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Amino Acid Transport in *Mycoplasma*

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The uptake of L-histidine by *Mycoplasma fermentans* and L-methionine by *M. hominis* was found to be dependent on temperature and *p*H and to follow saturation kinetics. Several metabolic inhibitors inhibited this uptake. The transport system for L-methionine was highly specific. The L-histidine transport system was less specific, and the uptake was competitively inhibited by L-arginine and L-lysine. L-Histidine accumulated in the intracellular pool of *M. fermentans* at a concentration about 200 times that found in the medium. Efflux of accumulated L-histidine was demonstrated at 37 C, but not at 0 C. The rate of efflux was greatly accelerated by addition of L-histidine to the medium. The findings indicate that the *Mycoplasma* cell membrane contains specific transport systems resembling the permease systems of other microorganisms.

The fact that the minute *Mycoplasma* cells lack many biosynthetic pathways and depend on the supply of many nutrients from the growth medium may indicate the presence of numerous transport systems in these organisms. Since *Mycoplasma* cells are bounded by a cell membrane only (15) and metabolites need not pass through a cell wall, they offer a distinct advantage over bacteria in transport studies. So far, this advantage has not been exploited, and little information is available on transport mechanisms in *Mycoplasma*. Earlier reports indicate that *M. laidlawii* accumulates potassium ions by an energy-dependent mechanism (17) and that acetate accumulation in the intracellular pool of this organism exhibits saturation kinetics (18). Perhaps the most attractive hypothesis concerning the special transport mechanisms of *Mycoplasma* cells is that elaborated by Smith (20). According to this hypothesis, cholesterol and carotenoids, besides their structural function as components of *Mycoplasma* membranes, also act as carriers of glucose and acetate through the membranes. Identification of cholesteryl glucoside and cholesteryl acetate in membranes of fermentative *Mycoplasma* species, of carotenyl glucoside in membranes of *M. laidlawii*, and of cholesterol esterase and β-glucosidase activities in *Mycoplasma* membranes was adduced in support of this hypothesis. Extensive evidence available on the presence of amino acid transport systems in microorganisms has prompted an investigation on amino acid transport in the fermentative *M. fermentans* and the nonfermentative *M. hominis* strains.

MATERIALS AND METHODS

Organisms and growth conditions. M. fermentans 15474 and *M. hominis* type 1, 15056, were obtained from the American Type Culture Collection, Rockville, Md. The organisms were grown statically in 500 ml of a modified Edward medium (13) containing 2% (v/v) PPLO Serum Fraction (Difco). For growth of *M. hominis*, the medium was supplemented with L-arginine to a final concentration of 20 mM. This supplement increased the yield of organisms twofold, as observed by Schimke et al. (19) in other mycoplasmas possessing the arginine dihydrolase pathway. The organisms were harvested by centrifugation (13,000 × *g* for 15 min) after 14 to 18 hr of incubation at 37 C. *M. fermentans* cells were washed once and resuspended in 0.4 M sucrose containing 0.01 M $MgCl_2$ (referred to as sucrose-magnesium solution). *M. hominis* cells were washed and resuspended in a solution composed of 0.4 M sucrose, 0.01 M K_2HPO_4, 0.01 M $MgCl_2$, and 0.05 M tris(hydroxymethyl) aminomethane (Tris)-chloride, *p*H 7.6. To measure the accumulation of amino acids in the intracellular pool, chloramphenicol was added to the growth medium at a final concentration of 200 μg/ml 3 hr before harvest, and the organisms were washed and resuspended in the sucrose solutions containing 200 μg of chloramphenicol per ml. Protein in cell suspensions was determined by the method of Lowry et al. (8).

Measurement of amino acid uptake. Uptake of ^{14}C-L-histidine by *M. fermentans* was tested in a mixture (total volume, 1 ml) consisting of washed cells (0.2 mg of cell protein), 0.05 M Tris-maleate buffer, *p*H 5.4, 0.28 M sucrose, 7 mM $MgCl_2$, and 2.5 × 10^{-6} M ^{14}C-L-histidine (ring-2-^{14}C, 40 mc/mmole; The Radiochemical Centre, Amersham, England). The reaction mixture for ^{14}C-L-methionine uptake by *M. hominis* consisted of washed cells (0.2 mg of cell protein), 0.05 M Tris-phosphate buffer,

