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Intestinal Glycoproteins of Germfree Rats

II. Further Studies on the Chemical Composition of Water-soluble Extracts from Intestinal Mucus

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Injection of glucose-^{14}C into germfree rats was followed, after 24 h, by removal of the entire gut and preparation of the aqueous non-dialysable extracts from contents of the small intestine, cecum, colon, and feces. The extracts were hydrolysed by acid for the release of sugars, which were then separated by paper chromatography and finally subjected to radioautography. In each of the extracts the following sugars were found to be radioactively labelled: galactose, mannose, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid. Arabinose and xylose were not radioactive, and the presence of these sugars is ascribed to extraneous polymer carbohydrate in the intestinal contents. This material remained in the germfree rat intestinal tract even after a ten day dietary adjustment period during which the animals were fed a chemically defined diet, containing glucose as the only sugar.

The extracts of cecum and colon were similar in their contents of carbohydrate, protein, and sulphate. In comparison, the extract of the small intestine contained more protein, less carbohydrate, and no sulphate.

In an initial study [1] it was found that aqueous non-dialysable extracts of intestinal and fecal mucus from germfree rats, fed a chemically defined diet, consisted of carbohydrate and protein. The extracts contained the common glycoprotein sugars galactose, mannose, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid.

The main amino acids present were serine and threonine, suggesting that a major part of the extracts might consist of mucin-type glycoproteins. This was consistent with the fact that mild alkali treatment of the material caused a marked decrease in the contents of serine and threonine.[2] In addition the extracts also contained significant quantities of the pentose sugars xylose and arabinose. There has been a number of reports on the occurrence of arabinose [3]

and xylose [4–6] in mammalian mucopolysaccharides and glycoproteins, but so far these pentoses have never been encountered as constituents of mucins. It has been clearly demonstrated that xylose is a structural component of mucopolysaccharides like heparin and chondroitin sulphate, were it serves as the point of linkage between carbohydrate and protein.[7,8]

However, in the case of other classes of glycoproteins the occurrence of pentose sugars as an integral part of the molecular structure has never been firmly established by chemical proof. At present it is thought that the occasional finding of pentoses in glycoproteins is due either to artefact formation, *e.g.* during acid hydrolysis, or more probably, to impure glycoprotein preparations. Our inability to completely remove the pentose-containing material from the glycoprotein fraction by ion exchange or gel chromatography necessitated an investigation into the origin of xylose and arabinose.

The monosaccharide components of mammalian glycoproteins, including the xylose residues of the mucopolysaccharides, are normally formed from D-glucose.[9] Thus by administering radioactively labelled glucose to the animals the label will appear in all the different sugars incorporated during glycoprotein biosynthesis. By using this method it was shown that the water-soluble, non-dialysable extracts from contents of different intestinal segments and of the feces from rats given glucose–^{14}C intraperitoneally were all radioactive. The relative levels of radioactivity in the four extracts were decreasing from the small intestinal to the fecal extract as measured on the lyophilized samples under identical conditions by the Geiger-Müller counter (Table 1).

Table 1. Relative amounts of radioactivity in non-dialysable extracts from the contents of the small intestine, cecum, colon, and from the feces. The measurements were done under identical conditions on lyophilized samples (10 mg) by a Geiger-Müller counter. Correction is made for background radiation, and the counts are adjusted to the nearest hundred.

Extract from	Counts per minute
Small intestine	2000
Cecum	1400
Colon	1300
Feces	600

The measurements can be regarded only as semiquantitative, but the trend is clear. The corresponding low-molecular weight fractions present in the respective dialysates, and also the lipid fractions (the combined acetone, chloroform-methanol, and ether extracts) all contained label but much less than the high-molecular weight material.

Thus it seems that the major part of the label is incorporated into macromolecular substances, and that the bulk of the radioactively labelled compounds secreted into the intestinal tract is not excreted from the animal during the 24 h after the injection of glucose-^{14}C.

Previous studies have demonstrated that epithelial cells of the rat small and large intestine incorporate labelled carbohydrate into glycoproteins [10–13] which are then gradually secreted into the intestinal lumen. Judging from the results obtained it seemed appropriate to collect the intestinal contents about 24 h after the injection of labelled sugar.

