though a definite secondary response effect was noted.

Textbooks usually do not differentiate completely between resistance and immunity; such indefinite factors as nutrition, heredity and environment as nonspecific factors of resistance are usually mentioned. While nonspecific factors may be important, now the inference can be drawn that resistance to a particular organism may be due to previous infection by an immunologically distinct organism that is related to the extent of inducing a state of accelerated antibody response. This relationship could be indicated by complement-fixation or perhaps by gel-diffusion, as was found for adenovirus-infectious canine hepatitis virus(4) and for mucosal disease and swine fever viruses(5). Heterotypic responses also have been noted within the human adenovirus group following vaccination (6,7) and complement-fixing antibodies for poliovirus have been observed in individuals following ECHO 9 infection(8). While serological studies have not been made, cross-protection among certain of the group A arboviruses has been found(9-12). Much of the variation in clinical response to infectious agents may result from some previous infection that has induced resistance and, by accelerating antibody production, has reduced severity of clinical illness. If this is true, then this resistance concept introduces an entirely new means of controlling diseases.

Summary. Pigs inoculated with VD (virus diarrhea) virus survived inoculations of virulent HC (hog cholera) virus that killed littermates that had not received VD virus. Survival was signalized by accelerated antibody production both to HC and VD viruses. In reciprocal tests, HC virus produced secondary response in calves. Reciprocal neutralization tests between VD virus and HC virus showed they did not share a common antibody. Protection appeared related to accelerated antibody production that resembled the secondary response induced by inactivated viral antigen.

1. Jenner, E., Pamphlet Vol. 4232, Army Medical Library, Washington, D. C.

2. Downie, A. W., McCarthy, K., *Brit. J. Exp. Path.*, 1950, v31, 789.

3. McCarthy, K., Downie, A. W., *Lancet*, 1953, v1, 257.

4. Carmichael, L. E., Baker, J. A., PROC. SOC. EXP. BIOL. AND MED., 1962, in press.

5. Darbyshire, J. H., *Vet. Record*, 1960, v72, 331.

6. Hilleman, M. R., Flatley, F. J., Anderson, S. A., Luecking, M. L., Levinson, D. J., *J. Immunol.*, 1958, v80, 299.

7. Van der Veen, J., Prins, A., *ibid.*, 1960, v84, 562.

8. Lenette, E. H., Schmidt, H. J., Magoffin, R. L., *ibid.*, 1961, v86, 552.

9. Stamm, D. D., Kissling, R. E., *ibid.*, 1957, v79, 342.

10. Parks, J. J., Price, W. H., *Am. J. Hyg.*, 1958, v67, 187.

11. Casals, J., *Trans. N. Y. Acad. Sci.*, 1957, v19, 219.

12. Hearn, H. J., Jr., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 607.

Received October 27, 1961. P.S.E.B.M., 1962, v109.

Effect of Bacterial Endotoxin in Germ-free Mice. (27200)

MAURICE LANDY, JAMES L. WHITBY,* J. GABRIEL MICHAEL,† MARK W. WOODS AND WALTER L. NEWTON

National Cancer Institute and Institute of Allergy and Infectious Diseases,‡ Bethesda, Md.

There has developed an increasing awareness that the characteristic reactivity of mammals to endotoxin may be a consequence of the continued presence of Gram-negative bacteria in the intestinal tract from shortly after birth. Animals reared in an environment without contact with living bacteria provide a means for determining whether the intes-

* Present address: Queen Elizabeth Hospital, Birmingham, England.

† Present address: House of the Good Samaritan, Boston, Mass.

‡ National Inst. Health, Public Health Service, U. S. Dept. of H.E.W.

tinal flora contribute to endotoxin hyperreactivity. To our knowledge, no information on this issue has been reported, possibly because of the very limited availability of germ-free animals.

During an investigation on natural antibodies to Gram-negative bacteria in which we explored factors relating to their appearance in animals, an opportunity arose to compare some of the effects of endotoxin in conventional and germ-free mice.

