Gnotobiotic animals in nutrition research

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Recognition that creatures in a conventional environment carry a large burden of metabolically active micro-organisms in their gastro-intestinal tracts makes untenable the classic concept of nutrition as a simple relationship between the diet and its consumer. Instead it must be regarded as a series of complicated interactions between the diet, its consumer and his resident microflora. To understand the interrelationships between the three components it is necessary to study each one in isolation. The idea of a host devoid of its usual microbial associates, although forecast by Pasteur as long ago as 1895, remained largely hypothetical until recent advances in gnotobiotic technique made it a practical possibility.

The technique depends upon the fact that an embryo developing inside a hen's egg or a mammalian uterus is almost always microbiologically sterile so long as the dam is free from disease. If transferred aseptically to a sterile container and reared there on sterile food and water the bird or mammal will remain 'germ-free', that is free from any other detectable form of life. The precise meaning of the term depends upon the extent of the tests applied to detect contamination. In practice it usually implies freedom from bacteria, fungi, protozoa, and internal and external parasites. A gnotobiote is an animal in which all the life forms present are known, that is it may be germ-free or associated with any number of strains of organism of known identity.

In the germ-free animal any nutritional process can be investigated without interference from the micro-organisms commonly inhabiting the gut in a conventional environment. The extent to which that process is modified by microbial action can then be examined by introducing known components of the gut microflora. However, the results of such studies cannot always be taken at their face value since, in a gnotobiotic system, the biological characteristics both of the animal and the associated microbes may deviate from the so-called 'normal' pattern displayed when the full conventional microflora is present.

Characteristics of the germ-free animal

Before the gnotobiote can be successfully applied as a research tool its peculiarities must be recognized. Given an adequate diet and comfortable living conditions the germ-free animal grows and thrives as well or even better than its conventional counterpart. As might be expected, the absence of microbial challenge results in

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modification of the defence mechanisms, but this aspect is outside the scope of the present discussion. Of more immediate concern in nutritional studies are small but important alterations to the physical and physiological properties of the gastro-intestinal tract. The gut contents of the conventional animal are generally more acid, particularly in the areas of greatest bacterial proliferation (Gordon, 1968; Ford, 1971), and their oxidation-reduction potential less positive (Wostmann & Bruckner-Kardoss, 1966). The small intestine of a germ-free animal usually weighs less per unit of length than that of its conventional counterpart. The difference is partly accounted for by a smaller proportion of lymphoid tissue but mainly by a reduction in connective tissue, particularly the lamina propria. The villi tend to be longer and more even, and the time taken for migration of the mucosal epithelial cells from the crypts to the villus tips is considerably extended. The brush border is more even, with longer microvilli, providing a potentially greater surface for absorption (Kenworthy, 1967). In vitro studies have shown greater uptake by everted sacs of germ-free intestine of xylose (Heneghan, 1963), glucose and several vitamins of the B complex (Ford & Coates, 1971) but no difference in the uptake of L-leucine (Riedel, Scharrer & Lösch, 1972). As yet no comparative absorption studies have been reported in intact animals. Nevertheless the morphological characteristics of the germ-free gut suggest that it may be a slightly more efficient organ of digestion and absorption than its conventional counterpart. Some workers have chosen to regard the conventional gut, even in health, as being in a chronic state of mild inflammation. It is still a matter for debate whether this condition is brought about by the presence of the general microbial population or whether it is a response to specific subpathogens commonly present in the conventional gut microflora.

One hitherto unexplained anomaly that occurs in germ-free rodents and rabbits is a gross enlargement of the caecum. It is associated with an accumulation of mucopolysaccharide materials and the presence of kallikreins in the caecal contents, a loss of intestinal muscle tone and an influx of water into the caecal sac (Wostmann, Reddy, Bruckner-Kardoss, Gordon & Singh, 1973). The phenomenon has not so far been reported in any of the other species that have been produced germ-free. It is unfortunate that the rat is such a favourite subject for nutritional studies, because in the germ-free state its caecum may represent 25% or more of its body-weight, therefore conclusions based on comparative growth rates or balance studies may be invalid. Furthermore, some of the differences in physiological performance between germ-free and conventional rats appear to be indirect results of the enlarged caecum rather than direct effects of the absence of specific micro-organisms. For example, the rate of passage of food through the small intestine is slower in germ-free than in conventional rats, but the difference largely disappears after caecectomy (Sacquet, Garnier & Raibaud, 1970). In the germ-free chick, which does not exhibit caecal enlargement, the transit time of food through the gut is no different from that in conventional birds (Ford, 1971).