Each of the aqueous non-dialysable extracts was hydrolysed under the appropriate conditions for the release of neutral sugars, amino sugars, and sialic acid, respectively. Paper chromatography of the acid hydrolysates and subsequent radioautography, apparently, revealed label in all the sugars except arabinose, xylose, and the tiny amount of glucose present.

Larger quantities of the extracts were hydrolysed and the sugars fractionated by chromatography on thick filter paper. Each of the monosaccharides was isolated separately and then subjected to paper chromatography in at least three different solvent systems followed by radioautography. In every instance the dark spots visible on the X-ray film coincided both with the location and the shape of the spots revealed by the use of the appropriate spray reagents for sugars. The results clearly indicated that galactose, mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, and *N*-glycolylneuraminic acid had become radioactively labelled after glucose-^{14}C injection. On the other hand paper chromatography of the isolated arabinose and xylose and subsequent radioautography revealed no trace of radioactivity, even when applying more than the normal amount of sugar to the chromatogram. Neither the trace amount of glucose isolated showed any sign of being radioactive.

No labelled carbohydrate could be detected in the intestinal and fecal extracts obtained from the animal that had received an injection of xylose-^{14}C. This finding is consistent with the general conception that xylose is not utilized as such by mammalian cells, but only after being formed by enzymic decarboxylation of uridine diphosphate glucuronic acid to uridine diphosphate xylose.[14,15]

The occurrence of non-labelled carbohydrate as part of the high-molecular weight intestinal contents from the animals that had received glucose-^{14}C suggests that this non-radioactive fraction is not produced by the animal. Thus the intestinal mucus of the rats that had been fed the synthetic diet for ten days probably contained exogenous polymer carbohydrate.

Extracts prepared separately of the intestinal contents from more than ten animals fed the chemically defined diet for ten days invariably proved to contain pentoses. The most likely source of a high-molecular weight carbohydrate contaminant would be the rat food used prior to the synthetic diet. The ordinary germfree rat diet was subjected to the general extraction procedure, and the non-dialysable extract obtained was analysed quantitatively for carbohydrate. Similarly, extracts of the contents of the small intestine, cecum and colon from the rats fed the synthetic diet for ten days were prepared and analysed under identical conditions. The extracts from the three segments of the gut all contained the same sugars although in somewhat different amounts and molar proportions (Table 2).

The rat food extract differed markedly from the intestinal extracts in its carbohydrate composition. It consisted mainly of glucose, xylose, and arabinose

Table 2. Composition of water-soluble, non-dialysable extracts from the contents of small intestine, cecum, and colon, and of the corresponding extract obtained from the ordinary germfree rat diet.

	Extract from							
	Small intestine		Cecum		Colon		Rat food	
	%[a]	Rel.am.[b]	%[a]	Rel.am.[b]	%[a]	Rel.am.[b]	%[a]	Rel.am.[b]
Arabinose	0.6	0.6	1.6	0.5	0.8	0.5	9.5	0.6
Xylose	1.0	1.0	3.0	1.0	1.5	1.0	15.3	1.0
Fucose	5.0	4.7	6.4	2.0	6.2	3.8	0.0	
Mannose	3.6	3.1	3.2	0.9	2.2	1.2	0.8	0.05
Galactose	11.0	9.4	15.1	4.2	14.7	8.2	1.7	0.1
N-Acetylgalactosamine	7.3	5.1	10.6	2.4	10.8	4.9	0.0	
N-Acetylglucosamine	11.5	8.4	12.3	2.8	12.2	5.5	0.0	
Sialic acid[c]	5.2	2.6	5.0	0.8	8.0	2.6	0.0	
Glucose	0.0		0.0		0.0		61.0	3.4
Total carbohydrate	45.2		57.2		56.4		88.3	
Protein	53.9		33.0		32.2		8.5	
Sulphate	0.0		0.4		0.3		0.0	
Total	99.1		90.6		88.9		96.8	

[a] Per cent of the lyophilized material, dried over phosphorus pentoxide *in vacuo*. [b] Molar proportions relative to xylose. [c] The content of sialic acid is calculated as *N*-acetylneuraminic acid.

and was devoid of fucose, hexosamine, and sialic acid. The molar ratio of xylose to arabinose is strikingly constant in all the four extracts. This makes it reasonable to conclude that the polymer material containing the two pentoses originates from the rat food and that this food component passes mainly unaltered through the gastrointestinal tract. On the other hand the greater proportion of the glucose-containing polymer in the rat food is obviously utilized by the animals.

