Reaction of Enzymes of *Lactobacillus bifidus* var. *pennsylvanicus* with Bifidus Factor: Effect of Monosaccharides. (22662)

CATHARINE S. ROSE AND PAUL GYÖRGY.
Nutritional Laboratory of the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia.

Activity as an essential growth factor for *L. bifidus* var. *pennsylvanicus*(1) is exhibited by a number of substances of high or low molecular weight, but all containing N-acetyl-D-glucosamine. Among the low molecular weight compounds with comparably high microbiological activity are such different types as the alkyl-N-acetyl-β-D-glucosaminides and 4-O-β-galactopyranosyl - N - acetyl - D-glucosamine; active large molecules include the mucopolysaccharides of human milk and the blood group substances. Free N-acetyl-D-glucosamine itself has some growth-promoting effect but is of the order of 1 to 2% as active as the bound forms. A cell-free extract from *L. bifidus* var. *pennsylvanicus*(2) inactivates the various forms of bifidus factor with release of N-acetyl-D-glucosamine and the other constituent monosaccharides. Watkins and Morgan(3) found that the action of an enzyme preparation from *Trichomonas foetus* on blood group substances was inhibited by the presence of certain simple saccharides acting, presumably, in competition with corresponding structures in the blood group substances. These experiments suggested that further information about the essential structure and mode of action of the "bifidus factor" might be obtained by studying the effect of simple sugars, particularly those which are components of the bifidus factor, on the activity of the enzyme preparation from *L. bifidus* var. *pennsylvanicus*. In other experiments the direct effect of these sugars on the growth of *L. bifidus* var. *pennsylvanicus* was tested. A number of sugars, notably D-galactose, N-acetyl-D-glucosamine and L-fucose were found to inhibit the enzymatic decomposition of the mucopolysaccharides from human milk and hog gastric mucin. Most striking was the very marked effect of fucose with the bifidus factor of human milk. An inhibitory effect of fucose could also be observed *in vivo*. It markedly inhibited the growth of *L. bifidus* var. *pennsylvanicus* when the source of the bifidus factor was human milk but not when other forms of bifidus factor were used.

Materials and methods. Most of the enzymatic studies and *in vivo* microbiological tests were made with the bifidus factor of human milk and hog gastric mucin. In addition to skimmed milk itself, a deproteinized non-dialyzable fraction and a slowly-dialyzable fraction were tested. Hog gastric mucin was suspended in water and centrifuged to remove any insoluble portion. No purified fractions were used. For comparison with these high molecular weight forms of bifidus factor N-acetyl-D-glucosamine, 4-O-β-galactopyranosyl-N-acetyl - D-glucosamine and ethyl - N-acetyl-β-D-glucosaminide were used. The ethyl glycoside was present as approximately 20% of an α-β mixture. The α-form is inert in the test. The preparation of the cell-free extract of *L. bifidus* var. *pennsylvanicus* has been described(2). The lyophilized material contains about 20% of protein. In most of the experiments the concentration of the enzyme preparation was 2 mg per ml for milk or preparations from milk and 5 mg per ml for mucin. These concentrations gave approximately equal rates of inactivation of the two substrates. Human milk was tested in a 1 to 4 dilution, mucin and the non-dialyzable preparations from milk at 5 mg per ml and the dialyzable milk fraction at 7.5 mg per ml. Inhibitors were tested first at a level of 4%. In later experiments active substances were studied at higher and lower concentrations. All experiments were carried out at pH 6.0 in phosphate buffer. Samples were incubated at 37° under toluene. Aliquots were withdrawn at desired intervals, diluted to a concentration suitable for assay and heated 10 minutes in boiling water to inactivate the en-

TABLE I. Effect of L-Fucose and D-Galactose on Microbiological Inactivation of and Release of N-Acetylhexosamine from Bifidus Factor by Means of Bifidus Enzyme.

		Control		Fucose		Galactose	
Substrate	Hr	Activity,* % loss	N-acetyl-hexosa-mine,† %	Activity, % loss	N-acetyl-hexosa-mine, %	Activity, % loss	N-acetyl-hexosa-mine, %
Non-dialyzable fraction from human milk	1	10	30	3	19	2	10
	6	72	68	20	44	27	42
	19	89	90	53	54	64	70
	48	93	93	60	75	78	90
Hog gastric mucin	1	28	24	10	10	4	8
	6	31	50	16	34	4	29
	19	48	72	31	51	25	34
	48	89	89	73	76	64	64

* Both substrates had an initial microbiological activity of approximately 2 units/mg.
† N-acetylhexosamine values are calculated as % of total amount which is released when substrate is completely inactivated.

zymes. N-acetylhexosamine was determined by a modification of the method of Aminoff *et al.*(4). The procedure for the microbiological assay of bifidus factor has been reported(5). The basal medium containing enzymatically digested casein corresponded to that of Hassinen *et al.*(6) modified by the addition of 1 g of "Tween 80" per liter of double strength medium. The effect of sugars, particularly fucose, was tested not only with *L. bifidus* var. *pennsylvanicus*, but also with the regular strain which does not require bifidus factor(7).

