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IV. Toxic Constituents of the Castor Bean*

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Introduction

The most familiar substance obtained from castor beans is, of course, castor oil. Because of the extensive use of this substance and its derivatives in industry, the raising and processing of castor beans is a huge industry all over the world. Castor oil is, however, a material of very low toxicity and will not be examined further in this presentation. The materials which will interest us are soluble in water and insoluble in the oil. Industrially, it is necessary to ensure their absence in the oil since even traces of them carry with them enzymes which rapidly destroy the oil and thus render it economically worthless. There has been some confusion on this point in the popular mind. Castor oil does not owe its purgative properties either to the toxin or to the allergen which will be discussed in this paper, since both are absent in the oil.

As indicated, there are two substances of extreme physiological potency in the aqueous juice of the castor bean. The first to be recognized was the toxin, ricin. Its toxicity was known to the ancients but the isolation of the active principle in reasonably pure form was performed by Stillmark.[1] The second active substance is the castor bean allergen, whose action was recognized by Alilaire in 1914.[2]

Ricin

Ricin has been a toxin of considerable historical interest, since the pioneers in the study of toxicity and immunity frequently

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used it as their model substance.[3] It is a haemorrhagin, inducing multiple capillary haemorrhages. It does not require the degree of organization characteristic of a vascular system for its action, however, since we have demonstrated that it is also toxic to single cells such as protozoa. While not so potent as the bacterial exotoxins, it is much more potent than alkaloids or inorganic poisons. Injection of ricin in sublethal doses produces immunity, after a suitable incubation period, due to the formation of an antitoxin. It is also possible to make a toxoid for this purpose.

In the isolation and purification of the toxin, no chemical or biochemical assay has been found suitable. It is necessary, then, to rely on determinations of toxicity as the means of assay. Two general methods have been used for this purpose, determination of the dose which kills 50 per cent of the animals tested and interpolation on a dose–survival time curve. For routine assays, the dose–survival time method is much more convenient, requiring only a single test on each of a few animals for each assay. Survival times with minimum lethal doses of ricin approach one week and, if the LD_{50} or MLD method were to be used, it would be necessary to adjust the dose for each assay a sufficient number of times to secure some deaths and some survivors. Because of the internal damage done by ricin, it is often difficult to tell whether animals which have survived for a week and then die have died due to the action of the toxin alone or whether there are superimposed infections which have added their effects to those of the toxin. By using the dose–survival time method it is possible to cut survival times to the neighbourhood of 24 h, thus eliminating the possibility of secondary infections and, at the same time, markedly speeding the assays.

The appearance of a typical dose–survival time curve for ricin is shown in Fig. 1.

Using the assay method indicated, numerous factors which influence the yield of toxin isolated were catalogued and the isolation of a potent crude toxin was made possible. Using such material as their starting substance, Kunitz and MacDonald[4] succeeded in crystallizing ricin.

The juice of the castor bean contains a number of enzymes, among them lipases and phosphatases. Crystalline ricin is free

of both of these types of enzymatic activity. No special prosthetic groups have been found on the toxin. A very small amount of carbohydrate seems to be inseparable from it. Otherwise it appears to be a normal protein. Its ultraviolet absorption spectrum is typical of the aromatic amino acids.

Levy and Benaglia[5] have shown that ricin is irreversibly denatured at 50–80°, the exact temperature being highly dependent upon the acidity of the solution. Denatured ricin resembles

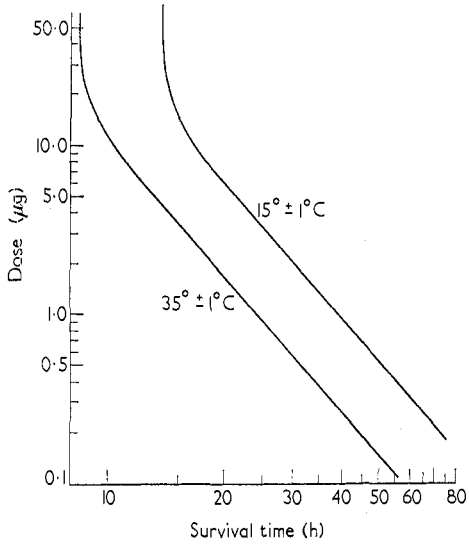

Fig. 1. Dose–survival time curve of ricin

cooked egg white in its appearance. The live toxin is surface-active, as can be seen by a simple demonstration. If a small beaker containing a ricin solution is stirred and then a pinch of talc is dropped on the surface, the talc remains stationary on the ricin film covering the surface, even though the underlying liquid continues to rotate.