*p*H 7.6, 0.28 M sucrose, 7 mM $MgCl_2$, 0.01 M Na butyrate, 0.01 M Na acetate, 0.02 M L-arginine, and 4×10^{-6} M ^{14}C-L-methionine (methyl-^{14}C, 25 mc/mmole; The Radiochemical Centre, Amersham, England). Chloramphenicol (200 μg/ml) was added to the uptake reaction mixture to prevent incorporation of the labeled amino acids into protein, thus allowing the measurement of the intracellular accumulation of histidine and methionine. The cells were incubated in the uptake mixture for 15 min at 37 C with gentle shaking before addition of the labeled amino acid. Incubation was then continued for another 15 or 30 min at 37 C. To estimate total uptake and accumulation of amino acid in the intracellular pool, the reaction was stopped by the addition of 3 ml of ice-cold sucrose-magnesium solution to the reaction mixture. To estimate the incorporation of the amino acid into macromolecules, 3 ml of cold 10% trichloroacetic acid was added to the reaction mixture and the mixture was incubated in ice for 30 min. All samples were passed through membrane filters (type HA, 0.45-μ pore size; Millipore Corp., Bedford, Mass.) previously soaked in the tested unlabeled amino acid (10^{-3} M). Filtration was carried out by suction, through application of a negative pressure of 60 mm of mercury. The filters were washed with 25 ml of 0.25 M NaCl, and precipitated samples were washed with 25 ml of cold 5% trichloroacetic acid containing the tested unlabeled amino acid at a concentration of 10^{-3} M. The filters were air-dried and transferred to scintillation vials containing 10 ml of scintillation liquid [POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene), 0.1 g; PPO (2,5-diphenyloxazole), 3 g; in 1 liter of toluene]. Samples were counted for 5 or 10 min in a Packard Tri-Carb liquid scintillation counter. In all experiments, correction was made for nonspecific adsorption on cells and filters by subtraction of the radioactivity of appropriate controls at zero-time.

Intracellular state of accumulated amino acid. Cells of *M. fermentans* or *M. hominis*, treated with chloramphenicol as described above, were incubated for 10 min at 37 C in 2.5×10^{-6} M ^{14}C-L-histidine or 4×10^{-6} M L-methionine, respectively. The cells were then sedimented by centrifugation at 11,000 $\times$ *g* for 15 min and washed in a cold 0.25 M NaCl solution containing 0.01 M $MgCl_2$. The sedimented cells were resuspended in 4 ml of deionized water and boiled for 20 min. Cell debris was removed by centrifugation at 13,500 $\times$ *g* for 20 min. The supernatant fluid was separated and freeze-dried. The dried material was dissolved in 0.2 ml of the corresponding unlabeled amino acid solution (2 mg/ml). Samples (0.02 ml) of this solution were applied to Whatman no. 1 paper and chromatographed, by use of a mixture of *n*-butyl alcohol-acetic acid-water (4:1:5) for L-histidine and phenol-water (4:1) for L-methionine. After 9 hr of ascending chromatography, the papers were dried and sprayed with 0.5% ninhydrin in acetone. The color was developed by heating the paper at 85 C for 5 min. Localization of the radioactive material on the chromatogram was carried out in a Packard Radiochromatogram Scanner.

Estimation of amino acid efflux. Efflux of ^{14}C-L-histidine from *M. fermentans* was examined in cells preloaded with the radioactive amino acid. The cells were loaded with ^{14}C-L-histidine in the uptake mixture described, at 37 C for 30 min. At the end of the incubation period, the uptake mixture was diluted 1:1,000 with a solution prewarmed to 37 C, containing all the ingredients of the uptake mixture except cells and labeled amino acid. Incubation was continued at 37 C, and 20-ml samples were withdrawn at various time intervals and passed through 0.45-μ Millipore filters. The filters were washed with 10 ml of 0.25 M NaCl and dried, and their radioactivity was determined.

RESULTS

The unique features of the *Mycoplasma* cells made it necessary to check the applicability of the standard techniques used in transport studies in bacteria. Membrane filters are routinely used for the rapid separation of cells from unbound substrate, but their applicability to *Mycoplasma* had first to be investigated in view of reports that these minute and plastic organisms passed through filters known to retain other bacteria (12). Suspensions of *M. fermentans* and *M. hominis* cells were filtered through Millipore filters of different pore diameters, with the application of various degrees of negative pressure. Cell suspensions containing 0.2 mg of cell protein per ml passed rapidly through filters of 0.45-μ pore diameter at a negative pressure of 60 mm of mercury. Viable counts performed on the filtrate showed that the cells did not pass through the filter. Cell protein in the filtrate was less than 1% of that in the original suspension. Filtration became slow with suspensions containing more than 0.5 mg of cell protein per ml, as the filter pores were clogged by the plastic microorganisms.