Materials and methods. Two endotoxin preparations were employed. The one used in most of the experiments was derived from *Salmonella enteritidis* by the aqueous ether procedure(1) and kindly supplied by Dr. Edgar Ribi. It contained 1.1% N, 0.6% P, 76% polysaccharide and 3% fatty acid ester; the LD_{50} for mice was 270 μg. The other preparation, derived from *Shigella flexneri,* was purchased from the Difco Laboratories, Detroit, Mich. For injection into mice in the germ-free units, saline solutions of these products in sealed glass ampoules were sterilized by boiling for 5 mintues on 3 successive days; the controls received the same preparations.

The germ-free mice (GF) used in these studies were from the Laboratory of Germfree Animal Research colony which was derived originally from the germ-free colony maintained at Notre Dame since 1954. The colony is maintained on a steam-sterilized semi-synthetic diet in the Reyniers Germfree System and is checked routinely to assure absence of contamination with viable organisms, according to technics described earlier(2,3).

The conventional animals used as controls (CVN) were from a colony derived originally from the germ-free colony and periodically seeded with breeders from the latter. The CVN mice were maintained on the same sterilized diet, bedding, water, etc., as the GF animals but they were kept in a general animal room and were thus exposed to bacterial contamination from other animals and from laboratory personnel. Periodic checks indicated that these animals consistently harbor various bacterial species such as *Escherichia coli, E. freundii, Aerobacter aerogenes, streptococci, Staphylococcus spp.* and enterococci.

TABLE I. Effect of Endotoxin on Glycolytic Metabolism.

Mice	Treatment with *S. enteritidis* endotoxin	Rate of aerobic acid formation* Spleen†	Rate of aerobic acid formation* Macrophages‡
Germ-free	None	+ 2	+ 8
	5 μg i.v. 24 hr previously	+30	+23
Conventional	None	− 2	+17
	5 μg i.v. 24 hr previously	+24	+25

* Aerobic acid formed in excess of respiration (net h readings × vk_{CO_2}) expresed as μl CO_2/hr.

† 100 mg freshly minced tissue/vessel.

‡ 2 × 10^7 cells/vessel.

CAF_1 mice from the NIH animal unit were included in a number of the experiments. All the animals were used at approximately 2 months of age. The mice were randomized into experimental groups so as to control the possible influence of litter, sex, age and diet. More than 100 germ-free mice were used in these comparisons; illustrative data are given in the tables.

Metabolic activity of host cells. Groups of GF and CVN mice were given 5 μg of *S. enteritidis* endotoxin i.v. and 24 hours later these groups as well as untreated GF and CVN control mice were killed by cervical dislocation. Peritoneal macrophages were obtained by washing out the peritoneal cavity with Krebs Ringer glucose bicarbonate and suspensions of spleen cells were prepared. The rates of aerobic and anaerobic glycolysis of standardized macrophage and spleen cell suspensions were determined. Glycolytic activity of cells obtained from untreated CVN and GF animals did not differ significantly (Table I). Cells from the endotoxin-treated animals displayed elevated glycolysis as previously reported(4) but no difference in the magnitude of enhancement by endotoxin was apparent between germ-free and conventional animals.

Lethality. Groups of GF, CVN controls and CAF_1 mice were injected i.p. with graded doses of *Sh. flexneri* endotoxin and the number of mice in each group which succumbed to the endotoxin was recorded at 3-hour intervals for a total of 72 hours. The rate at

TABLE II. Lethal Effect of Endotoxin.*

Mice	Exp. No.	Mortality data, No. dead/No. tested, μg 50	100	200	400	800	LD_{50} (μg)
Germ-free	1	0/5	0/5	1/5	3/5	5/5	320
	2	0/5	0/5	1/5	5/5	5/5	260
Conventional	1	0/5	0/5	1/5	5/5	3/5	300
	2	0/5	2/5	1/5	2/5	5/5	300
CAF_1	1	0/5	0/5	2/5	5/5	4/5	250

* Endotoxin: *Shigella flexneri*, Difco Labs.

which deaths occurred did not differ appreciably for the 3 sets of mice. The results of 2 such experiments (Table II), show that there was no significant difference in endotoxin LD_{50} for germ-free mice, mice of the same strain reared in a conventional environment, and CAF_1 mice.

