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FERROUS IRON UPTAKE BY *BIFIDOBACTERIUM BIFIDUM* VAR. *PENNSYLVANICUS*: THE EFFECT OF METALS AND METABOLIC INHIBITORS

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Abstract—1. Ferrous iron uptake studies in *Bifidobacterium bifidum* var. *pennsylvanicus* were carried out in a well-defined salt solution termed "modified Hanks solution" at both high iron concentrations (LAFIUS conditions) and low concentrations (HAFIUS conditions).

2. Various divalent metals, Mn^{2+}, Zn^{2+}, Ni^{2+} and Cu^{2+}, inhibited iron uptake under HAFIUS conditions in a non-competitive manner, and in a pseudo-competitive manner under LAFIUS conditions. Cr^{2+} had no effect. Co^{2+} inhibited iron uptake competitively under HAFIUS conditions.

3. Metabolic affectors that inhibited iron uptake both under HAFIUS and LAFIUS conditions were: tetraphenylphosphonium chloride, diethylstilbesterol, vanadate, carbonylcyanide-*m*-chlorophenylhydrazone, and a mixture of valinomycin and nigericin. Substances that stimulated iron uptake were KCl, valinomycin, and nigericin.

4. Iron uptake under LAFIUS conditions in piperazine-buffered modified Hanks solution was higher than that in the acetate-buffered solution, and acetate inhibited iron uptake in the piperazine buffer. HAFIUS showed no difference.

5. It is concluded that iron uptake in bifidobacteria is driven by an ATPase-dependent proton-motive force and that both the pH gradient and membrane potential are involved in this process. Mn^{2+}, Zn^{2+}, Ni^{2+}, and Cu^{2+} may be transported via LAFIUS, but not HAFIUS. HAFIUS may transport only Co^{2+} in addition to Fe^{2+}.

INTRODUCTION

Gastrointestinal tract of the breast-fed infant differs from that of the bottle-fed infant in that the microbial flora of the former is composed largely of the fermentative bifidobacteria, whereas that of the latter resembles the flora of the adult (Beerens *et al.*, 1980; Biavati *et al.*, 1984). Bifidobacterial flora in breast-fed infants contributes toward their protection against gastrointestinal and even systemic disease (Welsh and May, 1979). The reason why bifidobacteria are beneficial is because they produce large quantities of acetate buffer, which maintains the pH of the gut at around pH 5. The relatively low pH inhibits the proliferation of pathogenic and putrefactive microorganisms (Bullen *et al.*, 1976). Our laboratory has also proposed that the low pH encourages bifidobacteria to absorb ferrous iron, thus creating an iron-poor environment where iron-requiring pathogens cannot proliferate (Bezkorovainy *et al.*, 1986). Bifidobacteria grow in the gastrointestinal tract of breast-fed infants because human milk contains substances that encourage such growth (György, 1953; Beerens *et al.*, 1980).

Our laboratory has been attempting to elucidate the mechanisms of iron uptake by *Bifidobacterium bifidum* var. *pennsylvanicus*, a human milk requiring strain of *Bifidobacterium bifidum*. These microaerophilic anaerobes take up ferrous iron at pH 5.0 in preference to ferric iron in a saturable and energy-dependent process both from the medium in which they have grown and from a simple salt-lactose solution termed the modified Hanks solution (Bezkorovainy, 1984; Bezkorovainy *et al.*, 1986b). The uptake of iron exhibits a dual mechanism, one operating at low- and the other at high iron concentrations. These have been termed HAFIUS and LAFIUS respectively (Bezkorovainy *et al.*, 1986). We have proposed that the function of a membrane-type ATPase is associated with iron uptake by these microorganisms. This conclusion was based on the action of various metabolic inhibitors and protein modification reagents on the bifidobacteria (Bezkorovainy *et al.*, 1986a; Topouzian and Bezkorovainy, 1986).

