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Note

Inhibition of concanavalin A by (ethylenedinitrilo)tetraacetic acid

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The saccharide-binding ability of concanavalin A (con A) depends on the presence of essential, metal ions coordinated to the protein[1]. In order that con A may complex with saccharides, two metal-binding sites must be sequentially filled. The first site, S1, can be occupied with any of several transition metals, such as Co^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}. The second site, S2, is highly specific for the calcium ion[2]. Both metal-binding sites are located near the surface of the protein, and are ~500 pm apart[3]. When the metals are removed, con A becomes more susceptible than before to thermal denaturation and proteolysis[4,5]. Paradoxically, concentrations of Ca^{2+} or Mn^{2+} greater than ~0.1M appear to inhibit[6] the precipitation between con A and dextran NRRL B-1355-S. Several authors have observed difficulty in removing metal ligands from con A. Sherry *et al.*[7] found that, after extensive dialysis of con A against mM (ethylenedinitrilo)tetraacetic acid (EDTA) at pH 5.6, the protein remained partially active, and Agrawal and Goldstein[6] discovered that, when Mn^{2+} was added to metal-deficient con A, the resulting metalloprotein was largely refractory to the addition of EDTA, as measured by precipitation of dextran. Doyle *et al.*[8] also noted that con A is highly resistant to EDTA at pH 7. Other reports have shown that it is necessary to dialyze con A at low values of pH in order to lower the content of metals to a satisfactory level[1–3]. In experiments designed to study the kinetics of the interaction of Ca^{2+} with con A, we observed that EDTA readily abolishes the saccharide binding to the protein at pH 5.3. This report examines some of the effects of EDTA on the ability of con A to complex with polysaccharides.

EXPERIMENTAL

Con A was prepared by affinity chromatography on Sephadex G-100, according to Agrawal and Goldstein[9]. All solutions of con A were prepared gravimetrically, and then standardized by u.v. absorption at 280 nm, assuming that[9] $E_{1\%,\,1\,cm} = 11.4$. Salts were of analytical grade and purchased from Fisher Scientific Co. (Cincinnati, Ohio). Rabbit-liver glycogen (Type III, Sigma Chemical Co., St. Louis, MO) was

extracted twice with 44 per cent phenol for 30 min at 66° to remove traces of contaminating protein. The glycogen was then dialyzed against water, and freeze-dried. De-ionized, subsequently glass-distilled, water was employed for the preparation of all solutions.

In some experiments, con A was quantitated by the method of Lowry *et al.*[10], with con A as the standard. Metal ions (Ca^{2+} and Mn^{2+}) were quantitated by atomic absorption, by use of an Instrumentation Laboratory, Inc., Model 253 atomic absorption spectrophotometer. Prior to the metal analysis, con A preparations were hydrolyzed for 2 h at 100° in 2M HCl.

The extent of the interaction between con A and glycogen was measured turbidimetrically[11], by use of rounded cuvets (1.25 cm diam.), in a Coleman Junior 11 spectrophotometer at 450 nm. All readings were made at room temperature. Blanks contained con A and glycogen plus 20 μmol of methyl α-D-mannopyranoside per mL.

RESULTS AND DISCUSSION

Solutions of con A were mixed with various concentrations of EDTA at pH 5.3. Samples were then mixed with glycogen, and the turbidities recorded. The results, shown in Fig. 1, indicate that con A is readily inactivated by the chelating agent. The

