STUDIES ON THE ABSORPTION OF *ESCHERICHIA COLI* ENDOTOXIN FROM THE GASTROINTESTINAL TRACT OF DOGS IN THE PATHOGENESIS OF "IRREVERSIBLE" HEMORRHAGIC SHOCK [1]

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Shock resulting from hemorrhage, which is not reversed by retransfusion, is observed both in man and in experimental animals (1–3). The pathogenesis of this phase of hemorrhagic shock has been the object of extensive investigation (3–11). Endogenous toxic factors liberated in response to prolonged hypoxia, such as VDM (vaso-depressor material), have been implicated (8). More recently, the importance of various bacterial toxins liberated during the course of hemorrhagic shock has been emphasized and it has been postulated that the irreversibility of vascular collapse is principally the consequence of the deleterious action of these substances (6, 7, 9, 11). Several lines of evidence support this thesis.

The oral administration of neomycin or chlortetracycline to dogs before subjecting them to a standardized technique of hemorrhage reduced the mortality from 80 per cent to 20 per cent (7, 10, 12). This effectiveness of neomycin, which is essentially not absorbed from gastrointestinal tract, suggested the importance of the Gram-negative bacterial flora within the lumen of the alimentary tract in the genesis of "irreversibility" (11). The frequent development of extensive necrosis of the mucosa of the small intestine during "irreversible" hemorrhagic shock (1) could enable movement of bacteria or their toxins across an intestinal barrier which is ordinarily impermeable. The observations of Nelson and Noyes of an increased absorption of partially purified *Clostridium botulinum* toxin from the gastrointestinal tract of dogs during "irreversible" hemorrhagic shock lends support to this hypothesis (13). Furthermore, endotoxin liberated by the breakdown of various Gram-negative bacteria, in itself, produces shock. Ebert, Borden, Hall and Gold demonstrated accentuation of the physiological changes associated with endotoxin shock in dogs subjected to the additional stress of minor hemorrhage (14). The converse, extreme increases in the lethality of endotoxin administered after nonlethal hemorrhage, has been observed by Schweinburg and Fine in rabbits (15). Zweifach and Thomas also observed the conversion of a nonlethal episode of hemorrhagic shock in rats into a typical "irreversible" syndrome by the continuous administration of nonlethal doses of endotoxin (16). Such indirect evidence strongly supports the view that bacteria and their products are, in large part, implicated in the pathogenesis of irreversibility during hemorrhagic shock, and more specifically that Gram-negative bacteria through the action of endotoxin are especially concerned in this process.

The studies to be reported here were designed to assess the absorption of radioactive Boivin type endotoxin from various portions of the gastrointestinal tract of dogs subjected to "irreversible" hemorrhagic shock and to evaluate possible increases in the susceptibility of dogs subjected to hemorrhage to small amounts of endotoxin. The development by Braude, Carey, Sutherland and Zalesky (17, 18) of a method for labeling endotoxin with Cr^{51} permits detection in quantities too minute for biological or serological assay (19). Such studies should present direct evidence of the role of bacterial toxins of the type liberated by bacteria normally within the gastrointestinal tract

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in the genesis of "irreversibility" to prolonged hemorrhage.

MATERIALS AND METHODS

A strain of *Escherichia coli* isolated from a patient with fatal bacteremia was used in preparation of endotoxin of the Boivin type according to a previously described technique (19). The isolated endotoxin was either lyophilized or frozen for storage. Six separate lots of endotoxin prepared from this strain of *E. coli* were used.

The radioactive labeling technique generally followed that described by Braude, Carey, Sutherland and Zalesky (17). Precautions were taken to dilute and dialyze the endotoxin in solutions buffered at pH 7.0 to prevent loss of toxicity. Aliquants of endotoxin were diluted in saline solution buffered at pH 7.0 with 0.067 M phosphate buffer. Approximately 1.0 mc. of sterile $Cr^{51}Cl_3$ (Abbott Laboratories, Oak Ridge, Tenn.; specific activity, approximately 1.0 mc. per mg.) for each 300 mouse LD_{50} doses was added to a solution of endotoxin. The endotoxin-$Cr^{51}Cl_3$ mixture was well mixed and incubated at room temperature for 24 hours, then dialyzed with stirring in a bath containing 5 liters of 0.067 M phosphate buffer at room temperature. The bath was changed daily until the dialysate was virtually free of Cr^{51}. This required approximately 48 hours; dialysis was continued 24 to 48 hours longer. The radioactivity of the final endotoxin preparations ranged from 45,300 cpm per mouse LD_{50} to 163,200 cpm per mouse LD_{50}, with the exception of Lot No. 17 in which only 0.3 mc. was added to 300 mouse LD_{50} doses, the resultant final radioactivity being 13,500 cpm per mouse LD_{50}. The radioactivity of these preparations was in the same range (approximately 7,000 counts per second per milligram) as that of the preparations of Braude, Carey, Sutherland and Zalesky labeled with $Cr^{51}Cl_3$ (17).

