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Effect of L-Asparagine on Growth of *Mycobacterium tuberculosis* and on Utilization of Other Amino Acids

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L-Asparagine controls the utilization of other amino acids by *Mycobacterium tuberculosis* (H37Ra) in aerated, liquid synthetic media. In a mixture containing asparagine and either L-alanine or L-glutamic acid, amino acid utilization is diphasic, with asparagine being utilized first. Short-term growth rates and cell yields are diminished and mimic those seen with asparagine alone. Catabolite repression is the probable regulatory mechanism responsible for this effect of asparagine. In contrast, in the presence of aspartic acid, asparagine stimulates growth and increases utilization of aspartic acid.

Other workers have documented differences in the nitrogen requirements among various species of mycobacteria (2, 3, 5). Generally, comparisons were made of growth rates on complex media during stationary incubation. Previously, we used a liquid synthetic medium and rotary incubation to compare the effect of several nitrogen sources on the growth of two widely divergent strains of mycobacteria, the saprophyte *Mycobacterium smegmatis* 607 and the avirulent human strain *M. tuberculosis* (H37Ra) (6). It was shown that under these conditions asparagine does not function efficiently as a source of nitrogen for growth of *M. tuberculosis* (H37Ra). This paper explores the marked effect of L-asparagine on growth of H37Ra and on utilization of other amino acids in an aerated, liquid synthetic medium.

MATERIALS AND METHODS

Organism. *M. tuberculosis* (H37Ra) was obtained from the National Communicable Disease Center, Atlanta, Ga.

Media. Our basal medium was modified Sauton medium (9) and contained (in grams/liter): dibasic potassium phosphate, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; citric acid, 0.2; ferric ammonium citrate, 0.05; and glycerol, 10. Various amino acids were added at 5 μmol/ml of medium. Tween 80 was added to a final concentration of 0.02%. The pH was adjusted to 7.4 with sodium hydroxide. The media were dispensed in 200-ml amounts in 500-ml Erlenmeyer flasks and autoclaved at 121 C for 20 min.

Experimental cultures. *M. tuberculosis* (H37Ra) was carried in modified Sauton medium in which NH_4Cl was substituted for L-asparagine (Asn), the usual nitrogen source of this medium. Inoculum cells from actively growing cultures were harvested by centrifugation (4 C), washed twice in cold Sauton medium minus a nitrogen source (SBS-G), and suspended in SBS-G. In the experiments to be reported, the nitrogen sources, added singly or in combination at a level of 5 μmol/ml, included L-alanine (Ala), L-glutamic acid (Glu), L-aspartic acid (Asp), NH_4Cl, and L-Asn. Each experimental medium was inoculated to an optical density (OD) of 0.015 to 0.025 (0.02 to 0.03 mg of dry cells/ml) at 600 nm as measured with a Coleman Junior Spectrophotometer. The milligrams dry weight of cells was determined from a curve relating milligrams dry weight to OD. Growth was measured turbidimetrically. Samples (7 ml) were removed aseptically and homogenized with a Teflon tissue grinder, and the OD was determined in matched tubes (18 by 150 mm) at 600 nm. At appropriate intervals, samples of culture were passed through membrane filters (pore size 0.45 μm; Millipore Corp., Bedford, Mass.) under negative pressure. The filtrates were examined for amino acid content with a Beckman-Spinco amino acid analyzer, model 120C.

RESULTS

Figure 1 shows the growth rate of H37Ra with either Asn, Asp, Glu, or Ala. Each nitrogen source was added to a level of 5 μmol/ml. The addition of Asn to most other amino acids markedly retarded the growth rate. The inhibitory effect of Asn was more pronounced with Ala than with Glu. Although not shown, Asn also inhibited growth with NH_4Cl. Conversely, Asn stimulated the growth rate and cell yield when added to Asp. The inhibitory effects of Asn on growth with two or three additional amino acids combined was very marked (Fig. 2). When Asn was added to either an individual amino acid (Fig. 1) or to the combined amino acids (Fig. 2), growth resembled that seen with Asn alone.

