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THE GERMFREE ANIMAL IN NUTRITIONAL STUDIES

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HISTORICAL DEVELOPMENT

The value of the gnotobiotic approach to nutritional studies was recognized early. In 1885, Louis Pasteur made the following statement (102).

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> For several years during discussions with young scientists in my laboratory, I have spoken of an interest in feeding a young animal (rabbit, guinea pig, dog or chicken) from birth with pure nutritive products which have been artifically and totally deprived of the common microorganisms.
>
> Without affirming anything, I do not conceal the fact that if I had the time, I would undertake such a study, with the preconceived idea that under these conditions life would have become impossible.
>
> If this work could be developed simply, one could then consider the study of digestion by the systematic addition to the pure food, of one or another single microorganisms or diverse microorganisms with well defined relationships.

Pasteur's doubt about the feasibility of such studies was refuted within 10 years. In 1895, Nuttal & Thierfelder (96) reported obtaining the first germfree[1] (GF) guinea pigs by caesarian derivation into a sterile environment. Survival up to 13 days was achieved on a diet of sterilized cow's milk.

Following Pasteur's original concept, Schottelius obtained the first GF chicken in 1898 (131). By 1912, it was quite clear that chickens could live in the GF state much beyond the age believed possible by those who saw the microbial flora as an essential associate of the host (21). At approximately the same time, Kuster was able to rear GF goats and conducted the first morphological and metabolic study in the GF state (78).

After World War I, Glimstedt, interested in morphology and histology of the lymphoid tissue, resumed work with GF guinea pigs at the University of Lund, Sweden (42). His work reflects the advances made in the knowledge of nutrition—GF guinea pigs now often survived up to 2 months. In 1954, Miyakawa et al reported successful GF rearing of the guinea pig for a period of 5 months (92).

Definite proof that higher organisms could function adequately in the absence of microbial associates was offered by Reyniers and co-workers at the Lobund Laboratory, University of Notre Dame. During the 1940s, this group reared rats (120) and chickens (121) in the GF state to successive generations. At the same time, Gustafsson reestablished the tradition of GF work at the University of Lund. In 1948, he reported successful rearing of GF rats (51) and in 1956 reproduction within the GF system was achieved (52). The fact that during the fifties reproducing colonies of GF mice and GF rats could be established indicated that knowledge of nutritional requirements had advanced to the point that adequate, sterilizable diets could be composed. It had taken nutrition 50 years to complete the "germfree

[1]In the case of the rat, the term germfree implies the absence not only of bacteria, parasites, and fungi, but also of virus detectable by established procedures. In germfree mice certain viruses are transmitted vertically: B-type virus in some strains associated with mammary carcinoma; C-type virus in all strains associated with lymphatic leukemia; and LCM virus in Haas strain mice, associated with an immunoproliferative syndrome. In other germfree species the viral status has been insufficiently documented.

experiment," although the basic technology had been available since the turn of the century.

THE GERMFREE ANIMAL MODEL

General Aspects

Many GF species have been studied, not all under ideal conditions. Only for those species for which nutritional requirements are known in great detail is it possible to compose diets that after the required sterilization will cover requirements adequately. With this restriction in mind, it can be stated that GF animals appear to grow and reproduce at least as well as their conventional (CV) counterparts (16, 74). Whenever adequate conditions prevail, GF animals appear to live longer and in good health (46, 107).

Because of limited space, this review concentrates on mice, rats, gerbils, rabbits, chickens, and guinea pigs. Some data on dogs and pigs are included. The reader is referred to reviews by Gordon & Pesti (47), Combe et al (23), Pleasants (104, 105), Coates (17), and Wostmann (152) for information outside the scope of this review.

Morphology and Body Composition

As far as gastrointestinal morphology and physiology are concerned, experimental animals appear to fall into two classes: animals whose cecum is greatly enlarged in the GF state (rat, mouse, gerbil, rabbit, guinea pig), and animals that do not show much enlargement of the comparable organ (dog, pig, chicken).

CECAL ENLARGEMENT Cecal enlargement starts during the suckling period and does not appear to result from nutritional deficiency (155). It is caused in first instance by the accumulation of mucus, a mixture of macromolecular, sulfate-containing glycoproteins that normally are degraded by the microflora of the lower gut (167). These negatively charged macromolecules not only attract water into the cecal lumen, but also limit Na^+-dependent water transport out of the cecum by reducing the availability of Cl^- (3, 48). The result is a fluid cecal content that in extreme cases may reach 20% of body weight (even higher in guinea pigs and gerbils). Normally the site of intense microbial digestive action, the GF cecum becomes a reservoir of pharmacologically active materials that may become blood-born and affect the animal in ways as yet little understood (5).