Studies were performed on each lot of endotoxin after "labeling" with $Cr^{51}Cl_3$ to ascertain that toxicity had not been lost in the labeling procedure. The different lots were standardized on the basis of mouse toxicity and expressed as mouse LD_{50} doses. The LD_{50} of these lots of Cr^{51}-endotoxin varied from 0.08 ml. to 0.3 ml. when given intraperitoneally to 18 to 20 gram albino mice of the Bagg strain. Comparative studies of the toxicity of a representative lot of endotoxin were performed on an aliquant from Lot No. 3. The LD_{50} of the original solution was 0.08 ml.; this volume contained 176 μg. when lyophilized. The nitrogen content of Lot No. 3 was found to be 2.8 per cent by weight as determined by Nesslerization after digestion with selenium-sulfuric acid. This nitrogen content is in the range found by Webster, Sagin, Landy and Johnson for a typical Boivin endotoxin and represents in part glucosamine and amines present in the lipid component (20). The nitrogen content of this preparation is much higher than reported by Braude, Carey, Sutherland and Zalesky, who employed biuret and ninhydrin to test for the presence of proteins (17).

The toxicity of an unlabeled portion of Lot No. 3 when given by systemic intravenous injection was estimated in 12 normal dogs. Two mg. per Kg. killed 10 of 12 animals within 24 hours. The exact LD_{50} was not determined; however, 2.0 mg. per Kg. was chosen as a conservative estimate. The LD_{50} for an average 15 Kg. dog was in the range of 150 mouse LD_{50} doses. These toxicity values are in general agreement with values obtained by other workers, the LD_{50} for 20 gram mice ranging from 0.25 mg. to 1.5 mg., while deaths occurred in dogs following the administration of 5 to 10 mg. per Kg. (21–23).

Radioactivity of various samples was measured in a well-type scintillation counter (Nuclear, Model DS5-5) with an efficiency of approximately three per cent for Cr^{51}. The standard counting interval was 2 minutes in early studies and 10 minutes in later studies. The counting error at the 0.95 level was 23 to 32 cpm for 2 minute counts and 10 cpm for 10 minute counts.

Radioactivity was measured in arterial blood samples (2.0 ml.) drawn at hourly intervals during the shock procedure. At the end of the five hour period of shock, the animals were sacrificed by administration of excess pentobarbital. A midline thoracoabdominal incision was made in the control and shock animals through which a urine specimen was obtained from the bladder using a syringe and needle; portions of the following organs were then removed: adrenal, spleen, liver, paracecal mesenteric lymph node, kidney, muscular portion of diaphragm, lung and trachea. Next, gastrointestinal contents were removed at the following locations: stomach, 24 inches below the pylorus, 24 inches above the ileocecal valve, and descending colon. Approximately 1 gram pieces of the various tissues were weighed to the nearest milligram. The tissues were then digested by adding 2 ml. of concentrated HNO_3 and heating. Foaming was controlled by the addition of a small amount of antifoam (Dow Corning Silicone Defoamer, Antifoam A). The radioactivity of the various samples was measured the day following the shock procedure. The radioactivity for each sample was corrected to counts per minute per gram wet weight.

Experimental procedure

Mongrel dogs of both sexes, averaging 15.0 Kg. body weight (range, 9.1 to 24.5 Kg.) were dewormed, immunized against canine hepatitis and distemper, and kept under standard dietary and environmental conditions at least 21 days prior to the experiments. The dogs were fasted 18 hours before the experiments, which were conducted in an air-conditioned operating room. Control and shock animals were studied in each experiment.

A. Techniques of administration of labeled endotoxin. In the shock studies, labeled endotoxin was administered by two techniques. In the first, a cecostomy or ileostomy was performed; using strict aseptic technique, a midline incision was made, a small polyethylene catheter (outside diameter, 2.4 mm.) was inserted into either the cecum or terminal portion of the ileum employing a Witzel-type repair, and the free end of the catheter was closed and

sutured beneath the skin. At the time of the study, 14 to 21 days after the primary enterostomy, a small skin incision was made and the free end of the catheter located. This technique made it possible to place Cr^{51}-endotoxin into specific areas of the gastrointestinal tract.

In the second technique, dogs were anesthetized lightly with sodium thiopental (19 mg. per Kg.) and under direct laryngoscopy a Levine-type gastric tube was passed into the stomach. Cr^{51}-endotoxin dissolved in 16 to 20 ml. of saline solution followed by 20 ml. of water was gavaged into the stomach. The gavage was performed 2 to 10.5 hours before starting the bleeding, depending upon the portion of the gastrointestinal tract in which the greatest Cr^{51}-endotoxin concentration was desired. By 10.5 hours the maximum radioactivity was present in the large intestine.

The dogs received from 53 to 133 mouse LD_{50} doses with an average of 96 mouse LD_{50} doses through either stomach or enterostomy tube.

Of the two methods used for the administration of endotoxin, gavage through a stomach tube proved more satisfactory than injection through either an ileostomy or cecostomy. It was difficult to be certain in the enterostomy technique that a portion of the labeled endotoxin had not leaked into the peritoneal cavity from which systemic absorption would occur. The only problem encountered in the interpretation of results following gavage of labeled endotoxin was aspiration during the five hour period of study. This was controlled by using endotracheal intubation in all dogs and by determining the presence of radioactivity in the trachea and lungs. Dogs were excluded from tabulation which were found to have higher levels of radioactivity in the tracheal secretions than in lung.

