

THE IMMUNOCHEMISTRY OF ALLERGENS

X. ANAPHYLACTOGENIC PROPERTIES OF ALLERGENIC FRACTIONS FROM CASTOR BEAN*†

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ALLERGIC sensitivity to castor bean was first described by Alilaire¹ who believed the allergen to be identical with the toxalbumin, ricin. Barnard² reported the preparation of nontoxic, allergenic extracts from castor bean without giving experimental details. According to Barnard the allergen was water soluble, alcohol precipitable, heat stable, and nondialyzable. Grabar and Koutseff³ later separated a nontoxic, allergenic fraction from castor bean which they called "ricinallergene." Their preparation was water soluble, heat stable, and dialyzable. Further reviews of the literature on castor bean allergy are given by Ratner⁴ and Jones.⁵

Recent reports⁶⁻⁸ from this laboratory described the isolation and fractionation of an allergenic, protein-polysaccharidic component, CB-1A, from castor bean. Fraction CB-1A represented 1.8 per cent of the defatted bean and was isolated by the procedure developed for isolating a similar allergenic fraction from cottonseed.⁹ Fraction CB-1A was further purified by a series of procedures involving chromatography, electrophoresis, and solvent fractionation.⁷ The final product, CB-65A, contained 19.0 per cent nitrogen and was essentially free of carbohydrate. The allergenic potency of CB-65A was not diminished by the prolonged purifications, an indication that the allergenic property was inherent in the protein and was not due to an entrained contaminant.

Chemical properties and amino acid content of the castor bean allergen were remarkably similar to those of the previously described cottonseed allergen.⁶ Both were characterized by relatively high proportions of arginine and cystine. However, CB-1A contained no tryptophan, in contrast to the cottonseed allergen which contained 1.4 per cent of this amino acid.

Physical properties of CB-1A indicated low molecular weight in comparison with other natural proteins. The proteic component of CB-1A, like that of CS-1A from cottonseed¹⁰ readily dialyzed through semipermeable membranes.⁶

This report presents the results of a study of the anaphylactogenic properties of CB-1A and its subfractions.

EXPERIMENTAL

To determine the antigenic relationship between the isolated allergenic fractions and the whole protein complex of castor bean, it was necessary to induce anaphylactogenic sensitivity in guinea pigs to unfractionated extracts of the whole seed and to the purified allergenic fractions. Sensitization with CB-1A

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presented no difficulty because this fraction was nontoxic and highly antigenic. Sensitization with unfractionated extracts of castor bean presented difficulties owing to the toxic albumin component, ricin.

Sensitization With CB-1A

A series of 7 guinea pigs, weighing 300 ± 10 grams each, were given single intra-abdominal injections of saline solutions of CB-1A in doses ranging from 0.1 to 10 mg. of the air-dried solid. These doses contained 0.017 to 1.67 mg. of nitrogen. None of these animals showed any symptoms of toxicity during the 21-day incubational period. The animals were then challenged by intravenous administration of CB-1A with doses ranging from 0.005 to 1.0 mg. All but the one animal receiving the lowest challenging dose died in anaphylactic shock. Intravenous injection of 1 mg. of CB-1A in normal control animals had no effect. These results showed that CB-1A was nontoxic and anaphylactogenic.

Sensitization With Castor Bean Extracts

For 300-gram guinea pigs the lethal dose of a 1:20 unfractionated aqueous extract of castor bean by subcutaneous injection was about 0.005 ml. or 3.3 μ g. of protein nitrogen (PN). Single sublethal doses of the extract caused ulceration at the site of injection but did not induce anaphylactic sensitization.

Alum-precipitated dilutions of castor bean extract were employed with the prospect that the adjuvant might delay absorption of the ricin and enhance the sensitizing capacity of the extract. Results recorded in Table I show that the minimum lethal dose was the same for both untreated and alum-treated extracts. Sublethal doses of the alum-treated extract induced anaphylactic sensitivity only in occasional instances. Zoss, Koch, and Hirose¹¹ have similarly reported that alum adjuvant did not significantly delay absorption of allergenic components of ragweed pollen extracts.

