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The Gnotobiotic Animal as a Tool in the Study of Host Microbial Relationships

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INTRODUCTION

Gnotobiotic animals, living either in the absence of or in association with known viable heterologous agents, represent an extension of the microbiologist's pure culture concept to all biological forms. The gnotobiotic experiment offers considerable potential as a tool in the study of host microbial relationships because (i) it portrays the host either when free from germs and left to its own resources or when modified by known microbial or other associates, (ii) it permits the study of interflora relationships within the host organism, and (iii) it may be used in the study of any external or endogenous factor (e.g., in nutrition, immune reactions, responses

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to various forms of injury) where the pure actions of such factors, affected or unaffected by associates in the host, are of interest. In the latter sense, the gnotobiotic experiment represents microbiological standardization of the animal stock in the same fashion as it is commonly practiced in terms of animal strain, diet, housing, and various conditions of the physical environment.

Historically, the concept of gnotobiotic experimentation is credited to Pasteur's efforts in 1885 (223). This is unquestionably correct in the microbial sense of our definition. However, the recognition of the need to work with pure systems in biological experimentation, from which Pasteur appears to have drawn his analogy [quoted by Ducluzeau (71)], can be traced further back to the experiments of Boussingault (33) on nitrogen fixation of plants grown in a "sterile" soil which were carried out during the first half of the past century. Various reviews on gnotobiotic experimentation (e.g., 164, 178) have commented in detail on Pasteur's keynotes of our subject. It is less well known that Schottelius, a pioneer in the area of gnotobiotics who in his younger years spent some time in Pasteur's laboratory, has in one of his papers (290) expanded the master's scanty published remarks. In addition to outlining explicitly the actual gnotobiotic experiment, Pasteur speculated that on elimination of microbial associates, as is the case in the germ-free experiment, life of the animal host would become impossible. Apparently, he based this idea on the concept of the "survival of the fittest" in evolution. Following this principle, he assumed that in the course of phylogenesis microbial associates have become synergists which are indispensable in the life of the host. At the same time, the opposite view was held by Nencki (209) and later Metchnikoff (190, 191), who considered microbes to be antagonists to the well being of the host. Definite proof that normal life of higher organisms is possible in the absence of germs was offered first by Reyniers and his co-workers at Lobund Laboratories, University of Notre Dame, in the 1940's, who reared rats (267) and chickens (268) in the germ-free state to successive generations. This event, besides settling unequivocally the original question, made the gnotobiotic animal available as a practical tool for research for the first time. The breeding of germ-free animal colonies (mainly rats and mice) was successfully initiated in the 50's by the groups of Reyniers at the University of Notre Dame and of Gustafsson at the University of Lund.

Since the beginning of experimental work with germ-free animals, certain characteristics and distinct types of response became apparent in this form of life. The underdevelopment of some elements of the cellular and humoral defensive system (*see below*) seemed to be a plausible consequence of the greatly reduced exposure to antigens. By the same token, the germ-free animal's increased resistance to some stressor agents [e.g., X irradiation as reported by Wilson and Piacsek (344)] or the indication of longer life span (*see below*) have been attributed to relief from the conventional microbial burden. In these instances, the contribution of the flora appeared as a complicating or as an outright detrimental factor in the conventional host's life. On the other hand, it could be contended, the presence or absence of the flora can not be too far reaching in its overall effects on the host in view of the normal growth, development, and reproduction which were observed in germ-free animals when compared to their conventional controls. This reassurance came particularly after the conditions of the germ-free experiment could be standardized and a plentiful supply of normally born, mother-suckled, adult germ-free rats and mice became available for investigation. In addition, many morphological and functional details, originating mainly from parts of the body that are not in immediate contact with microbes, proved similarity between germ-free and conventional animals. Finally, there were observations, some of them quite old, which suggested that there is something basically different, or perhaps even wrong, with germ-free animals. Although ultimately caused by the absence of bacteria, these differences could not be readily explained in terms of the lack of microbial stimulation and a resultant tissue underdevelopment, as was the case in the defensive system. There is an expanding group of observations indicative of more or less pronounced departures from conventional standards in structure, chemical composition, and function of various organs in germ-free animals. Some of these differences did not appear to influence particularly the economy of the host, e.g., the ones resulting from the sparing effect of germ-free life on intestinal degradation of digestive enzymes (*see below*). Other changes seemed patently detrimental to the animal's health, e.g., the greatly enlarged cecum and its consequences. These observations have indicated that the animal host left to its own resources is not entirely self-sustained and that the normal microbial flora or some of its components or products are needed for maintenance of the physiological normality of the host. The resemblances to the germ-free state in conventional hosts treated with antibiotics [underdeveloped defensive elements, Gordon et al. (108); cecal anomalies, Meynell (192)]

have shown that the synergistic aspects in the host microbial associate relationship can be disrupted also in conditions of conventional life.

The entire literature of gnotobiotics is incorporated in fewer than 2,000 publications. This makes it a rare field in experimental biology and medicine where all pertinent material can be reviewed in the original text by the interested student. The older references are expressly recommended for perspective, and reviews and summaries have been particularly helpful; they include: Küster (164), Glimstedt (94), Reyniers (261–263), Reyniers and Trexler (266), Gustafsson (118), Phillips and Smith (230), Gordon (96, 98), Miyakawa (200), Luckey (178, 179), Trexler (322, 323), Podoprigora (243), and Pollard (244). The compilation of gnotobiotic literature with its yearly supplements by Teah (312) is a great asset to the reader.

This article is intended to portray both the potential and the limitations of research with gnotobiotic animals. A large portion of this material, representing comparative studies between germ-free and conventional control animals, is about the effects due to the total flora. A smaller number of studies deal with the effects of identified members of the flora on the host organism. The least-studied interactions are those between members of the flora and the modification of the flora by the host organism. We have chosen to select from the literature data that give assurance of the main independent variable of the experiment being the absence or presence of bacteria with no suspected concurrence of inadequate diets, housing, or management in the animal's history. Such conditions are best fulfilled in mammals that originated from established, self-reproducing gnotobiotic colonies. Birds may be included in this group, as with them difficulties arising from cesarean birth and from hand-feeding artificial formulas do not exist. With the present state of the arts, this means the rat, mouse, and chicken. These species, generally speaking, take precedence in this review over other animals that have only recently been reared to a second generation in the germ-free state, e.g., the rabbit, guinea pig, and dog, or which are available only in cesarean-born, hand-fed form, e.g. the sheep, pig, and cat (*see below*).

Some special areas of gnotobiotic research have been reviewed recently and are not included, i.e., those involving viral associations (246), neutralization of the flora by antibiotic treatment (325), animal nutrition (43), effects of radiation (28), and carcinogenesis (281).

GNOTOBIOTIC TERMINOLOGY AND CRITERIA

The terminology of gnotobiotic experimentation now in use derives primarily from an article of Reyniers et al. (269). The Institute of Laboratory Animal Resources of the National Academy of Sciences in Washington, D.C., has commented on this topic in a recent publication (95), as follows: "A number of attempts have been made to systematize the terminology used in gnotobiotic technology but no single system is universally accepted. In this publication the nomenclature has been limited to terms that by general usage are familiar to, and understood by, most workers in this field. *Gnotobiotic*: A word derived from the Greek "gnotos" and "biota" meaning known flora and fauna. *Gnotobiote* (Gnotobiotic animal): one of an animal stock or strain derived by aseptic cesarean section (or sterile hatching of eggs) which are reared and continuously maintained with germfree technics under isolator conditions and in which the composition of any associated fauna and flora, if present, is fully defined by accepted current methodology. *Germfree animal* (axenic animal): A gnotobiote which is free from all demonstrable associated forms of life including bacteria, viruses, fungi, protozoa and other saprophytic or parasitic forms. *Defined flora animal:* A gnotobiote maintained under isolator conditions in intentional association with one or more known types of microorganisms."

Commensal organisms living with the host are described as *associates* (usually microbes populating the gastrointestinal tract, bronchial tree, and the integument). The term *contaminants* often appears in literature but is considered less desirable as it carries a value judgement. No word has been introduced as yet for distinguishing between animal hosts living by themselves or together with homologous or heterologous animal associates. If the host's life included a germ-free episode after birth, it is referred to as *ex-germ-free*. The animals of ordinary life are commonly named *conventional. Conventional controls* to gnotobiotic animals are usually specimens of the same genetic background which are fed the same sterilized diet but which live in an open environment. For this reason, they are sometimes referred to as *open conventional* in contrast to *isolator conventional* animals which are maintained in enclosures similar to those of the gnotobiotic experiment. *Conventionalized* are ex-germ-free animals that have been associated with the flora of conventional controls. Since in some species (mainly in rodents) cesarean birth and hand-feeding artificial formulas cause

anomalies which persist later in life, it is necessary sometimes to distinguish between these and normally born, mother-suckled animals. For this purpose *cesarean born* or *first generation* and *normal born* or *successive generation* expressions are used. The expression *normal flora* usually refers to undefined microbial associates of healthy conventional animals. These words, generally speaking, constitute a commonly accepted working nomenclature. For the interpretation of other expressions that are occasionally used, the proponent's publications should be consulted.

Sterility of the germ-free host, i.e., the total absence of foreign viable associates, cannot be assured by absolute criteria. This is caused by the possibility of a preexistent contamination of the host at the source (i.e., in the course of fetal development), by microbial invasion of the isolator (especially by insufficient sterilization of air, food, and utensils), and by the difficulty of detecting certain associates in higher organisms. The criteria and procedures for sterility testing in general use are derived primarily from the work of Wagner (327) and were recently reviewed by Fuller (89). In this context, according to the previously mentioned publication of ILAR (95), "The germ-free animal may be defined as a gnotobiote that is free of all demonstrable microbial associations as determined within the limitations of the detection procedures available. Animals can be raised with good degree of assurance that they are free from bacteria and related forms, including yeasts, fungi, protozoa and metazoan parasites. The viral status of these animals is open to question because latent viruses may be present but remain undetected." Gustafsson (119), Pollard (246, 247), Parker et al. (222), and Pollard and Kajima (248) used an array of testing procedures and failed generally to show viral agents in various tissues of germ-free rats and mice. The activation of occult leukemia virus by whole body X irradiation was indicated in a variety of germ-free mice by Pollard and Matsuzawa (250). Recently Ashe et al. (11) reported the presence of a viral agent in the submaxillary gland of germ-free rats. The incidental presence of virus in the conventional parent stock and its placental transfer to successive germ-free offsprings may be responsible for the inconsistency in these findings.

The apparatus and procedures currently in use that permit the maintenance of gnotobiotic status in experimental animals have been documented amply (116, 119, 198, 264, 321). These include, in essence, apparatus for sterile transfer of the mature fetus from the uterus or egg into presterilized (by steam or germicide) steel or plastic isolators, supplied with air filters and rubber gloves. Routine colony procedures include holding and breeding of gnotobiotic animals in isolators and sterilization of diet, drinking water, bedding, and other utensils (by autoclaving, irradiation, or filtration, when applicable). Gnotobiotic animals and sterile supplies are transferred via sterile locks.

The flexible plastic isolator, the simplified associated apparatus and methods (321), the availability of germ-free animals and sterilizable diets from commercial sources, together with the possibility of shipping these animals in disposable isolators have brought gnotobiotic experimentation within the reach of most if not all laboratories.

THE MICROBIAL ASSOCIATES

Anthony Standen, the philosopher-humorist of scientific research, commented in 1952 on the reasoning of early workers in the gnotobiotic field (305). He stated that they devoted a great part of their lives to discovering the way to keep bacteria from the living host and predicted that, having learned how, they will put them right back in. He was unquestionably correct in his forecast.

The student whose main interest lies in the host tends to consider the flora as a single entity with a number of resultant effects on the animal body. In this sense the flora is a "package deal" or, borrowing Csáky's euphemism (54), the flora is an "organ" of the body. But the microbiologist, who deals with and is impressed by the immense heterogeneity in composition and in effects of the flora, will deem this type of reasoning an oversimplification. Such discrepancies, stemming from the philosophical approach of various disciplines, are frequently identified in our literature.

Two questions of special interest emerge concerning studies of the flora. Is the "sum-total" of flora effects generated through summation, mutual potentiation, or inactivation of microbial elements? Are the effects of the flora to be classified as synergistic, indifferent, detrimental, or all of these?

For more comprehensive information on the voluminous literature on the microbial floras in animal hosts, the reader is referred to the works of Rosebury (272), Haenel (130), Dubos et al. (69), Moore et al. (204), Smith (301), and Donaldson (65).

Development and Characteristics of the Normal Flora

After natural birth from a conventional mother, the newborn animal has a large and mixed microbial population established in the intestinal tract and in other exposed areas. These microbes are received without selection from the mother and from the immediate environment [Rosebury

(272)]. Proportions and numbers are quite variable. In a study of mammals (including man) during the first weeks of life, Smith and Crabb (300) found a certain degree of similarity of the floras among the species. As the animals matured, characteristic qualitative differences developed which appeared to be quite host-specific. Pesti (226) found a numerous and predominating population of *Escherichia coli* in the lower bowel of the pig during the first 24 hr of life. During the first week, the count of *E. coli* remained high, and then clostridia gradually increased in numbers. After the age of 4 weeks, lactobacilli and enterococci became the predominant forms, and the numbers of *E. coli* and clostridia began to decrease. In various segments of the gut of young animals, the duodenum and the jejunum were populated primarily by low numbers of lactobacilli and enterococci, whereas the counts in the ileum, cecum, colon, and rectum were considerably higher and included *E. coli*. In the colon, clostridia were also detected. In older animals (to 1.5 years of age), this trend persisted, with *E. coli* and clostridia found in the upper segments of the intestine. These observations support those of Wilbur et al. (340) who studied the flora of young piglets.

Raibaud et al. (251) found a constantly large and stable population of lactobacilli in the intestine of newborn rats. In addition, *Veillonella*, *Actinobacillus*, *Streptococcus*, and *E. coli* appeared before weaning. After weaning, the numbers of *E. coli* declined. Raibaud et al. (252) also reported that in adult rats lactobacilli, streptococci, enterobacteria, clostridia, yeasts, and molds are the predominant intestinal species.

Dubos et al. (70) found that postnatally the gut of healthy mice is populated by large numbers of lactobacilli, streptococci, and bacteroides. The former two occur in the stomach and in all segments of the intestinal tract, the latter only in the large bowel. All three groups of bacteria were concentrated mainly in the layer of mucus that normally covers the surface of the intestines. They have exacting nutritional requirements and multiply only under anaerobic conditions. The size and the location of the microbial population remained essentially unchanged throughout the life span of the mice (to ca. 2 years). Mice derived from various genetic strains and colonies were found to be similar. In NCS mice (287) which are maintained under strict conditions of sanitation, virtually only lactobacilli, streptococci, and bacteroides could be demonstrated in the intestinal tract, whereas in other less protected groups of mice, *E. coli*, other coliforms, *Proteus vulgaris*, *Pseudomonas*, and clostridia could also be recovered from the intestinal lumen. These authors hypothesize that lactobacilli, streptococci, and bacteroides constitute the original autochthonous associates which lived in symbiosis with the host in the course of evolution. The autochthonous group together with the accidentally acquired associates are considered the indigenous microbiota of a given community. The true and potential pathogens are found among the accidentally acquired elements of the flora. It is suggested by these authors that similar conditions exist in other animal species.

