Aspartate metabolism in *Mycobacterium avium* grown in host tissue and axenically and in *Mycobacterium leprae*

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Aspartokinase activity was detected in extracts from *Mycobacterium leprae* (recovered from armadillo liver) and in *Mycobacterium avium* grown axenically and *in vivo*. Homoserine dehydrogenase activity was only detected in *M. leprae* and in *M. avium* grown axenically. Activities, when detected, were 50 to 70% lower in *M. leprae* or *M. avium* grown *in vivo* than in axenically grown *M. avium*. In these two pathogenic mycobacteria, aspartokinase and homoserine dehydrogenase are subject to feedback inhibition by methionine – an additional regulator over those observed for the enzymes from *Mycobacterium smegmatis*. Intact mycobacterium incorporated carbon from [U-¹⁴C]aspartate into the aspartate family of amino acids (threonine, isoleucine, methionine and lysine) though the rate of incorporation in *M. avium* grown *in vivo* was about half that in *M. avium* grown axenically.

Introduction

In order to grow and divide intracellular bacteria must, perforce, obtain their nutrients from the host cells inside which they reside. Asparagine, glutamate, aspartate and various other amino acids and metabolic precursors and intermediates are available in host tissues in significant quantities and various micro-organisms, including mycobacteria, can accumulate these substances to concentrations many times higher than in the environment (see Holden, 1962; Sritharan *et al.*, 1987) and thus could use them directly rather than having to synthesize them *de novo*. This certainly seems to be the case with mycobacteria growing *in vivo*, which can assimilate and utilize exogenous fatty acids (Wheeler & Ratledge, 1988) and nucleotides (Wheeler, 1987, 1989) the key enzymes involved in the biosynthesis of these compounds being depressed simultaneously.

Asparagine is a preferred source of nitrogen for many mycobacteria (Lyon *et al.*, 1967). In *M. smegmatis*, the reason for this preference is probably the high activity of aspartokinase so that aspartate, derived from asparagine, can be rapidly utilized in various aspects of amino acid metabolism (Sritharan *et al.*, 1989). Asparagine is also a carbon source, being linked to carbohydrate metabolism through its deamination to oxaloacetate. The objective of this study was to investigate the aspartate pathway in the obligate intracellular parasite *M. leprae*. As *M. leprae* has not been cultivated

axenically, the regulation of the aspartate pathway was also studied in *M. avium*, a Mycobacterium which can be grown axenically and inside a mammalian host. Results obtained with *M. avium* were then used to interpret the findings with *M. leprae*.

Methods

Mycobacterial strains, growth and harvesting. *M. avium* CR1/69 (from the AFRC Institute for Research in Animal Diseases, Compton, Berkshire, UK) was grown axenically in glycerol/salts medium (Ratledge & Hall, 1971) with asparagine as sole nitrogen source at 37 °C in 100 ml medium shaken (200 r.p.m.) in 250 ml flasks. Suspensions were prepared for uptake studies by washing the bacteria three times in buffered Tween 80 (0.1%, w/v, Tween 80 in 1.5 mM-MES, pH 7.0).

For *in vivo* grown mycobacteria, *M. leprae* was grown in Nine-banded armadillos (*Dasypus novemcinctus* Linn.) and *M. avium* CR1/69 was grown in C57 Black mice (Wheeler, 1987). Bacteria were isolated from infected tissues by a method (World Health Organization, 1980) that yields highly purified suspensions and is applicable for metabolic studies on host-grown mycobacteria (Wheeler, 1984, 1987).

Uptake studies. Intact washed mycobacteria ($2 \times 10^8 - 5 \times 10^9$) were added aseptically to the incubation medium described below and incubated at 37 °C with orbital shaking (~160 r.p.m.) in siliconized screw-capped, glass vials for 4 h for *M. avium* and 24 h for *M. leprae*. The incubation mixture (Lyon *et al.*, 1967), filter-sterilized, was routinely as follows: 100 µmol potassium phosphate buffer (pH 6.8), 500 µmol glycerol, 2 µmol MgSO₄ and 100 U penicillin G (for > 4 h incubations) in 1 ml. Changes in pH and energy source (replacing glycerol) for some incubations are indicated in the text. A single radioactive amino acid (1 µmol; 18.5 kBq; diluted with unlabelled amino acid as required) was added to each incubation. The specific activities of the amino acids (obtained from Amersham) were as follows: L-[U-¹⁴C]aspartic acid, 7400 GBq mol⁻¹; L-[methyl-¹⁴C]methionine, 463 GBq mol⁻¹; and L-[U-¹⁴C]lysine, 5500 GBq mol⁻¹.

