Translocation and Fate of ^{32}P-Labeled Salmonella in Mice[1] (35092)

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Salmonellosis is a true food-borne infection and its onset is related directly to growth of salmonella bacteria from small to very large numbers in the host tissues. Symptoms associated with the disease are not very characteristic, but include severe gastroenteritis, diarrhea, vomiting, cramps, fever, and mortality is low (1). Onset of infection is evident within 8 to 48 hr after ingestion of the *Salmonella* organism and usually lasts from 24 to 48 hr (2). However, the duration of infection in mice varies under different experimental conditions. Massive doses of bacteria (3, 4) and postinjection of metabolic inhibitors and intermediates are among the important factors that may alter the course of infection and the survival time of infected mice (5–7). Shands (8) stated that there was no apparent difference in the toxicity of equal number of *S. typhimurium* when grown in enriched medium or in a chemostat. Prost and Riemann (1) stated that salmonellosis most probably is caused by a joint action of bacterial cell and endotoxin and that the degree to which these two factors are involved varies with individual type, or even strain, of *Salmonella*. These authors again pointed out that the pathogenesis of *Salmonella* depends not so much on the properties of the microorganism, as on the environment and the physiological condition of the host. Hawbecker (3) showed that salmonellosis has a complex and variable character. Buxton (9) reported that the factors which influence the course of the pathological process of this disease are not fully known. To attain some insight into this problem, a study was made to determine the toxigenesis and infection of salmonella in mice. Advantage was taken of the striking differences in the course of infection between mice fed *per os* viable cells of *S. enteritidis* obtained from either the negative acceleration or the early decline phases (3, 10). The present studies were undertaken to determine the translocation and fate of ^{32}P-labeled *Salmonella* in mice.

Material and Methods. Animals. White Swiss/ICR female, pathogen-free (6–8 weeks old weighing 30–35 g), housed in air-conditioned quarters (24°) in isolated cages and offered water and commercial pellets *ad libitum* were used. Pregnant mice, two weeks after conception were also employed.

Bacteria. S. enteritidis was kept by serial transfer in tryptic soy broth (TSB-Difco Laboratories) and was periodically passed through mice to maintain virulence. Salmonella were grown in TSB and harvested by centrifugation at 6000*g* from two different physiological phases of growth; a negative acceleration (18 hr) and an early decline (36 hr).

Mouse toxicity test. The lethal toxicity of the test cultures was determined by injecting mice intraperitoneally (ip) with 1 ml of saline suspension containing decreasing amounts of cells under test. The latter was confirmed by plate count in TSA. Deaths occurring within 48 hr were scored and the LD_{50} was calculated according to the method of Reed and Muench (11).

^{32}P-labeled Salmonella. Viable cells of *S. enteritidis* obtained from either growth phase

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were washed twice in saline and then resuspended in a sterile basal synthetic medium (pH 7.0) consisting of: (weight per 100 ml): NH_4Cl, 200 mg; $MgCl_2$, 1 mg; Na_2SO_4, 2.6 mg; glucose, 500 mg; KCl, 1 mg; and radioactive phosphate, 250 μCi (as ^{32}P-inorganic phosphate). The cell suspension was incubated at 37° for 1.5 hr and the labeled cells were harvested by centrifugation in sterile tubes at 12,000*g* for 10 min. The supernatant fluid was then poured off and the pellet was rinsed twice by filling the centrifuge tube with sterile saline and pouring it off immediately without disturbing the pellet. The rinse was sufficient to remove supernatant fluid adhering to the walls of the centrifuge tube, but the wash was required to remove the "water space" content of the cells. The washed pellet was then resuspended to a final concentration of 10^8 cells/ml for feeding to mice and the number of viable cells was always checked by plating an appropriate dilution on TSA. The specific activity of the ^{32}P-labeled cells, reported as counts min^{-1} mg^{-1} of dried cells, was determined after drying a 1-ml sample of the saline cell suspension to a constant weight and counting the radioactivity on an Abacus, Baird Atomic Model 123 G-M system. The specific activity of the test culture in all cases was greater than 1×10^4 counts min^{-1} mg^{-1} of dried cells and the number of viable cells per milligram of dry weight was 2×10^9 bacteria.

