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Isolation and Characterization of Viscumin, a Toxic Lectin from *Viscum album L.* (Mistletoe)*

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A toxic protein, viscumin, was isolated from extracts of mistletoe by affinity chromatography on acid-treated Sepharose 4B. Viscumin was selectively bound to the column and could be eluted with lactose. It migrated in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate corresponding to M_r = 60,000. In addition, two bands migrating corresponding to M_r = 29,000 and 32,000 were found. After treatment with 2-mercaptoethanol, only 2 bands (M_r = 29,000 and 34,000) were found. Apparently, viscumin consists of two chains which, in some of the molecules, are disulfide-linked. Protection experiments with antiserum against viscumin indicated that the major part of the cytotoxic activity in mistletoe extracts is due to viscumin. Gel filtration experiments on Sephacryl 200 indicated that, at low concentrations, viscumin occurs as a monomer and at higher concentrations as a dimer. Viscumin was found to inhibit protein synthesis in cell-free systems. When the two constitutent peptide chains of viscumin were eluted from polyacrylamide gels and tested for ability to inhibit cell-free protein synthesis, this property was found to be associated with the fastest migrating chain, here denoted the A chain. The heavier chain was denoted the B chain. The A chain was found to inhibit protein synthesis by inactivating the ribosomes catalytically. Reconstitution experiments with isolated ribosomal subunits from untreated and A chain-treated ribosomes showed that the 60 S ribosomal subunit was selectively inactivated.

The poisonous properties of mistletoe *(Viscum album L, (Loranthaceae))* have been known since ancient times (1). In pagan times mistletoe was considered a holy plant, and in Nordic mythology the famous death of Baldr was caused by an arrow of mistletoe (2). Extracts from mistletoe have been used against a variety of diseases, and such extracts are still in use as constituents of herbal remedies. An extract of *V. album,* marketed under the name of Iscador, is widely used in the treatment of cancer (3–4), but convincing evidence that it possesses antitumor properties has not been presented. In some cases the administration of mistletoe extracts has resulted in severe intoxications and damage to the liver (5).

Earlier work concerning characterization of the biologically active components of *V. album* has been reviewed by Luther (1). The best characterized component, denoted lectin I, appears to bear some resemblance to the plant toxins abrin, ricin, and modeccin, which have been studied extensively in our laboratory (for review see Ref. 6). Thus, evidence has been presented that lectin I, like these other plant toxins, consists of two disulfide-linked chains (7, 8), and that it inhibits protein synthesis in cultured cells, as well as in a lysate from rabbit reticulocytes (9).

We have now set out to isolate and characterize the toxic principles of mistletoe. The possible relationship in structure and mechanism of action between the mistletoe toxin and abrin, ricin, and modeccin which occur in unrelated plants is an interesting biological problem *per se.* Moreover, it is of considerable practical importance to test the biological and possible cancerostatic properties of the pure isolated toxins of mistletoe extract and to relate these to the composition of Iscador (10), a mistletoe extract that has been used in medicine for a long period of time.

Here we demonstrate that mistletoe extract indeed contains a cytotoxin with similar structure and mechanism of action as abrin, ricin, and modeccin. We propose to denote the cytotoxin viscumin which is analogous to the designation of the related plant toxins. Our data indicate that viscumin accounts for the main part of the cytotoxicity of crude mistletoe extracts.

EXPERIMENTAL PROCEDURES

Extraction and Purification of Viscumin—Mistletoe grown on Norway maple *(Acer platanoides)* was stored at −20 °C until use. After thawing, the green parts of the plant were chopped into slices of approximately 1-mm each and then transferred to a mortar and frozen in liquid nitrogen. The frozen material was transferred to a Warring blender placed in a well ventillated hood (because of production of toxic dust!) and ground into a fine powder which was transferred to an Erlenmeyer flask. Approximately 10 volumes of 10 mM Tris-HCl (pH 8.3), containing 100 mM lactose, were added and the suspension was stirred with a magnetic stirrer at 4 °C overnight. The lactose was included to avoid binding of viscumin to carbohydrate components in the homogenate. The suspension was filtered through cheesecloth and then centrifuged at 10,000 × *g* for 10 min in a Sorvall centrifuge. The pellet was discarded. The supernatant was applied to a column (5 × 20 cm) of DE-52 equilibrated with 10 mM Tris-HCl (pH 8.3). After washing with the same buffer, the bound toxin was eluted with 0.2 M NaCl in the same buffer. The eluted material was adjusted to 70% saturation with $(NH_4)_2SO_4$ and stirred at 4 °C for 3 h. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min and dissolved in a small volume of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2). Further steps in the purification procedure are described in Fig. 1.

Radioiodination—Labeling with ^{125}I was carried out essentially as described by Fraker and Speck (12). Briefly, 50 μg of protein in 25 μl of H_2O were mixed with 50 μl of 0.2 M Na borate (pH 8.4) and added to a tube, coated with 1,3,4,6-tetrachloro-3*a*,6*a*-diphenylglycouril, immersed in an ice bath. Then 0.5 mCi of $Na^{125}I$ was added. After 10 min the mixture was diluted with 500 μl 10 mM Na phosphate (pH 7.4) in 0.14 M NaCl and passed through a column of Sephadex G-50 (medium), equilibrated with the same buffer. The specific activity of

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the viscumin obtained was 300 cpm/ng.

Gel Filtration—A column (1 × 40 cm) of Sephacryl 200 superfine was equilibrated with 10 mM Na phosphate (pH 7.4) in 0.14 M NaCl, 0.1 M lactose. Lactose was added to prevent the toxin from binding to galactose residues in the column. The column was eluted at a speed of 5 ml/h.

Polyacrylamide Gel Electrophoresis—Electrophoresis in the presence of sodium dodecyl sulfate was carried out in 10% polyacrylamide gel slabs or in cylindrical gels (0.25 × 6 cm) as previously described (13). The electrophoresis was carried out at a constant voltage of 100 V until the marker dye, bromphenol blue, reached the bottom of the gel. Cylindrical gels were cut into 1-mm slices that were eluted with 100 μl of 0.1% Triton X-100 containing 50 μg/ml of rabbit hemoglobin.

