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Oligosaccharides and Polysaccharides specifically Utilizable by Bifidobacteria[1)]

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Oligo- and poly-saccharides which are utilized only by *B. infantis* and *B. breve* and not by other intestinal bacteria such as *E. coli*, *L. acidophilus*, and *S. faecalis* were investigated *in vitro* for the purpose of producing bifidus-flora in human intestines. Raffinose, stachyose, and inulin were found to be specifically utilized by *B. infantis* and doubling time of the cells with them as energy source was as short as with glucose and lactose. Inulin of molecular weight higher than *ca.* 4500 was not utilized by *B. infantis*. Oligosaccharides more than trisaccharide from inulin and tri- to penta-saccharides from dextran obtained by acid hydrolysis followed by Sephadex gel chromatographic separation were utilized specifically by *B. infantis*, but oligosaccharides from amylose and cellulose were found nonspecific for *B. infantis* and *B. breve*. Suitability of sugars for producing bifidus-flora *in vivo* is discussed.

Keywords——oligosaccharides; polysaccharides; bifidus-flora; *Bifidobacterium infantis*; *Escherichia coli*; *Lactobacillus acidophilus*; *Streptococcus faecalis*; raffinose; stachyose; inulin

Bifidobacteria, main constituents of normal intestinal flora of man, are considered to prevent the man from some bacterial diseases.[3)] Breast-fed infants have bifidus-flora[4)] in their intestines where bifidobacteria are predominant over other bacteria, while bottle-fed infants have a flora where the number of bifidobacteria decreases[4)] or that of other bacteria increases.[5)] Therefore, a great effort has been made to produce bifidus-flora in the intestines of bottle-fed infants. The vitamin-like substances for bifidobacteria, *i.e.*, glucosamine derivatives,[6)] Bifidus factor II,[7)] pantetheine derivatives,[8,9)] and nucleotides[10)] were found somewhat effective *in vivo*. On the other hand, Yoshioka[11)] claimed that the energy source (sugars) was more important for growth of bifidobacteria in intestines, showing that a strain of bifidobacteria (biotype I/II by Dehnert's grouping[12)]) and *Bifidobacterium bifidum* var. *pennsylvanicus* were able to grow markedly in media prepared from feces of bottle-fed infants only by supplement with lactose.

We tried to find suitable sugars which will increase the number of resident bifidobacteria in human intestines and, for this purpose, *B. infantis* and *B. breve* were used as representa-

1) A part of this work was presented at the 97th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1977.
2) Location: *Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan.*
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tives of bifidobacteria; *Escherichia coli*, *Lactobacillus acidophilus*, and *Streptococcus faecalis* as those of other competitive bacteria for sugars in intestines.

Experimental

Organisms——*B. infantis* S-12 and *B. breve* b S-46 were kindly supplied by Dr. Mitsuoka, The Institute of Physical and Chemical Research, Wako, *E. coli* K-12, *L. acidophilus* IAM 1084, and *S. faecalis* IAM 10067 were supplied by The Institute of Applied Microbiology, University of Tokyo.

Chemicals——G.R. grade of sulfuric acid and phenol were purchased from Kanto Kagaku Co., Inc., Tokyo, and *p*-anisaldehyde from Toyo Kasei Co., Tokyo. Lactulose was supplied by Chugai Seiyaku Co., Tokyo, and amylose by Nikken Kagaku Co., Tokyo. Inulin was purchased from E. Merck (G.R.), Difco Laboratories, and Wako Pure Chemical Industries Ltd., Osaka (E.P.), and cellulose (over 300 mesh) from Toyo Roshi Co., Tokyo. Dextran 40 was of the grade listed Japanese Pharmacopoeia VIII. Pectin, purchased from Nakarai Chemicals Ltd., Kyoto, was dialyzed against running water and lyophilized. Including above sugars, all mono- and oligo-saccharides used here were pure on TLC analysis, and polysaccharides did not contain any detectable oligomers.

