Microbiological Studies on Growth Factor for *L. bifidus* var. *pennsylvanicus*.* (21988)

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For *L. bifidus* var. *pennsylvanicus*(1) human milk furnishes a specific and essential growth factor (factors), named in short the "bifidus factor" (BF). In addition, this particular variant of *L. bifidus* is able to utilize only preformed pantethine but not pantothenic acid, even when the latter is offered in great excess. The growth of *L. bifidus* var. *pennsylvanicus* is further enhanced by a supplementary factor, present in human milk, cow's milk, insulin and pancreatic extract. For some mutants (or variants) this supplementary factor may act as an essential nutrient. The chemical characteristics and distribution of the supplementary factor appear to be closely related to those of strepogenin. The supplementary factor is different from the specific "bifidus factor"(2). Chemically the bifidus factor as it occurs in human milk belongs in the group of N-containing carbohydrates. In human milk the presence of a great variety of oligo- and polysaccharides has been demonstrated(3). Their total quantity in fresh human milk may be estimated to be around 0.4% which is by no means a negligible amount with respect to total solids. The bifidus factor in human milk is present in both low molecular dialyzable and high molecular, non-dialyzable form(4). The bifidus factor may be adsorbed from deproteinized human milk on charcoal and eluted by acetic acid(3). Such milk eluates contain also dialyzable and undialyzable fractions(4). The carbohydrates of human milk as obtained in the acetic acid eluates from charcoal adsorbates are composed of glucose, galactose, fucose and N-acetyl-D-glucosamine, in various combinations(3). Kuhn and his associates have found in human milk one non-N-containing triose (fucosido-lactose) and a group of N-containing oligosaccharides, consisting of a tetraose, 2 pentaoses and one hexaose. They have also determined the composition and exact structure of several of these oligosaccharides and of some of their breakdown products, such as of 2 bioses and 2 trioses obtained from the tetraose(5-10). In addition to the neutral oligo- and polysaccharides, human milk also contains acidic oligo- and polysaccharides, which occur again in dialyzable, low molecular and non-dialyzable, high molecular form. The acidic constituent of these saccharides has been isolated, chemically identified and tentatively named gynaminic acid(11). It is probably N-acetyl-neuraminic acid(11-13). Upon hydrolysis the oligo- and polysaccharides containing gynaminic acid yielded glucose, galactose, fucose and N-acetyl-D-glucosamine, in addition to gynaminic acid(11). The common link in all these N-containing oligo- and polysaccharides is the presence of N-acetyl-D-glucosamine. This is equally characteristic for all microbiologically active compounds obtained from other natural sources(14). As a matter of fact, N-acetyl-D-glucosamine, as well as N-acetyl-D-galactosamine and even inorganic ammonium salts have been found, in high concentration, active, probably as precursors of the bifidus factor(14). All chemically well defined oligosaccharides of human milk,† various disaccharides obtained from different sources and also through enzymatic or chemical synthesis, and finally a series of synthetic derivatives of N-acetyl-D-glucosamine have been tested microbiologically.

The present report summarizes the results of these studies.

Methods. The microbiological assays were carried out as previously described(1), with the semisynthetic basal medium(15,16) containing an enzymatic digest of casein ("NZ case peptone," Sheffield). The supplements were used in varying concentration and were

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† Most compounds from human milk, as well as from other sources, and a large number of synthetic compounds were kindly furnished by Professor Richard Kuhn, Heidelberg.

TABLE I. Saccharides Isolated from Human Milk.

No.	Rf (lactose)	Name	Composition	Ref.	Microbiological activity, mg/unit Autoclaved	Sterile
V	1.0	Lactose			Inactive	Inactive
IV*	.73	N-free triose	Fucose | Glucose>galactose	10		Inactive
IIIc*	.36	Lacto-N-tetraose	Glucose>galactose>N-acetyl-D-glucosamine-galactose	3, 5, 6	2.4	1.7
	1.49	Lacto-N-biose I	N-acetyl-D-glucosamine-galactose (3-0-β-D-galactopyranosyl-N-acetyl-glucosamine)	6, 8	Inactive	1.5
	1.12	Lacto-N-biose II	Galactose>N-acetyl-D-glucosamine	6, 7	.6	.2
	.51	Lacto-N-triose I	Galactose>N-acetyl-D-glucosamine-galactose	6, 7	1.2	.75
	.70	Lacto-N-triose II	Glucose>galactose>N-acetyl-D-glucosamine	6, 7	.2	.2
IIIb*	.27	Lacto-N-fuco-pentaose II	Fucose | Glucose>galactose>N-acetyl-D-glucosamine>galactose	9	.75	.26
IIIa*	.19	Lacto-N-fuco-pentaose I	Fucose | Glucose>galactose>N-acetyl-D-glucosamine>galactose	9	.3	.25
IIc*	.11	Lacto-N-difuco-hexaose	Fucose, Fucose | Glucose>galactose>N-acetyl-D-glucosamine>galactose	9	1.1	.38
	Rf (gynaminic acid)					
II	.58	Acid saccharide II	Gynaminic acid; galactose; glucose; N-acetyl-D-glucosamine; fucose	11	.4	.4
I	.15	*Idem* I	Similar to II but higher in mol. wt	11	.15	.15

