I. EVALUATION OF DETOXIFIED CASTOR MEAL ON RATS

II. TOXIC AND HEMAGGLUTINATING ACTIVITY OF RICIN

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Submitted to the faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
May, 1961

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ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to George R. Waller, Assistant Professor of Biochemistry, for his guidance and counsel during the course of these investigations and the preparation of this manuscript; Willis D. Gallup, Professor, Department of Biochemistry, for the reading of this manuscript; James B. Corcoran, Associate Professor, Department of Veterinary Pathology, for his help in post-mortem examination and preparation of histological slides of different tissues of the rats; Duane A. Benton, present address Roswell Park Memorial Institute, Buffalo, New York, for his counsel in the preparation of rat rations; Donald C. Abbott, Assistant Professor, Department of Biochemistry, for his assistance in running starch gel electrophoresis; Carrol Heninger, Technical Assistant, for her assistance in the chemical analysis of castor meal samples; Carol Townsend, for her work on the microbiological assay of the amino acids of castor meal; Richard Danke, Research Assistant, for his help in the determination of carotene in rat rations; and Jary Mayes and Douglas Morgan for their assistance in the handling of rats.

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CHAPTER I

INTRODUCTION

The seeds of the castor plant, Ricinus communis, L., have long been used as the source of castor oil. The press cake obtained after removal of the oil, called castor meal, contains about 50 to 55 per cent protein. The extremely toxic protein, ricin, and a very powerful allergen present in castor seeds renders the protein-rich castor meal unsuitable for use as an animal feed. The utilization of castor meal as a feed depends upon its successful detoxification and deallergenation. The nutritive value and possible toxic effects of two detoxified and deallergenized and two detoxified castor meals were investigated in feeding experiments with rats.

The toxicity and hemagglutinating properties of a crude ricin preparation were evaluated in 16 varieties of castor seeds. The results indicated that the two phenomena were unrelated. Pathological studies were performed to investigate the toxic effect of ricin. An attempt was made to separate the toxic and hemagglutinating factors in this crude preparation by $(NH_4)_2SO_4$ fractionation, starch gel electrophoresis and column chromatography.

The literature review was focused mainly on the utilization of castor meal and its principal toxic constituent, ricin.

CHAPTER II

LITERATURE REVIEW

The castor plant (Ricinus communis, L.) is reported to have originated in India (31). It is now widely cultivated in many of the temperate and tropical parts of the world. It has been grown exclusively for its oil which is found in the seeds. The oil content of the seeds varies from 45 to 55 per cent (31, 49). Castor oil is predominantly the triglyceride of ricinoleic acid (12-hydroxy-9-octadecenoic acid). It is used mainly for medical and industrial purposes.

The castor plant produces a very toxic protein, ricin, a mildly toxic alkaloid, ricinine, and a very potent allergen which has been shown to be a protein-polysaccharide complex (75). The amounts of these water soluble, physiologically active components in the plant probably vary; a recent report showed that about 1.0 per cent of ricin, 0.1 per cent ricinine and 0.22 per cent of dialyzed allergen are present in laboratory prepared castor meal (85).

Castor seeds are composed of about 25 per cent husk and 75 per cent kernel. After removal of the oil usually by the use of a hydraulic press followed by solvent extraction, the residue is called castor meal. Proximate and mineral analyses of castor meal (27, 31, 85) suggest that it could serve as a protein concentrate for animal feeds. Castor meal from decorticated seeds contains approximately 55 per cent protein (31, 49, 85). The presence of ricin and the allergen renders the protein-rich castor meal unsuitable for feeding. Castor beans are poisonous for

carnivores as well as herbivores (51). Small quantities of castor beans in cattle feed were reported to have caused abortions (10) and the death of cattle (65). In rat feed 10 to 15 per cent castor beans produced death in 1 to 5 days (47). A few cases of feeding castor meal to cattle in India without any deleterious effect have been observed.[1] The smallest dose of castor beans found lethal to guinea pigs was 0.179 gm/kg; 12.29 gm was lethal for a man weighing 150 pounds (20). Fatal poisoning of man by eating 2 to 5 beans has also been reported (48). Many other cases of castor bean poisoning resulting in sickness and death have been recorded in literature.

A number of methods, physical, chemical and biochemical are available for detoxification of the castor meal. They are as follows: 100° C moist or 130° C dry heat (50), boiling the meal repeatedly with water (70), autoclaving for 15 minutes at 125° C (38), boiling with organic solvents such as $CHCl_3$ (70), repeated extraction with hot 95 per cent ethanol (9), heating an alkaline dispersion of castor bean to 80 to 95° C (71), hydrolysis with HCl (43), treatment with proteolytic enzymes, autolyzed yeast and autolyzed azotobactor (42). There are a few recorded reports of feeding castor meal after detoxification to the pig (4, 66), fowl (84) and cow (12) without any toxic or harmful effect.

Dixson in 1887 (19) isolated a toxic protein from castor beans. The name, ricin, was given to this protein by Stillmark (77) who showed that it was an albumin. During the next several years much of the work on castor beans and castor proteins was devoted to attempts to determine if the toxicity of ricin was to be attributed to the protein itself or to

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[1] Personal observation.

some other factor intimately associated with it. Osborne, Mendel and Harris (64), in their classical work, were the first to isolate ricin as the protein to which the toxic properties of the seed were ascribed, although 11 years earlier Osborne (62) observed that the albumin appeared to be so intimately associated with the toxic substance that he felt that it was the toxic substance itself. At least six proteins have been shown to be present in castor meal by application of salting out, fractionation, paper electrophoresis and moving boundry electrophoresis methods (58). The euglobin fraction of the protein mixture was nontoxic, but the remaining globulin was found toxic. Five components in the globulin fraction were detectable using the Tiselius electrophoresis. These were partially separated by fractional precipitation with $(NH_4)_2SO_4$. All of the globulin fractions so obtained were extremely toxic to mice (18). Most of the interest in the proteins of castor beans has centered on the toxic fraction (25, 28, 64) and on the allergenic fraction (17, 25, 75, 85).

Several methods for the isolation of ricin from a saline (varying concentration) extract of castor meal followed by salting out with $(NH_4)_2SO_4$, Na_2SO_4 or $MgSO_4$ have appeared in the literature (18, 24, 28, 32, 40, 57, 64). Crystalline preparations of ricin (32, 40) have been reported to be homogenous; however, there is evidence that ricin may be a mixture of more than one component (18, 21, 23, 32, 36, 40, 85). The first strong evidence to this effect was found by the solubility test on crystalline ricin, which was otherwise electrophoretically and ultracentrifugally homogenous (40). The elemental analysis by different workers has produced close agreement on the percentage of carbon, hydrogen, oxygen, nitrogen and sulfur present. Ricin contains no phosphorus (31). The determination of sulfur in several ricin preparations showed that the

sulfur content declines with increasing purity (28). The specific rotation (optical) of ricin was found to be $[\alpha]_{20}^{D} = -27.5°$ (28), but $[\alpha]_{20}^{D} = -26.5°$ has also been reported (32). It is significant to note that the rotation also diminishes with the increasing purity of ricin preparations. Thus it was proposed that the optical activity and the sulfur content would be good criteria for the chemical determination of the purity of ricin (28). The ultraviolet absorption curve of ricin essentially coincides with that of other proteins; the absorption maxima have been reported variously, at 276 mμ (56) and 279 mμ (40). Ricin is least soluble within the range of pH 5 to 8 (40). The isoelectric point of ricin has been reported to be between pH 5.2 and 5.5 (29, 32, 56); the latest report suggests 6.6 (17). Ricin generally crystallizes in the form of rosettes of fine needles and large prismoidal crystals (40). The molecular weight of ricin has been reported as 77,000 or 85,000 (32); however, 36,000 has also been reported (40). Very recently Corwin (17) referred to the molecular weight as 50,000. Complete precipitation of ricin can be achieved by 0.15 per cent trichloroacetic acid (40). The study of influence of temperature and pH upon the denaturation of ricin showed that denaturation follows first order kinetics (44). Ricin is fairly stable below 60° C (25, 44) and between pH 3.8 to 10 (44). Isoleucine and methionine are reported to be the terminal amino acids with free amino groups (28).

Ricin has a strong proteolytic enzymic action which is not inactivated by cell cathepsins (55). On long standing (2 1/2 years) ricin loses its activity (21) with the appearance of new components in solution (28). The autolysis of ricin is perhaps due to its proteolytic activity (28). The proteolytic action of ricin on casein, oval albumins, serum

albumins, pepsin and ribonuclease has been shown but maximum activity is obtained at a different pH optimum for each substrate (56).

Ricin is extremely toxic to animals when ingested orally, subcutaneously or intravenously. It manifests its greatest effect when given intravenously (63). Given intraperitoneally 0.02 mg ricin killed kilogram weight rabbits in less than 72 hours (13). The effects of ricin do not appear until several hours after administration. This lag has been termed the "latent period" (78). The physiological effects of ricin that have been reported are as follows: diarrhea, extreme prostration (39, 51), hemorrhagic condition of the intestines, renal congestion, hyperemia of spinal medulla and brain, severe panophthalmitis (51), a state of hyperplastic myelosis caused by stimulation of the bone marrow followed by hemolytic anemia with hyperchromia, neutropenia, eosinophillia (68), inflamation of eyes (22, 51, 74), edema of eyes, nose, throat and lungs, adbominal pains, vomiting (22, 39). degeneration of the hypothalamus, adrenals, anterior hypophysis, ovaries, testies, periphereal nerve ganglia, lymphatic ganglia in the thymus (54), and necrotic lesions in various vital organs of guinea pigs on repeated injections (53), punctiform hemorrhages of intestinal tract and congestion of kidneys (39). Introduction of ricin into hen eggs during the development of the embryo produced a developmental anomaly designated as strophosomia (2); by the sixth day the embryo had entirely disintegrated, but the heart continued to beat and develop until the ninth day (3). Ricin was shown to inhibit the activity of an isolated rabbit heart and to inhibit the growth of chick embryonic heart, liver and stomach in tissue cultures (78).

A characteristic effect of ricin is to agglutinate red blood corpuscles. The agglutination involves a combination of the toxin

with the corpuscles; ricin then can be separated from the agglutinated blood cells and the free ricin is able to unite again with new erythrocytes (35, 45). The ricin combines with the stroma and not with the hemoglobin (45) Coagulation of blood corpuscles with ricin takes place only within the pH range of 5.6 to 5.8 and 8.9 to 9.1 (29). An insignificant degree of hemagglutination occurs when massive amounts of ricin are injected into rabbits. Thus the small number of red cells that agglutinate in vivo can not account for the toxicity of ricin (34). The suggestion has also been made that the toxic property of ricin and its agglutination of red blood cells are associated with a strong agglutinative action on protoplasm (11). Ricin causes agglutination of substances other than red blood cells, for example, suspensions of carmine and cholesterol (26). Ricin, like rennet, curdles milk (30). The erythrocytes from the dove, guinea pig and rabbits are agglutinated, but those of goat and sheep are not (50). When ricin is injected into rabbits secondary hemolysis occurs which is produced by the breaking of red cells and not by traces of ricin in the serum (7). The variation of hemagglutinating power of ricin with varying temperature has been shown experimentally with frogs (52).

Increasing concentration of such body fluids as saliva, urine and spinal fluid (5), serum (34) inhibits hemagglutination. Metallic copper destroys the hemagglutinating potency of ricin (82), but iodine does not (14). By treating ricin with formalin the hemagglutinating activity was reduced twice as much as its toxicity. Similarly with Sakaguchi's reagent (α-napthol and hypobromite) this property was reduced to a vanishing level without affecting toxicity appreciably (17). The condensation reaction of acetyl acetone and glyoxal with ricin, between pH 2.5 and 5, destroyed

the agglutination power by approximately 80 per cent without altering the toxicity (17). Ninhydrin did not affect the hemagglutinating power (17). The agglutinating principle is inactivated fairly easily under proteolytic digestion, where as the toxic principle is more resistant (23, 79). Digesting ricin with pepsin and pancreatin for 3 to 8 hours showed that less than 1 per cent of the agglutinating power and at least 90 per cent of the toxicity of the starting material was retained (17).

Ricin is digested with difficulty by proteolytic enzymes (36); the toxicity diminishes with the extent of digestion (23, 41, 79, 83). When fermented with the yeast Saccharomyces ellipsoideus it lost a considerable amount of its toxicity (16). Ricin loses its toxicity in varying degrees when treated with supersonic waves, 555 kilocycles (80), ultraviolet light (13) $Ca(OH)_2$ (76), heavy metals like zinc, silver (36), and copper (82), ethyl alcohol (25), oxidizing agents like congo red (13), dilute $KMnO_4$ solution (13), dilute H_2O_2 solution (8) and dilute iodine solution (14). Nevertheless the toxicity of ricin may partially be restored after the treatment with dilute iodine solution by subsequent action of $Na_2S_2O_3$ solution (14). Boiling ricin in an aqueous solution destroys its toxicity (13, 60). Effects of numerous other substances including amino acids, alkali, lipids and bacteria on ricin have been studied but no general grouping of results could be given (72).

It has been reported that the toxin and the agglutinin are present in practically constant amounts for a given weight of vegetable substances obtained from different varieties of the same species (1).

Inquiry into the possible relationship between agglutinating power and toxicity commenced with Stillmark (77) who first noticed the phenomenon of hemagglutination associated with ricin. Loss of toxic and hemagglutinating activity of ricin to a different extent by enzymatic digestion has already been discussed. Heating a solution of the protein to 55° C destroyed the agglutinating properties but the toxicity was lost only at boiling temperature (61). An entirely independent line of evidence that agglutination and toxicity are separate actions was provided by the observation that the antibody to the toxic action is not an antibody to the agglutinating action (15). Chemical differentiation of the agglutinating and toxic actions of ricin have been discussed, which indicate that the amino groups and guanidinium groups are responsible for the toxic and hemagglutinating effects respectively (17). A report has also been made to the effect that the toxic effect of ricin is linked with tissue cells and the antigenic action linked with red blood cells (80). In summary, these results show that these two properties of ricin are unrelated and independent.

In the past the mechanism of agglutination has been explained as follows: a) ricin in an acidic role combines chemically with cell stroma, acting as a base, forming a complex which can be decomposed with the liberation of free ricin (45), b) ricin attaches by adsorption to red cells in direct proportion to the lipoid content of the cell. Corwin (17) reconciled the two points of view by reversing the roles of acid and base of ricin and cell stroma and substituting phospholipids for lipoid materials in general. His experimental work showed: a) ricin is a polyvalent cation (guanidinium residues on the surface of the

molecule) and b) red cells are capable of maintaining a negative charge in a solution of considerable acidity and can be treated as polyvalent anions (phosphate groups). His explanation was anionic red cells in a water solution of cationic ricin, displaces the hydrophilic anions on the ricin surface and form bridges between ricin molecules; the latter, in turn being polyvalent cations, forms bridges between red cells. The whole system thus becomes an insoluble three dimensional polymer.

The mechanism by which ricin produces its toxic action is not known. The pathological changes caused by the action of the toxin have already been described earlier. Exhaustive studies of the biochemical changes produced by its action have been recorded (81). The outstanding findings within 24 hours of intraperitoneal injections of ricin to rats were decreased levels of plasma protein and glucose, and increased concentration of blood NPN, lactic acid and urea. The lactate/pyruvate ratio remained constant. In the liver there was a marked decrease in adenosine polyphosphates (no differentiation was made between ADP and ATP) and phosphocreatinin. From these and other data Thompson (81) explained the toxic action of ricin as being an interference with some metabolic process in the liver, possibly in the Krebs cycle. In spite of these studies, no adequate explanation for the high toxicity, caused by a small amount of the toxin, has been put forth. The latest report in this connection indicates that the ϵ-NH_2 group of lysine participates in the geometry necessary for the antibody formation as well as being responsible for its toxicity (17) which confirmed an earlier report (59).

PART I

CHAPTER III

EVALUATION OF DETOXIFIED CASTOR MEAL ON RATS

Method and material used

Male rats used in all of the feeding experiments were from the Sprauge-Dawley strain. They were kept in individual cages and allowed to eat and drink ad libitum unless otherwise stated. The inorganic salt mixture (67) shown in Table I, dry vitamin mixture[2] shown in Table II, corn oil (Mazola corn oil) and sucrose (table sugar) were mixed with every ration. The quantity of the above mentioned ingredients in each feed mixture will be shown with individual experiments. Two drops of a fat soluble vitamin mixture[2] as shown in Table III were given to each rat once a week, unless otherwise stated.

Description of the castor meals used:

1. The Southern Utilization Research and Development Division of the USDA treated castor beans essentially as follows:

NaOH dissolved in water was added to rolled castor meals (Brazilian castor seeds) to give 1 per cent on dry weight basis. The moisture content at this point was approximately 16 per cent. The temperature was raised and held at 200° F 30 to 45 minutes. The moisture content at this stage was approximately 9 per cent. The castor meats were crisped, re-rolled and extracted with hexane to remove the oil. The residual lipid content was 4.9 per cent. The pH of an aqueous

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[2]From Dr. D. A. Benton, Roswell Park Memorial Institute, Buffalo, New York.

dispersion was 9.4. The meal was analyzed for its allergen content (CB-1A) by an immunological "precipitin" method recommended by Stephens and Coulson. The "ring" test indicated 0.39 per cent and the "precipitin" test (48 hours) indicated 0.78 per cent of allergen. The meal was not tested for its ricin content but similarly prepared meals had tested zero to trace of ricin when the hemagglutination test was used.[3]

Future reference to this meal will be made as SURDD. The analysis of this meal is shown in Table IV.