B. Technique and features of shock procedure. At varying intervals after gavage or immediately after injecting endotoxin into the enterostomies, control and shock animals were lightly anesthetized with sodium thiopental (19 mg. per Kg.) and an endotracheal tube inserted. Then, using strict aseptic surgical technique, a sterile polyethylene cannula (1.8 mm. inside diameter) was inserted via the right femoral artery into the aorta. A two side-arm manifold (consisting of two three-way stop cocks) was connected to the cannula. A U-type mercury manometer was connected to one side-arm by means of sterile plastic tubing. A sterile disposable plastic donor set and sterile pyrogen-free 1,000 ml. glass reservoir (a standard 1,000 ml. saline solution bottle emptied just prior to use) was connected to the second side-arm. The remaining connection on the manifold was used to obtain blood samples. The dead space in the entire system was filled with sterile saline solution containing 0.4 mg. per ml. of sodium heparin. Each dog received 2.5 mg. per Kg. of sodium heparin intra-arterially, and 40 mg. of sodium heparin was injected into the reservoir. Control and shock dogs were subjected to the same operative procedure and received the same medications except that a reservoir was not connected to the cannula in the control animals.

After complete recovery from the short period of anesthesia, the dog to be "shocked" was allowed to bleed spontaneously into the sterile reservoir system until the mean arterial blood pressure was 40 mm. Hg. Under constant observation, the blood pressure was maintained at 40 mm. Hg using manual reinfusion under pressure when necessary. The period of shock was five hours, unless the animal died during this interval. No additional anesthesia was required in the shock dogs. Control animals usually required an additional 100 mg. of sodium thiopental at one and three hours after the start of the experiment. The significant features of the shock procedure are tabulated in Table 1. The mean values for the per cent of the maximum bled volume which had to be reinfused during the five hour period of hemorrhage to maintain the mean arterial blood pressure at 40 mm. Hg, the per cent "uptake," correspond quite clearly to the values observed by Fine, Frank, Schweinburg, Jacob and Gordon (7).

Hemorrhagic shock which is associated with a 40 per cent "uptake" of the maximum volume of shed blood is generally accepted as being "irreversible," even if at that time the entire volume of shed blood is reinfused. Hemorrhage into the lumen of the gastrointestinal tract, usually most marked in the duodenum but often

TABLE I

Observations on the shock procedure in dogs *

Route of toxin administration	No. animals	Maximum bled volume	Uptake†	Deaths during procedure
		ml./Kg.	%	
Gastric tube	16	53.6	38	3
Cecostomy	9	53.5	53	1
Ileostomy	1	47.5	35	0
Mean for all groups‡	26	53.3 ± 2.4	43 ± 6	4

* Dogs were allowed to bleed rapidly into a reservoir until the mean arterial blood pressure reached 40 mm. Hg. This blood pressure was maintained for a five hour period with further bleeding or reinfusion as required. Includes five dogs in which intraperitoneal leakage of labeled endotoxin occurred during injection through cecostomy tube.

† Per cent of maximum bled volume reinfused during the five hour experimental period to maintain mean arterial pressure at 40 mm. Hg.

‡ Mean plus or minus standard error of the mean.

TABLE II

Absorption and distribution of radioactivity from the gastrointestinal tract of normal dogs following the administration of free chromic^{51} chloride

No. animals	Radioactivity in tissue (mean *cpm/Gm.* ± *S.E.M.*)						
	Blood*	Urine	Liver	Spleen	Kidney	Adrenal	Lung
7	77 ± 29.4	6,623 ± 3,100	57 ± 27.9	28 ± 18.5	56 ± 29.4	37 ± 18.1	52 ± 11.9

* Five hour blood sample; earlier values were similar.

found extending to the ileocecal region, was observed in the majority of the shocked animals

C. Determination of the diffusibility of free Cr^{51} ions across the gastrointestinal mucosa and systemic distribution in normal dogs. Sterile chromic^{51} chloride diluted with saline was administered by gavage to seven lightly anesthetized dogs to determine whether free Cr^{51} crossed the gastrointestinal mucosa of the normal dog and, if so, the distribution of radioactivity in various body tissues. The volume and total radioactivity administered was similar to that used with labeled endotoxin (20.0 ml. containing approximately 10,000,000 cpm). The technique of administration, duration of study, and tissue analyses were the same as those used in the shock preparation. Significant radioactivity was detected in the tissues of each dog (Table II). The radioactivity in the blood probably accounts for the radioactivity detected in the other tissues. Very high levels of radioactivity were noted in the urines of these animals (6,623 ± 3,100 cpm per ml.). In two animals radioactivity was not detected in the gastrointestinal tract at the segment 24 inches below the pylorus; in the remainder, radioactivity was detected at this level or within the ileum. The radioactivity in the urine of the former animals was 470 cpm per ml., while in the latter, the radioactivity averaged 9,085 cpm per ml. of urine, suggesting that absorption was greater below the duodenum.