Hydrolysis of Polysaccharides——Inulin (0.5 g, Merck) was dissolved in 10 ml of hot distilled water. The solution was cooled to 50°, added with 0.5 ml of 2 N HCl, and incubated at 50° for 15 min. Dextran 40 (0.5 g) or amylose (0.5 g) was partially hydrolyzed in 10 ml of 0.4 N HCl at 100° for 50 min or 20 min, respectively. These solutions were neutralized with 1 M Na_2CO_3 and concentrated under a reduced pressure. Cellulose (5 g) was kneaded with a mixture of H_2O (3.5 ml) and concentrated H_2SO_4 (7.0 ml), and allowed to stand at room temperature for 2 days. The sticky solxtion was diluted with 100 ml of H_2O, neutralized with 10% NaOH, filtered through a filter paper, concentrated to *ca.* 50 ml, added with 50 ml of MeOH, filtered to remove Na_2SO_4, concentrated again to a syrupy solution, and diluted to 15 ml with H_2O. All portions of the hydrolyzate of inulin, dextran, and amylose or 2 ml of cellulose hydrolyzate were subjected to Sephadex G-15 gel chromatography.

Chemical Determination of Sugars——Quantitative determination was made with phenol-sulfuric acid method,[13] and number of sugar units was tested if necessary in the following way: Samples were spotted on pre-coated TLC plates of silica gel 60 (Merck), developed with a mixture of propanol–H_2O (85: 15, v/v) and sugars were detected with *p*-anisaldehyde–sulfuric acid reagent.[14]

Utilization of Sugars by Microorganisms——The assay media (as double strength) contained per liter, for *B. infantis* and *B. breve*, 16 g of Bacto casamino acids (Difco), 10 g of Bacto yeast extract (Difco), 6.4 g of polypeptone (Daigo Eiyo Kagaku Co., Osaka), 0.8 g of cysteine, 3.0 g of NaCl, and 2.0 g of Tween 80 (adjusted to pH 6.8 with NaOH); for *E. coli*, 27.2 g of KH_2PO_4, 4.0 g of $(NH_4)_2SO_4$, 0.4 g of $MgSO_4 \cdot 7H_2O$, and 1.0 mg of $FeSO_4 \cdot 7H_2O$ (adjusted to pH 7.4, CR medium[15] without carbon source); for *L. acidophilus*, 10 g of trypticase peptone (BBL), 1.0 g of Na_2HPO_4, Bacto yeast extract (Difco), 2.0 g of Tween 80, beef extract (Kyokuto Seiyaku Kogyo Co., Tokyo), and 0.8 g of cysteine (adjusted to pH 7.0); and for *S. faecalis*, 10 g of trypticase peptone (BBL), 7.0 g of Na_2HPO_4 and 5.0 g of NaH_2PO_4.

The aqueous sugar solution and the assay medium were autoclaved separately. The mixture of equal volumes of them was inoculated with 10^4 to 10^5 cells of each bacterium. *B. infantis* and *B. breve* were cultured at 37° for 4 days in N_2–CO_2 (9: 1), because they grow in ileum and colon under anaerobic conditions. Other bacteria were cultured at 37° for 2 days in air, because they are considered to consume oxygen to bring intestinal cavity anaerobic. The culture medium was well mixed and the increase in absorbance at 650 nm (light path: 1 cm) was measured as an indication of growth response to the sugars. Changes in absorbance of four bacteria in relation to glucose concentrations are shown in Fig. 1.

