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Characterization of Two Plant Lectins from *Ricinus communis* and Their Quantitative Interaction with a Murine Lymphoma†

Garth L. Nicolson,* John Blaustein,‡ and Marilynn E. Etzler

ABSTRACT: Two plant lectins were isolated from *Ricinus communis* beans and purified by affinity chromatography on agarose gels using D-galactose or β-lactose in the elution buffer. The *R. communis* agglutinins were separated by gel filtration on Sephadex or Bio-Gel polyacrylamide columns into two peaks, RCA_I and RCA_{II}. RCA_I and RCA_{II} were judged to be highly purified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and their molecular weights were estimated to be 118,000–120,000 (RCA_I) and 60,000–65,000 (RCA_{II}). The molecular weight of RCA_I was confirmed by gel filtration on Sephadex G-200, but estimates of the molecular weight of RCA_{II} by gel filtration on Sephadex G-100 and Bio-Gel P-100 gave results varying from 36,000 to 50,000. The elution of RCA_{II} on Sephadex columns was affected by the presence of D-galactose suggesting that RCA_{II} interacts weakly with Sephadex. RCA_I and RCA_{II} possessed both similar and unique biochemical properties. They had similar amino acid compositions, tryptic fingerprints, and pH and temperature stabilities. In double diffusion experiments a partial spur of identity between RCA_I and RCA_{II} was formed with anti-RCA_I and with anti-RCA_{II}, indicating the two lectins share certain antigens and have some unique antigens in their structures. The specificities of RCA_I and RCA_{II} were determined by inhibition of quantitative precipitation of hog blood group A + H substance. RCA_I and RCA_{II} were inhibited by a number of different mono- and oligosaccharides similar to D-galactose, but RCA_I differed from RCA_{II} because it was not inhibited by *N*-acetyl-D-galactosamine, favored β-linked terminal oligosaccharides over α-linked oligosaccharides, and was inhibited better by L-rhamnose than D-galactose. RCA_I and RCA_{II} were broken down into subunits by β-mercaptoethanol and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, RCA_I into 29,500 and 37,000 molecular weight subunits and RCA_{II} into 29,500 and 34,000 molecular weight subunits, respectively. This suggests that these lectins may share one type of subunit and each have one unique type of subunit in an $\alpha_2\beta_2$ structure (RCA_I) and an $\alpha\beta'$ structure (RCA_{II}). The *R. communis* agglutinins were labeled with ^{125}I and their interaction with a murine lymphoma (S49) was compared. [^{125}I]RCA_{II} effectively competed with RCA_I for RCA_I binding sites on the cell surface, but [^{125}I]RCA_I could not completely compete with RCA_{II} for RCA_{II} binding sites, consistent with their known oligosaccharide binding specificities. Concanavalin A did not compete for RCA_I or RCA_{II} binding sites, but wheat germ agglutinin and *Dolichos biflorus* agglutinin competed somewhat for both RCA_I and RCA_{II} binding sites. This suggests a close steric relationship between these lectin binding sites on the S49 cell surface. [^{125}I]RCA_I bound rapidly to S49 cells at 4° and reached a plateau level in approximately 10 min. [^{125}I]RCA_I binding could not be blocked by the addition of α-methyl D-mannoside or *N*-acetyl-D-glucosamine to the labeling solution, but a β-lactose effectively inhibited binding. The concentration dependency of β-lactose inhibition of [^{125}I]RCA_I binding suggests that there are heterogeneous binding sites for RCA_I on S49 cells.

Since Stillmark's (1889) discovery that a hemagglutinin could be isolated from castor beans (*Ricinus communis*), plant lectins or agglutinins have been isolated with a wide variety of carbohydrate-binding specificities (reviews: Mäkelä, 1957; Boyd, 1963; Sharon and Lis, 1972). Although the *Ricinus communis* lectins were among the first isolated, these proteins were not considered interesting by hematologists because of their inability to discriminate between common blood group types in hemagglutination assays (Mäkelä, 1957; Boyd, 1963).

Recently the lectins of *R. communis* were isolated and purified by affinity chromatography on agarose gel columns (Nicolson and Blaustein, 1972; Tomita *et al.*, 1972) and shown to be quite different in molecular weight and specificity (Nicolson and Blaustein, 1972). One of the lectins (*ca.* 120,000 daltons) has been called *R. communis* hemagglutinin (Waldschmidt-Leitz and Keller, 1969, 1970; Drysdale *et al.*, 1968), RCA_{120} (Nicolson and Blaustein, 1972), or Ricin I (Tomita *et al.*, 1972), while the smaller lectin (*ca.* 60,000 daltons) has been called Ricin (Kabat *et al.*, 1947; Lin *et al.*, 1970b), Ricin D (Ishiguro *et al.*, 1964), *R. communis* toxin (Waldschmidt-Leitz and Keller, 1969, 1970; Drysdale *et al.*, 1968; Gürtler and Horstmann, 1973), RCA_{60} (Nicolson and Blaustein, 1972), or Ricin II (Tomita *et al.*, 1972). Although the smaller molecule has been called by a variety of names, it appears that they refer to the same protein (Gürtler and Horstmann, 1973). Ricin D (Ricin, RCA_{60}, Ricin II, or toxin) is extremely toxic to animals (Kabat *et al.*, 1947; Lin *et al.*, 1970b; Ishiguro *et al.*, 1964; Waldschmidt-Leitz and Keller, 1970; Nicolson and Blaustein, 1972). It suppresses the growth of ascites tumor cells (Lin *et al.*, 1970a,b) probably by inhibiting protein synthesis (Lin *et al.*, 1971, 1972; Olsnes and Pihl, 1972a,b).

Evidence will be presented here that supports earlier proposals that these agglutinins are chemically unique and inter-

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act with some similar and some different receptors on lymphoma cell surfaces. To alleviate confusion on the nomenclature of these molecules, they will be called RCA_I[1] (120,000 mol wt) and RCA_{II} (60,000 mol wt) (after Tomita *et al.*, 1972) from their elution off Sephadex G-100 or Bio-Gel P-150.

