FURTHER OBSERVATIONS ON THE METABOLIC REQUIREMENTS OF LACTOBACILLUS BIFIDUS VAR. PENNSYLVANICUS[1]

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During the past few years a large number of strains of *Lactobacillus bifidus* var. *pennsylvanicus* have been isolated in our laboratory from the stools of breast-fed and bottle-fed infants (György, 1953) and from the vaginal secretions of pregnant women (Harrison *et al.*, 1953). This specific variant showed only scant or undetectable growth in the regular semisynthetic medium. However, it could easily be propagated in the basal medium after the addition of human milk or of nitrogen containing oligo- and polysaccsacharide fractions isolated from human milk (Gauhe *et al.*, 1954), of gastric mucin (Tomarelli *et al.*, 1954), or of other sources of such nitrogen containing carbohydrates.

The original basal medium contained in addition to chemically well defined constituents, such as amino acids, purines, vitamins, and salts, an enzymatic digest of casein. When acid hydrolyzed casein was substituted for the enzymatic casein digest, growth in the presence of human milk was much reduced. Furthermore, such sources of the "bifidus factor" as purified fractions from human milk and gastric mucin, highly active as supplements to the original medium, were not able to support growth of strains of *L. bifidus* var. *pennsylvanicus* in the medium containing acid hydrolyzate of casein.

The present report deals with the analysis of these observations and with the continued study of the metabolic requirements of *L. bifidus* var. *pennsylvanicus* under various experimental conditions. Particular attention was paid to the behavior of substrains within the specific group of *L. bifidus* var. *pennsylvanicus* as well as to the response of the variant to supplements autoclaved with the medium or added to it aseptically.

MATERIALS AND METHODS

Microbiological assays were carried out as previously described (György *et al.*, 1954). The basal medium (Norris *et al.*, 1950; Hassinen *et al.*, 1951) was used either with an enzymatic digest of casein ("NZ case peptone", Sheffield) or with an acid hydrolyzed casein (G.B.I., "vitamin-free") in equal concentration of 2.5 per cent. The original medium contained 400 μg of calcium pantothenate per liter. This was omitted in later experiments. The pantethine content of the medium containing enzymatically digested casein was adequate for maximum growth of the organisms; the medium containing acid hydrolyzed casein was supplemented with 50 μg of pantethine per liter.

Growth of the organisms was estimated by titrimetric assay of the acid produced. The specific growth promoting activity for *L. bifidus* var. *pennsylvanicus* was expressed in microbiological units related to a particular sample of human milk as reference standard.

Two strains of *L. bifidus* var. *pennsylvanicus* were studied: the original strain (212A) and one of its mutants (5S). Each of these was representative of several other strains. A strain (105C) of *L. bifidus* which did not require the specific "bifidus factor" essential for the growth of *L. bifidus* var. *pennsylvanicus* and a strain (Jackson, B_6) of *L. parabifidus* (Norris *et al.*, 1950; Williams *et al.*, 1953) were included in the study for comparison. In a previous publication (György *et al.*, 1954) strains of *L. bifidus* which require the "bifidus factor" were given the variety name Penn; however, the variety name pennsylvanicus is better from a nomenclatural standpoint.

The supplements used included skimmed human milk, skimmed cow's milk, charcoal eluates from human milk (Gauhe *et al.*, 1954), hog gastric mucin (Wilson and Bruno, 1950), 4 - O - β - galactopyranosyl - N - acetyl -D - glucosamine[2] (Tomarelli *et al.*, 1954), methyl and ethyl N-acetyl-β-D-glucosaminides,[3] N-acetyl-D-

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[2] Kindly furnished by R. M. Tomarelli, Wyeth Laboratories, Mason, Michigan.

[3] Prepared by Dr. F. Zilliken in our laboratory.