We next examined the effect of Asn on cell

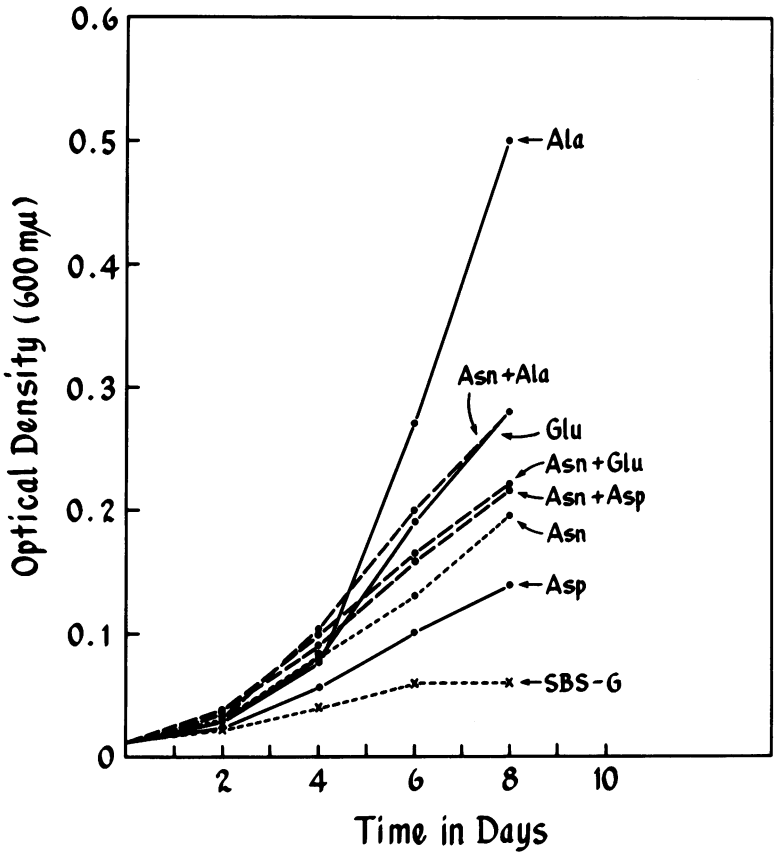

FIG. 1. *Effect of Asn on the growth rate of M. tuberculosis (H37Ra) in modified Sauton medium used in combination with Asp, Glu, or Ala. Methodology is outlined in Materials and Methods. SBS-G is basal medium alone.*

yield and amino acid utilization when added at different periods of incubation. This was determined by experiments with (i) control media containing each amino acid as the single source of nitrogen, (ii) media similar to (i) but also containing an equimolar quantity of Asn, and (iii) media similar to (i) but to which an equimolar amount of Asn, or one of the supplemental amino acids, was added 48 h after initial incubation. In this manner we were able to determine not only the initial effect of Asn but also its effect on 48-h cultures which contained Ala, Glu, Asp, or NH_4Cl. In the same manner, the converse situation was also examined, i.e., the effect of the supplemental nitrogen sources on growth with Asn and on its utilization.

Details of a study with Ala and Asn are shown in Table 1 and Fig. 3. The increase in cell mass during the second and third 48-h periods was significantly greater with Ala than with Asn (Table 1). Utilization of Ala correlated well with the increase in cell mass, being nearly complete in 144 h. On the other hand, utilization per unit of cellular material synthesized was far greater with Asn than with Ala (Table 1). A particularly large difference was seen during the first 48-h period (50.0 μmol of Asn versus 4.3 μmol of Ala utilized per mg of dry cells). Utilization of Asn was 100% at 192 h (Fig. 3). These data indicate a rapid but inefficient expenditure of Asn. The inhibitory effect of Asn on cell yield in mixtures containing Asn and Ala is also shown (Table 1). Asparagine added to Ala at time zero markedly reduced the final cell yield from that observed with Ala alone. The rate of utilization of Ala and Asp and the effect of Asn are shown in Fig. 3 and 4. Utilization of Asn, as judged by the concentration of extracellular Asn, was not affected significantly by simultaneous or late additions of Ala or Asp. Nearly 100% utilization of Asn was seen at 144 h. The curve representing utilization of Asn added to a 48-h culture containing supplemental amino acids shows an increase in the rate of Asn utilization. The more