2. The Pacific Vegetable Oil Company treated the solvent extracted Pacific Hybrid 6 castor beans (grown in Bakersfield, California) under high pressure steam (200 pounds per square inch, unofficial report). This preparation was made from whole castor beans and hence contained much less protein and much more crude fiber. Future reference to this preparation will be made as PVO meal. The analysis of this meal is shown in Table IV.

3. The Baker Castor Oil Company prepared a pilot plant batch of castor meal based on the SURDD procedure outlined above. The material was pulverized in a hammer mill. A hemagglutination test indicated that the meal was toxic. The sample sent to this laboratory was numbered F-0460008, and will be referred to as Baker No. 1 hereafter. The analysis of this meal is shown in Table IV.

4. The Baker Castor Oil Company also provided a 100 pound sample of castor meal detoxified by an undisclosed commercial process which they developed and are now using. This material was numbered F-0558055A and will be referred to as Baker No. 2 hereafter. The analysis of this meal is shown in Table IV.

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[3] Personal communication from Southern Utilization Research Development Division, United States Department of Agriculture, New Orleans, Louisiana, to G. R. Waller, 1958.

Experiment Number 1

The first experiment was designed to determine if castor meal prepared from alkali treated castor beans was toxic to rats. The SURDD meal was added at three different levels to a complete diet containing vitamin-free casein at a level of 25 per cent. The purpose was to provide a nutritionally adequate diet so that the addition of castor meal would provide a accurate measure of its toxicity. This avoided confusion between toxicity and nutritional deficiency. Soybean meal was used in control diets. Seven rats were used in each of the first seven trials in an experiment of a completely randomized design. The eighth trial, which was not a major objective in this experiment, involved five rats. The composition of the eight feed mixtures is shown in Table V. Two essential amino acids, DL-methionine and DL-tryptophan, were supplemented in all tests to overcome any possible deficiency.

Results and Discussion

The results of this experiment, presented in Table VI, on the growth study of rats did not show any marked difference between trials. Growh response by different trials was essentially the same except for trial number 4. The slower rate of growth of the rats on trial 4 was noticeable after the second week. This feed contained 29.25 per cent of SURDD castor meal, which was the highest level fed. The slower growth of the rats on this trial could be explained by assuming that this castor meal was slightly toxic. It probably would not begin to show toxicity until it reached the high percentage of 29.25 by weight, the highest percentage in all the trials. Possibly a palatability problem existed at this high percentage. A decrease in palatability at levels of about 20 per

TABLE V

DIETS FOR EXPERIMENT NO. 1

Trials / Ingredients %	1	2	3	4	5	6	7	8
Casein	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Castor Meal	---	9.75	19.5	29.25	---	---	---	30.3
Soybean Meal	---	---	---	---	10.0	20.0	30.0	---
Sucrose	64.7	54.95	45.2	35.5	54.7	44.7	34.7	34.4
Salt Mix.	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Corn Oil	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Dry Vitamin	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
DL Tryptophan	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
DL Methionine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Dietary Protein Total %	20.87	25.87	30.87	35.87	25.57	30.27	34.97	30.87
Protein From Test Material; as % of Total Protein	---	19.32	32.39	41.81	18.38	31.05	40.32	32.39
as % of Diet	---	5.0	10.0	15.0	4.7*	9.4*	14.1*	10.0

*This difference in total % of protein with trials 2, 3 and 4 was due to an error in protein analysis in soybean meal which was corrected.

TABLE VI

GROWTH OF RATS ON EXPERIMENT NO. 1

Trial	Average Initial Weight (gm)	Gain in Body Weight After Week- 1	2	3	4	5	6	Total Gain in Weight (gm)	Average Daily Gain (gm)
1	108.2	47.8	42.8	40.2	33.3	31.8	24.8	220.7	5.25
2	108.1	55.4	46.5	32.7	26.8	18.4	28.8	208.6	4.96
3	105.3	54.2	47.1	39.5	28.1	27.7	25.3	221.9	5.28
4	107.1	51.6	36.8	27.1	20.8	26.9	21.6	184.8	4.40
5	109.1	52.0	43.9	39.5	31.3	29.7	23.6	220.1	5.24
6	102.1	54.1	44.6	39.1	38.1	28.8	23.1	221.8	5.28
7	103.6	53.6	41.4	35.7	23.6	24.1	24.2	202.6	4.82
8	109.7	52.2	37.4	29.4	18.2	29.0	8.9	175.1	4.16

cent castor meal in the diet was shown previously (4, 12, 66); (c) the high protein content, 35.87 per cent, in this trial was perhaps detrimental. Neither sample of castor meal, SURDD or PVO at the levels given in the feed up to about 30 per cent by weight showed any toxicity when compared with soybean meal. Growth of rats was normal. Pathological examination of the rats fed on these castor meals revealed no lesions in the internal organs.

Experiment Number 2

Experiment number 2 was devised to study the nutritive value of the SURDD and PVO castor meal samples. In the previous experiment when castor meal supplied up to 10 per cent of the protein in the feed or 32.39 per cent of the total dietary protein no detrimental effect was produced on the growth of the rats. In this experiment castor meal served as the sole source of protein at two protein levels, 12.5 and 25.0 per cent. Casein and soybean meal were used in the control diets. The ingredients used were the same as those in experiment number 1. Castor meal has been shown to be low in lysine, tryptophan and methionine (38). This finding was confirmed by microbiological assay of SURDD meal for amino acids. In setting up these trials tryptophan was added to bring the level up to the required amount. Table VIII shows the composition of essential amino acids required (69) and the amount of these amino acids present in the castor meal diets. A slight modification was made in preparing the feed mixtures. The percentage of dry vitamin mixture was reduced from 2.0 to 0.25.[4] Methionine, which was supplemented in experiment number 1, was omitted. Table VII shows the composition of the eight different rations

[4]Personal communication from Dr. D. A. Benton.

TABLE VII

DIETS FOR EXPERIMENT NO. 2

Trials / Ingredients %	1	2	3	4	5	6	7	8
Casein	14.95	29.91	---	---	---	---	---	---
Soybean Meal	---	---	26.58	53.16	---	---	---	---
Castor Meal	---	---	---	---	24.36	48.72	37.87	75.75
Sucrose	76.78	61.83	65.16	38.58	67.32	42.90	53.80	15.86
Salt Mixture	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Corn Oil	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin Mixture	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-Tryptophan	---	---	---	---	0.06	0.12	0.06	0.12
Dietary Protein, Total %	12.5	25.0	12.5	25.0	12.5	25.0	12.5	25.0

Trials 5 and 6 were made of SURDD meal.

Trials 7 and 8 were made of PVO meal.

TABLE VIII

ESSENTIAL AMINO ACIDS PRESENT AND REQUIRED IN FEED

	Per Cent Amino Acid Present in SURDD Castor Meal	Percentage of Amino Acid Present in Feed		Per Cent Amino Acid Required in Feed*	Form
		at 12.5% Protein	at 25.0% Protein		
Valine	6.0	0.75	1.50	0.7	DL
Leucine	6.3	0.787	1.57	0.8	L
Isoleucine	3.4	0.675	1.35	0.5	DL
Phenylalanine	4.2	0.525	1.5	0.7	DL
Tryptophan	0.8	0.16	0.32	0.2	DL
Threonine	3.6	0.45	0.9	0.5	DL
Methionine	2.1	0.26	0.52	0.6	DL
Arginine	10.6	1.32	2.64	0.2	L-HCl
Histidine	1.9	0.237	0.47	0.4	L-HCl
Lysine	2.8	0.35	0.70	1.0	L-HCl
Tyrosine	2.8	0.35	0.70		

*Ramasharma, G. B., et al., J. Nutrition, 38, 177 (1949).

prepared for this experiment. Five rats were used in each of the eight trials in a completely randomized design.

Trials 5, 6, 7 and 8 were made with castor meal supplemented with DL-tryptophan. Rats on these trials showed a loss of body weight in one week. This result was followed by a similar observation after ten days. Modification of the feed was made to see if this condition could be reversed. A deficiency of one or more essential amino acids in the diets of trials 5, 6, 7 and 8 was suspected. A survey of the requirements of essential amino acids in the feed and the amount already present (Table VIII) was found to provide a possible answer to the problem. Lysine and methionine were noted to be the most deficient essential amino acids at the 12.5 per cent protein level of the castor meal diet. Phenylalanine was another deficient essential amino acid. Trials 5, 7 and 6, 8 had 0.35 and 0.70 per cent tyrosine respectively. It is known that tyrosine stimulates rat growth only when phenylalanine is furnished in sub-optimal amounts (86). Hence, in this case the tyrosine would replace the need for phenylalanine. The diets from trials 5, 6, 7 and 8 were then modified by splitting each into three parts. In every case one part was supplemented with the calculated quantity of L-lysine·HCl, the second part with DL-methionine and the third part was left unsupplemented. The animals from each of these trials 5, 6, 7 and 8 were also divided accordingly by taking two rats on the L-lysine·HCl supplemented, two on DL-methionine supplemented and one on unsupplemented (i.e., the original) feed. On the tenth day after starting the experiment these modified diets were given to the respective rats. On the 24th day one rat from trial 3 was found to be losing hair on its hind quarter, another rat on trial 5 also showed nearly the same kind of condition. Four rats in trial 8 had lost a total of 35.7 gm body weight in that week.

The explanation for this loss and loss of hair by some rats was found to be due to benzene hexachloride contamination of the feed. The feed mixer was used by someone 3 days before observing the first sign of poisoning from BHC. The mixing equipment contained a trace of BHC residue which contaminated the feed. As soon as it was realized that the BHC poisoning had occurred those batches of feed were discarded.

Figure 1 shows growth of rats on 12.5 per cent and 25.0 per cent protein diets prepared with casein and soybean. Figures 2 and 3 represent the results of SURDD castor meal diets at 12.5 per cent and 25.0 per cent protein level. Similarly Figures 4 and 5 represent the growth of rats on PVO castor meal at 12.5 per cent and 25.0 per cent protein diets. Table IX shows the over-all result.

Results and Discussion

The results of trial 8 will not be discussed because of toxication due to BHC. In trials 6 and 7 the effect of methionine supplementation with tryptophan appeared to be detrimental when compared with supplementation with tryptophan only. Lysine showed the best result as a supplement. The nutritive value of SURDD supplemented with tryptophan and lysine at the 25.0 per cent protein level compared closely with casin and soybean meal at 12.5 per cent protein. However, castor meal did not support the growth of rats when protein in the diet was entirely from this source. The results of this experiment can only be considered as preliminary. Nothing conclusive could be taken from this experiment, since only two rats were on lysine and methionine supplemented feed and only one on unsupplemented feed.

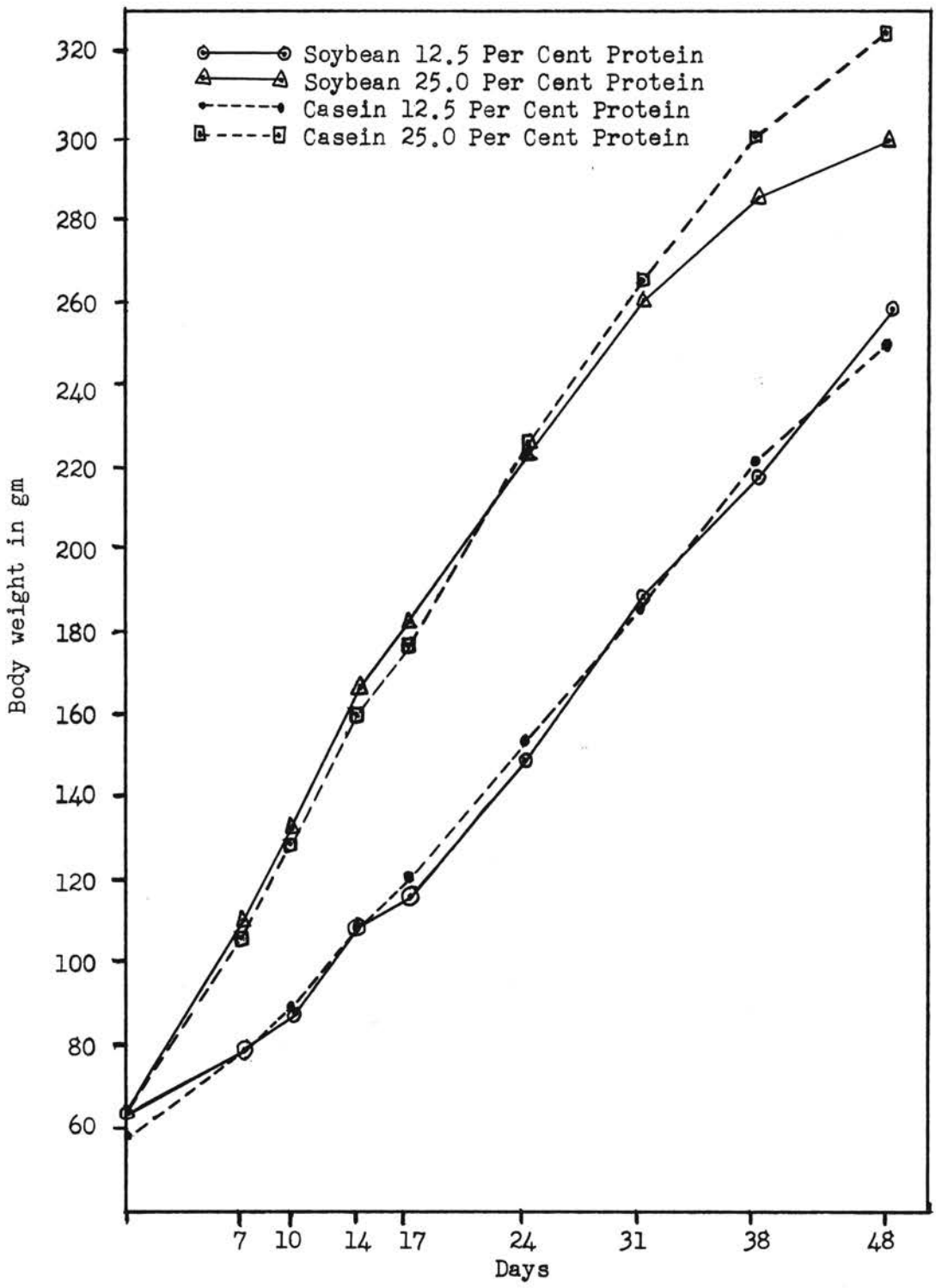

Figure 1. Trials 1, 2, 3 and 4.

