Purification and Some Properties of Glutamine Synthetases from *Bifidobacteria*

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Glutamine synthetases [L-glutamate; ammonia ligase (ADP forming), EC 6.3.1.2] were purified, respectively, 570-, 220- and 80-fold with 12, 5 and 0.5% yields from cell-free extracts of *Bifidobacterium bifidum* a, *B. breve* 203 and *B. pseudolongum* a. The purification procedure consisted of cell disruption, ammonium sulfate fractionation and column chromatographies on DEAE-cellulose, Sepharose 6B and hydroxylapatite. The purified enzymes were judged to be homogeneous on polyacrylamide disc gel electrophoresis. They had similar molecular weights of about 500,000. The optimum pH was 6.0~6.5 for their biosynthetic reactions with 30 mM Mg^{2+} , and 6.5 for their γ -glutamylhydroxamate forming (transferring) reactions. The K_m values for L-glutamate, ammonia and ATP in their biosynthetic reactions were, respectively, 18~25 mM, 0.56~0.69 mM and 1.5~2.0 mM with 30 mM Mg^{2+} as a cofactor. When 7.5 mM Mn^{2+} was substituted for 30 mM Mg^{2+} , the K_m for L-glutamate decreased by 8.6~23-fold. The optimum pH for the biosynthetic reactions and the susceptibility of the enzymes to some metabolites also varied with the kind of divalent cation added.

Bifidobacteria, gram-positive anaerobes first isolated from a breast-fed infant by Tisser,¹⁾ are the predominant organisms in the intestinal flora of humans and some other animals.²⁾ In contrast with some intestinal bacteria which produce noxious substances for the host, bifidobacteria are supposed to take part in reducing putrefactive bacteria and their toxic metabolites in the intestine. Therefore, the organisms have been used in the treatment and prophylaxis for some diseases in humans and animals.^{3~5)}

We have investigated the enzymatic features of sugar metabolism in bifidobacteria.^{6~9)} However, there is little information available on their nitrogen metabolism, which might be related to their functions in the intestine. Our previous paper¹⁰⁾ on the distribution of glutamine synthetase and glutamate dehydrogenase, which might participate in ammonia assimilation by *Bifidobacterium bifidum* a, emphasized the need for further characterization

of highly purified preparations. The present paper deals with the isolation and properties of glutamine synthetases from *B. bifidum* a (human origin), *B. breve* 203 (human origin) and *B. pseudolongum* a (pig origin).

Glutamine synthetases [EC 6.3.1.2] have been found and studied in a variety of bacteria,^{11~18)} yeast,¹⁹⁾ algae,^{20,21)} higher plants²²⁾ and animals.^{23,24)} Stadtman and Ginsburg²⁵⁾ and Holzer and Duntz²⁶⁾ concluded that glutamine synthetases are controlled in gram-negative enteric bacteria by the following complex of mechanisms: (1) repression of enzyme synthesis by alteration in the nitrogen supply to the cell, (2) modulation of enzyme activity in response to divalent cations, (3) feedback inhibition by end products, (4) covalent modulation by adenylation, and (5) energy charge. The properties of the bifidobacterial enzymes were discussed mainly from the standpoints of (2) and (3).

MATERIALS AND METHODS

Microorganisms and cultivation. *B. bifidum* a and *B. pseudolongum* a were kindly supplied by Dr. Mitsuoka (University of Tokyo and The Institute of Physical and Chemical Research). *B. breve* 203 was isolated from human feces in our laboratory and identified by the method of Mitsuoka.²⁷⁾ A colony of each organism that appeared on a BL-agar plate with the steel wool method²⁷⁾ was inoculated into 5 ml of Briggs Liver broth²⁷⁾ and then incubated at 37°C for 3 days according to the same method. The culture was then transferred to 500 ml of the medium described previously¹⁰⁾ in a 500-ml Sakaguchi flask, followed by incubation for 3 days at 37°C. The resultant culture was inoculated into 30 liters of the medium in a 30-liter jar fermentor and then incubated at 37°C for 2 days with stirring at 50 rpm under an atmosphere of CO₂. The cells were harvested by centrifugation (10,000 × *g*) and stored at -20°C.