Preparation of Antiviscumin—A sample of viscumin (0.2 mg in 1 ml of 0.14 M NaCl, 10 mM Na phosphate, pH 7.4) was adjusted to contain 3% formaldehyde and stored at room temperature for 3 days. Then 0.2 ml was mixed with Freund's complete adjuvant and injected subcutaneously into a rabbit. After 3 and 6 weeks the injections were repeated. Serum was collected 1 week after each of the latter injections.

Measurement of Toxicity to Mice—Increasing amounts of protein were injected intravenously into small groups of mice (2 to 5 mice), and the number of mice surviving after 7 days was scored. The amount of protein required to kill 50% of the mice, LD_{50}, was estimated.

Measurement of Toxicity to Cells in Culture—Mouse 3T3 cells or mouse 501.1 cells were seeded out in 24-well tissue culture trays (5×10^4 cells/well) and incubated overnight. On the next day, the indicated amounts of toxin were added and the cells were incubated as described in legends to the figures. Inhibition of protein synthesis was measured after removal of the medium and addition of serum-free medium containing 21 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.7) instead of bicarbonate and 50 nCi of [^{14}C] leucine (342 mCi/mol). The incorporation of radioactivity during 1 h was measured as previously described (14).

Hemagglutination—Human erythrocytes were washed twice in 0.14 M NaCl, 10 mM Na phosphate (pH 7.1) and diluted to 10^8 erythrocytes/ml. To each well of 96-well microtiter plates with hollow bottom, 10 μl of 0.14 M NaCl in 10 mM Na phosphate (pH 7.1), containing 0.1 mg/ml of bovine serum albumin, were added, and then serial dilutions (1:2) of the protein fractions were made. One drop of the erythrocyte suspension was added to each well and, after mixing, the plates were stored at room temperature to allow the erythrocytes to sediment. In the absence of agglutination, the erythrocytes formed a small button in the center of the well, whereas agglutinated cells were deposited as a film covering the whole bottom. The lowest concentration of added protein giving visible agglutination was determined.

Protein Synthesis in Rabbit Reticulocyte Lysate—Rabbit reticulocyte lysate, prepared by the method of Lingrel (15), was supplemented as described by Pelham and Jackson (16) and then stored in small aliquots in liquid nitrogen. Protein synthesis was measured in 23-μl samples containing 0.1 μCi of [^{14}C]leucine (342 mCi/mol), with and without toxin, as described in legends to figures. The incubation was terminated by adding 1 ml of 100 mM KOH and the trichloroacetic acid-precipitable radioactivity was measured as previously described (17).

Polymerization of Phenylalanine—The incubation mixture consisted of the indicated amounts of ribosomes or ribosomal subunits in 100 μl of buffer (50 mM Tris-HCl (pH 7.4), 60 mM KCl, 4 mM $MgCl_2$, 1.2 mM spermidine, 9 mM 2-mercaptoethanol) containing 10 μg of poly(U), 500 μM GTP, 10 μl of pH 5 supernatant and 92 mCi of [^{3}H] phenylalanyl-tRNA (7 Ci/mmol/well). After incubation for 15 min at 37 °C, the heat-stable, acid-precipitable radioactivity was measured as previously described (18).

Isolation of Ribosomes and Ribosomal Subunits—Rabbit reticulocyte ribosomes were prepared as earlier described (17). Ribosomal subunits were prepared by incubating ribosomes with 100 μM GTP, 1 mM puromycin, and 300 mM KCl for 30 min at 37 °C. The subunits were separated by sucrose gradient centrifugation as described (17).

RESULTS

Purification of Viscumin—The green parts of mistletoe were extracted as described under "Experimental Procedures." The purification of the toxin was monitored by assaying the ability of the different fractions to kill mice and to inhibit protein syntheis in mouse 3T3 cells. Also, the ability of the extract to agglutinate human erythrocytes was followed. The inhibitory effect on cells is described in more detail in the following paper (19).

The crude extract was first passed through a column of DEAE-cellulose to which several proteins, including the most toxic one, denoted viscumin, were bound (Table I). The toxic activity was eluted with 0.2 M NaCl, whereas most of a dark brown protein, which represents a major contaminant, remained bound. The eluted material was adjusted to 70% saturation with respect to $(NH_4)_2SO_4$ and the precipitated material which contained essentially all the cytotoxic activity was applied to a column of acid-treated Sepharose 4B (Fig. 1*A*). Most of the protein was eluted with NaCl. When 0.1 M lactose was added, a small peak was eluted that contained most of the cytotoxic activity.

If the batchwise chromatography on DE-52 was omitted and the crude extract, after dialysis to remove the lactose, was applied directly to a Sepharose 4B column, as described by Franz *et al.* (8), the yield of viscumin was much lower. Possibly, the DE-52 column chromatography removes some non-dialyzable carbohydrates in the crude extract which otherwise interferes with the binding of viscumin to the Sepharose 4B. It should be noted that although Ziska *et al.* (11) reported more than 5 times higher yield of toxin than we obtained, the toxicity to mice of their material was much lower than that of viscumin isolated here (see Ref. 9). The reason for this discrepancy is not clear.

In Table I the purification factor and the recovery of toxicity and hemagglutinating activity in the different fractions are given. It can be seen that the toxin was purified approximately

TABLE I

Purification scheme for viscumin

Protein fraction	Total protein	Toxicity to mice		Toxicity to cells		Hemagglutinating activity	
		LD_{50}	Recovery	ID_{50}[a]	Recovery	Lowest agglutinating concentration	Recovery
	mg	*μg/kg*	%	*ng/ml*	%	*ng/100 μl*	%
Crude extract[b]	1,300	235	100	53	100	4,700	100
First DE-52 (flow through)	450			7,880			
First DE-52 (eluted with 0.2 M NaCl)	290			8.4	68		
Sepharose 4B (flow through)	210			198.0	4	18,700	4.1
Sepharose 4B (lactose eluate)	7.8	2.4	59	0.48	66	40	70

[a] ID_{50} is the amount of protein required to reduce the incorporation overnight of [^{14}C]leucine by mouse 3T3 cells to 50% of the control value.
[b] From 115 g of mistletoe (net weight).