Savage et al. (285) reported on the establishment of the microbial flora of the gastrointestinal tract of mice by using histologic examination of tissue to supplement bacteriological assay. In conventional, normally born, mother-suckled mice, large numbers of gram-positive rods and streptococci were layered on the stratified squamous epithelium of the lower esophagus and the nonglandular mucosa of the stomach within the first days of life. In approximately 1 week, this microbial layer revealed that these organisms were lactobacilli and streptococci, similar to those found in the lumen. Similar bacteria were observed in histologic specimens of the small intestine; however, they were confined to the lumen and layering on the villus epithelium was not evident. Gram-positive and gram-negative rods and streptococci were observed on histologic examination of the colon and cecum during the first week of life. Afterwards, these groups were gradually replaced by fusiform rods which formed a dense layer in the mucus of the epithelium. These fusiforms eventually outnumbered all other bacteria in the colon and cecum by a factor as high as 1,000. Although bacteroides and clostridia could be cultured in the lower gut 12 days after birth, they could not be identified with the fusiform rods of the histological specimens. In addition, Brownlee and Moss (36) and Savage and Dubos (284) observed an indigenous yeast in the stomach of conventional rats and mice, respectively.

The reports to date generally suggest that, of the great variety of bacteria which reach the gut lumen after birth, many fail to survive and are eliminated. In the host approaching adulthood, those bacteria persist which have become adapted to a symbiotic life with the host; Rosebury (272) suggests that success attends those that "reach an area in sufficient numbers and find growth conditions to their taste and the obstacles not unsurmountable." To these, supplementary and transient, occasionally pathogenic, groups of microbes are added with the progress of age.

The existence of a flora closely linked to the gastrointestinal mucosa via a layer of mucus alters the concept that the bacterial population may be randomly distributed throughout the

tract. The proposal "that it is necessary to think of body surfaces as distinct microenvironments in which virtually pure cultures of a few species of microorganisms interact with their host and the adjacent microbial population," as suggested by Savage et al. (285) will influence future thinking and the planning of gnotobiotic models which deal with the physiology of the intestinal membrane.

Attempts to Establish a Microbial Flora in Germ-Free Animals

There is ample evidence that the normal indigenous floras of conventional animals may be readily transplanted to germ-free animals of the same species. Gordon and Wostmann (109) have found that germ-free rats receiving an oral inoculum of the cecal contents of a conventional control will change their morphological and functional characteristics from the germ-free to the conventional pattern within a few weeks. Similar changes will take place in germ-free animals when they are simply brought in physical contact with conventional controls. Examination of cecal contents a few hours after oral seeding showed a total bacterial count and the count of the more common specific bacterial groups normally found in the cecum to be quite comparable to bacteriological data obtained from conventional rat ceca. Generally, no loss of ex-germ-free animals by infections or otherwise occurred in these processes. The transfer of germ-free animals to quarters of conventional counterparts is a standard breeding procedure in the maintenance of close genetic linkage between the two colonies.

Another group of studies deals with the problem of assembling in vitro known elements of the flora which, when introduced into germ-free animals, will duplicate the distribution and the effects of the conventional flora on the host. Such studies dealing with an "artificial, known normal flora" are considered as a basis for critical work on the host-normal flora relationship. Earlier attempts in this area were reviewed by Luckey (179). Skelly et al. (298) inoculated the cecal flora of conventional mice into germ-free mice and continued this process of conventionalization from ex-germ-free mice to germ-free mice for a total of five successive oral passages. The cecal flora was cultured on each occasion. At the first passage, the ceca of the ex-germ-free mice contained *E. coli*, *Aerobacter*, *Proteus*, *Bacteroides*, *Micrococcus* and *Clostridium* species. From the fourth passage onwards, *Proteus* and micrococci were absent. The enlarged ceca (a main characteristic of germ-free rodents) were found to be reduced to conventional proportions in all instances. Successively, isolates of a *Clostridium* or of two *Bacteroides* strains were seeded into separate groups of germ-free mice. Two to four days after insemination, the weights of the ceca were reduced to those of conventional controls and persisted until term, 3 weeks after inoculation. The *Clostridium* isolate was identified as *Clostridium difficile*. The same species obtained from ATCC (90556) yielded results similar to those produced with the isolate. Association of germ-free mice with aerobic bacteria cultured from conventional mice did not reduce the cecum. However, Hudson and Luckey (148) found that an organism isolated from conventional rat feces (*Streptococcus* sp.) reduced the cecum of germ-free rats to about 60% of the original value within 24 hr.

Schaedler et al. (289) transferred known elements of the relatively simple flora of NCS conventional mice maintained at high levels of sanitation into germ-free mice. Oral inoculation with *Lactobacillus* sp., a *Streptococcus*, and a *Bacteroides* from NCS mice produced an ex-germ-free animal with essentially the same numbers and the same distribution of microorganisms as was found by Dubos et al. (70) in NCS mice. In these gnotobiotic animals, particularly in their microbially similar offspring, "the ceca appeared normal with regard to structure, shape and size." In an extension of the previous experiment, Schaedler et al. (289) monoassociated orally germ-free mice with a slow lactose-fermenting coliform bacterium which multiplied throughout the entire gastrointestinal tract. After passage in the now monoassociated host, the predominant organism isolated from the gnotobiotes was a lactose-fermenting *E. coli* type. When the slow lactose fermentor was fed to NCS (conventional) mice, it failed to multiply and it retained its slow lactose-fermenting characteristics. When "NCS-like", ex-germ-free mice (associated with *Lactobacillus*, *Streptococcus*, and *Bacteroides* spp.) received the slow lactose fermentor, it populated the entire gut but it did not change its lactose-fermenting characteristic. When tetra-associated, ex-germ-free mice were inoculated with the feces of conventional NCS mice, their intestinal coliform population fell rapidly to levels found in the coliform-seeded NCS conventional mouse group. The authors state that "it appeared as if NCS mice contributed (to the ex-germ-free mice) a transmissible agent, as yet unidentified, capable of affecting the composition of the gastrointestinal flora." The observed change in lactose-fermenting characteristics of the coliform, by passage as a monoassociate in ex-germ-free mice is as yet unexplained. Sasaki et al. (282) showed that various bacterial species which compose the normal flora of conventional mice persist well as monoassociates in the gut of ex-germ-free mice over a period

of several weeks. By using a combined oral inoculum of normal flora elements, including *E. coli*, streptococcus, lactobacillus, clostridium, and bacteroides, these authors found that all organisms populated the gut of ex-germ-free mice at high levels and no antagonism was observed among them. Pesti et al. (228) used a number of microorganisms which have been implicated in the elimination of germ-free cecal anomalies (*C. difficile*, *Lactobacillus casei*, *Bacillus subtilis*, *Lactobacillus* sp.). Their observations failed to identify a microbe which as a single associated species is capable of eliminating all anomalies of germ-free mice. They have suggested, however, that these may be selectively redressed by various associates (e.g., *C. difficile* neutralized the bioactive alpha pigment of the germ-free gut; *L. casei* changed the oxidation-reduction potential of cecal contents to conventional-like values; *see below*).

Thus it was shown that the undefined, indigenous intestinal flora and a variety of its known elements can be readily established in germ-free animals. In this respect, the germ-free animal proves to be a good selective, although not universal, microbial culture medium. In spite of the progress made in the study of the flora, the identity of elements which can convert all characteristics of germ-free animals to those of conventional animals is still unknown.

Dietary Effects

The diet, forming an essential part of intestinal contents (i.e., of the culture medium within the host), has long been known to affect the composition of the intestinal flora. The most common example is the aciduric intestinal flora of human babies fed mother's milk as compared to the high count of gram-negative bacilli in infants fed cow's milk (129). After weaning, the infant appears to retain a relatively stable level of intestinal flora on a wide variety of dietary regimens (131, 132). Graber et al. (114) reported only slight changes in the intestinal flora of rats fed high-fat diets in comparison to normal controls maintained on a balanced ration. In another study, Graber et al. (113) found an essentially stable flora in pathogen-free swine fed high-lipid or high-sucrose diets.

Dubos and Schaedler (68) observed that, when young NCS (i.e., pathogen-free) mice were fed semisynthetic diets containing casein or wheat gluten as sole source of protein (supplemented with all known growth factors), lower numbers of viable lactobacilli appeared in the feces when compared with feces from mice fed complete diets containing natural products. Another change in the mice fed with a semisynthetic diet was the disappearance from the stools of lactobacillus types showing rhizoid morphology. Upon switching these animals to a complete diet composed mainly of natural ingredients, the number of fecal lactobacilli increased. On intravenous injection of cultures of *Klebsiella pneumoniae* or *Staphylococcus aureus* to mice fed semisynthetic diet, these authors found higher mortality than among similarly challenged mice fed the complete natural diet. After intraperitoneal administration of endotoxin (derived from *E. coli*), there was a rapid increase of enterococci and coliform bacilli in the intestine of mice fed semisynthetic diet but not in those fed the natural diet. Wilkins and Long (342) feeding a complete diet to adult conventional mice found thick layers of fusiform bacteria, presumably the autochthonous associates of the host, lining the mucosa of the cecum and colon. The replacement of this ration by a liquid, chemically defined diet [a modification of the diet of Greenstein et al. (115)] resulted within days in the loss of the fusiform bacteria and in the appearance of gram-positive cocci and gram-positive rods throughout the lower bowel.

Fitzgerald et al. (84) observed the effect of coprophagy on the flora of the lower bowel and on body weight of conventional rats fed fat-free and -rich diets. Prevention of coprophagy by the use of tail cups caused a decrease of lactobacilli and an increase of coliforms in the feces. Weight gain was reduced in the cupped animals by ca. 30%. The nature of the diet did not influence the results.

It appears that the animal host is more susceptible to diet-conditioned flora changes during the preweaning period than it is after weaning. The development of the animal defenses may have a stabilizing effect. If the host is well supplied with nutrients at adult age, dietary changes may not affect the main components of the flora. On the other hand, feeding partly or completely synthetic diets, in which essential nutrients are less well balanced than in natural diets, might precipitate a major shift of the flora within the host. Competitive utilization of scarce nutrients by the flora and the host might cause departure from what is a more stable equilibrium when the nutrient supply is more plentiful. Under these circumstances, it appears that a vicious circle is initiated at the expense of the host and its authochthonous associates. The tail-cupping experiment offers an interesting illustration of how the disruption of a natural recycling process of food can trigger a detrimental chain of events in this context.

Indirectly related to these issues are "germ-poor" animals of polar regions which have received attention in literature since the turn of the century (171). Sieburth (297) reported that the extremely low intestinal flora population of various birds in the Antarctic can be traced to an

antibiotic substance (acrylic acid) originating from plankton, which, forming part of their natural diet, may be responsible for the permanently low microbial counts. This illustrates that, with mild, selective antibiotic supplementation of the diet, it might be possible persistently to modify the intestinal flora in conditions of conventional life, retaining synergists and eliminating antagonists without affecting adversely the health of the host.

Effects of Closed Environment

Work with germ-free animals must of necessity be conducted within isolators. This requires that microbially exposed control animals (in addition to matching the germ-free group in animal, dietary, and other environmental detail) must also be maintained within germ-free-type isolators. However, early in this work problems arose, particularly in attempts to maintain similar "normal" conventional flora in both open and isolator-housed groups. Nelson (208), working with guinea pigs, and Reback (253), using rats locked in germ-free-type isolators, found that changes in the intestinal flora and the well-being of the host occurred which depended on the length of the confinement and the degree of sanitation practiced. These findings suggested that the isolator-type internment of conventional animals, the development of an abnormal, "locked" flora, and, through this, an impairment of the animal's health, are related and possibly unavoidable phenomena.

Reyniers et al. (270) in a study with newly hatched conventional chicks locked in steel isolators and maintained for ca. 50 days in the same conditions as germ-free and open conventional counterparts found that the development of the locked flora could be prevented if open conventional chicks ("visitors") were placed daily in the isolator. (The purpose of this was to reseed elements of the normal flora.) At term, the body weight and organ weights of the interned chicks were found to be within normal limits. Gordon et al. (104), observing a rigid hygienic regimen, kept conventional rats from the age of 30 to 100 days in isolators without visitors. At the end of the experiment the locked rats showed normal body weight, organ weights, and red blood counts. Only the weight of the spleen and the white blood count of these animals showed low, germ-free-like values. The only modification of the intestinal microbial population of isolator-conventional rats was slightly elevated counts of micrococci. Pesti and Gordon (227), reporting on 4- and 24-month-old conventional mice, found that the intestinal population of clostridia and staphylococci grew considerably from young to old age in mice maintained in the open environment. In the case of isolator-conventional mice, the counts of these microorganisms in the old group remained at the low levels which were observed in the young group.

These findings suggest that the isolator-type environment does not produce an "abnormal flora" nor is it detrimental to the health of the confined animals. Poor hygienic regimen in the isolators may affect both the host and the flora alike, but this is not associated with "life in the isolator" per se. Actually, the barriers of the isolator may prevent the appearance of pathogens to which older animals seem to be attractive hosts. Under these circumstances, the isolator-conventional animal (provided it is kept clean) will derive only advantages. The excellent health status and the virtual absence of pathogenic flora found by Schaedler et al. (287) in their NCS mice housed in an ultraclean environment support this speculation.

The possibility that life might be affected by internment in plastic isolators was indicated by Fujiwara et al. (88). These authors found that the development of the chloroplast in leaves of plants sprouting in flexible plastic isolators was impaired and suggested that a plasticizer substance emanating from the material was implicated. Following this lead, one of the authors (L.P.) studied the effect of polyvinyl plastic on the growth of a variety of bacteria. Although adding sterilized minced plastic to the culture medium did cause growth depression of some microorganisms (staphylococci), the separation of the plastic from the medium by a few millimeters of air prevented this effect in all instances. Effects of this nature on animal life have not been studied; however, the overall similarity found between comparable gnotobiotic animals housed in plastic and steel isolators makes improbable the existence of such effects.

Factors Affecting Microbial Passage into the Host

Gnotobiotic experimentation has given clues as to direct, short term microbial passage into the host (in contrast to invasive microbial growth). It has been suspected that the oral cavity is a major site for parenteral penetration of microbes. This was suggested by the substantially reduced weight of submandibular lymph nodes of germ-free rats as compared to conventional controls (104). Blood engorgement of these nodes, frequently observed a few hours after conventionalization of germ-free animals, supported this assumption. The work of Thonard et al. (314) has suggested that the gingival crevice may be a site of parenteral penetration of antigenic agents.

Abrams and Bishop (4) and Abrams (1) observed the passage of *Salmonella typhimurium* from the intestinal lumen to the mesenteric lymph nodes of germ-free and conventional mice. They concluded that microbial passage (translocation) across the wall of the stomach and of the small intestine over a period of hours is determined by the size of the intraluminal population and not by the nature of the intestinal wall as conditioned by the germ-free or conventional state. On oral insemination of similar numbers of organisms, larger intraluminal populations developed in ex-germ-free mice than in conventional controls, resulting therefore in greater translocation in the former group. This suggests that the resident floras of the conventional animals exerted an inhibitory effect on the multiplication of the *Salmonella* organism. Bowel obstruction caused by ligating the gut aborally from the site of insemination resulted in high salmonellae populations in both ex-germ-free and conventional mice, indicating that the inhibitory effect of the resident flora is impaired or lost under these circumstances. These findings agree with those of Miller and Bonhoff (194) who described marked multiplication of *S. enteritidis* in the intestine of mice in which motility was blocked pharmacologically. Wolochow et al. (349), studying intestinal translocation of a variety of microorganisms in conventional rats, confirmed the observation of Abrams and Bishop (4) on the parallelism between the size of the microbial population and the degree of transfer across the intestinal wall. Their work further suggested that in noninfectious processes the physical dimensions of the microorganism may be a determining factor in intestinal penetration. Canada and Strong (38) demonstrated the consistent presence of *C. perfringens* in the livers of some monoassociated ex-germ-free and conventional mice.

