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THE INFLUENCE OF CHEMICAL AND OTHER AGENTS UPON THE TOXICITY OF RICIN:

By

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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CONTENTS

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I. HISTORICAL :

(a). The Chemical Nature of Ricin.

Although Ritthausen (34) and Vines (39) had investigated the proteins of the castor bean Ricinus communis, they did not observe that the proteins of the beans were responsible for the toxic effects produced when they were given to animals.

The report by Warden and Wadell (40) points out that the toxic principles known to be present in certain seeds were toxic proteins. Their work was with Abrus precatorius and they obtained a protein substance which they named abrin.

In 1886 Dixon (12) obtained a precipitate of a protein nature when he neutralized an extract of the castor bean. He found that this material was very toxic. Further research on the toxic properties of the castor bean was carried on by Stillmark (38) in 1888 and he ascribed these properties to the protein which he named ricin.

In 1898 Cushny (11) studying the toxic properties of ricin gave further proof that these were to be assigned to the proteins. If the proteins were removed from his solutions the toxicity was also removed. He noted , however, that a solution which showed strong toxic effects might be too dilute to give the ordinary tests for proteins. He concluded that ricin was a protein or else was so intimately associated or combined with protein that it could not be separated from it.

Many investigators, including Brieger (8), Müller(27), Jacoby and others, have questioned the protein nature of ricin. Müller allowed the ferment pepsin to act on the toxin for several hours and claimed to have obtained a product with only a slight decrease in toxicity, but that he was not able to make a separation of the toxic principle from the protein. Jacoby (19) believed that the toxin was not a protein and he reported that he obtained ricin free from protein by tryptic digestion which had not lost it's toxic properties but which did not give the protein reactions.

Osborne, Mendel and Harris (30) developed a method for the isolation of ricin which gives a very toxic principle. The most toxic fraction contained about 70 per cent. of albumin which could be coagulated, and they believed that the toxin was associated with this protein, if it was not identical with it. They digested some of the toxic material with trypsin and pepsin and found that the toxic principle and the protein content ran parallel and that the toxicity was destroyed only as the protein was digested. Since ricin

was so very toxic to laboratory animals, these authors suggested that Jacoby's solutions did not contain sufficient toxic material to give the protein reactions although they contained enough to kill animals

Karrer, Smirnoff, Ehrensperger and Van Slooten (20) performed digestion experiments on ricin using trypsin as did Osborne and his associates. They tested the toxicity of the digestion mixture and of the undigested residue as the digestion progressed and found that the toxic principle was destroyed as the protein was hydrolized. These authors also assigned the toxic properties of ricin to the protein as did Osborne and his associates.

(b) The Biological Action of Ricin

The toxic action of ricin on animals has been studied by Dixon (12), Stillmark (38), Jacoby (19), Cushny (11), and others.

The fact that ricin had the power of agglutinating a suspension of red blood corpuscles, causing them to settle, leaving a clear solution was discovered by Stillmark. He believed that the toxin caused the agglutination of the corpuscles <u>in vivo</u> and that these agglutinated masses of red cells congested the capillaries. Ehrlich (14) believed as did Stillmark that the toxin caused agglutination of the red cells and that these formed thromboses and resulted in inflammation of the intestinal mucosa. Stepanoff (37) performed experiments showing that ricin when injected

intravenously into the animal body is eliminated by the intestines.

Flexner (16) observed that ricin effects individual cells to a different degree. Lymph cells were affected most and the extent of the cell injury in an animal organ was proportional to the activity or the activities of the organ coming in contact with the toxin. He agreed with Stepanoff that the severity of the intestinal lesions or inflammations during ricin poisoning was very probably associated with the function of elimination. Calmette and Delarde (10) confirmed the work of Stepanoff that the intestinal tract eliminated the toxin,ricin.

Since ricin was so very toxic and since it also had the power of agglutinating the red blood cells of most experimental animals as well as man, the theory was formulated that there were two groups or separate bodies in this toxin. Stillmark, who discovered the agglutinating action of ricin, believed the toxic substance and the agglutinating substance to be the same. Cushny (11) and Muller (27) were among the first investigators who doubted that ricin was composed of one substance which had these two properties. Muller performed experiments with ricin digested with pepsin and reported that the solution was highly toxic but that it would not agglutinate corpuscles. Jacoby (19) confirmed Muller's experiments.

Rehns (32) working with ricin found that when he absorbed the toxin with red cells, the supernatant liquid was not toxic and that the cells after being washed were toxic if

injected into animals. Field (15) employed the method of Osborne and his assosiates for preparing ricin and obtained a very toxic principle which had lost its toxicity at the end of two and one half years but retained its agglutinating properties to a marked degree. He believed that ricin was composed of two substances or that it contained two toxic groups, one of which was stable, the other labile. Michaelis and Steindorff (26) found that ricin could agglutinate other cells than red blood corpuscles. They agglutinated kidney and spleen cells of guinea pigs. Reid (33) found that ricin agglutinated the brain cells of dogs, hedgehogs, pigs and guinea pigs and it also agglutinated liver, kidney, spleen and small intestinal cells. The ability of the cells to attract the toxin depends on the lipoidal content. The brainis high in lipoids and is easily affected. Reid performed experiments which showed that the adsorption of ricin by the brain cells as well as by the blood corpuscles left sufficient poison free to kill dogs. This is contrary to Rehn's (32) report. Liebermann (23) discovered that he could liberate the ricin agglutinin from the agglutinated blood cells by the addition of acid and that it would unite again with new erythrocytes. He believed that ricin was an acid or of an acid nature and that it combined with the stroma of the red cells which played the role of a base. Reid (33) used dilute hydrochloric acid to liberate ricin from agglutinated brain cells of dogs. He believed that the ricin was fixed on the cells by a physico-chemical adsorption. Gunn (18) tested

the agglutinating action on other substances than animal cells and he found that it agglutinated cholesterol and carmine suspensions. He reported that the agglutination by ricin was, partly at least, non specific and that it was of the nature of the precipitation of one colloid by a colloid of opposite sign.

Jacoby (19) found that an immune serum when mixed with a clear solution of ricin formed a precipitate and that also when a normal serum was mixed with ricin a faint clouding took place. Michaelis and Steindorff (26) obtained a precipitate with ricin in normal rabbit serum and found that an excess of serum hindered the precipitation but that an excess of ricin did not. The present author also noted that some sera from normal rabbits gave precipitates when treated with dilute solutions of ricin.

Ehrlich (14) immunized animals against ricin by the method of oral administration. He employed albino mice. He made little cakes containing definite amounts of ricin and fed these to mice. He started with very small doses and increased them as the experiment continued. Several days were allowed to elapse between doses. By this method of immunization he was subsequently able to inject subcutaneously many times the lethal dose of ricin without killing the mice. He demonstrated also that he could produce passive immunity by injecting blood from immunized mice into normal mice.

The typical symptoms which accompany intoxication by ricin together with the pathological changes produced in

the tissues by the toxin, have been described in detail by Flexner (16), Müller (27), Oppenheimer (29), Osborne, Mendel and Harris (30) and others. The following pathological changes were most frequently noted : There were punctiform hemorrhages in the peritoneal cavity, scattered over the omentum and along the covering of the intestine. Often the peritoneal cavity contained a considerable amount of a somewhat opaque fluid. Osborne and his associates noted that there was a reddening of the subcutaneous tissue at the point of inoculation, occasionally a noticeable oedematous condition and the liver showed numerous light colored spots. My findings agree very well with descriptions given by Flexner and the authors quoted. It has been noted in this investigation that if death resulted within the first day after the injection, the pathological changes were not so pronounced as when the animal lived two or three days.

The study of ricin was undertaken to see if something concerning the nature of the chemical constitution of this toxin could be determined and to see if an immunity to ricin could be developed by injecting the toxin after it was treated chemically or physically. This included the following investigations:

1. The preparation of the pure toxic principle.
2. The ultimate analysis of the toxin.
3. The toxicity of my preparations on laboratory animals.
4. The action of ricin on red blood cells.
5. The detoxification of ricin:

(a). by means of oxidizing agents.

(b). by means of moist heat.

(c). by means of dyes.

(d). by means of ultra-violet light.

6. The production of immunity in laboratory animals by injecting them with:

(a). untreated ricin.

(b). ricin which has been coagulated by means of moist heat.

(c). ricin which has been oxidized by means of potassium permanganate.

Unless otherwise stated the ricin or the treated ricin in this research was introduced into the body subcutaneously with due antiseptic precautions.

EXPERIMENTAL PART

II. THE PREPARATION OF RICIN.

The raw material from which the ricin was prepared was castor bean press cake (cold pressed) and had been at the laboratory for about two years before being used. The pressing process broke the beans up into such small pieces that no attempt was made to make a separation of the shells from the beans. The whole mass was ground in a mill. The process used from here on is similar to the one employed by Osborne, Mendel and Harris(30).

The method stated briefly was as follows: The castor bean meal was extracted with ether to remove any excess oil. The oil free meal was then extracted with 10 per cent. sodium chloride solution for 48 hours. This solution was filtered and dialysed for four or five days against running tap water at 6° - 10°C. The dialysed solution was filtered again to removethe precipitated globulin. Enough ammomium sulfate was added to the filtrate to make a solution which contained approximately 45 per cent. of the amount required for complete saturation with this salt. After standing, the precipitate was filtered off by suction as quickly as possible and dissolved in water and precipitated by saturation with ammonium sulfate and the precipitate was filtered off and dissolved in water; after which an equal quantity of saturated ammonium sulfate was added. It was allowed to stand several hours and the precipitate was again removed by suction. The dried

precipitate contained less ammonium sulfate by this method than it otherwise would. The partially dried precipitate was dissolved in water and enough saturated ammonium sulfate solution was added to make a one-third saturated solution. The precipitated material was sucked very dry by means of a Buchner funnel and dissolved in water and dialysed for about two weeks in running tap water at 6° - 10°C. The dialysed solution was filtered and the filtrate evaporated at room temperature by means of a fan. This is the most toxic fraction of the castor bean. Toluene was added to all aqueous solutions in the process.

The above process has been repeated several times and and each specimen so obtained labeled with a Roman numeral corresponding to the number of the preparation. My preparations were all made in the fall and winter months when the temperature of tap water was much lower than in the summer and spring months.

III. ULTIMATE ANALYSIS OF RICIN.

The following is an analysis of one of my preparations of ricin which was dialyzed against tap water.

Carbon	51.18 %
Hydrogen	6.8
Nitrogen	16.42
Sulphur	2.32
Ash	2.94

This analysis shows the composition to be that of a typical protein containing a rather large amount of sulphur presumably in combination in part with the ammonium radical and which was not removed by dialysis.

Another sample of ricin which was prepared sometime after the above and which was dialyzed against distilled water instead of tap water , in the last step of the preparation, contained only 0.94 % ash.

IV. THE TOXICITY OF MY PREPARATIONS OF RICIN ON LABORATORY ANIMALS.

The lethal dose of the ricin thus prepared was determined for white mice, white rats, guinea pigs and rabbits.

The influence of dietary conditions on the physiological resistance to ricin has not been studied in this investigation. That is, no attempt has been made to alter the protein content, or the vitamine content of the diet. The diet used for white mice and white rats was:

rolled oats
cracked corn
bread (white or rye) or table scraps
whole milk (sweet)
carrots
water.

The diet used for rabbits and guinea pigs was :

rolled oats
cracked corn
clover hay or alfalfa hay
cabbage or carrots
water & salt

Table 1 shows the toxicity of three samples of ricin on various laboratory animals.

Insert Table 1

Previous investigators have injected their samples of ricin subcutaneously, intravenously, intraperitoneally , intramuscularly and it has been fed to animals. This fact possibly accounts for some of the differences in potency of ricin that have been reported. That is, the toxin is probably not absorbed at the same rate by all the above methods of administration. For example, Field (15) reported a preparation many times more toxic than that prepared

Table 1.