Assay. The enzyme activity was measured in three reactions. (1) Transferring reaction: a mixture (1.0 ml) containing 100 mM imidazole-HCl buffer (pH 6.5), 35 mM L-glutamate, 10 mM hydroxylamine-HCl, 1.0 mM MnCl₂, 0.4 mM ADP, 10 mM potassium arsenate and 0.05 ~ 1 unit of the enzyme was incubated at 37°C for 10 min, and the reaction was terminated by the addition of 2 ml of an acidic FeCl₃ solution (10% FeCl₃: 24% TCA: 6N HCl: H₂O = 8:2:1:13). γ -Glutamylhydroxamate formed was determined colorimetrically at 540 nm. (2) Biosynthetic reaction: a mixture (1.0 ml) containing 100 mM imidazole-HCl buffer (pH 6.5 for the *B. bifidum* a enzyme, and pH 6.0 for the *B. breve* 203 and *B. pseudolongum* a enzymes), 50 mM L-glutamate, 25 mM NH₄Cl, 7.5 mM ATP, 30 mM MgCl₂ and 1 ~ 4 units of the enzyme was incubated at 37°C for 10 ~ 30 min. The reaction was terminated by the addition of 1 ml of 3N H₂SO₄ and then the inorganic phosphate liberated was determined by the method of Fiske and Subbarow.²⁸⁾ (3) Synthetic reaction: a mixture (1.0 ml) containing 100 mM acetate buffer (pH 5.5), 50 mM L-glutamate, 25 mM hydroxylamine-HCl, 7.5 mM ATP, 30 mM MgCl₂ and about 1 unit of the enzyme was incubated at 37°C for 30 min. The reaction was terminated and the product determined by the same procedures as for the transferring reaction.

One unit of enzyme activity was defined as the amount of enzyme forming 1 μ mol of γ -glutamylhydroxamate per 10 min in the transferring reaction. Specific activity was expressed as units per mg protein.

Protein was determined by the method of Lowry *et al.*²⁹⁾ with egg albumin as a standard. It was also determined from the absorbance at 280 nm of eluates obtained on column chromatography.

Purification of glutamine synthetases. All procedures were carried out at 0 ~ 15°C.

Step 1. Preparation of a cell-free extract: cells of *B.*

bifidum a (about 800 g wet weight) from 300 liters of culture broth were suspended in 0.3 M acetate buffer (pH 6.5) containing 20 mM EDTA and then disrupted with a Kaijo Denki ultrasonic oscillator (20 kHz, at 0 ~ 15°C for 1 hr). The cell debris was centrifuged off at 12,000 × *g* for 30 min, and the supernatant was dialyzed at 5°C for 48 hr against 0.05 M acetate buffer (pH 6.5) containing 3 mM EDTA. The crude extract was used as the starting material for the enzyme purification.

Step 2. Ammonium sulfate fractionation: the extract was fractionated with ammonium sulfate at pH 6.0. The fraction (45 ~ 60% saturation) in 0.05 M acetate buffer (pH 6.0) was dialyzed against the same buffer.

Step 3. 1st DEAE-cellulose column chromatography: the dialyzed solution was applied to a DEAE-cellulose column (5.4 × 42 cm) equilibrated with 0.05 M acetate buffer (pH 6.0). The column was developed by increasing the NaCl concentration stepwise in the buffer, and the enzyme was eluted with the buffer containing 0.3 M NaCl. The active fractions were concentrated by the addition of ammonium sulfate (80% saturation). The precipitate was dissolved in and then dialyzed against 0.05 M acetate buffer (pH 6.0).

Step 4. 2nd DEAE-cellulose column chromatography: the dialyzed enzyme solution was applied to a DEAE-cellulose column (2.2 × 25 cm) equilibrated with 0.05 M acetate buffer (pH 6.0). Elution was performed by increasing the NaCl concentration linearly from 0.15 to 0.5 M. The enzyme was eluted at a NaCl concentration of about 0.3 M, and the active fractions were precipitated by the addition of ammonium sulfate (80% saturation).