This report seeks to clarify several issues. Firstly, since it has been shown that the uptake of iron by bifidobacteria is not mediated by a siderophore-type carrier (Bezkorovainy *et al.*, 1986b), the possibility of an indirect electrogenic pump mechanism for iron transport is explored. Secondly, the possibility that a non-specific divalent cation transport system is responsible for iron uptake is investigated. And thirdly, further differences between HAFIUS and LAFIUS are documented. Since our previous report has eliminated non-specific binding of iron as being responsible for the iron uptake phenomenon observed in bifidobacteria, and has thus established specific energy-dependent transport system as the most likely basis therefore (Bezkorovainy *et al.*, 1986b), the terms "binding" and "uptake" are used interchangeably herein and designate iron accumulation by the bifidobacteria.

MATERIALS AND METHODS

Materials

The preparation of the Norris bacterial growth medium and the modified Hanks solution at pH 5 has been previously described (Bezkorovainy *et al.*, 1986a,b). The modified Hanks medium using piperazine instead of acetate as buffering agent at pH 5.0 was prepared by substituting the sodium acetate by 8.4 g piperazine/l solution and adjusting the pH to 5.0 with concentrated HCl.

Aldrich Chemical Co. (Milwaukee, Wis.) supplied the following chemicals: tetraphenylphosphonium chloride (TPPC), carbonyl-cyanide-*m*-chlorophenyl-hydrazone (CCCP), nickel chloride (II), chromium chloride (II), and cobaltous chloride. Sigma Chemical Co. (St. Louis, Mo), supplied the following: ferrous sulfate, piperazine, manganous chloride, valinomycin, nigericin, diethylstilbesterol, sodium thiocyanate, and the lactate assay kit (Cat. No. 826-B). More common chemicals such as sodium orthovanadate, $ZnSO_4$, $CuSO_4$, Cu_2Cl_2, $MgCl_2$ and others came from Fisher Laboratories (Chicago, Ill.).

Radioactive ferrous iron in the form of $^{59}Fe^{2+}$-citrate, was purchased from ICN Corp. (Irvine, Calif.). It was utilized to label stock ferrous sulfate solutions as previously described (Bezkorovainy *et al.*, 1986a).

Methods

The basic iron uptake experiments have been previously described (Bezkorovainy, 1984; Bezkorovainy *et al.*, 1986a; Bezkorovainy *et al.*, 1986b). In all cases, before adding the radioactively labeled iron solution, the test samples were preincubated at 37°C for 15 min. All incubations with labeled iron described were done for 1 hr at 37°C, since previous kinetic studies have demonstrated that iron uptake is essentially complete after 1 hr (Bezkorovainy *et al.*, 1986a; Bezkorovainy *et al.*, 1986b).

Lactate production during the iron uptake experiments was performed using the Sigma kit designed to perform blood lactate determinations. It is based on the conversion of NAD to NADH by lactate dehydrogenase with the concomitant production of a chromophore at 340 nm. Between 110 and 180 μmol of lactate/ml cell suspension were produced by controls during the incubation times, which contain about 0.6 mg of bacterial dry wt. Lactate production was deemed to be representative of the integrity of the cellular metabolic machinery.

Acid production by bifidobacteria was measured as a function of time in the presence and absence of iron. For this purpose, a jacketed vessel with a capacity of about 15 ml was constructed, which could be maintained at 37°C by circulating water heated by a Haake pump. The cell suspension representing some 24 mg dry bacterial weight in 10 ml of the modified Hanks solution at pH 5.0 (buffered by 0.005 M acetate rather than 0.1 M acetate), with or without iron, was maintained in the jacketed vessel for 20 min, and the pH was continuously back-titrated with 0.1 M NaOH to pH 5.0 with a syringe-type microburet. Since bifidobacteria are anaerobes and possess no oxidative phosphorylation mechanism, no effort to exclude oxygen was made. Controls elaborated between 33 and 55 μmol of protons during this time period. pH was measured with the Radiometer/London Co. pH meter using the expanded pH scale.