Feedings. Two groups of mice (60 in each) were fed the labeled cells by gavage. The first group received the 18-hr culture and the second was fed the 36-hr culture. A third group consisting of 30 mice received 1 ml of saline each and served as controls. The mice were anesthetized with diethyl ether and the saline suspension containing the labeled cells was then fed, by gavage, as described by Gay (12) and Hawbecker *et al.* (10).

Sampling. (a) *Microbiological assay.* At definite intervals following feeding, 1 ml of blood was obtained by cardiac puncture from both control and infected mice. The mice were then sacrificed; and spleen, liver, and cecum were aseptically excised, placed in sterile petri dishes, and chilled immediately. The tissues and blood samples were examined for viable test culture (13) and typical colonies were then isolated and examined both biochemically and serologically (14, 15) to insure definite identity of the culture recovered from infected mice.

(b) *Radioactive assay.* Samples of blood and organ tissue (spleen, liver, and cecum) were digested in 5 ml of concentrated nitric acid for 12 to 16 hr at room temperature (24°). Aliquots of digested samples were placed in planchets, dried to constant weight, and counted in a Nuclear Chicago C115 automatic low background G-M system. The counts were corrected for background and the specific activities were expressed as counts min^{-1} mg^{-1} of blood or dried tissue.

Translocation of Salmonella across the intestinal wall. The passage of intra-intestinal viable salmonella cells across the intestinal barrier was followed by staining the organism with fluorescent antibodies and examining ultrathin sections of the intestine with a Reichert-Fluoropan microscope. All fluorescent conjugate and tissue staining techniques were those of Thomason *et al.* (16, 17). Two groups of mice (10 in each) were fed the test cultures. Each mouse of the first group received 10^8 cells of 18-hr culture and the second 10^8 cells of 36-hr culture. A third group consisting of 10 mice each, received 1 ml of saline and served as controls. All mice were sacrificed 3-hr PF (postfeed or time interval following feeding of the test culture), 2 in. of the duodenum were removed, placed in 10% cold formalin, and fixed for 1 hr. The fixed sample was then frozen on a section block of a cryostat freezing microtome and ultrathin sections 2, 6, and 18μ were prepared, placed on Trident fluorescent microscope slides, dried, and fixed for 10 min in 95% ethanol. The sections were examined histopathologically or subjected to fluorescent staining using the direct technique (18). The histopathological evaluation was made by Dr. W. L. Chapman, School of Veterinary Medicine, University of Georgia.

Detection of Salmonella in the Fetus. Ten pregnant mice were fed 10^8 ^{32}P-cells of 18-hr test culture. The mice were sacrificed after

3-hr PF and the fetuses were excised aseptically. Five fetuses were digested in nitric acid for 16 hr and the radioactivity present counted, whereas the other five were subjected to bacterial analyses for viable salmonella.

Results and Discussion. Effect of phase of growth on toxicity. Two groups of mice, 60 in each, were inoculated ip with various numbers of viable *S. enteritidis*. The first group received cells harvested from the negative acceleration phase culture and the second from the early decline culture. The $LD_{50/48hr}$ for the former culture was 7.1×10^8 cells and that for the latter was 4.5×10^9 cells indicating that the young cells were slightly more toxic and/or virulent. Badakhsh and Herzberg (19) showed that the endotoxic activity of *S. typhimurium* resides in the cell extract rather than in the residue. It is possible that the difference in $LD_{50/48hr}$ noted between the 18- and 36-hr culture may be due to some changes in the cell wall of the old culture mediated either during incubation or following feeding. Fukushi *et al.* (20) showed that method of cultivation and extraction procedure produced differences in the chemical composition and biological activity of extracted endotoxins of *S. enteritidis*. In addition, the cell-wall composition of this organism may be altered by changes in growth rate (21).

