relatively inexpensive living model for screening potential therapeutic agents, meager information is available on the nature of endotoxin shock in the embryo. The susceptibility to endotoxin has been ascribed to the release of catecholamines from the adrenal medulla (1). Associated with this is the undeveloped function of the adrenal cortex so that a deficiency of endogenous glucocorticoid and susceptibility to endotoxin can be surmounted by administering the hormone, or one of its analogues. Cationic cyclic polypeptide antibiotics such as Polymyxin B sulfate, Colistin sulfate and tyrocidine hydrochloride have been reported to protect embryos against lethal doses of endotoxin(9). Finkelstein(2) pointed out that endotoxin causes hypoglycemia and death in the embryo, which can also be accomplished by injecting 4 μg of insulin. It had been postulated that circulatory stasis caused by "sludging" of blood led to the hypoglycemia but heparin did not alter the outcome, which has been confirmed in our laboratory. Other agents reported not to effect endotoxin lethality are cholesterol, deoxycorticosterone, l-dehydrocortisone, heparin and phenoxybenzamine. Although phenoxybenzamine does not protect against endotoxin in the embryo, this adrenergic blocking agent does protect against a lethal dose of epinephrine or norepinephrine(2).

The protection provided by Isoproterenol in the embryo centers attention on the role of adrenergic substances in endotoxin activity. Isoproterenol is a β-adrenergic stimulator having both inotropic and chronotropic actions, induces vasodilation and hyperglycemia, and affects other autonomic end organs(10). Although embryos tolerate large doses of Isoproterenol without apparent ill effect the mechanism of action in the chick embryo against endotoxin remains unknown.

Summary. The susceptibility of the chick embryo to endotoxin is confirmed. Embryos tolerate up to 20 μg of Isoproterenol when injected intravenously. Isoproterenol in amounts of only 0.1 μg significantly protect embryos against 0.05 μg of endotoxin, which is greater than an LD_{50} dose. The mechanism of the protective action of Isoproterenol is not known.

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Increased Resistance to Endotoxin in Germ-Free Guinea Pigs.* (31988)

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Reactivity to bacterial endotoxins can be modified not only by experimentally acquired infection with various heterogeneous microorganisms(1) but also by the type of bacteria present in the gastrointestinal tract. Schaedler and Dubos(2) found that Rocke-

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feller NCS mice lacking *E. coli* and *Proteus* in their intestinal contents were more resistant to the lethality of endotoxin than strains of mice with these bacteria in their gut. Feeding *E. coli* to NCS mice or immunizing them with endotoxin abolished this resistance. Similarly, Jensen *et al*(3) found that NIH-conventional mice, containing 10 to 100 times as many coliform bacilli as did Lobund-conventional mice, were approximately 2 to 3 times more susceptible to endotoxin lethality than the Lobund strain.

The significance of the bacterial flora in determining reactivity to endotoxin has been further evaluated by comparing the responses of germ-free animals to those of conventional animals. The studies reported have given conflicting results. Landy *et al*(4) found that germ-free mice and conventional mice with coliform bacteria and *Aerobacter* in their intestinal contents give similar responses after either *S. enteritidis* of *Sh. flexneri* endotoxin as concerns lethality, the stimulation of increased glycolysis in spleen cells and peritoneal macrophages, increased resistance to *S. typhosa* infection, or formation of antibody against Gram-negative bacteria. In contrast, Jensen *et al*(3) demonstrated that germ-free mice were more resistant to the lethality of *E. coli* endotoxin than conventional mice but that such resistance was abolished when a mixed intestinal flora was established. In the opposite direction, an increased susceptibility to endotoxin in the germ-free state was suggested by the observations of Matsuzawa and Wilson(5) that axenic mice responded to endotoxin with a greater fall in the pO_2 of various tissues than the fall observed in conventional animals.

To investigate further the contribution of naturally acquired microflora to reactivity to endotoxin, studies were done in another animal species with measurements of additional aspects of the host response to endotoxin. Because of their availability in this laboratory, germ-free guinea pigs were used to study the effects of endotoxin on body temperature, circulating white blood cell count, and lethality. The results indicate that the axenic state may influence endotoxin reactivity, but to a limited extent.