Animal No.	Animal	Sample ricin No.	Age	Weight grams.	Dose per kilo body weight. milligram.	Effects.
105	Albino mouse	V	2.5-3 mo.	25	0.08	Died 65 hours
106	,, ,,	V	,, ,,	25	0.04	,, 3.5 days
107	,, ,,	V	,, ,,	25	0.02	,, 4 ,,
108	,, ,,	V	,, ,,	25	0.016	Lived
109	,, ,,	V	,, ,,	25	0.008	,,
110	,, ,,	V	,, ,,	25	0.004	,,
302	Albino rat	III	adult	222	0.045	Died 40 hours
303	,, ,,	III	,,	222	0.045	,, 35.5-45 ,,
301	,, ,,	VI	,,	222	0.045	,, 40 ,,
355	,, ,,	VI	,,	250	0.040	,, 39.5 ,,
391	,, ,,	VI	,,	325	0.030	,, 43 ,,
392	,, ,,	VI	,,	285	0.035	,, 50-51.5 ,,
393	,, ,,	VI	,,	302	0.026	,, 36 ,,
394	,, ,,	VI	,,	270	0.029	,, 50-51.5 ,,
102	guinea pig	III	adult	660	0.0075	Died 80- 90 hours
111	,, ,,	III	50 da.	575	0.0208	,, 34.5-46. ,,
112	,, ,,	III	50 ,,	525	0.0095	,, 18 days
113	,, ,,	III	3 mo.	800	0.0200	,, 46.5 hours
114	,, ,,	III	adult	1100	0.0200	,, 46.0 ,,
115	,, ,,	III	,,	750	0.0210	,, 46.0 ,,
116	,, ,,	III	,,	1150	0.0190	,, 62 ,,
117	,, ,,	III	,,	1000	0.0200	,, 60-64 ,,
118	,, ,,	III	33 da.	307	0.0162	Lived
104	rabbit	V	adult	2225	0.020	Died 32 hours
201	,,	VI	3 mo.	1700	0:029	,, 24-35 ,,
202	,,	VI	3 ,,	1500	0.026	,, 24-35 ,,
218	,,	VI	2 ,,	1300	0.0074	,, 38-46.5 ,,
236	,,	VI	2 ,,	1100	0.009	Lived
240	,,	VI	1 ,,	600	0.033	Died 16 ,,
243	,,	VI	1 ,,	540	0.027	,, 24 ,,
244	,,	VI	1 ,,	505	0.019	,, 9-21 ,,
245	,,	VI	1 ,,	510	0.031	,, 23 ,,
247	,,	VI	1.5,,	975	0.010	,, 112 ,,

by Osborne and his associates but he injected his preparation of the toxin intramuscularly while they introduced their ricin subcutaneously. My preparations were injected subcutaneously. There were considerable differences in potency of my various samples as can be seen by inspecting table 1. My preparations were not quite as potent as those obtained by Osborne and his associates but exceded that of the preparations obtained by many of the quoted authors. The extremely toxic preparation obtained by Osborne and his associates was probably due to the wide experience of the authors and especially Dr. Osborne with proteins.

V. THE ACTION OF RICIN ON RED BLOOD CELLS:

The agglutinating power of the preparations of ricin was tested as follows: The influence of ricin on blood in vitro was exhibited in the agglutination of the erythrocytes followed by the precipitation or sedimentation of the flocked masses upon the bottom of the vessel. If the red blood cells had been washed with isotonic saline as many as five or six times the supernatant liquid was practically colorless after the sedimentation with the ricin. I have observed the action of the toxin upon red cells of man and the horse.

This property of the agglutination of erythrocytes has been investigated by Pfeiler and Engelhardt (31), by Brioux and Guerbert (9) and by others in regard to con-

tamination of feed stuffs with toxic substances as castor beans. They were able to detect as low as 0.5 per cent. of castor beans in feed stuff by this method.

Osborne, Mendel and Harris found that bloods of different species showed different degrees of sensitiveness toward the toxin, ricin. Lau (22) has made the statement that blood of fish is entirely resistant to ricin. Fraenkel's (17) observations showed that the immunity, so to speak, is only relative and that larger quantities of ricin cause agglutination. Kobert (19) was unable to agglutinate corpuscles from the Sipunculus.

Horse blood was defibrinated and the red blood cells were washed with physiologic saline solution until they were free from serum and then they were added to 9.9 c.c. of physiologic saline solution which contained 2 milligrams 1 milligram and 0.5 milligram of ricin and a control with saline solution was run also in each case. The contents of the tubes were well mixed and a series was placed in the ice box at 1° - 3°C. A second series was kept at room temperature 20° - 26°C and a third series was placed in the incubator at 36° - 38°C.

I found that the temperature of the incubator hindered the agglutination of the red cells as did G. de Macco (24) and that the temperature of the room and the ice box favored it. The results obtained at the three above tempertaures are given in table 2.

Insert Table 2

Table 2.

The Effects of Various Temperatures on the Agglutination of Red Blood Cells by Ricin.

Temperature	No. of tube.	Red cells in 9.9c.c. isotonic saline.	Ricin used. mgm.	Agglutination+ in 2 hours.	Agglutination+ in 24 hours.
		c.c.			
1° - 3°C	1	0.1	2.0	complete	complete
1° - 3°C	2	,,	1.0	3/4	,,
1° - 3°C	3	,,	0.5	slight	1/2
1° - 3°C	4	,,	CONTROL	none	none
20° - 26°C	1	,,	2.0	complete	complete
20° - 26°C	2	,,	1.0	3/4	,,
20° - 26°C	3	,,	0.5	slight	3/4
20° - 26°C	4	,,	CONTROL	none	none
36° - 38°C	1	,,	2.0	1/2	3/4
36° - 38°C	2	,,	1.0	slight	1/3
36° - 38°C	3	,,	0.5	very slight	slight
36° - 38°C	4	,,	CONTROL	none	none

Foot note table 2.

+ At the end of 2 hours a sterile glass rod was used to stir the contents of each tube and a drop of each suspension was examined with a microscope. An arbitrary method of measuring the agglutination was employed which was to estimate the per cent. of free cells in a number of fields as compared with the cells that were agglutinated. If there were no free cells or at most one or two in a single field, the agglutination was termed complete. The controls were not agglutinated in any case.

The results in table 2 show that my preparation was able to cause the agglutination or sedimentation of the red cells of the horse. Also it might be added here that the above experiment was carried out with practically the same results for red cells of man. My preparations seemed to be quite as active as those of other authors quoted. The above experiment proves that my preparation had the property of agglutinating red cells. This property affords a means of controling the amount of activity of preparations.

VI. THE DETOXIFICATION OF RICIN.

The purpose of this part of the investigation was to obtain a reliable method of detoxifying ricin and to standardize it so that the resulting material would still have the properties necessary to produce immunity to ricin when the resulting solution was injected into animals. Several new methods of treating ricin to destroy its toxic properties are reported. Two of the methods have been employed to produce immunity to ricin in animals and one of these, the treatment with potassium permanganate has given very reliable results. This is taken up on page 63

A. The Detoxification of Ricin by Means of Oxidizing Agents.

This part of the research was carried out with the idea that oxidizing agents might destroy the toxic principle of ricin and leave unaffected its power of producing immunity to ricin. All of the oxidizing agents employed destroyed the toxic properties of ricin but there seemed to be considerable difference in the speed of the reaction or reactions taking place, also there was some difference in the amonut of detoxification. The toxic part of ricin seems to be more readily oxidized than the antigenic part but even the antigenic part can be oxidized so that it produces little or no immunity to ricin. Since the toxic properties of ricin were destroyed, experiments were performed to see whether solutions of the modified toxin would not produce immunity to ricin if injected into animals. This is taken up on page 63.

(1). Oxidation of Ricin by Means of Potassium Permanganate.

At first twentieth normal potassium permanganate was used to oxidize dilute solutions of ricin. The permanganate was added drop at a time a nd a few minutes were allowed to elapse before another addition was made. The reaction was very quick and a brownish black precipitate was formed which was of a flocculent nature. This method of treatment was found to be an efficient means of detoxifying ricin if the process was continued until the pink color remained for about 15 - 30 seconds after an addition of the oxidizing agent. One cubic centimeter of N/20 $KMnO_4$ was found to detoxify 40 c.c. of a solution of ricin (1 c.c. = 0.02 milligram)

The above method of treatment served alright for dilute solutions of ricin but in order to detoxify larger amounts of ricin and still be able to inject the resulting solution into animals immediately after the treatment it became necessary to employ concentrated solutions of permanganate. Concentrated solutions of both ricin and permanganate were used. In these cases the precipitates were very heavy and often a gel-like mass was the result. The gel-like masses resembled clots but they were not very rigid and were easily broken up by means of a stirring rod or by merely rotating the container.

The following experiment serves to show that the reaction between ricin and $KMnO_4$ took place very quickly and that it went practically to completion. A solution

which contained 300 milligrams of ricin in 10 c.c. of water was treated with 2.5 c.c. of a concentrated solutionof $KMnO_4$, of which one cubic centimeter contained 70 milligrams of dry potassium permenganate, and the whole resulting solution and suspension was injected subcutaneously into a rabbit within two minutes and thirty seconds after they were mixed. The rabbit was an adult and weighed 2560 grams. The injections were started one minute and twenty seconds after the solutions were mixed. This quantity of ricin if untreated would have been sufficient to kill about 5000 rabbit of the same weight as this rabbit. The animal was never particularly sick and she never failed to eat or drink as rabbit often do when toxic solutions are injected. If the toxin was not completely oxidized to a non toxic substance, it was nearly so as 0.05 milligram of untreated ricin readily kills rabbits of this size within three to five days.

The detoxification of ricin by means of $KMnO_4$ and the production of immunity in animals to ricin which have been injected with the oxidized solutions of the toxin is taken up in detail on page 63.

(2). The Oxidation of Ricin by Means of Hydrogen Peroxide (30% or Superoxol +).

In the following experiment superoxol was used to oxidize ricin in aqueous solution. This concentrated solution of H_2O_2 was used in order to speed up the reactions

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+ Superoxol is a trade name for 30% H_2O_2.

taking place and also because it did not greatly increase the volume of the resulting solutions. It was found that superoxol detoxified ricin but that the reactions taking place were very slow when the two solutions were merely allowed to be mixed. If the solution, after being mixed, was agitated by some means the process of oxidation seemed to be accelerated considerably. This was accomplished by several means such as shaking by hand, by stirring with a platinum spiral which was rotated by a motor and by a mechanical shaker. Small glass beads were used to increase the liberation of the oxygen.

The concentration of the hydrogen peroxide was watched with extreme care as it was found to be very toxic. If the solution contained too much free hydrogen peroxide, a liberation of oxygen occurred immediately after the injection and a gas pocket was formed which caused the animal to jump around and finally caused convulsions. Mice often died during the convulsions within the first fifteen minutes after the injection. The results obtained by injecting albino mice with ricin which had been treated with superoxol by the above methods are included in table 3.

Insert Table 3

Foot note to Table 3.

Mice Nos.7 and 8 had convulsions very soon after they were injected and No. 7 died while in a convulsion. There was a large pocket of gas which formed immediately after

Table 3

The Effects Produced by injecting Albino Mice with Ricin that has been treated with Superoxol are recorded.