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Controls contained bacteria killed with formalin (4%, v/v, at 25 °C for 18 h), and their background activity was always subtracted when presenting uptake by live organisms.

Analysis of incubation products. At the end of each incubation, the samples were tested for contamination with fast-growing bacteria by plating 10 µl samples on to nutrient agar plates. Further samples were taken to measure the following. (a) *Assimilation by intact bacteria.* The bacteria were collected on Whatman GF/C filters under vacuum and washed four times at 4 °C with 15 ml buffered Tween 80 each time. The filters (with the cells) were air-dried and radioactivity was determined as described below.

(b) *Evolved $^{14}\text{CO}_2$.* $^{14}\text{CO}_2$ evolved during incubations was trapped in 400 µl 0.5 M-NaOH; the NaOH was contained in a separate vial from the incubation mixture inside the same sealed universal (25 ml) vial.

(c) *Incorporation into the amino acid pool.* The labelled bacteria were washed three times in buffered Tween 80 by centrifugation and resuspension then extracted, first with boiling water (200 µl per mg dry wt of cells) for 30 min and then with the same volume of 60% (v/v) ethanol at 4 °C for 60 min. The two aqueous extracts were pooled, dried gradually at 50 °C and then redissolved in 100 µl 0.1 M-HCl for subsequent separation by TLC.

(d) *Incorporation into proteins.* The residue obtained after extracting the soluble pool was subjected to alkaline hydrolysis with barium hydroxide (15 mg per mg dry wt of cells) at 120 °C for 24 h in sealed ampoules. The pH of the hydrolysate was adjusted to 6.0 with 1 M- H_2SO_4 and the hydrolysate heated at 100 °C for 5 min in a boiling water-bath. The contents were centrifuged and the precipitate was washed once with distilled water. The supernatant and the washing were pooled and dried at 50 °C. The residue was redissolved in 100 µl 0.1 M-HCl for further analysis.

Separation and identification of amino acids. Samples (10 µl) of the pool extract or alkaline hydrolysate were separated by two-dimensional TLC using Silica Gel G plates (Uniplat; Analtech). The plates were developed first with butanol/glacial acetic acid/water (4:1:1, by vol.) dried, and then developed with ethanol/sp.gr. 0.880 NH_4OH (77:23, v/v) in the second dimension. The amino acids were identified by spraying plates with fluorescamine (Roche; 30 mg dissolved in 100 ml 10%, v/v, triethanolamine in acetone) and visualized under UV light (Imai *et al.*, 1974). The areas corresponding to particular amino acids were marked, scraped off the plates and their radioactivity determined.

Determination of radioactivity. NaOH including trapped $^{14}\text{CO}_2$ was mixed with Bray's scintillant. Intact cells and their fractions were first solubilized with 0.5 ml Soluene 100 (Packard) at 50 °C for 60 min, neutralized with 50 µl glacial acetic acid and mixed with Bray's scintillant. Scrapings of silica gel from TLC plates were first mixed with about 1 g Cabosil (Koch-Light) and then with Bray's scintillant. The radioactivity was determined by liquid-scintillation counting.

Preparation of cell-free extracts. Cell-free extracts, usually desalted, were prepared as described previously (Sritharan *et al.*, 1987). Protein was estimated by the method of Bradford (1976).

Enzyme assays. Aspartokinase (EC 2.7.2.4; ATP: L-aspartate 4-phosphotransferase) was assayed by the FeCl_3 method (Stadtman, 1961) except that the final colour was developed with 3 ml FeCl_3 (5%, w/v) instead of 1.5 ml in the published method. This modification obviated the need for centrifugation. A_{520} values were determined and enzyme activity was calculated using $\epsilon = 0.35 \text{ mm cm}^{-1}$. L-Aspartate was routinely included at 20 mM, and only varied – between 12.5 mM and 200 mM – when K_m values for aspartate were determined; ATP was routinely included at 20 mM, and only varied – between 5 mM and 50 mM – when K_m values for ATP were determined. Specific activity of the enzyme is expressed as µmol aspartohydroxamate formed min^{-1} (mg protein) $^{-1}$.

