

study suggest that it may be greater than one year.

It is unknown if the uniformly low CF antibody response in man to the commercial RMSF vaccine reflects an equally low degree of protection afforded against the disease. Minimum requirements for potency of RMSF vaccine lots released for distribution include demonstration of capacity to protect guinea pigs against challenge with virulent organisms (5). Studies of the protective effect of the vaccine in man would seem desirable to evaluate its efficacy and to establish the degree of correlation of protection with laboratory tests.

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Protein and Carbohydrate Composition of Cecal Contents of Gnotobiotic Rats and Mice* (32977)

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One of the peculiarities of certain germfree animals is the enlarged cecum which can average up to 20% of the body weight. In conventional animals this part of the intestinal tract is about 1% of the body weight and its "normality" is attributed to the resident microbial flora. Previous studies have indicated that the material accumulating in the gnotobiotic cecum is higher in water content than that from conventional animals (1), and contains high molecular weight mucins (2). Recently Combe *et al.* (3-5) have fractionated the cecal contents of gnotobiotic rats and have demonstrated the presence of mucoproteins and large increases in free amino acids and urea over that found in the conventional cecum.

The present investigation was initiated to provide information concerning the total protein and carbohydrate content of cecal material obtained from the gnotobiotic rat and mouse. Such base line information is necessary in order to evaluate the phenomena responsible for cecal enlargement and could lead to a judicious selection of microbes with which to reduce cecal size.

Materials and Methods. Animals. Gnotobiotic CDF rats and CD-1 Swiss mice, were obtained from the Charles River Breeding Laboratories (North Wilmington, Massachusetts) and were housed in plastic germfree isolators. Comparable aged conventional animals of the same strain were kept in the animal room. Gnotobiotic rats and conventional rats were fed autoclaved diet L356 (General Biochemicals Corp.) (6) and water *ad libitum*. The gnotobiotic mice were fed Purina diet 5010 and conventional mice were

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TABLE I. Weight of Cecal Contents.

Item	Species: Condition:	Mouse		Rat	
		Gnotobiotic	Conventional	Gnotobiotic	Conventional
Diet		5010	Purina	L356	L356
No. of animals		6	20 ^a	7	23 ^a
Body wt. (gm)		33 ± 0.5 ^b	36.8 ± 1.4	96.1 ± 3.2	141.7 ± 3.2
Wt. of cecal contents (gm)		2.34 ± 0.16	0.37 ± 0.03	17.1 ± 1.1	1.47 ± 0.07
Cecal contents (as % of body wt.)		7.1	1.0	17.8	1.0

^a Grouped in lots of 4 or 5 animals.

^b ± SE of the means.

maintained on Purina lab chow and water *ad libitum*.

Fractionation procedures. All animals were sacrificed by neck fracture. The cecum was removed, weighed, the cecal contents were expressed, and the cecal sac was reweighed. The contents of each gnotobiotic cecum were analyzed individually, but it was necessary to pool the cecal contents of 4 or 5 conventional animals for analysis because of their small size. Cecal contents were centrifuged at 8000 g for 10 min at 4°C and the pellet or insoluble fraction was separated and stored at -60°C. It was analyzed at a later time for total carbohydrate by the anthrone procedure (7) and nitrogen which was determined by micro-Kjeldahl digestion followed by micro-diffusion analysis of NH₃ (8). The supernatant or soluble fraction was appropriately diluted and analyzed for trypsin (9), chymotrypsin (10), total Lowry positive material (11), and total carbohydrates (7). One-ml samples of the soluble fraction were treated with enough trichloroacetic acid (TCA) to give a final concentration of 10% TCA and the precipitate formed was separated by centrifugation and analyzed for protein. Ethanol was added to the TCA supernatant to give a final concentration of 80%. The resulting precipitate was analyzed for protein and carbohydrate. Larger samples of the soluble fraction were dialyzed for 48 to 72 hours and then placed on a Sephadex G100 column. The fractions collected were analyzed for protein by absorption at 280 mμ and for carbohydrate by the anthrone procedure. Known amounts of bovine albumin, chymotrypsinogen, and cytochrome C were used to determine the

column parameters and thus to estimate the molecular weight of cecal macromolecules (12).

Results. The cecal contents of gnotobiotic mice accounted for 7.1% of the total body weight (Table I), whereas the contents of 20 conventional mice of the same size and age represented only 1% of the body weight. The cecal contents of gnotobiotic and conventional rats comprised 17.8 and 1.0%, respectively, of the total body weights. The differences between the gnotobiotic rat and mouse could represent species variation as well as dietary differences.