Applications of gnotobiotes to nutritional problems

The nutritionist is concerned to know what part micro-organisms play in the digestion and metabolism of nutrients during their passage through the alimentary canal and the effects, if any, on the host. Studies with gnotobiotes have already provided a considerable amount of information on the subject (see reviews by Coates, 1968, and Kellogg & Wostmann, 1968), but much remains to be learnt. The examples given below illustrate the type of problem that can be investigated with germ-free animals and the factors that must be taken into account in relating the results to practical nutrition in man or domestic animals.

Intestinal disaccharidases. Several workers have been interested to determine whether the intestinal disaccharidases are of animal or bacterial origin. Siddons & Coates (1972) found no differences between germ-free and conventional chicks in the total maltase, sucrase or palatinase activities of the small intestine. Most of the activity was located in the intestinal wall, and lactase was virtually absent. There was, however, appreciable lactase activity in the large intestinal contents of conventional birds. When chicks were reared on a diet containing lactose as the main carbohydrate source many in the germ-free environment died in the first 2 weeks; their conventional counterparts survived longer, although they grew poorly. It was concluded that disaccharidases in the small intestine were entirely produced by the host but that lactase in the large intestine was of bacterial origin. Further, since the conventional birds survived longer on a lactose diet they must have been able to utilize at least part of the products of bacterial lactase digestion. In similar studies with rats Reddy & Wostmann (1966) also showed that disaccharidases in the small intestine were not microbially produced and, in fact, found higher disaccharidase activities in the germ-free animals. They postulated that the slower rate of turnover of mucosal epithelium in the germ-free intestine would result in more mature cells with, possibly, a higher content of enzyme.

Synthesis of vitamins. Microbial synthesis of the vitamins B and K has been demonstrated in rats and chicks by several workers. Conventional chicks, kept out of contact with their own excreta and reared on diets devoid of any one vitamin of the B complex, had appreciable quantities of the missing vitamin in their caecal contents whereas negligible amounts were found in corresponding samples from germ-free birds (Coates, Ford & Harrison, 1968). The signs of deficiency were as severe in the conventional birds, indicating that the microbially-synthesized vitamins were not utilized. This is not surprising since the principal site of bacterial activity in the chick is the lower gut, distal to the absorptive area. Further, the vitamins may have been bound inside bacterial cells, making them unavailable for absorption. Conventional chicks had higher liver levels of thiamine than did their germ-free counterparts although their growth and survival was no better, a finding in accord with earlier work in rats (Wostmann, Knight, Keeley & Kan, 1963). Studies in conventional rats showed that after oral administration of $^{35}SO_4^{2-}$ large amounts of ^{35}S-labelled thiamine were formed in the caecal and colon contents but little radioactivity was detected in the tissues. Much of the thiamine in the caecal contents was firmly cell-bound and thus probably unavailable for absorption even after recycling by coprophagy. It seems unlikely therefore that the higher levels of thiamine in the livers of conventional chicks and rats resulted from absorption of the bacterially synthesized vitamin. Instead, the thiamine content of the liver may depend on its metabolic activity; absence of micro-organisms, and consequently of the need to detoxify their products, would create a lower demand for energy and might thus account for the lower content of thiamine (Wostmann *et al.* 1963).

Germ-free rats given a diet without vitamin K rapidly develop a haemorrhagic condition but conventional rats on the same diet do not (Gustafsson, 1959). By association of vitamin K-deprived germ-free rats with organisms from conventional rats the signs of deficiency were reversed (Gustafsson, Daft, McDaniel, Smith & Fitzgerald, 1962). Since one of the organisms was isolated from the oral cavity it seems feasible that some synthesis of vitamin K takes place in the upper part of the alimentary tract and the product might then be available to the host. However, conventional rats fitted with tail cups to prevent coprophagy also developed the signs of deficiency. Thus it must be assumed that the main synthesis takes place in the lower gut and the vitamin is not available to the host without recycling.