Anaphylactic sensitization was accomplished by injections of unfractionated extracts that had been heated for one hour at either 80° or 100° C. These extracts induced strong sensitivity to the heat-stable allergenic protein and only occasionally a weak sensitivity to other proteins. Heating the dry, de-

TABLE I. COMPARATIVE TOXICITY IN GUINEA PIGS OF PLAIN AND ALUM-PRECIPITATED CASTOR BEAN EXTRACT

DOSE*		RESULTS	
1:20 EXTRACT (ML.)	PROTEIN NITROGEN μ G.	AQUEOUS EXTRACT	ALUM-TREATED EXTRACT
0.0025	1.7	Necrosis	Necrosis
0.0025	1.7	Necrosis	Necrosis
0.005	3.3	Necrosis	Necrosis
0.005	3.3	Died in 91 hrs.	Died in 91 hrs.
0.01	6.7	Died in 42 hrs.	Died in 42 hrs.
0.01	6.7	Died in 42 hrs.	Died in 66 hrs.
0.02	13.0	Died in 42 hrs.	Died in 66 hrs.
0.02	13.0	Died in 42 hrs.	Died in 42 hrs.
0.04	27.0	Died in 21 hrs.	Died in 42 hrs.
0.04	27.0	Died in 21 hrs.	Died in 42 hrs.

*The dose was diluted to 1 ml. for subcutaneous injection. The animals weighed approximately 300 grams.

fatted meal to temperatures up to 127° C. for one hour did not diminish the toxicity of extracts prepared from it. These observations with respect to the effect of heat on the toxicity of ricin confirm recorded findings.⁵

Subcutaneous injections of the heat-detoxified extracts led to formation of ulcers 2.5 to 3.5 cm. in diameter at the sites of injection. Carmichael¹² reported similar experience with ricin detoxified by sodium ricinoleate and by other means.^{13, 14}

Anaphylactic sensitization to unheated, aqueous extract of castor bean was finally accomplished by a succession of immunizing injections. Guinea pigs were injected subcutaneously, first with sublethal doses of the extract, and later with doses of increasing concentration. Table II shows a typical series of immunizing-sensitizing injections. Animals injected by this procedure gained weight continuously except on the day after each of the early injections. The last dose administered contained 17 to 34 times the dose that proved fatal by single injection. The Schultz-Dale tests on uteri from these animals on the twentieth day after the final injection showed anaphylactic sensitivity to CB-1A and to other proteins contained in the aqueous extract.

Schultz-Dale Experiments

The Schultz-Dale experiments were conducted by the same technique as described in previous reports from this laboratory.¹⁵ The method of sensitization and the dosage used are indicated in the tables and figures recording the results. Dilutions used in the Dale tests were calculated on the basis of the protein-nitrogen unit (PNU).*

The results tabulated in Tables III and IV and in Figs. 1 to 3 are typical of the results obtained in the Dale tests.

TABLE II. IMMUNIZATION TO CASTOR BEAN EXTRACT
(RECORD OF GUINEA PIG 5304)

SCHEDULE OF INJECTIONS			PN IN DOSE* (μg.)	BODY WEIGHT (GRAMS)	REACTION
NO.	DAY	INTERVAL			
1	1st	—	0.9	205	Necrosis
2	15th	14	1.8	294	Necrosis
3	25th	10	3.6	382	None
4	31st	6	7.2	441	None
5	38th	7	28.7	475	Necrosis
6	49th	11	115	520	Necrosis

Schultz-Dale test conducted on twentieth day after final injection; body weight, 546 grams.

*Protein nitrogen in dose.

Antigenic Relationship of CB-1A to Castor Bean Extract and to Heat-Detoxified Extracts

The antigenic relationship of CB-1A to heated castor bean extract (UCB-H) is shown in the first two experiments recorded in Table III. Guinea Pig 5243 was sensitized by a single subcutaneous injection of UCB-H containing 0.5 mg. PN. One uterine horn from this animal responded with a maximal contraction when CB-1A was added to the bath. After neutralization of antibodies for this fraction was established, addition of UCB-H induced only a very slight contrac-

*One PNU is equivalent to 0.00001 mg. of phosphotungstic acid-precipitable nitrogen.

tion of the muscle. The second horn of the same uterus responded maximally to first addition of UCB-H and this reaction desensitized the tissue to subsequent addition of CB-1A.