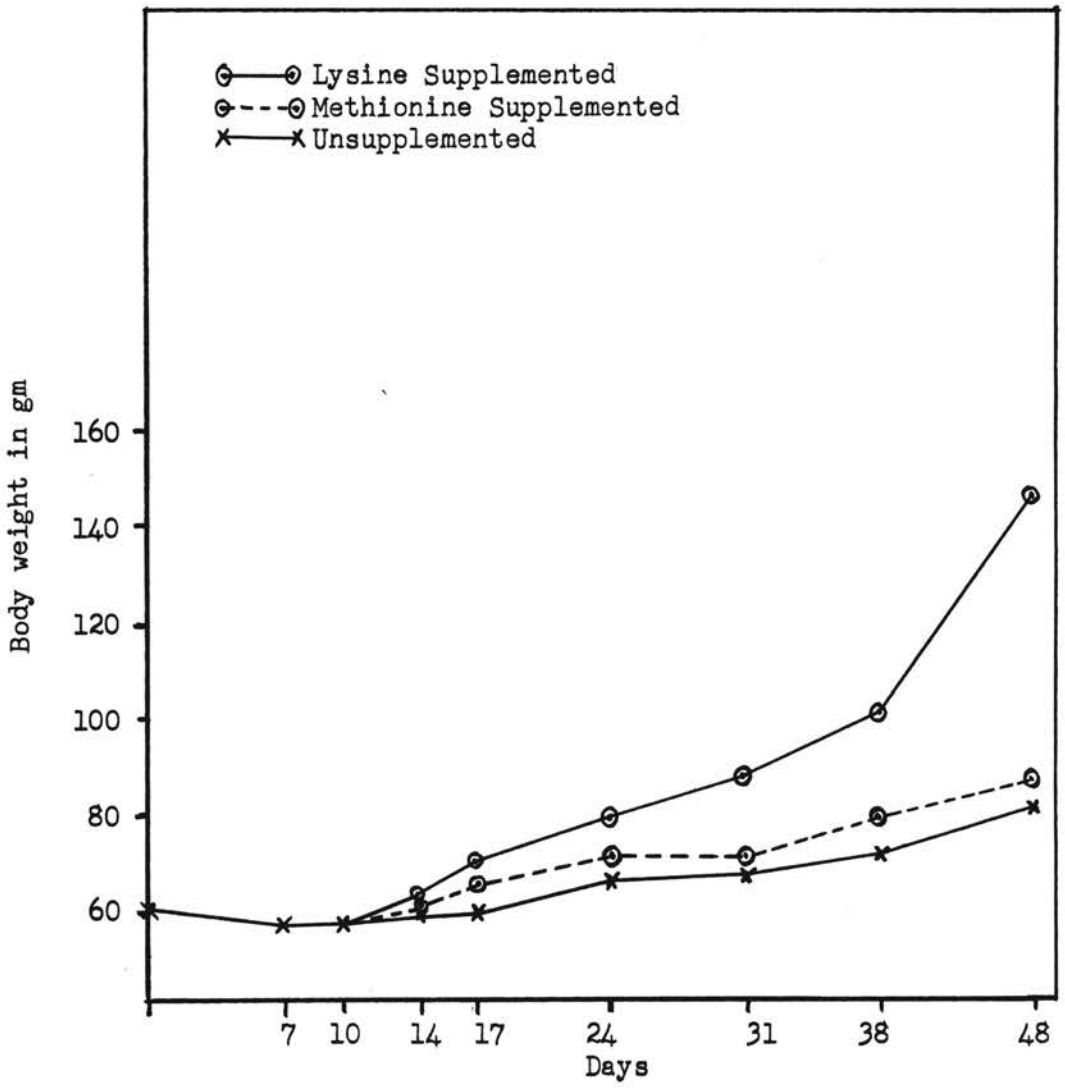

Figure 2. Trial 5 SURDD - 12.5 per cent Protein

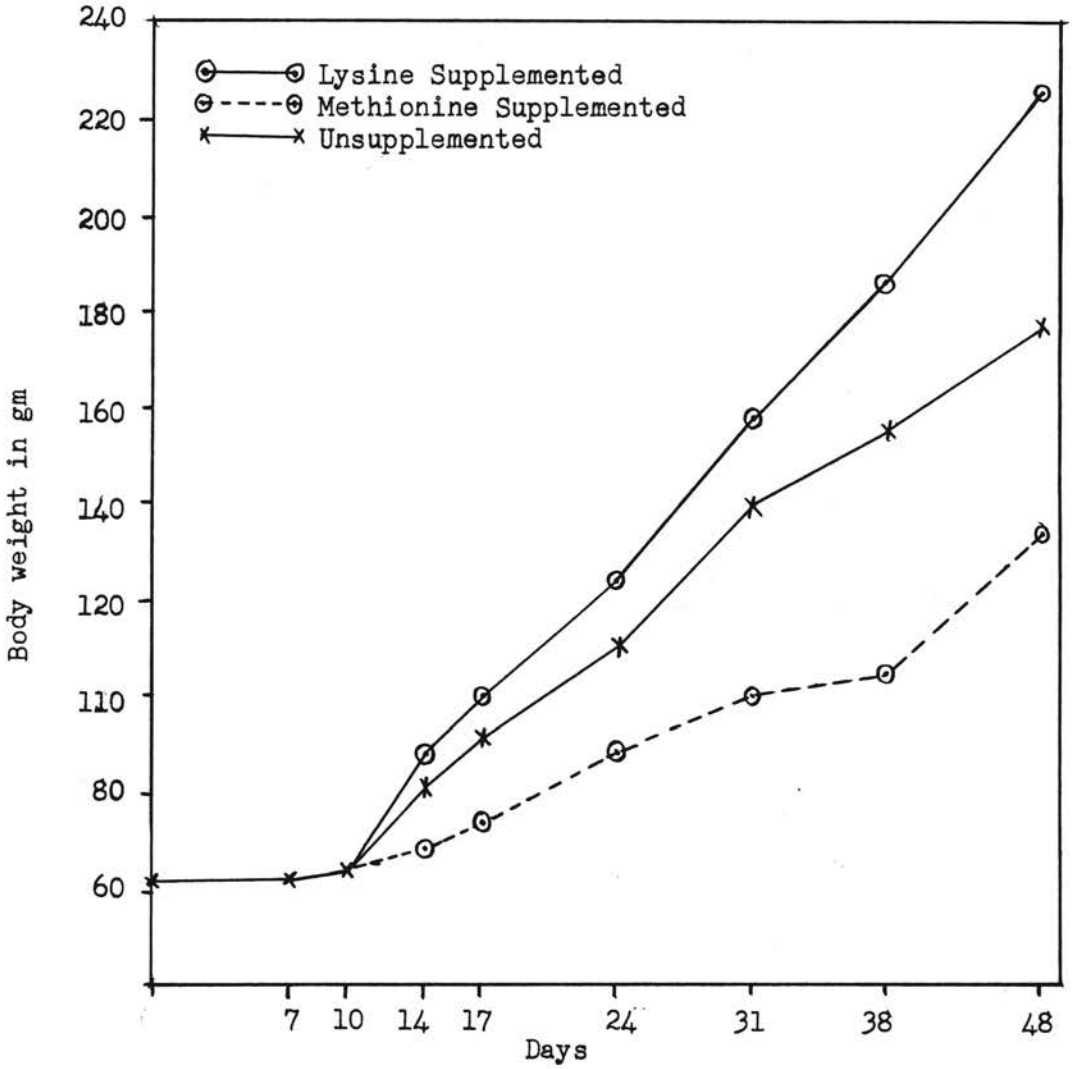

Figure 3. Trial 6 SURDD - 25.0 per cent protein.

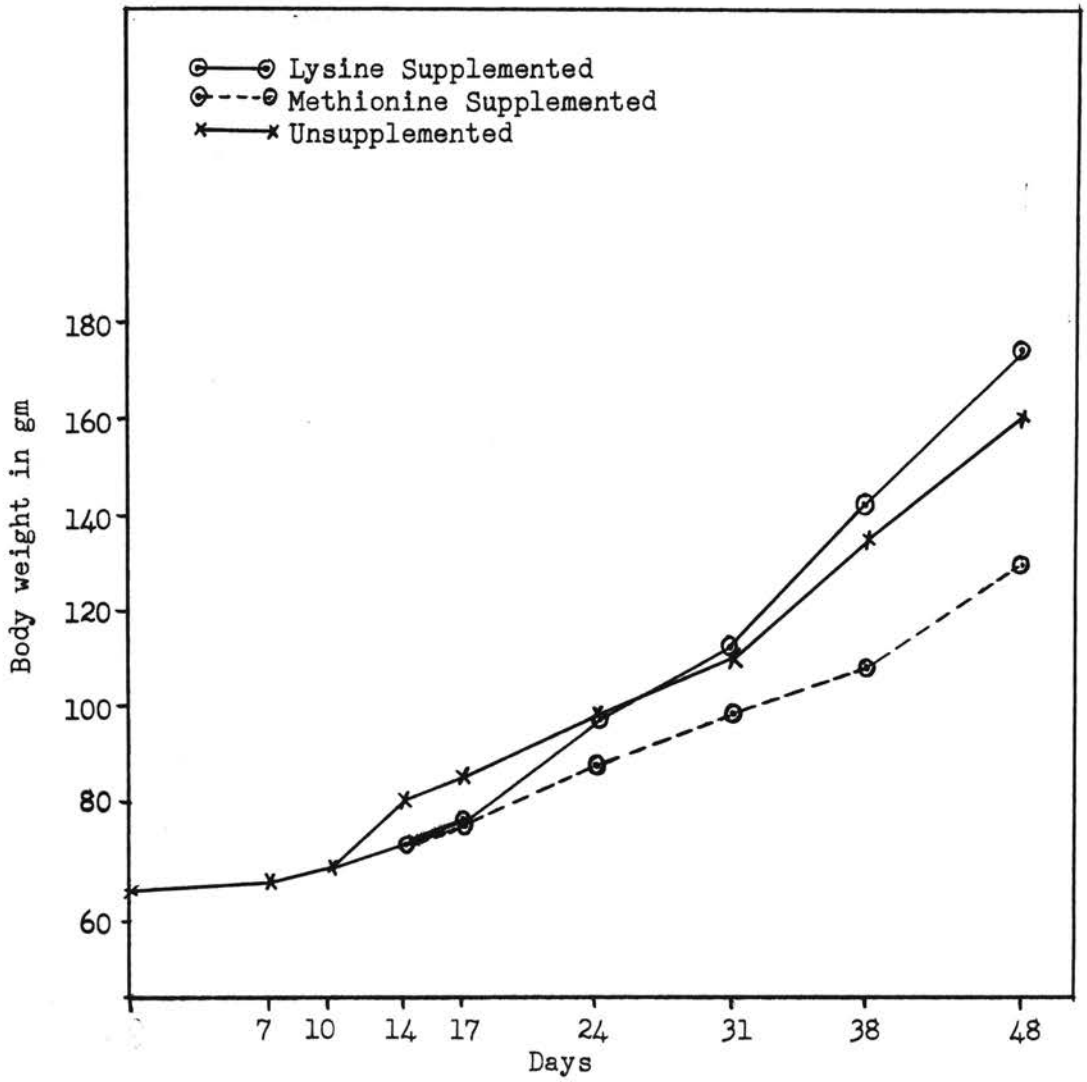

Figure 4. Trial 57, PVO on 12.5 percent protein.

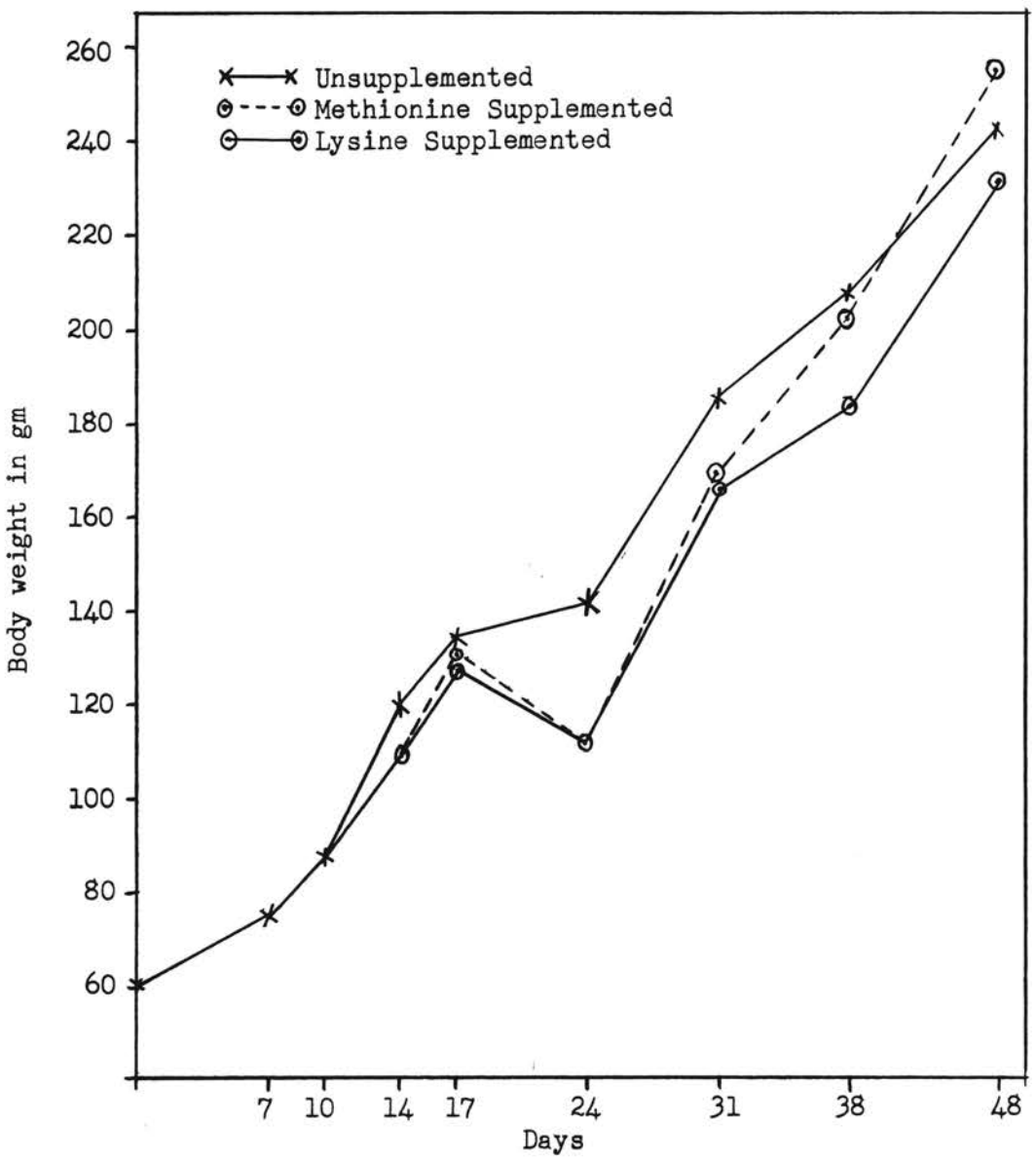

Figure 5. Trial 8 PVO - 25.0 per cent protein

TABLE IX

GROWTH OF RATS ON EXPERIMENT NO. 2

Trial No.	Protein Source	% Protein	Remark on Supplementation With Amino Acids*	Number of Rats	Average Weight Gained Per Week
1	Casein	12.5	no	5	27.7
2	Casein	25.0	no	5	39.0
3	Soybean	12.5	no	5	29.0
4	Soybean	25.0	no	5	35.1
5	SURDD	12.5	no	1	3.0
5	SURDD	12.5	Lysine	2	11.9
5	SURDD	12.5	Methionine	2	3.1
6	SURDD	25.0	no	1	17.9
6	SURDD	25.0	Lysine	2	25.3
6	SURDD	25.0	Methionine	2	10.6
7	PVO	12.5	no	1	14.3
7	PVO	12.5	Lysine	2	16.3
7	PVO	12.5	Methionine	2	9.7
8	PVO	25.0	no	1	26.7
8	PVO	25.0	Lysine	2	25.0
8	PVO	25.0	Methionine	2	28.4

*Supplements were added on the eleventh day of feeding.

Experiment Number 3

This experiment was set up to study the nutritive value of the two castor meal samples, obtained from Baker Castor Oil Company, as a preliminary study before working with sheep.

Seven rats were used in each of the six trials in a completely randomized design. The nutritive value of these castor meal samples was studied at 12.5 per cent and 25.0 per cent protein level, casein being used as the control. The amino acid content of the two Baker castor meal samples was assumed to be the same as that of SURDD. Three essential amino acids, lysine, tryptophan and methionine supplemented the diets at the level recommended (69). The composition of these diets is in Table X.

Ration of trial 4 depleted before the 6th week; hence results were recorded up to 5 weeks and 5 days. The other trials were continued for 6 weeks and 5 days. Administration of the fat soluble vitamin mixture was stopped after the 23rd day; however, cod liver oil[5] was given to all rats, 2 drops every other day from the 45th day.

The growth data are given in Table XI and Figure 6.

Results

All 14 rats on trials 3 and 6 were found dead within 78 to 96 hours after starting feeding. These two feed mixtures were prepared with Baker No. 1. About 28 hours before any of these rats died, they had started showing signs of distress and became very inactive. The average loss of body weight at death for each rat was found to be 39.5 gm, while 91.5 gm was their average body weight initially. The rats on trials 2

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[5] E. R. Squibb and Son, New York.

TABLE X

DIET FOR EXPERIMENT NO. 3

Trials / Ingredients %	1	2	3	4	5	6
Casein	14.95	---	---	29.91	---	---
Castor Meal	---	22.49	32.1	---	44.99	64.21
Sucrose	76.78	68.62	58.55	61.83	46.37	27.16
Salt Mixture	4.00	4.0	4.0	4.0	4.0	4.0
Corn Oil	4.00	4.0	4.0	4.0	4.0	4.0
Vitamin Mixture	0.25	0.25	0.25	0.25	0.25	0.25
DL-Tryptophan	---	0.1	0.1	---	---	---
L-Lysine	---	0.65	0.65	---	0.3	0.3
DL-Methionine	---	0.33	0.33	---	0.07	0.07
Dietary Protein %	12.5	12.5	12.5	25.0	25.0	25.0

Trials 2 and 5 were prepared with Baker No. 2.

Trials 3 and 6 were parpared with Baker No. 1.

