FATE OF PARTIALLY PURIFIED C^{14}-LABELED TOXIN OF *CLOSTRIDIUM PERFRINGENS*

PAUL D. ELLNER[1]

Department of Microbiology, College of Medicine, University of Florida, Gainesville, Florida

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ABSTRACT

ELLNER, PAUL D. (University of Florida, Gainesvillle). Fate of partially purified C^{14}-labeled toxin of *Clostridium perfringens*. J. Bacteriol. **82**:275–283. 1961.—A study was made of the fate of *Clostridium perfringens* toxin in susceptible animals. Labeled toxin was prepared by growing *C. perfringens* type A in a complex casein hydrolyzate medium containing a tryptic hydrolyzate of C^{14}-labeled algal protein. The toxin was purified about 30-fold (in terms of lecithinase activity) by ammonium sulfate and acetone precipitations. The tagged toxin was injected intravenously into mice and rabbits, and the disappearance from the blood stream, deposition in organs and appearance in urine and expired air determined by measurement of radioactivity. Experimental data showed that toxin disappears rapidly from the bloodstream following intravenous injection, with radioactivity appearing in the urine and expired air shortly thereafter (10 to 20 min). The organs primarily responsible for the uptake of toxin from the blood are the liver (72%), lungs (15%), kidney (8%), and spleen (5%). The toxin is not bound to skeletal muscle. Fractionation of the liver into subcellular particles by centrifugation showed the radioactivity to be concentrated in the mitochondrial fraction. These experiments indicate that the toxin is rapidly removed from the circulating blood, is metabolized, and breakdown products excreted in the urine, with 1 carbon fragments eliminated as CO_2 in the expired air.

The lecithinase or α-toxin of *Clostridium perfringens* is generally considered to be the most significant factor in the pathogenesis of toxemia caused by type A strains of this organism. Detailed studies of the fate of this toxic enzyme have been hampered by technical difficulties concerned primarily with the purification of lecithinase, which in turn has been hindered by the relatively poor yields of toxin obtained in culture. Some of the factors influencing toxin production have been studied (Adams, Hendee, and Pappenheimer, 1947; Jayko and Lichstein, 1959) and several methods for the partial purification of lecithinase have been described (Van Heyningen and Bidwell, 1948; Roth and Pillemer, 1953; Habermann, 1959).

A number of studies have been made of the pathological changes induced by the toxin with relatively impure preparations (Zamecnik, Nathanson, and Aub, 1947; Berg, Levinson, and Wang, 1951, Furr et al., 1952; Aikat and Dible, 1956). The over-all purpose of the current study was to investigate the fate of purified *C. perfringens* lecithinase in susceptible and immune animals by employing labeled toxin. The present paper describes studies with partially purified toxin labeled with C^{14}.

MATERIALS AND METHODS

Ten strains of *C. perfringens* type A were tested for lecithinase activity. These included a freshly isolated strain (HS-1) as well as strains obtained from various sources (S-107, 3895, 5053, Medical Research Council, London; 146, 814, L. S. McClung, Indiana University; AGS, PB6K, University of Maryland School of Medicine; SR-12, H. Noyes, Walter Reed Army Institute of Research). All strains were streaked for purity on half-antitoxin plates of lactose-egg-yolk-milk agar (Willis and Hobbs, 1959) and were maintained in the spore stage at 5 C in spore medium (Ellner, 1956) rather than by daily transfer. Strain 814 produced the highest yield of lecithinase and was employed for all subsequent studies.

Inoculum. The inoculum was prepared from spore stock for each individual batch of toxin by inoculating tubes of thioglycolate broth which

[1] Present address: Department of Medical Microbiology, College of Medicine, The University of Vermont, Burlington, Vermont.

had been steamed for 10 min and cooled just prior to inoculation. Growth was apparent after about 18 hr at 37 C and 1 ml was used to inoculate bottles containing 150 ml of trypticase-soy broth. The bottles of broth were incubated for 8 hr and then used to inoculate large batches of toxin medium.