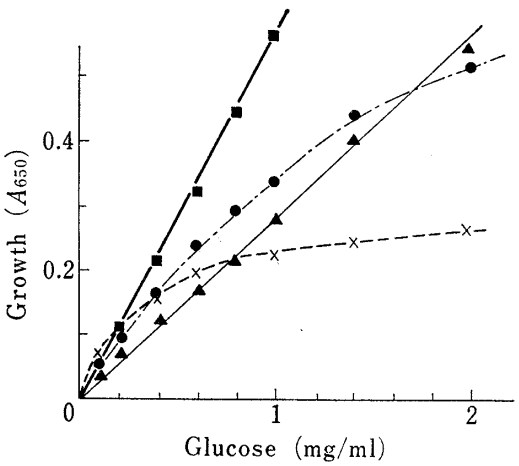

Fig. 1. Growth Response of *B. infantis*, *E. coli*, *L. acidophilus*, *and S. faecalis* to Glucose

—■—: *B. infantis*; ---×---: *E. coli*;
—▲—: *L. acidophilus*; —●—: *S. faecalis*.

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Results

B. infantis utilized maltotriose, raffinose (tri-), stachyose (tetra-), and inulin (polysaccharide), but did not utilize melezitose (trisaccharide), cycloheptaamylose and all polysaccharides other than inulin, as shown in Table I. It is certain that inulin polymer was utilized, because non-enzymic hydrolysis of inulin in the medium was not observed.

Table I. Utilization of Oligo- and Polysaccharides by *B. infantis*[a]

Remarkable[b]	Negligible[b]
Maltotriose, Raffinose[c]	Melezitose[c]
Stachyose	Cycloheptaamylose
Inulin[c]	Amylose (MW=4000)
	Soluble starch[c]
	Dextran 40, Pullulan
	Cellulose, Mannan, Xylan
	Pectin, Gum arabic
	Heparin sodium
	Chondroitin sulfate

a) Sugar concentrations were *ca.* 4 mg/ml.
b) Remarkable: absorbance more than 0.10. Negligible: absorbance less than 0.05.
c) As already reported by Dehnert.[12]

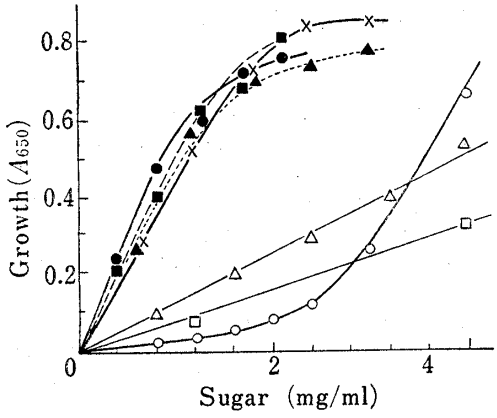

Fig. 2. Growth Response of *B. infantis* to Sugars

Sugar concentration is expressed as the weight of constituent monosaccharides. For example, lactulose (342 mg) in constituted by two monosaccharides (galactose, 180 mg and fructose, 180 mg), and 1 mg/ml means that 0.95 mg ($1\times342\div(180\times2)$) of lactulose is contained per ml. Inulin concentration is shown by weight of inulin itself.
—×—: galactose; ---▲---: glucose; —■—: lactose; —●—: lactulose; —△—: inulin (Merck); —□—: inulin (Difco); —○—: fructose.

Table II. Growth Response of *B. infantis* to Some Sugars[a]

Sugar added[b] (mg of constituent monosaccharides)		Wet weight of cells (mg) obtained after culture
G	150	131
Lactose	150	127
Sucrose	150	135
Raffinose	150	140
G+F	50+50	68
Ga+G+F	50+50+50	91
Inulin (Merck)	500[c]	93

a) The cells were cultured for 4 days in 100 ml of medium added with each sugar.
b) G: glucose, Ga: galactose, F: fructose.
c) Weight of inulin itself.

Lactose, lactulose, sucrose, and raffinose, which are constituted from fructose, galactose, and/or glucose, showed almost equal growth activities per monosaccharide molecule to galactose and glucose, while utilization of fructose itself was very poor at low concentrations (Fig. 2 and Table II). Merck inulin showed *ca.* 20% of growth activity of lactose, and Difco and Wako inulin did *ca.* 14%. Fractionation of inulin (Merck) demonstrated that inulin of molecular weight lower than 4500 was utilizable (Fig. 3).