Materials and Methods

Methyl glycosides, β-lactose, iodoacetamide, *p*-chloromercuriphenylsulfonic acid, EDTA, and Tris-HCl were obtained from Sigma Chemical. Monosaccharides were obtained from Calbiochem and Pfanstiehl and oligosaccharides from Nutritional and Sigma Biochemicals. All inorganic chemicals and organic solvents were products of Baker or Mallinckrodt Chemical. Acrylamide was a product of Matheson Coleman and Bell. Ninhydrin and Coomassie Blue were purchased from Mann Research. Blue dextran and Sephadex G-100 and G-200 were obtained from Pharmacia, and Bio-Gel A-0.5m and P-100 were from Bio-Rad.

Hog blood group A + H substance was isolated from hog gastric mucin powder (Wilson Laboratories) as previously described (Kabat, 1956). Blood group A active and blood group H active pig submaxillary mucins (A^+-PSM and H-PSM) were a gift from Dr. Don M. Carlson of Case Western Reserve University.

Isolation and Purification. Procedures generally follow those of Nicolson and Blaustein (1972). Local wild *R. communis* beans (100 g) were blended and extracted in 0.2 M NaCl–0.005 M sodium phosphate buffer (pH 7.2) (PBS) for 2–3 hr. After filtering and centrifugation at 17,000*g* for 30 min, the clear supernatant was removed without disturbing the floating lipid layer and adjusted to 0.6 saturation with ammonium sulfate and left for 2–3 hr at 4°. After centrifugation the pellet was dissolved in PBS buffer, extensively dialyzed to remove ammonium sulfate, and applied to a 5 × 65 cm affinity column of Sepharose 4B or Bio-Gel A-0.5m agarose. After washing with 2–3 l. of PBS to remove unbound protein, the agglutinins were eluted with 0.2 M D-galactose or 0.2 M β-lactose in PBS (Nicolson and Blaustein, 1972). The two agglutinins eluted in one peak (containing approximately 500 mg of protein per 100 g of beans) off either of the agarose columns and were easily separated on a 4.5 × 60 cm Sephadex G-100, Bio-Gel P-100, or P-150 column by elution with PBS. The first peak (RCA_I) corresponded to a protein of approximate molecular weight 120,000, while the second peak (RCA_{II}) corresponded to a lower molecular weight protein (see Results).

Gel Electrophoresis. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate generally followed the procedures of Fairbanks *et al.* (1971). Gels were made according to Davis (1964) and Fairbanks *et al.* (1971) that were 5.6 or 7.0% acrylamide and 1% sodium dodecyl sulfate in 0.1 M Tris-HCl–0.2 M sodium acetate (pH 7.4). Some of the buffers contained 1% β-mercaptoethanol. Gels were electrophoresed at 8 mA per tube and stained with 0.25% Coomassie Brilliant Blue and destained electrophoretically in 7.5% acetic acid–5% methanol. The following proteins were used as molecular weight standards: human γ-globulin, ovalbumin, and bovine serum albumin from Miles Laboratories, trypsinogen and catalase from Worthington Biochemical, and cytochrome *c* from Sigma Chemical.

Molecular Weight Estimation by Gel Filtration. Several gel filtration systems were used to estimate the molecular weights of RCA_I and RCA_{II}: Sephadex G-200 (1.5 × 150 cm column), Sephadex G-100 (1.5 × 100 cm column), and Bio-Gel P-150 or P-100 (1.5 × 100 cm column). The columns were equilibrated with PBS or 0.14 M NaCl–0.01 M sodium phosphate (pH 7.4) (0.15PBS). The same standards as those used for gel electrophoresis were used for calibrating the columns.

Fingerprint Analysis. Preparations of each lectin (5 mg of RCA_{II}; 10 mg of RCA_I) were dialyzed and lyophilized. The samples were suspended in 8 M urea–0.5 M Tris–0.2 M β-mercaptoethanol for 1 hr at 37° to reduce disulfide bonds Aminoethylation was performed by the addition of ethylenimine (14 M) to create a greater number of cleavage sites for trypsin in the form of "pseudo-lysine" (Raftery and Cole, 1963). The samples were then dialyzed, first against distilled H_2O and then 0.01 M NH_4HCO_3, pH 8.2. They were then digested with trypsin (50 μg of trypsin per 5 mg of protein) overnight at 37°. After lyophilization, the samples were resuspended in a 1% NH_4OH solution and applied to the paper (Raftery and Cole, 1966).

The map was developed by first chromatographing against Weigert–Garren buffer (38% redistilled pyridine, 24% 1-butanol, and 8% glacial acetic acid) followed by two-dimensional electrophoresis at 3.5 kV for 3.6 hr in 10% redistilled pyridine–10% glacial acetic acid–20% 1-butanol buffer (pH 4.7). The samples were stained with a 1% solution of ninhydrin in acetone (Weigert and Garen, 1963).

Hemagglutination Assay. Hemagglutination was performed in microtiter test trays as previously described (Nicolson and Blaustein, 1972). Initial hemagglutination titers of RCA_I and RCA_{II} equal to 32–128 were serially diluted and mixed with equal volumes of a suspension of rabbit erythrocytes (2 × 10^8 cells/ml) and the agglutination in each well was scored after 45 min at 20°. Inhibition of hemagglutination was scored as the ratio of titers between experimental and control samples, if differences greater than one serial dilution (the approximate limit of accuracy of the assay) were obtained.

Amino Acid Analysis. Samples of purified RCA_I and RCA_{II} were hydrolyzed with 6 M HCl in sealed tubes under nitrogen for 24 and 48 hr at 110°. The hydrolyzed samples were analyzed on a Spinco-Beckman Model 120 amino acid analyzer. Total protein was determined by the Lowry (Lowry *et al.*, 1951) or ninhydrin procedures (Schiffman *et al.*, 1964). Sulfur-containing amino acids were oxidized prior to analysis by the performic acid technique of Hirs (1956). Tryptophan was not determined.

Immunochemistry. Antisera against RCA_I or RCA_{II} were produced in rabbits by several doses of the purified lectins in incomplete Freund's adjuvant (starting with 2 μg of antigen and working up to 100 μg of antigen). Each dose was administered in six–eight subcutaneous injections every other week. Rabbits were bled 1–3 weeks after the final injection.

Double immunodiffusion was performed by the method of Ouchterlony (1948) in 1% ionagar (Consolidated Laboratories, Inc.) in PBS containing β-lactose.

