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Different Biological Properties of the Two Constituent Peptide Chains of Ricin, a Toxic Protein Inhibiting Protein Synthesis[†]

Sjur Olsnes and Alexander Pihl*

ABSTRACT: The toxic protein ricin was purified to homogeneity and its constituent peptide chains were isolated. The purification involved extraction from castor beans and chromatography on a CM-52 column and subsequently on a Sepharose-4B column. The LD_{50} dose in mice was 0.2 μg. The isolated toxin had only a slight hemagglutinating activity. After reduction of the toxin with β-mercaptoethanol in the presence of galactose the two constituent chains were isolated by chromatography on a DE-52 and a CM-52 column. The smaller peptide chain (the A chain) strongly inhibited protein synthesis in a cell-free system from rabbit reticulocytes, whereas the larger peptide (the B chain) lacked this ability. Human erythrocytes pretreated with intact ricin or with B chain were agglutinated by an antiserum directed specifically against ricin, whereas erythrocytes pretreated with A chain were not agglutinated under the same conditions. Only the B chain was bound to a Sepharose column, a binding which could be abolished by the presence of galactose. The results indicate that the toxic action of ricin is associated with the A chain and that the B chain functions as a carrier moiety which binds the toxin to the cell surface, a binding which probably involves galactose-containing receptors.

Ricin, a highly toxic protein present in castor beans, inhibits protein synthesis in a cell-free system even when it is present in extremely small concentrations (Olsnes and Pihl, 1972a). As previously reported ricin consists of two peptide chains held together by disulfide bonds (Olsnes and Pihl, 1972b; Olsnes, 1972). Recently, we have shown that after treatment with β-mercaptoethanol the ability of ricin to inhibit protein synthesis in a cell-free system is increased 50- to 100-fold, whereas its toxic effect in living animals and in cells in culture is virtually abolished (Olsnes and Pihl, 1972c), suggesting that the two chains have different functions. Similar findings were obtained with abrin, a toxic protein present in the seeds of *Abrus precatorius L*, an entirely different plant (Olsnes and Pihl, 1972c,d).

In the present paper we have described the purification of ricin to homogeneity, the separation of its constituent peptide chains, and the study of their properties. Evidence is presented that, as in the case of abrin, the smaller peptide chain inhibits protein synthesis in a cell-free system, whereas the larger chain appears to bind the toxin to the cell surface.

Materials and Methods

Materials. Castor beans (the seeds of *Ricinus communis L.*)
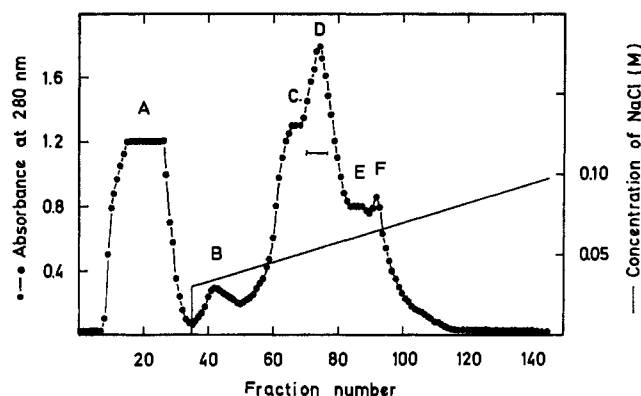
FIGURE 1: CM-cellulose chromatography of proteins extracted from castor beans. Crude ricin (300 ml), prepared as described under Materials and Methods, was applied to a 1.6 × 40 cm column of CM-52 (Whatman) equilibrated with 5 mM sodium phosphate (pH 6.5) and eluted at a speed of 80 ml/hr with a 1600-ml linear NaCl gradient (from 30 to 100 mM) in the same buffer.

TABLE I: Toxicity in Mice and Hemagglutinating Activity of Different Ricin Fractions.