The second experiment shown in Table III records the results obtained on the uterine muscle of Animal 5120, previously sensitized with CB-1A. The heated extract UCB-H and the allergenic fraction CB-1A each induced contraction on first addition and neutralized the tissues to each other. The observations recorded in these two tests are typical of several similar experiments and indicate that CB-1A was the principal antigen remaining in the heated extract.

The last two experiments recorded in Table III show the antigenic relationship of CB-1A to unheated, unfractionated castor bean extract (UCB). Animal 5304 was sensitized by the series of immunizing-sensitizing injections of UCB shown in Table II. One horn of the uterus of this animal responded with a maximal contraction to a dose of 1000 PNU of CB-1A. Addition of 10,000 PNU of CB-1A induced no further response. However, subsequent addition of 10,000 PNU of UCB induced a nearly maximal contraction. In the second horn of the same uterus the first addition of UCB induced a maximal contraction and desensitized to CB-1A. The kymograph record of this experiment is shown in Fig. 1.

TABLE III. ANTIGENIC RELATIONSHIP OF CB-1A TO HEATED AND UNHEATED CASTOR BEAN EXTRACTS

ANIMAL	SENSITIZING ANTIGEN	INCUBATION PERIOD (DAYS)	HORN NO.	PREPARATION ADDED	PROTEIN NITROGEN UNITS	UTERINE CONTRACTIONS
5243	UCB-H* 0.5 mg. PN s.c.†	23	1	CB-1A	10,000	++++
				UCB-H	10,000	+
				Histamine		++++
			2	UCB-H	10,000	++++
				CB-1A	10,000	0
				Histamine		++++
5120	CB-1A 0.17 mg. PN i.a.‡	21	1	CB-1A	10,000	++++
				UCB-H	10,000	0
				Histamine		++++
			2	UCB-H	10,000	++++
				CB-1A	10,000	0
				Histamine		++++
5304 (see Fig. 1)	UCB§ (see Table II)	20	1	CB-1A	1,000	++++
				CB-1A	10,000	0
				UCB	10,000	+++
				Histamine		++++
			2	UCB	10,000	++++
				CB-1A	10,000	0
				Histamine		++++
4535	CB-1A 0.17 mg. PN i.a.	26	1	CB-1A	1,000	++++
				UCB	1,000	0
				Histamine		++++
			2	UCB	1,000	++++
				CB-1A	1,000	0
				Histamine		++++

*USB-H = unfractionated castor bean extract heated 1 hour at 100° C.

†s.c. = subcutaneous injection.

‡i.a. = intra-abdominal injection.

§UCB = unfractionated castor bean extract.

In the final experiment recorded in Table III, Guinea Pig 4535 was sensitized with CB-1A. In the uterine horns from this animal UCB and CB-1A induced reaction on first addition and each desensitized to the other.

The last two experiments showed that the immunizing-sensitizing series of injections with castor bean extract induced sensitivity in guinea pigs to CB-1A and to other proteins of the bean and that the specificity of CB-1A was distinct from that of the other proteins.

Dialyzability of the Allergenic Protein

A previous report⁶ has shown that the allergenic component of CB-1A dialyzed readily through cellophane membranes. In one experiment 41 mg. of a solid fraction was recovered from the dialyzate of 100 mg. of CB-1A. The dialyzate fraction, CB-1A-D, contained 18.9 per cent total nitrogen as compared with 18.4 per cent in CB-1A. CB-1A-D retained full allergenic potency.⁶ CB-1A-D was also anaphylactogenic as shown by the first two experiments recorded in Table IV. In the first experiment Animal 5208 was sensitized with CB-1A. In the uterine muscle from this animal CB-1A and CB-1A-D cross-reacted and desensitized each to the other. In the second experiment Guinea Pig 5242 was sensitized with the dialyzate fraction, CB-1A-D. In the uterine muscles from this animal, fractions CB-1A and CB-1A-D likewise crossreacted and desensitized to each other. These experiments revealed no significant difference in specificity or potency between the dialyzate fraction and CB-1A.

