A STUDY OF THE PURIFICATION AND PROPERTIES OF RICIN

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(Received for publication, February 26, 1947)

Ricin, the highly toxic, hemagglutinating protein of the castor bean, has always been of great interest to the chemist and the immunologist (1–4), partly because it may readily be obtained in considerable quantities. Earlier methods of preparation were carried out before the development of modern physicochemical and immunochemical methods for characterizing proteins and data are therefore lacking on the purity and homogeneity of the products obtained.

In the present report a summary is given of a portion of a study carried out during 1943–45 in consultation and collaboration with other laboratories engaged in parallel investigations which, it is hoped, will soon be published. Since results and materials were freely exchanged, some of the data included here were obtained on material from other laboratories, as indicated in the text.

EXPERIMENTAL

Purification of Ricin—A partially purified sample of ricin (Lot A) was supplied by Dr. O. H. Alderks of The Procter and Gamble Company. It had been prepared according to Dr. A. H. Corwin of Johns Hopkins University by extraction of pressed castor beans with water at pH 3.8, precipitation of the toxin by saturation with sodium chloride, solution of the precipitate in water, and reprecipitation at pH 8 by saturation with sodium sulfate. Most of this material was obtained from castor beans without selection as to color. Other samples which had been prepared from white, gray, reddish gray, or black beans were identical in all properties examined.

7.5 gm. of Lot A were dissolved in water and the insoluble matter was centrifuged off (71 mg. of N). The solution and washings (650 mg. of N), at a volume of 300 ml., were treated with Na_2SO_4 solution saturated at 37°. Precipitation was interrupted after 175 ml. had been added. The precipitate (Fraction B1) was centrifuged off and the supernatant treated with an additional 95 ml. of warm saturated Na_2SO_4 solution. The precipitate (Fraction B2) was centrifuged off, leaving in the supernatant Fraction B3 which was precipitated by saturation with Na_2SO_4. Fractions B1

and B2 were each dissolved in water and freed from insoluble material. Fraction B1 was reprecipitated twice from a volume of 150 ml. by addition of 87.5 ml. of warm saturated Na_2SO_4 solution. The material in the supernatants from these reprecipitations was designated Fraction B1′. Fraction B2 was not further purified. Fraction B3 was taken up in a small volume of water and freed of a small amount of toxin (Fraction B3a) by addition of 45 per cent by volume of saturated Na_2SO_4 solution, leaving the supernatant,

TABLE I

Fractionation of Lot A by Sodium Sulfate

	Fraction B1	Fraction B1′	Fraction B2	Fraction B3a	Fraction B3b
Yield, *mg. N*	303	27	35	8	202
$[\alpha]_D$,* *degrees*	−28	−32	−32	−39	−59
Minimum hemagglutinating dose, γ *N*	0.3	0.3	0.3	0.3	1000
Intraperitoneal toxicity for mice					
N injected					
γ					
0.01	0/3†	0/3		0/3	
0.1	3/3 <96 hrs.‡	3/3 <96 hrs.	3/3 5 days	1/3 <95 hrs.	
1	3/3 <24 "	3/3 <24 "	3/3 <70 hrs.	3/3 <45 "	0/3
10	3/3 <24 "	3/3 <24 "	3/3 <20 "	3/3 <20 "	0/3
100					0/3
1000					0/3

* Optical rotation calculated for protein = N × 6.25.

† The number of deaths within 10 days to the number of mice injected.

‡ Longest life period of any mouse which succumbed.

Fraction B3b. Yields, optical rotation, hemagglutinating power, and toxicity for mice are given in Table I. It is evident that most of the toxin was concentrated in Fraction B1, which showed the lowest specific rotation. Fraction B3b, which contained about 30 per cent of the original soluble N, had less than 0.0001 of the toxicity of Fraction B1 and was readily dialyzable through cellophane membranes.

