MUCUS IN INTESTINAL CONTENTS OF GERMFREE RATS*

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One of the differences between animals reared under germfree conditions and conventional[1] animals is the considerable enlargement of the germfree cecum. It has been shown that the gross enlargement of the cecum is due to an increase in the amount of the cecal contents as well as to an increase in the weight of the cecal wall. When germfree animals were contaminated with a normal intestinal flora the amount of cecal contents as well as the weight of the cecum wall were reduced (1, 2). Skelly *et al.* (3) found that the contamination of germfree mice with *Clostridium difficile* or with two strains of *Bacteroides* was followed by a decrease within 14 days of the cecum size to that of the conventional animals.

Diets with various carbohydrates, which are absorbed slowly or not at all have been shown to produce cecum enlargement. In the study by Fournier *et al.* (4) the ceca in rats were twice as large when lactose, L-xylose, or glucosamine was substituted for glucose in the diet. The thickness of the epithelial layer of the cecum wall was increased in these rats with enlarged ceca. A retention of mucus in the dilated and sometimes branched Lieberkühn crypts in the enlarged ceca of germfree rats has also been described (14).

Since the enlargement of the cecum in germfree animals could at least in part be caused by retention of mucus, which is normally metabolized by intestinal bacteria, we have studied the excretion of hexosamine and nitrogen in the feces and the nature of mucus matter in the intestinal contents of germfree and conventional rats.

Material and Methods

Animals.—The germfree animals were reared according to Gustafsson (5, 6) and fed the semisynthetic diet "D7" and water *ad lib.* The diet was sterilized by autoclaving at 121°C

[1] The expression "conventional" refers to normal animals, raised and maintained under ordinary conditions, without any attempt at altering their indigenous microbial flora.

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for 20 minutes. The animals were kept in metabolism cages within the germfree isolator. Conventional animals of the same strain were housed in metabolism cages in the animal room and given the same sterilized diet.

Fecal Analysis.—Feces were collected daily and kept frozen until analysis. The thawed feces were homogenized in 30 ml of water with a Vir-Tis homogenizer (Telcolab Corp., New York) and suitable aliquots taken for analysis.

Analysis of Cecal Wall and Contents.—The wet weights of the cecal walls and of the cecal contents were determined separately immediately after killing the animals. The whole cecal wall and a weighed aliquot of the contents were then freeze-dried and weighed. From these figures the water content of the cecal wall and of the cecal contents could be calculated. Chemical analyses were performed on the freeze-dried material unless otherwise stated. The dried preparations were soaked in 30 ml of water for 1 hour and then homogenized with a Vir-Tis homogenizer for 2 minutes. Aliquots were taken from these homogenates for analysis.

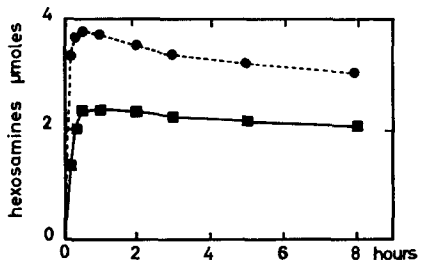

FIG. 1. Liberation of hexosamines from cecal contents by acid hydrolysis (refluxing 4 M HCl, hexosamine concentration 0.5 to 1 per cent). ●--●, germfree; ■—■, conventional.

Chemical Methods.—

Total hexosamines: The Elson-Morgan procedure (7) as modified by Boas (8) was used for analysis of total hexosamines. Recrystallized glucosamine hydrochloride (Hoffman-La Roche and Co., Basle, Switzerland) was used as a standard and the results expressed as equivalents of glucosamine hydrochloride. In the analyses performed on the fractions obtained by gel filtration (see below) the resin treatment used in the Boas procedure was omitted. Samples were hydrolyzed prior to analysis in refluxing 4 M hydrochloric acid. In preliminary experiments the optimal conditions for hydrolysis of fecal samples were determined by using different refluxing times. Fig. 1 shows the effect of hydrolysis time on the yield of Elson-Morgan color. A refluxing time of 60 minutes was chosen for routine use. If hydrolysis was carried out on a boiling water bath, the heating time had to be prolonged to 2 hours.

