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Accumulation of Endogenous Carbohydrate-Containing Compounds in the Cecum of the Germfree Rat* (33885)

WALTER J. LOESCHE
(Introduced by J. T. Irving)
Forsyth Dental Center, Boston, Massachusetts 02115

Considerable amounts of protein- and carbohydrate-containing material are found in the enlarged ceca of germfree rats and mice (1). Much of the protein is endogenous in origin accumulating in the cecum apparently due to a failure in its degradation and reabsorption in the terminal small intestine (2).

The cecal carbohydrates could be derived from either unabsorbed dietary carbohydrates or undergraded intestinal mucins. The present investigation attempted to determine the contribution of dietary carbohydrate to the cecal contents. For this purpose germfree rats were starved or given a diet containing glucose as the sole carbohydrate. Under conditions of starvation any carbohydrate containing material found in the cecum would be endogenous in origin and most likely mucinous in nature. On the high glucose diet, any unabsorbed dietary carbohydrate could be readily estimated by measuring the free glucose level in the cecum.

Materials and Methods. Young (30–35 day old) gnotobiotic CDF female rats were obtained from the Charles River Breeding Laboratories (North Wilmington, Mass.) and were housed in plastic germfree isolators. The animals were maintained on irradiated diet 585 (3) modified to contain 66% glucose as the sole dietary carbohydrate and 1% chromic oxide to serve as a dietary marker. After 2.5 weeks ingestion of this diet the animals were distributed so as to give comparable weight animals in both the fed and starved groups. All animals were fed for another 3 days and then the control animals were sacrificed; and food but not water was removed from the animals scheduled to be starved. Starved animals were sacrificed at 1 and 4 days and other starved animals died overnight between days 5 and 6.

All animals were killed by neck fracture and the cecum was removed, weighed and the cecal contents were expressed. The contents of each cecum were analyzed individually for total soluble protein and carbohydrate; high molecular weight proteins and glycoproteins (10% trichloroacetic acid precipitate); mucoproteins and mucopolysaccharides (80% ethanol precipitate) and low molecular weight carbohydrate and peptides (TCA and ethanol soluble material) by the fractionation procedure used previously (1). The dry weight of the insoluble residue was determined as well as its carbohydrate and chromic oxide content (1, 4). Galactose was used as the standard in the anthrone procedure because glucose was found to be a minor component of the cecal carbohydrates. Free glucose was measured enzymatically with glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, N.J.) with suitable controls to correct for the contribution of the cecal pigment to color development.

In other experiments the soluble fractions

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TABLE I. Effect of Starvation on Body Weight and Weight of Cecal Contents.

Condition	Fed	Starved		
		days: 1	4	6[a]
No. of animals	4	4	4	2
Body wt (g)	94.1 ± 6.4[b]	91.2 ± 3.1	89.1 ± 5.4	73.2 ± 0.5
Wt of cecal contents (g)	9.8 ± 0.7	10.1 ± 1.3	12.3 ± 2.9	15.4 ± 0.2
Cecal contents (as % of body wt)	10.4	11.8	13.8	21.2
H_2O (%) in cecal contents	79.5	77.1	77.2	79

[a] Animals died during night between days 5 and 6.

[b] ± Standard error of the means.

of 3 axenic animals ingesting the modified 585 diet were pooled as were similar fractions from animals ingesting diet L356. Four vol of ethanol were added and the precipitate obtained was resuspended in H_2O and again precipitated with ethanol. This ethanol precipitate was harvested by centrifugation at 10,000 and 25,000*g* thereby giving 2 fractions for each diet. The protein and carbohydrate content of each fraction was determined and the fractions were then diluted to give 3–8 mg of material/ml. Samples were hydrolyzed for 16 hr at 100° with 0.5 *N* HCl to release the neutral sugars (5). After passage through a Dowex 1 × 8 (HCO_3^-) and Dowex 50w (H^+) columns to remove acid and ions the neutral sugar fraction was concentrated and spotted on MN cellulose thin-layer plates. After development with either chloroform–acetic acid–H_2O (3:3.5:5.5, v/v) (6) or *n*-butanol–pyridine–0.1 *N* NCl (5:3:2, v/v) (7) solvents the spots were detected with either a *p*-anisiline (8) or diphenylamine spray (7). Fucose was also determined on both the hydrolyzed and unhydrolyzed fraction by the thioglycollic acid method (9). Glucuronic acid was sought for by the carbazole method (10).

