Effect of Germfree Status on ^{64}Cu Excretion by the Rat* (33776)

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There are very little data in the literature concerning mineral metabolism in germfree animals and studies to determine the mineral requirements of these animals are apparently nonexistent. With few exceptions, it has been generally assumed that the mineral supplements used for conventional animals are adequate and correct for germfree animals. Conversely, these few exceptions have indicated that intestinal microflora may play a rather important role in mineral nutrition. Based uopn the uptake of orally administered ^{47}Ca by the tibia, Edwards and Boyd (1) suggested that the germfree chick absorbed calcium more rapidly than its conventional counterpart. Reddy *et al.* (2) proposed that the rates of metabolism of iron and copper are lower in the germfree rat than in conventional rats, whereas, the rate of manganese metabolism appeared to be similar in the two groups. Subsequently, Reddy *et al.* (3) reported that a diet which supported normal growth and reproduction in conventional rabbits was inadequate for germfree rabbits. The germfree rabbits consuming this diet developed anemia, bone fragility, had irregular pregnancies, and were unsuccessful in nursing their young. These conditions were corrected either upon conventionalization of the animals or by supplying more iron from natural ingredients (in lieu of iron salts). In a recent report, Reddy *et al.* (4) indicated that germfree conditions increased the absorption of calcium and magnesium, but not phosphorus, in rats. In the present study, a modified balance technique was employed in an effort to determine whether or not gross differences exist between germfree and conventional rats in their ability to absorb and/or excrete copper.

Material and Methods. Eight germfree (GF) and eight conventional, pathogen-free (PF) male weanling rats,[1] CDF strain, were used in each of two experiments. The GF rats were housed in individual, stainless steel, wire-floor cages inside a sterile plastic chamber and the PF rats were kept in individual stainless steel, wire-floor cages in an open colony room. For routine microbiological monitoring of the GF chambers, diet and feces, three media were used: fluid thioglycolate (anaerobic), trypticase soy broth (aerobic) and Sabouraud liquid (fungus). All animals (GF and PF) received the same steam-sterilized diet and water *ad libitum*. The diet consisted of (%): vitamin-free casein, 20; corn starch, 66; alphacel, 3; corn oil, 5; mineral-mix,[2] 5; and vitamin-mix,[3] 1.

After the rats had become adjusted to the diet (a minimum of 3 weeks), each animal was intubated with 0.5 ml of an aqueous solution containing 10–25 μCi (Expt. 1) or approximately 20 μCi (Expt. 2) of radioactive copper (^{64}Cu) as the acetate.[4] For this amount of radioactivity, half of the animals in each experiment (four GF and four PF) received a physiological dose of copper

* The Principles of Laboratory Animal Care as promulgated by the National Society for Medical Research were observed.

[1] Obtained from Charles River Breeding Laboratories, Inc., N. Wilmington, Massachusetts.

[2] Mineral-mix contained (g/kg of diet): $Ca(H_2PO_4)_2 \cdot H_2O$, 21.8; $CaCO_3$, 9.3; K_2SO_4, 4.8; $MgSO_4$, 2.4; $Na_2HPO_4 \cdot 7H_2O$, 1.3; NaCl, 0.98; $FeSO_4 \cdot 7H_2O$, 0.15; $MnCO_3$, 0.125; ZnO, 0.018; $CuSO_4$, 0.015; KI, 0.00023; and corn starch, 9.1118.

[3] Vitamin-mix contained (g/kg of diet): riboflavin, 0.03; thiamine·HCl, 0.06; biotin, 0.001; folic acid, 0.01; niacinamide, 0.05; nicotinic acid, 0.05; vitamin B_{12}, 0.003; DL-Ca-pantothenate, 0.3; inositol, 1.0; ascorbic acid, 2.0; choline chloride, 2.0; pyridoxine·HCl, 0.02; pyridoxamine·$(HCl)_2$, 0.005; *p*-aminobenzoic acid, 0.05; menadione K, 0.10; vitamin E (alpha-tocopherol), 0.5 (1 mg = 1.1 IU); vitamin A, 0.02 (10,000 IU); vitamin D, 0.002 (1000 IU); and corn starch, 3.799.

[4] Obtained from Tracerlab, Waltham, Massachusetts (Expt. 1) and Cambridge Nuclear Corp., Cambridge, Massachusetts (Expt. 2).