Glucosamine-galactosamine ratio: Chromatographic separations of glucosamine and galactosamine were performed on columns of dowex 50 X8 with 0.3 M hydrochloric acid as eluant as described by Gardell (9). Authentic glucosamine and galactosamine were used for standardizing the columns.

Nitrogen: Total nitrogen was determined by the microKjeldahl procedure after digestion with sulfuric acid, copper sulfate, and hydrogen peroxide.

"Soluble" and "insoluble" material in the cecal contents: Fresh cecal contents obtained immediately after killing the animals were added to ten volumes of 0.9 per cent saline and carefully stirred with a glass rod. The suspension was then centrifuged for 2 hours at 20,000 *g* at a temperature of 20°C. The material remaining in the clear supernatant after this procedure will be referred to as "soluble" and the material in the sediment as "insoluble." Aliquots were taken from the supernatant and from the sediment, rehomogenized in saline, for nitrogen and hexosamine analysis.

Gel filtration: Gel filtration experiments were performed with sephadex G-100 (140 to 400 mesh) and G-25 (Pharmacia, Uppsala, Sweden). The dry gel was equilibrated with 0.9 per cent saline overnight and poured into glass columns (1.6 x 50 cm). The columns were washed with several volumes of 0.9 per cent saline before use. Fresh cecal contents were diluted with one volume of 0.9 per cent saline and centrifuged at 20,000 *g* for 2 hours. Sodium chloride, 4 M, was then added to the supernatant to give a final concentration of about 1 M. One ml of this solution, corresponding to about 5 mg of hexosamine, was put onto the column by layering it underneath the sodium chloride solution on the top. The column was eluted with 0.9

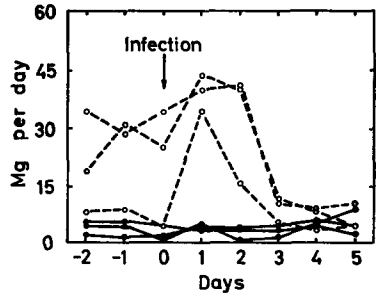

FIG. 2. Daily excretion of hexosamine in feces of conventional rats (●—●) and rats in the germfree and exgermfree state (O--O).

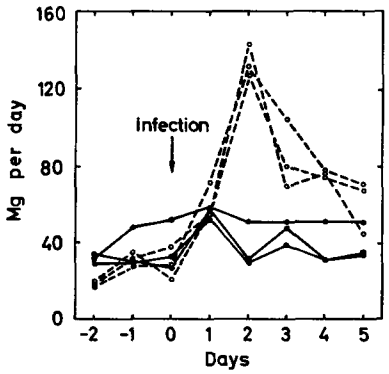

FIG. 3. Daily excretion of nitrogen in the feces of conventional rats (●—●) and rats in the germfree and exgermfree state (O--O).

per cent saline and the eluate collected in fractions of 4 ml. The fractions were analyzed for protein by the Lowry (10) procedure. For analysis of hexosamines by the Elson-Morgan procedure the fractions were hydrolyzed in 4 M hydrochloric acid in a boiling water bath, lyophilized, and redissolved into water.

Incubation of germfree cecal contents with intestinal microorganisms: Fresh cecal contents from germfree rats were diluted with an equal volume of 0.25 per cent (w/v) solution of Difco yeast extract in water. Tubes with 8 ml of the mixture were inoculated separately with: (*a*) *Clostridium difficile* which according to Skelly *et al.* (3) reduced the enlarged cecum in germfree mice, (*b*) a mixture of sporeforming bacteria which in other experiments in our laboratory reduced the cecum of germfree animals, and (*c*) feces from conventional animals. After incubation for 3 days at 37°C under anaerobic conditions the amounts of soluble and

