JOURNAL OF BACTERIOLOGY, Sept. 1969, p. 629-635

Vol. 99, No. 3
Printed in U.S.A.

Salmonella Suppression by Known Populations of Bacteria in Flies

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Received for publication 11 April 1969

Survivorship of *Salmonella typhimurium*, *Streptococcus faecalis*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* was studied in dibiotic and tribiotic interactions in vitro and in various regions of the digestive tract of the blow fly, *Calliphora vicina*. In dibiotic interactions, *Salmonella typhimurium* dominated *Streptococcus faecalis* and was dominated by *P. mirabilis*, but in neither case was it eliminated from the larval gut. In tribiotic interactions, there was synergic suppression and a definite trend toward elimination of *Salmonella typhimurium* from the gut. This trend approaches but does not match the total exclusion of *S. typhimurium* from the gut of maggots with a normal flora. Bacterial survival in the gut of the fly is discussed in relation to doubling time, sweep-out rate of the maggot and prepupal gut, and the midgut bactericide.

Two factors—the acid midgut of the maggot and ecdysis of the maggot's gut lining during metamorphosis—are known to adversely effect microbial survival in the developing fly. However, the persistence of *Salmonella* populations in monobiotic flies, contrasted by their elimination from flies with a normal flora, raises a possibility that microbial antagonisms are also involved (3, 4, 5). The significance of the normal gut flora in host resistance to enteric pathogens is well recognized in laboratory animals (10) but not in insects, although it was suggested more than 50 years ago by the work of Ledingham (8) and Bacot (1). In vivo antagonisms are a widespread biological fact and have been shown to occur between closely related strains and types of virus (14, 17), between different viruses (11), between a virus and bacteria (15, 19), between different strains and species of bacteria (10), and even between nematodes (9) and parasitic wasps (2). Hardly anything is known about microbial antagonisms in insects despite their importance in transmission of plant and animal diseases.

In this study, we have attempted to describe the microecology of the fly gut at dibiotic and tribiotic levels of bacterial interaction. By comparing in vivo and in vitro interactions, we have sought to identify the normal microbiota which, singly or in combination, antagonize *S. typhimurium* after its ingestion by the blow fly, *Calliphora vicina*.

MATERIALS AND METHODS

Bacteriological procedures. Bacteria and their induced level of antibiotic resistance were: *S. typhimurium*, 1,000 μg of streptomycin sulfate per ml (Wyeth); *Proteus mirabilis*, 100 μg of terramycin per ml (Chas. Pfizer & Co., Inc., Brooklyn, N.Y.); *Pseudomonas aeruginosa*, 2,000 μg of keflin per ml (Eli Lilly & Co., Indianapolis, Ind.); and *Streptococcus faecalis*, 500 μg of neomycin per ml (Pfizer). The first two species were used previously (3, 5); the latter two were obtained from laboratories of the Illinois State Department of Public Health. Neomycin was prepared from bulk powder and was autoclaved; the other antibiotics were reconstituted from sterile powder. Incorporation of an antibiotic into Brain Heart Infusion (BHI) Agar (Difco), at the above concentrations, permitted uninhibited outgrowth of the resistant species and complete suppression of the others. MacConkey's agar plus streptomycin proved more selective for enumeration of *Salmonella typhimurium*. Colonies were routinely confirmed biochemically and by slide agglutination (*S. typhimurium*). Bacteria from 24-hr, 37 C slants were suspended in saline and turbidometrically adjusted; they were inoculated in various combinations into flasks of 100 ml of BHI broth kept at 37 or 27 C and into embryonated chick on which gnotobiotic maggots were feeding. Duplicate 1-g samples of the chick were homogenized in 2 ml of saline and censused by means of dilution plates of antibiotic agar held at 37 C for 48 hr. Bacterial counts are corrected to include dilution factors.

Entomological procedures. The blow fly, *C. vicina* (Robineau-Desvoidy), was trapped locally and kept on sugar, water, and meat. About 100 disinfected fly eggs were introduced into a 1-liter flask which contained a 20-day-old chick embryo in sterile condition. A day later, various combinations of bacteria were introduced. Gnotobiotic fly stages were reared with a specific microflora at 27 C and were externally disinfected as previously described (3, 5). Specific

stages studied were mature maggots, prepupae, pupae, teneral, and 3-day-old adults. Specific structures censused for bacteria were crop, midgut, hind-midgut, hindgut, and pupal case. Specimens were triturated in 1 or 2 ml of saline and dilution plates were handled as described above.