Fate and Distribution of ^{32}P-labeled Salmonella. Spleen, liver, and cecum of mice fed 10^8 ^{32}P-cells of 18- or 36-hr cultures were examined for their specific radioactivity after 2-hr PF, and at various intervals. The average specific activity of the Salmonella preparation fed was $6 \times 10^4 \pm 300$ counts min^{-1} mg^{-1} of dried cells. The results (Table I) showed that feeding 10^8 cells obtained from early decline phase (36 hr) produced a rapid increase of the specific activity in spleen of infected mice reaching 227 counts min^{-1} mg^{-1} of tissue on the 2nd hr PF, followed by rapid decline to less than 20 counts min^{-1} mg^{-1} of tissue on the 8th hr. The liver and cecum of these mice responded in a similar manner; the specific activity in the former decreased from 328 counts min^{-1} mg^{-1} of tissue at 2 hr to 25 at 8-hr PF. On the other hand, feeding the negative acceleration phase (18 hr) culture produced a gradual increase in the specific activities in all tissues throughout the 6 hr of study. Blood analyses of mice receiving 10^8 ^{32}P-cells indicated similar trend to that of the spleen; 200 counts min^{-1} mg^{-1} of blood at 2-hr PF, 45 counts min^{-1} mg^{-1} of blood at 6 hr, 20 counts min^{-1} mg^{-1} of blood at 8 hr, and 10 counts min^{-1} mg^{-1} of blood by 12-hr PF. The distribution of radioactivities (counts min^{-1} mg^{-1}) in blood, spleen, and liver, at

TABLE I. Effect of Culture Age and Time Postfeeding (PF) on the Distribution of Radioactivities in Organ Tissues.

Mouse treatment[a]	Time PF (hr)	No. of mice fed	Radioactivities (counts min^{-1} mg^{-1} of tissue)[b] Spleen	Liver	Cecum
Negative acceleration phase	2	12	61 ± 19	72 ± 23	1480 ± 460
	3	12	46 ± 17	51 ± 11	715 ± 213
	4	12	118 ± 15	118 ± 11	2780 ± 1110
	5	12	143 ± 57	143 ± 37	2098 ± 1360
	6	12	103 ± 32	147 ± 49	2110 ± 1900
Early decline phase	2	10	227 ± 48	328 ± 61	11,170 ± 6720
	3	10	115 ± 52	200 ± 65	3550 ± 1980
	4	10	85 ± 14	176 ± 48	2730 ± 1180
	5	10	54 ± 55	32 ± 10	550 ± 283
	6	10	216 ± 68	234 ± 60	4820 ± 1950
	8	10	20 ± 5	25 ± 8	20 ± 6

[a] Mice were fed 10^8 viable ^{32}P-labeled *S. enteritidis* having a specific activity of $6 \times 10^4 \pm 300$ counts min^{-1} mg^{-1} of dried cells.

[b] Mean ± SD.

each specific interval, varied slightly within the range of standard deviation. In the cecum, higher specific activities were observed at all times, apparently due to accumulation of labeled cells. This indicated that the organisms persisted in the cecum and slowly, but consistently, migrated to other parts of the body. This was confirmed by bacteriological analyses. The test culture was also recovered in large numbers per unit weight of spleen and liver tissues after 1-, 2-, 3-, and 4-hr PF in mice fed the 36-hr culture and up to 72-hr PF in mice fed the 18-hr culture. The number of viable Salmonella recovered from blood, spleen, liver, and cecum in mice fed 36-hr culture was very small at 6 hr and declined to zero after 8- and 12-hr postinfection.

The incidence of positive culture recovery of viable cells from blood (counts of 30 colonies of more/ml) in mice fed the negative acceleration culture was 39% at 2 hr, increasing to 56% at 3–5 hr and decreasing thereafter to 25% at 6-hr PF. On the other hand, percentage positive recovery of Salmonella from the blood of mice fed early decline phase culture was 7% at 2-hr and 20% at 6-hr PF. Both the microbiological and the radioisotopic studies substantiated the toxigenic and bacteriological findings previously reported by Hawbecker (3) and Hawbecker *et al.* (10). It appears that feeding 10^8 cells of either phase of growth yielded salmonella infection before 4-hr PF and that the 18-hr culture challenge caused by the test organism continued beyond 6 hr and viable salmonella persisted in various organs for 72 hr. It can be stated that the 18-hr culture exhibited a greater invasive potential than the 36-hr culture evidenced by the rapid and continual increase of the radioactivity in the organ tis-

