14. Csáky, T. Z., Ho, P. M., Arch. gen. Physiol., 1966, v291, 63.
15. Verzár, F., Schweiz. Med. Wochschr., 1935, v65, 569.
16. Newey, H., Sanford, P. A., Smyth, D. H., J. Physiol., 1966, v186, 493.

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Detoxification of Endotoxin by Perfusion of Liver and Spleen.* (32118)

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This communication reports a study of the capacity of the liver and spleen to detoxify bacterial endotoxins introduced into the circulation. The evidence for detoxification is data showing 1, a correlation between survival rate and degree of accessibility of the liver to the endotoxin; 2, a loss of toxicity after perfusion through spleen, as determined by bioassay, and 3, disappearance of the toxic moiety after perfusion through spleen, as determined by immuno-assay. *In vitro* data showing detoxification of endotoxin interacted with splenic homogenate are also presented.

Experimental. A. *Effect on survival rate of a change in accessibility of the liver to the endotoxin.* Three groups of normal adult white rabbits were subjected to laparotomy under pentobarbital anesthesia (30 mg/kg) for exposure of a branch of the superior mesenteric vein. Each rabbit of the first group received one MLD/80 (1 mg/kg) of *Salmonella enteritidis* endotoxin into this vein. In the second group the same amount of endotoxin was injected into the renal artery instead of the superior mesenteric vein. In the third group the same amount of endotoxin was injected into a vein of the ear instead of the superior mesenteric vein. The laparotomy incisions were then closed and the survival rate, *i.e.*, the percentage alive and well 7 days later, observed.

Results. Table I shows that endotoxin injected *via* the superior mesenteric vein resulted in a survival rate of 85% as compared to 11% when it was injected *via* a systemic artery or vein.

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These results signify that the lethality of endotoxin is reduced or eliminated if it is given so that all or most of it must traverse the liver before reaching the systemic circulation. It follows that the normal liver can extract enough of an MLD/80 of endotoxin on its first passage through as to lower the circulating titer of the toxic moiety to a sublethal level. To determine whether the RE cells or the parenchymal cells in the liver, or both together, were necessary to achieve the detoxification, endotoxin was perfused through the spleen, which, apart from lymphocytes, contains only RE cells. The degree of detoxification of the endotoxin was determined as follows:

B. *Toxicity of bacterial endotoxin before and after perfusion through the dog's spleen.* An abdominal incision was made under ether anesthesia. Maximum accessibility of the splenic parenchyma to the endotoxin was achieved by abolishing vasoconstriction with 1% procaine injected into the splenic pedicle. Five mg of *S. enteritidis* endotoxin (labelled with Cr^{51}), suspended in 50 ml of normal dog plasma, was infused into a marginal artery in a period of 12 minutes, during which flow through the splenic artery was unimpeded. All of the venous effluent during this period and for 5 minutes thereafter was collected in a single pool in an ice bath, centrifuged in the cold, and its total plasma volume determined from its hematocrit. The spleen was then excised, washed free of blood with phosphate-buffered saline solution, and homogenized at 4°C in a volume of this solution equal to twice its own weight.

The homogenate was tested for toxicity by injecting 0.5 ml aliquots intraperitoneally into

TABLE I. Effect of Route of Administration on Lethality of Bacterial Endotoxin.*

No. of exp.	No. of rabbits	Route of administration	Type of Anesthesia	Survival rate in % after 24	96	144 hr
1	9	Ear vein	Nembutal	33	21	11
2	10	Renal artery	"	40	10	10
3	11	Mesenteric vein	"	100	85	85

* One mg/kg b.w. *Salmonella enteritidis* injected in each animal. All animals had a laparotomy incision under Nembutal anesthesia, regardless of site of injection of endotoxin.

TABLE II. Effect of Perfusion of Endotoxin Through Spleen on Mortality Rate in Pt Mice.

No. of exp.	Before Perfusion: Dose of endotoxin gammas	Before Perfusion: Mortality rate %	After Perfusion: Dose of endotoxin *gammas	After Perfusion: Dose of endotoxin †gammas	After Perfusion: Mortality rate %
1	50	100	6.7	8.6	80
	5	90	5	6.5	0
	1	80	1	1.3	10
	.3	10	.3	.4	0
2	50	100	5.5	9	40
	5	90	5	8	30
	1	30	1	1.6	10
	.3	10	.3	.5	30
3	50	100	5.5	10.5	0
	5	90	5	9.1	20
	1	10	1	1.8	10
	.3	0	.3	.6	0

* Calculated on the assumption that the Cr^{51} activity per test dose of effluent corresponds to the same amount of Cr^{51} activity per test dose of infusate.