Effects of the absence of a gut microflora on the host's nutrition

Although no precise comparisons of dietary requirements have been made between germ-free and conventional animals, interesting differences have been observed in their utilization of some nutrients.

Vitamins. Germ-free rats given a vitamin A-deficient diet survived much longer than their conventional controls, all of which were dead within 50 d whereas some of the germ-free rats were still alive after 272 d on the diet (Bieri, McDaniel & Rogers, 1969). The longer survival was ascribed to lack of exposure of the germ-free animals to infection. It is also possible that their requirement was less, since vitamin A is concerned in maintaining the integrity of epithelia and the rate of turnover of epithelial mucosa is much slower in the germ-free state. The phenomenon may also be an indirect result of caecal enlargement since no difference in survival time between germ-free and conventional chicks deprived of vitamin A has been observed in our own laboratory or elsewhere (Rogers, Bieri & McDaniel, 1971).

Prolonged survival also occurred in germ-free guinea-pigs on a scorbutogenic diet, and only minor signs of scurvy were seen (Levenson, Tennant, Geever, Laundy & Daft, 1962). This finding may indicate an increased demand for vitamin C under the 'stress' of a microbial burden. Miyakawa (1966) found smaller adrenals in germ-free animals which suggests that the presence of micro-organisms may lead to a greater need for adrenal steroids and thus to a greater demand for vitamin C. We have seen indications of an increased need for pantothenic acid by conventional chicks, which could be explained in the same way (Coates *et al.* 1968).

Essential fatty acids. In rabbits given diets marginally adequate in essential fatty acids very much higher proportions of polyunsaturated fatty acids in the triglyceride, phospholipid and sterol ester fractions were found in the livers of germ-free animals (Coates, Moore & Turvey, 1969). This is compatible with the known ability of gut micro-organisms to hydrogenate unsaturated fatty acids and indicates a lower dietary requirement for essential fatty acids by germ-free animals.

Iron. There is evidence with rats and guinea-pigs that dietary iron may be less available in the absence of a gut microflora, and in germ-free rabbits a hypochromic anaemia was observed on two diets that had proved adequate for their conventional controls (Reddy, Pleasants, Zimmerman & Wostmann, 1965). In view of the more positive redox potential in the intestine devoid of micro-organisms it seems likely that a greater proportion of iron would have been in the ferric form and therefore less readily utilized.

Calcium. In germ-free rats a very high incidence of urinary calculi, which were completely absent from their conventional controls (Gustafsson & Norman, 1962), indicates that calcium metabolism may be disturbed in the absence of micro-organisms. It is not clear whether there is a greater uptake of calcium from the germ-free gut, or more retention in the tissues. Either is feasible. The absorption of calcium is facilitated by bile salts and hence may be modified in the germ-free animal in which the endogenous bile acid pattern has not been altered by the action of micro-organisms in the gut. Increased retention could occur as a result of a disturbed electrolyte balance consequent upon the enlarged caecum.

It is not yet possible to say what relevance these findings in germ-free animals have to nutrition in a conventional environment. Those that are peculiar to one species, or are part of a general reaction to the total absence of microbes, will be of academic interest only. If any of the observed phenomena prove to be effects of specific components of the conventional gut microflora they may be of importance in practical nutrition, particularly if the presence or activities of the responsible organisms can be controlled by dietary treatment.

Choice of an experimental model

Experience with gnotobiotes emphasizes that the structure of the alimentary tract and the position of the sites of microbial activity largely determine whether or not the effects of that activity will be felt by the host. It is therefore unwise to draw general conclusions from results in a single species, and more comparative studies between species with different gut structure and food habits would be helpful. It is particularly difficult to apply the results in gnotobiotic laboratory animals, especially rodents, to human nutrition. Recent advances in clinical technique have made gnotobiotic human subjects a practical possibility. Infants with suspected immunodeficiencies have been delivered germ-free and maintained so for long periods. Their faecal bile acid pattern is similar to that of germ-free rats (Kellogg, 1973). Patients at high risk from casual infections have, by isolation and decontamination, had the population of their alimentary tract reduced to a few defined strains of organism. In such subjects valuable information could be obtained concerning the influence of the gut microflora on nutritional processes in man.

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