Purification Step	Fraction No.	LD_{50} (μg of Protein)[a]	Hemag-glutinating Act. (Units/ μg of Protein)[b]
CM-52 chromatography (Figure 1)	20 (A)	3.0	0.1
	42 (B)	0.8	0.5
	65 (C)	0.3	1
	73 (D)	0.2	3
	86 (E)	0.6	100
	92 (F)	3.0	100
Sepharose 4B chromatography (Figure 2)	11 (G)	>10.0	0
	29 (H)	0.2	2

[a] Increasing amounts of protein were injected intraperitoneally into groups of mice weighing 25 ± 3 g and the LD_{50} was calculated according to Litchfield and Wilcoxon (1949). Only deaths occurring within 72 hr were recorded. [b] One unit represents the amount necessary to give visible agglutination in 1 ml of suspension of erythrocytes as described earlier (Olsnes and Pihl, 1973).

were kindly provided by Deutsche Rizinus-Oelfabrik Boley & Co., Krefeld-Uerdingen, West Germany. CM-52 and DE-52 cellulose (Whatman) were obtained from W.&R. Balston Ltd., Springfield Mill, Maidston, Kent, England. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. [^{14}C]Leucine (uniformly labeled, specific activity 331 Ci/mol) was obtained from The Radiochemical Centre, Amersham, England.

Extraction of Ricin. Decorticated castor beans (100 g) were defatted by grinding in 100 ml of ether and centrifuged at 3000*g* for 10 min. The supernatant was discarded and the pellet was resuspended and ground once more in 100 ml of ether. This procedure was repeated four times. The final pellet was suspended in 400 ml of distilled water and the pH was adjusted to 4.0 by adding dilute acetic acid. The suspension was homogenized at maximum speed in an Omnimixer (Sorvall) at 0° in ten sequences of 1 min each with 30-min intervals. The homogenate was then centrifuged at 8000*g* for 10 min and the pellet was discarded. The supernatant was dialyzed for 2 days against distilled water and then against 10 mM Tris-HCl (pH 7.7) for 24 hr. The dialyzed material was centrifuged at 8000*g* for 20 min, the pellet was discarded, and the supernatant was centrifuged once more in the same way. The final supernatant is referred to below as crude ricin.

Polyacrylamide Gel Electrophoresis. A solution containing 0.5–1.0 mg/ml of protein was made up to contain 1% sodium dodecyl sulfate and 0.1 M sodium phosphate (pH 7.1). Sucrose was added to a final concentration of 0.1 M and then 10–50 μl was layered on to polyacrylamide gels (13% acrylamide–0.2% dimethyl bisacrylamide) and the electrophoresis was carried out as described by Weber and Osborne (1969). In some instances the proteins were treated with 1% β-mercaptoethanol at room temperature for 1 hr before the electrophoresis. Destaining and molecular weight determinations were carried out as described earlier (Olsnes, 1971).

Preparation of Antiserum Against Ricin. The procedure was the same as that used for the preparation of an antiserum against abrin (Olsnes and Pihl, 1973). Due to the high toxicity of the intact ricin, the immunization of rabbits was started with a toxoid prepared by formaldehyde treatment of ricin. Booster doses of toxin were given after 3 and 6 weeks, and the rabbits were bled one week after the last injection. Before use the serum was incubated at 56° for 30 min to inactivate complement and then treated with human erythrocytes

to adsorb antibodies directed against human red blood cells as earlier described (Olsnes and Pihl, 1973).

Cell-Free Protein Synthesis. Rabbits were made anemic with phenylhydrazine; the red cells consisting mainly of reticulocytes were collected, washed with isotonic buffer, and lysed with distilled water containing 10^{-5} M hemin. Stroma-free lysate (200 μl) was supplemented with [^{14}C]leucine and the other 19 unlabeled amino acids and an energy donating system, in a final volume of 0.5 ml as described in the figure legends. The procedure is essentially that described by Lingrel (1972). The system was incubated at 30°. Samples (10 μl) were removed after different periods of time and poured into 1 ml of 0.1 N KOH and incubated at room temperature for 60 min. Trichloroacetic acid was then added to a final concentration of 10% (w/v) and the heme was converted into a colorless compound by the addition of 1% H_2O_2. The precipitated protein was collected on Gelman glass fiber filters type A, 5 ml of scintillation solution was added, and the radioactivity was counted in a Beckman LS-130 scintillation system as previously described (Olsnes and Pihl, 1972a).