The toxicity of the fractions was estimated by intraperitoneal injection of 0.5 ml. of 10-fold serial dilutions of solutions standardized on the basis of nitrogen content. Female mice weighing 20 ± 3 gm. were used, three being injected with each dilution. Death or survival for 10 days was used as the end-point.

The hemagglutinating potency was measured as the minimum quantity of nitrogen giving definite agglutination (+) of 0.2 ml. of a 4 per cent suspension of washed human group O erythrocytes. All tests were carried out in a volume of 1 ml. and were read after incubation at 37° for 1 hour.

Crystalline Ricin—Ricin was obtained in crystalline form by Kunitz[1] and also by Cannan.[2] Samples of three or four times crystallized material were made available through the courtesy of Dr. R. K. Cannan, Dr. M. Levy, and Dr. A. E. Benaglia of New York University.

Characterization of Crude and Purified Ricin—A comparison of the properties of crude ricin (Lot A), of Fraction B1, and of crystalline ricin is given in Table II. Both Fractions B1 and crystalline ricin showed a lower optical rotation than did the crude ricin; they were both electrophoretically and ultracentrifugally homogeneous, while Lot A showed two components in the ultracentrifuge and three components by electrophoresis. From sedimentation and diffusion, Fraction B1 was found to have a molecular weight of roughly 85,000 for a partial specific volume of 0.75. The sedimentation constant of crystalline ricin was slightly lower, leading to a molecular weight of 77,000, a value not considered significantly different from that of Fraction B1, since precise temperature control was not possible during the measurements of sedimentation. From these data a frictional ratio, f/f_0, of 1.2 was found for Fraction B1 and the cystalline product, indicating a ratio of length to width of about 4 or 5 for a cylindrical or ellipsoidal shape and an unhydrated molecule. The mobilities of Fraction B1 and crystalline ricin were determined over a pH range of 4.0 to 8.5. The respective isoelectric points were calculated as 5.2 and 5.4 to 5.5, values that were practically the same.

A more precise comparison of the toxicity of these preparations was obtained by determining the minimum quantity of nitrogen (1 toxic unit) necessary to kill a 20 gm. mouse in 24 hours (modification of a method described by Dr. A. H. Corwin[3] of Johns Hopkins University). Ten mice were used for each sample to be assayed. Assays were carried out at New York University and at the University of Chicago through the courtesy of Dr. R. K. Cannan. Data in Table II indicate that samples of Lot A and Fraction B1 were only one-third and two-thirds as toxic as crystalline ricin.

The hemagglutinating potencies of Lot A and Fraction B1 were about the same within experimental error, 0.3 γ of N giving minimum agglutination of 0.2 ml. of a 4 per cent suspension of washed human erythrocytes when the tests were carried out in saline. The hemagglutinating power of

[1] Kunitz, M., personal communication.

[2] Cannan, R. K., personal communication.

[3] Corwin, A. H., personal communication.

TABLE II

Properties of Ricin Preparations

	Lot A	Fraction B1	Crystalline ricin
$[\alpha]_D$, *degrees*	−41.5	−28 to −30	−26
Sedimentation constant, *S*, and relative concentrations of components	5.1 (67%) 0.8 (33%)	5.3* (100%)	4.8 (100%)
$D_{20} \times 10^7$ cm.² per sec.		6.0†	
Mol. wt.‡		85,000	77,000§
f/f_0		1.2	1.2§
Approximate ratio of length to width‖		4–5	4–5
Electrophoretic mobility ($u \times 10^5$) and % components			
pH			
8.5	−1.0 (19%) −1.9 (77%) −4.1 (4%)	−2.2 (100%)	−2.2 (100%) −3.1 (Trace)
7.4	−1.3 (96%) −3.5 (4%)	−1.2 (100%)	−1.0 (100%)
6.05		−0.6 (100%)	−0.3 (100%)
5.0			+0.5 (100%)
4.9		+0.2 (100%)	
4.0			+2.1 (100%)
Isoelectric point, *pH*		5.2	5.4–5.5
Minimum hemagglutinating dose, γ *N*			
In saline	0.3	0.3	3–5
" 1:100 normal rabbit serum	0.3	0.3	0.5
Toxic unit per 20 gm. mouse, γ *N*¶	0.76	0.40	0.25
Crystalline ricin content from toxicity data, *i.e.* toxic N %	33	63–67	100**