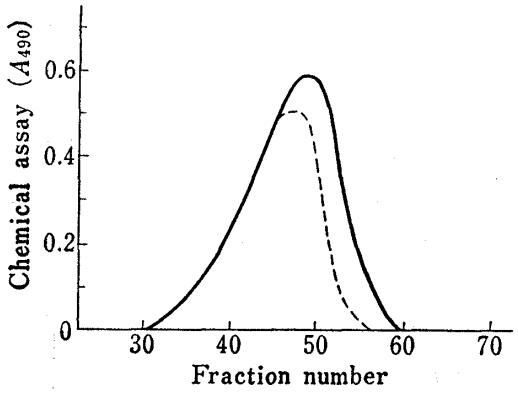

Fig. 3. Gel Permeation Chromatograms of Inulin and Its Fermented Residue by *B. infantis*

Column: Sephadex G-50 fine, 16 mm×100 cm.
Eluent: 0.02 M phosphate buffer, pH 6.8.
Sample: commercial inulin (Merck, ——) 50 mg; its fermneted residue by *B. infantis* (------).
Chemical assay: 50 μl of fractions (2.4 ml each) to phenolsulfuric acid method.
Calibration: blue dextran=fraction 24; amylose (MW≒4000)=fraction 54; heptaamylose (MW=1152)=fraction 63.

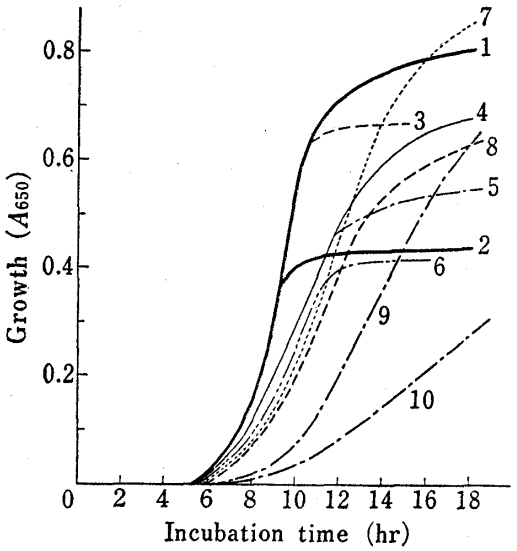

Fig. 4. Time Course of Growth of *B. infantis* with Some Sugars

Cells used were precultured with lactose except in the case of fructose, which was precultured with fructose.
1, stachyose 0.2%; 2, stachyose 0.1%; 3, raffinose 0.2%; 4, glucose 0.2%; 5, inulin (Merck) 1.0%; 6, isomaltose 0.18%; 7, maltotriose 0.2%; 8, lactose 0.2%; 9, fructose 0.2%; 10, fructose 0.1%.

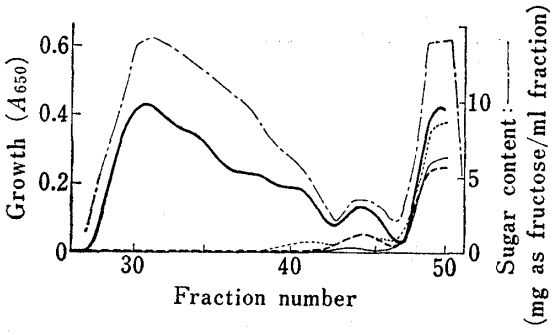

Fig. 5. Growth Response of *B. infantis*, *E. coli*, *L. acidophilus*, *and S. faecalis* to Fractions of Inulin Hydrolyzate through Sephadex G-15 Column

Column: Sephadex G-15 fine, 16 mm×100 cm.
Eluent: 0.02 M phosphate buffer, pH 6.8.
Chemical assay: 5.0 μl of fraction (2.4 ml each) was used.
Bioassay: 500 μl of fraction was used.
——, growth of *B. infantis*; – – – –, growth of *E. coli*; ——, growth of *L. acidophilus*; ------, growth of *S. faecalis*
Calibration: blue dextran=fraction 29; maltotriose=fraction 40; fructose=fraction 49.