RESULTS

The effect of various metals on iron uptake by bifidobacteria

Table 1 summarizes the effects of various divalent metals on the uptake of iron by bifidobacteria both under the HAFIUS and LAFIUS conditions. Inhibitor metal concentrations were chosen at levels that would allow the maintenance of iron uptake levels relatively close to those of the controls. Lactate production was measured in these incubation media, and it is seen that in the most cases, lactate production was not significantly affected by the metals. The various metals had different effects on iron uptakes. Cr^{2+}, Ca^{2+}, and Cu^{1+} had no effect on iron uptake under the conditions reported herein. Other metals under HAFIUS conditions, with the exception of Co^{2+}, showed a non-competitive type inhibitory activity, the case with Ni^{2+} being a typical example (Fig. 1A). Under LAFIUS conditions, again with the exception of Co^{2+}, a rather peculiar behavior was observed, again presented as an example with Ni^{2+} in Fig. 1B. At low iron concentrations, a very profound inhibitory activity was observed, whereas at higher iron concentrations, the two curves seemed to merge. In addition, reciprocal plots in the presence of the inhibitor metal were not straight lines. For lack of another designation, we have termed this behavior of the inhibiting metals as pseudo-competitive. In case of Co^{2+}, a typical competitive behavior was observed under HAFIUS conditions, and a non-competitive behavior was seen under LAFIUS conditions. It thus might be interesting to investigate whether or not bifidobacteria synthesize cobalamin. Monovalent copper and Ca^{2+} appeared to have no effect on iron uptake under LAFIUS conditions, whereas Mg^{2+} was inhibitory as previously reported (Bezkorovainy *et al.*, 1986a). The basis for the inhibitory effects of Mg^{2+} but not Ca^{2+} have not yet been investigated.

The effect of various substances on iron uptake by bifidobacteria

Table 2 summarizes the effects of various metabolic inhibitors, ionophores, and other compounds on iron uptake by bifidobacteria under HAFIUS and LAFIUS conditions. Some of these, such as diethylstilbesterol, vanadate, and CCCP have been used before (Bezkorovainy *et al.*, 1986a). Lactate production was also measured to assess the effects of these compounds on the overall cellular metabolic machinery. Membrane-type ATPase inhibitors, orthovanadate and diethylstilbesterol, were both inhibitory to iron uptake, though the latter also inhibited lactate production and its specificity in regard to ATPase may be suspect in this case. TPPC, which is supposed to discharge the membrane potential (Serrano, 1980), was a potent iron uptake inhibitor. It also inhibited lactate production by the bacteria, though under LAFIUS conditions, the decline in iron uptake was more rapid than that of lactate production as a function of TPPC concentration. Thiocyanate, which can traverse plasma membranes at will and contribute to the discharge of the membrane potential, did inhibit iron uptake without affecting lactate production. However, it is an iron chelator, and its effect on iron uptake may be, at least in part, due to that property (see Topouzian and Bezkorovainy, 1986). CCCP, which discharges proton gradients across membranes, was inhibitory to iron uptake without substantially decreasing lactate production. Large concentrations of K^+ increased iron uptake under LAFIUS but not HAFIUS conditions without affecting lactate production.

Most surprising were the effects of valinomycin and nigericin. Valinomycin is a K^+ ionophore, which

Table 1. Iron uptake by bifidobacteria in the presence of various metals. All incubations were done in the modified Hanks solution at pH 5.0. Iron uptakes by control cell suspension vary from one batch of cells to another