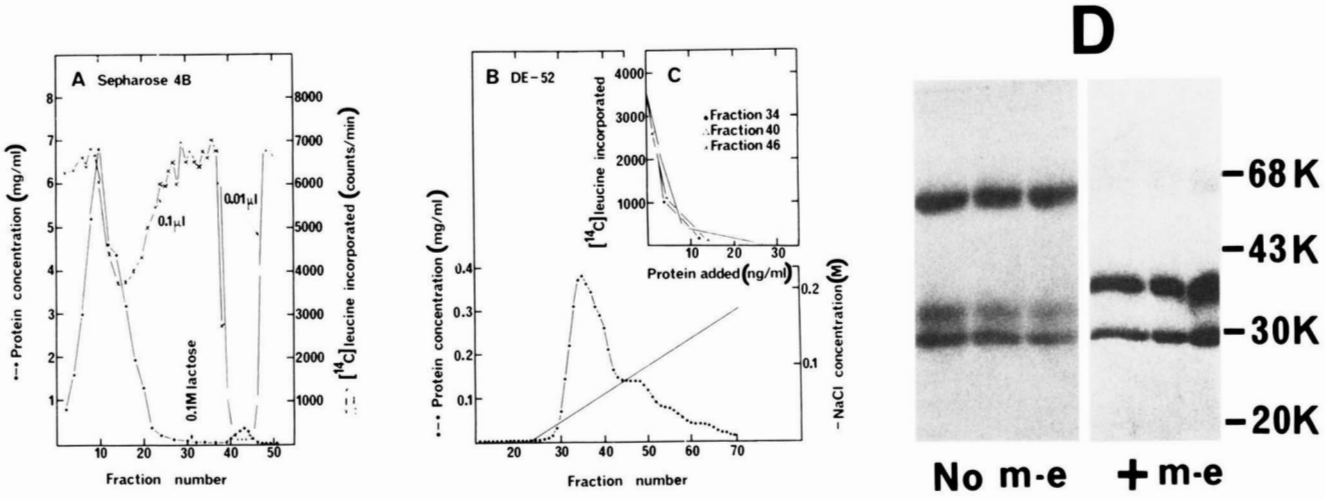

FIG. 1. **Purification of viscumin.** Viscumin was extracted and partially purified by DEAE-cellulose chromatography as described under "Experimental Procedures". *A*, the $(NH_4)_2SO_4$ precipitate was dissolved in a small volume of 10 mM Na phosphate (pH 7.3) in 0.14 M NaCl, dialyzed for 2 h against the same buffer, and then applied to a column of Sepharose 4B, (11) (2.5 × 40 cm), which had been pretreated for 3 h at 37 °C with 0.2 N HCl, and then washed extensively with 10 mM Na phosphate (pH 7.3) in 0.14 M NaCl. After application of the material, the column was washed with 10 mM Na phosphate (pH 7.3) in 0.14 M NaCl and then, as indicated by an *arrow*, eluted with 0.1 M lactose in the same buffer. The protein concentration in each fraction was measured with the Bio-Rad assay with bovine γ-globulin as a standard and the ability of an aliquot of each fraction to inhibit protein synthesis in mouse 3T3 cells was measured. ●, protein concentration; X, [^{14}C]leucine incorporated in cells treated with 0.1 µl of each fraction; △, [^{14}C]leucine incorporated in cells treated with 0.01 µl of each fraction. *B*, the material eluted with lactose from the Sepharose 4B column was dialyzed against 10 mM Tris-HCl (pH 8.3) and applied to a column of DE-52 (0.8 × 15 cm) equilibrated with the same buffer. The column was then eluted with a 100 ml gradient of 0–0.2 M NaCl in the same buffer. The protein concentration in each fraction was measured (●). *C*, increasing amounts of protein from the fractions eluted from the DE-52 column (*B*) were added to mouse 3T3 cells and the incorporation of [^{14}C] leucine was measured on the next day. *D*, aliquots from each of the three fractions in *B* were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before (the *3 left lanes*) and after (the *3 right lanes*) treatment with 5% 2-mercaptoethanol (*m-e*). The *lanes* represent (from the *left*) fractions 34, 40, and 46 from the DE-52 column in *B*. The presented data are from the same slab gel stained with Coomassie blue. The molecular weight markers were bovine serum albumin (M_r = 68,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), and trypsin inhibitor (M_r = 20,000).

80 times and that the recovery of toxicity to mice, cytotoxicity, and hemagglutinating activity was in the range 59 to 70%.

When the material eluted from the Sepharose 4B column with lactose was again adsorbed to a DEAE-cellulose column and eluted with a NaCl gradient, a main peak with a broad shoulder was obtained (Fig. 1*B*). When protein from different fractions was tested for cytotoxicity (Fig. 1*C*), no difference was found.

Also sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence and presence of 2-mercaptoethanol did not show any differences between the three fractions in Fig. 1*B*. Thus, in the absence of reducing agents, in all cases a major band migrating corresponding to M_r = 60,000 and two bands migrating corresponding to M_r = 32,000 and 29,000 were found (Fig. 1*D, three left lanes*). In the presence of 2-mercaptoethanol (*three right lanes*), only two bands migrating corresponding to M_r = 34,000 and M_r = 29,000 were found. Comparison of the *panels* shows that the lighter chain migrated at the same rate in the absence and presence of reducing agent. In the reduced sample there was no band migrating corresponding to M_r = 32,000. Since no new bands with a more rapid migration rate appeared in the gel, it is most likely that the material migrating corresponding to M_r = 32,000 in the unreduced sample was present in the band migrating corresponding to M_r = 34,000 in the reduced sample. Such a decrease in the migration rate could be due to reduction of one or more *intra*chain disulfide bridges and a corresponding unfolding of the polypepteide chain. In the related toxins, abrin and ricin, the heavy chains (B chains) contain internal disulfide bonds, whereas the light chains (A chains) do not (6). It should be noted that in all cases the bands of the viscumin sample are somewhat diffuse, possibly indicating microheterogeneity.