The following experiment demonstrated the allergenic protein in the dialyzate of the aqueous extract of castor bean. Fifty milliliters of a 1:20 aqueous extract of castor bean containing 0.63 mg. PN per ml. was enclosed in a cellophane tube and suspended in 50 ml. of saline saturated with toluene. After 48 hours the dialyzate (49 ml.) contained 0.044 mg. PN. Hence, nearly 7 per cent of the protein in the extract dialyzed through the membrane within 48 hours. Two guinea pigs were given single subcutaneous injections of 2.3 ml. of the dialyzate solution (UCB-D) containing 0.1 mg. PN. No evidence of inflammation or toxicity appeared during the 33-day incubational period. On the other hand, doses of the original extract containing 0.002 mg. PN regularly induced necrosis at the site of the injection; doses above 0.003 mg. PN caused death. Hence, the toxalbumin was quantitatively retained by the membrane.

Results of the Dale tests, recorded as the third experiment in Table IV and illustrated in Fig. 2, show that guinea pigs injected with UCB-D were sensitive to CB-1A and sensitive also to another antigen in the dialyzate. Crossed reactions between CB-1A and UCB-D in uterine muscle from Animal 5707 previously sensitized with CB-1A are shown in the last experiment recorded in Table IV.

The foregoing experiments show that there were at least two distinguishable antigens present in the dialyzate from castor bean extract. One of these dialyzable antigens was immunologically identical with the allergenic fraction, CB-1A. Identification of CB-1A in the dialyzate from the aqueous extract proved that this protein is native to castor bean and is not a cleavage product resulting from the chemical manipulations used to isolate it.

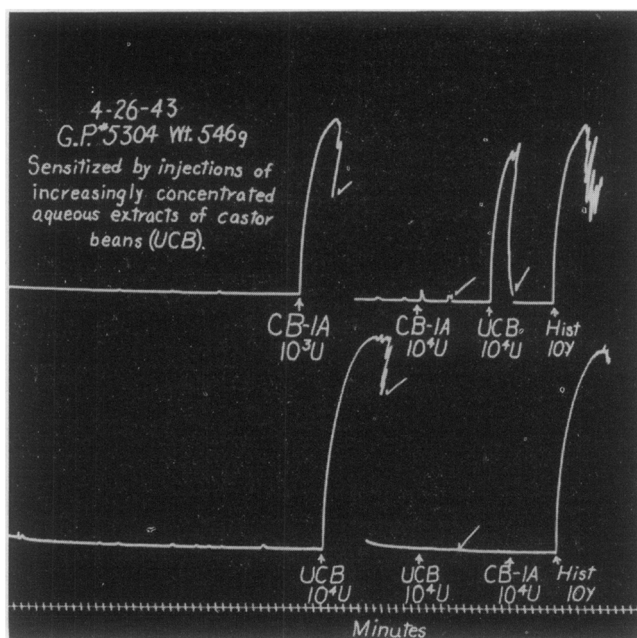


Fig. 1.—The antigenic relationship of CB-1A to unfractionated castor bean extract (UCB).

