# Water Movement Across the Cecal Wall of the Germfree Rat[1] (34657)

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(Introduced by T. Z. Csáky)

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Perhaps the most striking feature of rodents reared under germfree conditions is their enormously enlarged cecum which contains semiliquid material. In contrast, their small intestine does not display macroscopically visible anomalies. Since the excessive cecal fluid is not sufficiently absorbed during its further passage through the colon, the animals exhibit a mild chronic diarrhea. The observations that germfree animals drink more water (1) and cannot endure thirst and starvation for as long a period as conventionally raised controls (2) can be explained on this basis. All of these features disappear within 3–4 weeks after orally seeding the normal flora (3). Although long known and of central importance in germfree life, the disturbed cecal water absorption has not been satifactorily explained. The few available membrane transport experiments on this subject deal primarily with the small intestine (4, 5). This motivated the present studies performed *in vivo* in the cecum of germfree and conventional rats.

Net water movement across epithelial membranes is predominantly brought about by two mechanisms: It is either due to local osmotic forces created within the membrane by the active absorption of solutes, mainly of sodium chloride [solute-coupled water transport (6–8)], or it is the consequence of osmotic pressure differences across the whole complex of structures between lumen and blood plasma (osmotic water flow). Both mechanisms have been demonstrated in the lower bowel of conventionally reared animals (9–11). Therefore, in this work, water movement across the cecal wall was studied after replacing the normal content either by (a) saline isotonic with blood, or (b) a hypo- or hypertonic solution.

*Methods and Materials.* Germfree and conventional male rats of the same strain (Fischer 344, Charles River, CD-F), age (14 months) and weight (300–430 g) were kept on steam-sterilized L-462 diet (12) fed *ad libitum*. The day before the experiment the animals were fasted overnight with free access to water. The germfree rats were maintained in the flexible plastic isolators of Trexler (13), and the conventional controls in the open environment of the same air-conditioned room. Standard germfree rearing techniques were used (14). The germfree rats remained in the isolators until the injection of the anesthetic (urethane, 150 mg/100 g of body wt, sc), at which time they were exposed to the laboratory environment. Through an abdominal midline incision, the ileum and colon were ligated immediately at their emergence from the cecum, leaving intact the major blood vessels of this area. Through a small opening cut into the apex of the cecum its contents were removed and the lumen was rinsed with isotonic saline. From this point on, two different perfusion techniques were used (see below). The body temperature of the animals was kept at 36–38° by means of a heating pad and lamp throughout the experiment. Volume changes of the cecal fluid were measured both volumetrically and with $^{14}$C-polyethylene glycol (PEG)[3] added to the experimental solutions as a marker substance, using liquid scintillation counting. The fluids were assayed for sodium (Baird-Atomic flame photometer)

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[3] Polyethylene-1,2-$^{14}$C-glycol, mol wt ca. 4000, New England Nuclear Corp., Boston, Mass.

and for osmolality (Advanced Instruments osmometer). To obtain a measure of the surface area, the cecum was excised at the end of the experiment and cut open along its mesenteric border. It was spread out, its configuration was traced on a dry sheet of paper of uniform thickness, and the weight of the cut-out tracings was taken. This was then referred to the weight of a piece of the same paper of which the area was known. Dry weight determinations were done after removal of all visible fat from the organ.

*Results. Solute-coupled water transport.* In these experiments, a known amount of 0.9% NaCl solution containing $^{14}$C-PEG was introduced into the cecal sac through the apical opening, which was ligated afterwards. The cecum was then placed back into the abdominal cavity. No external fluid stirring was employed. This procedure closely followed the normal *in vivo* conditions, except that the cecum represented a ligated pouch. After 3 hr, the fluid remaining in the cecum was quantitatively withdrawn and analyzed.

Table I shows the results of the volume measurements. It is clear that absorption of fluid took place in both groups of animals but that it was significantly larger in the germfree rats. In terms of absolute amounts, the germfree rats absorbed about 20 times as much as the controls, the fluid uptake in both groups being essentially independent of the volume initially present in the sac. Expressing the results per unit surface area or dry weight, the saline transport still differed by a factor of 6 (both parameters were about 3 times larger in the germfree rats than in the conventional controls; see legend of Table I). Upon calculating the water transport from the increase in PEG concentration, the results were practically the same, with virtually complete recovery of PEG in both groups.