Experiment 1: S. typhimurium and Streptococcus faecalis in chick embryo and blow fly. A 1-ml amount of 10^9 of each organism was introduced to a chick embryo on which day-old sterile maggots were feeding. Samples of chick were censused on days 5, 9, and 19. Intact maggots, prepupae, pupae, and puparia were censused on days 5, 9, 13, and 19, respectively. Crop, midgut, and hindgut of mature maggots and prepupae were also analyzed. Data of duplicate experiments have been combined.

Experiment 2: Salmonella typhimurium, Streptococcus faecalis, and P. mirabilis in broth, chick embryo, and blow fly. About 10^8 of each organism was introduced into 100 ml of broth at 37 C and into chick-maggot medium. Broth was sampled on days 0, 4, 8, 11, and 15; chick on days 3 and 6; and crop, hind-midgut and hindgut of maggots and prepupae on days 3 and 6, respectively. Data of duplicate experiments have been combined. Flies which emerged on day 13 were dissected, and the gut, fly minus gut, and puparium were tested. New flies were also fixed to paraffin in petri dishes and aseptically kept on 5% proteose peptone and 5% sucrose solution, with twice-daily feedings. On the 3rd day, eight flies were dissected and the three bacteria were censused in crop, fore- and mid-midgut combined, hind-midgut, hindgut, and the rest of the fly.

Experiment 3: Salmonella typhimurium, P. mirabilis, and Pseudomonas aeruginosa in broth, chick embryo, and blow fly. Inocula of 3.2×10^8, 2.2×10^8, and 3.7×10^8, respectively, were introduced into 100 ml of broth and into chick-maggot medium. The broth was kept at 37 C and was sampled on days 0, 7, 12, and 16. Chick was sampled on days 7, 12, and 16. Crop, hind-midgut, and hindgut of 5-day-old maggots and 7-day-old prepupae were also tested.

Statistical analysis was based on the Mann-Whitney index for comparison of the medians of related samples, with significance expressed as P (18).

RESULTS

Experiment 1: S. typhimurium and Streptococcus faecalis in chick embryo and blow fly. Five days after introduction of the two bacteria, the population of *S. faecalis* in the chick had fallen 3 logs and was 4 logs lower than that of *Salmonella typhimurium;* during days 9 to 19, *S. typhimurium* levelled at $10^{10.5}$, whereas *Streptococcus faecalis* climbed slowly to 10^8 (Fig. 1). The same dominance relationship, but at lower densities, occurred in the maggot and in its isolated crop and midgut (Fig. 1, 2). *S. faecalis* came close to extinction in the prepupa. Presumably the initial density of *Salmonella typhimurium* enabled it to remain dominant throughout the tract, although its losses were relatively greater. Notable is the declining density of both populations toward the posterior part of the tract and the subsequent multiplication of *S. typhimurium* in the hindgut of the prepupa (Fig. 2). The approximate 4-log difference between the two populations persisted through metamorphosis (Fig. 3).

Experiment 2: S. typhimurium, Streptococcus faecalis, and Proteus mirabilis in broth, chick embryo, and blow fly. After 4 days in broth, the hierarchy in descending order was *P. mirabilis*, *S. faecalis*, and *Salmonella typhimurium*. The last was eliminated between days 8 and 11, whereas the first two organisms remained on a plateau until day 15 (Fig. 4). Extinction of *S. typhimurium* took 10 days longer when the broth was held at 27 instead of 37 C. At the lower temperature, *P. mirabilis* remained at $10^{8.5}$, and *Streptococcus faecalis* at 10^7 until day 25 when the experiment terminated. Although all three populations were severely reduced in the midgut of the maggot, only organisms of *Salmonella typhimurium* and *Streptococcus faecalis* were virtually eliminated from the hindgut of most maggots and prepupae (Fig. 5). Extinction was intensified during metamorphosis so that guts of 20 flies, at emergence and 3 days later, yielded neither of these two types. Pupal cases were uniformly contaminated with all three types of bacteria, yet 9 of 13 flies which emerged from these puparia yielded *P. mirabilis* exclusively (Fig. 6). This organism was present in a total of 12 of 13 teneral flies, but only in 2 of 13 of their guts. Flies from the same group were aseptically maintained for 3 days, at which time 4 of 8 possessed heavy burdens of *P. mirabilis* throughout their digestive tracts (Table 1).

