THE MOUSE INTESTINAL MICROFLORA WITH EMPHASIS ON THE STRICT ANAEROBES*

By ADRIAN LEE,‡ PH.D., JAMES GORDON, M.D., CHI-JEN LEE, SC.D., AND RENÉ DUBOS, PH.D.

(From The Rockefeller University, New York 10021)

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The mammalian fetus is essentially free of cultivable microorganisms at the time of birth, as shown by the regularity with which so-called germfree animals can be obtained by cesarian section. However, several bacterial species become established in the gastrointestinal tract immediately after birth. We have reported earlier that the various species of the microflora colonize the different parts of the gastrointestinal tract according to a definite time sequence and with a highly selective anatomical localization (1–5).

One of the limitations of our earlier studies was the failure, for lack of adequate cultural techniques, to enumerate quantitatively the strict anaerobes. As shown in references 6 and 7, this limitation has been partly overcome by the use of exacting anaerobic conditions in all phases of the bacteriological studies, including the collection of specimens. Some of the strict anaerobic species isolated from the mammalian intestine have been tentatively classified (7–9). They include a variety of bacillary forms that will be referred to in the present paper as fusiform or tapered rods to indicate their gross cellular morphology, and also unidentified anaerobic spirochete-like organisms.

The object of the present study is to define (*a*) the time and conditions of emergence of the strict anaerobes in the large intestine and cecum, and (*b*) the ecological interrelationships between these organisms and the other members of the intestinal microflora.

Materials and Methods

All experiments were carried out with specific-pathogen–free mice of the COBS-CD-1 strain produced by Charles River Breeding Laboratories, Inc., North Wilmington, Mass. For studies on the sequential development of the intestinal flora, female COBS-CD-1 mice were obtained when 2 wk pregnant, and were from then on maintained in our laboratory. The COBS-CD-1 strain is a specific-pathogen–free line with a relatively stable and well defined

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‡ Present Address: University of New South Wales, Kensington, New South Wales, Australia.

intestinal flora. Details regarding the derivation and maintenance of this strain are available in reference 7.

After arrival in our laboratory, the mice were housed in plastic boxes fitted with Isocaps (Isocages from Lab Cages, Inc., Kennett Square, Pa.) with wood shavings for bedding. Pregnant mice were housed individually. They were fed a diet of D & G pellets (Dietrich and Gambrill, Frederick, Md.) and either acid-water (10) or tap water ad lib. The dates of birth of the litters were noted and any litters of more than 10 were adjusted to that number. Weaning took place on day 21.

The so-called "milk diet" used in certain experiments consisted exclusively of powdered whole milk (Klim, The Borden Company, New York) compacted into pellets; it was fed to the mother as the sole source of food, along with water ad lib., from the time of parturition to the time of weaning, 21 days after birth.

The animals were killed under chloroform or ether anesthesia at times indicated in the tables or figures; the preparation of the specimens from the intestinal tract was carried out as described in earlier references. The various bacterial species were enumerated on selective agar media as described in references 1 and 7.

In some experiments, the numbers of strict anaerobes were evaluated by microscopic examination of stained preparations. Films of the cecal homogenates were prepared as for a blood smear; they were fixed by heat and stained by the Gram technique. So as to eliminate subjective error, a rigid "blind" system was adhered to. Large numbers of slides (300) from mice at all ages studied were identified by numbers, mixed, and counted by the investigator over a period of days. When all slides had been assayed, the results were computed and the slides decoded. Unbiased results from each individual litter could then be examined. At least five high power microscope fields were counted for each specimen (1000 organisms).

RESULTS

(*a*) *Colonization Pattern of the Mouse Intestine by the Microflora.*—Our earlier studies of the intestinal flora were carried out chiefly with NCS mice. More recent studies with COBS mice have given essentially the same results which need not be described here in detail, except as they relate to the strict anaerobic flora.

Table I gives the numbers of organisms recovered from the cecal homogenate of COBS mice by quantitative enumeration on selective agar media. Results for the first 6 days of life are not presented in the table since they correspond closely to those reported earlier and since no anaerobic fusiform-tapered rods or spirochetes could be seen by microscopic examination, or recovered on plates, before the 10th day of life.