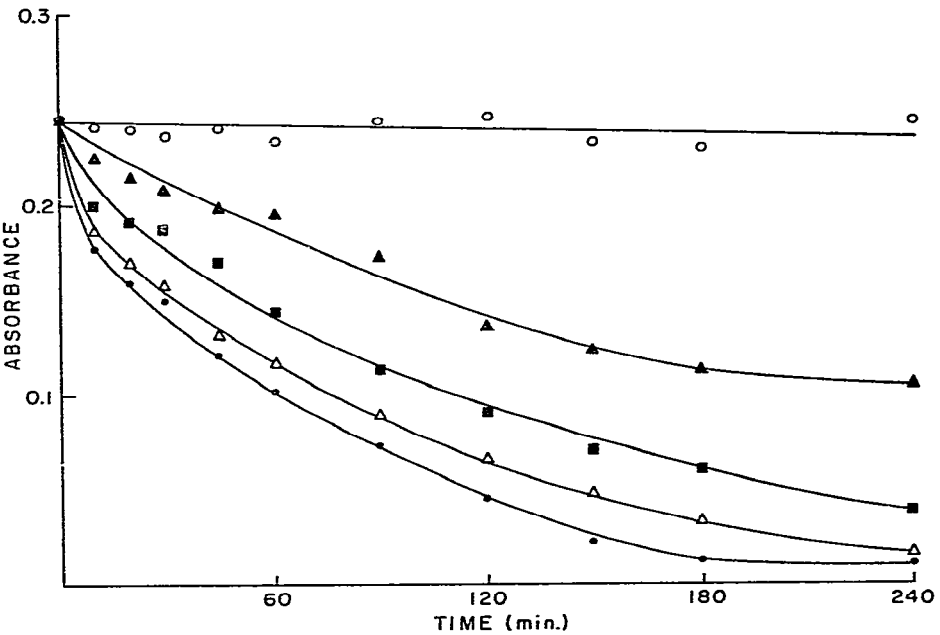

Fig. 1. Inhibition of precipitation of con A–glycogen by EDTA. [Solutions of con A (1.0 mg/mL) in 0.1M sodium acetate, 0.2M NaCl, pH 5.3, were incubated in the presence of various concentrations of EDTA. At the indicated times, the lectin (2.0 mL) was then mixed with glycogen (2.0 mg; 2.0 mL), and the turbidities determined after 10 min as described. Control, —○—; 50 mM EDTA, —●—; 1 mM EDTA, —△—; 0.2 mM EDTA, —■—; and 0.1 mM EDTA, —▲—.]

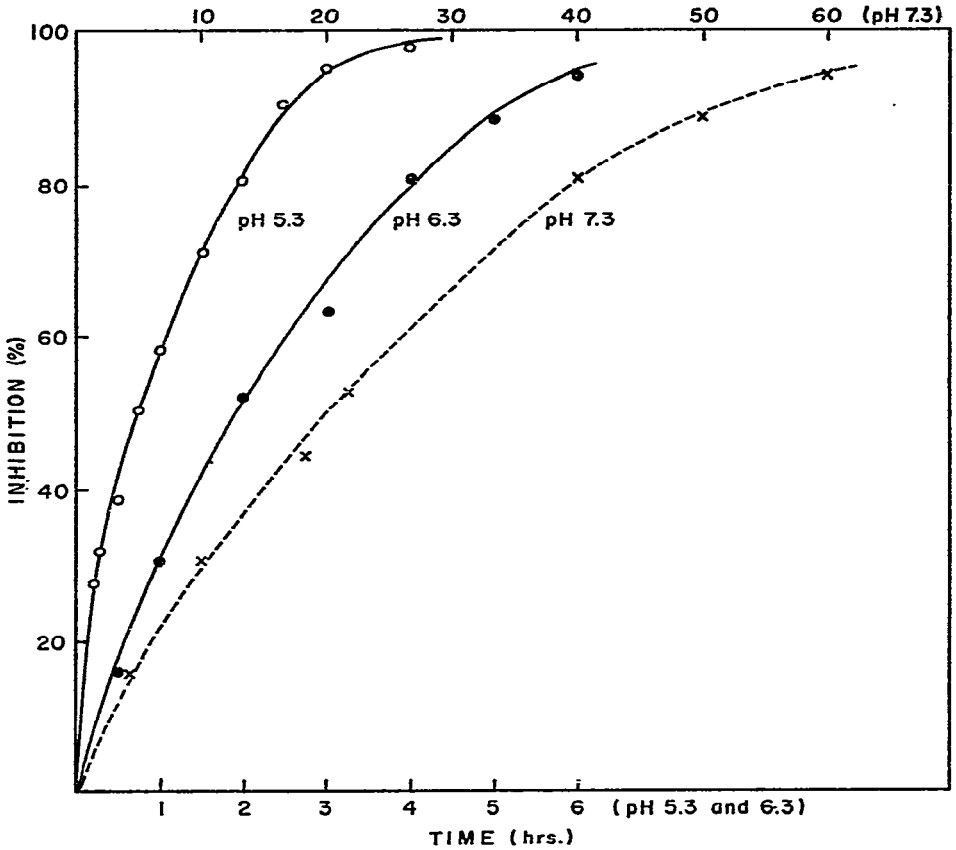

Fig. 2. Effect of hydrogen-ion concentration on the inhibition of con A by EDTA. [At pH 6.3, con A (2 mL; 2.35 mg) was mixed with glycogen (1.25 mg; 2 mL) in 0.05M phosphate–0.2M NaCl. At pH 7.3, con A (1.10 mg; 2 mL) was mixed with glycogen (2.50 mg; 2 mL) in 0.05M phosphate–2M NaCl. The data for pH 5.3 were derived from Fig. 1. The concentration of EDTA was 50 mM.]

rate of inactivation is dependent on the concentration of the EDTA. When con A was dialyzed against 50 mM EDTA at pH 5.3 (100 mL of con A dialyzed against 5 L of EDTA with three changes of dialysis medium), the metal contents were found to be 0.13 mol of Ca^{2+} and 10 mmol of Mn^{2+}, respectively, per mol of con A. The metal-deficient protein was incapable of precipitating glycogen. Native con A contained 0.85 mol of Ca^{2+} and 0.89 mol of Mn^{2+}, respectively, per mol of protein. Thus, the inactivation of con A by EDTA is a result of the removal of metals from the protein.