TABLE 1

The requirement of Lactobacillus bifidus var. pennsylvanicus for pantethine

Supplements to Basal Medium		Acid Production (ml of 0.1 N)	
		Medium A	Medium B
None		0.7	0
Human milk (skimmed)	0.02 ml	7.3	3.1
	0.06 ml	12.2	7.5
	0.2 ml	14.7	11.7
Charcoal eluate from human milk	0.1 mg	2.8	0.3
	0.3 mg	10.7	0.5
	1 mg	11.7	0.3
Pantethine, 0.1 μg			0.3
Pantethine, 0.1 μg + Charcoal eluate from human milk	0.1 mg		2.0
	0.3 mg		8.2
	1 mg		10.4
	3 mg		10.9

All amounts are per 10 ml tube.

Medium B differed from medium A only in containing acid hydrolyzed rather than enzymatically hydrolyzed casein. Both media contained 4 μg of calcium pantothenate per tube.

glucosamine, pantethine,[4] pancreatin (Viobin Corp.), and various fractions obtained from cow's milk by precipitation.[5]

RESULTS

The pantethine requirement of L. bifidus var. pennsylvanicus. Substitution of acid hydrolyzed casein for the enzymatic digest in the medium considerably reduced the growth promoting effect of human milk for *L. bifidus* var. *pennsylvanicus* and completely obviated the effect of purified fractions of the bifidus factor, such as eluates prepared from human milk, hog gastric mucin, N-acetyl-D-glucosamine, 4-O-β-galactopyranosyl-N-acetyl-D-glucosamine and the alkyl N-acetyl-β-D-glucosaminides. These results indicated the presence of a particular growth factor present in the enzymatic casein digest and, to a lesser extent, in human milk.

[4] Kindly furnished by Boehringer and Son, Ingelheim/Rhine, Germany, and Parke Davis, Detroit, Michigan.

[5] Prepared by G. Braun in our laboratory.

It has been shown (Tomarelli *et al.*, 1954) that various strains of *L. bifidus* respond better to pantethine than to pantothenic acid. In the light of this observation, the assumption was made that for *L. bifidus* var. *pennsylvanicus* both the specific bifidus factor and pantethine represent essential nutrients. This hypothesis was borne out by experiments in which pantethine was added in various amounts to the basal medium containing acid hydrolyzed casein. Whereas supplements of charcoal eluate of human milk did not support growth of *L. bifidus* var. *pennsylvanicus* in this medium, a good growth response was obtained when 0.1 μg of pantethine was added with the milk eluate (table 1). Pantethine in the absence of bifidus factor did not stimulate growth.

The original basal medium contained 4 μg of calcium pantothenate per tube (10 ml). Two questions arose: (a) whether the calcium pantothenate present enhanced the effect of pantethine, and (b) whether a larger amount of calcium pantothenate could replace pantethine. These questions have been put to test using a strain of *L. bifidus* var. *pennsylvanicus*, a regular strain of *L. bifidus*, and a strain of *L. parabifidus*. The results obtained are summarized in table 2. It is apparent that the strains of *L. bifidus* var. *pennsylvanicus* and of the regular *L. bifidus* tested were unable to utilize calcium pantothenate even when it was given in very large doses and required pantethine for growth. The pantethine requirement was not reduced when calcium pantothenate, up to 20 μg per tube, was present in the medium. In contrast the strain of *L. parabifidus* tested required a much larger amount of pantethine than of calcium pantothenate. Strains like *L. bifidus* var. *pennsylvanicus* 212A and regular *L. bifidus* 105C may be used for the microbiological assay of pantethine, even in the presence of free or bound pantothenic acid. In a number of food products used as supplements in our study, pantethine determinations were made with strain 105C as test organism. One-tenth μg of pantethine was found in about 0.33 ml of skimmed human milk or skimmed cow's milk, in 50 mg of hog gastric mucin, in 10 mg of "NZ case peptone", and in 12.5 mg of pancreatin. Charcoal eluates obtained from human milk contained only an insignificant trace of pantethine in 10 mg.