* *Cf.* Ref. 3,

TABLE II. Disaccharides of N-acetyl-D-glucosamine from Other Sources than Human Milk.

Name of compound	Origin	Ref.	Microbiological activity, mg/unit Autoclaved	Sterile
4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine	From hog stomach mucin	17	.08–.1	.06
	Enzymatically synthesized	18, 19, 20	.08–.1	.06
	From meconium	21	.08–.1	.06
	Chemically synthesized	22	.08–.1	.06
3-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine	Enzymatically synthesized	23	Inactive	1.5
6-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine	Chemically synthesized	24	1.7	.95
	Enzymatically synthesized	18, 20	8	.90
6-0-β-D-(2′-deoxy-2′-acetamino)-glucopyranosyl-D-galactose	Chemically synthesized	25	.17	.17
6-0-β-D-(2′-deoxy-2′-acetamino)-glucopyranosyl-D-glucose	*Idem*	25	.10	.07
N-N′-diacetyl-chitobiose	From chitin	26	.8–1.8	1.2–2.2

either autoclaved with the medium or were added to it sterile (Seitz-filtered). The microbiological activity was expressed in mg per unit of the compound tested, related to the reference standard of skimmed human milk (1).

Results. Table I summarizes the results of the microbiological assays carried out with chemically well characterized compounds isolated from human milk. The Rf-values for the neutral oligosaccharides are based on that of lactose as unity(3), those for the acid saccharides are related to that of gynaminic acid (11). As expected, lactose and the N-free triaose (fucosido-lactose) were microbiologically inactive. Great variation of activity was found for all N-containing oligosaccharides tested. Among the neutral oligosaccharides only lacto-N-triose II and lacto-N-fuco-pentaose I have shown high activity, regardless whether they were autoclaved with the medium or added to it sterile. High activity was also found for the acid saccharides, both in autoclaved and in sterile form. A few other compounds were highly active when used Seitz-filtered, but showed greatly reduced activity after autoclaving with the medium. It deserves special emphasis that 3-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine, which is widely distributed in the N-containing oligosaccharides of human milk(6,8) exhibited no activity when autoclaved with the medium, and only very slight activity as sterile supplement. In contrast (Table II), 4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine showed the highest microbiological activity of all the naturally occurring derivatives of N-acetyl-D-glucosamine. This disaccharide, originally isolated from hog mucin (17), is present in human milk in only low concentration, especially when compared with 3-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine. It has been obtained also from meconium(21), and through enzymatic(18,19) and chemical(22) synthesis, always with identical microbiological activity. In Table II several other disaccharides of N-acetyl-D-glucosamine are listed, some of them showing good microbiological potency.

In addition to the N-containing oligosaccharides, several synthetic derivatives of glucosamine were tested for microbiological activity (Table III). The α-anomers of the alkyl glyosides were found to be inactive. Unexpectedly very high activity was encountered with the β-alkyl compounds, in particular with the ethyl- and n-propyl-homologues. Even the benzyl and phenyl derivatives were strongly active. The activity of methyl-N-acetyl-β-D-glucosaminide was greatly enhanced(28) by the addition of the α-anomer and brought to the level of the pure ethyl- and n-propyl-compounds. On the basis of molecular weight the activity of these alkyl-derivatives of N-acetyl-D-glucosamine was comparable with that of 4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine. In this connection it may be added that in the course of

TABLE III. Synthetic Derivatives of N-Acetyl-D-Glucosamine.