Results. Galactose and fucose inhibited the enzymatic splitting of the polysaccharides of both human milk and gastric mucin. A typical experiment is shown in Table I. Both release of N-acetylhexosamine and loss of microbiological activity were delayed. In other experiments the effect was shown to be proportional to the amount of galactose or fucose present. Similar results were obtained with lactose which is hydrolyzed by the bifidus enzyme preparation. The effect of N-acetyl-D-glucosamine could not be as conveniently studied. The amounts of sugar used as inhibitor were much larger than those which would be released from the substrates so that N-acetyl-hexosamine could not be measured. Also, since N-acetyl-D-glucosamine is a growth stimulant it was necessary to remove it from the reaction mixture by dialysis before estimation of microbiological activity. The results of the experiments carried out indicated that N-acetyl-D-glucosamine did act as an inhibitor of the bifidus enzyme.

Galactose and N-acetyl-D-glucosamine had a greater inhibiting action with mucin than with human milk fractions. In many experiments in which the release of N-acetylhexosamine was followed the galactose effect with human milk was limited chiefly to the first hours of the experiment whereas with mucin it was quantitatively greater and more prolonged. With N-acetyl-D-glucosamine the rate of inactivation of milk polysaccharides was only moderately slower than that of the controls. With mucin the effect of N-acetyl-D-glucosamine seemed more persistent. Even after prolonged incubation about 30% of the initial activity was left while the control was completely inactive (Table II). Fucose, however, was most active with the milk polysaccharides and the extent of inhibition was greater than any observed effect of galactose or N-acetyl-D-glucosamine. This observation was confirmed in repeated tests. The effect appeared to depend primarily on the concentration of fucose rather than on a competitive action with the substrate. Marked inhibition of release of N-acetylhexosamine had been shown with 4% fucose and 0.5% of the non-dialyzable fraction of milk. With lower levels of fucose, 0.06 to 2%, inhibition was demonstrated with levels of substrate from 0.15 to 5%.

Of a number of other sugars tested only methyl-α-L-fucopyranoside and l-acetamino-

TABLE II. Effect of N-acetyl-D-glucosamine on Inactivation of Bifidus Factor by Bifidus Enzyme.

Substrate	Hr	Activity (%) Control	N-ac-glucosamine
Non-dialyzable fraction from human milk	1	79	88
	6	32	41
	24	17	30
	48	<5	11
	80	<5	<5
Hog gastric mucin	1	82	89
	6	62	62
	24	49	57
	48	<5	56
	80	<5	31

lactose were active. D-Glucose, L-fructose, L-rhamnose and L-arabinose were essentially without effect.

In the microbiological assay of samples from the enzyme tests it became apparent that, in some cases, the presence of L-fucose was inhibiting the growth of *L. bifidus* var. *pennsylvanicus*. When this observation was tested systematically, with varying amounts of fucose in the medium and with different forms of bifidus factor, it was found that inhibition occurred when bifidus factor was supplied as human milk or fractions from human milk, but not when other forms of bifidus factor were used (Table III). The effect was appreciable with 0.1% of fucose in the medium and increased with larger amounts of fucose. The effect was greater with suboptimal levels of bifidus factor. Fucose was equally effective whether it was autoclaved with the medium or added aseptically to previously autoclaved medium. Methyl-α-L-fucopyranoside which inhibited the bifidus enzyme had no effect *in vivo*. Up to a level of 0.5% fucose did not inhibit or retard the growth of the regular, non-milk-requiring strain of *L. bifidus*. The medium for *L. bifidus* contains lactose as carbohydrate. The organism does not grow as well with either glucose or galactose. These monosaccharides were tested for possible inhibitory effect. No inhibition was found when they were added in concentration up to 1%.