The various bacterial populations, expressed in numbers of colonies recovered per gram of cecal homogenate, exhibited the following time sequence: Lactobacilli were present by day 2, increased in numbers over the next 7 days, reached a peak level of 10^9, and maintained that level throughout the balance of the experiment (day 32); enterococci were present at day 2, increased in numbers over the next 10–12 days, reached a peak of 10^8, and then decreased over the next 4 days to a level of 10^4 which was maintained through day 32; coliform bacilli appeared on about day 10, rose over the next 6 days to a peak of 10^8, and then declined to a level of 10^5 over the next 4 days.

Bacteroides appeared on day 14 and quickly rose to a level of 10^{10} which

persisted through day 32. The tapered anaerobes began to appear on the plates about the same time as the bacteroides; microscopic examination of cecal suspensions revealed that they increased from approximately 10^4 on day 11–13, to a peak of 10^{11} within 4 additional days. From then on they remained the dominant group to the end of the experiment on day 32. Quantitative bacteriological determinations in other experiments have revealed that the populations of bacteroides and of strict anaerobic fusiform-tapered rods persist at extremely high levels (10^{10} and 10^{11} respectively) throughout the whole life span of the mouse, at least under conditions of health and with adequate nutrition.

The different species of tapered anaerobes appeared in fairly regular sequence. One type that could be recognized with consistency was a medium-sized rod

TABLE I

Development of the Cecal Flora of COBS Mice

	Cultivable organisms* at indicated age (in days)									
	8	10	12	14	16	18	20	24	28	32
Enterococci	6*	7	8	8	7	5	5	4	4	4
Lactobacilli	8	9	9	9	9	9	9	9	9	9
Coliforms	0	3	4	7	8	7	6	6	6	5
Bacteroides	0	0	0	9	10	10	10	10	10	10
Tapered anaerobes	0	0	0	7	10	11	11	11	11	11
Spirochete-like organisms	—‡	—	—	+	+	+	+	+	+	+

* Log_{10} of numbers of colonies recovered per gram of cecal homogenate on suitable agar media.

‡ Rated as + if detectable microscopically in cecal homogenate.

with flagella. This organism usually was detectable by microscopic examination of cecal suspensions some 2–4 days after the appearance of small and medium-sized nonflagellated tapered rods.

The spirochete-like organisms also were among the strict anaerobes that appeared around day 12–14. Their appearance coincided closely with that of the tapered anaerobes and bacteroides; they reached their maximum concentration over a period of 8–10 days.

The strict anaerobes thus constituted by far the most numerous bacterial populations in the cecum, whether enumerated by microscopic or cultural techniques. Microscopic examination suggested indeed that their preponderance was even more marked than is indicated in Table I, a fact readily explained by the great difficulty experienced in recovering them quantitatively on agar media.

As will be emphasized repeatedly in this paper, the populations of coliform bacilli and of enterococci decreased sharply at the time when the populations of strict anaerobes reached their maximum levels.

(*b*) *Colonization of the Intestine by Strict Anaerobes.*—Numerous experiments

with germfree mice, maintained under a great variety of conditions, and contaminated with various components of the normal intestinal flora, have shown convincingly that the strict anaerobes are not present in the fetus before birth.[1] Study of the origin of the fusiform-tapered rods was facilitated by the fact that addition of vancomycin to the drinking water of adult mice reduces their population below detectable levels, i.e., to less than 10^4 organisms per gram of cecal homogenate.

In order to test the hypothesis that the tapered rods were derived from the mother at birth, vancomycin was added to the drinking water of pregnant COBS mice in a concentration of 50 mg:200 cc water. Administration of this drug began either 1 wk before birth of the litter, on the day of birth, 5 days after birth, or 10 days after birth. Once begun, administration of the drug was continued until weaning. To prevent any direct drinking of water containing vancomycin by the young, the young were separated from their mother after day 16 of life during daytime and allowed to drink tap water without the drug; at night, the mother was returned to the litter and all water removed.

TABLE II

Effect of Vancomycin in Mother's Drinking Water on the Development of Cecal Flora of Young Mice

Vancomycin	Group	Day* of first appearance of tapered rods	Last day* with $>10^7$ coliforms/gram homogenate
Controls	A	13	18
1 week before birth	B	23	>30
Day of birth	C	16	24
Day 5	D	14	22
Day 10	E	13	19

* Average for group of five mice.