Quantitative precipitin reaction with 1.0 ml. 1:20 pooled rabbit anti-Fraction B1 serum

N added	Extinction (Folin-Ciocalteau) of 1.0/2.5 ml. aliquot of washed ppt.		
γ			
0.5	0.067	0.103	0.09
1	0.117	0.175	0.175
2	0.206	0.36	0.335
3	0.276	0.51	0.47
5	0.43	0.70	0.68
7.5	0.593	0.84	0.87
10	0.71	0.97	0.93
15	0.79		

TABLE II—*Concluded*

N added	Extinction (Folin-Ciocalteau) of 1.0/2.5 ml. aliquot of washed ppt.		
N to give extinction of 0.6, γ	7.6	4.0	4.0
Immunologically active ricin in preparation, %	53	100	100**
Detoxified N in preparation, %††	20	33–37	0**

* Average of 5.3, 5.4, 5.2, and 5.4 for several preparations at concentrations of 0.77, 0.53, 0.50, and 1.05 mg. of N per ml. At 2.0 mg. of N per ml. S was 5.0.

† Average of 5.98 and 6.08 for two preparations, corrected to two significant figures.

‡ For a partial specific volume of 0.75.

§ The diffusion constant is assumed to be the same as for Fraction B1.

‖ From f/f_0, for cylindrical or ellipsoidal shape.

¶ Determined at the University of Chicago.

** Assumed.

†† Immunologically active ricin minus crystalline ricin content from toxicity data.

crystalline ricin, however, was found to be only about one-tenth that of Fraction B1. Gilman and Phillips[4] observed that addition of normal serum increased the hemagglutinating potency of crystalline ricin. This was confirmed (Table II). By fractional precipitation with acetone it was found possible to obtain fractions with as little as one-fortieth of the hemagglutinating power of Fraction B1. Addition of serum or of 0.5 per cent of formalin restored hemagglutinating potency. These data suggest that hemagglutinating power, while a characteristic of ricin itself, is influenced by the presence of other substances.

Antisera to Fraction B1 and to crystalline ricin were prepared by immunization of rabbits with a formalinized toxoid prepared as follows: A solution containing 0.5 mg. of N of Fraction B1 per ml., buffered at pH 7.4 with 0.02 M phosphate and 0.15 M NaCl and containing 0.5 per cent formalin (1 part of 37 per cent formaldehyde solution to 200 parts of buffered ricin solution at pH 7.4), was kept at 37° for 5 days (in some instances 5 per cent formalin was used). This procedure resulted in about a 100- to a 1000-fold reduction in toxicity when 0.5 per cent formalin was used, and about a 1000-fold reduction in toxicity with 5 per cent formalin. Complete detoxication could not be obtained with formalin, as had previously been reported by Heymans (5). Marked loss of antigenicity occurred with formalin at pH 8.5 and above. Before use, the formalinized toxoid was precipitated by addition of enough 1 per cent alum to insure maximum flocculation. Because of the incomplete detoxication and the extreme susceptibility of the rabbit to ricin it was found necessary to

[4] Gilman, A., and Phillips, F. S., personal communication.

give each rabbit subcutaneous injections of 2.5, 5, and 5 γ of toxoid N at 5 day intervals to induce some immunity before intravenous injections were started. Each rabbit then received four intravenous injections weekly for 4 weeks, as follows: two injections of 0.01 mg. of N, two of 0.03, four of 0.05, four of 0.15, and four of 0.50 mg. of toxoid N. Rabbits were bled by cardiac puncture 5 days after the last injection. A later course of intravenous injections could be given with 0.5 or 1 mg. of N per injection. The animals became so resistant to the toxic effects of ricin that immunization could be continued with equal doses of an alum precipitate of undetoxified ricin.