Extreme cecal enlargement may prevent pregnancy from being carried to term. Enlargement depends to an extent on dietary composition, with high fiber content resulting in larger ceca. With diets presently in use, ceca of GF mice, rats, rabbits, and guinea pigs are four to seven times as large as in

comparable CV animals. In animal species where the cecal sac is not prominent, enlargement does not occur, although an accumulation of mucus in the lower gut may lead to localized fluidity of the contents and to the presence of bioactive materials similar to those found in the cecal contents of GF rats and mice (6).

Cecal enlargement is the most striking anomaly in the GF rodent, rabbit, and guinea pig. But other, less-striking morphological differences, affecting mostly intestinal structure and lymphoid tissue (45), do occur.

INTESTINAL MORPHOLOGY The GF intestinal tract usually weighs less than the tract of the CV animal (45, 90). This deficit results mainly from a reduction in lamina propria tissue. In GF rats and mice, surface area of the small intestine is reduced to approximately two thirds of CV levels (1, 44, 90). In pigs and dogs, on the other hand, the surface area of CF and CV animals was approximately comparable (63, 64).

ORGAN WEIGHTS Hearts, lungs, and livers of GF rodents usually were somewhat smaller than those of comparable CV animals (13, 45). Insufficient data are available to extend this statement to other species. As expected, lymphoid organs in GF animals tend to be smaller than in CV animals (9, 45, 98).

BODY COMPOSITION Small differences in body composition have been reported. Levenson states that the GF rat carcass contains somewhat less fat (13.2 vs 18.5%) than the CV animal (84). Yamanaka et al found no such difference between GF and CV mice, but their data indicate a somewhat higher N content per 100 g (body weight) in the GF (168). Livers of GF and CV rats were comparable in gross chemical composition (84).

Function and Metabolism

HEART AND LIVER As mentioned earlier, GF mice and rats have smaller hearts than their CV counterparts. They also have a smaller cardiac output (49) and, with similar body weight, use less oxygen per unit of body weight (85, 157, 159). In our laboratory we have not been able to detect any difference in body temperature (157, 159), although others claim that GF rats have slightly lower body temperatures than CV rats (85). The lower O_2 use for presumably normal function (growth, reproduction, etc) appears to be reflected in lower regional blood flow to major organs like liver and intestinal tract (43), organs that in the CV rat bear the brunt of what Sprinz has called the stress of "physiological inflammation" (139). Livers of GF animals tend to be smaller than those of CV animals (45, 152). The thiamine concentration of the liver of GF rats and GF chickens is substantially lower

than in CV animals, a difference not noted in muscular tissues (18, 164). Since thiamine pyrophosphate is a cofactor of several of the enzyme systems supporting ATP production, this again suggests a decreased metabolic load of the GF liver. In vitro, however, GF liver slices consume oxygen to the same extent as liver slices of CV rats (167). Liver mitochondria of GF rats showed O_{O_2} and P/O values comparable to those of CV rats (134). It would appear that homeostatic controls, as yet beyond our understanding, reduce the in vivo metabolic function of the liver of the GF rat. As an apparent adaptation, the entry enzymes to the hexose monophosphate shunt and succinic acid dehydrogenase, the latter a controlling enzyme in the tricarboxylic acid cycle, show reduced activity. Also, GF rats demonstrate a higher than CV activity of the citrate lyase and fatty acid synthetase complex (115).

The lower metabolic level of the GF rodent, indicated by reduction in O_2 consumption and in liver thiamine, is not translated into lower caloric intake. Carefully conducted balance experiments reveal, at least in the case of the rat, that extraction of metabolizable energy by GF and CV animals is comparable (160).

THYROID The deficit in oxygen consumption mentioned above logically lead to a study of thyroid function. GF rats 3–4 months old had serum T_3 and T_4 values comparable to CV rats and $T_{1/2}$ and T_4 was 16–18 h in each instance (133, 160). Older CV rats (24 and 30 months) showed the well-known decline in serum T_3 and T_4 concentrations described in the literature (77), with values ranging from 50 to 70% of young adult values. Older GF rats, however, never showed a reduction in serum T_3 and T_4 values (157). A similar situation was found to exist with GF and CV mice (157). Thus, under these conditions, O_2 consumption did not relate directly to these parameters of thyroid function.

The above indicates that basic differences in homeostasis exist between GF and CV rodents. The absence of a decline in thyroid function, coupled with the greater lifespan of the GF rat and mice, suggests that freedom from the stress of physiological inflammation could directly or indirectly affect the pace at which the animal lives. The effect might be analogous to that of balanced undernutrition.