rapid net disappearance of Asn is probably a response to the high cell density. The shape of the curve for Ala utilization (Fig. 3) suggests that seen for induced systems. A definite lag preceded significant utilization. Asn, added initially or at 48 h, inhibits but does not completely block utilization of Ala (Table 1 and Fig. 3). The rate of utilization of Ala added to Asn after 48 h was more rapid than seen with Ala alone, and indicates a lack of inhibition when Asn disappears (Fig. 3).

Similar results were seen with Asn-Glu and Asn-NH_4Cl combinations. Asparagine retarded the growth response of H37Ra both to Glu and to NH_4Cl. Table 2 shows the marked inhibitory effect of Asn on growth and on utilization of Glu by H37Ra. In this experiment, Asn and Glu were employed at levels approaching 5 and 20 μmol/ml, respectively. It is evident that, when combined with Glu, Asn not only inhibited growth but also blocked utilization of Glu during the first 4 days of incubation. In fact, a net gain in the extracellular Glu concentration was observed. (That extracellular amino acids, including Glu, Asp, and Ala, accumulate during the growth of H37Ra in Sauton medium containing Asn was reported previously by this laboratory [6].) Growth during this period mimicked that of the Asn culture. After disappearance of the extracellular Asn, the cell yield increased concomitantly with Glu utilization.

Figure 1 shows that addition of Asn to Asp considerably enhanced the growth rate over that seen with Asp alone. Data in Table 3 confirm these growth curves. Early addition of Asn to Asp brought a delayed increase in cell yield over that obtained with Asp alone. An immediate and significant increase in cell yield was seen when Asn was added to a 48-h Asp culture. The utilization data shown in Table 3 and Fig. 4 do not show disappearance of Asp commensurate with these increases in cell yield. However, utilization of Asp may be masked by the increase in concentration of extracellular