One interesting property of ricin, both crude and crystalline, is its ability to cause non-specific agglutination.[1] The addition of a minute amount of ricin causes washed red cells to agglutinate; just as readily, it causes milk to curdle.

The Castor Bean Allergen

The chemistry of the castor bean allergen has been most extensively studied by Spies and Coulson.[6, 7] These investigators have shown that it is stable to boiling water. Thus a crude preparation containing both toxin and allergen can be heated to coagulate the toxin, and the filtrate from the coagulum will have the allergen in solution.

The results of a preliminary analysis on the allergen are shown in Table I.

Table I. Amino acid analysis of castor bean allergen. (Percentage of total nitrogen)

Nitrogen in the form of	%
Humin	0·1
Ammonia	13·6
Cystine	5·0
Histidine	1·0
Arginine	26·6
Lysine	3·2
Glutamic acid	8·6
Tyrosine	1·1
Tryptophan	0·0
Monoamino acid fraction	19·3
Dicarboxylic acid fraction	8·6
TOTAL	87·1

The Toxin–Allergen Compound

We attempted to perform the converse separation, reasoning that a sufficient number of recrystallizations of the toxin would remove the allergen, which was thought to be an impurity. While it is true that crude toxin contains more allergen than the recrystallized material, repeated crystallization does not lower the allergen content below a minimum of approximately 14 per cent. It was found that the allergen could be removed slowly from the solution by dialysis, although even after four weeks' dialysis about 2 per cent remained behind. This process gives the purest allergen which has been prepared to date, since it has undergone a

process of repeated crystallization. However, all attempts to crystallize the allergen by itself or the toxin from which allergen had been removed were fruitless. We concluded from these studies that the crystalline toxin is a molecular compound between the toxic component and the allergenic component of the castor bean. This concept was further substantiated by the observation that the reconstitution of the toxin–allergen mixture permitted crystallization of the protein. Allergen purified by dialysis in this manner is undoubtedly identical with the 'peptide C' of Moule.[8] A recent report states that the castor bean allergen has been separated into twelve fractions, immunologically identical, but experimental details are not yet available.[9]

Electrophoretic studies have yielded further information about this compound. The isoelectric point of crystalline toxin is 6·6. The allergen associated with it has a very low isoelectric point.[3, 7] It can be shown that the toxin has free guanidinium groups, making it cationic, and that the allergen has free carboxyl groups, making it anionic. Hence we may picture the nature of the combination between the two substances as a salt of a substituted guanidino base and a substituted carboxylic acid. This explains the impossibility of completing the purification of the toxin by repeated crystallization. Because of the marked difference between the two isoelectric points, however, electrophoretic separation of the two active substances is quite feasible and permits sharper purification than is the case with dialysis.

A sample of ricin was crystallized four times, then extracted 25 times with 0·1 per cent sodium sulphate and finally crystallized again. This sample, like other crystalline ricin preparations, still caused fatal shock to guinea pigs sensitized only to the allergen. A solubility test was performed. As conducted, the solubility plot should have a slope of 45° and a sharp break to horizontal at saturation. It should then remain horizontal as more of the saturating phase is added. The experimental curve had a slope of 35° 52′ but then had a sharp break at saturation and remained horizontal. The deviation of 9° 08′ from the theoretical angle of 45° is interpreted as being due to the formation of a small amount of insoluble material by denaturation, a process which we have been unable to avoid completely in the recrystallization of this protein. With this exception, the solubility curve indicates that

the substance is a pure chemical individual. Thus this type of thermodynamic reasoning shows that the combination of toxin with allergen forms a single chemical individual, a substantial confirmation of our theory that the crystalline toxin is a molecular compound.

Agglutinating Power and Toxicity

Stillmark[1] first noticed the phenomenon of haemagglutination associated with ricin. That observation marked the commencement of an inquiry into the possible relationship between agglutinating power and toxicity.

An examination of various plant agglutinins shows that their agglutinating power does not parallel their toxicities. Thus phasin, obtained from ordinary navy beans, is a powerful agglutinin but its toxicity is vanishingly small. Tested on pigeon blood, ricin is a weaker agglutinin but it is an infinitely more powerful poison.