Quantitative precipitin analyses using RCA_I and RCA_{II} against hog blood group A + H substance were done by a microprecipitin technique (Kabat and Mayer, 1961). Tubes containing 250-μl final volume were incubated for 1 hr at 37° and kept for 48 hr at 20°. Total protein in each sample was

[1] Abbreviations used are: A^+-PSM, blood group A positive pig submaxillary mucin; Con A, concanavalin A; H-PSM, blood group H positive pig submaxillary mucin; PBS, 0.2 M sodium chloride–0.005 M sodium phosphate buffer (pH 7.2); 0.15PBS, 0.15 M sodium chloride–0.01 M sodium phosphate buffer (pH 7.4); RCA_I, *Ricinus communis* agglutinin of mol wt 120,000; RCA_{II}, *Ricinus communis* agglutinin of mol wt 60,000.

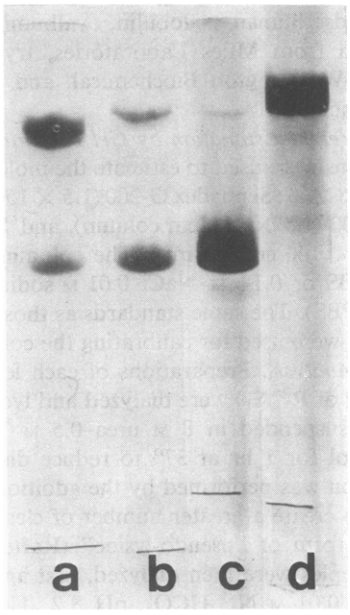

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of affinity-purified *R. communis* agglutinins: (a) lectins eluted off Bio-Gel A-0.5m with D-Gal; (b) lectins eluted off Bio-Gel A-0.5m with β-lactose; (c) peak II (RCA_{II}) off Sephadex G-100; (d) peak I (RCA_I) off Sephadex G-100.

determined by ninhydrin analysis (Schiffman *et al.*, 1964) of the washed, digested precipitates.

Cells. Rabbit blood was obtained by ear puncture and the red blood cells were separated and washed by centrifugation. Mouse S49.1TB.2 lymphoma (S49) cells were obtained from Dr. R. Hyman and were grown in Dulbecco's modified Eagles medium (Vogt and Dulbecco, 1963) supplemented with 10% calf serum (Hyman *et al.*, 1971).

Quantitative Labeling. RCA_I and RCA_{II} were labeled with iodine-125 using the iodine monochloride technique of McFarlane (1958). Six to ten milligrams of the lectin were iodinated in 0.1 M D-galactose–0.2 M glycine buffer (pH 8.0). The agglutinating activity of the ^{125}I-labeled lectins after extensive dialysis to remove D-galactose was unchanged from the initial titers. The specific activities of the ^{125}I-labeled proteins varied from 0.5 to 1 $\times 10^6$ cpm/μg of protein before dilution with unlabeled protein to the desired specific activity for each experiment.

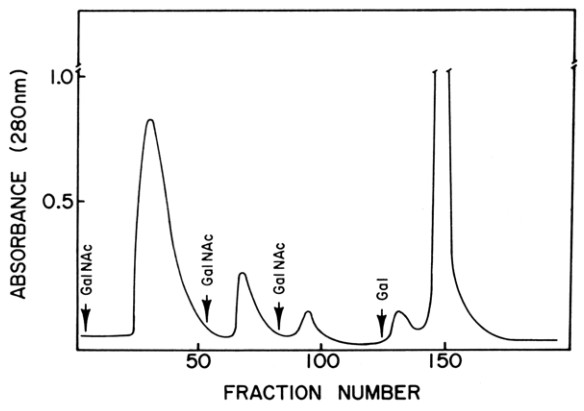

FIGURE 2: Affinity separation of *R. communis* agglutinins. A mixture of RCA_I and RCA_{II} was applied to a Sepharose-4B column and eluted by several additions of 0.01 M D-GalNAc to the chromatography buffer. When most of the D-GalNAc binding protein was eluted from the column, 0.01 M D-Gal was added to elute the remaining agarose-bound D-Gal binding lectin.

S49 cells at final concentrations of 6–8 $\times 10^6$ cells/ml were incubated for 10 min at 4° with a saturating concentration of $[^{125}I]RCA_I$ or $[^{125}I]RCA_{II}$ (usually 100 μg/ml for 10 min was saturating, *cf.* Nicolson, 1973) in a total volume of 0.25 ml. After the incubation, the cells were washed twice by centrifugation at 4°, and the final cell pellet was counted in a Packard γ scintillation counter. Controls were treated in the same manner except that the incubation and wash solutions contained, additionally, 0.05 M β-lactose or 0.05 M β-lactose plus 0.05 M *N*-acetyl-D-galactosamine for RCA_I and RCA_{II}, respectively. Specific binding was calculated as average radioactivity bound to quadruplicate cell samples, minus average radioactivity bound to triplicate cell samples with the saccharide inhibitor(s) present. Background labeling of $[^{125}I]RCA_I$ or $[^{125}I]RCA_{II}$ in the presence of saccharide inhibitors averaged 10% and was never more than 15% compared to the cell samples labeled without inhibitors present.

Results

Purification and Properties of RCA_I and RCA_{II}. In a previous paper we used an agarose gel as an absorbant for RCA_I and RCA_{II} and eluted the lectins with 0.2 M β-lactose or 0.2 M D-galactose (Nicolson and Blaustein, 1972). Separation on Bio-Gel P-100, P-150 or Sephadex G-100 or G-150 yielded peaks I (RCA_I) and II (RCA_{II}) which were judged to be highly pure on sodium dodecyl sulfate–polyacrylamide gels (Figure 1). These lectins could also be separated by their slightly different binding specificities on an agarose affinity column, because the smaller lectin (RCA_{II}) exclusively binds *N*-actyl-D-galactosamine (Nicolson and Blaustein, 1972) and can be selectively eluted with this sugar without eluting RCA_I. Subsequently, RCA_I can be eluted with D-galactose (Figure 2). The D-galactose eluted RCA_I was not contaminated with detectable amounts of RCA_{II} as shown by the lack of hemagglutination inhibition with *N*-acetyl-D-galactosamine. The lectins were quite stable after elution off the affinity column and aliquots were usually stored frozen until needed. After thawing and separation by gel filtration and dialysis, the lectins were stable at 4° for a few weeks; however, RCA_{II} tended to aggregate with time after 2 weeks at 4°.