Antiricin sera may be standardized by measuring their capacity to neutralize the toxic effects of ricin on intraperitoneal injection into mice or by assay of their inhibition of the hemagglutinating power of ricin. Assays of the potency of a large number of rabbit antisera and of several horse and goat antisera by both methods gave parallel results within experimental error, providing strong support for the concept that hemagglutinating power and toxicity are properties of the same protein molecule.

A comparison of the immunochemical properties of Fraction B1 and crystalline ricin was afforded by determination of their relative capacities to precipitate with rabbit antiricin sera. These analyses were carried out by addition of increasing amounts of a given preparation of ricin to a measured volume of antiserum. After 1 hour at 37° and 1 week in the ice box the precipitates were centrifuged off, washed twice in the cold with saline, dissolved in water with the aid of 1 or 2 drops of 0.5 N NaOH, and made up to 2.5 ml. Aliquot portions were analyzed by a modification of the Folin-Ciocalteau tyrosine method as described by Heidelberger and MacPherson (6). Extinction readings in the photoelectric colorimeter plotted against the amounts of antigen nitrogen used gave a smooth curve up to the point of complete precipitation of antibody. The curves obtained with different preparations of ricin could be compared with respect to their capacities to precipitate antiricin by determining the ratios of the amounts of antigen required to give the same extinction. From Table II it is evident that equal amounts of Fraction B1 or crystalline ricin nitrogen were of equal potency, within experimental error, in precipitating antiricin from an antiserum to Fraction B1. Similar results were obtained with an antiserum to crystalline ricin. The extinction values obtained with equal amounts of Lot A, however, were uniformly lower. Comparison of the amounts of Lot A and of crystalline ricin or Fraction B1 nitrogen required to give an extinction reading of 0.6 indicates that 7.6 γ of N of Lot A were required in contrast to 4.0 γ of N of fraction B1 or crystalline ricin. With respect to its content of material immunochemically reactive as ricin, Lot A would be only 4.0/7.6 or 53 per cent pure. Comparisons made at any extinction reading in the antibody excess range may be used.

After removal of the specific precipitates, supernatants were divided in half and tested for the presence of antibody or antigen. With the antiserum to Fraction B1 as well as with antiserum to crystalline ricin, it was found that a given supernatant did not contain both antigen and antibody, even when crude products such as Lot A were used. Supernatants contained excess antigen or antibody, and in some instances neither antigen nor antibody could be detected. As demonstrated by Kendall (7), this provides strong evidence that the antisera contained only antibody to a single antigen, in this instance antibody to crystalline ricin or its immunological equivalent.

Although crystalline ricin and Fraction B1 were immunochemically identical on a nitrogen basis, in addition to having the same electrophoretic and ultracentrifugal properties within experimental error, Fraction B1 was only about two-thirds as toxic as crystalline ricin. This was taken to indicate that a portion of the ricin in Fraction B1 was non-toxic. Lot A also contained more ricin (53 per cent) by immunochemical analysis than could be accounted for by toxicity assays. A number of other crude ricin-containing fractions were analyzed by both methods and, in each instance, it was found that about one-third to one-half of the immunochemically measured ricin was non-toxic.