Mouse No.	Weight grams.	Solution of Ricin and Superoxol.	Treatment used.	Time treated hours.	Dose ricin mgs.	Dose per kilo	Effect.
1.	24	5c.c.(1c.c.= 0.02mg.ricin +0.2c.c. Sup.	Shaken by hand	1	0.005	0.21	Died 4.5 days.
2.	24	5c.c. isotonic saline +0.2c.c. Superoxol.	,,	1	0.25 c.c.	Control	Normal
3.	23	5c.c. (1c.c.= 0.02mg.ricin) +0.05c.c. Sup.	Mixed	3	0.01	0.43	Died 4.5 days.
4.	23	5c.c. (1c.c.= 0.02mg. ricin) + 0.1c.c. Sup.	,,	3	0.01	0.43	,, 65 hrs.
5.	23	5c.c. isotonic saline + 0.05c.c. Superoxol.	,,	3	0.5c.c.	Control	Normal
6.	23.	5c.c. isotonic saline + 0.1c.c. Superoxol.	,,	3	0.5c.c.	,,	,,
7.	24	10c.c. (1c.c.= 0.02 mg. ricin) + 0.4c.c. sup.	shaker	24	0.01	0.42	Died 5 minutes convulsions.
8.	24	,,	,,	,,	,,	,,	Lived had a convulsion after injection.
9.	24	,,	,,	,,	0.005	0.21	Lived-normal.
10.	24	,,	,,	,,	,,	,,	,,
11.	23	5c.c.(1c.c. = 0.02 mg. ricin) + 02c.c. Sup.	stirred	26	0.01	0.43	,,
12.	24	,,	,,	,,	0.005	0.21	,,
13.	23	,,	,,	,,	,,	0.215	,,
14.	24.5	10c.c. (1c.c.= 0.02 mg ricin)	shaker	24	0.005	0.20	Died 8 days
15.	24	,,	,,	,,	,,	0.21	,, 70-92 hrs.
16.	22	,,	,,	,,	,,	0.225	,, 48-68 hrs
17.	25	Controls			,,	0.20	,, ,, ,, ,,
18.	25	,,			,,	,,	,, 4 days
19.	25	,,			,,	,,	,, 63.5-67.5hr
20.	29.5	,,			,,	0.165	,, ,, ,, ,,

the injection in each of these two cases. No. 8 was able to walk around in a few minutes after the injection and was apparently normal in eight days.

The superoxol was effective in detoxifying ricin but the speed of the reaction or reactions taking place was much slower than it was in the case with potassium permanganate or with some of the other agents that were used and which are discussed in this section of the thesis.

Since the mice lived that were injected with the exception of No.7 solutions which had been agitated by the stirrer and the mechanical shaker it was decided to inject some of each of these solution into albino rats. The results are given in table 4.

Insert Table 4

By inspecting table 4 it can be seen that the superoxol did detoxify the ricin and that it was possible to inject several lethal doses of the toxin without causing death.

(3). Oxidation of Ricin by Means of Ozone.

A Siemens ozone apparatus was used to generate the ozone from oxygen +. The ozone was passed through solutions of ricin for different lenghts of time to see if the toxic principle would be destroyed. The solutions

+ The oxygen was prepared by the Linde Company.

Table 4

The Effects Produced by injecting Albino Rats with Ricin that has been Treated with Superoxol are recorded.

Rat No.	Weight Grams	Solution of Treatment Ricin and Superoxol.	Time treated hrs.	dose Ricin Mgs.	Dose per Kilo Mgs	Effect
1.	90	5 c.c (1c.c= 0.02 Mgm. ricin) + 0.2 c.c. Superoxol. stirred	26	0.02	0.222	Lived-Normal
2.	85	10 c.c. (1c.c.= 0.02 mgm. ricin) + 0.4 c.c. Superoxol. shaker	24	0.01	0.117	,, ,,
3.	220	Control		0.01	0.045	Died 40 hrs
4.	270	Control		0.08	0.029	,, 51 ,,

became opaque very quickly and a precipitate was formed. A physiologic saline solution containing 0.02 milligram of ricin per c.c. was treated with ozone at first. Ten cubic centimeters of the above solution were put into Erlenmeyer flasks (150 c.c. each). The circuit was connected and oxygen was allowed to flow through the apparatus for several minutes before the solution of ricin was treated. This was done in order to insure a constant flow of ozone. The apparatus which was supported by a ring stand was lowered so that the exit tube dipped beneath the surface of the solution of ricin and the gas was allowed to flow through at the rate of two bubbles per second. This step was controled previously with a flask of water. A slight foam was formed when the ozone passed through the solution but it was not permanent as it disappeared very quickly. The solutions were treated for different lenghts of time. One flask was left open for a short time and then the contents were poured into another flask in order to decrease the concentration of ozone in the atmosphere above the solution. The other flasks were stoppered immediately after the gas passed through the solutions. The solutions were placed in the ice box 3° - 7°C and they were left there for six hours. Small quantities of these solutions were removed and injected into albino mice and the results of the experiments are given in table 5.

Insert Table 5

This method of oxidizing toxin is not as easily controled

Table 5

The Solutions of Ricin were treated with Ozone and injected into Albino Mice.

(10 c.c of a physiologic saline solution of ricin, lc.c. = 0.02 mg. ricin, were used each of the first two treatments mice Nos. 1 to 4 and a solution of ricin 1 c.c. = 0.2 mg. was employed in the last experiment mice Nos. 5 to 7).

Mouse No.	Weight Grams	Treatment	Dose Ricin mgs.	Dose per Kilo.	Fatal Doses injected.	Effect
1.	24	1 min. ozone gas left above solution.	0.01	0.41	2	Died 60 hrs
2.	21	2 min. ozone gas was removed.	,,	0.476	2	Lived no ulcer
3.	20.5	2min. ,, ,,	,,	0.488	2	,, ,,
4.	20.5	,, ,, ,, ,,	,,	0.488	2	,, ,,
5.	21	,, ,, ozone gas left above solution.	0.1	4.76	20	,, ,,
6.	20.5	,, ,, ,, ,,,	,,	4.88	20	,, ,,
7.	22	,, ,, ,, ,,	,,	9.08	40	,, ,,
8.	27	Control	0.005	0.185	1	Died 110 hrs
9.	26	,,	,,	0.19	1	,, 96 ,,
10.	25	,,	,,	0.20	1	,, 80 ,,
11.	23	,,	,,	0.215	1	,, 42 ,,
12.	24	,,	,,	0.205	1	,, 27 ,,

as the method which employes the oxidizing agent $KMnO_4$. There were so many factors such as the per cent. of ozone that was generated from the oxygen, the solubility of the ozone in physiologic saline solution and the temperature at which the experiment was conducted that might influence the degree of detoxification. Mouse No. 7, however, received forty lethal doses of ricin which had been treated with ozone and an ulcer did not develop. The animal appeared normal.

Further experiments on the detoxification of toxins by means of ozone and the production of immunity to the particular toxin is planned

(4). The Action of Chlorine on Ricin.

The following experiment shows the effect of chlorine on ricin. One cubic centimeter of a physiologic saline solution of ricin which contained one milligram of the toxin was put in a 50 c.c. graduated flask and 10 c.c. of the saline solution were added. A fresh saturated aqueous solution of chlorine + gas at 25°C was made and 10 c.c. of this were added to the flask containing the solution of ricin. There was a very slight opaqueness to the solution on standing for 24 hours. The solution was diluted to 50 c.c. and quantities were injected into albino mice and the results are recorded in table 6.

Insert Table 6

+ The chlorine used was generated from manganese dioxide and HCl. It was washed with H_2SO_4 and water.

Table 6.

The Effects of Injecting Albino Mice with the Solution of Ricin that was Treated with Chlorine Water. Controls are included.

Mouse No.	Weight Grams	Dose of Treated Ricin Milligrams.	Dose per Kilo Millligrams.	Effect.
1.	24	0.005	0.21	Lived.
2.	24	0.005	0.21	,,
3.	24	0.01	0.42	,,
4.	25	0.01	0.40	,,
5.	24	0.02	0.84	,,
6.	24	0.02	0.84	,,
		CONTROLS untreated ricin.		
7.	27	0.01	0.37	Died 28 hours
8.	25	0.005	0.20	,, 30 ,,
9.	25	0.005	0.20	,, 42 ,,
10.	25	0.005	0.20	,, 47 ,,
11.	25	0.005	0.20	,, 49 ,,

Since mice Nos. 5 and 6 table 6 lived after receiving several lethal doses of the toxin which had been treated chlorine, it was decided to use concentrated solutions of ricin.

Chlorine gas was employed instead of chlorine water for treating the concentrated solutions of the toxin. This made it possible to inject the resulting solutions without first evaporating some of the water. The gas was bubbled through the solutions of ricin in the sunlight and in the absence of light. The former condition favors the entrance of the halogen in the side-chain and the latter in the ring structure. The chlorine seemed to react very rapidly with the toxin and a white precipitate was formed immediately if the solution contained as much as two milligrams of ricin per c.c. The toxicity of solutions containing 0.02 milligram per c.c. was destroyed within five minutes, with the gas passing through at the rate of two bubbles per second, so that several lethal doses could be injected without killing the animal. The toxicity, however, was not destroyed when a solution containing 0.3 gram of ricin in 20. c.c. was treated for five minutes with the gas flowing at the above rate. The resulting solution was injected into a rabbit weighing 2400 grams and it died between 7 and 16 hours after the injection. Another solution containing 0.3 gram of the toxin was given four treatments of fifteen seconds each, two in the sunlight and two in the absence of light, on successive days. The solution was injected into a rabbit weighing 2150 grams and it was found dead 8 hours after the injection.

A solution containing 0.39 gram of ricin, in 20 c.c. of water, was treated four times with the chlorine gas as the solution that was injected into the above rabbit weighing 2150 grams except that the treatments lasted for ten minutes each. This solution was injected into a rabbit weighing 2500 grams and the symptoms which occur after injections of untreated ricin did not develop. This quantity of ricin if untreated is enough to kill more than 5,000. rabbits weighing as much as this one did. The detoxification was practically complete by this process. It is very probable that much larger quantities of the toxin could be treated by the above process and injected at once into a rabbit without killing it.

(5). The Action of Bromine on Ricin.

Since chlorine was efficient in destroying the toxic principle of ricin, bromine was employed to see if it would produce the same effect. At first, physiologic saline solutions of ricin were treated with an aqueous solution of bromine. A yellowish to buff colored precipitate was formed immediately in solutions which contained two milligrams of the toxin per c.c. Bromine destroyed the toxicity of the ricin very readily when it was in a dilute solution and when the treatments were carried on in the sunlight and in the absence of light.

The following experiment shows that bromine detoxified ricin. The experiment was divided into two parts.

Part I.

One milligram of ricin in 1 c.c. of water was added to a 50 c.c. graduated flask and 10 c.c. of isotonic saline were added. Two c.c. of a half saturated solution of bromine, at 25°C, were added. The solution was made up to 50 c.c. with isotonic saline after standing for six hours. The solution was allowed to stand for ten days at 20° - 26°C before it was injected into mice.

Part II.

One milligram of ricin in 1 c.c. of water was added to a 10 c.c. graduated flask and 4 c.c. of a bromine solution, as used in part I, were added. The solution was allowed to stand for two days at 20° - 26°C before it was injected into mice.

Table 7 gives the results obtained by injecting mice with the solutions of ricin which were treated with bromine water. The solution from Part I was injected into mice Nos. 1-3 and the solution from Part II was injected into mice Nos 4-7.

Insert Table 7

By inspecting table 7 it is readily seen that mouse No. 6 received 20 lethal doses of ricin and that mouse No. 7 received 40 fatal doses. These mice were not particularly affected by the injections and probably could have received many more doses of the treated toxin without being killed.

Table 7.

The Effects Produced by Injecting Albino Mice with Ricin that has been treated with Bromine Water. Controls are included.

Mouse No.	Weight Grams.	Dose of Ricin Treated with Bromine. mg.	Dose per Kilo. Milligram.	Effect. Lived -	Normal
1.	24	0.005	0.21	..	..
2.	24	0.01	0.42	..	..
3.	24	0.02	0.84	..	..
4.	22	0.05	2.27	..	..
5.	22	0.05	2.27	..	..
6.	23	0.10	4.34	..	..
7.	22	0.20	9.08	..	..
		Controls untreated Ricin			
8.	24	0.005	0.21	Died 30-35 hrs	
9.	24	0.005	0.21	.. 30-35 ..	
10.	24	0.005	0.21	.. 48 ..	

