(13) to spare essential protein tissues rather than to mobilize substrate.

Summary and Conclusions. A study of plasma growth hormone and glucose, and body temperature, was conducted in six infants undergoing deep hypothermic cardiovascular surgery. Pronounced hyperglycemia occurred during hypothermia when the infants received exogenous glucose, confirming earlier reports of diminished plasma glucose utilization at low body temperatures. Although growth hormone levels were high during ether anesthesia, they fell or changed little with cooling, and did not appear related to core temperature or to plasma glucose concentration. These findings indicate that changes in growth hormone levels in operated hypothermic infants did not relate to cold stress, alterations in plasma glucose concentration, or diminished glucose utilization.

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Fractionation of an Esterase from Calf Spleen Implicated in the Detoxification of Bacterial Endotoxin* (32946)

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The rapid uptake and detoxification of bacterial endotoxin by dog spleen *in situ* has been effectively demonstrated (1,2). Furthermore, crude extracts of splenic tissue have been shown to possess a strong detoxifying action against endotoxins *in vitro* (2,3). The present investigation was prompted by the recent finding that nonspecific carboxylic esterases from normal serum were implicated in the degradation and inactivation of endotoxin (4). The fractionation of calf spleen extracts and the identification of an esterase apparently responsible for the detoxification of endotoxin are described.

Materials and Methods. Whole calf spleens were removed from exsanguinated animals and either frozen immediately or carefully perfused with saline before freezing. Subsequently, frozen spleens were quickly thawed

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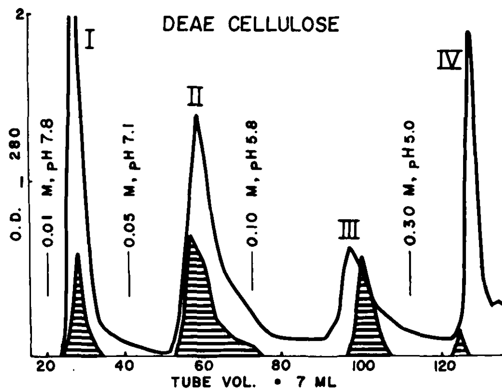

FIG. 1. Fractionation of 20 ml of spleen extract (100,000g supernatant) on 30 gm of cellulose. The hatched areas denote esterase activity.

and homogenized in 4 volumes of cold saline. The extracts were centrifuged at 20,000g for 1 hour and the decanted supernatants were recentrifuged at 100,000g for an additional hour. The final supernatants were dialyzed for 18 hours in the cold against 0.01 M Na-phosphate buffer, pH 7.8.

Step-wise fractionation on DEAE cellulose was carried out with Na-phosphate buffers according to the indications given in Fig. 1. The eluted tube fractions were monitored by UV absorption at 280 mμ. Aliquots of 0.05 ml from each tube were tested concomitantly for esterase activity. These aliquots were incubated with 2 ml of a saturated solution of 2-naphthyl acetate for 35 min at room temperature. Filtered diazo blue solution was added to each tube and after 15 min standing, the azo dye color was read at 540 mμ. The esterase activity associated with the four principal fractions is indicated by the hatched areas in Fig. 1.

The endotoxin-inactivating potency of the crude 100,000g supernatant and of the various fractions was monitored by an *in vitro* immunodiffusion method (4). This method is based on the destruction of a major precipitating antigen ("C antigen") associated with the lethal property of endotoxins. The disappearance of this antigen from immunodiffusion patterns correlates fully with the loss of the lethal action of endotoxins as ascertained in 3 different biological assays (2, 5, 6). The endotoxin employed was a Boivin preparation derived from the Danysz strain of *Salmonella enteritidis*. The preparation of the antiendotoxin serum used in this study is described elsewhere (4). In all immunodiffusion experiments the endotoxin incubates were put into circular agar wells and antiserum was placed in elongated troughs.

Results. Data on the fractionation of the crude extract on DEAE cellulose is given in Fig. 1. Each major fraction was pooled and concentrated to the initial volume of the 100,000g supernatant being fractionated. Approximately 80% of the total detoxifying activity recovered from the cellulose column was eluted with Fraction II: the remaining activity was divided between Fractions I and III. Aliquots of Fraction II were dialyzed briefly against water, concentrated tenfold by air-jet pervaporation and passed through G-200 Sephadex under the conditions described in Fig. 2. When effluent fractions were tested for esterase and endotoxin-inactivating properties, only Fraction II was active. Fractions I and III were negative with regard to both activities (Fig. 3). Optical density measurements indicated that approximately 95% of the total protein in the crude supernatant extract was removed by the two fractionation procedures.