TABLE XI

GROWTH OF RATS ON EXPERIMENT NO. 3

Trial	Average Initial Weight (gm)	Change in Body Weight After Days- 5	12	19	26	33	40	47	Total Gain or Loss	Gain in Weight Per Day
1	83.1	+ 8.4	+30.3	+28.3	+29.1	+ 9.1	+15.4	+1.6	+122.2	2.60
2	82.8	-17.2	+ 2.4	+17.2	+19.4	+16.5	+ 5.0	+5.1	+ 48.4	1.029
3	82.7	dead in 78 to 96 hours								
4	87.8	+15.7	+53.1	+38.6	+45.5	+ 7.7	- 2.2	Feed Ran Out	+158.4	3.960
5	82.6	-13.4	+22.6	+23.4	+37.2	+17.3	- 4.4	-9.4	+ 73.3	1.559
6	85.9	dead in 78 to 96 hours								

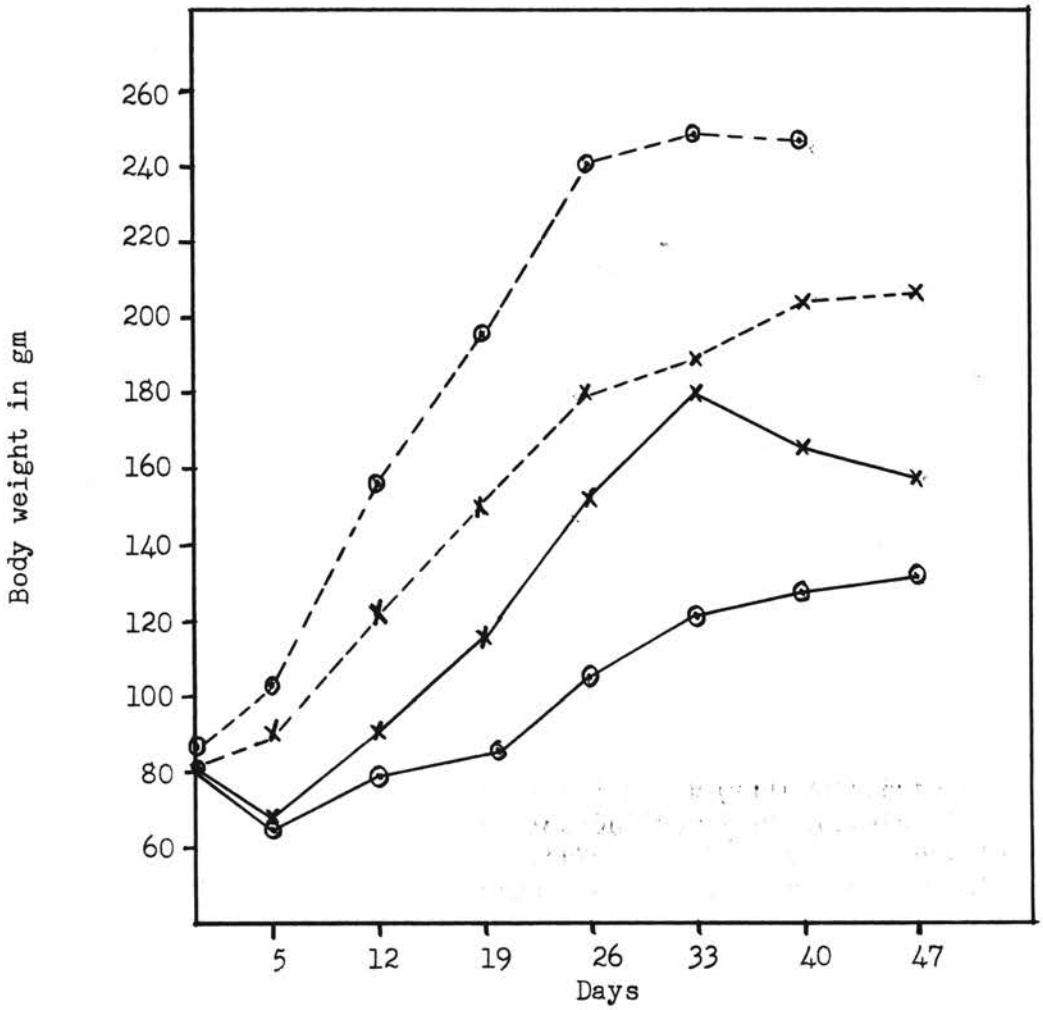

Figure 6. Trials 1, 2, 4 and 5.

X----X Casein, 12.5 Per Cent Protein
⊙----⊙ Casein, 25.0 Per Cent Protein
X——⊙ Baker No. 2, 12.5 Per Cent Protein
X——X Baker No. 2, 25.0 Per Cent Protein

and 5 showed a decrease of weight in the first week. After this period, these rats started gaining body weight slowly. Rats on trials 1, 2 and 4 showed a slower rate of gain of body weight and rats on trial 5 showed a decrease in body weight after the supply of fat soluble vitamin was cut off. The appearance of the rats, especially their eyes, showed ophthalmia in varying stages of severity indicating vitamin A deficiency. The carotene content of casein and the castor meal samples was determined (37), and it was found to be nil. At this stage cod liver oil was given to all rats. The beneficial effect of oral administration of cod liver oil was noticeable within 48 hours after administration.

The carcasses of the 14 rats on trials 3 and 6 had darkened and putrefaction was evident when found 18 hours after death. This prevented pathological investigations. In an effort to investigate the cause of death of these rats identical conditions were duplicated under which the 14 rats died. Three rats were kept without any food as control. The results are reported in Table XII.

Results

Rats with number I and "unmarked" were observed to have roughened hair coat and a collection of reddish brown material in each eye and in the nostrils. Gross pathological survey did not reveal anything remarkable. Histopathological examinations of the tissues of lung, heart, liver, spleen, kidney, stomach, small intestine, caecum and brain did not reveal any lesions which could be regarded as indicating death to be due to the ingestion of a toxic substance. A similar examination of 7 additional rats, number Y, 68, 69, 70, 71, 72 and 73, fed on the same castor meal resulted in findings essentially the same as the two animals described above. Rat No. Z

TABLE XII

RESULTS FROM DUPLICATING THE CONDITION UNDER WHICH RATS IN TRIALS 3 AND 6 OF EXPERIMENT 3 DIED

Rat Number	Protein Source	% Protein	Initial Weight gm	Final Weight gm	Loss in Weight gm	Time Taken To Die
I	Baker No. 1	25.0	87.7	66.8	20.9	95 hours
unmarked	Baker No. 1	25.0	104.7	79.3	25.4	95 hours
Y	Baker No. 1	25.0	78.8	49.0	29.8	69 hours
Z	Baker No. 1	25.0	204.0	170.0	34.0	Did not die in 96 hours
71	Baker No. 1	25.0	37.0	26.3	10.7	48 to 63
72	Baker No. 1	25.0	51.5	36.2	15.3	44
73	Baker No. 1	25.0	34.0	25.1	8.9	40 to 44
68	Baker No. 1	12.5	50.0	32.8	17.2	72 to 79
69	Baker No. 1	12.5	77.3	52.8	24.5	72
70	Baker No. 1	12.5	37.0	28.5	8.5	48
74	No food - only water		37.0	24.0	13.0	48 to 63
75	No food - only water		66.0	42.4	23.6	48 to 63
76	No food - only water		36.0	24.4	11.6	48 to 63

did not die in 96 hours and so was returned to normal diet and it recovered fully. Rats number 74, 75 and 76 were put on fasting to note the loss of body weight and any other possible clue to this problem, since all of these rats ate very little feed. These rats, after death, were submitted to the same type of examination as those fed with castor meal, but no diagnostically significant lesions appeared by either gross or microscopic observation. Upon both gross and histological examination no differences were noted between castor meal fed rats and starved rats. The loss of body weight at death and time taken to die were very nearly the same for the rats without food and the rats on castor meal. Refusal of the diet made from Baker No. 1 was also observed which indicated that this meal was unpalatable to the rats with the result that they died from starvation.

Experiment Number 4

Experiment number 4 was designed to find if there was any trace of toxic material left in the castor meal and also to study the adaptability of comparatively younger rats on diets prepared with Baker No. 2. Here the nutritive value of the castor meal was compared with casein and standard Rockland diet.[6] Four trials were designed with 5 rats in each. The protein level was 12.5 per cent in all trials except trial 1 which was made of Rockland diet (24.25 per cent protein). To test if there was any toxicity left in the castor meal, due to the presence of ricin, a sample of Baker No. 2 was autoclaved at 120° C for 20 minutes. Ricin, if present in the castor meal, would be destroyed by this method (38). Young rats, after weaning were used in trials 4 and 5. The experiment was carried out for six weeks. After that time all animals were put on Rockland diet.

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[6] Obtained from Arcady Farm Milling Company, 223 West Jackson Boulevard, Chicago, Illinois.

TABLE XIII

DIETS FOR EXPERIMENT NO. 4

Trial	Ingredients	% Protein
1	Pure Rockland diet	24.25
2	Casein, same as trial number 1 of experiment 3	12.5
3	Baker No. 2, same as trial number 2 of experiment 3	12.5
4	Same as trial number 3, except the castor meal was autoclaved before making feed mixture	12.5

Results and Discussion

The Table XIV and Figure 7 show the growth response of these 4 different trials. The control diets indicated that 12.5 per cent casein die not promote growth nearly as well as the Rockland diet. In the first 6 week period rats on trials 3 and 4 did not gain significantly in weight. The smaller weaned rats, average body weight 29.8 gm, could only survive on trial 3, gaining 6 gm body weight in 6 weeks. It is of interest to note that the average body weight of rats on trial 4 was 22.8 gm more than that of rats on trial 3 at the beginning of the experiment. In 6 weeks, this difference changed to 26.7 gm. These two figures were nearly the same which indicated almost identical growth rates in the trials 3 and 4. The castor meal sample did not improve the growth of rats even after autoclaving. This suggested that Baker No. 2 was free of ricin.

TABLE XIV

GROWTH OF RATS ON EXPERIMENT NO. 4

Trial	Average Initial Weight (gm)	Change in Body Weight After Weeks- 1	2	3	4	5	6	9	Total Weight Gain in 6 Weeks (gm)	Average Daily Weight Gain (gm)
1	55.6	+38.5	+40.2	+36.5	*	+35.8	+ 7.1	+ 13.8	148.1	3.52
2	57.1	+17.5	+12.1	+ 6.7	+25.0	+13.3	+15.9	+ 75.0	92.5	2.20
3	29.8	- 3.2	- 0.2	+ 1.6	+ 1.4	+ 4.8	+ 1.6	+117.6	6.0	0.14
4	52.6	- 3.0	+ 1.6	+ 2.4	+ 0.6	+ 2.2	+ 6.1	+129.1	9.9	0.23

*A reading was not taken.

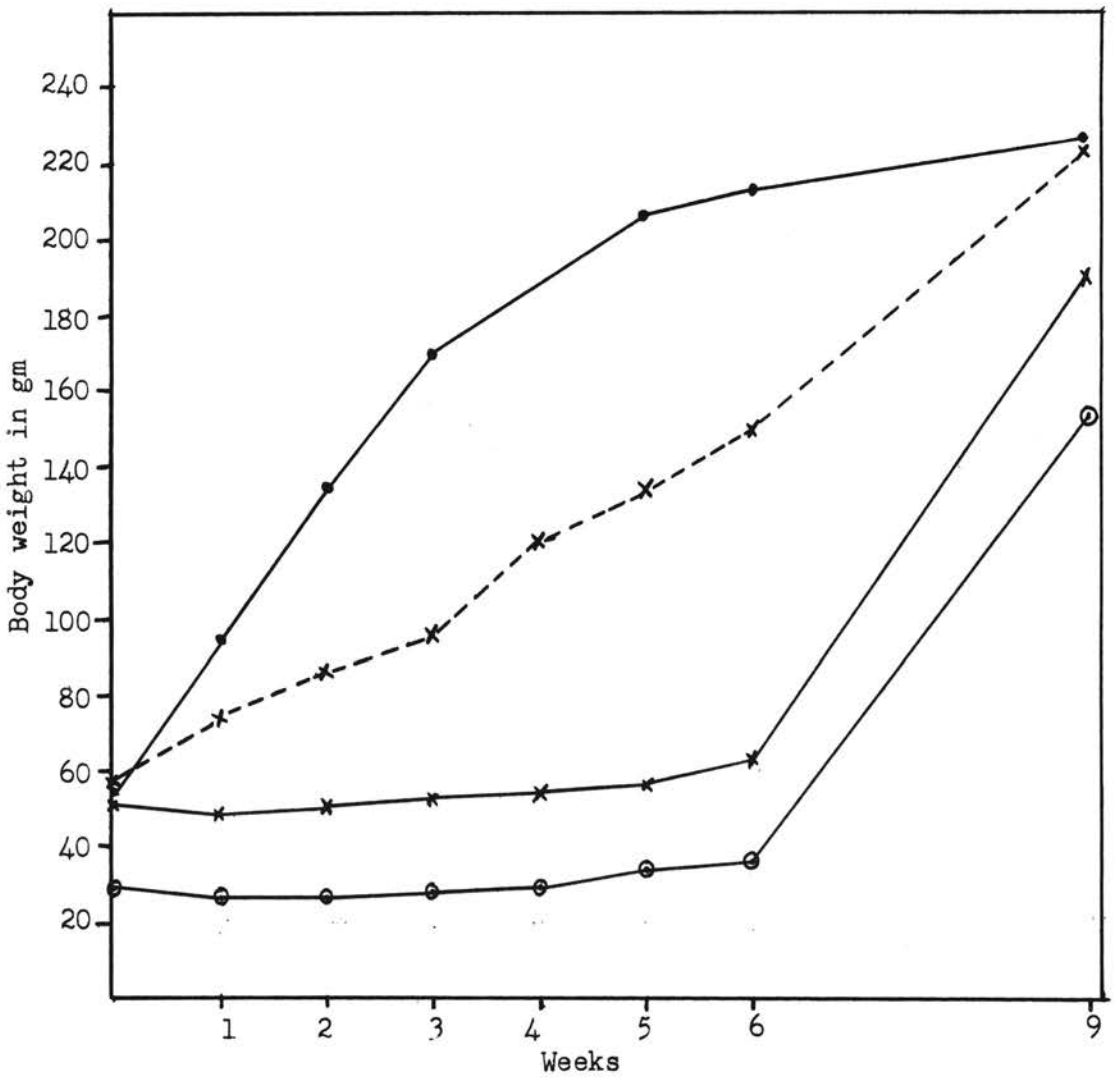

Figure 7. Trials 1, 2, 3 and 4.

Rockland Diet, 24.25 Per Cent Protein
Casein, 12.5 Per Cent Protein
Baker No. 2, 12.5 Per Cent Protein
Baker No. 2 Autoclaved, 12.5 Per Cent Protein

PART II

CHAPTER IV

DETERMINATION OF HEMAGGLUTINATING ACTIVITY AND TOXICITY OF DIFFERENT VARIETIES OF CASTOR BEAN

A. Photometric determination of hemagglutinating activity of ricin

It was considered desirable to determine if there was a difference in the quantity or activity of ricin present in different varieties of castor beans. If it were possible to locate a ricin-free variety of castor bean or a variety with a very low ricin content, it might then be possible for the plant breeders to produce an entirely new variety. The castor meal from such a bean could then be used as an animal feed without going through any detoxification processes.

It was mentioned earlier, in the literature review section, that the toxic and hemagglutinating properties of ricin have been attributed to one protein. The method used by industrial companies to measure the toxicity of castor meal is based on the hemagglutination test. Based on this line of reasoning, a photometric procedure was developed to establish a rapid, accurate and sensitive method of assay for ricin. The objective was to evaluate the activity of a crude ricin preparation by determining the point of 25 per cent hemagglutination of a washed suspension of easily available erythrocytes. The present approach was based on a method proposed by Liener (46) for the detection

of the soya bean hemagglutinin and on a hemagglutination test developed by Cahan.[7]

1. Selection of a method and materials

Photometric (turbidometric) measurements were made in an Evelyn Photometric colorimeter. All readings were made at a wave length of 720 mμ which was selected to reduce the absorption of light by the trace hemolysis that occurred. Preliminary experiments were conducted to determine the most suitable type of blood, to see what anticoagulant was most desirable, to determine the time of incubation and to determine the need of shaking during incubation. Blood obtained from three different sources was evaluated: 1) human blood, "type O," rejected due to aging, obtained from the Stillwater Hospital blood bank, 2) chicken blood, and 3) rabbit blood. Lithium citrate, heparin and Alsevers solution (33) modified to include Phenergan[8] (46) were tried as anticoagulants. Freshly drawn rabbit blood and lithium citrate were found to provide the best combination. An incubation period of 2 hours was found to be the best considering rapidity of the process and the reproducibility. After 2 hours of incubation, agitation of the incubation tubes was found necessary because heavy settling had occurred. A mechanical shaker, moving only in a horizontal plane, was used to prevent the settling of blood cells, but it was not satisfactory. By gently inverting the stoppered tubes through a 180° angle, four times by hand and then letting the tubes stand for 5 minutes, percentage transmittence readings

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[7] Mimeograph circular No. RF-27 from Southern Utilization Research and Development Division (1958).

[8] Trade name for N-(-2'-dimethylamino-2'-methyl) ethylphenothiazine Wyeth, Inc., Philadelphia, Pennsylvania.

from 93 to below 50 were obtained. Due care was taken not to agitate the settled cells after the 5 minutes settling period had elapsed before the readings were taken. These readings were reproduceable and duplicate readings checked within 2 per cent.

2. Preparation of red blood cells

Whole rabbit blood was drawn aseptically by heart puncture. One ml of 11.4 per cent lithium citrate was used for every 15 ml of whole blood. Citrated blood was transferred to a graduated centrifuge tube and centrifuged at 1000 to 1200 rpm for 3 minutes. The red blood corpuscles settled to the bottom and the supernatant was pipetted off. Physiological saline solution (0.85 per cent NaCl in glass distilled water) was added in the proportion of 1 volume of packed blood cells to 1 volume of saline. The cells were redispersed and centrifuged at 1000 to 1200 rpm for 3 minutes, and the supernatant was drawn off. This washing step was repeated 4 to 5 times until a clear supernatant was obtained. A trace of hemolysis was noticeable occasionally. Based on the volume of packed cells in the centrifuge tube, sufficient saline was added to give a final cell concentration of 4 per cent. Red blood cells so prepared could be preserved at 4° C for one day without any noticeable change occurring in the hemagglutination reaction.

