BIOCHEMICAL STUDIES OF RICIN, THE TOXIC PROTEIN OF CASTOR SEEDS

by

RUTH ANN SCROGGS

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Bethany Nazarene College

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Thesis Approved:

George R. Waller
Thesis Adviser

K. E. Ebner

Franklin R. Leach

Dean of the Graduate School

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CHAPTER I

INTRODUCTION

The castor seeds of Ricinus communis L. have long been known to be very toxic, as few as two or three seeds being fatal to mammals. The toxicity is a protein, ricin, whose structure is unknown, although the amino acid composition of certain preparations has been determined. Castor seeds have been grown primarily for their oil, but there is no ricin present in castor oil.

Various attempts have been made to isolate, purify and determine the mechanism of action of ricin. These attempts have included methods whereby the toxin could be separated from a very powerful allergen, which is also a protein found in castor seeds. Ricin exhibits proteolytic activity and also the property to agglutinate red blood cells. A serious problem in working with ricin is the lack of a suitable assay method.

This investigation was initiated to determine if there were selective toxicities for specific organs of the rat, especially the liver and the lymphatic system. Several fractionation procedures were used to separate proteins and each fraction was tested for toxicity, pathological damage and hemagglutination. Some studies were performed to determine if there were any changes in red blood cell volume and if any hemoglobin appeared in the serum or urine. The effect of ricin as a possible inhibitor of crystalline fumarase was tested. If ricin did inhibit fumarase, this inhibition could possibly be used for the development of an in vitro assay.

CHAPTER II

LITERATURE REVIEW

Proteins of the castor plant, Ricinus communis L., have been studied since the 1800's. Dixon (15) was the first to isolate a toxic protein fraction from castor seeds; Stillmark (60) named the toxin ricin. Another protein found in castor seeds is an allergenic factor, which is distinct from ricin (58). These two proteins have received the most interest because of their biological activity. Both ricin and the allergen preparations consist of more than a single component (14, 16, 18, 21, 27, 32, 33, 46, 49, 70).

Ricin is apparently toxic to all species of mammals, although the toxicity varies with the species. Guinea pigs appear to be the most susceptible. Ricin is toxic when ingested orally, injected subcutaneously or intravenously, with the latter exhibiting the greatest effect (52). A "latent period" of at least 15 hours intervenes before toxic symptoms appear even when quantities greater than a fatal dose have been administered (52). A dose of 0.5 μg of ricin per kg of body weight injected subcutaneously into a rabbit was fatal in seven days (28, 53, 54). A reported value for a dose to man was 0.18 g per kg of body weight, which corresponds to about six seeds; however, less concentrations have been fatal (9). Ricin is not as toxic as bacterial exotoxins, but more potent than alkaloids or inorganic poisons (10).

The most widely used method for the isolation of ricin has been extraction of oil-free seeds with a 10 percent NaCl solution, followed

by $(NH_4)_2SO_4$, Na_2SO_4 or $MgSO_4$ salting out (14, 21, 24, 25, 27, 31, 47, 53, 55). Other procedures such as extraction with water, followed by acidification with acetic acid and neutralization with NaOH (40) have been used. Kunitz and McDonald (31) obtained ricin crystals either in the form of rosettes of fine needles or as large prismoidal crystals. The crystalline form of ricin depended upon the method of crystallization. Crystalline ricin was reported to be homogeneous in the ultracentrifuge and electrophoresis, but solubility data indicated at least two components (27, 31). Other evidence has also been found indicating that ricin is more than one component (12, 14, 16, 18, 19, 27, 28, 31, 70).

An elemental analysis of ricin shows that it contains no phosphorus (26). High contents of arginine have been found (28, 29 47). It was found that as the purity of ricin was increased, the amount of sulfur decreased (24). The measurement of the sulfur content or the decrease in optical or specific rotation upon purification has been proposed as a criterion of purity (24). However, various specific rotation values have been reported and they appear to be dependent upon the method of preparation (24, 27, 28).

Ricin shows ultraviolet absorption similar to other proteins with maxima at 276 mμ (46) and 279 mμ (31). Various isoelectric points have been found between pH 5.2 - 5.8, although pH 6.0 (46) and pH 6.6 (10) have been reported. The toxin is stable between pH 3.8 - 10, and below 60° (22, 35) and least soluble between pH 5 - 8. The reported molecular weight of the protein has also varied. Kabat (27) reported values between 77,000 to 85,000; Kunitz (31), 36,000; and Corwin (10), 50,000. The denaturation of ricin by temperature and pH follows first order kinetics (35). Although the structure for ricin is unknown, amino acid composition

of certain preparations has been determined (28, 29, 46, 70). In an investigation by Holasek (24), methionine and isoleucine were reported to be N-terminal amino acids; however, the preparation was not pure enough to make this finding definite.

In addition to toxicity, ricin exhibits a proteolytic activity which is not inactivated by cell cathepsins (45). Ricin may lose its toxicity upon long standing (2 1/2 years) (16), with new components appearing in the solution (24). This autolytic or self-digestive action may be due to the proteolytic activity (24). On the contrary, purified dry ricin preparations suffer no deterioration in their physiological activities after being kept for many months (52). LeBreton and Moulé (34) have suggested that the toxicity of ricin may be due to proteolytic activity, while Funatsu (19, 20) has reported that proteinase activity was not crucial for the toxic action of ricin. Ricin preparations exhibit proteolytic activity on other proteins such as pepsin, serum albumin, ribonuclease, and casein, with maximum activity being pH dependent (46).

Digestion of ricin by other protolytic enzymes is difficult, but when given enough time the toxicity diminishes with the extent of digestion (18, 28, 34, 62, 69). The toxicity of ricin can also be reduced with $Ca(OH)_2$ (59); heavy metals such as zinc, silver (28) and copper (67); ethyl alcohol (22); supersonic waves, 555 kilocycles (63); ultraviolet light (6); and oxidizing agents as dilute $KMnO_4$ (7), dilute H_2O_2 (15) and dilute iodine solution (7). Ricin toxicity lost by treatment with iodine can be partially restored by treating with $Na_2S_2O_3$ (4, 7). Ricin fermented with yeast also loses a considerable amount of toxicity (8). Acetylation and benzolation of the free amino groups causes a decrease in toxicity. Also, guanidylation at the ϵ-NH_2 group of lysine gives a

marked decline in toxicity (50). When heated to boiling in aqueous solution toxicity is destroyed (6, 51).