Results

Isolation of Ricin. The crude ricin obtained after extraction and dialysis (see Materials and Methods) was first purified by chromatography on a CM-cellulose column. As shown in Figure 1 most of the applied protein was adsorbed to the column under the conditions used (5 mM sodium phosphate, pH 6.5) and was eluted by a linear NaCl gradient. The different peaks as well as the unadsorbed material were tested for toxicity in mice. The results in Table I show that the most toxic material was present in peak D.

The toxin was further purified by affinity chromatography on a Sepharose column (Nicolson and Blaustein, 1972; Olsnes and Pihl, 1973). It is apparent (Figure 2) that in the absence of galactose, only small amounts (fraction G) of the protein applied passed through the Sepharose column. After elution
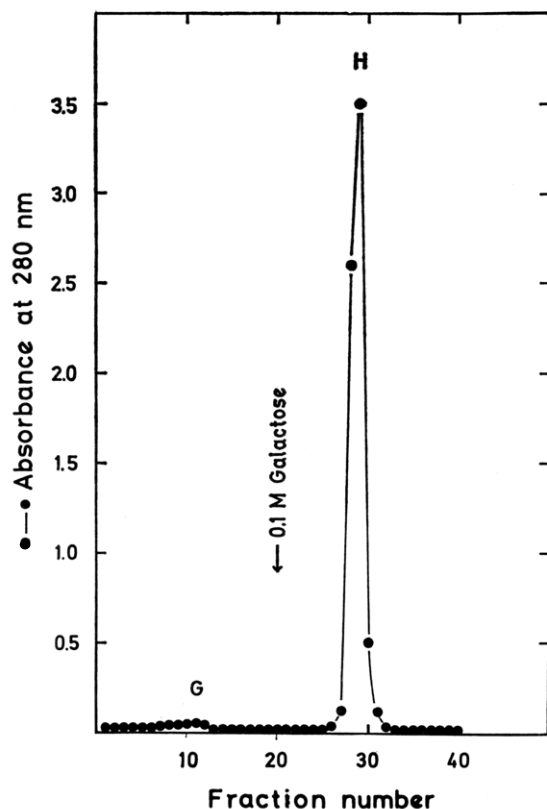
FIGURE 2: Affinity chromatography of ricin on a Sepharose 4B column. The fractions from peak D (Figure 1), indicated with a bar, were pooled and applied to a 1.6 × 20 cm column of Sepharose 4B equilibrated with 10 mM Tris-HCl (pH 7.7). The column was washed with 100 ml of the same buffer and the toxin was eluted with 0.1 M D(+)-galactose in 10 mM Tris-HCl (pH 7.7) at a speed of 40 ml/hr.
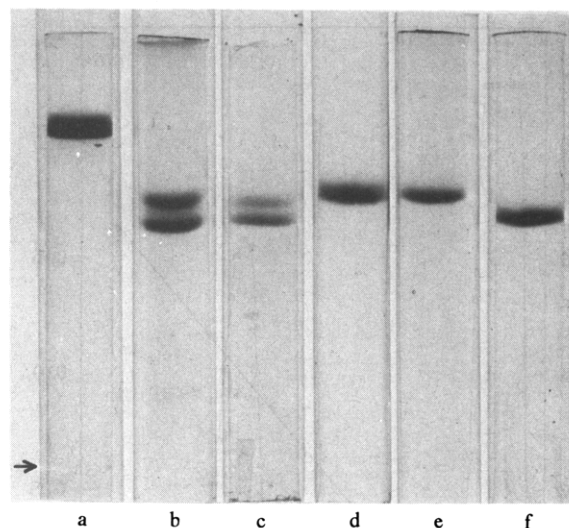

