ISOLATION OF CRYSrALLINE RICIN*

BY M. KUNITZ AND MARGARET R. McDoNALD

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

(Received for publication, March 24, 1948)

1. INTRODUCTION

A crystalline material possessing powerful toxicity has been isolated from crude extracts of castor bean meal. The crystalline material is a protein of the globulin type. It is soluble in acid or alkaline solution and is least soluble in the range of pH 5.0 to 8.0. The ultraviolet light absorption spectrum of the crystalline protein is similar to that of a typical protein. The toxicity of the isolated crystals is higher than that of the mother liquor freed of the crystals. On repeated recrystallization the toxicity of the mother liquor approaches that of the crystals. Ultracentrifuge and electrophoresis measurements of a sample of three times recrystallized material showed that the material is fairly homogeneous. Solubility measurements, however, indicate that the crystalline protein even after several recrystallizations apparently consists of a solid solution of more than one component. The method of separation of the crystalline protein into its possible components is still unavailable.

2. Method of Isolation of Crystalline Ricin

The crystallization of ricin from crude extracts of castor bean meal proceeds best in the presence of sodium sulfate or ammonium sulfate.

(a) Crystallization in the Presence of Sodium Sulfate.—A solution of crude Na₂SO₄-ricin in water yields crystals of toxic protein when stored for several weeks at about 5°C. The details are as follows:

10 gm. of dry powder of Na₂SO₄-ricin is suspended in 30 to 40 ml. water. It is best to add the powder slowly to the measured amount of water so as to allow the powder to become wet gradually. The mixture is stirred until uniformly dispersed. It is then filtered clear on fluted paper. The filtration, if slow, is allowed to proceed overnight in the cold room at about 5°C. The solu-

* This paper is based on work done for the Office of Scientific Research and Development under Contract OMR-129 with The Rockefeller Institute for Medical Research.

The experiments referred to were first reported July 15, 1944.

1 The isolation of a non-toxic crystalline globulin from castor bean has been reported by Rittausen in 1881 (1) and by Osborne in 1892 (2).

2 This preparation is an aqueous extract of castor bean meal, precipitated by saturation with sodium sulfate (3). The precipitate is filtered off and dried.
tion is adjusted with 1 M sodium hydroxide or hydrochloric acid to pH 6.8* and stored at 5°C. A slight precipitate of rosettes of very fine needles generally appears within 10 days (or longer). The bulk of the precipitate increases gradually until it reaches a maximum in 6 to 8 weeks. Occasional stirring accelerates the rate of formation of crystals, which is also greatly increased on inoculation with a large amount of crystals. The crystals are filtered or centrifuged. The yield is about 0.5 gm.

Recrystallization.—The crystalline precipitate is suspended in a volume of water equal to about one-fourth of the volume of water used for the first crystallization. Enough 1 M hydrochloric acid is added slowly with stirring until the crystals dissolve. The solution is filtered clear on fluted paper and the paper is washed with a small amount of 0.01 M hydrochloric acid. The filtrate and washings are titrated to pH 6.8 with 1 M sodium hydroxide, but the addition of sodium hydroxide is interrupted at the first appearance of turbidity even if pH 6.8 is not reached. The solution is stored at 5°C. A heavy crop of crystals generally is formed within 24 hours and the crystallization is complete in 2 or 3 days.

(b) Crystallization in the Presence of Ammonium Sulfate.—10 gm. dry Na₂SO₄-ricin powder is stirred up with 30 ml. of water. The mixture is filtered on fluted paper; the residue on the paper is washed with about 10 ml. of water. Enough solid ammonium sulfate is added to the combined filtrate and washings so as to bring the solution to 0.8 saturation (5.6 gm. per 10 ml.). The precipitate formed is filtered with suction. The filter cake is weighed and then dissolved in water in proportion of 1.3 ml. of water to 1 gm. of filter cake. Saturated ammonium sulfate is added slowly until a slight turbidity appears which is removed by filtration through folded paper. The filtrate is titrated to pH 6.8 with 1 M sodium hydroxide. A heavy precipitate gradually forms. The suspension is centrifuged at a temperature not higher than 10°C. The residue is dissolved in about 3 ml. of water and stored at 5°C. A heavy amorphous precipitate forms in a few hours. The amorphous precipitate gradually changes into fine needles. The suspension is filtered or centrifuged after 2 or 3 weeks.

The method for recrystallization is the same as described in section (a).

The mother liquors from the various crystallizations generally yield more crystalline protein when brought to 0.8 saturation with solid ammonium sulfate. The precipitate formed is dissolved in about an equal weight of water and stored at 5°C. A heavy crop of crystals is formed within 2 or 3 weeks.

3. Form of Crystals

The material generally crystallizes in the form of rosettes of fine needles (Fig. 1 a). Large prismoidal crystals appear if recrystallization takes place slowly from dilute solution (Fig. 1 b).

* The pH is checked by the drop method on a test plate.
4. Toxicity Tests

The toxicity of the first crystals was tested by Professor A. H. Corwin. 1 mg. of the crystalline protein was found to have a toxicity equivalent to 2 mg. of protein of the crude Na₂SO₄-ricin, whereas the toxicity of 1 mg. of protein in the mother liquor of the same crystals was equivalent to only 1.3 mg. protein of Na₂SO₄-ricin. This indicated a sharp fractionation in favor of the crystals and is evidence that the toxicity is really a property of the crystalline protein.