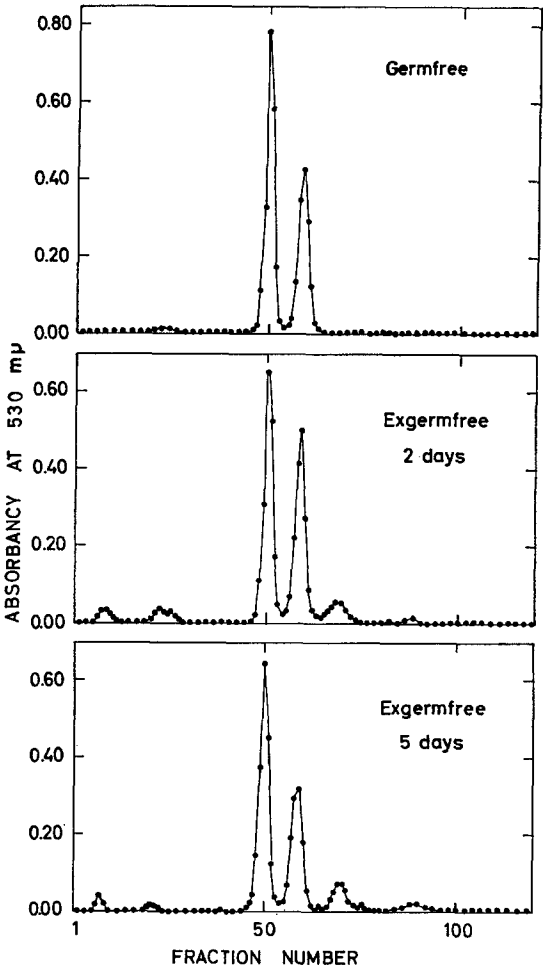

FIG. 4. Separation of hexosamines in acid hydrolysates of feces on cation exchange resin dowex 50 (X8, 200 to 400 mesh, 1.2 x 54 cm, H^+-form). Column eluted with 0.30 M hydrochloric acid. Fraction volume 4.80 ml. Glucosamine fractions 47 to 53, galactosamine fractions 56 to 61.

non-soluble hexosamine and nitrogen were determined and the supernatant was fractionated by gel filtration on sephadex G-100.

RESULTS

Fecal Excretion of Total Hexosamines and Nitrogen.—The daily excretion in the feces of total hexosamines and of total nitrogen was determined in three germfree and in three conventional animals. The germfree rats were then infected by feeding 1 ml of a mixture consisting of cecal contents from conven-

tional animals diluted with an equal volume of yeast extract (0.25 per cent w/v). The fecal excretion of total hexosamines and of total nitrogen was then followed for a period of 5 days after the infection. The results of this experiment are shown in Figs. 2 and 3. It can be seen that the excretion of hexosamines was

TABLE I

Analysis of Cecal Contents in Germfree and Conventional Rats

Animal No.	Body Weight	Contents				Hexosamines			Nitrogen		
		Wet weight	Wet weight in body weight	Dry weight	Water content	Total amount	Total amount in dry weight	Total amount per 100 gm body weight	Total amount	Total amount in dry weight	Total amount per 100 gm body weight
Germfree											
	gm	*gm*	*per cent*	*gm*	*per cent*	*mg*	*per cent*	*mg*	*mg*	*per cent*	*mg*
1	61	7.57	12.4	1.01	87	107	10.6	175	66	6.5	108
2	63	7.01	11.1	0.97	86	96	9.9	152	57	5.9	90
3	79	6.01	7.6	0.87	86	65	7.4	82	65	7.5	82
4	98	8.51	8.7	1.34	84	113	8.4	115	112	8.4	114
5	203	10.89	5.4	1.90	83	137	7.2	68	127	6.7	63
6	242	11.38	4.7	2.16	81	128	5.9	53	111	5.0	46
7	243	7.91	3.3	1.46	82	83	5.7	34	82	5.6	34
Average			7.6			104	7.9	97	103	6.5	77
Conventional											
8	34	0.39	1.1	0.08	79	1.4	1.7	4.3	7.4	9.1	22
9	44	0.50	1.1	0.10	79	1.2	1.2	2.7	7.4	7.6	17
10	45	0.46	1.0	0.10	79	1.3	1.4	2.9	8.4	8.8	19
11	48	0.55	1.1	0.12	79	1.9	1.6	4.0	8.7	7.6	18
12	59	0.71	1.2	0.15	79	2.2	1.5	3.7	11.0	7.5	19
13	210	1.91	0.9	0.36	81	4.2	1.2	2.0	21.0	5.9	10
14	218	2.11	1.0	0.34	84	3.3	1.0	1.5	18.0	5.5	8
15	305	2.95	1.0	0.28	90	5.3	1.9	1.7	23.0	8.9	8
Average			1.1			2.6	1.4	2.9	13.0	7.6	15

