with commercial nickel catalysts. Under suitable conditions the acid is reduced predominantly to monounsaturated acids with only a slight increase in saturated acids. An alkali-conjugation reaction mixture may be hydrogenated without isolating the conjugated acids. One set of conditions found suitable for hydrogenation is as follows: 10 g. of conjugated linoleic acid, 7 g. of sodium hydroxide, 250 ml. of water, and 0.05 g. of nickel placed under 40 p.s.i. hydrogen pressure and heated at 140°C. for 1 hour. Acids prepared from this reaction mixture have an iodine value of about 90. Oxidation and chromatographic analyses of the resultant dibasic acids indicate that with alkali-conjugated linoleic acid, 1,2, 1,4, and 3,4 addition of hydrogen take place with equal ease. The reduced acids contain 66% *trans* acids. With *trans,trans* conjugated linoleic acid, 1,4 addition takes place to a greater extent that 1,2 and 3,4 addition, and the reduced acids are all *trans*.

REFERENCES

1. Allen, R. R., Am. Oil Chemists' Soc., *33*, 301 (1956).
2. Bradley, T. F., and Richardson, D., Ind. Eng. Chem., *34*, 237 (1942).
3. Brice, B. A., Swain, M. L., Herb, S. F., Nichols, P. L. Jr., and Riemenschneider, R. W., J. Am. Oil Chemists' Soc., *29*, 279 (1952).
4. Jones, E. P., and Stolp, J. A., J. Am. Oil Chemists' Soc., *35*, 71 (1958).
5. Lemieux, R. V., and von Rudloff, E., Can. Jour. Chem., *33*, 1701 (1955).
6. Nichols, P. L. Jr., Herb, S. F., and Riemenschneider, R. W., J. Am. Chem. Soc., *73*, 247 (1951).
7. Nijkamp, H. J., Anal. Chim. Acta, *10*, 448 (1954).
8. Tsuchiya, T., and Kinomura, S., Repts. Govt. Chem. Ind. Res. Inst. Tokyo, *46*, 249 (1951); C. A. *46*, 4815 (1952).
9. Tsuchiya, T., Bull. Govt. Chem. Ind. Res. Inst. Tokyo, Spec. No. 3, March 1950; C. A. *46*, 8397 (1952).

[Received March 28, 1958]

Isolation of Ricin, Ricinine, and the Allergenic Fraction from Castor Seed Pomace from Two Different Sources[1]

GEORGE R. WALLER, Biochemistry Department, Oklahoma State University, Stillwater, Oklahoma, and S. S. NEGI, Indian Veterinary Research Institute, Mukteshwar-Kumaun, V. P., India

IN INVESTIGATIONS involving poisonous materials, it is necessary to curtail the handling as much as possible to avoid the associated hazards. Precaution is necessary with castor seeds because in handling them one is exposed not only to the extremely toxic protein ricin but also to the possibility of acquiring sensitivity to the allergenic fraction which has been described as a protein-polysaccharide complex. Besides ricin and the allergenic fraction, there is a mildly toxic alkaloid, ricinine. Methods for the individual isolation of one or the other of these factors in castor seeds or pomace have appeared in the literature (3, 4, 7, 10, 16). The classical work on the isolation of ricin is that of Osborne, Mendel, and Harris (15). More recent works on the isolation and purification of ricin are those of Kabat et al. (7) and Kunitz and McDonald (10). In spite of the fact that crystalline preparations of ricin have been reported to be homogenous, there is evidence that ricin may be a mixture of more than one component (2, 7).

The procedure used in the preparation and purification of ricin in this report is patterned after one used by Corwin of Johns Hopkins University as reported by Kabat et al. (7). The method of isolation of the allergenic fraction in castor seeds has been developed by Spies and Coulson (16). Their procedure was followed in this work. The principle used in the isolation of the alkaloid ricinine was essentially that of Evans (3).

