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A study of dogfish shark serum components participating in the inactivation of endotoxin

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A STUDY OF DOGFISH SHARK SERUM COMPONENTS

PARTICIPATING IN THE INACTIVATION OF ENDOTOXIN

by

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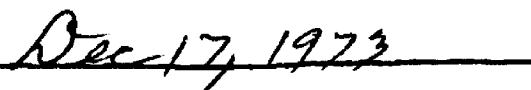
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ABBREVIATIONS

C	degrees Centigrade
C1...9	complement components
EAF	endotoxin altering factor
$\underline{g}$	gravity
IEP	immunoelectrophoresis
i.p.	intraperitoneal
i.v.	intravenous
KDO	ketodeoxyoctonate
LD_{50}	50% lethal dose
LPS	lipopolysaccharide
nm	nanometer
PBS	phosphate buffered saline
UDP	uridine diphosphate

CHAPTER I

INTRODUCTION

When bacterial endotoxin is extracted by the aqueous phenol method of Westphal, et al. (51) and injected parenterally into susceptible animals, varied host reactions result (22). Previous incubation of the endotoxin with plasma or serum from many animals resulted in a reduction or loss in ability of this endotoxin to elicit these host reactive properties (25, 39). Attempts to characterize humoral systems involved in the detoxification of endotoxin have been, for the most part, unsuccessful (30).

Von Eschen (49) studied the relationship of phylogeny to humoral endotoxin altering activity and found the Class Chondrichthyes to be the lowest level of animal to possess this ability. Because shark serum presented a system less complicated than that of higher animals, a study was designed to examine, characterize, and partially purify components of shark serum responsible for the inactivation of endotoxin.

It was hypothesized that endotoxin was responsible for complications in gram negative bacterial infections. Myerowitz, et al. (23) reported the following facts concerning bacillemia in human

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patients. Morbidity and mortality increased from infections with gram negative bacilli if the bacilli entered the bloodstream. Of every 1000 admissions to Peter Bent Brigham Hospital, Boston, in a 1971 study, 10.7 cases of bacillemia arose, or 8.6 episodes per 10,000 patient hospital days. Of these cases, 25.2% were fatal. This included patients admitted to the hospital for bacillemia and those who had an episode of bacillemia during or shortly after their hospital stay.

Endotoxin is present in the outer membrane of gram negative bacteria and is extractable by a variety of procedures (1, 8, 20, 26). The endotoxin is capable of eliciting a characteristic spectrum of host reactive properties (6, 22). Endotoxin is lipopolysaccharide in structure and is a heat stable macromolecular complex.

Most recent evidence (20, 52, 53) indicates that this macromolecular complex is a heteropolymer composed of three basic regions; O-specific chains, core polysaccharide, and lipid-A. The O-specific chains vary greatly between different kinds of gram negative bacteria. They are composed of sugar constituents, approximately 30 of which have been identified. The core polysaccharide is composed basically of ketodeoxyoctulosonic acid (KDO), phosphate, and heptose units. Most cores have 5 sugar constituents and are also variable in different gram negative bacteria. Lipid-A is relatively uniform in most gram negatives studies. It is composed of glycosidically linked glucosamine disaccharide units with the free hydroxyls substituted with long chain fatty acids. Mutants of the Re type of gram negative enteric bacteria contain only the glycolipid portion of the molecule (9). This is

mainly lipid-A-KDO. Westphal (50) reported that lipid-A was the toxic principle, and Rietschal, et al. (28) then found that KDO acted only as a carrier molecule. Galanos, et al. (9) showed that when lipid-A was complexed with bovine serum albumin, the complex acted as a potent pyrogen and was toxic for mice. Evidence has been cumulated (1, 26, 27, 37, 38) that the physical structure of endotoxin can be related to the toxic activity.

Shands (37, 38) examined current knowledge on the physical structure of endotoxin. The isolated LPS complex was found to be approximately 2.8-3.9 nm by 116.0-122.5 nm and 5×10^5 to 1×10^6 daltons in molecular weight. LPS particles were visualized in a bimolecular leaflet structure. Electron microscopy revealed a trilaminar structure for the isolated endotoxin of most gram negative bacteria. In aqueous environment, endotoxin was a bilayer of polysaccharide with hydrophobic nonpolar lipids at the interior. O-specific chains were hypothesized to project from the polysaccharide jacket into the aqueous environment. Anionic detergents were shown to fragment the LPS. It was postulated that hydrophobic forces held the quaternary structure of the LPS complex together.

Conflicting reports have been made as to the heat stability of the endotoxin altering factor. In human serum, a heat stable system of endotoxin detoxification was shown by Cluff (2) and Von Eschen (49). Keene, et al. (17), Skarnes (40), and Ho and Kass (12), however, found an endotoxin alteration system which was heat labile.

Hegemann (11) first observed that the pyrogenicity of endotoxin
could be reduced by incubation in serum. Skarnes and Chedid (42) ob-
served that endotoxin was still circulating in the bloodstream many
hours after injection and stated that survival from endotoxemia was
largely due to some detoxifying action of circulating plasma. Landy,
et al. (19) found that prior incubation with human serum decreased
endotoxic capacity to produce leukopenia, respiratory difficulty,
terminal vascular collapse in rabbits, tumor damaging potential, and
the local Shwartzman reaction. Ho and Kass (12) and Yoshioka and
Johnson (54) reported a loss in the ability of endotoxin incubated
with human serum to mediate a pyrogenic response. Stauch and Johnson
(45) reported a loss in ability of endotoxin to precitate with its
homologous antiserum, with maximal loss after about 4 hours of incu-
bation, and the formation of lighter components after incubation with
human plasma. Landy, et al. (18) suggested that endotoxin incubated
with serum was split into smaller haptenic molecules. Skarnes, et al.
(44) and others (2, 19, 32, 54) also reported a loss in many toxic
and immunologic properties after incubation with human serum.

Early work on characterization of endotoxin altering factors
indicated that endotoxin altering activity resided in the beta-globulin
fraction of human serum (2, 55). Ho and Kass (12) found that the endo-
toxin modifying substance was in the alpha and beta-globulin fractions
of human serum. When serum was fractionated by the cold ethanol ex-
traction method of Cohn, endotoxin altering activity was found in

fractions III_o and IV_1 (54). Skarnes (39, 41), Skarnes and Rosen (43), and Skarnes, et al. (44) demonstrated that detoxification of endotoxin was accompanied by complexing of the endotoxin with two alpha-globulins, a heat stable anionic-dependent lipoprotein and a heat labile globulin with esterase activity for naphthyl acetate.

Skarnes, et al. (44) initially suggested that complement did not play a role in the detoxification of endotoxin. However, Johnson and Ward (15), using 1000 times less endotoxin than Skarnes, reported that the methods of complement detection previously used had not been complete or specific and that complement was involved in the detoxification reaction. Also, May, et al. (21) showed that C4 deficient guinea pigs were more susceptible to the toxic effects of endotoxin. Serum of mice deficient in C5, and C6 deficient rabbit serum, exhibited reduced capacity to detoxify endotoxin (14, 15).

Keene, et al. (17) reported that rate of the inactivation of endotoxin by human serum was affected by time, temperature, pH, and amount of serum used in the incubation mixture. Below physiological temperature, serum degraded endotoxin slowly. As the temperature was raised to 90° C, the highest temperature assayed, the reaction moved progressively faster. A pH of 9.0-10.0 was optimum for endotoxin detoxification, with a progressive loss in rate at higher and lower pH. Little or no degradation of endotoxin was seen after 1-2 hours incubation of serum and endotoxin at physiological pH and temperature. Keene, et al. (17) interpreted these kinetic studies as indicating an

enzymatic degradation of endotoxin.

Early evidence suggested that the inactivation of endotoxin involved multiple steps (29). Skarnes (40) suggested a two step alteration of endotoxin. First the endotoxin is broken down to toxic subunits and then is enzymatically degraded. The second step might involve the protein with esterase activity proposed by Skarnes. The presence of ester-bound fatty acids on the lipid-A moiety has been shown to determine degree of endotoxic activity (24). However, no breakdown products of this type of enzymatic breakdown have ever been demonstrated (30).

Rudbach, et al. (31) proposed a nonenzymatic two step alteration of endotoxin. Initially, the endotoxin molecule is disaggregated to nontoxic subunits. This is followed by a binding of the subunits to certain plasma proteins, preventing reassociation. Rudbach and Johnson (33, 34) then showed that after alteration by plasma, endotoxic activity could be restored by methods not likely to reverse an enzymatic reaction. An artificial system to dissociate endotoxin with sodium deoxycholate was used to demonstrate the first step of the reaction as described above (27, 31). Termina, et al. (46, 47) showed reduced antigenicity and a reduced ability of endotoxin dissociated with sodium deoxycholate to protect mice against infection with Salmonella typhi, to provide dermal Shwartzman reactions, to induce pyrogenic tolerance, to kill chick embryos, and to act as an adjuvant.

Since direct attempts at isolation and purification of the proteins participating in the endotoxin altering reaction have proven

unsuccessful (30), an indirect approach was attempted. For example, Von Eschen (49) has shown that the capacity of serum to detoxify endotoxin was not present throughout the phylogenetic scale, but arose initially in the Class Chondrichthyes. Use of the less complicated humoral system of the dogfish shark, a chondrichthyian, might allow elucidation of the components involved in the detoxification of endotoxin.

One step in characterizing the endotoxin altering reaction is to identify proteins which bind to endotoxin during its inactivation. Therefore, concern was focused on these proteins rather than the dissociating agent.

In the following study, the endotoxin altering reaction in shark serum was partially characterized, isolated and purified. The following methods were employed: 1) kinetic studies; 2) esterase, hemolytic complement, and bacteriocidal assays: and 3) separation and purification of endotoxin altering components by ammonium sulfate precipitation, DEAE cellulose and Sephadex G-200 chromatography, and sucrose density ultracentrifugation. It was hoped that elucidation of the components participating in the endotoxin altering reaction in shark serum would lead to further understanding of the mode of action and identity of endotoxin altering factors in general.

CHAPTER II

METHODS AND MATERIALS

Endotoxin preparation

The Braude strain of E. coli 0113 was supplied by Dr. K. C. Milner of the Rocky Mountain Laboratory in Hamilton, Montana. Starter cultures of the E. coli were grown in shake flasks at 37° C for 24 hours in the M-9 medium and then transferred to stationary aerated glass containers for 24 hours at 37° C (30). Cells were harvested, washed in cold saline (0.15 M NaCl), and cell walls prepared by differential centrifugation following disruption of the bacteria at 16,000 psi in a Ribi Fractionator Model RF-1 (8). Endotoxin was extracted from the cell walls by the aqueous phenol extraction method of Westphal, et al. (51). Complete method of endotoxin preparation is diagrammed in Figure 1. With the assistance of Dr. K. C. Milner, Jim Kyle, Ronnie Brown, and Dr. Jim Cutler of the Rocky Mountain Laboratory, 650 milligrams of endotoxin were prepared by this method and designated Lot Ec 181.

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Lyophilized preparation of E. coli 0113 was placed on trypticase soy agar for 18 hours at 37° C.

↓

A typical colony was chosen and incubated in a 100 ml flask of M-9 medium[a] for 24 hours at 37° C.

↓

Six flasks, each with 100 ml of M-9, were inoculated from this one flask.

↓

Each flask was used as inoculum for 10 liters of M-9 in 10 gallon carboys and incubated statically for 18 hours at 37° C with sterile air bubbled through the medium.

↓

E. coli 0113 cells were harvested with the use of the Sharples Centrifuge, washed 3 times in cold saline (0.15 M NaCl) and suspended in cold water.

↓

E. coli 0113 cells were fractionated with the Ribi Fractionator Model RF-1 at 16,000 psi.

↓

Cell walls were separated from cytoplasm and cell debris by differential centrifugation at 27,000 x g for 2 hours in the Sorvall RC-2B Centrifuge.

↓

Cell walls were washed 6 times in cold water by resuspension and centrifugation and finally resuspended in 300 ml cold water.

↓

300 ml liquid phenol at 56°C was added to the 300 ml cell wall suspension, and the mixture heated at 56° C for 30 minutes with stirring.

↓

The suspension was cooled to 4° C and centrifuged in the Sorvall RC-2B at 27,000 x g for 1 hour.

Poor phase separation resulted.

↓

Water layer was removed

↓

300 ml distilled water added to the phenol-interface mixture and heated at 56° C for 30 minutes with stirring.