Medium. The composition of the medium employed for toxin production is given in Table 1. The pH was adjusted to 4.0 with HCl and the medium placed in a boiling water bath for 90 min. After cooling the pH was adjusted to 7.6 with 5 N NaOH, and the medium inoculated immediately. The type of casein hydrolyzate used was found to be of considerable importance since significant variations in toxin production occurred when other casein hydrolyzates were employed. The use of maltose as a carbohydrate source was found to result in equivalent yields of toxin as compared with starch, dextrin, or amylopectin. Maltose was chosen since it is dialyzable.

Production of C^{14}-labeled toxin. Labeled algal protein was obtained by growing *Chlorella pyrenoidosa* with $C^{14}O_2$ as the sole carbon source (Ellner, 1959). The protein fraction of the algae was suspended in phosphate buffer pH 8.5 and trypsin added. The mixture was maintained for 24 hr at 37 C with occasional additions of NaOH to maintain the pH. Ten per cent trichloroacetic acid was added to precipitate the residual protein and the enzyme, and after removal of the precipitate by centrifugation, the supernatant fluid was treated with ether to remove the trichloroacetic acid. The residual ether was removed in a flash evaporator and the supernatant fluid added to the toxin medium prior to sterilization. After sterilization and adjustment of the pH, the toxin medium was inoculated and incubated at 37 C for 12 hr.

Partial purification of toxin After removal of the bacterial cells by centrifugation, the crude toxin was precipitated from the supernatant fluid by the addition of solid $(NH_4)_2SO_4$ to 50% saturation. The crude toxin rose to the top as a scum which was skimmed off after standing several hours at 5 C. The crude toxin was immediately dissolved in ice cold water, disodium ethylenediaminetetraacetate (EDTA) added to 0.005 M, and the solution dialyzed overnight at 5 C against 0.03 M sodium phosphate buffer, pH 6.0. The dialyzed solution was chilled in an ice bath and 2 volumes of acetone cooled to −15 C added slowly with stirring. The mixture was stored at −15 C for several hours. The white precipitate which formed was centrifuged in the cold and dissolved in the pH 6.0 buffer. This solution was dialyzed in the cold against the same buffer until the dialyzate gave a negative test for acetone with sodium nitroferricyanide. After the addition of glycerol to 2.7% the solution was stored at 5 C and constituted the partially purified toxin.

TABLE 1. *Composition of medium*

	g/liter
Na_2HPO_4	2.32
KH_2PO_4	0.50
$MgSO_4 \cdot 7H_2O$	0.17
Ascorbic acid	0.25
NZ Amine type A*	16.00
Myosate (BBL)	4.00
Maltose	5.00

* Sheffield Chemical Company.

Lecithinase assay. Borate-calcium-gelatin-saline diluent (I) was prepared by dissolving H_3BO_3, 11.66 g; $Na_2B_4O_7 \cdot 10\ H_2O$, 1.14 g; NaCl, 8.13 g; $CaCl_2 \cdot 2H_2O$, 1.13 g; gelatin, 2.0 g; and Merthiolate, 0.10 g; in 1,000 ml of distilled water. Egg-yolk substrate was prepared by grinding the yolk of one fresh egg (after removing the yolk sac) with a mortar and pestle until uniform in appearance. The ground yolk was added to 500 ml of diluent I and 20 g of kaolin and emulsified for about 30 sec in a Waring Blendor. The material was centrifuged at 2,500 rev/min for 30 min, the supernatant fluid passed through an asbestos clarifying filter pad, and stored at room temperature. One-tenth milliliter of the toxin to be assayed (diluted with diluent I if necessary) was added to 0.9 ml of diluent I. Two milliliters of egg-yolk substrate were added, and the contents of the tube mixed and incubated in a water bath at 42 C for 60 min. Ten milliliters of saline containing 0.1 unit of antitoxin per ml were added, the contents mixed, and results read against a blank in a spectrophotometer at 590 mμ. A stable glycerinized reference toxin was always run with each set of unknowns. One LV unit is arbitrarily defined as the amount of toxin producing a turbidity of 0.001 optical density units. Figure 1 shows the curve obtained using varying amounts of the reference toxin.