Step 5. 1st Sepharose 6B column chromatography: the precipitate dissolved in a small volume of 0.05 M acetate buffer (pH 5.5) was filtered through a Sepharose 6B column (3.2 × 110 cm) equilibrated with the same buffer. The active fractions were pooled and then concentrated with ammonium sulfate (80% saturation).

Step 6. 2nd Sepharose 6B column chromatography: the precipitate obtained was dissolved in 0.05 M acetate buffer and then filtered through the same column as in step 5. The active fractions were concentrated with ammonium sulfate (80% saturation).

Step 7. Hydroxylapatite column chromatography: the concentrated enzyme solution was dialyzed against 0.05 M acetate buffer (pH 5.5) and then applied to a hydroxylapatite column (1.65 × 4 cm) equilibrated with the same buffer. After washing the column with 1 M acetate buffer (pH 5.5) containing 1 M NaCl, the enzyme was eluted with 0.05 M pyrophosphate buffer (pH 5.5).

The glutamine synthetases of *B. breve* 203 and *B. pseudolongum* a were purified by a similar procedure to that for the *B. bifidum* a enzyme with a slight modification: the acetate buffer (pH 5.5, 6.0 or 6.5) used for the *B. bifidum* a enzyme was replaced by imidazole-HCl buffer (pH 6.0) except for in the hydroxylapatite column chromatography step.

Gel electrophoresis. Disc gel electrophoresis of the enzymes was carried out at 5.0 or 7.5% gel concentration according to the procedure of Davis.³⁰⁾

Chemicals. DEAE-cellulose and ATP were purchased from Sigma Chemicals Co. and Sepharose 6B from Pharmacia Fine Chemicals. Hydroxylapatite was prepared by the method of Tiselius *et al.*³¹⁾ Other chemicals were analytical grade commercial reagents.

RESULTS

Purification

The glutamine synthetases of *B. bifidum* a, *B. breve* 203 and *B. pseudolongum* a were purified 570-, 220- and 80-fold with 12, 5 and 0.5% yields, respectively (Table I). As shown in Fig. 1, each preparation gave a single protein band on polyacrylamide gel electrophoresis, but a slight impurity was observed in the *B. bifidum* a enzyme preparation.

TABLE I. PURIFICATION OF GLUTAMINE SYNTHETASES FROM BIFIDOBACTERIA

Preparation	Protein (mg)	Specific activity (units/mg)	Total activity (units)
A) <i>B. bifidum</i> a			
Cell-free extract	29,700	3.9	115,500
Ammonium sulfate (45~60%)	9,370	9.9	92,800
1st DEAE-cellulose	720	93.4	67,400
2nd DEAE-cellulose	88	439	38,700
1st Sepharose 6B	34	1085	36,700
2nd Sepharose 6B	14	1527	21,100
Hydroxylapatite	6	2213	13,300
B) <i>B. breve</i> 203			
Cell-free extract	53,000	3.3	175,000
Ammonium sulfate (45~70%)	19,500	8.3	162,000
DEAE-cellulose	5,600	27.5	154,000
1st Sepharose 6B	1,600	53.1	85,000
Hydroxylapatite	217	341	75,000
2nd Sepharose 6B	11	732	8,100
C) <i>B. pseudolongum</i> a			
Cell-free extract	44,200	13.2	583,000
Ammonium sulfate (45~60%)	7,850	64.6	507,000
DEAE-cellulose	464	198	91,000
1st Sepharose 6B	309	219	67,000
2nd Sepharose 6B	23	739	17,000
Hydroxylapatite	3	996	2,690

Molecular weight

The molecular weights of the enzymes were determined to be about 500,000 by gel filtration on Sepharose 6B with catalase (molecular weight, 230,000), glutamate dehydrogenase (350,000), apoferritin (480,000) and thyroglobulin (669,000) as standard proteins.