FIGURE 3: Polyacrylamide gel electrophoresis of ricin and its constituent peptide chains. Different protein fractions were analyzed by electrophoresis in 13% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate: (a) ricin (Figure 2, fraction H); (b) ricin, treated with 1% β-mercaptoethanol; (c) fraction I in Figure 5; (d) fraction J in Figure 5; (e) fraction K in Figure 6; (f) fraction L in Figure 6. The arrow marks the position of the tracking dye (Bromophenol Blue) at the end of the electrophoresis.

with 0.1 M galactose a sharp protein peak appeared. Table I shows that the material which passed through the column (fraction G) was virtually nontoxic in mice, whereas the toxicity of the material eluted with galactose (fraction H) corresponded to the LD_{50} dose of 0.2 µg/25-g mouse. This value is close to that reported by Ishiguro et al. (1964), who purified ricin by a more complicated procedure.

The purity of the toxin was studied by electrophoresis in polyacrylamide gels. As shown in Figure 3A the material eluted with galactose from the Sepharose column (Figure 2) gave one main band and two faster moving faint bands. When the toxin preparation was treated with 1% β-mercaptoethanol it was quantitatively converted into two protein bands. From the migration rates relative to those of known markers, the molecular weights of the two peptide chains were estimated to be 32,000 and 34,000. The migration rates were the same as those of the faint bands observed in Figure 3A. The results indicate that fraction H eluted from the Sepharose column contained pure ricin, contaminated with slight amounts of its constituent peptide chains.

The different fractions were then examined with respect to their ability to inhibit protein synthesis. Protein (10 ng) was added to a cell-free system prepared from an unfractionated reticulocyte lysate, and the incorporation of [14C]leucine into protein was measured. The fractions were pretreated with β-mercaptoethanol, since this strongly increases the ability of ricin to inhibit protein synthesis in a cell-free system (Olsnes and Pihl, 1972c). The data in Figure 4 show that the protein in fractions D and H had the highest ability to inhibit protein
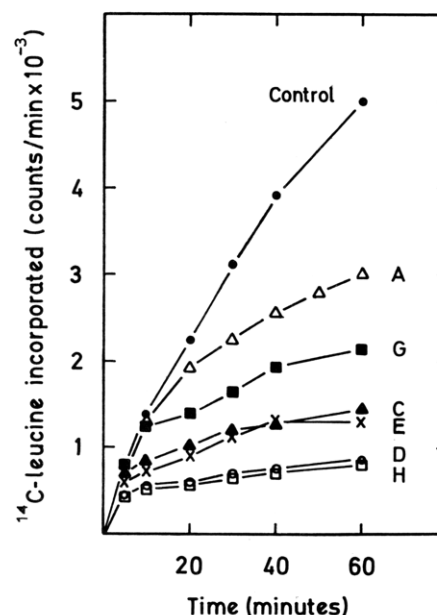