TABLE I. Effect of Environment upon Excretion of ^{64}Cu by Rats.[a]

Environment	Feces		Urine	
	Normal Cu[b]	High Cu[b]	Normal Cu[b]	High Cu[b]
Expt. 1				
Germfree	6.13 ± 3.61[c]	11.63 ± 8.34	1.59 ± 1.06	0.88 ± 0.52
Pathogen-free	16.91 ± 7.18	33.14 ±19.74	1.00 ± 0.29	0.79 ± 0.74
Expt. 2				
Germfree	4.28 ± 2.32	11.79 ± 3.74	0.70 ± 0.12	0.81 ± 0.36
Pathogen-free	13.27 ± 3.67	21.37 ± 8.64	0.49 ± 0.14	0.14 ± 0.02

[a] Values represent the percentage of the orally administered dose of ^{64}Cu which was excreted in 24 hr following dosage.

[b] Amount of carrier copper in the isotopic dose: normal Cu = 10–20 μg; high Cu = 250–300 μg.

[c] Mean of four observations with the standard deviation.

(10–20 μg), whereas, the other half of the rats received a relatively high dose of copper (250–300 μg). Immediately following intubation with the isotope, the rats were equipped with tail cups and placed in plastic metabolism cages where feed and water were again provided *ad libitum*. Urine and feces were collected for 24 hr and radioactivity determinations[5] were made on the collected samples. The relatively short half-life of ^{64}Cu (12.8 hr), the difficulty in obtaining an isotope of higher specific activity, and the desire to stay within the above-indicated copper dosage ranges made longer collection periods mechanically unfeasible. During the intubation process and collection period, the GF rats were maintained in the sterile environment.

Records were kept of feed consumption and body weight gains. At the end of the first experiment, five GF and two PF rats were sacrificed and ceca weights were determined. At this time, one of the remaining GF rats was "conventionalized" by placing it in the open colony room. Two days later, this rat was sacrificed and its cecum was weighted. The data were statistically treated by the analysis of variance method (5).

Results and Discussion. The effect of germfree status upon the excretion of ^{64}Cu is summarized in Table I. Two points are particularly evident: (a) the markedly reduced fecal excretion of the isotope by the GF rats, and (b) the very low level of urinary ^{64}Cu excretion. The reduced fecal excretion of ^{64}Cu ($p < .05$ in Expt. 1 and $p < .01$ Expt. 2) by the GF rats would seem to indicate that copper absorption is greater under axenic (germfree) conditions. Further, the low level of radiocopper in the urine would indicate that most of the absorbed copper had been retained. Although the amount of radioactivity (cpm) in the urine was not large enough to permit meaningful statistical analyses, the indicated trend is for greater urinary ^{64}Cu excretion by the GF rats. If this interpretation is accurage, the present data would appear to be in contradiction to that of Reddy *et al.* (2) who proposed a lower rate of copper metabolism in germfree rats.

The data concerning growth rates and feed consumption are summarized in Table II. It appears that the PF rats grew a bit more rapidly and consumed more diet than the GF rats. However, when feed efficiencies were calculated, these slight differences between GF and PF rats virtually disappeared.

The data concerning ceca weights are presented in Table III. As can be observed from these data, the GF rats had tremendously enlarged ceca, a prominent characteristic of germfree rats. During the course of the first experiment, from which the ceca data were obtained, the GF rats became mildly contaminated with what was believed to be

[5] Packard Auto-Gamma sodium iodide scintillation detector, Packard Instrument Company Inc., La Grange, Illinois.

TABLE II. Effect of Environment on Growth Rate and Feed Consumption.[a]

Environment	Growth rate (g/day)	Feed consumption (g/day)	Feed efficiency (g of diet/g of gain)
Expt. 1			
Germfree	3.27	8.91	2.73
Pathogen-free	3.66	10.18	2.78
Expt. 2			
Germfree	3.84	8.89	2.33
Pathogen-free	4.38	11.43	2.61

[a] Each value represents the mean of eight observations.

a single species of microorganism. Despite this contamination, the cecum size would seem to indicate that the GF rats had retained their germfree characteristics to a large degree since GF rats consuming a 3% fiber diet normally have ceca weights of 5–10% of the total body weight (6). Under multicontamination conditions, the cecum rapidly returns to normal, conventional size as can be seen for the "conventionalized" rat. The similarity of the ^{64}Cu excretion data for the rats in both experiments (Table I) further suggests that the GF rats in Expt. 1 metabolized copper in a "germfree manner" even though they were mildly contaminated.