Assay of other components. θ-Toxin was assayed by the 50% hemolysis method of

Roth and Pillemer (1955). α-Hemolysis was determined in the same manner, substituting diluent I for the reducing buffer and utilizing a secondary incubation at 5 C for 15 min. Collagenase was determined by the method of Bidwell and van Heyningen (1948) using Azocoll (Wellcome Laboratories) as the substrate. The supernatant fluids were read in the spectrophotometer at 525 mμ. One Q unit is the amount of enzyme liberating twice as much dye as the buffer control. Hyaluronidase was measured in turbidity reducing units by the method of Tolksdorf et al. (1949). Deoxyribonuclease was determined by the method of Kunitz (1950). Adams (1947) method was used to estimate the combining power or avidity of the toxin, expressed in Lb units. Determinations of the LD_{50} dose were made by injecting the toxin intravenously into Swiss mice (15 to 20 g), and calculated according to the method of Reed and Muench (1938). The end point was death in 48 hr. Protein was measured by the method of Lowry et al. (1951).

Agar gel diffusion. Fractions were tested in agar gel, using antitoxin prepared by immunizing rabbits with a 70% $(NH_4)_2SO_4$ precipitate of the crude toxin treated with formalin and alum.

Animal procedures. Mice were injected intravenously with the labeled toxin and placed in closed containers permitting the collection of respiratory CO_2, urine, and feces. Rabbits were lightly anesthetized with Nembutal and cannulas placed in the carotid artery, trachea, and both ureters. The labeled toxin was injected into an ear vein, and samples of blood, urine, and respiratory CO_2 collected at timed intervals until death occurred.

Assay of tissues for radioactivity. Tissues were removed from animals immediately after death or killing, washed briefly in ice-cold water, and weighed. Tissues (other than blood and urine) were homogenized in a Lourdes Multi-mixer followed by a further treatment with a Teflon tissue grinder. The protein material was precipitated by the addition of an equal volume of 10% trichloroacetic acid and centrifuged. After measuring the volume, the supernatant fluid was extracted with ether to remove the trichloroacetic acid, samples plated on aluminum planchets, and dried under an infrared lamp. The tissue sediments were washed three times with 95% alcohol, dried, and weighed. A portion was emulsified with 1% Triton X-100, plated on tared planchets, and dried, weighed, and counted. Subcellular fractions were separated by differential centrifugation in 0.88 M sucrose (Schneider and Hogeboom, 1950).

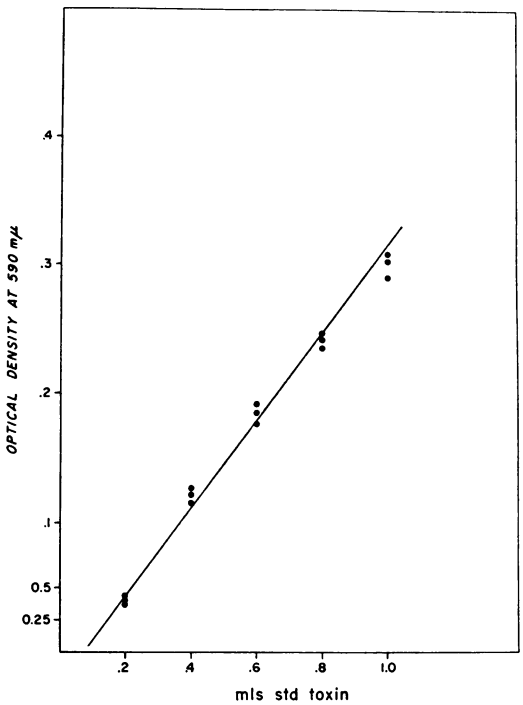

FIG. 1. *Assay curve obtained using increasing amounts of a 1:300 dilution of a standardized reference toxin.*

Blood was collected at timed intervals from the cannulated carotid artery and placed in graduated centrifuge tubes containing an anticoagulant (EDTA, 1 mg/ml of blood). After centrifugation, the plasma was removed, and an approximation of hemoglobin content determined by reading in the spectrophotometer at 590 mμ. The plasma was treated with an equal volume of 10% trichloroacetic acid as described above, and radioactivity determined in both the soluble and insoluble portions. The sedimented erythrocytes were washed three times in 0.9% NaCl, treated with an equal volume of trichloroacetic acid, and both soluble and insoluble portions counted. (Even after extensive intravascular hemolysis had occurred, sufficient red cells remained for assay purposes.)

