

Isolation and Properties of Abrin: a Toxic Protein Inhibiting Protein Synthesis

Evidence for Different Biological Functions of Its Two Constituent-Peptide Chains

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A new procedure is reported for the purification of the toxic protein abrin and for the isolation of its two constituent peptide chains.

Abrin was extracted from *Semen jequiriti* and purified by chromatography on a DEAE-cellulose column and on a Sepharose-4B column. The purified toxin behaved as a single protein on polyacrylamide-gel electrophoresis. The LD₅₀ dose in mice was about 70 ng indicating that the preparation was about 6-times as toxic as the most active abrin previously described.

After reduction of the toxin with 2-mercaptoethanol in the presence of galactose the two constituent peptide chains of abrin were isolated by chromatography on a DEAE-cellulose column.

The smaller peptide, denoted the A-chain, inhibited strongly 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 abrin or with B-chain were agglutinated by an antiserum against abrin, whereas erythrocytes pretreated with A-chain were not agglutinated under these conditions.

The results indicate that the toxic action of abrin is associated with the A-chain and that the B-chain functions as a "carrier" moiety necessary for binding of the toxin to the cell surface.

In previous papers we have reported on some of the properties and the mechanism of action of abrin, a highly toxic protein present in *Semen jequiriti*, the seeds of *Abrus precatorius* L. [1–3]. Abrin consists of two peptide chains held together by disulfide bonds which can be split by treatment with 2-mercaptoethanol [1].

Abrin appears to exert its toxic effect by inhibiting protein synthesis. Thus, in abrin-treated Ehrlich ascites cells, the protein synthesis is reduced more strongly than the RNA and DNA synthesis [4].

In the present paper studies on the function of the isolated constituent chains of abrin are reported. Abrin was purified to homogeneity, the constituent peptide chains were separated and their effect on protein synthesis in a cell-free system from rabbit reticulocytes was studied. Evidence is presented that the ability of abrin to inhibit protein synthesis *in vitro* is associated exclusively with the smaller peptide. Experiments involving the use of antibody to abrin suggest that the larger peptide is required for the binding of abrin to intact cells, possibly to galactose-containing receptors on the cell surface.

Definition. LD₅₀, the dosage giving 50% deaths.

MATERIALS AND METHODS

Materials

Semen jequiriti was obtained from Norsk Medisinaldepot (Oslo). DEAE-cellulose (DE-52, Whatman) was obtained from W. & R. Balston Ltd (Springfield Mill, Maidstone, Kent, England); Sepharose-4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); [¹⁴C]Leucine (uniformly labelled, specific activity 331 Ci/mol) was obtained from The Radiochemical Centre, (Amersham, England).

Extraction of Abrin

40 g decorticated *Semen jequiriti* were allowed to swell overnight in 5% acetic acid and 4 °C and ground in a porcelain mortar. The material was then suspended in 500 ml 5% acetic acid at homogenized at maximum speed in an Omnimixer (Sorvall) at 0 °C in 10 sequences of 1 min with 5–10 min intervals to prevent rise in temperature. The homogenate was then centrifuged at 8000 × *g* for 10 min and the pellet was discarded. The supernatant was dialyzed for two days against distilled water and then against 10 mM Tris-HCl pH 7.7 for 24 h.

The dialyzed material was centrifuged at $8000 \times g$ for 20 min, the pellet was discarded and the supernatant centrifuged once more in the same way. The final supernatant is referred to below as crude abrin.

Polyacrylamide-Gel Electrophoresis

A toxin solution containing 0.5 to 1.0 mg/ml protein was made up to contain 1% sodium dodecyl-sulfate and 0.1 M sodium phosphate pH 7.1 and then 25–50 μ l were layered onto polyacrylamide gels (13% acrylamide, 0.2% dimethyl-bisacrylamide). The electrophoresis was carried out as described by Weber and Osborn [5]. In some instances the proteins were treated with 1% 2-mercaptoethanol at room temperature for 1 h before the electrophoresis. When the protein concentration was too low the protein was precipitated in the presence of 10% trichloroacetic acid and dissolved in a smaller volume [6]. Protein content was measured according to Lowry *et al.* [7] with bovine serum albumin as standard. After electrophoresis the gels were allowed to stay in 20% trichloroacetic acid overnight and then stained with 0.1% Coomassie-blue [6] and destained by transverse electrophoresis. Molecular weights of the proteins were estimated by electrophoresis in the presence of standards of known molecular weights according to Weber and Osborn [5], as earlier described [1].