Here an attempt has been made to develop a method for simultaneous extraction of these three fractions from one lot of the material. Preliminary steps in the isolation of any of these factors are to shell, grind, remove the oil, and in some cases decorticate the castor seeds. Two samples of castor seed pomace, one a commercially prepared sample and the other prepared in the laboratory, were subjected to the following procedure.

Experimental Procedure

Extraction and Isolation. The general plan followed in this work is shown in Figure 1. The different steps involved are as follows.

Extraction of Oil from Castor Seeds. The shelled castor seeds were ground, pressed, and extracted with ethyl ether. This was done only on the laboratory-prepared pomace.

Extraction of Castor Seed Pomace. The oil-free pomace was extracted with five volumes of water acidified with HCl to a pH of 3.8. This was effected by shaking the contents in a large, wide-mouthed bottle during a 24-hr. period. The contents were allowed to settle and then were filtered through linen cloth (about 45 threads per square inch). The residue was treated with 3 volumes of distilled water, shaken for 2–4 hrs. and filtered through the same cloth. A second treatment with water was given to the pomace.

The three filtrates were combined to give filtrate I. The remaining residue was termed insoluble residue I.

Filtrate I. This filtrate contains all the ricin and portions of ricinine and allergenic fractions that are soluble in cold, dilute HCl. It was evaporated to a small volume by vacuum distillation below 40°C. Ricin I was obtained by saturating the filtrate with NaCl and was then separated by filtration. The filtrate after separation of ricin I is termed filtrate II.

Filtrate II. A precipitate of ricin II was obtained by saturation of this filtrate with sodium sulfate. The filtrate after separation of ricin II is termed filtrate III.

Insoluble Residue I. The residue of castor seed pomace left after the extraction of ricin was treated with five volumes of water between 70°–80°C., thoroughly shaken for 1–2 hrs., allowed to settle, and filtered through linen cloth. The residue was washed twice more with hot water and filtered through the same cloth. The combined filtrate and washings gave filtrate IV. The residue is termed insoluble residue II.

Insoluble Residue II. This castor pomace is free of the toxins and allergens that are removed by the aqueous and acidified extracts.

Filtrates III and IV. Filtrate IV contained ricinine and the allergenic fraction. Filtrate III also contained dilute HCl soluble fractions of these constituents along with excessive amounts of sodium chloride and sodium sulfate. These filtrates were combined and evaporated to dryness on a steam hot plate.

The dry mass was extracted three times with hot 95% ethyl alcohol. The extract contained ricinine. The remaining insoluble material was termed insoluble residue III.

Insoluble Residue III. This residue was extracted twice with 25% ethyl alcohol. This extract contained the allergenic fraction contaminated with excessive amounts of sodium chloride and sodium sulfate. The remaining insoluble material which contained considerable amounts of NaCl and Na_2SO_4 as well as non-extractable material from the castor pomace was discarded.

Purification of Ricin. The preparations, Ricin I and Ricin II, were dissolved in water and reprecipitated by saturation with sodium sulfate at pH 8. After reprecipitation twice more with the same salt they were dissolved in water and dialyzed against tap water for 48 hrs. and distilled water for 12 hrs. to remove salts. In both cases a soluble and an insoluble protein were observed in the collodion bag. The water-insoluble proteins were filtered on a sintered glass crucible (M). Both fractions were dried separately in an oven below 40°C. and finally in a desiccator over phosphorus pentoxide at room temperature. The dry, water-insoluble proteins were brown, and the dry, water-soluble proteins were whitish grey. Crystalline proteins were observed in each preparation.

Purification of Ricinine. The ricinine extract was evaporated to dryness on a water bath. It was purified by repeated crystallization from hot water and toluene. White prismatic crystals were obtained which had a melting point of 193–197°C. at atmospheric pressure. Evans (3) reported the melting point of ricinine at 193°C.