3. Crude ricin solution (CRS) was prepared as follows:

Four to five decorticated seeds (about 1 gm) were crushed in a mortar. Special handling was necessary to prevent the spreading of the allergen from the dry, powdery castor meal into the laboratory atmosphere. One gm of the decorticated and crushed seeds was wrapped in filter paper and extracted with ether for 16 hours in a Goldfisch extractor to remove the oil. From 73 to 76 per cent of oil was

extracted by this treatment. The crushed, low lipid castor seeds (castor meal) was air dried for 16 hours to remove the ether. It was then extracted with 50 ml of 0.85 per cent saline in a micro Waring blender for 3 minutes at 4° C. The solution was filtered through Whatman No. 42 paper. This filtrate was stored at 4° C for future use and will be referred to as the crude ricin solution (CRS). The pH of the CRS was 6.8 to 7.0. The protein (N x 6.25) content of CRS was determined by modified Kjeldahl method (6).

4. Procedure used for measuring the hemagglutinating activity of ricin

Duplicate experiments were carried out for each variety of castor bean. Seven serial dilutions of CRS were made for each set. Eight tubes were used in each set of dilutions. In the first tube 0.2 ml of CRS and 19.8 ml of saline were mixed. From this mixture 5.0 ml was transferred into a second tube, 3.5 ml into a third tube, 5.0 ml into a fourth tube, 2.0 ml into a fifth tube, 1.5 ml into a sixth tube, and 1.0 ml into an eighth tube. The required volume of saline was added in tubes 3, 5, 6, and 8 to bring the volume up to 5.0 ml in each tube. Five ml of saline was added to tubes 4 and 7. Tube 4 was mixed thoroughly, then 5.0 ml of this mixture was withdrawn and pipetted into tube 7. Tube 7 was shaken to mix the diluted CRS and saline, and 5.0 ml of this mixture was withdrawn and discarded. Thus, the final dilutions of CRS in the seven tubes (2 to 8) were 1:100, 1:143, 1:200, 1:250, 1:333, 1:400, and 1:500 respectively, all tubes having exactly 5.0 ml in volume. The 4 per cent blood corpuscle suspension was diluted with saline to 30 per cent transmittance (optical density = 0.52) at 720 mμ. The RBC suspension was stirred continuously with a magnetic stirring bar. To each of the

seven tubes containing diluted CRS 5.0 ml of the RBC suspension (30 per cent T) was mixed and incubated for 2 hours at 25° C. The transmittance of each tube was read after shaking and letting the tubes stand for 5 minutes. By plotting per cent transmittance from each tube against the reciprocal of the dilution smooth curves were obtained. Figure 8 shows a typical set of such curves.

In the control tube, prepared with 5.0 ml RBC suspension plus 5.0 ml saline, per cent T = 52 ± 2 (O.D. = .284) was constant for the 2-hour incubation period. Very light settling of the RBC occurred during this period in the control. It is evident that increasing concentration of CRS would cause more clumping of the suspended red blood cells. After the incubation period, therefore, the concentration of cells remaining in suspension should bear a nearly direct relationship to the quantity of ricin present in the diluted CRS. To measure the point at which 25 per cent of the red blood cells were agglutinated by ricin, the end point was taken as 61 per cent transmittance (optical density = 0.213), and this value was obtained from the graphs drawn for each CRS sample. Quantitative estimation of ricin present at the dilution corresponding to 61 per cent T was made as shown in Table 15. The two variables mainly governing the extent of hemagglutination were 1) quantity of ricin present and 2) activity or potency of the ricin present. The dilution 100 to 500 was used because below 100 dilution the hemagglutination was so strong that nearly a clear supernatant almost devoid of any red cells remained in the path of the light beam of the photometer.

B. Evaluation of toxicity of CRS

Rats were injected subcutaneously in the abdominal region

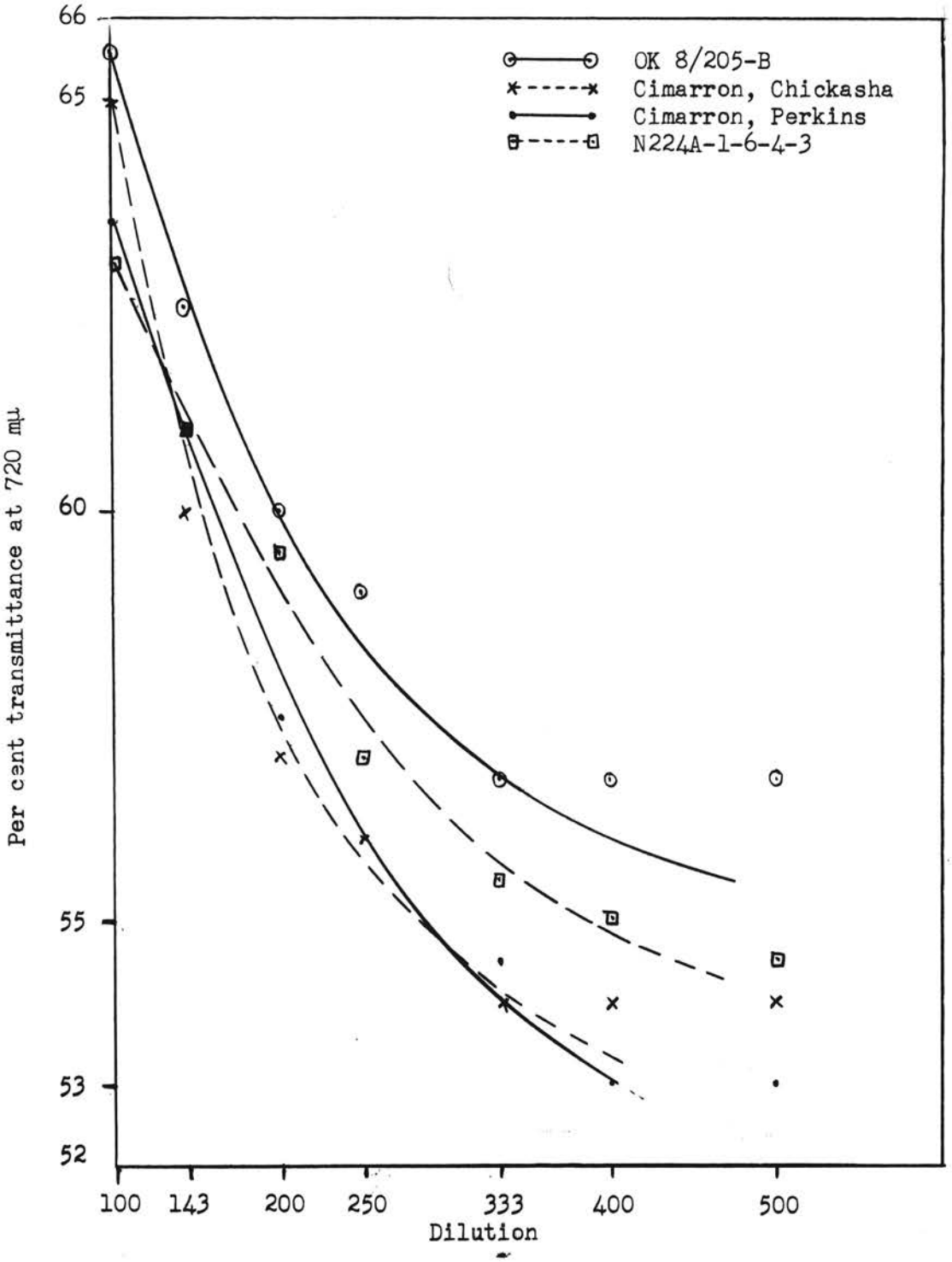


Figure 8. Hemagglutinating activity of selected varieties of castor seeds.

TABLE XV

RESULTS OF HEMAGGLUTINATION AND TOXICITY ASSAY OF CASTOR SEED VARIETIES

Variety							
1	2	3	4	5	6	7	8
No.	Name	Place Grown	Year Grown	Mg Prot./ Ml in CRS	γ Prot. Req. for 25% Hem. a	MLD γ Prot./ 100 gm Body Wt. b	Ratio a:b
5	Cimarron	Perkins, Okla.	1958	1.55	54.97	4.4	12.49
6	Cimarron	Chickasha, Okla.	1952	1.86	48.80	5.1	9.56
7	US 74	Blackwell Lake, Okla.	1951	1.23	78.88*	3.85	20.48
8	N224A-1-6-4-3	Paradise, Okla.	1955	1.48	49.9	4.0	12.47
9	Custer	Perkins, Okla.	1958	1.73	52.88	2.18	24.25
10	USDA 65	Perkins, Okla.	1958	1.54	44.21	2.04	21.67
11	US 3/384-8	Perkins, Okla.	1958	1.28	90.94*	3.61	25.19
12	Baker 296	Perkins, Okla.	1958	1.37	45.38	2.14	21.20
13	Dawn	Perkins, Okla.	1958	1.61	35.35		
14	Interspread Dawn	Perkins, Okla.	1958	2.03	46.65		
15	OK 8/201-B	Stillwater, Okla.	1958	1.75	50.04		
16	OK 8/205-B	Stillwater, Okla.	1958	1.31	36.58		
17	Cimarron Hybrid	Perkins, Okla.	1958	1.67	38.49		
18	US-51 Hybrid	Perkins, Okla.	1958	1.69	43.73		
19	Baker Hybrid 45	Perkins. Okla.	1958	1.73	50.37		
20	Pacific oilseeds Hybrid 6	Perkins, Okla.	1958	1.84	48.96		

*Values obtained by extrapolation. Varieties 13 to 20 were not tested on rats.

with graded doses of CRS. The minimum lethal dose (MLD) within 48 hours was found. These values given as the MLD should not be confused with LD_{50} values, i.e., the dose which would kill 50 per cent of the animals tested with one particular dose. The results are given in Table XV.

1. Results and discussion

The figures in column 6 indicate the γ of protein per 100 gm body weight needed to kill the rats within 48 hours after subcutaneous injection. The last column shows the ratio of γ of protein causing 25 per cent hemagglutination to the γ of protein required as MLD. Of the 16 varieties tested for hemagglutinating potency, variety No. 13 required 35.35 γ protein, the least quantity for 25 per cent hemagglutination, hence it was considered to be the most active. Variety No. 10 was similarly the most potent among the eight varieties tested for toxicity and hemagglutinating potency. This variety was also the most toxic since 2.04 γ, the least amount of protein, was required to kill 100 gm body weight rat in 48 hours. The ratios in column 6 came under at least two groups, 1) a:b = approximately 10 and 2) a:b = above 20.

Out of the 16 varieties tested, not a single sample showed the absence of hemagglutinating potency. The absence of a consistent relation between hemagglutination and toxicity made it impossible to evaluate toxicity based on hemagglutinating activity. The ratios of 10:1 and 20:1 indicated that the active groups on ricin causing these two effects were not produced by the plants in the same ratio. Most probably, two proteins produced in different quantities by castor plants are responsible for these two unrelated effects.

CHAPTER V

PATHOLOGICAL STUDIES AFTER RICIN ADMINISTRATION

CRS as previously described was injected subcutaneously in the abdominal region of rats. The first pronounced symptom observed in every case within 24 hours of injection of even sublethal doses was the accumulation of red tinged material at the nostrils and eyes. The other symptoms were convulsions and diarrhea followed by extreme relaxation and convulsions. Rats with sublethal doses showed all these symptoms to some degree. They recovered and were normal in appearance and behavior.

The gross pathological changes most frequently found at necropsy were: (a) red tinged material around the eyes and nostrils; (b) reddening of gastrointestinal tract from pyloric region of the stomach to the ileo-caecal junction, containing a red tinged mucous material; (c) large deep red mesenteric lymph nodes with multiple hemorrages in adjacent mesenteric adipose tissue; (d) liver dark brown in color with fine greyish white granular mottling on surface and throughout organ; (e) normal sized spleen reddish black in color; (f) pancreas a mottled red color; and (g) a diffuse edematous to necrotic yellowish swelling of abdominal wall at site of injected ricin solution. Less frequently noted lesions were red mottling of one or both lungs; enlarged red

thymus gland; white granular material in bladder with plugging of urethra; increased amount of clear straw colored fluid in thoracic and abdominal cavities; and reddening and enlargement of various lymph nodes. In all animals the kidneys and heart were essentially normal in appearance. In those cases in which the brain was examined, it was normal.

The following tissue specimens were placed in 10 per cent formalin solution upon autopsy; tissue from site of injection, fat tissue from mesenteric lymph nodes, kidney, thymus, lung, small intestine, large intestine, pancreas, eye ball and adjacent structure, cerebrum, adrenals, heart, and liver. Histological sections of the fixed tissues were prepared by mounting in paraffin and cutting with microtome at 5 microns thickness. These were stained with hematoxylin and eosin. The microscopic findings from the above mentioned organs and tissues were as follows:

Tissue from site of injection - The dermis and epidermis appeared normal; subcutis contained areas of fat undergoing necrosis. Blood vessels were distended and many had erythrocytes outside of the lumen. Masses of pink granular material containing dark droplet like masses of degenerated nuclei were observed in various other parts of the sections. These masses were confined within what appeared to be lymph vessels. Slight edema was present throughout.

Fat tissue from mesenteric lymph nodes - Erythrocytes were found loosely scattered between fat cells. In a few areas the capillaries were intact but most of them appeared to be undergoing disintegration with loss of erythrocytes into intracellular spaces.

Kidney - The cells lining the tubules swollen with lumen were not apparent, or if present they contained pink granular material.

Thymus - It revealed marked interlobular edema and small hemorrhages were scattered through parenchymal tissue.

Lungs - Mild congestion with a few small areas of septal cell proliferation was observed. This was probably a normal condition in these animals.

Small intestine - The duodenum had a few scattered areas of congestion at the tips of the villae with some necrotic areas.

Large intestine - Congestion of submucosa and mucosa was observed.

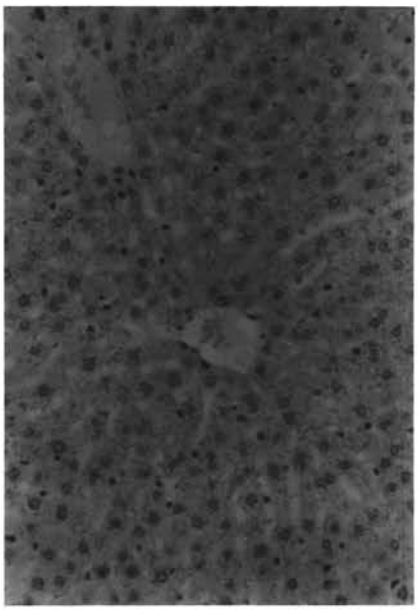
Pancreas - In some sections there was a uniform loss of acinar structure with no marked degenerative changes but with cellular outline lacking. The tubules were distended containing a smooth pink staining material. There was no evidence of congestion.

Eye ball and adjacent structures - These were found to be essentially normal.

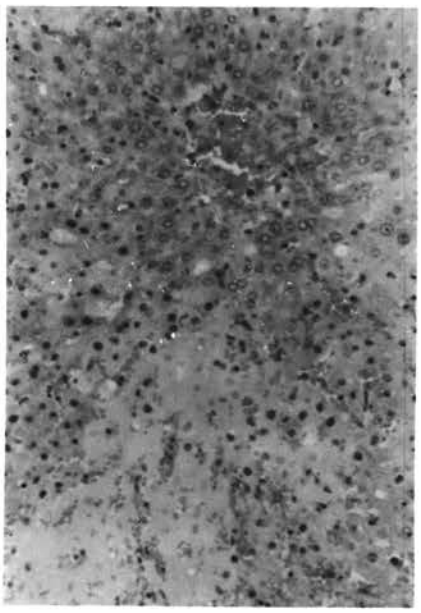
Cerebrum - There was no noticeable change from the normal.

Adrenals - A mild congestion of medullary area was observed.

Liver - Between one-half and two-thirds of the tissue had undergone very acute focal degeneration. These areas had lost their structural outline but contained apparently intact sinusoids filled with normal appearing erythrocytes. Many parenchymal cells which do not show complete loss of outline displayed vacuoles in cytoplasm. In several of the degenerating areas there was observed many various sized, round, evenly stained pink masses resembling hylin droplets. Photograph 1 is a section of liver from a normal rat. Two central veins, organized rows of liver cells, i.e., hepatic chords and nuclei (dark patches), are visible. In the section of liver taken from a ricin injected rat, Photograph 2, the central vein is present almost intact. Approximately half of the cells



Photograph 1
Section of liver from a normal rat
X35



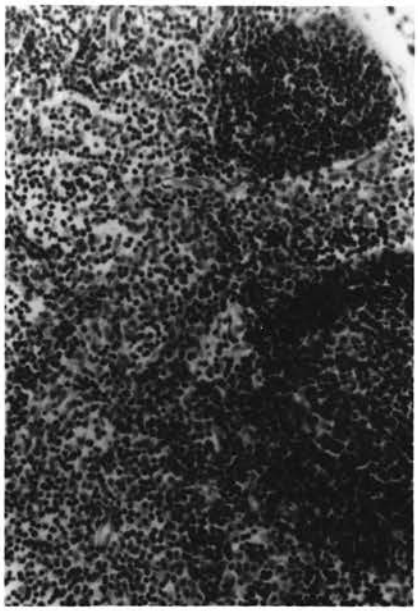
Photograph 2
Section of liver from
ricin injected rat
X35

Photograph 3
Section of mesenteric lymphatic node
from ricin injected rat
X35

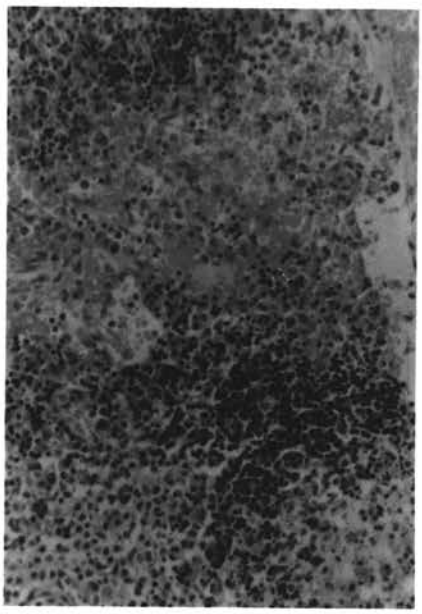
appear dead. No organized rows of cells can be found. The reddish areas are trapped blood.