The data in Fig. 1*B* clearly indicate that the viscumin eluted from the Sepharose 4B column is heterogenous with respect to charge as has previously been shown to be the case with modeccin (20). Since there was no apparent difference in toxic activity of the different fractions, in the following experiments we used the whole peak of viscumin eluted from Sepharose 4B with lactose, as in Fig. 1*A*, without further fractionation.

Approximately 4% of the cytotoxic activity in the crude extract did not bind to the Sepharose 4B column (Table I). Most of it was, however, bound to a column containing desialylated fetuin in the same way as shown earlier for modeccin (20). The protein could then be eluted with lactose (data not shown). It migrated corresponding to M_r = 55,000 in the absence of reducing agents, whereas after treatment with 2-mercaptoethanol, two bands (M_r = 26,000 and 30,000) were found. The material was only ~1% as toxic as viscumin both to cells in culture and to mice.

Evidence That Viscumin Represents the Main Toxic Component in Mistletoe Extract—Several toxic components have been reported to be present in mistletoe extracts (1, 21, 22). In view of the claim that mistletoe extracts have cancerostatic properties (3, 4, 23–25), it was important to establish how much of the total cytotoxic activity in mistletoe extracts can be attributed to viscumin. For this purpose we prepared an antiserum to the purified viscumin and measured its ability to protect 3T3 cells against pure viscumin as well as against crude mistletoe extract. The results in Fig. 2*A* show that 10 µl of the antiserum displaced the inhibition curve by viscumin to the right, corresponding to an increase in the ID_{50} dose (the dose required to reduce protein synthesis overnight to 50% of the control value) by a factor of about 280. With crude extract 10 µl of antiserum protected against approximately 200 times the ID_{50} dose. These results indicate that viscumin or immu-

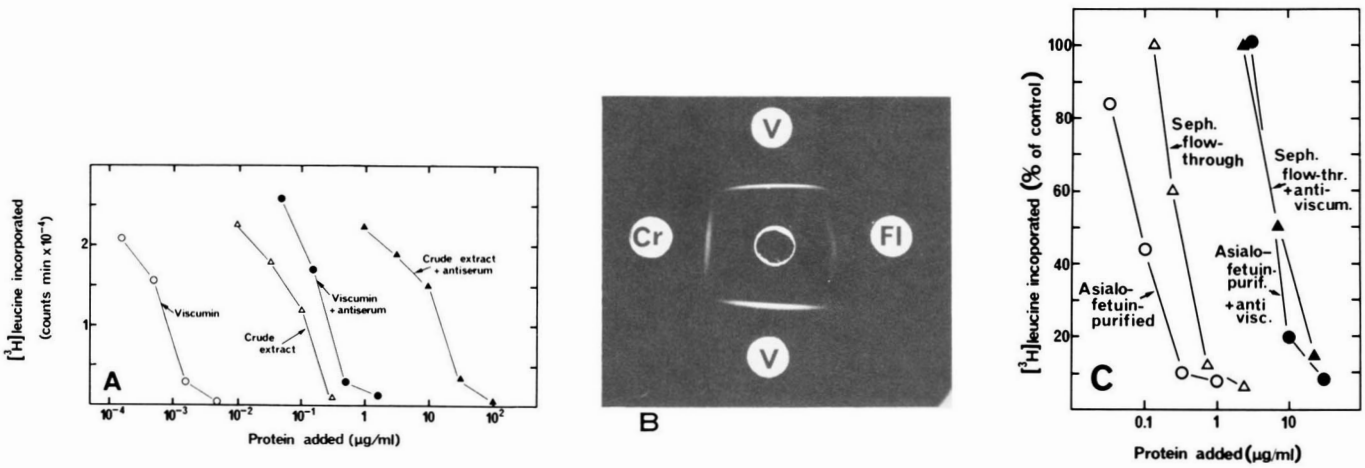

FIG. 2. **Specificity of antiviscumin and its ability to protect 3T3 cells against pure viscumin and crude extract from mistletoe.** *A*, increasing amounts of purified viscumin and crude extract from mistletoe were added to duplicate tubes containing 100 µl of 0.14 M NaCl, 10 mM Na phosphate (pH 7.4), and 0.1 mg/ml of bovine serum albumin. Then 10 µl of antiviscumin serum were added to one set of tubes and, after mixing, the tubes were incubated at room temperature for 1 h. Then the samples were added to 3T3 cells growing in 24-well disposable trays. The fetal calf serum in the medium had been preincubated at 56 °C for 30 min to inactivate complement. The cells were incubated at 37 °C overnight, and then their ability to incorporate [^{3}H]leucine was measured. *B*, immunodiffusion of antiviscumin serum against purified viscumin, crude extract, and viscumin proteins that did not bind to Sepharose 4B. The immunodiffusion was carried out into 1% agar containing 0.14 M NaCl, 10 mM Na phosphate (pH 7.4), 0.1 M lactose, and 0.02% NaN_3. Antiviscumin serum was added to the *center well*, and the *peripheral wells* contained: *V*, pure viscumin; *Cr*, crude mistletoe extract; *Fl*, the flow. *C*, increasing amounts of total protein that did not bind to the Sepharose 4B column (Sepharose flow through) and of the protein eluted with lactose from a column of desialylated fetuin (asialofetuin-purified) were treated with and without 10 µl of antiviscumin serum as in *A* and then added to cells. The rate of protein synthesis after incubation overnight was measured. The results are given as percentage of the control values (no toxin added) which were ~20,000 cpm.

nologically related material accounts for most of the cytotoxic activity of mistletoe extract. The fact that antiviscumin protected against pure and crude viscumin to about the same extent, indicates that in the extract there is not much material other than viscumin that binds and consumes the antibody. If other cytotoxins are present in the mistletoe extract, they must either be immunologically related to viscumin and cross-react with it or they contribute less than 0.5% (1/200) of the total cytotoxicity of the extract.

