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Biology of the Bifidobacteria

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INTRODUCTION

In much of the early literature, in retrospect, it is now apparent that many species of *Bifidobacterium* were all designated *Lactobacillus bifidus*. When the species identity of such organisms is in doubt, therefore, the term "bifidobacteria" will be used in the present paper. However, in cases where the change would significantly alter the original author's observation no change will be made in his designation. Bifidobacteria were first described in 1899 and 1900 by Tissier (166, 167) and named by him *Bacillus bifidus communis* or simply *B. bifidus*. This organism was a gram-positive, curved, and often bifid rod which was the predominant organism in the stools of breast-fed infants. In bottle-fed infants, however, gram-positive, straight, unbranched rods were the predominant organisms and were identified as *B. acidophilus*, the organism first isolated in 1900 by Moro (105) also from stools of breast-fed infants. For the next 50 years investigators were concerned with the organism in the human intestinal tract, with devising culture media for isolation and maintenance of the organism on subculture, and with the significance of the organism in the health of the newborn. It was determined that the numbers of bifidobacteria were fewer in the stools of bottle-fed infants and that, when weaned, infants harbored bifidobacteria only in small numbers. It was shown that when an adult diet was consumed, the stools of the infant shifted to the gram-negative bacillary flora of the adult.

Scientific interest in the organism was promoted by clinical observations in Europe that breast-fed infants were apt to be less susceptible to infections than bottle-fed babies and by speculation that bifidobacteria in the colon might play a role in the nurslings' resistance to infection. During this period, however, pasteurization of cow's milk was more widely employed, and measures to minimize bacterial contamination during handling and distribution of milk

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were made more effective. Artificial formulae for bottle feeding with cow's milk were also greatly improved. In economically advanced countries, therefore, the bottle-fed infant now has much less exposure to bacterial infection than does his predecessor.

Meanwhile the importance of maternal antibodies transferred to the infant by nursing in promoting passive resistance to both bacterial and viral infections was gradually appreciated.

Continuing investigation of bifidobacteria was also occasioned by scientific interest among bacteriologists in the nature and classification of this organism. Difficulties were soon experienced in culturing organisms seen in the gram-stained preparations of stools. The culture media in common use were unsatisfactory for isolation and maintenance of the organisms. For this reason uncertainty continued among investigators about whether organisms designated *L. bifidus* were identical in different laboratories and whether the organisms kept as stock strains were identical to those in nurslings' stools. Although there were dissenters, most workers classified the bifid organisms as belonging to the genus *Lactobacillus*, but the taxonomic relationship to the better studied *L. acidophilus* was not well established. The literature up to 1950 has been amply reviewed by Weiss (187), Weiss and Rettger (188), Orla-Jensen et al. (118), Orla-Jensen (117), and Olsen (114).

In 1950, Norris et al. (112) reported the composition of a culture medium which was partially defined chemically and which was satisfactory for the primary isolation and laboratory maintenance of at least some strains of bifidobacteria. Because of the suspicion that an ingredient of breast milk might be essential for the growth of some strains of bifidobacteria, György (50) and György and Rose (55) added defatted breast milk to the Norris medium. They isolated a milk-requiring strain of the organism and designated it *L. bifidus* var. *pennsylvanicus*. Since these reports, much more has been learned about the structure, cultural requirements, metabolism, and immunological characteristics of this organism. The number of known strains differing in part from one another has greatly increased. Their relationship to the lactobacilli, corynebacteria, and actinomyces, furthermore, has not been clarified to such an extent as to engender widespread acceptance.

This paper reviews the knowledge of bifidobacteria which has accumulated, especially during the past two decades. Emphasis will be placed on structure, nutritional requirements, classification, and possible ecological significance.

MORPHOLOGY

In his original description of bifidobacteria, Tissier (166) described curved rods and rods with ends split to give the characteristic Y-shape which led to the designation of "bifid." In addition, bizarre small branches and bulbous or swollen ends were seen less frequently. He and Moro (105) concurred that the organisms were different from *L. acidophilus* which was a straight rod or coccobacillus, sometimes occurring in chains, and which was more easily isolated from stools in the presence of oxygen.

Various workers generally confirmed the observations of Tissier on the structural variations of bifidobacteria and their differences from *L. acidophilus*. These included Herter (65), Blühdorn (14), and Kendall and Haner (78). Others expressed doubt that bifidobacteria and *L. acidophilus* were different organisms and believed that the latter likewise became pleomorphic and bifid when exposed to certain environmental conditions. Supporting this position were Rodella (135), Roos (136), and Stitt (159). Cruickshank (20) found that bifid forms were rare in direct examination of stools and, on culture, that the appearance of branching was often the result of overlapping of organisms to create the illusion of branching. Weiss (187) agreed with this point of view and with the conclusion that branching was rare. He also favored the view that both *L. acidophilus* and bifidobacteria produce rudimentary branching under unfavorable conditions. In 1935 Eggerth (31) described the characteristics of gram-positive rods isolated from the stools of both breast-fed and bottle-fed infants and found them to be similar to those described by Tissier.

Eggerth (31) noted an increase in branched forms in 4- to 6-day-old cultures. In some strains six to eight lateral branches, as well as compound branching, were observed. He described also cultures which became so granular that they had the appearance of a chain of close, irregular cocci and noted a decrease in metachromatic granules with age. Eggerth also described two types of bifidobacteria, group I and group II, based on certain biochemical characteristics.

By this time it was apparent that a serious problem to be solved was the inadequacy of available culture media for the primary isolation and continued maintenance of bifidobacteria. Attempts to improve media for this purpose by Weiss (187), Weiss and Rettger (189),

Blaurock (11), Boventer (15), and Tomarelli et al. (168, 169) did not accomplish the desired result. It was not until the medium of Teply and Elvehjem (165) for folic acid assay was modified, first by Tomarelli et al. (170) and then by Norris et al. (112), that bifidobacteria could be consistently isolated from stools and body secretions and could be carried indefinitely as stock cultures in the laboratory. This medium (hereafter called Norris medium) was selective, because it was inhibitory for other intestinal organisms except for enterococci, probably because of its acetate content. Its use therefore facilitated the isolation of bifidobacteria in pure culture. The aforementioned addition of defatted breast milk to this medium by György et al. (54) was also used for primary isolation. At least one strain of bifidobacteria requiring milk for its growth was isolated. The composition of the Norris medium currently in use is given in Table 1.

By using the new medium, Norris et al. (112)

TABLE 1. *Composition of Norris medium*[a]

Component	Quantities per liter
Adenine	17.4 mg
Alanine	200.0 mg
p-Aminobenzoic acid	10.0 mg
Ascorbic acid[b]	1.0 g
Asparagine	100.0 mg
Biotin	5.0 μg
Calcium pantothenate	200.0 μg
Cystine	200.0 mg
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	10.0 mg
Folic acid	10.0 μg
Guanine	12.4 mg
Lactose	35.0 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	200.0 mg
Manganese sulfate ($MnSO_4 \cdot H_2O$)	6.7 mg
Nicotinic acid	600.0 μg
N-Z case	5.0 g
Potassium phosphate, diabasic (K_2HPO_4)	2.5 g
Pyridoxine hydrochloride	1.2 mg
Riboflavine	200.0 μg
Sodium acetate, anhydrous	25.0 g
Sodium chloride	10.0 mg
Sorbitan monooleate (Tween 80)	0.5 ml
Thiamine hydrochloride	200.0 μg
Tryptophan	200.0 mg
Uracil	10.0 mg
Xanthine	10.0 mg
Human milk[c]	2%

[a] Autoclaved for 10 min at 15 lb at 121 C after adjustment to pH 6.8. For plates, add 17.4 g of agar (see references 50, 54, 111, 112, and 138).

[b] Ascorbic acid was added after autoclaving.

[c] Used to enhance growth of some strains.

found that two morphological types of gram-positive rods were in infant stools. One was a curved rod, sometimes bifid, and occasionally branched. It was microaerophilic and did not produce gas in liquid medium. The second was uniformly straight, unbranched, and often coccoid. Long chains were sometimes observed. This organism did produce copious amounts of gas and tolerated atmospheric oxygen. Because the two were frequently associated on primary cultures, the relationship of one to the other posed a problem. When supposedly pure cultures of the bifid organism were transferred in the laboratory, the straight-rod organisms occasionally appeared unexpectedly and sometimes completely replaced the bifid organisms in liquid cultures. By use of a micromanipulator, a single-cell clone of bifid organisms was established as a stock strain, the so-called Jackson single-cell 8, by Norris et al. (112). After serial transfers at 48-h intervals for several weeks, straight-rod organisms suddenly appeared in the culture on plating. Because precautions had been taken to prevent contamination, it was hypothesized that the straight rods were derived from the bifid forms by mutation. This hypothesis found support at a later date when two bifid strains, which were observed by Williams et al. (195) for a period of time, acquired an agglutinating antigen characteristic of the straight-rod strains and not found in bifids. Subsequently, these strains became aerobic, straight rods which no longer branched. These observations suggested that the surface antigen changed in advance of the morphological change to straight rods. On the basis of this work, Norris et al. (112) proposed that the naturally occurring bifidobacterium was a curved rod, often bifid, sometimes branched, and microaerophilic. It was designated *L. bifidus* (Tissier). The straight rod, unbranched strain, which was aerobic and which was thought to be derived from *L. bifidus* by mutation, was designated *L. parabifidus*. It was believed that bifid organisms described by these workers were the same as those described by Tissier, but that he and others were unable to maintain them as bifid and branched rods in stock culture because of the lack of a suitable culture medium. They also resembled those strains described by Orla-Jensen (115), Orla-Jensen et al. (118), Blaurock (13), Boventer (15), and Weiss and Rettger (189) and classified by the latter authors as *L. bifidus*, type II, or *L. parabifidus*. The proposal of Norris et al. in effect transposed the designations of Weiss and Rettger (189).

In 1955 Gyllenberg (44) studied the bifid or

branched form and the straight-rod form. He described the formation of straight rods from coccoid granules in the branched form. A cycle was described in which the organism developed into a highly branched form and into mycelial branched filaments. Some mycelial cells were observed to swell and contain intensely stained coccoid granules which, it was believed, gave rise to the straight rods. The author did not observe the conversion of short, unbranched rods to branched or bifid organisms. Although similar observations were made on several other strains, none was a single-cell isolate, and the possibility of contamination of stock strains at the time of isolation was not eliminated.

In a later study, Gyllenberg (46) noted that other reports described bifid bacteria abruptly changing from microaerophilic branched organisms to straight rods capable of aerobic growth. He suggested that there are two possible explanations: (i) the straight rods must be considered variants or mutants of the branched type, or (ii) the straight rods are contaminants of bifidobacteria cultures. Gyllenberg studied the cultural characteristics and the conditions which gave rise to nine straight-rod forms from bifid bacteria. It was discovered that the nine strains were almost identical in most of their characteristics and that they differed from the branched form in many ways. They resembled an authenticated *Corynebacterium acnes*. Investigations on the source of the *C. acnes* led the author to conclude that *C. acnes* of intestinal origin is sometimes a "latent contaminant" of bifidobacteria cultures and is capable, on repeated transfers, of overgrowing and replacing bifidobacteria in liquid cultures. The author emphasized, however, that the unbranched variants described by Norris et al. (112) were not *C. acnes*, but were probably lactobacilli.

In view of the uncertainties, Norris (unpublished data) continued the investigation of the relationship of unbranched to bifid organisms. Strain Jackson single-cell 8 has been maintained in this laboratory in Norris medium with periodic agar transfers for more than 22 years. For most of this time, characteristics of the organism have remained stable. The appearance of aerobic, gas-producing, straight rods has not occurred spontaneously. Various alterations of the physical and chemical environment have been applied to the organism without eliciting the appearance of the straight-rod form. In the case of a number of more recently isolated strains of bifidobacteria from breast-fed infants' stools, spontaneous transformation to straight rods has not been observed. Other authors, furthermore, were unable to confirm the derivation of the straight rods from bifid organisms by mutation. The preponderance of evidence at this time, therefore, favors the explanation that straight rods of the *Lactobacillus* type when found with cultures of bifidobacteria are contaminants rather than derivatives of these organisms.