higher (10 to 32 mg/day) in germfree than in conventional rats (2 to 7 mg/day) (Fig. 2), while no significant difference was found in the nitrogen excretion (30 mg/day) between the germfree and the conventional animals (Fig. 3).

Infection of the germfree animals with intestinal microorganisms from conventional rats resulted in an increase in the excretion of nitrogen and hexosamines during the 2 to 3 days immediately following the infection. After this transition period the excretion of hexosamines diminished and finally reached

the level found in the control animals. The excretion of nitrogen which reached a peak of about 120 mg per day on the 2nd day after the infection had not returned to the basal level when the experiment was terminated.

Fig. 4 shows a separation on a dowex 50 column of the hexosamines obtained after hydrolysis of feces from one of the rats in this experiment in (*a*) the germfree state, (*b*) during the transition period, and (*c*) in the more stabilized period

TABLE II

Analysis of Cecal Wall of Germfree and Conventional Rats

Animal No.	Dry weight	Total hexosamines	Hexosamines in dry weight	Nitrogen	Nitrogen in dry weight
			Germfree		
	mg	*mg*	*per cent*	*mg*	*per cent*
1	73	0.75	1.0	9.3	13.0
2	112	1.11	1.0	16.0	15.0
3	109	1.38	1.2	15.0	13.0
4	155	1.10	0.7	21.0	14.0
5	178	2.03	1.1	15.0	9.0
6	156	1.20	0.8	17.0	11.0
7	135	1.15	0.8	17.0	13.0
Average	131	1.20	1.0	16.0	13.0
			Conventional		
8	14	0.06	0.4	2.6	19.0
9	24	0.16	0.7	4.5	19.0
10	22	0.16	0.7	5.1	23.0
11	26	0.23	0.9	4.2	16.0
12	26	0.19	0.7	4.2	16.0
13	67	0.60	0.9	7.7	12.0
14	66	—	—	—	—
15	68	0.37	0.6	9.0	13.0
Average	39	0.30	0.7	5.3	17.0

5 days after infection. The two main Elson-Morgan positive compounds were glucosamine (fractions 47 to 53) and galactosamine (fractions 56 to 61), which were present in a ratio of 1.3 to 1.5 in all three periods. The peak containing Elson-Morgan positive material which appeared after galactosamine in fractions 66 to 72 has been found only in infected animals and has not been further identified. Incompletely hydrolyzed material, approximatively 5 per cent of the total, appeared early in the chromatograms from the exgermfree animals.

Cecal Contents and Cecal Wall.—Table I gives the wet and dry weights of the cecal contents and also the total amounts of hexosamines and nitrogen in

the cecal contents for a series of germfree and conventional rats of varying body weight. The content of hexosamines and nitrogen has also been calculated in per cent of dry matter and, for comparison of different animals, as amount per 100 gm of body weight.

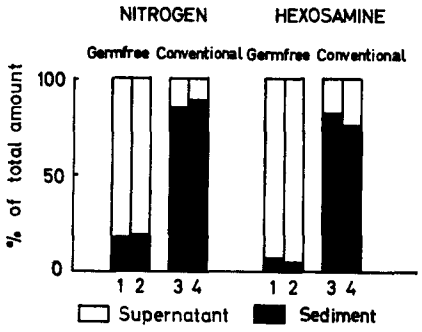

FIG. 5. Distribution of nitrogen and hexosamine in cecal contents between 20,000 *g* sediment and supernatant of germfree (*1*, *2*) and conventional (*3*, *4*) rats.