Due to the fact that an aqueous solution of bromine diluted the solution of ricin during the above process, it was decided to pass bromine gas through the more concentrated solutions of the toxin. Saturated bromine water was put into a distillation flask and rubber connections were made to two Woulff bottles. The first bottle contained sulphuric acid and the second one contained water. A basin of boiling water was used to heat the flask. This method gave a good supply of bromine gas and it was a quick mens of obtaining washed bromine.

The washed gas was passed through the solution of ricin in the sunlight and in the absence of light as it was in the treatment of ricin with chlorine.

A solution of ricin containing 0.3 gram in 20 c.c. was put in a 250 c.c. pyrex flask and treated with bromine gas by the above method. The flask was kept at 20° - 26°C during the entire treatment. Six treatments of five minutes each were employed. The flask was stoppered after each treatment, and they were alternated in the sunlight and in the absence of light on six successive days. The resulting solution was allowed to stand in the ice box for five days and then it was injected into a rabbit weighing 2400 grams. The rabbit was never sick and the symptoms of ricin poisoning did not develop. The amount of ricin injected into this rabbit if untreated would kill more than 5,000. adult animals weighing as much as it did.

(6). The Action of Iodine on Ricin.

Since chlorine and bromine were so efficient in destroying the toxic principle of ricin, iodine was added in order to see whether it had any effect on the toxin. The iodine was dissolved in distilled water and this solution was added to an aqueous solution of ricin. One cubic centimeter of the solution of ricin, containing one milligram of the toxin, was treated with 4 c.c. of an aqueous solution of iodine. There was 0.30 milligram of iodine in each c.c. The treated toxin was left in the sunlight during the first part of the treatment. That is, it was allowed to stand 2.5 hours in the sunlight at 23° - 25°C and then 5 c.c. more of the solution of iodine was added. The solution was allowed to remain in the sunlight for two more hours and then it was placed in the ice box at 2° - 5°C for 22 hours. The ice box was not illuminated. At the end of 22 hours, the solution was taken out of the ice box and placed in the sunlight for 1 hour at 25°C and then white mice were injected with this iodized solution of ricin. Controls were run at the same time on the iodine solution and on a solution of ricin. The results are included in table 8.

Insert Table 8.

The iodine seemed to react very much slower with ricin than chlorine or bromine. It, however, detoxified the ricin as the quantity injected into mouse No. 5 was sufficient if untreated to kill twenty adult mice.

Table 8.

The Effects produced by injecting Albino mice with Ricin that has been treated with an aqueous Solution of Iodine. Controls were run on the Ricin and on the Solution of Iodine.

Mouse No.	Weight Grams	Dose of treated Ricin. Milligrams.	No. of Lethal Doses.	Dose per Kilo Milligram	Effect
1.	24	0.005	1	0.21	Lived-Normal
2.	24	0.01	2	0.42	,, ,,
3.	22	0.025	5	1.135	,, ,,
4.	23	0.05	10	2.17	,, ,,
5.	23	0.10	20	4.34	,, ,,
		Controls untreated Ricin			
6.	24	0.005	1	0.21	Died 74.5hrs.
7.	22	0.005	1	0.22	,, 60 ,,
8.	22	1c.c. solution of iodine		(0.30 mg) iodine	Lived-normal

Since the mice lived that were injected with the iodized solutions of ricin as treatedin the above experiment, I made other experiments to see whether less iodine would destroy the toxic properties.

Experiment No. I

Flask No. I. One milligram of ricin,in 1 c.c. of water, was added to a 10 c.c. graduated flask and 4.5 c.c. of an aqueous solution of iodine was added. Each c.c. contained 0.3 mgm.

Experiment No. 2

Flask No. II. One milligram of ricin, in 1 c.c. of water, was added to a 10 c.c. graduated flask and 2.5 c.c. of the above solution of iodine were added.

Both flasks were placed in a light room for two hours at 25°C. There was no sunlight. They were put in the ice box for 16 hours at 1° - 3°C. The ice box was not illuminated. The flasks were taken out of the ice box and placed in the sunlight for 2 hours at 25°C and both were filled to the mark with distilled water. Quantities of these solutions were injected into white mice and the results are recorded in table 9. Controls were run on the toxin and on the aqueous solution of iodine.

Insert Table 9

Since iodine did destroy the toxicity of ricin in the above experiments, a concentrated solution of the toxin was treated so that larger quantities of the detoxified product could be injected into animals. Fifty milligrams of ricin were put into a 25 c.c. graduated flask and 5 c.c. of distilled water were

Table 9.

Futher Results produced by injecting Albino Mice with Ricin that has been treated with aqueous solutions of Iodine.

Controls are included.

Mouse No.	Weight Grams	Dose of Treated Ricin Milligram.	Flask No	No. of Fatal Doses.	Dose per Kilo Milligrams.	Effect.
1.	25	0.05	I	10	2.00	Lived-normal
2.	22	0.15	,,	30	6.81	,, ,,
3.	24	0.20	,,	40	8.32	,, ,,
4.	31	0.10	II	20	3.22	,, ,,
5.	22	0.20	,,	40	9.08	,, ,,
	Controls untreated ricin.					
6.	25	0.005		1	0.20	Died 34-46 hrs
7.	25	0.005		1	0.20	,, 34-46 ,,
8.	20	0.005		1	0.25	,, 34-46 ,,
9.	25	1 c.c. solution of iodine (0.30 mg. iodine).				lived Normal
10.	24	0.5 c.c. ,,	,,	,,	,,	,,

added. Eighty milligrams of iodine, in 20 c.c. of water, were added. 100 mgm. of KI were used to dissolve the iodine. The treatment was allowed to continue for twelve weeks at 20- - 26°C.

A mouse, weighing 20 grams, was injected with 0.25 c.c. of the above solution of the iodized ricin or the equivalent of 100 lethal doses of ricin if it were not treated. The mouse lived and was apparently normal.

B. The Effects produced by treating Ricin with Dyes and the Physiological Behavior of Animals injected with the resulting Solutions.

Since it had been found that ricin agglutinated or precipitated various substances such as cholesterol, lecithin and carmine, it was decided to test the toxin with congo red, an acid stain and neutral red, a basic stain. One half of a gram of each of the above dyes was dissolved in 500 c.c. of sterile, distilled water and the resulting solutions were filtered through dry quantitative filter paper. An aqueous solution of ricin, containing 1 mgm. per c.c., was made. One c.c. of each dye was treated with one c.c. of the solution of the toxin. There was no precipitate in 24 hours in either case.

The effects of the dyes on the toxic principle of ricin were tested by means of injections of the treated toxin into white mice. The quantities of the two dyes were varied but the dose of the toxin was kept constant.

The results obtained by injecting white mice with the treated toxin are included in tables 10 and 11. Controls were run on the toxin and on the solutions of the dyes.

Insert Table 10

Insert Table 11

There was some detoxification of ricin by means of the acid stain, congo red, as can be seen by inspecting table 10 even though all of the animals that lived had ulcers. The basic stain, neutral red, seemed to have very litte if any detoxifying properties on ricin. Mice Nos. 5 and 8 table 11 seemed to have some protection against the toxin but they were killed as were all the rest that received injections of fatal doses of ricin which had been mixed with this stain.

C. The Detoxification of Ricin by means of Boiling.

An aqueous solution of ricin, 1 c.c. = 1 mgm., was heated and a partial coagulation occurred at 56°C. This solution was allowed to stand at 1°-3°C over night and then it was centrifuged. The supernatant liquid was removed and heated slowly and further coagulation occurred at 70°-75°C. Since the above solution of ricin did not coagulate at on temperature, I decided to boil the solutions to insure complete detoxification. This

Tablel 10

Ricin in as aqueous solution was treated with different amounts of congo red and the solutions were injected into adult white mice. A lethal dose of Ricin (0.005 mgm) was used in each injection. Controls were run on the toxin and on the congo red.

Mouse No.	Weight Grams	Dose ricin Mgm VI	Congo Red mgm.	Time Mixed hours	Temp.	Effect
1	27.5	0.005	14	1	23C	Lived small ulcer
2	20	,,	12	25	2-5 C	,, ,, ,,
3	20	,,	12	,,	,,	,, ,, ,,
4	23.5	,,	14	38	,,	,, ,, ,,
5	22	,,	10	,,	,,	,, large ,,
6	23	,,	15	17	,,	,, small ,,
7	22	,,	15	,,	,,	Died 102-129 hrs
8	20	,,	15	24	,,	,, 48-55 ,,
9	20	,,	15	,,	,,	,, 24-31 ,,
10	23	,,	15	40 min	25 C	lived large ulcer
11	22	,,	15	,, ,,	,,	,, ,, ,,
12	20	Control	12			,, no ,,
13	22	,,	12			,, ,, ,,
14	21	Control	15			,, ,, ,,
15	23	,,	15			,, ,, ,,
16	21	0.005	Control			Died 41-52 hr
17	21	,,	,,			,, ,,
18	21	,,	,,			,, 53 hr.

Table 11

Ricin in aqueous solution was treated with different amounts of neutral red and the solutions were injected into adult white mice. A lethal dose of ricin (0.005 mg.) was used in each injection. Controls were run on the toxin and on the neutral red.

Mouse No.	Weight grams	Dose ricin Milligram	Neutral red mgs	time mixed hours	Temper-ature	Effect
1.	20	0.005	14	25	1-5 C	Died 44-47.5hrs
2.	20.5	,,	12	,,	,,	,, 48-59 ,,
3.	20	,,	10	,,	,,	,, ,, ,, ,,
4.	20.5	,,	8	,,	,,	,, ,, ,, ,,
5.	20	,,	15	24	,,	,, 90- 108 ,,
6.	20.5	,,	,,	,,	,,	,, 24-31 ,,
7.	20	,,	14	,,	,,	,, 71-88 ,,
8.	22	,,	,,	,,	,,	,, 115-139 ,,
9.	23	,,	,,	38	,,	,, 52-56 ,,
10.	22	,,	10	,,	,,	,, 57-78 ,,
11.	23	,,	15	17	,,	,, 41-52 ,,
12.	23	,,	,,	18	25 C.	,, 41-52 ,,
13.	23	,,	,,	,,	,,	,, 55-77 ,,
14.	20	Control	,,			Lived no ulcer
15.	21	,,	10			,, ,,
16.	22	,,	15			,, ,,
17.	23	0.005	Control			Died 55-77 hrs
18.	25	,,	,,			,, 63.5-67.5 hr
19.	20	,,	,,			,, 59 ,,
20.	21	,,	,,			,, 74 ,,

was done and the solutions were found to be non toxic when as many as 500 fatal doses were injected into a rabbit. After the detoxification of ricin by boiling had been confirmed by the author it was decided to investigate the effect of injecting rabbits and guinea pigs with increasing doses of the boiled toxin. This is taken up in detail on page 54

D. The Action of Ultra-violet Light on Ricin.

The light from a Bang lamp was used by Dreyer and Haussen (13) in studying ricin among other substances and they found that it lost its power of agglutinating blood cells when so treated or at least this property was weakened.