To study the specificity of the antiserum we first carried out immunodiffusion studies (Fig. 2*B*). With crude mistletoe extract one main line was found which showed reaction of identity with pure viscumin. In some cases an additional faint line was present. The fraction that did not bind to the Sepharose 4B column showed two lines with about equal intensity. The one showed reaction of identity with viscumin. The other line showed reaction of partial identity with viscumin. Other immunodiffusion studies (not shown) indicated that this material is identical with the protein which was isolated on the column of desialylated fetuin. This was also supported by experiments where the total material which did not bind to the Sepharose 4B column was labeled with ^{125}I, then treated with antiviscumin serum, and the immunocomplexes were adsorbed to staphylococci and analyzed by polyacrylamide gel electrophoresis (data not shown). The reason why two precipitin lines were seen in the material not bound to the Sepharose 4B column (Fig. 2*B*) is probably that this fraction had a low concentration of material reacting with the antiserum and therefore considerably more material was applied to the immunodiffusion gel without overloading it.

We then tested the ability of antiviscumin to protect cells against the material not bound to the Sepharose 4B column. The data in Fig. 2*C* show that 10 µl of antiserum were able to displace the inhibition curve to the right by a factor of only 30. This indicates that antiviscumin has lower ability to neutralize this material than viscumin itself. The antiserum displaced the inhibition curve of material eluted from the asialofetuin-Sepharose 4B column to the right by a factor of about 50. The data indicate that the material not adsorbing to the Sepharose 4B column contains traces of cytotoxic material which are not neutralized by antiviscumin.

TABLE II

Ability of antiviscumin to protect mice against purified viscumin and crude mistletoe extract

Antiviscumin added	LD_{50} in mice[a]	
	Pure viscumin	Crude mistletoe extract
	ng/mouse	*µg/mouse*
None	48	4.7
10 µl	840	36

[a] Increasing amounts of pure viscumin or crude mistletoe extract were added to Eppendorf tubes containing 50 µl of 0.14 M NaCl, 10 mM Na phosphate (pH 7.4), 0.1 mg/ml of bovine serum albumin with and without 10 µl of antiviscumin serum. After mixing, the tubes were stored at room temperature for 30 min. The immunoprecipitate formed was removed by centrifugation and the supernatant was injected intravenously into mice. Survival after 7 days was scored and the LD_{50} dose was estimated.

We also tested the ability of antiviscumin to protect mice against pure viscumin and against crude mistletoe extract. The data in Table II show that 10 µl of antiviscumin protected against approximately 840 ng of pure viscumin. This value is much higher than that found in the experiment with 3T3 cells (Figs. 2*A*), where 10 µl of antiviscumin neutralized about 280 ng of viscumin. The reason for this discrepancy is not clear. From Table II it appears that 10 µl of antiviscumin protected against approximately 20 LD_{50} doses of pure viscumin and against approximately 8 LD_{50} doses of crude extract. It is thus clear that the major part of the toxicity to animals is due to viscumin and immunologically related material.

It should also be noted that when an amount of crude extract containing approximately 30 LD_{50} doses was injected, the animals died rapidly within few minutes, whereas such rapid death was never seen after injection of 30 LD_{50} doses of pure viscumin. In this case mice died no sooner than the next

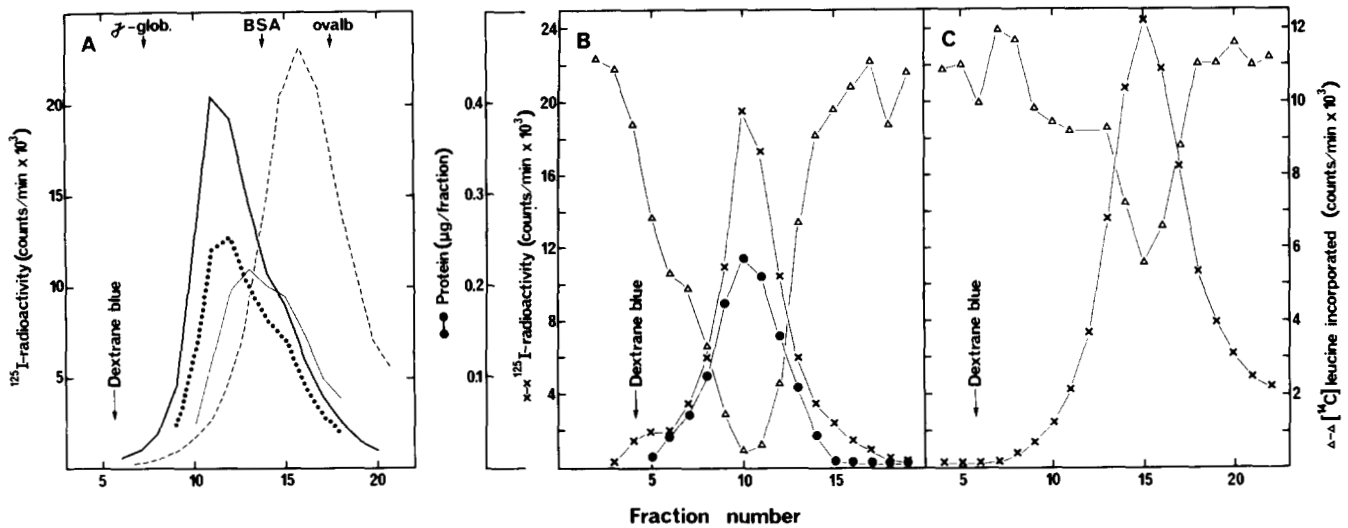

FIG. 3. **Gel filtration studies of viscumin.** The indicated amounts of unlabeled and ^{125}I-labeled viscumin were filtrated through a column of Sephacryl 200 superfine (1 × 40 cm), equilibrated with 10 mM Na phosphate (pH 7.4) in 0.14 M NaCl, 0.1 M lactose. *A*, ^{125}I-labeled viscumin (1.5 μg in 200 μl) was applied to the column alone (- - -), or after mixture with 5 μg (——), 20 μg (···), or 200 μg (——) of unlabeled viscumin. The fractions were collected and the radioactivity in each fraction was measured. *glob.*, globulin; *BSA*, bovine serum albumin; *ovalb*, ovalbumin. *B*, 1 mg of viscumin was mixed with 1.5 μg of ^{125}I labeled viscumin and filtered through the Sephacryl 200 column. In each fraction the protein content and the radioactivity were measured. From each fraction, 0.01 μl was added to wells of microtiter plates, each of which contained 5 × 10^4 mouse 501.1 cells. The cells were incubated overnight and their ability to incorporate [^{14}C]leucine was measured as described under "Experimental Procedures". ●, protein concentration; X, radioactivity; △, [^{14}C]leucine incorporated. *C*, ^{125}I-labeled viscumin (1 μg) was filtered through the Sephacryl 200 column. The fractions were collected and 50 μg of rabbit hemoglobin were added to each fraction to prevent adsorption of the toxin to the glassware. The radioactivity in each fraction was measured and then 10 μl of each fraction were added to mouse 501.1 cells. The next day the ability of the cells to incorporate [^{14}C]leucine was measured. X, ^{125}I radioactivity; △, [^{14}C] leucine incorporated in cells.