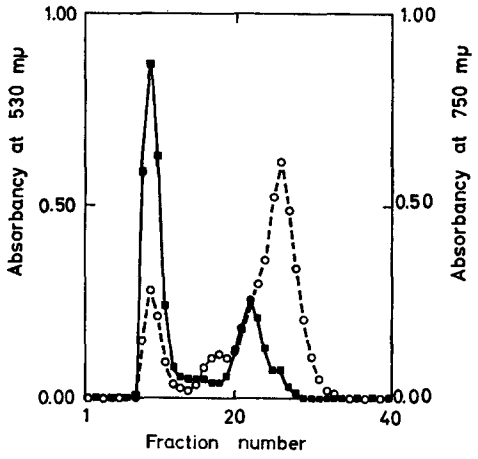

FIG. 6. Gel filtration through sephadex G-100 of soluble fraction of cecal contents from a germfree rat. Hexosamine (■—■) assayed by Elson-Morgan procedure at 530 mμ. Protein (O--O) assayed by Lowry procedure at 750 mμ. Column eluted with 0.9 M sodium chloride.

In the germfree rats the cecal contents accounted for 7.6 (3 to 12) per cent of the total body weight with the higher figures occurring in young animals. In the conventional rats, however, the cecal contents accounted for only 1.1 (0.9 to 1.2) per cent of the body weight. The percentage of water in the cecal contents appears to be somewhat higher in the germfree than in the conventional animals.

The total amount of hexosamines found after hydrolysis of cecal contents was 104 (65 to 137) mg per animal in the germfree rats as compared to 2.6 (1.2 to 5.3) mg in the conventional rats. The hexosamines accounted for 7.9 per cent of the dry weight of the cecal contents in the germfree and for 1.4 per cent of the dry weight in the conventional animals.

The total nitrogen in the cecal contents of germfree and conventional animals was 103 (57 to 127) mg and 13 (7.4 to 23) mg respectively. The mean nitrogen content expressed as per cent of dry matter was 6.5 and 7.6 in the two series.

Table II gives the weights of lyophilized cecal walls of germfree and conventional rats and the results of their analyses for total hexosamines and

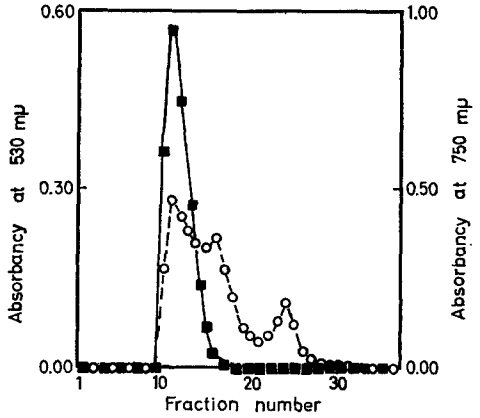

FIG. 7. Gel filtration through sephadex G-25 of soluble fraction of cecal contents from a germfree rat. Hexosamine (■—■) assayed by Elson-Morgan procedure at 530 mμ. Protein (O--O) assayed by Lowry procedure at 750 mμ. Column eluted with 0.9 M sodium chloride.

nitrogen. The weight of the lyophilized walls was 131 (73 to 178) mg in the germfree series and 39 (14 to 68) mg in the control series. The total hexosamine content of the hydrolyzed tissues was 1.2 mg in the germfree as compared to 0.3 mg in the conventional animals, while the corresponding figures for total nitrogen was 16 and 5.3 mg respectively.

Fractionation of Cecal Contents.—The distribution of nitrogen and hexosamines between the "soluble" and "insoluble" fractions obtained by centrifugation of cecal contents at 20,000 *g* is shown in Fig. 5. In the germfree animals hexosamines and nitrogen occurred mainly in a soluble form, whereas in the cecal contents obtained from conventional animals the major part of hexosamines and nitrogen was present in the insoluble 20,000 *g* sediment.