Similarities and differences were found when the two lectins were characterized. The purified lectins have very similar, but distinctly different amino acid compositions (Table I). RCA_I has higher contents of lysine and valine, while RCA_{II} has more alanine and arginine. Fingerprints of the purified proteins were obtained by tryptic digestion followed by paper electrophoresis and two-dimensional chromatography. The separated peptides were identified by ultraviolet absorption and ninhydrin staining (Figure 3). The majority of the ninhydrin spots corresponded quite well; the ratio of identical to unique ninhydrin spots was approximately 1:1 for the two lectins. Three ultraviolet spots corresponded for the two fingerprints, with RCA_{II} possessing two additional ultraviolet spots. These results imply a close structural similarity between the lectins.

Antisera produced in rabbits against the affinity-purified RCA_I and RCA_{II} showed cross-reaction with the other lectin. In immunodiffusion (double diffusion) experiments in the presence of D-galactose a partial spur of identity was detected between RCA_I and RCA_{II} with both anti-RCA_I and anti-RCA_{II} (Figure 4). This indicates that these lectins share

TABLE I: Amino Acids Analysis of *R. communis* Agglutinins.

Amino Acid	Mol % Amino Acid[a]	
	RCA_I	RCA_{II}
Alanine	6.40	7.38
Arginine	5.31	6.25
Aspartic acid	11.88	12.09
Half-cystine	9.83	9.81
Glutamic acid	9.86	9.81
Glycine	7.02	7.01
Histidine	1.12	0.99
Isoleucine	6.70	6.30
Leucine	8.23	8.45
Lysine	2.34	1.74
Methionine	0.66	0.78
Phenylalanine	3.46	3.27
Proline	5.31	4.91
Serine	6.86	6.81
Threonine	7.38	6.95
Tryptophan	N.D.[b]	N.D.[b]
Tyrosine	2.30	2.68
Valine	6.12	5.59

[a] Average of four determinations. [b] Not determined.

certain antigens and also have some unique antigens in their structures.

Molecular Weights of RCA_I and RCA_{II} and Their Subunits. The molecular weights of RCA_I and RCA_{II} were determined by electrophoresis on 5.6 and 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate (Fairbanks *et al.*, 1971) and gel filtration on Sephadex and Bio-Gel polyacrylamide columns. The molecular weight estimates are summarized in Table II. RCA_I was determined to be of mol wt

TABLE II: Molecular Weight Determinations of *Ricinus communis* Agglutinins and Their Subunits.

Method	RCA_I	RCA_{II}
Sephadex G-200	118,000–120,000	36,500
Sephadex G-100		36,000
Sephadex G-100 + galactose		50,000
Bio-Gel P-100 + galactose		40,000
Sodium dodecyl sulfate–polyacrylamide gel (5.6%)	118,500	60,000–64,000
Sodium dodecyl sulfate–polyacrylamide gel (5.6%) in mercaptoethanol	37,000 and 29,500	34,000 and 29,500
Sodium dodecyl sulfate–polyacrylamide gel (7.0%)	120,000	65,000

~118,000–120,000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Sephadex G-200 gel filtration. The molecular weight determination of RCA_{II} was not consistent and varied between 36,000 and 65,000 daltons depending on the method used. Gel filtration on Sephadex was affected by the presence of the inhibitory monosaccharide D-galactose suggesting that RCA_{II} interacts weakly with Sephadex gels.

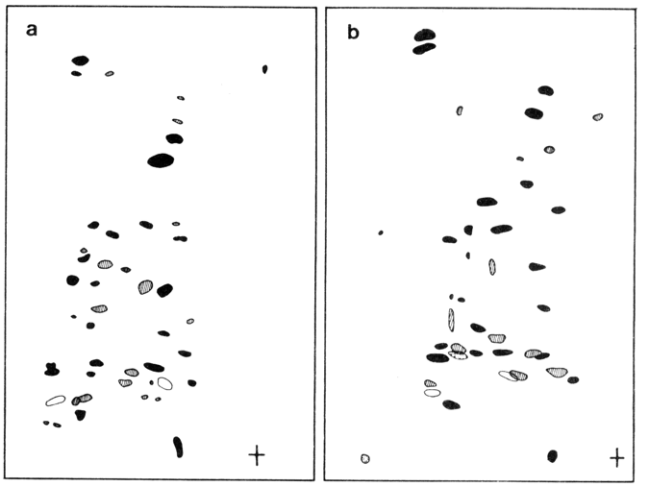

FIGURE 3: Tryptic fingerprint of *R. communis* agglutinins. Lectins were aminoethylated and digested with trypsin. Samples were applied to paper and chromatographed in one dimension and electrophoresed in the second dimension. Peptides were identified by ninhydrin staining and ultraviolet absorption: (a) RCA_I; (b) RCA_{II}; (■) identical ninhydrin-positive peptides; (▨) unique ninhydrin-positive peptides; (□) identical ultraviolet-positive peptides; (□) unique ultraviolet-positive peptides.

Electrophoresis on 5.6 or 7.5% sodium dodecyl sulfate–polyacrylamide gels reliably and consistently gave higher molecular weights for RCA_{II}, in the range of 60,000–65,000, consistent with the findings of others (Ishiguro *et al.*, 1964; Gürtler and Horstmann, 1973). When β-mercaptoethanol was included in the electrophoresis buffer, both of the lectins were broken down into smaller units with time. RCA_I yielded on sodium dodecyl sulfate–polyacrylamide gels components of mol wt 120,000, 90,000, 60,000, 37,000, and 29,500, respectively, within 5 min of β-mercaptoethanol treatment (Figure 5). Thirty minutes after β-mercaptoethanol addition, the 120,000 and 90,000 mol wt components disappeared with a concomitant increase in the 37,000 and 29,500 mol wt components. Further treatment with β-mercaptoethanol yielded only these latter two bands and a very faint band correspond-

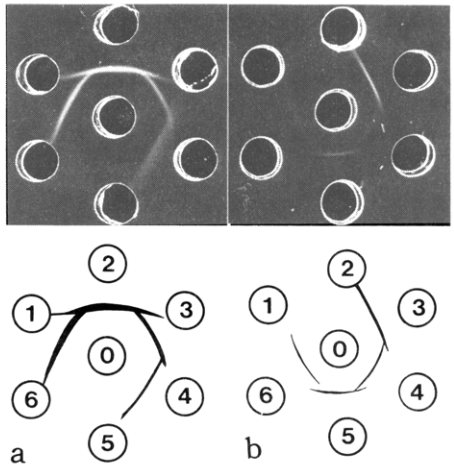

FIGURE 4: Immunodiffusion of *R. communis* agglutinins and anti-agglutinins in gels containing D-Gal: (a) anti-RCA_I in center well: 1, RCA_{II} (200 μg/ml); 2, RCA_I (200 μg/ml); 3, RCA_{II} (100 μg/ml); 4, RCA_I (100 μg/ml); 5, RCA_{II} (50 μg/ml); 6, RCA_I (50 μg/ml); (b) anti-RCA_{II} in center well: 1, RCA_{II} (200 μg/ml); 2, RCA_I (200 μg/ml); 3, RCA_{II} (100 μg/ml); 4, RCA_I (100 μg/ml); 5, RCA_{II} (50 μg/ml); 6, RCA_I (50 μg/ml).