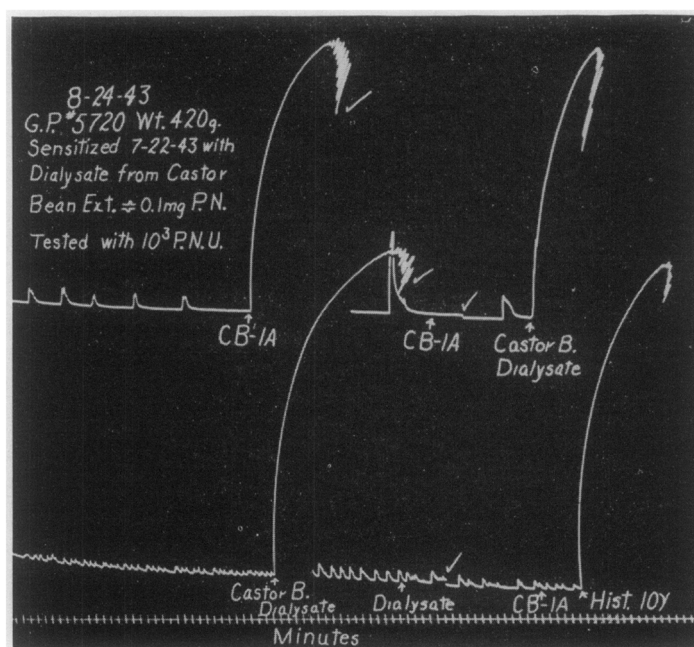


Fig. 2.—Crossed reaction between CB-1A and the dialysate from castor bean extract (UCB-D) in the uterus of an animal sensitized to UCB-D.

TABLE IV. ANTIGENIC RELATIONSHIP OF CB-1A TO THE DIALYZATE FROM CB-1A AND FROM CASTOR BEAN EXTRACT

ANIMAL	SENSITIZING ANTIGEN	INCUBATION PERIOD (DAYS)	HORN NO.	PREPARATION ADDED	PROTEIN NITROGEN UNITS	UTERINE CONTRACTIONS
5208	CB-1A 0.17 mg. PN i.a.	21	1	CB-1A-D*	1,000	++++
				CB-1A	1,000	0
			2	Histamine		++++
				CB-1A	1,000	++++
5242	CB-1A-D 0.17 mg. PN i.a.	23	1	CB-1A-D	1,000	++++
				CB-1A	1,000	0
			2	Histamine		++++
				CB-1A	1,000	++++
5729	UCB-D† 0.1 mg. PN s.c.	33	1	CB-1A	1,000	++++
				UCB-D	1,000	++++
			2	UCB-D	1,000	++++
				CB-1A	1,000	0
5707	CB-1A 0.1 mg. PN s.c.	33	1	Histamine		++++
				UCB-D	1,000	++++
			2	CB-1A	1,000	0
				UCB-D	1,000	++++

*CB-1A-D was the dialyzate from CB-1A.

†UCB-D was the dialyzate from unfractionated castor bean extract.

Antigenic Relationship of CB-65A and CB-1A

One of the objectives in the isolation of the carbohydrate-free fraction, CB-65A,⁷ was to determine the possible role of the polysaccharide in the immunological specificity of the allergen. The immunological specificity of CB-65A was investigated by the Schultz-Dale reaction and by its precipitative capacity with antiserum.

Fig. 3 shows the crossed reactions of CB-1A, CB-60C,* and CB-65A in the uterine muscle from Animal 5160, previously sensitized with CB-1A. Reaction with and desensitization to either CB-60C or CB-65A completely desensitized the tissue to subsequent test with CB-1A.

Comparative precipitin tests with fractions CB-1A and CB-65A were conducted by the antigen-dilution method with rabbit antiserum for CB-1A, which was described in an earlier report.¹⁶ Results recorded in Table V show that both CB-1A and CB-65A titrated to maximal antigen dilution of 1:51,200. Hence, there was no difference in the precipitative capacity of the protein-polysaccharidic fraction, CB-1A, and the carbohydrate-free fraction, CB-65A.

These experiments showed that the carbohydrate portion of the allergenic protein does not influence antigenic specificity.

*CB-60C was a precursor fraction of CB-65A and contained 0.47 per cent carbohydrate.⁷

TABLE V. PRECIPITIN REACTIONS OF CB-1A AND CB-65A WITH RABBIT-VS-CB-1A SERUM

ANTIGEN	ANTIGEN-DILUTION 1:						
	3,200	6,400	12,800	25,600	51,200	102,400	204,800
CB-1A	1+	2+	3+	1+	±	0	0
CB-65A	2+	2+	3+	1+	±	0	0

CB-1A and CB-65A gave no precipitate with normal rabbit serum.