Name of compound	Ref.	Microbiological activity, mg/unit Autoclaved	Sterile
Methyl-N-acetyl-α-D-g*	27,28,29	Inact.	Inact.
Ethyl-N-acetyl-α-D-g	29	"	"
n-Propyl-N-acetyl-α-D-g	29	"	"
Benzyl-N-acetyl-α-D-g	†	1.5	—
Phenyl-N-acetyl-α-D-g	‡	Inact.	—
Methyl-N-acetyl-β-D-g	28,29,30,33	.16-.2	—
Ethyl-N-acetyl-β-D-g	29,30	.04-.06	.05
n-Propyl-N-acetyl-β-D-g	29,30	.04-.06	.06
Isopropyl-N-acetyl-β-D-g	†	.100	.09
n-Butyl-N-acetyl-β-D-g	30†	.065	
Benzyl-N-acetyl-β-D-g	30†	.095	
Phenyl-N-acetyl-β-D-g	†	.05-.07	.05
N-formyl-D-g	†	2.5	
Methyl-N-formyl-β-D-g	33†	2.5	
Aniline-N-acetyl-D-g	†	Inact.	
p-Anisidine-N-acetyl-D-g	†	"	
p-Aminobenzoic acid ethyl ester N-acetyl-D-g	†	.7	

* g = glucosamine.
† Kindly furnished by Dr. R. Kuhn, Heidelberg.
‡ " " " Dr. S. Roseman, Ann Arbor.

the last 2 years the sensitivity of the strain of *L. bifidus* var. *pennsylvanicus* to pure N-acetyl-D-glucosamine autoclaved with the medium has shown a distinct, gradual deterioration from 2.3 mg per unit to 4.5-7.0 mg. No such reduction in sensitivity was noticed for the bifidus factor, as it is present in human milk, the active disaccharides, and the alkyl-derivatives of N-acetyl-D-glucosamine.

Discussion. In reviewing the results of the microbiological tests reported it is apparent that the various oligo- and polysaccharides and other derivatives of glucosamine have shown considerable variation in their activity as bifidus factor. The most potent compound of natural origin was the disaccharide 4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine. However, considering the relatively low concentration of this compound in human milk (5,6), it is difficult to assign to it the role of *the* specific bifidus factor. Further, the specificity of this disaccharide as bifidus factor is at variance with the fact that alkyl-glycosides of N-acetyl-D-glucosamine, especially the ethyl- and n-propyl derivatives show approximately the same high activity. Chemically the alkyl-glycosides and the disaccharide are not strictly comparable.

It is of additional interest that in previous studies crude eluates from charcoal adsorbates of skimmed human milk or fractions obtained by alcohol precipitation of deproteinized milk (3,31) have often been found to have very high microbiological activity (about 250 γ per unit), comparable to the activity of above named simple derivatives of N-acetyl-D-glucosamine. Purified blood group substances with an activity of ca. 150 γ per unit were obtained from ovarian cyst fluid (32).

In the light of all these microbiological findings it is not possible yet to identify the bifidus factor with *one* specific compound.

Summary. 1. The results of microbiological assays on a large number of oligosaccharides obtained from human milk and on simple derivatives (disaccharides or simple synthetic compounds) of N-acetyl-D-glucosamine are reported. The high microbiological activity of 4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine and of ethyl- and n-propyl-N-acetyl-β-D-glucosamine has not been exceeded by any other compound tested. 2. The "true" specific bifidus factor has either still to be found or several related compounds may act as precursors or in synergistic combination.

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Effects of β-Sitosterol and Ferric Chloride On Accumulation of Cholesterol in Mouse Liver.* (21989)

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Since repeated experimental evidence has associated high cholesterol levels with atherosclerosis, various attempts have been made to lower blood and organ cholesterol content. The effects of several substances on cholesterol absorption have been studied using various experimental animals. Soybean sterols, consisting mainly of β-sitosterol, have been shown to decrease the accumulation of dietary cholesterol(1-5). Studies on dihydrocholesterol treated animals have shown that this substance is also effective in reducing cholesterol absorption(6-8). Moreover, it has been found that, in the cockerel, precipitation of bile acids by ferric chloride results in a reduction of blood cholesterol(9,10). On the other hand, in the case of the mouse and rat, evidence has been produced that soybean sterols fail to lower organ and blood cholesterol(11,12).

The object of this experiment is to study: first, the effectiveness of β-sitosterol in prevention of liver cholesterol accumulation; and second, the effectiveness of ferric chloride in a mammalian species.

Methods. Exp. I. Dietary β-sitosterol. Webster strain female albino mice weighing 15 to 20 g were used. Throughout the experiment all mice were fed *ad lib.* a basal fat-free, cholesterol-free diet, described in a previous paper(7), supplemented by adequate amounts of vit. A and D. After a 2-week preliminary period on this basal diet, the mice were divided into 7 groups. The controls, Group I, were continued on the basal diet. For the other 6 groups the basal diet was supplemented with 1% cholesterol. Cholic acid was also added to the various diets as follows: Groups II and V received 0.25%; Groups III and VI, 0.5%; and Groups IV and VII, 1%. In addition, diets for Groups V, VI, and VII contained

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