For the selection of the amino acids most suitable for transport studies in *M. fermentans* and *M. hominis*, the uptake of L-histidine, L-methionine, L-phenylalanine, L-valine, L-glutamine, L-alanine, glycine, and L-leucine was tested. Highest uptake values were obtained with L-histidine for *M. fermentans* and with L-methionine for *M. hominis*. The uptake of these amino acids was therefore selected for detailed investigation.

Factors influencing uptake. Figure 1 demonstrates the steep decline in L-histidine uptake by *M. fermentans* cells harvested after the logarithmic phase of growth. A very similar curve was obtained for the uptake of L-methionine by *M. hominis* cells harvested at different ages of culture. Usually, 48-hr cultures had lost all ability to take up the amino acids.

Uptake of L-histidine by *M. fermentans* showed a sharp optimum at *p*H 5.4. The *p*H curve of L-methionine uptake by *M. hominis* showed a less-

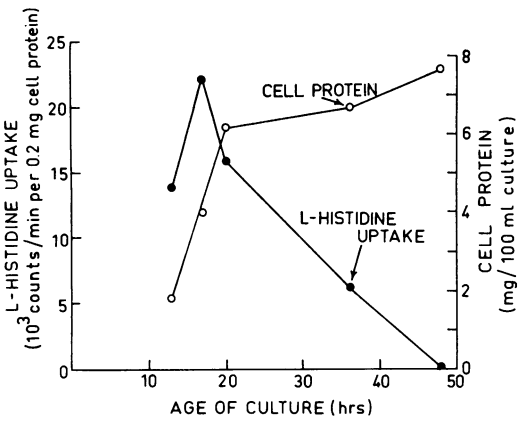

FIG. 1. *Uptake of ^{14}C-L-histidine by Mycoplasma fermentans at various growth phases.*

defined *p*H optimum, and uptake values did not significantly vary within the *p*H range of 6.8 to 8.0.

The uptake of the two amino acids by the mycoplasmas was temperature-dependent. There was no uptake of either amino acid below 10 C. Optimal temperature for L-histidine uptake by *M. fermentans* was 37 C, and the Q_{10} calculated between 27 and 37 C was approximately 3.0. The optimal temperature for the uptake of L-methionine by *M. hominis* was 42 C (Fig. 2), and the Q_{10} for the accumulation of this amino acid in the intracellular pool between 32 and C 42 was about 3.0.

Intracellular state of accumulated amino acid. Under the test conditions, only a negligible portion of the L-histidine in *M. fermentans* was incorporated into macromolecules (less than 1% of total uptake), and no chloramphenicol treatment was needed to study the accumulation of this acid in the intracellular pool (*see also* Fig. 3a). The uptake values of L-methionine by *M. hominis* were much lower than those of L-histidine by *M. fermentans*, and a significant portion of the transported L-methionine was incorporated into macromolecules, necessitating the use of chloramphenicol to study its accumulation in the pool.

Chromatography of hot-water extracts of the mycoplasmas after the uptake of ^{14}C-L-histidine or ^{14}C-L-methionine showed that all of the radioactivity was located in the spot of the corresponding amino acid. Thus, under the conditions tested, L-histidine and L-methionine accumulated in the intracellular pool as such, without being converted to any other material.

Kinetics of amino acid uptake. The rate of L-histidine uptake into *M. fermentans* cells is shown in Fig. 3a. Uptake was linear with time during the first 7 min and then declined in rate, reaching a plateau after about 15 min. The concentration of ^{14}C-L-histidine in the pool at steady state was calculated to be about 200 times as high as its external concentration. This calculation was based on data of S. Rottem (*to be published*) estimating the intracellular water volume in *Mycoplasma* cells to be 5.7 μliters per mg of cell protein. The rate of L-methionine uptake by *M. hominis* was slower than that of L-histidine by *M. fermentans*, and the intracellular pool reached its steady state only after 30 min of incubation at 37 C. Incorporation of the transported amino acid into macromolecules was almost linear throughout the reaction period (Fig. 3b). The concentration of ^{14}C-L-methionine in the pool at steady state was calculated to be about three times as high as its concentration in the uptake mixture.