Susceptibility to infection. GF, CVN and CAF_1 mice were each randomized into 6 groups of 5 mice; 2 groups of each set of mice were injected i.p. with 10 μg of *S. enteritidis* endotoxin and 24 hours later these and the untreated mice were challenged with graded doses of a washed, saline suspended 4-hour culture of *S. typhosa* Ty2. The mortality data are summarized in Table III and show that the number of *S. typhosa* required to produce a fatal infection is similar for germ-free and control mice. It is also evident that prior treatment with endotoxin elicited increased resistance in germ-free mice as it does in control animals.

Level of bactericidal antibody in serum. Individual and pooled serum specimens from GF, CVN controls, NIH and NCS§ mice were assayed for antibody against *S. typhosa* 0901 and *E. coli* 0127 by a procedure developed in this laboratory(5). Serial dilutions of mouse serum (0.1 ml) and the complement source (0.3 ml of a 1:5 dilution of pooled normal human serum that had been absorbed with the test organism) were interacted for 1 hour at 37°C with the bacterial inoculum of 1×10^6 organisms; survivors were enumerated by plating. Under these test conditions bactericidal activity is a function of the concentration of specific antibody in mouse serum. Results representative of a number of assays presented in Table IV show that the amount of typhoid and coli antibody in the serum of adult germ-free mice does not differ appreciably from that of animals reared in a conventional environment. The NCS Rockefeller mice maintained free of coliform organisms were also found to have undiminished levels of antibody against *E. coli* 0127.

TABLE III. Susceptibility of Mice to Infection with *S. typhosa*.

S. typhosa Ty2 challenge $\times 10^6$	Prior treatment of mice	Mortality data, No. dead/No. tested, Germ-free	Conventional	CAF_1
12	None	—	—	5/5
6	"	4/5	4/5	2/5
3	"	0/5	1/5	1/5
1.5	"	0/5	1/5	0/5
12	*S. enteritidis**	0/5	3/4	0/5
6	"	0/5	0/5	0/5

* 10 μg *S. enteritidis* endotoxin 24 hr previously.

TABLE IV. Level of Bactericidal Antibody in Mouse Serum.

Mice	Bactericidal titration of mouse serum, ml required to effect 50% reduction in bacterial count* *S. typhosa*	*E. coli*
Germ-free	.006	.0003
Conventional	.006	.0002
Rockefeller (NCS)	.006	.0003
NIH	.006	.0001

* Inoculum: 1×10^5 bacteria.

Increase in bactericidal antibody after endotoxin. We have recently reported that in mice, administration of endotoxin results in an increase in the level of antibody to various Gram-negative bacteria(6,7). Serum specimens from GF and CVN mice, obtained prior to and after i.p. injection of 5 μg of *S. enteritidis* endotoxin were assayed in parallel for antibody against *S. typhosa*. The results of this comparison (Table V) show that a) the germ-free and conventional mice have similar levels of typhoid antibody and b)

§ A strain of Swiss mice at Rockefeller Institute, maintained essentially free of coliform organisms. Serum specimens from this strain were kindly supplied by Dr. Curtis Williams, Jr.