Experiment 3.: Salmonella typhimurium, P. mirabilis, and Pseudomonas aeruginosa in broth, chick embryo, and blow fly. *S. typhimurium* was eliminated from broth by day 13; *Proteus mirabilis* and *Pseudomonas aeruginosa* remained at $10^{6.5}$ and 10^7, respectively, to terminal day 16. Census of the chick showed the same order of dominance as in broth during the 1st week. This hierarchy was obscured in maggot crops by overlapping population counts of the three organisms. Only *S. typhimurium* fell sharply in crops of prepupae, and its progressive elimination from the tract is seen in Fig. 7. *Proteus mirabilis* was least destroyed by midgut passage and thus remained dominant in the posterior gut with *Pseudomonas aeruginosa* multiplying slightly.

DISCUSSION

The microbial composition of the blow fly maggot's medium seems not to effect the insect,

for it is able to develop and reproduce normally under a wide range of breeding conditions, from axenic and monobiotic to natural. Nevertheless, the microbial community may profoundly influence population density and even survival of its own constituents. Thus, competitive exclusion of *Salmonella* species from maggots is so effective that survival is generally achieved only with simplification or elimination of the normal microbiota. This has been well demonstrated in higher animals as well (10). Although the gnotobiotic gut is obviously not a natural microbial habitat, it is one of the best ways known to study microbial succession and antagonisms in a system whose complexity would otherwise defy analysis.

The medium upon which the maggot feeds for the first 5 days of its life is a batch culture. Microbial input into its gut is nonselective, for there

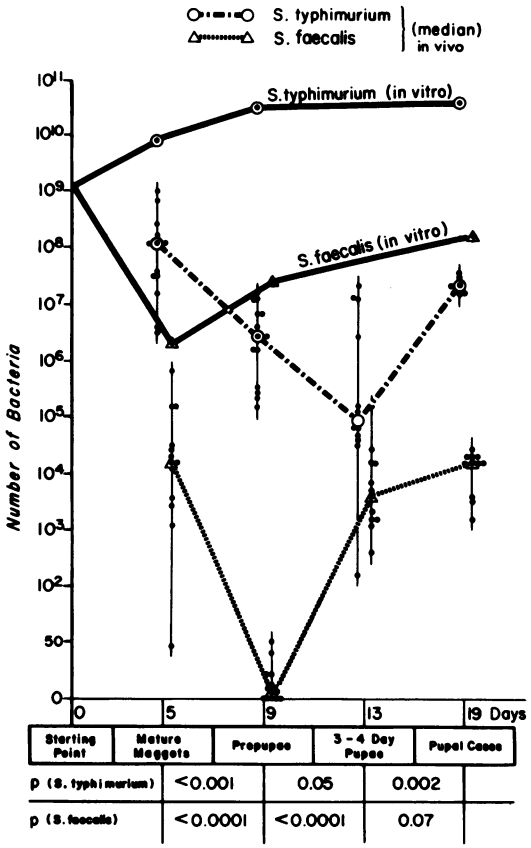

FIG. 1. *Dibiotic interaction of Salmonella typhimurium and Streptococcus faecalis in vitro in chick medium (solid lines), and during development of the blow fly, C. vicina (broken lines), starting with the same inoculum. Figures at bottom indicate level of significance between median populations of a bacteria in two successive fly stages.*