FIGURE 4: Effect of different protein fractions on protein synthesis in a cell-free system from rabbit reticulocytes. The fractions indicated with capital letters in Figures 1 and 2 were diluted in 10 mM Tris-HCl (pH 7.7) to a protein content of 100 µg/ml. Then β-mercaptoethanol was added to a final concentration of 1% and the samples were incubated at room temperature for 30 min. Subsequent dilutions were made in 10 mM Tris-HCl (pH 7.5) containing 10 µg/ml of bovine serum albumin. Protein (10 ng) was then added to a cell-free system containing, in a final volume of 0.5 ml, 200 µl of lysate (30 mg of protein), 10 mM Tris-HCl (pH 7.4), 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 15 µg/ml of creatine phosphokinase, and amino acids (except leucine) in concentrations varying from 0.01 to 0.1 mM and 1.25 µCi of [14C]leucine. The incubation was carried out at 28°. Samples (10 µl) were removed from each tube as indicated and the acid-precipitable radioactivity was measured as described under Materials and Methods: (△) A, (Figure 1, fraction 20; (▲) C, Figure 1, fraction 65; (O) D, Figure 1, fraction 73; (X) E, Figure 1, fraction 86; (■) G, Figure 2, fraction 11; (□) H, Figure 2, fraction 29; (●) control.
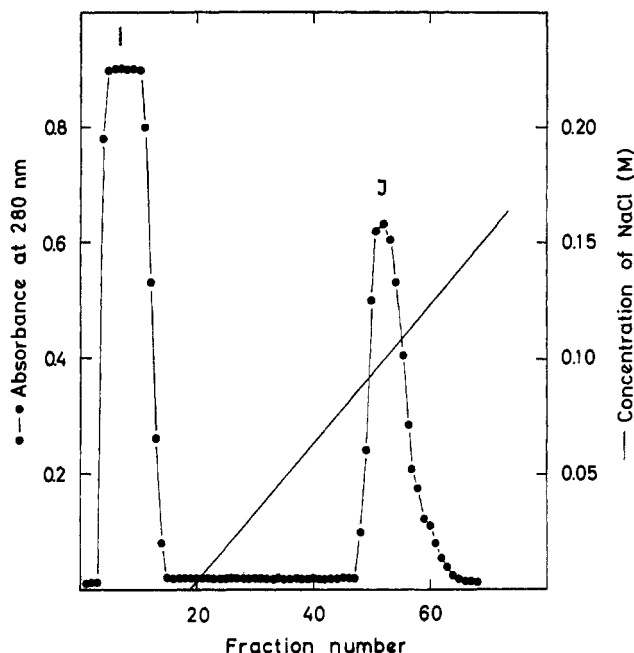
FIGURE 5: Chromatography of β-mercaptoethanol-treated ricin on a DEAE-cellulose column. A solution of ricin (fraction H, Figure 2) was made up to contain 0.5 M galactose and 5% β-mercaptoethanol. The preparation was incubated at room temperature overnight and then chilled to 0° and the pH was adjusted to 8.5 with 0.1 M Tris. The material was applied to a DE-52 column, equilibrated with 0.1 M Tris-HCl (pH 8.5), and eluted with an 800-ml linear NaCl gradient (from 0 to 0.15 M NaCl) in the same buffer. Due to the high absorbance of mercaptoethanol in some fractions the absorbance of the protein in the fractions was measured after precipitation in the presence of 10% w/v trichloroacetic acid. The precipitate was dissolved in 1 ml of 0.1 N NaOH and the absorbance at 280 nm was measured.

synthesis. The same fractions showed the highest toxicity in mice (Table I). Thus, the ability of ricin preparations to inhibit protein synthesis in a cell-free system correlated well with
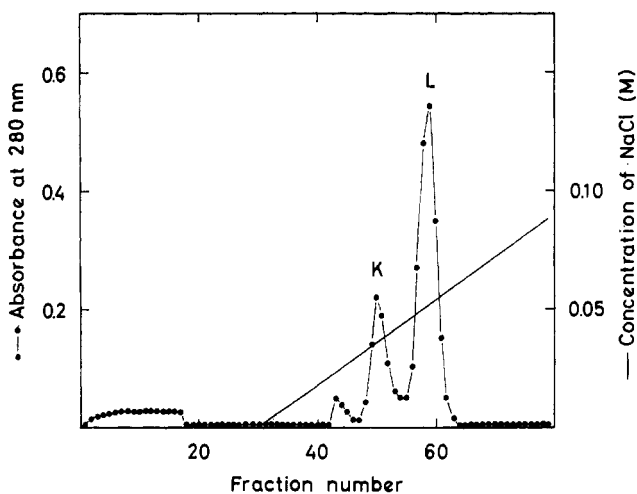