Purification of Allergen. This procedure was based on the properties of the allergen as observed by Spies, Coulson, et al. (16, 17): it was a) water-soluble, b) stable to boiling water, c) not precipitable by basic lead acetate, d) soluble in 25% ethanol, and

TABLE I

Yields of Ricin, Ricinine, and Allergenic Fraction from 3 kg. of Air-Dried Castor Seed Pomace
(Moisture-free basis)

	Commercial pomace	Laboratory pomace
	g.	*g.*
Quantity of pomace	2751	2850
Ricin I (from sat'd NaCl pptn.)		
Water-insoluble	0.75(A)	1.29(C)
Water-soluble	2.14(B)	1.44(D)
Ricin II (from sat'd Na₂SO₄ pptn.)		
Water-insoluble	Combined with A	1.14(E)
Water-soluble	Combined with B	24.22(F)
Total ricin	2.89	28.09
Percentage of ricin	0.105	0.99
Ricinine	0.60	3.00
Percentage ricinine	0.02	0.10
Allergenic fraction:		
Before dialysis	13.28(P)	28.27(R)
After dialysis	2.16(Q)	6.22(S)
Percentage of dialyzed allergen	0.08	0.22

e) insoluble in 75% ethanol. Final purification of the allergen was by dialysis. In the process of dialysis the allergen loses its carbohydrate moiety. The protein retains the allergenic property since the loss of the carbohydrate moiety which presumably acts as a hapten does not result in a decrease in allergenic potency (1, 17).

Results. The yields of the different fractions from 3 kg. of air-dried castor seed pomace are recorded in Table I. The different ricin fractions are termed A to F and the allergenic fractions P to S. The nitrogen content of the ricin preparations were: A = 16.82%; B = 17.23%; C = 15.86%; D = 15.18%; E = 16.27%, and F = 16.77%, with an average nitrogen content of 16.52%. The nitrogen content of the undialyzed allergenic fractions were P = 13.81%, R = 13.90% and for the dialyzed allergenic fractions, Q = 17.01% and R = 16.61%. These nitrogen values are expressed on an ash-free and moisture-free basis.

When dissolved in 1N NaCl solution, all of the ricin preparations left behind a small insoluble residue. Nevertheless in qualitative *in vitro* tests all the fractions were observed to have a strong hemagglutinating activity towards a suspension of rabbit erythrocytes. The strong toxicity of these preparations was quantitatively confirmed by intraperitoneal injections in rats. The details of these tests are given in Table II.

The allergenic preparations were completely soluble in the distilled water and in 1N NaCl solution. Three of the allergenic preparations were tested in guinea pigs. In preliminary studies each of the three preparations (Q, R, and S) were used to sensitize two guinea pigs (of an average weight of 392 g.) by subcutaneous injection of the allergen. Two weeks after sensitization they were administered a shocking dose by cardiac puncture. In this series of tests it
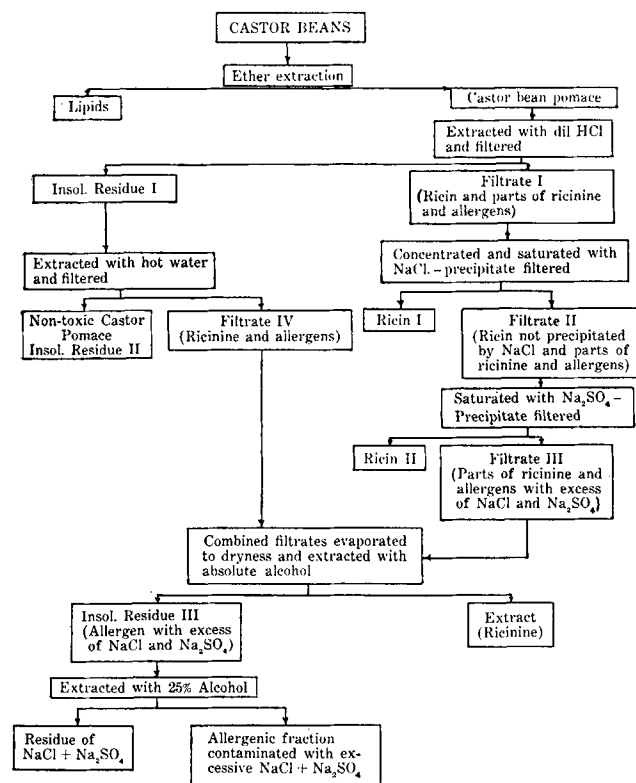

FIG. 1.