Pleomorphism and Branching

Hayward et al. (63) noted that in unfavorable culture media organisms were so greatly branched that resemblance to rods was lost. Other organisms were unbranched but were swollen and irregular. As the strains became adapted to artificial culture by transfers, the branched and swollen forms became less frequent until rods predominated. During the period before satisfactory culture media were devised, highly branched and bizarre distortions of the organism were often observed. These changes usually occurred just prior to death of the culture on serial transfer and were therefore usually interpreted to be involution forms of the organisms (122). Sundman and Björksten (161) also considered bizarre swelling of the organisms as involution forms, and swelling of rods was sometimes so extreme as to resemble a bladder which measured as much as 4 μm in diameter with a crosslike structure on the surface. The bladder-like forms were not easy to detect in stained preparations, but were clearly visible in wet mounts with phase-contrast microscopy. Two types of agar were used, Norris agar and tomato agar (161), but the involution form was observed only when grown in tomato agar. The authors concluded that, although tomato agar permitted abundant growth, it appears to be nutritionally deficient. All of the strains tested produced these forms on tomato agar. The abnormalities of structure appeared after repeated subculture. Sundman and Björksten also confirmed that although bifidobacteria have a tendency to show morphological variation when grown in vitro, they are mostly rod-shaped in the natural habitat. They suggested as an explanation for this phenomenon that bifidobacteria probably have a more complicated pathway of cell wall synthesis than many organisms. As later studies indicated, there is justification for this conclusion (see Physiological and Biochemical Characteristics section). This study thus reveals the importance of defining the composition of the culture media used when studying the structure of bifidobacteria.

The first definitive study of the actual cause of pleomorphism in this organism was made by Glick et al. (41) in 1960 when they demon-

strated the formation of bizarre forms of *L. bifidus* var. *pennsylvanicus* (*B. bifidum*) resulting from a deficiency of an *N*-acetyl-amine sugar which is an essential growth requirement and a precursor for cell wall synthesis. It was found that by varying the concentration of this precursor branching could be eliminated. This observation led the authors to the conclusion that the bifid character of the organism might be a consequence of an inadequate supply of this factor.

In 1968 Kojima et al. (82) studied pleomorphism of a bifidobacterium based on the observations of Homma et al. (70) that certain carbonates induce bifid formation. They confirmed the work of Homma et al. and also noted that similar effects are obtained with sodium chloride, sodium sulfate, sodium nitrate, tribasic sodium phosphate, sodium acetate, and chlorides of other univalent cations. This effect was not observed with chlorides of Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+}. They also noted that $CaCl_2$ or $MgCl_2$ in combination with NaCl reduced the pleomorphism-inducing effect of the latter. Observations of the effect of rhamnose and polyethylene glycol, which were used to alter the osmolarity of the media, led the authors to conclude that the pleomorphic effects of a univalent cation could not be explained solely on the basis of osmotic pressure. In a set of experiments they concluded that Na^+ caused a much greater increase in cell mass than in the number of viable bacteria on culture, because the former increased by as much as 25 times, whereas the latter increased by only 1.5 times. In 1970 Kojima et al. (83) found that the calcium ion content in intact cells and in the cell wall preparations of the bifid form were significantly lower than those of the bacilloid form. They also found that the addition of chelating agents to the culture medium induced bifid formation. When $CaCl_2$ was added to such Ca^{2+}-deprived cultures, the bifid form was reversed to the bacilloid form. They found that glucose and the total amount of sugars were more abundant in the bifid form than in the bacilloid form. Methionine and phenylalanine were observed in the bifid form but were undectable in the bacilloid form. The authors concluded that although the mechanism of induction of pleomorphism is not clarified, it appears that the presence of Ca^{2+} ions plays a principal role in its prevention. In the same year Kojima et al. (84) studied electron micrographs of bifidobacteria grown in medium enriched with NaCl to induce the branched form and in calcium-enriched medium to induce the bacilloid form. Cross walls were observed in the bacilloid form but not in the bifid form. They noted that these observations lend further support to the indispensable role of Ca^{2+} ions for cytokinesis in bifidobacteria.

Husain et al. (72) recently investigated a mucoid variant of bifidobacteria which has shown a stable, straight-rod or bifid morphology over a long period of time. They observed that when this organism was grown in a defined minimal medium the organism showed profuse branching. It was observed that, by the addition of four amino acids, alanine, aspartic acid, glutamic acid, and serine, the organism could be converted to its bacilloid form. Therefore, by manipulating these amino acids, their strain of bifidobacteria could be maintained indefinitely in either a bifid or highly branched form. Previous workers (22, 176) have demonstrated, furthermore, the presence of these amino acids in the cell walls of bifidobacteria. These observations imply that these amino acids are required to preserve the organism in the straight rod or bifid form. Their absence, however, has not been demonstrated in highly branched variations of the organism. Husain et al. also confirmed the work of Kojima et al. (82) that pleomorphism or branching of the organism was induced by sodium chloride. However, the reversal of this effect by the addition of Ca^{2+} ions, as reported by Kojima et al. (84), was not observed by these authors. Three cell wall precursors, *N*-acetyl-D-glucosamine, α-ϵ-diaminopimelic acid and muramic acid failed to inhibit branching when added to the minimal medium. They concluded that the proposal of Kojima et al. (84), that branching in bifidobacteria was principally due to its inability to form cross walls when grown in a medium deficient in Ca^{2+} ions, was an oversimplification of a more complex phenomenon. Reports such as these have been influential in eliminating the designation of variants or subspecies solely on the basis of morphological grounds.

In our laboratory, observations concerning the morphology of bifidobacteria are in agreement with those of most workers quoted. We have found that in stools of infants the bifidobacteria are curved, gram-positive rods which are sometimes bifid. When cultured for as long as 22 years on the Norris medium, this structure is maintained, but when grown on other media, such as tomato agar and thiogylcollate, branched and bizarre forms are often observed. Most evidence at hand, therefore, indicates that the curved bifid rod phase of bifidobacteria can be maintained in the laboratory for indefinite

periods of time on a nutritionally optimal medium. The branched phase of the organism can also be preserved for long periods of time in a medium which lacks certain amino acids. The implication of these observations is not that branching is necessarily an involution or degenerative form of the organism, but that it is an adaptive reaction to a less than optimal nutritional environment.

Morphological Relationships with Other Organisms

Although bifidobacteria were included in the genus *Lactobacillus* for many years, partly because of morphological resemblance to other members of this genus, branching when it occurred was greater than that of lactobacilli. Reservations about the taxonomic position of bifidobacteria were, therefore, engendered on morphological grounds.

Negroni and Fischer (110) compared the structure of bifidobacteria with *Actinomyces bovis*. Their illustrations of the bifid and branched phases show more pleomorphism than in *A. bovis* and confirm previous reports on the pleomorphic characteristics of the former. Bifid splitting of the bacillary ends, simple and complex branching, and swelling of the bacilli were illustrated. It was concluded that the two organisms were similar but not morphologically identical. Frank and Skinner (35) also compared these organisms and agreed that pleomorphism is greater in bifidobacteria than in *A. bovis*. Simple branching and pleomorphism in strains of anaerobic diphtheroids were less frequent than in bifidobacteria. Hayward et al. (63) described the marked pleomorphism which was found in cultures of bifidobacteria soon after isolation. Branched and unbranched rods, irregularly septate, were illustrated. Some were bifid. Some were swollen in the middle of the bacillus, and others had terminal swellings. Short, lateral branches asymmetrically placed also were numerous. As the culture became adapted to artificial media after repeated subculture, the pleomorphism became less frequent and the rod form predominated. The authors concluded that the organisms had morphological features in common with both actinomyces and corynebacteria, and they thought that the resemblance to corynebacteria was greater. Only in the case of well-adapted cultures was the resemblance to lactobacilli close. Sundman et al. (162) compared the structure of strains of bifidobacteria with representatives of the genera of *Actinomyces*, *Butyribacterium*, *Corynebacterium*, *Lactobacillus*, and *Propionibacterium*. On tomato agar their strains of bifidobacteria showed the pleomorphic variation described by others. Marked branching and bladder-like and protoplastic-like swellings were numerous. Reversion to the straight-rod form occurred regularly when such organisms were transferred to a nutritionally more complex medium. Morphological similarities were noted among all the groups studied, but relationship of the bifidobacteria with any of them was not determined. Three of four morphological subtypes of bifidobacteria were thought to resemble *Butyribacterium* most closely; the fourth, *L. bifidus* var. *pennsylvanicus* (*B. bifidum*), had structural features in common with *Actinomyces*. Beerens et al. (5) noted that the morphology of the bifidobacteria is not always characteristic and also noted the importance of biochemical and cultural properties for the indentification of these organisms.

By light microscopy, therefore, investigators are in agreement that the bifidobacteria have certain morphological features which are not unique but which, taken together, are of aid in distinguishing these organisms from others. On certain media, notably tomato broth and agar, a high degree of branching, which is sometimes complex, is observed. Swelling of the bodies of the organism and bladder-like globular swellings, which are often terminal, are observed less often. Septa, when present, are often infrequently or irregularly placed, as if branching may occur in lieu of cell division. When the organisms are transferred to more complex media, they revert to the straight or curved-rod form which is sometimes bifid. The observations of Tissier, therefore, have been confirmed and expanded.

Ultrastructure

Although the morphology of bifidobacteria has concerned many workers, very little attention has been given to their ultrastructure. In 1963 Overman and Pine (120) studied electron micrographs of several species of *Actinomyces* and included one micrograph of *L. bifidus* var. *pennsylvanicus* (*B. bifidum*). This appears to be the first published electron micrograph of a sectioned organism of a member of the genus *Bifidobacterium*. Of the organisms studied, no membrane complexes were observed in *L. bifidus* var. *pennsylvanicus* or *A. bovis*. However, "dark bodies" were observed which were suggestive of bacteriophage. The authors believed that the lack of "membrane coils" in *L. bifidus* var. *pennsylvanicus* indicated that the organism is

more primitive than *L. acidophilus* and that it is more closely related to *A. bovis*.

Kojima et al. (84) published electron micrographs of branched organisms and of a bacilloid form of bifidobacteria and noted a lack of cross walls in the former. Because the former were cells grown in a Ca^{2+}-deficient medium and the latter were grown in a Ca^{2+}-enriched medium, they believed that the electron micrographs support their contention that Ca^{2+} ions are important in the formation of cross walls and in the prevention of pleomorphism in bifidobacteria.

Exterkate et al. (34) used electron micrographs to study the ultrastructure of lysozyme-treated *L. bifidus* var. *pennsylvanicus* (*B. bifidum*). They noted that in these cells the cytoplasmic membrane was much easier to distinguish because it was separated from the electron-dense layer by a bright zone. It was also noted that incubation with lysozyme resulted in a complete disappearance of the cell wall of *L. bifidus* var. *pennyslvanicus*, but that the cell walls of the branched form of the organism showed no changes after lysozyme treatment.

For electron micrographs, *B. bifidum* (*L. bifidus* var. *pennsylvanicus*) was grown in the Norris medium supplemented with breast milk for 48 h. Organisms were fixed and embedded by the method of Higgins (personal communication). The method of fixation is similar to that described by Reyn et al. (134). Our procedure differed from theirs in that the organisms were fixed in 3% glutaraldehyde, followed by additional fixation with Kellenberger osmium fixative. For embedding, Epon 812 was used instead of Vestopal. Grids were stained with uranyl acetate and counterstained with lead citrate.

Photomicrographs (Fig. 1) show bifid and nonbifid forms. Although a nuclear area and a distinct cell wall are present, the character of other intracellular structures is uncertain.

It has been demonstrated in the past that the composition of the growth medium greatly affects the structure of this organism. Before any definite conclusions can be drawn concerning the ultrastructure of the organisms, therefore, additional comparative studies of bacillary, bifid, and branched phases of the organisms are needed.