Stimulated by the above research, I tried the effect of ultra-violet light upon ricin in order to see whether it would destroy the toxic principle. A Cooper Hewitt quartz mercury lamp was the source of the light. The distance between the quartz tube and the table which supported the solutions varied from 18 to 30 cm. in the different experiments. The temperature of a thermometer lying on the table, by the side of the solutions being subjected to the treatment, varied from 29° - 35°C. in the different treatments while the room temperature varied from 18° - 22°C. The voltage employed ranged from 60 to 80 and the current varied from 3.4 to 4 amperes. The light was always allowed to operate for at least fifteen

minutes before starting any experiments in order to insure constant current, voltage etc. At first the solutions of ricin, in 10c.c. quantities, were treated in open petri dishes with all the rays from the light for different lengths of time and the shortest time required to destroy the toxicity was determined. One minute was sufficient to destroy the toxicity so that when two lethal doses were inject into albino mice the typical symptoms following poisoning by ricin did not occur. Solutions which were treated for five minutes caused no ulcers or sloughing off of the skin when they were injected in quantities equivalent to three lethal doses if they had not been treated.

Since the toxicity of ricin was destroyed by means of all the rays from the quartz mercury lamp, I decided to see which wave length detoxified it. The filters+ use were aqueous solutions of ammonium sulphate, calcium chloride, acetic acid and lead acetate. The filters did not allow the light of short wave length to pass. For example filter No. 5 cut out all wave lengths shorter than 230 μμ.

+ The Institute of Industrial Research, University of Cincinnati, very kindly allowed me to use their filtersand other apparatus for these experiments. The author wishes to express his thanks to Dr. Herman Schneider, Mr. G. Sperti and Mr. R. B. Withrow. for their cooperation and assistance in making it possible to perform these experiments.

The filters which were used and their corresponding values in wave-lengths and Angstrom units, are given in table 12.

Insert Table 12

The two photographs+ page 46 show the effect produced by passing the iron and mercury spectrum through these filters. The top line, being the complete mercury spectrum on each photograph, is added for comparison. Starting from the top next to the complete spectrum the spectra are in pairs. The first of each pair is an exposure of five seconds and the second is an exposure of sixty seconds. The first photograph includes 2030 - 2450 Angstrom units and the second photograph 2480 - 2750 Angstrom units. The first four pairs of lines on photograph No. 1 are of the iron spectrum and all the others are of the mercury spectrum.

A solution of ricin, 1 c.c. = 0.02 mgm., was put in crystallizing dishes which were fit in brass cylinders with a lid of brass which had an opening for the passage of the rays. A cylinder with one end closed by means of a quartz plate served as a container for the filters. The solutions of ricin were agitated every few minutes during the exposure of the light. Table 13 shows the effects produced by injecting solutions of ricin which had been treated for different

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+ Grateful acknowledgement is made of the asistance of Professor J. B. Homan for making these photographs and sketches in this thesis.

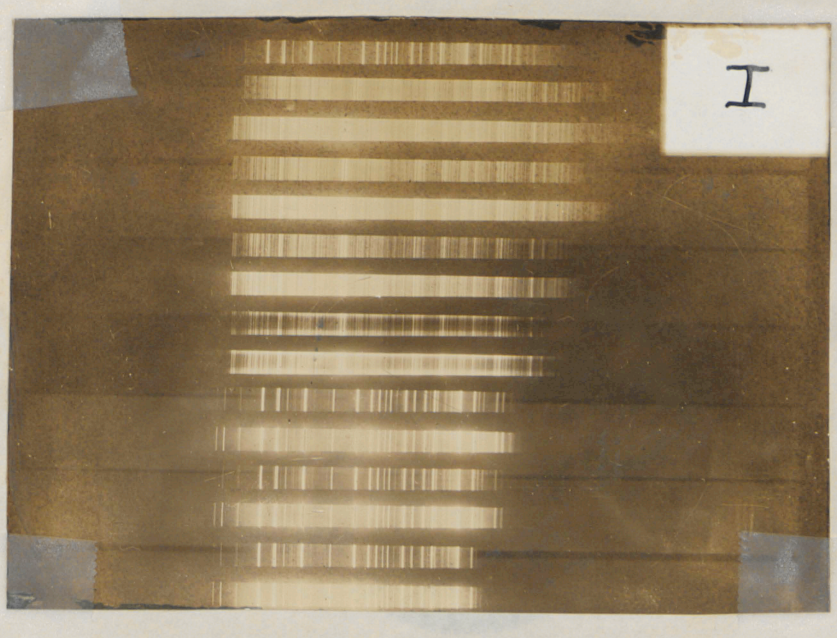
e See page47 for photographs of this apparatus.

Table 12

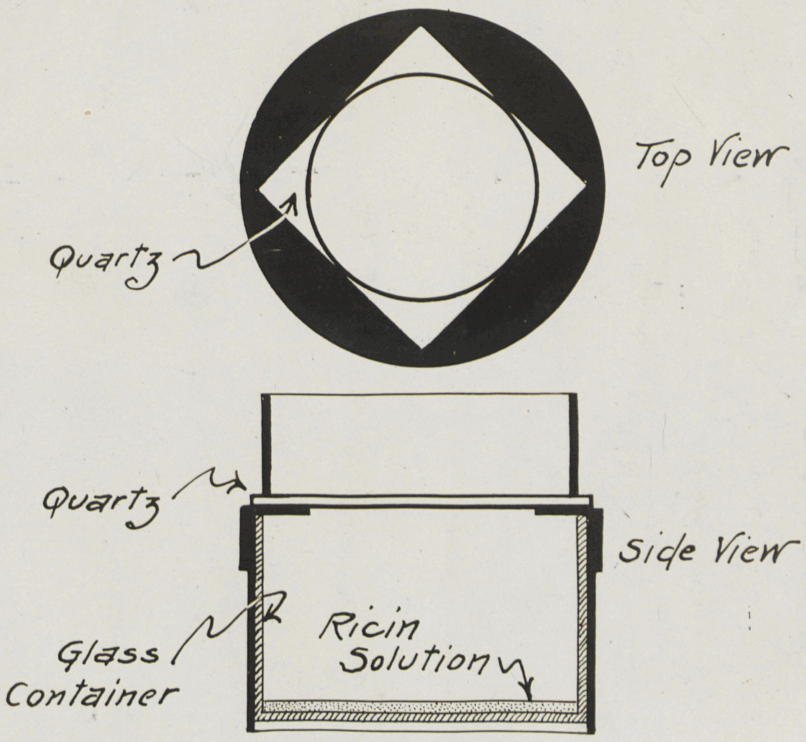
Filter No.	Salts and Acetic acids.	Concentration		Angstrom Units.
1	Ammonium Sulphate	Concentrated	203	2030
2	Strontium Chloride	Concentrated	212	2120
3	Calcium Chloride	1-2	222	2220
4	Acetic Acid	1-500	225	2250
5	Acetic acid	1-50	230	2300
6	Acetic acid	1-20	237	2370
7	Lead acetate	1-1000	245	2450
8	Lead Acetate	1-600	248	2480
9	Lead Acetate	1-400	254	2540
10	Lead Acetate	1-200	257	2570
11	Lead Acetate	1-100	265	2650
12	Lead Acetate	1-40	270	2700
13	Lead Acetate	1-20	275	2750

This table was prepared by the University of Cincinnati Institute of Industrial Research. I reproduced it with the permission of that Department

The photographs No. I and No. II show the effect of passing the ultra-violet light through the filters.



Photograph of apparatus used to contain the ricin solutions and the filter solutions in the ultra-violet light experiments.



lengths of time with the rays from the mercury vapor lamp after they had passed through the filters. White mice were used for these injections.

Insert Table 13

It is evident from the data in table 13 that the rays which destroyed the toxin were between 225 and 254 μμ in length.

The quartz mercury vapor lamp generated considerable ozone in the surrounding atmosphere during the above treatments. Since I found that ozone destroyed the toxicity of ricin when it was bubbled through a solution of the toxin, I performed experiments to see whether it was possible that the effect of the ultra-violet light was due to ozone and not to certain specific rays.

Solutions of ricin, 1 c.c. = 0.02 mgm., were put directly beneath the mercury vapor lamp but were covered with paper boxes which had the sides and ends cut and elevated so that a circulation of gases could take place. One of the boxes had the sides removed except for a little at each corner in order to support it above the dish. A paper chamber was arranged on one side of the light so that the ozone would not diffuse away so rapidly. The concentration of the ozone in the chamber was very much greater than it was in the atmosphere surrounding the boxes as tested by moist KI papers. Table 14 shows the effects produced on white mice that were injected with ricin which was treated with the ozone formed by the lamp or solutions of ricin which were allowed to stand in the atmosphere surrounding the mercury vapor lamp.

Mouse No.	Age Months	Weight Grams	Filter used in Angstrom	Time treated	Dose mgm.	Local reaction	Effect
941	2	21	2030	1 hr.	.005	no ulcer	lived
942	2	20	2120	1 hr.	.005	" "	"
943	2	19	2220	1 "	.005	" "	died 9 days
944	2	20	2250	1 "	.005	" "	lived
945	2	20	2300	1/2 "	.005	" "	"
946	2	19	2300	1/2 "	.01	ulcer	"
947	2	20	2370	1/2 "	.005		died 6 days
948	2	19	2370	1/2 "	.01		" 68 hrs.
949	2	20	2450	1/2 "	.005	"	lived
950	2	20	2450	1/2 "	.01	"	"
951	2	20	2480	1/2 "	.005		died 6 days
952	2	21	2480	1/2 "	.01		" 4 "
953	2	20	2540	35 min.	.005		" 88 hrs.
954	2	21	2540	35 "	.01		" 71--81 hrs.
955	2	20	2570	35 "	.005		" 86 "
1001	2	20	2250	1.5 hrs.	.01	no ulcer	lived
1002	2	20	2250	1.5 "	.015	ulcer	"
1003	2	20.5	2300	1.5 "	.005	"	"
1004	2	20.5	2300	1.5 "	.01	"	"
1005	2	20.5	2370	1.5 "	.005	"	"
1006	2	20.5	2370	1.5 "	.01	"	"
1007	2	20.5	2450	1.5 "	.005	slight ulcer	died 13 days
1008	2	21	2450	1.5 "	.01		" 100 hrs.
1009	2	20	2540	1.5 "	.005		" 90 "
1010	2	20	2540	1.5 "	.01		" 50 - 60 hrs.

Table 13.

Insert Table 14

From the preceding table it is evident that the ozone, generated from the mercury vapor lamp, did not affect the ricin very markedly at least in one hour and fifteen minutes either under a box which had the sides cut off for circulation of the atmosphere or in the paper chamber. One minute in all the rays of the light was sufficient time to destroy the toxicity of ricin so that quantities equivalent to several times the lethal dose did not cause the usual toxic symptoms or death. Since an atmosphere containing more ozone than is normally present around a quartz mercury lamp did not appreciably affect the toxicity of the solution of ricin even in 1 and 1/2 hours, it is very probably a fact that the ozone played only a slight part in the de - toxification of the toxin when the solution was treated with all the rays of light from the quartz mercury lamp. The chance that ozone affected the toxin in the brass containers is very slight, since ozone is formed by wave-lengths shorter than 200 (41) (36) and filter 225 μμ (2250 Angstrom units), allowed rays to pass through which destroyed the toxin.

Mouse No.	Age Months	Weight grams	Dose mgm.	How treated	Time treated	Effect
832	2.5 - 3	22	.005	under paper box	30 min.	died 66 hrs.
833	2.5 - 3	22	.01	" " "	30 "	" 60 "
834	2.5 - 3	22	.005	" " "	40 "	" 100 "
835	2.5 - 3	23	.01	" " "	40 "	" 61 "
918	2.5	20.5	.005	" " "	1 hr.	" 42 - 46 hrs.
919	2.5	20.5	.005	" " "	1 " 15 "	" 55 - 57 hrs.
836	2.5	22	.005	in paper chamber	30 "	" 74 hrs.
837	2.5	23.5	.01	" " "	30 "	" 48 - 59 hrs.
838	2.5	21	.005	" " "	40 "	" 49 - 59 hrs.
839	2.5	21	.01	" " "		" 57 hrs.
920	2.5	21	.005	" " "	1 hr.	" 58 - 62 hrs.
921	2.5	21	.005	" " "	1 " 15 "	" 74 - 96 hrs.