It was not possible to assess with precision how the drug given the mother affected the different species of tapered anaerobes in the young. As seen in Table II, however, there was a clear effect on the populations of coliform bacilli and of anaerobes considered as a whole.

There were striking differences between mice whose mothers had been on vancomycin for 1 wk before their birth (Group B) and those whose mothers were given the drug on the day of birth (Group C). In Group B, the fusiform-tapered rods did not appear in detectable numbers until an average of 10 days later than in the controls and 7 days later than in Group C. Indeed, one litter in Group B did not acquire these organisms until 9 days after weaning and one did not acquire them at all. Even allowing for a possible effect of the vancomycin received by the babies from the mother's milk, the disparity in the

[1] Schaedler, R. W. Unpublished observations.

intestinal microflora between Groups B and C shows that the composition of the microflora of the offspring is profoundly influenced by that of the mother at the time of birth. When the administration of vancomycin was delayed until the day of birth or until day 5 or 10 of life, it had little noticeable effect on the appearance of the fusiform-tapered anaerobes in the cecum.

Administration of vancomycin also greatly retarded the establishment by the fusiform-tapered rods of their numerical superiority over other morphological types of bacteria in the cecum. The alterations were even more significant than what was indicated by microscopic examination because certain species of anaerobic rods present in the normal mouse did not appear at all in the drug group, while others not usually seen reached high concentrations. With the experimental procedures used, the latter phenomenon could result in a normal count of fusiform-tapered rods as judged by microscopic examination, even though a major qualitative change had occurred in the microflora. Two examples of such abnormalities were clearly recognized. The medium-sized tapered rod with flagella appeared by day 16 in all controls, but in Group B this organism appeared on day 20 in one litter, day 32 in another, and not at all in three other litters. The high count of tapered rods on the slides and plates for two litters in Group B turned out to be due to a short sporeformer that was present in a concentration of 10^{10} per gram of homogenate, although this particular organism had never been cultured from any of over 200 mice studied in this laboratory. The administration of vancomycin had no discernible effect on the time of appearance or the number of anaerobic spirochete-like organisms.

(*c*) *The Effect of Strict Anaerobes on the Fate of Coliform Bacilli in the Intestine.*—Earlier publications from this laboratory have provided suggestive evidence that the strict anaerobes of the mouse intestine play an important role in the physiology of this organ and furthermore control the populations of other members of the microflora. When lactobacilli, enterococci, and bacteroides are introduced into germfree mice, they multiply extensively throughout the intestinal tract, but do not correct the large cecal size characteristic of the germfree state. Cecal enlargement was corrected only in part by contamination with coliform bacilli even though these organisms achieve very large populations in germfree mice (11). In contrast, cecal size returned to normal and the populations of coliform bacilli and enterococci rapidly fell to very low levels, when germfree mice which had been infected with the latter two bacterial species were contaminated with the cecal contents of normal specific-pathogen-free mice.[1] Microscopic and histological studies have shown that these profound and rapid changes (reduction of cecum size and spectacular fall in numbers of coliform bacilli and enterococci) were associated with the establishment of a very large population of fusiform-tapered rods which existed in intimate association with the mucosal layer of the cecum and the large intestine.

The findings reported in the preceding paragraph are compatible with results obtained by adding vancomycin to the drinking water of animals, as described in section (*b*) of the present paper.

As seen in Table II, the animals of Group B, the mothers of which had received vancomycin in their drinking water from 1 wk before parturition until weaning time, exhibited an abnormal behavior with regard to coliform bacilli. In all of them, the coliform population remained at levels of 10^8–10^9 bacilli per gram of cecal homogenate for at least 8 days after weaning, instead of decreasing dramatically on day 16–20 of life as usual. The coliform population also persisted at high levels for an unusually long period of time in animals of Groups C and D, but not as long as in Group B (Table II). In contrast, the coliform population in the animals of Group E followed a course similar to that in the control group. In brief, the greater the delay in the appearance of the anaerobic tapered rods as a result of vancomycin treatment, the longer the coliform bacilli persisted at a high level in the large intestine.