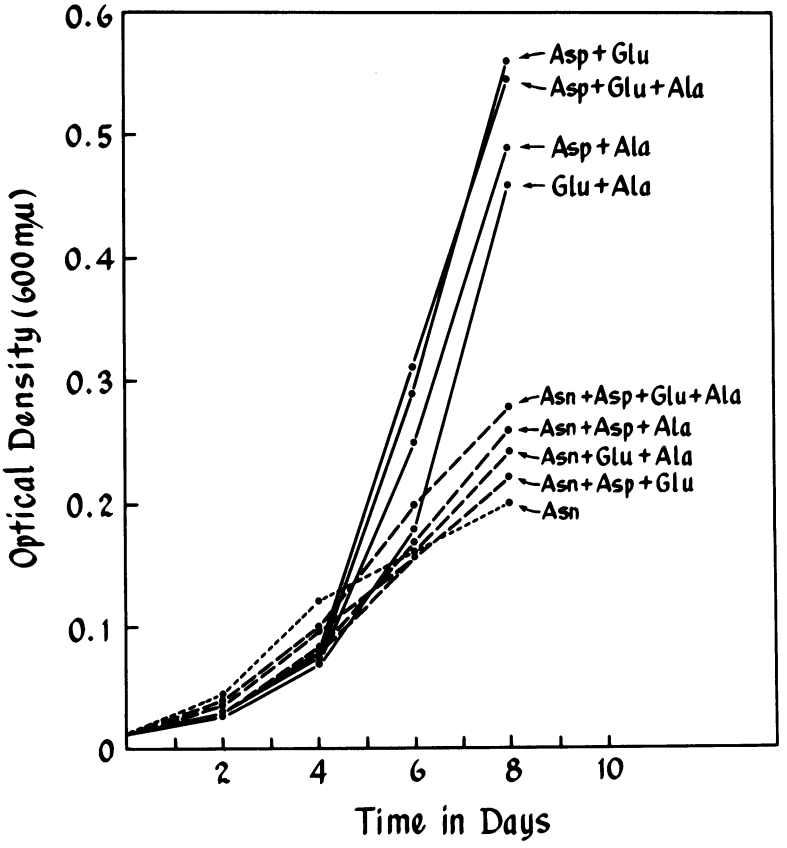

FIG. 2. *Effect of Asn on the growth rate of M. tuberculosis (H37Ra) in modified Sauton medium containing combinations of Asp, Glu, and Ala. Methodology is outlined in Materials and Methods.*

TABLE 1. *Effect of L-Ala and L-Asn on growth and on amino acid utilization by M. tuberculosis (H37Ra)*[a]

Nitrogen source (time added in h)	Initial concn (μmol/ml)	Cell yield[b] (mg of dry cells/ml)			Amino acid utilization[c] (μmol/mg of dry cells)			Percent of total
		0-48 h	48-96 h	96-144 h	0-48 h	48-96 h	96-144 h	
Ala (0)	5.0	0.06	0.20	0.58	4.3	7.6	5.1	95
Asn (0)	6.4	0.06	0.09	0.12	50.1	20.6	11.1	87
Ala (0)	5.4				3.7	4.2	6.1	46
+		0.08	0.21	0.21				
Asn (0)	6.4				33.9	11.1	5.3	96
Ala (0)	5.0				4.9	3.2	4.9	51
+		0.06	0.28	0.28				
Asn (48)	5.5					13.4	6.1	99
Ala (48)	6.9					13.6	7.4	48
+		0.08	0.12	0.23				
Asn (0)	6.5				47.0	11.0	6.2	99

[a] See Materials and Methods for methodology of cultivation.
[b] The figures denote the quantity of cells produced during each 48-h period of growth.
[c] Filtrates were obtained by passing cultures, at the intervals specified, through 0.45-μm membrane filters. Quantitation of Ala and Asn was determined with a Beckman Spinco amino acid analyzer, model 120C. The μmoles of each amino acid utilized was related to milligrams dry weight of cells produced during each 48-h period.

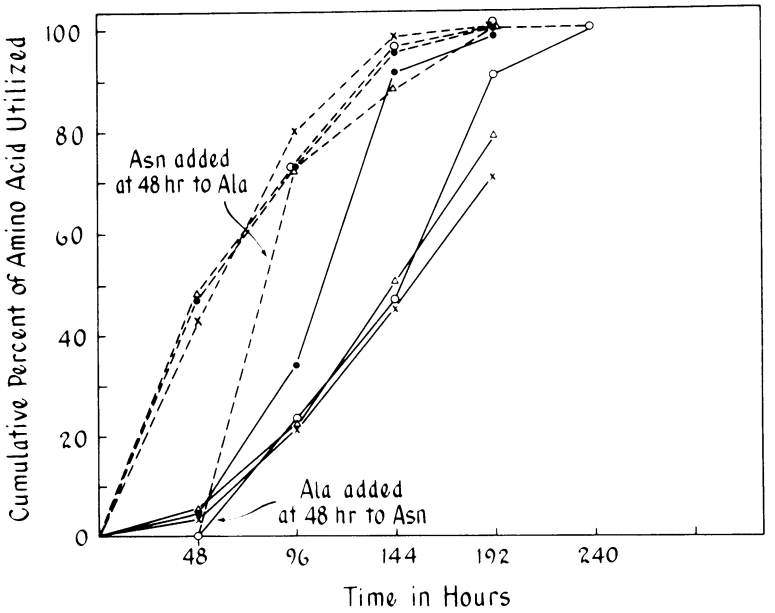

FIG. 3. *Effect of L-Ala and L-Asn on amino acid utilization by M. tuberculosis (H37Ra). Solid lines* (——) *represent utilization of Ala:* (●) *Ala at time zero,* (×) *Ala plus Asn at time zero,* (△) *Ala at time zero plus Asn at 48 h, and* (○) *Asn at time zero plus Ala at 48 h. Broken lines* (- - -) *represent utilization of Asn:* (●) *Asn at time zero,* (×) *Asn plus Ala at time zero,* (△) *Asn at time zero plus Ala at 48 h, and* (○) *Ala at time zero plus Asn at 48 h.*