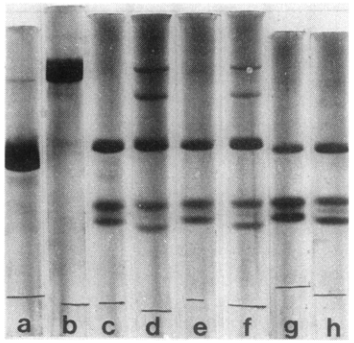

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of peptides formed by β-mercaptoethanol treatment of *R. communis* agglutinins: (a) untreated RCA_{II}; (b) untreated RCA_I; (c) RCA_{II} treated 5 min with β-mercaptoethanol; (d) RCA_I treated 5 min with β-mercaptoethanol; (e) RCA_{II} treated 15 min; (f) RCA_I treated 15 min; (g) RCA_{II} treated 60 min; (h) RCA_I treated 60 min.

ing to a 34,000 mol wt component (Figure 6a). When RCA_{II} was similarly treated with β-mercaptoethanol, the 60,000 mol wt component was reduced with the concomitant appearance of two bands of mol wt 34,000 and 29,500, respectively (Figure 6b). These lower molecular weight bands could not be further broken down in β-mercaptoethanol and represent the subunits of RCA_{II} (Figure 6).

These results suggest that each of the lectins may be composed of two types of subunits linked by S–S bridges and further that they appear to share one of these subunits, the 29,500 mol wt component. The fact that a faint band corresponding to the 34,000-dalton component was obtained when RCA_I was reduced and electrophoresed in sodium dodecyl sulfate (Figure 6a) could have been due to minor contamination of RCA_I by RCA_{II}. In fact, as mentioned above, purified RCA_{II} appears to aggregate into larger molecular weight components with time, initially to a 120,000 mol wt dimeric component.

Immunochemical Specificities. Each of the two lectins

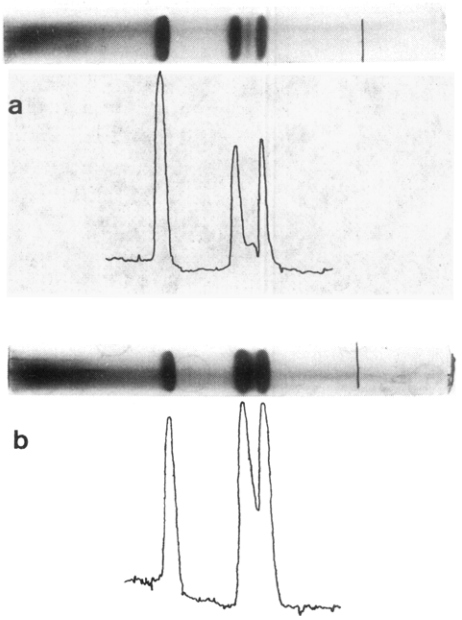

FIGURE 6: Subunits of *R. communis* agglutinins produced by β-mercaptoethanol treatment and analyzed on sodium dodecyl sulfate–polyacrylamide gels: (a) RCA_I; (b) RCA_{II}.

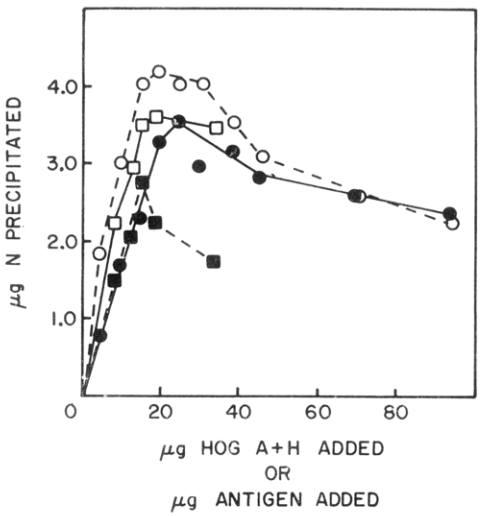

FIGURE 7: Quantitative precipitation of hog A + H blood group substance or hog submaxillary mucin substances (PSM) with *R. communis* agglutinins: (○- - -○) A + H blood group substances with RCA_I (4.77 μg of N/tube); (●—●) A + H blood group substances with RCA_{II} (5.48 μg of N/tube); (□—□) H-PSM with RCA_I + RCA_{II} = 6.44 μg of N/tube; of (■—■) A^+-PSM with RCA_I + RCA_{II} = 6.44 μg of N/tube.

precipitated with hog blood group A + H substance (Figure 7). A mixture of RCA_I plus RCA_{II} gave similar precipitin curves when combined with A^+-PSM or H-PSM (Figure 7), indicating the ability to precipitate is not due to the blood group A active determinant.

Various substances were tested for their ability to inhibit the precipitation of hog blood group A + H substance with the two *Ricinus communis* lectins. RCA_{II} was inhibited by a number of different monosaccharides including D-Gal, D-fucose, D-GalNAc, L-rhamnose, and L-arabinose (Figure 8A). RCA_I was not inhibited by D-GalNAc and also differed from RCA_{II} in that L-rhamnose was a better inhibitor than D-galactose (Figure 8B). The methyl α- and methyl β-D-galactopyranosides were about equal in their ability to inhibit RCA_{II}, whereas with RCA_I, the methyl β-D-galactopyranoside was more than a twofold better inhibitor than the methyl α-D-lactopyranoside. Melibiose and raffinose inhibited the precipitation of both *R. communis* lectins; however, their relative inhibitory abilities were different. Lactose was the best inhibitor of the substances tested for both lectins, in agreement with previous results (Nicolson and Blaustein, 1972).