GASTROINTESTINAL FUNCTION Presence or absence of the physiological inflammation imparted by the CV microflora is most obvious in the gastrointestinal tract. The differences in morphology mentioned earlier translate, to an extent, into differences in function. Cecal enlargement especially appears to lead not only to reduced intestinal transit time (124, 143), but also to locally reduced motility (2) and to a delay in gastric emptying

(2, 124). Villi of GF animals may be smaller [rat (44), mouse (1)] or larger [dog (63), pig (64)], but a consensus exists that the renewal rate of their intestinal mucosa is much slower than in CV animals (1, 75, 90, 123). As a result, the average age of the mucosal cell of the GF animal will be as much as twice that of its CV counterpart. This could well be the cause of the higher concentration of brush border enzymes like lactase, maltase, sucrase, and alkaline phosphatase found in GF rats (71, 110), mice (170), and chickens (135). Less obvious is the cause for the increased activity of Mg^{2+}-dependent and $(Na^+ + K^+)$-stimulated ATPase found in intestinal tissue of GF mice (169).

The progressive inactivation of pancreatic enzymes upon passage through the tract is much reduced in the absence of bacteria (83, 111), leading to higher concentrations of these enzymes in cecum and lower gut of the GF animal. In the lower gut especially, the absence of a microflora may lead to a slightly higher pH of the intestinal contents, whereas E_h values may easily be 300 mV more positive (103, 156) and may lead to a difference in availability of Fe (114, 116).

The aforementioned differences in morphological, physical, and biochemical characteristics could be expected to lead to differences in absorption. Available data indicate that the GF small intestine allows somewhat faster passive and carrier-facilitated absorption (36, 62, 65, 79), although not all studies indicate a clear-cut difference (22, 143, 147).

A special aspect of intestinal function is depicted by the intestinal bile acids, end products of the catabolic conversion of cholesterol. In CV animals, the microflora will deconjugate, dehydroxylate, and oxidize primary bile acids, leading to a variety of secondary bile acids with different reutilization characteristics. In general, these modifications enhance excretion of bile acids and thereby increase catabolism of cholesterol.

In the absence of a microflora, the primary, conjugated bile acids remain intact, and more bile acids are reabsorbed from the lower gut, resulting in higher bile acid pools in the enterohepatic circulation. This is reflected by the much higher bile acid concentrations in the small intestine (87, 125). In turn, this affects lipolytic enzyme action and transport across the "unstirred layer" and may well be the cause of the increased absorption of Ca and Mg observed in GF rats (39, 112), presumably via increased micelle formation with mineral-carrying lipid complexes (25). Although no large differences have been found, GF rats (25), rabbits (171), and chickens (12) appear to utilize dietary fats somewhat better than their CV counterparts, presumably because of the aforementioned improved absorption of Ca and Mg salts of fatty acids.

GF animals also lack microbial hydrogenation of the various unsaturated fatty acids in the lower gut. GF rats will excrete significantly more of the

unsaturated acids with the feces (31). GF lambs (81) and rabbits (17) had significantly

It has not been established whether this PUFA (polyunsaturated-fatty acids) sparing aspect of the GF state affects requirements in any substantial way.

The presence of an intestinal microflora notably affects the metabolism of amino-N (127). Microbial action causes proteolysis of both dietary and endogenous protein, and the amino acids so released undergo enzymatic conversion to amines and ammonia, the latter also the end product of microbial breakdown of urea and uric acid. Ammonia may be reincorporated either by intestinal bacteria or by the liver into amino acids. In the absence of bacteria, far less ammonia is released from exogenous and endogenous proteins (145). Although this reduces loss of nutritionally valuable amino acids, it also implies absence of bacterial formation of both nonessential and essential amino acids. In the rabbit (172) and in the chicken (97) bacterial formation of essential amino acids was shown to be of at least some value to the host.

THE GERMFREE ANIMAL IN NUTRITIONAL STUDIES

Studies Focusing on Systemic Requirements

Under normal conditions, the intestinal microflora appears to produce a variety of B vitamins (18, 24) and vitamin K cogeners (91). Although in general CV animals do experience vitamin deficiencies,

acid or vitamin K, for example, microbial production usually is sufficiently available to cover systemic requirements of many species (e.g. rats, especially). In those instances, only GF studies can estimate systemic requirements. And in the case of those factors for which systemic synthesis is not excluded, GF experimentation is crucial to establish the extent to which the animal could cover its systemic requirements.

In 1951 duVigneaud et al reported that GF rats would incorporate deuterium oxide given with the drinking water into the methyl groups of choline (28). The breakdown of dietary choline to trimethylamine and trimethylamine oxide, on the other hand, was found to be a purely microbiological process (108), which is one reason why the GF rat appears to be less sensitive to choline deficiency

similar experiments established that the GF rat could incorporate ^{14}C from I-^{14}C-labeled glucose into inositol (37).