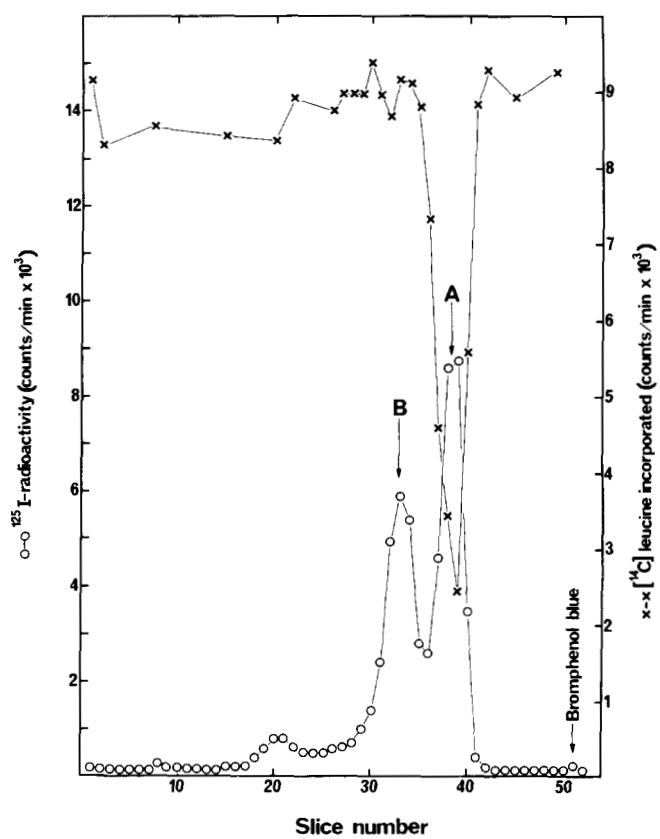

FIG. 4. **Ability of isolated chains of viscumin to inhibit protein synthesis in rabbit reticulocyte lysate.** ^{125}I-labeled viscumin (7 × 10^4 cpm, 15 μg) was made up to contain 0.3% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 0.1 M sucrose. The sample was incubated at room temperature for 1 h and then submitted to polyacrylamide gel electrophoresis in 10% gels (0.25 × 6 cm). When the bromphenol blue marker had reached the lower edge, the gel was sliced into 1-mm slices and the radioactivity was measured (○). Each slice was then extracted for 2 days at 4 °C with 100 μl of 0.1% Triton X-100 containing 50 μg/ml of rabbit hemoglobin. Samples (0.3 μl) of each fraction were then added to the cell-free system from a rabbit reticulocyte lysate and the incorporation of [^{14}C]leucine after 10 min was measured (X).

day. This difference could be due to the presence of other toxins, like viscotoxin (21, 22), in the crude extract. This is supported by the fact that such rapid death was also seen in mice treated with high amounts of crude extract where viscumin had been precipitated with antiviscumin and the immunocomplexes removed before injection. In this case all animals which did not die within the 1st h, survived.

Dimerization of Viscumin—When a small sample of ^{125}I-labeled viscumin (1.5 μg) was filtered through a Sephacryl 200 column, it was eluted corresponding to a molecular weight of approximately 57,000 (Fig. 3*A*). In contrast, when a larger sample (200 μg) of unlabeled viscumin was added, the radioactivity eluted corresponding to a molecular weight of approximately 100,000. After addition of an intermediate amount of viscumin (20 μg), the radioactivity eluted as a main peak corresponding to a M_r = 100,000 with a shoulder of approximately 60,000. When 5 μg of viscumin were filtered through the column, a broad peak was found. The results indicate that at high concentrations, viscumin exists as a dimer and at low concentrations as a monomer. Previously similar findings have been made with so-called lectin I, isolated from mistletoe (7). Even at very high concentrations of viscumin (5 mg/ml) we found no evidence for formation of still heavier complexes.

In attempts to study if the monomer, the dimer, or both forms of viscumin are toxic to cells, we first filtered a large amount (1 mg) of viscumin through the Sephacryl 200 column and measured the ability of each fraction to inhibit protein synthesis in cells. As shown in Fig. 3*B*, there was good correlation between the elution pattern of the protein and the distribution of inhibitory activity, showing that the viscumin

dimer is toxic. We then filtered a small sample (1 μg) of ^{125}I-labeled viscumin, which in this case eluted corresponding to a M_r = 57,000 (Fig. 3*C*), and tested each fraction for its ability to inhibit protein synthesis in the very sensitive mouse 501.1 cells. Both the radioactivity and the ability to inhibit protein synthesis in cells was now shifted to a position corresponding to a M_r = 57,000. The results show that viscumin is cytotoxic whether it is eluted as a monomer or dimer.

Ability of Isolated Chains of Viscumin to Inhibit Cell-free Protein Synthesis—It was earlier shown that viscumin inhibits cell-free protein synthesis and that the inhibitory activity was strongly increased after treatment with 2-mercaptoethanol (9). In three related plant toxins, abrin, ricin, and modeccin, in all cases the ability to inhibit cell-free protein synthesis resides in the shorter chains, denoted the A chains (6). To test if this is the case also with viscumin, we attempted to separate the two constituent polypeptide chains in their native form, but were unable to do so without the use of denaturing agents. However, as in the case of abrin, ricin, modeccin, diphtheria toxin, and *Shigella* toxin (6, 26), the ability of viscumin to inhibit cell-free protein synthesis was not reduced after treatment with sodium dodecyl sulfate. We therefore treated the toxin with sodium dodecyl sulfate and 2-mercaptoethanol and separated the chains by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The gel was sliced and the protein in each slice was eluted and tested for its ability to inhibit protein synthesis in a rabbit reticulocyte lysate. As shown in Fig. 4, only material eluted from the slices containing the light chain was able to inhibit cell-free protein synthesis. No such activity was associated with the heavy chain.