Temperature and pH Stabilities. Temperature sensitivities of the two lectins were determined by incubating the proteins in PBS for 3 hr at 0, 5, 20, 37, 50, 65, 85, and 100° and then assaying hemagglutinating activity at 20°. Both lectins were stable to mild temperature denaturation and were about 50% inactivated at 65° and 100% inactivated at 85° in 3 hr.

The sensitivity of pH was determined by dialyzing (three changes) the lectins into 0.1 M buffers (Gomori, 1955) set to various pH values for 3 hr (20°) and then dialyzing back (three changes) into 0.15PBS. Hemagglutination titers indicated that both lectins were quite stable and lost no activity in the buffers set to pH values ranging from 3 to 9.

Quantitative Labeling to S49 Cells. Incubating S49 murine lymphoma cells for 10 min at 4° with concentrations of [^{125}I]RCA_I greater than 100 μg/ml resulted in maximal labeling with little detectable endocytosis of lectin molecules (Nicolson, 1973; Nicolson and Lacorbiere, 1973) and back-

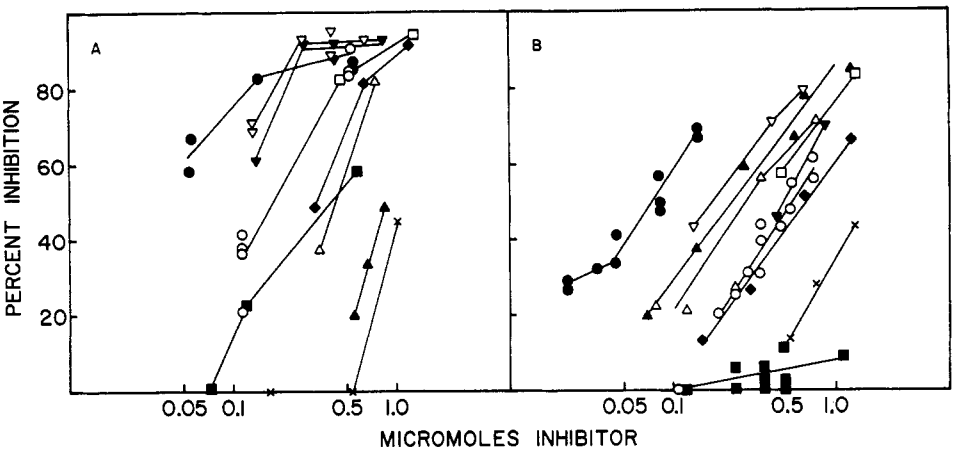

FIGURE 8: Inhibition by monosaccharides, methyl glycosides, and oligosaccharides of *R. communis* precipitation of hog A + H blood group substance: (A) hog A + H substance (19.85 μg) with RCA_{II} (6.67 μg of N); (B) hog A + H substance (15.01 μg) with RCA_I (4.95 μg of N): (●) β-lactose; (○) D-Gal; (▼) Me-α-D-Gal; (▽) Me-β-D-Gal; (▲) L-rhamnose; (△) raffinose; (◆) D-fucose; (□) melibiose; (■) D-GalNAc; (×) L-arabinose. No significant inhibition was obtained with the following compounds when tested up to the amount indicated: D-galactitol (1.37 μmol), D-glucose (1.34 μmol), L-glucose (1.4 μmol), D-lyxose (1.3 μmol), and D-galactosamine (0.41 μmol).

ground labeling of approximately 10% (Table III). Similar results were obtained when S49 cells were labeled with

TABLE III: Competition of *Ricinus communis* Agglutinins for Binding Sites on S49 Lymphoma Cells at 4°.

First Incubation[a]	Second Incubation[a]	cpm Bound (per 10^6 Cells)[b]
$[^{125}I]RCA_I$		11,170
$[^{125}I]RCA_I$ + β-lactose[c]		840
$[^{125}I]RCA_I$	RCA_{II}	11,770
RCA_{II}	$[^{125}I]RCA_I$	3,350
RCA_{II}	$[^{125}I]RCA_I$ + β-lactose[c]	1,050
$[^{125}I]RCA_{II}$		16,660
$[^{125}I]RCA_{II}$ + β-lactose[c]		1,320
RCA_I	$[^{125}I]RCA_{II}$	9,380

[a] Final concentration 100 μg/ml. [b] For each incubation 6×10^6 cells were incubated with the lectins (100 μg/ml) for 10 min at 4° and then washed twice. [c] Incubations contained 0.1 M β-lactose.

$[^{125}I]RCA_{II}$ (Table III). When S49 cells were prelabeled with saturating concentrations of RCA_{II} and then labeled with $[^{125}I]RCA_I$, the amount of cell bound radioactivity was dramatically reduced. This indicates that RCA_{II} can effectively compete for RCA_I binding sites on the S49 cell surface. When the labeling sequence was reversed and cells were saturated with RCA_I and then labeled with $[^{125}I]RCA_{II}$, a considerable amount of radioactivity was cell bound indicating RCA_I does not compete as effectively for RCA_{II} binding sites as the converse (Table III). Considering the broader binding specificity of RCA_{II}, this was the expected result.

The proximity of RCA_I and RCA_{II} binding sites to other lectin sites was investigated by first saturating cells with concanavalin A (inhibited by α-D-mannosyl-like residues [Agrawal and Goldstein, 1967; So and Goldstein, 1967]), wheat germ agglutinin (inhibited by *N*-acetyl-D-glucosamine-like residues [Burger, 1969] and sialic acid [Allen *et al.*, 1973]) or *Dolichos biflorus* agglutinin (inhibited by *N*-acetyl-D-galactosamine residues [Etzler and Kabat, 1970]) and then labeling the cells with either $[^{125}I]RCA_I$ or $[^{125}I]RCA_{II}$. Saturation with Con A did not inhibit the binding of either *R. communis* lectin, but saturation with wheat germ or *D. biflorus* agglutinin inhibited somewhat the binding of either lectin. $[^{125}I]RCA_{II}$ was inhibited more by *D. biflorus* agglutinin than $[^{125}I]RCA_I$ labeling, as expected (Table IV). These results indicate that Con A binding sites are distinct from RCA_I and RCA_{II} binding sites, while there appears to be some steric overlap with wheat germ and *D. biflorus* agglutinin binding sites. Some of the RCA_{II} binding sites are probably also *D. biflorus* binding sites on the S49 cell surface.