Accordingly, it was considered of interest to try to establish whether this non-toxic form of ricin existed in the intact castor bean or whether it was a result of chemical treatment in the course of purification. A number of different extracts of castor beans were assayed for their content of material immunochemically reactive as ricin and by Dr. A. E. Benaglia for toxicity and heat-coagulable protein. The results are given in Table III. Samples 272 and O were obtained from intact castor beans. The hulls were removed and an aqueous extract was prepared. Samples 217, 218, and M were extracts of castor beans pressed to remove oil; Sample 217 had been defatted with carbon tetrachloride-petroleum ether. In all instances, even in the aqueous extracts of shelled castor beans, ratios of toxic N to immunologically reactive N ranged from 0.46 to 0.64, indicating the presence of considerable non-toxic ricin. In these extracts the immunologically active nitrogen accounted for most of the heat-coagulable protein.

Since crystallization effects a separation of the toxic and non-toxic forms of ricin, it could be anticipated that the mother liquors, after removal of as much crystalline ricin as possible, should contain a lower proportion of toxic N to immunologically reactive N than the crude products. Sample 216, obtained from Dr. R. K. Cannan, Dr. M. Levy, and Dr. A. E. Benaglia, was such a mother liquor. The data in Table III show that the ratio of toxic to immunologically active N in this product was only 0.32, as compared with values of 0.46 to 0.64 for the unfractionated samples.

Estimation of immunologically reactive N by the quantitative precipitin method has been useful in following enzymatic digestion of ricin by pepsin

TABLE III

Correlation of Immunologically Active and Toxic Nitrogen in Ricin-Containing Crude Fractions of Castor Beans

Sample No.	272	O	217	218	M	216	Crystalline ricin
Total N, *mg. per ml.*	3.38	1.14	4.46	2.74	5.38	12.35	
Coagulable N, *mg. per ml.*	0.92		1.58	0.72	2.04	10.32	
	(27%)	(23%)	(34%)	(26%)	(38%)	(84%)	100%
T.u.,* γ N per 20 gm. mouse†	2.2	2.6	2.6	2.6	1.9	1.6	0.35
$100 \times \frac{t.u., crystalline}{t.u., sample}$ = % *toxic N*	15.5	13.4	13	13	18	21	100‡

Quantitative precipitin reaction with 1.0 ml. 1:20 pooled rabbit anti-Fraction B1 serum

N added	Extinction (Folin-Ciocalteau) of 1.0/2.5 ml. aliquot of solution of washed ppt.						
γ							
0.5							0.09
1							0.175
2							0.335
2.5	0.101	0.113	0.135	0.119	0.113	0.315	
3							0.47
5	0.226	0.255	0.243	0.233	0.228	0.538	0.68
7.5	0.328	0.350	0.368	0.294	0.373	0.705	0.87
10	0.429	0.48	0.47	0.42	0.475	0.81	0.93
15	0.581	0.62	0.63	0.545	0.575	0.97	
20	0.695	0.74	0.735	0.655	0.735	1.14	
30	0.84	0.89	0.895	0.805	0.89	1.30	
40	0.98	1.07	1.04	0.95	1.05	1.40	
N to give extinction of 0.6, γ	15.8	14.5	14	17.2	14.4	6.0	4.0
Immunologically active N, *% of total N*	25	28	28.5	23	28	66	100‡
Ratio, toxic N to immunologically active N	0.62	0.48	0.46	0.57	0.64	0.32	1.0

* Toxicity unit.

† Determined at New York University.

‡ Assumed.

and trypsin. Digestion of ricin by pepsin at pH 4 or by trypsin at pH 7.4 or 8.5 was found to take place slowly, 1 to 2 weeks being required for the appearance of 40 to 50 per cent of products of low molecular weight. The

data in Table IV show that the disappearance of substance immunochemically reactive as ricin corresponded closely to the appearance of material of low molecular weight and to loss of toxicity.