↓

continued

Centrifugation was repeated at
27,000 x g for 1 hour in the
Sorvall RC-2B Centrifuge.

Phenol phase was
discarded.

The aqueous phases were dialyzed against distilled water at $4^{\circ}C$ to
remove phenol, as indicated by the lack of detectable phenol odor.

Endotoxin was precipitated from the water by addition of sodium acetate
($NaC_2H_3O_2$. $2H_2O$, Fisher Scientific Co.) to a final concentration of
0.15 M and absolute ethanol to a final volume to volume concentration
of 68% while stirring at 4° C.

The solution was kept at 4° C overnight to allow precipitation.

The precipitate was separated in the Sorvall RC-2B Centrifuge at
1000 x g for 30 minutes.

Pellet from centrifugation was resuspended in 300 ml distilled water
and then dialyzed against distilled water to remove traces of ethanol.

Lyophilization yielded the purified endotoxin.

FIG. 1. Extraction of endotoxin from E. coli 0113.

---

[a]M-9 medium was prepared by dissolving 1 g NH_4Cl, 6 g Na_2HPO_4,
5 g NaCl, 3 g $KHPO_4$, 0.1 g MgS and 5% glucose in 1000 ml distilled
water.

Dogfish shark serum

With the advice of Dr. Douglas Anderson of the U.S. Naval Air Station in Seattle and assistance of Edward Goldberg, dogfish shark were caught by trawling in Puget Sound at a depth of 400 to 600 feet. Bleeding was effected by caudal amputation of 140 sharks into 500 ml flasks kept in an ice bath. The blood was allowed to clot overnight while kept cold. It was centrifuged at 1000 x g for 30 minutes at 4° C, and the serum then decanted into 10 ml vials and frozen $(-20^o$ C).

Antisera

Anti-E. coli 0113 serum was prepared by inoculating rabbits with increasing doses of heat killed E. coli 0113 cells. The rabbits were inoculated repeatedly by varying routes for at least 48 days. They were bled by cardiac puncture and the blood was allowed to clot at room temperature for 1 hour and overnight at 4° C. Centrifugation at 1000 x g for 30 minutes at 4° C separated the clot from the serum. The serum was then divided into 10 ml vials and frozen $(-20^o$ C). Six days later, they were boosted with 1 ml intraperitoneally and 1 ml subcutaneously of a 1 mg/ml suspension of heat killed E. coli 0113 cells in phosphate buffered saline. The next day, the rabbits were boosted with 1 ml of the same suspension injected intravenously. One week later, they were again bled. From this point, the rabbits were inoculated every other week, as described, and bled on the alternate

weeks. When approximately 400 ml anti-E. coli 0113 serum had been collected, all samples with hemagglutination titers of 5120 or above were pooled, heat inactivated at 56° C for 30 minutes, decanted into vials and frozen (-20° C).

Anti-shark serum was prepared by inoculating rabbits intramuscularly with increasing doses of whole shark serum emulsified with complete Freund adjuvant. Rabbits were boosted for 48 days. On day 49, they were bled by cardiac puncture of 30 ml blood. Seven days later, they were boosted by an intramuscular injection of 1 ml of an emulsion of 3 ml shark serum in 3 ml complete Freund adjuvant. The rabbits were bled seven days after being boosted. Rabbits were boosted every other week, and bled on alternate weeks. The serum was treated as was the anti-E. coli serum except that the anti-shark serum was not heat inactivated.

Anti-endotoxin altering factor (anti-EAF)

The endotoxin, shark serum and PBS (phosphate buffered saline-0.15 M NaCl, 0.0033 M PO_4, pH 7.4) mixture was incubated for 5 minutes at 37° C. The complex was precipitated with rabbit anti-endotoxin serum. It was hypothesized (30) that early in incubation of endotoxin and shark serum, shark serum components participating in the second step of the endotoxin altering reaction as described by Rudbach, et al. (31) might be bound to the endotoxin. If so, these shark serum components (EAF) could be precipitated with the rabbit antibodies and endotoxin.

Immunization of rabbits with this complex should then stimulate anti-
bodies to the EAF of shark serum. Therefore, the precipitated
material was collected by centrifugation at 1000 x g for 30 minutes
at 4° C. After washing twice in cold PBS, the material was resus-
pended in PBS and frozen (-20° C).

Anti-EAF serum was prepared by inoculating rabbits with in-
creasing doses of the endotoxin-EAF suspension. Rabbits were boosted
and bled by the schedule described for anti-shark serum in the pre-
vious section. The anti-EAF rabbits were boosted with 1 ml of an
emulsion of 3 ml endotoxin-EAF suspension in 3 ml complete Freund
adjuvant. Treatment of the serum was the same as for the anti-shark
serum.

Standard incubation mixture-endotoxin
and test serum mixture

To 1.7 ml shark serum was added 200 ug of endotoxin dissolved
in 0.1 ml distilled water and 0.2 ml PBS. The mixture was then
incubated at 37° C for 4 hours (Figure 2), and then assayed for
unreacted endotoxin.

Quantitative nitrogen precipitation assay

Complexing and precipitation occurred if endotoxin and

homologous antiserum were combined. If the endotoxin had been altered by incubation with serum, a reduction in the amount of precipitate was observed. Figure 3 outlines the procedure for the precipitation of endotoxin as described by Rudbach, et al. (35). To 0.5 ml of the standard incubation mixture was added 0.5 ml anti-E. coli serum, 10 units of heparin in 0.1 ml distilled water, and 0.4 ml PBS. This mixture was incubated for 2 hours at 37° C, 4°C for 24 - 48 hours and then centrifuged at 1000 x g for 30 minutes at 4° C. The precipitate was washed twice with cold PBS. As a control, an antiserum blank of 0.5 ml anti-E. coli serum, 0.9 ml PBS and 10 units heparin was dissolved in 0.1 distilled water and treated as the other tubes.

The amount of nitrogen present was determined by a modification of the Nessler nitrogen assay (16) following a Kjeldahl digestion. Initially, 0.2 ml of digestion mixture was added to each tube. The digestion mixture consisted of 0.181 g sodium selenite (Na_2SeO_2 . 5 H_2O, J. T. Baker Chem. Co.), and 0.262 g copper sulfate ($CuSO_4$. 5 H_2O, J. T. Baker Chem. Co.), both of which were dissolved separately in distilled water, and then mixed to form a green gelatinous suspension which cleared upon addition of 56.0 ml sulfuric acid (H_2SO_4, J. T. Baker Chem. Co.). Total volume of the digestion mixture was then brought to 200 ml with distilled water. The tubes were then placed in

1.7 ml shark serum + 200 ug endotoxin[a] + 0.2 ml PBS[b]

Incubate at 37° C for 4 hours

Assay for endotoxin (Figure 3)
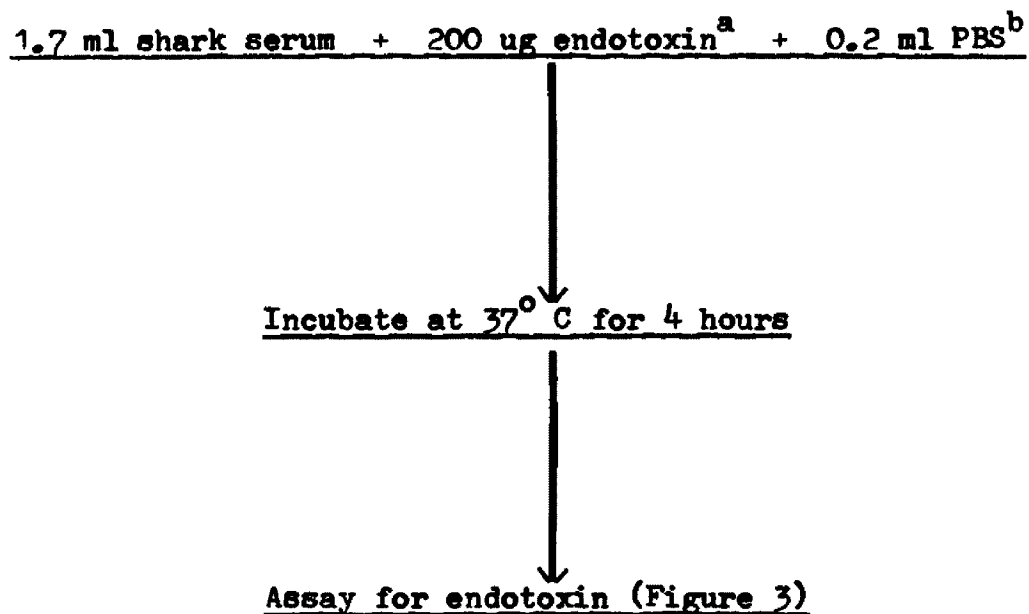
FIG. 2. Preparation of the standard incubation mixture.

[a]0.1 ml of a 2 mg/ml suspension of endotoxin in PBS was added to the incubation mixture.

[b]PBS = phosphate buffered saline

0.5 ml of endotoxin-shark serum incubation mixture (Figure 2)	0.5 ml of anti-serum to E. coli 0113	10 units of heparin in 0.1 ml distilled water	0.4 ml PBS[b]

Incubate at 37° C for 4 hours and 4° C for 18-24 hours

Centrifuge 1000 x g at 4° C for 30 minutes

Discard supernatant fluid

Wash precipitate twice in 1 ml cold PBS

Nitrogen determination
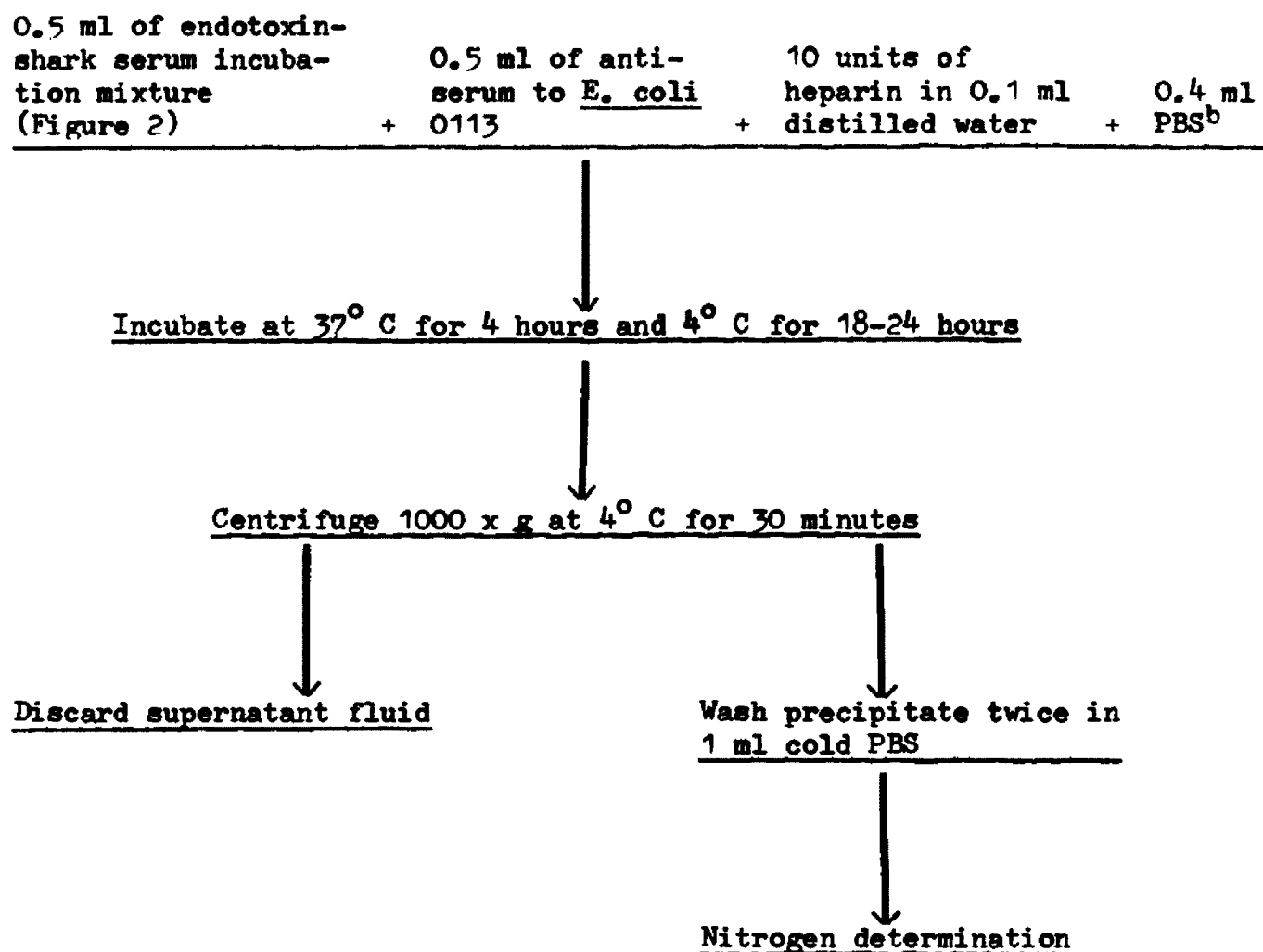
FIG. 3. Procedure for quantitative precipitation of endotoxin by homologous antiserum.