The rate of L-histidine and L-methionine uptake by the mycoplasmas depended on the external concentration of the amino acid, according to Michaelis-Menten kinetics. The K_m value for L-histidine uptake, calculated from the Line weaver-Burk plot, was 8×10^{-5} M and V_{max} was about 17 mμmoles of L-histidine per mg of cell protein per min, whereas the K_m for L-methionine uptake

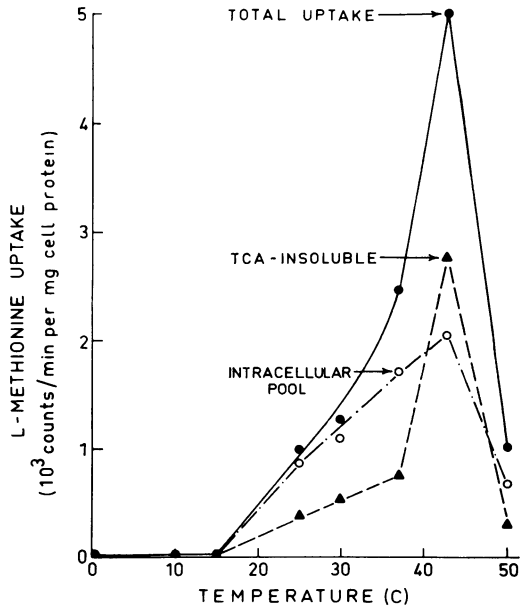

FIG. 2. *Temperature dependence of ^{14}C-L-methionine uptake by Mycoplasma hominis. Accumulation in the intracellular pool was estimated in cells treated with 200 μg of chloramphenicol per ml; total uptake and incorporation into macromolecules (trichloroacetic acid-insoluble) were determined in untreated cells as described under Materials and Methods.*

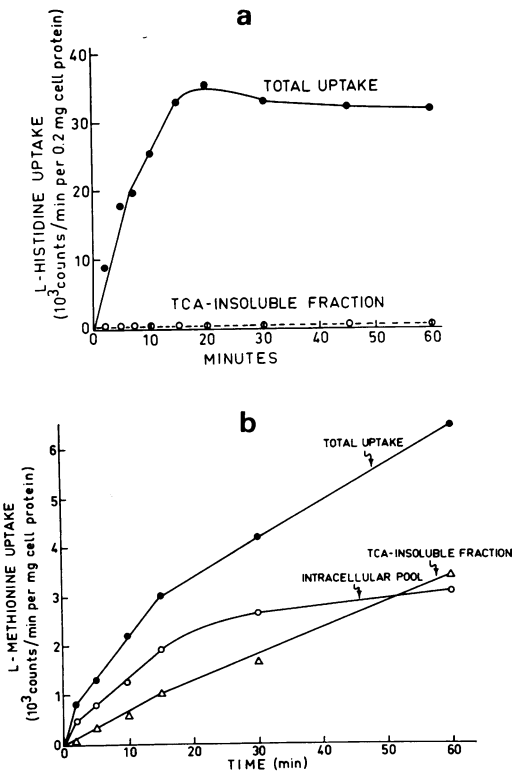

FIG. 3. *Time course of* ^{14}*C-*L*-histidine uptake by Mycoplasma fermentans (a) and* ^{14}*C-*L*-methionine uptake by Mycoplasma hominis (b). Accumulation in intracellular pool was estimated in cells treated with 200 μg of chloramphenicol per ml; total uptake and incorporation into macromolecules (trichloroacetic acid-insoluble fraction) were determined in untreated cells as described under Materials and Methods.*

was 3×10^{-5} M and V_{max} was 0.04 mμmole per mg of cell protein per min.