Metal	Metal conc. (mM)	System	Iron conc. (μM)	Iron uptake (n/mol/pellet) With metal	Iron uptake (n/mol/pellet) No metal (control)	Type of inhibition	Lactate production (% control) With iron	Lactate production (% control) Without iron
Mn^{2+}	0.038	HAFIUS	1.9	2.2	2.6	NC		
			3.8	3.2	3.6			
			4.8	3.6	4.3			
			7.6	4.4	5.5			
			9.5	4.8	7.1			
	0.38	LAFIUS	49	27	52	PC	107	—
			97	54	92			
			192	99	129			
			380	146	157			
Zn^{2+}	0.038	HAFIUS	1.9	1.8	3.4	NC		
			3.8	2.8	5.1			
			4.8	3.4	5.6			
			7.6	3.9	7.1			
			9.5	4.0	7.6			
	0.038	LAFIUS	48	11	44	PC		
			96	19	81			
			144	38	110			
			192	43	122			
			380	126	156		90	71
Co^{2+}	0.19	HAFIUS	1.9	0.60	2.3	C		
			3.8	1.1	3.2			
			4.8	1.3	3.6			
			7.6	1.8	4.5			
			9.5	2.2	5.7			
	0.37	LAFIUS	47	7.7	23	NC		
			94	12	45			
			141	19	62			
			188	28	74		100	—
			376	49	102			
Cr^{2+}	0.38	HAFIUS	2.0	2.1	2.2	None		
			3.9	2.8	2.7			
			4.9	3.0	2.9			
			7.8	3.1	3.5			
			9.7	4.0	4.1			
	0.38	LAFIUS	47	40	41	None		
			94	60	61			
			141	76	73			
			187	80	79			
			374	103	95		100	100
Ni^{2+}	0.019	HAFIUS	1.9	0.48	2.3	NC		
			3.8	0.76	3.3			
			4.8	0.79	4.0			
			7.6	1.1	4.5			
			9.5	1.4	7.4			
	0.019	LAFIUS	47	5.4	20	PC		
			94	11	36			
			141	21	45			
			187	30	57			
			374	60	81		96	91
Cu^{2+}	0.0039	HAFIUS	1.9	0.55	2.4	NC		
			3.8	1.2	5.3			
			4.8	1.4	6.5			
			7.6	2.0	9.6			
			9.5	2.0	12			
	0.0039	LAFIUS	47	12	24	PC		
			95	29	44			
			143	42	61			
			190	57	74		80	—
			380	88	103			
Cu^{1+}	0.0037	LAFIUS	47	30	32	None		
			94	48	48			
			141	57	56			
			187	63	63		100	—
			374	72	74			
Mg^{2+}	0.09	LAFIUS	180	64	62	ND		
	0.72		180	64	28			
Ca^{2+}	7.2		180	64	67	None		

NC—non-competitive; C—competitive; PC—pseudo-competitive; ND—not determined.

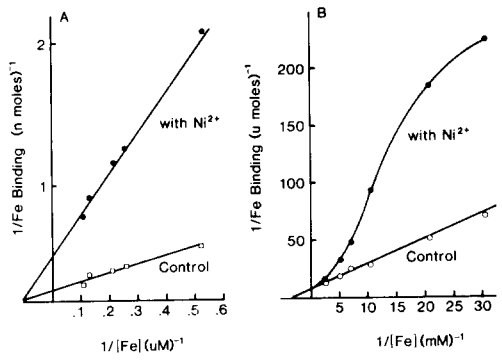

Fig. 1. The effect of Ni^{2+} on the binding of iron by bifidobacteria. Frame A indicates the high affinity system (HAFIUS), and frame B indicates the low affinity system (LAFIUS). Nickel concentrations were 18.6 μM in both cases. All incubations were carried out for 1 hr at 37°C.

would equalize K^+ concentration inside and outside the cell. It is thus said to be capable of depolarizing membranes. Nigericin, on the other hand, short-circuits the proton gradient without disturbing the membrane potential, by exchanging H^+ (outside) with K^+ (inside). Used together, they eliminate both the pH-gradient and membrane polarization. In Table 2, it is seen that both valinomycin and nigericin increased microbial iron uptake, in spite of the fact that nigericin depressed lactate production by about 50%. Used together, iron uptake was severely curtailed. The stimulatory effects of either valinomycin or nigericin were especially dramatic under HAFIUS conditions, where iron uptake of as much as seven times the control was observed in the presence of valinomycin. The reason why combining K^+ with valinomycin actually depresses iron uptake is unclear.