Since the work of Mueller in 1899,[10] several investigators have reported that the agglutinating power of ricin can be destroyed by enzymatic digestion without loss of toxicity. Field[11] reported complete destruction of toxicity with very little loss in agglutinating power. Enzymatic digestions of ricin were carried out by Fuchs and Falkensammer[12] who found that a 3- to 8-hour digestion with pepsin appreciably decreased the agglutinating power, while the toxicity was unaffected. The same effect was reported for a one-hour digestion with pancreatin. We have confirmed these results by obtaining lyophilized samples of digested ricin which have at least 90 per cent of the toxicity of the starting material and less than 1 per cent of the agglutinating power.

Olmer and Sauvan[13] reported that heating a solution of the protein to 55° destroyed the agglutinating properties but that toxicity was lost only at boiling temperature.

An entirely independent line of evidence that agglutination and toxicity are separate actions was provided by the carefully controlled studies of Clarke.[14] Correlating and extending earlier contradictory observations, Clarke showed that antiricin serum in low concentrations is a powerful promoter of agglutination. In higher concentrations, where it exerts an inhibiting effect on agglutination, normal serum not containing the antibody is as effective

as the antiserum as an inhibitor. Thus the antibody to the toxic action is not an antibody to the agglutinating action.

Chemistry of Agglutinating Groups

We have extended the argument from biological to chemical grounds. A preliminary chemical differentiation of the groups responsible for the agglutinating and toxic actions of ricin was secured by the use of formaldehyde, on the one hand, and ninhydrin on the other. Formaldehyde reacts with both amino groups and guanidinium groups and destroys both toxicity and agglutinating power. Ninhydrin reacts only with amino groups and destroys only toxicity, not agglutinating power.

It we assume that agglutinating action is related to the presence of guanidinium groups, a further chemical differentiation between toxic and agglutinating activity may be obtained with formaldehyde alone. Since the action of formaldehyde on guanidinium groups will not be strongly pH-dependent while the concentration of free amino groups will decrease approximately tenfold for each pH unit, if the reaction mixture is acidified, acidification should accomplish a selective destruction of agglutinating activity. It was found possible to bear out this prediction and reduce agglutination about twice as much as toxicity by exposure to formaldehyde in the acid range.

Acetylacetone and glyoxal are reagents that are reasonably specific for guanidinium groups,[15] when the condensation is carried out between pH 2·5 and 5. Since ricin is quite stable to acid, condensations with these agents were performed. Approximately 80 per cent of the agglutinating power was destroyed without altering the toxicity.

The Sakaguchi reagent[16] (α-naphthol with hypobromite) reacts with some guanidino compounds, arginine among them. Although the hypobromite probably destroys some amino groups, our experiments indicate that the agglutinating power of dilute ricin solutions can be decreased nearly to the vanishing point, while 50 per cent of the toxicity remains. In summary, evidence from several sources shows that guanidinium groups are required for the agglutinating action while amino groups are not.

Mechanism of Agglutination

A start on the investigation of the mechanism of agglutination was made by von Liebermann[17] who concluded that ricin combines with erythrocytes, forming a complex which can be decomposed with the liberation of free ricin. He postulated ricin in an acidic role, combining chemically with cell stroma, acting as a base. We see that by reversing the roles of acid and base, this explanation would be acceptable today, with the cell stroma supplying phosphate groups to combine with the guanidinium groups of the ricin.

The explanation of von Liebermann was contradicted by the conclusions of Reid[18] who believed that the toxin was attached by adsorption to red cells and others in direct proportion to the lipoid content of the cell. The two points of view can be reconciled if we substitute phospholipids for lipoid materials in general. In the course of a qualitative experiment with sodium 2-ethyl-1-hexyl sulphate, a commercial anionic wetting agent, parallel experiments were performed with 1 per cent ricin and 1 per cent egg albumin, both in saline. In the case of the egg albumin, precipitation could be obtained with the wetting agent only in the range more acidic than pH 5. This indicates that increased alkalinity removes the positively charged groups on egg albumin which bind the anionic wetting agent. Ricin, on the other hand, gave precipitates with the wetting agent at pH values ranging from 3 to 9. This indicates that the positive charges responsible for the binding of the wetting agent are approximately 10,000 times as stable as those acting in the case of egg albumin. This is the approximate factor of the difference in basicity between amino and guanidino groups and thus serves to implicate the guanidinium ions as the ones acting in the case of ricin.