The binding sites for RCA_I on S49 cells appear to be heterogeneous. Labeling of these cells with $[^{125}I]RCA_I$ occurs very rapidly and reaches a plateau level in approximately 10 min at 4° (Figure 9). Inhibitory and noninhibitory saccharides were included in the $[^{125}I]RCA_I$ incubation media, and the amount of cell-bound lectin was determined after a 10-min incubation at 4°. *N*-Acetyl-D-glucosamine and α-methyl D-mannoside had very little effect on the binding of $[^{125}I]RCA_I$ over a broad concentration range, but β-lactose effectively inhibited cell binding of $[^{125}I]RCA_I$ (Figure 10). The non-

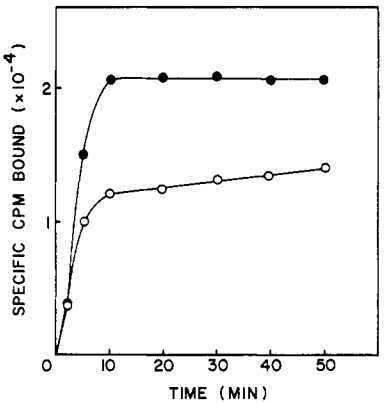

FIGURE 9: Time course of binding of $[^{125}I]RCA_I$ to 6×10^6 S49 lymphoma cells at 4°: (○) 40 μg/ml; (●) 100 μg/ml.

TABLE IV: Competition of Concanavalin A, *Dolichos biflorus*, and Wheat Germ Agglutinin for RCA_I and RCA_{II} Binding Sites on S49 Lymphoma Cells at 4°.

First Incubation			Second Incubation[b]	cpm Bound (per 10^6 Cells)
Lectin	Concn (μg/ml)	Inhibitor[a]		
			[^{125}I]RCA_I	40,200
		0.1 M β-lac	[^{125}I]RCA_I	5,280
			[^{125}I]RCA_{II}	36,500
		0.1 M β-lac	[^{125}I]RCA_{II}	4,870
Con A	200		[^{125}I]RCA_I	43,390
Con A	200		[^{125}I]RCA_{II}	35,330
Con A	200	0.1 M α-MeMann	[^{125}I]RCA_I	41,800
Con A	200	0.1 M α-MeMann	[^{125}I]RCA_{II}	35,900
Wheat germ agglutinin	100		[^{125}I]RCA_I	28,400
Wheat germ agglutinin	100		[^{125}I]RCA_{II}	32,200
Wheat germ agglutinin	100	0.1 M D-GNAc	[^{125}I]RCA_I	41,790
Wheat germ agglutinin	100	0.1 M D-GNAc	[^{125}I]RCA_{II}	37,800
D. biflorus agglutinin	200		[^{125}I]RCA_I	31,250
D. biflorus agglutinin	200		[^{125}I]RCA_{II}	20,900
D. biflorus agglutinin	200	0.1 M D-GalNAc	[^{125}I]RCA_I	39,150
D. biflorus agglutinin	200	0.1 M D-GalNAc	[^{125}I]RCA_{II}	37,380

[a] β-lac, β-lactose; α-MeMann, α-methyl D-mannoside; D-GNAc, *N*-acetyl-D-glucosamine; D-GalNAc, *N*-acetyl-D-galactosamine. [b] Final concentration 100 μg/ml.

linear inhibition of [^{125}I]RCA_I binding with β-lactose concentration suggests that heterogeneous RCA binding sites exist on S49 cells.

Discussion

Affinity chromatography on agarose gels was successfully used to purify two lectins from *R. communis* beans (Nicolson and Blaustein, 1972; Tomita *et al.*, 1972). Using similar procedures, agarose gels have been used to purify lectins from *Semen jequiriti* (Olsnes and Pihl, 1973), *Abrus precatorius*, and *Momordia charantia* (Tomita *et al.*, 1972), so its usefulness for D-galactose binding lectins is well established. There are several related reports indicating that lectins which bind D-glucose can be purified by affinity techniques on Sephadex (Agrawal and Goldstein, 1967; Howard and Sage, 1969; Entlicher *et al.*, 1970).

The two *R. communis* lectins were judged to be highly pure by several criteria and appear to be composed of one identical and one distinct type of subunit. The larger lectin (RCA_I, mol wt ~120,000) was broken down into two major peptides of approximate mol wt 29,500 and 37,000, respectively, by reduction with β-mercaptoethanol and denaturation with sodium dodecyl sulfate. The smaller lectin (RCA_{II}, mol wt ~60,000) yielded peptides of approximate mol wt 29,500 and 34,000, respectively, on β-mercaptoethanol–sodium dodecyl sulfate–polyacrylamide gels. Evidence in support of the possibility that RCA_I and RCA_{II} share one type of peptide, the 29,500 mol wt peptide, and have one unique type of peptide is that the molecules have similar amino acid compositions and saccharide-binding specificities, several identical tryptic peptides, and the purified lectins cross-react immunologically with antisera made against either purified lectin molecule. Similar results on the sodium dodecyl sulfate–polyacrylamide gel analysis and tryptic fingerprints of these proteins have been obtained recently by Gürtler and Horstmann (1973). From the molecular weights of the subunits obtained after reduction and electrophoresis on sodium dodecyl sulfate–polyacrylamide gels, we propose that RCA_I is probably a tetramer of the $\alpha_2\beta_2$ type and RCA_{II} is a dimer of the $\alpha\beta'$ type. A discrepancy in this proposal is that the molecular weight determinations of intact RCA_{II} were somewhat variable, depending on the method used (see Table II). On 5.6 or 7.0% sodium dodecyl sulfate–polyacrylamide gels we obtained a molecular weight of 60,000–65,000, while on Sephadex G-100 or Bio-Gel P-100 we obtained molecular weights from 36,000 to 50,000. However, the lower molecular weights of RCA_{II} obtained on these columns were due, in part, to the interaction of the lectin with the gels. This was partially overcome by addition of D-galactose to the elution buffer, so we place more value on the subunit molecular weight determinations made by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Since the lectins are glycoproteins and certain glycoproteins have been shown to exhibit anomalous migration in sodium dodecyl sulfate–polyacrylamide gels (Glossmann and Neville, 1971; Segrest and

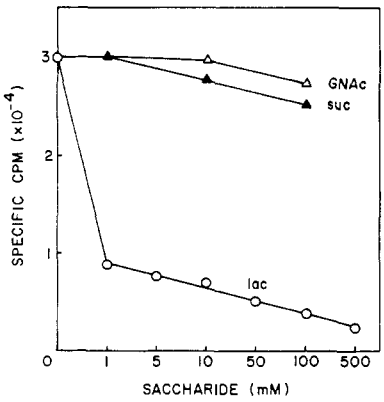

FIGURE 10: Inhibition of binding of [^{125}I]RCA_I (100 μg/ml) to 6 × 10^6 S49 lymphoma cells by various saccharides: (○) β-lactose; (▲) sucrose; (△) D-GalNAc.