Preparation of Antiserum against Abrin

Due to the high toxicity of the intact toxin the immunization of rabbits was started with a toxoid prepared by formaldehyde treatment of abrin in the following way: 5 mg abrin in 3 ml 0.05 M sodium phosphate pH 7.5 was made up to contain 1% previously neutralized formaldehyde and incubated at 37 °C for three days. Then the mixture was filtered through a Sephadex G-25 column (0.8 \times 30 cm), equilibrated with 0.05 M sodium phosphate pH 7.5 to remove excess formaldehyde. The protein-containing fractions were pooled and mixed with an equal volume of Freund's complete adjuvant. Then doses containing 0.5 mg toxoid were injected intramuscularly into rabbits. After 3 weeks a mixture of 0.05 mg intact toxin and 0.5 mg toxoid was injected subcutaneously and after a further 3 weeks, 0.5 mg intact toxin was injected subcutaneously. One week after the last injection the rabbits were killed by heart puncture and the serum was collected and stored in small samples at -20 °C until use. Before use the serum was incubated at 56 °C for 30 min to inactivate complement and then mixed with an equal volume of washed human erythrocytes to adsorb antibodies directed against human red blood cells. After incubation at room temperature for 1 h the erythrocytes were removed by low-speed centrifugation and the serum was collected.

Cell-Free Protein Synthesis

The cell-free protein synthesizing system was an unfractionated rabbit reticulocyte lysate prepared as described by Lingrel [8]. At different periods of time 10- μ l samples were removed and poured into 1 ml 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 colourless 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 [9] was added and the radioactivity was counted in a Beckman LS-130 scintillation system.

RESULTS AND DISCUSSION

Isolation and Properties of Abrin

Since the published methods for the purification of abrin [10,11] are rather cumbersome, efforts were made to work out a simpler procedure.

The crude abrin obtained after extraction and dialysis (see Materials and Methods) was further purified by chromatography on a DEAE-cellulose column. As shown in Fig. 1 the adsorbed proteins were eluted with NaCl as four peaks, two of which, C and D, were poorly separated. The different peaks as well as the unadsorbed material were tested for toxicity in mice. The results in Table 1 show that the material in peak B was 4 to 20-fold as toxic as the material in the other peaks.

Different fractions were then analyzed with respect to their ability to inhibit protein synthesis in a cell-free system from rabbit reticulocytes. Since treatment of abrin with 2-mercaptoethanol increases greatly its inhibiting activity [2], 2-mercaptoethanol was added to the different fractions. It is apparent (Fig. 2) that again the material in peak B exhibited the highest potency, whereas that in peaks C, D and E had a much lower inhibitory effect in the cell-free system. Fraction A which was relatively toxic *in vivo* (Table 1) was almost as effective as fraction B in inhibiting protein synthesis. By and large the data indicate that the toxic effect *in vivo* is correlated with inhibitory effect on protein synthesis *in vitro*.

In the course of the work it was accidentally observed that abrin is strongly bound to Sepharose-4B, a binding which could be abolished by galactose. The material from peak B in Fig. 1 was therefore subjected to chromatography on a Sepharose-4B column. It is seen from Fig. 3 that some material (fraction F) passed through the column and that the bound material was eluted as a single sharp peak with 0.1 M galactose.