Homoserine dehydrogenase (EC 1.1.1.3; L-homoserine: NAD(P) $^{+}$ oxidoreductase) was assayed by the standard method (Datta & Gest, 1970) which we used previously (Sritharan *et al.*, 1987). DL-Homoserine was routinely included at 20 mM and only varied – between 10 mM and 70 mM – when K_m values for homoserine were determined; NADP was routinely included at 4 mM and only varied – between 0.15 mM and 4 mM – when K_m values for NADP were determined. Specific activity of the enzyme is expressed as µmol NADH produced (mg protein) $^{-1} \text{ min}^{-1}$.

Results

Aspartate metabolism in intact *M. leprae*

Uptake of L-[U- ^{14}C]aspartic acid was more rapid at pH 6.8 and 8 than at pH 5.5 or 7.2 (Table 1). As aspartate transport is an energy-dependent process in mycobacteria (Israeli *et al.*, 1977), various compounds were tested as energy source for this process in *M. leprae* at pH 5.5, as this pH value is close to the pH inside the macrophage in which *M. leprae* resides (Lowrie, 1983). Aspartate uptake was most rapid in the presence of pyruvate, though differences were not significant ($P > 0.05$; Student's *t*-test), and slowest in the presence of glucose ($P < 0.05$) (Table 1). Very little radioactivity was incorporated into amino acids of the aspartate family (threonine, isoleucine, methionine and lysine) from [U- ^{14}C]aspartate (Table 2), totalling 6 Bq per 10^9 organisms in 24 h. The reason for the failure to detect any incorporation into isoleucine may be that the limit of detection is about 0.5% of the radioactivity in the pool, only a half to a third of that detected in threonine and asparagine. Only 38% of the incorporated radioactivity was recovered in the amino acids listed in Table 2; the remainder was not sought, but could be in carboxylic acids derived from deamination of the amino acid.

Table 1. Effect of pH and energy source on aspartate uptake by *M. leprae*

Intact *M. leprae* organisms ($\sim 10^9$) were incubated with 18.5 MBq [U- ^{14}C]aspartate, buffer, MgSO_4 and penicillin as in Methods, together with one energy source (10 µmol) in 1 ml. Incubation mixtures were shaken at 37 °C for 24 h. Values for aspartate uptake are Bq per 10^9 *M. leprae* organisms in 24 h, $\pm$ SEM. Six incubations were done using duplicates with three separate suspensions of *M. leprae*.

Energy source	pH	Uptake of ^{14}C from aspartate
Glycerol	5.5	43 $\pm$ 13
	6.8	105 $\pm$ 10
	7.2	72 $\pm$ 13
	8.0	102 $\pm$ 20
Glucose	5.5	19 $\pm$ 5
Succinate	5.5	35 $\pm$ 13
Pyruvate	5.5	63 $\pm$ 22

Table 2. Distribution of label from [U-¹⁴C]aspartate into other amino acids of the aspartate family in *M. leprae* and *M. avium*

Intact *M. leprae* organisms ($\sim 10^9$) were incubated with [U-¹⁴C]aspartic acid under the standard conditions (pH 6.8, with glycerol) described in Methods and shaken at 37 °C, for 4 h in the case of *M. avium* and 24 h in the case of *M. leprae*. Amino acids of the soluble pool were extracted and analysed as in Methods. 'Aspartate taken up' includes carbon assimilation and metabolism to CO₂. Eight incubations were done for *M. avium* grown axenically, six for *M. avium* grown in mice, and four for *M. leprae* obtained from armadillo tissue. Values are expressed as a percentage of the radioactivity associated with the extractable pool of amino acids, or as total radioactivity taken up or in pool amino acids.