Asp previously demonstrated during the metabolism of Asn by H37Ra (6). Growth of cultures which received Asp at 48 h resembled that seen with Asn alone (Table 1). On the other hand, utilization of Asp was stimulated significantly.

DISCUSSION

Asparagine is quickly but inefficiently utilized by *M. tuberculosis* (H37Ra) in aerated, liquid synthetic medium (6). The present stud-

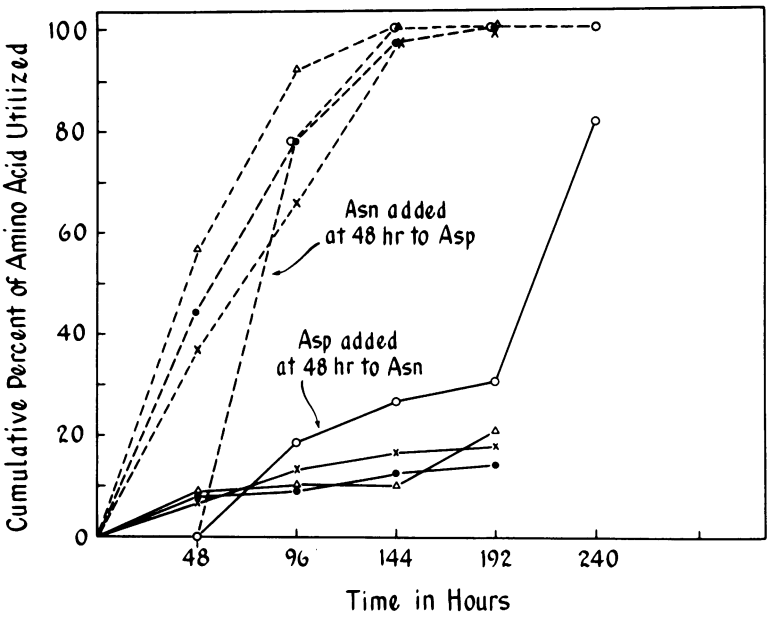

FIG. 4. *Effect of L-Asp and L-Asn on amino acid utilization by M. tuberculosis (H37Ra). Solid lines* (——) *represent utilization of Asp:* (●) *Asp at time zero,* (×) *Asp plus Asn at time zero,* (Δ) *Asp at time zero plus Asn at 48 h, and* (○) *Asn at time zero plus Asp at 48 h. Broken lines* (- - -) *represent utilization of Asn:* (●) *Asn at time zero,* (×) *Asn plus Asp at time zero,* (Δ) *Asn at time zero plus Asp at 48 h, and* (○) *Asp at time zero plus Asn at 48 h.*

TABLE 2. *Effect of L-Asn on growth and on utilization of L-Glu by M. tuberculosis (H37Ra)*

Nitrogen source (time [h] added)	Initial concn (μmol/ml)	4 days		8 days	
		OD[a]	μmol/ml[b]	OD	μmol/ml
Asn (0)	3.96	0.1	1.09	0.23	0.19
Glu (0)	22.7	0.69	18.5	1.1	5.8
Asn (0) + Glu (0)	4.2 25.5	0.12	1.8 31.4	0.54	0 19.7

[a] Optical density.

[b] Total micromoles per milliliter remaining in culture filtrate.

ies suggest that Asn also controls the utilization of Ala and Glu when added either at time zero or 48 h after initiation of growth. On the other hand, the supplemental amino acids have little effect on utilization of Asn.