Energy requirement of the uptake systems. The addition of glucose to the uptake mixture had no effect on L-histidine uptake by *M. fermentans*. Starvation of the cells by incubating washed cells in the sucrose-magnesium solution for 30 min at 37 C decreased their ability to take up L-histidine by 30 to 34%. Incubation for 60 min decreased this ability by over 50%. The addition of glucose (0.02 M) to the uptake mixture did not restore the activity lost. This finding was somewhat surprising because *M. fermentans* is supposed to utilize glucose (4). Manometric experiments failed to show oxygen uptake by washed or unwashed *M. fermentans* cells suspended in 0.05 M phosphate buffer at *p*H 5.7 or 7.8 with glucose as substrate. Cells of *M. laidlawii*, another fermentative *Mycoplasma* species, showed a high rate of oxygen uptake when tested under the same conditions. Nevertheless, when grown on Edward medium containing 2% glucose and 0.005% phenol red, *M. fermentans* produced acid under both aerobic and anaerobic conditions.

More successful were the efforts to demonstrate an energy requirement for the uptake of L-methionine by *M. hominis*. Omission of L-arginine, acetate, and butyrate from the uptake mixture considerably reduced the uptake of L-methionine (Table 1).

Specificity of the transport systems. The transport of L-histidine by *M. fermentans* was inhibited by the basic amino acids L-arginine, L-lysine, and L-ornithine, and, to a smaller extent, by several amines derived from these amino acids. The D isomer of L-histidine also showed some inhibitory effect (Table 2). The nature of the inhibition by L-lysine and L-arginine has been further investigated and was found to be competitive (Fig. 4). The K_i for L-lysine inhibition, calculated from the Lineweaver-Burk plot, was 6×10^{-4} M—about 7.5 times the K_m for L-histidine uptake. The K_i of L-arginine inhibition was very close to the K_m of L-histidine uptake, indicating the high affinity of L-arginine for this

TABLE 1. *Effect of* L*-arginine, acetate, and butyrate on* ^{14}*C-*L*-methionine uptake by Mycoplasma hominis*

L-Arginine	L-Methionine uptake (counts per min per mg of cell protein)	
	With acetate and butyrate	Without acetate and butyrate
μmoles/ml		
0	480	320
0.4	1,930	1,060
4.0	3,800	2,450
20.0	3,850	2,350

TABLE 2. *Per cent inhibition by basic amino acids and amines of* ^{14}*C-*L*-histidine uptake by Mycoplasma fermentans*

Amino acid or amine	Molar ratio of inhibitor to L-histidine		
	10	100	1,000
L-Arginine	54	99	ND[a]
L-Ornithine	55	90	99
L-Lysine	44	51	ND
D-Histidine	12	36	85
L-Citrulline	9	40	61
Cadaverine	21	40	49
Putrescine	0	29	64
Histamine	0	4	49

[a] Not done.

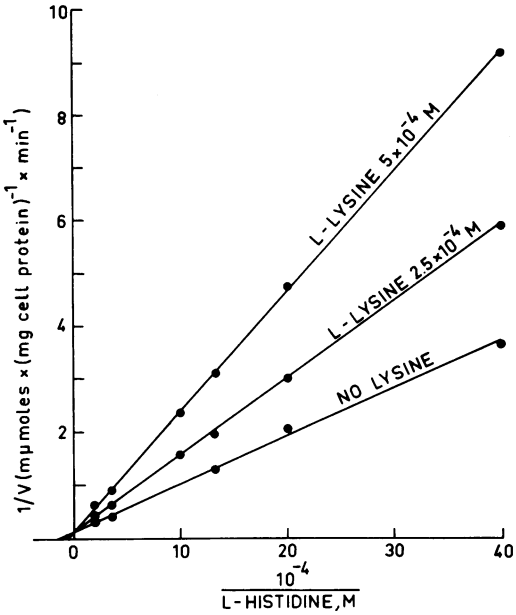

FIG. 4. *Competitive inhibition by* L*-lysine of* ^{14}C-L*-histidine uptake by Mycoplasma fermentans. The reciprocal of initial velocity (V) of* L*-histidine uptake (mμmoles of* L*-histidine taken up per mg of cell protein per min determined during the first 5 min of uptake) is plotted against the reciprocal of the external* L*-histidine concentration.* L*-Lysine was added to the uptake mixture at a concentration of* 2.5×10^{-4} *or* 5×10^{-4} M.

transport system. A series of another 15 amino acids, tested in concentrations 100 times that of L-histidine, were without effect on the amino acid uptake. The transport system for L-methionine in *M. hominis* exhibited a high degree of specificity. Thus, all the amino acids tested, including the stereoisomer D-methionine L-ethionine, were without effect on L-methionine uptake in concentrations 100 times that of L-methionine.