The two peaks were analyzed with respect to toxicity in mice and the inhibitory effect on protein synthesis in the cell-free test system. Only the

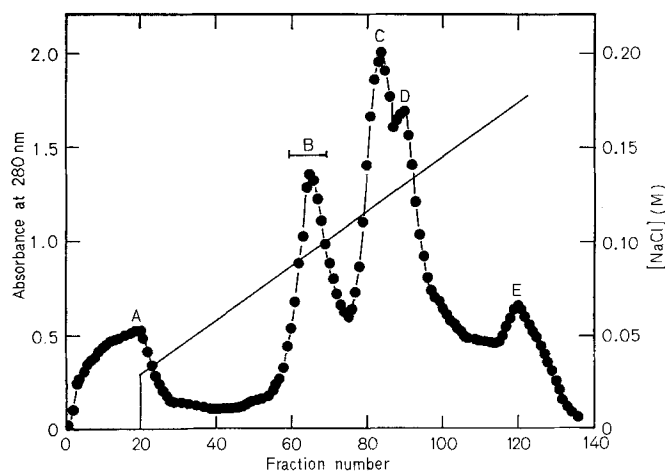


Fig. 1. DEAE-cellulose chromatography of proteins extracted from *Semen jequiriti*. 300 ml crude abrin obtained as described under Materials and Methods was applied to a column (1.6 × 40 cm) of DEAE-cellulose equilibrated with 30 mM NaCl in 10 mM Tris-HCl pH 7.7 and eluted at a speed of 80 ml/h with a 1600-ml linear NaCl gradient (from 30 to 180 mM) in the same buffer. (●) Absorbance at 280 nm; (—) Concentration of NaCl

Table 1. Toxicity in mice of different fractions after chromatography of crude abrin

Increasing amounts of protein were injected intraperitoneally into groups of mice weighing 25 ± 3 g and LD_{50} was calculated according to Litchfield and Wilcoxon [18]. Only deaths occurring within 72 h were recorded

Purification step	Fig.	Fraction number	Peak	LD_{50} μg protein
DEAE-cellulose chromatography	1	20	A	0.6
		65	B	0.15
		84	C	0.9
		90	D	1.1
		120	E	3.1
Sephacrose-4B chromatography	3	12	F	> 10.0
		35	G	0.07

material that did bind to the column (fraction G) proved to be toxic in mice (Table 1), and was capable of inhibiting protein synthesis (Fig. 4).

The purity of the product obtained by the affinity chromatography was checked by polyacrylamide electrophoresis (Fig. 5). Whereas the material in peak B of Fig. 1 (obtained before Sepharose chromatography) showed several weak bands in addition to the main protein band (Fig. 5A), the toxin eluted from the Sepharose-4B column moved as a single band (Fig. 5B), corresponding to a molecular weight of 65000. When the toxin was treated with 2-mercaptoethanol before electrophoresis, all the material