Another property exhibited by ricin is the agglutination of red blood cells. An extensive review has been given by DasGupta (11) on its agglutination property. The ratio of toxicity to hemagglutinating activity was not constant with several varieties of castor seeds, suggesting that the two properties might not be attributed to a single protein (11). Takahashi et al. (64, 65) isolated a protein hemagglutinin from Ricinus sanguineus seed which was distinct from the toxic protein.

Some of the pathological effects of ricin that have been observed are as follows: congestion, hemorrhagic lesions and cellular degeneration of the hypothalmus, adrenals, anterior hypophysis, ovaries and testis; degeneration of peripheral nerve ganglia; a variable amount of vasodilation in the parathyroids and pancreatic insular tissue; degeneration of lymphatic ganglia in the thymus; and a hyperfunctional aspect, without lesions, in the thyroid (41, 42); diarrhea and extreme prostration (30, 38); hemorrhagic conditions of the intestines, renal congestion, hyperemia of spinal medulla and brain and severe panophthalmitis (38). Also reported are inflammation of eyes (17, 38, 57), edema of eyes, nose, throat and lungs, abdominal pains and vomiting (17, 30). In rabbits injected with ricin a secondary hemolysin was observed due to breaking down of red blood corpuscles (13). Ricin was also found to inhibit the activity of the isolated heart of the rabbit (3). Even in high dilutions (10^8 and 10^7), ricin inhibited the growth of chick embryonic heart, liver and stomach in tissue culture. The spleen appeared to be more resistent, and a latent period was observed before the toxic effect appeared (61). Strophosomia has been produced by injecting ricin into hen eggs during

development of the embryo (1). By the sixth day the embryo had disintegrated, but the heart continued to beat and develop until the ninth day (2). DasGupta (12) has reported marked degenerative changes in the mesenteric lymph nodes, spleen, mucosa of the small intestine, and liver, in rats injected with crude ricin solutions. However, in preparations that had been chromatographed, the liver and lymphoid tissues were preferentially attacked (12).

A change in blood and bone marrow cells in ricin injected rabbits has been observed. Ricin causes a destruction as well as a stimulatory effect on bone marrow. In mature cells, the degenerative effect predominated, while in younger cells the maturation promoting effect was greater (39). Mosinger (43, 44) has shown that ricin produces sarcomalytic effects in rats with implanted tumors and blastostimulative effects after prolonged administration. He suggested that ricin might serve as an anticancer agent.

As no chemical or biochemical assay has been found suitable, determinations of toxicity have been used as a means of assay. Two methods have been used: determination of the LD_{50} or interpolation on a dose-survival time curve (10).

Although ricin has been studied for many years, the mechanism of action of ricin is still unknown. Thomson (66) has suggested that ricin may interfere with some metabolic process in the liver, possibly in the Krebs cycle. Assays of homogenates made from the livers of normal and poisoned rats showed no difference in activities except for fumarase which decreased in poisoned rats to 66 percent of the normal values. He also suggested that doses causing death after several days may involve an entirely different mechanism of action than those rats which die

rapidly. Mosinger (44) has reported that damage to organs as well as necrosis of the sarcoma are due to a direct action of ricin and not because of neuro-endocrine related effects. Thus ricin may become of importance as an anticancer agent if the mechanism of action can be determined and controlled.

CHAPTER III

EXPERIMENTAL METHODS

A. Preparation of Defatted Castor Seeds

Decorticated castor seeds, Ricinus communis L., of the Cimarron variety (Oklahoma State University, Agronomy Farm, Stillwater, Okla.), were pressed (Carver Press, Model B, Fred S. Carver, Inc., Summit, N.J.) at 20,000 lbs/sq in. for 2-3 hours to remove most of the castor oil. The pressed seeds were extracted with ether in a Soxhlet extractor for 48 hours to remove any remaining oil. The defatted seeds were stored at -13 to -15° and used as source material for further experiments.

B. Column Chromatography Preparations

1. Amberlite CG-50 Column

Amberlite CG-50, Type I, a synthetic weakly acidic cationic exchange resin (Rohm and Haas Co.) 100-200 mesh, was cycled by treating alternately with 0.1N HCl, distilled water, 0.1N NaOH, and washed to pH 6.5 to 7.0 with distilled water and transferred to a 25 ml burette, 1 x 25 cm, fitted with a glass wool plug and 0.5 cm of small glass beads. The column was then equilibrated to the pH of the buffer by continuous elution with buffer.

2. DEAE-Cellulose Column

DEAE-cellulose (Sigma Chemical Co.) which had been cycled in the same manner as the Amberlite resin, was washed several times with 0.005 M sodium phosphate buffer, pH 6.5, and then slurried into a column, 1 x 20 cm, which was fitted with a coarse sintered glass disc. A Nichrome wire was used to slurry the material in the column during packing. The column

was equilibrated with the phosphate buffer until the effluent was of the same pH as the buffer.

3. Sephadex Column

Sephadex G-25 (Pharmacia, Uppsala, Sweden) was suspended in 0.1N NaCl solution and washed repeatedly until the fines were removed and then washed several times with 0.05 M sodium phosphate buffer, pH 6.5, before being packed into a column 2.5 x 30 cm, fitted with a medium sintered glass disc. The material was slurried in as previously described and equilibrated to the pH of phosphate buffer.

C. Protein Determinations

Protein content of the various fractions was determined by the method of Lowry, Rosebrough, Farr and Randall (36). Absorbancy at 280 mμ was used to monitor protein content of fractions separated by column chromatography.