(*d*) *Effect of Diet on the Ecology of the Intestinal Microflora.*—The dramatic changes that occur in the intestinal microflora of mice around day 12–16 after birth coincide with the beginning of intake of solid food. At that time, the young animal progressively changes its diet from 100% maternal milk to a mixture of milk and whatever kind of acceptable solid food is available in the cage. In the following experiment, an attempt was made to follow the evolution of the intestinal flora in mice maintained on a diet consisting exclusively of whole milk.

Whole milk (compacted in the form of pellets) was fed to the mother as exclusive source of food from the time of parturition until weaning of the young, 21 days after birth. The intestinal fusiform population was followed by microscopic assay, and the other bacterial species were enumerated by cultural methods.

As the purpose of the experiment was to study the relationship of fusiform-tapered rods to the other components of the microflora, an attempt was made to determine the percentage of the former organisms in the total bacterial population, rather than their absolute numbers. The fusiform population was assayed by the microscopic technique described in Materials and Methods; its size relative to the total microbial population of the intestine is represented in Figs. 1 and 2.

The mice readily accepted the milk diet, and the weight gains of their young were not significantly different from those of the control animals fed D&G pellets. The numbers of lactobacilli and bacteroides were also identical in the two groups of animals. In contrast, the two groups differed profoundly with regard to the numbers of fusiform and coliform bacilli in their intestinal flora.

In the control animals (Fig. 1) the numbers of coliform bacilli began to fall as usual at the time that the fusiform population reached its highest level, around the 16th day of life. The findings were strikingly different in the milk