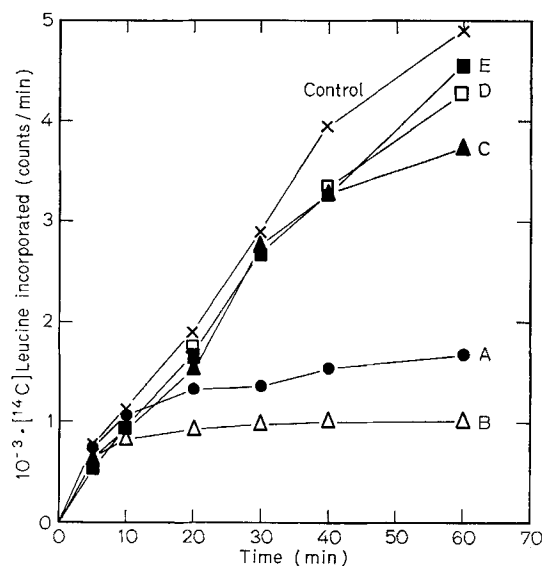


Fig. 2. Effect of different protein fractions on protein synthesis in a cell-free system from rabbit reticulocytes. The peak fractions in Fig. 1 were diluted in 10 mM Tris-HCl pH 7.7 to a protein content of 10 μg/ml. Then 2-mercaptoethanol was added to a final concentration of 1% and the samples were incubated at room temperature for 30 min. Subsequently the samples were diluted 10-fold in 10 mM Tris-HCl pH 7.5 containing 15 μg/ml of bovine serum albumin and 10 μl from each sample (containing 10 ng of the original protein) was added to the cell-free system. The system contained in a final volume of 0.5 ml: 0.2 ml lysate, 10 mM Tris-HCl pH 7.4, 10 mM ammonium acetate, 2 mM magnesium acetate 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 15 μg/ml creatine phosphokinase and amino acids (except leucine) in concentrations varying from 0.01 mM to 0.1 mM and 1.25 μCi [14 C]leucine. The incubation was carried out at 28 °C. After different periods of time, samples were removed and the acid-precipitable radioactivity was measured as described under Materials and Methods. (●) Fraction 20 (A); (Δ) fraction 65 (B); (▲) fraction 84 (C); (□) fraction 90 (D); (■) fraction 120 (E); (×) control (no protein added)

was converted into two peptides (Fig. 5C) moving at rates corresponding to molecular weights of 30000 and 35000, in agreement with our previous findings [1]. The fact that the single protein band was quantitatively converted into two pure peptides by treatment with 2-mercaptoethanol strongly indicates that the toxin eluted from the Sepharose column represented a pure protein. The LD_{50} of this protein was found to be 0.07 μg/25 g mouse (Table 1), indicating that the abrin here isolated was about 6-times more toxin than the most active abrin previously described [11]. In the present work lethality was scored after 3 days. However, if deaths occurring within one week were included, the LD_{50} was found to be as low as 0.04 μg/25 g mouse. This corresponds to about 10 toxin molecules per cell in the animal assuming even distribution.

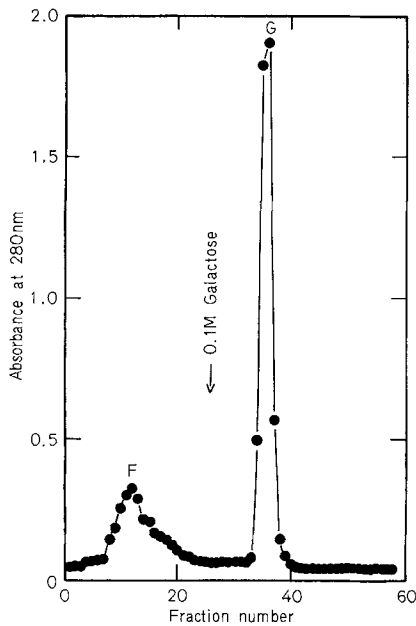


Fig. 3. Affinity chromatography of abrin on a Sepharose-4B column. The fractions from peak B (Fig. 1) indicated with a bar, were pooled, diluted three-fold and applied to a column (1.6 × 20 cm) of Sepharose 4B equilibrated with 10 mM Tris-HCl pH 7.7 containing 0.03 M NaCl. The column was then washed 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/h

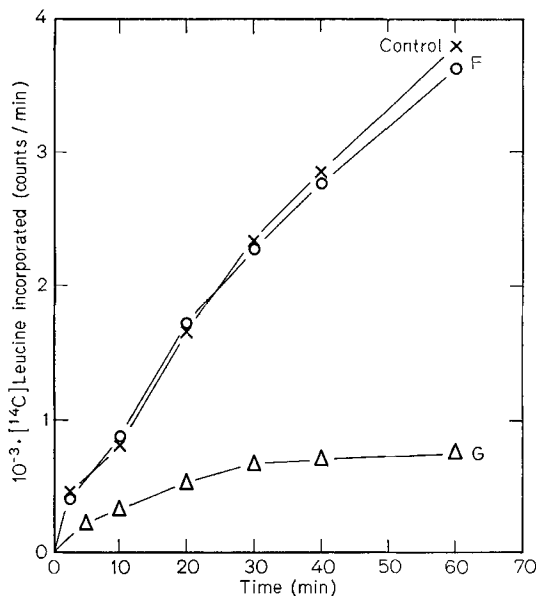


Fig. 4. Inhibitory effect of different protein fractions on protein synthesis in a cell-free system. The peak fractions in Fig. 3 were diluted, treated with 2-mercaptoethanol and their inhibitory effect on protein synthesis was studied as in Fig. 2. (O) Control (no protein added); (X) 100 ng protein from fraction 12 (F); (Δ) 10 ng protein from fraction 35 (G)