Detoxifying potency of the crude extract was found to be substantial. The 100,000g supernatant obtained from 1 gm of whole spleen (5 ml) was capable of inactivating 0.5–1 mg of endotoxin according to the

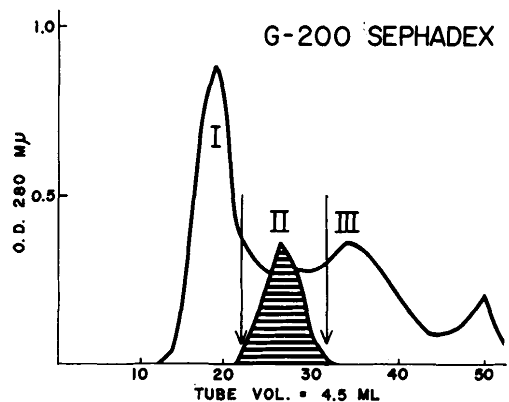

FIG. 2. The fractionation of cellulose fraction II on G-200 Sephadex. The column bed was 1.7 × 75 cm and the eluant was physiologic saline at pH 7.1.

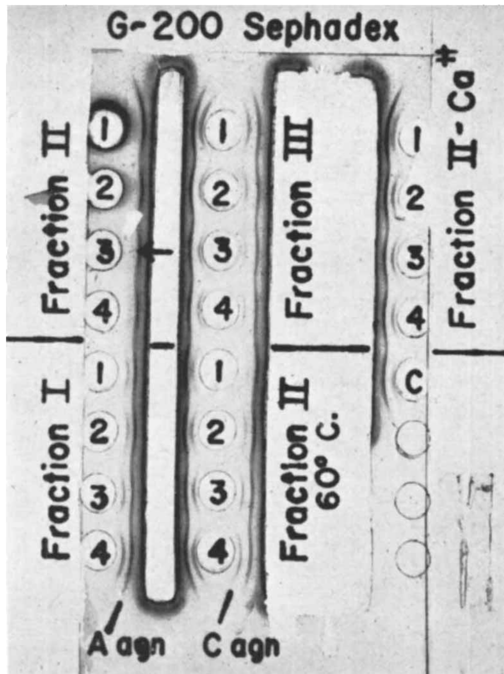

FIG. 3. Detoxifying potency (loss of "C antigen" precipitation) of G-200 Sephadex fractions. The circular wells, 1–4, received 0.04-ml aliquots of the following reactants previously incubated for 2 hours at 37°C. (1) 0.30 ml; (2) 0.25 ml; (3) 0.20 ml; (4) 0.15 ml of the indicated fractions, together with 12 μg of endotoxin and 10^{-3} M sodium citrate, pH 7.1. Incubation volumes were made constant (0.35 ml with weakly buffered. Six μg of nontoxic, acid-hydrolyzed hapten (7) were added to each reaction mixture after incubation to obtain a better separation of the inner C antigen precipitation arcs from those of the faster diffusing A antigen. Each well then contained 1.3 μg of endotoxin and 0.7 μg of hapten. Antiendotoxin serum was put into the elongated troughs. The horizontal arrow (upper left) indicates the detoxifying titer of Fraction II: in this instance, 0.20 ml was the minimum amount capable of abolishing C antigen precipitation. The endotoxin control, well C, was incubated in buffered saline. The immunodiffusion pattern was stained with 1% azocarmine.

immunodiffusion assay (Fig. 4). This value is close to that previously obtained with similarly prepared dog spleen extracts (2). Approximately 50% of the potency of the crude extract was present in the final Sephadex Fraction II.

The association of esterase activity with detoxifying activity, as obtained in both fractionation procedures indicated that an esterase was the essential enzyme in spleen extracts. However, more convincing evidence was obtained to demonstrate that an esterase–endotoxin interaction actually occurred during the detoxification reaction. Endotoxin (100 μg) was incubated for 1 hour at 37°C with 1 ml of crude extract or Sephadex Fraction II in the presence of mM sodium citrate, pH 7.1. Small amounts (0.2 ml) of these incubation mixtures were placed in agar wells and diffused against the specific antiendotoxin serum. Following development of the