Jayarum et al. (4) purified two L-asparaginases from cell-free extracts of *M. tuberculosis* (H37Ra) possessing different pH optima, 9.0 and 9.6. The pH 9.6 enzyme accounted for two-thirds of the asparaginase activity of this strain and was found to be inhibited about 22% by L-aspartic acid. They proposed, based on these findings, that the concentration of aspartic acid regulates utilization of L-asparagine by H37Ra and thereby contributes to the slow growth of the organism. Our studies on the effect of adding Asp to Asn on growth of strain H37Ra and on utilization of Asn do not lend support to this hypothesis (Fig. 1 and 2 and Table 3).

Analysis of culture filtrates showed an accumulation of extracellular amino acids (Asp, Glu, Ala, and Lys) during growth of H37Ra in media containing Asn as the sole amino acid added; Asp was the first to be detected (6). These particular amino acids are rapidly utilized after the disappearance of Asn from the medium. The results shown here reveal a rapid but inefficient metabolism of Asn and its preferential utilization in the presence of the added supplemental amino acids. Also, when Asn is added to the other amino acids the growth rate is the same as that seen with Asn alone.

We have observed two phenomena in this study: (i) when incorporated with Ala or Glu, Asn inhibits growth and utilization of the supplemental nitrogen sources; and (ii) when incorporated with Asp, Asn appears to stimulate growth of H37Ra and utilization of Asp. The inhibitory effects of Asn on growth and on utilization of Ala are much less than that of Asn on Glu. In the former case, Asn inhibits but does not completely stop growth, nor does it com-

TABLE 3. *Effect of* L-*Asp and* L-*Asn on growth and on amino acid utilization by M. tuberculosis (H37Ra)*[a]

Nitrogen source (time added in h)	Initial concn (μmol/ml)	Cell yield (mg of dry cells/ml)			Amino acid utilization (μmol/mg of dry cells)			Percent of total
		0–48 h	48–96 h	96–144 h	0–48 h	48–96 h	96–144 h	
Asp (0)	5.4	0.06	0.11	0.03	4.8	1.1	6.5	11
Asn (0)	6.8	0.06	0.11	0.08	57.1	17.8	15.2	99
Asp (0)	6.0				4.3	3.1	0.79	12
+		0.07	0.11	0.11				
Asn (0)	5.6				30.0	17.8	12.3	97
Asp (0)	5.4				6.7	0.99	1.3	14
+		0.06	0.22	0.17				
Asn (48)	7.1					26.7	7.5	100
Asp (48)	7.4					15.9	2.9	25
+		0.08	0.10	0.09				
Asn (0)	5.8				41.5	19.1	12.42	97

[a] Methodology used was the same as that recorded for Table 1.

pletely block utilization of Ala. However, the effect of Asn on Glu is more complete. Growth is significantly reduced and utilization of Glu is completely blocked. Both of these inhibitory effects disappear after utilization of Asn. We suspect that catabolite repression (7) is the regulatory mechanism whereby Asn inhibits growth and utilization of Ala and Glu. The role of catabolite repression in regulating carbohydrate metabolism by microorganisms has been reviewed by Paigen and Williams (8). Beggs and Lichstein (1) have presented evidence that catabolite repression may also function in amino acid metabolism. Our work indicates that the utilization of certain amino acids, in particular Glu, by *M. tuberculosis* (H37Ra) in the presence of Asn may be diphasic (diauxie), with Asn being utilized first. The phenomenon of diauxie is generally recognized as a modification of catabolite repression (8). Identification of the effector(s) responsible for the phenomenon of repression by Asn has not been made.

In reference to the stimulation of growth and on utilization of Asp in the presence of Asn we have no good explanation. It is conceivable that Asp derived from the deamidation of Asn may induce utilization of externally supplied Asp. Our studies show that when Asp is supplied as the sole source of nitrogen at 5 μmol/ml of medium, growth of H37Ra and utilization of this amino acid are minimal. Utilization of Asp is not increased by doubling its concentration. One may also speculate that Asn induces an Asp transport system. Work on factors affecting transport of Asp by *M. tuberculosis* (H37Ra) is in progress.

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