[a]10 units heparin in 0.1 ml distilled water.

[b]PBS = phosphate buffered saline

[c]Anti-serum to E. coli 0113 was prepared in rabbits by injection of heat killed E. coli 0113 cells.

a cold tube furnace and heated to 290° C. They were removed from the furnace, cooled, and one drop of 30% hydrogen peroxide added to each. The tubes were heated to 290° C and if color was still present, another drop of hydrogen peroxide was added and the tubes heated an additional 15 minutes. After the tubes were cooled, 2 ml of distilled water was added and mixed. Next, 2 ml of Nessler reagent was added and the tube contents again mixed. Nessler reagent consists of a solution of 1.75 g gum ghatti and 750 ml water which has been refluxed for 4 hours, to which has been added 5 g potassium iodide (KI, J.T. Baker Chem. Co.) and 5 g mercuric acid (HgI_2, J.T. Baker Chem. Co.) dissolved in 25 ml distilled water. Nessler reagent was allowed to stand for 3 days, filtered through a thick layer of glass wool, and diluted to a volume of 1000 ml. After addition of the Nessler reagent, 3 ml of 2 N sodium hydroxide (NaOH, J.T. Baker Chem. Co.) was added and the solutions mixed. This final mixture was allowed to stand for 15 minutes and the absorbancy read on a Klett-Summerson Colorimeter, Model 800-3, with a 500 nm green filter. The machine was corrected to read zero with a mixture containing 2 ml distilled water, 2 ml Nessler reagent, and 3 ml 2 N NaOH. Nonspecific nitrogen in the precipitate was determined from a control of 0.9 ml PBS, 0.5 ml antiserum, and 0.1 ml of heparin (10 units). By comparison to a 30 ug standard, the amount of nitrogen was determined by the following formula:

$$\text{ug nitrogen} = \frac{\text{corrected Klett reading x 30}}{\text{Klett reading of 30 ug nitrogen standard}}$$

Once the amount nitrogen had been determined, the value was interpolated into a standard curve of ug nitrogen precipitated against ug endotoxin added (Figure 4) and the unreacted endotoxin determined. Varying amounts of endotoxin were added to 0.5 ml antiserum, 10 units of heparin

contained in 0.1 ml distilled water, and sufficient PBS to give a total volume of 1.5 ml. The tubes were incubated at 37° C for 2 hours, overnight at 4° C and then the nitrogen content determined. A standard curve was calculated for each antiserum pool prepared (Figure 4), starting with AS-4 (antiserum-pool 4).

Quantitative radioisotope precipitation assay

Endotoxin with ^{51}Cr label (42) was prepared as outlined in Figure 5. Activity of the endotoxin preparation was 2.38 mg per ml of 0.001 M disodium phosphate and 4.92×10^4 counts per minute per ml. A standard curve was prepared by adding varying amounts of ^{51}Cr-endotoxin to 0.5 ml antiserum and a sufficient volume of PBS to bring the mixture to a total of 1.5 ml. The mixtures were then incubated for 2 hours at 37° C and overnight at 4° C. Tubes were centrifuged at 1000 x g for 30 minutes at 4° C. The precipitates were washed twice in cold PBS and measured for radioactivity in a Nuclear Chicago Model 8775 Scaler and crystal scintillation detector well. The standard curve of counts per minute detected against ug ^{51}Cr-endotoxin added is shown in Figure 6.

Shark serum was tested for the ability to alter endotoxin by adding 107.5 ug ^{51}Cr-endotoxin in 0.05 ml 0.001 M disodium phosphate to 0.85 ml serum and 0.1 ml PBS. A control of 0.05 ml ^{51}Cr-endotoxin and 0.95 ml PBS was prepared to represent 100% recovery of endotoxin. The mixtures were then incubated for 4 hours at 37° C. A half ml of anti-E. coli serum and sufficient PBS to give a total
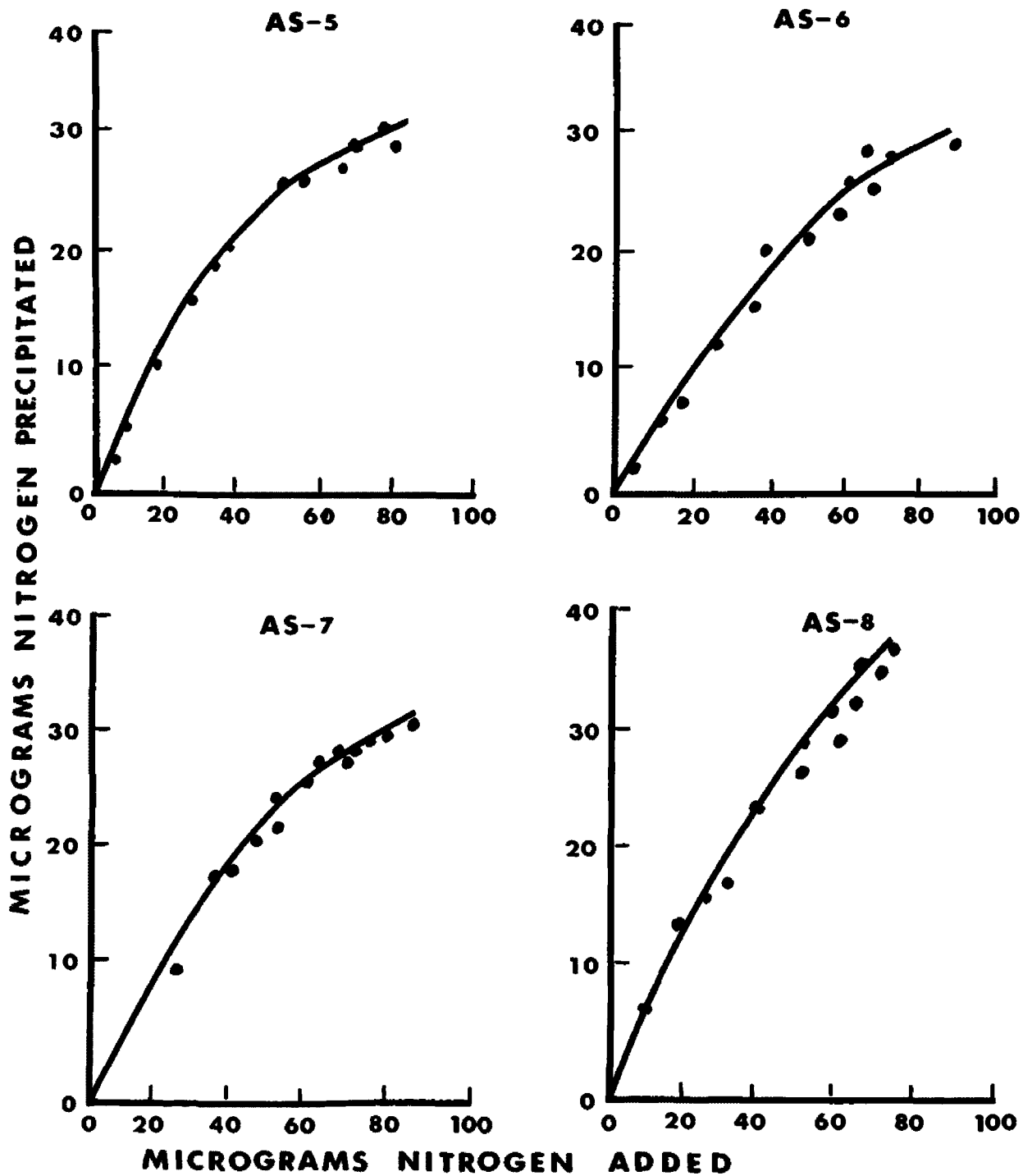
FIG. 4. Standard nitrogen precipitation curves prepared with 4 antisera pools from rabbits immunized with heat killed cells of E. coli 0113.

10 mg endotoxin was dissolved in a 5 ml solution containing 1 milli-curie $^{51}Cr^a$ at room temperature and incubated at 37° C for 48 hours.

The solution was dialyzed against 0.001 M disodium phosphate (Na_2HPO_4, J. T. Baker Chem. Co.).

The dialysate was changed daily and checked for radioactivity.

When the radioactivity measured in the dialysate was constant for three days the contents of the dialysis tube were transferred to a small vial for storage.

0.1 ml of the ^{51}Cr labeled endotoxin was measured for radioactivity.

FIG. 5. Preparation of ^{51}Cr labeled endotoxin from E. coli 0113 and ^{51}Cr labeled sodium chromate.

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^{a}The sodium chromate had activity of 0.21 millicuries/ml, or 43.8 millicuries/mg solid.

^{b}Specific activity was 4920 counts per minute per 0.1 ml over background (265 counts per minute) and 2.38 mg endotoxin per ml 0.001 M phosphate.
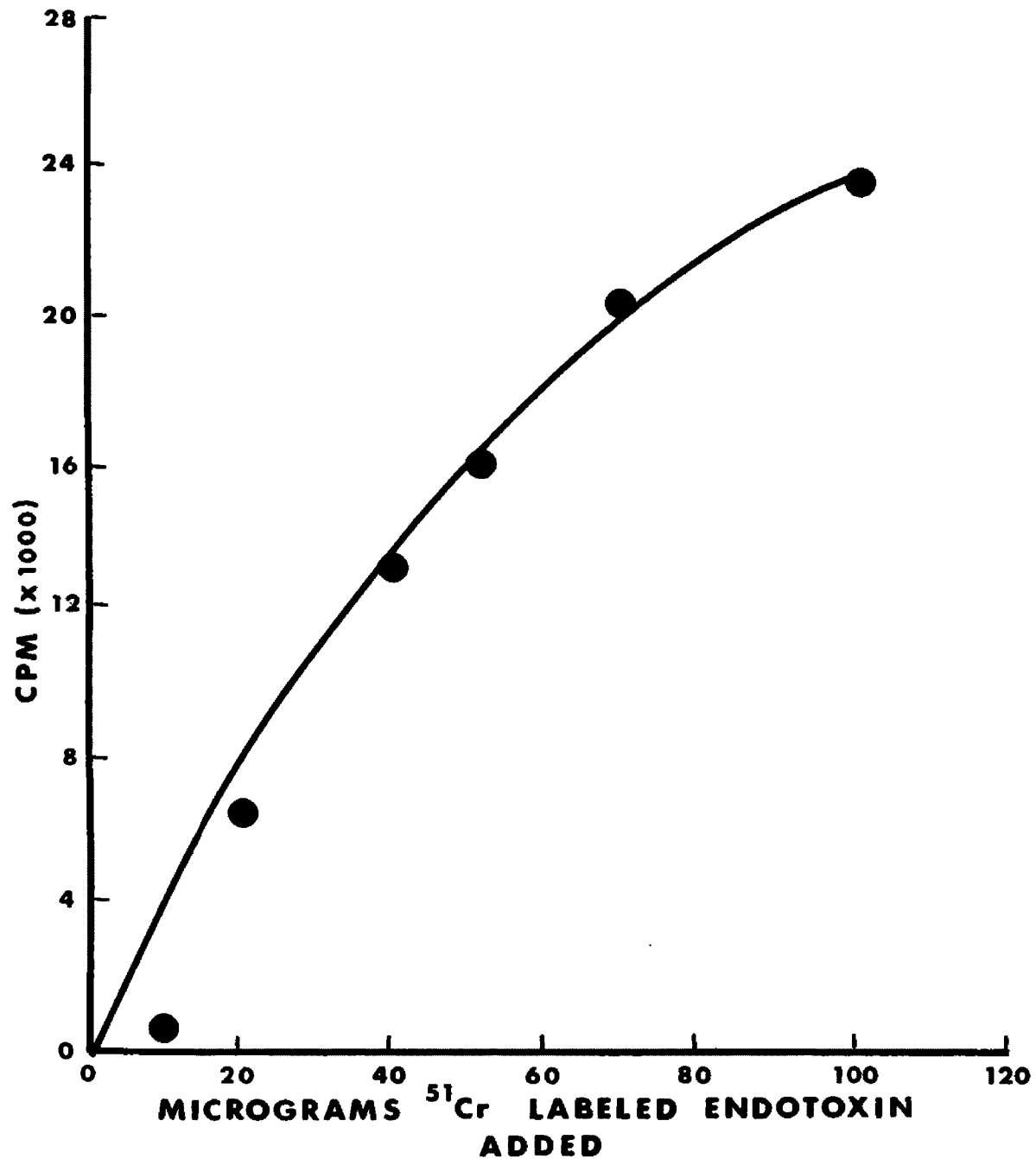
FIG. 6. Standard quantitative radioisotope precipitation curve for ^{51}Cr-labeled endotoxin, prepared with antisera pool AS-6.