At timed intervals urine was collected as it flowed from both ureteral cannulas, precipitated with trichloroacetic acid, and counted as above.

TABLE 2. *Characterization of toxin fractions*

	Crude filtrate	50% $(NH_4)_2 SO_4$ ppt	Acetone ppt
Protein; mg per ml	2.62	0.340	0.120
Activity per mg protein:			
LV units	400	6667	13,167
α-Hemolytic units	18.9	185.8	600
θ-Hemolytic units	9.6	26.3	1041
Collagenase; Q units	7.1	94.3	10.8
Hyaluronidase; turbidity reducing units	4.0	407	133
Deoxyribonuclease; units	0	0	0
Avidity; Lb units	—*	—	54
LD_{50}	1	17	33
Lines on agar-gel diffusion plate	6	5	3
Purification factor		17	33
Counts/min $\times 10^3$ per ml	442	546	333

* Not done.

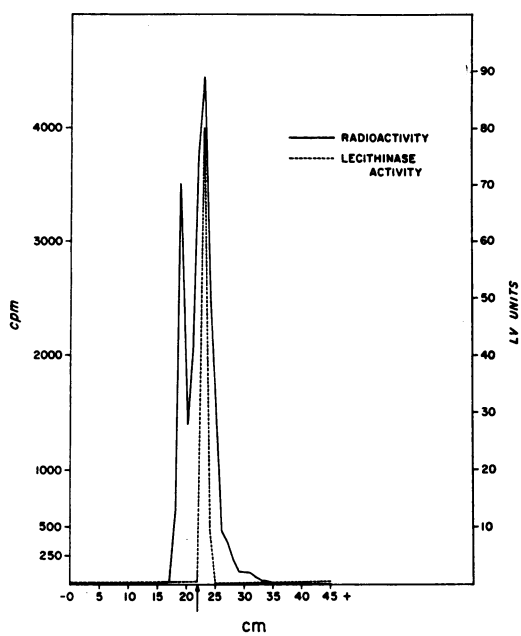

FIG. 2. *Starch block electrophoresis of labeled toxin preparation showing radioactivity and lecithinase activity in a single peak. Barbital buffer, pH 8.6, μ = 0.1, 10 ma 400 v, 18 hr at 5 C.*

Counting of all tissues was done in the Geiger region with an Atomic Scaler, model 1091, equipped with a Packard gas flow counter. All counts were made with 95% probability of 4% error or less. Respiratory CO_2 was collected in NaOH and precipitated as $BaCO_3$. After washing and drying of the precipitate, the CO_2 liberated from the $BaCO_3$ by $HClO_4$ was counted in a Cary vibrating reed electrometer.

RESULTS

Characterization of the toxin. As shown in Table 2, approximately a 30-fold purification of the toxin was obtained in terms of lecithinase activity. It can also be seen, however, that the preparation still contained theta hemolysin as well as collagenase and hyaluronidase activity. The toxin was subjected to starch block electrophoresis (Fig. 2) and the starch fractions analyzed for radioactivity and lecithinase activity. Two distinct peaks of radioactivity were found; one of which coincided with enzymatic activity thus indicating that the lecithinase was indeed labeled with C^{14}.

Preliminary mouse experiments. Radioactivity was detected in the expired CO_2 and in the urine. Organs showing activity (Table 3) were the liver, lungs, spleen, heart, and kidneys. Stripping film radioautographs of the tissues also showed activity to be associated with the erythrocytes.

Rabbit experiments. It can be seen from Fig. 3 that 90% of the toxin disappeared within 5 min from the circulating blood. The slight increase seen after about 15 min may represent a secondary release from damaged tissues. The relative amounts of toxin, as determined by radioactivity, is shown in Fig. 4. In addition, after a lag of about 15 min, intense intravascular hemolysis occurred in a one-step manner.