It appears that in the normal host the activity of the gut and the population size of the flora influence the noninfectious passage of microbes from the gastrointestinal lumen into the tissues. Considerable translocation may result when a microbe is present as a monoassociate in the host, as suggested by Abrams and Bishop (4). This may be caused by the lack of microbial competitors and by the impaired propulsive movements that are inherent with the germ-free and often also with the ex-germ-free gut.

THE ANIMAL HOST: GERM-FREE OR MODIFIED BY MICROBES

Rats and mice are most generally used for gnotobiotic experimentation. They are obtained from reproducing colonies of germ-free parent animals and descend from a handful of germ-free male and female ancestors which were cesarean born and artificially reared to maturity at the Universities of Notre Dame and of Lund about 15 years ago. This stock was expanded by allowing cesarean-delivered animals to suckle lactating germ-free foster mothers. The preference for these animals in experimental work stems from their apparent normality and uniformity, the availability of adequate, sterilizable solid rations, and from the ease of obtaining them from commercial sources. The time-tested host in earlier gnotobiotic work had been the chicken, for which germ-free hatching procedures and complete diets were elaborated. The use of mammals other than rats and mice has increased lately and these represent, for the most part, cesarean-derived and artificially reared germ-free stock.

Gnotobiotic studies of the normal animal host ask two main questions. The first of them represents a continuation of the question originally asked in germ-free work "Is life possible without the flora?". This is now modified to "Is normal life possible without the flora?". The other question asks "What are the effects of the microbial flora or some of its elements on the animal host?". In both instances, but particularly in the latter, the use of conventional control animals is an integral part of the experimental design.

Nutrition, Digestion, and Metabolism

Nutritional and metabolic characteristics of gnotobiotic animals are discussed because they appear to be essential to assessing the effects of the microbial flora on the animal host.

Rats and mice. A number of solid diets are adequate for rearing germ-free rats and mice and their microbially associated controls (118, 168, 267, 350). Wostmann and Kellogg (358) have designed a diet in which nutrients, vitamins, and salts are given in separate units which may be selected according to the needs of the experiment. These rations are either semisynthetic or contain natural ingredients and may be sterilized by heat or radiation. An excess of heat-labile compounds in the diets compensates for the losses in these ingredients incurred in the course of sterilization. Growth of the germ-free animals fed these diets is comparable to that of conventional controls and they reproduce well into successive generations. Rearing of germ-free rats and mice during the preweaning period on artificial sterile formulas (after cesarean birth) is rather complex (118, 267) and seldom practiced although Miyakawa (201) has reported recent progress.

Of considerable interest is the use of chemically defined liquid diets, low in antigenic components, which are based on the original formulation of Greenstein et al. (115) and elaborated by others

(238, 240, 364). Reddy et al. (259) grew germ-free rats from birth to maturity, using membrane-filtered, chemically defined water-soluble diets based on amino acids and glucose. Pleasants et al. (241) reared reproducing colonies of germ-free mice on such a diet into the fifth generation.

Desplaces et al. (61) have shown that the metabolic rate and fixation of radioactive iodine in the thyroid of germ-free rats were significantly lower than in conventional controls. Wostmann et al. (356) corroborated this finding in reference to the metabolic rate. They have also shown that the surgical removal of the enlarged cecum in germ-free rats (37) changes their metabolic rate to conventional values. Since cecal enlargement is linked to the absence of the intestinal flora, it is assumed that that the reduced metabolic rate of germ-free rats portrays missing microbial regulatory function (*see below*).

Luckey (179) found no difference in food intake between comparable groups of germ-free and conventional rats. Combe et al. (48) and Gordon (101) reported a 10 to 20% excess of food consumption among germ-free rats and mice, respectively. In the latter work, germ-free mice were found to drink more water in an apparent compensation for the increased loss of liquid via the chronic, mild diarrhea of these animals (*see below*). Levenson and Tennant (173), Evrard et al. (78), Luckey (179), and Reddy et al. (259) found increased fecal nitrogen excretion in germ-free rats in comparison to conventional controls.

Despite the absence of microbial protein in the intestines and the apparently increased fecal nitrogen excretion of germ-free animals, little difference was found in the per cent nitrogen of intestinal contents between germ-free and conventional rats (169). Because of increased secretion or reduced degradation (or both), substantially greater amounts of varied substances have been found in the intestinal contents or feces of germ-free animals. These include free amino acids, urea (48, 49, 78), hexosamines (174), mucoproteins, and mucopolysaccharides (175, 176).

The amounts of trypsin and chymotrypsin were found consistently elevated in bowel contents or feces of germ-free animals (31, 175). Reddy et al. (254) repeated these observations and added that pancreatic trypsinogen and chymotrypsinogen levels closely aligned in germ-free and conventional rats. This suggested that in normal life the microbial flora is responsible for the partial inactivation of these enzymes.

Concerning the catabolism of proteins in the absence of bacteria, Combe et al. (48) and Combe and Sacquet (49) observed very little ammonia in cecal contents of germ-free rats. These observations corroborated indirectly the earlier work on guinea pigs by Warren and Newton (336), who reported fourfold ammonia values in the portal blood of conventional in comparison to germ-free guinea pigs. Levenson et al. (172), administering parenterally ^{14}C-labeled urea to germ-free and conventional rats, could retrieve only traces of $^{14}CO_2$ in the exhaled air of the germ-free animals, whereas, in the samples taken from the conventional controls, labeled CO_2 was present in ample quantities. These observations suggested that the primary end product of protein catabolism in the host is urea and that ammonia in essence is a microbial artefact. This view is supported by Ducluzeau et al. (72) who demonstrated that monoassociation of germ-free rats with various species of *Lactobacillus, Actinobacillus, or Staphylococcus* induced the formation of ammonia from urea in the cecum of these animals. Two other genera, *Lactobacillus* and *Proteus*, though clearly ureolytic in vitro, proved ineffective in vivo.

The scanty work done in carbohydrate metabolism of germ-free animals concerns carbohydrate digestive enzymes of the intestinal tract, which originate from the host rather than intestinal bacteria (57, 256). In conventional conditions, the microbial flora appears to be responsible for the partial inactivation of these enzymes, either directly or indirectly (257).

Evrard et al. (78, 79) and Hoet et al. (143) found that in germ-free rats the fecal fatty acids were typically unsaturated and of the long-chain, even-numbered type. In conventional controls, saturated acids prevailed and cyclical and branched-chain fatty acids (that are commonly attributed to bacterial synthesis) could be identified.

Evrard et al. (79) have shown in germ-free rats fed a purified corn-oil type diet that the only sterols demonstrable in the feces were cholesterol and certain phytosterols. The former obviously was an endogenous product; the latter originated from the diet. The feces of conventional control rats were found to contain, in addition, a variety of coprostanol analogues and other sterols. This change, entailing among others reduction and oxidation of the steroid moiety, must therefore be considered a result of intestinal microbial action. Through similar mechanisms, the intestinal flora may be able to modify the cholesterol metabolism. Wostmann and Weich (366) found the cholesterol levels of the liver to be substantially elevated in germ-free rats. Interestingly, the cholesterol content in the aorta at old age was significantly higher in conventional rats than in germ-free controls, suggesting possibly vascular impairment in the host by prolonged microbial exposure. Danielsson and Gustafsson (58) found

elevated cholesterol levels in the blood serum of germ-free rats in comparison to conventional controls. Wostmann and Kellogg (359) recently identified a species of *Clostridium* which increased the cholesterol turnover in ex-germ-free rats to the rate corresponding to that of conventional controls. This organism approximately doubled the excretion of neutral fecal sterols, however, without the formation of coprostanol or its analogues.

Cholic acid reaches the intestinal lumen in conjugated form and is generally known to undergo deconjugation and a series of modifications by the microflora which results in a variety of steroid metabolites in the gut. Many of these, being complexed in the intestine, are excreted via the feces. Gustafsson et al. (121) found orally administered, labeled taurocholic acid in unchanged form in the feces of germ-free rats. The daily excretion of bile acids appeared to be reduced, and the half-life of labeled cholic acid was five times longer in the germ-free rats over conventional controls. Monoassociation of germ-free rats with *C. perfringens* left the half-life time of the labeled cholic acid at the same levels but caused deconjugation of the acid. Gustafsson et al. (127) reported that germ-free rats monoassociated with *E. coli* showed a similar reduction of fecal cholic acid excretion, but they observed a continuous formation of 7-ketodeoxycholic acid in the cecum, which is commonly considered the result of microbial modification.

The role of bacteria, and possibly of virus, in the absorption and malabsorption syndrome of lipids and sterols is an interesting and well documented field (80, 81, 280) whose discussion exceeds the limitations of this review.

The degradation of bilirubin by the intestinal flora has been shown by Gustafsson and Swenander-Lanke (128).

Wostmann et al. (362) reported consistently lower levels of thiamine, paralleled by reduced blood flow, in the liver of germ-free animals. They speculated that, in conventional animals, the elevated levels of this vitamin and of blood flow portray the stimulatory effects of the intestinal flora on the chemical energy and oxygen requirements of this organ. In another experiment, Wostmann et al. (361), feeding labeled precursors of thiamine to conventional rats, found considerable amounts of labeled thiamine in contents of the lower bowel but only small amounts of it in the liver and in other organs. This suggested that, in conventional hosts, microbially synthetized thiamine is not available for absorption, probably because it remains bound to the microbial cell component.

Daft et al. (56) noted that germ-free rats fed a folic acid-deficient diet developed some symptoms of folic acid deficiency, whereas the same diet had essentially no effect on conventional controls. It appeared that the deficiency symptoms could be prevented by monoassociation of germ-free rats with species of *Aerobacter*, *Alicaligenes*, *Proteus*, or *Escherichia coli*. Conventional rats maintained on a pantothenic acid-deficient diet were capable of utilizing this vitamin synthesized by the intestinal flora only if it was recycled by coprophagy (thus creating more favorable conditions for absorption of the microbial pantothenic acid). Germ-free rats or conventional controls supplied with tail cups developed the symptoms of pantothenic acid deficiency when fed the same diet.

Germ-free rats maintained on a diet free from vitamin K promptly developed symptoms typical of deficiency disease (120). When these animals were changed to conventional state, or when their diet was supplemented with vitamin K, the prolonged prothrombin time returned to normal values and the hemorrhagic diathesis ceased. Gustafsson et al. (122) and Wostmann et al. (362) have shown that vitamin K_1 was most effective in reversing the symptoms of the deficient germ-free rats, whereas various menadiol salts were effective only in much higher concentrations. It has been found that *E. coli* or an unidentified sarcina-like micrococcus were able to reverse the symptoms of deficiency (122). Thus it appears that vitamin K requirements can be fully satisfied by the flora of the animal host.

The assumption that a microbial factor exists in the antagonism between A and K vitamins (187) was tested by Wostmann and Knight (360). Using the customary intake range of vitamins A and K, they were unable to demonstrate any effect on the vitamin K requirement of germ-free rats. Only when the dietary levels of vitamin A were increased by a factor of 10 or more (and the rations contained the normal amount of vitamin K) did the symptoms of K deficiency develop. Thus, their work failed to support previous speculations that an excess of vitamin A interferes with the microbial synthesis of vitamin K in normal subjects.

Other animal species. In this section a brief general description of additional animal species is given, which are more or less well established in gnotobiotic research. The results obtained with these animals in specific fields of investigation are interspersed with those of rats and mice in various chapters of this review.

The guinea pig is particularly adapted to germ-free studies because as a neonate it does not require a period of suckling and it may be weaned directly onto semisolid natural diets. Consequently, young cesarean born guinea pigs have

frequently been used since the beginning of work in our area (94, 146, 197, 212, 235, 311). By using improved rearing methods and diets, these animals have been maintained in the germ-free state to four successive generations (242). Germ-free rabbits have been reared to successive generations by feeding cesarean born offspring with milk formulas (239) and by weaning the animals onto complete diets (255). The problems encountered in maintaining a colony of germ-free guinea pigs and rabbits are similar. The growth of both these species in the germ-free state is reported (239) inferior to that of conventional controls. Along with rats and mice, germ-free guinea pigs and rabbits tend to develop cecal enlargement and intestinal volvuli.

Germ-free pigs may be obtained by cesarean section (166, 337, 339). Most reported work refers to the early postnatal period because germ-free piglets are considered excellent experimental animals at this age when their growth is comparable or even better than that of conventional controls.

Sheep and goats have been reared as first generation germ-free animals to approximately 4 months of age (299), following an older report (164).

Dogs have been obtained as first generation germ-free animals and reared to maturity (138). Recently, successful reproduction to second generation has been achieved in germ-free beagle dogs (139). Cats (271) and monkeys (348) were reared as first generation germ-free animals to approximately 10 months of age. The growth of dogs, cats, and monkeys under these circumstances was comparable to that of conventional controls.

The chicken is considered as one of the most valuable germ-free hosts because of the simplicity of the techniques involved, its excellent growth and development record, and its being a uniform and rugged experimental animal (42, 270). Second generation germ-free chickens have hatched from the eggs laid by germ-free parent animals (268). Quails are the competitors of the chicken in gnotobiotic experimentation because of their small size and their earlier maturation (42, 265). Turkeys have also been obtained in the germ-free state (180).

Structure and Function of Various Organ Systems

A comparison of morphologic characteristics of germ-free and conventional animals was first undertaken by Glimstedt (94) in guinea pigs. He observed the weight of various organs, following the commonly accepted principle that any departure from the conventional values may indicate a functional change of the organ, and managed a limited survey of the entire animal. This was later extended to the chicken (270) and to the rat (104). In general, the organs could be divided into two groups: (i) those which harbor or are in close association with the microbial flora of conventional life showed mostly reduced weight in germ-free animals, and (ii) those organs remote from bacteria showed practically no difference between germ-free animals and their conventional counterparts. Paraphrasing the expression of Claude Bernard (30), we see a division of the organs into groups associated with either the "external" or the "internal" environment. Into the former fall primarily some elements of the cardiovascular and respiratory systems, the gastrointestinal tract, and the integument. The latter includes the musculature and the nervous system. Pleasants (239) reviewed some of these areas. In the following, a summary of the essential findings are given.

Cardiovascular system. The weight of the heart, the total blood volume, and cardiac output were reduced, whereas ,red blood cell count and hematocrit values were found elevated in germ-free rats (111). Regional blood flow determinations indicated reduced values for germ-free rats (in some instances as much as 45%) in organs that are normally in close contact with the flora (skin, bronchial circulation, digestive tract, and liver). Organs of the internal environment (kidneys, spleen, thymus) showed only smaller, if any, differences between the opposing groups (101). These phenomena are apparently reflected in the oxygen supply of some tissues, as indicated by Matsuzawa and Wilson (188) who reported reduced oxygen partial pressures in the liver and in the subcutaneous tissue of germ-free mice.

