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THE FATE OF RADIOLABELED BOTULINAL TOXIN WITHIN THE ANIMAL BODY

by

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INTRODUCTION

Botulism is a very rare, but highly fatal disease caused by the ingestion of a specific antigenic exotoxin produced by Clostridium botulinum. To date six antigenically distinguishable toxins have been identified and are labeled A, B, C, D, E and F (Lamanna and Carr, 1967). These materials are neurotoxins which paralyze certain motor nerves in animals by suppressing the release of acetylcholine at the cholinergic synapses (Brooks, 1954; Mitchell and Silver, 1963). Extremely small quantities of toxin are required to kill animals. Putnam et al., 1946 estimated that the lethal dose for mice was 20 million molecules of type A toxin. The specific toxicities range from 0.6×10^{10} to 3.8×10^{10} per gram of toxin (Schantz, 1964). Chemical characterization of the toxins indicated they are simple proteins composed entirely of amino acids (Buehler et al., 1947; Lamanna and Carr, 1967). The fate of botulinal toxin within bodies of animals has been studied by many workers (Dack, 1926; May and Whaler, 1958; Hildebrand et al., 1961). In these studies toxin was detected in animal tissues by bioassay methods. Zacks et al. (1962) used ferritin labeled botulinal toxin and electron microscopy to demonstrate localization of toxin in neuromuscular junctions of mice. Iodine 131 has been used successfully to radiolabel proteins used for in vivo and in vitro studies (Masouredis et al., 1951; Tekman and Dalglish, 1954; Melcher et al., 1955;

Francis et al., 1955a, 1955b; Weigle and Dixon, 1959; Weigle et al., 1960). Kirilenko et al. (1965) reported on results of experiments in which tetanal toxin was radiolabeled with iodine 131 and then injected into mice. The animals were sacrificed at selected time intervals and the major organs and tissues of the animals were analyzed in a radiation counter. The assumption was made that radioactivity in tissues demonstrated the presence of toxin. Radioactivity counted at selected time intervals yielded absorption patterns for the tissues examined. These authors made no attempt to compare the distribution patterns obtained for tetanal toxin with patterns for similarly treated non-toxic proteins.

The main purpose of this work was to compare the fate of radiolabeled botulinal toxin in animals with the fate of non-toxic proteins which were radiolabeled and introduced into rats in identical fashion as the toxin. This was done to determine if any differences existed between distribution patterns established for peritoneally inoculated toxic and non-toxic proteins.

REVIEW OF LITERATURE

The Organism and Toxin Production

Botulism is a true food poisoning caused by the ingestion of food containing toxin produced by Clostridum botulinum (Frazier, 1967). For centuries eating of certain preserved foods has been related to the incidence of botulism. Botulism was recognized by German physicians as a specific food illness in the latter part of the eighteenth century (Dolman, 1964). The organism responsible for botulism was isolated in 1894 by a Belgian physician named van Ermengen (Dolman, 1964). According to Bengston (1924) van Ermengen described the organism as being a large sporeforming bacillus, 4 to 6 microns in length, 0.9 to 1.2 microns wide and as having rounded extremities. The spore was described as being generally terminal and somewhat wider than the rod and resistant to ordinary staining reactions. Motility was slight and there were four to eight very fine flagella. The organism gave a positive gram staining reaction. The bacterium was characterized as being a strict anaerobe which grew on glucose agar produced turbidity and gas in glucose broth, but not in lactose or sucrose broths. Gelatin was liquified, but milk protein was not coagulated or otherwise changed. The unpleasant odor of butyric acid occurred in various media. Acid media prevented growth, while slightly

alkaline media encouraged growth and toxin production. The optimal growth temperature was in the range of 20 to 30 C. Filtrates of foods in which C. botulinum had grown killed guinea pigs and mice when administered orally. Certain animals such as dogs, cats, chickens and rats withstood more toxin than did mice, guinea pigs and rabbits.

Van Ermengen originally suggested the name Bacillus botulinus for this organism. Bengston (1924) studied 15 strains of these organisms and suggested the names Clostridium botulinum and Clostridium parabotulinum. The seventh edition of Bergey's Manual of Determinative Bacteriology (1957) suggests that the name C. botulinum be applied to non-ovolytic strains of the organism and C. parabotulinum be applied to ovolytic strains.

Bengston (1924) grouped C. botulinum into types based on serological reactions of their toxins. To date six antigenically distinguishable toxins have been identified and are designated A, B, C, D, E and F. Type C toxin can be further divided into subtypes alpha and beta. Each toxin type is antigenically unique and very little cross neutralization is observed between toxin types and specific antitoxin. Antitoxin type C subtype alpha protects against subtype beta, but the reverse is not true (Lamanna and Carr, 1967).

Much work has been done on determining the effect of temperature, pH and salt concentration on the growth of

C. botulinum and toxin production. The present discussion will be limited to C. botulinum types A, B, E and F, which are generally regarded as the causative agents of human botulism.

In general, the optimal temperature for growth and toxin production of C. botulinum types A and B is in the range of 20 to 30 C (Dolman, 1964). Ohye and Christian (1966) reported that C. botulinum type A and B grew at temperatures ranging from 20 to 40 C. and type E grew in a range of 10 to 40 C. The optimal temperatures for growth and toxin production of types A and B were reported by these authors to be 40 C, while the optimal temperature for type E was reported to be 35 C. Dolman (1964) and Riemann (1963) reported that C. botulinum type E was able to grow and produce toxin after 8 weeks incubation at 5 to 6 C. The probable lower limit of growth was suggested to be 2 C.

Ohye and Christian (1966) observed growth of these three types of C. botulinum at pH 6.0, 7.0 and 8.0; none of the three types, however, demonstrated growth at pH 5.0 or 9.0. The optimal pH for types A, B and E was reported to be in the range of pH 7 to 8. At optimal temperature and pH appreciable differences were observed in the water requirements of the three types. The minimal levels of water activity (a_w) supporting growth were reported to be 0.950, 0.940, and 0.970 for types A, B, and E respectively. These values correspond to NaCl concentrations of 8.0%, 9.4% and 5.1%. The effect

of lowered incubation temperature was to elevate water requirements for all three types. The pH at which NaCl tolerance was greatest was reported to be 7.0 for types A and B. The growth and toxin production of type E were reported to be more adversely affected by low temperature in combination with high salt concentrations and low pH than was the growth of types A and B. Segner et al. (1966) reported that NaCl in concentrations of 4.5% to 5.0% were generally sufficient to inhibit growth of C. botulinum type E. The lower concentration of NaCl was required to inhibit growth when the organism was incubated at 8 C and 10 C, while a concentration of 5% NaCl was required to inhibit growth of organisms incubated at 30 C. The organism did not exhibit growth at pH values below 5.03. These data contradict earlier reports by Dolman (1964) that C. botulinum type E was able to grow in NaCl concentrations as high as 10% in an acid medium below pH 4.5.

Walls (1966) reported that C. botulinum type F is capable of growth and toxin production at temperatures as low as 4 C. The optimal temperature for growth and toxin production for type F is reported to be 30 C.

Heat resistance of spores of C. botulinum has been of great concern to investigators for many years. Esty and Meyer (1922) did much of the early work on determining accurately the thermal resistance of botulinal spores and their work was used as a basis for setting up standards for thermal

processing of foods. Ito et al. (1966) reported that the D values of C. botulinum type A strain 62 A and 12885A at 112.8 C were 1.23 minutes and 1.09 minutes, respectively; type B strains 213 B and 32B had D values of 1.32 minutes and 0.15 minutes, respectively when heated at the same temperature of 112.8 C. Type E when heated at 77.0 C gave D values of 1.95 minutes, 1.55 minutes, 1.23 minutes and 0.77 minutes for strains Saratoga E, Minneapolis E, 1304 E and 8E, respectively. Type E botulinal spores are destroyed in a fraction of a minute at 100 C and their rate of destruction need not be considered in calculating process times and temperatures designed to kill the more heat resistant types A and B (Perkins, 1964).

Chemical Nature of Botulinal Toxin

Five of the six types of botulinal toxins have been obtained in relatively pure form. These five types are A, B, C, D, and E. Type F has not yet been obtained in a highly purified form. Most of the chemical characterization of botulinal toxin has been done with type A since it is the only one available in crystalline form. Toxin types B, C, D, and E have been observed in amorphous form only (Schantz, 1964).

Type A botulinal toxin was found by Buehler et al. (1947) to be composed entirely of amino acids and, therefore, the toxin is classified as a simple protein. They reported that

Table 1. Comparison of some properties of purified preparations of botulinal toxin (Schantz, 1964)

Type	Molecular weight	Specific Toxicity LD_{50} per gram	Isoelectric point
A	900,000[a]	3.8×10^{10}	pH 5.6[a]
B	60,000[b] 9,000 to 10,000[c] 500,000[d]	3.8×10^{10}	pH 5.6
E	19,000[e]	0.6×10^{10}[f]	-

[a]Putnam et al., 1946.
[b]Lamanna and Glassman, 1947.
[c]Gerwing et al., 1966.
[d]Wagman and Bateman, 1951.
[e]Gerwing et al., 1961.
[f]Duff et al., 1956.

the total nitrogen content for type A toxin was 16.2% and the total sulfur content was 0.436%. The amino acid content of botulinal toxin types A and B is summarized in Table 2.

Some workers have attributed toxicity to the presence of certain key amino acids. Boroff et al. (1966) proposed that tryptophane residues in the molecule are critical to the maintenance of reactive sites responsible for toxicity, and that these residues might also be involved as antigenic determinents in the formation of antitoxin. Lamanna and Carr (1967) point out that purified botulinal toxin type B does not possess tryptophane. If it were the key amino acid responsible

Table 2. Amino acid composition of toxins (Lamanna and Carr, 1967)

Amino acid	Botulinal toxin			
	Type A		Type B	
	%	Residues	%	Residues
Glycine	1.05	166	5.50	5
Alanine	3.12	394	4.57	4
Valine	4.45	506	4.39	4
Leucine	8.91	708	7.06	7
Isoleucine	10.33	820	7.38	7
Serine	3.60	374	7.07	7
Threonine	7.19	642	4.92	5
Aspartic acid	17.34[a]	1,370	16.00	16
Glutamic acid	13.67	953	7.13	7
Lysine	6.78	477	6.38	6
Histidine	0.91	60	0.61	1
Arginine	4.14	239	2.10	2
Proline	2.19	203	2.39	3
Phenylalanine	1.04	64	4.35	4
Tyrosine	12.18	672	4.14	4
Tryptophane	1.69	82		
Methionine	0.93	64	1.88	2
Cysteine	0.23	20	1.02	1
Half cystine	0.45	40		
Total kinds	19		17	
Total moles		7,754		85
% amino acids	100.20		86.9	
Total amide ammonia	2.13			
Molecular weight		894,589		9,531

[a]Calculated as asparagine.

for biological activity of botulinal toxin it would be expected to appear in all types of the toxin. Dolman and Gerwing (1966) have suggested cysteine may be a key amino acid in type E toxin. The -SH groups make cysteine a very active amino acid, except at acid pH level, where botulinal

toxins likewise tend to be stable. Versene, a chelating agent that protects -SH groups from reacting with cations, was found to be an important aid in maintaining biological activity of type E toxin. Since cysteine residues are found in toxin types A, B, and E, the authors concluded that if a cysteine-specific reagent were found to destroy the biological activity of these toxins, it would follow that this amino acid must be critically involved in the biological activity of the toxin.

Attempts have been made to explain the high toxicity of the botulinal toxin molecule on the basis of arrangement of amino acids. Schantz et al. (1960) reported that type A toxin in pure form will fluoresce when excited by ultraviolet light. When the toxicity is lost the fluorescence of the molecule is lost as well. This suggested that the structure responsible for toxicity was also responsible for its fluorescence (Boroff and Fitzgerald, 1958). Schantz et al. (1960) reported that if the toxic activity of the molecule is destroyed in 6 M urea, fluorescence is not lost. The suggestion that fluorescence and toxicity were due to a common structure within the molecule was invalidated by this finding.

Cartwright and Lauffer (1958) observed that type A toxin is stable at 40 C in a solution adjusted to pH 6.9, but that the toxicity is lost in a matter of minutes at 50 C. A strongly alkaline pH destroys the toxin rapidly with 90% of the toxin being destroyed in 1 to 2 minutes at pH 11.2

(Spero, 1958). Ketene (Schantz and Spero, 1957) halogens (Brazis et al., 1959) nitrous acid (Spero and Schantz, 1957) protein denaturants (Stefanye et al., 1964) formaldehyde and photooxidants (Weil et al., 1957) are all agents which have been shown to destroy or alter the biological activity of the botulinal toxin molecule.

Lamanna and Carr (1967) concluded that the toxicity of botulinal toxin cannot be attributed to a single amino acid. Destruction or modification of one of a number of amino acids could result in loss of biological activity. The toxin appears to be a single polypeptide chain without prosthetic groups whose maintenance of integrity of tertiary structure appears necessary for toxicity.

The question arises whether a molecule as large as that of type A botulinal toxin with a molecular weight of 900,000, can pass through the intestinal barrier. Sedimentation coefficients determined for crystalline type A toxin before and after exposure to residence in the small intestine of a rat gave no evidence that the toxin in the intestine broke down into smaller sized particles or sub-units before passing the intestinal barrier (Heckly et al., 1960). Wagman and Bateman (1951) demonstrated with the aid of an ultracentrifuge that under proper conditions of ionic strength and pH, toxin molecules disassociate to form smaller toxic sub-units with a molecular weight of 40,000 to 100,000. Wagman (1954)

reported an estimated molecular weight of the toxic sub-unit based on the sedimentation rate in an ultracentrifuge to be 10,000 to 20,000. A dialyzable toxic unit with a molecular weight of 3,800 was obtained from a pepsin digest by Wagman (1963). Gerwing et al. (1965) used electrophoresis and ultracentrifigation to obtain a homogeneous type A toxic unit with a calculated molecular weight of 12,000. Chromatographic separation on DEAE cellulose has given molecular weights of 12,000, 9,000 and 18,600 for types A, B and E respectively (Gerwing et al., 1964, 1965, 1966).

Gerwing et al. (1965) suggested that purification procedures used to obtain pure toxin for molecular weight determinations may cause low molecular weight toxin to polymerize into an aggregate of much larger molecular weight. Schantz and Spero (1966) argue strongly against this premise. They reported estimates by ultracentrifigation of the molecular weight of type A toxin was the same in culture as that of purified toxin (900,000) and that all other types B through F also must have molecular weights that approach the weight of type A.

Lowenthal and Lamanna (1951) and Lamanna and Lowenthal (1951) reported that botulinal toxin type A was closely associated with a hemagglutinating component which was separable from the toxin. Meyer and Lamanna (1959) suggested that preparation of botulinal toxin type A on DEAE cellulose may

completely separate the hemagglutinating component from the toxin molecule. Removal of the hemagglutinating component would yield a toxin with a lower molecular weight.