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

Nutrition and Atmospheric Growth Requirements

Nutrition. Bifidobacteria are not a nutritionally homogeneous group, and nutritionally different types of bifidobacteria have been reported (47, 48, 54, 62, 197). Prior to the studies of Tomarelli et al. (168–170), very little information was available concerning the growth requirements of these organisms. Norris et al. (112) described a semisynthetic medium, supplemented with most of the factors known to stimulate the growth of lactobacilli, which would support the growth of bifidobacteria in the bifid phase. The growth stimulatory activity of each of the constituents of this medium was investigated by Hassinen et al. (62) in order to determine the minimal nutritional requirements of four strains of this organism. It was found by these workers that in a relatively simple medium, containing cysteine (or cystine), these strains utilized ammonium salts as a source of nitrogen. This is, perhaps, the most striking difference in the nutritional requirements of these strains from most lactobacilli, which generally have more complex nutritional requirements. Of the B-group vitamins, only biotin and calcium pantothenate were required; purine and pyrimidine compounds were not essential; and the cysteine (cystine) requirement was not replaceable by methionine, homocysteine, or related compounds.

A strain which showed only scant or undetectable growth in the regular synthetic medium (112), but which could be propagated easily in the basal medium after the addition of human milk, was isolated from the stools of breast-fed and bottle-fed infants and from the vaginal secretions of pregnant women (50, 61). It was named *L. bifidus* var. *pennsylvanicus* (54), the present-day *B. bifidum*.

The essential factor in human milk, which was lacking in cow's milk (bifidus factor), was subsequently identified as *N*-acetyl-D-glucosamine-containing saccharides. These growth factors were shown to be used by this organism as a substrate for cell wall synthesis (41, 113). Bifidus factor and related subjects have been studied extensively by György (53), György and Rose (56), György et al. (57, 58), O'Brien et al. (113), Pope et al. (125), Rose and György (138), and Zilliken et al. (198–201). Certain *N*-substituted derivatives of D-glucosamine, e.g., *N*-benzoyl - D - glucosamine, *N* - carboethoxy - D - glucosamine, and *N*-caproyl-D-glucosamine, and *O*- and *N*-β-glycosides of *N*-acetyl-D-glucosamine also promote growth of this microorganism (87, 139). When the first two compounds, radioactively labeled in the carbohydrate moiety, were added to the culture medium, marked radioactivity was measured in the muramic acid and D-glucosamine of the bacterial cell walls (86). These two growth factors, representing synthetic derivatives of

D-glucosamine, can be considered, therefore, to act as a source for the glucosamine unit which is essential for cell wall synthesis. Some of these *N*-substituted glucosamine derivatives exhibit a growth-promoting activity exceeding that of the naturally occurring *N*-acetyl-D-glucosamine. A possible explanation of the greater growth-promoting activity of these synthetic derivatives was given by Veerkamp (172), based on his experimental observations. It was considered that the slower conversion of these compounds to glucosamine-6-phosphate, as compared with the less active *N*-acetyl-D-glucosamine, and an inhibition of deamination of the resulting glucosamine-6-phosphate (to fructose-6-phosphate) by the 6-phosphate of these derivatives enabled the organism to use glucosamine-6-phosphate more efficiently for cell wall mucopeptide synthesis. Later studies by Veerkamp (171) also established that the slight growth-promoting activity of *N*-acetyl-D-glucosamine compared with other *N*-substituted glucosamines is especially caused by its rapid conversion to fructose-6-phosphate, which is for the most part degraded via acetyl phosphate to acetate and lactate.

Atmospheric growth requirements. Investigators have been concerned with the question of whether bifidobacteria are strictly anaerobic. In studying branched and unbranched strains of

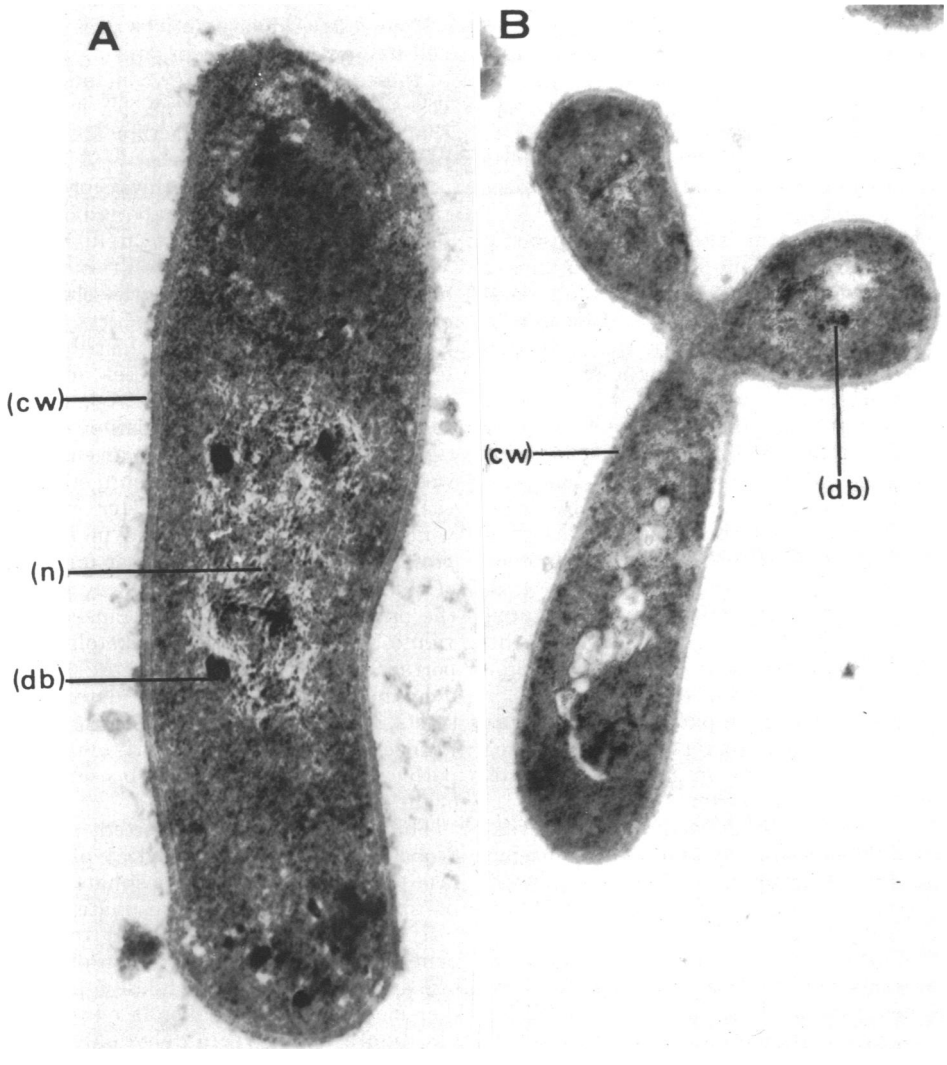

FIG. 1. *Electron micrographs of glutaralydehyde-osmium tetroxide-fixed, uranyl acetate-lead citrate-stained B. bifidum. A, An unbranched phase; B, the bifid phase demonstrating the presence of a nuclear area (n), dense bodies (db), and a distinct cell wall (cw).* ×60,000.

bifidobacteria, Norris et al. (112) noted differences in their requirements for atmospheric CO_2, and sensitivity to atmospheric O_2, when they were grown on solid media or in broth tubes. Although Mayer et al. (98) claimed that bifidobacteria could be grown under aerobic conditions, with concomitant production of catalase activity, it is now generally accepted that bifidobacteria are strictly anaerobic and do not possess catalase activity (30, 153, 154).

In a detailed study of the factors determining the degree of anaerobiosis of bifidobacteria strains, de Vries and Stouthamer (181) determined the degree of sensitivity of 12 bifidobacteria strains to O_2 by measuring the size of the zones of inhibition obtained when they were grown in deep agar cultures under air and by measuring growth in aerated cultures. None of the strains tested grew on agar plates under aerobic conditions, thereby confirming the observations of earlier workers (25, 112, 153). However, great differences in sensitivity to oxygen were found for different bifidobacteria strains, as was evident from the size of the zones of inhibition obtained.

It was also shown that the principal reason for anaerobiosis was different for different strains of bifidobacteria. Some strains, which had small zones of inhibition in agar stabs, appeared to possess a weak catalase activity. Hydrogen peroxide did not accumulate in the presence of air or, if traces were formed, H_2O_2 was removed by catalase. It is also likely that reduced-form nicotinamide adenine dinucleotide oxidase of these strains did not form H_2O_2 at all. The absence of growth on agar medium probably resulted from the fact that these strains grew only below a certain oxidation-reduction potential.

In the case of some strains of bifidobacteria, accumulation of H_2O_2 was found to be the principal reason for the requirement for anaerobic conditions. Hydrogen peroxide causes a block in the fermentation pathway by inactivation of fructose-6-phosphate phosphoketolase, which is a key enzyme in the carbohydrate metabolism of this organism.

Other strains of *Bifidobacterium*, however, which had the largest zones of inhibition in agar medium did not accumulate H_2O_2. These were found to ferment glucose only when cysteine or ascorbic acid was added. The presence of O_2 was not lethal for these strains. It was concluded by these workers (18) that O_2 prevented growth and fermentation of these strains by establishing too high an oxidation-reduction potential. It is obvious from their study that, even in a group of such closely related microorganisms, the principal reason for anaerobiosis may be different for different members of the same group.

Dehnert (26) noted the effects of the culture media when studying this organism and noted its strict anaerobic requirements. Kandler (75) in a comparative study of the lactobacilli also noted that the bifidobacteria were strict anaerobes and, therefore, were different from most of the other organisms he studied.

Biochemical Characteristics

Carbohydrate metabolism. Kuhn and Tiedemann (85) gave some suggestions concerning the pathway of carbohydrate fermentation in bifidobacteria. They reported the formation of labeled acetate and lactate from [*1*-^{14}C]glucose. Aldolase activity was detected in cell-free extracts of the organism. On the basis of these observations, they concluded that glucose is fermented via the glycolytic system and that both acetate and lactate are formed via phosphoenol pyruvate.

More recently, a new pathway for the fermentation of hexoses has been reported in bifidobacteria by Scardovi and Trovatelli (147) by de Vries et al. (178), and by de Vries and Stouthamer (180). The pathway was elucidated by chemical analysis of end products, determination of radioactive carbon distribution after fermentation of [*1*-^{14}C]glucose, and assay of enzymes in cellular extracts. A key feature of the pathway is the participation of fructose-6-phosphate phosphoketolase, an enzyme which was first discovered by Schramm et al. (152) in the obligate aerobe *Acetobacter xylinum* and which has also been reported in *Leuconostoc mesenteroides* (42, 71). Another unique aspect of this new fermentative pathway is that it is the only one in which both transketolase and xylulose-5-phosphate-phosphoketolase play important roles. Aldolase and glucose-6-phosphate dehydrogenase could not be detected in extracts, which rules out the glycolytic system and hexosemonophosphate shunt, characteristic pathways for degradation of glucose in members of the genus *Lactobacillus*.

Thus, hexoses are converted to fructose-6-phosphate which is cleaved by phosphoketolase to yield erythrose-4-phosphate and acetyl phosphate. Erythrose-4-phosphate and fructose-6-phosphate are converted to 2 mol of pentose phosphate by the action of transaldolase and transketolase. Xylulose-5-phosphate is then cleaved by phosphoketolase to yield acetyl phosphate and glyceraldehyde-3-phosphate. Glyceralde-3-phosphate is converted to pyruvate, which can either be reduced to lactate or

cleaved, presumably by a phosphoroclastic reaction, to acetate and formate. Thus, in cases where all of the pyruvate was cleaved rather than reduced, no lactate was formed. Because this pathway does not occur in any other species of the genus *Lactobacillus*, de Vries and Stouthamer (179), in agreement with some previous workers, suggested that classification of the bifidobacteria in this genus is not justified. The same alternative route of glucose dissimulation has been established in *L. bifidus* var. *pennsylvanicus* by Veerkamp (171). The electrophoretic behavior of fructose-6-phosphate phosphoketolase from the different members of the genus *Bifidobacterium* was studied by Scardovi et al. (146) in the search for an additional tool for the speciation of this genus. They found that the electrophoretic mobility of this enzyme, although not a useful characteristic for the differentiation of species in the genus, was closely related to their ecology.