Addition of piperazine but not NH_4^+ to the iron uptake system was stimulatory to iron uptake under

Table 2. Effect of various substances on iron uptake by *Bifidobacterium bifidum* var. *pennsylvanicus*. All data are in percent of control

		Iron uptake				Lactate produced		
		HAFIUS		LAFIUS		With iron		
Substance	Final conc.	Iron con. (μM)	Iron uptake	Iron conc. (μM)	Iron uptake	[Fe]μM	Lactate	No iron
NaSCN	18.5 mM	18.6	100	190	103	190	90	110
	37 mM	18.6	65	190	41			
	74 mM	18.6	23	190	16			
TPPC*	0.09 mM	18.6	58	190	49	190	73	—
	0.18 mM	18.6	51	190	29	190	63	—
	0.72 mM	18.6	22	190	9.3	190	14	14
KCl	47 mM	9.3	108	190	104			
	96 mM	9.3	108	190	124	190	102	—
	192 mM	9.3	111	190	169			
	384 mM	9.3	111	190	210			
DES	49 μM	18.6	113	190	99	—	—	—
	98 μM	18.6	60	190	41	190	37	—
	196 μM	18.6	45	190	25	190	29	19
CCCP	9.8 μM	9.3	105	190	77	—	—	—
	19.6 μM	9.3	90	190	43	—	—	—
	49 μM	9.3	34	190	15	190	90	81
	98 μM	—	—	190	16	—	—	—
Vanadate	392 mM	—	—	190	9.8	190	81	62
	980 mM	—	—	190	8.9			
	95 μM	18.6	33	190	—	—		
	476 μM	18.6	28	—	—			
	952 μM	18.6	25	—	—	—		
Valinomycin	1.8 μM	9.3	294	190	179	9.3	100	108
Valinomycin + KCl	1.8 μM & 90 mM	9.3	60	190	63	190	97	—
Nigericin	0.97 μM	9.3	354	143	150	9.3	64	62
						190	64	55
Valinomycin + nigericin	1.8 μM & 0.97 μM	9.3	33	190	24	9.3	53	52
DMSO†	0.01 ml	9.3	96	190	110	9.3	100	100
Piperazine	0.043 M	9.3	104	190	119			
	0.086 M	9.3	98	190	134	190	111	111
	0.174 M	9.3	73	190	138			
	0.260 M			190	98			
$(NH_4)_2SO_4$	0.75 M	—	—	190	109	—	—	—
Acetate‡	36 mM	9.3	98	190	77	—	—	—
	144 mM	9.3	88	190	65	—	—	—
Acetate + Lactate§	0.086/0.060	9.3	100	190	80	—	—	—
	0.12/0.084	9.3	100	190	80	—	—	—
	0.2/0.13	9.3	125	190	94	—	—	—

*TPPC—tetraphenylphosphonium chloride; DMSO—dimethyl sulfoxide; DES—diethylstilbesterol; CCCP—carbonylcyanide-*m*-chlorophenylhydrazone.

†DMSO was the solvent for all water-insoluble reagents. Maximum quantity used was 0.01 ml per incubation flask (total vol 5.1–5.6 ml).

‡The modified Hanks solution was buffered at pH 5.0 by 0.1 piperazine.

§Mixture of 2 M Na-acetate and 1.4 M lactate at pH 5 was added to simulate end products of bifidobacterial metabolism (Pine, 1977). Control is modified Hanks solution buffered with 0.05 M acetate 0.035 M lactate.

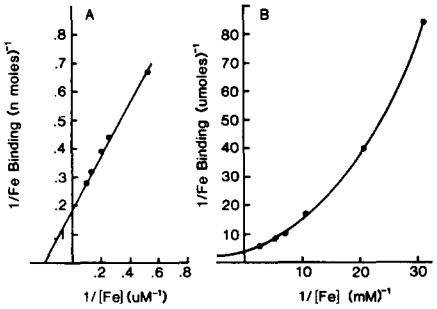

Fig. 2. The effect of iron concentration on iron binding by bifidobacteria carried out in 0.1 M piperazine buffer at pH 5.0. The buffer contains per liter of solution 8.6 g piperazine, 0.4 g KCl, 8 g NaCl, 0.14 g $CaCl_2$, and 2 g lactose adjusted to pH 5.0 with HCl. Frame A indicates the high affinity system (HAFIUS), and frame B indicates the low affinity system (LAFIUS). All incubations were carried out for 1 hr at 37°C.