D. Fractionation Procedure

In the early phase of this work, the fractionation procedure of DasGupta (11) was used. The need for an improved fractionation procedure became apparent since the final step in this procedure contained at least four components. Thus, a new fractionation scheme was developed in an attempt to further fractionate the components, and was used for the remainder of the studies. The procedure is as follows:

All operations were performed in a cold room (4-8°) unless otherwise stated. Defatted castor seeds were extracted with 0.85 percent NaCl solution (50 ml/g of seeds). A paste was made by grinding seeds wetted by a small volume of the NaCl solution in a mortar and pestle. The paste was transferred quantitatively to a micro Waring blender, using the remaining volume of NaCl solution and blended for three minutes at a variac (The Superior Electric Co., Bristol, Conn.) setting of 50. The solution

was filtered through eight layers of cheese cloth and then centrifuged for 20 minutes at 10,000 x g in a Lourdes centrifuge, Model LR, at 0-4°. The cold supernatant solution was adjusted to pH 5.0 using 2N HCl and 0.1N HCl for final adjustment. If the solution was at room temperature when the pH was adjusted, little or no precipitation occurred. The solution was then allowed to sit for at least 5-6 hours in the cold, 4-8°. If centrifuged immediately after pH adjustment, more material precipitated upon sitting. The solution was centrifuged at 13,000 x g for 20 minutes. The clear supernatant solution was then heated in a stainless steel beaker to 65° within 2-3 minutes and maintained at this temperature for 10 minutes, with stirring. The solution was cooled immediately in an ice bath and centrifuged 20 minutes at 10,000 x g. The supernatant solution was then brought from 0 to 33 percent saturation with respect to $(NH_4)_2SO_4$ (Mann Laboratories) using 196 g/liter of solution. After 20-30 minutes of stirring, the solution was centrifuged for 20 minutes at 10,000 x g and the supernatant solution was brought from 33 to 50 percent $(NH_4)_2SO_4$ saturation by the addition of 107 g/liter solution. After 20-60 minutes of stirring, the solution was centrifuged 20 minutes at 10,000 x g. The supernatant solution was decanted and the precipitate dissolved in a minimum volume of 0.05 M sodium phosphate buffer, pH 6.5. The solution was desalted by using a Sephadex G-25 column. The column was eluted with 0.05 M phosphate buffer, pH 6.5, and 10 ml fractions were collected until a noticeable turbidity was observed when one drop of effluent was added to 1-2 ml of 1 percent $BaCl_2$ solution. The protein was chromatographed on an Amberlite CG-50, Type I column at pH 6.5 and 0.05 M with respect to sodium phosphate buffer. A linear gradient elution was used with 500 ml of 0.05 M sodium phosphate buffer, pH 6.5, and 500 ml of 1.0 M NaCl in 0.05 M phosphate buffer, pH 6.5. Five ml fractions were

collected at room temperature by an automatic fraction collector (Instrumentation Specialties Co., Inc., Lincoln, Nebr.). Material not retained on the Amberlite column was further chromatographed on a DEAE-cellulose column at 0.005 M sodium phosphate buffer, pH 6.5. The column was eluted with a linear gradient from 0.005 M to 1.0 M sodium phosphate buffer, pH 6.5, using 250 ml of each. The column was then eluted with 50 ml of 0.1N NaOH. Protein content, toxicity, pathological damage and hemagglutinating activity were determined on protein fractions obtained from each step.

E. Evaluation of Toxicity

Male, albino rats of the Sprague-Dawley strain (Holtzman Co.) ranging from 75 to 180 g, were used as test animals. The rats were injected either intramuscularly in the right hind leg, or intraperitoneally with varying levels of the protein fractions. Neither an LD_{50} value nor a minimum lethal dose was established. Doses were based on μg/100 g body weight of the rat (11).

F. Pathological Examination

Gross observations were made of pathological changes. Selected tissue and bone marrow were prepared and observed for microscopic findings by the Veterinary Pathology Department, using accepted methods.

G. Starch Gel Electrophoresis

The method of Smithies (56) was used for horizontal starch gel electrophoresis. A 13 percent preparation of starch (Connaught Medical Research Laboratories, Toronto, Canada) was most suitable. The mobility of various protein fractions was studied at pH 5.0 using 0.03 M sodium phosphate buffer. Four volts/cm (voltage was checked periodically) were applied across the gels for 20-24 hours in a cold room, 4-8°. The gels were stained with a saturated solution of Amido Black dye (Eastman Kodak Co.)

in methanol, water and acetic acid (50:50:10) and decolorized by a methanol, water, acetic acid (50:50:10) solution.

H. Determination of Hemagglutinating Activity

A washed suspension of fresh (not over 24 hours) rabbit red blood cells in 0.85 percent NaCl solution was used to test the hemagglutinating activity of the different ricin fractions. The increase in absorption at 720 mμ after an incubation period of two hours was measured (11).

I. Hematocrit and Hemoglobin Studies

1. Hematocrits

Hematocrits were determined for a group of rats (18 for the first experiment and 24 for the second) which had been injected with various protein fractions at a level of 7.5 μg/100 g body weight. A normal hematocrit value for each animal was established by averaging the values of blood samples taken several hours before injection and just prior to injection. The blood was obtained by cutting off a small piece of the tail with a sharp scapel and then forcing blood out rapidly to prevent clotting. The samples were collected in heparinized capillary tubes (Scientific Products, No. 4417-2). The samples were then centrifuged in a hematocrit centrifuge (International Equipment Co.) for four minutes at 11,000 RPM. The ratio of packed red cells to serum was then measured using a hematocrit reader (International Equipment Co.). In the first experiment samples were collected every six hours and in the second, every four hours except between 11:00 P.M. and 7:00 A.M. Samples were collected more frequently just before the death of the animal.

2. Hemoglobin Studies

Urine samples were collected every 12 hours or until death in metabolism cages from rats which had been injected with 7.5 μg/100 g body

weight. Hematest Reagent Tablets (Ames Co., Elkart, Ind.) and Hemastix Reagent Strips (Ames Co.) were used to test the urine for hemoglobin.

J. Fumarase Experiments

Fumarase activity was assayed according to the procedure of Massey (37) except that the temperature was not maintained at 20°. Various ricin fractions were tested for inhibition.