Polysaccharide formation. Occurrence of mucoid strains of bifidobacteria has been reported by several workers. A mucoid variant of bifidobacteria was first described by Malyoth and Bauer (91, 92). Later, Norris et al. (111) reported that when strains of bifidobacteria isolated from the stools of breast-fed infants were carried in their chemically defined medium (112) by periodic transfer, mucoid colonies occasionally appeared among the smooth parent colonies. The cultural requirements of the mucoid strains appeared to be the same as those of the parent type, and differences in agglutination reactions between them were not detected by Williams et al. (195). Although the mucoid organisms appeared to be variants of the smooth parent type, their reversion to the nonmucoid type was not observed. Aqueous solutions of the polysaccharide produced by these strains were highly viscous. Reports on the composition of these extracellular polysaccharides have been few. Vogel (177) found that the component sugars of the capsular polysaccharide of a strain of bifidobacteria were glucose, xylose, uronic acid, an unidentified pentose, and an unidentified hexose exhibiting an R_f value suggestive of a methylated sugar. Wang et al. (184) found that the extracellular polsaccharide of a mucoid but non-encapsulated variant of bifidobacteria (Jackson M) was composed of four sugars: D-glucose, D-galactose, 6-deoxy-L-talose, and D-galacturonic acid. This polysaccharide was named "Bifidan." It is reasonable to suppose that different strains of bifidobacteria elaborate extracellular polysaccharides of differing chemical composition, which may be the reason for dissimilarities in the composition of "Bifidan" and the polysaccharides studied by Vogel (177).

Colonies of the mucoid variant described by Malyoth and Bauer (91, 92), incubated anaerobically on solid media, were said to be hygroscopic and became watery on exposure to atmospheric conditions at room temperature. Norris et al. (111) presented evidence indicating that the extracellular mucoid substance produced by their mucoid strain (Jackson M) was a highly polymerized polysaccharide which underwent depolymerization with loss of relative viscosity when exposed to atmospheric conditions. This viscosity-reducing activity was found by Wang et al. (185) to be proportional to the concentration of ascorbic acid and was enhanced by atmospheric oxygen. Hydroperoxy radicals were considered responsible for the viscosity reduction activity.

Extracellular dextranase and intracellular α-1 $\rightarrow$ 6 glucosidase. Although several species of molds have been shown to produce extracellular dextranases, very few bacterial species have been reported to produce this type of enzyme. Hehre and Sery (64) reported the production of extracellular dextranases by members of the genus *Bacteroides*, and Bailey and Clarke (1) gave an account of an extracellular dextranase secreted by several strains of *B. bifidum*. Hydrolysis of dextran by the dextranase from the latter source was unusual in that it did not liberate glucose or isomaltose, which are the main products from the action of all other known dextranases on dextran. Instead, the enzyme hydrolyzed dextran, by random cleavage of the α-1 $\rightarrow$ 6 glucosidic links, to a mixture of isomaltotriose, isotetraose, isopentaose, and higher isomalto dextrins. All extracts, prepared from a rumen strain of bifidobacteria grown on dextran, were shown to contain an α-1 $\rightarrow$ 6 glucosidase (2).

Deoxyribonucleic Acid Base Composition

The mean base composition of the deoxyribonucleic acid (DNA) of bacteria has been shown to have important taxonomic significance (66, 140). Precise comparisons of DNA base compositions have therefore assumed great importance.

Werner and Seeliger (192) and Sebald et al. (153) studied the guanine plus cytosine (G + C) contents of 28 strains of *Bifidobacterium* by using a chemical method employing hydrolysis of purified DNA. These workers included a representative organism of each of the groups preposed by Reuter (132). They found the average G + C values to be 60.1 $\pm$ 0.33% for

Bifidobacterium and to be less than 50% for *Lactobacillus*. This difference in values was believed to constitute a valid method for distinguishing between these genera and also for differentiating *Bifidobacterium* from *Propionibacterium*, *Catenabacterium*, and *Corynebacterium*, which also had G + C values lower than that of *Bifidobacterium*. The relationship of *Bifidobacterium* and *Actinomyces* was not clarified. It was believed that DNA base composition, in addition to morphological and biochemical characteristics, supported the contention that *Bifidobacterium* should be classified as an independent genus.

Gasser and Mandel (37), by using a chromatographic method, studied the DNA base composition of the genus *Lactobacillus* and included five strains of *Bifidobacterium*. With present-day nomenclature, these strains of bifidobacteria would be representative of *B. infantis* and *B. breve*. These strains were selected because they represented the mean and extreme values obtained in previous studies by the hydrolysis method (153, 192). They found that the five strains of *Bifidobacterium* had an identical base composition of 58% G + C which was higher than for *Lactobacillus*.

However, the work of Scardovi et al. (150, 151) and Corciani et al. (19) on the DNA homology relationships among species of the genus clearly indicated that the genus *Bifidobacterium* is not as genetically homogeneous as might be deduced from the narrow range of the DNA base composition of its members. Genetic relatedness among the strains representing several named and unnamed species of the genus *Bifidobacterium* was assessed by means of DNA-DNA hybridization in competition experiments by using the technique of Johnson and Ordal (73). Several genetically distinct groups were recognized among the various proposed species of *Bifidobacterium*. *B. bifidum* was, however, found to be clearly distinct genetically from the other bifidobacteria and, hence, its designation as a species was validated at the genetic level (150).

Cell Wall, Lipid, and Phospholipid Composition

Cell wall composition. Biochemical composition of bacterial cell walls is important in the classification of gram-positive anaerobes (21, 23, 128). The cell wall composition of eight strains of bifidobacteria was examined by Cummins et al. (22). An unusual degree of variation was found among the strains which, at first, were thought to be a single bacterial species. Alanine, glutamic acid, and lysine were present in fractions from all strains, as were glucosamine and muramic acid. Either one or two, but not more, of the amino acids serine, glycine, threonine, and aspartic acid were detected in significant quantities. Hexoses in the cell wall also varied. Rhamnose appeared to be present either in fairly large amounts or only in minute quantities. The acid-resistant peptide of lysine and aspartic acid, previously noted in cell wall hydrolysates from lactobacilli, was not present in those strains of *B. bifidum* which contain aspartic acid. This observation suggests that the cell walls are structurally different from those lactobacilli in which these peptides occur. Absence of arabinose and α-ϵ-diaminopimelic acid, which are characteristically present in the cell walls of *Corynebacterium*, and differences in the composition of cell hydrolysates from those of any groups examined in *Actinomycetales* (23) led these workers to state that "as far as cell wall composition is concerned, these eight strains do not correspond to any group of gram-positive organisms previously examined."

The composition of the cell wall of *L. bifidus* var. *pennsylvanicus* (*B. bifidum*) was studied by Veerkamp et al. (176). The cell wall mucopeptide was found to contain serine, alanine, aspartic acid, glutamic acid, and muramic acid. In addition, ornithine was identified as the basic amino acid component. This observation was considered significant because Cummins et al. (22) did not report its occurrence in their experiments. It was suggested, however, that ornithine could have been mistaken for lysine, for the two homologous amino acids do not separate easily in most solvent systems used in paper chromatographic methods. Rhamnose, glucose, and galactose were detected in large amounts in the cell wall preparation. Phosphorus and glycerol were also present.

Although the amino acid composition of the murein (peptidoglycan, mucopeptide) is supposed to be useful as a chemotaxonomic criterion (23), the determination of the qualitative or quantitative amino acid composition of the cell wall is no longer considered sufficient to characterize the type of murein unequivocally. It was pointed out by Kandler (76) that the amino acid content of two strains may be identical, but that the amino acid sequence, or even the type of cross-linkage, may be quite different.

In their study of the amino acid sequence of murein (peptidoglycan) of six strains of bifidobacteria, Kandler et al. (77) showed that the peptidoglycan contained muramic acid, glucosamine, alanine, glutamic acid, lysine, and

glycine at a molar ratio of 1:1:2:1:1:1. The analysis of the peptides obtained by partial acid hydrolysis indicated that the amino acid sequence of the tetrapeptide is identical to that of most bacteria (L-Ala-D-*glu*-L-*lys*-D-Ala). Glutamic acid was present as an amide. Glycine was involved in the cross-linking of adjacent mucopeptides by forming a bridge between the ϵ-amino group of lysine and the carboxyl group of a C-terminal D-alanine. About 50% of the glycine is N terminal, indicating that only 50% of the possible cross-linkages are realized. The murein of these strains of *B. bifidum* resembles the murein of staphylococci, but differs by the number of glycine molecules. Although a pentameric glycopeptide occurs in the murein of staphylococci, only one molecule of glycine is involved in the cross-linkage of the murein of the *B. bifidum* strains studied.

Reporting results of a survey of the distribution of the various murein types within the lactobacilli and related organisms, Kandler (76) stated that all of the species of some groups (*Thermobacterium*, *Pediococcus*) contained only one type of murein, but that the species of other groups (*Bacterium*, *Bifidobacterium*, *Leuconostoc*) contain several types. The murein types of the various strains in the genus *Bifidobacterium* were found to correlate very well with the species of this organism which was defined by Reuter (132). Kandler thought that the murein type may be a valuable criterion for the separation of species within the genus *Bifidobacterium*.

The various murein types of several species of *Bifidobacterium* have been described in the literature. Holzapfel et al. (69) studied the amino acid sequence of the ornithine- and lysine-containing mureins of six strains of *B. globosum*. They found that murein contained MurNAc, glycine NH_2NAc, glutamine, alanine, and diamino acids in a molar ratio of 1:1:1:5:1. No teichoic acid was found. They found the tetrapeptides attached to the muramic acid to be equal to other mureins: L-alanine-D-glutamine-L-lysine (or ornithine)-*O*-alanine and believed that the evidence indicated that 10 to 20% of the interpeptide bridges were not cross-linked. Some of the peptide units were thought also to be incomplete.

Koch et al. (80) studied the amino acid sequence of the threonine- and serine-containing murein of 18 strains of *B. longum* and one strain of *B. lactentis* and found them to contain murine, glycine, glutamine, alanine, ornithine (or lysine), threonine, and serine in a molar ratio of about 1:1:1:4:1:1:1. The peptide subunits attached to the muramic acid were: L-alanine-D-glutamine-L-ornithine-D-alanine, and the interpeptide bridge consisted of β-D-aspartyl-L-serine. They found 60% of the subunits to be cross-linked. Teichoic acid was not found. They noted that all strains of other species of *Bifidobacterium* investigated contained different types of murein. The murein of *B. bifidum* contained an Orn-Ser-Asp-type murein which differentiated *B. bifidum* from the other bifidobacteria.

The structure of the cell wall peptidogylcan of *B. bifidum* var. *pennsylvanicus* was studied in detail by Veerkamp (175). It appeared to contain equimolar amounts of *N*-acetyl-D-muramic acid, *N*-acetyl-D-glucosamine, D-isoglutamine, D-(iso)asparagine, D-serine, L-ornithine, L-alanine, and D-alanine. Analysis of peptides obtained by acid hydrolysis indicated that the tetrapeptide linked to muramic acid has the structure: *N*-α-L-alanyl-γ-D-isoglutamyl-L-ornithyl-D-alanine. Seryl asparagine is involved in the cross-linking of adjacent tetrapeptides by forming a bridge between the 5-amino group of ornithine and the carboxyl group of C-terminal D-alanine. About a quarter of the (iso)asparagine is N terminal, indicating that only 75% of the possible cross-linkages are realized. The peptidoglycan structure of *L. bifidus* var. *pennsylvanicus* (*B. bifidum*) differs, therefore, from that of other bifidobacteria and that of lactobacilli by the structure of this specific cross-linking depiptide.