In a study of the microcirculation of the mesocecum of germ-free rats, decreased motor activity and considerable degree of hyporesponsiveness to epinephrine and vasopressin (approximately 20 to 1) were found in vascular smooth muscle (16); however, the response to angiotensin was essentially similar in both germ-free and conventional animal groups. During a search for the cause of this difference, a pigment ("alpha") was isolated by chromatography from the cecal contents of germ-free rats and mice which reduced the sensitivity of vascular and intestinal muscles to epinephrine (106). A similar pigment could be isolated also from conventional controls; however, it was biologically inactive. The epinephrine inhibitory effects of germ-free alpha pigment were found to be similar to those of ferritin or apoferritin in their reduced form, as originally described by Mazur and Shorr (189). Trypsinization did not change the bioactivity of these substances, whereas incubation with antiferritin serum or with an inoculum of cecal microflora in vitro inactivated them. Recently, a

close spectral alignment was found between trypsinized ferritin, apoferritin, and germ-free alpha pigment (182). It is speculated that alpha pigment originates from the intestinal epithelial cells, whose iron-carrier protein ferritin is known to reach the intestinal lumen in sizable quantities on desquamation of these cells (50, 53). In the germ-free gut, digestive enzymes break up the cells and then the large ferritin molecule to the smaller, absorbable, yet still active alpha pigment. The previously mentioned hyporesponsiveness of mesocecal microvessels to epinephrine (16) may be explained on the basis that alpha pigment reaches the local circulation. This speculation is supported by the work of Reddy et al. (258) who found elevated levels of ferritin iron in the livers of germ-free rats. It is conceivable that alpha pigment taken up in the circulation is involved in the reduction of metabolic rate observed in germ-free rodents (*see above*) by inhibiting the calorigenic effect of endogenous epinephrine. This is supported by the observation that in germ-free rats, on surgical removal of the cecum, i.e., of the large pool of alpha pigment, the metabolic rate was found elevated to conventional values in spite of the fact that the animals maintained their germ-free status (356). The inactivation of alpha pigment by the intestinal flora is irreversible (106); the mechanism of this process is unknown. The suggested negative feedback effects of undegraded alpha pigment in germ-free animals is an illustration for synergistic actions of the intestinal flora on the host organism.

Gastrointestinal tract (the upper segments). Among the few differences which exist in this area between germ-free and conventional animals, the most consistent is the lower weight of the small intestine (270). This seems to result mainly from the reduction of the lamina propria tissue. In consequence, the mucosal surface area of the germ-free small intestine is reduced (102). In the intestinal epithelium of germ-free animals, lower renewal rates have been found (2). In these cells a reduction in all stages of the generation cycle has been observed (170). The mitosis indexes of epithelial cells in the duodenum and ileum were uniformly low in germ-free rats and uniformly high in conventional rats (117). The possibility that the flora is directly involved in this phenomenon (e.g., by increasing cell-attrition) is improbable because of the great differences in microbial population between the duodenum and ileum of conventional animals. It seems more probable that, by an unknown mechanism, the proliferative capacity of these cells is uniformly depressed in the germ-free state and elevated in the conventional state. The passage time of intestinal contents through the entire gastrointestinal tract of germ-free rats has been found increased (5). Concerning intestinal absorption, Heneghan (135), using xylose as test substance, found generally enhanced uptake from the small bowel in germ-free rats. He speculates that the small intestine of germ-free animals, which is not burdened by an excess of defensive cells, represents a more efficient absorptive membrane than its conventional counterpart.

Enlarged cecum of germ-free rodents. In 1896, Nuttal and Thierfelder (213), commenting on the lower bowel of their young germ-free guinea pigs wrote, "Der Blinddarm war stark aufgetrieben unt mit brauner, käsig geronnener Flüssigkeit schwappend gefüllt." Cecal enlargement was observed to develop in baby rats during lactation (354) and persists during the life span of the animal. Excessive forms of cecal enlargement, volvuli at the ileo-cecal-colic junction, and progressive loss of intestinal muscle tone are among the prime lesions observed at natural death of these animals (105). The cecal contents average 6 to 10% of the body weight, reaching 20 to 25% in extreme cases. They are considerably more liquid than in conventional controls. Germ-free cecal contents show a more alkaline reaction by approximately 1 *p*H unit (355) and a more positive oxidation-reduction potential (233, 355).

The apparent reduction of smooth muscle tone in the germ-free lower bowel may have several coexisting causes. Staley (303), searching for pressor substances of microbial origin by the use of rat cecal muscle strips in vitro, found that the state of contraction of germ-free preparations was reduced to approximately two-thirds that of conventional controls. Bacterium-free filtrates prepared from conventional rat cecal contents caused a tone increment of the germ-free cecal muscle to near conventional values, whereas strips from conventional cecal were relatively insensitive to this treatment, supporting the original assumption.

Changes in autonomic innervation or reduced sensitivity to endogenous transmitter substances may also play a role in these phenomena. Dupont et al. (73) found structural differences in the primary plexus of Auerbach and a greatly increased size of myenteric neurones in the cecal wall of germ-free rats. Reduced levels of diphosphopyridine nucleotide diaphorase activity in these cells indicated lower oxidative energy metabolism in germ-free animals at this site. Strandberg et al. (309) observed that normal, spontaneous muscle contractions of rat cecal strips in vitro could not be observed in germ-free

specimens and that these strips were less sensitive to epinephrine and acetylcholine than conventional controls.

The possibility that in absence of the flora the endogenous, muscle-depressant substances accumulate in the gut must also be considered. In the lower bowel of germ-free rats and mice, higher levels of a hypotensive kinin-releasing protease have been observed. This enzyme has tentatively been identified as fecal kallikrein (97, 100) or trypsin (12). A relationship between microbial degradation of this enzyme and cecal reduction of ex-germ-free rats has been suggested. In rats monoassociated with *S. typhimurium*, the transient reduction of the cecum was paralleled by a reduction and successive increase of the enzyme (347). Germ-free mice immunized and resistant against pathogenic effects of *S. typhimurium* still showed the transient reduction of cecal size and the lowering of the enzyme (346), suggesting that immunological phenomena are not implicated in cecal reduction by microbes. The progress of cecal enlargement in aging germ-free mice was accompanied by a parallel increment of the cecal kinin-releasing enzyme (99).

Other autacoid substances of the germ-free intestine. Beaver and Wostmann (27) reported that the histamine levels in intestinal contents were lower in germ-free rats and mice than in conventional controls. They speculated that the high luminal values in conventional animals result from microbial decarboxylation of histidine. The study of Gustafsson et al. (123) suggested that histamine of microbial origin is not readily available for absorption to the conventional host since the quantity and distribution of this amine in the tissues of germ-free and conventional rodents are essentially similar. Gordon (97) has reported that cecal supernatant fluid of germ-free rats was toxic on intraperitoneal injection into mice, whereas similar material from conventional controls was relatively nontoxic. It appears that the cecum of a germ-free mouse contains five- to eightfold of the dose which is lethal to germ-free or conventional recipient mice within 1 to 3 hr. The nature of the toxic substance is unknown (although it is suspected that the active form of alpha pigment is implicated).

Water absorption inhibition in the germ-free lower bowel. The more liquid contents of the cecum and colon and a chronic mild diarrhea are widespread characteristics of germ-free animals. In addition to appearing in rodents (118, 267), it has been observed in germ-free specimens of other species [chicken, Reyniers et al. (270); dog, Heneghan (137); pig, Miniats (196)].

In the absorption of water from the lower small intestine, Csáky (54) found that in germ-free rats there was an initial lag but that the ultimate rate of water absorption was no different in germ-free and conventional animals. This finding suggested that in intestinal contents of germ-free animals a substance may be present which inhibits water absorption and which, when washed out, results in the restoration of normal water absorption. In conventional life, intestinal bacteria destroy the water absorption inhibitory substance.

Loeschke and Gordon (177), replacing the cecal contents of germ-free rats in vivo with saline, found that water absorption from the germ-free cecum was five to six times greater per unit of mucosal surface area than in conventional controls. These observations suggested that the impaired water absorption from the germ-free lower bowel is conditioned by the nature of its contents and not by an inherent defect of the mucosa. (The mechanism of the improved performance of the germ-free intestinal mucosa when "relieved" from the contents remained unexplained.)

Following the lead that mucus and related substances are present at elevated levels in germ-free intestinal contents (48, 54, 174, 176) and might be implicated in this phenomenon, Gordon and Nakamura (107) determined colloid osmotic pressure (i.e., the water retentive power) in intestinal contents. They found that in germ-free rat cecal contents the colloid osmotic pressure averaged 60 to 70 mm of Hg higher values than in blood plasma, whereas in conventional animals these values were essentially similar. Thus the excess of unabsorbable macromolecules in the germ-free gut explained in part the impaired water absorption. This conclusion is supported by the work of Hoskins (147) who found that the enzyme activity in the feces of germ-free rats which is able to destroy ABH (O) blood group (mucopolysaccharide-type) antigen was absent although it was present in conventional controls. It was also indicated by this study that the source of the activity was an obligate anaerobic bacterium. Wilkins and Gordon (341) reported that, in addition to mucus-like substances, undegraded dietary components also contribute to the "colloid pool" of the germ-free lower bowel.

It appears that the impaired water absorption caused by the lack of the intestinal flora is conditioned also by another mechanism. Asano (8–10) observed slightly reduced sodium and very low chloride ion concentrations in germ-free rat cecal contents. Feeding chloride-yielding resin to these animals increased the chloride

levels in gut contents and considerably improved water absorption from the lower bowel. Thus, the deficit of these ions in the germ-free gut, which are needed for maintaining "solute coupled water transport" across the intestinal mucosa (55), might also be involved in the impairment of water absorption in these animals. The mechanism by which the flora increases the availability of these ions in gut contents is unknown.

Other organ systems. Only a few and isolated observations were made on other organs of germ-free and conventional animals. The work of Reyniers et al. (270) made an attempt to account for the weight of all tissues in chickens. In addition to the characteristics already described, they found that the weight of the skin was consistently reduced by approximately 10% in the germ-free group. For all of the weight reductions observed in various organs of these animals, no organ system appeared to stand out with a decided weight increment in comparison to conventional controls, with the possible exception of the skeleton, which indicated higher values in the germ-free group.

Histologic studies of the lungs of old germ-free mice (98) showed, in general, considerable thinness of the alveolar and of the adjacent capillary wall and a paucity of reticuloendothelial elements similar to that seen in young germ-free or conventional mice. In old, normal, conventional controls the alveolar wall was two to three times thicker, holding an abundant amount of wandering cell elements. Studying the "washout time" of intravenously administered labeled xenon in old rats, better ventilatory clearance was consistently found in the germ-free group in comparison to conventional controls (291). These observations suggest that the age-induced impairment of the lung's respiratory surface is at least in part caused by the presence of the flora in the respiratory tree.

Flora effects on liver function have previously been mentioned (*See above*). Blood urea nitrogen as well as the concentrating ability of the kidneys (ratio of urine and blood plasma osmolality) and cortical or medullary blood flow were found essentially similar in germ-free and conventional rats (324).

Among endocrine functions, the reduced iodine fixation by the thyroid in germ-free rats has already been mentioned (61). Reports on the adrenal glands of germ-free animals are conflicting. Wakabayashi et al. (334) reported lower weight of these glands and evidence of cortical hypofunction in germ-free rats. Gordon et al. (104) found slightly elevated adrenal weights and levels of urinary corticoids in similar animals. Chakhava et al. (41) observed increased adrenal weights and ascorbic acid concentrations along with elevated levels of glucocorticoids in the blood of young germ-free guinea pigs. It is possible that differences in species, strain, housing, and diets used (including various degrees of nonspecific stress phenomena induced) contributed to the inconsistency of the results. Recently, Ericsson and Gustafsson (77) studied the fate of parenterally injected labeled steroids and found more of the sulfurylated forms in the urine and feces of germ-free rats, whereas in conventional controls the free forms prevailed. The authors speculate that the sulfohydrolase activity of the intestinal flora is responsible for this difference.

Body Defenses

As mentioned in the introduction of this paper, one of the original incentives for gnotobiotic experimentation was the study of body defenses. The early work comparing germ-free and conventional guinea pigs carried out by Glimstedt (94) in Hellman's laboratory in Lund aimed largely at answering the question: is the lymph follicle a germinal center (i.e., a constant producer), or does it respond mainly to extrinsic stimuli with proliferation of lymphoid tissue in the form of a reaction center? The germ-free animal, it was thought, would clarify the issue because of its lack of exposure to antigenic stimulation by viable microbes. Glimstedt's work, incidentally, favored the latter alternative.

In the years that followed, the concept of the germ-free animal as an "antigenic virgin" was made less useful by several practical considerations. One of these is that freedom from foreign associates is not assured by absolute criteria, but it is limited by the effectiveness of the tests for sterility (*see above*). Furthermore, virtually all available animal diets contain microbial or other antigenic substances, which persist in some form after sterilization and may lead to oral immunization (*see below*). Finally, the possibility of nonspecific stress reactions modifying the body defenses of germ-free animals must be considered. Stress, causing loss of lymphoid tissue, among other effects, may be precipitated by inadequate nutrition and the effect would be hard to differentiate from a deficit due to lack of antigenic stimulation. Deficiencies in germ-free diets caused by rigorous steam sterilization of both milk formulas and solid rations have abounded in the past (267) and still present problems. Nowadays, almost complete freedom from antigenic stimulation (save for that caused by auto-antigenicity), with minimal effects from nutritional stress may be observed in germ-free rodents which are fed chemically defined antigen-free liquid diets. In this area the colostrum-

deprived germ-free neonatal pig has also proven useful. Using more practical rations, we must be satisfied with compromise regarding dietary antigens.

Cells, tissues, and organs associated with defense functions. No pronounced differences have been detected in the number, distribution, and phagocytic ability of macrophages in lymph nodes and spleens of germ-free and conventional mice (19, 21). Phagocytic indexes studied in the liver and spleen of these animals with carbon clearance method were similarly undifferentiated (63, 317).

The functional similarity of macrophages originating from both groups of animals was suggested by Heise and Myrvik (134), who found similar concentrations of lysozyme, acid phosphatase, and cathepsin in macrophages obtained from the peritoneal cavity of germ-free and conventional rats. However, when these cells originated from the lung alveolar wall, then the concentrations of these enzymes were found decidedly lower in the germ-free group. Recently, Perkins et al. (224) have shown that, on intraperitoneal injection of a sterile irritant (gelatin), conventional mice respond with significantly greater production of phagocytic cells (termed histiomonocytic) than do germ-free mice. However, the "engulfing efficiency" of individual cells for foreign erythrocytes (opsonized sheep red blood cells were used) in vitro was essentially the same, regardless of whether the cells originated from germ-free or from conventional animals.

The total white blood cell count in germ-free rodents occasionally shows lower values in comparison to open conventional controls; there has not been a consistent pattern in the differential count. In one series of observations (104), when germ-free rats were compared to isolator conventional controls, the quantitative and qualitative white blood cell counts were found to be identical in the two groups of animals. However, germ-free chickens up to 1 year of age showed consistently reduced total white blood cell counts, which were due to a lower number of lymphocytes (270).

Undeveloped lymphoid tissue and smaller lymph nodes are characteristic of young, first generation germ-free guinea pigs (3, 94, 197, 202). The same is found in germ-free rats and chickens, but smaller lymph nodes are found mainly in areas which are normally closely associated with the microbial flora (104, 110, 270). According to other workers (19, 20), the difference between the lymphatic systems of germ-free and conventional mice was not impressive.

Recently, active germinal centers (3, 19) and all phases of the cytopoietic activity of the lymph nodule have been traced in germ-free animals (316, 318). All authors agree that the lymphatic nodules of germ-free animals are, in general, less clearly demarcated, show fewer mitoses, are smaller, and occur more rarely than in conventional counterparts.

Abrams and Bishop (3) found in the intestinal mucosa of young germ-free guinea pigs, that "the population of wandering cells in the lamina propria is much less dense than in controls. Although varying in absolute number, plasma cells in particular were less numerous in this location in germfree animals." These findings have been repeated in the same animals (302) and in the gut of germ-free rats and chickens (103).