K. Effects of Ricin on Mitochondrial Respiration

Rat liver mitochondria were prepared and their oxygen uptake was measured according to procedures given in Umbreit, Burris and Stauffer (68). Rat liver homogenates were prepared by using 3 ml 0.25 sucrose/g of rat liver and then fractionated by differential centrifugation to obtain mitochondria. A 1:3 dilution of mitochondria in 0.25 M sucrose was used for the experiments. The reaction mixture contained the following[1]: 10 μMoles substrate, 500 μg hexokinase (Crude Type, Sigma Chemical Corp.), 3 μMoles $MgCl_2$, 150 μMoles nicotine adenine dinucleotide (NAD) (Sigma Chemical Corp.), 3 μMoles ethylenediaminetetraacetic acid tetrasodium salt (EDTA) (Sigma Chemical Corp.), 10 μMoles potassium phosphate buffer, pH 7.4, and 0.1 ml ricin solution which had been fractionated through the Amberlite chromatography step of the old fractionation procedure (11) and represented the peak from the first protein eluted from the column with 0.05 M sodium phosphate buffer, pH 6.5. The center well contained 0.1 ml 6N KOH absorbed on a filter paper wick. The total volume in the flask was 3.0 ml, which included the KOH solution. The following substrates were used: fumarate, succinate, α-ketoglutarate and glutamate (California Biochemical Corp.). The addition of 10 μMoles of pyruvate greatly stimulated the oxidation

[1]Experimental Biochemistry (University of Illinois Laboratory Manual, Stipes Publishing Company, Champain, Illinois, 1959) p. 201.

rate of fumarate. The substrate plus ricin was tipped into the main compartment after five minutes in Experiment A and after ten minutes in Experiment B.

CHAPTER IV

RESULTS AND DISCUSSION

Attempts to repeat the fractionation procedure of DasGupta (11) were unsuccessful in that a protein fraction which was specific for the liver was not found. The elution pattern obtained by DasGupta (11) from an Amberlite CG-50, Type I column showed approximately 33.5 percent of the protein in peak one which caused marked degenerative changes in the liver and 57 percent in peak two which caused marked lymphoid damage. Although the same lot of castor seeds was used, the elution pattern differed in that: (a) a peak was obtained which corresponded to the reported peak one, but it did not show the marked degenerative change in the liver; and (b) the remainder of the protein came off continuously in the next 500 ml collected at concentrations of 0.023 to 0.204 mg/ml (which were not tested for toxicity).

A. Fractionation

1. Comparative Extraction

Since a number of other investigators had used a 10 percent NaCl solution for the extraction of ricin from castor seeds, a brief study was made to determine if a higher yield of protein could be obtained than when using a 0.85 percent NaCl solution. It was found that only 10-15 percent more protein was extracted. The higher salt concentration caused peritonitis in rats and consequently was not used in this study.

2. Heat Treatment

Advantage was taken of the heat stability of ricin below 65° in developing a new fractionation procedure. According to the study of Levy and Benaglia (35) on the effect of pH and heat on crystalline ricin, maximum stability to heat was observed to be from pH 4.6 to 5.1. Therefore, the crude ricin solution was adjusted to pH 5.0 prior to heat treatment. Table I shows the effect of heat treatment on the protein content and toxicity of a crude ricin solution which had been adjusted to pH 5.0.

TABLE I

EFFECT OF HEAT TREATMENT ON PROTEIN CONCENTRATION AND TOXICITY

Time at 65° C (minutes)	Protein Concentration (mg/ml)	Toxicity (Death in 48 hours)
Not Heated	11.0	Not Lethal
0	6.5	Lethal
1	6.1	Lethal
3	5.8	Lethal
5	6.1	Not Lethal
10	6.4	Lethal

Five 1 ml aliquots of ricin fractionated through the pH step were pipetted into 12 ml heavy duty glass centrifuge tubes. The tubes were suspended in a stainless beaker by means of a wire rack. The water in the beaker was heated to 65° with stirring. When the water had reached 65° (approximately 5-10 minutes) the zero-time sample was removed and other samples were removed at 1, 3, 5 and 10 minutes. Each tube was cooled in a beaker filled with ice. The solutions were then centrifuged for 10 minutes at 6000 RPM in an International clinical centrifuge. Protein was determined as described in Experimental Methods. Toxicity was determined by injecting a single rat intraperitoneally with 15 μg protein/100 g body weight and the animals were observed for 48 hours to determine time of death. If the animals had not expired, they were sacrificed.

The rat injected with a ricin solution not heated showed symptoms of ricin poisoning which included listlessness, reddening around the eyes and nose, and loss of appetite. The animal was sacrificed but no degenerative changes were observed. Similar findings were observed for the rat injected with the preparation which had been heated for five minutes. The lesions suggested by the action of ricin were very slight in this series. Since the soluble proteins were lethal after 10 minutes of heating at 65° this step was continually used in fractionating further ricin preparations.

3. Ethanol Treatment

The effect of ethanol was briefly investigated and the results are shown in Table II.

TABLE II

EFFECT OF ETHANOL ON PROTEIN CONCENTRATION AND TOXICITY

Sample	Total Protein mg	Toxicity, Gross Appearance
Control	11.0	Sick, sacrificed
50% Ethanol Supernatant Fraction	4.6	Normal, still living 3 months later

To 1 ml of protein solution fractionated through the pH step was added 0.5 ml of absolute ethanol, which caused the solution to become turbid. Addition of another 0.5 ml of an ethanol caused the formation of a flocculent precipitate. The solution was centrifuged for 10 minutes at 6000 RPM in an International clinical centrifuge. Protein content was determined as given in Experimental Methods. Toxicity was determined by injecting a single rat with 15 μg protein/100 g body weight and the rat was observed for 48 hours. The rat injected with ricin not treated with ethanol was the same as previously discussed (Table I).

The toxicity of the solution was lost upon addition of ethanol. Several attempts were made to solubilize the precipitate but these were unsuccessful and it was not determined if the precipitate was toxic. This approach was not further pursued.