† Calculated on the basis of dilution of the infusate and on the assumption that no endotoxin was extracted by spleen.

each of 10 PT mice, and the mortality rate observed (1).

The infusate and effluent were tested for toxicity in pertussis-treated mice as follows: Four dilutions of the infusate were prepared on the basis of the weight and Cr^{51} activity of the endotoxin in the original preparation. The toxicity of each dilution was assayed by injecting 0.5 ml intraperitoneally into each of 10 pertussis-treated mice. Equivalent dilutions of endotoxin in the effluent pool were prepared and tested for toxicity on the basis of their Cr^{51} activity, and on the assumption that none of the endotoxin in the infusate was retained by the spleen (Table II). A reduction in the mortality rate from 80% to 20% or less in a group of 10 PT mice was taken to indicate significant detoxification.

The infusate and effluent were also tested for toxicity by the immuno-diffusion technic (2) as follows: Aliquots (0.2 ml) of the various dilutions of infusate, containing 5, 2, and 1 gamma of endotoxin respectively, were placed in a row of wells on one side of a central trough containing antiserum specific for the endotoxin. 0.2 ml aliquots of corresponding dilutions of endotoxin in the effluent based on radioactivity counts were placed in a row of similar wells on the opposite side of the central trough (Fig. 1). A 2-day period of diffusion was allowed for development of precipitation bands. The plates were dried and radioautographed as described elsewhere (3). The precipitation bands representing the toxic moiety ("C antigen") of the endotoxin (2) in the infusate served as controls for the presence or absence of corresponding bands in the effluent. Absence of such bands in the effluent was taken to signify absence of toxicity.

Results. Three perfusion experiments were performed. In the first experiment 78% of the injected radioactivity was recovered in the effluent plasma and 16% in the splenic homogenate. In the second experiment the corresponding figures were 60% and 15%, and in the third, because of the loss of a considerable amount of effluent through a leak in

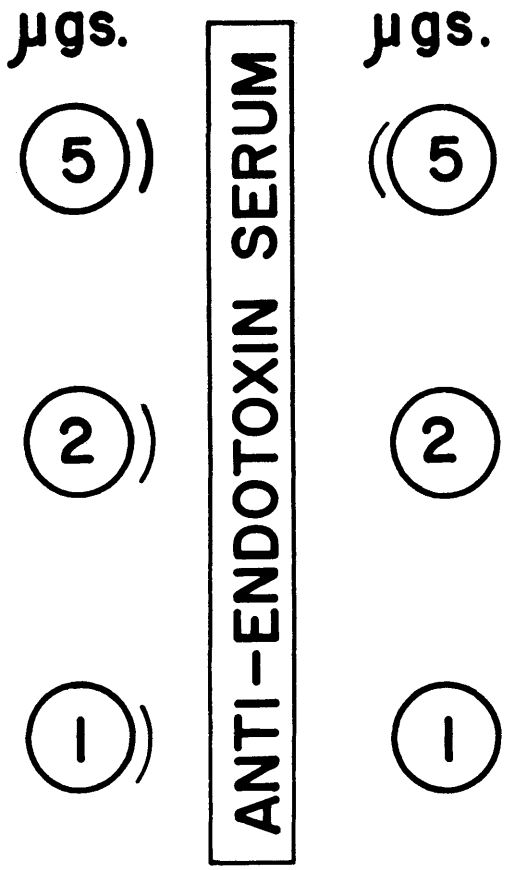

FIG. 1. Radioautograph of immunodiffusion pattern of Cr^{51} endotoxin in dog plasma before (left) and after (right) splenic perfusion. Only the precipitation arc associated with toxicity and label ("C antigen") has marked the film.

a branch of the splenic vein, 50% and 3% respectively. The volume of effluent averaged some 6 times (290 ml) that of the infusate.

1. *Detoxification of endotoxin in the effluent and in spleen by the PT mouse test.* As already stated dilutions of effluent prepared for toxicity tests in PT mice were made 1, on the basis of Cr^{51} content, and 2, on the assumption that the spleen retained none of the endotoxin, thus weighting the test dose in favor of lethality. Table II shows the change in the mortality rate of PT mice as a result of perfusion through the spleen. Averaging the data for each test dose in the 3 experiments shows that on the basis of the lethality data for the infusate there was not more than 1 gamma of potent endotoxin per 0.5 ml of undiluted effluent, and that the total amount of potent endotoxin in the 290 ml of effluent from each spleen did not exceed 600 gamma.