Gamma globulin-bearing plasma cells (tested with fluorescence technique) were virtually absent in mesenteric lymph nodes of germ-free mice, whereas they were numerous in conventional controls. Gamma globulin-containing plasma cells were absent from the spleens of both germ-free and conventional mice (144). In a similar comparison, plasma cells from the submaxillary lymph nodes of mice aligned with those of the mesenteric lymph nodes of the previous study, whereas plasma cells from the axillary and popliteal nodes behaved similarly to those of the spleen (19). Recently Crabbé et al. (51) used fluorescence techniques to show that the intestinal mucosa of conventional mice is rich in plasma cells synthesizing immunoglobulin (IgA); germ-free mice showed only 10% or less of this type of cell. They later indicated that germ-free mice would produce IgA-containing plasma cells if the antigen (horse spleen ferritin) was administered by mouth; parenteral administration led to IgM- and IgG_1-containing plasma cells (52).

Olson and Wostmann (215) attempted to determine, with the help of ^3H-thymidine and differential counts, the quantity of potential antibody-producing cells in cervical and mesenteric lymph nodes of germ-free and conventional mice. The lymph nodes of germ-free animals contained as little as one-twelfth the number of blast and potential antibody-producing cells as their conventional counterparts. The mode and quantity of label incorporation in cells, when it was actually taking place, was similar in both groups. In another experiment, the same authors (216) stimulated mice with intraperitoneal injections of 7*S* human gamma globulin or with a vaccine prepared from killed cultures of *S. typhimurium*. Potential antibody-producing cells showed a greater increase in germ-free mice after the challenge with either antigen in com-

parison to conventional controls. This finding suggested that, although germ-free hosts have fewer competent cells, the greater increase after challenge reflects their "uncommitted" nature which is due to the lack of previous antigenic stimulation. Bosma et al. (32) and Nordin (211), attempting to assay the immune response in germ-free and conventional mice on parenteral challenge with sheep erythrocytes, found that antibody formation in the spleen proceeded in essentially the same manner in both groups of animals.

Thus, it appears that the available stock of immunocompetent cells of germ-free animals is smaller in comparison to conventional controls, particularly in organs of the external environment. The quantity of potential antibody-producing cells of germ-free hosts, however, seems to be only slightly reduced or at the conventional levels, depending on the nature of the parent organ and on the criteria used for testing cell response. The existence of qualitative differences, in this sense, between the two groups of animals can be excluded.

Wilson et al. (343) reported a slightly smaller weight of the thymus in germ-free mice. Bealmear and Wilson (24) observed the lymphocyte population in the thymus of mice to ca. 5 months of age. They found in the germ-free group a lower number of small lymphocytes in the cortex and fewer cells of this type migrating from the cortex into the medulla in comparison to conventional controls. These observations suggested that the presence of the microbial flora influences the event which causes the spread of thymic lymphocytes to other lymphoid organs.

The thymus of germ-free chickens has provided analogous findings (316, 318) although the deficit in weight of this organ could not be demonstrated (270). The bursa of Fabricius (cloacal tonsillar organ of birds which shows certain analogies with the thymus) loses weight significantly in the germ-free state (270). The histological appearance of the bursal lymphatic follicles did not change. Broadly speaking, the deficit of lymphoid tissue of germ-free animals appears to apply also to these organs.

Humoral defensive elements. Thorbecke et al. (318) observed that germ-free chickens have undeveloped lymphatic tissue with fewer plasma cells and lower gamma globulin content in their blood serum in comparison to conventional controls (25 to 50% of the conventional values). The levels of beta and gamma globulins in the serum have been found reduced also in germ-free rats, whereas other fractions of serum proteins are within normal limits with the possible exception of an albumin increase (124, 357). Grabar et al. (112) using immunoelectrophoretic techniques showed that the serum of germ-free rats contained qualitatively the same major antigenic components which are observed in the serum of conventional rats. Injection of germ-free rat serum into rabbits produced the entire spectrum of anti-gamma antibody at low levels which is otherwise found on immunization with conventional rat serum. Fahey and Sell (82) observed that germ-free or specific pathogen-free (SPF)-type mice had low levels in all four major components of serum immunoglobulins that are characteristic for this species, but particularly in the case of IgA. Arnason et al. (7), working with mice, found reduced levels of alpha$_2$ and gamma globulins in the germ-free group in comparison to conventional controls. In the case of one gamma globulin component (IgM), however, similar levels were found in both groups of animals.

The composition of the diet seems to have little effect on the rat (364) although effects of the dietary regimen on defensive elements have been reported in other animal species. Of these, the guinea pig and the mouse are best documented (214, 294, 365). The original reports of Šterzl et al. (306, 307), that colostrum-deprived sterile piglets had no trace of antibody, introduced another model for studying "antigenic virgins." Kim et al. (157), delivering piglets in germ-free form, depriving them of colostrum, and giving them only pyrogen-free distilled water, found their sera to be completely free from detectable immunoglobulins. The absence of maternal antibodies appeared to be related to the more impervious placenta which characterizes this species. On oral or parenteral administration of antigens, these piglets proved immunologically fully competent.

Concerning the synthesis and metabolism of immunoglobulins, Nash et al. (207) found that, on parenteral or oral administration of ferritin, germ-free mice were capable of developing circulating antibodies as well as gamma globulins without affinity for the antigen (non-antigen globulins). Sell and Fahey (295) determined total gamma globulin and observed the turnover of labeled homologous and heterologous gamma globulin in germ-free and conventional mice. In this work, the following trends were indicated. The rate of synthesis of gamma globulin in conventional mice was approximately 50 times higher than in germ-free mice. The greatly reduced serum concentrations of gamma globulin found in germ-free mice reflected the lower rate of synthesis. The ability to catabolize heterologous gamma globulin was found essentially similar in germ-free and conventional mice.

Fahey and Sell (82), using immunoelectrophoresis techniques, studied the catabolism of five classes of immunoglobulins in mice. Three classes of 7S components appeared to be affected in their catabolism by the levels of immunoglobulins in the serum and to share a common control mechanism. The turnover rate for these components was found slowed in germ-free mice with low levels of gamma globulins, whereas in conventional mice hyperimmunized with hemocyanin the turnover rate was accelerated. The two other classes (IgA and IgM) showed a faster turnover rate, independent of the 7S components, which suggested that their catabolism is not influenced by their concentration in the serum. In germ-free and conventional guinea pigs, Sell (293) found essentially the same rate of catabolism of gamma globulin and in either case no significant loss of this component into the intestinal tract. The greatly reduced levels of gamma globulin found in the serum of guinea pigs suggest lower rates of synthesis for this serum component in the germ-free state.

Wagner (328) has reported preliminary data on properdin, complement, and complement component titers in the serum of rats and observed that the levels of all three approached the lowest limits of the conventional normal range in the germ-free group. It was noted, however, that the sera of germ-free rats showed positive agglutination titers with *M. epidermidis* (an organism usually demonstrable in the diet prior to sterilization); therefore, these rats could not be considered as minimally stimulated animals. These observations on properdin were later confirmed (126). Newton et al. (210) compared complement levels of germ-free and conventional guinea pigs with similar results and found also an age-conditioned increment of complement in both groups.

Immune responses. No systematic studies on autoimmunity have been conducted to date in normal germ-free animals. However, the consequences of necrotizing liver lesions in germ-free and conventional mice have been studied after intraperitoneal injection of carbon tetrachloride (7) which led to the development of anti-liver antibodies in the serum of both groups of animals. Similar studies with the hepatotoxic agent of butter yellow (3'-methyl-4-dimethyl aminoazobenzene) indicated that both groups of animals, particularly the germ-free, developed liver injury, and both groups showed hypergammaglobulinemia with increased activity of lymphatic tissue (23). These experiments suggest that the germ-free host can give an autoimmune response but the lack of the use of chemically defined diets in these experiments leaves the possibility that foreign antigens play a part.

The presence of antibodies against tumor antigens has also been considered as an autoimmune response of the host. Pollard (245) found in germ-free mice that fibrosarcomas developing on injection of sterile methylcholanthrene caused increased activity of lymphatic tissue in the regional nodes of the involved area. In germ-free rats, on the other hand, in which mammary tumors were induced by feeding the same carcinogen, regional lymph nodes did not show a response. The possibility that under these circumstances the "unmasking" of a latent viral agent may have contributed to the indicated immunologic response of lymphatic tissue was suggested by the fact that, in general, germ-free mice may be and germ-free rats may not be considered carriers of virus. Olson and Burnstein (217), studying the experimental allergic encephalomyelitis by injecting autoclaved rabbit spinal cord-Freund's emulsion in germ-free and conventional rats, found on various immunological tests similar antibody responses in both groups. The incidence of paralysis, however, was lower among germ-free animals.

Thus, these very limited observations with germ-free animals concur with the concept that the host is capable of producing autoantibodies. Problems of central interest in this area, e.g., the action by which tolerance to "self" is normally achieved, or the mechanisms by which autoimmune disease is precipitated, have not been taken up to date with this highly suitable tool.

Germ-free animals that have not undergone any infection or overt antigenic stimulation show, nevertheless, detectable levels of antibody against a variety of antigens: in chickens, hemagglutinins for rabbit erythrocytes (326, 328) in mice, antibodies to certain strains of *E. coli* or to *S. typhosa* (151, 193) and antibodies to staphylococcus antigen A (45). In general, in germ-free animals, the levels of antibody were much lower than in conventional controls.

A good example of the complexity of the issues involved in this area is given by the work of Stollerman et al. (308) who tested germ-free mice and colostrum-deprived piglets for the presence of antibodies to streptococci. On intraperitoneal administration of standardized cultures of group A streptococci lacking both M protein and capsules, the median lethal doses (LD_{50}) for germ-free and conventional control mice were essentially similar. However, when encapsulated and M protein-containing forms of the same strain of microorganisms were injected, the germ-free mice showed greater susceptibility than the conventional controls. Inter-

estingly, resistance could be imparted to germ-free mice if they received, prior to the challenge, an injection of whole globulin from conventional mice. Administration of gamma globulin of the same source alone proved ineffective. In vitro studies conducted on phagocytosis of avirulent group A streptococci with the blood of germ-free mice indicated that leukocytes from these animals destroyed the streptococci as readily as leukocytes from conventional controls. Opsonins against the avirulent streptococci were readily demonstrable in the blood of the colostrum-deprived piglets (whose serum contained only minute amounts of gamma globulin).

The indispensability of antigenic stimulation for the rise of elements that are considered to correspond to natural antibodies of the host has been indicated to date by the work of Kim and Watson (159), who aimed to study whether the "Jerne background cells" detectable by plaque assay technique (which are considered to correspond to natural antibody of the host) rise spontaneously or only by antigenic stimulation. Colostrum-deprived germ-free piglets fed Mull-soy diet failed to develop plaque-forming cells to sheep erythrocytes for a period of 2 months. However, on parenteral challenge by this antigen, the piglets proved fully immunocompetent. This true primary response to sheep erythrocytes was reduced when the piglets were exposed to unrelated antigen prior to the challenge. This suggests to the authors the presence of an "uncommitted multipotent system in these immunologically virgin animals."

Lysozyme levels in serum, saliva, and tears of germ-free and conventional rats and mice were studied by Makulu and Wagner (183). The results show that the lysozyme levels in the serum and in the tears were quite similar in all experimental groups. In saliva, on the other hand, substantial levels of this agent were found in the conventional animals, whereas in the saliva of the germ-free stock it was absent. The missing salivary lysozyme correlated with the absence of leukocytes in the saliva of germ-free animals and they seem to be the source of lysozyme. Broader issues in this area, e.g., natural resistance versus natural immunity, canonot be discussed because of a paucity of experimental data.

Germ-free and conventional chickens appear to respond with antibody formation on intravenous injection of bovine serum albumin and human gamma globulin (328, 363). Germ-free rats injected with bovine serum albumin by Wostmann (351) showed perceptible reactions only if the albumin was mixed with Freund's incomplete adjuvant. Albumin or adjuvant by itself was ineffective. On combination of the two, gamma and particularly the beta globulin fractions in the serum of the germ-free rats rose. At the same time, slight but unmistakable levels of anti-bovine serum albumin precipitating antibody became demonstrable which appeared to coincide with the elevation of gamma globulin in the serum. In germ-free and conventional mice, a glycoprotein or bovine serum albumin proved to be equally potent antigens without the addition of Freund's incomplete adjuvant (237). A special case of milk allergy in infant germ-free rabbits provides further evidence of ability for germ-free hosts to respond to foreign protein (44).

An excellent review on the actions of endotoxin which includes aspects of gnotobiotic experimentation has recently been written by Kováts (162). The majority of systematic works done in this area in gnotobiotic and related animals concerns host susceptibility and resistance to endotoxin.

Thorbecke and Benacerraf (317), studying the blood clearance of heat-killed *E. coli* in germ-free mice, found it perceptibly slower than in conventional controls. These observations suggested, in general, that the exposure of a host to microbial agents may modify its response to endotoxin. Schaedler and Dubos (286, 288) studied a variety of strains of mice. One of these strains (NCS) had an intestinal flora consisting predominantly of lactobacilli and devoid of *E. coli*. All other strains contained, among others, *E. coli*. A massive intraperitoneal dose of endotoxin (400 to 500 μg of lipopolysaccharide prepared from *E. coli* or *K. pneumoniae*) produced no mortality among the "*coli*-free" NCS mice although a variety of lesions (e.g., inhibition of influx of polymorphonuclear leukocytes into the peritoneal cavity) developed in these animals. High mortality was observed among all of the "*coli*-associated" mouse strains. Pretreatment of NCS mice with a vaccine which consisted of organisms from which the endotoxin used had been prepared protected them from the developopment of endotoxin-induced lesions. This pretreatment, on the other hand, facilitated the development of infections or even of fulminating septicemias when the NCS mice were successively challenged with unrelated, viable pathogens (staphylococci were used). The simultaneous administration of the pathogens and even a minute dose of endotoxin to these pretreated mice protected them largely from the lethal effects of the pathogen's challenge. These observations suggest that a lack of exposure to endotoxin will cause the host to be resistant to the lethal effects of endotoxin. This challenge, however, will render the host more susceptible

to the invasion of pathogens, probably by the impairment of its natural defenses (illustrated by the inhibition of reticuloendothelial elements). Jensen et al. (153) corroborated these findings and observed that germ-free mice monoassociated with viable *E. coli* remained fully resistant to the lethal effects of endotoxin injections in the same fashion as germ-free, or "*coli*-free" (SPF), mice were. These results suggest that the concurrence of other microbial effects (present in the conventional but absent in the *E. coli* monoassociated or "*coli*-free" SPF gut) is needed for the potentiating effect of *E. coli*. Landy et al. (167) observed that in mortality to *Shigella flexneri* endotoxin and in pathological responses to *Salmonella enteritidis* endotoxin no essential differences could be demonstrated between germ-free and conventional mice harboring *E. coli*. The conflicting findings in this area may, in part, be resolved by the work of Sacquet and Charlier (279) who found a considerable animal-strain dependence among germ-free, "*coli*-free" (SPF-type), and conventional mice in their sensitivity to the untoward effects of endotoxin.

It would appear that the lethal effect of parenterally administered endotoxin is dependent on the previous sensitization of the host by intestinal microorganisms which can serve as a source for endotoxin, possibly in association with other microbes. Whether the development of this sensitivity to endotoxin of conventional or other microbially associated animals is connected with their resistance to infections remains to be seen. Informative experiments may involve antigenically virgin neonatal animals. In a study conducted by Kim and Watson (158), colostrum-deprived, germ-free newborn piglets which were free from immunoglobulins responded to graded doses of endotoxin with a considerably lower LD_{50} than did the colostrum-fed controls. (The latter showed a variety of immunoglobulins in their blood within hours after feeding.) These data suggested to the authors that endotoxin (which in this instance was prepared from *S. abortus equi*) has a true primary or intrinsic toxicity which is not dependent on classical antigen-antibody reactions. The colostrum-imparted protection is thought to result from an antiendotoxin, distinct from O antibody, comparable to the 19*S* class of protective antibody.