4. Manganous Chloride Treatment

The addition of 0.05 percent 1.0 M $MnCl_2$ to a ricin solution fractionated through the pH and heat steps showed that some precipitation occurred. The resulting supernatant solution retained its toxicity. No further investigations were made.

5. Removal of Salt

The protein fraction obtained from the 33-50 percent saturation with $(NH_4)_2SO_4$ was dialyzed against 100 volumes of 0.05 M sodium phosphate buffer, pH 6.5. It was found that the removal of chloride and sulfate ions were not complete after dialyzing for one week. Therefore, Sephadex gel was used to desalt the preparation in a shorter time period.

6. Column Chromatography

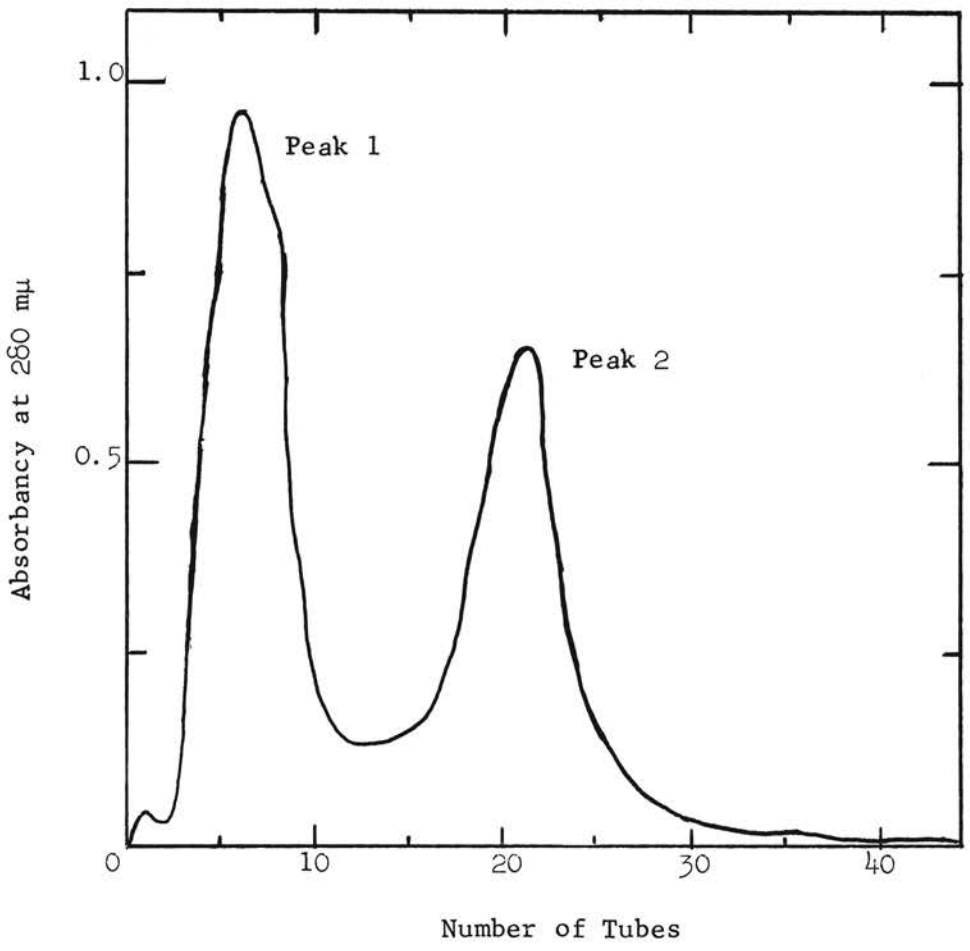
a. Amberlite Chromatography

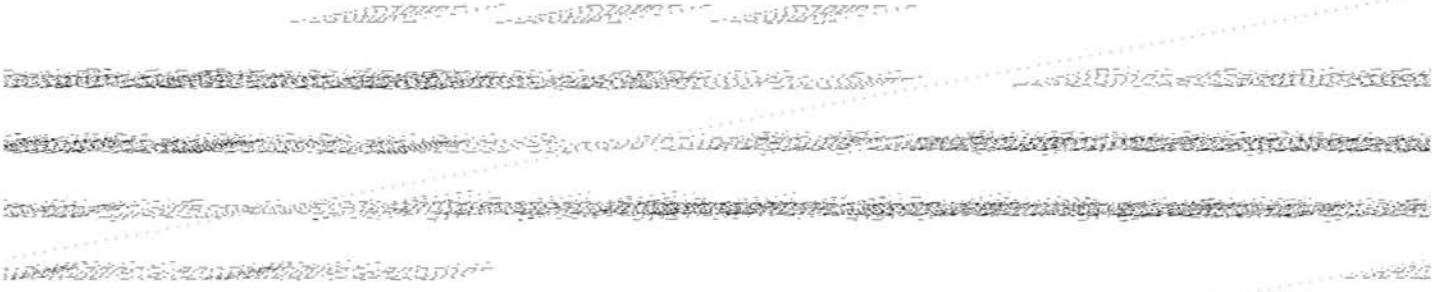
Following desalting, the preparation was chromatographed on an Amberlite CG-50, Type I column. The elution pattern is shown in Figure 1.