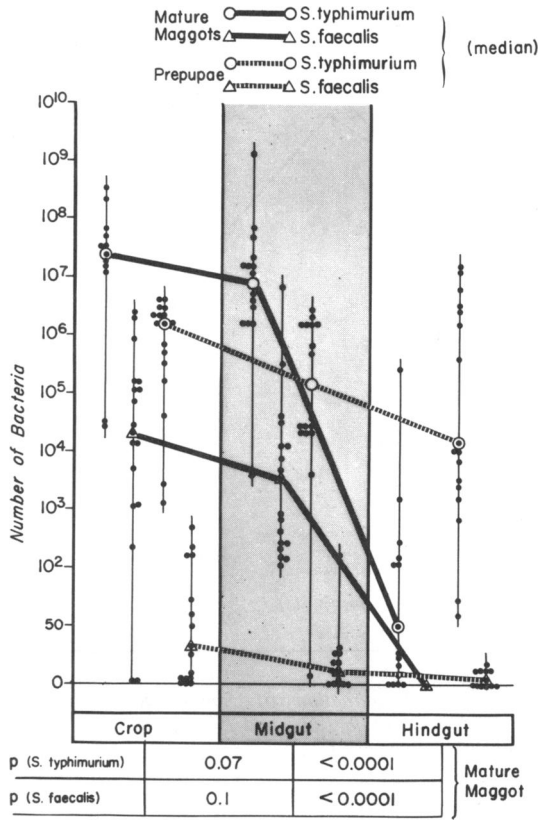

FIG. 2. *Census of Salmonella typhimurium and Streptococcus faecalis in three gut regions of dibiotic blowfly maggots and prepupae.*

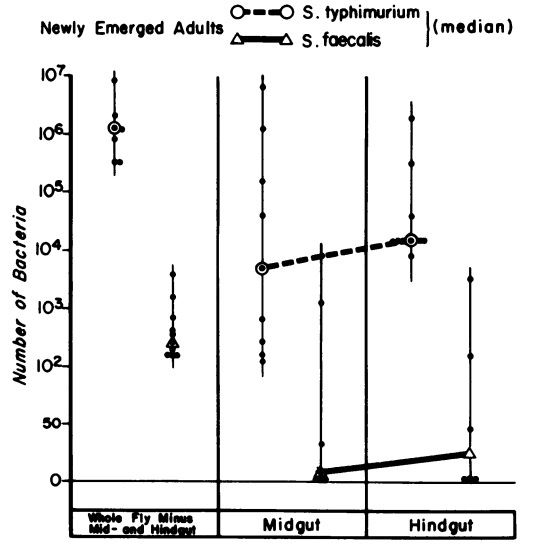

FIG. 3. *Levels and distribution of Salmonella typhimurium and Streptococcus faecalis in newly emerged dibiotic blow flies.*

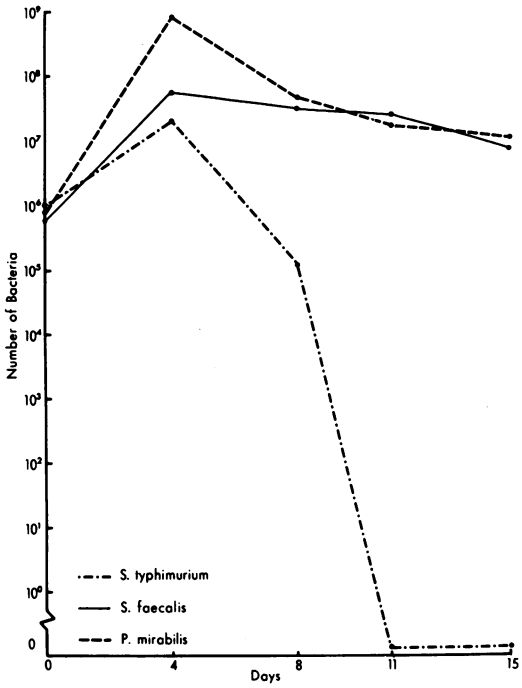

FIG. 4. *Interaction of Salmonella typhimurium, Streptococcus faecalis, and Proteus mirabilis in static broth culture (37 C).*