Requirement for supplementary factors by L. bifidus var. pennsylvanicus. In the presence of sufficient amounts of pantethine, the medium

TABLE 2

Pantethine and calcium pantothenate requirement of variants of Lactobacillus bifidus

Organism	Pantethine	Calcium Pantothenate	Ratio of Activity Pantethine/ Calcium Pantothenate
L. bifidus (strain 105C)	3 μg maximum growth 0.3 μg almost maximum growth 0.01 μg perceptible effect	1,000 μg; no effect 20 μg; no potentiating effect with pantethine	>100,000
L. bifidus var. *pennsylvanicus* (strains 212A and 5S)	0.3 μg maximum growth 0.1 μg less than maximum growth	1,000 μg; no effect 20 μg; no potentiating effect with pantethine	>10,000
L. parabifidus (strain Jackson)	10 μg maximum growth	0.2 μg maximum growth	0.02

containing acid hydrolyzed casein will support satisfactory growth of strain 212A of *L. bifidus* var. *pennsylvanicus* provided the essential microbiological growth factor is present in the medium in adequate amount. However, the growth achieved with human milk or with eluates from human milk given in graded doses is still below that observed with the same supplements when the enzymatic casein digest is used in the medium. In addition, the growth curve obtained with the milk eluates shows a distinctly lower slope in the medium with acid hydrolyzed casein than in the medium containing enzymatic casein digest. A mutant, 5S, of strain 212A shows even greater

TABLE 3

The requirement of strains of Lactobacillus bifidus var. pennsylvanicus for supplementary factors in addition to "bifidus factor" and pantethine

Supplements to Basal Medium		Acid Production (ml of 0.1 N)			
		Medium A		Medium B	
		Strain 212A	Strain 5S	Strain 212A	Strain 5S
None		1.0	1.1	0.3	0.5
Human milk (skimmed)	0.02 ml	5.8	3.1	3.4	1.3
	0.06 ml	13.6	8.5	9.1	2.6
	0.2 ml	19.2	19.0	15.2	5.0
Cow's milk (skimmed)	0.06 ml	1.9	1.4	0.6	0.6
	0.2 ml	2.3	1.4	1.0	1.1
	0.6 ml	4.9	2.2	3.1	2.3
Eluate from human milk	0.1 mg	4.9	2.0	2.3	1.3
	0.3 mg	11.8	6.0	8.0	1.8
	1.0 mg	18.3	17.0	10.3	2.4
Cow's milk, 0.2 ml +	0.3 mg			11.9	3.2
Eluate from human milk	1.0 mg			17.1	4.6

The data are expressed as amount per 10 ml tube.

Medium A contained enzymatically digested casein, medium B, acid hydrolyzed casein. Both media contained 0.5 μg pantethine per tube.

dependence on the supplementary factor. In medium containing acid hydrolyzed casein supplemented with human milk eluate there is practically no growth, and even with supplements of skimmed human milk, the acid production is much lower than in the control tubes inoculated with the original strain, 212A. Thus it appears that both strains, and particularly strain 5S, require, in addition to pantethine and the specific bifidus factor, a supplementary factor (factors) which is present in the enzymatic casein hydrolyzate and, to some extent, also in skimmed human milk but is absent from the acid hydrolyzed casein. This supplementary factor is independent from the specific bifidus factor. This conclusion is supported by the fact that human milk eluate was found to be free from the supplementary factor and, on the other hand, skimmed cow's milk containing only a negligible quantity of bifidus factor may exert a very pronounced effect as supplementary factor. The relevant data of one experiment are summarized in table 3.