volume of 1.5 ml was added to two tubes containing 0.2 ml and 0.4 ml of the standard incubated mixture. These tubes were incubated for 2 hours at 37° C and 18-24 hours at 4° C. The tubes were centrifuged at 1000 x g for 30 minutes at 4° C. Radioactivity was measured as in the standard curve preparation. These values were interpolated into the standard curve to determine amount of unreacted endotoxin. Amount inactivation was calculated by the following formula:

$$\% \text{ inactivation} = 100 - \frac{\text{ug unreacted endotoxin}}{100\% \text{ recovery of endotoxin}}$$

Mouse lethality assay

Rocky Mountain Laboratory mice, 3-6 weeks old, were used in determining an increase in LD_{50} of endotoxin after incubation with serum. The standard incubation mixture was increased four-fold in volume, and following the incubation, serial two-fold dilutions of the mixtures were prepared in PBS. All mice were injected intravenously with 1 mg lead acetate $(Pb(CH_3CO_2)_2$, J. T. Baker Chem. Co.) dissolved in 0.2 ml distilled water to potentiate endotoxin poisoning (4, 5, 36). Five mice were put aside as a control group against death by lead acetate. After bleeding had stopped from the lead acetate injection, 5 mice each were injected intraperitoneally with 0.5 ml of two-fold serial dilutions of the incubation mixtures. Deaths were recorded at each of the following 3 days. The Reed-Meunch Method (3) was used to calculate the LD_{50}. The LD_{50} of mice injected with the endotoxin and

PBS mixture dilutions was recorded as the 100% recovery value of endo-
toxin. Percent inactivation was determined by the following formula:

$$\% \text{ inactivation } = 100 \; (\frac{LD_{50} \text{ of endotoxin and PBS}}{LD_{50} \text{ of endotoxin and test serum}})$$

Dialysis of shark serum against
deionized distilled water

Shark serum was placed in dialysis tubing and dialyzed against
deionized distilled water. The water was changed at frequent inter-
vals and conductivity of the dialysate measured on the YSI Model 31
conductivity bridge. When the dialysate reading was that of the de-
ionized distilled water, the serum was removed from dialysis and cen-
trifuged at 1000 xg for 30 minutes at 4° C. Supernatant fluid was
decanted into vials and frozen (-20° C). The precipitate was redis-
solved in PBS, divided into vials and also frozen (-20° C).

Addition of sodium citrate to the
standard incubation mixture

Sodium citrate ($Na_3C_6H_5O_7$, J. T. Baker Chem. Co.) in some cases
was added to the standard incubation mixture to a final concentration
of 0.1 M. Other than the addition of 0.2 ml of 1 M citrate in place
of the 0.2 ml PBS, all else remained the same.

Dowex cation exchange

Dowex 50W-X8 in the hydrogen form was charged with 7.5% sodium

chloride and then washed free of excess ions with distilled water. Ions in the washings were detected with the conductivity bridge. A sample of 20 ml shark serum was then applied to a 1.5 x 20 cm column of the charged resin and allowed to flow at a rate of 1 ml per 3 minutes. The eluting buffer was PBS. The pH of the eluted shark serum was adjusted to 7.2, the original pH of the shark serum. Precipitated material was removed by centrifugation at 1000 x g for 30 minutes at 4° C. Shark serum was decanted into vials and frozen (-20° C). In all remaining sections in methods and materials serum was used that had been passed through Dowex 50W-X8 in this manner.

Immunoelectrophoresis

Immunoelectrophoresis (IEP) was performed on 7.62 cm x 2.54 cm microscope slides coated with 2.5 ml of 2% ion-agar in veronal buffer 0.05 M, 0.01% merthiolate, pH 8.2). Antigen wells were displaced 2.54 cm to the cathode side of center. A constant current of 4 milliamps per slide was applied for 2.5-3 hours. Troughs were then prepared, antisera added, and immunodiffusion allowed to proceed for 24 hours. Slides were read with oblique lighting against a dark background.

Ammonium sulfate precipitation

In an attempt to separate the shark serum proteins on ammonium sulfate ((NH_4)$_2SO_4$, J. T. Baker Chem. Co.) fractionation was undertaken. Desired concentrations of ammonium sulfate were obtained by adding saturated ammonium sulfate as calculated by the following formula:

$$X = V(C_2 - C_1) \quad (100 - C_2)$$

The volume of ammonium sulfate to be added is X with C_2 the desired concentration, C_1 the existing concentration, and V the volume of solution to which the ammonium sulfate is to be added. At each concentration, the precipitated fraction was removed by centrifugation at 1000 x g for 30 minutes at 4° C, resuspended in PBS, decanted into vials and then frozen $(-20^\circ$ C).

Sephadex exclusion chromatography

Sephadex G-200 gel (Pharmacia Fine Chem.) was swollen in cold PBS to give a volume of approximately 450 ml. After air bubbles were removed from the PBS and the Sephadex slurry, the slurry was added slowly and continuously to 10.4 cm from the top of the chromatographic column (Pharmacia Chem., Uppsula, Sweden, 100 cm high, 2.5 cm in diameter). The column was packed using a pressure head of 20 cm and reversed flow. The gel was then washed with approximately one liter of cold PBS. Serum to be passed through the column was dialyzed overnight at 4° C against cold PBS followed by centrifugation at 1000 x g for 30 minutes at 4° C. The serum was then added through the lower end of the column by a needle and syringe, care being taken not to allow introduction of air bubbles into the gel. Up to 40 ml of sample was added, with 5 ml fractions being collected. The fractions were then analyzed for protein by absorption readings of ultraviolet light at 280 nm (Beckman DU-2 Spectrophotometer). When possible, samples were read directly as they came off the column by the Gilson UV recorder.

Cellulose ion exchange chromatography

DEAE cellulose (J. T. Baker Chem. Co.) was placed in distilled water to allow swelling and washed to remove fines. Once all of the fine material had been removed, the cellulose was washed with 1% sodium hydroxide for 1 hour. Excess sodium hydroxide was removed by washing with distilled water. The slurry was then washed with tris-HCl (0.005 M, pH 8.0) until the pH of the washings equalled that of the washing buffer. Air bubbles were removed from a 1:1 (volume to volume) slurry of the cellulose and buffer which was loaded into the chromatographic column (Fischer-Porter Co., 84 cm high, 2.5 cm in diameter) slowly and continuously to a height of 70-80 cm. The material to be chromatographed was dialyzed against the tris-HCl buffer while the column continued to wash, also with the tris-HCl buffer. Five ml of shark serum was carefully layered onto the top of the column, run into the column, and then eluted with a linear gradient of 0.0 to 0.5 M potassium chloride (KCL, J. T. Baker Chem. Co.) in the tris-HCl buffer. Fractions of 10 ml were collected.

Sucrose density gradient ultracentrifugation

A discontinuous gradient of sucrose was added to each of 8 40 ml polyalloyl ultracentrifuge tubes. Sucrose in distilled water was added to each centrifuge tube in the following amounts and sequence: 4 ml of 60%, 6 ml of 50%, 6 ml of 40%, 6 ml of 30%, 6 ml of 20%, and 5 ml of 10%.

Four ml of a 1:5 dilution of shark serum in PBS was slowly layered over the gradients. The assembled tubes which were then checked for leakage were placed in a Ti 60 rotor and centrifuged at 60,000 rpm for 10 hours at 4° C in the Beckman L2-65B ultracentrifuge. After the centrifugation, 1 ml fractions were collected from a small hole carefully placed in the bottom of the centrifuge tubes.

Esterase activity

Esterase activity was determined by the Skarnes modification (39) of the Uriel method (48).

Six 40 ml test tubes were prepared, each containing 10 mg of beta naphthyl acetate $(CH_3COOC_{10}H_7$, J. T. Baker Chem. Co.) dissolved in 1.0 ml acetone $(CH_3COCH_3$, and 25 ml of 0.15 M tris buffer, pH 7.2. Varying amounts of shark serum were added to the tubes as follows: 1.0 ml, 0.5 ml, 0.25 ml, 0.1 ml. A tube without shark serum was included as a control. Sufficient tris buffer (.015 M, pH 7.2) was added to each tube to bring the volume to 27 ml. The tubes were incubated at 37° C for 1 hour. Diazo blue B (tetrazotized di-o-anisidine, K and K Laboratories, Inc.) was then added to a final concentration of 0.2 mg per ml. Esterase activity was detected visually by a red-violet coloration of the solution.

Hemolytic complement

Serial two-fold dilutions of shark serum were prepared in saline

supplemented with 0.005 M Mg^{++} and 0.0015 M Ca^{++}. Fresh sheep red

blood cells were washed three times in PBS and then sensitized by adding

cells to a final concentration of 2.5% in a 1:200 dilution of rabbit

hemolysin (BBL Laboratories). One drop of the sensitized cells was

added to 0.5 ml of each shark serum dilution. Control tubes were pre-

pared as follows: (1) 0.5 ml diluent and 1 drop sensitized cells;

(2) 0.5 ml guinea pig serum and 1 drop sensitized cells; (3) 0.5 ml

shark serum and 1 drop 2.5% normal sheep red blood cells. All tubes

were incubated at 37° C for 1 hour and overnight at 4° C. The tubes

were read visually for complete lysis of the red blood cells.

Bacteriocidal assay

A 24-hour culture of E. coli 0113 in trypticase soy broth (Difco

Co.) was prepared and diluted 1:10^5, 1:10^6, and 1:10^7 in sterile saline.

The following mixtures were then made in 3 ml test tubes with all three

culture dilutions: 1) 0.5 ml shark serum, 0.4 ml culture dilution,

and 0.1 ml sterile saline; 2) 0.5 ml shark serum heated at 56° C for

30 minutes, 0.4 ml culture dilution, and 0.1 ml guinea pig complement

(BBL Laboratories); 3) 0.5 ml sterile saline, 0.4 ml culture dilution,

and 0.1 ml guinea pig complement; and 4) 0.6 ml sterile saline and 0.4

ml culture dilution. Tubes containing only complement, shark serum, or

saline were also prepared to check for bacterial contamination. The

contents of each tube was mixed thoroughly and incubated at 37° C for

1 hour. After incubation, pour plates of trypticase soy agar at 45° C

were prepared with the contents of each tube. Bacterial counts were made after the plates were incubated in an inverted position at 37° C for 24 and 48 hours.

CHAPTER III

RESULTS

Ability of shark serum to inactivate endotoxin

Sharks were caught by trawling at a depth of 400-600 feet in
Puget Sound and bled by caudal amputation. Due to the depth at which
the sharks were caught and the speed at which they were brought to
the surface, blood was pushed into the muscle tissue. This made
bleeding of the fish extremely difficult. During collection, sea
water and mucous from the outside of the fish dripped into the col-
lection flask, adding to the problem. When serum was removed from
the clot by centrifugation, it contained a dark material. This ma-
terial was nitrogenous and continually precipitated from the serum.

The quantitative nitrogen precipitation assay could not be used
because of this nonspecific precipitate. Therefore, ^{51}Cr-labeled
endotoxin was used in the standard incubation mixture, and radio-
activity was quantified in the precipitate rather than the nitrogen.
With this assay, 100% inactivation of endotoxin by untreated shark
serum was detected (Table 1). However, such an incubation mixture
resulted in only a 28% reduction in the LD_{50} for mice of the endotoxin.