The distribution pattern of radioactivity throughout the body noted in two dogs given chromic^{51} chloride into a foreleg vein was similar to that observed following absorption from the gastrointestinal tract.

D. Distribution of labeled endotoxin in shocked and normal dogs following splanchnic or systemic administration. The distribution of radioactivity throughout the body following the systemic or portal intravenous or intraperitoneal injection of Cr^{51}-labeled endotoxin into dogs during shock was compared with the distribution following systemic intravenous injection or the absorption of free chromic^{51} chloride from the gastrointestinal tract. The radioactivity following the parenteral administration of Cr^{51}-labeled endotoxin was highest in the liver, spleen and lungs, while the radioactivity in the urine was minimal in contrast to the distribution noted with free chromic^{51} chloride (Table III). The distribution of radioactivity in normal dogs after the systemic or portal intravenous administration of Cr^{51}-endotoxin was the same as during shock and is essentially the same as noted in rabbits (16). The differences in the renal excretion of Cr^{51}-endotoxin and free $Cr^{51}Cl_3$ suggest that high levels of radioactivity in the urine of animals receiving Cr^{51}-

TABLE III

*Distribution of radioactivity following the parenteral administration of Cr^{51}-labeled endotoxin to dogs during shock**

Dog no.	Route of injection	Radioactivity in tissue (*cpm/Gm.*)						
		Blood	Urine	Liver	Spleen	Kidney	Adrenal	Lung
2371	Intravenous	16	46	1,430	760	32		160
2125	Intraperitoneal	24	0	1,575	150	0	75	22

* Representative animals, which were maintained at a blood pressure of 40 mm. Hg for five hours. Cr^{51}-endotoxin was administered two hours after the onset of shock. The quantity of radioactivity (endotoxin) administered was not the same for each animal, but was approximately 0.10 of the estimated LD_{50} dose for the dog.

TABLE IV

Relationship between radioactivity of liver tissue and quantity of Cr^{51}-labeled endotoxin administered by portal vein injection

Animal no.	1	2	3	4	5	6
Total radioactivity injected (*cpm*)	399,000	199,500	149,620	99,750	74,810	37,410
Radioactivity in liver* (*cpm/Gm.*)	540	356	290	224	112	48

* Average radioactivity determined in duplicate samples from both the right and left lobes of the liver.

TABLE V

Radioactivity of $Cr^{51}Cl_3$-endotoxin preparations

Endotoxin lot no.	Radioactivity* (*cpm/mouse LD_{50}*)	No. shock dogs given each lot	Minimum detectable quantity of endotoxin† (Fraction of LD_{50}) Mean	Range
3	74,270	3	0.0018	0.0011–0.0030
8	64,200	2	0.0021	0.0013–0.0035
14	163,200	5	0.0008	0.0005–0.0014
15	96,750	5	0.0014	0.0008–0.0023
17	13,500	4	0.0108	0.006 –0.016
31	45,360	2	0.0014	0.0008–0.0023

* Counts per minute per volume of $Cr^{51}Cl_3$ endotoxin which was determined to be LD_{50} for 18 to 20 Gm. mice of Bagg strain.

† Minimum detectable quantity of endotoxin was based on the assumptions that liver weight was 2 per cent of body weight, that 47 per cent of radioactivity localized in the liver following portal vein injection, and that the LD_{50} for the dog was 150 mouse LD_{50} doses. The radioactivity necessary to exceed the 0.95 counting error of 32 cpm or 10 cpm depending on counting interval was calculated. The mean was based on 15 Kg., the range 9.1 Kg. to 24.5 Kg., which were the weights of the dogs in these studies.

labeled endotoxin is an indication that the Cr^{51} label had been split from the endotoxin.

E. Determination of lower limit of detectability of labeled endotoxin following injection into portal vein. The minimal quantity of Cr^{51}-labeled endotoxin which could be detected in body tissue was ascertained by the injection of varying amounts under direct vision into the portal veins of six lightly anesthetized (sodium thiopental, 19 mg. per Kg.) normal dogs. The animals were sacrificed after a period of 30 minutes and the radioactivity present in the liver, spleen, adrenals, kidneys, lungs, blood and urine was determined. The variability in cpm per gram wet weight between various lobes of the liver was within the counting error of 32 cpm. Assuming the liver weight to be two per cent of the total body weight, 47.3 ± 2.4 per cent of the total injected radioactivity was present in the liver. The radioactivity present in the other tissues was variable and was not well correlated with the quantity of Cr^{51}-labeled endotoxin injected. A linear regression was calculated for the relationship between radioactivity per gram of liver (x) and the total radioactivity injected into the portal vein (y), $y = 705x - 24{,}500$ with the correlation coefficient $r = +0.9617$ (Table IV). This linear regression enables calculation of radioactivity absorbed into the portal vein based upon the radioactivity per gram of liver. Hepatic radioactivity had to exceed 35 cpm per gram weight to utilize these calculations. Calculation of the minimum quantity of endotoxin detectable was based upon the assumptions that liver weight was 2 per cent of body weight, that 47 per cent of radioactivity localized in the liver following the portal vein injection of Cr^{51}-endotoxin, and that the LD_{50} for the dog was 150 mouse LD_{50} doses (Table V). The radioactivity necessary to exceed the counting error of 32 cpm or 10 cpm, depending on the counting interval, was calculated. The mean values and the range of minimum detectable quantities of endotoxin were based on the weights of the dogs employed; the range was 9.1 to 24.5 Kg. with a mean of 15.0 Kg.