TABLE IV

Digestion of Crystalline Ricin by Pepsin and Trypsin

Total N precipitable by	Peptic digest	Control	Tryptic digest	Control
Trichloroacetic acid, %	47	100		
Half saturation with Na_2SO_4, %	47	100	56	98
Non-dialyzable N, %			58.5	100

Quantitative precipitin curves of digested and untreated materials with 1.0 ml. 1:20 dilution of pooled rabbit antiricin serum

N added	Extinction (Folin-Ciocalteau) of 1.0/2.5 ml. aliquot of solution of washed ppt.			
γ				
1	0.088	0.154	0.093	0.193
2	0.141	0.305	0.192	0.384
4	0.302	0.568	0.368	0.65
6	0.40	0.75	0.466	0.825
8	0.56	0.875	0.613	0.94
10	0.68	1.03	0.715	1.10
15	0.83	1.15	0.87	1.19
20	0.99	1.22		
N to give extinction of 0.6, γ	8.8	4.0	7.7	3.9
Immunologically active N, %	46	100	51	100
Toxicity for mice				
0.05 γ N injected			1/3 6 days	3/3 < 45–95 hrs.
0.10 " " "	2/3 < 70, 95 hrs.	3/3 < 45–70 hrs.		

After dialysis of the digested samples the non-dialyzable N showed 93 per cent of the precipitating power of crystalline ricin with antiserum.

DISCUSSION

By fractional precipitation with sodium sulfate it is possible to obtain a highly toxic protein (Fraction B1) which is electrophoretically, ultracentrifugally, and immunochemically homogeneous, and identical by these criteria with samples of crystalline ricin.[1,2] However, Fraction B1 is only two-thirds as toxic as the crystalline product. Assays were made of a variety of crude extracts of castor beans and of partially purified products for toxicity and for material immunochemically identical with ricin. These showed that there was uniformly less toxic nitrogen present than

immunologically active ricin nitrogen. The amount of substance immunochemically identical with ricin accounted for most, if not all, of the heat-coagulable protein in these materials. It may, therefore, be inferred that the castor bean contains two proteins, one of which is toxic, the other non-toxic, but which are immunochemically, ultracentrifugally, and electrophoretically identical. The toxic ricin in the castor bean extracts and in purified products, including Fraction B1, accounts for about one-half to two-thirds of the total ricin. It is possible that further studies at various stages in the development and germination of the castor bean might throw light on the relationships and mode of formation of toxic and non-toxic ricin.

The quantitative precipitin method could not be used to standardize antiricin sera on the basis of their content of specifically precipitable nitrogen, since ricin was found to precipitate normal serum protein as well as other proteins. The washed specific precipitates from antiricin sera consisted of ricin, antiricin, and coprecipitated normal serum protein, but this did not interfere with the use of calibration curves of antiricin sera for the estimation of the amounts of total ricin in various preparations.

Alternative possibilities to the existence of toxic and non-toxic ricin in the non-crystalline preparations have been considered but were rejected as contrary to the evidence. For example, if it is assumed that, although crystalline ricin precipitates the same amount of total nitrogen from antisera as does the less pure Fraction B1, the precipitate consists of more antibody and less non-specific protein, as might be expected, the antibody would be exhausted by less crystalline ricin than by Fraction B1. Actually the equivalence zone is found at the same level of added antigen in both instances. This also disposes of the possibility that crystalline ricin precipitates less antibody and more non-specific protein.

These studies provide an additional instance of the value of quantitative immunochemical methods, especially when used in conjunction with other physicochemical and biological techniques, in the characterization and estimation of proteins (for other studies *cf.* (8–10)).

SUMMARY

1. Highly toxic, hemagglutinating preparations of ricin can be obtained by fractional precipitation with sodium sulfate. These products are electrophoretically, ultracentrifugally, and immunochemically homogeneous and identical in these respects with crystalline ricin, but are only two-thirds as toxic. The toxic and non-toxic forms can be separated by crystallization.

2. Immunochemical and toxicity assays of crude extracts of castor beans indicate that these contain both forms of ricin.

3. Ricin was found to have an isoelectric point of about 5.2 to 5.5 and a molecular weight by sedimentation and diffusion of about 77,000 to 85,000 for an assumed partial specific volume of 0.75.

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