Jackson, 1973), we examined the migration of RCA_I and RCA_{II} in 5.6 and 7.0% polyacrylamide gels. There were only slight changes in the calculated molecular weights of these proteins; thus, the carbohydrate moieties do not significantly interfere with the electrophoretic migration of RCA_I and RCA_{II}, and the molecular weights of 118,000–120,000 and 60,000–65,000 for RCA_I and RCA_{II} are probably fairly accurate.

In a previous study using hemagglutination inlhibition, we found that RCA_I and RCA_{II} were inhibited by saccharides containing terminal nonreducing D-galactose residues, but RCA_{II} was additionally inhibited by *N*-acetyl-D-galactosamine (Nicolson and Blaustein, 1972). Quantitative precipitation experiments with hog blood group A + H substance confirmed this difference in specificity. Also, RCA_{II} was found to have a wider range of effective saccharide binding specificities than RCA_I. RCA_I preferred α over β linkages (methyl α- compared to methyl β-D-galactopyranosides) greater than 2:1, but RCA_{II} was inhibited equally by saccharides with α or β linkages. It was reported by LeBreton and Moule (1949) that RCA_I contains a protease activity. We could not demonstrate proteolytic activity in either purified *R. communis* lectin, nor did we find breakdown products from the lectins upon standing for 1 month at 5°. In order to check this more carefully, samples of RCA_I and RCA_{II} were sealed and incubated for 1–2 days at 37°. At that time they were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. No degradation products were observed in the gel samples.

Saccharides with the arrangement of hydroxyl groups at the C-2, C-3 and C-4 positions similar to D-galactose were all strong inhibitors of RCA_I and RCA_{II}. These findings are in general agreement with the hemagglutination inhibition data of Drysdale *et al.* (1968) using partially purified *R. communis* extracts. We found that L-rhamnose was a strong inhibitor of *R. communis* lectins, and a better RCA_I inhibitor than D-galactose. The stereochemical similarity between L-rhamnose and D-galactose is not immediately obvious; however, if L-rhamnose is inverted and the C-4 position aligned with the C-2 position of D-galactose, the same hydroxyl configurations are obtained at the C-2, C-3, and C-4 positions. From the inhibition data it is assumed that these are structurally the most critical positions for successful saccharide binding.

The difference in saccharide-binding specificities between RCA_I and RCA_{II} explains the differences in competition for binding sites on a murine lymphoma cell line. RCA_{II} could effectively compete for RCA_I binding sites, but the converse was not entirely possible. Concanavalin A binding sites were found to be distinct from the binding sites for the *R. communis* lectins, but two other lectins (wheat germ and *D. biflorus* agglutinins) partially blocked *R. communis* agglutinin binding sites. This is similar to the finding with SV40-transformed 3T3 cells where the sites for Con A and wheat germ agglutinin were found to be distinct by competition experiments (Ozanne and Sambrook, 1971).

The lectins from *R. communis* have been used in a variety of useful tasks as molecular probes for D-galactose-like residues in complex cellular carbohydrates. RCA_I has been used to analyze cell surfaces for quantitative changes in D-galactose-like residues during growth and cell contact of normal and transformed tissue culture cells (Nicolson and Lacorbiere, 1973) and after trypsin and neuraminidase treatment of cells (Nicolson, 1973). Ferritin conjugates of RCA_I have been used to study the distribution and asymmetry of oligosaccharides on plasma membranes (Nicolson and Singer, 1971, 1973; Nicolson, 1972) and other cell membranes as well (Hirano *et al.*, 1972).

R. communis binding sites have been found on a variety of cell types (Pardoe *et al.*, 1969; Drysdale *et al.*, 1968; Tomita *et al.*, 1970; Nicolson and Yanagimachi, 1972; Uhlenbruck and Herrmann, 1972; Moscona, 1971; Nicolson, 1973) and also on cell organelles (Nicolson *et al.*, 1972; Hirano *et al.*, 1972; Henning and Uhlenbruck, 1973).

One of the more interesting properties of RCA_{II} is its extreme toxicity (Stillmark, 1889; Kabat *et al.*, 1947; Ishiguro *et al.*, 1964; Lin *et al.*, 1970a,b; Waldschmidt-Leitz and Keller, 1969, 1970; Osborne *et al.*, 1905; Ralph and Nakoinz, 1973). Recently Lin and his collaborators have used an *R. communis* protein (RCA_{II}) to inhibit the *in vivo* growth of Ehrlich ascites tumor cells (Lin *et al.*, 1970a). Lin *et al.* (1971, 1972) and Olsnes and Phil (1972a,b) found that RCA_{II} strongly suppressed cell protein synthesis by interfering with the completion of ribosome-bound initiated peptide chains similar to the action of diphtheria toxin (Olsnes, 1972). RCA_I appears to be only a few per cent as active as RCA_{II} in suppressing cell-free protein synthesis (G. L. Nicolson, M. Lacorbiere, and A. Hunter, manuscript in preparation); thus, the ability to block ribosome peptide chain elongation may be conferred by the unique 34,000 mol wt β' subunit of RCA_{II}. Whether RCA_{II} exerts its toxic effect on cells by binding to the plasma membrane and mediating a trans membrane effect across the membrane similar to the action of hormones or by a two-step process which requires binding to the cell membrane as a first step to being transported inside the cell where the toxic effects are directly upon a ribosome–protein synthesis system remains to be elucidated, although we have recently found that the time required for the maximal binding of RCA_{II} to the cell surface (~5 min) does not correlate with the time required to inhibit protein synthesis (~30–60 min). This latter time correlates well with the time required for binding, clustering, endocytosis, and subsequent release into the cell cytoplasm of ferritin-conjugated RCA_{II} (G. L. Nicolson, M. Lacorbiere, and A. Hunter, manuscript in preparation). These findings suggest that RCA_{II} acts directly on cell protein synthesis after being transported inside cells.

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