It has been reported (49) that untreated shark serum would not

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inactivate endotoxin and that heating the serum at 56° C for 30 minutes activated the endotoxin detoxifying system. These results were obtained with serum collected by cardiac puncture. As shown in Table 1, un- treated shark serum collected by caudal amputation did inactivate endo- toxin, whereas heating the shark serum at 56° C for 30 minutes routinely diminished or destroyed inactivating capability. It is hypothesized that proteases from the mucous covering of the shark altered the charac- teristics of the sera used in the present study; this could account for the apparent discrepancies between the two studies.

Dialysis of shark serum against deionized distilled water caused precipitation of the euglobulins. This procedure removed the non- specific precipitate from the shark serum and allowed the quantitative nitrogen precipitation assay to be employed. Table 2 indicates 56%, 74%, and 90% inactivation detected by the quantitative nitrogen precipitation assay after treatment of the shark serum in this manner. Addition of sodium citrate to the shark serum also prevented formation of the non- specific precipitate. Shark serum with citrate added gave 71% inacti- vation of endotoxin as detected by mouse LD_{50} and 100% inactivation of endotoxin in the quantitative radioisotope precipitation assay (Table 3). When this citrate-containing serum was heated at 56° C for 30 minutes, inactivating capability was once again either reduced or destroyed. The quantity of unreacted endotoxin recovered from a control containing endotoxin, PBS, and citrate was defined as the 100% recovery value.

TABLE 1. Capacity of shark serum to inactivate endotoxin.

Sample	% inactivation		
	Quantitative N precipitation[a]	Mouse lethality[b]	Quantitative radio-isotope precipitation[c]
Untreated shark serum + endotoxin	ND[d]	28	100
Heated[e] shark serum + endotoxin	ND	0	0
PBS[f] + endotoxin	ND	0	0

[a]Nitrogen quantitated to assay for unreacted endotoxin in standard incubation mixture and compared to a 100% recovery value.

[b]Decrease in lethality of mice for endotoxin is measured.

[c]Unreacted ^{51}Cr-labeled endotoxin in the standard incubation mixture is measured against a 100% recovery value.

[d]ND = not done.

[e]Heated at 56° C for 30 minutes.

[f]PBS = phosphate buffered saline.

TABLE 2. Capacity of shark serum dialyzed against deionized distilled water to inactivate endotoxin.

	% inactivation[a]		
Sample	Assay 1	Assay 2	Assay 3
Dialyzed shark serum + endotoxin	56	74	90
PBS[b] + endotoxin	0	0	0

[a]Inactivation determined by quantitative nitrogen precipitation assay. Nitrogen is quantitated to assay for unreacted endotoxin in the standard incubation mixture and compared to a 100% recovery value.

[b]PBS = phosphate buffered saline

TABLE 3. Capacity of shark serum to inactivate endotoxin after addition of sodium citrate.

| Sample | % inactivation | | |
	Quantitative N precipitation[a]	Mouse lethality[b]	Quantitative radio-isotope precipitation[c]
Shark serum + citrate[d] + endotoxin	71	50	100
Heated[e] shark serum + citrate + endotoxin	0	0	29
PBS[f] + citrate + endotoxin	0	0	0

[a]Nitrogen quantitated to assay for unreacted endotoxin in standard incubation mixture and compared to a 100% recovery value.

[b]Decrease in lethality of mice for endotoxin is measured.

[c]Unreacted ^{51}Cr-labeled endotoxin in the standard incubation mixture is measured against a 100% recovery value.

[d]Sodium citrate added to a final concentration of 0.1 M.

[e]Heated at 56° C for 30 minutes.

[f]PBS = phosphate buffered saline.

An example of the details of the data collected and the calculations of the mouse median lethality for endotoxin treated with shark serum and citrate is shown in Table 4.

Procedures which removed free ions from shark serum also removed the nonspecific precipitate and allowed the quantitative nitrogen precipitation assay to be used for detecting the alteration of endotoxin. Therefore, shark serum was treated with Dowex 50W-X8 cation exchange resin and tested for its inactivating capacity. Table 5 indicates the high and reproducible inactivating capability of shark serum treated in this manner. When shark serum was passed through the cation exchange resin, it was noted that pH had dropped from 7.2 to between 1.5 and 2.5. Adjustment to pH 7.2 was made with NaOH. After treatment with cation exchange resin, flocculent material appeared and was removed by filtration or centrifugation. Preheating the shark serum at 56° C for 30 minutes reduced its capacity to inactivate endotoxin.

Effect of varying incubation conditions
on the endotoxin altering reaction

Various amounts of shark serum were incubated with endotoxin. It was found that as little as 0.4 ml shark serum in the otherwise standard incubation mixture gave 50% inactivation (Figure 7). Maximal inactivation was caused by 0.8 ml shark serum, or 7.8 mg shark serum protein. Routinely, 1.7 ml shark serum was used in the standard incubation mixture.

TABLE 4. Lethality for mice of endotoxin incubated with shark serum[a] and citrate[b].

ug endotoxin	D[c]	S	CD	CS	Total	% Dead	LD$_{50}$	% Inactivation
50[d]	2	3	9	3	12	75		
25	2	3	7	6	13	54	22.6 ug	50
12.5	1	4	5	10	15	33		
6.25	2	3	4	13	17	23		
3.12	1	4	2	17	19	10		
1.56	1	4	1	21	22	4.5		
0.78	0	5	0	26	26	0		
0.39	0	5	0	31	31	0		
50[e]	4	1	15	1	16	94		
25	3	2	11	3	14	79	10.88 ug	0
12.5	2	3	8	6	14	57		
6.25	2	3	6	9	15	40		
3.12	2	3	4	12	16	25		
1.56	1	4	2	16	18	11		
0.78	1	4	1	20	21	5		
0.39	0	5	0	25	25	0		
50[f]	5	0	14	0	14	100		
25	3	2	9	2	11	82		
12.5	2	3	6	5	11	54	11.3 ug	0
6.25	2	3	4	8	12	33		
3.12	1	4	2	12	14	14		
1.56	1	4	1	16	17	5.9		
0.78	0	5	0	21	21	0		
0.39	0	5	0	26	26	0		
0.0[g]	0	5						

[a]Shark serum and endotoxin incubated at concentrations found in the standard incubation mixture.

[b]Sodium citrate added to a final concentration of 0.1 M.

[c]Abbreviations: D = died, S = survived, C = cumulated, LD$_{50}$ is the dose at which 50% of the mice died.

[d]Standard incubation mixture with citrate added.

[e]Standard incubation mixture with serum heated 56° C for 30 minutes with added citrate.

[f]100% recovery value of PBS, citrate, and 200 ug endotoxin.

[g]Mice given 1 mg lead acetate only.

TABLE 5. Capacity of shark serum treated with cation exchange resin to inactivate endotoxin.

| Sample | % inactivation | |
	Quantitative nitrogen precipitation assay[a]	Mouse LD$_{50}$[b]
Shark serum (CE)[c] + endotoxin + PBS[d]	55, 62, 64, 67, 70, 97[e]	75
Heated[f] shark serum (CE) + endotoxin + PBS	0, 12, 18[e]	14
Endotoxin + PBS	0	0

[a]Nitrogen quantitated to assay for unreacted endotoxin in the standard incubation mixture and compared to a 100% recovery value.

[b]Decrease in lethality of mice for endotoxin was measured.

[c]Shark serum was passed through Dowex 50W-X8 cation exchange resin.

[d]PBS = phosphate buffered saline.

[e]Multiple assays performed.

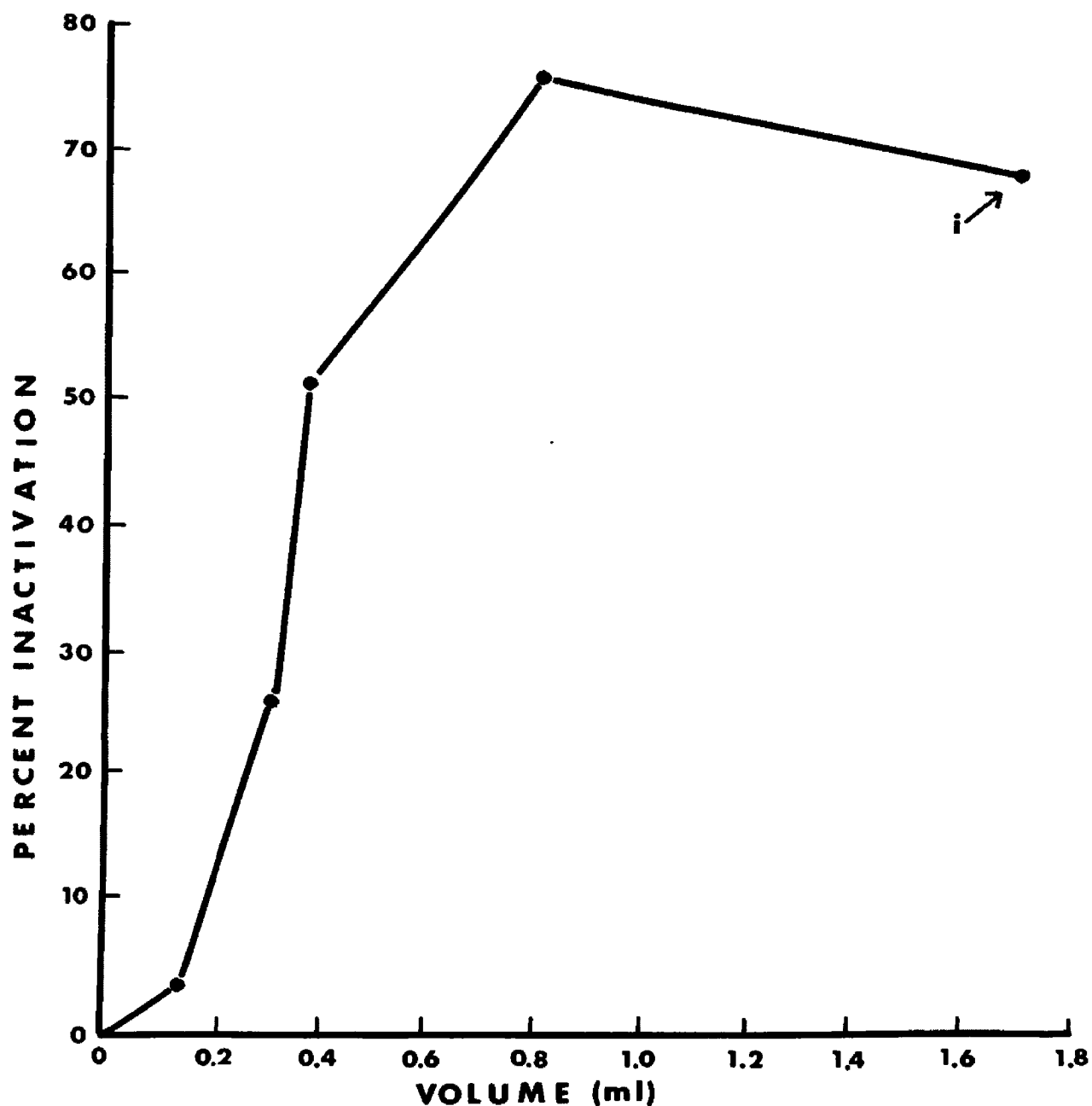[f]Heated at 56° C for 30 minutes.

FIG. 7. Effect of amount shark serum added to the standard incubation mixture. Percent inactivation determined by the quantitative precipitation assay. Except for varying amount shark serum added, incubation mixtures were handled in the standard manner. Amount shark serum used in the standard incubation mixture is indicated by i.

Figure 8 indicates that as little as 5 minutes of incubation of the mixture gave 50% inactivation of endotoxin and 1 hour allowed near maximum inactivation. Four hours of incubation was used routinely. This time period placed the inactivation on the plateau portion of the curve.

Temperature of maximum activity was 45° C (Figure 9). However, in all assays, 37° C was used as the temperature for incubation.

The pH for maximal inactivation was 7.2 (Figure 10). The pH used in all assays was 7.0 - 7.2

Studies of the endotoxin altering factor

by immunoelectrophoresis

Rudbach, et al. (31) proposed a two-step alteration of endotoxin by EAF (endotoxin altering factor) as previously described in this paper. The first step was disaggregation of the endotoxin. Anti-EAF serum used in this study was prepared against only those proteins binding to endotoxin, not the factors participating in the disaggregation step.