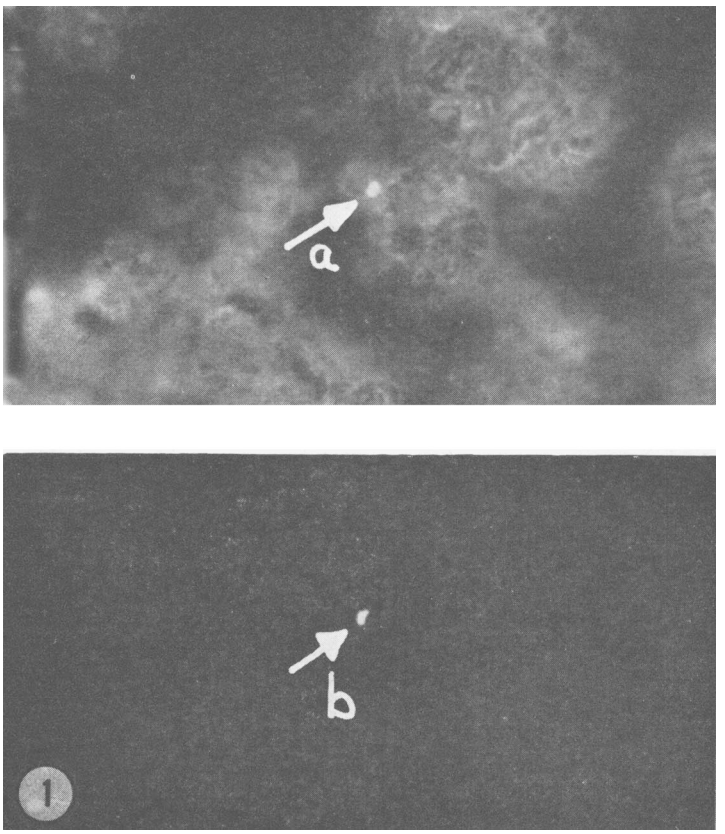

FIG. 1. Cross section (2 μ) of duodenum from infected mice (a), note bright "Salmonella-like" structure located in the submucosa (×950) similar to that of pure culture of *S. enteritidis* (b).

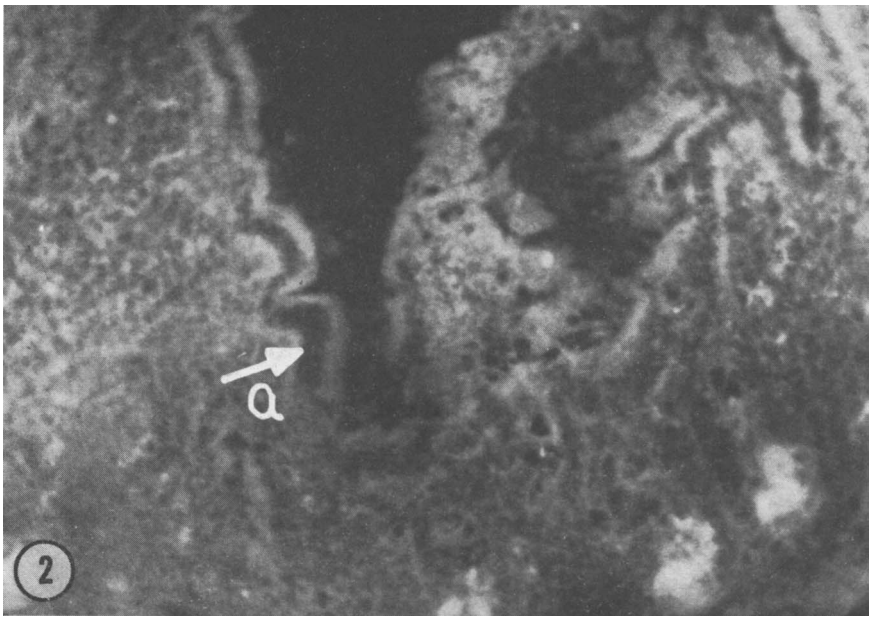

FIG. 2. Cross section of duodenum (18 μ in thickness $\times$160) from mice fed *S. enteritidis*, note the shape of villi (a).

sues during the 2- to 6-hr period. The ^{32}P-orthophosphate is known to be incorporated into the cell in the form of phosphorylated intermediates and it was assumed that this labeling may persist within the viable cell of both culture phases when fed *per os* to mice. However, the possibility that the 36-hr culture is easily destroyed cannot be completely eliminated. Therefore, the high specific activity caused by the 36-hr culture 2-hr PF may be due to phosphorylated intermediates or free ^{32}P released upon destruction of the labeled bacterial cells. However, further analysis should be made before definite conclusion can be drawn.

