

Rearing Germfree Rats on Chemically Defined, Antigen Low Diets¹

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The potentialities of the germfree animal as a research tool of unusual precision have been outlined by Reyniers and Trexler (7) and Glimstedt (2). These potentialities are especially notable in areas of study such as immunology and nutrition, in which any living microbes could introduce new variables. For the immunologist, one such variable might be the non-specific stimulation of the reticulo-endothelial system by bacterial endotoxins. For the nutritionist, expected variables would include the destruction or synthesis of a nutrient by bacteria, and changes in absorption due to modification of the intestinal wall in the presence of bacteria.

But the mere absence of a living microbiota is no guarantee of freedom from bacterial influence. Germfree chickens have been found by Wagner (10) to have a low titer of antibodies against *Micrococcus epidermidis* after 200 days of age. This organism is one of the more predominant forms found in the dry diet ingredients before autoclaving. In addition to this, any ordinary laboratory-type diet, especially when steam-sterilized, contains a great variety of molecules of uncertain nutritional and antigenic properties.

There would therefore be a marked gain in precision if animals already germfree could be maintained on chemically defined diets, purified as far as practicable from bacterial products and foreign macromolecules. Such a regimen would not be fully effective, however, if started only at weaning. The permeability of the intestine during the suckling period, and the presence in milk of both antibodies and potential antigens, argue for the use of antigen-low diet from birth. In nutritional studies, the persistence of body stores of some nutrients, and the influence of early nutrition on later life, also argue for the use of a chemically defined diet from birth.

Although nutritionally adequate mixtures of pure chemicals had been elaborated earlier (8), the first such mixture that appeared readily adaptable both to sterilization for the germfree environment and to use as an infant formula was developed by Greenstein (3). This adaptability came from the fact that the diet was completely water-soluble, being designed for eventual use in parenteral nutrition. The diet had supported normal growth in mice (1), and growth, reproduction and lactation in rats through several generations (3). It remained for us, then to adapt the diet to the germfree animal as such, and in particular, to the requirements of the germfree mammal during its suckling period.

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Apparatus and Method

Germfree Lobund strain rats at various stages during the suckling period, were transferred from their mothers' nest to plastic isolators of the Trexler (9) type and kept in a nest box similar to that of Gustafsson (5) except that heat came from a heating pad underneath the plastic

Weaned rats were housed in individual screen bottom cages within the plastic isolator. Bedding cannot be used because it is consumed greedily by the rats. The rats were offered soluble diet in Fisher animal drinking tubes. Distilled water was provided in overhead bottles with glass tubes. Towards weaning, the corn oil fed during the hand-feeding period was gradually eliminated, and Dextri-maltose gradually increased until it finally provided one half of the total solids of the diet.

Analyses of gastro-intestinal contents for osmolarity were carried out with a Mechrolab osmometer, model 301. bottom of the isolator. The force-feeding technique of Pleasants (6) was followed, except that the natural latex nipple there described had to be replaced by a silicone nipple² which would not be attacked by the oily component of the diet. The 2 hour feeding schedule used for milk formula by Pleasants (6) was replaced by a 30 minute feeding interval in the final successful experiments.

The original Greenstein diet (3) with its later modifications (4) gradually underwent further changes at our hands in order to meet the higher needs of the suckling rat for cysteine, tryptophan, zinc and sodium chloride. When weanling rats on this diet developed a hemorrhagic syndrome, the vitamin K_3 (menadione) of the diet was replaced by natural vitamin K_1 . As the harmful effects of a high osmotic value in the diet became apparent, the 65% dextrose of the diet was greatly reduced and only partially replaced by Dextri-maltose (maltose and dextrans obtained by enzymic action of barley malt on corn flour). To replace the missing calories, corn oil was then fed separately from the diet. Table I gives the formula which was ultimately successful.

Results

Pilot studies with conventional baby rats force fed the high dextrose diet were rendered useless by a massive growth of mycotic forms in stomach and esophagus, sometimes entirely closing the esophagus. The relatively high pH of an infant rats' stomach (about 6.0) may explain why conventional infant rats fared less well than the conventional weanling rats used by Greenstein (3).