Upon fractionation, 10 times as much protein, i.e., Lowry positive material, and from 27 to 200 times more carbohydrate were found in the soluble fraction of the gnotobiotic animals than in their conventional counterparts when the results were expressed per 100 gm of body weight (Table II). Carbohydrate accumulation on diet L356 was more pronounced than on diet 5010. The protein precipitated by 10% TCA accounted for 12-29% of the soluble protein from the axenic animals and was 36-44% of the soluble protein found in conventional animals. A considerable amount of protein was soluble in 10% TCA but was precipitated by 80% ethanol suggesting it was mucoprotein and/or mucopolysaccharide in nature. Considerable amounts of Lowry positive material still remained in the supernatant of the gnotobiotic animals after TCA and ethanol treatment indicating the presence of low molecular weight peptides. Such compounds of molecular weight of about 4000-6000 were demonstrated by Sephadex chromatography of the

TABLE II. Protein and Carbohydrate Composition of Cecal Contents (mg/100 gm of body wt.).

Item	Species: Condition:	Mouse		Rat	
		Gnotobiotic	Conventional	Gnotobiotic	Conventional
Soluble fraction (mg/100 gm of body wt.)					
Total protein		260 ± 43 ^a	25 ± 6	266 ± 23	25 ± 3
TCA ppt.		33 ± 4	11 ± 2	78 ± 9	9 ± 1
Ethanol ppt.		85 ± 7	10 ± 6	134 ± 16	11 ± 1
Total carbohydrates		134 ± 14	5 ± 1	227 ± 23	1.2 ± 0.2
Ethanol ppt.		87 ± 13	2 ± 1	74 ± 9	0.9 ± 0.1
Insoluble fraction (mg/100 gm of body wt.)					
Dry wt.		903 ± 54	165 ± 15	1597 ± 71	193 ± 6
Carbohydrate		257 ± 12	28.5 ± 0.8	827 ± 16	48 ± 6
Nitrogen		31.5 ± 5	4.4 ± 0.9	15 ± 2	6.7 ± 0.7

^a ± SE of the mean.

soluble cecal contents obtained from gnotobiotic rats (Fig. 1). Also observed in the Sephadex elution diagram were a large protein and carbohydrate band, which because they traveled with the void volume were of molecular weight size of 120,000 or more. The superimposition of these bands suggested that they contained mucoproteins and/or mucopolysaccharides. The fractions collected from these peaks could be precipitated with 80% ethanol and the precipitate was shown to contain protein. Also, if the cecal supernatant was treated with ethanol prior to addition to the column, this peak at the void volume was all but eliminated. Small amounts of protein of molecular weight 10,000–20,000 as well as various carbohydrate fractions including one of about 25,000–27,000 mol. wt. were also observed. The Sephadex patterns obtained from similar size samples of soluble cecal contents of conventional rats showed the same carbohydrate and protein bands at the void volume, but the amount recovered was only 5–10% of that which could be recovered from the gnotobiotic rat.

The dry weight of the insoluble fraction when expressed per 100 gm of body weight was 5–8 times greater in gnotobiotic animals than in the conventional animals. This was due mainly to an increase in carbohydrate especially in the gnotobiotic rat, though the nitrogen content was also elevated (Table II).

Thus far the data has been referred to 100

gm of body weight. When the results were referred to gm of wet weight of cecal contents some changes were observed (Table III). The dry weight of the insoluble fraction was higher in the conventional animals undoubtedly reflecting the presence of the microbial flora. The microbes also accounted for the higher pellet nitrogen content found in the conventional animals. The levels of protein in the soluble fraction did not differ appreciably between the gnotobiotic and conventional animals. However, the character of the protein was different as there were pronounced accumulations of trypsin and chymotrypsin in

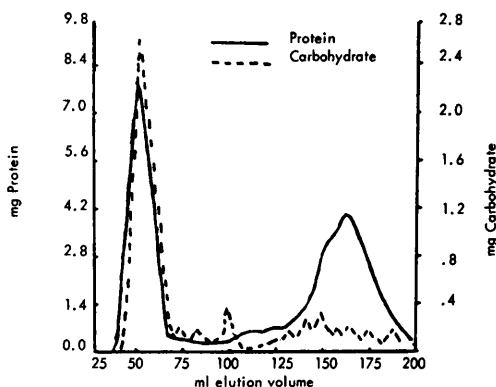


FIG. 1. Elution diagram of soluble cecal contents obtained from a gnotobiotic rat. A sample of soluble cecal contents containing 90 mg of protein was dialyzed for 72 hours against 0.01 M phosphate buffer, pH 7.5, placed on a G-100 superfine Sephadex column and eluted in the cold with 0.05 M Tris-HCl buffer containing 0.1 M KCl at pH 7.4.

TABLE III. Protein and Carbohydrate Composition of Cecal Contents Expressed per Gram of Wet Weight of Cecal Contents.