The bifidus factor activity of a given substance is unspecifically enhanced in the medium containing NZ case peptone as the supplementary factor. It is of interest that the enhancing effect is not of the same magnitude for various substances. Charcoal eluate of human milk, hog gastric mucin, and the disaccharide of galactose and N-acetyl-D-glucosamine isolated from gastric mucin have shown, in general, a relatively higher activity in the medium containing acid hydrolyzate of casein than have N-acetyl-D-glucosamine and alkyl N-acetyl-β-D-glucosaminides (table 4). The former group of compounds may be regarded as more closely related to the naturally occurring form of the bifidus factor. In the same experiments it was demonstrated that autoclaving, particularly in the presence of the medium, may alter the growth promoting activity of substances for *L. bifidus* var. *pennsylvanicus*. N-Acetyl-D-glucosamine decreased markedly in activity when it was autoclaved with the medium. A smaller, but consistent, increase of activity was observed with

TABLE 4

Differences in the activity of various substances as bifidus factor, with replacement of enzymatically digested casein in the medium by acid hydrolyzed casein

Substance	Treatment	Unit		Ratio of Activity in the Two Media
		Enzymatic hydrolyzate	Acid hydrolyzate	
		mg	*mg*	
Charcoal eluate from human milk	Autoclaved with medium	0.30*	0.30*	1*
	Not autoclaved	0.30	0.36	
	Autoclaved separately	0.25	0.26	
Hog gastric mucin	Autoclaved with medium	0.40	0.36	1
	Not autoclaved	0.47	0.46	
	Autoclaved separately	0.40	0.45	
4-O-β-D-Galactopyranosyl-N-acetyl-D-glucosamine	Autoclaved with medium	0.091	0.11	1
	Not autoclaved	0.075	0.075	
	Autoclaved separately	0.097	0.090	
N-Acetyl-D-glucosamine	Autoclaved with medium	2.2	8.0	3.5
	Not autoclaved	1.2	4.3	
	Autoclaved separately	1.3	5.5	
Methyl N-acetyl-β-D-glucosaminide	Autoclaved with medium	0.15	0.37	2.5
	Not autoclaved	0.17	0.37	
	Autoclaved separately	0.15	0.33	

* By definition, 0.3 mg of the charcoal eluate represents one unit of activity as bifidus factor in each medium, and the activity of the other substances is related to this unit. As shown in previous tables the unit represents a considerably smaller production of acid in the medium containing acid hydrolyzed casein than in that containing NZ case.

4 - O - β - galactopyranosyl - N - acetyl - D -glucosamine when it was sterilized by Seitz filtration and added to the medium without autoclaving.

Both human milk and cow's milk contain the supplementary factor which enhances the growth of *L. bifidus* var. *pennsylvanicus* strain 212A in the presence of the specific bifidus factor. For assay of this supplementary factor in the milk of the two species we used the medium containing acid hydrolyzed casein, with eluate from human milk as specific source of the bifidus factor. When human milk was used as a supplement, the amount of milk eluate was decreased in order to keep the total bifidus factor activity constant. The results are summarized in table 5. As a source of the supplementary factor, human milk appears to be less active than cow's milk: The effect of the supplementary factor becomes apparent with 0.06 ml of cow's milk and with 0.2 ml of human milk.

The medium containing enzymatic digest of casein does not contain the optimal amount of supplementary factor. The addition of skimmed cow's milk may enhance the activity of the specific bifidus factor, but only when the latter substance is present in suboptimal concentration. Conversely, in the medium with acid hydrolyzate of casein the enhancing effect of cow's milk is demonstrable throughout the whole range of the concentrations used.

The activity of skimmed cow's milk as supplementary factor remained the same whether it was autoclaved with the medium, autoclaved separately, or sterilized with ethylene oxide (Wilson and Bruno, 1950) and added to the medium aseptically.

Attempts were made to fractionate and isolate the supplementary factor in milk. A preparation of crude casein showed some enhancing effect on growth at levels of 3 to 10 mg per tube. Charcoal eluate from human milk was used as bifidus factor. The effect of the casein was more apparent as the amount of milk eluate approached that required for maximum growth. Mild acid hydrolysis of casein (Kodicek and Mistry, 1952) considerably increased its effect. In contrast, complete acid hydrolysis destroyed the supplementary factor in crude casein: even 100 mg had no activity. Vitamin-free casein (Labco) was less effective than crude casein. Considerable increase in activity of the supplementary factor in casein was also achieved by tryptic digestion.