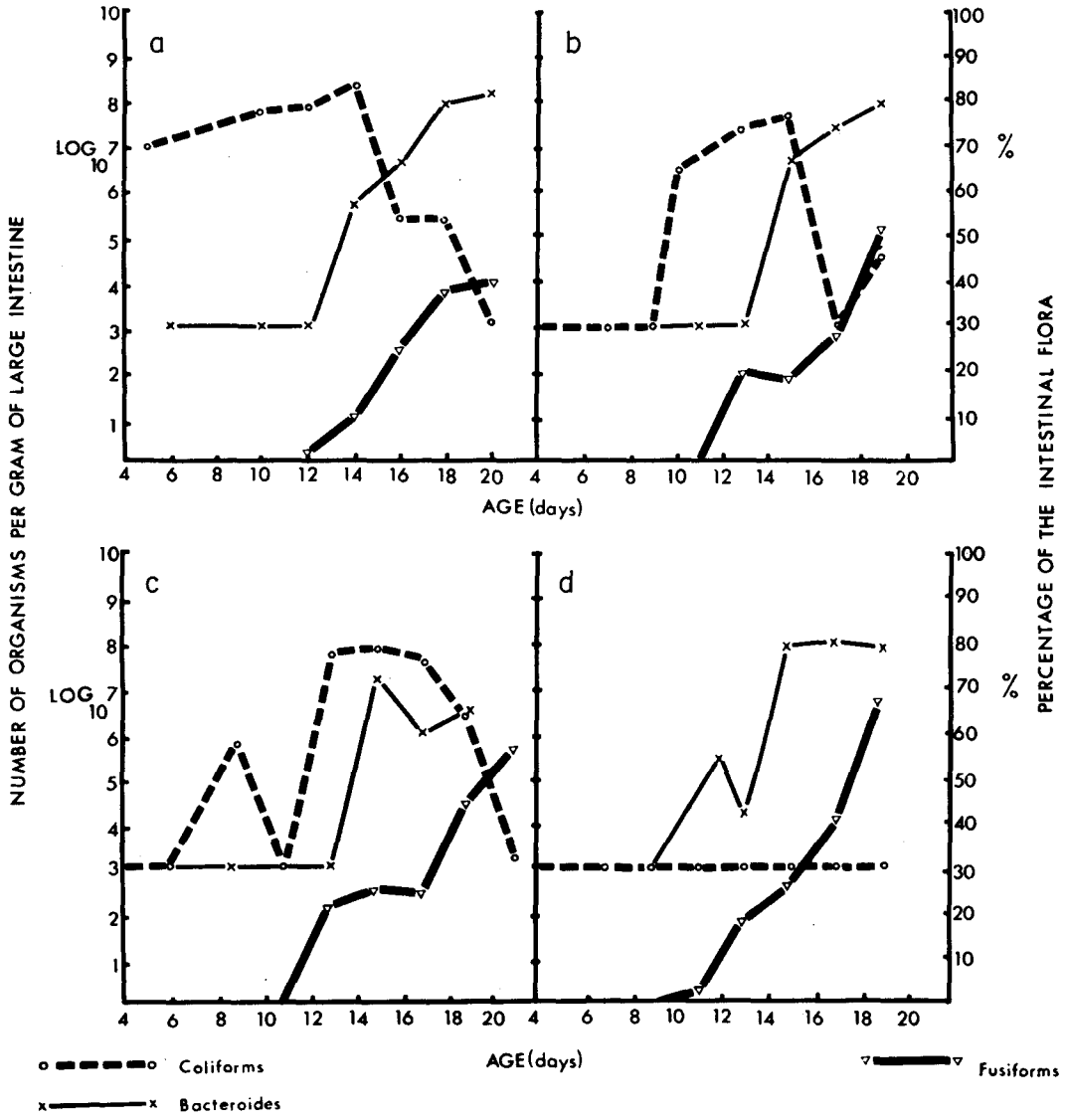

FIG. 1. The curves for a, b, c, and d illustrate the development of the bacterial flora in the large intestines of four individual mice on a normal diet (D&G pellets). The levels of coliform bacilli and bacteroides, determined by cultural methods, are shown as the numbers of organisms per gram of intestinal content. The fusiform populations are expressed as percentages of the total intestinal flora, on the basis of microscopic examinations.