FIGURE 6: Carboxymethylcellulose chromatography of the partially purified A chain. The material that did not bind to the DE-52 column (fractions 4–14 in Figure 5) was pooled and dialyzed against 5 mM sodium phosphate (pH 6.5) containing 0.1% β-mercaptoethanol and 10 mM galactose. After dialysis the material was applied to a 0.8 × 20 cm CM-52 column, equilibrated with 5 mM sodium phosphate (pH 6.5) containing 0.1% β-mercaptoethanol, and then eluted with an 800-ml linear NaCl gradient (from 0 to 0.1 M NaCl) in the same buffer.
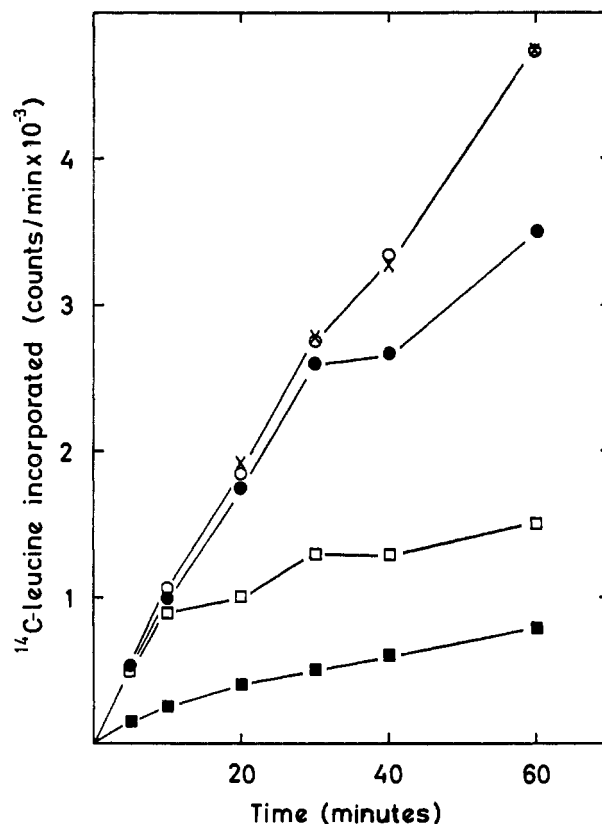
FIGURE 7: Inhibitory effect on protein synthesis of the isolated peptide chains of ricin. The cell-free system was prepared and the protein synthesis measured as in Figure 4: (□) 1 ng of A chain added (fraction L in Figure 6); (■) 10 ng of A chain added; (O) 10 ng of B chain added (fraction J in Figure 5); (●) 100 ng of B chain added, (X) control.

their toxicity in mice. It is seen (Figure 4) that fraction G had a moderate inhibiting effect on protein synthesis. This is probably due to the presence of small amounts of A chain (see below).

Some authors have attributed the toxicity of ricin to the hemagglutinating activity found in many preparations (Onozaki et al., 1972). The data in Table I clearly show, however, that the most toxic fractions had a relatively low hemagglutinating activity and, conversely, that fraction F, which had a high hemagglutinating activity, possessed only 7–8% of the toxic activity of fraction D. The results strongly indicate that the toxicity of ricin is not caused by cell agglutination in accordance with the findings of Ishiguro et al. (1964).

Isolation of the Two Peptide Chains of Ricin. The larger peptide chain of ricin (the B chain) was found to be rather unstable and it rapidly precipitated at room temperature. In the presence of galactose, however, the precipitation of the B chain was strongly retarded. In the purification of the two peptide chains advantage was taken of this finding. After treatment of ricin with β-mercaptoethanol in the presence of galactose, the mixture was applied to a DE-52 column. Part of the protein passed through the column (Figure 5, fraction I) and the protein which remained bound was eluted (fraction J) with a linear NaCl gradient. When analyzed by polyacrylamide gel electrophoresis fraction I was found to consist mainly of A chain, although some material moving at the same rate as the B chain was also present (Figure 3c). The material which did bind to the column and was eluted in peak J consisted of pure B chain (Figure 3d).

In the further purification of the A chain, fraction I, which did not bind to the DE-52 column (Figure 5), was applied to a CM-52 column and eluted with a linear NaCl gradient (Figure 6). Two peaks appeared, one (peak K) containing material moving at the same rate as the B chain (Figure 3e) and the other (peak L) containing pure A chain (Figure 3f). The material in peak K is not contaminating B chain but appears to represent a varient of A chain, slightly heavier than the material in peak L.