RESULTS

A. Absorption of Cr^{51}-labeled endotoxin from the gastrointestinal tract during "irreversible" hemorrhagic shock

The levels of radioactivity in various tissues in control dogs, which received Cr^{51}-labeled endotoxin but were not bled, exceeded the counting error in only 1 of 13 dogs (Table VI, Group B).

TABLE VI

Absorption of radioactivity from the gastrointestinal tract of normal dogs and dogs during hemorrhage following the administration of chromic^{51} chloride

Group	Experimental preparation	No. animals	Animals with significant hepatic radioactivity (no./total)*
A	Control-free $Cr^{51}Cl_3$	7	6/7†
B	Control-Cr^{51} endotoxin	13	1/13‡
C	Shock-Cr^{51} endotoxin	21	4/21§

* Significant radioactivity is that which exceeds two standard deviations (S.D.) of the counting error of the sample and background. Background was 273 ± 2 cpm. The S.D. of the sample in most experiments was 12 to 14 cpm. In one dog in Group B and three dogs in Group C, the counting interval was increased so the S.D. of the sample was 5 cpm.

† In the dog which had insignificant radioactivity in the liver, significant though minimal radioactivity was present in the urine.

‡ In each dog in which significant radioactivity was detected in the liver, significant radioactivity was also detected in the urine.

§ The differences between Groups B and C are not significant (method of Chi square).

The calculated quantity of endotoxin absorbed was 0.0009 dog LD_{50}. The radioactivity in the urine of this group exceeded 32 cpm in three animals, including the dog with evidence of hepatic absorption. In eight of the nine animals receiving labeled endotoxin by gavage in Group B, the maximum level of radioactivity was found in the gastrointestinal tract below the pylorus. The maximum radioactivity was in the jejunum in three dogs, in the ileum in four dogs, and in the colon in two dogs. In those animals with enterostomies, three had cecostomies and one an ileostomy.

In dogs subjected to hemorrhage (Group C, Table VI) the radioactivity within the gastrointestinal tract was in the same range of activity as noted in the control animals. Descent of the radioactivity within the gastrointestinal tract following gavage was as follows: stomach, one dog; jejunum, two dogs; ileum, eight dogs; and colon, five dogs. Four shock animals had satisfactory cecostomies and one an ileostomy. Radioactivity was detected in the tissues of 4 of the 21 shocked dogs (Table VI, Group C). The estimated quantity of endotoxin absorbed by the shocked dogs was 1.35 mouse LD_{50} doses, 1.04 mouse LD_{50} doses, 0.04 mouse LD_{50} doses and 0.009 mouse LD_{50} doses. The two larger values were observed in the two dogs which had received Cr^{51}-labeled endotoxin 10.5 hours prior to the onset of hemorrhage and in both, significant levels of radioactivity were present in the urine at the time of sacrifice 15.5 hours after the administration of endotoxin, 129 and 57 cpm per ml. of urine. This radioactivity was probably the result of splitting off of some of the Cr^{51} from the Cr^{51}-labeled endotoxin in the gastrointestinal tract and excretion of free Cr^{51}. The radioactivity detected in the other two dogs was at the lower limit of detectability, though in one the sample was counted for 10 minutes.

Four of the shocked dogs died during the five hour period of hemorrhage; however, none had absorbed the minimal quantity of radioactivity from which endotoxin toxicity could be calculated.

B. Alteration in the susceptibility to endotoxin of dogs subjected to hemorrhage

Alterations in the susceptibility of dogs, either during or after "irreversible" or "reversible" hemorrhage, to the hypotensive and lethal effects of endotoxin were studied. The endotoxin administered during "irreversible" hemorrhage was a portion of Lot No. 3; however, this portion was not labeled with $Cr^{51}Cl_3$. The effect of the intravenous administration of sublethal quantities of endotoxin on the blood pressure of a normal dog is shown in Figure 1. This pattern of response is uniform and has been observed by other workers (24). Varying quantities of this same lot of endotoxin were administered intravenously to seven dogs during a period of protracted hypotension (five hours at 40 mm. Hg) and during the immediate post-reinfusion period when a temporary pressor response was evident. The first dose of endotoxin was administered after two hours of hemorrhage, then repeated and increased at each subsequent hour (Figure 2). Four of the seven dogs received at least 0.10 LD_{50} of endotoxin, while three of the seven received 0.10 LD_{50} both before and after reinfusion. No change in mean arterial blood pressure or loss of vasomotor tone as measured by an increased rate of "uptake" from the reservoir was noted in these animals. The response depicted in Figure 2 is representative of the seven animals. Even after reinfusion, the administration of 0.10 LD_{50} did not result in the immediate fall in mean arterial blood pressure that was observed in normal animals. A similar lack of effect of a single sublethal injection of endotoxin during hemorrhage in rats was observed by Zweifach and Thomas (16).