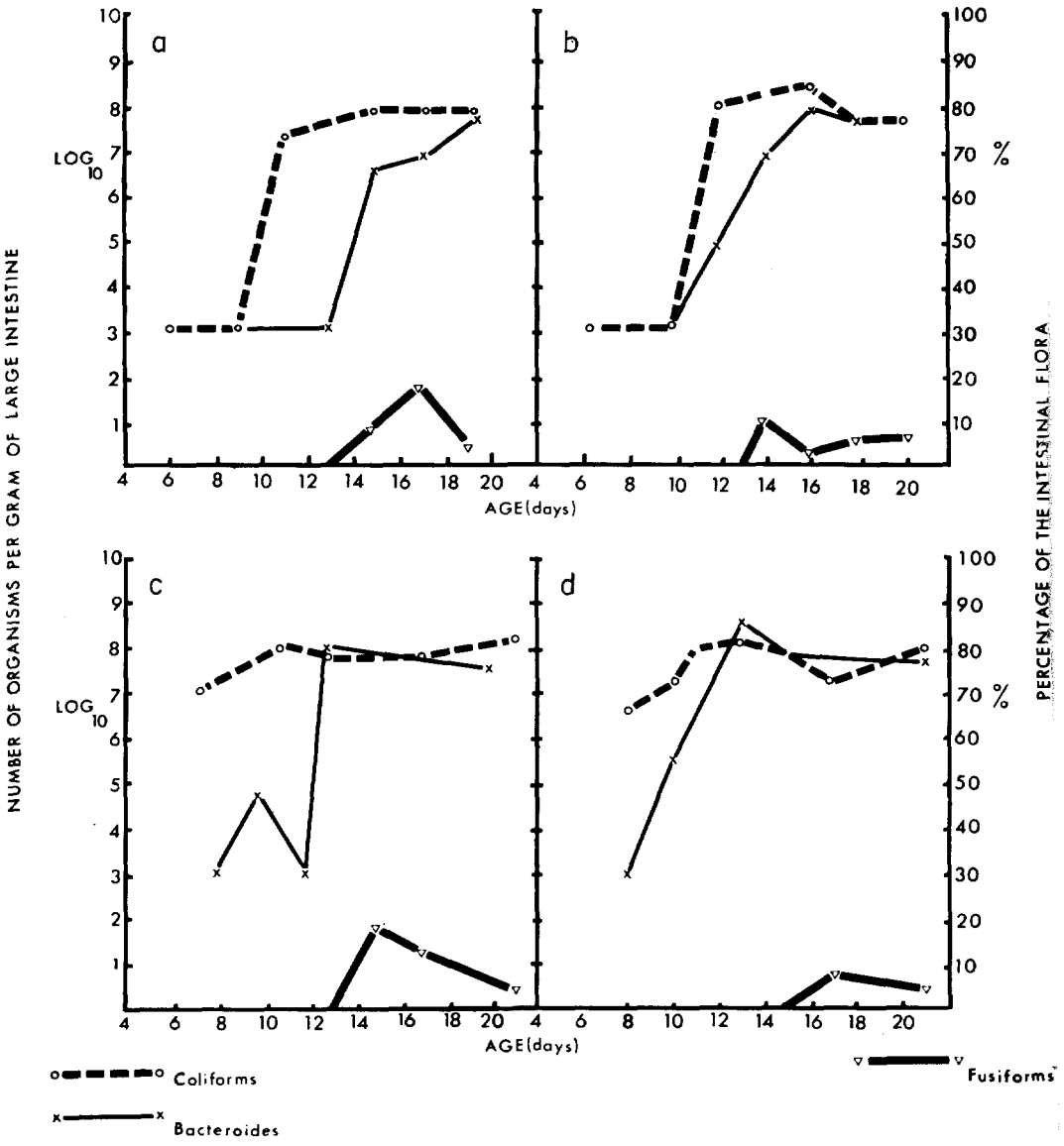

FIG. 2. The curves for a, b, c, and d illustrate the development of the bacterial flora in the large intestines of four individual mice on a whole milk diet. The levels of coliform bacilli and bacteroides, determined by cultural methods, are shown as the numbers of organisms per gram of intestinal content. The fusiform populations are expressed as percentages of the total intestinal flora, on the basis of microscopic examinations.

diet group (Fig. 2). In these animals, the coliform population persisted at a high level throughout the period of observation. As to the fusiform population, it remained extremely low until the end of the experiment in the milk diet group, to such an extent indeed that organisms of this group could not be detected visually in the cecal material of some of the young animals at weaning. Comparison of the results illustrated in Figs. 1 and 2, therefore, suggests again that some components of the fusiform population play an important role in controlling the levels of coliform bacilli in the mouse intestine.

(*e*) *Viral Infection and the Ecology of the Intestinal Microflora.*—Accidentally, in the course of an experiment, some of the mice in one of the animal rooms became victims of a disease (viral?) that caused diarrhea, poor weight gain, and in a few cases, death. Only very young mice were affected.

Although most of the animals in that room continued to gain weight normally, with little or no diarrhea, their intestinal microflora was somewhat

TABLE III

Intestinal Flora of 4 Baby Mice Suffering from Diarrheal Disease

Mouse	Weight gain	Coliform bacilli*	Bacteroides*	Tapered anaerobes*
	(*gm*)			
1‡	14.7	10^7	10^9	10^8
2‡	13.0	10^5	10^9	10^9
3§	7.1	10^9	10^{10}	10^7
4§	6.2	10^9	10^{10}	$\simeq 10^5$

* Log_{10} of number of colonies per gram of cecal homogenate.

‡ Mildly ill mice.

§ Severely ill mice.

abnormal. The levels of coliform bacilli, instead of falling as usual around day 16 of life, remained extremely high; there were still 10^8–10^{10} organisms of this group per gram of cecal homogenate on day 29 of life. However, no gross abnormality could be ascertained with regard to the numbers or colonial appearance of lactobacilli, bacteroides, or strict anaerobes.

Among the animals in which the accidental infection was more severe, as expressed by high mortality rates and poor weight gain of the survivors, the intestinal flora was more abnormal. In the survivors, the population of anaerobic tapered rods was decreased by more than a millionfold and it contained fewer morphologic types than usual; furthermore, the coliform population remained at high levels for long periods of time (Table III). These very sick animals exhibited therefore a clear correlation between low fusiform and high coliform populations.

In the following experiment, an attempt was made to study under controlled

conditions the effect of a known viral infection (12, 13) on the intestinal bacterial flora.

COBS mice, 2 days old, were contaminated orally with the enterovirus preparation described in reference 13. Others were kept as controls. In all groups, the litter size was reduced to eight. Animals were killed under ether anesthesia at various ages; the large intestine with cecum was removed and homogenized in 5 ml of charcoal water. The fusiform population was estimated by microscopic study of the homogenate; other bacterial species were enumerated on selective culture media.