Effect of metabolic inhibitors on uptake. Table 3 shows the inhibition of L-histidine uptake by a series of SH-blocking reagents and by some other inhibitors. Sodium fluoride, 2,4-dinitrophenol, thallium acetate, and ouabain, in concentrations up to 0.01 M, were without effect on L-histidine uptake. Very similar results were obtained on testing the effect of these inhibitors on L-methionine uptake by *M. hominis*.

Ethylenediaminetetraacetic acid (EDTA), 0.01 M, inhibited the L-methionine uptake of *M. hominis* by 41% in the usual uptake mixture. When magnesium was omitted from the washing fluid and from the uptake mixture, EDTA at the same concentration inhibited L-methionine uptake by over 80%. No effect of EDTA on L-histidine uptake by *M. fermentans* could be demonstrated.

TABLE 3. *Effect of metabolic inhibitors on* ^{14}C-L*-histidine uptake by Mycoplasma fermentans*

Inhibitor[a] (M)	Per cent inhibition[b]
p-Chloromercuribenzoate	
10^{-2}	100
10^{-3}	99
10^{-4}	97
N-ethylmaleimide	
10^{-2}	46
10^{-3}	22
Potassium iodoacetate	
10^{-2}	71
10^{-3}	20
Potassium cyanide	
10^{-2}	75
10^{-3}	18
Sodium arsenate	
10^{-2}	57
10^{-3}	37
Sodium azide	
10^{-2}	43
10^{-3}	25

[a] The cells were incubated in the uptake mixture containing the inhibitor for 15 min at 37 C before the addition of ^{14}C-L-histidine.

[b] Compared with uptake without inhibitor.

The uptake of this amino acid was not affected by the omission of Mg^{2+} from the uptake mixture. However, when *M. fermentans* cells were washed in the sucrose solution without Mg^{2+}, their ability to transport L-histidine was about 35% lower.

Efflux of L*-histidine from M. fermentans cells.* ^{14}C-L-histidine leaked out from preloaded cells upon incubation at 37 C. The rate of efflux was practically zero at 0 C (Fig. 5). On addition of nonradioactive L-histidine to the reaction mixture, the rate of efflux increased by about fivefold. The addition of *p*-chloromercuribenzoate decreased the stimulation of efflux by external L-histidine (Fig. 5).

DISCUSSION

The finding that HA 0.45-μ Millipore filters are suitable for the rapid collection of *Mycoplasma* cells from the uptake mixtures was somewhat unexpected in light of previous data. Mycoplasmas are regarded as "filterable" microorganisms, some cells of which may pass through membrane filters having a pore diameter as low as 0.22 μ (12). Yet, our experiments with *M. fermentans* and *M. hominis* have shown that these cells do not pass through filters of 0.45-μ pore diameter. There is little doubt that the degree of pressure, positive or negative, applied during filtration has a decisive effect on the results of *Mycoplasma* filtra-

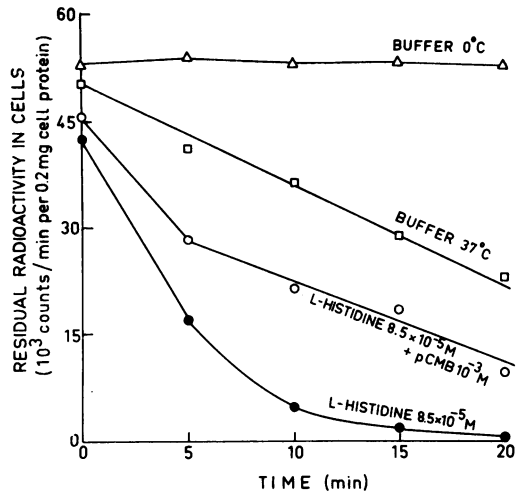

FIG. 5. *Kinetics of* ^{14}C-L-*histidine efflux from Mycoplasma fermentans cells. Efflux was measured as described under Materials and Methods with preloaded cells suspended in buffer at 37 C* (□) *or at 0 C* (△). *The effect on efflux of the addition to the buffer of unlabeled* L-*histidine with* (○) *or without p-chloromercuribenzoate* (●) *was tested at 37 C.*

tion. The highly plastic organisms may be squeezed through pores whose diameter is smaller than that of the organisms in their natural form. The low negative pressure applied in our experiments apparently prevented this squeezing effect. Furthermore, observations in a phase-contrast microscope indicate that the mean diameter of *M. hominis* and *M. fermentans* cells is about 0.4 μ, and, since most of them appear in chains and clusters, their passage through the 0.45-μ filter pores is prevented.