Materials and methods. Pregnant guinea pigs were obtained from commercial sources. Close to the estimated delivery time, a hysterectomy was done aseptically and the piglets were delivered into a sterile plastic isolator for rearing in a germ-free environment(6). They were fed an autoclaved diet of Purina Lab Chow, glucose, sodium chloride, yeast and water, supplemented with thiamine and ascorbic acid. Sterility of each isolator was monitored frequently by cultures in enriched media and thioglycollate broth. Control animals were obtained from the same sources but reared in the laboratory under conventional circumstances and given the same diet.

Salmonella typhosa endotoxin (Difco lipopolysaccharide *S. typhosa* 901) was chosen as representative of endotoxin with which the guinea pigs should have had no previous contact. It was suspended in pyrogen-free saline and stored at 4°C. Graded amounts (2 mg/kg, 4 mg/kg, 6 mg/kg) were injected intraperitoneally into separate groups of 5 or 6 animals of at least 8 weeks in age. To minimize any effect of the circadian change in reactivity to endotoxin, all injections were given in the morning and each experiment included concurrent testing of a germ-free and a conventional group.

Each animal had measurements of white blood count (WBC) and temperature prior to injection with endotoxin and again at 1, 3, 6 hours afterwards and was then observed over 48 hours for lethality. Blood counts were done by standard counting chamber techniques. Changes after endotoxin were calculated as percentages of the initial value. Temperatures were determined with a clinical thermometer kept in the rectum for 3 to 5 minutes. The curve for each animal was plotted on graph paper, with 1 inch on the ordinate representing 1°F change from the base-line temperature and 1 inch on the abscissa representing 1 hour after endotoxin. The extent and duration of temperature changes were quantitated by measuring with a planimeter the areas above and below the base-line temperature, assigning the area above as positive and the area below as negative. Addition of these gave the "tempera-

TABLE I. Temperature Indices After Endotoxin in Conventional and Germ-Free Guinea Pigs. (Temperature Index: sum of areas of temperature curve above (+) and below (—) pre-endotoxin temperature over 6 hours after endotoxin.)

2 mg/kg		4 mg/kg		6 mg/kg	
Conventional	Germ-free	Conventional	Germ-free	Conventional	Germ-free
— 27.1	+35.8	—123.1	+40.0	— 74.6	+30.1
— 48.7	+23.7	—130.6	+20.4	— 84.0	—24.4
— 78.0	+19.6	—138.1	+13.5	—120.6	—25.9*
—113.4	+10.1	—142.5	+ 6.9	—135.0	—61.1
—184.4	— 7.4	—163.5	— 2.8	—146.5	—65.1
	—11.9		—32.2	—189.0	—90.2

* 3 hr only.

ture index." A negative index therefore indicated that hypothermia had been the main response; the greater the negative value, the lower or more prolonged was the hypothermia.

Intradermal reactions to endotoxin were tested by injecting varying amounts of endotoxin contained in 0.1 ml at widely separated sites of the shaven abdomen. The sites were observed at 15 and 30 minutes and then daily for 3 days.

Results. Response of temperature to endotoxin. The temperature before endotoxin tended to be lower in germ-free animals (mean 101.3°F, range 100.4-102.6°) than in conventional animals (mean 102.4°, range 100.8-103.6°). The temperature index values for each dose of endotoxin are summarized in Table I. All conventional animals exhibited hypothermia, whereas germ-free animals had a higher threshold for production of hypothermia.

Response of WBC to endotoxin. Base-line WBC values were distinctly lower in 14 germ-free animals (mean 3700/mm^3, range 2300-5400/mm^3) than in 16 conventional animals (mean 10,600/mm^3, range 4800-18,700/mm^3). Table II summarizes the responses to endotoxin. All conventional animals exhibited prompt and persistent leukopenia, with the nadir at 3 hours. In contrast, germ-free animals had a higher threshold. In 6 germ-free guinea pigs the WBC rose at least 60%. It was not determined whether this increase was a response to endotoxin or simply a response to the stress of injection and handling.