Under normal conditions, CV animals hardly require a dietary source of folic acid, or K vitamins, since intestinal production is adequate, and the microbially produced vitamins are available on first passage. Similarly, part

of systemic vitamin B_{12} requirement may be met by microbial production (144). Studies with GF rats made it possible to establish a definite requirement for folic acid (24). Vitamin B_{12} requirement was established at 15–20 ng/day (144). However, it must be pointed out, in general, that systemic requirements of GF and CV animals are not comparable per se, since definite differences exist in intermediary metabolism (see previous section).

The Effect of the Microflora on Nutritional Requirements

ENERGY Early studies showed no consistent pattern of altered energy requirements for GF animals. Studies comparing GF and CV chickens suggested a slightly higher energy extraction by the CV animals (17). GF mice were found to eat more than CV mice, but they also excreted more fecal material (43). More carefully conducted experiments with rats revealed that GF rats excreted almost double the fecal dry material voided by CV rats, but their dietary intake was also 18% higher. As a result, both GF and CV adult Wistar rats retained approximately 607 kJ/kg/day (481 $kJ/kg^{0.75}$/day) from ingested food, amounting to 71% of the intake for the GF, and 80% of intake for the CV animal (160).

In considering these and following data, we have to keep in mind that most of the CV animal studies were conducted in modern, clean animal facilities, supposedly harboring a minimum of growth-depressing pathogens. Therefore they are not directly comparable to the field trials that indicate a greater energy efficiency can be obtained in livestock production by the use of antibiotics (60).

FEED EFFICIENCY AND THE SPARING ACTION OF ANTIBIOTICS The sparing action of antibiotics is well known. However, when Coates et al compared growth of CV chickens, CV chickens fed penicillin, and GF chickens, it became obvious that the antibiotic actually counteracted a growth-depressing effect of the intestinal microflora (19). Subsequent studies revealed that it was possible to depress growth of GF chickens by association with *Streptococcus faecalis* and administration of a cell-free fecal filtrate to 1-week-old chicks. This procedure resulted in a thickening of the intestinal wall, combined with increased lipid excretion with the feces, a syndrome that could be *largely* reversed by the administration of antibiotics. Eyssen & DeSomer argued that the above combination precipitated a malabsorption syndrome and concluded that the cell-free filtrate contained a viral agent (30). Similar results were reported by Lee & Dubos, who infected newborn mice with an intestinal filtrate of "dirty mice" (82).

However, the much-maligned virus was never found. In a recent paper on this matter, Fuller et al do find that *Streptococcus faecium* effectively

depressed growth, in associated chicks. Although a faecal filtrate further depressed growth, the authors state that "so far our efforts to demonstrate a virus by tissue culture or electron microscope have been unsuccessful" (38).

PROTEIN AND AMINO ACID REQUIREMENTS Corn is usually considered a poor source of protein for the mammal, because of its low content of tryptophan and lysine. In a classical experiment, Dubos & Schaedler (27) have shown that ex-GF mice populated with a controlled nonpathogenic microflora could survive and even grow on corn, whereas mice harboring a "normal" flora would lose weight and eventually die. Even when the diet contained protein of more adequate quality but in marginal quantity (e.g. 15% casein), growth of the "clean" mice was always much better than that of the conventional "dirty" mice. In the same vein, Gustafsson (53) has reported acceptable growth of GF rats fed diets containing 6–8% protein, an amount generally considered insufficient to sustain growth in the CV rat. In a refinement of these studies, Stoewsand et al (141) fed defined diets containing increasing amounts of lysine to GF mice, ex-GF mice associated with defined microflora (128), and CV mice. Both the GF and the selectively associated mice grew maximally at two thirds of the lysine level needed by the CV mice for optimal growth.

Reddy et al (119) have reported a slightly higher nitrogen retention in young GF rats, and other data seem to be in agreement. In addition, a somewhat faster absorption of certain amino acids seems to take place in the absence of an intestinal flora (65). But since dietary protein usually is utilized for at least 95%, it is doubtful whether or not the latter observation indicates a possible effect on protein requirements. However, the totality of the data available appears to point to a notable protein-demanding effect of certain commonly occurring members of the "normal" intestinal microflora.