To determine whether intact toxin or break-

TABLE 3. *Fate of labeled toxin in mice (avg of 10 mice)*

Organ	Counts: min: 100 mg dry wt	σ	Approx counts: min: total organ
Liver	1462	742	1170
Lungs	522	296	125
Spleen	413	339	87
Heart	207	149	37
Kidneys	124	50	71
Urine	6347	5965	
CO_2	5	1.9	

The following organs were counted but had no activity: stomach, large intestine, small intestine, pancreas, skin, muscle, brain, mesentery including lymph nodes, and feces.

Dose: 0.2 ml (0.024 mg protein, 316 LV units, 16.8 × 10^3 counts/min). Mean survival time: 34 min.

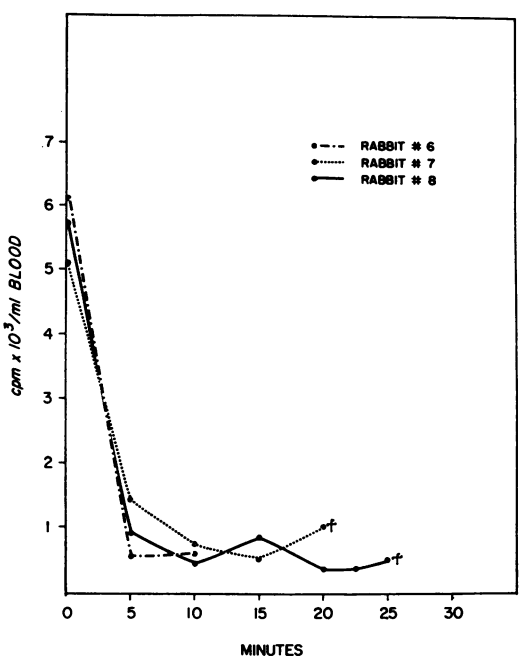

FIG. 3. *Uptake of toxin as measured by radioactivity from the circulating blood following intravenous injection of labeled toxin. Dose: 0.5 ml (0.6 mg protein, 790 LV units, 178 × 10^3 counts/min).*

down products, such as peptides or amino acids, were being measured as radioactivity, blood and other tissues were precipitated with trichloroacetic acid and the sediment and supernatant counted separately. Table 4 shows that in the plasma almost all of the activity was in the trichloroacetic acid precipitate. After 15 min, however, the erythrocytes showed considerable activity in the acid-soluble fraction.

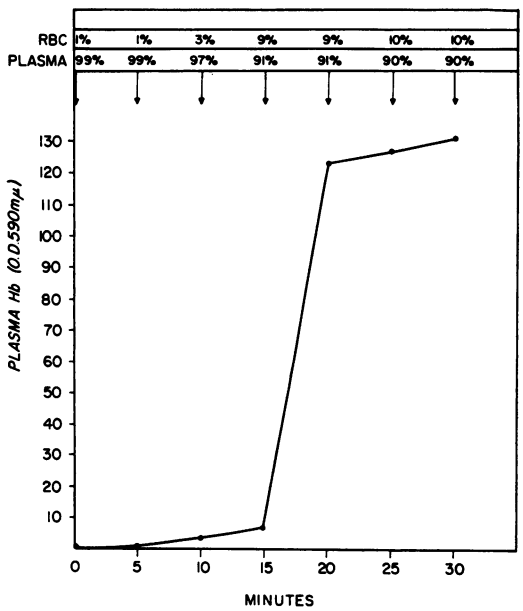

FIG. 4. *Appearance of intravascular hemolysis (plasma hemoglobin) following intravenous injection of labeled toxin. Also shown is the relative distribution of radioactivity in the plasma and erythrocytes at various times. Dose: 0.5 ml (0.6 mg protein, 790 LV units, 178 × 10^3 counts/min).*

TABLE 4. *Distribution of radioactivity in blood (avg of 3 rabbits)*

Time	RBC		Plasma	
	Per cent trichloroacetic acid soluble	Per cent trichloroacetic acid ppt	Per cent trichloroacetic acid soluble	Per cent trichloroacetic acid ppt
min				
0	0	100	2	98
5	0	100	4	96
10	0	100	4	96
15	74	26	0	100
20	65	35	0	100
25	32	68	0	100
30			3	97

Dose: 0.5 ml (0.6 mg protein, 790 LV units, 178 × 10^3 counts/min).