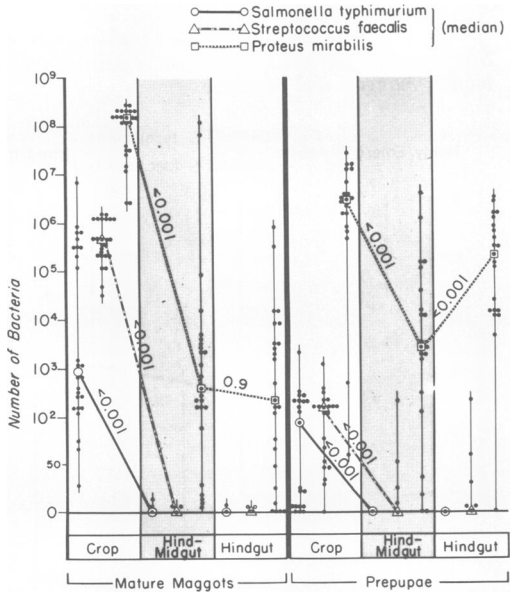

FIG. 5. *Survival of Salmonella typhimurium, Streptococcus faecalis, and Proteus mirabilis in three gut regions of tribiotic blowfly maggots and prepupae. Significance figures between successive medians are placed on interconnecting lines.*

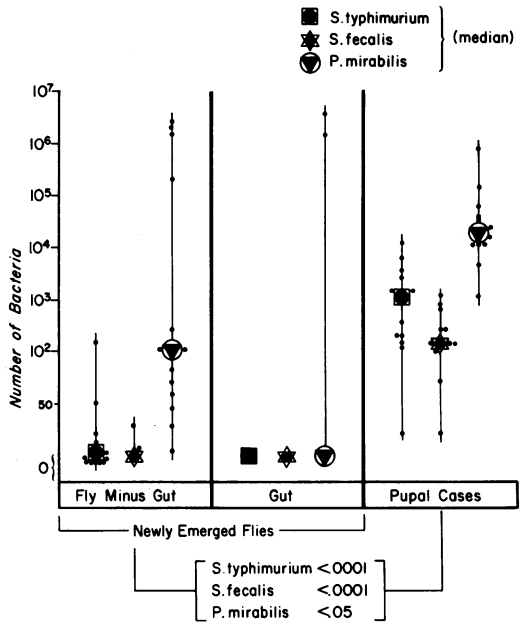

FIG. 6. *Levels and distribution of Salmonella typhimurium, Streptococcus faecalis, and Proteus mirabilis in newly emerged tribiotic blow flies.*

TABLE 1. *Distribution of Proteus mirabilis in 3-day-old blow flies gnotobiotically reared with Salmonella typhimurium, Streptococcus faecalis, and P. mirabilis*

Fly specimen	Crop	Fore- and mid-midgut	Hind-midgut	Hindgut	Fly minus gut
1	<1	<1	<1	<1	<1
2	<1	<1	<1	<1	<1
3	2×10^6	4×10^4	1×10^5	2×10^6	4×10^5
4	2×10^6	7×10^4	4×10^3	3×10^5	2×10^5
5	5×10^6	3×10^4	9×10^2	2×10^6	2×10^5 [a]
6	<1	<1	<1	<1	<1
7	8×10^6	1×10^5	4×10^2	4×10^4	2×10^3
8	<1	<1	<1	<1	<1

[a] A total of 112 *Streptococcus faecalis* was recovered here but none was recovered from the gut of this fly, nor from any other fly.

is a close correspondence in the bacterial census between the crop and the larval medium. This is to be expected since the crop is a storage organ with neither distinctive *p*H value nor digestive function of its own. Illustrations of such a nonselective input are found in the *S. typhimurium-Streptococcus faecalis* interaction (Fig. 1) and again when *P. mirabilis* is added to the other two (Fig. 5). The 100-fold difference between chick and crop counts (Fig. 1) disappears when one adjusts the chick sample of 1 g

or 1 ml to the volume of a fully engorged crop which is about 10 μliters.

The nature of the maggot and its medium enable us to monitor, but not control, input. Different densities of bacterial input were achieved only when the number of interacting species was altered, with highest densities occurring under monobiotic conditions. This type of manipulation is obviously unsatisfactory because it introduces additional variables. At best, conclusions based on density phenomena are tentative and will require re-examination when a means has been found to control how much a maggot ingests, a parameter easily controlled in the adult fly.