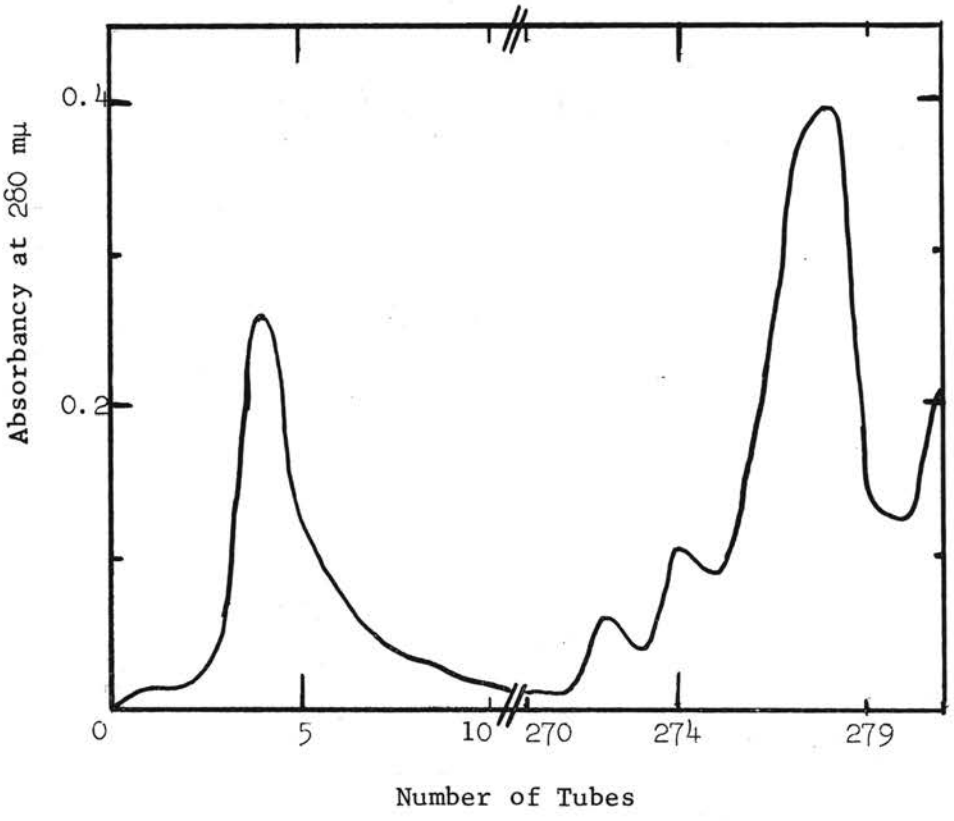
b. DEAE-Cellulose Chromatography

The first peak eluted from the Amberlite column was further chromatographed on a DEAE-cellulose column. The elution pattern is shown in Figure 2. The first peak obtained was toxic at a level of 5 and 10 μg protein/100 g body weight. The other protein eluent was not tested.

7. Hemagglutinating Activity


1.0
0.5
0
Peak 1
Peak 2
10
20
30
40
Absorbancy at 280 mμ
Number of Tubes




Absorbancy at 280 mμ
0.4
0.2
0
5
10
270
274
279
Number of Tubes

All preparations from both the new and old fractionation procedures showed a strong hemagglutinating activity. This confirms and extends the previous reports (11, 10, 19) that toxicity and hemagglutinating activity are closely associated in protein preparations obtained from the seeds of Ricinus communis L.

8. Starch Gel Electrophoresis

Starch gel electrophoresis was employed to test the homogeneity of the various fractions, especially the chromatographed preparations. The results are shown in Table III.

TABLE III

STARCH GEL ELECTROPHORETIC MOVEMENT OF RICIN PREPARATIONS

Ricin Preparation	Number of Distinct Bands	Movement in cm in 23 Hours
Unchromatographed (New Fractionation)	3	7.8
		7.4
		6.7
Amberlite Peak 1	1	7.4
DEAE-Cellulose (Tube No. 4)	1	7.6

This experiment was performed as given in Experimental Methods.

The results indicate only one band after Amberlite and DEAE-cellulose chromatography.

B. Pathological Observations - Gross and Microscopic

Table IV shows the gross and microscopic pathological changes of rats injected with the various preparations obtained from the fractiona-

TABLE IV

PATHOLOGICAL CHANGES IN RATS INJECTED WITH RICIN PREPARATIONS

PREPARATION	INJECTION SITE	TIME OF DEATH (hours)	PATHOLOGICAL CHANGES										
			LIVER		SPLEEN		LYMPH NODES		EDEMA AT INJ. SITE	INTESTINE	FLUID IN SEROUS CAVITIES	MESENTERIC LYMPH NODES	BONE MARROW
			Gross	Micro.	Gross	Micro.	Gross	Micro.	Gross	Gross	Gross	Gross	Micro.
Crude Ricin Solution	IP	<36	N	N	N	N	N	Dg-sl	N	N	+	R-sl	N
Crude Ricin Solution	IM	1 rat >48	E	N	N	N	N	N	+	R-sl	N	D	S+, C
Unchromatographed Ricin Solution	IP	24-36	N	N	N	N	R-sl	Dg-sl	N	N	+	N	N
Unchromatographed Ricin Solution	IM	36-48	N	N	N	N	N	Dg-sl	+	R++	N	N	S+, C+
Amberlite Peak 1	IP	<24	N	N	N	N	N	Dg-sl	N	N	+	R-sl	S+
Amberlite Peak 1	IM	24-36	N	Nc-sl	N	N	N	D+	N	R++	N	B	S-sl, C+
Amberlite Peak 2	IP	<36	N	N	Dg-sl	N	N	Dg-sl	N	N	+	R-sl	S-sl
Amberlite Peak 2	IM	24-36	N	N	N	Nc-sl	N	Dg+	+	R++	N	D++	S-sl

Level of dose in all cases: 7.5 µg protein/100 g body weight of rat
Number of rats injected: 3/preparation
Abbreviations used:
Normal = N; Enlarged = E; Necrosis = Nc; Degeneration = Dg
Degree: Slight = sl; Moderate = +; Marked = ++
Intraperitoneally = IP; Intramuscularly = IM
Color: Red = R; Black = B; Dark = D
Stimulation = S; Congestion = C

tion procedure outlined in this thesis. No marked degenerative changes of the liver were observed from any preparation; however, the protein (Amberlite peak one, IM injection) which corresponded to the same fraction as that previously obtained (11), did show a slight necrosis (microscopic examination) in one rat. The similarities in the pathological changes of the spleen, lymph nodes, mesenteric lymph nodes and bone marrow and intestine among all fractions used was striking. Slight differences which could be related to the injection site were observed, the noticeable difference being the fluid in the serous cavities of those rats injected intraperitoneally. Some evidence for erythropoietic stimulation of bone marrow was observed.