TABLE 5

Growth of Lactobacillus bifidus var. pennsylvanicus, as measured by production of acid, with human milk and cow's milk as sources of the supplementary factors.

Bifidus Factor* Units	Supplementary Factor								
	None	0.02 ml		0.06 ml		0.2 ml		0.6 ml	
		SHM	SCM	SHM	SCM	SHM	SCM	SHM	SCM
0.33	2.2	2.1	2.9						
1.3	7.7	7.9	7.4	6.9	9.5				
3.3	11.3	10.7	11.3	11.0	12.4	14.1	15.1		
13.0	12.4	10.8	12.3	12.0	14.0	14.7	15.9	17.2	17.3

SHM: skimmed human milk; SCM: skimmed cow's milk.

Acid produced is expressed as ml of 0.1 N per 10 ml tube.

The medium contained acid hydrolyzed casein and was supplemented with 0.5 μg of pantethine per tube.

* Human milk has a high level of bifidus factor (0.045 ml = 1 unit); cow's milk contains almost none. Charcoal eluate of human milk (0.3 mg = 1 unit) was added to give the desired level of bifidus factor for each amount of milk tested.

The presence of casein is not essential for the activity of milk as supplementary factor. Casein-free whey was as active as the whole milk. The precipitate obtained from whey, after half or full saturation with sodium sulfate, showed good activity as supplementary factor, even after exhaustive dialysis (4 times 24 hours against 10 volumes of distilled water). Five to 10 mg of this precipitate had just as high activity as 0.6 ml of cow's milk. Not all the supplementary factor in milk is nondialyzable. Approximately 50 per cent of the activity of both human and cow's milk may be recovered in the dialyzate after prolonged dialysis.

The distribution of the supplementary factor in milk, and in particular the activation of casein by mild acid hydrolysis or by trypic digestion, placed the supplementary factor in the category of strepogenin. This conclusion has been borne out by observations with crystalline insulin (Lilly) which, especially after mild acid hydrolysis, was found to have high activity for strain 212A. Very satisfactory enhancement was observed with 1.5 mg of insulin and even better with the same amount of mildly acid hydrolyzed insulin.

Yeast extract (Difco) and crude liver extract (Wyeth) showed only weak activity as supplementary factor, 30 mg of the dry residue being equivalent to about 0.2 ml of cow's milk. Purified liver extract (Parke Davis) and rice bran extract (Nopco) in doses up to 30 mg were inactive.

The supplementary factor activity of the medium containing enzymatic casein digest is due not only to the presence of the hydrolyzed casein but also to that of pancreatic extract. Pancreatin (Viobin) has been found highly active as a source of the supplementary factor for *L. bifidus* var. *pennsylvanicus*, 20 to 25 mg being equivalent to 0.6 ml of cow's milk. As in milk, the supplementary factor in pancreatin is present in dialyzable and in high molecular, nondialyzable form. When a solution of pancreatin, from which the small amount of protein had been removed, was dialyzed against 10 volumes of water for two twenty-four hour periods in the cold, approximately 50 per cent of the solids dialyzed out during the first twenty-four hours, 35 per cent during the second twenty-four hours while 15 per cent was nondialyzable. The nondialyzable fraction was the most active, 6 mg being equivalent to 0.6 ml of skimmed cow's milk. The corresponding values for the two dialyzates were 20 and 15 mg.

TABLE 6

The relative activities of pancreatic extract and of cow's milk as supplementary factor for strain 5S of Lactobacillus bifidus var. pennsylvanicus.