When con A was incubated with EDTA at higher pH values, a lowering in the rate of inactivation was observed (see Fig. 2). The rates of inactivation for all three pH values follow first-order kinetics, as shown in Fig. 3. At pH 5.3, the inactivation rate-constant (k) was determined to be 0.95 h^{-1}. At pH 6.3, k was 0.45 h^{-1}, whereas, at pH 7.3, it was 0.046 h^{-1}. Thus, at pH 7.3, the rate of inactivation is $\sim$1/20th of that at pH 5.3.

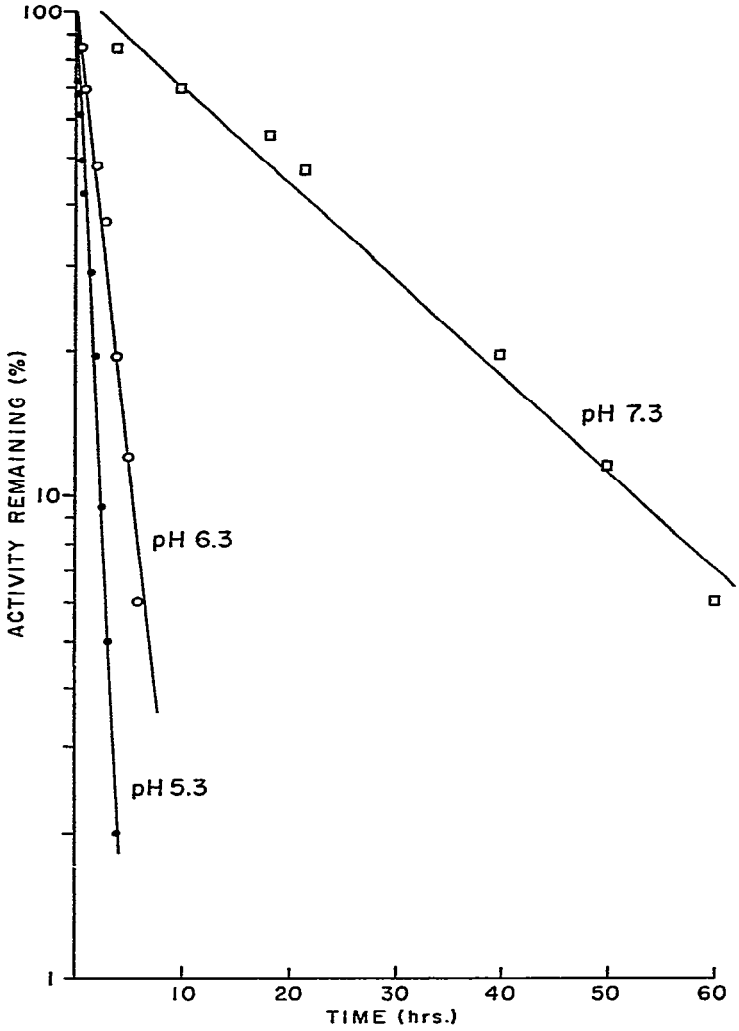

Fig. 3. First-order plots for the inactivation of con A by EDTA at different hydrogen-ion concentrations. [Data were derived from Fig. 2. The curves were determined from least-squares analyses.]

Following removal of Ca^{2+} and Mn^{2+} from con A by EDTA, it should be possible to reverse the inactivation by adding back an excess of metals to the protein. Accordingly, con A was incubated with 0.2 mM EDTA for 3 h. Following incubation, the protein was found to complex minimally with glycogen (see Fig. 4). The addition of either Ca^{2+} or Mn^{2+} partially restores the ability of con A to complex with glycogen. Addition of both metals in excess resulted in an enhancement of activity over that of the control. Similarly, native con A showed an increased ability to precipitate glycogen when the protein was supplemented with both Ca^{2+} and Mn^{2+}.