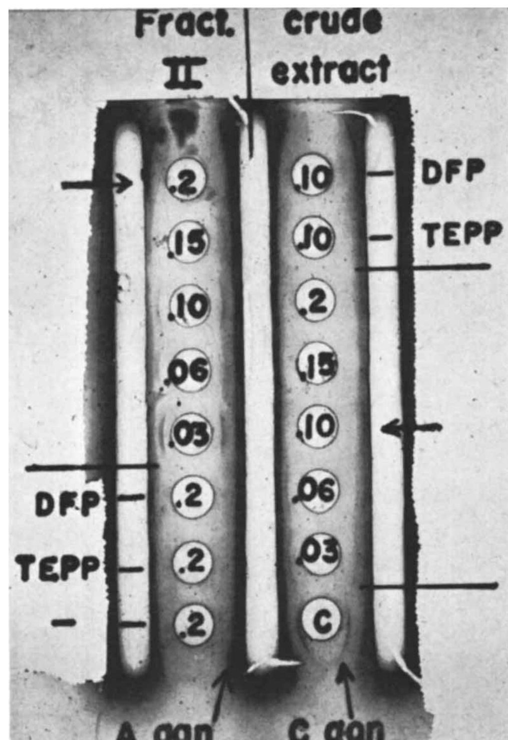

FIG. 4. Detoxifying potency (loss of "C antigen" precipitation) of Sephadex Fraction II (left side) and crude unfractionated extract (right side). The figures in each well denote the amounts of Fraction II or crude extract incubated during 2 hours with 12 μg of endotoxin and 10^{-3} M sodium citrate. Acid-hydrolyzed hapten (12 μg) was added to each reaction mixture following incubation. Each well received 0.04 ml of the indicated incubation mixture containing 1.3 μg of endotoxin and 1.3 μg of hapten. The two horizontal arrows indicate the detoxifying capacity of Fraction II and crude extract. The concentrations of DFP and TEPP are given in the text. The plate was stained with 1% azocarmine.

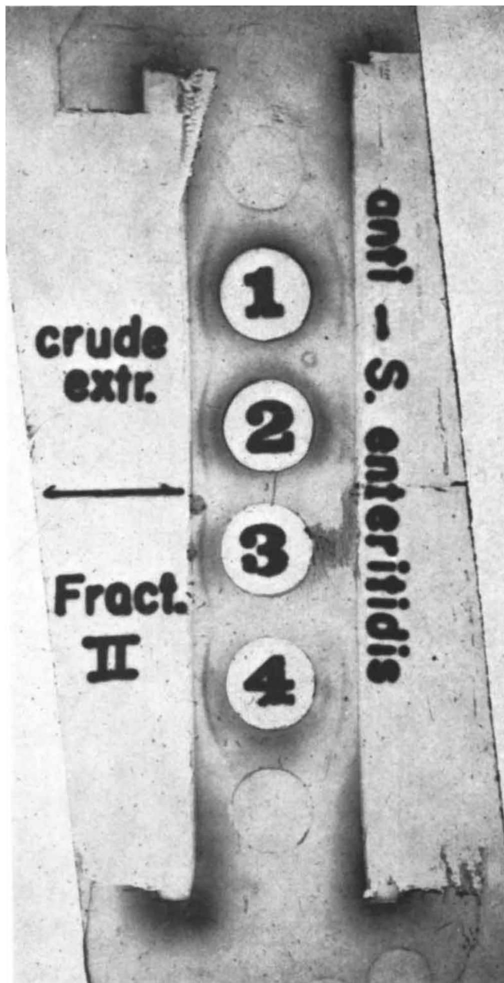

FIG. 5. Demonstration of esterase activity on precipitated endotoxin (A antigen). The precipitation arcs were colored by the formation of the azo dye complex.

precipitating arcs (36 hours at room temperature), the immunodiffusion patterns were washed overnight in cool saline. The agar plates were then covered with oversized sheets of filter paper and placed in an air stream for quick drying. Dried agar films were immersed in 0.1 *M* phosphate buffer (pH 7.1) saturated with 2-naphthyl acetate and incubated for 45 min at 37°C. The plates were rinsed with water and then immersed in a filtered solution of diazo blue for 5–10 min. By this means, esterase activity was readily demonstrated on the precipitated endotoxin arcs which assumed the characteristic blue-violet color of the azo dye (Fig. 5). Controls for this experiment consisted of identical mixtures to which $CaCl_2$, at a final concentration of 3×10^{-3} *M*, had been added before incubation was started. In the presence of this concentration of C^{2+}, the esterase–endotoxin complex does not form and detoxification does not occur (Figs. 3 and 6). The immunodiffusion pattern in Fig. 6 also shows the effect of anions on the detoxification reaction. The addition of citrate to incubation mixtures promotes detoxification and calcium completely inhibits this reaction. These observations were previously reported and discussed in studies of the plasma esterase–endotoxin interactions (4).