As anticipated from similar studies in other epithelial membranes, little change in osmotic pressure and sodium concentration of the saline occurred during the experiment. The fluid osmolality (arithmetic means and standard deviations are given throughout this paper) increased from an initial 288 to a final 300 ± 8.1 mOsm/kg in the germfree rats and to 301 ± 6.3 mOsm/kg in the conventional animals, while the sodium concentration decreased slightly from 156 to 138 ± 8.5 meq/liter and to 139 ± 10.0 meq/liter, respectively. Thus, net absorption of sodium occurred somewhat in excess of water, while other solutes must have entered the fluid from the cells and the plasma. The net sodium uptake calculated from the average val-

TABLE I. Sodium-Coupled Water Transport in Ceca of Germfree and Conventional Rats.[a]

| Germfree | | | | Conventional | | | |
|---|---|---|---|---|---|---|---|
| | Fluid transport | | | | Fluid transport | | |
| Initial fluid vol (ml) | (ml/3 hr) | (μl/cm² × hr) | (μl/0.1 g × hr) | Initial fluid vol (ml) | (ml/3 hr) | (μl/cm² × hr) | (μl/0.1 g × hr) |
| 10 | 6.7 | 29.5 | 530 | 3 | 0.5 | 8.2 | 175 |
| 15 | 7.4 | 31.9 | 748 | 5 | 0.7 | 7.7 | 160 |
| 15 | 8.7 | 34.4 | 612 | 5 | 0.4 | 6.7 | 117 |
| 20 | 8.6 | 34.0 | 637 | 7 | 0.2 | 2.1 | 48 |
| 20 | 8.6 | 31.0 | 633 | 7 | 0.4 | 4.7 | 106 |
| 30 | 10.5 | 41.2 | 833 | 10 | 0.3 | 3.9 | 85 |
| M ($n=6$) | 8.4 | 33.7 | 666 | M ($n=6$) | 0.4 | 5.5 | 115 |
| SD | 1.2 | 3.8 | 98 | SD | 0.2 | 2.2 | 43 |

[a] Values are given as arithmetic means (M) ± standard deviation (SD), number of observations ($n$). The surface area of cecal sacs was: germfree 83.2 ± 8.0 cm², conventional 26.2 ± 5.1 cm² ($n = 6$/group). The dry weight of cecal sacs was: germfree 424.9 ± 50.8 mg, conventional 121.6 ± 19.3 mg ($n = 6$/group).

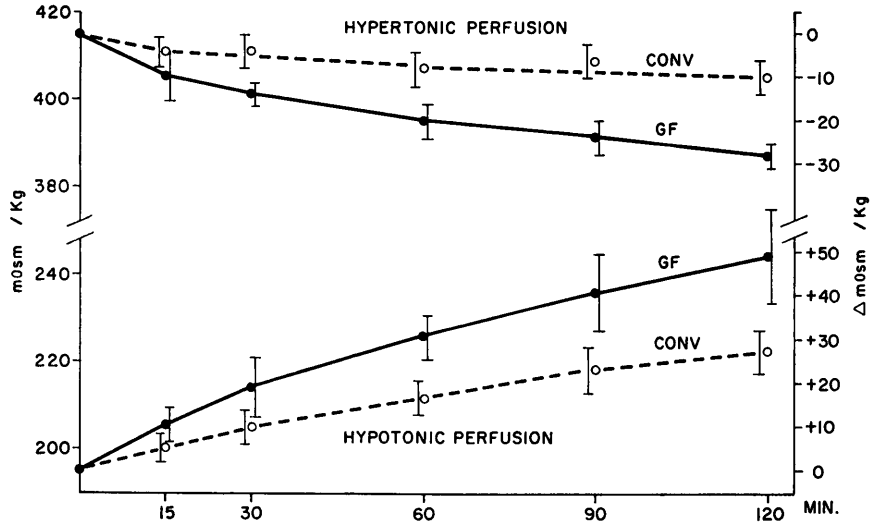


FIG. 1. Osmolality change of hypertonic and hypotonic perfusates in ceca of germfree (gf) and conventional (conv) rats. Arithmetic means ± standard deviations (vertical bars) in three rats per group. Absolute (left) and relative (right) osmolality changes are plotted versus time.

ues of water transport and sodium concentrations in the fluid (in meq/3 hr) was 1.5 in the germfree rats and 0.16 in the conventional group, showing about a tenfold difference.