LAFIUS conditions. In the absence of acetate, at iron concentrations of 190 μm, iron uptake was 77 nmol/pellet in the modified Hanks solution buffered with 0.1 M acetate, and 177 nmol/pellet in the modified Hanks solution buffered with 0.1 M piperazine. Piperazine had a slightly inhibitory effect under HAFIUS conditions. Acetate, used in the piperazine-buffered iron uptake system was inhibitory to iron uptake! Reciprocal plots representing uptake of iron by bifidobacteria in the piperazine buffer system are shown in Fig. 2. It is seen that whereas under HAFIUS conditions, the plot follows a straight line previously observed in acetate-buffered modified Hanks solution, the plot, under LAFIUS conditions, does not give a straight line as seen with the acetate buffer.

Proton changes as a function of the iron uptake process

Bacteria transport many substances across membranes using proton symport systems, as, for example is the case with hexose phosphates (Essenberg and Kornberg, 1975). An attempt was therefore made to determine acid production by bifidobacteria during the iron uptake process. The results are presented in Fig. 3. It shows that at high iron concentrations, there is less acid in the medium than in the controls. This may be interpretated as indicating a proton-iron symport system, however, the stoichiometry of 5–8 protons per atom of iron makes this very unlikely. Moreover, at low iron concentrations, there was a net increase in acidity of the medium compared to the controls. Lactate production remained constant at 125 μmol/ml cell suspension compared to 110 μmol/ml in the control. A complicating factor in these measurements is the fact that bifidobacteria constantly elaborate large quantities of acid as they elaborate acetate and lactate. It is thus necessary to measure small differences between suspensions producing large quantities of acid, which may, of course, introduce unacceptably high errors.

DISCUSSION

Results with the various transition metals indicate that there most probably is not a single common divalent cation transport system in bifidobacteria. The system responsible for transporting iron at low iron concentrations (HAFIUS) may also transport Co^{2+}, but with all other metals tested the inhibitor was of the non-competitive type, indicating that these inhibiting metals do not compete with iron for entry into the cell. In case of LAFIUS, the situation is more complex. If one accepts as fact that the reciprocal plots observed with Mn^{2+}, Zn^{2+}, Ni^{2+}, and Cu^{2+} are indicative of competitive inhibition, albeit not of the classical type, then it may be concluded that these metals may be transported by the same mechanism that transports iron. In experiments performed in the spent Norris medium, inhibition of iron uptake by Zn^{2+} was also deemed to be of the competitive type (Bezkorovainy, 1984).

Several differences were discovered between LAFIUS and HAFIUS, which may be summarized as follows: increasing extracellular $[K^+]$ affects LAFIUS, but not HAFIUS. The latter was also not affected by piperazine, whereas the former was. Acetate did not inhibit iron uptake under HAFIUS conditions, whereas it did under LAFIUS conditions (see Table 2). And lastly, the response to various metal inhibitors was quite different with the two systems (see Table 1).

It is well established that most solutes are driven across the membrane into microbial cells via the proton-motive force generated by primary proton pumps, and mathematically expressed by Mitchell and collaborators as follows (Mitchell and Moyle, 1969):

$$\Delta\rho = \Delta\psi - \frac{2.3\,RT}{F}\,\Delta\mathrm{pH},$$

where $\Delta\rho$ is the proton motive force, $\Delta\psi$ is the membrane potential, and ΔpH is the pH difference between outside the cell (acidic) and inside the cell (alkaline). There are four types of primary proton pumps: those associated with electron transport chains, those driven by bacteriorhodopsin, those dependent on membrane-type ATPase, and those associated with end product efflux. The latter is found in fermentative organisms producing large amounts of such products as lactate (Konings, 1985; ten Brink *et al.*, 1985; Simpson *et al.*, 1983). Bifidobacteria