Results. Apparently germfree rats do not perform well on a starvation regimen. In a preliminary experiment 4 of 8 young rats (24–31 days old) when subjected to starvation, died after 3 days and those that survived and were killed on day 4 were weak and apathetic. Both groups had greatly enlarged ceca which accounted for 21–30% of the body weight at autopsy. In the present experiment, 2 older animals (50 and 57 days old) did not survive 6 days of starvation and had ceca which averaged 21% of the body weight (Table I). In contrast the fed animals had ceca which averaged 10% of the body weight. Starved animals sacrificed at 1 and 4 days had ceca which averaged 12 and 14% of the body weight. The increased percentage contribution of the cecum to the body weight in the starved animals was due to a real increase in cecal size as well as to a loss of weight in the other body constituents. The magnitude of the latter is apparent when the cecal weight was substrated from the body weight. The increase in cecal size upon starvation was not associated with a change in water content as all ceca contained between 77 and 79% water.

When the cecal contents were fractionated, considerable amounts of carbohydrate-containing material was found in the ceca of the starved animals (data for the animals that died on day 6 were not included as they might reflect postmortem alterations). The total carbohydrate as measured by the anthrone method with galactose as a standard was 720 mg/100 g of body weight for the fed animals, but 956 mg/100 g of body weight in the animals starved for 4 days (Table II). It did not seem likely after this period of starvation that the cecal carbohydrate could be of dietary origin. This was supported by the fact that glucose was the sole dietary carbohydrate and only trace amounts of it could be found in the cecal contents. Even in the animals ingesting the high glucose diet, glucose accounted for less than 1% of the cecal carbohydrates (Table II). Thus, at least on this diet, endogenous mucins ac-

TABLE II. Amount of Free Glucose and Carbohydrate Found in Cecal Contents of Rats Fed a Diet Containing 66% Glucose.

Condition	Fed	Starved	
		days: 1	4
Glucose	3[a]	0.4	0.1
Carbohydrate	720	574	956
Glucose (% of total carbohydrate)	0.4	0.05	0.0

[a] (mg/100 g of body wt).

counted for about 99% of the carbohydrate-containing material. We have observed similar results with gnotobiotic mice on the same diet.

To verify the endogenous origin of the cecal carbohydrates additional experiments were performed to identify the sugar components in the cecal contents. The soluble cecal contents from axenic rats ingesting either the modified 585 diet or diet L-356 were pooled. The carbohydrate material precipitated by 80% ethanol contained 13–15% fucose and 3–4% hexuronic acids (Table III). This ethanol precipitate was then hydrolyzed under conditions favoring release of neutral sugars. Fucose, galactose, and mannose were readily identified by thin-layer chromatography. An unidentified reddish spot (*p*-anisidine spray) which migrated with an R_f similar to mannose with the chloroform–acetic acid–H_2O solvent and just behind fucose with the butanol–pyridine HCl solvent was also observed. A fifth spot which migrated in advance of fucose was observed in the 10,000*g* fraction from diet 585-fed animals. Otherwise the 10,000 and 25,000*g* fractions from animals fed either the modified 585 or L-356 diets were similar. The sugar profile obtained, together with the previous demonstration of sialic acid (2) suggested that the carbohydrate material accumulating in the cecum was glycoprotein in nature.

Starvation did not appreciably change other cecal constituents. Total soluble protein and carbohydrate levels after 4 days of starvation were comparable to values obtained with the fed animals (Table IV and V). However, animals starved for 1 day had uniformly lower values than either the fed animals or the 4-day starved animals. This might reflect the initial response of the animals to starvation as fewer digestive enzymes would be secreted into the intestine and subsequently emptied into the cecum. Less material entered the cecum as estimated by the decrease in the chromic oxide content of the 1-day starved animals. The situation is reversed in the 4-day starved animals in that all parameters were increased and approached and often surpassed the control values (Tables IV and V). Conspicuous was the increase in the dry weight of the insoluble pellet and in the chromic oxide content. As the latter could only be derived from the diet, its presence in these starved animals suggested a slowdown in cecal emptying. Such a slowdown in cecal emptying would permit the endogenous proteins, glycoproteins, and cellular material which enter the cecum daily, to accumulate in the starved animals and this might account for the increase in cecal size observed (Table I). Coprophagy did not appear to be significant in the 4-day starved animals as their stomach and intestinal tract contained only small amounts of chromic oxide containing material.

Discussion. When germfree rats were starved there was a net increase in cecal size. As it was unlikely that after 4 days of starva-

TABLE III. Chemical Composition of 80% Ethanol Precipitate.

