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MATERIALS AND METHODS

A. Materials

These studies were performed with samples of crude botulinum toxin and crystalline Type A toxin which were received from the U. S. Army Chemical Research and Development Laboratories. The crude toxin was received as a dry powder while the crystalline toxin was supplied as a suspension in 0.9 M ammonium sulfate. The antitoxins, monovalent Type A and monovalent Type B and bivalent A and B, which were used in the agglutination studies, were obtained from Lederle Laboratories.

Assays of toxin potency were made by intraperitoneal injection into Swiss-Webster mice (18-22 grams). Mice were also utilized for the demonstration of toxin in body fluids and organs. Sprague-Dawley albino rats were used in the tissue distribution studies.

B. Methods

1. Tissue Distribution Studies

The purpose of these studies was to determine the location of toxin in body tissues at various time intervals after administration. Very large doses of toxin, ranging from 1650 to several million mouse LD₅₀ units, were administered to albino rats by oral, intravenous, or intraperitoneal routes. The rats were sacrificed at times ranging from 30 minutes to four hours after administration of toxin; mouse assays were done on various tissues to determine the presence of toxin.

In one series of these experiments, efforts were made to study blood levels at various times and also to determine the location of the toxin among the various components of the blood. Rats were given 1650 or 50,000 units of toxin intravenously and sacrificed after from 15 minutes to four hours. Blood was drawn from the abdominal aorta; heparin was added to one sample and the other was allowed to clot. In each case, serum or plasma was separated from the cells and assayed in mice. In some instances, the red blood cells were resuspended in saline and assayed. At other times, the cells were washed three times with saline prior to assay. In one study, the washed red blood cells were hemolyzed in distilled water before injection into mice. All materials to be assayed for toxin were injected intraperitoneally into mice in a volume of 0.25 ml.

In another series of studies, tissue distribution of toxin was studied following oral or intraperitoneal administration to rats. A 1.0 ml. suspension of crude toxin, containing several million mouse LD₅₀ units, was administered to each rat. The rats were sacrificed at various intervals as before; serum, dilutions of serum, and kidney homogenates were assayed for toxin. The kidneys were thoroughly washed in saline and homogenized in 0.3 M PO₄ buffer. In some instances, the gastrointestinal contents were also assayed for toxin. Portions of the tract were isolated between ligatures, were removed from the rat and were then flushed with saline after removal of the ligatures. The material washed from the gastrointestinal tract was centrifuged, 0.25 ml. aliquots of the supernatant being injected into mice. The numbers of deaths occurring within 24, 48 and 72 hours were noted.

2. Recovery of Toxin from Tissue

Very large doses (approximately 1×10^{10} mouse LD₅₀ units) of Type A crystalline toxin were administered intravenously to rats. Following these large doses the rats died after three to five hours. After death, skeletal muscle was collected from various parts of the body and was prepared for column chromatography.

The first step of this preparation was homogenization of the muscle tissue in a 0.1 M PO₄ buffer of pH 7.3 in a Waring Blender. Homogenization was carried on for a half-hour, with rest periods to insure that the mixture was kept cold by immersion in an ice bath. A weighed amount of muscle was homogenized, at the close of the procedure being diluted to a 10% (w/v) mixture. After homogenization, the mixture was hand milled in a Potter-Elvehjem homogenizer.

The homogenate was filtered through cheese cloth and spun at 9000 x g in a refrigerated centrifuge. The supernatant was transferred to other tubes and spun at 145,000 x g for one hour in the cold. The second supernatant was frozen and lyophilized. The resulting dry powder was stored in a deep freeze at -20°C until used.

Approximately one gram of the dry powder was dissolved in 10 cc of 0.1 M PO₄ buffer, pH 7.4, and was dialyzed against this buffer for 24 hours, with two changes of the dialyzing medium. During dialysis the volume increased to approximately 15 cc, but only 10 cc was placed on a column of DEAE-Sephadex A-50 (coarse). The eluant consisted of a three step gradient. The top

(0.1 M PO_4 buffer, pH 4.50, plus 10% NaCl) slowly dropped into the middle solution (0.1 M PO_4 , pH 4.50) which in turn dropped into the bottom buffer (0.1 M PC_4 , pH 7.40). The eluant was monitored on a spectrophotometer set at 280 m μ .