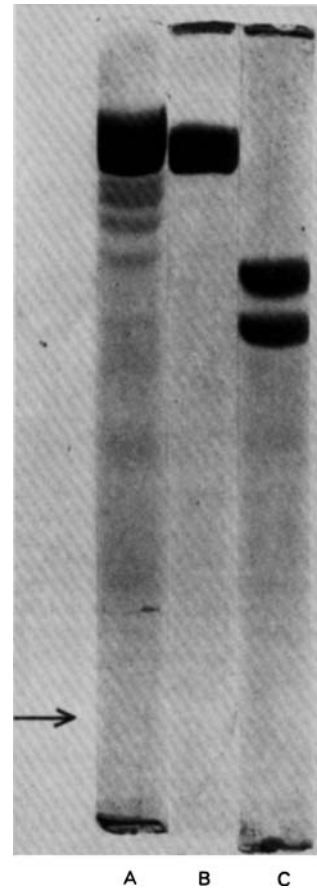


Fig. 5. Polyacrylamide-gel electrophoresis of intact and dissociated abrin. Protein from different fractions were treated with 1% sodium dodecylsulfate and analyzed by electrophoresis in 13% polyacrylamide gels in the presence of 0.1% sodium dodecylsulfate. The arrow marks the position of the tracking dye (bromphenolblue) at the end of the electrophoresis. (A) Fraction 65 from peak B of Fig. 1; (B) fraction 35 from peak G, Fig. 3; (C) the same fraction as in (B), after treatment with 1% 2-mercaptoethanol

It is known that *Semen jequiriti* also contains a potent hemagglutinin, which is extracted together with abrin, and it has been suggested that the toxic effect of abrin preparations might be due to the presence of the hemagglutinin [12, 13]. To localize this activity the material in the different peaks eluted from the DEAE-cellulose column (Fig. 1) was also tested for its ability to agglutinate washed human erythrocytes. It is shown in Table 2 that the material in peak B which possessed the highest toxicity (Table 1) contained virtually no hemagglutinating activity, whereas the far less toxic material in peaks C and D exhibited a strong hemagglutinating activity and that in peak E somewhat less. The data indicate that abrin as such does not agglutinate human erythrocytes.

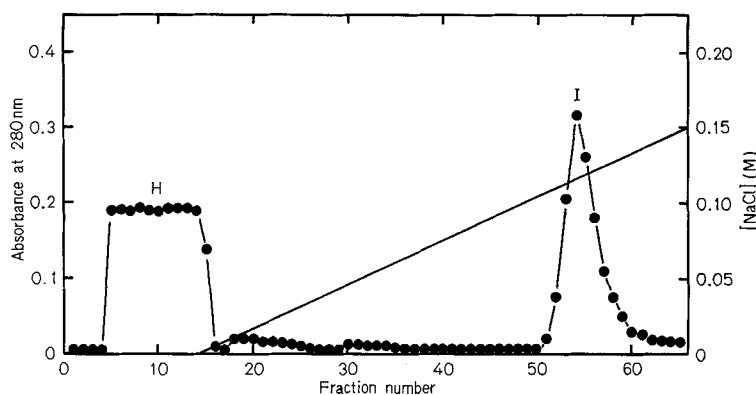


Fig. 6. Chromatography of 2-mercaptoethanol-treated abrin on a DEAE-cellulose column. A solution of abrin (Fraction G in Fig. 3) was made up to contain 0.5 M galactose and 5% 2-mercaptoethanol. The preparation was incubated at room temperature overnight, then chilled to 0 °C and pH adjusted to 8.5 with 0.1 M Tris. The material was applied to a DEAE-cellulose column, equilibrated with 0.01 M Tris-HCl pH 8.5

containing 1% 2-mercaptoethanol and eluted with a 500-ml linear NaCl gradient (from 0 to 0.2 M NaCl) in the same buffer. 1 ml samples from each fraction was made 10% (w/v) in trichloroacetic acid, and the precipitated protein was dissolved in 1 ml 0.05 N NaOH and the absorbance was measured. (●) Absorbance at 280 nm; (—) NaCl concentration