Bifidus Factor Units	Supplementary Factor		Acid Production (ml of 0.1 N)
0			0.2
3.3			1.1
3.3	Cow's milk (skimmed),	0.2 ml	2.6
3.6		0.6 ml	3.7
4.0		2.0 ml	5.2
3.3	Dialyzate of pancreatin,	5 mg	6.7
4.0		15 mg	13.3
3.0		50 mg	16.5

Supplements and acid production are expressed as amount per 10 ml tube.

The basal medium contained acid hydrolyzed casein and 0.5 μg of pantethine per tube.

Bifidus factor was supplied as a charcoal eluate of human milk. The bifidus factor in the pancreatic extract and the small amount in the cow's milk were taken into account in estimating the level of bifidus factor.

Milk, insulin, pancreatin, and various fractions prepared from these products may be used interchangeably as the supplementary factor for strain 212A. The supplementary factor acts only as an accelerator of growth and is not an essential nutrient. In contrast, for the mutant 5S of *L. bifidus* var. *pennsylvanicus*, the supplementary factor is indispensable for growth. This strain shows full response only to pancreatic extract, not to human or cow's milk or to insulin. Corresponding observations are summarized in table 6. The preparation of pancreatin used in this experiment was a dialyzate of pancreatic extract. The results obtained with milk and with pancreatic extract appear to present the conclusion that the mutant 5S responds more fully to the pancreatic extract as the supplementary factor than to skimmed cow's milk. Even with increased amounts of bifidus factor, skimmed cow's milk was unable to bring about full growth or even acceleration of growth beyond a limited level. In contrast, maximal growth was supported by pancreatic extract as the supplementary factor, when an optimal amount of bifidus factor was present. Thus, it seems plausible to assume that the mutant 5S requires more than one supplementary factor. Whereas the pancreatic extract contains all necessary supplementary factors, skimmed cow's milk must be deficient in at least one of the factors required by strain 5S.

No supplementary factor was needed by the regular strain of *L. bifidus* or for the strain of *L. parabifidus* when they were grown in medium containing acid hydrolyzate of casein.

DISCUSSION

Strains of lactobacilli preferring pantethine to pantothenic acid have been reported (McRorie *et al.*, 1950; McRorie and Williams, 1951; Craig and Snell, 1951; Tomarelli *et al.*, 1954). In general, strains with a preference for pantethine may utilize pantothenic acid when it is offered in the medium in large amounts (McRorie *et al.*, 1950; Craig and Snell, 1951). The strains of regular *L. bifidus* and of *L. bifidus* var. *pennsylvanicus*, selected for the present study, appear to be wholly dependent on pantethine and are unable to accept pantothenic acid as a substitute.

In contrast to the strains of regular *L. bifidus*

or *L. parabifidus*, the strains of *L. bifidus* var. *pennsylvanicus* that were studied required for optimal growth the presence of a supplementary factor (or factors) in addition to the specific bifidus factor. The supplementary factor was found in appreciable quantity in milk, in whey, in casein, in casein hydrolyzed enzymatically, or mildly, by acid, in insulin, and in pancreatin. For the original strain of *L. bifidus* var. *pennsylvanicus* the supplementary factor fulfills only the role of a growth accelerator. A more specific role of the supplementary factor (or factors) was exhibited by the mutant 5S of *L. bifidus* var. *pennsylvanicus*. In the absence of the supplementary factor this strain showed practically no growth even with an excess of the specific bifidus factor present. In contrast to the original strain of *L. bifidus* var. *pennsylvanicus*, human milk and cow's milk alone were unable to assure optimal growth of the mutant 5S. Full growth was achieved only with a preparation obtained from pancreatic extract, whereas the effect of cow's milk or insulin remained partial, reaching a suboptimal plateau. In consequence, it appears that this mutant requires several supplementary factors or else it will respond fully only to the specific present in pancreatic extract.