TABLE II

Toxicity of Ricin to Rats

Ricin fraction	Toxic level of ricin (gamma ricin/ 100 g. body wt.)
A	19.5
B	8.4
C	9.5
D[a]	......
E	17.0
F	21.4

[a] This sample accidentally got overheated and as a result was partially detoxified. 37 gamma failed to kill rats in seven days, but an additional dose of 500 gamma killed them in 48 hrs.

was ascertained that an approximate level of 100–150 gamma of allergen nitrogen per kilogram of body weight would be required to produce death by anaphylactic shock. After this preliminary study it was desired to test the allergenicity of these preparations in a more elaborate manner. Thirty-six guinea pigs of an average weight of 368 g. were divided into three main groups with three subgroups of four each. The animals were sensitized subcutaneously by graded doses of the allergens in 0.1N NaCl solution. Three weeks after sensitization they were administered a shocking dose by cardiac puncture. The results are shown in Table III.

TABLE III

Comparative Toxicity in Guinea Pigs of Salt-Containing and Salt-Free Castor Allergens

Group	Subgroup	Sensitizing dose gamma N/kg. body wt.	Shocking dose gamma N/kg. body wt.	Results
		Allergenic Fraction Q		
I	A	80	80	Moderate shock—no death
	B	160	160	3 animals died in 3 min. 1 animal died in 96 hrs.
	C_1	240	160	2 animals died in 5–10 min.
	C_2	240	240	2 animals died in 3 min.
		Allergenic Fraction R		
II	A	60	60	Moderate shock—no death
	B_1	120	90	1 animal died in 5 min. 1 animal died in 24 hrs.
	B_2	120	120	1 animal died in 5 min. 1 animal died in 2 hrs.
	C	180	180	2 animals died in 24 hrs. 2 animals died in 48 hrs.
		Allergenic Fraction S		
III	A	80	80	2 animals—moderate shock 1 animal died in 96 hrs. 1 animal died in 20 min.
	B	160	160	Died in 2–15 min.
	C	240	240	1 animal died in 40 min. 3 animals died in 24 hrs.

Evaluation of the Toxicity of Extracted Castor Pomace. The protein of castor pomace has been found to be deficient in tryptophan and low in methionine and lysine. Biological tests by Kodras et al. (9) have shown that castor protein did not support adequate growth in the rat when used as the sole source of protein.

Samples of the two different castor pomaces before and after extraction were fed to groups of rats to determine their relative toxicities. A qualitative *in vitro* test showed no hemagglutinating activity of the pomaces towards a suspension of rabbit erythrocytes. The unextracted pomaces were fatal to a group of white rats while no deaths resulted from feeding the extracted pomaces. The rats fed the extracted pomaces showed no weight loss over a period of eight weeks. The pomaces were fed at two levels: (1) a 1:1 mixture, and (2) a 2:1 mixture (castor pomace: Rockland Rat Diet—Complete).

Since the toxin-free pomace was nontoxic to rats and appeared to have some value as a feed for rats, some measure of its nutritive value was needed. Proximate and mineral analyses of the castor pomace samples before and after extraction of the toxic and allergenic principles are shown in Table V.

In general, these results follow the expected trend with respect to change in composition after removal of the toxic and allergenic principles. The pomace retains a sufficiently high protein value to be considered as a supplemental feed for livestock. The main problem encountered with the extracted castor pomace is one of palatability. Rats overcame their objection to the pomace in two to four days and thereafter ate the blended pomace as readily as the control rats ate the control laboratory feed.

Amino Acid Content of Ricin. Some preliminary observations of the amino acid content of ricin have been made. Microbiological procedures were employed for these determinations. They were essentially those of Henderson and Snell (5).

The values are compared with some of the more recent values reported in the literature. The amino acid analysis of the castor pomace is also shown for comparison. The amino acids are typical of those found in plant materials although there have been some recent reports (13, 14) that some less common and possibly unknown amino acids may exist in the ricin.