The effects of *E. coli*, because of its importance as a frequent element of the intestinal flora, have been repeatedly studied. Monoassociation of young germ-free guinea pigs with a viable *E. coli* originating from the human gut was found well established in the intestinal canal with the animals surviving in high percentage (87). Blood clearance and organ distribution of a strain of ^{32}P-labeled heat-killed *E. coli* were studied in germ-free and conventional mice nonimmunized or immunized with the same heat-killed microorganism (317). The results indicated that in nonimmunized mice the clearance of *E. coli* from the blood of germ-free mice was markedly slower than in conventional controls. Immunization effected a similar enhancement of the clearance rates for *E. coli* in both groups. The recovery of the microbial label from various organs of nonimmunized animals showed the same percentage values when germ-free and conventional mice were compared; after immunization, both groups of mice responded with a substantial increment of the bacterial label recovered from the liver, with a reduction of radioactivity in the spleen, lungs, kidney, and blood. Using a strain of *S. aureus* in nonimmunized mice in the same type of experiment, these authors found, contrary to the results with *E. coli*, similar and very rapid blood clearance of the microbial label in both germ-free and conventional mice. A variety of responses in germ-free and isolator conventional control mice after the injection of Formalin-killed *E. coli* into the foot pad were studied by Horowitz et al. (145). Particulate antigen appeared in the sinus macrophages of regional lymph nodes as early as 2 hr after the injection in both groups of animals and seemed to disintegrate more rapidly in the conventional controls. Circulating antibody to the *coli* antigen developed in all mice after 4 days, but the conventional controls attained higher titers. In view of the fact that the conventional mice in this experiment may have possessed preexisting antibody to *E. coli* of other serotypes, Bauer et al. (22) repeated this experiment with *Serratia marcescens*, an organism that was not harbored in their conventional stock of animals. The results, in essence, confirmed those obtained previously with *E. coli*. It was indicated that the delayed immune response in germ-free mice resulted in a more sustained and greater response than in conventional controls, presumably through a slower release of immunogenic antigen fragments. Less vigorous but qualitatively similar responses were recorded with ferritin, as a nonmicrobial antigenic challenge. These authors conclude that germ-free animals in responding to antigenic stimulation show considerable qualitative similarity to conventional controls. The conventional state of an animal, in respect to the challenge used, seemed to confer only minor advantages to the host.

Monoassociation of germ-free chickens with *Clostridium perfringens* or with *Streptococcus faecalis* by the oral route resulted within several weeks in levels of serum proteins, counts of reticuloendothelial elements in the gut wall, and

in homologous serum agglutinin titers which reached or were close to those seen in conventional controls (332, 333). In rats, similar microorganisms (and *L. casei* as well) demonstrably stimulated agglutining formation but had less clear effects on the serum electrophoretic pattern.

It has been uncertain whether bacteria present in the diet prior to sterilization and ingested in dead form by the animal are capable of eliciting antibody response on oral vaccination. Wagner (328) showed this to be the case in the chicken in reference to *M. epidermidis*. In comparison to conventional controls, germ-free chickens showed delayed antibody formation to this organism and in low titers.

In view of the known great complexity of the host's immune responses to bacteria and the fragmentary information available, there is, as yet, little on which to comment. On exposure to living or dead, potentially pathogenic or innocuous bacteria, the conventional animal is in a more advantageous state for mustering its defenses than its germ-free counterpart. As pointed out by Bauer et al. (22), this may result from the conventional animal's continual exposure to the flora. However, on reaching the ultimate state of adaptation to the challenge, there is no essential qualitative difference between the two groups of hosts, except for some delay of response in the ex-germ-free group. The apparent modification of bacterial pathogenicity in the positive or negative sense according to the presence or absence of other microbial associates is one of the more promising avenues of gnotobiotic experimentation.

Tissue Repair

The study of tissue repair in gnotobiotic animals has been undertaken mainly to assess the role of the microbial flora in wound healing and consider the changes in inflammatory response.

Puzzling species differences have been found in the repair of skin wounds in young, first-generation germ-free guinea pigs (199). In the germ-free animals, there was less formation of vascular tissue and the regeneration of the epidermis preceded that of the dermis. The development of the former occurred over a loose mesenchymal base rather than over granulation tissue as it did in conventional controls. This experiment has been repeated and confirmed in most of its findings, except that no differences in vascularization has been found in the newly formed tissue between germ-free and conventional animals (319).

On the other hand, both young adult and senescent germ-free and conventional mice showed no gross and histological differences in the healing of surgical or tooth extraction wounds (274, 275, 277). The inflammatory cell response, vascular characteristics, proliferation of fibrous tissue, and regeneration of the epidermis were the same in both groups of animals.

Collagen production (as measured by hydroxyproline content of polyvinyl sponges implanted in skin wounds) seemed to show no quantitative differences between germ-free and conventional mice (35).

The differences between guinea pigs and mice in the effect of the flora on tissue repair cannot be explained without further experimental evidence. Differences in animal species, in their nutritional status, nature and site of injury, and composition of the implicated flora may play a role. The observations in mice suggest that the presence of the flora does not exert a profound effect on healing in case of simple and clean wounds involving mainly epithelial and connective tissue.

Survival and Aging

The value of gnotobiotic animals for the study of aging has long been recognized, but its implementation was delayed by technical difficulties. Two attempts have been made to construct life tables and to determine lesions at natural death in germ-free and conventional animals. One study (105) was conducted in genetically closely linked Swiss Webster mice and included over 300 germ-free and the same number of conventional controls which were introduced into the colony at the age of 12 months (to eliminate the effect of early losses). At natural death, the ages of the mice were (means and standard errors in days are given): germ-free males, 723 $\pm$ 19; females, 681 $\pm$ 12; conventional males, 480 $\pm$ 10; females, 516 $\pm$ 10. This pattern of survival rates seemed to continue throughout the course of the experiment. In the second study (335), approximately 50 germ-free and the same number of conventional ICR mice were introduced into the colony after weaning. At natural death, the age of the mice was (using the same mode of expression): germ-free males, 556 $\pm$ 43; females, 535 $\pm$ 46; conventional males, 536 $\pm$ 41; females, 547 $\pm$ 45. In the first trimester of life, the survival rate was essentially the same in the germ-free and conventional control groups. In the middle third, the germ-free mice displayed an increased survival rate (e.g., 40% cumulative mortality was reached for the combined group of germ-free males and females only at the age of approximately 580 days, whereas for the conventional

controls this value was approximately 410 days). Increased mortality of the germ-free group at more advanced age resulted in the similarity of mean ages between the opposing animal groups when all animals participating in the study were considered. Among germ-free mice of both studies, the prevalent lesions at natural death were associated with the enlargement of the cecum. It appeared that the muscle tone of the gut became reduced to the point where propulsive movements were severely impaired or came to a complete standstill. Numerous cecal volvuli were observed. Occasionally, the gall bladder was severely distended. None of these lesions was observed among conventional controls in which inflammatory lesions prevailed. Degenerative changes of parenchymatous organs were observed in both groups of animals. Neoplastic diseases seemed to be evenly distributed among germ-free and conventional animals in both studies. In senescent germ-free rats fed improved diets which were killed in apparent state of health, no frank lesions were observed save for the low incidence of benign tumors (249).

The survival studies clearly indicate an advantage for the germ-free over the conventional mice, with the exception of the accelerated terminal loss of germ-free animals experienced by Walburg and Cosgrove (335) which is not understood. It is possible that this discrepancy was due to the different strains of animals and diets used in these studies or to the low animal population in the second study. In terms of meeting dietary requirements, both investigations agree that the presently available autoclaved diets are suboptimal for aging studies. The apparent similarity of survival between males and females in the germ-free group, in contrast to generally higher female survival in conventional populations, leads to the speculation that the latter characteristic is related to the female's known greater resistance to infection (338). Aside from such considerations, none of these studies can answer the broad question of whether germ-free animals live longer than their conventional counterparts. In view of the findings to date, though, such a query is almost meaningless. It is entirely conceivable that, for example, strains of animals prone to anomalies of the lower bowel, yet naturally resistant to infections, will do poorly in germ-free and better in conventional aging colonies and vice versa. The important fact emerging from this work is that the pattern of lesions conducive to death is completely different in animals exposed or unexposed to the microbial flora.

THE GNOTOBIOTIC ANIMAL IN THE STUDY OF DISEASE

Simple and reasonably inexpensive gnotobiotic techniques are of such recent development that logical extensions of the experiments into broad areas of disease have not been undertaken. The following section represents the meager yet promising collection of work available.

Shock, Intestinal Obstruction, and Thermal Trauma

It has been postulated that the development of shock initiated by various types of trauma is mediated primarily by a breakdown of bacterial defense mechanisms and an attendant vasculotoxic sequela caused by the passage of flora elements or of their products into the circulation (Jacob *et al.* (152, 292). Therefore, it was important that Zweifach et al. (368) were able to induce irreversible hemorrhagic shock in germ-free animals. These authors and others that followed them (104, 136, 206) found that survival and the responses to bleeding and to the hypotensive episode were essentially the same in germ-free and conventional rats. It is interesting to note that, in the study of Zweifach et al. (368), germ-free rats accidentally contaminated with *S. faecalis*, *M. pyogenes* var. *aureus*, *M. epidermidis*, and *Alcaligenes* sp., in spite of the fact that these microorganisms could be grown from the blood and from various organs after the shock episode, did not differ in their responses from the other animals of this series. In Heneghan's work (136), surprisingly, a group of germ-free rats contaminated with *S. albus*, a yeast, and a fungus displayed substantially greater resistance to shock than the germ-free or the conventional groups.

Microbial effects in ischemic shock have been studied in our animal model by limb tourniquet and by ligation of superior mesenteric artery. In the former approach, Einheber and Wren (75) found that 3 hr of limb ischemia was rapidly and uniformly lethal in germ-free and conventional mice. This work was repeated by Markley et al. (185) who obtained different results by finding that shock mortality of germ-free mice was significantly lower than in conventional controls. Furthermore, antibiotic-treated (sulfadiazine and streptomycin) conventional mice showed decreased mortality to limb ischemia in comparison to untreated conventional controls. On ligation of the superior mesenteric artery, Carter and Einheber (39) found essentially similar mean survival times and uniformly high mortality in germ-free and conventional mice. However, in case the occlusion of the artery was released after

a few hours duration, then in the germ-free group a markedly shorter survival time was indicated than in the conventional group.

Thus, the majority of the findings in this area are opposed to the concept that bacteria or bacterial products are implicated as primary factors in the pathogenesis of shock in rodents. The observation that during the shock episode certain types of microorganisms may penetrate and be present in various organs of the host with apparent impunity corroborates this assumption. Yet, the more rapid death of germ-free mice on occlusion release of the mesenteric artery suggests perhaps greater complexity of this issue. It is possible that in germ-free animals during the ischemic episode bioactive substances (which abound in the gut of these animals) permeate from the intestinal lumen into the tissues, passing on into the circulation after the occlusion release. This suggests that the underlying mechanisms of shock in our opposing animal groups are different, at least in part, whereas the net result may be the same as measured by survival or mortality.

Severe intestinal obstruction (closed loop obstruction, by placing two ligatures spaced 5 cm from each other on the ileum) with intact blood circulation maintained was found lethal both for germ-free and conventional rats; however, the mean survival was found to be considerably longer in the former group (6). A similar trend was found when a single obstructing ligature, strangulating both arterial and venous blood flow, was placed on the ileum of germ-free and conventional rats (47) or dogs (313). It is interesting to note that, in rats, when a full dose instead of a minimal of anesthetic (pentobarbital sodium) was used, the survival of the conventional group was prolonged to values found in the germ-free group. The effect of single intestinal obstructing ligatures combined with arterial, venous, or arteriovenous strangulation in germ-free and conventional rats was studied by Yale and Altemeier (367). The results showed that all rats with the obstructing ligature died; however, the mean survival time was about twice as long in the germ-free group in comparison to the conventional controls. Simultaneous strangulation of veins, arteries or both types of vessels supplying the test segment did not appear to affect profoundly the survival margins of these animals, except in case of venous strangulation which proved highly lethal. Strangulation of vessels alone, in reference to a test segment of similar length, without intestinal obstruction, was generally survived by germ-free rats, with the exception of venous strangulation, in which case relatively long survival time was noted. In conventional controls, venous strangulation of this type proved most lethal, with the two other types of vascular strangulation leading to death only after a more prolonged period of time.

These results, in general, emphasize the importance of the intestinal bacteria to the apparent severity of the trauma caused by intestinal obstruction, particularly when it is combined with venous strangulation. The indication that deeper anesthesia in the conventional animals relieves to some extent this trauma is of special interest. The absence of bacteria from the gut helps but does not afford protection in such situations, save for cases of isolated blockage of arterial blood supply.

In terms of thermal trauma, Markley et al. (186) found significantly less shock mortality and an indication of reduced late mortality in germ-free mice which were exposed to severe or moderate hot water burns, in comparison to conventional controls.

Wasting Syndrome

The wasting (or "runting") syndrome is a consequence of neonatal thymectomy of conventional animals that may be accompanied by depletion of lymphocytes, diminished capacity to produce serum antibody, and failure to reject tissue grafts. The use of germ-free animals aims at searching for a microbial variable in this phenomenon. Miller (195) originally indicated that the mortality of neonatally thymectomized mice was significantly reduced in a group of animals that were kept in "nearly pathogen-free conditions," whereas Wilson et al. (345) and McIntire et al. (205) showed that germ-free mice thymectomized 24 hr after birth did not, but conventional controls did, develop the wasting syndrome. Furthermore, thymectomy had no effect on the lymphocyte populations in lymph nodes of germ-free mice; spleen cell grafts were rejected, indicating that anti-host immune reactions can operate also in the germ-free host. Bealmear and Wilson (26) observed no particular depletion of granulo- or agranulocytic white blood cells in thymectomized germ-free and conventional mice.

The resistance of germ-free animals to the development of wasting syndrome elements was further demonstrated by the following experiment. Bealmear and Wilson (25) observed that methylcholanthrene-induced homologous tumor cells which were rejected upon transplantation into thymectomized germ-free mice, could find acceptance if such hosts were previously exposed to an immune-depressant dose of X irradiation. If, however, the thymectomized germ-free mice were permitted to recover from radiation and the transplantation was made at that time (2 months after irradiation), the grafts were then rejected. In thymectomized conventional mice, tumor homo-

grafts were generally accepted, even without X irradiation. This illustrates that the neonatal germfree host, after considerable reduction of its lymphoid tissue by thymectomy and after irradiation-induced, transient depression of its defenses, is eventually able to muster homograft rejection-type immune response. The action of the flora on the conventional host, "when it has been deprived of most of its lymphoid tissue, appears to overtax its immunologic defense and to induce a debilitated state in which it can not successfully respond to homotransplant." In general agreement with these findings are the results of Reed and Uotila (260), who observed lower mortality in a group of germ-free mice where wasting disease was induced by administration of cortisol acetate. Ekstedt and Nishimura (76), who were inducing runting (wasting) disease with parenteral injections of autoclaved staphylococci or streptococci, found germ-free mice more resistant than conventional controls. However, the germ-free mice could be induced to runt if the homologous antiserum was mixed to the antigen. The authors speculate that the complexed antigen-antibody possibly triggers the destructive response in susceptible lymphoid tissue. Differences in resistance of thymectomized mice to the wasting disease appear to be conditioned also by the animal strain (60). These observations support the concept of the existence of a microbial contribution to the post-thymectomy impairment of the host.