Duff et al. (1956) reported that botulinal toxin type E was unique in that its full biological activity was not attained until it reacted with trypsin. This activation phenomenon was reported by Sakaguchi and Sakaguchi (1966) to be the result of conversion of an inactive precursor into the fully toxic molecule by the action of trypsin. Activation with trypsin resulted in a 200 fold increase in toxicity, but prolonged exposure to trypsin resulted in destruction of the biological activity of the type E molecule. The nitrogen content and molecular weight, were the same for both the precursor and the trypsin-activated type E toxin. Bulatova (1965) reported that the precursor of type E toxin was serologically indistinguishable from the trypsin-activated toxin. Botulinal toxin type F is also reported to be activated by trypsin (Holdeman, 1966).

Pharmacological Action

The symptoms of botulism begin 18 to 96 hours after ingestion of the toxin and include: visual disturbance (lack of coordination of eye muscles, and double vision) inability to swallow and speech difficulty. Signs of bulbar paralysis are progressive and death occurs from respiratory paralysis,

or cardiac arrest. No fever is present. Gastrointestinal symptoms are not prominent. The patient remains fully conscious until shortly before death (Jawetz et al., 1962).

The toxin apparently suppresses the release of acetylcholine at the cholinergic synapses (Brooks, 1954; Mitchell and Silver, 1963). Lamanna and Carr (1967) reported that the toxin produces some lesion at the myoneural junction which renders it incapable of releasing acetylcholine in quantities sufficient to evoke a contraction of muscle fibers. Poisoned nerve terminals retain their normal content of acetylcholine but are prevented from releasing it from its binding sites. Botulinal toxin does not inhibit the release of cholinesterase.

Transmission of nerve impulses across individual synapses becomes impaired after an initial time lag of 20 minutes from the time the synapses come into contact with the toxin. Once the blockage of the nervous junction becomes complete it is irreversible by any known procedure (Brooks, 1954). Recovery from chronic botulism poisoning takes months for autonomic ganglia and muscle ganglia (Burgen et al., 1949). Botulism is characterized by its peripheral effects; the central nervous system seems to continue to function normally. The antigenic difference in the six types of toxin are reflected only in the animal species susceptibility, not in the mode of action of the toxins (Brooks, 1964).

Guyton and MacDonald (1947) reported that localized paralysis had been produced in lower hind leg muscles of

guinea pigs and rabbits by injections of small doses of botulinal toxin. Closed intra arterial injections of acetylcholine evoked an immediate contraction of paralyzed muscles.

Putnam et al. (1946) reported that for type A toxin the mouse lethal dose may be as little as 20 million molecules. Toxicity for a single type of toxin varies from one species of animal to another. The method by which the toxin gains entrance to the animal's body also affects the toxicity. Lamanna and Carr (1967) estimated that the oral dose of purified type A toxin may be 1000 times less toxic than an equal amount of purified toxin inoculated intraperitoneally into man.

Davies et al. (1953) reported on the comparative lethalities of botulinal and tetanal toxins when inoculated intravenously and directly into the medula oblongata portion of a rabbit's central nervous system. Botulinal toxin was found to be less toxic when inoculated directly into the rabbit's brain than when injected directly into the venous circulation. The opposite was true for tetanal toxin.

Dack (1926) found little toxin in the blood of rats which had been fed large doses of botulinal toxin. May and Whaler (1958) reported that when botulinal toxin was administered orally to rats and rabbits it was absorbed much more rapidly from the upper portion of the small intestine than from the

lower region of the intestine or from the stomach. In rabbits toxin migrated into the circulatory system by way of the thoracic lymph duct. Cannulation of the intestinal lymph duct and continuous collection of lymph following oral ingestion of toxin by rats gave partial or complete protection from toxemia.

Hildebrand et al. (1961) reported that in rabbits which were given type A botulinal toxin intravenously and which died rapidly from acute poisoning, the toxin was found in lymph and plasma with no tendency to diffuse into the erythrocytes. Toxin appeared in small quantities in urine and bile. Cerebrospinal fluid was generally free of toxin.

There is some indication that multiple injections of sublethal doses of type A toxin into guinea pigs, rabbits and mice produced death when the total dose was significantly smaller than one single lethal dose. Impairment of some general metabolic functions elicited by small sublethal doses of toxin may explain these observations (Matveev, 1959a; 1959b).

When administered orally, purified toxin is less toxic than toxin which is contained in a complex mixture such as in food. Coleman (1954) has suggested that purified toxin is probably more easily denatured by the digestive processes of an animal than impure toxin because of the loss of protective colloids from the pure toxin.

MATERIALS AND METHODS

Sources of Toxin and Antitoxin

Purified type A botulinal toxin was obtained from Dr. Edward Schantz, U.S. Army Biological Laboratory, Fort Detrick, Md. The toxin was received in two forms; one dissolved in 0.05 M sodium acetate buffer at pH 4.2 with a concentration of 3 mg of toxin per ml, and the other in the lyophilized state. The lyophilized toxin was prepared from a solution of purified toxin in 0.05 M sodium phosphate buffer, pH 6.8.

Type A equine antitoxin was obtained from the U.S. Public Health Service Communicable Disease Center (CDC) Atlanta, Georgia. This antiserum was received in the lyophilized state and was rehydrated in a 1:1 solution of sterile glycerin and distilled water. After rehydration the preparation contained 10 International Units of antiserum per ml. An International Unit (I.U.) is defined as the specific activity contained in a known weight of dried antitoxin for each C. botulinum type; the weight for type A is 0.1360 mg. (Bowmer, 1963).

Radiolabeling of Proteins

Crystalized and lyophilized bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.) fraction V rat serum albumin (RSA) (Pentex Incorp., Kankakee, Ill.) and purified type A botulinal toxin were radiolabeled by a method similar

to that described by Kirilenko et al. (1965). A volume of 0.1 ml of a solution of sodium iodide 131 (Nuclear Chicago catalog no IBS-3, Des Plaines, Ill.) was placed in a small glass test tube. A small crystal of elemental resublimed iodine was added to the solution of iodide and allowed to react for 20 minutes at 37 C. An isotropic exchange reaction took place during this time which allowed the formation of free iodine 131. The solution containing the free radioiodine was then drawn off with a micropipette. A volume of 50 microliters was added to 1 ml volumes of the proteins previously mentioned. The proteins were dissolved in Bacto-FA buffer pH 7.2 (Difco Laboratories, Detroit, Mich.). The protein solutions and iodine were allowed to react together for 4 hours at 4 C. Separation of unbound iodine was accomplished by filtration of the protein solution through a column 30 cm in length packed with Sephadex G50, fine (Pharmacia, Uppsala, Sweden) which was swelled in the same phosphate buffer used to dissolve the proteins. The protein material was placed on the column and then eluted with phosphate buffer, pH 7.2, flowing at a rate of 0.5 ml per minute. Two ml fractions were collected manually using a 10 ml graduated cylinder. The two ml aliquots were placed in glass tubes, 15 x 2.5 cm, (Picker Nuclear, Minneapolis, Minn.) and counted in a Picker Autowell II gamma radiation counter. The count rate was plotted against the number of milliliters

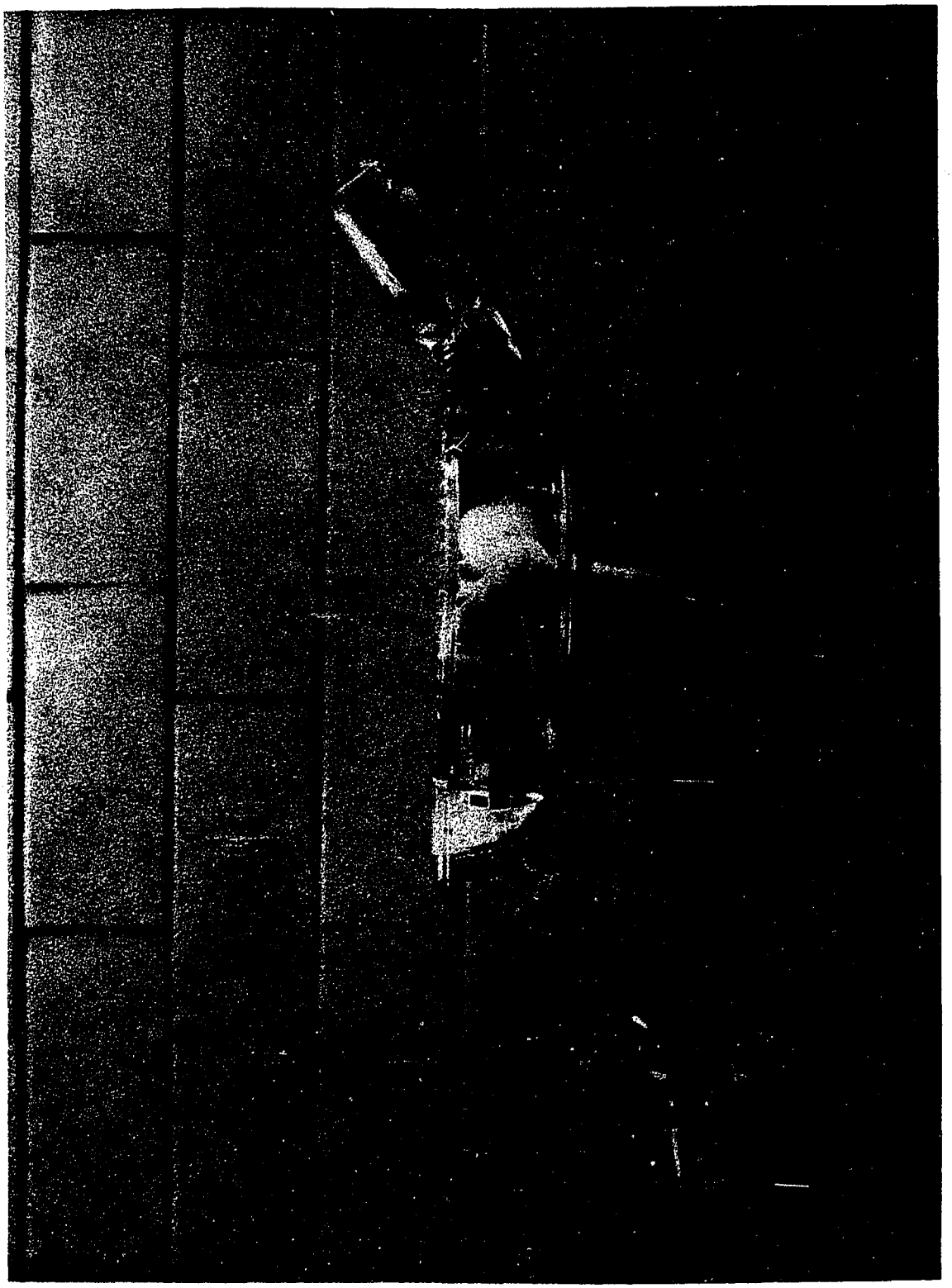
collected. The absorbance of the eluted material at 280 mu was measured on the Beckman DU. The absorbance values were plotted against the number of milliliters collected.

One ml of the radiolabeled complex collected from the column was precipitated with cold 3% trichloroacetic acid. The precipitated material was centrifuged at 12,350 x G for 30 minutes at 2 C in an RC2-B refrigerated centrifuge (Sorvall, Norwalk, Conn.) to insure complete separation of the precipitate from the supernatant. Both the precipitate and the supernatant were transferred to counting tubes and assayed for radioactivity.

Elimination Rate Determination

The rate at which radiolabeled toxin was eliminated from rats was determined by feeding the animals the radiolabeled material and then collecting all their excretory products for a period of time. Sublethal doses of radiolabeled toxin were placed on pieces of dry bread which were then coated with a thin layer of peanut butter. The toxic peanut butter sandwich was then fed to a rat which had fasted for 24 hours. This determination was performed on a pair of rats which were treated identically. After they had eaten the toxic sample the rats were placed in metabolism cages as shown in Figure 1. Urine and feces samples were collected at 12 hour intervals for a period of 48 hours and

Figure 1. Metabolism cage used to collect urine and feces from rats used in elimination studies.



placed in counting tubes. A volume of radioactive toxin equal to the volume consumed by each rat was also placed in a counting tube. This reference sample as well as the excretory samples were counted in the Picker gamma counter. The samples were counted in the same manner as the tissue samples. The counts were corrected for decay factor in the same manner as subsequently described. The counts for each 12 hour sampling of urine and feces were added together to obtain the total amount of radioactivity excreted by each animal within the 12 hour time interval. This totaled count was then divided by the count obtained for the reference sample to obtain the percent of radioactive material lost by excretion during each time interval.

The effect of passive immunity on the rate of toxin elimination was determined by injecting 0.8 IU of type A antiserum into the hind leg muscles of two rats 24 hours prior to feeding them toxic peanut butter sandwiches. The balance of this experiment was identical to the procedure described previously.

Samples of urine from both immune and non-immune rats were treated with cold 3% trichloroacetic acid and incubated at 4 C for two hours. The samples were spun at 12,000 x G for 30 minutes at 2 C in the Sorvall RC2-B centrifuge. The supernatant fluid was separated from any precipitate that was formed. Supernatant and precipitated material were

transferred to counting tubes and radioactivity assayed in the Picker counter. This procedure was used to determine if any of the radioactive material excreted in the rat's urine was in protein form.

The differences in excretion rates between rats which had been fed a radiolabeled protein and intraperitoneally inoculated with the same material were also determined. Two rats were fed peanut butter sandwiches on which radiolabeled bovine serum albumin had been placed. These animals were then placed in metabolism cages and their urine and feces collected and counted for a period of 48 hours. A second pair of rats was inoculated peritoneally with an equivalent dose of radiolabeled bovine serum albumin and then placed in metabolism cages. The counts from the excretory products were divided by the weight of the excreta in order to obtain the specific count for the excretion products.

LD_{50} Determinations

The LD_{50} bioassays were run to determine the amount of loss of toxicity of the botulinal toxin during the radiolabeling procedure. A toxic solution was divided into two equal parts, one part was radiolabeled with iodine 131, the other was not. The unlabeled toxic sample was then filtered through a 30 cm column packed with Sephadex G 50. The

buffer system and flow rate were identical as that described previously. Five ml fractions were collected manually using a 10 ml graduated cylinder. The fraction starting with the 15th milliliter and stopping with the 20th milliliter contained most of the proteinaceous material. This fraction was saved and used for the LD_{50} determination. The column was washed for several hours with phosphate buffer to insure removal of all protein material. The radiolabeled sample was then put on the column and eluted. The same fraction was saved and used for the LD_{50} determination. Both samples were diluted in ten fold dilutions with physiological saline. The dilutions ranged from 10^{0} to 10^{-9}. Five albino mice per dilution were peritoneally inoculated with 0.5 ml of toxic material. Non-radiolabeled and radiolabeled toxin were inoculated in this manner into 50 animals for each of the two toxin groups. The animals were observed for 96 hours and LD_{50} values were calculated using the method of Reed and Muench (1938).