Susceptibility to endotoxin was studied after reinfusion of blood following a period of hypotension which in itself was nonlethal. The endotoxin used was Cr^{51}-labeled endotoxin in which the radioactivity had decayed, pooled from Lots No. 14, 15 and 17. The mouse toxicity of the pooled Cr^{51}-labeled endotoxin was again determined prior to injection. The Cr^{51}-labeled endotoxin was administered intravenously into a foreleg vein four hours after reinfusion of the shed blood.

In addition, estimated sublethal quantities of this Cr^{51}-labeled endotoxin were administered to normal dogs to be certain that the estimated doses were in fact sublethal (Table VII, Control Group). While the dosage was not lethal, the dogs which received an estimated 0.10 LD_{50} nevertheless developed symptoms of toxicity consisting of un-

TABLE VII

Susceptibility of dogs to the intravenous administration of endotoxin following hemorrhage of short duration ("reversible") *

Experimental procedure†	No. of animals	Duration of hemorrhage (*hrs.*)	Dosage of endotoxin‡ (dog LD_{50})	Mortality (no./total)
Control	5		0.01	0/5
	4		0.10	0/4
Reversible hemorrhage				
Group A	4	2	0	3/4
	4	2	0.01	4/4
Group B	3	1.5	0	1/3
	3	1.5	0.01	2/3
	6	1.5	0.001	1/6
Group C	2	1	0	0/2
	3	1	0.001	0/3

* Dogs were allowed to bleed to a mean arterial blood pressure of 40 mm. Hg and maintained at this level for periods of one or two hours, rather than five hours as in the "irreversible" shock group. The shed blood was reinfused after the shock period. Four hours after reinfusion, $Cr^{51}Cl_3$-endotoxin was injected into a foreleg vein. The mouse toxicity of the pooled $Cr^{51}Cl_3$-endotoxin was determined prior to injection.

† Control group consisted of normal dogs.

‡ The endotoxin dosage was based upon the assumption that one LD_{50} for the dog is 10 mouse LD_{50} doses per Kg. body weight. Control dogs received intravenous saline.

steadiness, collapse, wretching, vomiting and diarrhea.

Initially, a degree and duration of hypotension (40 mm. Hg for two hours) which has been "reversible" for other workers after reinfusion was used (18). This degree of hypotension was lethal in three of four control animals, so that an alteration in the degree of sensitivity to Cr^{51}-labeled endotoxin could not be ascertained (Table VII, Group A). Another group of animals was bled to a blood pressure of 40 mm. Hg for one and one-half hours (Table VII, Group B). This degree of hypotension was lethal for one of three control dogs. The administration of an estimated 0.01 LD_{50} of Cr^{51}-labeled endotoxin resulted in the death of two of the three shocked animals. The administration of an estimated 0.001 LD_{50} of Cr^{51}-labeled endotoxin to animals hypotensive for one and one-half hours was lethal for one of six dogs, while a similar amount of endotoxin was not lethal for three dogs maintained at 40 mm. Hg for one hour.

A number of the enterostomy procedures were not technically satisfactory for interpretation of absorption of labeled endotoxin because of leakage of radioactivity into the peritoneal cavity. However, these unsatisfactory experiments gave some further indication of the degree to which susceptibility to Cr^{51}-labeled endotoxin was altered in the dog during irreversible shock. In six shocked animals and in seven control animals radioactivity was present in the liver equivalent to the absorption of 0.13 to 65 mouse LD_{50} doses of endotoxin. Only two of the six shocked animals died; these two had absorbed smaller amounts of labeled endotoxin than other dogs in the group (1.0 and 1.3 mouse LD_{50} doses). None of the control animals which absorbed radioactivity equivalent to relatively small quantities of endotoxin (0.4 to 12.2 mouse LD_{50} doses) died during the period of study.

Thus, these experiments furnish no evidence of increased susceptibility to the repeated injection of endotoxin during shock or to the single injection of 0.001 LD_{50} of endotoxin following reversible hemorrhagic shock. Increased susceptibility to a single injection of 0.01 LD_{50} of endotoxin following a degree of hemorrhage, which in itself was occasionally lethal, was not clearly demonstrated.

DISCUSSION

Indirect evidence along several lines has implicated the importance of endotoxin from Gram-negative intestinal bacteria in the pathogenesis of "irreversibility" during prolonged hemorrhage (7, 10–12, 15, 16). Direct evidence to support this hypothesis has been dependent upon the development of techniques which permit the detection of endotoxin. The technique of isotopic

labeling of endotoxin with Cr^{51} ions seemed to be sufficiently sensitive to warrant its use in evaluation of the role of the absorption of endotoxin in "irreversible" shock. If endotoxin liberated from the bacterial flora within the lumen of the alimentary tract is an important factor in irreversible shock, either significant absorption of endotoxin from the gastrointestinal tract should be demonstrable during the development of "irreversibility," or a profound increase in the susceptibility of the shocked animal to amounts of endotoxin undetectable by Cr^{51}-labeling should be demonstrable.