TABLE V

Amino Acid Content of Ricin

Amino acid	Castor pomace Kodras et al. (10)	Ricin Fraction C	Ricin Moule (13)
Glycine	....	2.0	7.6
Valine	6.6	2.9	....
Leucine	7.2	3.8	....
Isoleucine	5.3	3.6	....
Phenylalanine	4.2	2.3	....
Tryptophan	0.6	0.8	2.7
Threonine	3.6	2.3	....
Cystine	....	1.6	3.0
Methionine	1.5	0.9	0.6
Arginine	11.0	12.7	8.8
Histidine	2.5	0.9	1.7
Lysine	3.1	1.5	4.0
Aspartic acid	4.6	10.3	....
Glutamic acid	18.0	6.8	....
Serine	....	8.2	....

Values reported are g./16 g. N.

TABLE IV

Proximate and Mineral Analysis of Castor Pomaces Before and After Extraction
(Percentage: Moisture-Free Basis)

Sample description	Dry matter (Air-dried)	Ash	Protein	Fat	Fiber	Ca	P
Commercial-pomace							
Before extraction	93.24	7.17	41.56	1.27	34.22	.615	.842
After extraction	97.26	4.30	41.07	1.34	37.49	.350	.429
Laboratory pomace							
Before extraction	95.25	7.11	46.72	6.92	25.71	.765	1.107
After extraction	97.29	3.56	39.71	11.13	34.81	.582	.465

Discussion

A repeatedly purified ricin preparation used by Kabat et al. (7) proved fatal to a rat within 24 hrs. in a dose of 7.8 gamma/100 g. They prepared ricin by saturation of the dilute HCl extract of castor beans with sodium chloride and purified the precipitate by repeated dissolution in water and repeated precipitation with sodium sulfate. Thus their preparation corresponded to the ricin (C) fraction obtained in this investigation. This particular preparation also appears to be highly toxic in our studies with the lowest tested dose of 9.5 gamma killing a 100-g. rat in 48 hrs. Ricin (B) is slightly more toxic. From the observations presented here it will appear that considerable ricin activity is present in other fractions as well. All of the ricin fractions strongly exhibited erythrocyte hemagglutinating activity and were toxic to rats. Delphaut (2) has separated electrophoretically at least five components in the globulin fraction of castor pomace, which were extremely toxic to mice.

The quantity of ricin isolated from the commercially processed pomace is much less than that from the pomace prepared in the laboratory. Though the source of castor beans in the two cases is not the same, it appears that a considerable portion of the ricin has been detoxified during factory processing. The quantity of crude ricin as determined in the laboratory prepared castor bean pomace is approximately 1% of the fat-free material. Different values for the ricin content have been reported by different authors depending on the source of the castor beans, the particular fraction isolated, and the purity of the sample. According to Osborne et al., (15) ricin comprises about 1.5% of the oil-free meal. Funck (4) reports 2.8–3.0% ricin in seeds of Ricinus communis. Moriyama (11) records an average yield of only 0.5%. The nitrogen content of the ricin preparations averaged 16.52% and compared favorably with 16.56% reported by Jones (6, 8, 9) and 16.32% by Moule (12). Since Moule's (12) pure ricin was prepared differently from those reported in this work, it is not surprising to find some difference in amino acid values. Recently Mourgue (13) found agmatine and two unidentified compounds containing guanidino radicals in a crude toxic protein preparation from castor pomace. Certainly more information would be desirable to help elucidate the composition of this highly toxic protein.

The comparative data for the yield of allergens from the two pomace samples are also indicative of the effectiveness of industrial processing in reducing the potency of the allergen. The yields of the allergens are somewhat lower than those reported by Spies, Coulson, et al. (1, 16, 17). These authors report a yield of dialyzed allergen of 0.74% from domestic castor pomace. A similar comparison would give a yield of 0.18% for Brazilian castor pomace. The figures for the purified allergens obtained from the commercial and laboratory samples are 0.08% and 0.22%, respectively. Coulson and Spies (1) found a nitrogen value of 18.9% on the dialyzed allergen, which may be compared with our value of 16.8%.