Substrate specificity and effects of divalent cations

Table II summarizes the specificities of the enzymes toward substrates and divalent cations in their biosynthetic reactions. The bifidobacterial enzymes were specific for L-glutamate as an amino acid. Hydroxylamine could be substituted for ammonia: the *B. bifidum* a, *B. breve* 203 and *B. pseudolongum* a enzymes showed 59, 13 and 10% reactivity toward ammonia, respectively. Methylamine and ethylamine were slightly effective. ATP was replaceable by GTP in the reaction with the *B. bifidum* a enzyme (15% reactivity) but not in the reactions with the *B. breve* 203 and

FIG. 1. Disc Gel Electrophoresis of Glutamine Synthetases from *B. bifidum* a, *B. breve* 203 and *B. pseudolongum* a.

The enzyme preparations were electrophoresed on polyacrylamide gel at a current of 2 mA for 90 min at pH 8.9. (A) *B. bifidum* a enzyme (10 μ g protein on 5% gel). (B) *B. breve* 203 enzyme (10 μ g protein on 7.5% gel). (C) *B. pseudolongum* a enzyme (30 μ g protein on 5% gel).

TABLE II. SUBSTRATE AND DIVALENT CATION SPECIFICITIES OF GLUTAMINE SYNTHETASES

Substrate analogues were substituted for usual substrates at their concentrations in the biosynthetic reaction mixture. In the divalent cation specificity experiment, 30 mM Mg^{2+} was replaced by divalent cations at concentrations of 3 and 30 mM. Inorganic phosphate was determined.

Substrate and divalent cation	Relative activity (%) of glutamine synthetase from		
	<i>B. bifidum</i> a	<i>B. breve</i> 203	<i>B. pseudolongum</i> a
L-Glutamate	100	100	100
D-Glutamate	0	0	0
L-Aspartate	0	0	0
D-Aspartate	0	0	0
DL- α -Aminoadipate ^a	0	0	0
DL- α -Aminopimelate ^a	0	0	0
Ammonia	100	100	100
Hydroxylamine	59	13	10
Methylamine	5	4	3
Ethylamine	2	4	3
ATP	100	100	100
GTP	15	0	0
UTP	3	4	0
CTP	2	0	0
ADP	2	0	0
AMP	0	0	0
Cations			
Mg ²⁺ (30 mM)	100	100	100
(3 mM)	14	4	10
Mn ²⁺ (30 mM)	250	156	160
(3 mM)	216	201	190
Co ²⁺ (30 mM)	19	6	14
(3 mM)	11	4	11
Others ^b (30 mM)	0	0	0

^a 100 mM.

^b Others: Ca^{2+} , Cu^{2+} , Hg^{2+} , Zn^{2+} .

B. pseudolongum a enzymes.

The bifidobacterial enzymes required Mg^{2+} , Mn^{2+} or Co^{2+} for their biosynthetic reactions, of which Mn^{2+} was the most effective, as is the case for the enzymes from some other bacteria.^{15,16, 32)}

The relationship between enzyme activity and the concentration of Mg^{2+} , Mn^{2+} or Co^{2+} was investigated in mixtures containing various concentrations of ATP (Fig. 2). For Mg^{2+} , the activities of the three enzymes were maximal when Mg^{2+} was in excess of ATP. The activities of the *B. breve* 203 and *B. pseudolongum* a enzymes were inhibited by a higher concentration of ATP. The same

phenomenon was found with Mn^{2+} and Co^{2+}

The activity with Mn^{2+} was generally maximal when the ratio of ATP to Mn^{2+} was 1.0, and decreased with deviation from this ratio. The decrease at a higher concentration of Mn^{2+} , however, was less remarkable than in the case of the enzyme from gram-positive *Bacillus subtilis*.³³⁾

The effect of Co^{2+} varied with the enzyme source. The maximum activity of the *B. bifidum* a enzyme was seen when Co^{2+} was in excess of ATP while those of the *B. breve* 203 and *B. pseudolongum* a enzymes were seen at the ATP- Co^{2+} ratio of 1.0.