TABLE V. Effect of Injection of Endotoxin on Bactericidal Activity of Mouse Serum for *S. Typhosa.*

Serum, ml	Bacterial survival in serum from Conventional mice: Untreated	Conventional mice: 24 hr after 5 μg *S. enteritidis* endotoxin	Germ-free mice: Untreated	Germ-free mice: 24 hr after 5 μg *S. enteritidis* endotoxin
	%			
.01	.8	.4	.8	.1
.0066	46	2.7	16	2.3
.005	75	33	71	27
.004	110	67	95	65

the elevation in this level brought about by endotoxin is of similar magnitude in germ-free and control mice.

Discussion. The present work has shown that as determined by a number of parameters there is little if any difference in type or magnitude of reaction to bacterial endotoxin, in animals in continued contact with viable Gram-negative bacteria and those denied such contact. Previous attempts to account for some of the aspects of endotoxin reactivity had postulated that animals not exposed to endotoxin-bearing organisms would react in an entirely different manner. This concept has recently received support in the report of Schaedler and Dubos(8) that mice freed of coliform organisms by antibiotics showed markedly reduced susceptibility to the lethal effects of endotoxin. The normal level of susceptibility could be restored, in part specifically, by immunization with heat-killed bacterial vaccine.

The present findings argue against such a concept. However, the demonstration of specific antibodies to Gram-negative species in germ-free mice at levels which did not differ greatly from those of normal animals‖ suggests that the reaction of the host to endotoxin may be modified in ways other than by direct exposure to viable Gram-negative organisms. If this is the case, the germ-free animal can provide at best only a partial answer to this issue. It is evident that the food and water fed germ-free mice, while sterile, may nonetheles contribute antigenic material and thus provide the necessary stimulus. The diets employed have not been formulated with this in mind. Even if this factor could be controlled, the available information on cross reactivity between polyglucose, vegetable seeds and Salmonella serotypes(11) suggests that it may not be possible to prepare a diet of natural foodstuffs which would be free of antigenic material, nor is there assurance that rearing germ-free mice on a completely synthetic diet would result in absence of these natural bactericidal antibodies.

As there is now no way to assess the effect of these natural antibodies on the host response to endotoxins, it is not feasible to approach experimentally the concept of hyperreactivity. Nevertheless, if the presence or absence of a bacterial flora is the only significant difference between animals of similar genetic constitution raised in a germ-free and conventional environment this microbial factor does not control susceptibility to endotoxin.

Summary. Germ-free and conventionally reared mice of similar genetic stock maintained on the same sterilized diet were compared as to their reaction to bacterial endotoxin. No significant difference was found in the following: elevation in levels of antibody to Gram-negative bacteria, stimulation of glycolytic activity of spleen cells and peritoneal macrophages, susceptibility to infection with *Salmonella typhosa,* production by endotoxin of increased resistance to infection, and LD_{50} of endotoxin.

‖ Benacerraf, *et al.,* have reported that the rate of clearance by mice of intravenously injected P-32 labelled *E. coli* is determined by the level of specific antibody(9). It was subsequently found that P-32 labelled *E. coli* was cleared at a similar rate by conventional and by germ-free mice(10).

1. Ribi, E., Hoyer B. H., Milner, K. C., Perrine, T. D., Larson, C. L., Good, G., *J. Immunol.,* 1960, v84, 32.

2. Reyniers, J. A., Trexler, P. C., and Ervin, R. F., *Lobund Rep. No. 1,* Univ. of Notre Dame Press, 1946.

3. Baer, P. N., Newton, W. L., *Oral Surg. Med. Path.,* 1960, v13, 1134.

4. Woods, M. W., Landy, M., Whitby, J. L., Burk, D., *Bact. Rev.,* 1961, v25, 447.

5. Landy, M., Michael, J. G., Whitby, J. L., *J. Bact.,* 1962, v83.

6. Michael, J. G., Whitby, J. L., Landy, M., *Nature,* 1961, v191, 296.

7. Whitby, J. L., Michael, J. G., Woods, M. W., Landy, M., *Bact. Rev.*, 1961, v25, 437.

8. Schaedler, R. W., Dubos, R. J., *J. Exp. Med.*, 1961, v113, 559.

9. Benacerraf, B., Sebestyen, M., Schlossman, S. *Abst. VII Int. Cong. Microbiol.*, 1958, 167.

10. Thorbecke, G. J., Benacerraf, B., *Ann. N. Y. Acad. Sci.*, 1959, v78, 247.

11. Heidelberger, M., Cordoba, F., *J. Exp. Med.*, 1956, v104, 375.

Received October 27, 1961. P.S.E.B.M., 1962, v109.