Properties of the Two Peptide Chains of Ricin. Experiments were then carried out to see if the two peptide chains of ricin had different biological properties. As shown in Figure 7 only the A chain strongly inhibited protein synthesis in a cell-free system, whereas the isolated B chain had virtually no effect. Thus, as little as 1 ng of pure A chain (fraction L in Figure 6) reduced the rate of the protein synthesis to about 30% of the control value, whereas 100 times as much B chain (fraction J in Figure 5) did not inhibit to this extent. The results indicate that the inhibitory effect of ricin on protein synthesis is exerted exclusively by the A chain.

In analogy with our earlier studies on abrin (Olsnes and Pihl, 1973) experiments were carried out to see whether the B chain might be involved in the binding of the intact toxin to the cell surface. In this experiment we used washed human erythrocytes as a model system. If ricin or one of its peptide chains will bind firmly to the surface of the erythrocytes, hemagglutination should occur upon subsequent addition of antiserum, directed specifically against ricin. As shown in Table II this did indeed take place. Furthermore, anti-ricin agglutinated erythrocytes pretreated with purified B chain (Table II), whereas no agglutination occurred with erythrocytes treated in the same way with purified A chain. Since precipitation tests showed that the antiserum used contained antibodies in about the same amount against the A and B chain, the results indicate that only the B chain of ricin is involved in the binding of the toxin to the surface of the erythrocytes. The fact that ricin binds to Sepharose which contains galactose residues and can be specifically eluted with galactose suggests that galactose-containing receptors on the cell surfaces may be involved in the binding of the toxin. The view that only the B chain is involved in this binding is consistent with the finding that the isolated A chain was unable to bind to the Sepharose column, whereas the B chain did have this ability and could be eluted from the column with a galactose-containing buffer (data not shown).

Discussion

The present finding that the smaller of the two constituent chains of ricin (the A chain) is capable of inhibiting protein synthesis in a cell-free system whereas only the B chain is bound to the surface of erythrocytes is quite analogous to our observations with abrin (Olsnes and Pihl, 1973). The two toxins as well as their constituent peptide chains have closely similar molecular weights. Moreover, we have recently been able to prepare in high yields toxic hybrid molecules consisting of A chains of either toxin with the B chain of the other one (Olsnes *et al.*, 1973[1]). Altogether these findings demonstrate striking structural and functional similarities between abrin and ricin, two proteins which are found in the seeds of two entirely different plants belonging to different orders (Leguminosae and Euphorbiaceae, respectively). The

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[1] Olsnes, S., Harper, A. A., and Pappenheimer, A. M., Jr., manuscript in preparation.

TABLE II: Ability of Antiricin to Agglutinate Erythrocytes Pretreated with Ricin or Its Constituent Peptide Chains.[a]

Toxin	Amount of Protein Added (μg)					
	0.04	0.1	0.2	0.5	1.0	1.0
Intact ricin (fraction H)	+	+	++	++	++	0[b]
A chain (fraction L)	0	0	0	0	0	0[b]
B chain (fraction J)	+	++	++	++	++	0[b]

[a] Increasing amounts of protein were mixed with 1.6×10^7 thoroughly washed human erythrocytes (blood group B⁻) in 1 ml of 10 mM Tris-HCl (pH 7.5) in 0.14 M NaCl. The mixture was incubated at room temperature for 1 hr. Antiricin serum (5 μl) was then added and the mixture was incubated at room temperature for 9 hr. One drop of the suspension was examined under the microscope to detect hemagglutination. The degree of agglutination was scored as follows: 0, no aggregation; +, a major part of the cells was present in small aggregates; ++, virtually all cells were present in aggregates of varying sizes. [b] Serum from a nonimmunized rabbit was used instead of antiricin.

possibility should be considered that these toxins represent regulatory proteins involved in the control of protein biosynthesis, and that for obscure reasons, they have accumulated in high amounts in the seeds of *Abrus precatorius* and *Ricinus communis.*

The extremely small amounts of abrin and ricin required to inhibit protein synthesis *in vitro* and *in vivo* indicate that the toxins act catalytically on some component necessary for protein synthesis (Olsnes and Pihl, 1972c). Recently we have shown that the toxins inactivate the ribosomes rather than some of the soluble components (Olsnes *et al.* 1973). It is therefore likely that the A chains of abrin and ricin represent enzymes which somehow modify the ribosomes. Since we have not been able to show any effect of abrin and ricin on protein synthesis in cell-free extracts from *Escherichia coli* (Olsnes *et al.*, 1973) our present evidence suggest that the toxins may inactivate specifically the eucaryotic type of ribosomes.