Substance (mg/ml)	Diet:	Modified 585		L-356	
	Fraction:	10,000*g*	25,000*g*	10,000*g*	25,000*g*
Protein		6.7	3.2	22.2	13.2
Carbohydrate		8.1	5.7	18.5	10.8
Fucose		1.2	0.9	2.7	1.4
Hexuronic acid		0.4		0.7	0.3

TABLE IV. Effect of Starvation on Chemical Composition of Rat Cecal Contents.

Condition	Fed	Starved	
	days:	1	4
	Soluble fraction (mg/100 g of body wt)		
Soluble protein	172 ± 26[a]	126 ± 14	184 ± 38
TCA ppt	21 ± 1	13 ± 3	37 ± 9
EtOH ppt	21 ± 1	20 ± 3	33 ± 5
TCA and EtOH soluble	83 ± 3	64 ± 9	112 ± 17
Soluble carbohydrate	272 ± 16	190 ± 24	284 ± 76
TCA ppt	26 ± 2	14 ± 4	38 ± 4
EtOH ppt	46 ± 2	44 ± 10	66 ± 14
TCA and EtOH soluble	144 ± 6	134 ± 18	114 ± 20
	Insoluble fraction (mg/100 g of body wt)		
Dry wt	792 ± 37	776 ± 72	1255 ± 167
Insoluble carbohydrate	446 ± 56	384 ± 48	672 ± 78
Chromic oxide	88 ± 4	65 ± 8	105 ± 20

[a] ± Standard error of the means.

tion any dietary material would still be present, the protein and carbohydrate found were presumably entirely of endogenous origin. On a diet containing glucose as the sole carbohydrate, glucose accounted for less than 1% of the cecal carbohydrates further indicating the endogenous origin of these compounds. Acid hydrolysis of the ethanol precipitable fraction yielded a variety of neutral sugars known to be found in glycoproteins (7), *i.e.*, fucose, galactose, and mannose. Sialic acid (2) and hexosamines (11) have been demonstrated previously in germfree cecal contents. This number of monomers suggested a diversity of glycoproteins were present in the cecal contents, and raised questions as to where in the gastrointestinal tract they were originating from and why they were present in the cecum. Some of these protein–carbohydrate complexes probably are secreted directly into the cecum by the mucosa cells lining the cecal

TABLE V. Effect of Starvation on Chemical Composition of Rat Cecal Contents.

Condition	Fed	Starved	
	days:	1	4
	Soluble fraction (mg/g of cecal content)		
Soluble protein	16.3 ± 2.1[a]	11.8 ± 0.6	12.4 ± 1.1
TCA ppt	2.1 ± 0.1	1.1 ± 0.2	2.5 ± 0.4
EtOH ppt	2.0 ± 0.1	1.8 ± 0.0	2.3 ± 0.1
TCA and EtOH soluble	8.0 ± 0.3	5.8 ± 0.3	7.8 ± 0.4
Soluble carbohydrates	26.3 ± 1.8	17.4 ± 1.2	18.6 ± 2.8
TCA ppt	2.6 ± 1.8	1.2 ± 0.2	2.6 ± 0.6
EtOH ppt	4.6 ± 1.8	3.8 ± 0.6	4.4 ± 0.4
TCA and EtOH soluble	14.0 ± 1.0	12.2 ± 0.6	7.8 ± 0.4
	Insoluble fraction (mg/g of cecal content)		
Dry wt	77.2 ± 8.4	72.0 ± 3.7	88.0 ± 2.6
Insoluble carbohydrate	44.0 ± 8.8	37.2 ± 7.2	47.8 ± 5.0
Chromic oxide	8.6 ± 0.9[a]	6.0 ± 0.3	7.2 ± 0.5

[a] ± Standard error of the means.

wall (11). But unless the cecal wall in the germfree animal has unusual secretory properties it is unlikely that this source could account for all the glycoproteins found. Another possibility is that the mucinous secretions occurring higher in the gastrointestinal tract are not degraded and absorbed in the germfree small intestine and therefore pass into and accumulate in the cecum. Previous observations with regard to nitrogen and sialic acid have shown that there was a net nitrogen and sialic acid accumulation as the intestinal contents passed from the distal small intestine to the cecum in germfree rats, whereas in conventional animals both nitrogen and sialic acids levels were greatly decreased in passage from the small intestine to cecum (2). This hypothesis would state that the majority of the cecal contents were derived from mucinous and protein secretions occurring elsewhere in the digestive tract and that their accumulation in the cecum with associated cecal enlargement is secondary to an absence in the germfree state of a bacterial flora which somehow mediates their degradation and reabsorption in the small intestine. The bacteria may provide the necessary degradative enzymes or an environment which sufficiently denatures the endogenous macromolecules so that host enzymes are capable of degrading them.