Table III summarizes the number of animals giving either a hypothermic or leukopenic response to endotoxin.

Lethality of endotoxin. Deaths occurring within 48 hours are summarized in Table III. At the dosages tested, germ-free and conventional animals were equally susceptible to the lethality of endotoxin.

Intradermal reactions to endotoxin. Four germ-free and 4 conventional guinea pigs were given intradermal injections of 1 μg, 5 μg, 25 μg, and 50 μg endotoxin. No reactions detectable either immediately or within 72 hours occurred in any animals.

Discussion. These experiments show that conventional guinea pigs react differently

TABLE II. Effect of Endotoxin on WBC in Conventional and Germ-Free Guinea Pigs.

Amount of endotoxin	Time after endotoxin (hr)	Change in WBC (% of pre-injection value)											
		Conventional						Germ-free					
		1	2	3	4	5	6	1	2	3	4	5	6
2 mg/kg	1	—71	—61	—77	—59	—44		+ 2	+20	+ 33	+ 38	—38	—49
	3	—82	—87	—86	—86	—63		+52	+61	+ 54	+136	—62	—78
	6	—60	—79	—69	—63	—58		+57	+59	+ 70	+ 85	—22	—75
4 mg/kg	1	—74	—77	—84	—78	—75			+43	+ 58	— 15		
	3	—83	—83	—82	—85	—81		+22	+60	+148	— 2	—73	—81
	6	—70	—68	—70	—69	—43		+ 3	+48	+ 66	— 15	—58	—65
6 mg/kg	1	—83	—70	—64	—82	—73	—35	—52	—58	— 64	— 40	—68	—61
	3	—75	—82	—79	—92	—71	—67	—72		— 90	— 52	—56	—57
	6	—76	—82	—70	—75	—42	—26	—74		— 35	— 72	—40	—17

TABLE III. Comparison of Reactions to Endotoxin in Conventional and Germ-Free Guinea Pigs.

Amount of endotoxin	Hypothermia		Leukopenia		Lethality	
	Conventional*	Germ-free*	Conventional*	Germ-free*	Conventional*	Germ-free*
2 mg/kg	5/5	0/6	5/5	2/6	0/9	1/8
4 mg/kg	5/5	1/6	5/5	2/6	3/8	1/10
6 mg/kg	6/6	5/6	6/6	6/6	4/10	5/7

* Animals reactive/animals tested.

to endotoxin than do germ-free guinea pigs lacking autochthonous flora. While both types of animals exhibited the expected responses to endotoxin of hypothermia, leukopenia, and lethality, there were clear-cut quantitative differences in their responses. Conventional animals required at least 3-fold less endotoxin for elicitation of hypothermia and leukopenia. However, the lethal dose was the same in both conventional and germ-free animals.

No clear explanation is available for this selective increased susceptibility in animals with autochthonous flora. Another difference between the conventional and germ-free animals besides the presence or absence of bacteria was the lower WBC in germ-free guinea pigs. In rabbits severe leukopenia (less than $1000/mm^3$) ameliorates or abolishes their febrile response to endotoxin(7). However, it is doubtful that the modest leukopenia in the germ-free guinea pigs (mean WBC $= 3700/mm^3$) was sufficient to alter the effect of endotoxin on body temperature.

Since antigen-antibody reaction may cause leukopenia and change in body temperature, it can be speculated that the increased susceptibility to leukopenia and hypothermia in the conventional guinea pigs resulted from hypersensitivity to endotoxin present in conventional animals but absent in the germ-free animals. These experiments do not allow evaluation of such a concept.