Discussion. The mucopolysaccharide of human milk, like the blood group substances, contains N-acetyl-D-hexosamine, D-galactose and L-fucose. It differs from the blood group substances in that it contains glucose and that the hexosamine is almost entirely glucosamine while all of the blood group substances have an appreciable proportion of galactosamine. Structure of a hexasaccharide unit of the milk polysaccharide has been reported by Kuhn(8) but the position of this unit in the substance as it occurs in milk is still to be worked out. The hexasaccharide contains 2 molecules of L-fucose in α-fucopyranosidic linkage. The L-fucose of the blood group substances is also present as an α-fucopyranoside, but its position in the whole oligosaccharide is not known and is probably not the same for all of the blood group substances (*cf* 9). Kuhn and Osman(10) have shown that the fucose-containing oligosaccharides of human milk have very low blood group O(H) activity not only in comparison with the blood group substance but also in comparison with simple α-fucopyranosides and L-fucopyranose.

In earlier experiments with the *L. bifidus* enzyme preparation loss of microbiological activity was known to be accompanied by increase of reducing sugar, and release of N-acetyl-D-glucosamine was revealed chromatographically. In the present experiments release of N-acetyl-D-hexosamine in a form measurable by the Morgan-Elson reagent, was followed quantitatively and was found to parallel closely the degree of microbiological inactivation with enzyme alone or in the presence of galactose or fucose. Approximately 75 γ of N-acetyl-D-glucosamine was released for each unit of activity lost. Of the 4 components of the mucopolysaccharides tested only glucose was without effect. The other sugars exhibited the type of inhibition which might be expected from the presence of an

TABLE III. Growth of *L. bifidus* var. *pennsylvanicus* with and without Fucose.

Supplement of bifidus factor	Acid production (ml of N/10) No fucose	0.15% fucose
Skimmed human milk, .06 ml	8.5	5.3
Non-dialyzable fraction from human milk, .5 mg	10.5	8.7
Dialyzable fraction from human milk, 1.5 mg	7.5	5.0
Hog gastric mucin, .5 mg	9.7	9.4

end product. Only in the case of fucose with the milk polysaccharide was there indication of a more specific effect. *In vivo* only fucose exerted an inhibitory effect. This inhibition was limited to the milk polysaccharide and could not be demonstrated with hog gastric mucin or low molecular weight forms of bifidus factor. Neither did fucose have any effect on the growth of strains of *L. bifidus* which do not require the bifidus factor. The position of fucose in the milk polysaccharide appears to give it distinctive properties with respect to activity as bifidus factor.

Summary. 1. The effect of the constituent monosaccharides on the enzymatic inactivation of high molecular forms of bifidus factor has been studied. The most marked effect was observed with fucose when it was used with human milk or purified fractions from human milk. 2. The significance of this effect of fucose on the inactivation of the bifidus factor *in vitro* was emphasized by the experiments *in vivo*. L-Fucose markedly inhibited growth of *L. bifidus* var. *pennsylvanicus* when the source of bifidus factor was human milk, but not when it was supplied as mucin or the simple N-acetyl-D-glucosamine-containing compounds.

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Chemical Inhibitors of Theiler's Virus.* (22663)

HAROLD E. PEARSON, DOROTHY L. LAGERBORG AND DONALD W. VISSER.
Departments of Public Health and Biochemistry and Nutrition, University of Southern California School of Medicine and the Laboratory Division, Los Angeles County Hospital, Los Angeles, Calif.

Certain amino acids, nucleoprotein derivatives and analogues as well as various other compounds, previously have been reported to inhibit propagation of Theiler's GD VII virus, *in vitro*(1-5). In the present study some additional chemicals have been tested. A preliminary report was given elsewhere(6).

Methods. Tissue cultures of minced brain tissue from newborn mice were made in 50 ml stoppered, Erlenmeyer flasks. Each flask contained 50-100 mg of tissue in 3 ml Simms' solution at pH 8-9 and approximately 100 intracerebral MLD_{50} of a tissue culture passage strain of Theiler's GD VII mouse encephalomyelitis virus. After incubation at 35-36°C for 2 days the pooled supernatant fluids obtained by centrifugation of the contents of 3 flasks were tested for viral content by hemagglutination of human red cells. Ordinarily titers of 1280-2560 are obtained by this procedure. The structure of the hydroxycytidine used as prepared in this laboratory has not been established with certainty. The Metuchen was kindly furnished by Dr. F. M. Berger, Wallace Laboratories, the thiosemicarbazone compounds by Dr. R. L. Thompson, Sterling-Winthrop Research Institute.

Results. Table I gives results of tests with various chemicals. Of the substituted pyrimidine nucleosides, the cytidine compounds are slightly more inhibitory than are the corresponding derivatives of uridine. Some of the naturally occurring deoxyribosyl nucleo-

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