The allergenicity tests in Table III do not show any significant difference in effectiveness between the dialyzed allergen and the salt containing allergen which agrees with an earlier report (1). All were found to be strong antigens, capable of producing death from anaphylactic shock. Death occurred in all cases when a concentration of at least 160 gamma allergen nitrogen per kilogram of guinea pig weight was administered.

The extraction of the ricin, ricinine, and the allergen leaves a nontoxic product which may have considerable value as a source of animal feed. At the present price of castor pomace (about $30 per ton) it offers a low-cost source of protein. There would be an additional cost of extraction of the toxic and allergenic principles, but it is not believed that this would increase the cost to more than $60 per ton. At this cost nontoxic castor pomace might compete with current sources of feed, such as soybean meal and cottonseed meal. At present castor pomace is used solely as a fertilizer.

Summary

A scheme of separation is described for the extraction of ricin, ricinine, and the allergenic fractions from the same lot of castor seeds, thereby considerably curtailing the hazard associated with the handling of the material.

The yield of ricin from the commercially prepared pomace was only one-tenth of that from the laboratory-prepared pomace, which contained approximately 1% of this toxic protein. Likewise the amount of dialyzed allergenic fraction present in commercial pomace is only about one-third of that present in the laboratory sample, which contained 0.22% of this constituent. The laboratory pomace contained five times as much (0.10%) of the alkaloid ricinine as did the commercial pomace. It appears that commercial processing of the pomace is effective in destroying a considerable portion of the toxic and allergenic activity in castor seeds. The pomace remaining from the extraction is nontoxic and may be used to provide a source of protein for feeding stuffs.

Acknowledgments

REFERENCES

1. Coulson, E. J., Spies, J. R., and Stevens, H., J. Allergy, 21, 34–44 (1950).
2. Delphaut, J., Mourgue, M., and Dokham, R., Compt. rend. soc. biol., 149, 1582–1585 (1955).
3. Evans, T., J. Am. Chem. Soc., 22, 39–46 (1900).
4. Funck, E., Osterr. Chem. Ztg., 45, 15–16 (1942).
5. Henderson, L. M., and Snell, E. E., J. Biol. Chem., 172, 15–29 (1948).
6. Jones, D. B., J. Am. Oil Chemists' Soc., 24, 247–250 (1947).
7. Kabat, E. A., Heidelberger, M., and Bezer, A. E., J. Biol. Chem., 168, 629–639 (1947).
8. Karrar, P., Smirnoff, A. P., Ehrensperger, H., Van Slooten, J., and Keller, Max, Hoppe-Seylers Ztschr. f. Physiol. Chem., 135, 129–166 (1924).
9. Kodras, R., Whitehair, C. K., and MacVicar, R., J. Am. Oil Chemists' Soc., 26, 641–644 (1949).
10. Kunitz, M., and McDonald, M. R., J. Gen. Physio., 32, 25–31 (1948).
11. Moriyama, H., Med. and Biol. (Japan), 10, 163–166 (1947). Igaku to Seibutsugako is the Japanese title. If necessary, see C. A. 47, 2228 (1953).
12. Moule, Y., Bull. Soc. chim. biol., 33, 1467–1472 (1951).
13. Mourgue, M., Barot, R., and Dokhan, R., Compt. rend. soc. biol., 147, 1449–1451 (1953).
14. Mourgue, M., Dokhan, R., and Reynaud, J., Bull. Soc. chim. biol., 38, 123–128 (1956).
15. Osborne, T. B., Mendel, L. B., and Harris, I. F., Am. J. Physiol., 14, 259–286 (1905).
16. Spies, J. R., and Coulson, E. J., J. Am. Chem. Soc., 65, 1720–1725 (1943).
17. Spies, J. R., Coulson, E. J., Stevens, H., and Shimp, H. J., J. Am. Chem. Soc., 66, 1798–1799 (1944).

[Received February 18, 1958]