TABLE III. ENHANCEMENT BY Co^{2+} OF GLUTAMINE SYNTHETASE ACTIVITY IN THE REACTION MIXTURE WITH A SUBOPTIMAL CONCENTRATION OF Mg^{2+}

The activity in the biosynthetic reaction was assayed in the presence of the metals shown in the table. The activity was expressed as a percentage of that with 4 mM Mg^{2+} alone. The buffers used were 100 mM imidazole-HCl buffer (pH 6.5) for the *B. bifidum* a enzyme and 100 mM imidazole-HCl buffer (pH 6.0) for the *B. breve* 203 and *B. pseudolongum* a enzymes.

Addition of metals	Relative activity (%) of glutamine synthetase from		
	<i>B. bifidum</i> a	<i>B. breve</i> 203	<i>B. pseudolongum</i> a
4 mM MgCl_2	100	100	100
4 mM MgCl_2 + 3 mM CoCl_2	535	326	657
4 mM MgCl_2 + 3 mM MgCl_2	226	281	293
3 mM CoCl_2	100	36	49
7 mM CoCl_2	185	93	114

FIG. 2. Effects of the Concentrations of ATP and Divalent Cations on the Activity of Glutamine Synthetases from (A) *B. bifidum* a, (B) *B. breve* 203 and (C) *B. pseudolongum* a.

The concentrations of ATP and (i) Mg^{2+} , (ii) Mn^{2+} or (iii) Co^{2+} in the biosynthetic mixture were varied as shown in the figure. The enzyme activity is expressed as inorganic phosphate (mm) liberated. Symbols: $\triangle$ — $\triangle$, ATP 3.75 mM; $\bullet$ — $\bullet$, ATP 7.5 mM; $\circ$ — $\circ$, ATP 15 mM.

Stimulation of enzyme activity by Co^{2+}

The activity of the bifidobacterial glutamine synthetases under Mg^{2+} -unsaturated conditions was remarkably enhanced by the addition of a small amount of Co^{2+} , as was

TABLE IV. OPTIMUM pH OF GLUTAMINE SYNTHETASE REACTIONS

The activities of the transferring, synthetic and biosynthetic reactions were assayed under the conditions given under MATERIALS AND METHODS. In the biosynthetic reaction, 30 mM Mg^{2+} was replaced with 7.5 mM Mn^{2+} or 30 mM Co^{2+} . The buffers used were 100 mM acetate buffer (pH 4.0~6.5) and 100 mM imidazole-HCl buffer (pH 5.5~8.0).

Reaction	Optimum pH of glutamine synthetase from		
	<i>B. bifidum</i> a	<i>B. breve</i> 203	<i>B. pseudolongum</i> a
Transferring reaction	6.5	6.5	6.5
Synthetic reaction	5.5	6.5	6.0
Biosynthetic reaction			
Mg^{2+} (30 mM)	6.5	6.0	6.0
Mn^{2+} (7.5 mM)	5.5	5.5	5.5
Co^{2+} (30 mM)	6.5	6.5	6.5

observed with the *Escherichia coli* enzyme³⁴⁾ (Table III). The addition of 3 mM Co^{2+} to biosynthetic reaction mixtures containing 4 mM Mg^{2+} resulted in a 3-7-fold increase in the activity. The stimulation was not due to the increase in ionic strength.

Effect of pH on the enzymes

As shown in Table IV, the reactions of the

bifidobacterial glutamine synthetases proceeded optimally in the acidic pH range of 5.5~6.5. It was noticeable that the pH optimum varied with the divalent cation in the mixture.

The enzymes were most stable at pH 5.0~6.0: the *B. bifidum* a enzyme retained 86% of its initial activity after incubation in 0.1 M acetate buffer (pH 5.5) for 10 min at 60°C, and the *B. breve* 203 and *B. pseudolongum* a enzymes retained, respectively, 50% and 42% in

0.1 M imidazole-HCl buffer (pH 6.0).

Km values

As summarized in Table V, the *Km* values of the enzymes for L-glutamate, ammonia and ATP in the biosynthetic reactions with Mg^{2+} and Mn^{2+} were mostly the same. It was of interest that the bifidobacterial enzymes showed lower *Km* values for ammonia than the enzymes from other bacteria,^{17,18,32,35} and that the value for L-glutamate decreased