In some experiments, it was observed that the addition of EDTA to con A–glycogen complexes did not significantly change the turbidities due to the dispersed

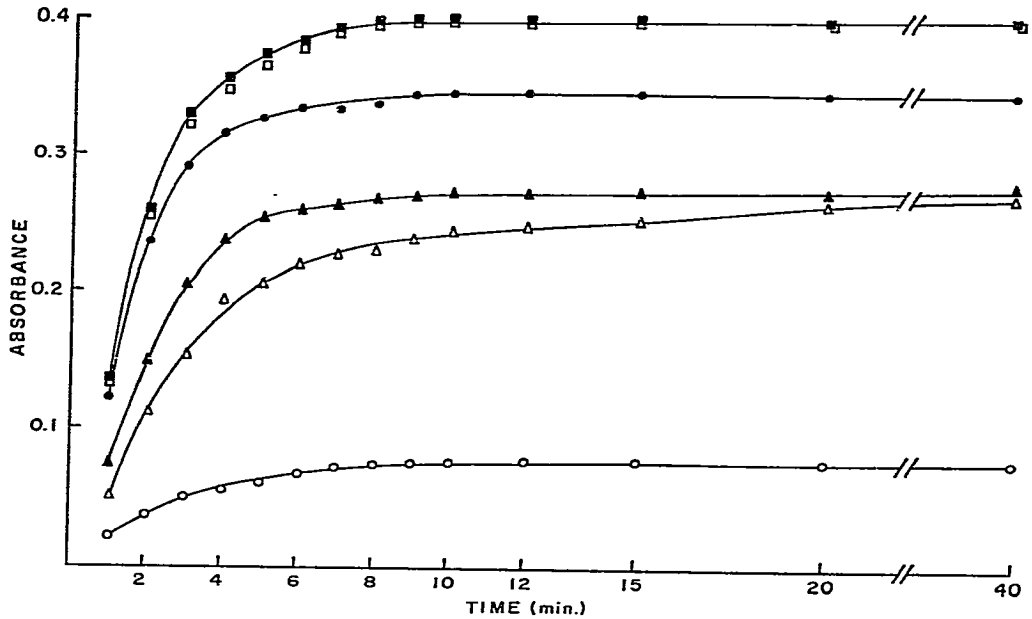

Fig. 4. Reversibility of the inhibition of con A by EDTA. [A solution (1.0 mg/mL) of con A in 0.1M sodium acetate–0.2M NaCl, pH 5.3, was incubated with 0.2 mM EDTA for 3 h at room temperature. Con A (2.0 mL) was then supplemented with metals, and mixed with glycogen in buffer (2.0 mL). The extent of complex-formation was measured turbidimetrically as a function of time. Control (no EDTA added), —●—; con A–EDTA (no metals added), —○—; con A–EDTA plus 50 mM Ca^{2+}, —△—; con A–EDTA plus 50 mM Mn^{2+}, —▲—; con A–EDTA plus 50 mM Ca^{2+} plus 50 mM Mn^{2+}, —□—; and control (no EDTA added) plus 50 mM Ca^{2+} plus 50 mM Mn^{2+}, —■—. It should be noted that the final concentration of EDTA after the addition of polysaccharide and metals was 0.1 mM.]

precipitates; this finding suggested that the metals could not be readily removed in pre-formed complexes. Accordingly, EDTA was added to tubes containing insoluble con A–glycogen complexes. At intervals, the precipitates were collected by centrifugation, washed, and analyzed for protein. The results, shown in Fig. 5, confirmed that EDTA is not effective in dissolving the complexes. After 20 h, ~70% of the protein had been solubilized; this contrasts with the results shown in Figs. 1–3, in which EDTA rapidly removed the ability of con A to bind glycogen at pH 5.3. A half-time of 0.7 h for dissolution of the lectin–glycogen complex would be expected, based on the determined rate of inactivation of 0.95 h^{-1} at pH 5.3. From Fig. 5, it may be seen that only ~6% of the complex was solubilized by EDTA at 0.7 h, emphasizing the protective effect of the polysaccharide on binding of metal.

The foregoing results may be summarized as follows: (*a*) EDTA readily inactivates con A at pH 5.3, but more slowly at lower hydrogen-ion concentrations; (*b*) the inactivation is a result of the loss of Ca^{2+} and of Mn^{2+} from the protein; (*c*) the inactivation is partially reversed by either Ca^{2+} or Mn^{2+}, and is fully reversed by both metal ions together; and (*d*) con A in pre-formed, con A–polysaccharide complexes is largely refractory to the action of EDTA.