Our findings with abrin and ricin are in many respects similar to those reported earlier on diphtheria toxin by other authors (for a review, see Olsnes, 1972). Diphtheria toxin inhibits protein synthesis by inactivating the elongation factors, EF_2. This activity is associated with one part of the toxin molecule, whereas the other part is involved in the binding of the toxin to the cell surface (Uchida *et al.*, 1971, 1972). After mild trypsin treatment of diphtheria toxin, the two fractions are held together by a single disulfide bond and can be separated after treatment with reducing agents (Gill and Dinius, 1971; Drazin *et al.*, 1971). The similarities between abrin, ricin, and diphtheria toxin suggest that the presence of a "carrier" moiety which binds the toxin to the cell surface may be found in many toxic proteins. It is not clear whether binding as such of the toxin molecule to the cell surface is sufficient to ensure that the A chains possessing the toxic action penetrate into the cell. Possibly the B chains are required to facilitate the penetration of the A chains through the membrane.

In castor beans a lectin is found which binds to galactose-

containing receptors on the cell surface in a similar way to ricin (Nicolson and Blaustein, 1972). A similar situation is observed in the abrus seeds (Olsnes et al., 1973[2]). It is an interesting possibility that the B chains of ricin and abrin represent monovalent lectins closely related to one of the constituent peptide chains of the agglutinin present in the seeds.

Acknowledgment

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Purification of Particulate Glucose-6-phosphatase[†]

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ABSTRACT: An ultracentrifugal method is described for the purification of glucose-6-phosphatase from a lyophilized microsomal fraction of rat liver. The method is based on a selective removal of protein and phospholipid by centrifuging the particles through successive layers of sucrose solution of increasing specific gravity, one layer containing buffer at pH 10 and another 0.4% deoxycholate at pH 7.5. The purified particles owing to the removal of phospholipids in the deoxycholate layer have an increased specific gravity and are recovered in the precipitate in a yield of up to 75% in terms of enzyme activity of the starting material and a six- to sevenfold increase in specific activity. Some properties of the purified particles are described. To date attempts to solubilize the enzyme with retention of activity have not been successful.

Glucose-6-phosphatase (EC 3.1.3.9), an enzyme bound to the endoplasmic reticulum of the liver and essential for blood glucose formation, has not so far been purified extensively, although the enzyme has been known for more than 30 years. Its association with the microsomal fraction in a liver homogenate and its dependence on phospholipids for activity have also been known for a long time (Hers et al., 1951; Beaufay and de Duve, 1954). Two reasons are chiefly responsible for this situation—one is the instability of the enzyme and the other that solubilization of the enzyme with retention of activity has not been accomplished.

Our attempt has been to purify the enzyme as a particle by selective removal of other proteins and phospholipids.

In a previous paper a chromatographic method for the separation of phospholipids from microsomal particles has been described (Garland and Cori, 1972). This method is briefly as follows. A lyophilized preparation (M_1), purified about 40-fold over a crude homogenate with respect to glucose-6-phosphatase activity, is dispersed by sonication in a small volume of pH 7.5 barbital buffer containing 0.4% deoxycholate. When applied to a column of Sepharose 4B equilibrated with the same buffer, the void volume contains most of the enzyme activity, whereas two-thirds of the protein inactive in the glucose-6-phosphatase test and more than 90% of the phospholipids appear in later fractions. The resulting enzyme preparation (M_2) required the addition of phospholipids for full activity and was purified about twofold over the starting material. Although the columns were run at 4°, some irreversible inactivation of the enzyme had occurred since the total recovery of activity in the different fractions rarely exceeded 50%. Deoxycholate even at 4° was found to inactivate the enzyme in a concentration- and time-dependent reaction. Concentrations of de-