Item	Species:	Mouse		Rat	
	Condition:	Gnotobiotic	Conventional	Gnotobiotic	Conventional
Soluble fraction (mg/gm of cecal contents)					
Total protein		36.7 ± 6.1 ^a	25.4 ± 5.7	15.0 ± 1.3	24.1 ± 3.2
TCA ppt.		4.7 ± 6.1	11.0 ± 2.4	4.4 ± 0.5	8.4 ± 1.1
Ethanol ppt.		12.1 ± 1.0	10.4 ± 6.5	7.5 ± 0.9	10.9 ± 1.6
Total carbohydrates		18.8 ± 1.9	5.1 ± 0.8	12.8 ± 1.3	1.2 ± 0.2
Ethanol ppt.		12.3 ± 1.8	2.2 ± 0.5	4.2 ± 0.5	0.8 ± 0.1
Insoluble fraction (mg/gm of cecal contents)					
Dry wt.		128.6 ± 7.7	164.1 ± 15.1	90 ± 4	186.4 ± 5.7
Carbohydrate		36.7 ± 1.7	28.4 ± 0.8	46.5 ± 0.9	46.2 ± 6.0
Nitrogen		4.5 ± 1	4.4 ± 0.8	0.9 ± 0.1	6.5 ± 0.7

^a ± SE of the means.

TABLE IV. Enzyme Levels in Cecal Contents.^a

Item	Species:	Mouse		Rat	
	Condition:	Germfree	Conventional	Germfree	Conventional
	No. of animals:	6	20	7	23
Trypsin					
mg of enzyme/100 gm of body wt.		4.54 ± 0.7 ^b	0.02 ± 0.01	13.38 ± 1.62	0.05 ± 0.01
mg of enzyme/100 gm of cecal contents		64.1 ± 9.4	1.5 ± 0.8	75.2 ± 9.1	5.1 ± 0.8
mg of enzyme/100 mg of TCA ppt.		13.8 ± 2.0	0.09 ± 0.05	17.1 ± 2.1	0.6 ± 0.1
Chymotrypsin					
mg of enzyme/100 gm of body wt.		1.78 ± 0.24	0.02 ± 0.01	9.94 ± 1.52	0.02 ± 0.01
mg of enzyme/100 gm of cecal contents		25.2 ± 3.4	1.5 ± 0.8	55.8 ± 8.5	1.6 ± 0.2
mg of enzyme/100 mg of TCA ppt.		5.4 ± 0.7	0.09 ± 0.05	12.7 ± 1.9	0.18 ± 0.02

^a Trypsin and chymotrypsin were estimated spectrophotometrically by the hydrolysis of benzoyl arginine ethyl ester (trypsin) and acetyl tryosine ethyl ester (chymotrypsin) (11, 12). The values obtained were compared to standard curves determined for known amounts of trypsin and chymotrypsin.

^b ± SE of the mean.

the gnotobiotic animals (Table IV). These elevated enzyme levels existed regardless of what they were referred to, i.e., body weight, cecal weight, or TCA precipitable protein. In the gnotobiotic animal trypsin accounted for 14–17% and chymotrypsin for 5–12% of the TCA precipitable protein. In conventional animals these enzymes were found in small amounts and collectively accounted for less than 1% of the TCA precipitable protein.

Discussion. The above results plus the findings of Combe (3–5) suggest that there is an accumulation of certain endogenous proteins,

mucoproteins, and mucopolysaccharides in the gnotobiotic cecum. In conventional animals these compounds enter the gastrointestinal tract in amounts which may be equal to or greater than dietary nitrogen and approximately 90% of the secreted or sloughed nitrogen is digested and reabsorbed (10). Reabsorption most likely occurs in the terminal ileum so that the material reaching the cecum and/or colon area represents the undigested or unabsorbed residue. In gnotobiotic animals the pattern is different as these endogenous sloughings and secretions tend to

accumulate in the cecum. This would suggest that microbes normally resident in the distal small intestine contribute in a significant way to their degradation. In the absence of microbes some of this endogenous nitrogen can not be recovered and accordingly, the need for absorptive surfaces in the lower small intestine should be less. It is of interest that Gordon (1) found a pronounced reduction of mucosal surface area in the mid and lower parts of the small intestine of gnotobiotic animals. Thus cecal enlargement may be secondary to the failure to degrade and reabsorb endogenous protein, mucoprotein, and mucopolysaccharides in the terminal small intestine.

The values found for trypsin and chymotrypsin differ markedly with those reported by Lepkovsky *et al.* (13). This may be due to the use of different substrates and to the fact that our assays were performed within 1-3 hours after sacrifice of the animals.

Summary. Cecal contents of gnotobiotic rats and mice have 10 times as much soluble protein and from 20 to 200 times as much soluble carbohydrate as their conventional controls. About 12-29% of the soluble protein was precipitated by 10% TCA and was shown to contain elevated levels of trypsin and chymotrypsin (i.e., from 90 to 200 more than conventional animals per 100 gm of body wt.). Another 30-50% of the protein was soluble in 10% TCA but could be precipitated by 80% ethanol. The material was most likely mucinous in nature as it contained carbohydrate and was of high molecular weight. The gnotobiotic ceca had 5-8 times more insoluble material per 100 gm

of body weight than that found in conventional ceca. The demonstration of endogenous enzymes and high molecular weight mucins suggested that there is an impairment in the digestion and reabsorption of endogenous nitrogen in the gnotobiotic animals.

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