Toxin Recovery from Tissues

A lethal dose of radiolabeled toxin was inoculated peritoneally into two white rats. Following death of the animals, the brain, lymph node in the neck, thyroid gland, blood, heart, lung, liver, spleen, kidney, stomach, small intestine, spinal column and tail were removed from each

animal and weighed. The tissues were counted in the Picker counter in the same manner as subsequently described. These tissues were then homogenized in 0.85% sterile saline in a Sorvall Omni-Mixer Homogenizer with micro-homogenizer attachment. Homogenization was generally accomplished in 3 to 4 minutes at top speed in the micro-homogenizer attachment. These samples were centrifuged at 12,350 x G for 30 minutes at 2 C in the Sorvall RC2-B centrifuge to settle particulate material. A volume of 0.2 ml of the supernatant material was inoculated peritoneally into an albino mouse. A second 0.2 ml of the supernatant material was mixed with an equal volume of type A antiserum and inoculated peritoneally into a white mouse. Death of the mouse not receiving the toxin-antitoxin mixture was considered to be caused by toxin extracted from the tissues. The remaining supernatant fluid was then treated with cold 3% trichloroacetic acid and allowed to precipitate for 3 hours at 4 C. The material was centrifuged at 12,350 x G for 30 minutes at 2 C in the Sorvall RC-2B centrifuge to insure separation of supernatant from precipitate. Supernatant, precipitate and particulate material were transferred to counting tubes and counted in the Picker counter. This procedure was performed in order to determine whether the radioactivity in the tissues was protein bound or in free or amino acid bound form at the time of death of the rat.

Determination of Purity of Toxin

The exact purity of botulinal toxin supplied by Dr. Edward Schantz is unknown. Purified crystalline type A toxin is reported by Schantz (1964) to be 98% pure. Toxin used in this work was analyzed for major impurities by using electrophoretic techniques.

Agar gel was used in the electrophoretic procedure. Speical Agar Nobel (Difco), was dissolved in pH 7.8 borate buffer. The concentration of agar used was 0.75 grams per 100 ml of buffer. Twenty-five ml of melted agar were pipetted onto a lantern slide and allowed to harden. A paper wick saturated with botulinal toxin was inserted into the agar either in the center or 1 cm from the positive pole of the lantern slide. The slide was then placed in a Beckman Spinco paper electrophoretic apparatus. Borate buffer pH 7.8 was placed in the buffer tanks on either side of the electrophoresis apparatus. Filter paper wicks were attached to either side of the slide and allowed to extend into the buffer reservoirs. A Beckman power supply was used to provide the electrical potential. Using constant current a setting of 3 milliamperes and 180 volts was applied. The wick containing the toxin was removed from the agar after 30 minutes at this power setting. The electrical potential was then maintained for 18 hours. This allowed for a migration of 2.5 cm from the sample slot. The plate was then

stained with Amido Schwartz stain (National Analine, New York) and destained with a solution consisting of 2 parts of methanol, 2 parts of distilled water and 0.45 parts of glacial acetic acid.

Autoradiography

Autoradiography experiments were performed in an attempt to locate any large concentrations of radiolabeled botulinal toxin in the alimentary canal of a rat which had died of botulinal poisoning. A lethal dose of radiolabeled toxin was placed on a piece of bread along with peanut butter and fed to a rat. Upon death of the animal the alimentary canal was removed from esophogus to anus and laid out on Kodak RP 54 Xray film. An aluminum foil envelope was used to protect the film from light. The digestive tract was allowed to remain on the film for varying periods up to eight hours to obtain good film exposure. A polaroid photograph was taken of the tissue layout so that the exposed portion of the film could be correlated with the tissue location.

The film was processed by the Student Health Service, Iowa State University and examined for the presence of dark areas which indicated the localization of radioactive material in the digestive tract.

Detection of Radioactive Material in Animal Tissues

Distribution patterns in rat tissues were established using the radiolabeled proteins. Two patterns were established using bovine serum albumin and rat serum albumin. A third pattern was established for purified type A botulinal toxin. The fourth pattern was determined for purified type A botulinal toxin introduced into rats which had received 0.6 International Units (IU) of type A antitoxin 24 hours earlier. The antiserum was introduced by intramuscular injection into the hind leg muscle of the rat.

With each distribution pattern determination 14 female albino rats were used. The rats which averaged in weight from 140 to 160 grams were obtained from Simonsen Laboratories, White Bear, Minn. Two rats were used for each sampling period. The sampling periods were 1, 2, 4, 8, 12, 24 and 48 hours from the time of introduction of the radiolabeled protein into the animals. In each determination the rats were anesthetized with ether and the radiolabeled protein was introduced by intraperitoneal inoculation. Sampling began 60 minutes after the last pair of rats was inoculated.

At each sampling period two rats were anesthetized with chloroform and exsanguinated by cardiac puncture. The animals were then stretched out on a small animal operating board and opened ventrally from chin to tail. The following

tissues were removed: brain, lymph node in neck area, thyroid gland, blood, heart, lung, diaphragm, liver, spleen, kidney, stomach, small intestine, a portion of the spinal column containing muscle tissue, bone and central nervous cord, and finally the last 3 to 4 cm of the tail. As each tissue was removed it was placed in a disposable 5 ml microbeaker. After the tissues from both animals were removed they were weighed.

The tissue samples contained in the microbeakers were folded and dropped into the bottom of the glass counter tubes and counted twice for periods of 60 seconds in the Picker counter. At the beginning of the counting periods the counter was allowed to count an empty tube for 10 minutes. This count divided by 10 was the background count which was then automatically subtracted from each of the successive counts by the Picker counter. The pair of values counted for each sample were averaged together at the end of the counting cycle. The average value for each of the samples was multiplied by a decay factor number. This number was obtained from the formula ($\text{Log } \frac{A_o}{A} = \frac{0.301\ t}{t^{\frac{1}{2}}}$) where t = the amount of time in hours from the time the radioisotope was made up until it was counted. This time value in Greenwich Mean Time (GMT) was given on each isotope container. The $t^{\frac{1}{2}}$ value is the half life of the isotope. In the case of iodine 131 $t^{\frac{1}{2}}$ = 8.05 days. After the count had been

corrected for decay factor it was divided by its tissue weight to obtain count per minute per gram of tissue. This value is the specific count of the tissue. The specific counts for each of the tissues from the duplicate animals in each sampling period were averaged together to obtain the average specific count for the pair of rats.

A volume of radiolabeled protein material equal to the volume used for injection of each of the animals was counted along with the tissue samples. This sample was used to determine the total number of counts received by each animal in each distribution pattern determination. This value was constant within treatment groups since all the animals received equal volumes of material, but varied among treatment groups. The value which was called the total radioactive doses per animal was used to correct all specific counts statistically in such a way that all animals in all treatment groups received theoretically equalized radioactive doses. Statistical adjustments were calculated using analysis of covariance. The reader is referred to Snedecor (1956) chapter 13 for formulae and directions for calculation of covariance.

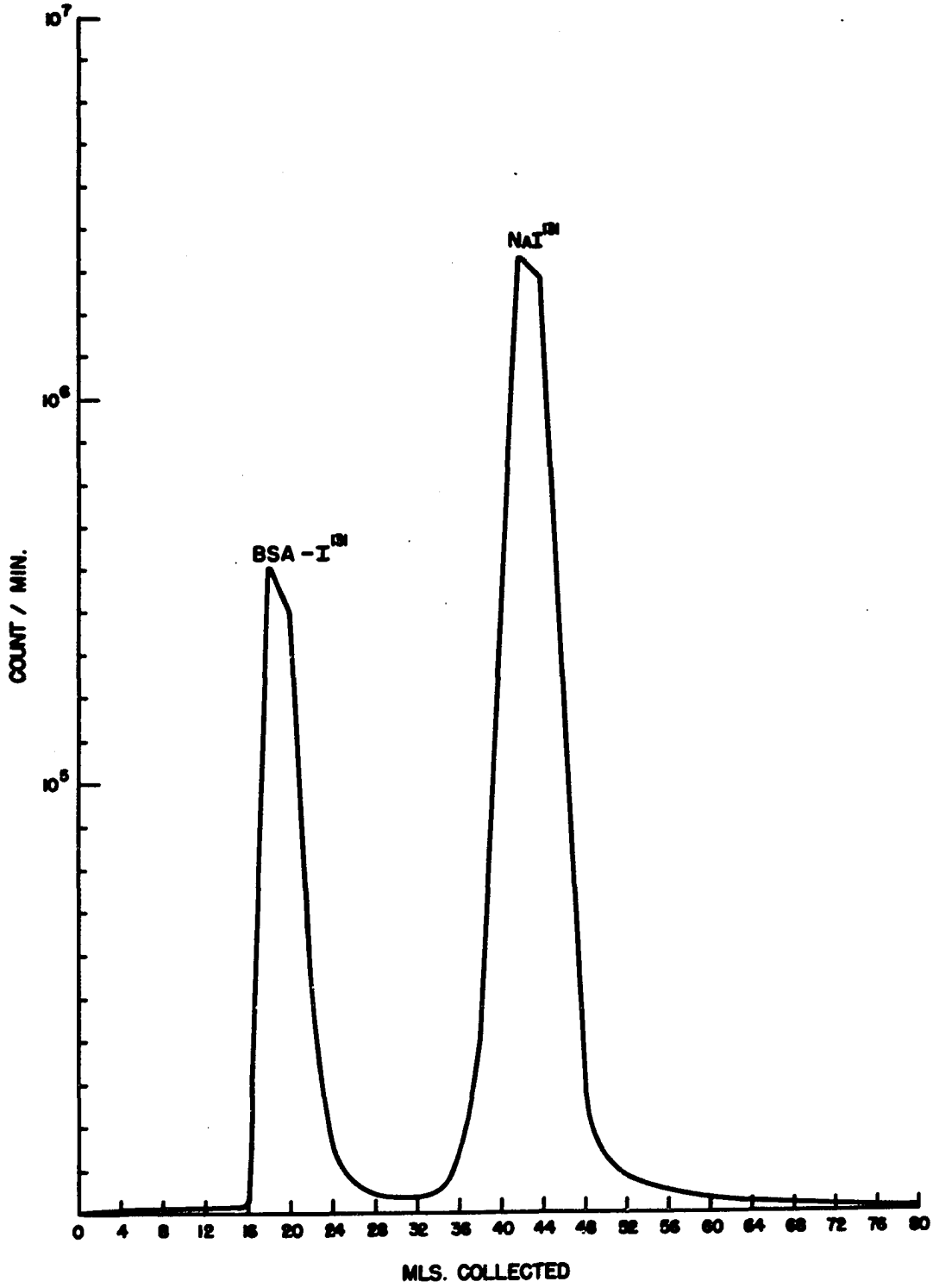
RESULTS AND DISCUSSION

Radiolabeling of Proteins

Radioactive iodine 131 was coupled to the proteins used in this study so they could be easily detected in animal tissues. The radiolabeling procedure was satisfactory for obtaining biologically active toxin and serum albumins with high radioactive counts. The method was simple and direct with the entire procedure consuming no more than 6 hours. Time is an important factor when using an isotope of short half life such as iodine 131 for radiolabeling. The addition of iodine to toxin solutions did cause significant loss in biological activity. The magnitude of loss of toxicity will be discussed in a subsequent section. Less harm was done to the toxin when both the iodine and the toxin solution were chilled to 4 C before mixing and the incubation of the combination was carried out at 4 C.

Filtration through Sephadex G50 was effective and rapid in removing the unbound iodine from the toxin solution. Figure 2 shows that when the number of milliliters collected from the column was plotted against the count for each fraction two distinct fractions were detected. The first peak which occurred between the 16th and 24th milliliters was equivalent to the void volume of the column as determined with dextran blue. The second peak which occurred between

Figure 2. Filtration of radiolabeled protein material through Sephadex G50. The number of milliliters eluted from the column is plotted against the radiological count for each fraction.

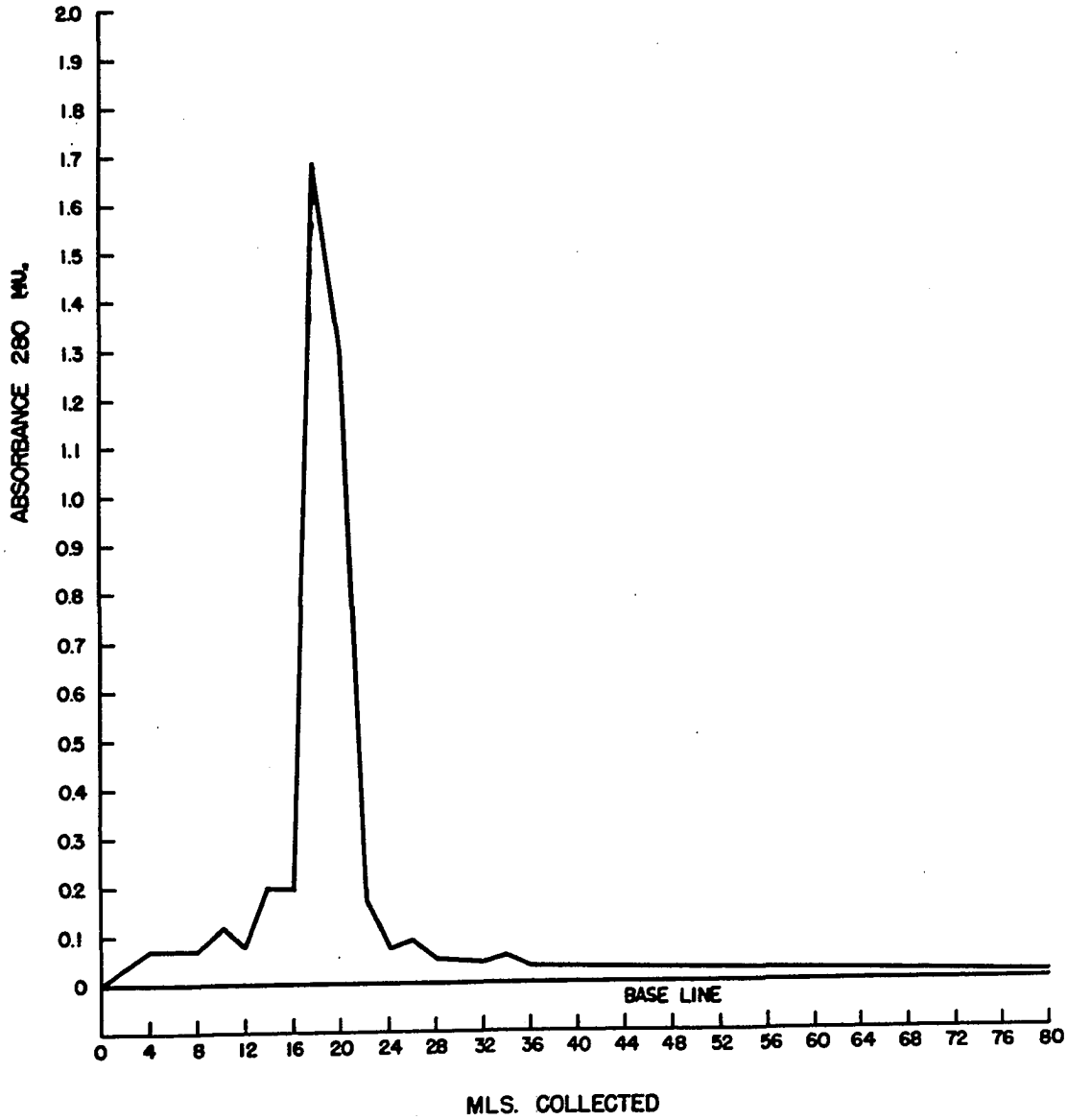

10^7
10^6
10^5
COUNT / MIN.
BSA - I^{131}
NaI^{131}
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80
MLS. COLLECTED

the 36th and 48th milliliters moved more slowly in the column, and apparently contained material with a molecular weight less than the exclusion limit of Sephadex G50.