Anaphylactogenic Potency of CB-1A and CB-65A

Previous studies^{17, 18} of corresponding allergenic fractions from cottonseed showed that the sensitizing capacities of those fractions were markedly influenced by the carbohydrate moiety. However, the carbohydrate did not affect either antigenic specificity or shocking capacity. It seemed pertinent, therefore, to determine the effect of the carbohydrate component on the sensitizing and shocking capacity of the castor bean fractions. For determination of the median sensitizing dose (SD50), guinea pigs weighing 300 ± 10 grams each were injected intra-abdominally at dosage levels ranging from 25 to 200 μ g. for CB-1A, and from 200 to 3,200 μ g. for CB-65A. Six to eleven animals were assigned to each

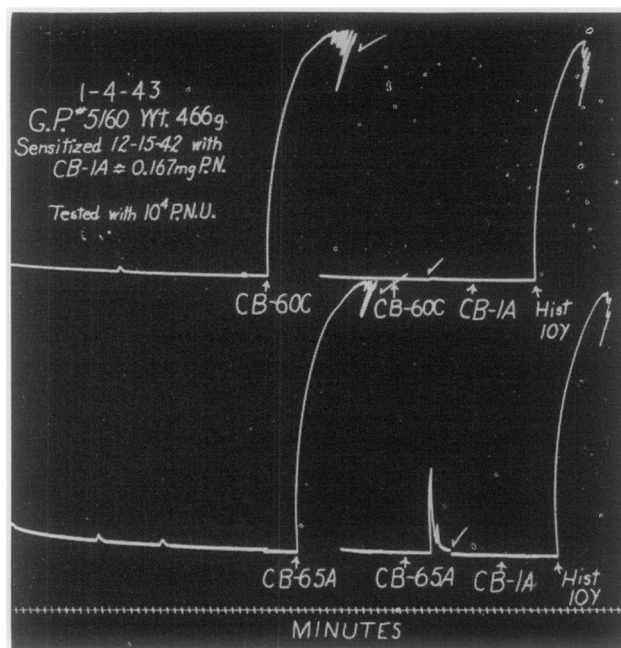


Fig. 3.—Antigenic relationship of CB-60C and CB-65A to CB-1A.

dosage level. Twenty-one days later each animal was challenged intravenously with 0.25 mg. of CB-1A in 0.5 ml. of saline. Details of the procedure and method of calculation of the results are given in a previous report.¹⁷ The data for calculation of SD50 for CB-1A and CB-65A are assembled in Table VI. Results were calculated on the basis of the air-dried solid for SD50, and also on the basis of nitrogen content for SD50_N. The SD50 for CB-1A and CB-65A, respectively, were 70 ± 13 μ g. and 820 ± 240 μ g. The SD50_N for CB-1A was 12 ± 2 μ g.; for

TABLE VI. DATA FOR CALCULATION OF SD50 FOR CB-1A AND CB-65A IN GUINEA PIGS

FRACTION	SENSITIZING DOSAGE-LEVELS: μ G SOLID							
	25	50	100	200	400	800	1600	3200
	MORTALITY RATIOS							
CB-1A	1/10	4/11	7/11	10/11				
CB-65A				0/6	2/6	4/6	4/6	5/6

CB-65A, $140 \pm 40 \mu\text{g}$. Hence, on a nitrogen basis CB-1A, containing carbohydrate, had a sensitizing capacity about 12 times greater than that of the carbohydrate-free CB-65A.

For determination of the median lethal dose (LD50) in sensitized animals, guinea pigs were sensitized at 300 grams body weight by a single intra-abdominal injection of 1 mg. of CB-1A. Twenty-one days later each animal received an intravenous challenging injection. Fraction CB-1A was tested at 5 dosage levels ranging from 1.3 to 6.75 μg . Fraction CB-65A was tested at only the 3 μg . dosage level. The data for calculation of LD50 are recorded in Table VII.

TABLE VII. DATA FOR CALCULATION OF LD50 FOR CB-1A AND CB-65A IN GUINEA PIGS SENSITIZED WITH 1 MG. CB-1A

FRACTION	CHALLENGING DOSE: μ G SOLID				
	1.30	2.00	3.00	4.50	6.75
	MORTALITY RATIOS				
CB-1A	1/10	4/10	6/10	8/10	9/10
CB-65A			6/10		

The LD50 of CB-1A derived from the complete dosage-response curve, was $2.6 \pm 0.4 \mu\text{g}$; the LD50_N was $0.43 \pm 0.06 \mu\text{g}$. For the one group of animals challenged with 3.0 μg . of CB-65A, the mortality ratio was 6/10. This ratio was identical with that observed when similarly sensitized animals were challenged with 3.0 μg . of CB-1A. In shocking capacity, therefore, CB-1A and CB-65A were essentially the same.