Electrophoretic studies on red cells have shown that they are capable of maintaining a negative charge in solutions of considerable acidity and can be treated as polyvalent anions. Having established that ricin is a polyvalent cation, we may assume that the red cells play a similar rôle in the agglutination process to that played by the anionic wetting agent in the preceding experiment. It is then reasonable to assume that, when the anionic red cells are placed in a water solution of ricin and electrolytes, the red

cells displace the hydrophilic anions on the ricin surface and form bridges between ricin molecules. The latter, in turn being polyvalent cations, form bridges between red cells. The whole system thus becomes an insoluble three-dimensional polymer.

Numerous tests have been made of the effect of alteration of the agglutination conditions by various reagents. So far all of these have proved to be consistent with the relatively simple hypothesis presented above.

One might object that the analysis of castor bean allergen as presented shows an even higher proportion of arginine than do the published analyses for ricin.[19] In spite of this we have established the fact that the allergen is not an agglutinin, even when prepared by a method that does not involve heating. The explanation must be sought in the relative geometries of the two molecules. Notwithstanding the arginine content of the allergen molecule, its isoelectric point is quite acidic, indicating that its surface properties are controlled by its carboxylate residues. Thus the arginine present must be buried within the molecule. In the case of ricin, the reaction with the wetting agent shows that the cations are on the surface, and the bridge-forming properties with red cells show that they are exposed on at least two sides of the surface. The latter fact can be demonstrated by still another experiment. If a small number of red cells is added to a large excess of ricin, no agglutination takes place. When washed, these cells are no longer anionic but are now cationic. Thus one side of the ricin molecules has reacted with the negatively charged phosphate groups on the cell surfaces, leaving the other side of the ricin molecules exposed on the surface of the altered red cells and presenting a net positive charge to the field. We thus find an explanation for the agglutinating power of ricin in the presence and arrangement of the guanidinium residues on the surface of the molecule.

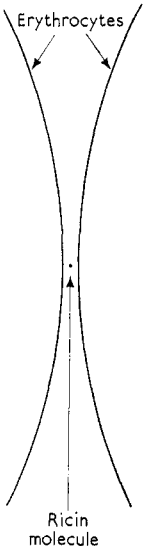

Fig. 2

It is interesting to represent the relative sizes of the red cell and the ricin molecule by a drawing to scale (Fig. 2).

Mechanism of Action of Ricin

The mechanism by which ricin produces its toxic action is not known. Starting with the classical work of Flexner,[20] the pathological changes caused by the action of the toxin have been carefully detailed. More recently exhaustive studies of the biochemical changes produced by its action have also been recorded by Thomson.[21] It has been shown that a weak proteolytic activity parallels the toxic action of the protein through a number of chemical and physical changes.[22] In spite of these studies, the large toxic effect caused by a small amount of the toxin has not been accounted for adequately. Chemical studies bearing on the nature of the toxophoric group and pharmacological studies on synergists and antagonists provide the background for our current studies on the mechanism of action of the toxin.

Chemistry of Toxoid Formation

In studying the formation of toxoid by the action of formaldehyde upon ricin, we have examined the effects of changes of six variables. These were: (1) the concentration of ricin; (2) the concentration of formaldehyde; (3) the time of incubation; (4) the temperature of incubation; (5) the alkalinity of the reaction mixture; (6) the nature of the buffering medium. The information gained from these variations is informative with respect to the underlying chemistry of the toxin.

Formaldehyde is capable of producing numerous chemical reactions involving a variety of amino acid side chains as reagents. For example, it can react with lysine amino groups, with tyrosine phenolic rings and with arginine guanidinium groups. We eliminate the guanidinium groups from consideration in the formation of toxoid because of the fact that they can be covered chemically without loss of toxicity. We also eliminate phenolic rings because ninhydrin, which is not believed to react with them, is capable of forming a quite satisfactory toxoid. Similar reasoning would make reaction with tryptophan indole groups and histidine imidazole groups unlikely. While this is not finally established, the evidence suggests that the terminal amino groups of lysine are the most probable sources of the reactive groups for toxoid formation.

Formaldehyde reacts with amino groups to form mono- and dimethylol ammonium groups. These reactions are extremely rapid and reversible. The reaction to form toxoids is very slow and essentially irreversible. Hence we may conclude that the toxoid reaction is not the formation of methylol ammonium derivatives. Effective toxoids have been prepared by incubations of 30–40 days at 38°. They have also resulted from incubations at 45° for as short a time as two days. Neither of these sets of conditions resembles the methylol ammonium reaction.