To locate the position of the botulinum toxin as it comes off the column, crystalline toxin alone was placed on the column and found peaked at the 350 ml. volume.

Mouse assays were made of the eluant at the 350 ml. volume of the following materials:

1. the dry powdered muscle extract
2. botulinum toxin alone
3. homogenized normal muscle to which toxin was added after homogenization
4. homogenized normal muscle tissue

Using the above procedures, lung tissue from rats that had succumbed to large intravenous doses of toxin was similarly homogenized, lyophilized and subjected to column chromatography. To eliminate the residual blood, muscle tissue from a rat that had died was thoroughly perfused with saline prior to study by the procedures outlined above. In both cases, mouse assays for toxin were done on the fractions eluted at 350 ml.

3. Toxin Characterization by Density Gradient Centrifugation

Crystalline Type A toxin was suspended in ammonium sulfate at a concentration of 20 mg/ml. This material was dialyzed against 0.1 M PO_4 to remove the ammonium sulfate and was then centrifuged. The final concentration was estimated to be 10 mg/ml, since on dialysis there was an increase in volume and a failure of some of the toxin to dissolve.

The density gradient, prepared with a device made by Buchler Instruments, Incorporated, consisted of sucrose dissolved in 0.1 M phosphate buffer, pH 7.3, in a concentration of 40% (w/v) at the bottom of the tube and 5% at the top of the tube. Various concentrations of crystalline toxin, dissolved in 3% sucrose, were layered on top of each tube. The tubes were placed in a 25.2 Spinco swinging bucket rotor and spun at approximately 100,000 x g for 21 hours. After centrifugation, the tubes were examined in a dark room with a point source of light.

After the visual examination, the tubes were punctured at the bottom, a series of samples (numbered consecutively from 1 to 25) of 2 ml. each being collected. Before entering the fraction collector, the contents was monitored by a spectrophotometer set at 280 m μ .

The series of 25 samples from the tube with the lowest concentration, approximately 0.1 mg/ml, was assayed in mice. Prior to injection, each sample was dialyzed against saline to remove the sucrose and potassium of the buffer. The mice were observed for 48 hours.

Muscle tissue extract, from a rat that had died following an injection of crystalline toxin, was similarly centrifuged through a sucrose density gradient. About 1 gram of the lyophilized powder was used. After centrifugation, 27 samples, of 2 ml. each, were collected from the bottom of the tube. Each of the samples was dialyzed against saline and assayed in mice.

4. Agglutination Studies

In the development of an agglutination procedure, studies were made of the reaction between latex particles, which had been sensitized with toxin, and various antitoxins.

Latex particles, suspended in saline and diluted to give an optical density of 0.300, were sensitized by adding dialyzed crystalline Type A toxin to the particle suspension and allowing the mixture to stand for 10 minutes at room temperature. These sensitized particles were then added to serial dilutions of the antitoxin to be tested.

In one set of experiments, latex particles, which had been sensitized with two different lots of toxin (designated as Lot 2 and Lot 3), were tested against normal horse serum, diphtheria antitoxin, tetanus antitoxin, monovalent A and B botulinum antitoxins, and bivalent A and B antitoxin.

While analyzing the results of these experiments, the possibility that the reaction was more likely a precipitin one rather than an agglutination was considered. To answer this question, the following two experiments were performed. In the first of these, serial dilutions of specific antitoxin were reacted with suspensions of toxin in saline without latex particles.

In the second, sensitized latex particles were centrifuged and the supernatant was saved. The sediment was resuspended in saline and centrifuged again; the second supernatant also was saved. The washed particles were resuspended in saline and used in the test. The two supernatants and the particles were added to serial dilutions of antitoxins.

RESULTS AND DISCUSSION

1. Tissue Distribution Studies

A summary of the results obtained in eight of the tissue distribution studies is given in the addenda. The toxin was given intravenously in studies 1 - 3, by stomach tube in studies 4 - 7, and by intraperitoneal injection in study 8.

Following intravenous administration of toxin to rats, undiluted plasma and serum remained lethal to mice for up to four hours after administration. Assay of serial dilutions of the fluid portion of the blood, however, suggested that some decrease in blood level had occurred by four hours. Clotting apparently did not remove any significant amount of toxin from the blood since serum appeared to be just as lethal to mice as plasma.