Figure 3 displays the plot of the number of milliliters eluted from the column against the absorbance at 280 mu as measured on the Beckman DU. The absorbance peak occurring between the 16th and 24th milliliters corresponded exactly with the first count peak in Figure 2. The first count peak evidently contained protein material with radioactive iodine bound to it.

The tightness of the bond between the protein and the iodine was demonstrated using trichloroacetic acid precipitation techniques. Bovine serum albumin and toxin to which radioactive iodine was bound were precipitated with cold 3% trichloroacetic acid. The supernatant and precipitate were collected and counted separately. The precipitated bovine serum albumin contained 99.5% of the radioactivity while 95.0% of the activity remained with the precipitated toxin. This demonstrated that the iodine was sufficiently tightly bound to the protein to withstand precipitation in trichloroacetic acid and did not dissassociate to any extent. Iodine 131 has been used successfully by other workers to radiolabel proteins and toxins for *in vivo* and *in vitro* immunological studies (Masouredis *et al.*, 1951; Tekman and Dalglish, 1954; Melcher *et al.*, 1955; Francis *et al.*, 1955a, b; Weigle

Figure 3. Filtration of radiolabeled protein material through Sephadex G50. The number of milliliters eluted from the column is plotted against the absorbance at 280 MU of each fraction.


ABSORBANCE 280 MU.
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
BASE LINE
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80
MLS. COLLECTED

and Dixon, 1959; Weigle et al., 1960 and Kirilenko et al., 1965).

LD_{50} Determinations

The biological activity of botulinal toxin type A was reduced by the radiolabeling procedure from 315,000 mouse units of toxin per ml to 315 mouse units per ml when iodine 131 was used as the radiolabel. This amounted to a reduction factor of 10^3 in biological activity. A precipitate formed in the toxic solution after iodine was added and the sample was incubated for several hours at 4 C. The precipitate did not form in the toxic solution into which no iodine was added. Brazis et al. (1959) reported that iodine in concentrations of 7.7 mg per liter of water was able to biologically inactivate 99.99% of partially purified type A botulinal toxin. In this instance, the toxin was reported to be inactivated in 60 seconds at pH 6.25 when the sample was incubated at 25 C. The actual amount of iodine on a weight per volume was not known since the radioactive iodine sample was calibrated only in terms of total activity in millicuries (mc); however, it was much less than the amount of iodine used by Brazis et al. (1959).

Despite the heavy loss of biological activity enough toxin was present initially in the concentrated purified sample to obtain a toxic solution with moderate biological

activity and high radioactivity after radiolabeling. The biological activity of the radiolabeled toxin sample used in the tissue distribution patterns was approximately 1000 mouse units per ml of solution.

Elimination Rate Determination

The toxin used for elimination studies was placed in a food sample which would be readily eaten by the rats and which would provide protection for the toxin. As reported earlier by Coleman (1954), pure toxin is partially destroyed when fed to animals without protective colloids present. Table 3 shows the percent of total orally fed radioactive toxin excreted from the rats in urine and feces. Non-immune rats eliminated 63% to 70.5% of the total radioactivity in a period of 48 hours. Passively immune rats in the experiment excreted the radiolabeled toxin more rapidly than the non-immune animals; after 24 hours 70% to 73% of the total activity was eliminated from the immune rats and after 48 hours the immune rats had eliminated 83% to 88%. Thus immune rats eliminated approximately the same amount of activity in 24 hours that was eliminated in 48 hours from non-immune rats.

The passively immune rats were inoculated intramusculary with type A antiserum 24 hours prior to ingestion of the toxin. This time period was assumed to be sufficiently long to allow circulation of the antitoxin throughout the

Table 3. Elimination of orally fed botulinal toxin from rats. Elimination values are in percent of total dose given to each animal

Time in Hours	Rat 1	Rat 2
Non-Immune Rats		
12	30%	31%
24	50%	55%
36	58%	62%
48	63%	70.5%
Immune Rats		
12	32%	31%
24	73%	70%
36	81%	77%
48	88%	83%

animal. Specific antibody would then be available to complex with the toxin which had been ingested and absorbed into the rat's circulation. The toxin-antitoxin complexes which formed in the animal were probably phagocytized. Phagocytic products probably included amino acids and short peptides containing radioactive iodine. These materials were excreted in the urine and feces of the rats. Degradation of toxin in the presence of specific antibody by phagocytosis may explain the increased rate of elimination of radiolabeled toxin from the passively immunized rats.

Cells responsible for phagocytosis are microphages and macrophages. Microphages include the polymorphonuclear leucocytes of the blood. Macrophages comprise the cells of the reticuloendothelial system and are represented by both fixed and wandering cells. Fixed macrophages line the endothelium of capillaries and of the sinuses of organs such as the spleen, bone marrow and lymph nodes. Wandering macrophages such as monocytes migrate through tissues and are not localized to one particular tissue. The liver also possesses phagocytic properties (Carpenter, 1965). These tissues did not localize higher amounts of radioactive toxin in the passively immunized animals than did non-immune animals in this work.

Treatment with trichloroacetic acid indicated less than 1.0% of the total radioactivity found in urine samples

was precipitatable. Radioactive iodine was probably not attached to protein, but rather to breakdown products such as amino acids and short peptides. No attempt was made to determine if urine and feces contained biologically active toxin in quantities lethal to mice.

The difference in excretion rates between rats which had been fed radioactive bovine serum albumin and intraperitoneally inoculated with the same material can be seen in Figures 4 and 5. The specific counts for urine and feces from rats fed radiolabeled BSA were much higher than the specific counts for excretory products from peritoneally inoculated rats. Higher concentrations of radioactive material were present in the excrement of rats fed the radiolabeled protein. Animals fed the radiolabeled protein apparently degraded and excreted the material in a shorter time than the animals which received protein by intraperitoneal inoculation. These observations were made with bovine serum albumin and not toxin. If the assumption was made, however, that radiolabeled toxin behaved in the same manner, it would explain why peritoneal doses of toxin were more potent than oral doses. The rats were able to digest and excrete a greater amount of the oral dose than the peritoneal dose of type A botulinal toxin.

Figure 4. Specific counts for urine and feces of rats given oral doses of radiolabeled bovine serum albumin.

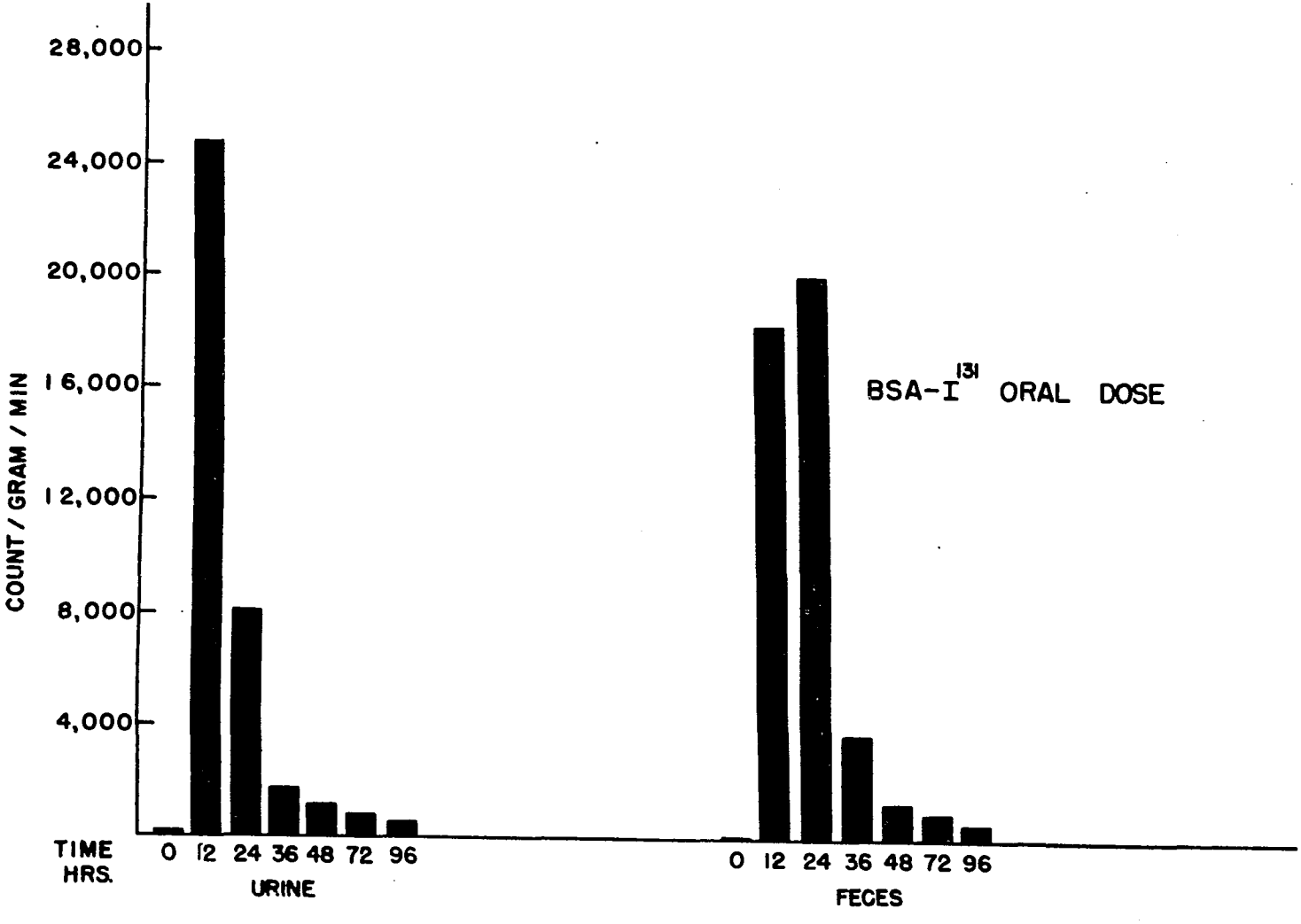
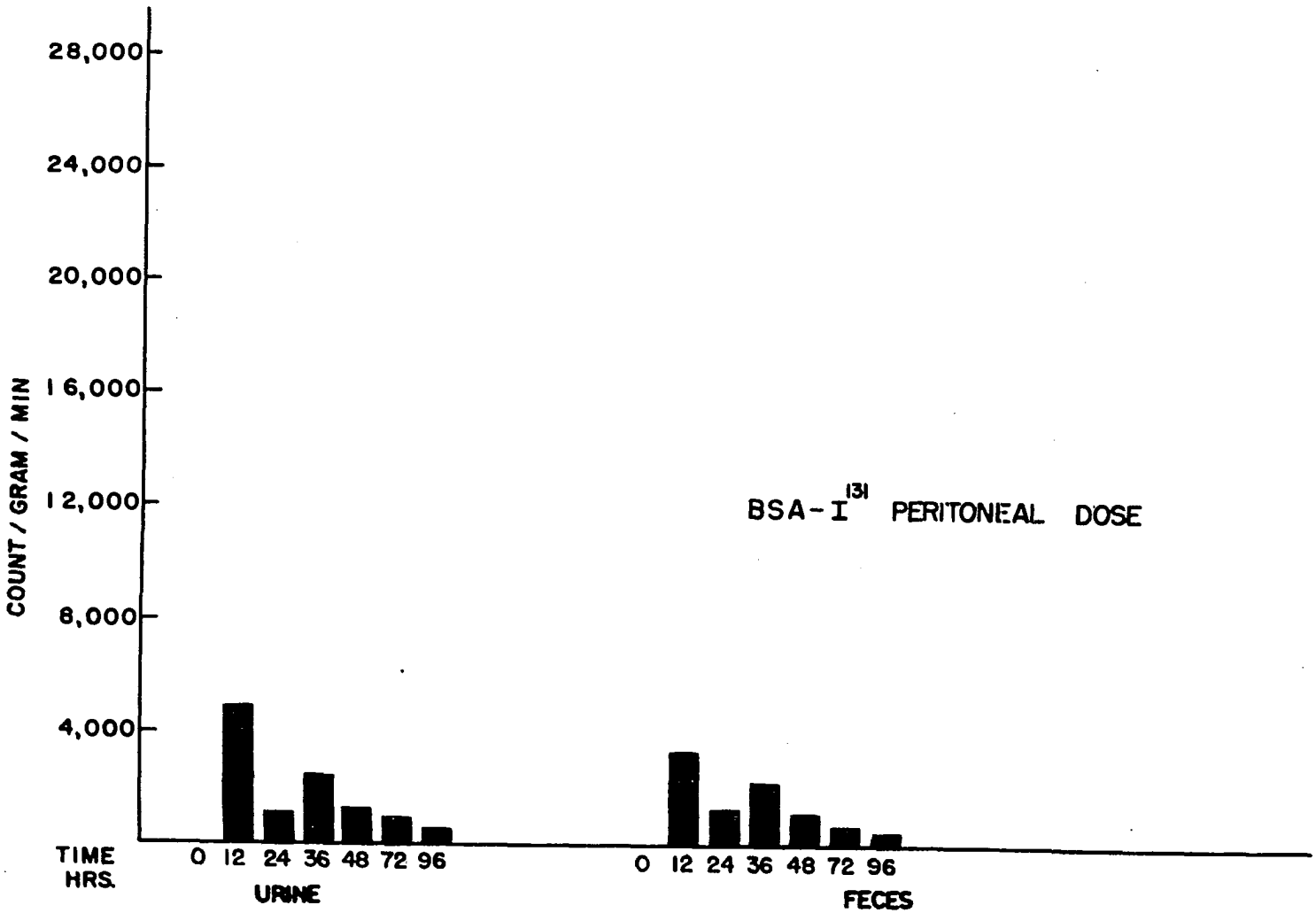

28,000
24,000
20,000
16,000
12,000
8,000
4,000
COUNT / GRAM / MIN
TIME HRS.
0 12 24 36 48 72 96
URINE
0 12 24 36 48 72 96
FECES
BSA-I131 ORAL DOSE

Figure 5. Specific counts for urine and feces of rats given peritoneal doses of radiolabeled bovine serum albumin.


28,000
24,000
20,000
16,000
12,000
8,000
4,000
COUNT / GRAM / MIN
BSA - I 131 PERITONEAL DOSE
TIME HRS.
0 12 24 36 48 72 96
URINE
0 12 24 36 48 72 96
FECES

Toxin Recovery from Tissues

The rats into which the radiolabeled toxin was inoculated died within 6 hours. The results of the experiment are seen in Table 4. Only extracts from one tissue, the blood sample, produced death in mice when inoculated peritoneally. Mice protected with specific antitoxin survived; thus, it was concluded that toxin was present in lethal quantities in the rat's blood. The reason for recovery of biologically active toxin from blood only is not clear. Toxin may have been liberated more easily from the blood because the sample was fluid and therefore much easier to homogenize. The homogenization of tougher tissues may have caused biological inactivation of the toxin. Toxin may not be readily freed from solid tissue as was the case for the fluid blood sample. Dack (1926) observed little toxic activity in the blood of rats which were fed large doses, but he did find toxin in the blood of rats inoculated peritoneally.