Lipid and phospholipid composition. A comparative study of the lipid content of cell, membrane, and cytoplasmic preparations from normally grown cells of *L. bifidus* var. *pennsylvanicus* (*B. bifidum*) and cells in which cell wall synthesis was inhibited by growth in a medium lacking human milk was undertaken by Exterkate and Veerkamp (33). The percentage of lipids in cells grown without human milk exceeded that of normally grown cells. The lipid content of the membrane, under the two conditions of growth, was not significantly different. The carbohydrate content in whole cell, membrane, and cytoplasmic lipids was considerably decreased after cell wall inhibition. The phosphorus content also showed a minor decrease. These changes were not due to differences in pH values in the media with or without human milk at the time of harvesting. Eighteen phospholipids were detected. Diphosphatidyl glycerol, phosphatidyl glycerol, and a polyglycerol phospholipid, identified as glycerophosphorylglyceroldiacylphosphatidyl glycerol, were the main phospholipids. Mono-, di-, and triacyl-bis-(glycerophosphoryl) glycerol, alanyl phosphatidyl glycerol, phosphatidic acid, and

two lyso derivatives, glycerophosphoryl glycerol and diacylphosphatidyl glycerol, were detected in smaller quantities. After cell wall inhibition, the amount of triacyl-bis-(glycerophosphoryl) glycerol was increased considerably. Phosphatidyl glycerol, glycerophosphoryl glycerol, and diacylphospatidyl glycerol (and its first lyso derivative) were decreased, but diphosphatidyl glycerol remained constant. The decrease in phosphatidyl glycerol, and to a lesser extent the increase in triacyl-bis-(glycerophosphoryl) glycerol, was a response upon exposure to a medium of lower acidity. Phospholipid composition was found to be greatly affected by the temperature at which the cells were grown. A nearly complete reversal between the polyglycerol phospholipids was observed after cell wall inhibition when cells were grown at 37 rather than at 23 C.

All glycolipids were galactolipids. Besides mono-, di-, and tri-galactosyldiglyceride, a monoacyl and diacyl derivative of monogalactosyldiglyceride and a monoacyl derivative of digalactosyldiglyceride were detected. Monogalactosyl- and digalactosyl-monoglyceride were present in small quantities. All galactolipids had the pyranosyl configuration. A proline-containing lipid without phosphorus was also detected.

Analysis of fatty acid composition of *L. bifidus* var. *pennsylvanicus* (*B. bifidum*) was made on different lipid fractions isolated from cells grown with or without human milk (173). No large differences were found in the fatty acid pattern between total, membrane, and cytoplasmic lipids and glyco- and phospholipids. Major constituents were the normal even-numbered saturated and monoenoic acids. The percentages of lactic acid and branched fatty acids were low. Fatty acid composition of *Bifidobacterium* and *Lactobacillus* was also compared (174).

Phospholipid composition of 10 strains of bifidobacteria of human intestinal origin, two strains of *B. bifidum* (as well as eight other strains of bifidobacteria), and nine strains of *Lactobacillus* were compared by Exterkate et al. (32). Included in the study were one strain each of *B. asteroides*, *B. coryneforme*, and *B. indicum* from honey bees and two strains from bovine rumen liquor, *B. ruminale* and *B. globosum*. Diphosphatidyl glycerol and phosphatidyl glycerol were prsent in strains of both genera. All *Bifidobacterium* strains contained as specific phospholipids a new polyglycerol phospholipid and its lyso derivatives earlier detected in *L. bifidus* var. *pennsylvanicus* (33). Also lyso compounds of diphosphatidyl glycerol and alanyl phosphatidyl glycerol were only present in this genus in variable amounts. Lysyl phosphatidyl glycerol was the only ninhydrin-positive phospholipid in seven *Lactobacillus* strains. In *L. delbrucki* and *L. helveticus* it was absent and partially replaced by an unidentified ninhydrin-negative phospholipid. The differences in phospholipid composition between bifidobacteria and lactobacilli, especially in the polyglycerol phospholipids and the aminoacyl phosphatidyl glycerol, may, therefore, be another means of differentiating these two genera.

CLASSIFICATION

Historically the identification of members of the genus *Bifidobacterium* in the literature is often difficult because many unrelated gram-positive rods, branched or unbranched, without spores, were assigned to the genus *Bacillus* and later to the genus *Lactobacillus*. During the first half of the present century, interest was centered on those organisms found in man which were designated *L. bifidus* and *L. acidophilus*. Less attention was paid to the possible occurrence of this genus or of related organisms elsewhere in nature until recent years. For this reason, the historical review is divided into two periods: from 1899 to 1957 and from 1957 to the present.

First Period

The distinction between *B. bifidus communis* or *B. bifidus* and *B. acidophilus*, first described and agreed to by Tissier and Moro (166, 167, 105, 106), was generally accepted, but close biological relationship between these organisms was also believed to exist. However, *B. acidophilus* was not assigned to the genus *Lactobacillus*, first characterized by Beijerinck in 1901 (4), unit 1920 when Holland (68) did so. The first edition of *Bergey's Manual of Determinative Bacteriology* (8) also classified the organism as *Lactobacillus acidophilus*, and from that time on this designation was in general use. The designation *B. bifidus*, meanwhile, was not changed for some years. The designation was changed to that of *Bacteroids bifidus* in the third edition of the *Manual of Tropical Medicine* (1919) by Castellani and Chalmers (17), and this designation was also the one given in the first through the fourth editions of *Bergey's Manual* (6–10). In the fifth edition (7) the organism was classified as *L. bifidus*. Placing the organism in this genus was based on morphological grounds because it was a gram-positive nonsporeforming bacillus and because its biochemical characteristics were consistent with those of the genus

Lactobacillus. In the United States, therefore, *L. bifidus* was the generally accepted designation which, however, was not so widely accepted in Europe. Orla-Jensen (116) in 1924 proposed that this organism be placed in a new genus *Bifidobacterium*. However, the designation *Bifidobacterium* was not generally accepted. In the European literature the terms *Bacillus* or *Bacteroides bifidus*, as well as *L. bifidus*, continued to be employed. Cruickshank (20) in 1925 reported serological similarities between *L. bifidus* and *L. acidophilus*. These similarities were also noted a few years later by Weiss and Rettger (188). Lehmann and Neumann (88) studied the organism in 1927 and classified it as *Bacterium bifidum*. Two years later the name *Tissieria bifida* was proposed by Pribram (129).

Beginning in 1930, investigators began noting the relationship of this organism to *Actinomyces*. Vuillemin (182) in 1931 called the organism *Nocardia bifida*; Nannizzi (109) later called it *Actinomyces bifidus*. Puntoni (130, 131) maintained that *L. bifidus* and *A. bovis* were the same organism and presented morphological observations, as well as serological studies, to confirm this relationship.

In 1933–1934, Weiss (187) and Weiss and Rettger (188, 190) studied various strains of bifidobacteria isolated from human feces as well as from other sources. They concluded that *L. bifidus* and *L. acidophilus* showed similar morphological and cultural characteristics and that fermentation patterns were not significantly different. They also found that serological differences between the two organisms were not great. They concluded that *L. bifidus* was a variant of *L. acidophilus* and should be classified in the genus *Lactobacillus*.

In the following year (1935), Eggerth (31) published his work on the gram-positive nonsporeforming anaerobic bacilli in human feces. He was one of the first investigators to realize that organisms, isolated from human subjects and classified as *Bacteroides bifidus*, were not homogeneous. After studying isolates from 85 stools of adults and breast-fed infants and testing them for their ability to ferment 12 carbohydrates, he concluded that these isolates could be divided into two major groups, based mainly on the fermentation of four key carbohydrates. These were arabinose, xylose, melezitose, and mannose. On the basis of these reactions he classified all organisms as *Bacillus bifidus* group I or II. All organisms isolated from infants were group I, whereas most isolates from adult stools were group II. Eggerth also expressed the opinion that these organisms should be placed either in the genus *Lactobacillus* or in a separate genus, possibly in the order *Actinomycetales*, but not in the genus *Bacteroides*. The name *Actinobacterium bifidum* was proposed by Puntoni (131) in 1937. Serological relationships between *Bacillus bifidus* and *L. acidophilus* were reinvestigated in the years 1936–1939 by Blaurock (11, 12) and Boventer (15). These workers failed to demonstrate the serological relationship between these two organisms, which had been reported by earlier investigators (20, 188).

The Orla-Jensens and Winther (118) published information which was not in agreement with the results of Weiss and Rettger (187, 188). The former authors considered *B. bifidus* a branched organism and *L. acidophilus* and unbranched one and believed the former should be classified as an *Actinomycetales*.

In 1938 Weiss and Rettger (189), having studied several of the Orla-Jensen strains, concluded that the organisms were unlike either *L. acidophilus* or *B. bifidus* isolated from nurslings' stools and were similar to *B. bifidus*, group II, of Eggerth (31). Weiss and Rettger were still convinced that their *L. bifidus* was a variant of *L. acidophilus* and was not identical with the organism originally isolated by Tissier. The latter was therefore considered to be the "true *Lactobacillus bifidus*." Weiss and Rettger proposed that, since there appeared to be at least two types of *L. bifidus*, the organisms should be classified as *L. bifidus*, type I, which usually becomes aerobic and unbranched after primary isolation, and *L. bifidus*, type II, or *L. parabifidus*, which usually remains anaerobic and branched. Several workers accepted the term *L. parabifidus*, as described by Weiss and Rettger, and in the sixth edition of *Bergey's Manual of Determinative Bacteriology* (16) both *L. bifidus* and *L. parabifidus* are listed. Also in 1938, Prévot (126) placed what he called *Bifidobacterium bifidum* (Tissier) Orla-Jensen in the family *Actinomycetaceae*, but in a genus different from *A. bovis*. In 1940 Lewis and Rettger (90) agreed with the conclusion of Orla-Jensen et al. (118) that at least some strains of *L. bifidus* were related to the actinomycetes. However, in 1942 King and Rettger (79) reaffirmed the position of Weiss and Rettger (189) that these organisms should be classified as lactobacilli. Olsen (114) in 1949 reviewed the literature and added his own observations. He disagreed with classifying this organism as *Bacterium bifidum* and believed that the organism should be classified in the genus *Corynebacterium* and should be called *C. bifidum*. In 1944 Negroni and Fisher (110) confirmed the relationship of the organism with

the actinomyces and placed it in the genus *Cohnistreptothrix* (*C. bifidus*), which was also the genus in which they classified *A. bovis*.

Norris et al. (112) in 1950 studied the morphology of bifidobacteria with a culture medium designed for this purpose. These workers disagreed with Weiss and Rettger (189) in the use of the term *L. parabifidus* to describe the branched anaerobic form and *L. bifidus* to describe a type that became unbranched and aerobic or microaerophilic. These workers proposed that the straight-rod type was a variant of the anaerobic type and that the term *L. bifidus* should be used for the branched form, whereas the term *L. parabifidus* should be used for the unbranched variant. Therefore *L. parabifidus*, as used by Weiss and Rettger, describes a branched organism and the term, as used by Norris et al., describes an unbranched bacillus. In 1953 Williams et al. (195) studied the antigenic relationships of *L. bifidus* and *L. parabifidus*, as defined by Norris et al. (112), and included some strains of *L. acidophilus*. These authors found it difficult to prepare a satisfactory antiserum for *L. parabifidus*, but did find a distinct antigenic component of the organism and noted much serological variation among most of the strains tested. They found no serological relationships between *L. acidophilus* with *L. bifidus* or *L. parabifidus*, and they concluded that serologically there was justification for use of the three separate species designations. In 1954 György et al. (54) described a variant of *L. bifidus* that required the addition of human milk to the medium to maintain the organism after primary isolation. György et al. (50) originally designated the organism *L. bifidus* var. *penn*. Because this designation was not acceptable by taxonomic standards, the designation was subsequently changed to *L. bifidus* var. *pennsylvanicus* (55). Morphologically *L. bifidus* and *L. bifidus* var. *pennsylvanicus* were indistinguishable. It was noted that the milk-dependent strain could convert to the regular *L. bifidus* type, but attempts to make the regular *L. bifidus* milk dependent failed. These authors suggested the possibility that *L. bifidus* var. *pennsylvanicus* could become the regular *L. bifidus*, which in turn could become the unbranched *L. parabifidus*.