*Osmotic water flow.* A different technical approach in determining the osmotic permeability of the cecum was needed, since such studies require a thorough fluid mixing. This is difficult to achieve within the lumen of the large and kidney-shaped cecum *in vivo*. The best way to accomplish it proved to be the following procedure: Two glass cannulae were attached at the opposing poles of the organ and connected via rubber tubing to a pump[4] which by means of two syringes simultaneously infused and withdrew an equal amount of fluid. The whole system, cecum plus syringes, contained 30 ml of fluid in all experiments, with a perfusion rate of 12.4 ml/min. Applying hydrostatic pressure to the mucosa was avoided as much as possible in such a design. Samples were withdrawn by means of a small, removable, glass cup attached to one of the rubber tubes connecting a cannula and a syringe. The fluid in the hypotonic perfusion studies was a modified sulfate Ringer's solution containing (mmole/liter): $Na_2SO_4$, 79.4; KCl, 4.8; $CaCl_2$ 2.6; $MgSO_4$, 1.9; and $^{14}$C-PEG. The total osmolality of this solution was 195 mOsm/kg. In the hypertonic perfusion experiments, the same fluid was used but brought to a total osmolality of 415 mOsm/kg by the addition of raffinose. Both solutions could be expected to exert an effective osmotic pressure very near to the theoretical value (reflection coefficient $\approx 1$). That no appreciable net sodium absorption occurred under these circumstances, and hence no net sodium-coupled water transport was indicated by the fact that a comparison of the initial and final fluid sodium concentrations closely reflected the water flow in both sets of experiments.

Figure 1 depicts the change in osmolality of the perfusion fluids in the hypo- and hypertonic experiments as a function of time. In both sets of animals, osmolality showed a graded increase and decrease, respectively, with more pronounced effects in the germfree group. Analogous curves, though with somewhat larger scattering, were obtained when the $^{14}$C-PEG concentration was plotted versus time. The plasma osmolality changed little during hypotonic perfusion (from an initial 308 ± 3 to a final 311 ± 3

[4] Dual infusion/withdrawal pump with variable speed, Model No. 600-000, Harvard Apparatus Co., Dover, Mass.

TABLE II. Osmotic Water Flow and Osmotic Water Permeability Determined in Ceca of Germfree and Conventional Rats.[a]

| Exp. | Osmotic water flow (ml/2 hr) | Osmotic water permeability ($\mu$l/cm$^2$ × hr × mOsm) | Osmotic water permeability ($\mu$l/0.1 g × hr × mOsm) |
|---|---|---|---|
| Hypotonic perfusion | | | |
| Germfree | −4.62 ± 0.90 | 0.33 ± 0.20 | 6.1 ± 1.9 |
| Conventional | −2.90 ± 0.80 | 0.64 ± 0.01 | 9.8 ± 2.5 |
| Hypertonic perfusion | | | |
| Germfree | +3.15 ± 1.73 | 0.27 ± 0.14 | 4.3 ± 2.3 |
| Conventional | +1.37 ± 0.70 | 0.28 ± 0.16 | 4.1 ± 2.1 |

[a] − = loss; + = gain of luminal fluid. Three rats per group, means ± SD. The osmotic permeability coefficients were calculated as $P_{os} = Q/A \cdot \Delta mOsm$, where $Q$ is the rate of osmotic water flow, $A$ the surface area (or dry wt) of the cecal sac, and $\Delta mOsm$ the mean osmolar concentration difference between lumen and plasma during the experiment.

and 320 ± 10 mOsm/kg in germfree and conventional animals) and increased somewhat in the hypertonic studies (from 308 ± 3 to 331 ± 10 and 324 ± 11 mOsm/kg, respectively).

Table II shows the volumetric data and the osmotic permeability coefficients. The latter were calculated in reference to the surface area or the dry weight (see legend of Table II for method of calculation). They are not significantly different between germfree and conventional animals ($p > 0.05$). The osmotic water permeability was somewhat smaller in the hypertonic perfusions than in the hypotonic, a phenomenon observed in most biological membranes ["non linear osmosis" (15)].