The counts for particulate material which settled after homogenization and the first centrifugation, the tricholoroacetic acid precipitated homogenate and the non-precipitated supernatant, suggested that in most of the tissue sampled the largest portion of the radioactivity was found either in the particulate or trichloroacetic acid precipitated material. Possibly, the radioactivity was still bound to

Table 4. Biological activity and distribution of radioactive material from tissue homogenates. All values are averages for two animals

Tissue	Weight in grams	Particular material count	Precipitated material count	Supernatant count	Toxic to mice
Blood	1.388	4180	1400	1840	+
Heart	0.855	830	250	350	-
Lung	1.515	1430	180	860	-
Diaphragm	0.417	5710	220	2740	-
Liver	2.644	850	3040	850	-
Spleen	0.484	110	210	540	-
Kidney	1.048	1510	340	880	-
Stomach	2.719	24250	430	1532	-
Small Intestine	1.244	360	180	1040	-
Spinal Column	1.392	570	120	500	-
Thyroid	0.071	6510	10220	500	-
Lymph Node	0.115	220	110	140	-
Brain	1.617	100	50	90	-
Tail	0.473	3000			-

protein. The small intestine appeared to be an exception to that observation. Most of the radioactivity for this particular tissue was found in the supernatant. The thyroid gland, in which one would expect to find free iodine revealed very little radioactivity in non-precipitated material.

The degree of homogenization depended on the elasticity and toughness of the tissues. Soft tissues like blood, lung, liver, small intestine and brain allowed more complete homogenization than the stomach, spinal column and tail. After several minutes homogenization at top speed on the Sorvall homogenizer these tissues were still in a chunk state instead of a smooth homogeneous mixture. The tail was too tough to be broken up by the homogenizer and thus the entire structure was counted and recorded as particulate count. No tissue extract from the tail was obtained.

Trichloroacetic acid generally causes precipitation of proteins by forming insoluble salts with them (White et al., 1964). This procedure using trichloroacetic acid was designed to show to what extent the radioactivity was associated with precipitatable protein molecules. Assuming that type A botulinal toxin has a molecular weight of 900,000 and is completely precipitated by trichloroacetic acid, the extent of its breakdown into small non-precipitatable products within the tissues sampled could be determined by this procedure. Since the molecular weight of type A toxin is

unknown when circulating throughout an animal, the possibility exists that biologically active toxin was present in the tissues, but was too small to be precipitated with trichloroacetic acid. Wagman (1963) reported a type A botulinal toxin sub-unit with a weight of 3,800 which still possessed biological activity. Heckly et al. (1960) found no evidence that type A botulinal toxin broke down into sub-units in the intestine of a rat. Schantz and Spero (1966) insisted that the weight of type A toxin was 900,000 and the purity of the toxin was not a significant factor in molecular weight determinations. Disagreement about the molecular weight of botulinal toxin has been previously discussed in the literature review.

The radioactive material found in the non-precipitatable samples might have been amino acids and short peptides to which iodine was bound. These materials were probably from the degradation of the botulinal toxin in the rat's body. Ability of the rat to excrete large quantities of radioactive material in the urine indicated that the toxin was being broken down into excretable products.

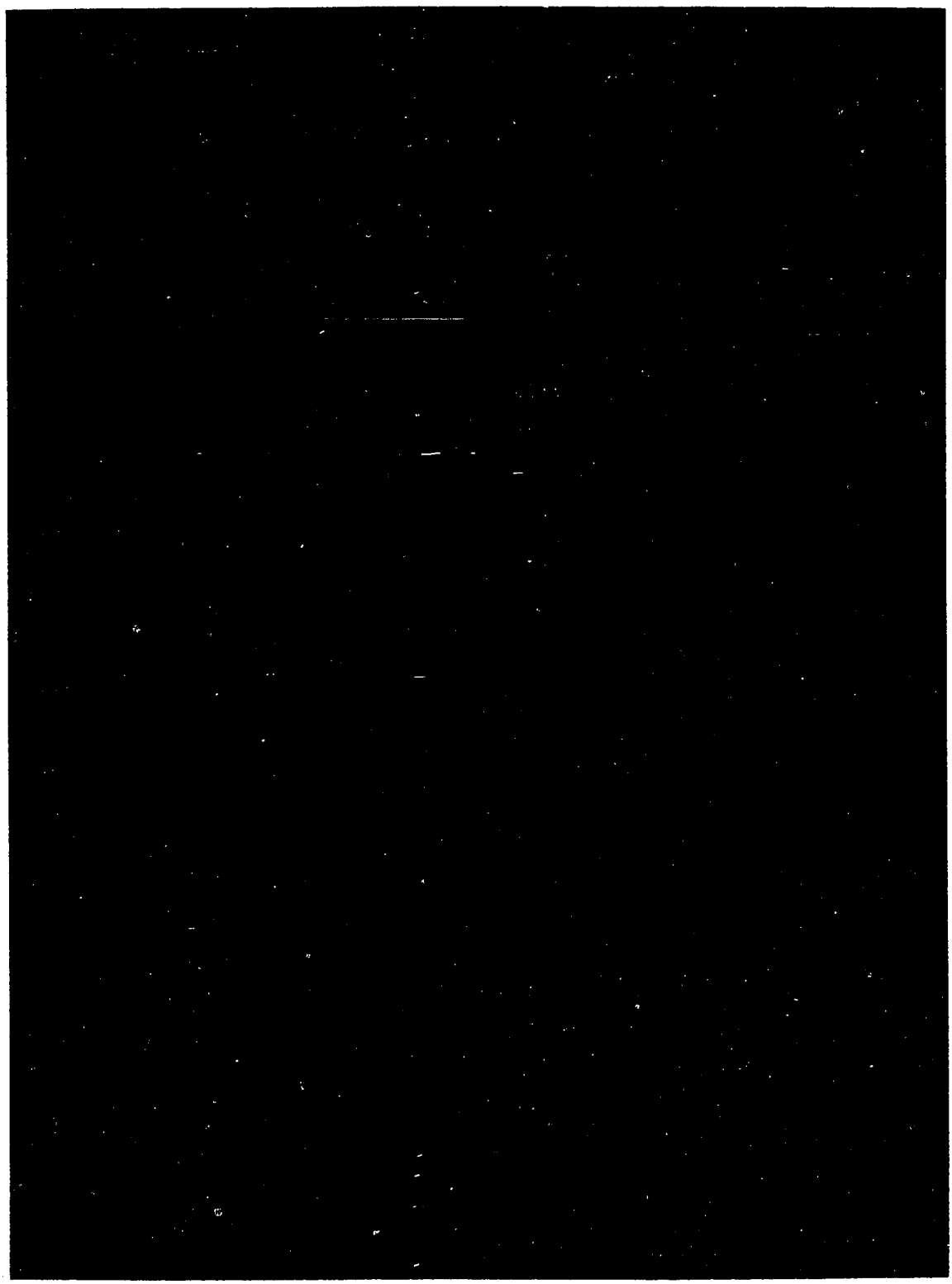
Determination of Purity of Toxin

Gel electrophoresis was used to determine if any major impurities were present in the purified botulinal toxin used in this work. Only one band was visible by this technique

after 18 hours continuous application of electrical potential (Figure 6). The toxic material migrated slowly and was about 2.5 cm from the sample slot after 18 hours. Since only one band was present it was concluded that either no major impurities existed in the toxin sample, or the methods used were not sufficient to promote separation. One of the unexplained aspects of this experiment was the observation that the toxin sample migrated toward the cathode. The isoelectric point for botulinal toxin was reported by Schantz (1964) to be at pH 5.6. At pH values above this, such as the pH of the buffer system used in the electrophoresis (pH 7.8) the toxin molecules should have had a negative charge and migrated towards the anode.

One explanation for the phenomenon is that the material was not toxin but some other protein with different electrical properties. The sample, however, was lethal to mice and type A antitoxin protected the mice. Another suggestion is that the isoelectric point of botulinal toxin is not at pH 5.6 as previously reported, but at a value above pH 7.8. The ionic strength and pH of the buffer system used may have caused the toxin to aggregate or disassociate in such a manner as to change the isoelectric point. Insufficient experimental work was done to legitimately challenge the established isoelectric point of crystalline, type A botulinal toxin and so this argument will not be promoted.

Figure 6. Electrophoresis plate used in toxin purity determination. Black bar indicates point at which sample was applied. One protein band is seen approximately in the center of the slide, 2.5 cm from the sample slot. The electrical poles are indicated on the slide by the symbols + and -.



A third suggestion is that the 0.05 M sodium phosphate buffer at pH 6.8 in which the toxin was lyophilized may interfere with the electrical properties of the toxin. Perhaps some hydrogen ion from the phosphate buffer was able to cling tightly enough to the toxin even in a sodium borate pH 7.8 system to impart a net positive charge to the toxin molecules causing the toxin to migrate towards the anode in a pH 7.8 system.

Autoradiography

Poor results were obtained in the autoradiography experiment. The first animals examined died 18 to 20 hours after ingesting the toxin and the autoradiographs displayed no evidence of localization of radioactivity in any portion of the digestive canal. During a second attempt, large doses of toxin were fed to the rats. These animals died within 7 hours and large amounts of radioactivity were found in the stomach and small intestine. The film was exposed underneath and approximately 2 to 3 cm on either side of the tissues and it was difficult to relate the location of the radioactivity with the location of the parts of the digestive system. This overexposure was caused in part by the long range high energy characteristics of the gamma radiation emitted by iodine 131. Because of the brief period between ingestion of the toxin and death, it could

not be concluded from the autoradiographs that the toxin was actually absorbed into the stomach and intestinal tissues. Examination of the stomach contents of one of these rats after death revealed that much of the peanut butter and bread on which the toxin was mixed prior to ingestion was still in the stomach.

Determination of Radioactive Material in Animal Tissues

All of the distribution patterns were determined with proteins that were inoculated peritoneally into rats. The normal route of entry of toxins and proteins into an animal's body is by the oral route. Nevertheless, this method of inoculation was used because of ease of introducing the radiolabeled material into a number of animals within a short period of time and of assurance that equal volumes of protein were received by each animal. Feeding radiolabeled proteins to rats was unsatisfactory because 14 rats would not eat equal amounts of material simultaneously. Often one or more hours elapsed between the time that the animals received the sample until all had consumed it. Introducing radiolabeled material into rats by stomach tube sometimes allowed a portion of the sample to be forced back up the esophagus and into the trachea and lungs. In addition, this method was found to be much too slow when large numbers of rats were inoculated.

To insure survival of all animals through the 48th hour of sampling a sublethal dose of toxin was given. Twice the amount of toxin given to the rats in this experiment constituted a lethal dose.

The total amount of radioactive material localized per gram of tissue during the seven sampling periods can be seen in Table 5. The group of rats receiving bovine serum albumin had high total localization of radioactivity in the thyroid gland, intestine, blood and diaphragm. Lesser amounts of radioactivity were accumulated in the spleen, kidney, stomach, lung, heart and tail. The tissues which accumulated the least radioactivity were the spinal column and brain. Radiolabeled rat serum albumin was found to localized in relatively high concentrations in the thyroid, stomach, kidney, spleen, blood, diaphragm and intestine. Less total localization was found in the liver, tail, heart and lung. Little accumulation of activity was found in the spinal column, lymph node and brain. The rats into which radioactive toxin was inoculated localized high amounts of activity in the thyroid, stomach, diaphragm, blood and spleen. The kidney, tail, liver, heart, spinal column and lung had less total accumulation. Localization of radioactive material was slight in the lymph node and brain. Passively immunized rats into which toxin was injected had high total localization of radioactivity in the thyroid,

Table 5. Total specific counts for tissues

Tissue	Bovine Serum Albumin	Rat Serum Albumin	Toxin	Toxin + Antitoxin
Blood	112154	63485	54348	51815
Heart	56406	35391	18754	17017
Lung	60359	34907	12190	10074
Diaphragm	88830	61912	88192	29966
Liver	44722	44671	26581	20566
Spleen	67805	66464	39872	26335
Kidney	63664	80460	38940	10290
Stomach	58225	100465	95971	72557
Intestine	115694	49591	47217	18628
Spinal Column	7477	8313	16765	15017
Lymph Node		5327	4950	7453
Thyroid	1669859	2716051	2076360	1602618
Brain	3852	2943	1604	1411
Tail	28229	41316	32169	31324

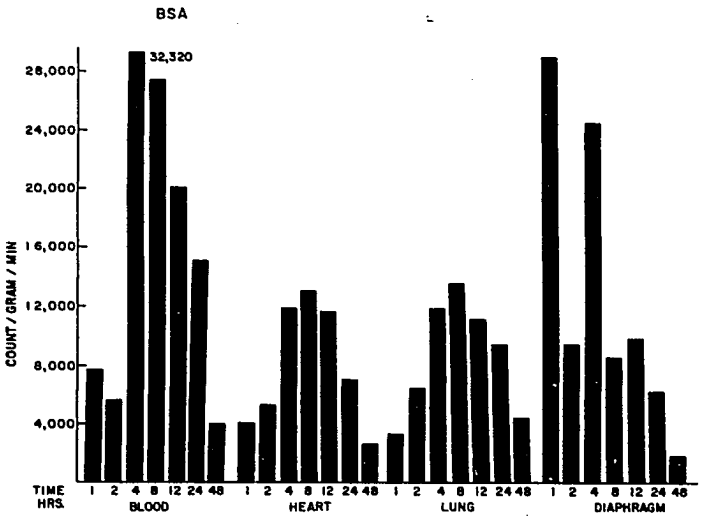
stomach, blood, tail, diaphragm and spleen. Less activity was accumulated in the liver, intestine, heart, spinal column, kidney and lung. Small amounts of radioactive material were found in the lymph node and brain.

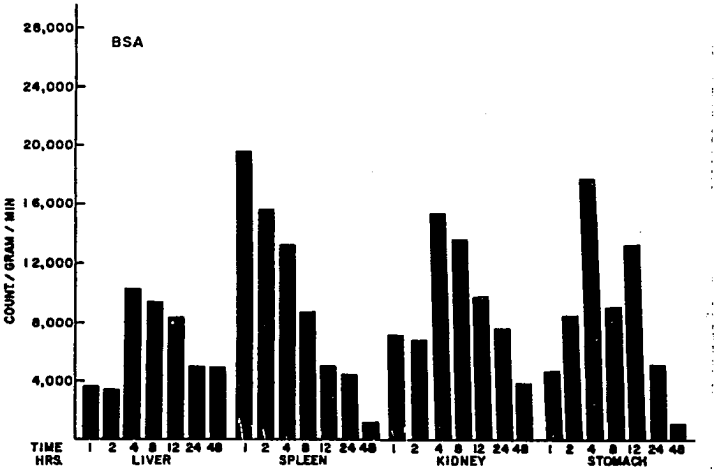
Radioactivity from the non-toxic and toxic proteins localized to a high degree in the thyroid, blood and diaphragm. Although localization was less pronounced for the group of rats receiving bovine serum albumin, all groups accumulated relatively high total specific counts in the stomach, spleen, intestine and tail. Very little localization occurred in the lymph node or brain with any of the groups.