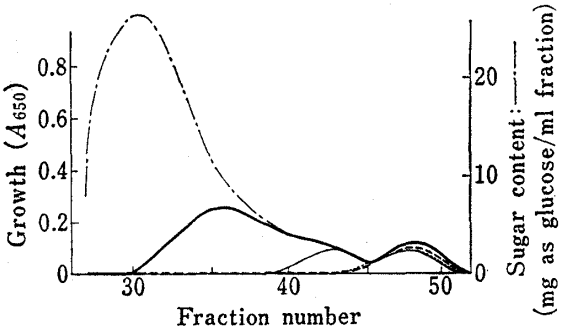

Fig. 6. Growth Response of *B. infantis*, *E. coli*, *L. acidophilus*, *and S. faecalis* to Fractions of Dextran Hydrolyzate through Sephadex G-15 Column

Condition of chromatography was the same as in Fig. 5.
——, growth of *B. infantis*; – – – –, growth of *E. coli*; ——, growth of *L. acidophilus*; ------, growth of *S. faecalis*.

Doubling time of *B. infantis* (Fig. 4) was calculated to be 1.0 hr with raffinose and stachyose, and 1.1—1.3 hr with glucose, lactose, isomaltose, and inulin, and was not affected by concentration of each sugar. These results indicated the suitability of these sugars for growth of bifidobacteria. Among the sugars utilized by *B. infantis*, raffinose, stachyose, and inulin were not utilized by *E. coli*, *L. acidophilus*, and *S. faecalis*. As for hydrolyzates of polysaccharides, *E. coli* and *L. acidophilus* utilized mono- and di-saccharides of inulin hydrolyzate, and *S. faecalis* mono- to tri-saccharides (Fig. 5). *B. infantis* utilized mono- to penta-

saccharides of dextran hydrolyzate, *E. coli* and *S. faecalis* monosaccharide only, and *L. acidophilus* mono- to di-saccharides (Fig. 6). Thus inulin of lower molecular weight, oligosaccharides more than trisaccharide of inulin hydrolyzate and tri- to penta-saccharides of dextran hydrolyzate were found to be specifically utilized by *B. infantis*.

On the contrary, oligosaccharides from amylose and cellulose were not specific for bifidobacteria. *B. infantis* utilized mono- to tetra-saccharides of amylose hydrolyzate, but *E. coli* and *S. faecalis* mono- to hexa-saccharides and *L. acidophilus* mono- and di-saccharides (Fig. 7). *B. breve* utilized di- to tetra-saccharides of cellulose hydrolyzate, and *E. coli* monosaccharide only, but *L. acidophilus* mono- to tri-saccharides and *S. faecalis* mono- to tetra-saccharides (Fig. 8).

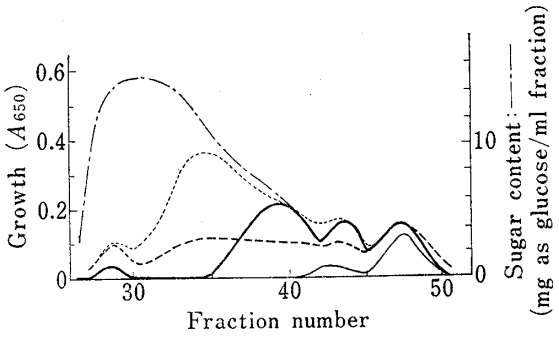

Fig. 7. Growth Response of *B. infantis*, *E. coli*, *L. acidophilus*, and *S. faecalis* to Fractions of Amylose Hydrolyzate through Sephadex G-15 Column

Condition of chromatography was the same as in Fig. 5.
—, growth of *B. infantis*; – – –, growth of *E. coli*;
——, growth of *L. acidophilus*; ------, growth of *S. faecalis*.