Shark serum exhibited a wide variety of proteins, most migrating slowly, with the notable absence of the albumin band (Figure 11a). When shark serum was electrophoresed and the pattern developed with anti-EAF serum, only two distinct bands appeared (Figure 11b). However, when the serum was assayed by crossed electrophoresis (separation of proteins by electrophoresis in two directions, perpendicular to each other) and developed with the anti-EAF serum, more protein bands appeared, indicating similarly migrating endotoxin binding proteins (Figure 11c). In all
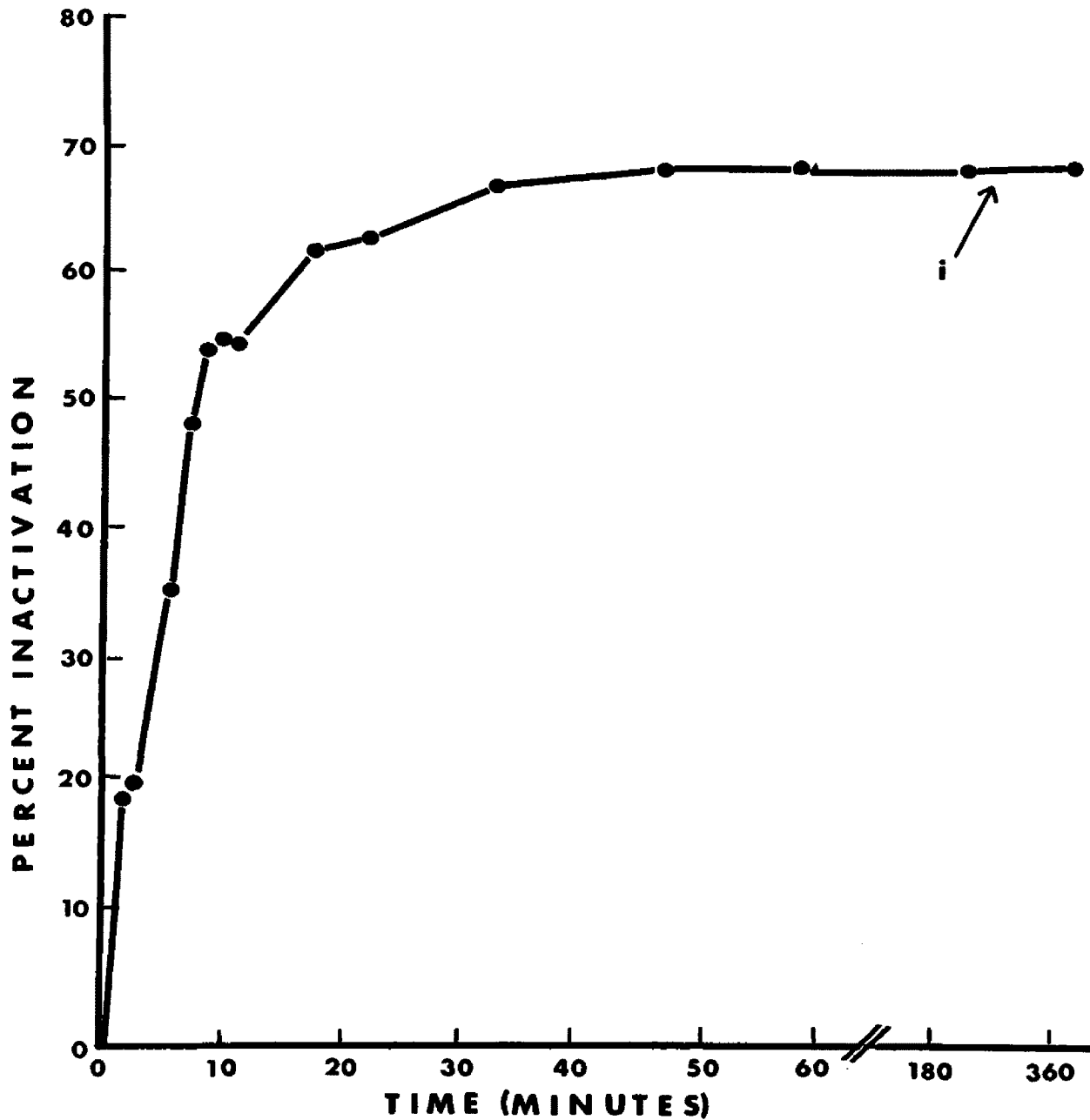
FIG. 8. Effect of time of incubation on the capacity of shark serum to inactivate endotoxin. Percent inactivation was determined by the quantitative nitrogen precipitation assay. Except for varying time, the incubation mixtures were handled in the standard manner. Incubation time used for the standard incubation mixture is indicated by i.
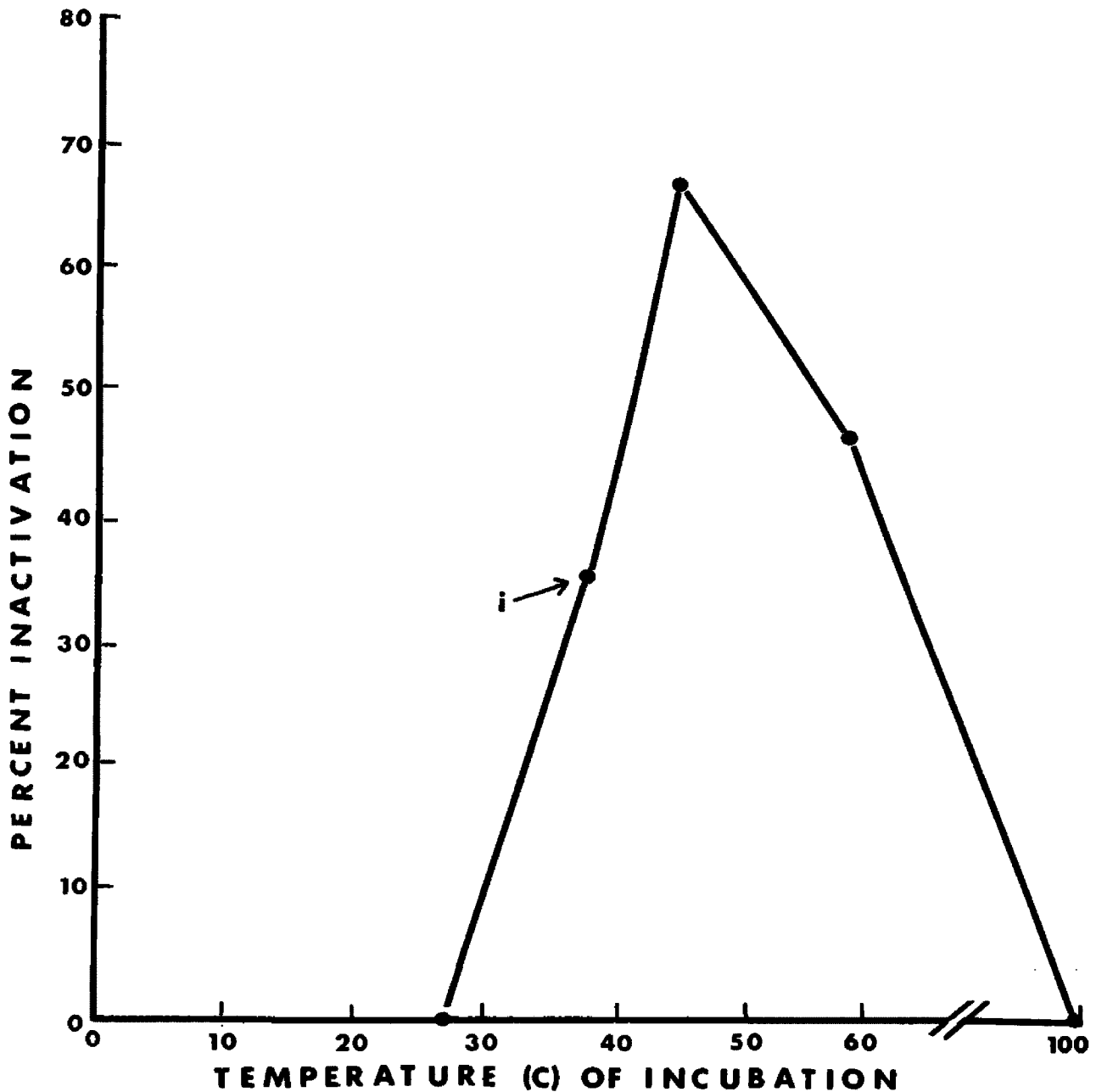
FIG. 9. Effect of temperature of incubation on the capacity of shark serum to inactivate endotoxin. Percent inactivation was determined by the quantitative precipitation assay. Except for varying incubation temperatures, incubation mixtures were handled in the standard manner. Temperature used in the standard incubation mixture is indicated by i.
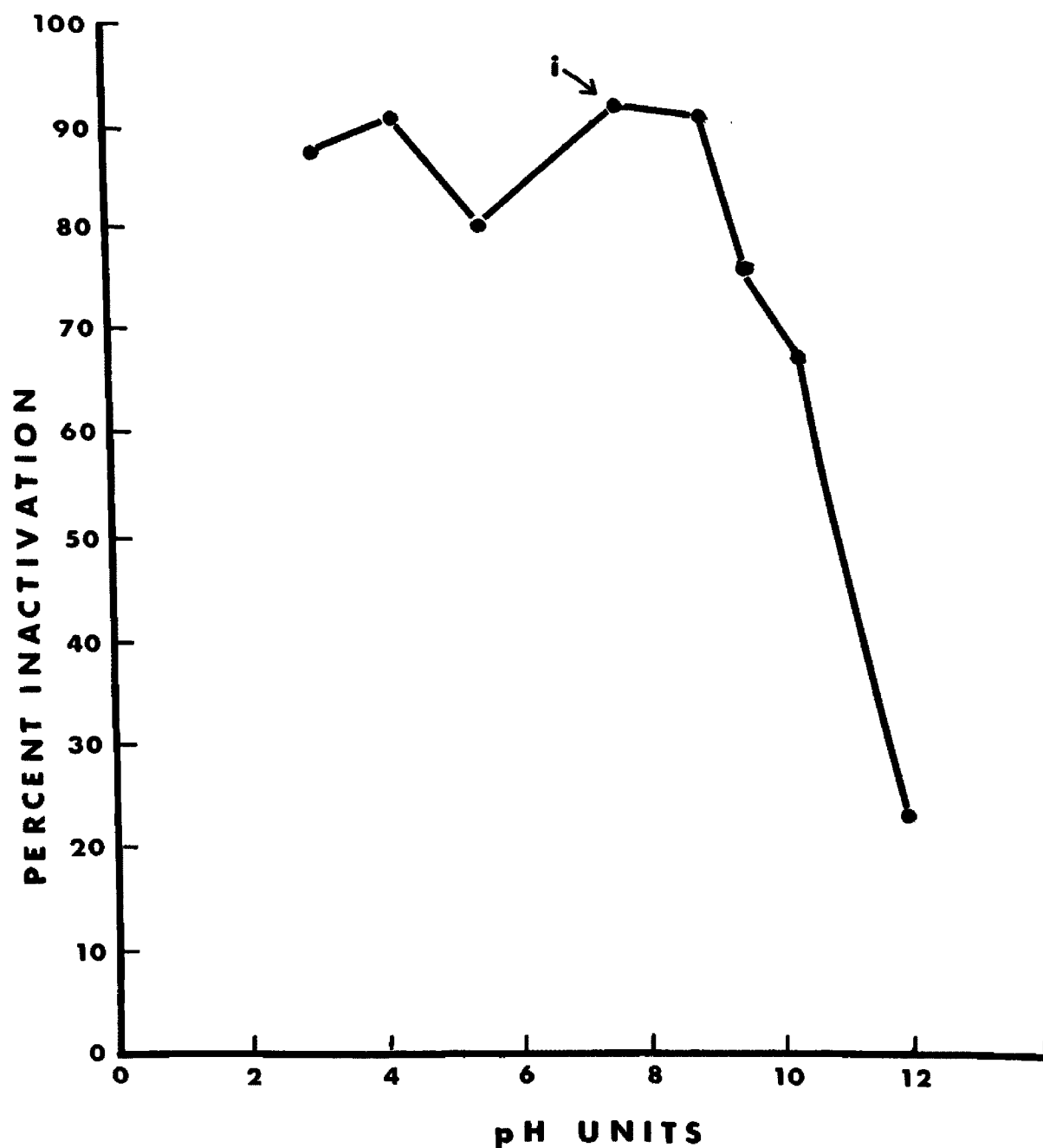
FIG. 10. Effect of pH of incubation on the capacity of shark serum to inactivate endotoxin. Percent inactivation was determined by the quantitative precipitation assay. Except for varying pH, the incubation mixtures were handled in the standard manner. pH used in the standard incubation mixture is indicated by i.
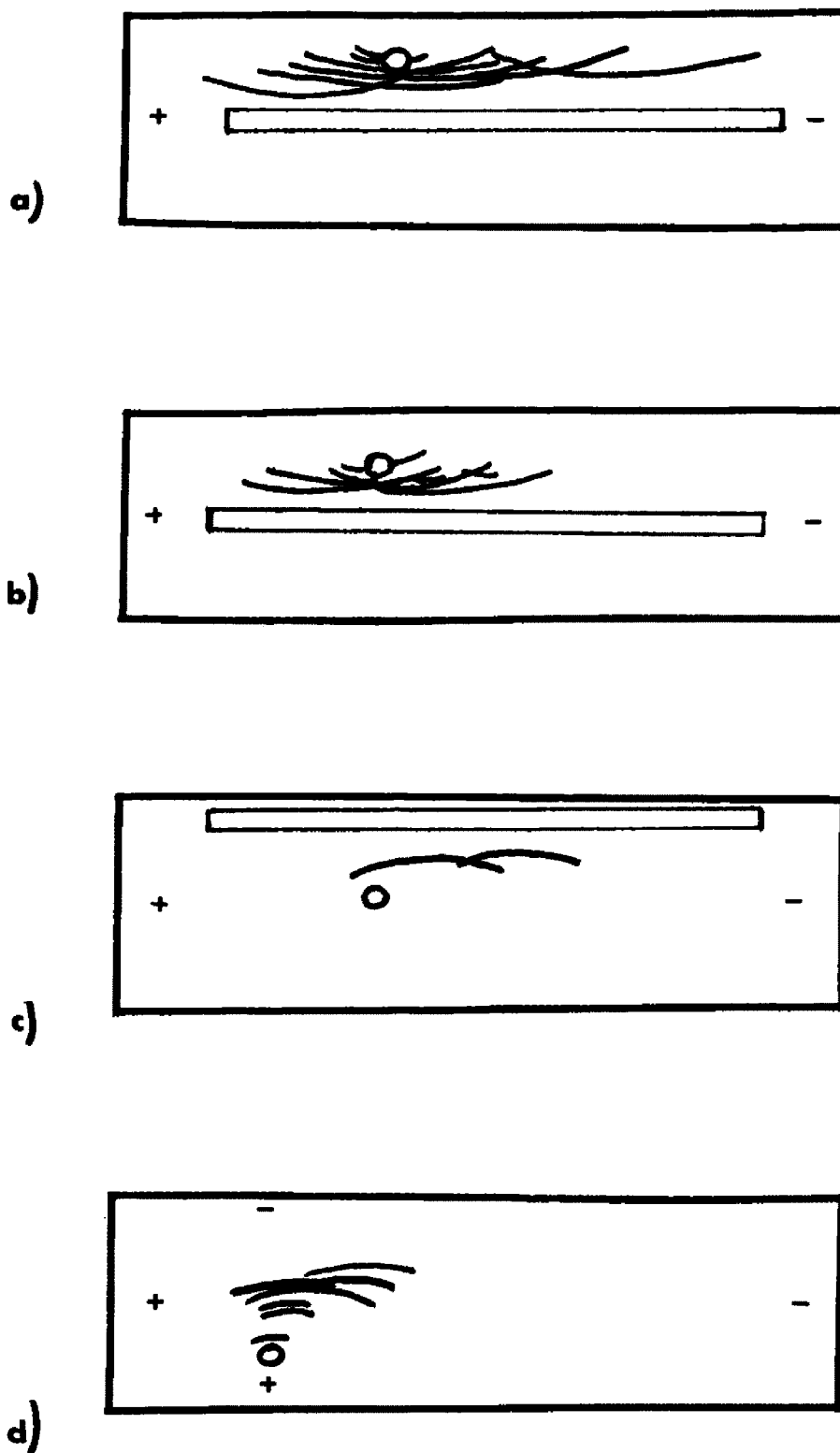
FIG. 11. Immunoelectrophoretic patterns of shark serum as assayed by anti-shark serum and anti-EAF serum. a) Human serum in the well with anti-human serum in the trough. b) Shark serum in the well with anti-shark serum in the trough. c) Shark serum in the well with anti-EAF serum in the trough. d) Shark serum in the well was electrophoresed in two directions perpendicular to each other with the agar impregnated with anti-EAF.