The eluted light chain was on a molar basis approximately as active in inhibiting protein synthesis in a rabbit reticulocyte lysate as viscumin treated with 2-mercaptoethanol in the absence of denaturating agents (data not shown). When tested for toxicity to mouse 501.1 cells, 60 ng/ml of isolated A chain did not inhibit protein synthesis under conditions where 1 ng/ml of intact toxin reduced protein synthesis to half the control value (data not shown).

In analogy with the notation used for the other toxins (6, 27) we propose to name the light chain of viscumin the A chain and the heavy chain the B chain. Since the ability of viscumin to bind to cells is lost after treatment with sodium dodecyl sulfate, we have so far been unable to ascertain whether binding to cell surface receptors is exclusively associated with the B chain, as is the case with abrin, ricin, and modeccin.

Inactivation of 60 S Ribosomal Subunits by Viscumin A Chain—Viscumin A chain acts by inhibiting the ribosomes. Thus, ribosomes isolated from a rabbit reticulocyte lysate pretreated with viscumin A chain had much lower activity in a polyphenylalanine-synthesizing system than ribosomes isolated from an untreated lysate (Fig. 5*A*). To test which of the ribosomal subunits is inactivated by viscumin A chain, the subunits of ribosomes from A chain-treated rabbit reticulocyte lysate were separated and tested for their ability to polymerize phenylalanine in the presence of the complementary subunit from untreated ribosomes. As shown in Fig. 5*B*, 40 S ribosomal subunits from A chain-treated ribosomes were as active as 40 S subunits from untreated ribosomes, whereas 60 S subunits from A chain-treated ribosomes were essentially inactive.

Evidence for Catalytic Activity of Viscumin A Chain—To test the possibility that viscumin A chain activates an inherent protein synthesis inhibitor rather than directly inactivating the ribosomes, we carried out the following experiment. Samples of rabbit reticulocyte lysate were incubated with and without A chain, then antiviscumin was added in some cases and each of the treated lysates was mixed with a sample of

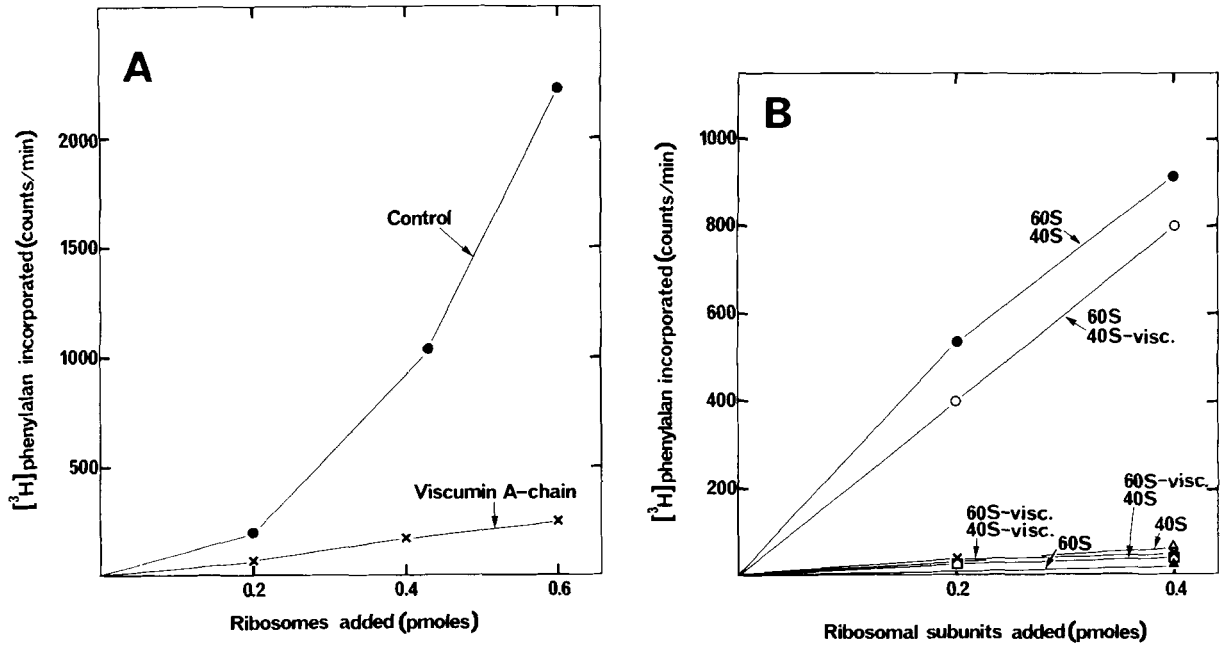

FIG. 5. **Ability of ribosomes and ribosomal subunits from toxin-treated and untreated rabbit reticulocyte lysate to support polyphenylalanine synthesis.** *A*, samples of rabbit reticulocyte lysate (1 ml each) were incubated with and without viscumin A chain (2.2 μg) for 30 min at 37 °C. Then the ribosomes were isolated as described under "Experimental Procedures," and their ability to support polymerization of [^{14}C]phenylalanine for 15 min was measured. ●, ribosomes from untreated lysate; X, ribosomes from lysate treated with viscumin A chain. *B*, reconstitution experiments. Ribosomes were treated and isolated as in *A*, and the ribosomal subunits were prepared. The ability of the individual and combined subunits to support polyphenylalanine synthesis was determined. ●, both subunits from untreated ribosomes; X, both subunits from viscumin-treated ribosomes; ○, 60 S subunits from untreated ribosomes, 40 S subunits from viscumin-treated ribosomes; ■, 60 S subunits from viscumin-treated ribosomes, 40 S subunits from untreated ribosomes; ▲, 60 S subunits from untreated ribosomes alone; △, 40 S subunits from untreated ribosomes alone.