Fig. 6 shows a separation on sephadex G-100 of soluble cecal contents from a germfree rat. It is seen that the protein- and the hexosamine-containing material is resolved into two main peaks. Seventy per cent of the hexosamines

occurred in the first peak which contains material with a molecular weight above 100,000 while only 30 per cent of the total protein appeared in this fraction. On chromatography on a column of sephadex G-25 the hexosamine-containing material appeared in the front which indicates a molecular weight above about 4000 (Fig. 7). The cecal contents from several conventional animals were pooled and the soluble part subjected to the same chromatographic procedure. Essentially the same results as for the germfree animals were obtained.

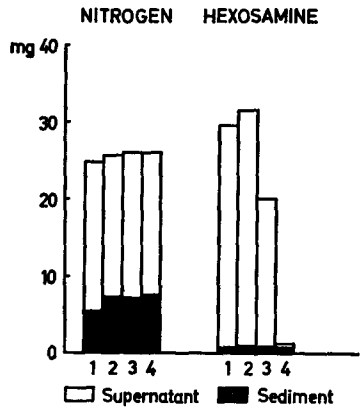

FIG. 8. Distribution of nitrogen and hexosamine between 20,000 *g* sediment and supernatant after *in vitro* incubation of germfree cecal contents at 37°C for 3 days. Inoculate: none, *1*; *Clostridium difficile*, *2*; sporeforming bacteria, see text, *3*; feces from conventional rats, *4*.

In Vitro Experiments.—Fig. 8 shows the results of one experiment out of four with the same outcome in which the cecal contents from germfree rats were diluted with yeast extract and incubated as follows (*a*) no addition, (*b*) *Clostridium difficile*, (*c*) a mixture of cecum reducing sporeforming bacteria, and (*d*) a fecal suspension from conventional rats. The total nitrogen content and its distribution between the soluble and insoluble fraction were the same in the control as in the three inoculated tubes. The total hexosamine content and its distribution were not changed by incubation with *Clostridium difficile*. The mixture of spore formers slightly reduced the total amount of hexosamine in the incubation mixture, whereas the conventional flora reduced the total amount of hexosamine to about one-twentieth of the initial amount. The amount of hexosamine in the insoluble fraction was about the same in the three inoculated tubes as in the control. The soluble fractions from these incubations were subjected to gel filtration on columns of sephadex G-100 (Fig. 9). The distribution of protein- and hexosamine-containing material between the high molecular (above 100,000) and low molecular fractions was essentially the same