Some evidence was obtained which indicated that the crude spleen extract was unstable in storage at —20°C. The stability of the Sephadex Fraction II was examined as follows. Frozen samples were thawed and tested for detoxifying potency after 1, 7, 14, and 30 days of storage at —20°C. No loss in potency was detectable after 1 week; a small decline was noted after 2 weeks, and about 40% of potency was lost after 1 month. Heating of freshly prepared Fraction II at 60°C for 30 min reduced the total esterase activity (Fig. 7) and the endotoxin-inactivating potency (Fig. 3) by approximately one-half. Whereas 0.20 ml of fresh Fraction II was sufficient to abolish antigen C precipitation, 0.30 ml of the heated fraction significantly reduced, but did not abolish, the precipitability of this antigen.

Experiments were performed to determine the effect of two common esterase inhibitors on the esterase and endotoxin-detoxifying activities of Sephadex Fraction II. Enzyme activity was measured in tubes by the release of 1-naphthol from 1-naphthyl acetate as determined by UV absorption at 328 mμ. Both diisopropylphospho-fluoridate (DFP) at 2×10^{-4} *M* and tetraethylpyrophosphate (TEPP) at 10^{-5} *M* reduced total esterase activity by one-third (Fig. 7). However, the endotoxin-inactivating potency was not affected by either of these inhibitors (Fig. 4).

Discussion and Summary. The use of the two successive fractionation procedures resulted in the preparation of an esterase-rich

fraction which was capable of detoxifying endotoxin. The partial inhibition of esterase activity—but not detoxifying power—by DFP or TEPP indicated that the final Sephadex Fraction II contained a cholinesterase-type enzyme not involved in the detoxification reaction. The concentration of DFP employed was sufficient to have inhibited the esterolytic activity of proteases such as trypsin or chymotrypsin, if present. Consequently, the bulk of the enzyme in Fraction II which was active in the detoxification of endotoxin is considered for the present to be an esterase of the nonspecific, carboxylic type. Most of the esterase activity present in DEAE-cellulose Fractions I and III (Fig. 1) was inhibited by DFP and these fractions possessed only a weak capacity to inactivate endotoxin.

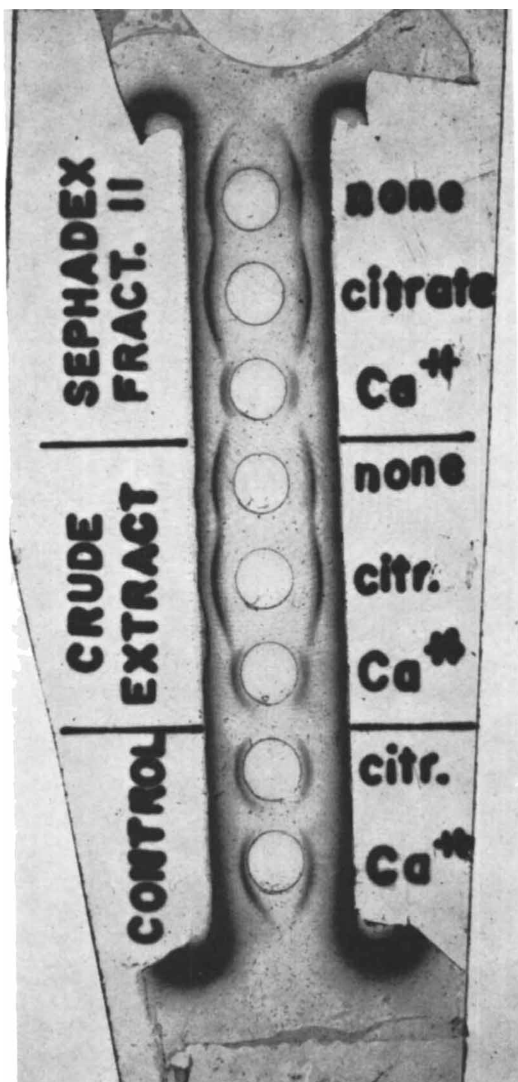

FIG. 6. The influence of anions and cations on the detoxification reaction. The 3 top wells each received 2 μg of endotoxin incubated for 1 hour with Sephadex Fraction II and the indicated ions. The next 3 wells contain 2 μg of endotoxin incubated for 1 hour with the unfractionated extract and the indicated ions. The bottom 2 wells contain 2 μg of endotoxin incubated in buffered saline to which either sodium citrate (10^{-3} M or $CaCl_2$ (3×10^{-3} M) had been added. No hapten was added to this immunodiffusion pattern: staining with 1% azocarmine.