Translocation of Salmonella across the intestinal wall. Both the radioisotopic and the bacteriological studies conducted in mice fed *S. enteritidis* revealed that 18-hr culture persisted in mice tissue. In order to demonstrate that the organism is able to migrate through the intestinal mucosa, ultrathin sections of the duodenum were stained with fluorescent antibodies (FA). Examination of thin sections (2–6 μ) of the duodenum of the infected mice revealed the presence of high fluorescent bacterial cells having a retention time of 3 min similar to that of pure salmonella cells. However, nonspecific staining was encountered in this study and, therefore, these high fluorescent bacterial cells can be considered only "salmonella-like" bacteria (Fig. 1). Ultrathin sections (18 μ) of the duodenum from 18- and 36-hr culture fed mice were examined histopathologically and the results revealed regressive changes in the surface epithelium of the mucosa of the mouse fed 18-hr culture (Fig. 2). Coagulation necrosis of the surface epithelium was common at the tips of the villi which appeared to be shortened and blunted. An increase in inflammatory mononuclear cells in the lamina propria was also noted. Mice fed 36-hr-old culture showed slight alteration in the surface epithelium of the intestinal mucosa, the villi contained areas of proliferation of the surface epithelium and limited numbers of mononuclear inflammatory cells in the lamina propria were detected. These observations indicated that the 18-hr culture produced a more severe enteritis compared to that of 36-hr phase.

There are several theories as to how the bacterial cells invade the host system. The

cells may irritate the intestinal wall causing sloughing of the lining which allows the cells to enter the lymph through the inflamed areas; the bacterial cells themselves may possess enzyemes which alter the intestinal permeability, or the stress by the presence of *Salmonella* may change the permeability of the intestines. Hamdy *et al.* (22) found that when either *S. enteritidis* or virulent *S. aureus* were established in the gut, the organism was able to traverse the serosa and longitudinal muscles of the gut of bruised chickens. Wolochow *et al.* (23) suggested that the size of the organism was the only one factor that determined whether a particular microorganism could pass through the intestinal wall into the lymph by a noninfectious process.

Detection of Salmonella in the Fetus. Radioactive and bacteriological analyses were conducted on fetuses obtained 3-hr postfeeding from pregnant mice fed *per os* 10^8 ^{32}P-cells of the 18-hr culture. Radioactivity was present to the extent of 500 counts min^{-1} mg^{-1} of fetus whereas viable organisms were not. The absence of *Salmonella* was also verified after enrichment techniques were performed. This was conducted by inoculating throughly homogenized fetus in tetrathionate broth followed by plating on brilliant green agar as previously reported (10). This indicated that the test organism was destroyed by phagocytosis and/or by the fluids of the uterus and that some uptake of free ^{32}P-orthophosphate or passage of ^{32}P-labeled organic metabolites occurred across the placental membrane.

Summary. The translocation and fate of both ^{32}P-labeled and unlabeled *Salmonella enteritidis* in mice fed, by gavage, 1 ml containing 10^8 cells of either an 18-hr or 36-hr-old culture was determined. Onset and duration of infection and the presence of viable cells of test culture in organs were confirmed by bacterial and radioisotope analyses. Mice fed 10^8 ^{32}P-18-hr culture had low specific activity on 2nd hr PF (liver, 72; spleen, 61; and cecum, 1480 counts min^{-1} mg^{-1} of tissue) increasing to 147, 103, and 2110, respectively, on 6th hr. Mice infected with ^{32}P-36-hr culture showed high specific activity (counts min^{-1} mg^{-1} of tissue) on 2nd hr postfeeding; liver, 328; spleen, 227; and cecum, 11,170 followed by a rapid decrease to 234, 216, and 4820 counts min^{-1} mg^{-1} of tissue on 6th hr, respectively. This radioisotope study indicated that the 18-hr-old culture was more invasive than the 36-hr culture. Ultrathin sections of duodenum, obtained from infected mice, and stained with fluorescent antibodies revealed the presence of high fluorescent bacterial cells having a retention time of 3 min similar to that of pure *S. enteritidis* cells. Histopathologically these sections showed regressive changes in surface epithelium of intestinal mucosa of mice fed 18-hr culture and coagulation necrosis was common at tip of the villi. The pathological data showed that 18-hr culture produced a more severe enteritis compared to 36-hr-old culture.

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