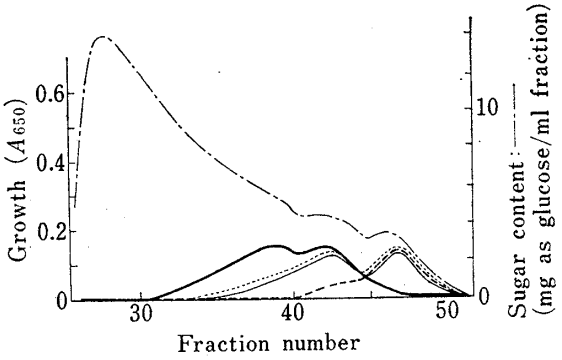

Fig. 8. Growth Response of *B. breve*, *E. coli*. *L. acidophilus*, and *S. faecalis* to Fractions of Cellulose Hydrolyzate through Sephadex G-15 Column

Condition of chromatography was the same as in Fig. 5.
—, growth of *B. breve*; – – –, growth of *E. coli*;
——, growth of *L. acidophilus*; ------, growth of *S. faecalis*.

Discussion

In the present work, we tried to find sugars which are utilized well by bifidobacteria and not by other intestinal bacteria. Glucose, galactose, fructose, maltose, lactose, sucrose, and lactulose[11,16] are known to be nonspecific, and oligosaccharides from amylose and cellulose were also found nonspecific for bifidobacteria. On the contrary, raffinose, stachyose, inulin of molecular weight less than 4500, oligosaccharides more than trisaccharide of inulin hydrolyzate, and tri- to penta-saccharides from dextran were found to be specifically utilized by bifidobacteria.

All species of bifidobacteria of human origin except *B. bifidum* utilize raffinose[17] and perhaps stachyose. Though raffinose was reported to be utilized by some intestinal strains of *E. coli* and stachyose has a possibility to be utilized by them, we think these sugars have higher specificity for bifidobacteria than lactulose, as Yoshioka suggested.[11]

It is known that molecular weight of inulin varies over a wide range,[18] although usually used as a homogenous polysaccharide to measure glomerular filtration ratio, or to test biological properties of bacteria. We also confirmed variation in molecular weight and inulin of lower molecular weight was found utilizable by *B. infantis*. Most strains of *B. infantis* and many strains of *B. adolescentis* utilize inulin without delay, and most strains of *B. breve* do after several tens of hours for adaptation. Though inulin is hydrolyzed by inulase produced

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by fungi,[19] rumen bacteria,[20] and *Saccharomyces fragilis*,[21] these bacteria are considered to live in human stomach and intestines in negligible numbers. Therefore, inulin of lower molecular weight seems to be very specific, and oligosaccharides from inulin seem to be at least as specific as raffinose and stachyose.

Hydrolysis and absorption by man are very important factor for sugars to reach the ileum and colon where bifidobacteria mainly live. Starch and its hydrolyzates are also unsuitable in this sense. Taeufel *et al.*[22] showed using rats that oligosaccharides from dextran are digested, lactose is hydrolyzed slowly, lactulose negligibly, and raffinose, stachyose, and verbascose not at all. They also showed that the amount of oligosaccharides absorbed by intestines without prior enzymic hydrolysis is considered to be less than a few percent. In man disaccharide from dextran was reported to be hydrolyzed by a mucosal enzyme.[23] Therefore, oligosaccharides from dextran seem unsuitable for our purpose, while it is not known whether other oligosaccharides from dextran are digested by man or not.

In conclusion, it is probable that bifidobacteria would increase in human intestines and become predominant by oral administration of raffinose, stachyose, inulin of lower molecular weight, or oligosaccharides more than trisaccharide of inulin hydrolyzate.

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