Whereas the spleen enzyme is considered to be a nonspecific esterase of the type found in normal serum, it differs from the latter in some important respects. The degradation and inactivation of endotoxin in serum or plasma was reported to occur as a two step reaction requiring two different esterases, one of which was a lipoprotein esterase (4). Although not yet established, it appears that in the spleen fraction only one esterase functions in the degradation and inactivation of endo-

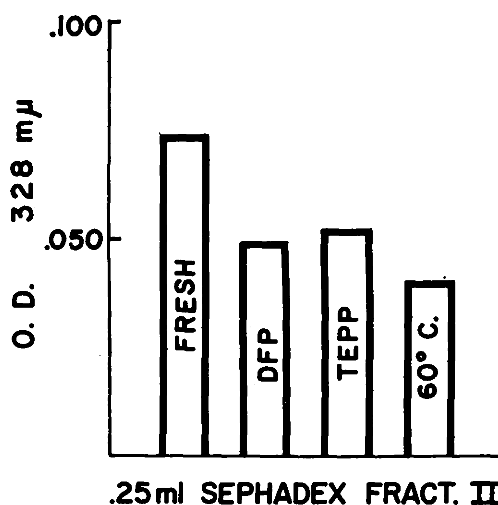

FIG. 7. Esterase activity of Sephadex Fraction II as measured by the release of 1-naphthol. Fraction samples were incubated for 30 min at 37°C with 2 ml of a saturated solution of 1-naphthyl acetate in 0.1 M phosphate buffer, pH 7.1. The results represent the average of 3 determinations in each case. Corrections were made for protein absorption.

toxin. The spleen esterase is not associated with a lipoprotein and no lipoprotein was detected in the isolated fraction. The spleen esterase also differs from the plasma esterases in chromatographic behavior and in heat stability.

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Cross Plaque Neutralization of Two Antigenically Closely Related Dengue Viruses (Type 2 New Guinea C and TH-36)* (32947)

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In 1958 an epidemic of hemorrhagic fever occurred in and near Bangkok, Thailand during the rainy season. The disease there was called Thai hemorrhagic fever. Over 2500 patients were hospitalized with about 10% case fatality rate. During the epidemic, Hammon *et al.* (1) isolated several viruses both from human sera and from *Aedes aegypti*. Among these isolates a prototype strain named TH-36 (representing a number of apparently identical isolates) was found to be antigenically closely related to dengue type 2 (D-2). However, further studies by the same investigators revealed some antigenic differences between these two viruses (2). In an attempt to explore further such possible differences, cross plaque neutralization tests were undertaken.

Materials and Methods. Virus stocks were 20% infected suckling mouse brain suspensions of D-2, New Guinea "C" strain, and of TH-36, both at the twenty-third mouse passage level. Each virus stock was diluted to contain approximately 300 plaque forming units (pfu) per 0.1 ml. The diluent was: Tris (hydroxymethyl) aminomethane, buffered salt solution with 0.4% bovine albumin, adjusted to pH 8.4 with 0.1 N HCl.

The D-2 and TH-36 hyperimmune mouse sera were prepared in 2-fold serial dilutions in the aforementioned diluent. The dilutions used, based on earlier trials were: 1:320, 1:640, 1:1280, and 1:2560. Serum-virus mixtures were prepared by mixing 1 ml of antiserum dilution with an equal volume of virus suspension. Controls consisted of (a) normal mouse serum (NMS) diluted 1:320, and (b) diluent, each mixed with an equal volume of virus suspension. All of the above sets of virus-serum and virus-control mixtures were incubated at 30°C for 1 hour, and then transferred to an ice bath.

Bottles (3-oz prescription type) used for plaque assays were seeded with approximately 3×10^5 versinized LLC-MK_2 cells, a continuous line of rhesus monkey kidney cells, at the thirtieth to forty-eighth serial passage level. The medium was Eagle's basal medium supplemented with 10% heat inactivated calf serum. This medium was changed on the second day, and the cells were inocu-

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