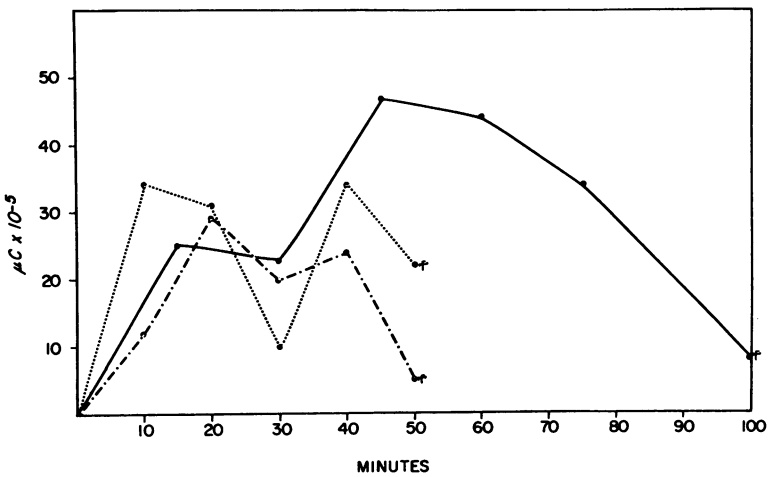

FIG. 5. *Appearance of radioactivity in the respiratory CO_2 of 3 rabbits following intravenous injection of labeled toxin. Dose: 0.5 ml (0.6 mg protein, 790 LV units, 178 × 10^3 counts/min).*

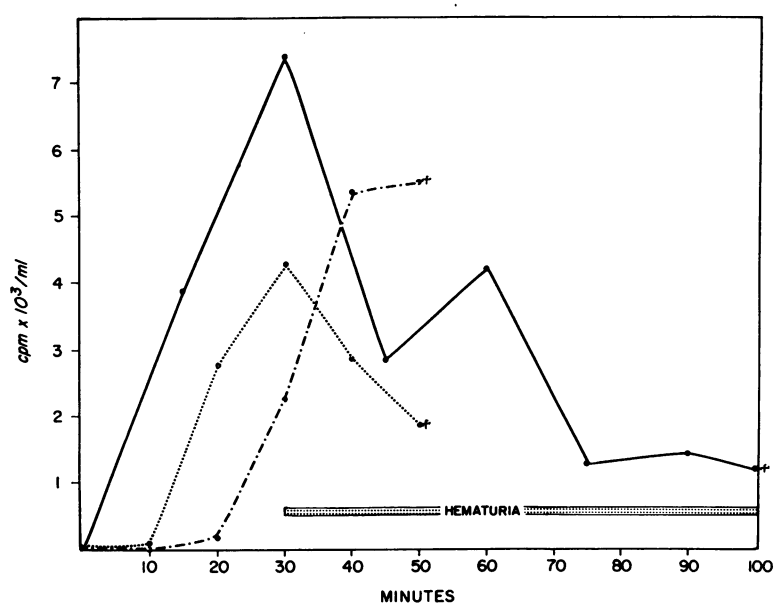

FIG. 6. *Appearance of radioactivity in the urine of 3 rabbits following intravenous injections of labeled toxin. Dose: 0.5 ml (0.6 mg protein, 790 LV units, 178 × 10^3 counts/min).*

Radioactivity appeared in the respiratory CO_2 (Fig. 5) and in the urine (Fig. 6) almost immediately after the intravenous injection of the toxin. Gross hematuria regularly occurred after about 30 min. In contrast to the small amount of the radioactivity in the acid-soluble fraction of the plasma, the urine showed considerable radioactivity in this fraction (Table 5).