Oral Diseases

The use of gnotobiotic animals in the study of oral diseases represents one of the oldest, best documented, and most fruitful applications of this biologic tool. A review on this area was written by Fitzgerald (83). Rats were mostly used; hamsters, cotton rats, and rice rats, which are especially susceptible to oral disease, are not yet available in gnotobiotic form.

Dental caries. The early observations of Orland et al. (220), which have shown that dental caries do not develop in germ-free rats regardless of the susceptibility of the strain, the nature of the diet fed, and the age of the animals, remain unchallenged. Various authors have shown that single species of bacteria indigenous to the gastrointestinal tract may be implicated in this process in rats fed high sugar, low fluorine diets. Orland et al. (221) observed that gnotobiotic rats harboring an enterococcus and a bacterium developed caries. Fitzgerald et al. (86) found that certain streptococci isolated from the intestinal tract of conventional rats were cariogenic. Other microorganisms which were observed to produce caries as monoassociates in ex-germ-free rats include the following: a variety of *L. acidophilus* (85), *S. faecalis* (330), and a strain of *L. casei* (273). Testing of caries-producing properties in gnotobiotic rats with microorganisms isolated from the human mouth was first attempted by Orland (219). A *Lactobacillus* sp. used by him proved cariogenic but only after the gnotobiotic rats were monoassociated with this bacterium for three generations. Gibbons et al. (90, 93) isolated three strains of anaerobic streptococci from human carious teeth which as monoassociates proved highly cariogenic in orally inoculated germ-free mice and rats. By a number of biochemical and serological criteria, these streptococci appeared similar to those found in the oral cavity of the conventional rat. On sucrose-agar culture medium, the bacteria displayed marked capsule formation resulting in adhesive, gelatinous growth. These authors speculate that in vivo a similar process could be responsible for the massive bacterial plaque and successive decay which has been observed on the teeth of rats inoculated with these bacteria. In general, all bacteria implicated in caries of gnotobiotic rats were strongly acidogenic although this characteristic does not make a microorganism cariogenic per se. It also appears from these studies that proteolytic properties of a bacterium are not needed to make it cariogenic. Shklair and Rosen (296) produced gross carious lesions in germ-free rats on a sucrose-type diet which were orally seeded with a nonsticky, dextran-forming streptococcus isolated from a human carious tooth. Recently, Rovin et al. (278) observed in a long-term experiment that silk ligatures placed around the molar teeth of conventional rats (Fischer strain, which is not considered particularly caries-susceptible) fed a low sugar, relatively high fluorine-containing diet (35 ppm), caused extensive decay in the ligated tooth. This process was found to spread to the neighboring, nonligated tooth. The molars of germ-free control rats proved resistant to caries even after ligation. These observations suggest that a mechanical stressor agent which traps bacteria and debris near the tooth may be an important contributing factor to cariogenesis.

The discovery that specific strains of bacteria are responsible for the development of caries in the susceptible host, combined with the knowledge that antibacterial antibodies are secreted into the saliva, suggested an attempt to immunize the host against tooth decay. Wagner (329, 331) extended his earlier work with *S. faecalis* to test the possibility of serological protection against tooth decay. Gnotobiotic rats fed a cariogenic diet, associated with this microorganism and given a course of parenteral immunization with the homologous bacterin in adjuvant, showed virtual elimination of the cariogenic activity. Thus,

among 16 immunized rats, 14 remained caries-free and 2 developed minimal or questionable lesions on challenge with viable microorganism. All 14 nonimmunized controls developed caries under these conditions.

From these studies, the microbial contamination of the oral cavity emerges as the prime etiological factor of dental caries. Susceptibility of the host and the composition of the diet (high sugar, low fluorine content) are important yet ancillary conditions for cariogenesis.

Periodontal disease. Periodontal disease, the chronic impairment of gingival tissue, appears to have inflammatory and degenerative components. It is expected that gnotobiotic experimentation will permit better differentiation between the two and clarify the role which the oral flora play in these processes.

Young adult germ-free Sprague-Dawley rats generally do not show periodontal lesions except for those that are foreign body reactions associated with the embedding of hairs. Gnotobiotic controls associated with various types of oral microorganisms, however, did develop purulent plaques, alveolar bone loss, and destruction of gingival tissue [*L. acidophilus*, (85); an anaerobic filamentous actinomycete, (154); a cariogenic streptococcus, (90); eight organisms common in human gingival debris, (92)]. Older germ-free Sprague-Dawley rats (approximately 18 months), on the other hand, did display periodontal lesions made evident by migration of the gingival epithelial attachment and bone loss (13, 92). In young and older germ-free Fischer rats, no periodontal lesions were observed except for those associated with impaction of hair. Even if silk ligatures were placed around the molars of similar germ-free rats at weaning, the only changes produced were grooves formed in the crevicular epithelium. No alveolar bone loss was observed in any of these animals (276). Periodontal bone loss has been reported in germ-free mice (14, 15).

Recently, the role of specific enzymes and of other bioactive substances has been considered in the pathogenesis of periodontal disease and tested in germ-free animals. A collagenase isolated from *Clostridium histolyticum* was found to cause changes suggestive of chronic gingivitis on prolonged intragingival administration to germ-free mice (315). A collagenase isolated from an oral bacteroid capable of causing lytic effects in the oral epithelium destroyed reconstituted collagen in vitro (91). Injection of *E. coli* endotoxin intragingivally into germ-free mice caused the rise of specific antibodies in the serum, spleen, and gingival tissue of these animals (314).

Gnotobiotic experiments have permitted differentiation between degenerative and inflammatory factors in periodontal disease. The former, which may develop in pure form in germ-free animals only, appears to be conditioned by the strain, age, and perhaps the diet of the host. Select microbial associates are involved in the latter.

Enteric Infections

Enteric infections constitute one of the oldest chapters of gnotobiotic work, as accidental and intentional association or contaminations were from the beginning a natural byproduct of this activity. Early speculations that germ-free animals are highly susceptible to infections by elements of the normal flora were not borne out (104, 125).

Historically, the first report on the use of frank pathogens in this area was that of Cohendy and Wollman (46) in 1922 who observed complete mortality in young germ-free guinea pigs orally monoassociated with *Vibrio cholerae*. An attempt to modify the course of the infection by seeding additional associates (*Agarbacterium mesentericum* or a strain of staphylococcus was used) failed. Recently, oral infection of the same host with *S. flexneri* was attempted by Formal et al. (87) and all animals died. This outcome could not be modified by vaccination with heat-killed *Shigella* leading to sizable antibody titers prior to the oral challenge. Contrary to this, the germ-free guinea pigs could be protected against the fatal outcome of the *Shigella* infection if they were previously associated orally with viable *E. coli*. At term only *E. coli* and no *Shigella* could be demonstrated in the gut. Pathological evaluation of these animals showed that the germ-free guinea pigs infected with *S. flexneri* developed acute ulcerative enterotyphlitis, which was not found in similarly infected conventional controls. Monoassociation of the germ-free guinea pigs with *E. coli* rendered the bowel similar to that seen in conventional controls both in terms of architecture and cellularity (302).

S. typhimurium was selected as a challenging microorganism by a number of workers because some of its strains (e.g., ND 750A) are pathogenic to mice on monoassociation, causing high mortality and a reversible reduction of the enlarged cecum. Histological changes occurred in the wall of the ileum; among these, proliferation of the epithelium in the Lieberkühn krypts and the reduction in the number of goblet cells was most conspicuous, suggesting some of the changes observed after the infection with *Shigella* (163). The mortality could be eliminated if the animals were immunized (and developed antibody titers) by intraperitoneal injections of formalinized *S. typhimurium* vaccine prior to the challenge with the viable microorganism. In spite of the dis-

crepancy in resistance to challenge between the nonimmunized and preimmunized mice, the transient reduction of the cecum was essentially the same in both groups of monoassociated animals. Wostmann (353) recently observed that oral infection of germ-free rats with *S. typhimurium* (750A) showed an increase of nonimmune gamma globulin fraction and rapid carbon clearance from the serum (expressing nonspecific response of the host) during the first week. During the second week, immune gamma globulin levels increased along with the appearance of homologous agglutinating antibody in the serum, whereas the nonimmune gamma globulin fraction and the carbon clearance were reduced to the prechallenge level. This suggested that germ-free rats can fully adjust to the association with a frank pathogen and that protection appears to be afforded first by natural immunity factors and later by the specific antibody response. However, *S. typhimurium* should not be regarded an obligate pathogen for germ-free rodents; e.g., Margard and Peters (184) showed that a strain of this microorganism as a monoassociate, over a period of several months, did not affect the health of ex-germ-free mice.

An excellent illustration of the use of gnotobiotic animals in the elucidation of host-flora and interflora relationships is provided by Sasaki et al. (283) who studied the persistence and antigenicity of *V. cholerae* and *Shigella* in relationship to superinfection with *E. coli*, *S. faecalis*, and *C. welchii* in the intestine of gnotobiotic mice. The "superinfectants," representing elements of the normal flora in mice, were found to coexist with one another, but antagonism occurred between them and the pathogenic organisms. *Shigella* was removed from the intestine by the combination of *E. coli* and *S. faecalis*, whereas the removal of the *Vibrio* required the addition of *C. welchii*. On monoassociation of the mice with *Vibrio*, IgM and IgG immunoglobulins were found in the sera, and IgA in the intestinal wall. Time relationships in the rise of these fractions suggest that the two former globulins correlate to coproantibodies, whereas the latter does not.

Several studies involve enteric infections with *E. coli* in neonatal pigs (160, 161, 337). These describe the development of high mortality diarrheal disease in germ-free piglets after oral infection with an enteropathogenic serotype of *E. coli*. Kenworthy and Allen (156) studied the morphogenic effect of one or more serotypes of *E. coli* (0140:K and 08:H) and of the conventional flora (such as develops on placing the animals in a normal pig environment) on the structure of intestinal villi, which are of uniformly slender shape in monoassociated pigs. The villi occasionally branched and became more flat on diassociation with the two serotypes, whereas the conventional flora of the gut resulted in club-shaped, stunted villi with a tendency to fusion. Using the pathogenic serotype of *E. coli* (0141:K), Kenworthy (155) found severe inflammatory changes in the intestinal mucosa of monoassociated piglets. Staley et al. (304), using light- and electron microscopy techniques and a readily invading serotype of *E. coli* (055:B) in the postpartum infection of germfree piglets, indicated two types of passage from the gut lumen into the tissues. In the early stages of the exposure (2 and 6 hr after infection), nonfimbriated bacteria were engulfed by intestinal epithelial cells (some of which appear to be endowed with this characteristic in neonatal pigs) and passed rapidly to the regional lymph nodes without causing special cytopathologic changes. The lymph nodes being ineffective microbial filters at this stage permitted the passage of bacteria into the general circulation. These early events were followed (20 hr after infection) by the attachment of fimbriated bacteria to the epithelial lining. (This process seems to be facilitated by the presence of mucus.) On penetration of the epithelial cells, bacteria appear to cause degeneration of the microvilli at this stage. All segments of the small intestine permit the penetration of bacteria with equal ease in the postnatal period. Drees and Waxler (66) seeded orally a different enteropathogenic serotype *E. coli* (0138:K, which is a nonfimbriated form) into germ-free piglets at the age of 1 to 2 or 40 to 120 hr and killed the animals 3 to 72 hr after infection. Using fluorescence and other techniques, they found in the early exposure group extensive mucosal damage, intestinal atony, penetration, and widespread distribution of bacteria in various organs. In the late exposure group, these symptoms were clearly less pronounced. In a subsequent publication, the same authors (67) reported electron microscopic findings in piglets which were infected with the same serotype of *E. coli* and which were comparable to the early exposure group of the previous paper. In these animals, degenerative damages could be demonstrated in the intestinal epithelium; however, this damage was more pronounced but did not differ qualitatively from the necrobiotic changes which are normally seen in this tissue of germ-free controls. Tlaskalová et al. (320), using a nonpathogenic serotype of *E. coli* (086) in the neonatal oral infection of germ-free piglets, could demonstrate by various methods antibodies against the microbial antigen in the lymphatic apparatus of the intestinal wall,

in the serum and, somewhat later, in the mesenteric lymph nodes and in the spleen.

The work on gnotobiotic piglets suggests that there are various mechanisms allowing passage of enteropathogenic microbes across the intestinal wall. During the postnatal period (when the intestinal membrane undergoes gradual reduction of permeability), the age of the host and the species and the serotype of the pathogen appear to be of prime importance. A bewildering array of strains has been used for experiments; more extensive work with a few selected serotypes would be of much greater value.

Ulcerative Colitis

In the pathogenesis of ulcerative colitis, an immunological component was indicated by the fact that antibodies against antigen prepared from sterile human fetal colon is present occasionally in the sera of patients suffering from this disease. However, the use of such extracts proved impractical because of their low concentration of the antigen. Adult colon tissue for this purpose is obviously useless because of its contamination with microbial antigen. Perlmann et al. (225), using indirect hemagglutination technique, found that extracts prepared from the intestinal wall of germ-free rats contain sizable quantities of an antigen which is active in similar manner to human colon antigen. Hammarström et al. (133) later recognized the confusing fact that human blood-group antigens, along with the colon antigen, are widely distributed in the gastrointestinal tract of germ-free rats and pointed to the need to remove isohemagglutinins from the patient's sera prior to testing with the rat's intestinal extracts. They found that such blood-group antigens were present particularly in the goblet cells and in the mucus of the germ-free gut. In a third publication from the same laboratory, Lagercrantz et al. (165), using germ-free rat intestine antigen, conducted extensive clinical studies on various groups of patients suffering mainly from gastrointestinal disorders. It was found that virtually all individuals participating in this study, including healthy controls, had titers of anticolon antibodies in their sera. However, the incidence of elevated hemagglutinin titers ($> 1:16$) was highest in the group of ulcerative colitis patients, and the difference between them and other groups was statistically significant.

These studies have established a component of autoimmunity in the etiology of ulcerative colitis. It appears that gnotobiotic techniques offer a potential in the diagnostic procedures of this disease.

Uremia

Einheber and Carter (74) undertook experiments with gnotobiotic rats to test current theories on the retention of toxic substances originating from the intestinal flora in the uremic host. Bilaterally nephrectomized and sham-operated food- and water-deprived animals were used. The results showed that the mean survival time of germ-free rats in experimental uremia was significantly longer (122 ± 6) than of the conventional controls (76 ± 4 hr). The survival time of additional mono-, di-, and tetra-associated ex-germ-free rats fell in the same order between the germ-free and conventional values. These groups included association with *S. albus; S. albus* and *P. mirabilis; S. albus, P. mirabilis, E. coli,* and *S. faecalis.* In the course of uremia, the cecal enlargement persisted in all gnotobiotic rats. All sham-operated, starved, and water-deprived controls of this experiment lived much longer, as expected; however, surprisingly, the survival time of the sham-operated germ-free group was significantly shorter than that of the conventional control group. It was also shown that in all sham-operated groups the males outlived the females by a significant margin. These observations have been further elaborated by Carter et al. (40). It would appear that blood urea nitrogen remained much lower and urea in cecal contents was much higher in the germ-free group in comparison to conventional controls. Lesions ranging from inflammation to frank ulceration of the intestines were found only in uremic conventional rats. On the other hand, cardiovascular lesions associated with uremia (myocardial and arterial necrosis and calcification) had earlier onset and higher occurrence in the germ-free group than in the conventional. Recently Visy et al. (324) conducted work in kidney-traumatized (unilateral nephrectomy, contralateral ligation of renal artery and release after 120 min) germ-free, conventional antibiotic-treated (bacitracin, streptomycin, and nystatin given orally), and conventional rats. In this study, the longer survival of the kidney-traumatized germ-free rats versus conventional controls has been confirmed; from this point antibiotic-treated rats aligned with the germ-free group. In the postoperative stage, the blood flow and concentrating ability of the kidneys were found at the normal levels, whereas these were considerably impaired in conventional controls. The clearance of labeled thiourea into urine and cecal lumen of germ-free and antibiotic-treated rats was markedly elevated in comparison to the low clearance of this label in conventional rats.