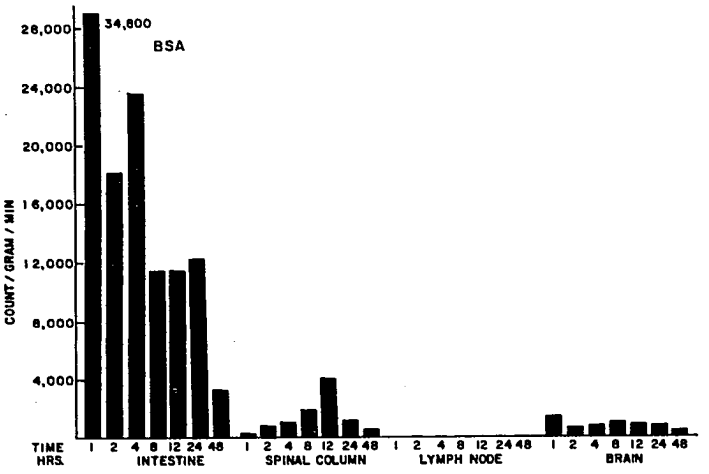
The main observable effect of passive immunity was the markedly reduced magnitude of the count in tissues such as the stomach, spleen, intestine diaphragm and thyroid over that observed for the non-immune rats which received only toxin. Data on excretion by immune and non-immune animals may partially explain this observation. The values reported by Kirilenko et al., 1965 for radiolabeled tetanal toxin in tissues were similarly reduced when animals were passively immunized prior to injection of toxin.

The distribution patterns shown in Figures 7 through 14 display many similarities among the four groups. The diaphragm and spleen in the four treatment groups have similar distribution patterns. The highest specific count for these tissues was observed at the one hour sampling period and the counts decreased with each successive sampling.

Figure 7. Tissue distribution patterns for rats receiving bovine serum albumin.


BSA
32,320
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
BLOOD
HEART
LUNG
DIAPHRAGM


BSA
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
LIVER
SPLEEN
KIDNEY
STOMACH


34,800
BSA
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
INTESTINE
SPINAL COLUMN
LYMPH NODE
BRAIN

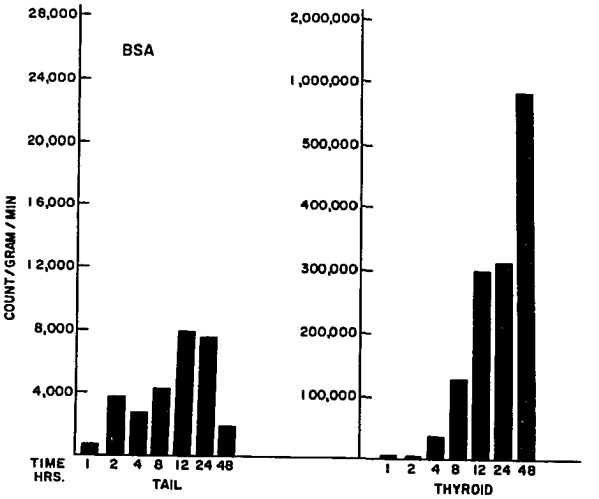
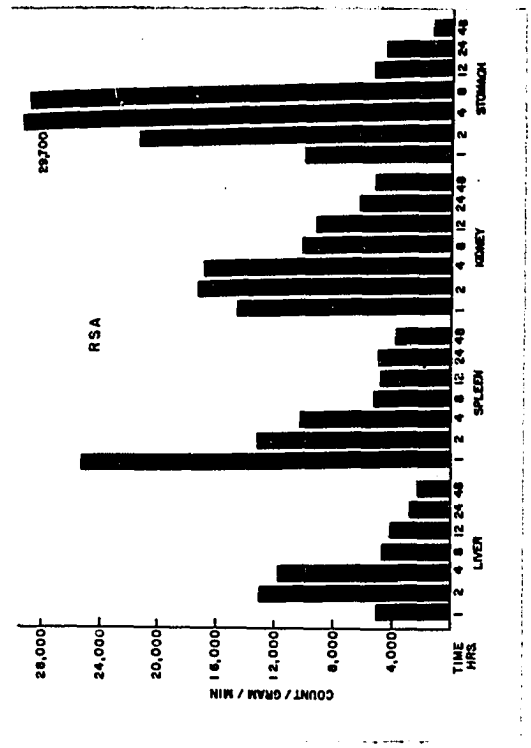
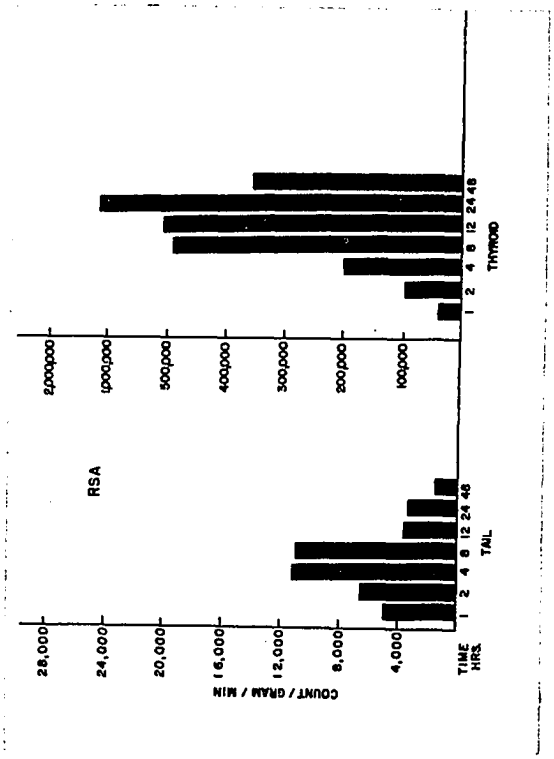
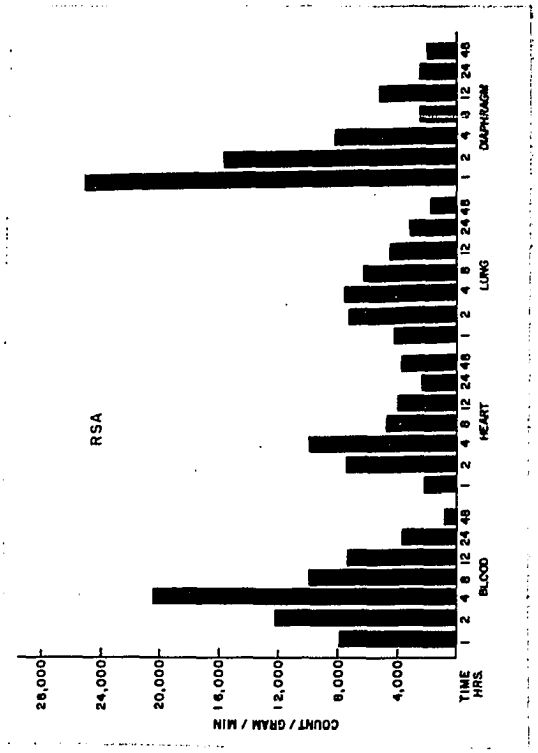

BSA
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
2,000,000
1,000,000
500,000
400,000
300,000
200,000
100,000
TIME HRS.
1 2 4 8 12 24 48
TAIL
THYROID

Figure 8. Tissue distribution patterns for rats receiving rat serum albumin.


RSA
COUNT / GRAM / MIN
TIME HRS
LIVER
SPLEEN
KIDNEY
STOMACH
23,700


RSA
COUNT / GRAM / MIN
TIME HRS
TAIL
THYROID


RSA
COUNT / GRAM / MIN
TIME HRS
BLOOD
HEART
LUNG
DIAPHRAGM

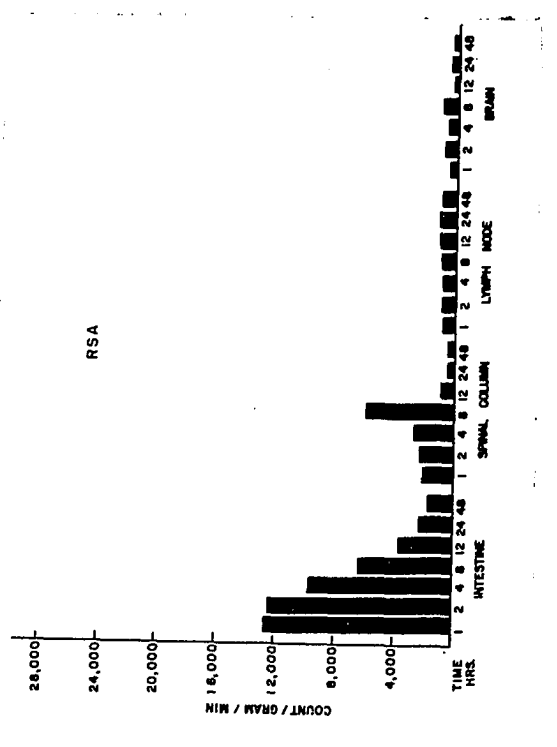
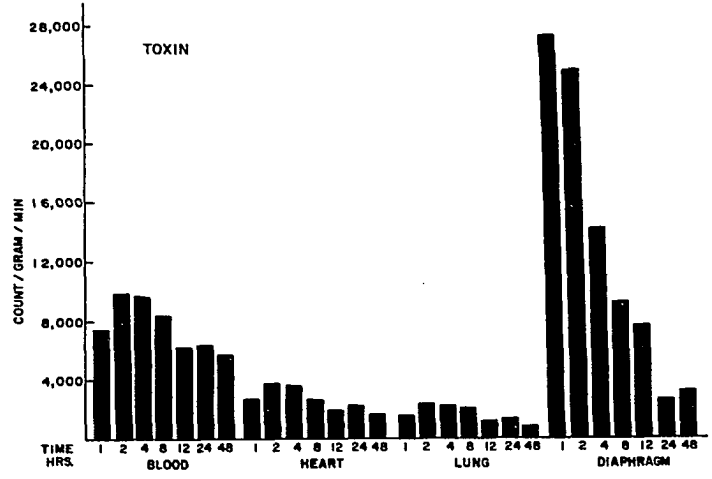
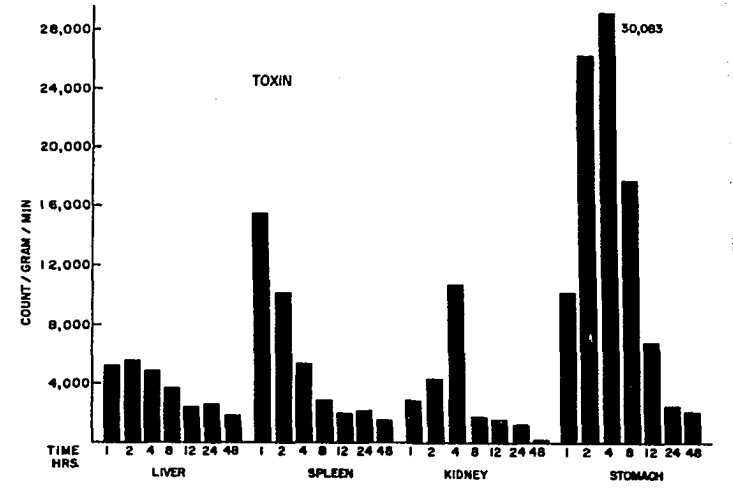
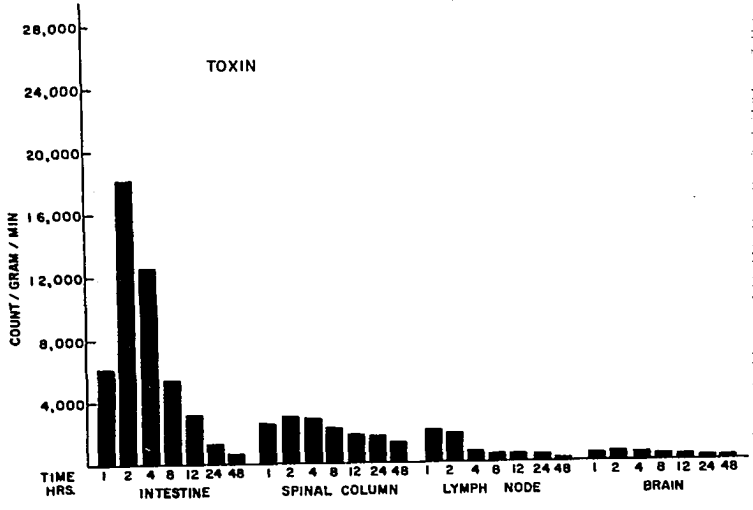

RSA
COUNT / GRAM / MIN
TIME HRS
INTESTINE
SPINAL COLUMN
LYMPH NODE
BRAIN

Figure 9. Tissue distribution patterns for rats receiving botulinal toxin.


TOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
BLOOD
HEART
LUNG
DIAPHRAGM


TOXIN
30,083
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
LIVER
SPLEEN
KIDNEY
STOMACH


TOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
INTESTINE
SPINAL COLUMN
LYMPH NODE
BRAIN

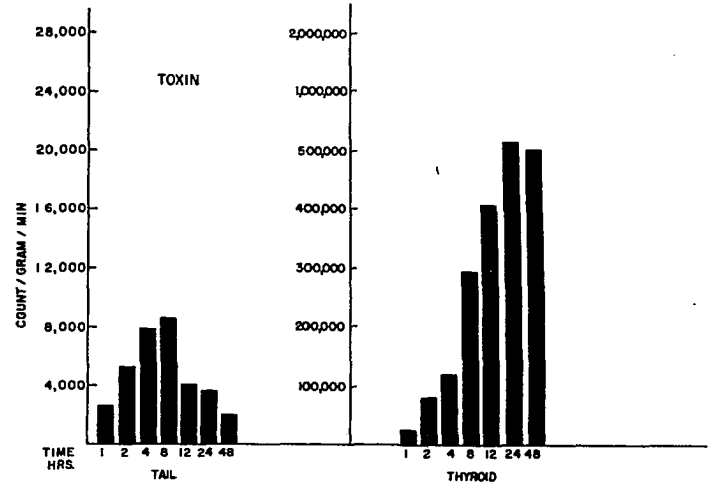
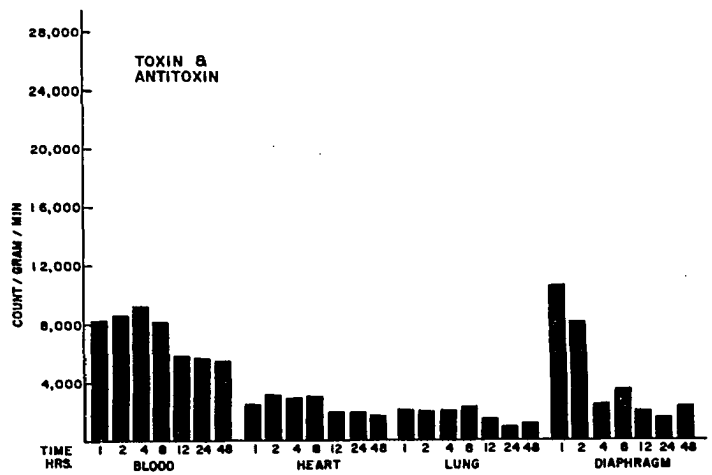
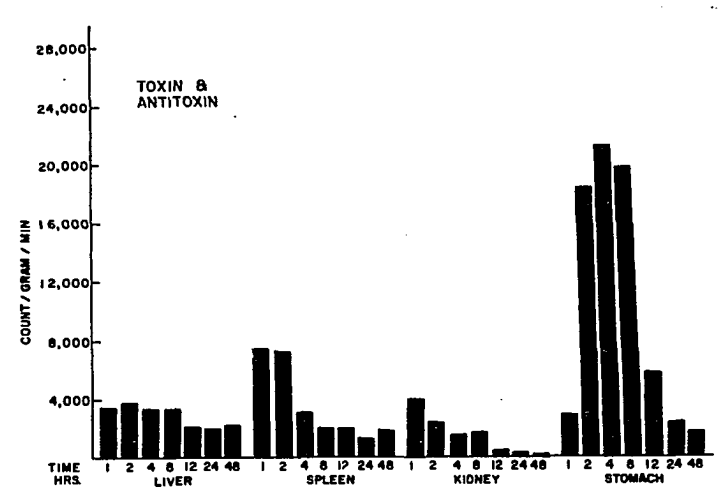
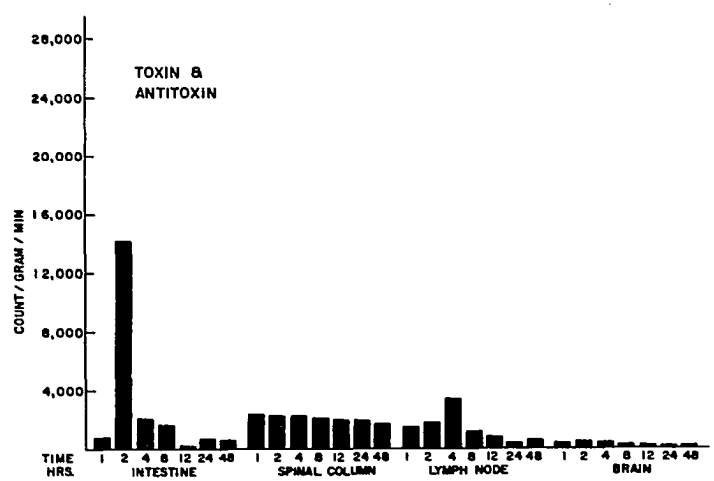

TOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
2,000,000
1,000,000
500,000
400,000
300,000
200,000
100,000
TIME HRS.
1 2 4 8 12 24 48
TAIL
THYROID

Figure 10. Tissue distribution patterns for rats receiving botulinal toxin plus specific antitoxin.


TOXIN &
ANTITOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME
HRS.
1 2 4 8 12 24 48
BLOOD
HEART
LUNG
DIAPHRAGM


TOXIN &
ANTITOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME
HRS.
1 2 4 8 12 24 48
LIVER
SPLEEN
KIDNEY
STOMACH


TOXIN &
ANTITOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME
HRS.
1 2 4 8 12 24 48
INTESTINE
SPINAL COLUMN
LYMPH NODE
BRAIN

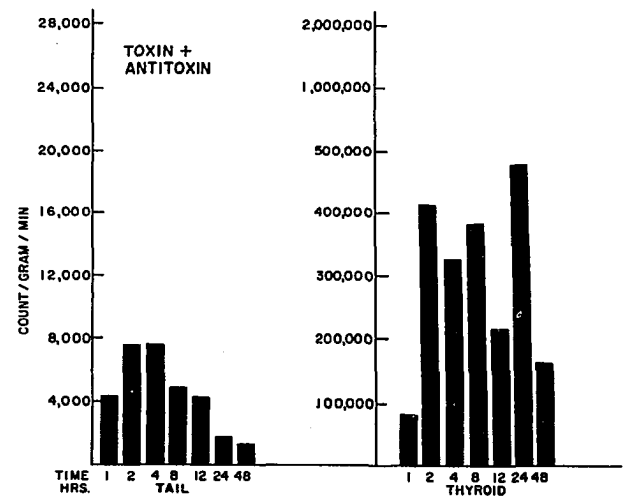
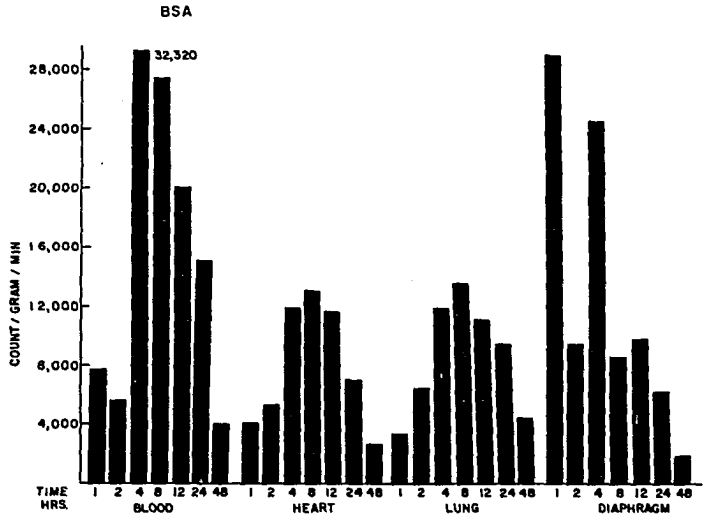
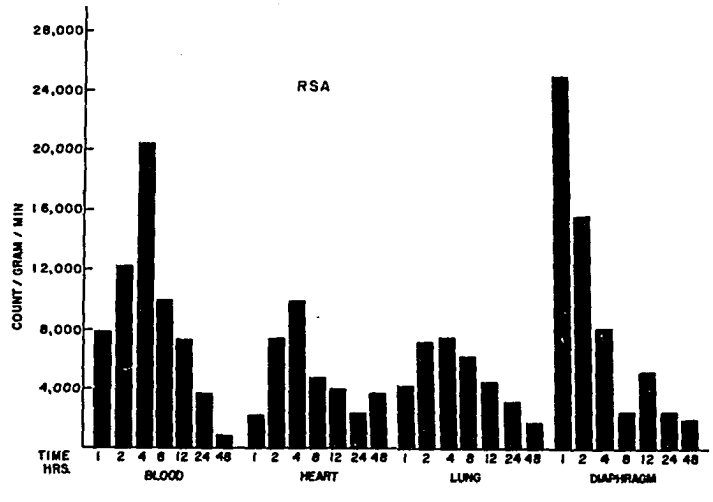
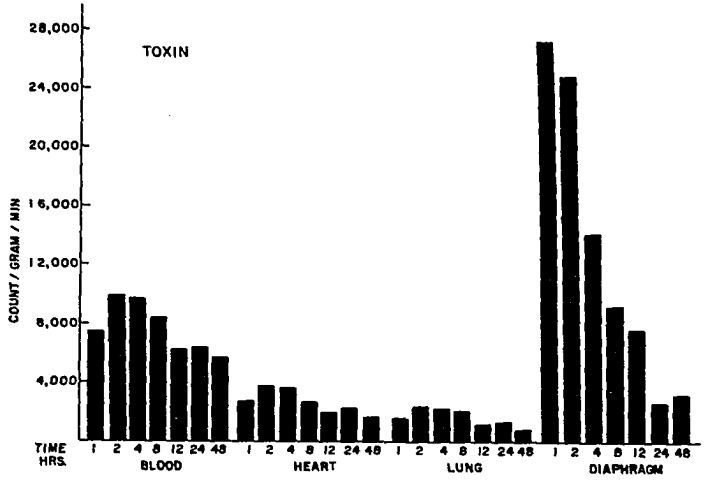

TOXIN +
ANTITOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
2,000,000
1,000,000
500,000
400,000
300,000
200,000
100,000
TIME
HRS.
1 2 4 8 12 24 48
TAIL
THYROID

Figure 11. Comparison of distribution patterns for the blood, heart, lung and diaphram of rats receiving bovine serum albumin, rat serum albumin, toxin and toxin plus antitoxin.


BSA
32,320
28,000
24,000
20,000
16,000
12,000
8,000
4,000
COUNT / GRAM / MIN
TIME HRS.
1 2 4 8 12 24 48
BLOOD
HEART
LUNG
DIAPHRAGM


RSA
28,000
24,000
20,000
16,000
12,000
8,000
4,000
COUNT / GRAM / MIN
TIME HRS.
1 2 4 8 12 24 48
BLOOD
HEART
LUNG
DIAPHRAGM


TOXIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
COUNT / GRAM / MIN
TIME HRS.
1 2 4 8 12 24 48
BLOOD
HEART
LUNG
DIAPHRAGM

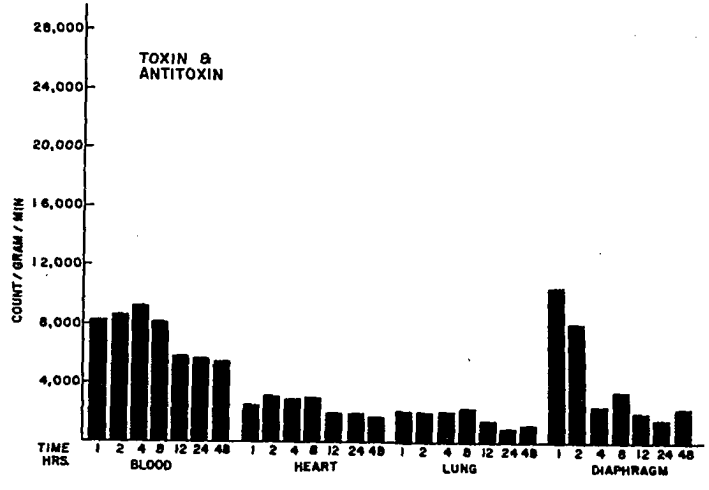
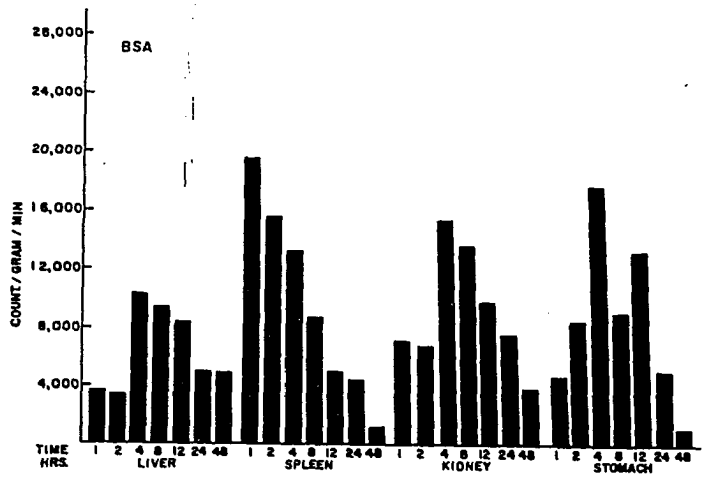
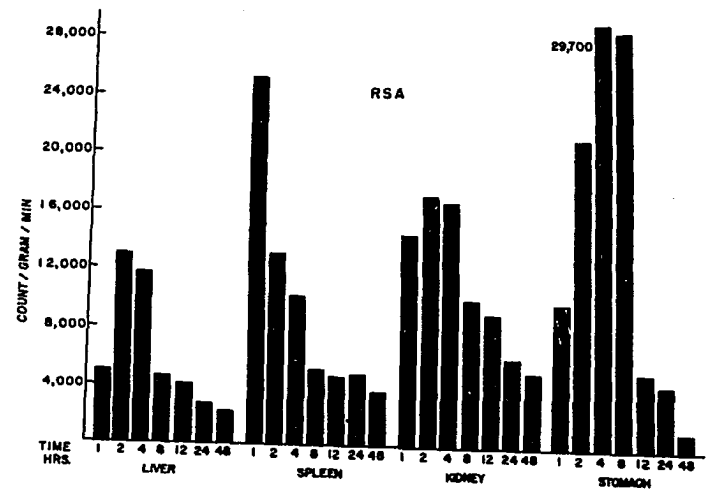
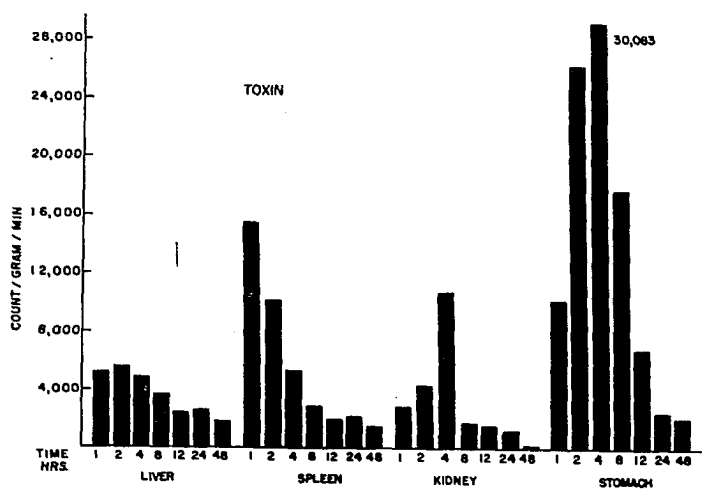

TOXIN &
ANTITOXIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
COUNT / GRAM / MIN
TIME HRS.
1 2 4 8 12 24 48
BLOOD
HEART
LUNG
DIAPHRAGM

Figure 12. Comparison of distribution patterns for the liver, kidney, spleen and stomach of rats receiving bovine serum albumin, rat serum albumin, toxin and toxin plus antitoxin.


BSA
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
LIVER
SPLEEN
KIDNEY
STOMACH


RSA
29,700
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
LIVER
SPLEEN
KIDNEY
STOMACH


TOXIN
30,083
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
LIVER
SPLEEN
KIDNEY
STOMACH

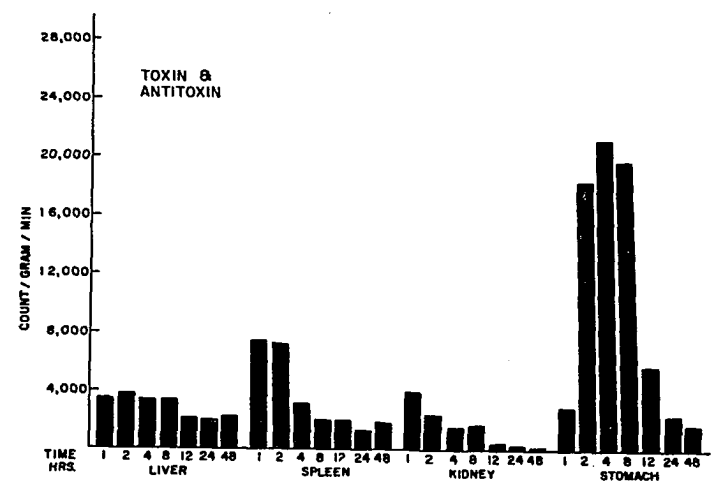

TOXIN &
ANTITOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
TIME HRS.
1 2 4 8 12 24 48
LIVER
SPLEEN
KIDNEY
STOMACH

Figure 13. Comparison of distribution patterns for the intestine, spinal column, lymph node and brain of rats receiving bovine serum albumin, rat serum albumin, toxin and toxin plus antitoxin.