Table 14.

VII. THE PRODUCTION OF IMMUNITY IN LABORATORY ANIMALS.

A. The Production of Immunity by means of Untreated Ricin.

The process of immunizing animals to untreated ricin was confirmed during this research. Three mice and one guinea pig were used for this experiment. The quantity of blood obtained from the white mice was so small that 0.5 c.c. of physiologic saline solution was added in each case after the clot had formed. The serum from each mouse was mixed with a lethal dose of ricin and allowed to stand for one hour at 2°C and then it was injected into young white rats. The serum from the guinea pig was mixed with a lethal dose of ricin and allowed to stand for one hour at 2°C and then it was injected into white mice. The results of these experiments are included in table 15.

Insert Table 15

The sera from the above animals were able to protect other animals from a lethal dose of rcin as can be seen by inspecting table 15. The results of this experiment affords another check on the purity of my preparation and it shows that my sample of ricin was similar to those of other authors quoted.

Table 15

Animal	No of Animal	Amount of Ricin injected	Time from first to last injection	Time from last injection to taking of blood.	Quantity serum to prevent death when mixed with lethal dose.
Mouse		3.54	47 days	11 days	.3 c.c. blood
,,		3.54	47 days	11 days	.15 ,, ,,
,,		3.54	47 days	11 days	.2 ,, ,,
Guinea pig	101	102.	60 days	11 days	.5 serum

B. The Production of Immunity in Animals by Heat Coagulated Ricin.

The fact that moist heat destroyed the toxic properties of ricin in solution was reported by (28), (38) and other authors. The present author has verified these reports. Since ricin was detoxified by this process, an attempt was made to see if solutions of the toxin, treated by boiling, would still have any antigenic properties. Although ricin coagulated upon boiling and there was a suspension of particles of the coagulum, it was possible to inject the material into animals.

An electric stirrer was used to keep the solution agitated while it was heated. The stirrer also influenced the size of the particles of the coagulum. If it was allowed to rotate at 3 - 5 revolutions per second, the particles were very coarse and if it was allowed to rotate at 10 - 25 revolutions per second, the particles of coagulum were quite small and were easily injected into animals by means of a No. 20 gauge needle.

Quantities of ricin were dissolved in 100 c.c. of water in a 600 c.c. pyrex beaker and they were heated on a sand bath by means of an electric hot plate. The speed of the electric stirrer was controled by means of a rheostat. The solutions were heated for an hour or more as can be seen by inspecting table 16 and 17 and they naturally lost in volume. This loss in volume was made up by adding distilled water from time to time. At the last

of each treatment, however, the solution was allowed to evaporate to a volume of 25-40 c.c.. This was done for convenience in making injections. Since the resulting solutions were mostly suspensions of coagulum, it was difficult to know exactly the quantity of treated toxin that was injected at each interval but the whole solution from a single treatment was injected into one animal and thus the approximate total injected into each animal was known. The term approximate is used since it was impossible to completely inject all of a coagulum as there was always a small amount left in the syringe. Tables 16 and 17 include the Nos. of the solutions, the time heated and the temperatures that they were heated at.

Insert Table 16

Insert Table 17

The solutions of ricin that were treated according to tables 16 and 17 were injected into rabbits and quinea pigs and the results are given in table 18.

Insert Table 18

Since rabbit No. 218 and guinea pigs Nos. 26 and 27 lived after they were injected with more than lethal doses of untreated ricin in each case, it is evident that an immunity to the toxin had been developed.

Table 16

Solution No. I		Solution No. II		Solution No. III		Solution No. IV		Solution No. V	
Time	Temp. C.	Time	Temp. C.	Time	Temp. C.	Time	Temp. C.	Time	Temp. C.
3:00P.M.	25°	12:00P.M.	25°	2:30P.M.	50°	3:20P.M.	56°	12:15P.M.	25°
3:05 "	55°	12.05 "	60°	2:35 "	59°	3:30 "	68°	12:30 "	80°
3:10 "	60°	12:10 "	68°	2:45 "	71°	3:35 "	76°	12:45 "	82°
3:12 "	62°	12:15 "	69°	2:55 "	80°	3:47 "	83°	12:50 "	89°
3:15 "	64°	12:25 "	75°	3:05 "	83°	4:00 "	83°	12:54 "	98°
3:20 "	67°	12:30 "	75°	3:20 "	80°	4:15 "	98°	1:00 "	85°
3:25 "	69°	12:40 "	80°	3:25 "	79°	4:23 "	98°	1:05 "*	83°
3:30 "	70°	12:45 "	82°	4:05 "	78°	4:30 "*	85°	1:10 "	85°
3:35 "	72°	12:50 "	78°	4:10 "	80°	4:40 "	88°	1:20 "	99°
3:40 "	71.5°	stopped		4:20 "	100°	4:45 "	98°	1:28 "	98°
3:45 "	75°	to re-		reheated		4:50 "	95°	1:30 "*	93°
3:47 "	80°	pair		above		5:00 "	93°	1:40 "	99°
3:50 "	88°	stirer		solution		5:05 "	84°	1:50 "	91°
3:55 "	89°	1:10P.M.	45°	12:10P.M.	70°	5:10 "	82°	1:55 "	96°
3:57 "	100°	1:20 "	60°	12:12 "	80°	5:18 "	80°	2:00 "	99.5°
4:00 "	94°	1:25 "	68°	12:14 "	90°	5:23 "	82°	2:10 "	90°
4:05 "	75°	1:30 "	80°	12:20 "	91°	5:28 "	90°	2:20 "	90°
4:10 "	50°	1:40 "	80°	12:25 "	94°	5:33 "	96°	2:30 "	95°
4:15 "	20°	1:55"	80°	12:30 "	94°	5:55 "	98°	2:35 "	86°
Reheated		2:15 "	79°	12:40 "	95°	6:05 "	70°	2:45 "	70°
above		2:30 "	80°	12:45 "	96°				
Solution		2:35 "	90°	12:50 "	95°				
12:10P.M.	70°	2:40 "	90°	12:55 "	96°				
12:12 "	80°	2:45 "	100°	1:00 "	95°				
12:14 "	90°			1:05 "	97°				
12:20 "	91°			1:10 "	98°				
12:25 "	94°			1:15 "	75°				
12:30 "	94°			1:20 "	66°				
12:40 "	95°								
12:45 "	96°								
12:50 "	95°								
12:55 "	96°								
1:00 "	95°								
1:05 "	97°								
1:10 "	98°								
1:15 "	75°								
1:20 "	66°								

* Water was added to make up to volume.

Foot note Table 16.

The first two solutions were reheated as can be seen by inspecting table 16. This was done because the first injection in each case caused a slight swelling. Therefore in order to avoid any toxic effects the solutions were subjected to the heat treatment a second time.

Table 17

Solution No VI		Solution No VII		Solution No VIII	
Time	Temp C.	Time	Temp C.	Time	Temp C.
10:15 PM	25° C	9:15 PM	25° C	4:05 PM	25° C
10:25 ,,	65° C	9:20 ,,	52° C	4:15 ,,	75° C
10:30 ,,	75° C	9:25 ,,	92° C	4:20 ,,	87° C
10:35 ,,	82° C	9:28 ,,	95° C	4:25 ,,	92° C
10:40 ,,	86° C	9:30 ,,	95° C	4:30 ,,	87° C
10:45 ,,	88° C	9:37 ,,	Boiling	4:40 ,,	88° C
10:55 ,,	91° C	9:43 ,,	,,	4:50 ,,	95° C
11:05 ,,	89° C	9:50 ,,	,,	5: ,,	96° C
11:10 ,,	Boiling	9:55 ,,	,,	5:15 ,,	96° C
11:15 ,,	,,	10: ,,	,,	5:25 ,,	92° C
11:20 ,,	,,	10:05,,	,,	5:27 ,,	Boiling
11:25 ,,	,,	10:10,,	,,	5:30 ,,	,,
11:30 ,,	,,	,,		5:35 ,,	88° C
11:35 ,,	,,				
11:40 ,,	,,				
11:45 ,,	,,				

Table 18

The solutions of ricin that were treated by boiling were injected into rabbits and guinea pigs. The animals were injected with fatal doses of the intreated ricin several days after a sample of blood was obtained.

Animal	Animal No.	Weight grams.	Solution injected No.	Ricin heated mgs.	No. of injections	Days from first to last injection.	Days till obtained bkood.	Untreated ricin mgm.	Effect
Rabbit	214	1550	I	300	5	28	15	0.05	lived
Rabbit	215	2050	III	300	5	34	14	0.05	,,
Rabbit	215°	1950	IV	330	4	28	14		
Rabbit	217+	2000	V	330	4	27	13		
Rabbit	218	2800	VI	500	4	18		0.15	lived
Guinea Pig	10	950	II	300	5	28	15	0,04	,,
,,	20	700	VII	365	4	21		0.05	Died 37 hrs
,,	26	700	VIII	330	5	37		0.08	Lived
,,	27	800	VIII	330	5	37		0,04	,,

Foot notes to Table 18.

o This rabbit developed slobers and she was killed ~~to~~ in order keep from infecting the colony, so I was unable to inject her with untreated ricin to see if she had any immunity to ricin. She was in the same cage with rabbit No.217 at the same time that she developed slobbers.

+ This rabbit developed slobbers 7 days after the last injection and she was in a state of coma on the 13th day when I took a sample of blood and killed her.

I was unable to procure mice at the time rabbit No. 218 and guinea pigs Nos. 20, 26 and 27 were treated but I injected them with the untreated ricin and all, with the exception of guinea pig No. 20, showed immunity to the ricin.

The sera from the animals in table 18 were mixed with ricin and injected into white mice. These results are included in table 19.

Insert Table 19

The sera from animals injected with solutions Nos. I, II and IV clearly showed properties of antiricin as these sera when mixed with a lethal dose of ricin did not cause an ulcer nor were they fatal. The sera from animals injected with solutions Nos. III and V, however, did not possess as strong properties of antiricin as the others. Nevertheless there seemed to be some evidence of immunity being developed in these animals as mice of the same age and weight would have died much sooner if they had been injected with the same quantity of ricin and the same amount of normal rabbit sera.

That the toxic group was destroyed by heating is evident as the first dose injected into each animal was large enough to have killed 300 animals of their age and weight if it had not been treated in some way to destroy its toxicity. Since the first dose given would have killed more than 300 animals had it not been treated, the toxicity of the ricin after the treatment was less than 1/300 of that of the original toxin.

Although this part of the investigation has been carried out only on a few animals, I feel that it is worthy of consideration and further work is projected.

Table 19

This table shows the effects on white mice of ricin which has beenmixed with the sera from the animals which were injected with the heated solutions of ricin.

Serum from		Mouse No.	Weight grams	Amount of serum used C.c.	Amount of Ricin added to the serum mg.	Local reaction	Effect
Rabbit-	214	732	26	0.5	0.005	ulcer	Lived
,,	,,	733	25.5	0.75	0.005	,,	,,
Rabbit	215	764	22.5	0.5	0.005		Died 57-68 hrs
,,	,,	764	22	0.75	0.005		,, 57-68 ,,
Rabbit	216	870	20.5	0.5	0.005	No ulcer	Lived
,,	,,	871	22.5	0.5	0.005	,, ,,	,,
,,	,,	872	23.5	0.75	0.005	,, ,,	,,
,,	,,	873	22	0.75	0.005	,, ,,	,,
Rabbit	217	846	21	0.5	0.005		Died 96 hrs
,,	,,	847	21	0.75	0.005		,, 73 ,,
,,	,,	848	21	0.75	0.005		,, 59.5-71,,
Guinea	10	885	20	0.5	0.005	No ulcer	lived
pig	,,	886	20	0.5	0.005	,, ,,	,,
,,	,,	887	20	0.75	0.005	,, ,,	,,
,,	,,	888	20	0.75	0.005	,, ,,	,,

C. The Production of Immunity in Animals with Ricin which had been oxidized by means of Potassium Permanganate.

Potassium permanganate was found to be very effective in destroying the toxic principle of an aqueous or physiologic saline solution of ricin as can be seen on page 19. Since this oxidizing agent was so efficient in detoxifying ricin it was decided to inject the resulting solutions and see if an immunity to the toxin could be developed.