Solubility of Bilirubin in Aqueous Solutions.* (27201)

RICHARD C. BURNSTINE† AND RUDI SCHMID

Department of Pediatrics, Boston University School of Medicine, and Thorndike Memorial Laboratory, Boston City Hospital, Harvard Medical School

Recent studies concerning the bilirubin-binding properties of serum proteins(1) have made it necessary to reexamine the solubility of unconjugated bilirubin in aqueous protein-free solutions of varying salt concentrations. In dialysis experiments, Martin(2) noted that only 0.1 mg % bilirubin was soluble in phosphate buffer of ionic strength 0.1. By potentiometric titration, Overbeek *et al.*(3) found that less than 0.5 mg % bilirubin was soluble at pH 7.4 in solutions with an ionic strength ranging from 0.003 to 0.006; but Lucassen(4) encountered difficulties in repeating these determinations unless acetone was added to stabilize the reaction mixture. On the other hand, Odell's studies(1) appeared to indicate that at pH 7.4, larger amounts of bilirubin may be soluble in protein-free ultrafiltrates possessing a higher ionic strength. Consequently, the solubility of bilirubin was determined in buffers of varying ionic strengths and pH.

Materials and methods. Pyrex™ glassware was used which was prepared as follows: (a) soaked in 0.4N NaOH for 15 minutes; (b) rinsed with distilled water × 5; (c) soaked in 1:100 solution of 7×™ for at least 18 hours; (d) rinsed with distilled water × 20; (e) soaked in 6N HCl for at least 24 hours; (f) rinsed with distilled water × 20; (g) soaked in 3% bovine albumin for 15 min.; (h) rinsed with distilled water × 25. For centrifugation in the Spinco Model L ultracentrifuge, the Lusteroid™ tubes were prepared as in steps c, d, g, h, and discarded after each experiment. The screw caps were first treated with Siliclad™ and then prepared in the same manner as the Lusteroid tubes .

Cold phosphate buffer solutions of 0.01, 0.05, 0.10 and 0.20 molarity, corresponding at pH 7.4 to an ionic strength of 0.024, 0.120, 0.240 and 0.479 respectively(5) were prepared at various pH values ranging from 7.1 to 7.9. In all subsequent steps the solutions were protected from direct light. Bilirubin (Pfanstiehl) was dissolved in cold sodium hydroxide of appropriate normality to give an approximate pigment concentration of 100 mg %. One ml of this freshly prepared sodium bilirubinate solution was added immediately to 10 ml of the corersponding buffer, mixed by inversion, placed in a Lusteroid™ tube and centrifuged for 60 minutes in the dark at room temperature at 30,000 rpm. (G_{avg} 78,410). The pH of the clear supernatant was determined in a Beckman Model G pH Meter, care being taken to standardize the instrument before each determination. An aliquot of the supernatant was analyzed for bilirubin concentration by the Malloy and Evelyn method(6), modified as follows: the aliquot of the supernatant was diluted with appropriate amounts of 60% aqueous methanol (v/v); the diazo reagent was made up in 0.6N HCl; optical density was determined at 555 mμ in a Beckman DU Spectrophotometer. The results obtained were compared with a standard curve, prepared by the same

* Aided in part by grants from The Medical Foundation, Inc., and U.S.P.H.S., A-1833.

† This work was carried out during the tenure of a Postdoctoral Fellowship from Division of General Med. Sciences, U.S.P.H.S.