Absorption of Cr^{51}-labeled endotoxin from various segments of the gastrointestinal tract, from the stomach to the colon, was not observed in 21 dogs subjected to a degree and duration of blood loss uniformly accepted as "irreversible" in at least 80 per cent of animals. Two of the animals were reinfused and absorption was not demonstrable during the pressor phase.

Likewise, if present, increased susceptibility to a single injection of endotoxin during the recovery phase of reversible hemorhage was in the range of 0.01 LD_{50}, which was well within the limits of detectability. Thus, the role of Boivin type bacterial endotoxin in the pathogenesis of the phenomenon of "irreversibility" following prolonged hemorrhage in dogs must be questioned.

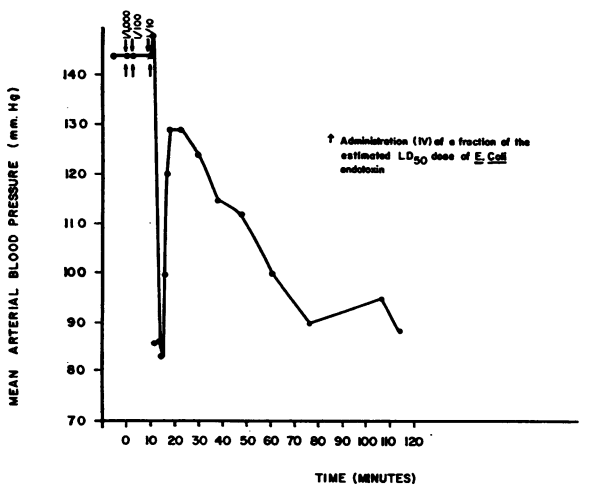

FIG. 1. THE EFFECT OF THE INTRAVENOUS ADMINISTRATION OF *ESCHERICHIA COLI* ENDOTOXIN IN VARYING QUANTITIES TO A NORMAL DOG

The fractions noted at each arrow represent a portion of the estimated LD_{50} for this dog. Following the administration of an estimated 0.10 LD_{50} or 15 mouse LD_{50} doses of unlabeled endotoxin an immediate fall in arterial blood pressure is noted.

These experiments might be criticized from several points. First, did the technique of labeling inactivate the endotoxin? Toxicity, as determined by lethality in mice, pyrogenicity in rabbits and the ability to produce shock and leukopenia in dogs, was demonstrated in various lots of endotoxin labeled using this technique. While six lots of labeled endotoxin were used in these studies, the quantity administered was determined on the basis of the mouse lethality of the labeled material.

Second, was the quantity of labeled endotoxin administered adequate? This varied from 53 to 133 mouse LD_{50} doses, with an average of 96 mouse LD_{50} doses. In conjunction with these experiments, studies on the quantitative bacterial flora of the gastrointestinal tract of normal dogs and of dogs subjected to "irreversible" shock were performed (25). The small intestine of the normal dog contained very few bacteria, approximately 1,000 per inch, of which two-thirds are Gram-positive, until the terminal six inches of ileum. The small intestine of animals subjected to "irreversible" shock contained approximately 45,000,000 viable aerobic bacteria per inch, of which Gram-negative organisms, especially *E. coli,* were predominant (25). The absolute quantity of endotoxin present in the gastrointestinal tract (80 inches) is difficult to determine. Landy and Johnson estimated that one billion organisms (Salmonella typhosa, 0–901) could contribute approximately 15 micrograms of lipopolysaccharide (endotoxin) with the LD_{50} for mice in the range of 250 μg. (21). From such rough approximations, the quantity of endotoxin administered definitely seemed adequate.

Third, did the Cr^{51}-labeling procedure alter the diffusibility of endotoxin across the gastrointestinal mucosa? This question is unanswered by the data. Likewise, the administration of barbiturate anesthesia in preparation for the shock procedure might have altered intestinal absorption. For this reason, sodium thiopental was used and the dogs were allowed to regain complete consciousness before starting the shock procedure.

Fourth, was the endotoxin administered representative? *Escherichia coli* is the predominant Gram-negative aerobic bacterium in the gastrointestinal tract of the dog; hence this bacterium was