Assuming that the spleen (average weight = 70 g) retained the remainder, *i.e.,* 4400 gamma, each 0.5 ml of splenic homogenate tested should contain 11 gamma. Since only one of 30 mice (3 groups of 10), each of which received 0.5 ml, died, there was less than one gamma of potent endotoxin in this amount of homogenate. Hence most of the endotoxin retained by spleen was detoxified. This conclusion also follows from the Cr^{51} data. For if the isotope in spleen were still attached to undetoxified endotoxin in the same proportion as in the infusate, the maximum amount of potent endotoxin present in the spleen should not exceed some 16% of the total infused, *i.e.,* 700 gamma or 1.7 gamma per 0.5 ml, as compared to less than one gamma per 0.5 ml on the basis of the data on the infusate.

The possibility that bioassay of the potency of endotoxin in splenic homogenate may be invalidated because binding to protein may mask toxicity was excluded by the finding that the MLD/80 in PT mice for 3 different doses of endotoxin is not lower when suspended for one hour in splenic homogenate at 4°C than in saline at 4°C.

2. *Detoxification of the endotoxin in the effluent by the immuno-diffusion technic.* Fig. 1 shows the presence of precipitation bands of the toxic moiety ("C antigen") in all 3 dilutions of the infusate. They are absent in 2 of the corresponding 3 dilutions of the effluent. The band is present in the well containing 5 gamma, but the amount present, as determined by the intensity of the labelled precipitation band, is approximately equivalent to that present in the well for one gamma of infusate. The results were the same in all 3 perfusion experiments.

C. *Toxicity of S. enteritidis endotoxin before and after interaction with a fraction of splenic homogenate.* Although the homogenates of spleen proved to be non-toxic in PT mice, the concentration of endotoxin per ml of homogenate may have been too low for the sensitivity of this bioassay. Therefore, further experiments were performed to examine the toxicity of endotoxin interacted *in vitro* with

partially purified extract of spleen, as follows:

1. A freshly excised normal dog's spleen was washed as free as possible of blood by perfusion with physiologic saline at 4°C. The filtrate obtained by gauze mesh was spun at 20,000 *g* for 15 minutes, and its supernatant was spun at 100,000 *g* for one hour, all at 4°C. It was then stored in the deep freeze until used. The detoxifying power of each of seven such supernatant preparations was tested as follows:

One ml of each preparation was interacted with 100 gamma of *S. enteritidis* endotoxin (MLD/80 in PT mice = 5 gamma) at 37°C for one hour. This reaction mixture was then diluted to 6 ml with phosphate-buffered saline in order to bring the endotoxin content to 8 gamma per 0.5 ml. This volume was injected intraperitoneally into each of 10 PT mice. A survival rate of 80% or better was taken to signify detoxification. Several of these reaction mixtures were also tested for the presence of the toxic antigen by the immuno-diffusion technic as described above.

Results. By the PT mouse test 4 of the 7 preparations were found to have detoxifying power of 100 gamma per ml; 2 were of twice this potency, and the seventh was 5 times as potent.

Aliquots of 3 splenic extracts having each of these 3 potencies were also tested for detoxifying power by the immuno-diffusion technic. According to this test (Fig. 1) one ml of the splenic extract that detoxified 100 gamma also eliminated the toxic moiety ("C antigen") from 100 gamma; one ml of the extract that detoxified 200 gamma also eliminated the toxic moiety from 200 gamma. However, the aliquot that detoxified 500 gamma removed only part of the toxic moiety.

Discussion. Numerous studies of detoxification of endotoxin *in vitro* have been reported. Some of these studies deal with the effect of plasma or serum, others with the effect of extracts of various tissues on endotoxin, as determined by a variety of bioassay technics (4,5,6,7). This study is concerned with the role of the liver *in vivo* and of the spleen *in vivo* and *in vitro* in detoxification. The data showing that the death of rabbits caused by an intravenously injected dose of endotoxin is prevented if the endotoxin is obliged to pass through the liver before reaching the systemic circulation, and the data obtained by the use of the PT mouse assay and immuno-diffusion technics for assessing detoxification before and after perfusion through the dog's denervated spleen *in vivo,* reaffirm the view that circulating endotoxin is extracted and detoxified by these organs(8,9). These data also demonstrate that the full detoxifying potential of the RE cells in liver and spleen is ordinarily not available because the vessels to these organs as a rule are not sufficiently open to allow maximum utilization of these cells. This had also been shown in a previous study demonstrating that a dose of endotoxin that is lethal when given *via* a systemic vein does not kill if the coeliac ganglion has been anesthetized (10). It is apparent that the full detoxifying potential of the liver and spleen is more than enough to deal with the challenges these organs are likely to encounter in nature.