No attempt was made to estimate the time required for the complete clearing of the pentose-containing diet from the intestinal tract. It seems likely that non-absorbable diet components are trapped, to some extent, in intestinal crypts, partly because of the reduced motility and propulsive activity of the germfree intestinal tract,[16] and partly because of the gel-like viscous mucoid material filling up the entire intestinal lumen.

Even if the analytical data presented in Table 2 are obtained on heterogeneous material consisting of a mixture of macromolecules, a comparison of the chemical composition of the three intestinal extracts still is informative. There is a significant difference between the extracts of the cecum and colon on one side and of the small intestine on the other side (Table 2). The small intestinal extract contains much more protein, no sulphate, and the sum of protein and carbohydrate makes up almost 100 % of the extract while these components account for only about 90 % of the extracts from the cecum and colon. This may be due to the presence in the cecum and colon mainly of mucin-type glycoproteins, giving an incomplete Lowry reaction because of the large number of carbohydrate side chains, thus resulting in a too low protein value.

Regarding the amount and composition of carbohydrate in the three extracts, again there is a rather close resemblance between the extracts of colon and cecum compared to that of the small intestine. The finding of sulphate ester groups in the two former extracts could be indicative of contamination with mucopolysaccharides, but all the intestinal extracts were devoid of uronic acid thus ruling out this possibility. On the other hand mucin glycoproteins frequently contain a small proportion of sulphate, and in the present case such substances would be the most probable source of the sulphate ester residues.

Fractionation experiments have revealed a considerable complexity in the composition of the extracts described in the present report. However, it appears that the intestinal mucin, which is regarded to be the predominant glycoprotein fraction of the germfree intestinal tract, can be obtained in a sufficient state of purity required for more detailed studies.

EXPERIMENTAL

Germfree rats of the CDF strain (The Charles River Breeding Laboratories, Wilmington, Mass., USA) were reared under the conditions described by Midtvedt and Trippestad.[17] Animals of both sexes, more than 40 days of age, were used in the experiments.

Two animals, fed the ordinary germfree rat diet, were given a single intraperitoneal injection of D-glucose-^{14}C, uniformly labelled, 0.5 mCi/animal. Similarly one animal, fed the same diet, was given a single intraperitoneal injection of D-xylose-^{14}C, uniformly labelled, 0.1 mCi. After 24 h the animals were anaesthesized by ether and sacrificed. The contents of the small intestine, cecum, colon, and the feces excreted during the 24 h were immediately collected separately under sterile conditions and stored at $-20°$.

In another experiment animals were fed the chemically defined diet under the same conditions as described previously.[1] After ten days the animals were sacrificed and the contents of the small intestine, cecum, and colon were collected and stored as above.

The conditions for the preparation of the intestinal extracts as well as for the acid hydrolysis and paper chromatography were as reported previously.[1] In addition neutral sugars were liberated from the polymer material by hydrolysis with 2 N sulphuric acid at 100° for 4 h. The deionized hydrolysates were subjected to preparative paper chromatography on Whatman No. 3 MM paper in either of the solvent systems, by volume: Ethyl acetate, acetic acid, formic acid, water, 18:3:1:4, or ethyl acetate, pyridine, acetic acid, water, 5:5:1:3. Three additional solvent systems were introduced for paper chromatography, by volume: Butanol, propanol, 0.1 N hydrochloric acid, 1:2:1; propanol, acetic acid, water, 7:1:2; ethyl acetate, pyridine, water, 8:2:1. Methanolysis was carried out by heating the lyophilized and dried sample, 1 mg, with 1 N hydrogen chloride in methanol at 85° for 20 h.

Gas-liquid chromatography of the derived trimethylsilylated methyl glycosides was performed on columns of 0.1 % OV-17 on glass beads using a Perkin-Elmer 900 gas chromatograph. The experimental details were as given by Reinhold.[18]

Radioactive compounds were located by a Geiger-Müller counter (Frieske & Hoepfner) or by radioautography.

When using radioautography the paper chromatograms were kept on X-ray film for three to four weeks.

Protein was estimated by the Lowry procedure,[19] bovine serum albumin being used as the standard. Sulphate was estimated by the barium chloroanilate method by Spencer.[20]

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