Another group of rabbits were killed 30 min after the intravenous injection of the toxin and the various organs removed, homogenized, and treated with trichloroacetic acid. It can be seen (Fig. 7) that the liver, lungs, kidney, and spleen accounted for a large portion of the toxin injected. The percentage excreted in the urine and expired air was not determined. Furthermore,

most of the activity was in the trichloroacetic acid sediment, with the outstanding exception of the kidney.

The one discrepancy between the findings in rabbits and those in mice was the apparent lack of activity in the rabbit heart. This led to electrocardiographic studies on a rabbit after intravenous injection of the toxin. Thirty-six minutes after the intravenous injection of the toxin, ST depression appeared in leads I, II, AVF, and V2–6, with flattening of the T waves throughout. This could be interpreted as inferior and anterior subendocardial injury. From 90 to 115 min, V2 showed elevated ST segments compatible with anterior epicardial injury. At the time of the convulsion (129 min), the QRS complex was prolonged indicating an intraventricular conduction defect. After the convulsion, a variety of rhythm disturbances appeared; first sinus bradycardia, then irregular ventricular rhythm with loss of P waves, suggesting atrial fibrillation, followed by A-V dissociation with grossly distorted and irregular QRS complexes.

The rapid, marked electrocardiographic changes led to a renewed attempt to demonstrate radioactivity in the rabbit heart by stripping film radioautography. After prolonged exposure, definite evidence of activity associated with the myocardial fibers was found.

TABLE 5. *Distribution of radioactivity in urine*

Time	Per cent trichloroacetic acid soluble	Per cent trichloroacetic acid ppt
min		
0–15	62	38
15–30	80	20
30–45	77	23
45–60	67	33
60–75	73	27
75–90	54	46
90–105	55	45

Dose: 0.5 ml (0.6 mg protein, 790 LV units, 178 × 10^3 counts/min).

TABLE 6. *Subcellular distribution of toxin in liver**

Fraction	Counts: min:100 mg	Per cent of subcellular fraction in tissue	Per cent of toxin in fraction
Whole liver	700		
Nuclei	272	15	12
Mitochondria	586	28	51
Microsomes	220	30	21
Soluble	116	35	13

Dose: 0.5 ml (0.6 mg protein, 790 LV units, 178 × 10^3 counts/min). Duration: 67 min.

* As measured by radioactivity.

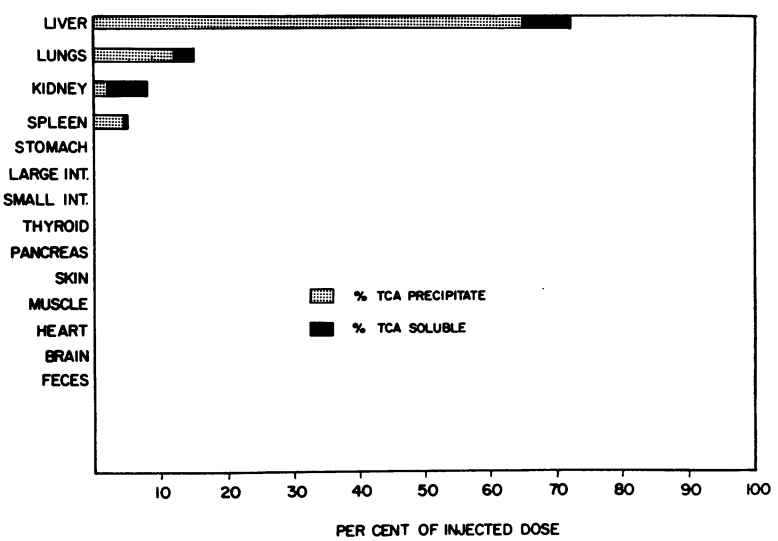

FIG. 7. *Distribution of radioactivity in the organs of rabbits 30 min after the intravenous injection of labeled toxin. Dose: 0.5 ml (0.6 mg protein, 790 LV units, 178 × 10^3 counts/min). TCA = trichloroacetic acid.*

To determine more specifically the location of the toxin in the tissues, rabbits were killed about 1 hr after the injection of toxin. The liver was removed, perfused with ice-cold saline, homogenized, fractionated by centrifugation in sucrose, and counted. It is apparent from Table 6 that there is a marked concentration in the mitochondrial fraction with the remainder of the activity fairly evenly distributed throughout the cell.