The amount of radiocopper excreted in the feces appears to be dependent upon the amount of carrier copper in the dose. The data in Table I show that when the dosage contained a high level (250–300 μg) of carrier copper, the percentage of isotope appearing in the feces was greater than when the dosage contained a normal amount (10–20 μg) of carrier copper. In both the GF and PF rats, this difference approached significance ($.05<p<.10$) in Expt. 1 and was significant ($p<.05$) in Expt. 2.

TABLE III. Effect of Environment on Cecum Weight.

Environment	Cecum wt. (% of body wt.)
Germfree	12.7[a]
Pathogen-free	2.7[b]
"Conventionalized" germfree	2.5[c]

[a] Average of five rats.
[b] Average of two rats.
[c] One rat.

One of the major criticisms of the few reported germfree mineral studies is that the quantities of minerals given are usually far from the required amounts, ranging from near the requirement for zinc to some 30 times the requirement for iron (2). For that reason, the diet used in the studies reported herein was designed to contain approximately 120% of the recommended mineral allowance (7). The growth rate data would seem to substantiate the contention that these quantities of minerals are adequate for GF as well as PF rats, even though there appears to be some difference in copper metabolism between the two environments.

Although the reduced fecal excretion of ^{64}Cu by GF rats would seem to indicate that copper absorption is enhanced by axenic conditions, there is another possibility which cannot be excluded at the present time. Since the GF rats have a voluminous cecem compared with their conventional counterparts, it is possible that the overall rate of passage of nutrients is reduced in the germfree environment.

Experiments are now underway to determine whether or not the presently observed phenomenon can be extended to trace elements other than copper. It is also hoped that the site of this effect can be elucidated using radioisotopes in which the half-life and specific activity do not impose mechanical and/or physiological limitations.

Summary. The fecal excretion of radioactive copper during the first 24 hr following oral dosage was significantly lower in germfree rats than in conventional rats. Urinary excretion of ^{64}Cu during the same period of time amounted to approximately 1% of

the oral dosage and was not significantly affected by germfree conditions. Growth rates indicated that a mineral level of 120% of the recommended daily allowances for conventional rats was adequate for germfree rats despite the fact that some of the minerals may be metabolized differently by rats in the two environments.

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A Simple Micro Tissue Culture Method for the Determination of Lymphocyte Cytotoxicity *in Vitro** (33777)

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Different techniques have been used to assess the cytocidal effect on target cells *in vitro* of lymphocytes from nonimmunized and immunized donors. Monolayer cultures in different types of culture tubes have most commonly been employed (1–3), but methods utilizing the formation of plaques in petri dishes also have been described (4), as well as inhibition of colony formation (5). Common to most methods is the requirement of rather large numbers of lymphocytes. As supply of animals and cells used for immunization is often limited, a micro scale test is desirable. Our experience with a simple micromethod is described in the present report.

Materials and Methods. Principle of the test system. Mice were immunized with cultured cells. Cell suspensions consisting of known numbers of lymphocytes prepared from the lymphnodes of normal, *i.e.*, nonimmune, or sensitized animals were added to previously established monolayer cultures of known numbers of target cells. At specified time intervals after addition of lymphocyte suspensions, the number of target cells remaining in the cultures were quantitatively determined.

Animals and procedure of immunization. Young adult C3H/Fib mice of both sexes were employed for this study. The animals were sensitized by subcutaneous inoculation bilaterally in both flanks of a total of 20×10^6 cells usually suspended in 0.2 ml of Tyrode's solution. In some instances the cells were suspended in complete Freund's adjuvant. Hyperimmunization of the animals was achieved by 5–7 inoculations given at 7–10-day intervals.

Target cells. The three cultured cell lines used in the assays originated from normal C3H cells. The C3H-M cells were obtained from the spleen of an adult C3H mouse and the C3H-L cells came from the lung of the same mouse, whereas the C3H-E cells were obtained from cultures of 19-day-old C3H mouse embryos (6). All three cell lines had been propagated continuously *in vitro* for 4 years at the time of the present experiments.

The cells were propagated in Roux flasks on a standard medium consisting of 80% Eagle's minimum essential medium (7) with a twofold concentration of the essential amino acids and a fourfold concentration of the

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