Frank and Skinner (35) restudied the morphological relationship of *L. bifidus* and *A. bovis*. They concluded that, based on several morphological pecularities such as primitive branching and similar appearance on gram-stained preparations when grown on several media, these two organisms were similar and were related at least at the genus level. They thought that the evidence justified designating the organism *Actinomyces bifidus*.

Pine and Howell (124), in an attempt to more clearly define the relationship of the bifidobacteria to the *Actinomyces*, compared the biochemical and physiological characteristics of several *Actinomyces* species with four isolates of bifidobacteria. They found the sugar of choice for growth of their *Actinomyces* species to be glucose or maltose, whereas lactose or maltose produced optimal growth of the bifidobacteria. None of the bifidobacteria strains reduced nitrate to nitrate, whereas the *Actinomyces* did possess this ability. It was also noted that the bifidobacteria fermented 50 to 89% of a 1% glucose medium, but the *Actinomyces* fermented an average of 34 to 59% of the glucose. Both groups of organisms produced the same fermentation products from glucose (lactic, acetic, formic, and succinic acids). Although the *Actinomyces* strains predominantly formed lactic acid with small amounts of acetic, formic, and succinic acids, the strains of bifidobacteria formed approximately equal amounts of lactic and acetic acids. The authors concluded that the last point suggested a major metabolic difference between *Actinomyces* and the bifidobacteria.

In evaluating the taxonomy of *L. bifidus* in 1955, Gyllenberg (45) stated that from the definition of the variant concept outlined by Smith et al. (157) the designation of *L. parabifidus*, proposed by Norris et al. (112) and Weiss and Rettger (189), is invalid because of the variability of the parent strain. Gyllenberg also noted that, unlike *L. parabifidus*, *L. bifidus* var. *pennsylvanicus* could be considered a true variant of *L. bifidus* and fulfilled the requirements of Smith et al. (157) for the variant concept.

To summarize, it was first thought that *L. bifidus* and *L. acidophilus* were closely enough related to justify their inclusion in the same genus. During much of the first period, technology was comparatively primitive, and methods of analysis were generally limited to the use of morphological observations and simple fermentation patterns. The results of studies by these methods did not reveal morphological and biochemical characteristics which were so distinctive as to convince workers that *L. bifidus* belonged in one genus or another. In the United States, for lack of definitive criteria, however, the organisms were regarded by the majority of workers as belonging to the genus *Lactobacillus*. In chronological order the various proposed generic designations of the organisms are as

follows: *Bacillus bifidus communis*, *Bacillus bifidus*, *Bacteroides bifidus*, *Lactobacillus bifidus*, *Bifidobacterium bifidum*, *Bacterium bifidum*, *Tissieria bifida*, *Actinomyces befidus*, *Actinobacterium bifidum*, *Corynebacterium bifidum*, and *Cohnistreptothrix bifidus*. Although the genus *Bifidobacterium* was originally proposed by Orla-Jensen (116) in 1924, it has only recently gained general acceptance. The genus *Bifidobacterium* will be recognized as an independent genus in the eighth edition of *Bergey's Manual of Determinative Bacteriology*.

Second Period

Prior to 1957 *L. bifidus* var. *pennsylvanicus* was the only generally accepted additional species or variant of bifidobacteria. In 1957 Dehnert (25) presented a scheme for the differentiation of five groups of bifidobacteria based mainly on the fermentation of 24 carbohydrates. Species were not designated, but the various groups were assigned a number. *L. bifidus* var. *pennsylvanicus* was placed in Dehnert's group II. In an effort to clarify the relationship of the bifidobacteria, recently proposed taxonomic schema are tabulated in Table 2. For completeness Eggerth's groups I and II are also listed, although the relationship to them of recently described species and types is often uncertain. It is seen that Eggerth's group I includes Dehnert's groups I through IV, and Eggerth's group II corresponds with Dehnert's group V. The work initiated by Dehnert was the first to recognize the existence of multiple biotypes of *Bifidobacterium* and was the basis of later studies which eventually led to the recognition of species and subspecies. In the same year Cummins et al. (22) examined the cell wall composition of several strains of bifidobacteria and concluded that these organisms differed from all previously tested gram-positive organisms. The taxonomic classification of bifidobacteria was therefore open to question and the subject of renewed investigation.

Sundman et al. (162) in a comparative study of several organisms concluded that the bifidobacteria in general were most closely related to *Butyribacterium*. However, they believed that *L. bifidus* var. *pennsylvanicus* closely resembled an *Actinomyces*. In 1961 Slack et al. (155) compared the serological grouping of bifidobacteria with *Actinomyces* and *Corynebacteria*, by using fluorescent antibody techniques, and found no relationship between the bifidobacteria and the other organisms. Lerche and Reuter (89) in the same year studied morphological and biochemical variants of the anaerobic lactobacilli and divided them into four groups, one with four variants. However, no attempt was made to designate species.

In 1963 Reuter (132) did biochemical and serological tests on isolates from 136 stools of 38 infants and compared his results with those of Dehnert (25, 27). From a study of these strains and of strains previously isolated from adults, Reuter devised the following scheme for identification. If a gram-positive anaerobic bacillus resembled lactobacilli except for morphologic variability, then the character of acids produced from glucose was examined. If the ratio of lactic to acetic acid was two to one, the strain was tested for its ability to ferment 11 additional carbohydrates. On this basis all strains were placed into one of ten groups (Table 1). Group I had four biotypes; groups II and III had two; and the remaining groups had no additional biotypes. After evaluating his data, he concluded that these organisms should be classified within the tribe *Lactobacilleae*, of the family *Lactobacillaceae*, and in the genus *Bifidobacterium*. He concluded that the genus *Bifidobacterium* should be divided into eight species and several species variants. He recognized the following species of *Bifidobacterium*: *bifidum* a and b, *infantis*, *pavulorum* a and b, *breve* a and b, *lactentis*, *adolescentis* a, b, c, and d, and *longum* a and b. The classification of Dehnert (25) was thus expanded by Reuter (132) with the designation of eight species and a number of variants on the basis of carbohydrate fermentation. This precedent of designating species on the basis of carbohydrate fermentation was to lead to recognition of additional species and to increased reliance on the pattern of carbohydrate fermentation for species designation. Many of these strains were characterized and deposited in the American Type Culture Collection by Reuter (133).

Between the years 1965 and 1969 biochemical studies were expanded to include the pathways of carbohydrate fermentation. Scardovi and Trovatelli (147) and de Vries et al. (178, 180) discovered a new pathway for the fermentation of hexoses in bifidobacteria, previously referred to, which does not occur in any species of the genus *Lactobacillus*. Veerkamp (17) demonstrated a similar pathway in *L. bifidus* var. *pennsylvanicus*. Many of the earlier classification schemes were reexamined by the original authors (28).

Also during these years, a study of the DNA base composition by Sebald et al. (153) and Gasser and Mandel (37) confirmed the earlier work of Werner and Seeliger (192) that mem-

TABLE 2. *Comparison of the major classification schemes of the genus Bifidobacterium*[a]

Eggerth, 1935 (31)	Dehnert, 1957 (25)	Reuter, 1963–64 (132)	Mitsuoka, 1969 (100)	Scardovi et al., 1971 (150)	Holdeman and Moore, 1972 (67)
I	I, II	*bifidum* a, IIIa	*bifidum* a	*bifidum*	*bifidum*
		bifidum b, IIIb	*bifidum* b		
	III	*infantis* V	*infantis* a	*infantis*	*infantis* SS *infantis*
			infantis b		*infantis* SS *liberorum*
	IV	*liberorum* IX	*liberorum*		*infantis* SS *lactentis*
		lactentis X	*lactentis*		*infantis*—other
	III	*breve* a, VII	*breve* a	*breve*	*breve*
		breve b, VIII	*breve* b		
			breve c		
		parvulorum a, IV	*parvulorum* a		
		parvulorum b, VI	*parvulorum* b		
II	V	*adolescentis* Ia	*adolescentis* a	*adolescentis*	*adolescentis* A
		adolescentis Ib	*adolescentis* b		*adolescentis* B
		adolescentis Ic	*adolescentis* c		*adolescentis* C
		adolescentis Id	*adolescentis* d		*adolescentis* D
		longum a	*longum* var. *longum* a	*longum* SS	*longum* SS *longum*
		longum b	*longum* var. *longum* b		
		longum IIa	*longum* var. *animalis* a		
		longum IIb	*longum* var. *animalis* b		
			pseudolongum a	*pseudolongum*	*pseudolongum*
			pseudolongum b		
			pseudolongum c		
			pseudolongum d		
			thermophilum a	*thermophilum*	*thermophilum*
			thermophilum b	*ruminale*	
			thermophilum c		
			thermophilum d		
				asteroides	*asteroides*
				"dentium"	*"dentium"*
				"angulatum"	
				"catenulatum"	
				suis	
				globosum	
				indicum	
				coryneforme	
					cornutum
					eriksonii

[a] SS, Subspecies.

bers of the genus *Bifidobacterium* were not homogeneous with members of the genus *Lactobacillus* or other related genera. Exterkate and Veerkamp (33) noted that bifidobacteria and lactobacilli also differed in their phospholipid composition. Snyder et al. (158) reexamined the serological relationships of bifidobacteria and members of *Actinomyces* and *Nocardia* together with other related genera. They confirmed the earlier observations of Slack et al. (155) that these organisms are not serologically related to the *Actinomyces*, *Corynebacterium*, or any other of the gram-positive filamentous bacteria tested. *Bifidobacterium* thus appears to be a serological entity. Werner (191) and Werner and Seeliger (193) confirmed and simplified Dehnert's classification by singling our five carbohydrates as being sufficient for identification of the five groups: arabinose, xylose, mannose, mannitol, and sorbitol.

The next major classification scheme was presented by Mitsuoka (100) in 1969. Four hundred and eighty-three strains of bifidobacteria, isolated from human as well as nonhuman sources, were studied and classified on the basis of physiological and biochemical characteristics (Table 2). Mitsuoka also compared his results with those obtained by Reuter (132). The species designations originated by Reuter were retained by Mitsuoka, who increased the number of variants. In addition, he described two

new species: *B. pseudolongum* and *B. thermophilum*. It was noted in this study that nonhuman were clearly differentiated from human strains by their temperature requirements (ability to grow at 46.5 C) and carbohydrate fermentation patterns. As a consequence, the 15 species and variants given by Reuter were increased to 27. Unlike the three previously mentioned classifications, the Mitsuoka scheme included animal strains.

Pine (123) in 1970, evaluating the serological relationships of the family *Actinomycetaceae*, agreed with Slack et al. (155) and others that no significant cross-reactions exist between bifidobacteria and any of the other organisms tested. In this same year Koch et al. (81) studied the murein composition of the cell walls of *B. bifidum* and demonstrated a particular type of murein (Orn-Ser-Asp type) which was typical for *B. bifidum* and which was not found in any other species of *Bifidobacterium*.

By a different approach Kandler (76) in 1970 studied the murein types in the cell walls of *Bifidobacterium* and found agreement with the species proposed by Reuter (132). Kandler suggested, therefore, that murein characterization may be a valuable criterion for the separation of species within the genus *Bifidobacterium*. Differing phospholipid composition of several strains of *Bifidobacterium* led Exterkate et al. (32) to propose that these differences can also be employed in classifying members of genus *Bifidobacterium*.