DISCUSSION

Crude filtrates of *C. perfringens* type A, grown under the conditions previously described, contain at least six antigenic proteins as measured by agar-gel diffusion. The proteins without known biological activity are readily removed by ammonium sulfate and acetone precipitations. However, the separation of the lecithinase from the biologically active proteins (θ-hemolysin, collagenase, and hyaluronidase) is more difficult. Preliminary experiments in our laboratory have indicated that the final purification of lecithinase may be accomplished by electrophoretic and ion exchange methods.

Both intrinsic and extrinsic methods of labeling the toxin are feasible. By growing the organism in a medium containing C^{14}-labeled amino acids and peptides, it has been possible to label the lecithinase. This method has the advantage of natural labeling, i.e., not altering the configuration of the protein molecule by the introduction of foreign groups. However, this method has proved extremely inefficient since radioactivity decreases and toxic activity increases as the purification proceeds. An alternative method of tagging the toxin would be trace labeling under very gentle conditions with I^{131}. Masouredis (1959) has shown that diphtheria toxin labeled with I^{131} was unchanged with respect to the LD_{50}.

The interpretation of the data presented is based on the assumption that radioactivity appearing in the trichloroacetic acid precipitate represents intact toxin, whereas activity appearing in the acid-soluble portion indicates smaller molecules or breakdown products of the toxin.

Immediately following intravenous injection of the labeled toxin, almost all of the radioactivity is found in the plasma, but with the passage of time the radioactivity appearing in the erythrocytes increases to a maximum of about 10%. This increase of activity in the erythrocytes probably represents continued binding of the toxin to the red blood cells. Although the activity in the plasma remains essentially trichloroacetic acid precipitable, acid-soluble radioactivity appears in the red cells after about 15 min. Since this coincides with the appearance of massive intravascular hemolysis, it may be that toxin breakdown accompanies the hemolysis.

The rapid disappearance of the toxin from the circulating blood is similar to that obtained with other antigenic proteins. This rapid uptake coupled with the appearance of radioactivity in the respiratory CO_2 and in the acid-soluble fraction of the urine is evidence that the toxin is rapidly metabolized, although the organ or organs responsible for metabolizing the toxin have not yet been determined. It should be noted, however, that intact toxin also appears to be excreted in the urine. This, as well as the occurrence of hematuria, points to the renal damage regularly occurring in this toxemia.

The secondary increases of radioactivity in the circulating blood, expired CO_2, and urine may represent a release of toxin from previously damaged tissues with renewed availability for metabolic breakdown and excretion. Although the liver, lungs, kidneys, and spleen are the only organs showing radioactivity, this is probably a reflection of their reticulo-endothelial function, and the fact that these tissues receive a large amount of blood. The complete absence of radioactivity in brain and central nervous tissue was somewhat unexpected in view of their high phospholipid content, but it is apparent that the toxin does not pass the blood-brain barrier.

The concentration of the toxin in the mitochondrial fraction of the liver suggests that the enzyme is specifically fixed to its phospholipid substrate of this subcellular organelle. This is in keeping with the findings of MacFarlane and Datta (1954) who showed the succinic dehydrogenase activity of mitochondrial preparations to be markedly inhibited by the α-toxin with a concomitant decrease of phospholipid content, and by Berg and Levinson (1959) who noted the destructive effect of the toxin upon the mitochondria of multiple organs, especially liver and kidney, using histochemical techniques. The presence of radioactivity in the other subcellular fractions may be due to degraded toxin or to contaminating radioactive proteins.

The significance of activity in the heart and the electrocardiographic changes is questionable since the preparation employed contained θ-hemolysin. In view of the antigenic relationship of the θ-hemolysin to streptolysin O and other oxygen-labile hemolysins and the well-known cardiotoxic action of streptolysin O, the question of cardiac damage due to the α-toxin must await further purification of the lecithinase.

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