These studies have further significance as concerns the general mechanism of endotoxin reactivity. It has been proposed that endotoxin reactions are mediated through hypersensitivity developing from exposure to endotoxin-containing bacteria in the host(8). The ability of germ-free animals never exposed to any bacteria to manifest typical reactions to endotoxin implies that hypersensitivity is not a necessary requisite for endotoxin reactivity. This is supported by the absence of intradermal reactions to endotoxin in these germ-free guinea pigs despite typical systemic responses. It is argued that hypersensitivity to endotoxin may still develop in germ-free animals as a result of intestinal absorption of endotoxin present in food and water. Intestinal absorption of radioactive-labelled endotoxin has been demonstrated but only when large amounts were given to either new-born pigs(9) or adrenalectomized mice(10) and not in dogs with hemorrhagic shock(11). It is therefore doubtful that the small amounts of endotoxin in the diet would be absorbed in amounts sufficient to influence endotoxin reactvity. Hypersensitivity may contribute to the host responses to endotoxin in conventional animals but such studies as these in germ-free animals suggest that hypersensitivity is not the primary mechanism of endotoxin reactivity.

Summary. *Salmonella typhosa* endotoxin produced hypothermia, leukopenia, and death in both germ-free and conventional guinea pigs. The lethal dose was identical in both, but hypothermia and leukopenia were produced in conventional guinea pigs by only one-third of the amount required in germ-free animals.

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Studies on Biliary Metabolites of Orally Administered Ethynylestradiol (EE) and its 3-Cyclopentyl Ether (EECPE-Quinestrol*). (31989)

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Preliminary experiments showed that measurable amounts of radioactivity appear in the bile of the rat shortly after oral administration of aqueous suspensions of isotopically labeled EE or EECPE. The observation that as much as 45% of the administered dose of EE and 27% of that of EECPE could be recovered from the 2½-hour bile samples indicated that the 2 compounds or their metabolites undergo an active enterohepatic circulation. It seemed, therefore, worthwhile to investigate the nature, distribution and biological activity of the radioactive substances appearing in the bile. The reabsorption of these substances was also studied.

Materials and methods. A) The chromatographically pure compounds tested were ethynylestradiol-6,7 ^3H with a specific activity of 0.93 $\mu c/\mu g$ and either ethynylestradiol-6,7 ^3H-3-cyclopentyl ether with a specific activity of 0.78 $\mu c/\mu g$ or a mixture of ethynylestradiol-6,7 ^3H-3-cyclopentyl ether with a specific activity of 0.78 $\mu c/\mu g$ and ethynylestradiol-3-cyclopentyl-1^{14}C ether with a specific activity of 0.0392 $\mu c/\mu g$.

B) *Rate of biliary excretion and preliminary characterization of biliary metabolites.* Male albino rats, 350-400 g body weight, were used. Under ether anesthesia a polyethylene tubing (Intramedic® PE 10) was inserted into the common bile duct and secured in place by means of silk ligatures.

*Non-proprietary name.

TABLE I. Excretion of Radio-Metabolites of EECPE and EE in Rat Bile.*

	Percentage of administered dose		
	EECPE		EE
Time (hr)	H^3	C^{14}	H^3
2½	27.0	19.8	45.5
5	37.4	22.0	58.0

* Three EECPE-treated rats per group; 2 EE-treated rats per group. Average bile volumes were: EECPE, 2½ hr = 1.85 ml, 5 hr = 2.78 ml. EE, 2½ hr = 2.0 ml, 5 hr = 4.25 ml.

Upon closure of the abdominal incision, this tubing was exteriorized. The animals were thereafter transferred to individual restraining cages as described previously(1). Aqueous suspensions of ^3H-labeled EE or ^3H-^{14}C-labeled EECPE were administered by gavage at the doses and in the $^3H/^{14}C$ ratio (EECPE) as specified in Table I. Bile samples were obtained at 2½ and 5 hours following administration. After determination of total radioactivity present, the bile samples were diluted to 10 ml with HOH and extracted 3× with 20 ml volumes of ether. Ten ml of the pooled ether extracts ("free fraction") were evaporated in a counting vial and 15 ml liquid scintillation cocktail† added for determination of radioactivity on a 3 channel Packard Tri Carb liquid scintillation spectrometer Model 3365 equipped with an external standard for quench correction.

†Formula: 7 g PPO, 0.3 g dimethyl POPOP, 100 g naphthalene in 1 l redistilled dioxane.