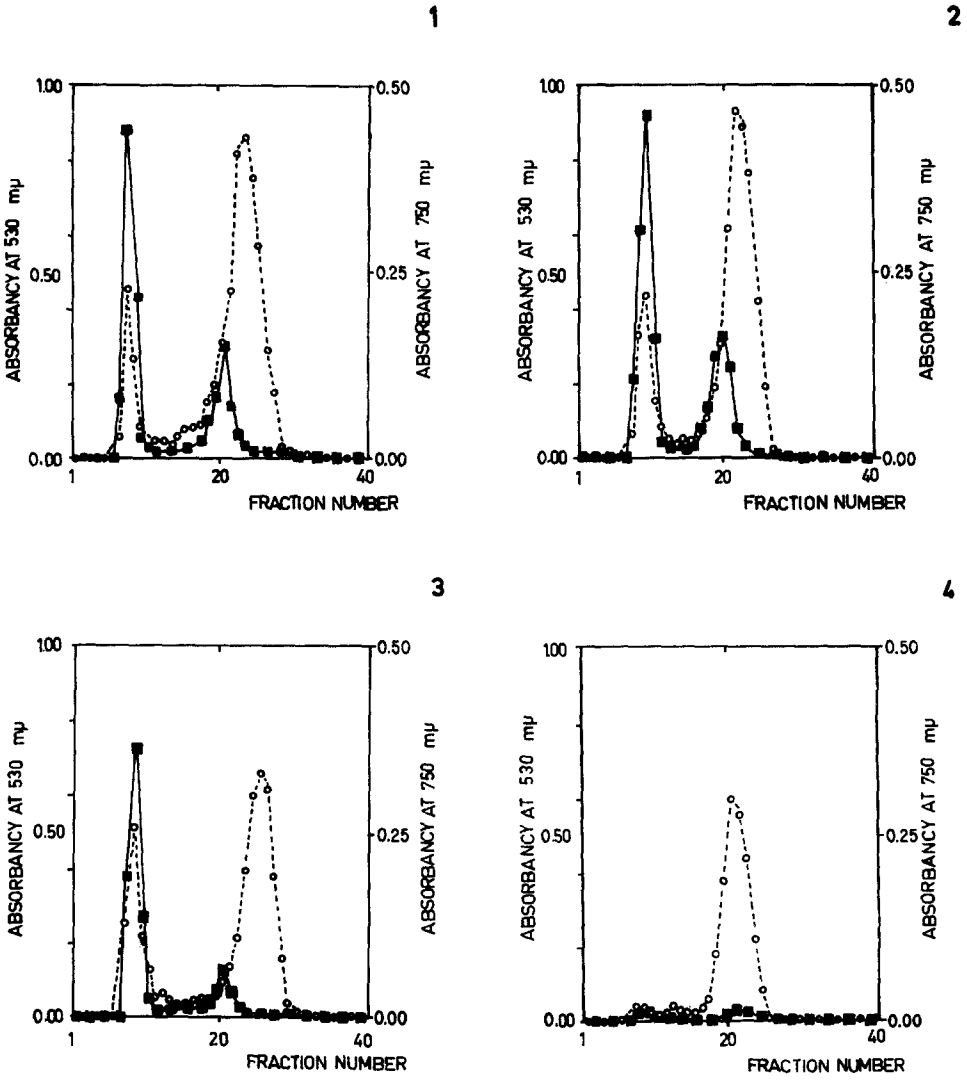

FIG. 9. Gel filtration through sephadex G-100 of supernatant from experiment shown in Fig. 8. Hexosamine (■—■) assayed by Elson-Morgan procedure at 530 mμ. Protein (○--○) assayed by Lowry procedure at 750 mμ.

in the control, in the two incubations with *Clostridium difficile*, and in the mixture of spore formers. In contrast practically no material, containing protein or hexosamine with a molecular weight above 100,000, was seen in the chromatogram of the soluble material after incubation with the conventional flora.

DISCUSSION

The enlargement of the ceca in the animals studied is of the same magnitude as found in germfree rats by earlier investigators.

In the present work determinations of total hexosamines were used as an index of the amount of mucus material present in the cecum. The percentage of hexosamines varies in different types of mucins, but determinations of hexosamines were considered to give an acceptable basis for comparing germfree and conventional rats. The enlargement of the cecum of the germfree rat is associated with an increased dry weight and with higher amounts of hexosamines and nitrogen in the cecal wall. There appears to be a proportionally somewhat higher increase in the hexosamines than in the nitrogen showing an accumulation of mucus material in the wall of the germfree cecum.

The cecal contents from germfree rats contains 30 to 40 times as much hexosamines as that from conventional animals. This is due not only to an increase in the total amount of contents in the cecum but also to a five- to tenfold increase in the concentration of hexosamines in the cecal contents.

The distribution of hexosamines and nitrogen between the soluble and insoluble fractions of the cecal contents is different in the germfree and in the conventional animals, which indicates a difference in the physicochemical nature of the hexosamine-containing molecules. Thus in the germfree rats only 5 per cent of the total hexosamines appeared in the 20,000 *g* sediment while in the control animals 80 per cent of the total hexosamines were present in this fraction. To what extent the insoluble fraction represents bacterial mucopolysaccharides or denatured intestinal mucins cannot be decided from the present data.