Mesenteric lymph node - Cortical lymphoid tissue in many areas underwent degenerative changes. The number of immature lymphocytes increased. All lymphatic sinuses, both cortical and medullary, were distended with blood instead of lymph. The afferent vessels were distended with clear lymph. The mesenteric fatty tissue surrounding the node contained massive hemorrhages. Photograph 3 is a section of mesenteric lymphatic node from a ricin injected rat. Unfortunately a good slide of a normal mesenteric lymphatic node could not be obtained for comparison. The upper part, that above the arched region, is outside of the lymph node. Normally this area has clear fat and no blood, but here the red stain is due to blood from ruptured vessels. Photograph 4 is a section of lymph node from a rat not injected with ricin preparation. Two reaction centers are conspicuous here. The deep blue spots are nuclei of lymphocytes and the gaps are sinuses. Photograph 5 is a picture of the section of a ricin damaged lymph node. In this case the reaction centers are undergoing degeneration. The reddish stain is blood, which is not found in normal tissue. Large dark patches are damaged lymphocytes and the clear areas the lymph sinuses.

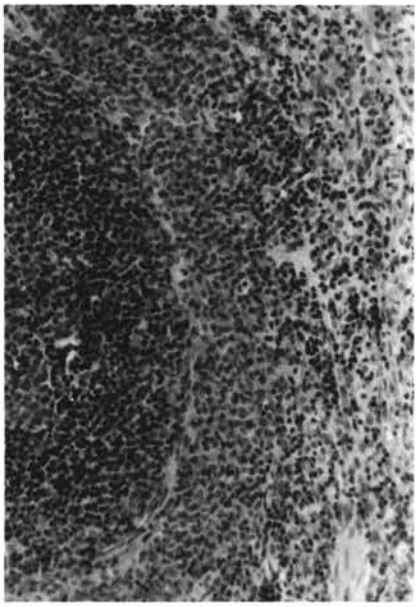
Spleen - The outstanding feature was the large number of highly immature lymphocytes. The reaction centers had lost their normal histological pattern and many contained small foci of degeneration with cells showing typical pyknosis and karyorrhexis. The venus sinuses were almost devoid of blood but did contain masses of lymphoblasts and endothelial cells many of which were showing mitosis. A section of spleen from a normal rat is pictured in Photograph 6. Here the reaction



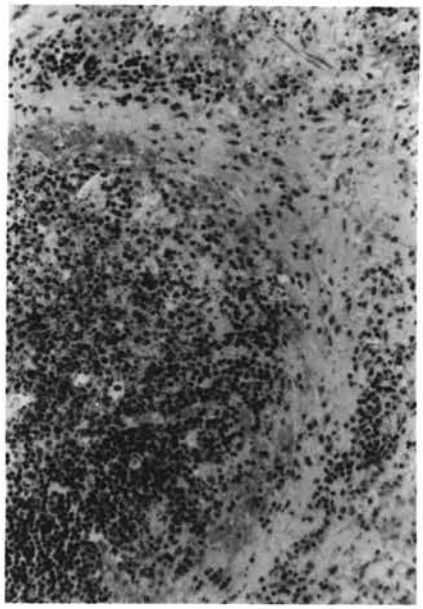
Photograph 4
Section of lymph node
from normal rat, X35



Photograph 5
Section of lymph node
from ricin injected rat, X35



Photograph 6
Section of spleen from normal rat
X35

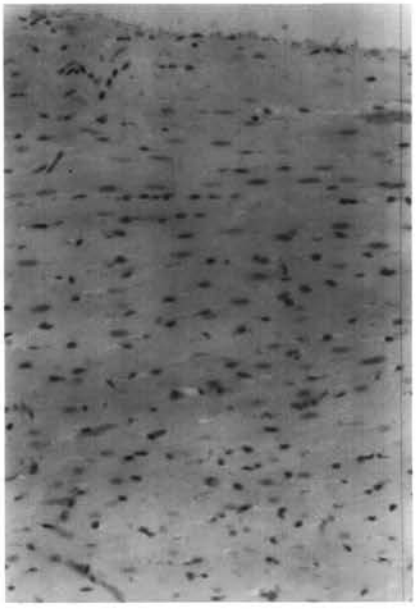


Photograph 7
Section of spleen from
ricin injected rat, X35

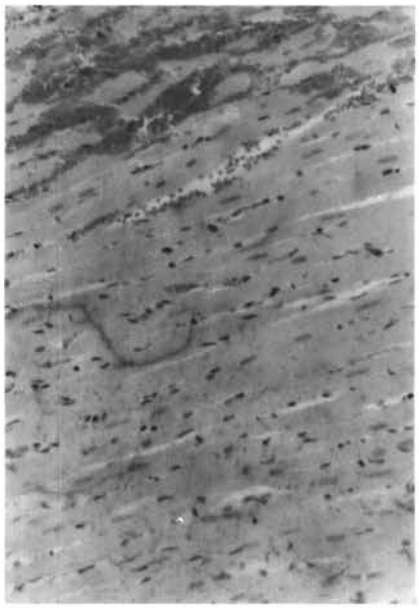
center is well organized; the blood sinuses are distinct. Photograph 7 shows the effect of ricin on the spleen. Many cells were dead and the blood sinuses were distended.

Heart - Congestion and sub-endothelial hemorrhages in valvular regions with a few small hemorrhages between muscle fibers in surrounding area were observed. Sections of a normal rat heart muscle and a rat heart muscle affected with ricin are shown in Photographs 8 and 9, respectively. In the normal heart compact muscle fibers are visible. Dark spots are nuclei and small red patches are blood vessels. Photograph 9 shows the hemorrhage identified by the red areas. The clear gaps are the spaces between muscle fibers which were found to be compact in the normal heart.

To determine the effect of oral ingestion of ricin, the protein fraction precipitated at pH 8.0 and 1/2 ammonium sulfate saturation, was mixed with drinking water and Rockland diet was fed. Every day the amount of ricin preparation in water was increased; first day 1 in 10, second day 2 in 10, third day 4 in 10, fourth day 8 in 10 parts of water until water was entirely replaced with ricin solution on the fifth day. The protein content of this solution was 11.72 mg/ml. No visible symptoms appeared even in 7 days from the day ricin solution was first administered to rats. The total quantity of water taken by these rats every day was normal. The quantity of water consumed was not measured. The same ricin preparation when injected at a dose of 5.0 - 5.9γ/100 gm body weight was found to be lethal. After the seventh day the rats were given pure water for drinking. No usual symptom was observed.



Photograph 8
Section of normal
heart from rat



Photograph 9
Section of heart from
ricin injected rat

The present work showed a difference in effects of ricin preparations when injected and when given orally. This was contradictory to the report (39) where characteristic pathological changes were observed after administering ricin intraperitoneally and in the feed.

Because of the internal damage done by ricin, it would be difficult to tell whether death of animals after 96 hours was due to the action of the toxin alone or whether there were superimposed infections which have added their effects; however, a brief investigation of this possibility was not confirmed. Corwin (17) has described a dose-survival time method to eliminate this type of secondary effect in evaluating toxicity.

CHAPTER VI

FRACTIONATION OF CRUDE RICIN SOLUTION

The nonhomogenous nature of crystalline ricin in solubility tests (40) and the present work (discussed on page 44) suggested the possibility of assigning the unrelated toxic and hemagglutinating properties to two different proteins Up to date literature does not show work on the chromatographic separation of proteins from castor seed (more particularly ricin). Recognizing the high resolving power now attainable, the utilization of a chromatographic technique for the separation of a protein mixture was considered desirable. To separate and identify the proteins which were possibly causing toxicity and hemagglutination independently, selection of a suitable adsorbent from conventional materials used for chromatography was the first stage of the problem.

Holasek (28) isolated ricin from a 10 per cent saline extract of low lipid castor meal. The most toxic fraction was obtained from the precipitate at the 1/3 to 1/2 saturation level of $(NH_4)_2SO_4$. This protein solution was repeatedly dialyzed against water and precipitated; in toxicity tests with rats it resembled closely the crystalline ricin obtained by Kunitz and McDonald (40). In the experiments discussed earlier, toxicity and hemagglutinating tests were made with crude ricin prepared by extracting low lipid castor meal with 0.85 per cent NaCl solution. In order to obtain results comparable with those obtained from the varietal tests, extractions of the crude ricin were made with 0.85 per cent NaCl solution.

A. Ammonium sulfate fractionation

Procedure

Saline extract of castor seeds was prepared as described on page 39. The CRS was centrifuged at 8000 rpm in a 5.75 inch centrifuge head for 40 minutes to remove the precipitate which passed through the filter paper. This precipitate was mainly starch. The starch-free extract was made up to the 1/3 saturation level with solid $(NH_4)_2SO_4$ (reagent grade, Matheson Coleman and Bell). The pH was adjusted to 8.0 with 0.1 N NH_4OH. The CRS at 1/3 saturation and pH 8.0 was allowed to settle. The pH of this suspension was found to change with time; however, pH 8.0 was maintained throughout the 24 hours precipitation period. The precipitated protein was centrifuged at 9000 rpm in a 5.75 inch head for 1 hour and then removed. Enough solid $(NH_4)_2SO_4$ was added to the clear supernatant to reach the 1/2 saturation level. After 24 hours of standing at pH 8.0 without stirring, a thick, floculated protein was observed. This protein was centrifuged as previously described. In a similar manner protein fractions were collected at 2/3 and full saturation with $(NH_4)_2SO_4$ at pH 8.0. Protein fractions precipitated at 1/3, 1/2, 2/3 and full saturation and the protein fractions residing in the corresponding supernatants were assayed for their toxicity by injecting graded doses into rats and for their hemagglutination activity by the test described previously. The protein concentration was determined from optical density readings at 280 mμ and 260 mμ using a Beckman DU spectrophotometer and nomograph.[9] The approximate percentage of protein in each fraction and the results of the assays are given in Table XVI. The protein precipitate obtained

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[9]From California Corporation for Biochemical Research, Los Angeles, California. As prepared by E. Adams.

TABLE XVI

RESULTS OF AMMONIUM SULFATE FRACTIONATION OF CRS

Protein Fraction	% Protein Precipitated From CRS	Toxicity in Rats γ Protein/100 gm Body Weight	Hemagglutinating Activity Visual Observation
Precipitate at 1/3 saturation	16.1	6.5 not lethal 7.2 lethal in 48 to 60 hours	high
Precipitate at 1/2 saturation	24.5	4.7 lethal in 24 to 36 hours 5.0 lethal in 24 to 36 hours	high
Precipitate at 2/3 saturation	7.0	4.3 lethal in 36 to 48 hours 4.8 not lethal	low
Precipitate at full saturation	13.9	5.1 lethal in 96 hours 5.5 lethal in 84 hours 5.8 lethal in 48 to 60 hours	high
Supernatant after 1/3 saturation	75.9	4.4 not lethal 6.6 not lethal	low
Supernatant after 1/2 saturation	47.5	4.8 not lethal 6.6 not lethal	low
Supernatant after 2/3 saturation	37.7	5.5 not lethal 5.9 not lethal	very low
Supernatant after full saturation	27.9	7.2 not lethal 11.1 not lethal	very low

at 1/2 saturation was dissolved in distilled water (pH 6.9). The solution, which had a very faint buff color, was filtered through Whatman No. 42 paper and dialysed[10] in a cellophane[11] bag against distilled water (pH 6.9) for 84 hours. The water in the dialyzer was changed every 12 hours. After 84 hours the water in the apparatus was found to be free of Cl^- and $SO_4^=$ ions. During dialysis a white granular sediment appeared in the bag during the first 24 hours. The clear supernatant obtained from the dialysis bag was filtered to remove the sediment and then lyophilized. The lyophilized material was white. This was stored at 4° C for further use.

The precipitated protein at 1/3 saturation was dissolved in distilled water and preserved at 4° C. Within 37 days a white precipitate appeared. The precipitated matter was removed by centrifugation, dissolved in water, filtered and tested on rats by subcutaneous injection. A dose of 17.8 γ protein/100 gm body weight failed to kill or produce any conspicuous symptoms. On the other hand, the clear supernatant when injected at the minimum dose tried (7.6 γ protein/100 gm body weight) was lethal within 29 to 43 hours.

A very brief study was made with the sedimented product found in the dialysis bag. This compound was insoluble in distilled water (pH 7.0), but was soluble in the dilute H_3PO_4 at pH 3.9 to 4.1. Most of the compound dissolved in aqueous NH_4OH at pH 8.8, but even after reaching pH 10.0 the solution did not clear up completely. A maximum stage of turbidity (visual observation) appeared near pH 7.0 whenever acidic solution was made basic or visa versa. The compound was dissolved in water at pH 9.0

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[10] Oxford Multiple Dialyzer Model B, Oxford Laboratory, San Francisco, California.

[11] Cellulose Casing, Visking Company, Chicago, Illinois.

(0.1 N NH_4OH), filtered, and the clear filtrate injected subcutaneously into rats. The minimum level injected was 16.6 γ protein/100 gm body weight. This was a lethal dose; the rat died in 48 to 60 hours. Another sample was prepared by dissolving the compound at pH 3.8 (0.1 N H_3PO_4). In a similar toxicity test 15.8 γ protein/100 gm body weight was the minimum dose tried and this proved lethal in 60 hours. In both cases control animals were injected with water at pH 9.0 (NH_4OH) and at pH 3.8 (H_3PO_4). No visible harmful effect was observed. The protein (sediment) solutions were 2 1/2 hours old before the injections were made.

B. Starch gel electrophoresis

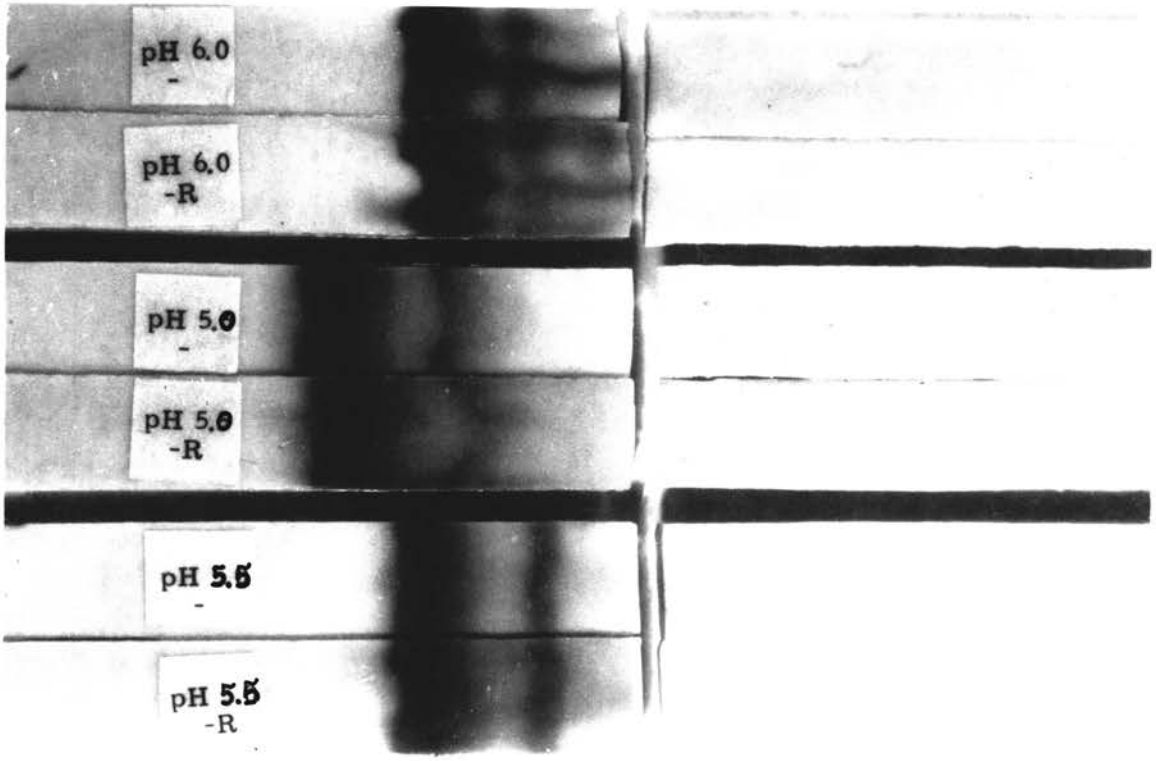
Starch gel electrohporesis was used to separate the proteins present in the fraction obtained at 1/2 saturation. Another purpose was to determine the behavior of ricin in this system since no work has been reported.