In study No. 1, red blood cells that had been separated from the plasma by centrifugation and resuspended in saline, were lethal to mice. In studies 2 and 3, red blood cells that had been washed in saline prior to injection, did not produce death in mice, but the saline used to wash the cells did kill mice. Injections of hemolyzed red blood cells, that had been washed in saline, did not produce any symptoms or deaths in mice. These results indicated that the toxin did not penetrate the blood cells, but was adsorbed on their surfaces and could be removed by washing with saline. The toxin was therefore carried both in the fluid portion of the blood and on the surfaces of the red cells.

When 1.0 ml. doses of crude toxin suspension, containing approximately 100 mg of toxin per ml., were given to rats by stomach tube, there was apparently very little absorption from the gastrointestinal tract. In three of these studies (Nos. 4, 5, 6), undiluted serum, obtained from rats sacrificed at from 30 minutes to four hours after administration of the toxin, did not produce death in mice. Occasionally, symptoms of intoxication were seen in the mice, indicating that some absorption had taken place. In study No. 7 though, serum obtained 30 minutes and one hour after the toxin had been given was lethal to mice. This discrepancy apparently stems from the difficulty in obtaining uniform samples of toxin from the stock suspensions. This difficulty also resulted in wide variations in determinations of the LD₅₀ of the toxin for the mouse.

In spite of the apparent poor absorption of toxin into the blood stream following oral administration, there appeared to be rapid accumulation of toxin in the kidneys. For example, in study No. 6 serum obtained at 30 minutes was negative in the mouse assay and yet kidney homogenates killed 4/4 mice injected. In study No. 7, sera obtained at two and four hours were both negative but homogenates of the kidneys from the same animals killed 4/4 mice in both instances.

Following intraperitoneal injection of toxin into rats, serum assays were positive within 30 minutes. The serum remained positive through the four hours, but an apparent decrease in serum level was observed in the four-hour sample. The gastrointestinal contents were lethal to mice within 30 minutes. Accumulation of toxin by the kidneys was indicated again since kidney homogenates killed 4/4 mice at all time intervals.

2. Recovery of Toxin from Tissue

The elution pattern of homogenized muscle tissue, monitored at 280 m μ , included at least eight peaks. The greater portion of the second peak was found to be myoglobin, but the other peaks were not identified.

The results of the mouse assays of the eluants of the various materials were as follows:

1. Powdered muscle extract from a rat that died following an intravenous injection of toxin (diluted 1:20) - - 2/3 mice died within 48 and 72 hours, respectively.
2. Botulinum toxin alone----3/3 mice died within 18 hours.
3. Normal muscle tissue to which toxin was added after homogenization---3/3 mice died within 18 hours.
4. Normal muscle tissue (entire homogenate) ---0/3 mice died.
5. Lung tissue from a rat that died following an injection of toxin---3/4 mice died.
6. Perfused muscle tissue from a rat that died ---6/6 mice died.

3. Toxin Characterization by Density Gradient Centrifugation

After centrifugation through the density gradient, the tubes were examined in a dark room with a point source of light. In the tubes containing the highest toxin concentrations, a thin band of turbidity was observed 3.5 to 4 cm from the bottoms of the tubes. In the tubes containing the lower toxin concentrations there was no discernable turbidity.

Among the samples collected from the tube with the highest concentration of toxin (approximately 1.0 mg/ml), there was increased optical density beginning with sample 11 and reaching a maximum at sample 15. The samples with the increased optical density correspond to the region of the tube in which the turbidity was observed.

Mouse assay of the 25 samples from the tube with the lowest concentration of toxin (approximately 0.1 mg/ml) gave the following results:

Sample #	1	2	3	4	5	6	7	8	9
Mouse Deaths	0/2	0/2	0/2	0/2	0/2	1/2	0/2	0/2	0/2
Sample #	10	11	12	13	14	15	16	17	18
Mouse Deaths	0/2	2/2	1/2	2/2	1/2	1/2	1/2	1/2	2/2
Sample #	19	20	21	22	23	24	25		
Mouse Deaths	0/2	2/2	1/2	1/2	2/2	1/2	2/2		

Mouse assay of the 27 samples from the tube containing the muscle extract gave these results:

Sample #	1	2	3	4	5	6	7	8	9	10
Mouse Deaths	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Sample #	11	12	13	14	15	16	17	18	19	20
Mouse Deaths	0/2	0/2	2/2	1/2	2/2	2/2	2/2	2/2	2/2	2/2
Sample #	21	22	23	24	25	26	27			
Mouse Deaths	2/2	1/2	2/2	2/2	1/2	2/2	2/2			

The results of these studies indicate that the toxin is not a homogenous substance. The material seen in the band 3.5 to 4 cm from the bottom of the tube with the highest concentration of toxin has a molecular weight of approximately 900,000. The material dispersed through the gradient above this band, which is detected by mouse assay, has molecular weights of a lower order.

Mouse assays of the samples from the tube containing pure crystalline toxin and assays of those from the tube containing the lyophilized muscle extract gave similar results. In both cases, the samples from the upper half of the density gradient had lethal activity in mice. Apparently, the lethal material extracted from muscle and pure crystalline toxin have the same distribution of molecular weights.

4. Agglutination Studies

Using latex particles sensitized with crystalline Type A toxin, positive reactions were observed with botulinum Type B antitoxin as well as with Type A antitoxin and bivalent A and B antitoxin, but not with normal horse serum, diphtheria antitoxin, or tetanus antitoxin. The results of these studies with two different lots of toxin were as follows:

Lot 2

Normal horse serum	No titer
Diphtheria antitoxin	No titer
Tetanus antitoxin	No titer
Botulinum Type A antitoxin	Three plus reaction at a dilution of 1:640*
Botulinum Type B antitoxin	Two plus reaction at a dilution of 1:320, negative at 1:640
Botulinum A and B antitoxin	Three plus reaction at a dilution of 1:320, negative at 1:640**

* This was the highest dilution of serum used in the test.

** This result is identical with other tests performed previously.

Lot 3

Normal horse serum	No titer
Diphtheria antitoxin	No titer
Tetanus antitoxin	No titer
Botulinum Type A antitoxin	Four plus reaction at a dilution of 1:640, negative at 1:1280
Botulinum Type B antitoxin	Four plus reaction at a dilution of 1:320, negative at 1:640
Botulinum A and B antitoxin	Three plus reaction at a dilution of 1:320, negative at 1:640

These tests indicated that there is a definite cross reactivity between Type A toxin and Type B antitoxin. This cross reactivity was also demonstrated with a precipitation reaction; precipitates were obtained in both Anti A and Anti B sera, using toxin from Lot 3.

When tests were done without latex particles, keeping the amount of toxin constant and adding serial dilutions of antitoxin, end point was reached at the same dilution as when sensitized particles were used. This suggested that the reaction was primarily one of precipitation rather than of agglutination. It had previously been assumed that agglutination had taken place because, in studies with the latex particles, the resulting aggregates adhered more tenaciously than the flocs resulting from precipitation. When the sensitized particles were centrifuged in saline, however, the particles no longer gave a positive reaction, but a precipitation occurred when the supernatant was tested against antitoxin. This confirmed the primarily precipitin nature of the reaction.

SUMMARY

Following intravenous administration of toxin to albino rats, there appeared to be a reduction in the concentration of toxin within the blood within four hours. Analyses of the gross components of the blood have indicated that the toxin is carried in the fluid portion of the blood. Some toxin may be adsorbed on the surface of the red blood cell, but does not penetrate it, and can be eluted from the surface with saline. Clotting did not remove the toxin from the serum.

When large oral doses of crude toxin were given to rats, there was apparently very little absorption from the gastrointestinal tract. Assays of plasma in mice at times up to four hours after oral administration were generally negative, although the contents of the gastrointestinal tract remained lethal to mice. In spite of the apparent poor absorption, there was indication of accumulation of toxin in the kidneys.

Following intraperitoneal injection of the toxin into rats, the mouse assay of the plasma became positive within 30 minutes. The contents of the gastrointestinal tract also became lethal within 30 minutes. There was apparent reduction in the blood level of the toxin within four hours and accumulation of the toxin by the kidneys.