Table V shows pathological changes observed in rats injected with ricin preparations representing the old (11) and new fractionation scheme. Degenerative changes, varying from slight to marked, occurred in the liver, spleen and kidney, and hyperemic, hemorrhagic and necrotic changes in the thymus glands from those preparations of the old fractionation scheme. The only similarity between ricin preparations of both schemes was the change in the lymph nodes (Tables IV and V).

In this study the crude ricin preparations as well as the chromatographed preparations did not show the extensive degenerative changes in the liver and the spleen as did the preparations of DasGupta (11). The basis for the difference is unknown; however, unpublished findings (71) have shown that the biological activity of ricin changes markedly on aging. The castor seeds in this study were from the same lot grown in 1958 as those used by DasGupta. However, the seeds were 4-5 years of age whereas they were 1-2 years of age when the earlier study was made.

C. Hematocrits and Hemoglobin Studies

TABLE V

COMPARISON OF PATHOLOGICAL CHANGES IN RATS INJECTED WITH RICIN FROM TWO FRACTIONATIONS

PREPARATION	NUMBER OF RATS INJECTED	LEVEL OF DOSE µg PROTEIN/100 g BODY WEIGHT	INJECTION SITE	TIME OF DEATH (hours)	PATHOLOGICAL CHANGES									
					LIVER		SPLEEN		MESENTERIC LYMPH NODES	KIDNEY		THYMUS GLAND		INTESTINE
					Gross	Micro.	Gross	Micro.	Gross	Gross	Micro.	Gross	Micro.	Gross
Crude Ricin Solution	1	15	IP	<24	N	N	D	Nc+,++	R-sl	N	D-sl	R	Hyp, Hem, Nc	N
Unchromatographed Ricin Solution*	1	10	IP	<24	M	Dg	E, D	Nc++	R-sl++	N	Dg	R-sl++	Hyp, Hem, Nc	N
Unchromatographed Ricin Solution*	1	10	IM	<24	N	Dg	D, M	Nc+++	R-sl	N	Dg	R	Hyp, Hem, Nc	N
Chromatographed Ricin Solution, Amberlite Peak 1*	2	15	IP	<24	N	N	D, M	Dg-sl	R-sl	N	N	R-sl	Hyp	R-sl
Chromatographed Ricin Solution, Amberlite Peak 1**	3	7.5	IP	<24	N	N	N	N	R, Dg-sl	N	N	N	N	N

* Old fractionation scheme
**New fractionation scheme
Abbreviations used:
Normal = N
Enlarged = E
Necrosis = Nc
Degeneration = Dg

Degree: Slight = sl
Moderate = +
Marked = ++
Extensive = +++

Intraperitoneally = IP
Intramuscularly = IM

Mottled = M
Hyperemic = Hyp
Hemorrhagic = Hem

Color: Red = R
Black = B
Dark = D

Figure 3 shows an increase in the packed red cell volume of those rats injected intraperitoneally (IP) with all ricin solutions tested. Since the crude ricin solution, which was not as toxic at the same dose level as other fractions (death was delayed or did not occur with the amount injected), caused as great an increase as more toxic fractions, the increase in the hematocrit value may not be related to the toxicity. Hematocrit values for rats which did not die (two with IM injections of crude ricin solution and one with IM injection of Amberlite peak two) were found to return to normal after 32 hours and 24 hours, respectively.

Hemoglobin assays on the serum from the blood samples used to determine the hematocrits were positive in those that showed a slight reddening. This indicated that some hemolysis had occurred. This tendency was noticed only in the 8-16 hour period following injection and not in every animal. It was not clearly established whether this hemolysis was due to the action of ricin or if it occurred after the blood sample had been taken. Hemoglobin assays on the urine of a group of rats which had been injected in a similar manner were negative. The basis for hemolysis has not been established.

The marked increase in hematocrit values observed only in those rats injected IP suggested that the route of the toxin influenced the maintenance of cell volume. The toxin might have produced some change in the vascular system causing a fluid leakage to occur since many rats injected IP contained increased fluid quantities in the serous cavity.

D. Studies on Mechanism of Action

1. Effect of Ricin on Mitochondrial Respiration

The results of ricin on mitochondrial respiration is shown in Table VI. The results show no inhibitory effect on oxygen uptake by