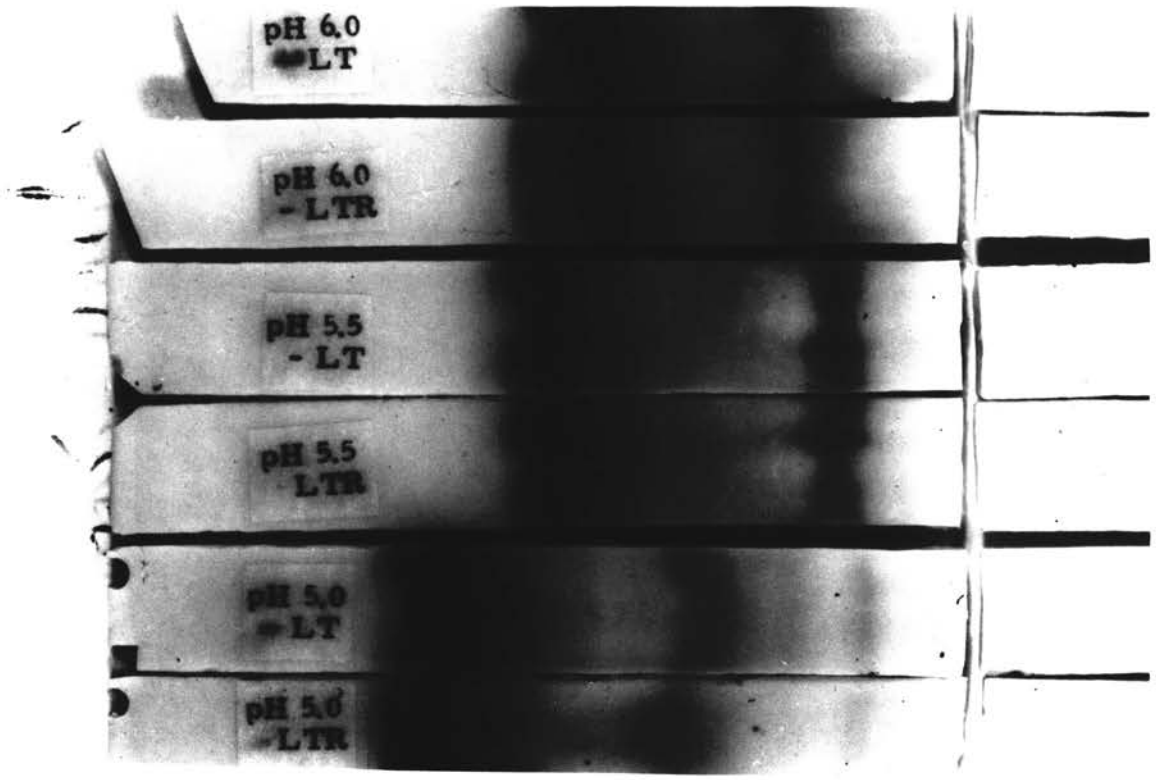
Procedure

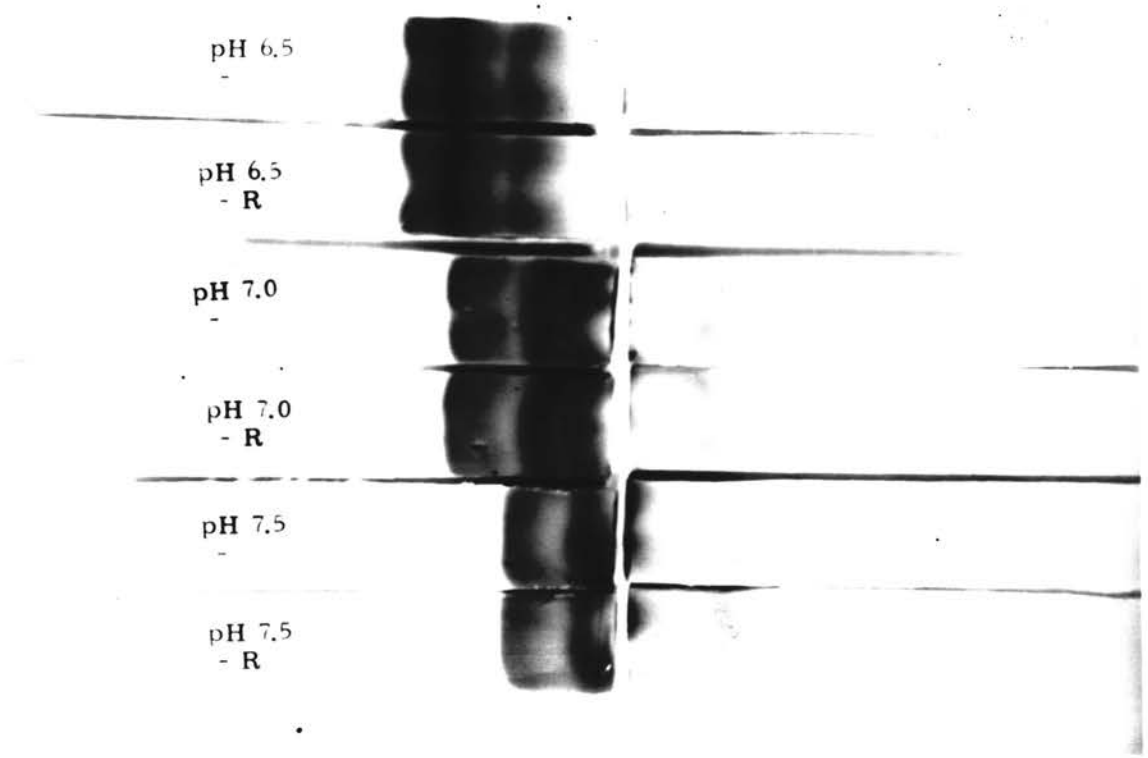
The procedure adopted in running starch gel electrophoresis was as reported by Smithies (73). The behavior of the protein fraction was studied at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 using 0.03 M sodium phosphate buffer at 4 volts/cm. Current varying between 56 to 60 milliamp was passed for 24 hours in one set and 45 1/2 hours or 48 hours in another set to note whether protein bonds separated further. Observations were limited to the pH range between 5 and 9 since under more severe conditions hydrolysis of starch and loss of activity of ricin occurs. The results are shown by Photographs 10, 11, 12, 13, 14 and 15.

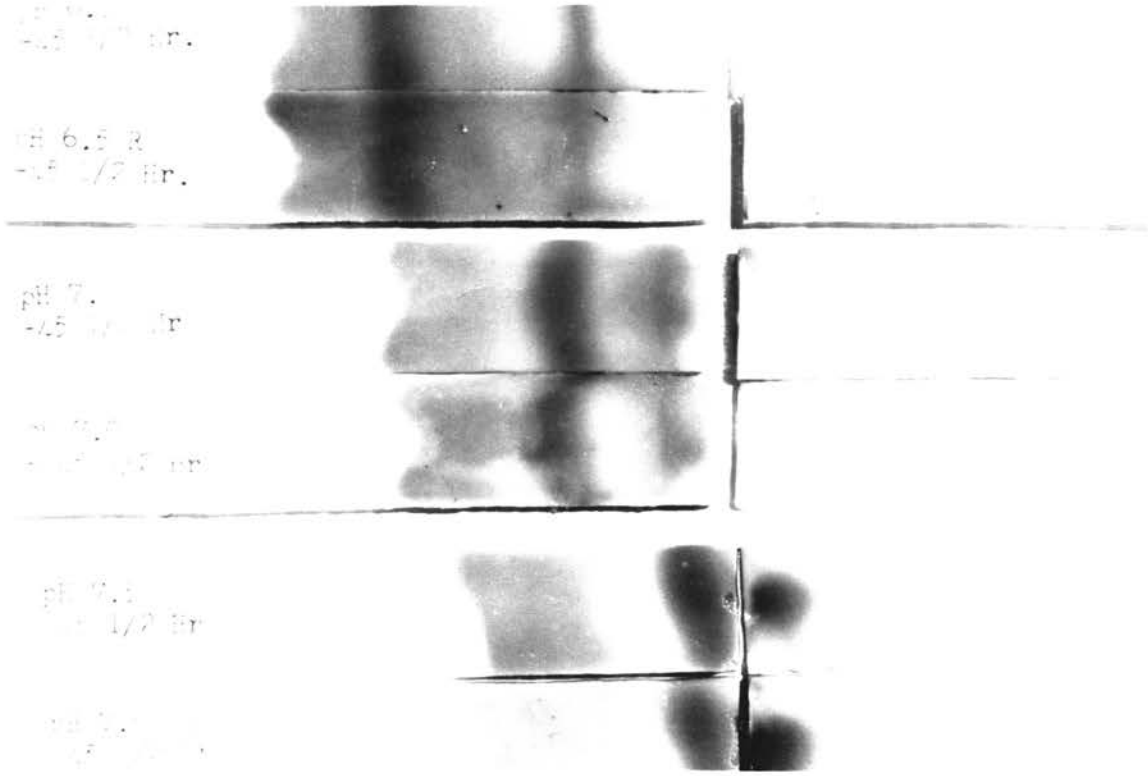
Results

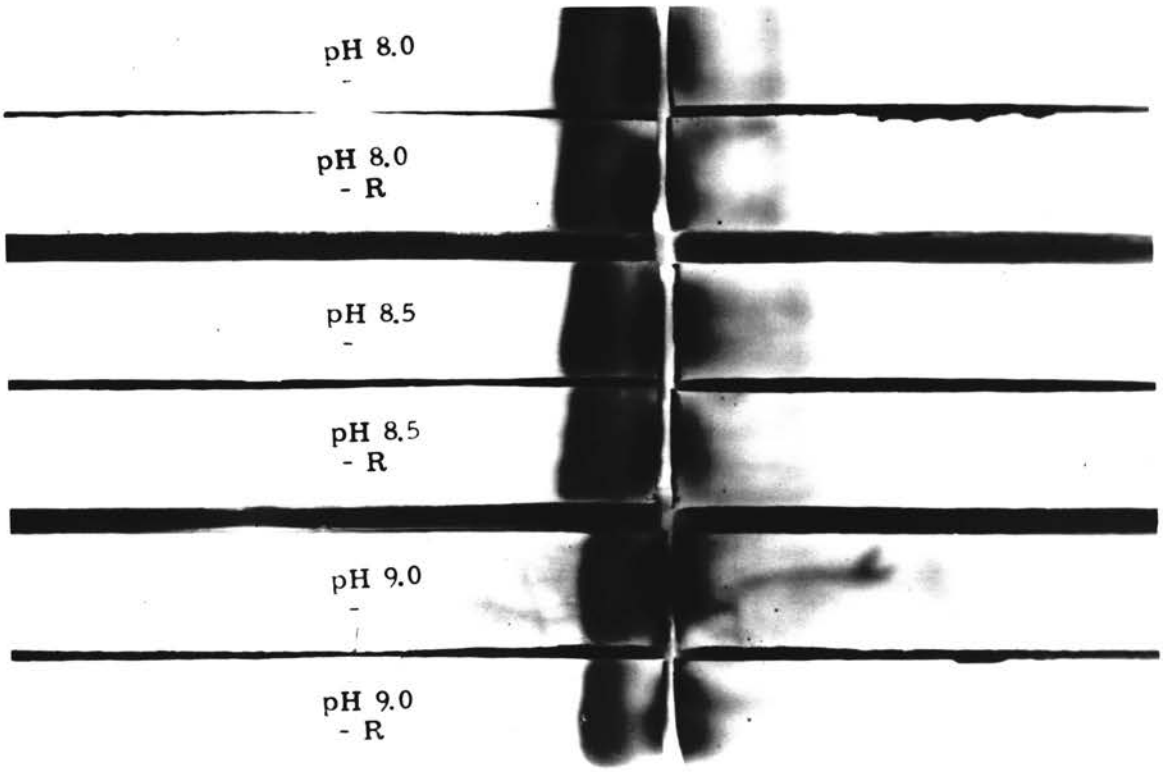
In all cases there were at least two bands on the cathodic

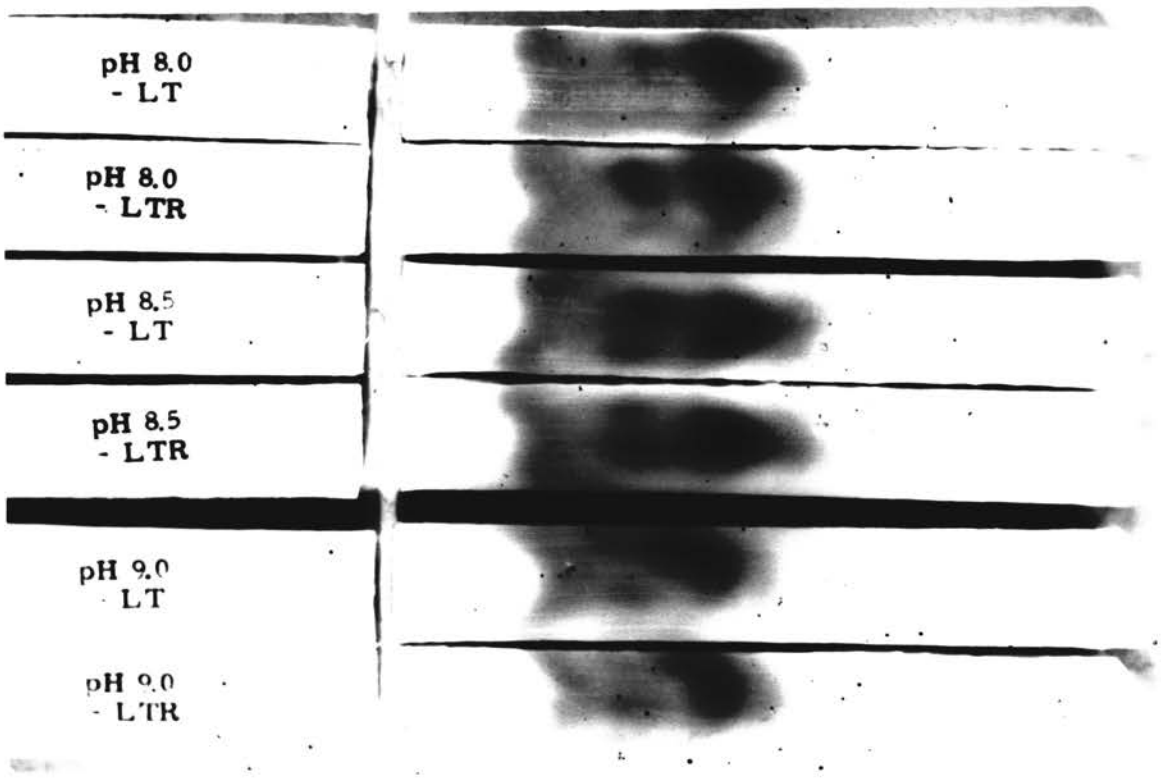

pH 6.0
-
pH 6.0
-R
pH 5.0
-
pH 5.0
-R
pH 5.5
-
pH 5.5
-R


pH 6.0
-LT
pH 6.0
-LTR
pH 5.5
-LT
pH 5.5
-LTR
pH 5.0
-LT
pH 5.0
-LTR


pH 6.5
-
pH 6.5
- R
pH 7.0
-
pH 7.0
- R
pH 7.5
-
pH 7.5
- R




pH 8.0
-
pH 8.0
- R
pH 8.5
-
pH 8.5
- R
pH 9.0
-
pH 9.0
- R


pH 8.0
- LT
pH 8.0
- LTR
pH 8.5
- LT
pH 8.5
- LTR
pH 9.0
- LT
pH 9.0
- LTR

· of all these bands on the cathodic
pH 5.0 to a minimum at pH 9.0. At pH
ıide and it moved more with an increase
ıe anodic side appeared at pH 7.5 but
lkalinity.
ı were run for 45 1/2 hours or 48 hours
ıe origin at pH 5.0, 5.5, 6.0, 6.5 and
and as expected. A third and narrower
ı 11) only on the cathode end. At pH
odic and anodic sides respectively.
Beyond pH 7.5 no spot or band was visible on the cathodic side and 2 spots moved nearly the same distance at pH 8.0, 8.5 and 9.0.

C. Column Chromatography

Since the fraction obtained at 1/2 saturation level with $(NH_4)_2SO_4$ contained most of the ricin activity (Table XVI), the proposed technique would have the possibility of fractionating ricin into its separate components.

Selection of a method and materials

In the preliminary runs DEAE cellulose[12] was used as adsorbent under the following conditions: DEAE cellulose was equilibrated with 0.2 molar phosphate buffer at pH 8.0; 0.01 molar phosphate buffer at pH 7.3; 0.01 molar phosphate buffer at pH 6.8; and 0.005 molar phosphate fubber at pH 8.0. In all cases most of the protein added, on the column, came off immediately after the hold up volume. After this first "peak"

[12]Obtained from California Corporation for Biochemical Research, Los Angeles, California; grade Cellex-D, exchange capacity 0.4 milliequivalents per gm.

no more protein could be eluted either by increasing molarity to 0.8 to 1.0 or pH until pH 10 to 11 was reached using approximately 0.1 N NaOH. Amberlite CG-50 Type I, a carboxyl-containing resin, was also made use of in these pilot runs. Four columns were run at pH 8.5, 8.0, 7.5 and 6.5 with 0.05 M phosphate buffer. From the columns equilibrated at pH 8.5, 8.0 and 7.5 the added protein came off the column as a single sharp peak immediately after the hold up volumes. A second peak was observed after 24 hold up volumes when the column equilibrated at pH 6.5 with 0.05 M phosphate buffer was eluted with 0.1 M buffer at pH 8.5. There was a trailing of eluted protein at this molarity and pH which stretched to 3 hold up volumes.