WATER-SOLUBLE VITAMINS No data are available that actually support the notion that GF rodents have different systemic requirements for the various B vitamins than do CV rodents. In the case of biotin, folic acid, vitamin B_{12}, and possibly pantothenic acid, the systemic requirements of the CV animal appear to be largely covered by intestinal production. In other instances, e.g. vitamin B_1 or B_6, true deficiencies occur in CV animals, although the severity of the deficiency appears to be somewhat ameliorated by the presence of a microflora (142, 164).

Thiamine concentrations in the liver of GF rats and GF chickens were lower than those found in comparable CV controls, but concentrations in other tissues were comparable (18, 164). Since the liver contains 40% of

body thiamine, this suggested a possible lower requirement of the more slowly metabolizing GF animal. However, maximal growth was obtained in both GF and CV rats on diets containing 1.1 μg of thiamine/g of diet (163). Since under the conditions of the experiment little coprophagy took place, this indicated that in the case of thiamine the presence or absence of an intestinal microflora had little effect on actual requirements for maximal growth. It could be speculated that in the CV rat a certain potential benefit from microflora-derived thiamine is balanced by a slightly higher requirement in the presence of that microflora.

Vitamin B_6 deficiency may decrease the tissue concentrations of riboflavin, niacin, biotin, and pantothenic acid and may reduce absorption of vitamin B_{12} (68). In the case of pantothenic acid and biotin, this secondary reduction was greater in GF than in CV rats. Again, available data suggest that when CV rats were fed diets deficient in vitamin B_6, the microflora makes some contribution to the vitamin B_6 status of the host (67). On the other hand, GF chickens given a diet deficient in pantothenic acid appear less affected by the deficiency than do their conventional controls (80). Studies with GF guinea pigs fed an ascorbic acid-deficient diet show that in the absence of a microflora the animals live longer, and signs of scurvy are less pronounced (86). It would seem that although in certain cases the microflora may make a small but crucial contribution to animals fed a deficient diet (e.g. vitamins B_1 and B_6), the stress of the CV state with its physiological inflammation in general leads to a somewhat higher systemic vitamin requirement of the host.

FAT-SOLUBLE VITAMINS The GF rat can maintain life on extremely low levels of vitamin A (122). Diets that contained only traces of the vitamin permitted survival up to 40 weeks, although typical symptoms of vitamin A deficiency were obvious and growth of the Sprague-Dawley rats was arrested at approximately 200 g. CV rats survived for only 8–10 weeks under these conditions. Although these data suggest a role of vitamin A in tissue integrity, and its related resistance to infection, studies with chickens could not confirm the vitamin A sparing condition of the GF state (17).

No differences in vitamin D and vitamin E requirements between GF and CV animals have become obvious. As mentioned earlier, GF rats have a well-defined requirement for K vitamins. Data obtained by both Gustafsson et al (56) and Wostmann et al (164) indicated a definite superiority of vitamin K_1 over vitamin K_3 for the GF rat and suggest a daily requirement on the order of 3 μg of K_1/day. On a molar basis, requirements for vitamin K_3 were approximately 10 times as high as for vitamin K_1 (164). Diets providing a daily intake of 1 μg of vitamin K_1 or less were suboptimal, as indicated by prolonged prothrombin times. In that range of intake, a definite antagonism between vitamin A and vitamin K became obvious (162).

MINERALS Early observations in GF rodents maintained on fortified casein-starch diets indicated a tendency to soft tissue mineralization. A strain of inbred GF C3H males showed calcification throughout the body, including lung and heart tissue (B. S. Wostmann, unpublished data). Calculi found in the pelvis of the kidney contained Ca, oxalate, and citrate (41, 57, 136).

More recent studies demonstrate a generally greater absorption and retention of Ca and Mg by the GF animal (39, 112, 171). Consequently, more of these minerals accumulate (30% more Ca and 40% more Mg) in the heavier skeleton (113). Presumably, the much higher level of intestinal bile acids in the GF rodent is at least one of the causes of increased Ca and Mg absorption, since it enhances micelle formation with mineral-carrying lipid complexes. This would be in agreement with the observation that fecal excretion of insoluble Ca soaps is decreased in the GF rat (25).

Zn requirements were also found to be reduced in the GF state. It has not been established, however, whether GF animals have lower requirements or whether for CV animals only part of dietary Zn is available due to irreversible binding by bacteria (137).

GF rabbits maintained on diets containing moderate amounts of Fe and Cu from natural ingredients, but relatively high amounts as supplemented salts, showed severe Fe-deficiency anemia, although Cu parameters were normal. Either conventionalization of the animals by exposure to a "normal" microflora sustaining a more negative E_h value or increase of Fe from natural ingredients (e.g. soybean meal) without increasing total Fe intake alleviated all symptoms within 4 weeks (116). In the GF rat no anemia has become apparent. However, storage and distribution of iron indicated differences in Fe and Cu metabolism between GF and CV rats. Mn metabolism appeared not to be affected by the GF state. The results suggest a generally lower rate of Fe and Cu metabolism in the GF animal (118, 172), although one study reported greater retention of intragastrically instilled Fe^{59} by GF than by CV rats (40).