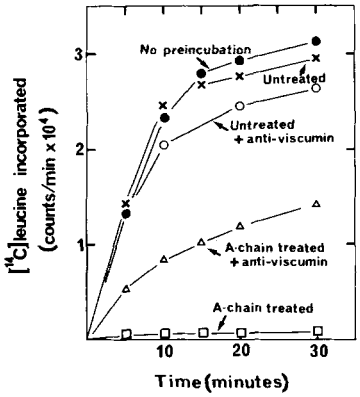

FIG. 6. **Ability of antiviscumin to prevent viscumin A chain-induced inhibition of protein synthesis in reticulocyte lysate.** Parallel samples (100 μl each) of rabbit reticulocyte lysate prepared and supplemented as described under "Experimental Procedures" were incubated for 5 min at 30 °C in the absence and presence of 220 ng of viscumin A chain and then chilled to 0 °C. To one of the parallel samples were added 5 μl of antiviscumin. From each sample, 40 μl were mixed with 40 μl of an unincubated lysate system containing 0.3 μCi of [^{14}C]leucine. The mixed lysates were then incubated at 30 °C. After the indicated periods of time 10-μl aliquots were transferred to tubes containing 1 ml of 0.1 M KOH and then the trichloroacetic acid-precipitable material was measured. The unincubated lysate was mixed with lysate which had been pretreated as follows: X, preincubated without viscumin A chain; ○, preincubated without viscumin A chain, antiviscumin added; □, preincubated with viscumin A chain; △, preincubated with viscumin A chain, antiviscumin added; ●, control, not preincubated lysate.

untreated lysate. Finally, the ability of the mixed lysates to incorporate [^{14}C]leucine was measured. The data in Fig. 6 show that a mixture containing lysate preincubated without A chain was approximately as active as lysate which had not been preincubated, whereas the sample containing the A chain-treated lysate and no antiserum was almost completely inhibited. The important finding is that the system containing A chain-treated lysate and antiviscumin incorporated [^{14}C] leucine at approximately half the control rate. This indicates that the antiviscumin serum prevented inactivation of the second part of lysate which was added after the antiserum. If an inherent inhibitor had been activated by viscumin A chain it would not have been inactivated by the antiviscumin serum. The data therefore provide evidence that the A chain exhibits its effect directly on the ribosomes.

DISCUSSION

The data presented here show that extracts from mistletoe contain a highly toxic lectin, here denoted viscumin, which is strikingly similar in structure and mechanism of action to three other toxic plant proteins previously described, abrin, modeccin, and ricin. The results confirm and extend those of previous investigators (7, 8, 11, 28). Thus, viscumin is probably identical with lectin I, previously described by Luther *et al.* (7) and Franz *et al.* (8). Like lectin I, viscumin was found to consist of two chains with M_r = 29,000 and 34,000 and it could be isolated by affinity chromatography on a column of acid-treated Sepharose by elution with lactose (11, 28). Moreover, viscumin inhibits protein synthesis in cultured cells as well as in a lysate from rabbit reticulocytes as previously found with lectin I (9).

It is shown here that the toxic activity of viscumin is associated exclusively with its smallest chain, the A chain, which acts by catalytically inactivating the 60 S ribosomal subunit. From the data in Fig. 4 it can be estimated that one A chain molecule inactivated at least 50 ribosomes within 10 min. Like abrin, modeccin, and ricin, viscumin binds to Sepharose 4B, and, as will be described in the subsequent paper (19), it apparently binds to carbohydrates containing terminal galactose residues. However, unlike abrin, ricin, and modeccin, viscumin is a potent agglutinin of red blood cells. This is probably due to its strong tendency to dimerize, as shown previously (7) and confirmed here. Although we have so far been unable to demonstrate that the B chain of viscumin is the binding moiety of the molecule, this appears highly probable. In a subsequent paper (19) we present evidence that viscumin enters cells by a mechanism similar to that operating in the case of abrin and ricin.

Viscumin appears to be heterogenous with respect to charge, as it does not elute from a DEAE-cellulose column as a single peak. This heterogeneity does not, however, appear to reflect heterogeneity with respect to activity. Previously we have found that also modeccin is heterogenous with respect to charge (20).

It is clear from the present and previous studies that in mistletoe extracts, also toxic proteins related to viscumin are present. Thus, it was found here that in a fraction that did not bind to the Sepharose 4B column, toxic material reacting with antiviscumin serum was present. It appears that this toxic material has a molecular structure similar to viscumin, but that the constituent polypeptide chains are somewhat lighter. Probably, this material, which accounts for approximately 4% of the total cytotoxic and hemagglutinating activity, is identical with lectins II and III, described by Franz *et al.* (8). These lectins were bound to immobilized γ-globulin. We have so far been unable to confirm this finding, but in our hands the material did bind to desialylated fetuin, as is also the case with modeccin (20).

The data presented here provide evidence that viscumin is the main toxin in mistletoe extract. This follows from studies both on mice and on cells in culture. After administration of large amounts of crude extracts where the viscumin had been neutralized by addition of antiviscumin, mice suffered rapid death which was probably due to the low molecular weight viscotoxins previously described (21, 22, 29, 30) and which acts by provoking circulatory collapse. With untreated crude extract such rapid death was only observed after injection of an amount at least 30 times higher than that which killed mice within 1 week. Furthermore, mice injected with crude extract treated with excess antiviscumin either died within the 1st h or survived. Thus these data indicate that less than 1/30 of the toxicity to mice in the crude extract can be accounted for by material not reacting with antiviscumin.

Since abrin and ricin have been found to possess cancerostatic properties (for review see Ref. 6), the fact that mistletoe extract contains similar proteins is of considerable interest. Experiments are now in progress to establish whether pure viscumin possesses cancerostatic properties and whether the commercial mistletoe preparation, Iscador, contains viscumin in biologically active amounts in the doses used. In the subsequent paper (19) we describe in more detail the toxic effect of viscumin on cells in culture.

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Note Added in Proof—After the submission of this manuscript Franz *et al.* (31) reported that the A chain of mistletoe lectin I inhibits cell-free protein synthesis and that the B chain binds to acid-treated Sepharose 4B.

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