All the evidence obtained in the present study points to the conclusion that the amino acid transport systems in *Mycoplasma* resemble those found in other bacteria. The transport of L-histidine in *M. fermentans* and L-methionine in *M. hominis* fulfills the criteria for active transport in microorganisms (7). Transport of the amino acids was dependent on *p*H and temperature, showed high substrate specificity, and followed saturation kinetics. The rate of the amino acid uptake depended on its external concentration, following Michaelis-Menten kinetics, which suggests that the transport process is enzymatic in nature. The K_m values calculated for the transport of the two amino acids were of the same order of magnitude as that found for similar transport systems in bacteria (1, 5, 10). L-Methionine uptake by *M. hominis* was shown to be energy-dependent. Furthermore, uptake in both *Mycoplasma* species was inhibited by several metabolic inhibitors known to inhibit energy-yielding reactions. Both L-histidine and L-methionine were accumulated in the intracellular pool of the cells. The level of L-histidine in the pool reached about 200 times its extracellular concentration, and this level compares well with values obtained for amino acid accumulation in other bacteria (6). The fact that the accumulated amino acid in the pool did not undergo any chemical modification, fits in with the current concept of the intracellular pool in microorganisms (3, 6).

Some points which might be relevant to further transport studies in *Mycoplasma* deserve some comment. The age of cells used in transport experiments is of importance for any microorganism, but seems even more so for *Mycoplasma*. Many of our experiments failed because the cells used were too old. The loss of enzymatic activity on aging is most pronounced in *Mycoplasma* (18). The steep decline in activity at the end of the logarithmic phase of growth may be due to damage of the delicate *Mycoplasma* cell membrane. At this phase of growth, the cells tend to swell, and some even lyse (16), losing cofactors essential for enzymatic activity. The Mg^{2+} requirement for L-methionine uptake is apparently associated with the stabilizing effect of this cation on the *Mycoplasma* membrane (14). Magnesium in addition to an osmotic stabilizer effectively prevents the deleterious effects caused by washing these fragile cells.

Chloramphenicol was effective in arresting protein synthesis in *Mycoplasma* (21) and could be used for uncoupling the transport of the amino acids from their utilization. However, in experiments on L-histidine uptake by *M. fermentans* no chloramphenicol was required because, under the test conditions, practically no protein was synthesized. On the other hand, protein was synthesized by *M. hominis* during L-methionine uptake. The difference might be explained by the different content of the uptake mixtures: the uptake mixture for L-histidine consisted exclusively of buffer, sucrose, and Mg^{2+}, whereas that for L-methionine also contained substantial amounts of L-arginine, acetate, and butyrate. *M. hominis* is known to degrade L-arginine via the arginine dihydrolase pathway, yielding adenosine triphosphate (2), and to oxidize acetate and butyrate (9). However, apart from supplying the energy required for the uptake process, these compounds may give rise to, or be contaminated with, small amounts of amino acids, thus allowing some limited protein synthesis to occur.

The ability of *M. fermentans* cells to accumulate appreciable amounts of labeled L-histidine in the intracellular pool made it possible to study the

efflux of this amino acid. The exit mechanism of amino acids from microorganisms is not yet clear and seems to differ in various systems (7). Efflux of L-histidine from *M. fermentans* was temperature-dependent, and was highly accelerated by extracellular L-histidine, so that the mechanism may correspond with the exchange theory based on the permease model of Kepes (7). *p*-Chloromercuribenzoate, which inhibits transport of L-histidine into the cells, also reduces the rate of efflux, possibly by inhibiting the exchange reaction.

Our findings show that *Mycoplasma* resembles other microorganisms in possessing specific transport systems for metabolites. The minute *Mycoplasma* organisms, which have smaller genomes than ordinary bacteria (11), economize on genetic information by the absence of many of the enzymatic systems usually present in other bacteria. This, however, has led to their dependence on the external supply of many nutrilites. The need for transport systems for these nutrilites is obvious, and, if these systems are indeed so specific as shown here for the few amino acids tested, then a significant portion of the cell genome must be diverted towards producing the specific carrier systems, and the net saving in genetic information is apparently smaller than was thought before.

ACKNOWLEDGMENTS

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