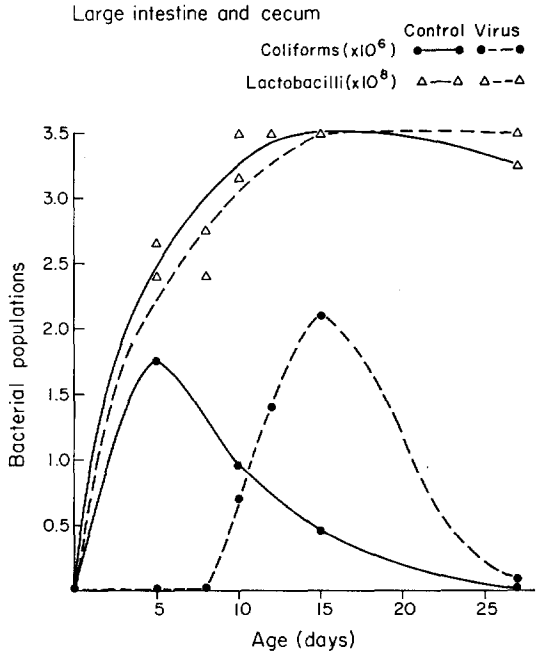

FIG. 3. Intestinal flora in large intestine and cecum of mice contaminated neonatally with enterovirus. Altogether 28 control and 35 contaminated males were used. Groups of 4 controls and 5 contaminated mice were sacrificed at 5, 8, 10, 12, 15, and 27 days of age. Large intestine with cecum was removed and homogenized in 5 ml of sterile charcoal water.

Neonatal infection with the enterovirus did not affect the development of the lactobacillus population, but retarded by approximately 3 days the appearance of coliform bacilli and also of the anaerobic tapered rods (Fig. 3). In this case, therefore, there was no evidence of an ecological interplay between fusiform and coliform populations. It appeared rather that neonatal infection with this particular enterovirus exerted an inhibitory effect on the multiplication of several bacterial species. Some evidence for this hypothesis was derived from the following experiment.

Control mice and mice neonatally infected with the enterovirus received 0.05 ml of broth culture of *Escherichia coli* by the oral route on day 10 of life, i.e., 8 days after the administration of virus. The animals were killed at ½ hr intervals after the bacterial infection and the numbers of coliform bacilli in the intestine were determined by cultural techniques (Fig. 4).

As seen in Fig. 4, prior infection with the enterovirus retarded significantly the multiplication of coliform bacilli in the intestine. This finding was confirmed by histological examinations.

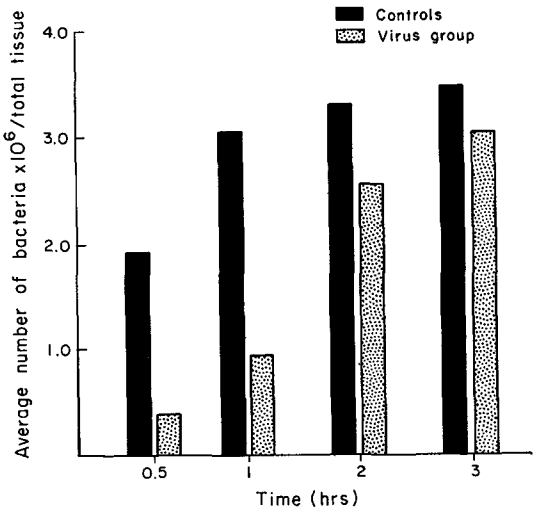

FIG. 4. Multiplication of *E. coli* in small intestine of mice contaminated neonatally with enterovirus. See text.

DISCUSSION

Microscopic examination of the mouse intestine, or of its contents, makes it clear that the immense majority of the bacterial population in normal healthy animals consists of Gram-variable rods with pointed ends. These organisms have been designated in the present paper fusiform bacilli or tapered rods. They are extremely abundant in the lumen, and even more in the layer lining the mucosa. Indeed, it is often impossible to see any other groups of bacteria in the crypts between the villi. The preponderance of this bacterial population makes it likely that its components play an important role in intestinal ecology.