TABLE V. *Km* VALUES FOR SUBSTRATES OF GLUTAMINE SYNTHETASES WITH Mg^{2+} OR Mn^{2+} AS THE DIVALENT CATION

The biosynthetic reaction was carried out with 30 mM Mg^{2+} or 7.5 mM Mn^{2+} . The activity with 30 mM Mg^{2+} was assayed at pH 6.5, 6.0 and 6.0 with 100 mM imidazole-HCl buffer for the *B. bifidum* a, *B. breve* 203 and *B. pseudolongum* a enzymes, respectively. The activity with 7.5 mM Mn^{2+} was assayed with 100 mM acetate buffer (pH 5.5). Inorganic phosphate liberated was determined.

Substrate	<i>Km</i> (mM) values of glutamine synthetase from					
	<i>B. bifidum</i> a		<i>B. breve</i> 203		<i>B. pseudolongum</i> a	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
L-Glutamate	25	1.1	21	1.3	18	2.1
Ammonia	0.56	0.77	0.59	0.69	0.69	0.71
ATP	2.0	2.0	1.7	2.3	1.5	2.5

TABLE VI. EFFECTS OF SOME METABOLITES ON GLUTAMINE SYNTHETASE ACTIVITY

The enzyme activity was determined with the mixture for the biosynthetic reaction in the presence of 30 mM Mg^{2+} or 7.5 mM Mn^{2+} . The substances were added to the mixture at the concentration of 8 mM. Other conditions were the same as in Table V. Inorganic phosphate was determined.

Addition (8 mM)	Relative activity (%) of glutamine synthetase from					
	<i>B. bifidum</i> a		<i>B. breve</i> 203		<i>B. pseudolongum</i> a	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
None	100	100	100	100	100	100
Glycine	98	75	100	68	78	59
L-Alanine	94	63	96	61	83	35
L-Threonine	100	96	100	100	91	82
L-Histidine	100	94	99	99	89	89
L-Tryptophan	99	97	100	98	100	100
L-Glutamine	98	91	100	96	74	91
ADP	38	57	18	57	23	48
AMP	83	99	40	83	56	51
Adenosine	10	73	7	56	19	45
CMP	100	98	100	92	82	65
GMP	—	—	93	82	94	64

TABLE VII. EFFECTS OF HEAVY METALS ON GLUTAMINE SYNTHETASE ACTIVITY

The metals were added at a concentration of 5 mM to the biosynthetic reaction mixture containing 30 mM Mg^{2+} or 7.5 mM Mn^{2+} . Other conditions were the same as in Table V.

Metal (5 mM)	Relative activity (%) of glutamine synthetase from			
	<i>B. bifidum</i> a		<i>B. pseudolongum</i> a	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
None	100	100	100	100
Zn^{2+} (sulfate)	71	30	23	59
Ca^{2+} (chloride)	56	35	33	92
Cu^{2+} (chloride)	26	61	15	55
Ni^{2+} (sulfate)	23	27	13	90
Fe^{2+} (sulfate)	15	16	32	37
Hg^{2+} (chloride)	3	70	44	86
Pb^{2+} (acetate)	2	16	—	—

strikingly when 7.5 mM Mn^{2+} was substituted for 30 mM Mg^{2+} .

Effects of some metabolites and heavy metals

The susceptibility of the bifidobacterial glutamine synthetases to some metabolites also varied with the divalent cation (Table VI). Like in the case of the *Bacillus subtilis* enzyme,³⁶⁾ their activities were more inhibited by L-alanine and glycine in the mixture with Mn^{2+} than with Mg^{2+} , while ADP, AMP and adenosine were more potent inhibitors in the reactions with Mg^{2+} than with Mn^{2+} . These findings suggested that the activities might be controlled by feedback inhibition and the energy charge according to the divalent cation present.

The effects of heavy metals on the enzyme activity also differed with Mg^{2+} and Mn^{2+} in the mixture (Table VII).