isolation and purification procedures, only presence or absence of these proteins was recorded.

Ammonium sulfate precipitation

As shown in Figure 6, endotoxin altering components were found in all fractions below 50% ammonium sulfate concentration. This placed the endotoxin altering components in the subalbumin and pseudoglobulin range of proteins.

Fractionation of shark serum by cellulose chromatography

Table 7 shows that endotoxin altering components were eluted in the first 90 of the 210 fractions collected. The type of gradient utilized caused the proteins to be eluted in the order of electro- phoretic migration. Therefore, the endotoxin altering proteins eluted in the gamma, alpha, and perhaps the beta globulin protein regions.

Fractionation of shark serum by Sephadex
exclusion chromatography

The protein elution curve of shark serum on Sephadex G-200 is seen on Figure 12. Three peaks appeared when fractions were analyzed at 280 nm. The first two peaks were high in protein concentration, in the 10 mg/ml and 5 mg/ml range, whereas the third peak registered only 0.35 mg protein per ml. Endotoxin altering factors could be demon- strated in the first 33 of the 54 fractions collected.

TABLE 6. Fractionation of shark serum using ammonium sulfate as the precipitating agent.

Shark serum fraction in % ammonium sulfate	Protein[a] (mg/ml)	Reaction with anti-EAF[b]
0 - 20	1.04	+
21 - 30	1.41	+
31 - 40	0.45	+
41 - 50	0.45	+
51 - 70	0.41	-
71 - 90	0.86	-

[a]Determined by nomograph (Warburg and Christian, 1942)

[b]Endotoxin altering factor (EAF) as determined by immuno-electrophoresis against anti-EAF serum.

TABLE 7. Fractionation of shark serum on DEAE cellulose.[a]

Fraction number[b]	Protein[c] (mg/ml)	Reaction with anti-EAF serum[d]
1 - 50	0.060	+
51 - 70	0.150	+
71 - 90	0.385	+
91 - 140	0.439	-
141 - 210	0.510	-

[a]10 ml fractions were eluted with a linear gradient of 0.0 - 0.5 M KCl in tris buffer.

[b]Each group of fractions was combined and concentrated to a 3 ml sample.

[c]Determined by nomograph (Warburg and Christian, 1942).

[d]Endotoxin altering factor (EAF) was determined by immuno-electrophoresis against anti-EAF serum.

REACTION WITH ANTI-EAF

+ + + + + + + + + + + − − − − − −
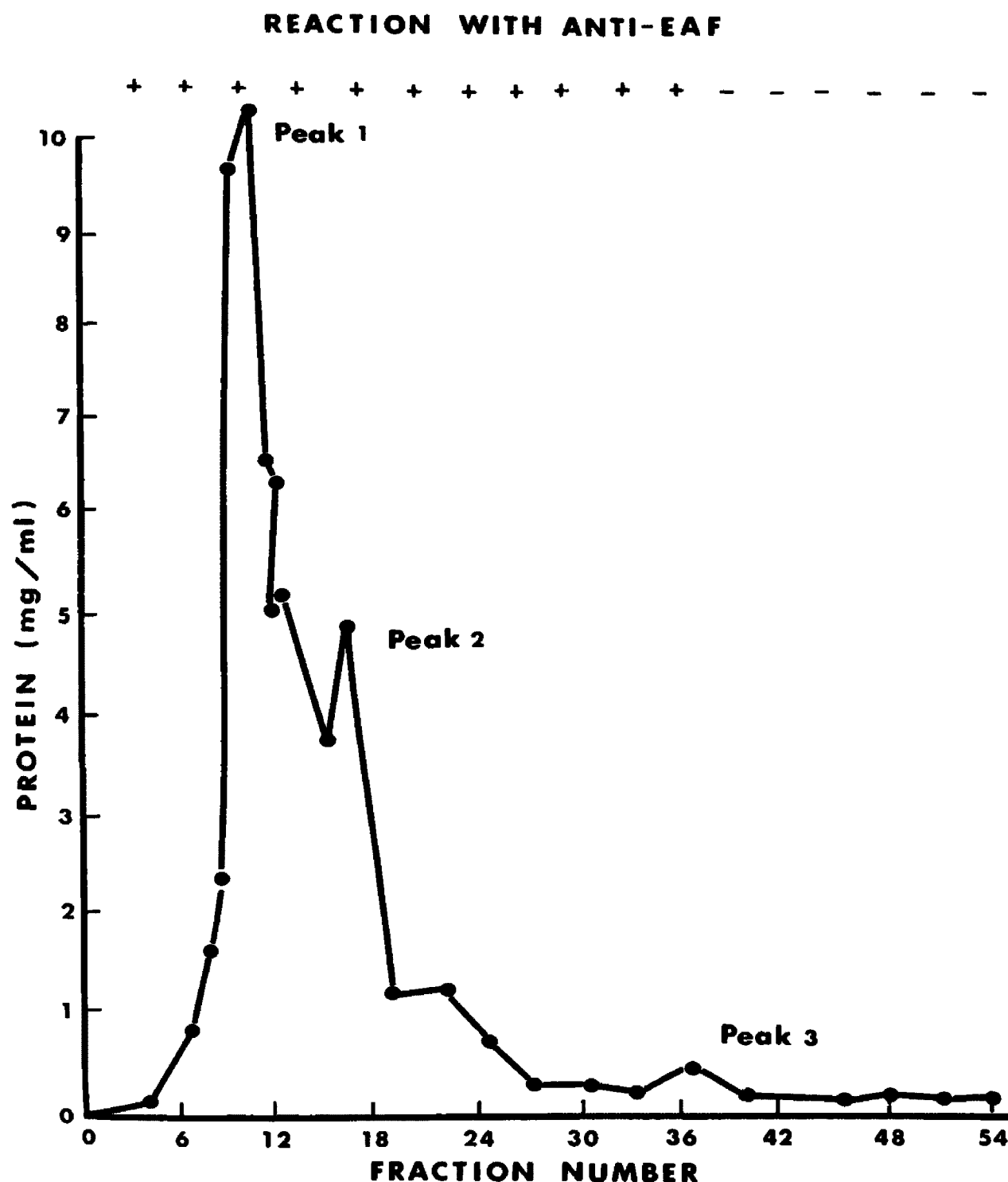
FIG. 12. Fractionation of shark serum on Sephadex G-200. Proteins were eluted with PBS, and 3 ml fractions were taken. Reaction with anti-EAF (endotoxin altering factor) was determined by electrophoresis.

Sucrose density ultracentrifugation of shark serum

Endotoxin altering factors could be detected in the first 16 of the 33 fractions collected from the bottom of the polyalloyl centrifuge tubes. This indicated the macromolecular size of the shark serum components participating in the endotoxin altering reaction. Since these proteins were spread through 16 fractions, perhaps shark serum components of different size act in the detoxification of endotoxin.

Use of sodium deoxycholate as an artificial
dissociant of endotoxin for the demonstration
of the endotoxin altering factor

Table 8 shows that when fractions from DEAE Cellulose, Sephadex G-200, and ultracentrifugation were recombined and concentrated to 4 mg/ml, none of the three pools had the capacity of inactivating endotoxin.

In the hypothesized two-step degredation of endotoxin proposed by Rudbach, et al. (31), endotoxin initially was dissociated by some factor in serum. This dissociating component might have been lost on attempts at isolation and purification. Therefore, sodium deoxycholate was added to dissociate the endotoxin (27). This procedure substituted for the first step in the alteration reaction. Then, with the artificially dissociated endotoxin, shark serum pools were assayed for ability to participate in the second step of the endotoxin alteration. This was the binding of dissociated endotoxin to plasma proteins,

TABLE 8. Capacity of shark serum to inactivate endotoxin dissociated by sodium deoxycholate.[a]

Method of preparation	Sample[b] tested	% inactivation[c]	
		artificially dissociated	not artificially dissociated
Untreated	Shark serum	67	78
Sucrose density centrifugation[d]	All fractions pooled	52	0
Sephadex G-200 chromatography[e]	All fractions pooled	55	0
DEAE Cellulose chromatography[f]	All fractions pooled	0	0

[a]Sodium deoxycholate added at a concentration of 3 mg per 200 ug endotoxin.

[b]When all fractions were pooled, they were also concentrated to the extent of normal shark serum concentration.