Toxin was recovered from skeletal muscle of rats that had succumbed to large intravenous doses of crystalline toxin. This was accomplished by homogenization, ultracentrifugation, and column chromatography. Density gradient centrifugation indicated that crystalline toxin was not homogenous and that the material extracted from muscle had the same distribution of molecular weights as the crystalline toxin.

In agglutination studies, Type B antitoxin reacted with Type A crystalline toxin. Normal horse serum, diphtheria and tetanus antitoxins did not produce any precipitation when reacted with Type A toxin.

ADDENDUM

SUMMARY OF TISSUE DISTRIBUTION STUDIES

1. Albin^o rats (90-100 gm) given 1650 mouse LD₅₀ units of toxin intravenously were sacrificed at 15 minutes, 30 minutes, 1 hour and 2 hours. Plasma, red blood cells (RBC) and serum from each time of death were injected intraperitoneally into mice.

Tissue Assayed	<u>Mortality in Mouse Assay</u>			
	15 Min.	30 Min.	1 Hr.	2 Hrs.
Plasma	4/4	4/4	4/4	4/4
RBC	2/4	3/4	2/4	3/4
Serum	4/4	4/4	4/4	4/4

2. Rats given 50,000 units of toxin intravenously were sacrificed after 30 minutes. Red blood cells were washed three times with 2.0 ml aliquots of saline. Mouse assays made of plasma, serial dilutions of plasma, suspensions of washed RBC, and washings.

<u>Tissue Assayed</u>	<u>Mortality (Mouse Assay)</u>	<u>Tissue Assayed</u>	<u>Mortality (Mouse Assay)</u>
Plasma	4/4	Washed RBC	0/4 NS
1:10 dil.	3/4	1st Wash	3/4
1:100 dil.	0/4 S	2nd Wash	1/4
1:1000 dil.	0/4 NS	3rd Wash	0/4 S

S = Symptoms of intoxication in mice (sunken flanks and belly drop)

NS = No symptoms

3. Rats given 50,000 units of toxin intravenously were sacrificed at 30 minutes, 1 hour, 2 hours, and 4 hours. RBC washed three times with saline, and a portion hemolyzed with distilled water. Mouse assays made of RBC collected at each time of death.

Tissue Assayed	<u>MORTALITY IN MICE</u>			
	30 Mins.	1 Hr.	2 Hrs.	4 Hrs.
Plasma	4/4	4/4	4/4	4/4
1:10 dil.	4/4	4/4	4/4	2/4
1:100 dil.	0/4 S	0/4 S	0/4 S	0/4 S slight
1:1000 dil.	0/4 NS	0/4 NS	0/4 NS	0/4 NS
Washed RBC	0/4 NS	0/4 NS	0/4 NS	0/4 NS
1st Wash	2/4	4/4	4/4	4/4
2nd Wash	0/4 S	0/4 S	4/4	1/4
3rd Wash	0/4 NS	0/4 S	0/4 S	0/4 S
Hemolyzed RBC	0/4 NS	0/4 NS	0/4 NS	0/4 NS

4. Rats given 1.0 ml. of crude toxin by stomach tube were sacrificed at 30 minutes and 1 hour. Serum, 1:10 dilutions of serum, and kidney homogenate assayed in mice at each time. Mice observed for 72 hours.

Time Rat Sacrificed Tissue Assayed	<u>Mortality in Mice at</u>		
	24 Hr.-	48 Hr.-	72 Hr.
<u>30 Minutes</u>			
Serum	0/4	0/4	0/4
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
Kidney homogenate	0/4 S	0/4 S	0/4
<u>1 Hour</u>			
Serum	0/4	0/4	0/4
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
1:1000 dil.	0/4	0/4	0/4
Kidney homogenate	0/4	0/4	0/4

S = Symptoms of intoxication in mice

NS = No symptoms

5. Rats given 1.0 ml. of crude toxin by stomach tube were sacrificed after 30 minutes, 1 hour, and 4 hours. Serum, 1:10 and 1:100 dilutions of serum, and kidney homogenates pertaining to each time of death assayed in mice. Mice observed for 72 hours.