DISCUSSION

Investigations at this laboratory have been concerned primarily with chemical and immunological characterization of the principal allergenic constituents of oil-bearing seeds. Application of the chemical principles developed for the separation of an active component of cottonseed^{9, 19} led to the isolation of similar allergenic constituents from kapok seeds,²⁰ castor beans,⁶⁻⁸ and a number of other oil-bearing seeds. These investigations have disclosed an unusual class of proteins of potential significance to the allergist. The physical and chemical properties of allergens of the CS-1A type correspond to those of secondary protein derivatives. Immunologic evidence justifying recognition of these natural proteoses as a subclass of native proteins has been presented in preceding reports of this series.^{10, 15-18, 20} This evidence was based chiefly on antigenic properties of the isolated protein fractions and on demonstration of the protein in the dialyzate from unfractionated, aqueous extracts of oil seeds.¹⁰ The present report provides evidence justifying recognition of the castor bean allergen as another example of this type of naturally occurring protein.

The antigenic component segregated in the allergenically active polysaccharidic-protein fraction, CB-1A, from castor bean diffuses through cellophane membranes that retain the polysaccharidic component.⁶ The same antigen was demonstrated by immunologic methods in the dialyzate from aqueous castor bean extract. The toxalbumin, ricin, was quantitatively retained by the membrane. This evidence justifies the conclusion that CB-1A is a preformed, native protein of comparatively low molecular weight and is distinct from other proteins of castor bean.

Fraction CB-1A is a strong antigen despite its small molecular size. In sensitizing capacity CB-1A is of the same order as CS-1A from cottonseed. On a nitrogen basis the median sensitizing dose for guinea pigs was 12 ± 2 μ g. as compared with 14.3 ± 2.9 μ g. for CS-1A. CB-1A was also a strong shocking agent. The median lethal dose of CB-1A in guinea pigs previously sensitized with 1 mg. of the antigen was 0.43 ± 0.06 μ g. nitrogen. The previously recorded¹⁷ LD50 of CS-1A in sensitized animals was 3.4 ± 0.35 μ g. nitrogen. However, those animals were sensitized with 5 mg. of CS-1A—a 5 times larger dose—and, hence, to induce fatal shock a larger dose would be required.²¹

The protein-polysaccharidic fraction, CB-1A, and the carbohydrate-free fraction, CB-65A, were about equally potent in eliciting fatal shock in hypersensitive animals and in precipitating rabbit antiserum. Moreover, these two fractions exhibited the same antigenic specificity in Schultz-Dale tests. The antigenic specificity was therefore attributable to the protein component. On the other hand, fraction CB-1A was about 12 times more potent in sensitizing guinea pigs than CB-65A. This enhancement of sensitizing capacity by the carbohydrate moiety corresponds to the effect of the polysaccharide of the cottonseed allergenic fraction.^{17, 18}

SUMMARY

1. The allergenic protein-polysaccharidic fraction, CB-1A, from castor bean was nontoxic and anaphylactogenic.
2. The purified, carbohydrate-free fraction, CB-65A, had the same antigenic specificity as CB-1A. Fractions CB-1A and CB-65A contained a preformed protein native to castor bean and this protein was distinct from other proteins of the seed.
3. The carbohydrate moiety of CB-1A did not influence antigenic specificity but did enhance the sensitizing capacity of the protein component.
4. The protein component of fraction CB-1A was dialyzable and a protein with the same specificity was readily demonstrable in the dialyzate from unfractionated castor bean extract. An unidentified antigen was also detected in the dialyzate from the extract.
5. Heat diminished the toxicity of aqueous castor bean extract but did not destroy an ulcerative factor.
6. Alum adjuvant did not diminish the toxicity of castor bean extract when injected in guinea pigs.

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