The distribution and growth promoting properties of the supplementary factor place it in the category of strepogenin (e.g., Sprince and Woolley, 1944) and, from the chemical point of view, in the class of peptides and of protein, with all the known difficulties of its further chemical characterization. The good activity of exhaustively dialyzed, high molecular material obtained by salt precipitation from whey is of special interest. It is reminiscent of recent observations by Sloane and McKee (1952*a*,*b*) on the growth promoting activity of a protein for *Staphylococcus albus* and of the report by Williams and Grady (1954) on a thermolabile protein, "lactein", from milk required for the growth of *L. bulgaricus*. In contrast to the latter, the protein-like substance required for *L. bifidus* var. *pennsylvanicus* has been found to be thermostable.

The requirement for the supplementary factor varies with the chemical nature of the bifidus factor used. For N-acetyl-D-glucosamine and for alkyl N-acetyl-β-D-glucosaminides the effect of the supplementary factor is more marked than for crude charcoal eluates prepared from human milk, hog gastric mucin, or the disaccharide obtained from hog gastric mucin. Thus, the closer the bifidus factor is chemically to the naturally occurring form, the less it is dependent for a growth promoting effect on the supplementary factor.

The supplementary factor is present both in human milk and cow's milk, but its concentration is slightly higher in cow's milk. Recently, a strain of *L. bifidus* not requiring the bifidus factor has been described (Gyllenberg *et al.*, 1953), which has shown optimal growth only in the presence of human milk or cow's milk or of various protein digests. This effect was ascribed to the presence of strepogenin. It should be noted, however, that the medium (Hassinen *et al.*, 1951) used by Gyllenberg *et al.* was deficient in many other nutrients possibly required by the organism. The effect observed, therefore, was not necessarily due to a strepogenin-like substance.

The interaction of the supplementary factor (or factors) represents an important possible source of error in assaying for the specific bifidus factor. For exact microbiological values of bifidus factor activity, it is necessary to enrich the medium with the supplementary factor as pancreatic extract or skimmed cow's milk depending upon the strain of *L. bifidus* var. *pennsylvanicus* used. For substances known to be free from supplementary factor, relative comparative values for the specific bifidus factor may be obtained by using the medium containing the enzymatic digest of casein. However, the level of the factor in this medium is below the optimum.

N-Acetyl-D-glucosamine and the disaccharide, 4 - O - β - galactopyranosyl - N - acetyl - D - glucosamine, had much less activity as bifidus factor when they were autoclaved with the culture medium than when they were added aseptically (after Seitz filtration) to autoclaved medium. In contrast, crude charcoal eluates obtained from human milk, mucin, and purified blood substances have shown no change in activity when they were autoclaved with the medium. It appears that free or open N-acetyl-D-glucosamine may interact with constituents of the basal meduim and that this reaction reduces the amount of N-acetyl-D-glucosamine available as precursor of the bifidus factor.

SUMMARY

Strains of regular *Lactobacillus bifidus* and *L. bifidus* var. *pennsylvanicus* were found to be able

to utilize only preformed pantethine but not pantothenic acid, even when the latter was offered in great excess. Such strains may be used for the microbiological assay of pantethine.

Two strains of *L. bifidus* var. *pennsylvanicus* studied required, in addition to pantethine and the specific "bifidus factor", a supplementary factor present in human milk, cow's milk, insulin, and pancreatic extract. This supplementary factor may be utilized as a high molecular, non-dialyzable compound, probably a protein. Casein was found active, but its activity was considerably increased after mild acid hydrolysis or tryptic digestion. Complete acid hydrolysis led to loss of activity. The chemical characteristics and distribution of the supplementary factor are closely related to those of strepogenin.

For a mutant (5S) of *L. bifidus* var. *pennsylvanicus* the supplementary factor proved to be essential for growth. Optimal growth of this mutant was only reached with pancreatic extract as the supplementary factor. In contrast to the original strain of *L. bifidus* var. *pennsylvanicus*, this mutant responded only partially to cow's milk, human milk, or insulin as supplementary factor.

The closer the bifidus factor is chemically to its natural form, the less it is dependent for its growth promoting effect on the supplementary factor.

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