Table 2. Hemagglutinating activity of the different protein fractions from Fig. 1

Protein, in the amounts indicated, was mixed with 1.6×10^7 thoroughly washed human erythrocytes (blood group B-) in 1 ml 10 mM Tris-HCl pH 7.5 in 0.15 M NaCl. The mixture was incubated at room temperature for 10 h and the sedimented cells were resuspended. One drop of the suspension was examined under the microscope to detect hemagglutination. The degree of agglutination was scored as follows: (+), a minor part of the cells was present in small aggregates; +, a major part of the cells was present in small aggregates; ++, virtually all cells were present in aggregates of varying sizes; +++, most of the cells were present in large conglomerates

Amount of protein added	Hemagglutinating activity of fraction (peak):				
	20 (A)	65 (B)	84 (C)	90 (D)	120 (E)
μg					
0.01	0	0	(+)	0	0
0.05	0	0	+	+	0
0.1	0	0	++	++	(+)
0.3	0	0	++	++	+
1.0	0	0	+++	+++	++
2.0	0	0	+++	+++	+++
5.0	0	(+)	+++	+++	+++

Isolation of the Two Peptide Chains of Abrin

After splitting of abrin by treatment with 2-mercaptoethanol, the larger peptide chain, denoted the B-chain, was found to be unstable and rapidly precipitated at room temperature, whereas the smaller A-chain, was stable and remained in solution for several weeks at room temperature. However, when the 2-mercaptoethanol treatment of abrin was made in the presence of galactose, the precipitation of the B-chain was strongly retarded. Advantage was taken of this finding to isolate the two peptide chains.

After treatment of abrin with 2-mercaptoethanol in the presence of galactose, the protein was adsorbed onto a DEAE-cellulose column and eluted with a linear NaCl gradient. It is shown in Fig. 6 that about half of the material did not bind to the column, whereas the other half was eluted as one main peak.

The material in the two peaks was analysed by polyacrylamide-gel electrophoresis in the presence of sodium dodecylsulfate with untreated and 2-mercaptoethanol-treated abrin as reference standards (Fig. 7). It is evident that the material in fraction H (Fig. 7C) represents the larger peptide (B-chain) of the toxin, whereas fraction I contained the smaller A-chain (Fig. 7D).

Properties of the Two Peptide Chains

Since 2-mercaptoethanol-treated abrin has a strong inhibitory effect on protein synthesis in a cell-free system [2], it is clear that the intact structure is not required for this effect. In order to see whether the ability of abrin to inhibit protein synthesis is associated with one or both of the two peptide chains, the purified A and B-chains were added separately to a protein-synthesizing system from rabbit reticulocytes. It is seen from Fig. 8 that the A-chain has a strong inhibitory effect. Thus, as little as 1 ng protein gave a clearly demonstrable inhibition. On the other hand, 10 ng of the B-chain gave no significant inhibition of protein synthesis and 100 ng B-chain gave only a very slight inhibition, probably due to contamination with some A-chain. The data thus indicate that the inhibitory effect of abrin on protein synthesis in the cell-free system is associated exclusively with the A-chain.