The toxicity of three times recrystallized ricin protein was measured in Dr. R. Keith Cannan’s laboratory. The toxicity of the crystals was 680 T.U. per mg. protein while the toxicity of the mother liquor was 520 T.U. per mg. protein.
The specific toxicity of five times recrystallized protein and of the mother liquor was determined in Dr. R. Keith Cannan's laboratory. No significant difference in the specific toxicity of the crystals and the mother liquor was found. This result shows that the present method of recrystallization no longer changes the composition of the crystalline protein sufficiently to be detected by differences in toxicity.

5. Some of the Protein Properties of Crystalline Ricin

Crystalline ricin appears to be a protein of the globulin type, with an isoelectric point reported (3) to be at pH 5.4 to 5.5. It is least soluble in the range of pH 5.0 to 8.0. It is precipitated in 0.15 M trichloracetic acid. Its ultra-

Additional information on the properties of crystalline ricin is to be found in the publication of Kabat, Heidelberger, and Bezer (3).
violet absorption spectrum (Fig. 2) resembles that of other proteins with a maximum absorption at a wavelength at 279 m\(\mu\) and a minimum at 250 m\(\mu\).

6. Purity of Three Times Crystallized Ricin

(a) Ultracentrifuge Method (Dr. M. A. Lauffer).—Solution used: 1.5 per cent solution of three times crystallized ricin in 0.2 M sodium chloride made up in 0.05 M acetic acid; final pH about 3.5. The solution was filtered clear on No.

![Ultraviolet absorption spectrum of crystalline ricin.](image)

The data were recorded by the Svenson schlieren method at 10 minute intervals. Only one moving boundary was observed throughout the 2 hours' centrifugation and the symmetry of the schlieren curves indicated fair homogeneity of the material.

Sedimentation constant corrected to water at 20°C. = 3.9 × 10^{-13} cm./sec./unit field. Molecular weight = 36,000 (assuming the particles to be spheres with a specific volume of 0.73).

(b) Electrophoresis (Dr. M. A. Lauffer).—Solution used: 0.63 per cent solution of three times crystallized ricin in 0.02 M sodium chloride made up in 0.05 M acetic acid. The same electrolyte solution was used to fill the upper compartments of the Tiselius apparatus. Current passed at the rate of 10 milliamperes for 1.5 hours. Boundary recorded at the end of the experiment by the Longworth schlieren scanning method. The electrophoretic pattern indicated the presence of only one moving component.
(c) Solubility Test.—Measurements were made of the solubility in 0.05 M acetate buffer pH 5.5 of four times recrystallized ricin in the presence of increasing amounts of crystals of ricin in suspension.

Experimental Procedure.—5 gm. of three times recrystallized filter cake were dissolved in 15 ml. 0.1 M acetic acid and filtered clear. The solution was brought to pH 5.5 by means of 15 ml. 0.1 M sodium hydroxide and left for several days at 5°C. for crystallization. The suspension of crystals was filtered on Whatman's No. 42 paper at about 20°C. The crystals were then washed twice by resuspending in 30 ml. 0.05 M acetate buffer pH 5.5 and refiltering on No. 42 paper.

![Graph](image)

Fig. 3. Solubility of three times crystallized ricin in presence of increasing quantities of solid phase.

The concentration of protein in the filtrates was found to be as follows:

- Mother liquor: 3.0 mg./ml
- First washing: 3.1 mg./ml
- Second washing: 3.3 mg./ml

The washed crystals were resuspended in 30 ml. 0.05 M acetate buffer pH 5.5. Increasing amounts of the concentrated suspension of crystals were then pipetted into 15 ml. test tubes each provided with a pyrex glass bead. The tubes were nearly filled with the same buffer solution, stoppered with one-hole rubber stoppers, and then plugged with short glass rods. Care was taken not to leave any air bubbles in the tubes. The suspensions were revolved mechanically with a slow motion for 18 hours at about 20°C., then filtered on small No. 42 filter papers. The filtrates, as well as the suspensions before filtration, were analyzed for protein by the copper-phenol method of Herriott (4). The data are given graphically in Fig. 3. The experiment shows that the solubility of the crystals of ricin is not independent of the total amount of the excess crystals in suspension, but continues to increase gradually in the presence of...
increasing amounts of the solid phase. The curve\textsuperscript{a} resembles the theoretical solubility curve of a solid solution of two or more components (5). Since more than one component is present it is possible that the agglutinating properties of the preparation are due to one protein and the toxic properties to another protein.

7. SUMMARY

A toxic crystalline protein has been isolated from crude extracts of castor bean meal. Ultracentrifuge and electrophoresis tests show the crystalline protein to become fairly homogeneous after three or four crystallizations. This is also confirmed by toxicity measurements. Solubility tests, however, indicate the presence of more than one protein component in the crystalline material, possibly in the form of a solid solution which cannot be separated into its components by repeated crystallization under the present technique.

REFERENCES


\textsuperscript{a} Such curves are frequently found with proteins which appear to be homogeneous by ultracentrifuge or electrophoresis methods. The constant solubility test is much more sensitive since it will detect the presence of a mixture or solid solution of proteins even though the various components have the same solubility. The electrophoresis or ultracentrifuge technique, on the other hand, can detect only proteins which have different rates of sedimentation or electrophoresis.