodinated α-toxin (see preceding paper in this issue), additional evidence was obtained that supports the view that binding and membrane damage are separate though sequential events. In that study we observed that iodination of α-toxin to approximately 1 iodine atom per mol of toxin does not alter its membrane binding capacity but does reduce the hemolytic activity for rabbit erythrocytes to 10% of that of native toxin.

Recently Kato et al. (1975) reported that pronase digestion of rabbit erythrocytes leads to a reduction of both the hemolytic sensitivity and the ^{125}I-labeled α-toxin binding capacity of the cells. Our own results confirm this observation and extend it in that a variety of other proteolytic and lipolytic enzymes tested were without effect on the cells. Moreover, incubation with a number of sugars failed to inhibit ^{125}I-labeled α-toxin binding to cells suggesting that saccharide moieties are probably not involved with toxin binding.

Although the nature of the high affinity binding site is only indirectly characterized, preliminary experiments (Cassidy and Harshman, unpublished) show that a ^{125}I-labeled α-toxin complex bound to rabbit erythrocyte membranes can be characterized after solubilization of the complex in the nonionic detergent Triton X-100. The solubilized ^{125}I-labeled α-toxin complex was found to be excluded from Sephadex G-200 and Bio-Gel P-200 columns and had an apparent sedimentation coefficient of 11.5 S in sucrose density gradients containing 1% Triton X-100. The 11.5S complex is formed only on exposure of ^{125}I-labeled α-toxin to rabbit erythrocytes and cannot be detected when ^{125}I-labeled α-toxin is exposed to either human erythrocytes or egg lecithin liposomes (see further discussion below). Based on these preliminary results and the sensitivity to pronase, we believe the radioactive complex solubilized from rabbit erythrocyte membranes exposed to ^{125}I-labeled α-toxin is a toxin–membrane protein complex.

Hatefi and Hanstein (1974) have shown that chaotropic ions can be useful in the selective solubilization of membrane proteins. Chaotropic ions such as SCN^-, I^-, and Br^- effectively block ^{125}I-labeled α-toxin binding to rabbit erythrocytes, and these ions are also very effective at reversing the toxin binding, even though the toxin binding site interaction is essentially irreversible in their absence. It should be noted that the order in which these ions affect toxin binding is identical with the order of their effectiveness in producing inhibition of a variety of unrelated enzymes (von Hippel and Schleich, 1969). Since the 50% inhibition point for these ions falls between 0.5 and 3 M, it is possible that the chaotropic ion effects on ^{125}I-labeled α-toxin binding and dissociation are due to the induction of reversible conformational changes in the toxin molecule or the rabbit erythrocyte membrane receptors which result in the reversible loss of the ability of α-toxin to be bound to the high affinity receptors in rabbit erythrocytes.

The unexpected finding that the binding of α-toxin to rabbit erythrocyte membranes is irreversible is rare, but not unique. Similar results have been reported by Bulger and Hess (1973) for the binding of α-bungarotoxin to the acetylcholine receptor receptor from *Electrophorus electricus*. It seems clear that part of the explanation for the apparent equilibrium observed with α-toxin binding is due to the loss of available unbound sites by apparent site-site interaction with occupied sites (Figure 12). Similar findings have been reported recently for insulin binding (Huang and Cuatrecasas, 1975). Perhaps most surprising is the extraordinarily good fit obtained when the binding data were examined using the Scatchard plot (Figure 10). Cohen's group (Carpenter et al., 1975) found a similar situation with epidermal growth factor, but in their case EGF degradation accounted for the results obtained.

Previous investigators (Freer et al., 1968, 1973) have reported that, at high concentrations, α-toxin may polymerize on membrane surfaces to form closely packed, ring-like structures 10 nm in diameter, which can be visualized in the electron microscope. These ring-like hexamer forms of α-toxin have been isolated and are reported to have an apparent sedimentation coefficient of 12 S. A number of our results have bearing on this observation. In unpublished work we have confirmed that, in the concentration range of 10–100 μg of α-toxin/ml, "ring forms" are formed on rabbit and human erythrocyte membranes. However, in the effective hemolytic range for rabbit erythrocytes of 0.1–0.2 μg of α-toxin/ml, no such ring aggregates were detected. It is important to note that membranes relatively resistant to α-toxin, such as human erythrocytes, liposomes, mycoplasma, and cultured mammalian cells, are disrupted by α-toxin in the high 10–100-μg range as contrasted to susceptible rabbit erythrocytes, which are lysed completely by exposure to the low range of α-toxin, 0.1–0.2 μg/ml. From another point of view, we have demonstrated that approximately 5000 high affinity binding sites exist per rabbit erythrocyte. It is possible to calculate that saturation of the membranes with ring-like arrays would consume approximately 1×10^7 α-toxin molecules. This is 10^3- to 10^4-fold higher than the estimate of ^{125}I-labeled α-toxin binding sites per cell. Based on these combined considerations, we suggest that ring forms of α-toxin may not be related to specific α-toxin binding by membranes, and that specifically susceptible cells are distinguished by a relatively small number of high affinity receptor sites. This concept is reinforced when one considers the lethal effect of α-toxin in mammals. The LD_{50} for α-toxin in the rabbit has been reported to be 5–7 μg/kg of body weight (Arbuthnott, 1970). Thus, the lethal circulating concentration of α-toxin in the rabbit is in the range of 0.002 to 0.02 μg/ml, several orders of magnitude lower than that required to disrupt nonsusceptible cells. If the specific target cells have high affinity receptors of the same order as do rabbit erythrocytes, effective concentration would occur with concomitant injury to the target tissue and death. All of these findings taken together permit the view that a common mechanism may exist for membrane injury by α-toxin action on all cell membranes. Thus, the differences in sensitivity to α-toxin may be traced to the absence or presence of specific high affinity toxin re-

ceptors, which in turn affect local concentrations of toxin on the cell's surface.

Dedication and Acknowledgment

We respectfully dedicate this paper to Professor Roger M. Herriott, Johns Hopkins University, on the occasion of his retirement as Chairman of Biochemistry, School of Hygiene and Public Health. It is a pleasure to acknowledge the helpful comments from and discussions with Drs. Sidney P. Colowick and Murray Smigel.

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