Time Rat Sacrificed Tissue Assayed	<u>Mortality in Mice</u>		
	24 Hr.	48 Hr.	72 Hr.
<u>30 Minutes</u>			
Serum	0/2 S	0/2 S	0/2 S
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
Kidney homogenate	0/4	0/4	0/4
<u>1 Hour</u>			
Serum	0/2 S	1/2 S	1/2 S
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
Kidney homogenate	0/4	0/4	0/4
<u>4 Hours</u>			
Serum	0/3 S	0/3	0/3
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
Kidney homogenate	0/4 S	1/4 S	1/4 S

6. Rats given 1.0 ml. of crude toxin by stomach tube were sacrificed at 30 minutes, 1 hour and 2 hours. Serum, 1:10 and 1:100 dilutions of serum, intestinal contents and kidney homogenate for each time of death assayed in mice. Mice observed for 72 hours.

Time Rat Sacrificed Tissue Assayed	<u>Mortality in Mice</u>		
	24 Hr.	48 Hr.	72 Hr.
<u>30 Minutes</u>			
Serum	0/2	0/2	0/2
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
Intestinal Contents	1/1	-	-
Kidney Homogenate	2/4	4/4	-
<u>1 Hour</u>			
Serum	0/2 S	0/2	0/2
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
Intestinal Contents	1/1	-	-
Kidney Homogenate	1/4 St	3/4 St	3/4
<u>2 Hours</u>			
Serum	0/2	0/2	0/2
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
Intestinal Contents	1/1	-	-
Kidney Homogenate	0/4 S	0/4	0/4

S = Symptoms of intoxication in mice.

7. Rats given 1.0 ml. of crude toxin by stomach tube were sacrificed after 30 minutes, 1 hour, 2 hours, and 4 hours. Serum, 1:10 and 1:100 dilutions of serum, stomach contents, intestinal contents, and kidney homogenate for each time assayed in mice. Mice observed for 72 hours.

Time Rat Sacrificed Tissue Assayed	<u>Mortality in Mice</u>		
	24 Hr.	48 Hr.	72 Hr.
<u>30 Minutes</u>			
Serum	4/4	-	-
1:10 dil.	4/4	-	-
1:100 dil.	4/4	-	-
Stomach Contents	4/4	-	-
Intestinal Contents	4/4	-	-
Kidney Homogenate	3/4	4/4	-
<u>1 Hour</u>			
Serum	4/4	-	-
1:10 dil.	0/4 S	3/4	3/4
1:100 dil.	1/4 S	1/4 S	1/4
Stomach Contents	4/4	-	-
Intestinal Contents	4/4	-	-
Kidney Homogenate	4/4		
<u>2 Hours</u>			
Serum	0/4	0/4	0/4
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
Stomach Contents	4/4	-	-
Intestinal Contents	4/4	-	-
Kidney Homogenate	4/4	-	-
<u>4 Hours</u>			
Serum	0/4	0/4	0/4
1:10 dil.	0/4	0/4	0/4
1:100 dil.	0/4	0/4	0/4
Stomach Contents	4/4	-	-
Intestinal Contents	4/4	-	-
Kidney Homogenate	4/4	-	-

S - Symptoms of intoxication in mice

8. Rats given 1.0 ml. of crude toxin by intraperitoneal injection were sacrificed after 30 minutes, 1 hour, 2 hours, and 4 hours. Serum, stomach and intestinal contents, and kidney homogenates for each time of death assayed in mice. Mice observed for 72 hours.

Time Rat Sacrificed Tissue Assayed	<u>Mortality in Mice</u>		
	24 Hr.	48 Hr.	72 Hr.
<u>30 Minutes</u>			
Serum	3/4	4/4	-
Stomach Contents	4/4	-	-
Intestinal Contents	3/4	4/4	-
Kidney Homogenate	4/4	-	-
<u>1 Hour</u>			
Serum	4/4	-	-
Stomach Contents	4/4	-	-
Intestinal Contents	4/4	-	-
Kidney Homogenate	4/4	-	-
<u>2 Hours</u>			
Serum	4/4	-	-
Stomach Contents	3/4	3/4	3/4
Intestinal Contents	0/4	0/4	0/4
Kidney Homogenate	4/4	-	-
<u>4 Hours</u>			
Serum	1/4 S	2/4 S	2/4 S
Stomach Contents	0/4 S	2/4 S	2/4 S
Intestinal Contents	4/4	-	-
Kidney Homogenate	4/4	-	-

S = Symptoms of intoxication in mice