As can be seen from the schemes presented so far, although other factors are taken into consideration, the use of carbohydrate fermentation patterns remains the main basis for species designation. Scardovi et al. (150) noted the taxonomic uncertainties created by such a system and noted that further separation of the species into subspecies or biotypes would only add to the confusion. In a previous report Scardovi et al. (146) described three types of electrophoretic behavior of fructose-6-phosphate phosphoketolase in various strains of *Bifidobacterium*. In this study it was found that the R_f of the enzyme did not correspond with the name of the species, but with the host of the organism. This observation suggested divergent evolutionary pathways in varying habitats of *Bifidobacterium*. In addition, by DNA-DNA hybridization competition studies, Scardovi et al. (145, 151) and Crociani et al. (19) reported the genetic relatedness among some proposed species of *Bifidobacterium* from the rumen of cattle and the intestines of honey bees. Scardovi et al. (150) in 1971 presented a summary chart of the recognized species and biotypes of *Bifidobacterium* along with the biochemical and physiological characteristics of each type. This chart included all the species and biotypes mentioned so far, and the following additional species were included: *B. asteroides*, *B. suis*, *B. ruminale*, *B. globosum*, *B. indicum*, and *B. coryneforme*, for a total of 34 species and biotypes. The additional species were of nonhuman origin. *B. ruminale* and *B. globosum* were first described by Scardovi et al. (149) in 1969 and were isolated from the bovine rumen. *B. asteroides*, *B. coryneforme*, and *B. indicum* from the alimentary tract of honey bees were first described by Scardovi and Trovatelli (148) and were possibly related to some of the organisms described earlier by White (194). *B. suis* was isolated by Matteuzzi et al. (96) in 1971 from the feces of pigs. Scardovi et al. (150) noted that *B. bifidum* had a distinct fermentation pattern in contrast to other species which had patterns resembling each other. They studied DNA homology relationships of 179 strains of *Bifidobacterium* isolated from man, bovine rumen, and other animal sources. These workers found all of the strains of *B. bifidum* to be homologous and also found *B. bifidum* and *B. bifidum* var. *pennsylvanicus* to be homologous with each other. Little similarity was found between *B. bifidum* and other species of *Bifidobacterium*. The authors expressed the opinion that the validity of this species is well established. They proposed also that *B. infantis*, *B. liberorum*, and *B. lactentis* be merged into the single species *B. infantis* on the basis of their homology studies and their evaluation of the fermentation patterns of these three species. Likewise, they suggested that *B. breve* and *B. parvulorum* be merged into a single species, *B. breve*. They also found *B. rumenale* and *B. thermophilum* to be similar. They noted similarities between *B. Pseudolongum* and *B. globosum*, but they did not feel that they had enough evidence to recommend that these species be merged. Several of the previous biotypes associated with these species were eliminated from their classification (Table 2). The possibility of the existence of three additional species, "dentium," "catenulatum," and "angulatum," was noted, but the authors thought that more evidence was needed to establish species status of these organisms.

DNA homology patterns, as employed by Scardovi et al. (150), appear to aid materially in identifying species, This methodology is a significant advance in determinative bacteriology and should help resolve much of the confusion previously encountered when species differentiation of *Bifidobacterium* was made principally

on carbohydrate fermentation patterns.

The latest taxonomic scheme for the genus *Bifidobacterium* is presented in the 1972 edition of the *Anaerobic Laboratory Manual* by Holdeman and Moore (67). This scheme is based mainly on the proposals made by Scardovi et al. (150) with the following modifications. *B. infantis* is divided into three subspecies: subspecies *infantis*, subspecies *liberorum*, and subspecies *lactentis*. In addition, a fourth category, "*B. infantis*—other," is included to give recognition to subspecies intermediate to their recognized subspecies. *B. adolescentis* is divided in groups A, B, C, and D on the basis of the work of Reuter (132). In this scheme, several nonhuman species designated by Scardovi et al. (150) are not included (Table 2). The species "*dentium*" is included as a separate category. In addition to the species previously mentioned, two additional species are recognized: *B. cornutum* and *B. eriksonii*.

Bifidobacterium cornutum was first described by Distaso (29) in 1912 as *Bacillus cornutus*. This was apparently the same organism described 7 years later by Casstellani and Chalmers (17) as *Bacteroides corntus*. Prévot (126) in 1938 was the first to classify this organism as a *Bifidobacterium* and assigned the name *B. cornutum*. This organism is found in the mouth and intestines of man and is frequently found in the horse. *B. eriksonii* was first described by Georg et al. (38) in 1964. These workers isolated two organisms, one from human pleural fluid and the other from a lung abscess, and designated them as strains X407 and X573. The following year Georg et al. (39) designated these organisms *Actinomyces erikosonii*. Holdeman and Moore (67) placed this organism in the genus *Bifidobacterium* in 1972.

In summary, the major classification schemes have been presented here and summarized in table form. It can be seen from Table 2 that the initial designations presented by Eggerth (31) and Dehnert (25) consisted of a number notation system only. The Dehnert classification was the first to divide the group of bifidobacteria into several groups on the basis of carbohydrate fermentation. Reuter (132) expanded this classification and gave actual species designation to many of the organisms. A period of naming additional species followed until the work of Scardovi et al. (150), in which DNA homology studies supported the elimination of some of these designations and made one of the first serious attempts to place the taxonomic classification of the bifidobacteria on a more concrete basis. This was followed by the latest scheme presented by Holdeman and Moore (67) which demonstrates the trend of decreasing the number of species. It is our opinion that the work of Scardovi is a significant advance in the methodology for the classification of bifidobacteria and that no new species should be accepted solely on the basis of a unique carbohydrate fermentation pattern.

Identification

Definitive identification of *Bifidobacterium* in the routine laboratory is difficult to accomplish. Although the usual biochemical and morphological methods can be employed for a tentative identification, the use of a method that identifies the fermentation product is necessary to render a final identification. The method for differentiating *Bifidobacterium* from other anaerobes, as described in the *Anaerobic Laboratory Manual* (67), is currently the most complete method available. The method relies on a gas chromatography analysis of the fermentation products of glucose for a final identification, which limits its use to laboratories equipped with this technique. However, laboratories without this technique can still obtain reliable information by depending on morphology and the usual biochemical methods. It is apparent that members of the genus *Bifidobacterium* can be confused with several related organisms. On primary isolation or in smears of feces the organism cannot always be differentiated from members of the *Actinomyces*, *Corynebacterium*, *Lactobacillus*, *Propionibacterium*, *Eubacterium*, *Butyribacterium*, *Ramibacterium*, *Cillobacterium*, and *Clostridium*. However, when the organisms are subcultured onto media, such as the one described by Norris et al. (112), bifidobacteria demonstrate typical bifid morphology which enables the experienced observer to eliminate almost all of the related gram-positive anaerobic organisms. Most strains of *Bifidobacterium* produce easily distinguishable porcelain white colonies on this medium which further aids in their differentiation from related genera. Almost all of the biochemical tests used for the differentiation of *Bifidobacterium* from related organism are negative or nonreactive. Until a wider spectrum of biochemical characteristics is devised, the identification of *Bifidobacterium* will remain difficult. One of the more practical approaches to a tentative differentiation of bifidobacteria from related anaerobic gram-positive nonsporeforming bacilli is the scheme presented by Moore and Holdeman (104). This method relies on a morphological scheme initially limited to the possibilities within the

group of organisms mentioned above. These authors place *Bifidobacterium* in the same morphological group as *Eubacterium*, *Lactobacillus*, and *Cillobacterium*. Members of *Clostridium* are eliminated by testing for lecithinase activity. Lack of catalase activity differentiates *Bifidobacterium* from *Corynebacterium* and *Propiniobacterium*. The differentiation among members of the actinomyces is more difficult but can be made in general by the thicker cell wall and more rapid growth of *Bifidobacterium*. By employing methods such as these, one can usually identify the typical *Bifidobacterium* and rely on reference laboratories for the final identification of the more unusual strains that may be encountered. It is hoped that in the future more definitive and practical biochemical methods will be developed to make possible an accurate identification of members of the genus *Bifidobacterium*.

ECOLOGY

Occurrence

Soon after Tissier described *B. bifidus* in the stools of breast-fed infants, it was recognized that the organisms became predominant in the stools between days 4 and 7 after birth. Although *L. acidophilus* was the most numerous organism in the stools of bottle-fed infants, bifidobacteria were also found in smaller numbers. It was believed that bifidobacteria were present in small numbers in stools of adults, but they were difficult to isolate because of the lack of a suitable culture medium or method of reducing oxygen tension. For the next half century little progress was made in extending these observations. When more selective culture media became available, interest in the occurrence and distribution of bifidobacteria was revived.

Mata et al. (93) reported the average figures for the incidence of bifidobacteria in the feces of breast-fed infants to be approximately 10^{11}, in weanlings 10^{10}, and in adults 10^{9} organisms per g (wet weight). The figures expressed by these authors are in general agreement with those expressed earlier by Gyllenberg and Roine (49), Smith and Crabb (156), Zubrzycki and Spaulding (202), Weijers and van de Kamer (186), and Werner (191). They are somewhat higher than those reported by Kalser et al. (74), Werner and Seeliger (192), Mossel (107), and Gorbach et al. (43). In a study of intestinal flora in a rural area of Guatemala, Mata and Urrutia (94) reported that bifidobacteria appeared on the first day of life in only a few infants. One-third of the babies they studied had these bacteria on the second day of life. By the end of the first week all infants had them in concentrations ranging from 10^{9} to 10^{11} organisms per g of feces. These workers also reported that *Bacteroides* and *Veillonellae* were not frequently found in the stools of breast-fed neonates, although their concentration ranged from 10^{8} to 10^{11} when found. When 12 breast-fed infants were studied throughout the first year of life, bifidobacteria continued to be the most numerous bacterium, amounting to 10^{10} to 10^{11} organisms per g of feces. With food supplementation the anaerobic gram-negative bacilli became more numerous and eventually outnumbered other bacterial groups. Mata and Urrutia (94) summarized their data by stating that nearly 100% of all bacteria cultured from the stools of breast-fed infants were bifidobacteria. During weaning there was a decrease by 1 log and a proliferation of *Bacteroides*. In adults, *Bacteroides* outnumbered all other groups. Although this study was a valuable aid in estimating the relative occurrence of various groups of intestinal organisms and their variation with age and other factors, no attempt was made at a finer classification of members of the genus *Bifidobacterium*. These organisms were simply referred to as the bifidobacteria group. There is a definite need for such information based upon one of the recent classification schemes.

Miller (99) reported on the fecal flora of seven Eskimo children. He reported *B. adolescentis* in concentration of 10^{8} to 10^{11} per g in all the specimens studied and noted that in one case members of the genus *Bifidobacterium* exceeded those of the *Bacteroides* group. In a study of the intestinal flora of adults, Moore et al. (103) reported that in some cases members of *Bifidobacterium* outnumbered *Bacteroides*, thus confirming the observations of some earlier workers on this point. Significant differences in incidence of bifidobacteria among individuals and possibly among certain restricted populations are evident.

An investigation of variation in the incidence of bifidobacteria among newborns was carried out in our laboratory (unpublished data). In the course of investigations, we noted fewer bifidobacteria in newborns in a large, urban university hospital than in a suburban hospital in the same area. In the past, bifidobacteria could readily be cultured from the stools of breast-fed infants at the Hospital of the University of Pennsylvania. Recently, difficulty was experienced in culturing these organisms from the stools of infants in the nursery. Gram-stained spreads of feces of breast-fed infants, 3 to 4 days old, were examined. Of 61 breast-fed infants

studied, only 20% had a significant number of gram-positive nonsporeforming bacilli. A similar study was conducted at a suburban hospital. By contrast, two-thirds of 21 breast-fed babies had significant quantities of these organisms. An explanation has not been found, although some unidentified environmental factor is probably responsible for the difference in incidence. Experiments such as these indicate that, although there is general agreement on the overall occurrence of the bifidobacteria, there may be significant variation among various groups within a particular population.