Fig. 7. Polyacrylamide-gel electrophoresis of abrin and its constituent peptide chains. (A) Untreated abrin; (B) abrin treated with 2-mercaptoethanol; (C) protein from fraction H (Fig. 6); (D) protein from fraction I (Fig. 6)

The binding of the two peptide chains to intact cells was then studied. In these experiments we used washed human erythrocytes as a model. If abrin or one of its peptide chains becomes bound to the cell surface, this would be expected to be revealed by hemagglutination upon addition of an antiserum against abrin. It is shown in Table 3 that addition of antiserum indeed agglutinated abrin-treated cells under these conditions, indicating that the toxin binds firmly to the erythrocyte surface. Since gel-precipitation tests showed that antiserum against abrin contains antibodies against both the A and the B-chain, similar experiments were carried out with the isolated peptide chains. It was found (Table 3) that anti-abrin agglutinated erythrocytes pretreated with B-chain, although to a lesser degree than if the cells had been pretreated with the same amount of intact toxin. On the other hand, virtually no agglutination was found when the erythrocytes had been pretreated with A-chain. The results therefore indicate that only the B-chain is involved in the binding of abrin to the cell surface.

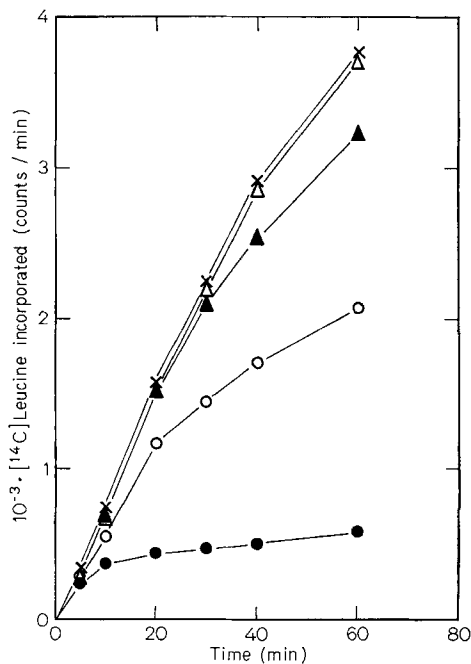


Fig. 8. Inhibitory effect on protein synthesis of the isolated peptide chains of abrin. The cell-free system was prepared and the protein synthesis measured as described in Fig. 2. (×) Control; (○) 1 ng A-chain added; (●) 10 ng A-chain added; (Δ) 10 ng B-chain added; (▲) 100 ng B-chain added

Table 3. Ability of anti-abrin to agglutinate erythrocytes pretreated with abrin or its constituent peptide chains. The amounts of protein indicated were mixed with erythrocytes as described in Table 2 and the mixture was incubated at room temperature for 1 h. Then 5 μ l of anti-abrin serum was added and the mixture was incubated for nine more hours. Hemagglutination was detected and scored as described in Table 2. As a control, an antiserum to ricin (an immunologically different protein) was added instead of anti-abrin

Amount of protein added	Hemagglutinating activity of toxin:		
	Intact abrin	A-chain	B-chain
μ g			
0.05	+	0	0
0.1	+	0	(+)
0.2	+	0	+
0.5	++	0	+
1.0	++	(+)	++
Control			
1.0	0	0	0

The present observation that only the smaller of the two constituent chains of abrin (the A-chain) is capable of inhibiting protein synthesis in the cell-free system, whereas only the B-chain is able to bind to the surface of erythrocytes confirms our previous suggestion [2] that the two chains of abrin may possess different functions.

Our findings with abrin and ricin, a toxic protein which behaves similarly to abrin [14] even though it is extracted from an entirely different plant, provide an interesting analogy to earlier studies of diphtheria toxin. Thus, after mild trypsin treatment, diphtheria toxin is split into two peptide chains which are held together with a disulfide bond [15]. The two chains have been shown to have different functions. The smaller chain inhibits protein synthesis whereas the larger chain appears to be necessary for the binding of diphtheria toxin to the cell surface [16, 17]. The similarities between these three toxins from entirely different sources suggest that other toxic proteins may also turn out to consist of a carrier part and a moiety possessing the toxic activity.

The high affinity of abrin for galactose residues in Sepharose suggests that galactose-containing receptors on the cell surface might be involved in this binding. Since the individual peptide chains have a low toxicity in living animals it appears that the B-chain functions as a carrier for the toxic A-chain which is unable to attach itself to the cell surface. It is also possible that the B-chain not only binds the toxin to the cell surface, but also facilitates penetration of the A-chain through the cell membrane.

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