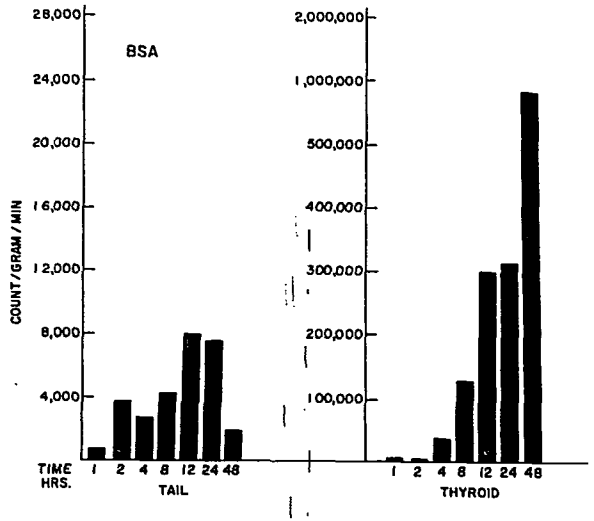
COUNT / GRAM / MIN
4,000
8,000
12,000
16,000
20,000
24,000
28,000
34,800
TIME HRS.
1 2 4 6 12 24 48
INTESTINE
SPINAL COLUMN
LYMPH NODE
BRAIN
BSA

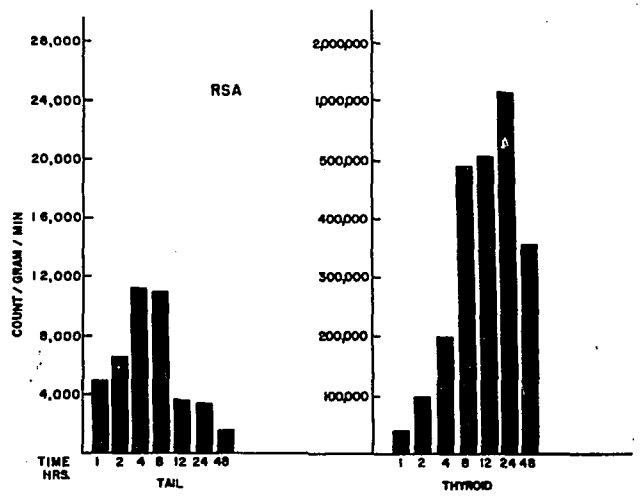
COUNT / GRAM / MIN
4,000
8,000
12,000
16,000
20,000
24,000
28,000
TIME HRS.
1 2 4 6 12 24 48
INTESTINE
SPINAL COLUMN
LYMPH NODE
BRAIN
TOXIN

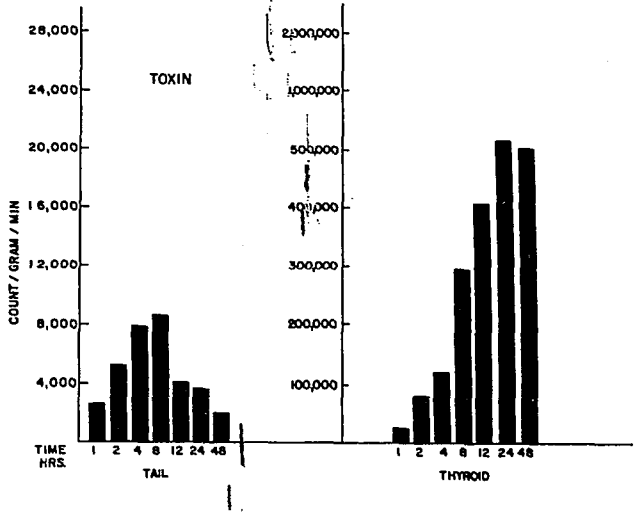
COUNT / GRAM / MIN
4,000
8,000
12,000
16,000
20,000
24,000
28,000
TIME HRS.
1 2 4 6 12 24 48
INTESTINE
SPINAL COLUMN
LYMPH NODE
BRAIN
RSA

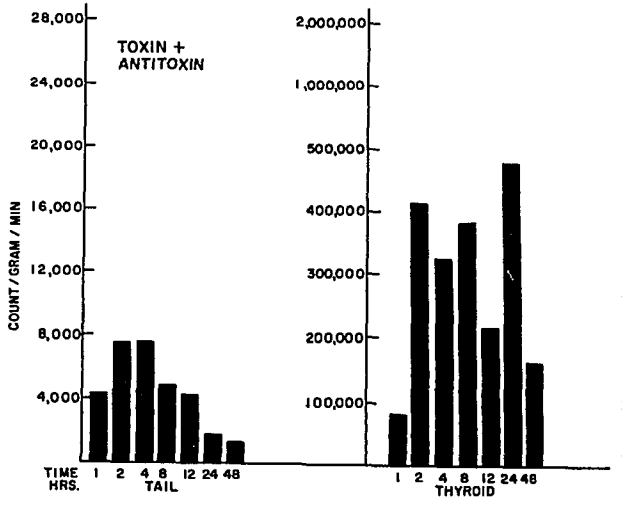
COUNT / GRAM / MIN
4,000
8,000
12,000
16,000
20,000
24,000
28,000
TIME HRS.
1 2 4 6 12 24 48
INTESTINE
SPINAL COLUMN
LYMPH NODE
BRAIN
TOXIN &
ANTITOXIN

Figure 14. Comparison of distribution patterns for the tail and thyroid of rats receiving bovine serum albumin, rat serum albumin, toxin and toxin plus antitoxin.


BSA
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
2,000,000
1,000,000
500,000
400,000
300,000
200,000
100,000
TIME HRS.
1 2 4 8 12 24 48
TAIL
THYROID


RSA
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
2,000,000
1,000,000
500,000
400,000
300,000
200,000
100,000
TIME HRS.
1 2 4 8 12 24 48
TAIL
THYROID


TOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
2,000,000
1,000,000
500,000
400,000
300,000
200,000
100,000
TIME HRS.
1 2 4 8 12 24 48
TAIL
THYROID


TOXIN + ANTITOXIN
COUNT / GRAM / MIN
28,000
24,000
20,000
16,000
12,000
8,000
4,000
2,000,000
1,000,000
500,000
400,000
300,000
200,000
100,000
TIME HRS.
1 2 4 8 12 24 48
TAIL
THYROID

This same general pattern was observed for the intestine in the groups of rats inoculated with radioactive serum albumin. The intestines of rats receiving toxin did not contain high amounts of radioactivity in the first sampling period, but the activity rose rapidly to a peak at 2 hours. The counts for each successive time period were reduced respectively. Blood, heart, lung, liver, stomach and tail displayed similar distribution patterns for all rats. Radioactive counts generally increased to a peak value at either 2 or 4 hours then declined during successive sampling periods. This pattern was duplicated for the kidney and spinal column in all but the group receiving antitoxin and toxin. In the passively immunized group of rats the highest degree of localization in these tissues occurred during the first hour and the count declined with successive sampling periods. The concentration of radioactive material localized in the thyroid gland increased steadily through the 24 hour sample and through the 48 hour for the rats receiving bovine serum albumin. The general distribution patterns were similar for all rats in all groups of rats.

Significance of Localization Patterns in Rat Tissues

Caution must be observed in interpreting data from this type of experiment. Biological variability is a major source of error when working with biological systems. Occasionally

identical organs taken from a pair of rats in a sampling period varied greatly in the amount of radioactivity localized within them. The animals were simultaneously inoculated peritoneally and killed at the same time. The entire organ was removed and in most cases the weights were nearly equal, yet one organ would demonstrate a high count while the other had a low count. Since only two rats were used in each sampling period an insufficient number of samples was available to calculate an estimation of variance. The counts reported on the bar graphs were averages which were greatly influenced by unusually high or low tissue radioactivity in a sampling period. The variations in specific count for a tissue where one count dropped below the values on either side of it as observed in Figure 7 is an example of the effect of biological variability.

Counting error is another factor which must be considered. Nuclear decay is a random process and a sample will rarely yield the same value when counted repeatedly for equal time periods. Long counting periods reduce the magnitude of error. Standardization of counting techniques allowed this type of error to become relatively constant which theoretically subjected all data to the same general magnitude of error.

The specific count for organs or tissues with high radioactive content and low weight, such as the thyroid

gland, were effected greatly by weight variations. The specific count for a thyroid gland could be reduced by 100% by changing the weight from 0.05 grams to 0.10 grams. Since the thyroid in a rat is very small, the larynx-thyroid complex was removed and counted as a unit. An additional 0.05 gram of tissue could easily have been carried along with the thyroid gland and caused this type of error.

When protein materials are peritoneally inoculated into an animal, they gain entry into the general circulation by way of the lymphatics draining into the thoracic duct which in turn drains into the subclavian vein (Raffel, 1961). The blood samples contained relatively high amounts of radioactivity shortly after peritoneal inoculation of the rat. Since the proteins were inoculated into the peritoneal cavity, an initially high concentration of radioactivity might be expected to be associated with the intestine because of adsorption of radioactive material onto this tissue at the injection site. This author cannot explain why rats which received toxin did not localize high amounts of radioactivity in the intestine during the first hour as did animals receiving serum albumin. Relatively high amounts of radioactive protein localized in the spleen within the first hour in all treatments. The spleen is one of the main filters in the circulatory system and is important in destruction of foreign material such as bacteria and worn

out blood cells (Romer, 1962). This may explain the initially high concentration of activity in this tissue. The reason for the diaphragm following this same pattern is not clear, but it's location in the body cavity may have permitted absorption of large amounts of peritoneally injected protein within the first hour. Large amounts of radioactive material located in the tissues of the stomach in many of the rats. Again the reasons for concentration of radioactivity in this tissue and not in the surrounding tissues is not understood. Large amounts of radioactivity were observed in the tip of the tail. This structure is composed mostly of bone and collagen (Greene, 1935). Perhaps the combination of material was effective in retaining radioactive proteins or their breakdown products. The highest specific count for any tissue was obtained for the thyroid glands in the rats. An examination of the bar graphs reveal that counts in excess of 500,000 were obtained for these glands. The patterns were generally similar in the fours treatments with a steadily rising count through the 24th hour. This tissue has an affinity for accumulating iodine (Romer, 1962). As the protein material broke down in the animal the iodinated products including amino acids and short peptides apparently localized in the thyroid. Very little radioactivity was found in either the brain or lymph nodes of the rats sampled. The radiolabeled proteins

either did not enter or were not retained in these tissue to any great extent.

The distribution patterns obtained for the toxic and non-toxic proteins appear to have more similarities than differences. Bovine and rat serum albumins, although the former is foreign while the latter is native to the rat, displayed almost identical distribution patterns. These in turn were similar to the patterns obtained for sublethal doses of toxin. The specific effect of passive immunity was a reduction of specific count values for some tissues over the counts obtained for the same tissues in rats receiving only toxin. The patterns obtained for immune animals retained many similarities to the other distribution patterns. No outstanding differences existed between the distribution patterns observed for toxin and nontoxic albumins. Other methods of inoculation such as intravenous, intramuscular, or oral may have produced distribution patterns differing from the ones observed for peritoneally inoculated proteins.

Alternate methods of radiolabeling might be more suitable for this type of study. For example, botulinal toxin with carbon 14 integrated into the carbon chain structure might be used to determine the fate of radio-labeled botulinal toxin within the animal body. With the radiolabel being an integral part of the toxin molecule

there would be no concern about the effect of adding iodine to toxin, or to the tightness of the bond between iodine and toxin once the protein was introduced into the animal.

CONCLUSIONS

1. Purified botulinal toxin can be radiolabeled with iodine 131 using isotopic exchange methods. The biological activity of the toxin, however, was reduced by a factor of 10^3 by the radiolabeling procedure.

2. The distribution of botulinal toxin within animals into which this material was inoculated was found not to be unique to the toxin, but similar to distribution patterns obtained for nontoxic serum proteins. Large amounts of radioactivity were found in the thyroid, blood, diaphragm, stomach, spleen, intestine and tail of most rats in all groups. Very little localization occurred in the lymph node or brain in any of the rats.

The main effect of passive immunity was a reduction in the degree of localization of radiolabeled toxin in the stomach, spleen, diaphragm, intestine and thyroid of the rats. Only the magnitude of localization, not the distribution patterns, was effected appreciably by passive immunization.

3. Large amounts of orally fed radiolabeled botulinal toxin were excreted by rats within 48 hours. Passively immune rats excreted in 24 hours the same amount of radioactive material excreted in 48 hours by non-immune rats. Orally fed, radiolabeled protein is excreted more rapidly by rats than the same material inoculated intraperitoneally.

SUMMARY

Botulinal toxin type A was obtained in pure form from Dr. Edward Schantz. The toxin was radiolabeled with iodine 131 using an isotopic exchange reaction. A small crystal of elemental iodine was added to a solution of sodium iodide 131 and allowed to react for 20 minutes. Free iodine 131 was obtained in the solution which was then added to the toxin; the mixture of toxin and radioactive iodine was incubated at 4 C for 4 hours. The unbound iodine was removed by filtration through Sephadex G50. Bovine serum albumin and rat serum albumin were also labeled by this procedure.

Radiolabeled toxin was fed to non-immune and passively immunized rats and their excretory products were collected and counted at 12 hour intervals for a period of 48 hours. Excretory rates were reported in terms of percent of the total ingested radioactive material eliminated by the rats during each 12 hour period. Passive immunity apparently speeds up the rate of excretion of toxin. A large percentage (70% to 73%) of the radioactive material was excreted by the passively immunized animals within 24 hours; in non-immune animals, the time required for elimination of a similar amount was 48 hours.

Distribution patterns were established for radiolabeled bovine serum albumin, rat serum albumin, botulinal toxin type A and botulinal toxin type A given to animals which were

passively immunized with type A antiserum. Fourteen rats were used in each distribution pattern determination. The animals were inoculated intraperitoneally with the radio-labeled material and two animals were sacrificed at each of the following time periods: 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 24 hours and 48 hours after injection. The following tissues were removed, weighed and analyzed for radioactivity: brain, lymph node in neck, thyroid gland, blood, heart, lung, diaphragm, liver, kidney, spleen, stomach, intestine, spinal column and tip of the tail. These tissues were analyzed for radioactivity in a Picker Autowell II gamma radiation counter. Tissue counts were corrected for decay factor and divided by the weight of the tissue to obtain the count per minute per gram of tissue. The counts were treated statistically to enable direct comparisons of the specific counts for the tissues. The distribution patterns obtained for the groups of rats were generally very similar. Large amounts of radioactivity was found in the thyroid, blood, diaphragm, stomach, spleen, intestine and tail of most rats. The main effect of passive immunity was to reduce the amount of localization of radio-labeled toxin in the stomach, spleen, intestine and thyroid of the rats.

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