The development of exacting anaerobic techniques has recently made it possible to cultivate on agar these fusiform-tapered rods. In the mouse as in man this group is made up of several different species and it constitutes 90% or more of the total cultivable bacteria of the intestine. Since several of the

bacterial types seen under the microscope have not yet been cultivated quantitatively, the strict anaerobic flora (including the anaerobic spirochetes) probably constitutes a percentage of the total viable flora much larger than 90%.

The strict anaerobes become the predominant constituents of the mouse intestinal flora only around day 16 of life, whereas other bacterial species reach their maximum level much earlier. But once they are established, the strict anaerobes remain at a constant high level in the large intestine and cecum (at least 10^{11} viable organisms per gram of tissue) throughout the life span of the animal. They seem to be parts of the autochthonous flora, as defined in reference 2.

There may be significance in the fact that the anaerobic tapered rods and spirochetes characteristically inhabit the mucous layer of the large intestine and cecum (3, 14) where they seem to constitute a barrier between the outside world and the intestinal mucosa. It has been suggested in earlier publications from this laboratory that at least some of these strict anaerobes play an essential and perhaps indispensable role in the maintenance of a healthy physiological structure of the intestine; this is indicated by the fact that they are uniquely effective in correcting cecal enlargement in germfree animals. The anaerobic fusiform-tapered rods also seem to interfere with the growth or survival of several other bacterial species in the intestine. In very young mice, for example, the populations of coliform bacilli and enterococci persist at extremely high levels until approximately the 16th day of life, but they fall precipitously as the population of strict anaerobes reach their maximum. In contrast, several situations that result in a drastic decrease in the numbers of the strict anaerobes have been found to be associated with an administration of the drug vancomycin, use of an abnormal diet, or an infection of presumably viral nature.

Under natural conditions the young animal almost certainly acquires the fusiform-tapered anaerobes from its mother. Evidence for this is found in the fact that treatment of the mother with vancomycin before parturition greatly delays or prevents the process of colonization of the intestine by strict anaerobes in her young. However, there must be other sources of these organisms since germfree mice usually acquire them rapidly after being removed from the germfree environment.

Although anaerobic organisms of the bacteroides, spirochete, and fusiform-tapered groups are certainly present in the intestine almost from the time of birth, they multiply to large populations only 14–16 days later. The factors that eventually trigger their multiplication have not been studied, and only hypotheses can be formulated in this regard. One may assume, for example, that anaerobes cannot multiply until highly reducing conditions have been established at the level of the intestinal mucosa by other microbial species. Since the appearance of the strict anaerobic flora coincides with the time when the animal begins to ingest solid particles of food, it is also possible that special metabolites are required for the growth of the very exacting anaerobic flora.

Complete understanding of intestinal ecology will probably require the contamination of germfree animals, on a diet of known composition, with pure cultures of the organisms which constitute the autochthonous flora, including the strict anaerobes considered in this and earlier publications (3, 7–9, 14). But incomplete as it is, the available knowledge seems to warrant the conclusion that strict anaerobes of the fusiform-tapered rod group exert profound effects on the anatomic structures of the mouse intestine, its physiological functions, and its microflora.

SUMMARY

The various components of the intestinal microflora in the mouse become established according to a definite time sequence; the strict anaerobes are the last groups of bacteria to reach their maximum population levels, 14–16 days after birth.

The multiplication of these strict anaerobes in the mouse intestine seems to depend upon the prior multiplication of other bacterial species, and coincides with the ingestion of food other than maternal milk. These two conditioning factors may correspond to the establishment of a suitably reduced Eh potential and to the provision of certain metabolites.

Once established, the strict anaerobes constitute by far the largest percentage of the total intestinal microflora; most of them are associated in a viable form with the mucosa. In normal animals they persist at very high levels throughout the life span. However, their populations can be drastically reduced by dietary manipulation of the animal, by administration of vancomycin, or by certain disease processes of the intestine.

The strict anaerobic bacteria seem to play an important, and perhaps essential role in the maintenance of the anatomic structures and physiological functions of the intestine. They also seem to hold in check several species of intestinal bacteria, in particular the coliform bacilli.

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