Initial attempts at hand feeding germfree rats, taken from their mothers within a few days after birth, were hampered by the development of severe intestinal distention. At this time the diet was high in dextrose. Most animals were lost by aspiration of diet into the lungs, either through misplacement of the feeding nipple or by regurgitation

2. The smaller of two nipples originally designed by the Walter Reed Army Institute of Research and obtainable commercially from the R. E. Darling Co., 4858 Cordell Avenue, Bethesda, Maryland.

from an overdistended gut. Unlike milk formulas, the soluble diet forms no curd to stabilize it within the confines of the stomach.

TABLE I
Composition of Diet

Essential amino acids		Non-essential amino acids	
l-Lysine HCl	2.48 gm.	l-Aspartic acid	2.74 gm.
l-Histidine HCl•H ₂ O	1.08 gm.	l-Proline	5.98 gm.
l-Tryptophane	0.80 gm.	Na-l-glutamate	12.04 gm.
l-Phenylalanine	1.80 gm.	Glycine	0.96 gm.
l-Isoleucine	1.00 gm.	l-Serine	3.10 gm.
l-Leucine	1.60 gm.	l-Alanine	1.50 gm.
l-Threonine	1.00 gm.	Ethyl-l-cysteinate•HCl	0.56 gm.
l-Methionine	1.20 gm.	Ethyl-l-tyrosinate•HCl	3.96 gm.
l-Valine	1.40 gm.	This combination is based on diet	
l-Arginine•HCl	1.50 gm.	26 of Greenstein et al. (3)	

Minerals

MgO	0.15 gm.	Dextrose	30.00 gm.
Glucanolate (for MgO)	1.30 gm.	Sucrose	8.00 gm.
CH ₃ COOK	2.12 gm.	B-mix 110	1.5 gm.
NaOH	0.90 gm.	Choline Cl	0.50 gm.
NaCl	0.76 gm.	Ethyl linoleate	0.80 gm.
Ca fructose (PO ₄) ₂	10.00 gm.	Tween-80	1.20 gm.
Salts 21	0.44 gm.	Ladek 50-E2	0.20 ml.
Na ₂ SeO ₃ sol. 5.5 mg%.	0.4 ml.	Distilled H ₂ O	320.0 ml.

0.44 gm. Salts 21 contains: 280 mg. Fe (NH₄)₂•(SO₄)₂•H₂O; 102.6 mg. ZnSO₄•H₂O; 52 mg. Mn(CH₃COO)₂•4H₂O; 6 mg. KI; 3 mg. Cu (CH₃COO)₂•H₂O; 1.8 mg. Co(CH₃COO)₂•4H₂O; 1.2 mg. (NH₄)₆Mo₇O₂₄•4H₂O.

1.50 gm. B mix 110 contains: 1 mg. thiamine•HCl; 1.5 mg. riboflavin; 1.26 mg. pyridoxine•HCl; 7.5 mg. niacin; 50 mg. i-inositol; 10 mg. calcium pantothenate; 0.06 mg. biotin; 0.1 mg. folic acid; 20 mg. of 0.1% triturate of vitamin B₁₂ in mannitol; 100 mg. ascorbic acid; 60 mg. p-aminobenzoic acid; 1.25 gm. dextrose carrier.

0.2 ml. Ladek 50-E2 contains: 2 mg. Vitamin A acetate; 1.4 microgm vitamin D₂ (calciferol); 10 mg. dl-alpha-tocopherol acetate; 0.44 mg. vitamin K₁; 0.19 ml. absolute ethanol carrier.

Notes: The order of addition described in Greenstein et al. (4) must be followed. The ethyl tyrosinate•HCl had to be dissolved very rapidly in its own weight of water, then added at once to the main solution.

The final diet was filtered through a GS Millipore filter (0.22 micron pore size) with glass fiber prefilter. The flask of diet was sealed off and passed into the plastic isolator through a lock sprayed with 2% peracetic acid. Within the isolator, this diet (28% solids w/v) was diluted to 14% solids for 3 day old rats. The level of solids was gradually increased to 19% solids for 20 day old rats.

Corn oil (0.05ml) was fed 4 times daily in place of a soluble diet feeding. In addition, the nipple tip was dipped in corn oil at each feeding of soluble diet for lubrication. This is estimated to have provided 0.10 ml. oil daily.

A 28% solution of Dextrin-maltose, w/v, was mixed with the diet after weaning to raise gradually the proportion of carbohydrate without increasing total solids.