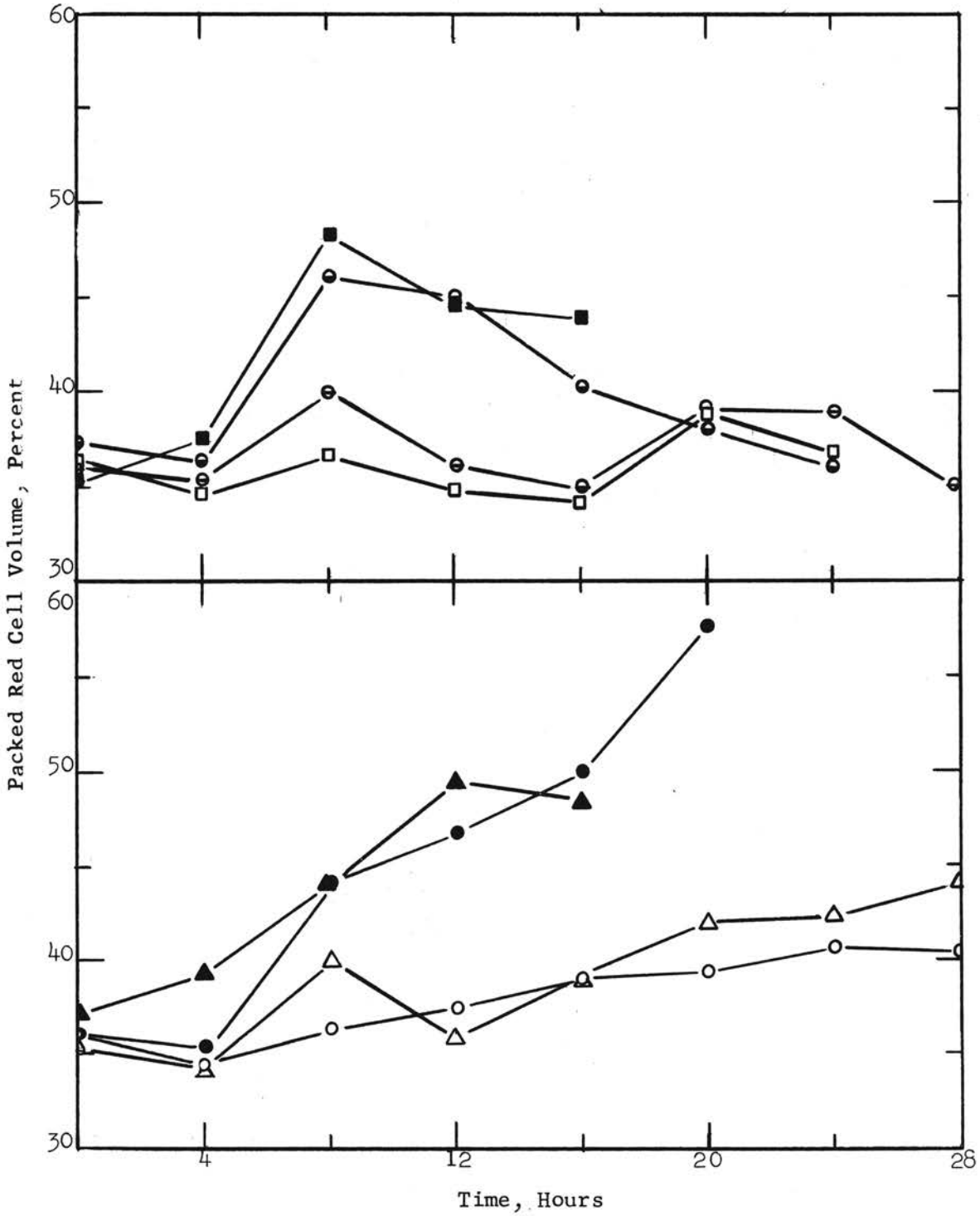

60
50
40
30
60
50
40
30
4
12
20
28
Packed Red Cell Volume, Percent
Time, Hours

TABLE VI

EFFECT OF RICIN ON MITOCHONDRIAL RESPIRATION

	Substrate 10 μMoles	Ricin μg	Initial Rate μMoles/10 Min.	Total Oxygen Uptake in 40 Min. μMoles
A.*	None		0.084	0.42
	None	58.7	0.00	0.098
	Fumarate		0.492	1.72
	Fumarate	49.5	0.332	1.58
	Fumarate	58.7	0.600	1.65
	Succinate		2.295	6.80
	Succinate	49.5	2.178	7.23
	Succinate	58.7	2.496	7.97
B.*	None			0.610
	Fumarate, Pyruvate**		1.083	4.28
	Fumarate, Pyruvate**	58.7	1.219	4.558
	Fumarate, Pyruvate**	117.4	0.935	4.510
	Succinate		0.867	4.284
	Succinate	117.4	1.375	4.015
	α-Ketoglutarate		0.880	3.69
	α-Ketoglutarate	58.7	0.988	3.30
	Glutamate		0.795	4.028
	Glutamate	58.7	0.915	3.599

* The temperature for Experiment A was 30°.
The temperature for Experiment B was 37°.
**10 μMoles of each were added
The experiment was performed as given in Experimental Methods.

ricin on any of the substrates tested. However, in certain experiments there was an increase in respiration in the presence of ricin.

2. Effect of Ricin on Crystalline Fumarase

The effect of ricin on the rate of crystalline fumarase is given in Table VII.

TABLE VII

EFFECT OF RICIN ON THE RATE OF CRYSTALLINE FUMARASE

Addition	Protein μg	Enzyme Units
None		10
0.85% NaCl		10
Crude Ricin Solution	42.24	9.5
Unchromatographed Ricin	85	10
Amberlite Peak 2	44.2	10
Amberlite Peak 1, After 5 Min. Incubation Period	56	9

Sodium fumarate (17 μMoles) was used except in the blank which contained the same components as the reaction cuvette. The total volume of each cuvette was 3.0 ml. The unit of activity was defined as the amount of enzyme which caused an initial rate of change of absorbancy at 300 mμ of 0.01/minute at room temperature and pH 7.3.

Since many of the ricin solutions tested contained NaCl and fumarase activity is sensitive to anions (37) the effect of 0.85 percent NaCl solution was tested for any inhibition of rate. No inhibitory affect was observed, by either the NaCl solution or by ricin.

CHAPTER V

SUMMARY

Various fractionation procedures were applied to extracts of seeds of Ricinus communis L. in an attempt to separate toxic protein fractions for specific organs. While castor seeds of the same lot studied two years previously had shown selective toxicity to the liver and lymphatic system, none of the preparations of this study showed the specific liver damage. The preparations were all toxic and demonstrated hemagglutinating activity.

The gross and microscopic pathological changes indicated marked degeneration of the lymphatic system and erythropoietic stimulation of bone marrow. The route of injection apparently influenced the pathological changes.

There was no effect of the various preparations on mitochondrial respiration or on the activity of crystalline fumarase.

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VITA

Ruth Ann Scroggs

Candidate for the Degree of

Master of Science

Thesis: BIOCHEMICAL STUDIES ON RICIN, THE TOXIC PROTEIN OF CASTOR SEEDS

Major Field: Chemistry (Biochemistry)

Biographical:

Personal Data: Born in Hobart, Oklahoma, August 22, 1939, the daughter of Virgil H. and Louise K. Scroggs.

Education: Attended grade school in Ft. Worth, Texas, and Sioux Falls, South Dakota; graduated from North Side High School, Ft. Worth, Texas, in 1957; received the Bachelor of Science degree from Bethany Nazarene College, Bethany, Oklahoma, with a major in Chemistry and minors in Biology and Mathematics, in May, 1961; completed requirements for Master of Science degree at Oklahoma State University in August, 1963.

Professional Experience: Research Assistant, Biochemistry Department, Oklahoma State University, 1961 to 1963.

Professional Organizations: American Chemical Society and Phi Sigma