The present findings do not preclude the possibility that on other diets, dietary carbohydrates could contribute to the cecal contents. Any insoluble, undigestible dietary carbohydrate, *i.e.*, starch, celluose could pass through the intestinal tract and accumulate in the cecum (12). In other experiments, (1) when comparable age germfree rats ingested diet L-356 which contains 5% fiber and 58% rice flour, the cecum was larger, *i.e.*, 17% of the body weight and contained higher amounts of soluble and insoluble carbohydrate than those reported here. The differences found could be dietary in origin.

The sudden demise of the starved rats is in accordance with the observations of Tennant *et al.* (13), in germfree mice. Why germfree animals should succumb so readily upon starvation is a matter of speculation. Germfree animals tend to ingest more food (14, 15), perhaps to compensate for their reduced capacity to recover, directly, the considerable amounts of endogenous nitrogen which enters their gastrointestinal tract. Thus upon starvation they would be subjected to a more severe nitrogen depletion than normal animals and accordingly would die sooner. The actual as well as relative increase in the cecal contents during starvation may also be contributory in this regard. Gordon (15) has shown that germfree cecal contents contain compounds which if injected exert adverse pharmacological effects upon the host. More of these substances would be present in a larger cecum and presumably could gain access to the systemic circulation.

Summary. Germfree rats died after 4–6 days on a starvation regimen. As the animals were losing weight the cecum was increasing in size. It was unlikely that after 4 days of starvation any dietary material would still be present in the cecum. Therefore, the protein and carbohydrate found were presumably entirely of endogenous origin. On a diet containing glucose as the sole carbohydrate, glucose accounted for less than 1% of the cecal carbohydrates further indicating the endogenous origin of these compounds. Acid hydrolysis of the ethanol precipitable fraction yielded neutral sugars characteristic of glycoproteins, *i.e.*, fucose, galactose, and mannose. The accumulation of glycoproteins in the cecum implied that their degradation and absorption in the small intestine was impaired in the germfree rat.

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Rat Leukemia Derived 9H Virus (9HV) I. Properties of the Virus and Evidence for the Development of Heterogeneity in Cell Culture*† (33886)

SAM EISENSTEIN, VICTOR V. BERGS, AND MAIJA BERGS
(Introduced by M. Michael Sigel)

Variety Children's Research Foundation, and Department of Microbiology, University of Miami School of Medicine, Coral Gables, Florida 33155

A recent communication from this laboratory described the isolation of a filterable hemagglutinating agent, negative for *Mycoplasma*, from leukemias produced in rats by cell-free extracts prepared from chemically induced rat mammary carcinomas (1). A serological relationship between this virus, designated "9H" and rat virus (RV) (2) was reported (1). In a more recent study (3) it was shown that inoculation of the 9H virus (9HV) into newborn rats resulted in peliosis hepatis, a condition characterized by hemorrhagic cysts in the liver. The development of similar lesions in the livers of rats inoculated with certain strains of RV was also observed in another laboratory (4). The present paper reports on the properties of 9HV propagated in the REL line of rat embryo cells (1) since its original isolation from leukemic tissues which in certain aspects differ from those hitherto reported for RV.

Materials and Methods. Chemicals, media, and cell cultures. Cesium chloride was obtained from the Matheson, Coleman and Bell Co., Norwood, Ohio. Bio-Glas-500, a powdered glass material of controlled pore size was purchased from Bio-Rad Laboratories, Richmond, California. The origin of the REL cell line and the maintenance medium (MM) for these cells have been described previously (1). Secondary rat embryo (SRE) cells were propagated in MM enriched with 15% bovine amniotic fluid. The cells were grown as monolayers in culture tubes for use in experiments and in culture bottles for further passage and for the preparation of virus stocks.

Virus. Stocks of 9HV were produced by infecting monolayers of either REL or SRE cells. Cytopathic effects (CPE), manifested by granulation, rounding, and vacuolation of the cells, were evident after 12–14 days of incubation at 37°. The cells and culture fluids were harvested and, following a tenfold concentration at 105,000*g* for 1 hr, the virus was placed in ampoules and stored at —70° until needed.

Virus titrations were done in REL cell cultures according to the procedure described by Dougherty (5). The hemagglutinating (HA)

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