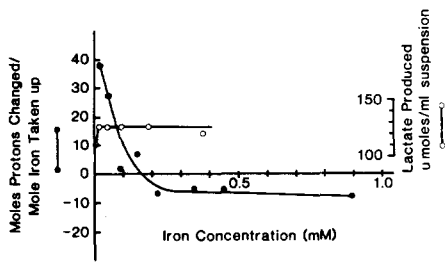

Fig. 3. Production of acid (protons) during the iron binding process by bifidobacteria. Closed circles represent moles of protons either in excess of control (positive side) or in deficit of control (negative side of x-axis) per mole bound. Open circles represent lactate produced as a function of iron concentration. Note: at 0-iron concentration, 110 μmol/ml of lactate was produced.

certainly do not possess an electron transport chain or rhodopsin. However, membrane-type ATPase and end-product efflux-generated proton-motive force are distinct possibilities, especially in view of the fermentative nature of bifidobacteria. It has been shown, however, that in non-growing cells, as is the case herein, at relatively low pH-values and in the absence of an end-product gradient across the membrane, the contribution of end-product efflux to the proton-motive force is minimal (ten Brink *et al.*, 1985). Under the iron uptake conditions reported herein, which occurs at pH 5 and in 0.1 M acetate buffer, acetate gradient across the bifidobacterial membrane is not large if it exists at all. Moreover, since the presence of additional acetate and lactate did not substantially alter iron uptake (Table 2), we may conclude that in acetate buffer the proton-motive force responsible for iron uptake by bifidobacteria is largely ATPase generated. This conclusion is consistent with the effects of such metabolic inhibitors as vanadate, dicyclohexylcarbodiimide (DCCD), and diethylstilbesterol reported herein and elsewhere (Bezkorovainy *et al.*, 1986a; Topouzian and Bezkorovainy, 1986).

Proton-motive force, $\Delta\rho$, consists of two components, the membrane potential $\Delta\psi$, and the pH gradient, and it is of interest to inquire as to which component is primarily responsible for the transport of iron. Proton ionophores, to wit, CCCP, 2,4-dinitrophenol, and CCTPM (Table 2 and Bezkorovainy *et al.*, 1986a) are strongly inhibitory of iron uptake without affecting lactate production (in case of CCCP). On the basis of this information it would be attractive to propose a proton-iron symport mechanism. However, the inconclusive proton-iron stoichiometry studies (Fig. 3) and the results with nigericin, which is supposed to collapse the pH gradient but not $\Delta\psi$, would tend to make this proposal too simplistic. In addition, there are the results observed with KCl, valinomycin, and TPPC, which tend to implicate $\Delta\psi$ and K^+ transport in this process as well. The most curious finding is the inhibition of iron uptake by a mixture of nigericin and valinomycin, where individually the two ionophores resulted in iron uptake stimulation, especially in case of HAFIUS. Thus from the data presented, the conclusion that can be made is that both components of $\Delta\rho$ are important for the iron uptake phenomenon.

The effects of piperazine and acetate on the iron uptake mechanism may be explained as follows: in the piperazine buffer, in the absence of acetate in the medium, a large acetate gradient exists between the cell interior and exterior, thus generating a significant proton-motive force via the end-product efflux mechanism. This complements the proton-motive force generated by the ATPase, resulting in a higher level of iron transport, compared to the situation where the ATPase only is operative. Addition of acetate to the piperazine buffer diminishes the acetate gradient thus diminishing $\Delta\rho$ and iron uptake. The peculiar curvature of iron uptake under LAFIUS condition in the piperazine buffer (Fig. 2B) may indicate that iron inhibits its own uptake at high concentrations under those conditions. In the case of HAFIUS, there was no increase in iron uptake when acetate was replaced by piperazine. This may indicate that the $\Delta\rho$ developed by the ATPase is, in contrast to the LAFIUS conditions, sufficient to drive iron uptake in bifidobacteria at a maximum rate at HAFIUS iron concentrations. In addition, under HAFIUS conditions, there does not seem to be an inhibition of iron uptake by itself (Fig. 2A).

Our studies have thus described a bacterial iron uptake system that does not depend on siderophore-type carriers and which most likely depends on a proton-motive force-associated electrogenic pump or pumps. This system appears to be quite complex requiring the function of both the proton gradient and membrane potential, and exhibits different behaviors in the presence of low amounts of iron (HAFIUS) as opposed to high amounts of iron (LAFIUS).

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