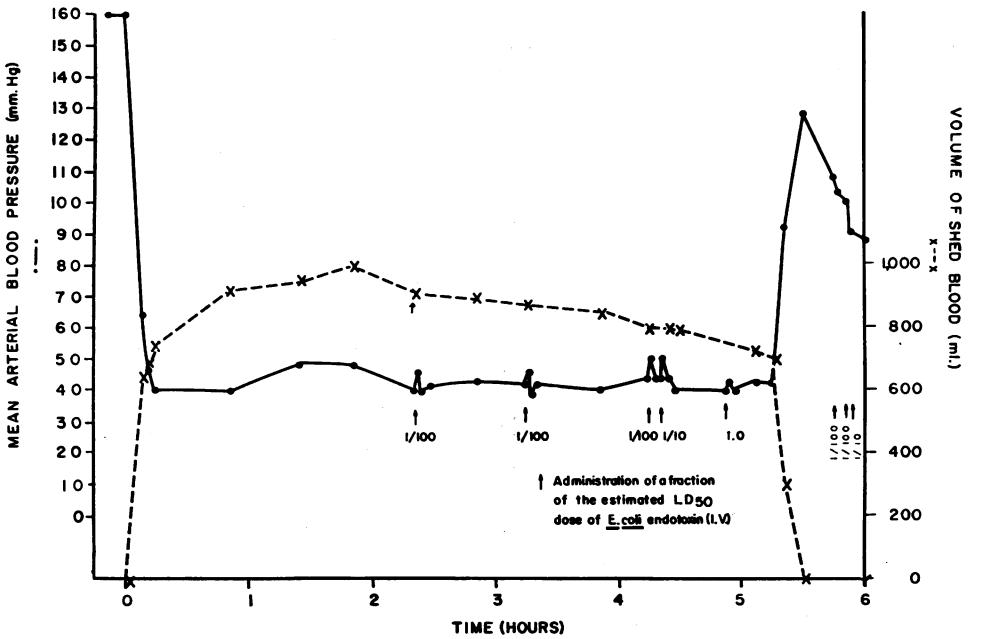

FIG. 2. THE EFFECT OF THE INTRAVENOUS ADMINISTRATION OF A PORTION OF THE SAME LOT OF *ESCHERICHIA COLI* ENDOTOXIN AS DEPICTED IN FIGURE 1 IN VARYING QUANTITIES TO A DOG DURING A PERIOD OF PROLONGED HYPOTENSION AND AFTER REINFUSION OF THE SHED BLOOD

The solid line depicts the mean arterial blood pressure. The dotted line represents the volume of blood in the reservoir. The rate and volume of blood which has to be reinfused during the hemorrhage is a measure of vasomotor tone. Note the rapid decline in blood pressure following the reinfusion of the shed blood. This response is typical in the "irreversible" phase.

used as the source of endotoxin. Endotoxin derived from various species of bacteria demonstrates immunological specificity; however, the physiological activities of various lipopolysaccharide extracts are similar (26). While Boivin type endotoxin is the prototype used in most studies, there may be other toxic components in bacterial cells which are lost from the Boivin type of endotoxin.

Lastly, is the source of bacterial endotoxin from bacteria which invade the gastrointestinal mucosa and multiply outside of the intestinal lumen? Lillehei demonstrated that irreversibility was prevented in 90 per cent of dogs by perfusing the bowel with arterial blood from a donor dog during a five hour period of hemorrhagic shock, yet there was no difference in the bacteriologic findings between survivors and nonsurvivors (27). Lymph nodes were obtained from the coeliac and paracecal areas of three shocked animals which demonstrated extensive necrosis of the bowel mucosa (25). These were homogenized and cultured. The nodes contained fewer than 100 viable aerobic bacteria per node. Similarly, in a study of blood cultures obtained from normal dogs and dogs in hemorrhagic shock, no difference in the total percentage of positive cultures, or the percentage of Gram-negative organisms was found, (28). Thus, the number of bacteria present in the small intestine may increase during shock; however, they do not seem to invade the damaged gastrointestinal mucosa and multiply outside of the intestinal lumen.

Since absorption from the gastrointestinal tract of a Boivin endotoxin derived from *Escherichia coli* could not be demonstrated, the role of this classical type of bacterial endotoxin in the pathogenesis of the phenomenon of "irreversibility" following prolonged hemorrhage in dogs must either be questioned or further extra-intestinal sources of Gram-negative bacteria, including laboratory equipment, must be sought (29). Landy and

Shear obtained lipopolysaccharide materials which possessed the biological properties of bacterial endotoxin from various mammalian tissues including mouse stomach (30). Tolerance could be induced to these lipopolysaccharides which was associated with cross tolerance to bacterial endotoxin. One could postulate that as a result of the mucosal necrosis which occurs during prolonged hemorrhage, lipopolysaccharides of endogenous origin are absorbed and contribute to the genesis of irreversibility. This hypothesis would account for protective effect of bowel perfusion in dogs, the resistance which rats rendered tolerant to bacterial endotoxin demonstrate to hemorrhagic or traumatic shock, and the observation that hemorrhagic shock in germ-free rats is identical to that observed in normal rats including necrosis of the gastrointestinal mucosa (*27*, 17, 31).

SUMMARY AND CONCLUSIONS

1. The possible role of absorption of endotoxin from the gastrointestinal tract in the genesis of the phenomenon of "irreversibility" in hemorrhagic shock was studied in dogs using endotoxin prepared according to the Boivin technique from a strain of *Escherichia coli* and labeled with $Cr^{51}Cl_3$. The technique was sensitive enough to detect approximately 0.001 of the LD_{50} dose of this endotoxin for normal dogs.

2. No absorption of Cr^{51}-labeled endotoxin from various portions of the bowel was observed in normal dogs or dogs subjected to "irreversible" hemorrhagic shock.

3. Increased susceptibility of dogs subjected to "reversible" hemorrhage and then injected with 0.001 LD_{50} dose of endotoxin was not demonstrated. If an increase in susceptibility did occur, it was in the range of 100-fold which was less than the minimal amounts of endotoxin detectable.

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