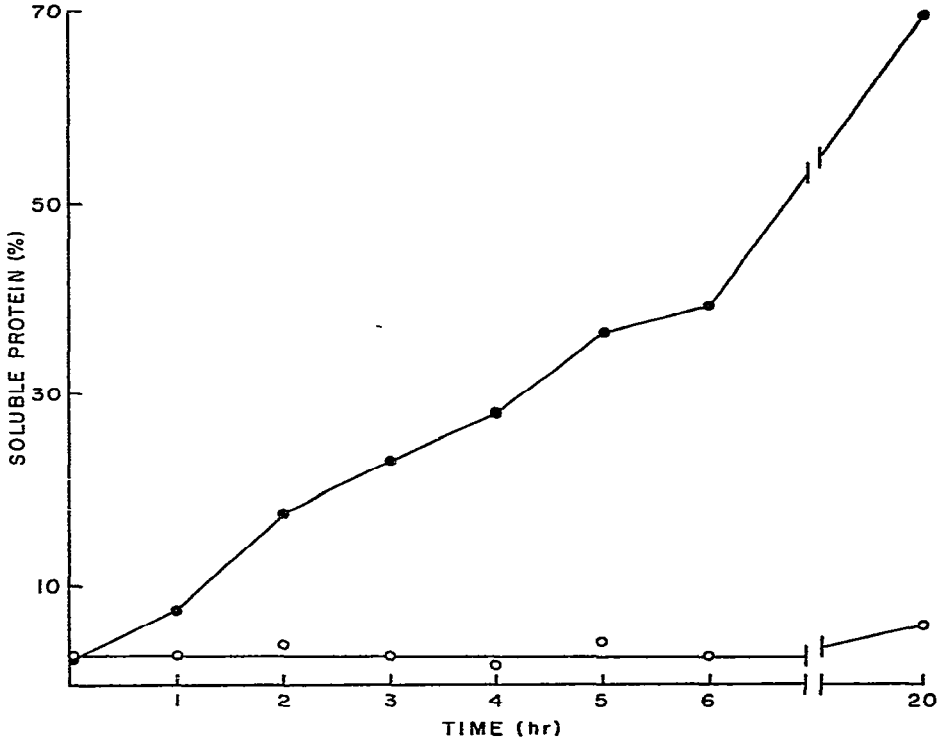

Fig. 5. Dissolution of con A–glycogen complexes by EDTA. [Con A (3.0 mg, 1.0 mL) and glycogen (2.0 mg, 2 mL) were incubated for 2 h at room temperature in 0.1M sodium acetate–0.2M NaCl, pH 5.3. Following incubation, 3.0 mL of buffer (—○—), or 100 mM EDTA (—●—), was added to the tubes. At the indicated times, samples were centrifuged, and the precipitates washed with 0.2M NaCl (10.0 mL). Finally, the precipitates were dissolved in 0.2M NaCl (10 mL) containing 20 μmol of methyl α-D-mannopyranoside, and analyzed for protein. At time zero, each tube contained 790 μg of protein in the precipitate.]

Sherry *et al.*[7] found that both Ca^{2+} and Mn^{2+} are removed from con A when the protein is incubated with EDTA at pH 5.6, although the removal is incomplete. These authors also showed that the association of Mn^{2+} for con A increases with decreasing hydrogen-ion concentrations. Thus, our observation that EDTA is less inhibitory at pH 7.3 than at pH 5.3 is probably due to the fact that con A binds metals more strongly at the higher pH.

The present data, and a previous study[12] from this laboratory, support the findings of Karlstam[13], who showed that "native" con A preparations lack stoichiometric amounts of Ca^{2+} and Mn^{2+}; thus, when Ca^{2+} and Mn^{2+} were added in excess to con A, the extent of interaction with glycogen was greater than that observed with the unsupplemented lectin (see Fig. 4).

The Mn^{2+} ion is "buried" in the interior of the con A molecule, but is exposed to solvent by a channel of ~600 pm cross section and 1.5 nm deep[14]. An EDTA molecule could be accommodated in a crevice of this size, and could interact with the

metal. Meirovitch and Kalb[15] and Koenig *et al.*[16] have shown that Mn^{2+} is readily accessible to solvent in solutions of con A. The Ca^{2+} ion is coordinated closer to the surface of the protein, and is probably susceptible to chelation by EDTA.

The finding that EDTA will not readily dissolve the lectin–glycogen complex (see Fig. 5) was unexpected; it is possible that, on binding with carbohydrate, the affinity of con A for Ca^{2+} and Mn^{2+} increases, as predicted by Hardman[3]. However, we have recently observed that site-saturating quantities of methyl α-D-mannopyranoside actually lower the affinity of con A for Ca^{2+} (unpublished results). A more plausible explanation is that EDTA is sterically excluded from the metal-binding sites when the lectin is complexed in an insoluble form with glycogen.

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