*Discussion.* The osmotic measurements were performed because an enhanced filtration permeability was regarded as one possibility to explain the increased water content of the germfree cecum. As the driving force for the osmotic water flow, osmolar concentration differences between the blood plasma and intestinal contents were considered. The supernatant is markedly hypertonic (about 400 mOsm/kg) in the terminal ileum, irrespective of the microbial status of the animals. In the cecum of conventional rats, the osmolality remains essentially the same as in the ileum, whereas it drops to values almost isotonic with plasma in the germfree cecum (1, 16). This decrease in the germfree group could have been the consequence of larger osmotic water permeability. The data presented do not seem to support this hypothesis.

It should be pointed out, however, that this conclusion would be based on the assumption that the results obtained under the present experimental conditions truly reflect the situation in the intact animal. Most importantly, it is assumed that the removal of the cecal content does not modify the tissue response to osmotic gradients. This is, of course, open to question since, for example, an increased amount of bioactive substances has been demonstrated in germfree cecal supernatants (17). Similarly, the osmotic water flow might be influenced by the vascular microcirculation which was found to be reduced in the cecal wall of germfree rats (1). Furthermore, it should be recalled that the osmotic water flow was referred to a gross measurement of the surface area (or to the dry weight), and that the osmotic water permeability was not statistically different in these terms. With respect to microstructure, the ceca of germfree and conventional rats may not be strictly comparable as suggested by some microscopic observations in mice (18) and rats (1).

Similar considerations are pertinent for solute-coupled water transport. There are indications, however, that sodium and chloride absorption is increased in the intact germfree cecum, too, since Asano (16, 19) has reported lower concentrations of both ions in cecal supernatants of germfree, as compared to *Clostridium difficile* monoassociated, or con-

ventional rats and mice. His finding corresponds to preliminary data obtained in this laboratory which show that water can be absorbed from isotonic NaCl/mannitol mixtures against considerably higher transmural concentration differences for sodium in ceca of germfree rats than of conventional controls. Thus, the enhanced net uptake found in our experiments seems to be paralleled by a more efficient uphill transport of sodium chloride in both the presence and absence of the cecal content. As long as measurements of potential differences and of unidirectional fluxes are not available, this cannot be attributed to increased active sodium "pumping," but the data are certainly suggestive for such a mechanism.

The finding that the net uptake of sodium chloride and water is augmented in the germfree cecum is of considerable interest not only because it helps us understand an important aspect of germfree life but also, in general, as an adaptation phenomenon which it apparently represents. Teleologically, a more efficient saline absorption would be of great value for the animal because it would reduce the loss of sodium and water connected with the large cecal volume.

If the osmotic permeability is unaltered and the sodium-coupled water transport even enchanced, additional elements must obviously be involved in the "anomalous" water absorption of the germfree cecum. It was recently reported that the colloid osmotic pressure of cecal supernatants of germfree rats is considerably higher than that of their blood plasma. In comparison, these values are essentially equal in conventional controls. Thus, the more liquid contents of the lower bowel may be explained on the basis of water attraction exerted by large molecular compounds in the lumen, to which the intestinal wall is impervious (20). One of the substances implicated in this phenomenon might be endogenous mucus or some of its components, which is known to accumulate in the germfree gut in considerable amounts (21–24) and which, in conventional life, is degraded by the intestinal flora. This explanation would be along the view of Csáky (5) who found that mucus from various parts of the alimentary tract inhibits intestinal water absorption. He suggested that the water-absorption inhibitory substance in the intestine of germfree animals, which could be removed by prolonged rinsing with saline, is nondegraded mucus which accumulates due to the absence of the intestinal flora.

*Summary.* The present experiments demonstrate that the isotonic saline absorption of cecal sacs *in vivo* is significantly larger in germfree than in conventionally-raised rats living on the same diet. The osmotic permeability of the cecal wall, on the other hand, is not statistically different in the two groups under the experimental *in vivo* conditions employed. While other findings are compatible with the assumption that the retention of water in the cecal lumen of the intact germfree animal is due to the presence of osmotically active substances to which the cecal mucosa is impermeable, the sodium-coupled water transport seems to be increased as a compensatory mechanism.

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