The first experiments were conducted on the hypothesis that it might be possible to oxidize the toxic principle of ricin and at the same time leave the antigenic principle unaffected. With the above thought in mind the principal variable noted in the experiments on the animals in table 20 was to completely detoxify the solutions so that the animals would not die as the size of the injections was increased. That is, the solutions of the toxin, after treatment with the $KMnO_4$, were injected into rabbits in in increasing doses and after a period of a few days, a sample of blood was taken and tested for the presence of antiricin. This was done by mixing the sera from these rabbits with lethal doses of ricin (for white mice) and injecting the resulting solutions into white mice.

Since the first hypothesis was that the antigenic properties would not be affected by the oxidizing agent, the first experiments were performed with solutions which

were treated with excess $KMnO_4$ over and above that necessary to detoxify them. These experiments, however, showed that there was a difference in the toxic and antigenic parts or groups of the toxin. The above concept was all right in part but I carried it too far to obtain a very high state of immunity in my first series of animals which are included in table 20.

The toxin was dissolved in isotonic saline solution and treated with an aqueous solution of $KMnO_4$ until a pink color remained about 30 seconds to one minute after an addition of the oxidizing agent. As explained previously the total amount of the oxidizing agent was not measured in the first series of experiments but care was exercised to see that the oxidation of the toxin was completed so that the animals would not be killed during the immunization process. Twentieth normal $KMnO_4$ was used for detoxifying the toxin for the first injections on each animal and then normal solutions were employed for the concentrated solutions of ricin. A precipitate of manganese dioxide or the tetravalent compound $Mn(OH)_4$ was formed and it was copius if both of the solutions were concentrated. The precipitate was of a flocculent

$$2KMnO_4 + 5H_2O = 2Mn(OH)_4 + 2KOH + 3O$$

nature and it remained in suspension for sometime after an agitation which made it possible to inject the solution and suspension without much difficulty. The additions of the oxidizing agent were made at intervals of five to thirty minutes. The solutions of ricin were made up fresh for each treatment and the number of injections ranged from three to five. The following protocol shows the process of immunization employed.

Protocol

Rabbit No. 201 weight 3200 grams.

		Ricin mgm.	
Nov.	22	5	+ $KMnO_4$ until permanent pink for 30-60 seconds
,,	27	15	,, ,, ,, ,, ,, ,, ,,
Dec.	2	40	,, ,, ,, ,, ,, ,, ,,
,,	10	90	,, ,, ,, ,, ,, ,, ,,
,,	20	190	,, ,, ,, ,, ,, ,, ,,

Table No. 20 gives the results obtained by injecting rabbits with solutions of ricin which have been treated with $KMnO_4$ as described above. That is, the animals in this table have been injected with ricin that was partially oxidized.

Insert Table 20

Samples of blood were taken from the above rabbits and the sera were mixed with lethal doses of ricin (for adult white mice) and the solutions were injected into white mice. The results are included in table 21.

Insert Table 21

The sera from rabbits109, 112, 113 and 201 protected mice against lethal doses of ricin as can be seen by inspecting table 21, which demonstrates that an immunity to ricin has been developed by means of the partially oxidized toxin.

Table 20

These rabbits were injected with solutions of ricin which were partially oxidized by means of potassium permanganate.

Rabbit No.	Weight Grams	Amount of ricin mgs.	Number of injections	Days from first to last injection.	Days from last injection until blood was taken.
109	2000	160	5	26	4
112	2300	140	5	33	7
113	2600	140	5	32	9
114	2600	105	3	20	24
115	2700	105	3	19	24
201	3200	340	5	28	17

Table 21

Sera from rabbits in table 20 were mixed with lethal doses of ricin (for white mice) and the solutions were injected into white mice.

Serum from Rabbit No.	Mouse No.	Weight Grams	Amount of Serum used c.c.	Amount of ricin milligrams	Effect
109	130	22	0.5	0.01	Lived - no ulcer
,,	131	22	0.5	0.005	,, ,, ,,
,,	132	22	0.5	0.005	,, ,, ,,
,,	133	21	0.5	0.005	,, ,, ,,
,,	134	21	0.5	0.005	,, ,, ,,
112	228	21	0.5	0.005	,, Ulcer
,,	229	22	0.5	0.005	,, ,,
113	305	24	0.5	0.01	Died 6 days
,,	306	23	0.5	0.005	Lived no ulcer
,,	307	23	0.5	0.005	,, ,,
114	382	23	1.0	0.005	Lived - ulcer
,,	383	23	0.5	0.005	Died 5 days
,,	384	23	0.5	0.005	,, 52-63 hrs.
115	387	23	0.5	0.005	,, 48 ,,
,,	388	22	0.5	0.005	,, 70 ,,
,,	390	23	1.0	0.005	,, 76 ,,
201	625	22	0.25	0.005	Lived - ulcer
,,	626	23	0.5	0.005	,, ,,

Since some of the rabbits in table 20, which had been injected with partially oxidized ricin, developed an immunity to ricinm I decided to experiment to see if the toxin could be completely deprived of its antigenic function. The toxin was dissolved in water and an aqueous solution of normal $KMnO_4$ was added a few drops at a time over a period of 13-24 hours. + The toxin was almost completely oxidized or at least it had desolorized practically all the oxidizing agent that it would under these conditions. The solutions were treated with enough of the oxidizing agent to make them a deep pink color just previous to their injection in each case. This was done to insure further oxidation if it were possible under the conditions that the toxin would be subjected to in vivo. The dises of the oxicized toxin were increased in size as the process of immunization continnued. Five injections were made in each animal. The results obtained by injecting rabbits with ricin which had been treated with $KMnO_4$ until it was completely oxidized or nearly so, are included in table 22.

Insert Table 22

Rabbits 229 and 230 were each injected with 0.05 milligram of ricin thirty six days after the last injection of

---

+ 0.3 gram of ricin decolorized 270 milligrams of potassium permanganate in 24 hours treatment when 2 or 3 drops of a N/2 solution were added at a time.

Table 22

These rabbits were injected with ricin that had been treated with potassium permanganate for several hours and until the solutions would remain pink for least 30 minutes after an addition of the oxidizing agent.

Rabbit No	Weight Grams	Amount of ricin injected mgs.	Time treated hours	No of injections	Days from first to last injection.	Days from last injection until blood was taken
204	2600	350	13-24	5	26	14
206	1500	400	,,	5	28	18
207	1700	400	,,	5	28	18
208	1500	340	,,	5	35	17
209	1575	340	,,	5	35	17
229	2150	300	,,	5	32	28
230	2225	300	,,	5	32	28
232*	2400	260	,,	5	33	18

* This rabbit was bred before giving her the last injection of the series. She gave birth to young but died on the following day.

the oxidized toxin and both animals died between 47 and 69 hours after the injection. That is, these rabbits had no protection against the toxin.

Blood was obtained from each rabbit in table 22 and the sera were tested for the presence of antiricin by the method used in table 21. The results are included in table 23.

Insert Table 23

By inspecting the data in table 23, it is evident that the sera obtained from the rabbits in table 22 failed to protect mice from lethal doses of ricin when the sera were used in amounts from 0.75 to 1 c.c. From the data in the above two tables, it appears that the toxin was oxidized so that it no longer had any antigenic properties.

From the data in tables 20 and 21 it is evident that it is possible to develop an immunity to ricin by means of several injections of the toxin that has been partially oxidized by $KMnO_4$. That is, if the injections were increased in size as the process was continued and if as much as 0.3 gram of the toxin was used. Since there was the possibility of oxidizing the toxin so much that it would not produce an immunity it became necessary to see exactly how small an amount of the $KMnO_4$ would be required to completely detoxify a given quantity of ricin. Also the amount of ricin necessary to produce an immunity had not been definitely determined.

Keeping the above two methods of treatment of ricin in mind, I decided to alter the process of immunization so that it would not take so long to get results. The

Table 23.

The sera from rabbits in table were mixed with ricin and injected into white mice.

Serum from rabbit No.	Mouse No.	Weight Grams	Amount of serum used. c.c.	Amount of ricin used. Milligram	Effect
204	676	23	0.5	0.005	Died 49 - 58 hrs.
"	689	20	0.25	"	" 52 - 55 "
"	690	20	0.5	"	" 47 "
"	691	22	0.75	"	" 72 "
206	705	22	0.5	"	" 90 "
"	706	20	0.5	"	" 44 "
"	735	23	1.0	"	" 80 - 90 "
"	746	22.5	1.0	"	" 45 "
"	747	24	1.0	"	" 77 - 89 "
207	707	20	0.5	"	" 48 "
"	708	20	0.5	"	" 27 - 36 "
208	737	22	0.5	"	" 52 - 54 "
"	738	22	1.0	"	" 113 - 115 "
"	750	23	1.0	"	" 53 - 63 "
"	751	22.5	1.0	"	" 64 "
209	739	22	0.5	"	" 63 - 67 "
"	752	22	1.0	"	" 114 - 120 "
229	1142	21	0.5	"	" 76 "
"	1143	20.5	0.75	"	" 77 "
230	1145	23.5	0.5	"	" 108 "
"	1146	23	0.75	"	" 55 - 64 "
"	1147	26	0.75	"	" 93 "
232	1148	23.5	0.5	"	" 55 - 64 "
"	1149	23	0.75	"	" 90 "
"	1150	23	0.75	"	" 72 - 75 "

possibility of producing an immunity to ricin by the method of one injection of a solution of the treated toxin presented itself.

The next experiments deal with the production of immunity to ricin by the method of one injection of the oxidized toxin. The weights of both the toxin and the $KMnO_4$ that were used were noted. The ricin was dissolved in 5 - 7 c.c. of distilled water and an aqueous solution of $KMnO_4$, 1 c.c. = 35 mgm. of the dry salt, was added. The oxidizing agent was added a drop at a time and the solution was agitate after each addition.

The first six rabbits that were injected in these experiments with the exception of one were given solutions that were treated less than fifteen minutes. Later on the solutions were treated very slowly and the process of treatment was continued several hours. The results obtained from single injections of the toxin which had been oxidized by $KMnO_4$ are recorded in table 24.

Insert Table 24

The sera obtained from the rabbits in table 24 were for the presence of antiricin by the method used in tables 21 and 23. The results are included in table 25.

Insert Table 25

Table 24

Rabbit No.	Weight grams	Dose Ricin mgs	$KMnO_4$ mgs	Time treated	Days till blood was obtained	Days till doseuntreated ricin was injected	Dose ricin mgs	Dose per kilo	Effect
231	2325 2650	300	100	11 min	14	32	0.05	0.021	Lived
234	2650	300	300	2 hrs-15mi.	14				Died +
242	2400	280	280	1 min	13	28	0.08	0.033	,, 24-46hrs
248	1600	330	160	13 min	14	26	0.05	0.031	Lived
249	1750	330	125	8 min	14	26	0.05	0.028	,,
250	2450	330	105	9 min	14				Died e
256	1800	300	60	2 hrs	15	18	0.05	0.027	Lived
257	1600	300	70	15 mih					Died 20-24hrs
271	2600	300	105	5 hrs	10	10	0.25	0.096	Lived
273	2900	300	70	5 hrs	10	12	2.50	0.80	,, swelling
275	2300	300	70	40 min	10	11	2.00	0.86	,, ,,
276	2600	300	35	2 hrs	10	10	2.50	0.96	,, ,,
277	2100	300	50	2 hrs	10	11	2.00	0.95	,, ,,
281	2300	300	70	6 hrs	10	12	2.00	0.86	,, ,,
282	1800	300	50	2 hrs		19	2.50	1.38	,, ,, ulcer
283	2000	300	35	2 hrs		21	5.00	2.50	,, ,, ,,
284	2000	300	35	6 hrs		43	6.00	3.00	,, ,, ,,

Table 25

Sera from rabbits in table 24 were mixed with ricin and the resulting solutions were injected into white mice.