Summary and conclusions. The mortality of a dose of a bacterial endotoxin in rabbits, which was 85% when injected into a systemic vein, was reduced to 11% when it was injected *via* a mesenteric vein, *i.e.,* so that it must traverse the liver before entering the systemic circulation. Bacterial endotoxin (*Salmonella enteritidis* MLD/80 = 1 mg/kg) in plasma was perfused through the denervated dog's spleen *in vitro.* Corresponding dilutions of infusate and effluent were assayed for detoxification in pertussis-treated mice, and by the immuno-diffusion technic. These assays showed substantial detoxification of the endotoxin in the effluent and of the endotoxin retained by the spleen. Because the degree of dilution of the effluent may have been great enough to reduce the endotoxin content per aliquot below the sensitivity of the bioassay technics, partially purified extracts of spleen were also interacted with endotoxin and detoxification by the same assay technics was demonstrated. These data show that the liver and spleen not only rapidly extract, but also rapidly detoxify bacterial endotoxins. Because of varying degrees of vasoconstriction of the circulation to these organs in the normal animal, the full detox-

ifying potential of these organs when challenged may not be available.

1. Rutenburg, S., Michael, J. G., Proc. Soc. Exp. Biol. & Med., 1964, v117, 301.
2. Skarnes, R. C., Ann. New York Acad. Sci., 1966, v133, 644.
3. Chedid, L. C., Skarnes, R. C., Parant, M., J. Exp. Med., 1963, v117, 561.
4. Ho, M., Kass, E. J., J. Lab. & Clin. Med., 1958, v51, 297.
5. Collins, R. O., Wood, W. B., Jr., J. Exp. Med., 1959, v110, 1005.
6. Trapani, R. J., Waradvekar, V. S., Landy, M., Shear, M. J., J. Infect. Dis., 1962, v110, 135.
7. Keene, W. R., J. Lab. Clin. Med., 1962, v60, 433.
8. Wiznitzer, T., Better, N., Rachlin, W., Atkins, N., Frank, E. D., Fine, J., J. Exp. Med., 1960, v112, 1157.
9. Rutenburg, S., Rutenburg, A., Smith, E., Fine, J., Ann. New York Acad. Sci., 1966, v133, 633.
10. Palmerio, C., Zetterstrom, B., Shammash, J., Euchbaum, E., Frank, E. D., Fine, J., New Eng. J. Med., 1963, v269, 709.

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The Presence of Diaminopimelic Acid in the Rickettsiae.* (32119)

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Diaminopimelic acid (DAP) is an amino acid peculiar to the mucopeptide of all bacteria with the exception of the Gram positive cocci. It is not found in yeast, fungi, protozoa, viruses, or in mammalian tissues(1,2). With the discovery of muramic acid in rickettsiae (3) it was a reasonable assumption that rickettsial cell walls contained a mucopeptide similar to that in bacteria. Diaminopimelic acid was first described in the cell wall of *Rickettsia mooseri* by Wood and Wisseman (4). The following report describes its presence in *Rickettsia prowazekii, Rickettsia mooseri, Rickettsia quintana* and *Coxiella burneti.*

Materials and methods. Rickettsial preparations. R. prowazekii, R. mooseri and *C. burneti* were grown in the yolk sac of embryonated eggs. *R. prowazekii* and *R. mooseri* were purified by a combination of differential centrifugation and treatment with DEAE cellulose by a batch process(5). Additional purification was performed with a sucrose density gradient(6). The cells were inactivated with 0.1% formalin, washed 3 times in distilled water, lyophilized and dried to constant weight over P_2O_5. *C. burneti* was obtained as a formalin killed, lyophilized product which had been purified by a somewhat different procedure(7). It was dried to constant weight.

R. quintana, Fuller strain, obtained from Dr. Weiss, Naval Medical Research Institute, was grown in an atmosphere at 5% CO_2 on Vinson's agar medium(8). After 5 days the cells were harvested, washed 3 times in physiological saline, and inactivated in 0.1% formalin. Finally, they were washed 3 times in distilled water, lyophilized and dried to constant weight.

General procedure. Rickettsial samples varied between 20 and 50 mg dry weight. The cells were first extracted in turn with 5 ml volumes of acetone, alcohol, and ether (5 min with shaking). The cells were then hydrolyzed in 6 N HCl (18 hours, 105 C, N_2 atmosphere). The hydrolyzed sample was evaporated to near dryness in a stream of N_2 (hot plate, 50-60°C). Residual HCl was re-

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