Dehnert (25, 27) reported that one biochemical type, Dehnert's group IV (*B. infantis*), predominates in the stools of breast-fed infants. Seeliger and Werner (154) reported that Dehnert's group III (a subspecies of *B. infantis*) was predominant in Bonn (West Germany), although group IV was also present. Petuely and Linder (121) noted the predominance of Dehnert's group IV in the stools of breast-fed infants. Werner (191) also reported the prevalence of Dehnert's group IV in breast-fed infants and stated that group IV has been found only in the stools of breast-fed infants or of infants fed maternal milk together with cow's milk. He confirmed the fact that this group has not been found in the stools of adults. Werner also reported that Dehnert's group I (*B. bifidum*), III (*B. breve*), and V (*B. adolescentis* and *B. longum*) were found in adult stools and noted the presence of groups III and V in the human appendix. Werner concluded that cooperative research in different parts of the world is needed to widen our knowledge about the global ecology of different species of *Bifidobacterium* and particularly of the source and mode of transmission of those in Dehnert's group IV.

Origin and Mode of Transmission

Several proposals have been made concerning the source of initial colonization of the newborn with bifidobacteria. Some early workers reported the isolation of bifidobacteria from the vagina: Blaurock (13), Roufogalis (141–143), and Montagna and Cataldi (101). Harrison et al. (61) studied the incidence of bifidobacteria in the vaginas of normal nonpregnant women and in women in the second and third trimesters of pregnancy. They noted a significant increase in incidence of the organism at term. Although these workers found similar cultural characteristics between the vaginal strains isolated from the stools of breast-fed infants, they were not successful in conclusively demonstrating the antigenic relationship between these two groups of organisms. They suggested that the occurrence of bifidobacteria in the stools of adults makes fecal contamination a possible source, either directly at the time of delivery or indirectly from other parts of the mother's body after birth.

Cataldi and Müller (18) expressed the view that colostrum and the skin of the breast were probable sources of bifidobacterium, particularly for infants born by Caesarean section. They reported the recovery of the organism from colostrum in 22% of the cases and also reported recovering the organism in 14% of the cases from the skin of the maternal breast immediately after delivery. Wyatt and Mata (196) found an average of 3×10^4 bacteria per ml in colostrum. The presence of lactobacilli was demonstrated in 17 of 51 samples of colostrum by them, but bifidobacteria were not specifically identified. Stolte (160) doubted that the infant acquired the organism by swallowing, because she could not demonstrate the presence of bifidobacteria in the mouths of newborn infants nor in the milk or about the breasts of the mother. She surmised that the portal of entry was the anus; however, the conclusion rests on the assumption that failure to isolate the organism from the mouth eliminates swallowing of the organism as a means of colonization.

It should be noted that (i) none of these studies has definitely determined the main source of colonization of the infant bowel with bifidobacteria; (ii) the identity of the infant's strain with that of the mother has not been established; and (iii) these studies were conducted prior to the proposal and acceptance of the current classification scheme presented in the previous section. With the use of more refined methods of isolation and of classification, it is hoped that a better understanding of the pathways of colonization of the infant intestine with bifidobacteria will be obtained in the future.

Interaction with Other Organisms

In an attempt to explain the significance of bifidobacteria in humans, several investigators studied the effects of this organism on other microorganisms. One of the first reports was that of Rose and György (137). These workers reported an inhibitory effect of *L. bifidus* var. *pennsylvanicus* (*B. bifidum*) on other strains of bifidobacteria and on *E. coli*. Whether bifidobacteria in the intestine of the infant can likewise inhibit other bacteria has not been determined.

Wagner and Starr (183) employed germfree

mice in the study of the interaction of bifidobacteria and *Salmonella typhimurium*. Colonies of animals mono-associated with either *S. typhimurium* or bifidobacteria in the intestine were first established. When mice mono-associated with bifidobacteria were pooled with mice mono-associated with the *S. typhimurium* group, the former group rapidly picked up the *S. typhimurium*. A di-associated state was thus established. However, there was a precipitous drop in the bifidobacteria population of the di-associated mice. In the mice from the original *Salmonella* population there was a low-level uptake of bifidobacteria, but after 14 days bifidobacteria were no longer recovered from these mice. There was no reduction in the number of the original *Salmonella* population. Although these workers were the first to use germfree animals in studying the interaction of bifidobacteria with other bacteria in vivo, their findings are not conclusive in determining that bifidobacteria in the colon protect the animal against infection from intestinal pathogens.

Mata et al. (95) studied *Shigella* infections in breast-fed Guatemalan neonates. Of 210 babies born in a village, 109 were breast-fed. Only four of these infants developed *Shigella* infections, a rate which was significantly less than the infection rate of the total population. Bifidobacteria were the predominant organism in the flora of the breast-fed babies. Of the four *Shigella*-infected infants, one was exclusively breast-fed and had a transient and a synptomatic infection. The infection was asymptomatic, but persistent, in two children receiving food supplementation. This infection was associated with severe diarrhea. These authors concluded that the intestinal microflora in breast-fed children favors resistence to *Shigella* infection or elimination of the agent when it is acquired. Mata and Urrutia (94) also studied enteropathogenic *E. coli* in breast-fed infants from a rural area of Guatemala. The authors noted no infections with enteropathogenic *E. coli* in the breast-fed neonates and concluded that the predominance of bifidobacteria was responsible for the low incidence of enteropathogenic *E. coli*.

Haenel (59) noted a reduction or absence of putrefactive bacteria such as *Bacteroides*, *Veillonella*, *Clostridium*, *Proteus*, and others in the stools of breast-fed infants. He attributed the inhibition of these organisms to unknown properties of human milk as well as to certain environmental conditions. The possibility of bifidobacteria playing an important role in inhibition was not pursued.

The viral inhibitory effect of bifidobacteria has been given little attention. Mayer (97) has presented some evidence for the inhibition of enteric viruses by bifidobacteria. He concluded that the disappearance of the bifidobacteria from the intestine is an indication that the infant has become more susceptible to enteric viral infections. Tassovatz and Kragouyevitch (164) and Damjanović and Radulović (24) have also presented data to support this contention. However, these experiments do not appear to be conclusive. Several experiments were conducted in our laboratory (unpublished data) to determine whether bifidobacteria, or one of their metabolites, produce interferon or some other antiviral substance. Monolayers of HeLa cells, known to be capable of producing interferon, were exposed to filtrates of broth cultures, adjusted to pH 7.0, of several strains of bifidobacteria. After overnight exposure of these monolayers to appropriate dilutions of the filtrates, the monolayers were challenged with a dilution of vesicular stomatitis virus (VSV) that produces a 50% cytopathogenic effect in 24 h. The experiment was attempted several times with VSV and the Mahoney strain of polio virus. No significant reduction in cytopathogenic effect was observed in any of these attempts.

Several attempts were made to demonstrate antiviral properties of a polysaccharide produced by the Jackson mucoid strain of bifidobacteria. Both purified and crude preparation of this polysaccharide were injected intravenously twice a day for 3 days into rabbits. The sera of these rabbits failed to show any antiviral activity when tested in the HeLa-VSV system described above.

In summary, evidence has been presented that strains of bifidobacteria may exert an inhibiting effect on one another and on other bacteria both in vitro and in vivo. The production of organic acids by the organism increases the hydrogen ion concentration in the growth medium. The increased acidity is probably inhibitory to some other bacteria. The organic acids may also exert a direct, toxic effect on other bacteria. György expressed this conclusion in several reports and suggested that fermentation products of bifidobacteria, lactate, acetate, and formate, may be directly toxic for other bacteria (51, 52, 55).

Effects of Bifidobacteria on the Host

Barbero et al. (3) studied the physiological effects of the intestinal flora on human infants. These authors confirmed the well-publicized fact that stools of breast-fed infants are more

acidic than are the stools of formula-fed infants. Stools of 236 infants fed exclusively either human milk or cow's milk (or one of its derivatives) were studied. Some babies from each group were intubated to study conditions at various locations in the intestinal tract. The duodenum was found to be relatively sterile. Bacteria were found in the ileum, and marked bacterial proliferation was noted in the cecum. The pH was highest in the cecum and tended to fall in the distal portion of the colon. The average pH in the stools of breast-fed infants was 5.5, and in formula-fed infants the average was 6.7. Barbero et al. concluded that low pH in breast-fed infants was due to the greater amounts of acid produced by bifidobacteria. These authors also found reducing sugars in varying amounts throughout the intestine. A low pH of the stools appears to be the only effect which, at this time, can be attributed to the presence of large numbers of bifidobacteria in the colon.

Muting et al. (108) induced a bifidobacteria intestinal flora in 20 patients with liver disease and noted a significant decrease in blood ammonia, free serum phenol, and free amino nitrogen. A decrease of free phenols and free amino acids in the urine was also noted. These authors concluded that bifidobacteria significantly contributed to these physiological changes and aided in the recovery of the patients.

Gall (36) noted that the intestinal bacteria have been reported to affect natural resistence to infection and have been implicated in the metabolism of cancer, serum proteins, cholesterol, hormones, vitamins, and the incidence of caries. Her study of the anaerobic intestinal flora indicated that (i) many of the major processes associated with digestion, including the metabolism of certain carbohydrates, fats, and proteins, and the production of certain B vitamins, can be carried out by these organisms; (ii) that symbiotic relationships may be important in maintaining the normal balance of microflora in the gut; and (iii) that organisms may exert a deleterious effect on the host by the production of cholesterol or by the formation of toxic amines by the decarboxylation of certain amino acids.

Savage (144), in summarizing the recent information on colonization of the gastrointestinal mucosa by bacteria, concluded that interaction between bacteria and the mucosa may be important in mammalian physiology and in the resistence of animals to certain infectious diseases. Donaldson (30) and Smith and Crabb (156) have made preliminary studies on the physiology and development of the intestinal flora in man and animals, but there has been no specific study of this nature on bifidobacteria.

It is evident from this discussion that the significance of bifidobacteria in the intestinal tract of man has not been defined. It appears that techniques with gnotobiotic animals, such as those employed by Syed et al. (163) and Gibbons et al. (40), will be useful in studying the physiological effects of bifidobacteria on the host. Difficulties in establishing pure cultures of bificobacteria and other anaerobes in the intestine were experienced by these authors, however, and have not yet been resolved. Studies on the effects of the bifidobacteria on the host outside of the intestinal tract has received little attention. Harrison and Hansen (60) have presented the possibility of some strains of bifidobacteria as secondary invaders in livers of turkeys, and *B. eriksonii* has been isolated from pleural fluid and a lung abscess (38). Thus, the organism appears to have possible pathogenic significance when isolated from sources other than the intestinal tract.

CONCLUDING REMARKS

The occurrence of bifidobacteria as the predominant organism in the stools of breast-fed infants was intriguing to investigators who had difficulting in isolating and maintaining the organisms in the laboratory. Effects at classification were impeded until the availability of nutritionally more appropriate culture media permitted more detailed studies. At present, the acceptance of the distinct genus *Bifidobacterium* appears to be justifiable on morphological, biochemical, and serological grounds. A number of species in this genus are now recognized, and it seems likely that more will be identified in the future.

The peculiar, if not unique, pleomorphic features of bifidobacteria have been studied but have not been adequately explained on biochemical grounds. Structural features seen by light microscopy have not been examined extensively by electron microscopy. Future research, it is hoped, will elucidate the biochemical pathways which are responsible, on the one hand, for keeping the organism in the curved-rod bifid phase and, on the other hand, for maintaining it in the compound-branching phase. The relationship of branching to a possible life cycle in the organism and to adaptation to an unfavorable environment needs further study. An understanding of the mechanism of branching in bifidobacteria might aid in elucidating branching in other microorganisms and also in higher forms of life.

The ecological significance of bifidobacteria in infants has attracted attention, but in spite of considerable research the organisms have not been shown to elaborate antimicrobial substances. It is possible, in the light of empirical observations on the better health of breast-fed infants, that bifidobacterium aids in the better nutrition of infants and, thus, indirectly contributes to a greater resistance to infection. As better techniques for study are devised, more definitive information on the importance of the bifidobacteria as part of the intestinal flora will be obtained. Now that it appears that bifidobacteria-like organisms occur elsewhere in nature, more research on related organisms should prove to be scientifically productive and should expand knowledge of the ecological significance of this organism in nature.

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