Serum rabbit no.231	Mouse No.	Weight	Amount of serum used c.c.	Amount of ricin used. milligrams	Local reaction	Effect		
231	935	21.5	0.5	0.005	No ulcer	lived		
,,	936	20	0.5	0.005	,, ,,	,,		
,,	937	23	0.5	0.005	,, ,,	,,		
234	1019	20	0.25	0.005		Died	57	hrs
,,	1020	20	0.25	0.005		,,	36-48	,,
,,	1021	21	0.50	0.005		,,	75	,,
,,	1022	20	0.50	0.005		,,	79-84	,,
242	1203	20	0.5	0.005		,,	55	
,,	1204	21	0.5	0.005		,,	31-43	,,
,,	1208	23	0.75	0.005		,,	55	,,
,,	1209	26	0.75	0.005		,,	72	,,
248	1268	23	0.5	0.005		,,	75-83	,,
,,	1269	22	0.5	0.005		,,	75-83	,,
,,	1270	21	0.75	0.005		,,	75-83	,,
249	1273	21	0.25	0.005		,,	98-108	,,
,,	1274	22	0.5	0.005	ulcer	Lived		
,,	1275	22	0.5	0.005	,,	,,		
250	1280	24.5	0.5	0.005		Died	98-108	,,
,,	1281	22.5	0.5	0.005	,,	Lived		
,,	1282	22.5	0.75	0.005	,,	,,		
256	1329	22	0.25	0.005	No ulcer	,,		
,,	1330	22	0.25	0.005	,,	,,		
,,	1331	22	0.5	0.005	,,	,,		
271	1521	22	0.5	0.005	ulcer	,,		
,,	1522	21	0.5	0.005	,,	,,		
273	1533	22	0.25	0.005	,,	,,		
,,	1534	22	0.25	0.005	,,	,,		
,,	1535	21	0.5	0.005	no ulcer	,,		
275	1536	22	0.25	0.005	ulcer	,,		
,,	1537	21	0.5	0.005	No ulcer	,,		
276	1542	22	0.25	0.005	No ulcer	,,		
,,	1543	22	0.25	0.005	No ulcer	,,		

By inspecting table 24, it can be seen that it was possible to detoxify ricin with about one tenth of its weight of $KMnO_4$. By varying the amount of $KMnO_4$ used for a certain amount of ricin it was possible to approach the minimum amount of the oxidizing agent necessary to just completely detoxify the toxin. That is , when a solution of the toxin was treated slowly with a dilute solution of $KMnO_4$, the toxicity was destroyed but the power to produce an immunity was retained

The time that each solution was treated was very probably a large factor in the destruction of the toxic properties and also in the destruction of the principle or parts of the protein responsible for the production of immunity. If the solutions of ricin had been treated over a longer period with less concentrated solutions of $KMnO_4$, the minimum amount of the oxidizing agent might have been much smaller than I have used.

The injections of the oxidized toxin coused swelling around the seat of the injection and in the cases where the smaller amount of the $KMnO_4$ were used, there was usually an ulcer which required from about one to three weeks to heal.

Since rabbits 271 to 284 inclusive in table 24 were given several lethal doses of ricin after an interval of 10 to 12 days after the injection of the oxidized ricin and since all of them lived, I feel that I have an entirely new method of producing immunity to foreign proteins. Also this method of immunization gives an antiserum which is able in very small quantities to protect mice against lethal doses of the toxin.

VIII Summary

Ricin was prepared in a very pure state and it possessed the two distinct characteristic properties or functions which have been assigned to ricin by Field (21) the agglutinating function and the toxic function. That is, red blood cells which had been washed several times with physiologic saline were agglutinated by ricin and also the ricin was found to be very toxic for laboratory animals when a dilute solution of it was injected subcutaneously.

Ricin was detoxified by various means with the hope of finding one method which could be used to produce an immunity to the toxin. The following oxidizing agents detoxified ricin.

(a) Potassium permanganate

(b) Hydrogen Peroxide (superoxol)

(c) Ozone

(d) Chlorine

(e) Bromine

(f) Iodine

(2) Ricin was detoxified by congo red.

(3) Ricin was detoxified by ultra-violet light.

That is, ricin was treated by the above chemical and physical agents and the resulting solutions were injected into animals. All of the methods of treatment that are listed above detoxified the ricin and this detoxifying powervaried according to the particular agent used, congo red

being able to prevent death when a small quantity was mixed with a lethal dose of the toxin. Potassium permanganate was so active that it rendered quantities of the ricin which were equivalent to more than 5,000 fatal doses so that it would not kill the animal when injected subcutaneously.

In order to show that my product of toxin was similar in another property to that obtained by previous workers I injected increasing doses of the untreated toxin into mice and one guinea pig and found that an antiserum was formed which protected mice and young rats against fatal doses of the toxin.

Although ricin had been detoxified previously by other investigators by boiling, they never attempted to produce an immunity to the toxin by injecting the resulting solutions. I found that a definite antigenic function remained after the boiling and an antiserum was produced. The animals also lived after they were injected with several fatal doses of untreated ricin.

Since the toxic properties of ricin were so readily destroyed by means of potassium permanganate and-se also since there were no particular complications resulting from subcutaneous injections of the treated solutions, I decided to thoroughly test the possibility of producing an immunity to the toxin by means of increasing doses of such solutions. My first hypothesis was to treat the solution of the toxin with an excess of the oxidizing agent over and above that necessary to destroy the toxic properties.

This was accomplished but I also found that the antigenic function was affected by this treatment. These experiments were followed by others in which I demonstrated that the antigenic function could be completely or nearly destroyed by this agent.

With the first experiments in mind in which I obtained an antiserum from increasing injections of the toxin which was oxidized with potassium permanganate I decided to try the effect of injecting a single large dose of a solution which had been oxidized by this method. A definite weight of the toxin was treated with different amounts of the permanganate . The solutions of the toxin were treated very gradually and the length of the treatment varied from a few minutes to several hours. It was found that antiserum was formed in the animals that received injections of 0.3 g. of the toxin which were treated with 100 milligrams of the permanganates and that if only 35-70 milligrams of the oxidizing agent were used that the animal had protection against ricin in amounts equivalent to 100-120 lethal doses.

I believe that I am submitting records of experimental results which offer considerable evidence in support of the view that there are two biological properties or function of ricin of major importance, a toxic property or function and an antigenic property or function.

The antibidies or protective principles engendered upon injecting the solutions of ricin treated by means of the smaller amounts of potassium permanganate were highly specific for untreated ricin.

IX CONCLUSIONS

1. Ricin has been detoxified by various oxidizing agents, such as ozone, 30% hydrogen peroxide, potassium permanganate, chlorine, bromine and iodine, by the dye, congo red, and by ultra-violet.
2. Ricin which was detoxified by boiling retained part of its antigenic properties as shown by the fact that when injected into rabbits and guinea pigs that their sera served to protect white mice from lethal doses of the toxin.
3. I found that it was possible to oxidize solutions of ricin with potassium permanganate so that the treated toxin served as an antigen with the production of an antiserum. That is, the antigenic properties of ricin were found to be less easily oxidized than the toxic properties.
4. I was able by long treatment of the toxin with excess of the permanganate to oxidize ricin so that it lost its antigenic function or at least it was greatly reduced in this activity.
5. Rabbits which received one large injection of ricin which was partially oxidized by means of potassium permanganate developed an immunity to ricin. They were not killed when as many as 100-120 fatal doses of the toxin were injected 2 or 3 weeks after they were given oxidized toxin. Sera from these rabbits in quantities as small as 0.25 c.c. readily protected mice against lethal doses of the toxin.

That is the antibodies or protective pronciples engendered were highly specific for ricin.

6: I believe that I have offered considerable experimental evidence in support of the view that ricin, a protein , has two separate and distince functions which possibly are due to two separate groups, a toxic function and an antigenic function.

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X. BIBLIOGRAPHY.

General Works

1. Ehrlich, P., Studies in Immunity.
2. Mann. Chemistry of Proteins.
3. Mathews, A. P., Physiological Chemistry. 4th Edition.
4. Osborne, T.B. The Vegetable Proteins. Monograph on Biochemistry. 1909, 1924 London.
5. Pauli, W. Colloid Chemistry of the Proteins. 1922.
6. Robertson, T.B., Principles of Biochemistry. 2nd Edition.
7. Schryver, S. B. The General Characters of the Proteins. Monograph on Biochemistry, 1909, London.

References

8. Briegger, L., Chem. Centr. 1904, 1, 1286.
9. Briux and Guerbert., Compt. rend. acad. agr. France. 6, 449. 1920.
10. Calmette and Delarde., Ann. Inst. Pasteur. 1896, 10, 675.
11. Cushny, A. R. Arch. f. Exp. Path. u. Pharmakol., 1898. 41, 439.
12. Dixon, T., Australian Med. Gazette. 1887, 6, 155.
13. Dreyer and Hausen., Compt. rend. 1907, 145, 234.
14. Ehrlich, P., Deutsche Med. Woch. 1891, 17, 976.
15. Field, C. J. Exp. Med. 12, 551.
16. Flexner, S., J. Exp. Med. ii, 197, 1897.
17. Fraenkel, A. Beitr. Chem. Physiol. Path. 1903, 4, 224.
18. Gunn, J. A., Proc. Physiol. Soc. J. Physiol. 1921, 54, LXXXVIII.
19. Jacoby, M. Arch, f, Exp. Path. u. Pharm. 1901, 46, 28.
20. Karrer, Smirnoff, Ehrensperger, Van Slooten and Keller., Z. Physiol. Chem. 1924, 135, 129.

21.	Kobert, R.	Ibid Vol. 8, 3080.
22.	Lau	Dissertation, Rostock. 1901
23.	Liebermann, L.V.	Arch. Hyg. 1907, 62, 276.
24.	di Macco, G.	Z. Immunitats. 1923, 38, 467.
25.	Mathews, A. P.	Am. J. Physiol. 1898, 1, 445.
26.	Michaelis and Steindorff.,	Biochem. Zeitsch. 1906, 41, 108.
27.	Müller	Arch. f. Exp. Path. u. Pharm. 1899, 42, 302.
28.	Olmer and Sauvan.	Comp. rend. Soc. Biol. 68, 638.
29.	Oppenheimer, C.	Toxine und Antitoxine 1904.
30.	Osborne, Mendel and Harris.	Am. J. Physiol . 1905, 14, 259
31.	Pfeiller and Engelhardt.	Exp. Sta. Records, 45, 413.
32.	Rehns.	Compt. rend Soc. Biol. 1902,54,89 and 212.
33.	Reid, G.	Am. Chem. Soc. Abst. 1914, 177.
34.	Rittenhausen, R.	J. Pr. Chem. (2) 25, 130.
35.	Rona and Gyorgy.	Biochem. Z. 1920, 105, 120.
36.	Schneider, H. and Sperti, G.	University of Cincinnati Institute Of Industrial Research, Series 4, paper.
37.	Stepanoff.	Ann. Inst. Pasteur, 1896, 10, 663
38.	Stillmark. H.	Chem. Centr. 1889, 11, 978.
39.	Vines, S. H.,	J. Physiol. 3, 93.
40.	Warden and Waddell.,	Calcutta, Bengal Secretarial Press 1884, 76.
41.	Lenard	Ann. der Physick 1900,1, 486