Cholesterol and Bile Acid Metabolism

Bacteria modify both the biliary bile acid pattern and the endogenous and exogenous cholesterol in the intestinal tract. Although bile acid composition in duodenum and jejunum largely resembles the biliary bile acid pattern, bacterial deconjugation and dehydroxylation start in the ileum and continue in the lower gut, not only modifying bile acids but, in doing so, affecting the patterns of their recirculation. In both the GF and the CV male Wistar rat, biliary bile acids consist of cholic acid and β-muricholic acid, although in somewhat different proportions. Fecal bile acids of the GF Wistar rat show a bile acid pattern only slightly different from its biliary composition. The CV rat, on the other hand, has a fecal pattern consisting

largely of nonconjugated secondary bile acids, dominated by hyodeoxycholic acid, ω-muricholic acid, deoxycholic acid, and certain keto-acids (87, 125). Comparison of intestinal concentration and fecal output shows that GF rats excrete approximately one half of the bile acids voided by CV rats, whereas the bile acid pool in their enterohepatic circulation is about three times higher (158). Thus, reabsorption of bile acids is more efficient in the GF rat, presumably on the average better than 99%. Secondarily, the resulting higher intestinal bile acid concentrations lead to an increase in cholesterol absorption, all leading to increases in the metabolic cholesterol pool of the GF animal (151). All in all, microbial modification of bile acids appear to reduce their reabsorption and promote their excretion, in turn reducing body cholesterol pools by reducing intestinal absorption and enhancing catabolic conversion of cholesterol to bile acids.

This concept has recently been confirmed in work with GF and gnotobiotic gerbils. The gerbil's serum cholesterol is much more sensitive to dietary cholesterol than the rat's (61). The gnotobiotic gerbils had been associated with a defined, murine-derived hexaflora that modified primary bile acids only to a minimal extent (<5%). Compared to CV gerbils, the hexaflora-associated animals had almost twice the serum cholesterol concentration, the difference being largely in the VLDL (very low density lipoproteins) and LDL (low density lipoproteins) fractions (8).

Cholesterol passing through the intestinal tract will normally be dehydrogenated by specific organisms (101) to coprostanol, a sterol less well absorbed than cholesterol (69). The absence of this reaction in the GF animal, in addition to a somewhat better intestinal absorption of cholesterol, will cause the GF rat to excrete only about 60% of the neutral sterols voided by its CV counterpart (73). Thus, the intestinal microflora promotes reduction of cholesterol pools by enhancing cholesterol elimination both directly and via the bile acid system. Similar results were obtained in studies with pigs (148). In this case it appears that formation of hyodeoxycholic acid from cholic acid enhances the elimination of acid sterols (154).

Because of the strong influence of the microflora on sterol metabolism, GF animals, mostly rats, have been used to study its various systemic aspects (32). Kellogg confirmed the serum cholesterol-lowering effect of safflower oil in GF rats (72). Gustafsson & Norman studied the effect of the physical characteristics of the diet on intestinal transit time and its effect on bile acid metabolism (58). They also established that in the rat increased cholesterol intake led to changes in liver microsomal metabolism that favored formation of chenodeoxycholic acid and its primary metabolites, α- and β-muricholic acids (54). In the case of the CV rat, the latter will then be converted to hyodeoxy- and ω-muricholic acids (32, 88, 126). These secondary bile acids are reabsorbed only to a limited extent and provide a convenient pathway for the elimination of excess cholesterol (153). Admin-

istration of hyodeoxycholic acid with the diet to GF rats resulted in hepatic formation of ω-muricholic acid, thus establishing that, for the rat, this secondary bile acid is a direct systemic precursor to ω-muricholic acid. For this reason the trihydroxy-bile acid ω-muricholic acid has been termed a tertiary bile acid (88). On the other hand, an increase in the metabolic cholesterol pool in the GF animal will lead to a compensatory reduction of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase (29). Cholestyramine feeding, by binding bile acids, strongly enhanced HMG CoA activity (55) and conversion of the resulting cholesterol to bile acids, especially cholic acid (4).

Dietary Effects on Immune Mechanisms

The GF animal provides an ideal model for this type of study because it makes it possible to separate dietary from microbial influences on the immune system. Both cellular and humoral responses appear adequate in all species of GF animals studied, but different authors report different primary response patterns, which may or may not differ from their counterparts (11, 99, 140). In all of these experiments, dietary stimulation appears to be a major variable.