Since Greenstein (3) had found no intestinal distention in conventional weanling rats fed water-soluble diet, it appeared necessary at this time to determine if distention of the small intestine and cecum on this diet was peculiar to the hand-feeding period or was a reaction of the germfree rat as such. For this purpose germfree rats were offered the diet only after weaning. Although these suffered severe intestinal distention during a transition period of several weeks, and the majority died from volvulus of the cecum, those which survived became adjusted to the diet and grew at about $\frac{2}{3}$ the normal rate of gain for periods up to 3 months. To see if the high osmolarity of the diet might be accentuating the general tendency of the germfree rodent to develop an enlarged cecum, most of the dextrose was replaced by the much higher molecular weight Dextrin-maltose. This change substantially reduced the death rate in weanling rats during the transition period.

Efforts at hand-rearing were then resumed, with emphasis on reducing the osmolarity of the diet by diluting with water, by using less total carbohydrate and by making up the calories with separate feedings of corn oil. However, on the old two-hour feeding schedule it still remained difficult to provide enough calories and protein with this diluted diet while avoiding excessive stomach distention and risk of diet aspiration.

Analysis of gastro-intestinal contents³ showed that even the diluted diet's osmolarity of 1.0 was considerably higher than the osmolarity of 0.35 to 0.4 found regularly in the small intestine of rats fed this same diet. Analysis of stomach contents at various intervals after feeding showed a rapid initial decrease of osmolarity to 0.7-0.8, followed by a slower rate of decrease thereafter. Apparently, water passed in less readily after an initial passage leading to severe distention of the stomach.

Until now the rats had routinely been fed at 2 hour intervals, a schedule carried over from much earlier experience with hand feeding of milk. A 30 minute interval was adopted and proved immediately successful. Feeding smaller amounts of the diet apparently did not impede the diluting and emptying of the stomach contents. The daily intake of food could now be doubled without creating severe gastro-intestinal distention. The incidence of diet aspiration dropped sharply. Out of 5 young, taken from their germfree mother at 3 days of age and fed every half hour on the diet in table I, one was sacrificed at 10 days of age. Its stomach contents, 30 minutes after feeding, showing an osmolarity close to that of the small intestine. The remaining four continued growing at the rate of 0.5 gm. per day during hand feeding, a rate of gain comparable to that of rats hand-fed on milk formula by Pleasants (6). They were weaned at 20 days of age and maintained to sacrifice at 59 days of age. Loss of fur and slow growth about the time of weaning indicated some general or specific inadequacy of the diet but resumption of fur growth at 6 weeks and gross normality of organs at sacrifice indicated no severe deficiency.

3. We are indebted to Dr. Dean Zimmermann for these and other analyses carried out in the course of diet development and also for valuable suggestions on diet ingredients.

Discussion

At this point, the most encouraging finding is the probability that no unknown nutritional factors are required by the infant rat. Although two poorly defined supplements, corn oil and Dextri-maltose, have been included in the diet in order to determine the influence of dietary osmolarity, it is planned to replace them as soon as practicable with chemically defined substitutes.

It is interesting that a method which quadruples forced entries into the esophagus should greatly reduce the risk of diet aspiration. This fact suggests that much of the earlier diet aspiration must have followed regurgitation from a stomach under extreme pressure. The osmolarity studies suggest that the stomach probably increased in volume for a time after each feeding as water was drawn in to dilute the hypertonic contents. Excessive distention of the stomach might also impair the stomach's ability to contract and force contents into the intestine. Frequent feeding of small amounts of food seems to have by-passed this particular problem by allowing for a certain amount of water to enter the stomach without its reaching a distended stage which interfered with emptying.

With the improved safety of the new schedule, we plan to go at once to the mechanically more difficult task of rearing rats on the diet from birth rather than from 3 days of age. As soon as practicable, the diet and technique will be tried on germfree newborn mice. Mice have proved very difficult to rear by hand (6) because of a high risk of diet aspiration. Virologists, however, prefer to see new strains of germ-free mice started by hand feeding rather than by cross-suckling on other germfree strains which could be harboring latent viruses. If the present diet should improve hand rearing of mice, it would also improve the usefulness of the germfree mouse for virus and cancer research.

Current success with the new diet and technique thus encourages us to hope that this type of diet will permit more precise determination of the needs of suckling animals, hasten the procurement of new germ-free species and strains, and improve the precision of the germfree animal as a research tool for the biological and medical sciences.

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