Under monobiotic conditions, equal-size populations of *Salmonella enteritidis* and *P. mirabilis* suffered a millionfold decrease of viable cells while passing through the acid midgut, indicating that the two species are equally sensitive to the bactericidal activity encountered here (3). Under dibiotic and tribiotic conditions, *S. typhimurium*, *Streptococcus faecalis*, and *Pseudomonas aeruginosa* suffered similar losses, suggesting that they, too, are equally susceptible. The data also suggest that a population must exceed $10^{5.5}$ organisms to pass through the midgut and implant in the hindgut. Survival data on *Proteus mirabilis* under monobiotic and two different tribiotic conditions show a direct relationship between population density and midgut kill as follows: 500 million killed at $10^{8.5}$, 100 million killed at 10^{8}, and 20 million killed at $10^{7.2}$. This destruction is rapid, probably taking a few minutes or less (4).

Flow rate in the gut of a maggot which is actively feeding at 23 C is 1 to 2 mm/min. It is roughly three times faster in an adult blow fly, and faster still in the house fly, in which protozoon cysts pass through the gut in as little as 5 min (20). Nevertheless, microbes are able to establish and multiply in the adult house fly's gut despite the more rapid flow (7). In fact, volume of flow may be more important than rate of flow as a condition for implantation. One need only compare the dissected guts of a maggot and an adult fly to observe the relatively enormous gut volume of the maggot. This gut volume is maintained as a result of continuous imbibition of liquid food during the first 5 days of maggot life. The term sweep-out rate is used here to connote the combined effect of both volume and rate of flow. A population will tend to be constant in a favorable region of gut if its doubling time equals sweep-out rate. This is apparently the case in the hindgut of the maggot where none of the bacterial populations showed significant gain or loss during the stage of active feeding.

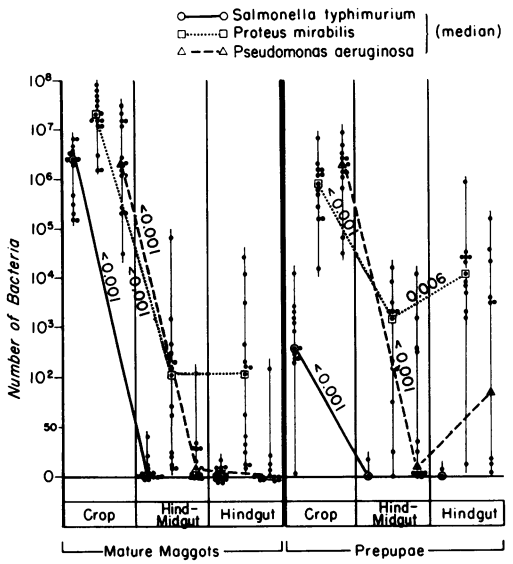

FIG. 7. *Survival of Salmonella typhimurium, Proteus mirabilis, and Pseudomonas aeruginosa in three gut regions of tribiotic blowfly maggots and prepupae.*

The prepupal gut is a static culture inasmuch as input and output cease and microbes are forced to exploit shrinking resources. By the end of the 3-day fast, crop volume has shrunk about 100-fold; the corresponding decrease in its flora may be density-related and nonspecific. Thus, median populations of *P. mirabilis* are reduced from $10^{8.2}$ to $10^{6.5}$, *S. faecalis* from $10^{5.7}$ to 10^{2}; *Salmonella typhimurium*, with lowest density, diminishes least (Fig. 5). With gut flow markedly diminished, doubling time exceeds sweep-out rate and multiplication of *P. mirabilis* (<0.001, Fig. 5; 0.006, Fig. 7) and *S. typhimurium* (<0.001; Fig. 2), and possibly also *Pseudomonas aeruginosa* occurs in the hindgut. Multiplication may occur from inocula possibly as low as 25 organisms (Fig. 2, 5, 6) and may depend on pockets of nutrient sequestered in folds of hindgut epithelium and also on substances released from dead bacteria. Under in vitro starvation conditions, a declining population may rebound as much as three orders of magnitude by exploiting the resource of dead bacteria (6).