An early study (130) had indicated the possibility of certain resistance factors occurring in practical type diets. This phenomenon became quite obvious during studies in which GF rats were mono-associated with *Salmonella typhimurium* ND 750 to study the mobilization of immune function in the immunologically "inexperienced" rat (150). Two well-proven practical-type diets were used, L-462 (148) and L-485 (74). Upon introduction of *S. typhimurium,* rats reared on diet L-462 suffered severe weight loss and a protracted period of diarrhea, and approximately 25% of the animals died. Reared on diet L-485, rats suffered little weight loss and much less diarrhea, and none of the animals died as a direct consequence of the association. No diet-related difference could be found in the immunologic and serologic parameters followed during the study. Apparently a dietary factor unrelated to the nutritional quality of the diet had bestowed a measure of protection to the immunologically inexperienced GF rat reared on diet L-485, although both the nature of the factor and its mechanism of action remain unknown.

Other experiments pointed to dietary effects of readily measurable immunological parameters. Feeding of bovine milk proteins was recognized as stimulating immune globulin production in GF rats and guinea pigs (149). Studies with GF mice showed major effects of dietary composition on the blood leukocyte pattern, and smaller but significant effects on the cellularity of the lymphoid system (165, 166). On the other hand, studies using hysterectomy-derived, colostrum-deprived GF miniature swine raised on a diet of Mullsoy (Syntex Laboratories, Palo Alto, Calif.) showed none

of the otherwise spontaneously occurring natural hemagglutinins (129). Serum IgG levels were very low (132), and the usual background of anti-SRBC (sheep red blood cell) hemolytic plaque-forming cells was absent (76). Thus, for controlled, quantitative studies of the immune system it is not only necessary to remove uncontrolled microbial stimulation, but dietary influences must also be considered and controlled, even if the diet can be considered nutritionally adequate.

Further control of dietary antigenicity has been obtained through the use of water-soluble, chemically defined amino acid-glucose diets (CD diets), which can be sterilized by filtration (106) (see next section). When serums of inbred C3H GF mice reared on 0.22-μm membrane-filtered CD diet were compared by immunoelectrophoresis to serums of comparable mice reared on the usual solid diets, the antigenicity of the latter diets became obvious. Sera of 75-day-old GF mice reared on solid diet showed reduced but substantial amounts of the various immune globulins. GF mice reared on membrane-filtered CD diet showed no immunoelectrophoretically demonstrable immune globulins at that age. Some residual antigenicity remained, however, since after 11 months on this diet these mice demonstrated appreciable amount of the various immune globulins (166). When additional ultrafiltration removed all molecules over 10,000 daltons, the now virtually antigen-free diet produced year-old GF C3H mice in which no serum immunoglobulins except a limited amount of IgM could be detected by direct immunoelectrophoresis or quantitative radial immunodiffusion (59, 165). As mentioned earlier, these mice showed totally adequate humoral [anti-SRBC (165; B. S. Wostmann, unpublished data)] and cellular [mitogen induced (146)] responses. Similar results have been reported in GF piglets raised on a "non-antigenic," synthetic diet. The authors report nondetectable gamma-globulin levels and the absence of surface IgG and IgA from the spleen lymphocytes of 5-month-old animals (89).

Thus, it appears to be possible to raise immunocompetent GF animals, protected from the stresses and potential pathogenicity of the "normal" microflora. Such an environment will be especially suited for the many studies gauging the effects of nutritional manipulation on immune potential. Recent investigations of the effects of vitamin A deficiency (20, 95), protein-caloric deficiency (7, 15, 100), deficiency of specific amino acids (78), and Zn deficiency (10, 35) are only a few examples.

The Microflora and the Etiology of Cancer

In recent years a strong correlation between nutrition and cancer of the colon has become obvious. A similar, though less-pronounced, correlation appears to exist between diet and breast cancer. In both instances, dietary fat seems to be a determining factor (14). Intestinal bacteria have been

implicated, especially in the case of colon cancer (26). Cycasin, a glycoside derived from cycad nuts, will produce tumors in liver and large intestine when fed to rats, but only in the presence of a CV microflora. In GF rats the aglycone of cycasin, methylazoxymethanol, normally released by bacterial action, will be as active as cycasin in CV rats (138).

Both Hill et al (66) and Reddy (109) have implicated bile acids in colon cancer. The latter study sees bile acids in a cancer-promoting role. Bile acids were instilled intrarectally into GF rats treated with methyl-N^1-nitro-N-nitrosoguanidine, thus avoiding modification of the administered bile acid by the local microflora. Although originally suspecting deoxycholic acid, found in high concentrations in stools of Western populations prone to colon cancer, these studies indicated that chenodeoxycholic acid and cholic acid also act as promotors, of approximately similar activity (117). Others, however, have failed to find the high fecal concentrations of neutral and acid sterol in high-risk human population groups upon which these studies were based (93).