[c]Inactivation determined by a decrease in lethality of mice for endotoxin.

[d]Centrifugation at 60,000 rpm in the Beckman L2-65B for 10 hours at 4° C.

[e]Eluted with PBS in 3 ml fractions.

[f]Eluted with a linear gradient of 0-0.5 M KCl in tris buffer with 10 ml fractions being taken.

preventing reassociation. Decrease in mouse LD_{50} for endotoxin was used to detect inactivating capability. The Sephadex pool gave 55% inactivation and the ultracentrifugation pool gave 52% inactivation of endotoxin (Table 8). The DEAE cellulose pool, however, still did not exhibit inactivating capability.

Possibly the DEAE cellulose fractions were too dilute for endotoxin altering activity to be detected. Furthermore, all fractions should be tested at similar concentrations for valid comparison. Therefore, fraction pools were concentrated to 4 mg/ml and retested for inactivation of endotoxin.

The pool of ultracentrifuge fractions 11-20 showed 22% inactivation, with slight inactivation being found in the pool of fractions 21-36. This suggested that the endotoxin altering components were middle range in size, not in the macroglobulin area. Inactivating capability was found throughout the Sephadex fractions. Whole shark serum at a concentration of 4 mg/ml gave 26% inactivation of endotoxin.

Purification of inactivating components was seen in Sephadex pools 1-7, 15-21, and 22-35 (Table 9). This indicated that endotoxin altering components were found in shark serum fractions corresponding to the human alpha and beta globulin region.

Complement, bacteriocidal, and esterase activity of shark serum

When sheep red blood cells were coated with rabbit anti-sheep antibodies, a hemolytic complement system was not found in shark

TABLE 9. Inactivation of endotoxin by shark serum from fractionation procedures using sodium deoxycholate.[a]

Method of serum preparation	Sample	Fraction pool[b]	% inac- tivation	x purifi- cation[d]
Untreated	Shark serum + endotoxin + sodium de- oxycholate + PBS[f]	NA[e]	26	NA
NA	Endotoxin + sodium de- oxycholate + PBS	NA	0	NA
Sephadex G-200 chromatography[g]	Fraction pool + endotoxin + sodium de- oxycholate + PBS	1 - 7 8 - 14 15 - 21 22 - 35 36 - 58	39 13 51 78 22	1.5 0 1.96 3 0
Sucrose density centrifugation	Fraction pool + endotoxin + sodium de- oxycholate + PBS	0 - 10 11 - 20 21 - 30	0 22 5	0 0 0

[a]Added at a concentration of 3 mg/200 ug endotoxin.

[b]All serum and pools at 4 mg protein/ml.

[c]Decrease in mouse lethality for endotoxin was measured.

[d]Determined by comparison to inactivation of endotoxin by 4 mg shark serum/ml.

[e]NA = not applicable.

[f]PBS = phosphate buffered saline.

[g]Eluted with PBS in 3 ml fractions.

[h]Centrifugation at 60,000 rpm in the Beckman L2-65B for 10 hours at 4° C.

serum. This does not negate the possibility of individual complement components being present. The hemolytic complement system may have been inactivated during collection of the shark serum.

Shark serum did exhibit a very low level of esterase activity. Only the first tube containing the greatest amount of serum, 1.0 ml, exhibited any esterase activity. Activity was detected visually by the presence of a blue-violet coloration of the reaction mixture.

Untreated shark serum showed a negligible amount of bacterio-cidal activity. Addition of guinea pig complement to the serum en-hanced the bacteriocidal activity in that 98% of the bacteria were killed (Table 10). Complement alone was not bacteriocidal to the E. coli cells. Growth on the controls was negative or negligible. These results suggested the presence of antibody, either natural or induced, in the shark serum.

TABLE 10. Bacteriocidal activity[a] of shark serum for E. coli 0113.

Sample	Bacterial count	% killed[b]
Diluent[c] + E. coli culture	2.5×10^{10}	0
Shark serum + diluent + E. coli culture	2.27×10^{10}	9
Complement[d] + diluent + E. coli culture	2.32×10^{10}	7
Heated[e] shark serum + complement + E. coli culture	5.9×10^{8}	98
Complement[f]	3	NA[g]
Shark serum[f]	20	NA
Diluent[f]	0	NA

[a]Determined by plate counts using 3 dilutions of a 24 hour culture of E. coli 0113 and comparison to various controls. Trypticase soy agar medium was used.

[b]Determined by comparison to 100% growth value of E. coli in diluent. Bacterial counts made at 24 and 48 hours.

[c]Diluent used was sterile saline (0.15 M).

[d]Guinea pig complement (BBL Laboratories).

[e]Heated at 56° C for 30 minutes.

[f]Controls for reagent contamination.

[g]NA = not applicable.

CHAPTER IV

DISCUSSION

The following objectives were proposed in this study:
1) to characterize the endotoxin altering reaction; 2) to purify
partially and to isolate components of shark serum binding to endo-
toxin during the endotoxin altering reaction; and 3) to establish
the similarity of the endotoxin altering reactions of shark serum
and human serum.

Although there have been many studies on the inactivation of
endotoxin by serum (2, 12, 39, 43, 54), the components responsible
have not been identified (30). It was reasoned from studies on the
phylogenetic level at which endotoxin inactivating capability arose
that a less complicated humoral system, such as that in the dogfish
shark, could be found from which those components of serum partici-
pating might be isolated. The dogfish shark is phylogenetically the
most primitive animal possessing a humoral detoxifying system.

Some investigators (45, 49) have found that most sera, in
addition to human, exhibited a heat stable endotoxin altering system.
Von Eschen (49) demonstrated that shark serum would not inactivate
endotoxin unless it had been heated at 56° C for 30 minutes. However,
others (12, 17, 40) found the system to be heat labile. In this
study, heating shark serum at 56° C for 30 minutes destroyed or

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diminished by at least 50% the ability of serum to detoxify endotoxin. The serum used in the study by Von Eschen (49) was collected by cardiac puncture while the serum for this study was collected by caudal amputation. It is quite likely that divalent cations from sea water, as well as proteolytic enzymes from the surface of the shark, gained entry into the blood collection flasks. Proteolytic enzymes from slime on the shark skin might have destroyed an hypothesized inhibitor (49) and interfered further with endotoxin inactivation upon heating.

It has been shown (2, 17, 29, 40) that divalent cations, specifically calcium, will inhibit the endotoxin alteration by human serum. The shark serum was treated by various methods to remove divalent cations, including dialysis against deionized distilled water, addition of chelating agents, and by passing the serum through a cation exchange resin. After these treatments, inactivating capability could be detected by the quantitative nitrogen precipitation assay, as well as the other assays for detecting inactivation.

Shark serum was assayed under various incubation conditions for optimum inactivation. These conditions were found to be pH 7.0 - 7.2, 45° C, 1-2 hours incubation time, and an incubation mixture that contained 40% serum. Keene, et al. (17) studied the conditions necessary for maximal rate of the endotoxin altering reaction in human serum. Maximal rate was recorded at pH 9.0-10.0 and an incubation time of 1-2 hours. The rate increased up to 90° C and an incubation mixture of 80% serum.

Ho and Kass (12) found endotoxin altering activity in the alpha and beta-globulin fractions of human serum. Cluff, et al. (2) demonstrated that the beta-globulin fraction from human serum was capable of detoxification of endotoxin. Skarnes (41) stated that two alpha globulins were necessary for the inactivation of endotoxin, a heat stable anionic dependent endotoxin binding lipoprotein, and a heat labile globulin with esteric activity for naphthyl acetate.

Shark serum was fractionated by a variety of methods. Ammonium sulfate fractions that were assayed for proteins binding to endotoxin with the anti-EAF serum showed the endotoxin altering components to be found in the portion of shark serum corresponding to the sub-albumin and pseudoglobulin fraction of human serum. Shark serum proteins from Sephadex chromatography that were binding to endotoxin were macroglobulins, whereas sucrose density ultracentrifugation indicated the size of these components to be in the middle range of shark serum protein size. Analysis of shark serum proteins fractionated by DEAE cellulose chromatography demonstrated that proteins binding to endotoxin in the endotoxin altering reaction were to be found in the shark serum fractions corresponding to the alph and beta-globulin fractions of human serum.

Recently, specific components of the hemolytic complement system have been implicated in the detoxification of endotoxin (7, 14, 15, 30). However, no hemolytic complement could be detected in shark serum. Gigli and Austin (10) reported that chondrichthyians have a fully active complement system, although detection was a function of finding the correct combination of assay reagents. The studies of specific components

of complement indicated that a complete hemolytic complement system was not necessary for specific complement components to participate in the endotoxin altering reaction. Johnson and Ward (14, 15) have shown that C5-deficient mice and C6-deficient rabbits exhibit decreased ability to withstand the toxic effects of endotoxin. These studies also show a decreased ability to mediate the in vitro detoxifying action of normal mouse and rabbit sera. Recently, Rudbach (30) has showed the capacity of purified human C3 to detoxify endotoxin.

Skarnes (39) has demonstrated that endotoxin can complex with an alpha lipoprotein from human serum. He found that this alpha-lipoprotein and also an alpha-globulin were involved with endotoxin alteration; both proteins possessed esterase activity. Other studies (41) also supported the theory of interaction between endotoxin and a plasma lipoprotein with esterase activity. Esterase activity also was detected in shark serum, although only at a low level. No correlation was made between this esterase activity and the inactivation of endotoxin.

Jaques and Jaquet (13) showed that immune serum against endotoxin could neutralize the toxic activity of the endotoxin as assayed in adrenalectomized rats. Immune plasma diluted 1:10 had the same detoxifying capacity as did normal undiluted plasma in terms amount endotoxin altered and the rate of the endotoxin altering reaction. Their investigations suggested antibodies, either normal or induced, played a role in the ability of serum to detoxify endotoxin. Ziegler,

et al. (56) showed that antisera directed against the lipid-A-KDO-polyheptose phosphate portion of endotoxin provided protection against the dermal and generalized Shwartzman reaction. Antibodies were demonstrated in shark serum by the very sensitive bacteriocidal assay. However, their participation in the detoxifying activity of the serum could not be established. Along this line, Ho and Kass (12) showed that human serum in which the antibodies to endotoxin were removed still retained endotoxin altering capability comparable to that before the antibody removal.

In conclusion, the objectives of this study were met in that: 1) the effect of physical variables on the endotoxin altering reaction by shark serum was determined; 2) esterase, hemolytic complement, and bacteriocidal antibody were studied; 3) serum components binding to the endotoxin during the endotoxin altering reaction were partially isolated and purified; and 4) the possibility was shown that endotoxin alteration by shark serum may be analogous to that found in human serum. It was hoped that by use of a less complex humoral system than that of human serum, components participating in the detoxification of endotoxin might be more clearly understood.

CHAPTER V

SUMMARY

Shark serum that had been treated by various methods which remove cations demonstrated a high degree of endotoxin altering activity. The endotoxin altering activity was greatest at pH 7.0-7.2, and at 45° C; 1-2 hours of incubation in a 40% serum mixture resulted in maximal inactivation of endotoxin. Esterase was detected in shark serum, although only at a very low level. No correlation could be made between this esterase activity and the detoxification of endotoxin. An intact hemolytic complement system was not found in this serum. However, this did not rule out the possibility of specific complement components acting independently in the endotoxin altering reaction. Antibodies were demonstrated in the shark serum against $\underline{E.}$ $\underline{coli}$ 0113; their significance could not be established. The endotoxin altering factor of shark serum was heat labile. Heating at 56° C for 30 minutes destroyed or diminished by at least 50% the ability of shark serum to detoxify endotoxin.

More than one component appeared to participate in the detoxification of endotoxin. One of these components, a protein, was separated from shark serum by ammonium sulfate precipitation, Sephadex G-200 exclusion chromatography, DEAE cellulose ion exchange chroma-

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tography, and sucrose density ultracentrifugation. Results of the fractionation procedures indicated that the protein endotoxin altering component of shark serum was 11-19S in size and could be found in fractions corresponding to the alpha or beta-globulin fraction of human serum. This component remained with the pseudoglobulin fraction during dialysis against deionized distilled water and during ammonium sulfate precipitation and sedimented with the macroglobulins in density gradient ultracentrifugation. Certain Sephadex fractions in these regions demonstrated a purification of the component.

The protein endotoxin altering component of shark serum was similar in size and electrophoretic mobility to that of human serum. It is apparent that the system of endotoxin alteration found in shark serum may be analogous to that found in human serum.

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