The primary purpose of these studies has been to identify members of the fly's normal microbiota which singly or in combination cause the extinction of *S. typhimurium*. This organism has now been studied in three dibiotic and two tribiotic interactions, both in the fly and in vitro, with results which may be summarized as follows. Dibiotically, *S. typhimurium* was at parity with *Escherichia coli*, dominant over *Streptococcus faecalis*, and dominated by *Proteus mirabilis*.

In none of these interactions was *Salmonella typhimurium* eliminated from prepupae, in contrast to its routine extinction from conventional prepupae. When a third organism was added to the interaction, we observed a synergic suppression of *S. typhimurium*. Thus, none of 10 prepupae in the *S. typhimurium-P. mirabilis* rearing were free of *S. typhimurium* (5), compared with 7 of 21 prepupae in the *S. typhimurium-P. mirabilis-Streptococcus faecalis* rearing. Metamorphosis almost completed the elimination of *Salmonella typhimurium*, only small numbers remaining in a few flies at emergence and none 3 days later (Fig. 6 and Table 1). Although less important medically, biologically it is equally noteworthy that the fate of *Streptococcus faecalis* parallels that of *Salmonella typhimurium*. *P. mirabilis* may also be disadvantaged in a tribiotic interaction, suggested by data which show that with addition of *Streptococcus faecalis* to the *Salmonella typhimurium-P. mirabilis* complex median densities of *P. mirabilis* in teneral adults are reduced from 10^6 to 10^2.

The contents of the colon and caecum of the normal mouse are weakly bactericidal against *S. typhimurium*, and this appears to be due to volatile fatty acids produced by the normal flora (13). This system operates under reduced conditions and may account for elimination of *S. typhimurium* from static broth in the two tribiotic interactions we have described; we are currently testing this possibility. A possible bactericidal role of fatty acids in the maggot gut is less certain if we consider, on the one hand, the good survival of *Streptococcus faecalis* in the same broth from which *Salmonella typhimurium* was eliminated and, on the other hand, the equally poor survival of *Streptococcus faecalis* in the gut. Nevertheless, fatty acids are known to be more bactericidal at low *p*H. It is unlikely that pyocyanin produced by *Pseudomonas aeruginosa* has an important role since *Salmonella typhimurium* was also suppressed in its absence.

Differential growth rates are a likely basis for competitive exclusion of enteric pathogens from a mixed flora, but here we are on strictly empirical ground. Doubling times for some of our test organisms under optimum, pure culture conditions have been reported in minutes as follows: *E. coli*, 17; some *Proteus* species, 21; some *Salmonella* species and *Streptococcus faecalis*, about 25; and *Pseudomonas aeruginosa*, 31 (12). Generation times determined in pure culture, however, have little meaning in mixed culture. For example, a BHI broth of *Proteus mirabilis* kept at 37 C for 24 hr had twice the numbers of a corresponding broth of *Salmonella typhimurium*, yet the difference was 13-fold when the organisms were grown together. Despite its shorter doubling time, *E. coli* did not exceed *S. typhimurium* when the two were grown together in chick-maggot medium, and their coequal populations persisted into the adult stage. *Pseudomonas aeruginosa*, with a slower doubling time, outgrew *S. typhimurium* in vivo and in vitro, whereas *Streptococcus faecalis*, with the same doubling time, was suppressed by *Salmonella typhimurium*. A population of faster-growing *Proteus vulgaris*, in mixed continuous culture with *Saccharomyces cerevisiae*, was made smaller than the latter by limiting niacinamide (16).

We should not neglect, in our consideration of biotic effects on population size of bacteria, the possible influence of blow fly maggots themselves, which alkalinize, aerate, liquefy, and, finally, consume both the medium and its flora. Whatever the physicochemical factors leading to supremacy or extinction of an exogenous microbial population, survival in the gut is favored by a large initial input. The maggot tract is a generally unfavorable microbial environment in which sweep-out rate, a midgut bactericide, starvation, and, finally, ecdysis, take their successive toll. Competitive inhibition of *Salmonella* in the breeding medium prevents buildup of the densities needed to overcome these maggot factors. The result is a newly emerged fly with little or no burden of pathogenic organisms.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-03498 from the National Institute of Allergy and Infectious Diseases.

We acknowledge the technical assistance of Robert Norton and Joseph Chiu.

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