ABSOLUTE DEFINITION IN NUTRITIONAL STUDIES

The Chemically Defined, Low-Molecular Weight, Water-Soluble, Filter-Sterilizable Diet for Germfree Rodents

Diets for GF animals obviously require sterilization, usually by heat or irradiation (161). Although the effects of sterilization on nutritional value and dietary antigenicity are much better known than at the start of the germfree era, much uncertainty remains. When solid diets are autoclaved, thiamine must be added to an extent that its level after sterilization exceeds the usually accepted requirements. Presumably part of the 50% or more of the thiamine inactivated during sterilization actually turns into an antimetabolite (148).

This lack of definition can be prevented by the feeding of water-soluble glucose-amino acid diets, fortified with water-soluble vitamins and minerals. Based on formulations originally described by Greenstein et al (50), these diets are sterilized by filtration (59, 84, 152) and thereby make total definition of intake possible. In principal, no bedding is provided for these animals. Since these diets also serve to eliminate dietary antigenicty (see Dietary Effects on Immune Mechanisms), no detergents are used to solubilize the lipid needed to cover PUFA requirements, and to serve as a vehicle for fat-soluble vitamins. Instead, the lipid fraction is filter-sterilized separately and fed as such.

Even with the rigorous definition provided by the use of ultrafilters that remove all material over 10,000 dalton, inbred GF C3H/HeCr (165) and

JeL:(ICR) (59) mice fed chemically defined (CD) diet grew at acceptable rates and reproduced. Their ceca were somewhat smaller than of comparable GF mice fed solid diets. However, although GF mice fed solid diets showed a lower O_2 consumption than did CV mice, the O_2 consumption of these GF mice was much higher than that of their GF counterparts fed solid diet, and even higher than that found in CV controls. Heart size of the various groups confirmed this picture. Apparently these amino acid-glucose diets require a more extensive, energy-demanding metabolism than the usual casein-starch or natural ingredient formulas (13). Other studies indicated that under GF conditions, inclusion of cysteine ethyl ester in these diets caused haemolytic anemia, azotaemia, and pancreatic acinar atrophy (84).

In our laboratory GF C3H mice maintained on CD diet have reproduced into the fourth generation (B. S. Wostmann, unpublished data). Although this seems to indicate that nutritional requirements are at least qualitatively met, reproduction fell off sharply during the reproductive life time of a female, and in later generations. Providing bedding material in the form of the purest available filter paper improved the situation to an extent, but breeding performance is still well below that of GF mice fed solid diets. This has directed attention to queuine as a possible dietary requirement.

The nucleoside queuosine is a hypermodified derivative of guanosine and is located in the first position of the anticodon of $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{His}$, and $tRNA^{Tyr}$. Its presence supposedly facilitates the proper translation of mRNA. The original transcript of $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{His}$, and $tRNA^{Tyr}$ contains a guanine residue, which is excised by hydrolysis of the N-glycoside bond and is replaced by queuine without cleavage of any phosphodiester bonds.

When GF- and CV-inbred C3H mice were maintained for 4 weeks on CD diet, the GF mice showed total depletion of queuine in $tRNA^{Asn}$ and $tRNA^{His}$, whereas CV mice showed normal values. In both the GF and CV mice, however, $tRNA^{Asp}$ and $tRNA^{Tyr}$ retained their queuine (34). More recent studies show that feeding CD diet to GF mice for a year or longer leads to queuine depletion of all four queuine-containing tRNA's. When queuine is administered intraperitoneally to depleted mice, synthesis of queuine-containing tRNA follows (W. P. Farkas, personal communication). This suggests that the requirement for queuine may be met by microbial synthesis, or must be provided with the diet. Its total absence may affect especially reproductive function.

CONCLUSIONS

The GF animal model is especially valuable in nutrition research when we try to evaluate the role of the microflora in the totality of the host-microflora

complex. It also makes it possible to study systemic metabolic interactions, like the interaction of vitamins A and K, in a system from which microbial interference—in this case an uncontrollable microbial production of K vitamins—is excluded. Combined with the total definition of dietary intake made possible by the CD diets, the GF model provides the utmost in control of experimental variables.

It must be borne in mind, however, that the animal without its conventional microflora has become a changed animal, in which different homeostatic conditions prevail. Only by taking these differences into account can we make intelligent use of an animal model that makes it possible to control virtually every conceivable external parameter.

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