One might assume that the time lapse for reaction would be consumed in self-condensation of the formaldehyde. Our experiments show that preliminary treatment of the formaldehyde with buffer before addition of the toxin produces no increase in effectiveness of toxoid formation nor decrease in time in the reaction. We may conclude that the time is actually consumed in a slow reaction between the formaldehyde and the toxin.

Two hypotheses for the reaction with formaldehyde are that it consists in a linking of two molecules of ricin by methylene bridges or, alternatively, that there is an internal linking between groups in the same molecule. Condensation between two molecules would be favoured by high concentrations of toxin while dilute solutions would favour internal condensation. Experiment shows that the most satisfactory concentrations are approximately 1 per cent. In a molar sense, these are very dilute. Taking a molecular weight of 50,000 for ricin,[8, 23] they are of the order of 0·0002 molar. Since an increase of concentration above this point is deleterious to toxoid formation, it seems safe to draw the conclusion that the reaction in question is probably intramolecular rather than intermolecular. We should, therefore, expect no multiplication of molecular weight by the process of toxoid formation. The critical determination of the molecular weight of this toxoid has not yet been performed.

The most satisfactory pH for the reaction is 9·0. At pH 10·0 solutions lose their toxicity faster initially but lose antigenicity with even greater relative speed. Carbonate buffers give better immunizing properties than borate buffers. The interpretation is not immediately clear.

All processes which we have tried for the formation of toxoids cause a diminution in antigenic power. Our best toxoids have

about 10 per cent of the immunizing power of the live toxin. It is interesting to note that prolonged incubation with the relatively selective ninhydrin reagent also produces a marked loss in antigenic power. This indicates that the lysine amino groups participate in the geometry necessary for antibody formation as well as in that responsible for toxicity, although to a markedly different degree.

Potentiators and Inhibitors of the Toxic Action of Ricin

Because of the highly lethal nature of ricin, an antidote to its action would be desirable as a protective measure. In addition, a study of potentiators and antagonists might give clues to the mechanism of action of the toxin. In fact, although several antagonists have been found, none of the substances is suitable as an antidote. With respect to mechanisms, we already know from pathological studies that the toxin causes capillary haemorrhages. Both potentiators and antagonists consistent with this finding have been found but none of these adds materially to our understanding of mechanisms.

Miscellaneous substances which affect the survival time of animals intoxicated with ricin are listed in Table II. It should

Table II. Potentiators (+) and antagonists (−) for ricin toxicity

Substance	Administration	
	Before	After
Epinephrine	−	+
Sodium nitrite	+	0
Tripellenamine	+	+?
Rutin	+?	
Potassium thiocyanate		++
Nicotinic acid		++
Adenylic acid		+
Atropine		++
Neostigmine		0
Urea		−
Glucose		0
Sodium glycerophosphate		−
Thiourea		+++
Vitamin E		0

be noted that the order in which the toxin and the second agent are administered is of critical importance.

The most effective antagonists to the action of ricin turned out to be amino acids and their derivatives. A list of these substances together with their actions is given in Table III.

Table III. Amino acid potentiators (+) and antagonists (−) for ricin toxicity

Substance	Administration	
	Before	After
Histamine		+ +
Glycine (early)		− − −
Glycine (late)		+ +
Glutathione (early)	0	− − − −
Glutathione (late)		+
Alanine		−
Asparagine	+ +	−
Aspartic acid	− ?	+ + + +
Glutamine	+	
Glutamic acid	+	+ + +
Lysine (9 × isotonic)	−	
Lysine (3 × isotonic)	+	
Proline		+
Arginine		−
Glycylglycine		+

Of all the substances tested, glutathione was the most effective antagonist but glycine was not far behind. Either of these substances must be administered in a massive dose to secure effective action. The dose must be given before symptoms of intoxication set in. If administration is delayed until after the onset of symptoms, either substance exerts a powerful deleterious action, probably due to the inability of damaged capillaries to cope with the hypertonic solutions necessary for effective action. It is conceivable that this problem might be solved by an intravenous drip method of administration, using a much weaker solution.

In the present state of knowledge we must conclude that there

is no effective antidote for the action of ricin. It seems probable that the intelligent way to attack the problem will be to learn more about the biochemical mechanism of action of the toxin first and then to approach the problem of antidote design from a more rational point of view. These are the lines of current investigations on the ricin problem in the Hopkins Laboratories. By these studies on the toxic constituents of the castor bean we hope to learn more about the processes by which toxins and allergens in general produce disease.

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