Information on the particle size of the hexosamine-containing material was obtained through the experiments in which the soluble fraction from cecal contents was subjected to gel filtration on sephadex of different cross-linking No major difference was found between germfree and control rats as regards the molecular weight distribution of the hexosamine-containing molecules, since in both cases 70 per cent of the material had a molecular weight above 100,000. The total amount of this high molecular weight mucus material was, however, 500 to 1000 times higher in the germfree than in the conventional animals due to the combination of a difference in *total* amount of mucus and a difference in *relative* amount of soluble material. The mucus material present in the cecum represents a secretion from the cecal wall but may also in part originate from higher levels of the intestinal tract. The knowledge of the effect of digestive enzymes and intestinal microflora on the mucus secretions of the intestinal tract is rather incomplete. It is generally stated that digestive proteolytic enzymes do not attack these substances in the gut (11). This is an agreement with the finding that the germfree cecum contains hexosamine-rich material of high molecular weight although the digestive enzymes retain their

activity throughout the intestinal tract in the germfree rats (Borgström *et al.*, 12).

The experiment in which the excretion of hexosamines and nitrogen was studied showed that the germfree animal excretes a larger amount of hexosamine-containing material per day than the conventional animal, while no appreciable difference in the excretion of total nitrogen appears to exist between the two types of rats. The infection of the animals was followed by a large increase in the excretion of hexosamines and nitrogen during the following days. It appears likely that this additional excretion represents the elimination of mucus material and proteins retained in the germfree ceca. The individual differences in the excretion and the short duration of the experiment, however, do not permit any detailed quantitative assessment of the observed effects.

The presence of an increased excretion of hexosamine-containing material and large amounts of mucus material in the cecum of the germfree rat could be due either to an increased synthesis or to a decreased breakdown of mucus in these animals. Through the series of *in vitro* experiments it was clearly demonstrated that intestinal microorganisms attack the mucopolysaccharides of the type found in the cecum of the germfree rat. In consequence a decreased rate of breakdown of mucus appears as one of the possible explanations for the increased concentration of mucins in the ceca and feces of germfree rats.

The cecal enlargement in the germfree rats could thus be explained by an accumulation of mucus with a high molecular weight, which is not removed through the attack of intestinal microorganisms and therefore dilates the cecum. It appears possible, however, that other factors than the absence of mucin-degrading microorganisms may contribute to the cecal enlargement in germfree rats. It is of interest that the isolated strains of bacteria capable of reducing the cecal size *in vivo* did not show any capacity to degrade the mucus *in vitro* in a test system, where a full intestinal flora was highly active.

SUMMARY

The fecal excretion of total nitrogen and of total hexosamines has been determined in germfree and conventional rats. Germfree rats excreted more hexosamines than the conventional rats, while no difference in the nitrogen excretion was found. Infection of the germfree rats with a normal flora resulted in a temporarily increased excretion of hexosamines and nitrogen over a period of 2 to 3 days after which they reached the level of the conventional animals.

The contents of the germfree cecum contained 65 to 137 mg of hexosamines and 57 to 127 mg of nitrogen as compared to 1.2 to 5.3 and 7.4 to 23 mg in conventional animals. The high figures for hexosamines were due to an increase in the total amount of contents in the cecum and to a fivefold increase in the concentration of hexosamine-containing material.

Studies on the distribution of hexosamine-containing cecal contents between

sediment and supernatant after centrifugation at 20,000 *g* for 2 hours demonstrated that 5 to 10 per cent of the hexosamines occurred in the sediment in the germfree rats, while 75 to 85 per cent was found in this fraction in the conventional rats. The soluble part of the cecal contents in germfree as well as in the conventional rats contained 70 per cent of hexosamines in molecules with a molecular weight above approximatively 100,000 as found by gel filtration experiments on sephadex gels.

The higher weight of the germfree cecal wall was reflected in a high total amount of nitrogen and hexosamines.

Isolated strains of bacteria capable of reducing the cecal size *in vivo* did not show any capacity to degrade the mucus *in vitro* in a test system, where a full intestinal flora was highly active.

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