These observations suggest that the presence of the normal microbial flora has an adverse effect on the survival of the uremic host. This effect develops probably by microbial invasion via intestinal lesions and subsequent impairment in clearance of nitrogenous materials. An advantage of the germ-free animal, in addition to escaping microbial invasion under these circumstances, is that its enlarged cecum acts as an effective dialysis membrane for the elimination of nitrogenous waste. Contrary to this, germ-free animals are penalized by more severe cardiovascular lesions, the cause of which remains obscure. The shorter survival time of sham-operated germ-free animals dying of thirst and starvation may be associated with the absorption of bioactive substances which have been found in their bowel (97).

Mycoplasmosis

Lutsky and Organick (181) studied the pathogenicity of *Mycoplasma pulmonis*, *M. salivarius*, and *M. pneumoniae* after intranasal administration to germ-free and conventional mice. Over 60% of the germ-free mice inoculated with *M. pulmonis* developed pneumonia, essentially similar to the natural disease seen in mice. The two other mycoplasmas, although demonstrable for some time in the airways of the ex-germ-free mice, proved noninfective and eventually disappeared completely. In the case of conventional mice, a high incidence of pneumonia was observed with all species, *M. pulmonis* being the most pathogenic. In a successive study, Organick et al. (218) reported the stages in the *M. pulmonis*-induced lesions. First, the passage of the microorganism from the bronchial surfaces to the alveoli was observed; here, the microorganisms were taken up largely by phagocytic polymorphonuclear cells. In later stages of the disease, "ring forms" were observed within granular pneumocytes. These bodies are thought to represent intracytoplasmatic developmental stages of the mycoplasma.

These observations represent a concise initial step in the study of host specificity in mycoplasmoses with gnotobiotic techniques. The pneumonia which developed in conventional mice inoculated with various types of mycoplasma may portray a broader susceptibility of the conventional host for this disease, the cause of which is not clear.

Tuberculosis

Miyakawa and Kishimoto (203) were the first to carry out a gnotobiotic experiment with *M. tuberculosis*, using oral administration of strain H37 Rv in germ-free and conventional mice. Their main finding was a delayed disappearance of the tubercle bacilli from the colony of ex-germ-free mice. Other studies (149, 310) of germ-free and conventional mice given BCG by the intravenous route showed no significant differences in the numbers of the bacilli that could be recovered from the spleens or lungs of the animals up to 35 days after infection.

An extensive and rigorous study by Hobby et al. (141) charted the effects on intravenous administration of *M. tuberculosis* H37 Rv and the Vallée strain of *M. bovis* in young germ-free and conventional mice. Both groups of mice were susceptible to infection with the human and bovine tubercle bacilli and both showed a considerable degree of uniformity, with animals dying at 2 to 4 weeks (50% survival times: germfree, 18.9 days; conventional, 19.1 days). The pathological picture and the distribution of the organisms were the same for all groups. In ex-germ-free mice, the per cent mortality plotted against the time of death showed consistently similar slopes, whereas conventional controls showed considerable variation from group to group. Intravenous administration of live BCG 4 weeks prior to challenge with the bovine tubercle bacilli almost doubled the 50% survival time in the ex-germ-free mice but did not protect them against the fatal outcome of the experiment. In the conventional control mice, the increase of survival time on pretreatment with BCG was less conspicuous. This indication that the immunological responses of germ-free mice to antituberculosis vaccines may be greater than in conventional controls was further tested by Hobby et al. (142) in the same model, using the same number of batches of BCG for immunization of mice, and challenging them successively with *M. bovis*. Their results indicated that in germ-free mice immunization with most batches of BCG significantly increased the survival time in comparison to nonimmunized germ-free controls after challenge. In this mode of testing, germ-free mice gave consistently reproducible results. In conventional animals, no such pattern was apparent and batches of BCG repeatedly tested gave responses with widely varying degrees of significance. It was also shown that the number of viable cells in the immunizing dose of BCG apparently bears no relation to the protective effect imparted by it to the animal, germ-free or conventional. Thus the use of gnotobiotic animals in this area is motivated mainly by the greater uniformity which is displayed by these hosts on infection with tubercle bacilli or on administration of related immunogenic agents. Hobby and

her co-workers, in the listed publications, as well as elsewhere (140), suggest that the presented findings are compatible with the undeveloped yet fully responsive defensive elements of germ-free animals (i.e., hosts which are more richly supplied with "unoccupied antigenic receptor sites").

Yeast Infections

The use of gnotobiotic animals was prompted by observations which indicated that on reduction of the intestinal flora by certain antibiotics the incidence of yeast infestations increases in a wide variety of hosts. Balish and Phillips (17) selected birds for their studies as these animals appear to be natural hosts for yeasts. *Candida albicans* was seeded orally in 1-week-old germ-free and conventional chickens maintained on a complete diet. The challenge seemingly did not impair the animal's health. At sacrifice, approximately 1 month after, it was found that the entire intestinal canal of all experimental animals had been colonized. Largest numbers of colonies could be grown from the crop and ceca of ex-germ-free birds (approximately 100-fold in comparison to conventional controls). Hyphal forms predominated in the ex-germ-free chickens; in the conventional counterparts only yeast forms were observed. The crop of the ex-germ-free birds displayed evidence of infection, with dense growth of hyphae and blastospores within the epithelium, whereas the conventional controls were normal. The diet seems to be important because, in another run of animals, conventional chickens maintained on a somewhat unbalanced diet (presumably low in vitamins, yet which was capable of maintaining growth) and challenged with *C. albicans* showed a systemic invasion by the challenging organism in the yeast form, frequently with a lethal outcome. The ex-germ-free chickens, on the other hand, remained essentially healthy and developed only crop lesions similar to those of the first experiment. Elements of the intestinal flora appear to protect conventional chickens maintained on a nutritionally adequate diet against infection and invasion by *C. albicans* (18). Establishing a mono-flora association with *E. coli* in young ex-germ-free chickens afforded full protection against subsequent challenge with *Candida*. This was not the case when *S. faecalis* was used under similar circumstances.

Similar experiments have involved the establishment of *C. albicans* in various strains of germ-free and conventional mice (229). One susceptible strain of ex-germ-free mice showed considerable loss of body weight, preponderance of hyphal colonization of the intestine, and evidence of infection in the stomach, suggestive of the crop infection of challenged ex-germ-free chickens. Conventional mice remained normal, showing colonization of their gut mainly by the yeast form.

The findings suggest that the normal flora in susceptible hosts, maintained on adequate diets, will prevent the morphogenesis of *C. albicans* to hyphal forms and, through this, allow only mild infections with the organism. In this protective action only certain elements of the flora, e.g., *E. coli*, might be involved. In nutritionally deficient conventional animals, the picture is considerably changed by the inability of the host to prevent the systemic invasion by yeast cells and perhaps other pathogens.

The situation in histoplasmosis is remarkably similar. Del Favero and Farrell (59) have studied the effects of intratracheal inoculation of *Histoplasma capsulatum*, commonly considered pathogenic for dogs, in a limited number of 8-week-old germ-free and conventional pups. The animals were autopsied at intervals up to 32 days after the inoculation, and all were found to have developed systemic granulomatous inflammation in the lungs, liver, spleen, tonsils, and various lymph node groups associated with the airways and with the gastrointestinal tract, as early as 7 days after the infection. In most instances, the presence of *Histoplasma* could be shown in these organs. The incubation time, signs, and nature of the lesions corresponded with those of the naturally occurring disease observed in conventional dogs. The only difference was a less severe course of the disease in the ex-germ-free group.

Protozoal Infections

Amebiasis. Phillips and co-workers (231, 234, 236) in their pioneering work in the 50's and early 60's have shown that intracecal seeding of germ-free guinea pigs with *Entamoeba histolytica* failed to induce the severe enteritis, which is normally observed in conventional controls. Enormous inocula did lead to relatively mild, well contained lesions in ameba-monoassociated animals. Seeding of the germ-free guinea pigs with *E. coli* or *Aerobacter aerogenes*, in addition to *E. histolytica*, resulted in enteritis-like lesions, even when a small inoculum of the ameba was used. More recently, Phillips and Gorstein (232) reported studies on young germ-free guinea pigs inoculated with *E. histolytica* by the same route. Shortly after monoassociation, these animals were given, in separate groups, an oral inoculum of each of the following species of bacteria: *E. coli*, *C. perfringens*, *S. faecalis*, *S. aureus*, *L. acidophilus*, *B. subtilis*, and an incompletely identified micrococcus. The animals were observed

for an approximate 2-month period after inoculation. All of the ex-germ-free guinea pigs, associated with the ameba only, remained essentially healthy. All animals exposed to various ameba-bacterium combinations developed lesions in the lower bowel; among these, the combination of the ameba with *B. subtilis* proved most pathogenic. The lesions observed in the lower bowel of these animals (necrotizing ulceration in the cecum) were suggestive of the enteritis of conventional animals infected with the ameba. In the case of the micrococcus or *E. coli*, no fatalities occurred, and, particularly in the case of the latter bacterium, only low incidence of superficial lesions of the cecum were observed. The other microorganisms occupied an intermediate position between these two extremes. Bacterially monoassociated control guinea pigs without ameba failed to show adverse signs, with the exception of the *B. subtilis* group in which frank inflammation of the cecal wall was observed. These studies extend our knowledge on the potentiating effects among elements of the flora which may be conducive to lesions in the animal host. Among various elements of the flora, *B. subtilis* particularly, reputedly an innocuous associate, emerges as a considerably aggressive agent for the guinea pig.

Histomoniasis. The problem of the intestinal flora possibly modifying the pathogenicity of protozoa was studied also in the case of the flagellate *Histomonas meleagridis*, the alleged etiologic agent of blackhead disease in turkeys. Doll and Frakner (64) inoculated germ-free and conventional turkeys with *H. meleagridis* by the oral route. All monoassociated turkeys survived and displayed no symptoms of the disease. Virtually all (11 out of 12) infected conventional turkeys developed typical lesions of the ceca and liver and died within 21 days. Bradley and Reid (34) repeated these experiments and aimed at the identification of the microbial associates which render the histomonas pathogenic in conventional turkeys. Young germ-free and conventional turkeys were orally or rectally seeded with *H. meleagridis*. *E. coli* was the principal associate which was given orally. Other monoassociates additional to *H. meleagridis* included *C. perfringens*, *B. subtilis*, or *P. mirabilis*. The former two associations combined with the histomonas produced the typical disease; the latter did not. Since *C. perfringens* and *B. subtilis* are not indigenous in turkeys, their role in the disease was not further considered. In the case of *E. coli*, the authors attempted to use classic criteria for correlating the disease to its causative agents. They found (i) that both oral or rectal insemination of *E. coli* and *H. meleagridis* produced in ex-germ-free turkeys the typical symptoms of enterohepatitis; (ii) *E. coli* could be recovered in pure culture from the diseased turkeys, and *H. meleagridis* was retrieved from diseased organs in exclusive association with *E. coli*; (iii) the pure culture of these two recovered microorganisms produced an experimental infection similar to the naturally occurring disease; (iv) from lesions of the experimental disease, the two microorganisms could be isolated in pure form. This indicated that the combination of *H. meleagridis* and *E. coli* represented the etiological pattern for infectious enterohepatitis of turkeys.

CONCLUSION

During the last decade, gnotobiotic animals have gradually become more readily available and techniques have been simplified to the point of general utility. In mapping out the characteristics of germ-free animals and of microbial modifications of the host, a number of essential and plausible studies have been undertaken. These referred mainly to nutrition, metabolism, and the functioning of body defenses. In differentiating the role of the flora from nonmicrobial elements as etiological agents in various disease processes, a search has been undertaken and notable basic information has been gathered. Yet successes were slow in materializing for areas of broad importance after the breakthrough achieved in rearing germ-free animals through successive generations. Although gnotobiotic experimentation offered suggestive leads in the study of cancer and cardiovascular disease, it has not gone to the root of these problems, thereby failing to contribute to the prevention and cure of these ailments. Thus, the relative lack of major successes in work with gnotobiotic animals has tended to dampen the initial enthusiasm.

There may be several reasons for this. It is possible that our microbial associates, after all, do not play a very important role in the household of the normal host. With the improvement of rearing methods and diets, we find similarity between germ-free and conventional animals in an increasing number of details. Germ-free and conventional animals are similar in the sense that both are essentially normal and adapted to their respective environments. In addition, the effects of germs on the host, if present, can be traced mainly to organs that are directly exposed to the flora. When such effects are demonstrable beyond the immediate area of contact, the difference between the germ-free and conventional observations is indicated primarily in quantitative and not in qualitative terms. At this point, one might argue that the physiological normality displayed

by the germ-free host in a homeokinetic sense, together with the apparent lack of fundamental flora effects, will result in an experimental model that does not permit particular insight into biological events of the host and its microbial associates.

However, elusiveness of success in gnotobiotic experimentation may also stem from the emerging and little understood complexity of our animal model. Initially, it was assumed that the germ-free animal is essentially a host without germs. Then it was learned that, in addition, it is a host minus responses to germs. Now it becomes clear that the lack of germs results in a complex of additive direct and indirect, local and generalized effects. An illustration for these is the reduced metabolic rate and cardiac output of germ-free animals which can be corrected by the surgical elimination of their greatly enlarged ceca early in life. Thus, the large cecum, which is a direct result of the absence of the intestinal microflora, appears to handicap and modify the host by by secondary, nonbacterial mechanisms. These, in turn, may affect other functions that are elected for study. There may be more such aspects, important and unknown, which form a part of life in the absence of germs. Under such circumstances, the design of an experiment and the interpretation of the results become major undertakings and users of the gnotobiotic animal find this "working in the dark" somewhat disconcerting. It still remains theoretically correct to regard gnotobiotic experimentation as an extension of the microbiologist's pure culture concept to all biological forms, yet in the case of multicellular organisms the complexity of interrelated functions considerably reduces the practical value of such generalizations.

To fill many gaps that exist in this area, more interest of researchers in this tool and much more work are needed. Perhaps future recognition of the patent value of gnotobiotic experimentation in special fields of investigation will act as a catalyst and make this approach a widespread utensil of research. Maybe the confinement of humans in space capsules (29), the use of clinical life island units (62, 150), issues of environmental pollution, or the ill-effects of the widespread use of antibiotics will create this stimulus. In this sense, we can suggest a comparison to the use of tissue culture techniques, which for many years was confined to a few laboratories until its value in studies on cytogenetics, growth of viruses, etc. had been recognized. In the meantime, the proponents of the gnotobiotic experiment may draw solace from the fact that in matters of host microbial relationship, this type of work will remain, by its very nature, the final arbiter. However, the answers thus provided may not be as simple as we had originally hoped. Fifty-odd years ago the scientific community did not especially care about genetic or dietary standardization of experimental animal colonies. Today we tend to smile at some results obtained with this type of work. What will our successors 50 years hence think of today's work which the scientific community carries out largely without microbiological standardization of experimental animals?

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