This indicated that the added protein was adsorbed by this particular resin at pH 6.5 and could be eluted under mild conditions. With this indication a more elaborate study was taken up. The temperature 22.5° C at which chromatography was done was safe for the protein-ricin.

Procedure

Amberlite CG-50 Type 1 washed with distilled water for 3 times was stirred with an excess of 0.1 N NaOH and allowed to stand over night. After rinsing several times with water an excess of 0.15 N HCl was added and left for 6 hours. The HCl was decanted and the resin was rinsed several times with water. The resin was converted to the Na phase again, as above, rinsed and placed in the column. A 36 x 2.2 cm glass tube filled with a coarse sintered glass disc was used as the column. The column was back washed. The buffer used was a 0.05 M solution of Na_2HPO_4-NaH_2PO_4 at pH 6.5. This buffer was allowed to flow through the column at a rate of 10 ml/27 minutes, until the pH of the effluent was the same as that of the influent, namely, 6.5. Three ml of protein solution (57.6 mg

protein), dissolved in the same buffer, was introduced onto the column and permitted to flow down through the column at the same rate. After the solution had all passed into the column, elution was started with 0.05 M pH 6.5 buffer. By means of an automatic fraction collector[13] 10 ml of effluent was collected in each tube. After 510 ml of this buffer had passed through the column, stepwise gradient elution was started by changing the molarity of the buffer to 0.1 M; 250 ml of each of the 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M and 1.0 M buffer at pH 6.5 was used. NaCl was used to increase the molarity of the buffer. After 250 ml of 1.0 M buffer at pH 6.5 had passed through the column, the pH of the 1.0 M buffer was raised to about 11 (0.1 N NaOH) to strip the column. The molarity of the buffer in the tubes was determined by titration of Cl^- against standard $AgNO_3$ solution, $K_2Cr_2O_3$ being used as indicator. The eluted protein fractions were measured for their protein and nucleic acid content, (reading optical density and using nomograph) and were assayed for their toxic and hemagglutinating activity (as previously described). The results are given in Table XVII.

Results and Discussion

Figure 9 shows the appearance of 3 major and 1 minor peaks. The first peak contained 33.48 per cent, the second peak 57.27 per cent, the third peak 0.31 per cent and the fourth peak 0.31 per cent of the total quantity of protein added to the column. The fourth peak was richer in nucleic acid than the other peaks. The protein that emerged in the first peak had retained its hemagglutinating activity. The toxicity of this fraction had decreased as compared to the protein added to the column

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[13] Packard Instrument Company, LaGrange, Illinois.

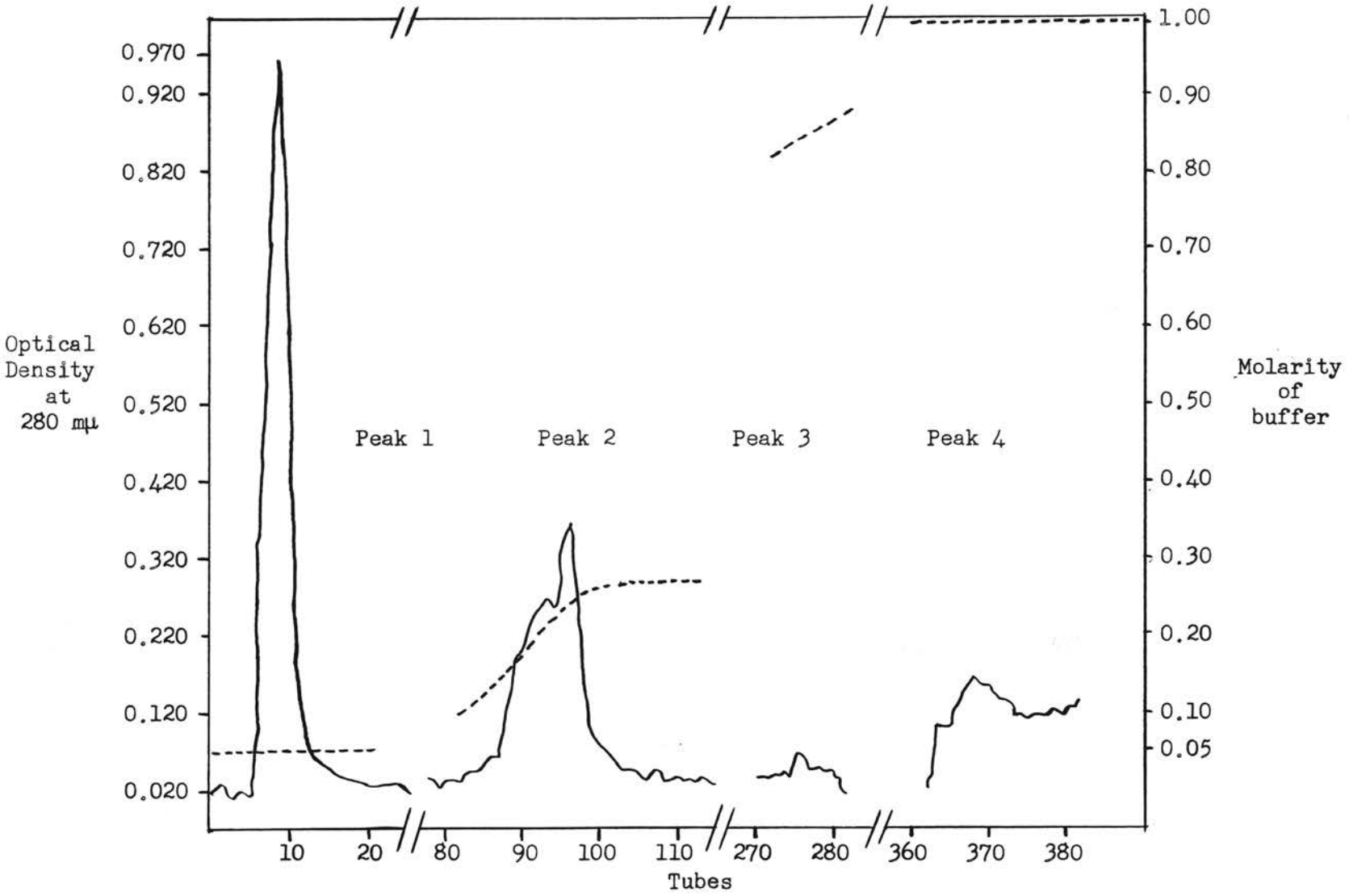
Optical Density at 280 mμ
0.970
0.920
0.820
0.720
0.620
0.520
0.420
0.320
0.220
0.120
0.020
Peak 1
Peak 2
Peak 3
Peak 4
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.05
Molarity of buffer
10
20
80
90
100
110
270
280
360
370
380
Tubes

TABLE XVII

PROTEIN FRACTIONS FROM THE COLUMN

Tube No.	Effluent Protein mg/ml	pH	Molarity	γ Protein Injected Per 100 gm Body Weight		γ Protein Required for 25% Hemagglutination Activity
7	0.07	6.5	0.05	7.33	Not lethal	No activity
8	0.42	6.5	0.05	8.26	Dead in 36 hours	High activity**
				9.69	Not lethal	
				10.14	Not lethal	
				13.41	Not lethal	
				15.21	Dead in 36 hours	
				16.03	Dead in 36 hours	
9	0.66	6.5	0.05	4.54	Dead in 44 hours	9.1
				7.60	Dead in 36 hours	
				13.80	Dead in 36 hours	
				15.00	Not lethal	
				15.34	Not lethal	
				17.09	Not lethal	
10	0.44	6.5	0.05	9.05	Dead in 72 hours	9.4
				9.84	Dead in 36 hours	
11	0.15	6.5	0.05	6.85	Dead in 44 to 46 hours	Not tested
				8.56	Not lethal	
				8.92	Dead in 72 hours	
				9.28	Dead in 36 hours	
				9.84	Not lethal	
				11.53	Dead in 72 hours	
94	0.27	6.5	0.22	9.64	Dead in 36 hours	Not tested
95	0.34	6.5	0.23	11.05	Dead in 36 hours	7.5
96	0.40	6.5	0.24	7.66	Dead in 36 hours	5.4
97	0.25	6.5	0.25	8.58	Dead in 36 hours	2.4
Crude ricin* kept at 22.5° C		6.5	0.5	8.24	Dead in 72 hours	Very high activity**
Crude ricin* kept at 4° C		6.5	0.5	6.97	Dead in 36 hours	Very high activity**

*Same preparation was used for the column.

**Visual observation.

(Table XVII). The protein fraction in the second peak was more potent in both respects than the protein in the first peak and was similar to the protein added. The protein from the third peak was inactive in its hemagglutinating property and in its toxicity. The fraction from the fourth peak could not be assayed because of its high alkalinity.

The inconsistency in the results of the toxicity assay may be due to the use of different strains of rats. Pathological examinations were performed on the rats which were injected with the protein from each of the two major peaks. It was observed that: a) the protein from the first peak caused marked degenerative changes in the liver with minor congestion and necrosis in the lymphoid tissue; b) the protein from the second peak caused marked destruction of the lymphoid tissue with mild to extensive congestion and slight necrosis of the liver. This suggested the presence of at least two proteins with different loci for toxic action or with two kinds of toxic effects.

The ammonium sulfate fractionation of castor seeds extracted with 0.85 per cent saline confirmed the results obtained by Holasek (28) who used 10 per cent NaCl solution for extraction. The protein fraction precipitated at the 1/2 saturation level with $(NH_4)_2SO_4$ at pH 8.0 was found to be the most toxic and to have the highest hemagglutinating activity. This was the saturation level at which 24.5 per cent of protein in CRS was precipitated; consequently, it was considered the most suitable for the isolation of ricin. This fraction was a mixture of at least four different proteins. One of these four, precipitated during dialysis. Holasek (28) had observed that a precipitate formed during dialysis of the protein fraction obtained at 1/2 saturation with ammonium sulfate but without any experimental support reported that this precipitate did not contain any ricin. Waller and Negi

(85) also had observed the appearance of an insoluble protein in the dialysis bag; it dissolved in 1 N NaCl solution and was toxic at 19.5 γ protein/100 gm body weight of rat. The sedimented protein can be said to be a euglobulin since it was insoluble in distilled water but soluble in acidic, alkaline and salt solution.

The three other proteins were separable by starch gel electrophoresis at pH 5.0. Results of column chromatography also revealed the presence of at least three proteins. The very narrow band that appeared at pH 5.0 during 45 1/2 hours (Photograph 11) of electrophoresis most probably corresponded to the small peak obtained by column chromatography (Figure 9), which was found to be inactive. Thus the activity of ricin probably was distributed between the two protein fractions represented by the two major peaks (Figure 9) and the two broad bands in Photograph 11. Amberlite CG-50 Type 1 resin, at pH 6.5 with 0.05 M Na-phosphate buffer, was found suitable to permit separation of proteins while retaining their hemagglutinating activity and toxicity.

The available information from the literature and the present work could be correlated and extended to explain the hemagglutinating activity and toxicity of ricin. Two protein molecules differing only in the absence or the presence of the free guanidino group which is responsible for hemagglutinating activity of ricin (17) could be the two components of ricin. These molecules would vary in their net charge, the one containing the extra guanidino group being more basic. The two protein molecules each carrying a slightly different charge at the same pH could be mutually associated in such a manner as to render their separation very difficult. Such an association would not be expected to separate readily by salting out and ultracentrifugation techniques; however, it is possible that it

could be separated by application of chromatography and electrophoresis. The different molecular weights, isoelectric point values and solubility tests results, could be explained on this basis of the existence of these two protein molecules. It is expected that they would be produced in varying proportions by the castor plants. With the ϵ-NH_2 group of lysine which is required for toxicity (17) both of these molecules would be toxic. However, their toxic effects can be expected to be different, as was found in this study, because of the extra guanidino group in one of the molecules. When not associated, the protein molecule containing the guanidino group would have both hemagglutinating activity and toxicity, while the other protein molecule would have only toxicity. These two molecules together, as found in nature, would show the properties attributed to ricin.

CHAPTER VII

SUMMARY

I. The toxicity and nutritive value of two castor meals that had been detoxified and deallergenized and two that had been only detoxified were investigated with male rats. Addition of castor meal, prepared from alkali treatment of the seeds, at the highest level (30 per cent by weight) to a nutritionally adequate diet resulted in a slight depression of growth. However, at lower levels this effect was not observed. Histopathological examination of rats fed on these different castor meal samples did not reveal any evidence of toxicity. Growth on autoclaved and unautoclaved meal was almost identical which indicated the absence of any toxin.

The palatability of castor meal was probably a major factor in the slow growth of the rats. Rats on a particular sample (Baker I) of detoxified and deallergenized castor meal were observed to refuse the feed and die of starvation.

Castor meal as a sole source of protein in the diet was able only to sustain life. Supplementation with lysine, tryptophan and methionine improved the nutritive value of the meal, which confirmed previous reports. However, after supplementing with these three amino acids the meal remained inferior to casein. This observation was contrary to earlier reports. These preliminary results suggest the presence in castor meal of factors that reduce the availability of its protein. Experiments in which the daily feed intake is controlled might provide an answer to the palatability problem. A critical study of the need for supplementation with essential

amino acids, other than the three mentioned, should be made to find if it is a problem of amino acid deficiency. Nitrogen balance procedures could be used to determine the availability of protein from castor meal.

II. The study of 16 varieties of castor seeds showed that it was not possible to measure the toxicity of ricin by evaluating its hemagglutinating activity. The ratio of toxicity to hemagglutinating activity was not constant which suggested that these two properties can not be attributed to a single protein and that at least two proteins produced in different quantities by castor plants were required to account for such variation. Crude ricin was found more toxic (minimum lethal dose) to rats than the partially purified protuct. This observation suggested that during purification cofactors which were necessary for the maximum activity of ricin were lost.

Rats which had been injected subcutaneously with crude ricin preparations showed marked degenerative changes in the mesenteric lymph nodes, spleen, mucosa of the small intestine and liver. The liver was the most severely damaged. Two proteins separated by column chromatography attacked preferentially the liver and lymphoid tissues. Crude ricin preparation given orally was the least toxic and produced no visible pathological changes in rat tissues.

$(NH_4)_2SO_4$ fractionation of the crude ricin solution yielded a protein fraction at the 1/3 to 1/2 saturation level which contained the highest hemagglutinating activity and toxicity. This fraction contained at least four proteins as shown by dialysis and starch gel electrophoresis. Preliminary results with Amberlite CG-50 (a cation exchange resin) indicated this fraction could be separated into two major and one minor components. The possibility of good results from DEAE and other cellulosic adsorbents are to be explored with varying molarity, pH and buffer system.

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A P P E N D I X

TABLE I

SALT MIXTURE*

Ingredients	Quantity in gm
$CaCO_3$	400.000
$CaHPO_4$	82.660
K_2HPO_4	430.000
$MgSO_4 \cdot 7H_2O$	136.000
NaCl	223.330
Ferric Citrate	36.660
KI	1.066
$MnSO_4 \cdot H_2O$	5.066
$CuSO_4 \cdot H_2O$	0.400
$ZnSO_4$	0.400

*Phillips, P. H., and Hart, E. B., J. Biol. Chem., 109,657 (1935).

TABLE II

DRY VITAMIN MIXTURE*

Ingredients	Quantity in gm
Calcium Pantothenate	1.000
i-Inositol	1.000
Biotin	0.005
Folic Acid	0.010
Vitamin B_{12} (0.1% Manitol Preparation)	0.001
Niacin	1.000
Vitamin B_1 (Thiamine Hydrochloride)	0.250
Vitamin B_{12} (Riboflavin)	0.300
Vitamin B_6 (Pyridoxal)	0.150
para-Amino Benzoic Acid	1.000
Vitamin K (Menadione)	0.025
Powdered Sugar	115.259
	120.000

Two ml. of 75 per cent choline chloride solution in water was added to each kilogram of feed mixture. This was done because of the extreme hygroscopic nature of choline chloride.

*From Dr. D. A. Benton. Present address: Roswell Park Memorial Institute, Buffalo, New York.

TABLE III

FAT SOLUBLE VITAMIN MIXTURE*

Ingredients	Quantity in gm
Vitamin A, Ester Concentrate	7.0
Vitamin D (Viosterol)	0.5
Vitamin K (2-Methyl-1,3-Napthoquinone)	0.4
Vitamin E (α-Tocopherol)	1.0
Corn Oil	41.1
	50.0

*From Dr. D. A. Benton.

TABLE IV

PROXIMATE ANALYSIS OF CASTOR MEAL SAMPLES

(Percentage on as is basis)

Sample Description	Dry Matter	Ash	Protein	Fat	Crude Fiber	N.F.E.
Castor Meal SURDD	83.27	11.59	51.31	0.19	9.35	10.83
Castor Meal PVO	90.19	5.78	33.00	0.92	24.56	25.93
Baker No. 1 F-0460008	94.45	7.59	38.93	1.47	18.39	28.07
Baker No. 2 F-0558055A	91.75	7.48	55.56	2.06	12.77	13.88

Protein contents of casein and soybean meal were 83.56 and 47.02 per cent respectively.

VITAE

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Master of Science

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II. TOXIC AND HEMAGGLUTINATING ACTIVITY OF RICIN

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