DISCUSSION

Bifidobacteria are the predominant bacteria in the intestine of humans and some animals, and are supposed to be beneficial as to the host's health because they reduce putrefactive bacteria and their metabolites.³⁷⁾ Biochemical

information on the metabolic activity of nitrogenous compounds might be valuable for consideration of the function of the organisms in the intestine. A preliminary experiment¹⁰⁾ on their glutamine synthetases and glutamate dehydrogenases suggested that these enzymes might participate in ammonia assimilation by these organisms. The present work was undertaken on glutamine synthetase to clarify its role in ammonia assimilation.

The glutamine synthetases from *B. bifidum* a, *B. breve* 203 and *B. pseudolongum* a were similar in many properties, such as molecular weight, specificity and K_m values for substrates, optimum pH, requirement for divalent cations and susceptibility to metabolites, but showed a few differences in the reactivity to GTP (Table II), the effect of Co^{2+} and inhibition by an excess of ATP (Fig. 2). Therefore, it seems that the enzymes of these *Bifidobacterium* species are essentially not different.

Bifidobacterial glutamine synthetases show several characteristic properties compared with other bacterial enzymes. Firstly, as shown in Table IV, they reacted optimally in the acidic pH range of 6.0~6.5 for the biosynthetic reaction with 30 mM Mg^{2+} , while the enzymes of other bacteria mostly have neutral and alkaline pH optima (e.g., pH 7.7 for the *Escherichia coli* enzyme,³⁵⁾ pH 7.2 for the *Bacillus subtilis* enzyme,³³⁾ pH 7~8 for the *Micrococcus glutamicus* enzyme,¹⁸⁾ and pH 8.5 for the *Gluconobacter suboxydans* enzyme¹⁷⁾). Secondly, the bifidobacterial enzymes showed lower K_m values for ammonia than the enzymes of many other bacteria.^{17,18,32,35)} This suggests that they might play a role in the ammonia assimilation by bifidobacteria, which form acetic acid and lactic acid during their growth. Thirdly, their affinity for L-glutamate increased by more than 10-fold (Table V), and their reactivity was enhanced (Table II) when Mn^{2+} was substituted for Mg^{2+} , indicating that Mn^{2+} might play a significant role in the reactions of the bifidobacterial enzymes.

As mentioned above, glutamine synthetases are known to be regulated in enteric bacteria

by a complex of five mechanisms. The present results indicate the possible regulation of bifidobacterial enzymes by divalent cations. The variation of the enzyme activity with the type and concentration of divalent cation in the reaction mixture (Fig. 2) and the activating effect of Co^{2+} with a suboptimal level of Mg^{2+} (Table III) were similar to those observed with the glutamine synthetases from *Escherichia coli*³⁴⁾ and other bacteria,^{17,21)} which are regulated by divalent cations. Furthermore, the changes in such enzymatic properties as optimum pH (Table IV), *K_m* value for L-glutamate (Table V) and susceptibility to some metabolites (Table VI) indicated the role of divalent cations in the regulation. The differences in the inhibitory effects of several heavy metals, depending on whether Mg^{2+} or Mn^{2+} was present in the reaction mixture (Table VII), might imply a conformational change caused by the divalent cation, as observed for enzymes from other sources.^{38,39)}

The results in Table VI at the same time suggest that the enzyme activity might be controlled by feedback inhibition and by the energy charge in the organisms.

Variation of the glutamine synthetase level in the cells in response to the nitrogen source in the culture medium (*i.e.*, the regulatory system of repression–derepression of the enzyme formation) and the adenylylation–deadenylylation system of the enzyme protein, which has been recognized in gram-negative bacteria and found recently in gram-positive *Streptomyces*⁴⁰⁾ and *Mycobacterium*,⁴¹⁾ were not investigated in this study due to the complex nutritional requirements of bifidobacteria. The recent establishment of a defined minimum medium for *B. bifidum* Es 5⁴²⁾ might make it possible to examine them.

In parallel with this study, we are working on the purification and characterization of the glutamate dehydrogenases from the same organisms. This enzyme is assumed to participate in the incorporation of ammonia together with glutamine synthetase.¹⁰⁾ Comparative discussion as to glutamine synthe-

tase and glutamate dehydrogenase will reveal the mechanism of ammonia assimilation by bifidobacteria.

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