Amino acid	Percentage radioactivity in each amino acid, $\pm$ SEM		
	<i>M. leprae</i>	<i>M. avium</i> grown axenically	<i>M. avium</i> grown in C57 Black mice
Aspartate	24 $\pm$ 5	71 $\pm$ 3	75 $\pm$ 4
Asparagine	1.7 $\pm$ 0.7	4 $\pm$ 0.7	1.5 $\pm$ 0.3
Threonine	1.0 $\pm$ 0.6	6 $\pm$ 2	0.8 $\pm$ 0.2
Isoleucine	0 $\pm$ 1.0	1.2 $\pm$ 0.9	1.8 $\pm$ 0.4
Methionine	5.0 $\pm$ 3.0	1.5 $\pm$ 0.6	0.6 $\pm$ 0.1
Lysine	5.7 $\pm$ 2.6	4 $\pm$ 1	2.4 $\pm$ 0.6
Total radioactivity in above amino acids (%)	37	92	82
Pool amino acids (100% radioactivity; Bq per 10 ⁹ bacteria)	45 $\pm$ 6	87 $\pm$ 3	98 $\pm$ 8
Aspartate taken up (Bq per 10 ⁹ bacteria)	105 $\pm$ 10	225 $\pm$ 7	155 $\pm$ 23

Table 3. Uptake of [U-¹⁴C]lysine and [methyl-¹⁴C]methionine by *M. avium*

Intact *M. avium* organisms ($\sim 10^9$) were incubated with buffer and MgSO₄ as in Methods. Values for uptake are Bq per 10⁹ *M. avium* in 4 h, $\pm$ SEM. Four incubations were done, all duplicates with two separate suspensions of *M. avium*.

Growth in:	Amino acid uptake	
	[¹⁴ C]Lysine	[¹⁴ C]Methionine
Glycerol + asparagine medium	36 $\pm$ 1	28 $\pm$ 3
C57 Black mice	105 $\pm$ 11	108 $\pm$ 1

Aspartate metabolism in *M. avium* grown in vivo and axenically

M. avium grown in vivo took up less [U-¹⁴C]aspartic acid than *M. avium* grown axenically. This reflects lower rates of both protein synthesis and evolution of CO₂ from aspartate in *M. avium* grown in vivo (Sritharan, 1988), but not the appearance of radioactivity in the amino acid pool (Table 2). However, the total radioactivity in the

amino acids of the aspartate family was lower – 6 Bq per 10⁹ bacteria in 4 h – in *M. avium* grown in vivo than in *M. avium* grown axenically, when it was 11 Bq per 10⁹ bacteria in 4 h (calculated from Table 2). In contrast, *M. avium* grown in vivo showed significantly higher (three to four times) uptake of exogenously supplied L-[methyl-¹⁴C]methionine and L-[U-¹⁴C]lysine (i.e. two of the amino acids synthesized from aspartate) than *M. avium* grown axenically (Table 3).

Aspartokinase in extracts of *M. avium* and *M. leprae*

Aspartokinase was present at a specific activity of 0.33 units (mg protein)⁻¹ in *M. avium* grown axenically. The enzyme had an apparent *K_m* of 29 mM for aspartic acid and 19 mM for ATP. Methionine was the major feedback inhibitor of this enzyme (Table 4) but a combination of two, or all three, of lysine, methionine and threonine (all at 2 mM) gave 40 to 43% inhibition, the maximum with any combination of two or three amino acids.

The specific activity of aspartokinase in *M. avium* recovered after growth in mice was about a quarter of the specific activity of aspartokinase in axenically grown *M. avium* (Table 4). Perhaps more significant was that this

Table 4. *Inhibition of aspartokinase in M. avium and M. leprae*

Aspartokinase activity was assayed at pH 9 and 30 °C in cell-free extracts as in Methods; the assay mixture contained 20 mM-aspartate and 20 mM-ATP. Values are means from four assays done with extracts prepared from three isolates of each source. The range of percentage inhibition was the mean $\pm$ 5%. Values for 100% activity (with no inhibitor) were 0.33 ± 0.036 units (mg protein)⁻¹ for *M. avium* grown axenically in asparagine medium, 0.085 ± 0.024 for *M. avium* grown in C57 Black mice and 0.097 ± 0.012 for *M. leprae* (variance term: SEM).

Organism/source	Inhibitor concn. (mM)	Percentage inhibition of aspartokinase activity by:			
		L-Threonine	DL-Homoserine	L-Methionine	L-Lysine
<i>M. avium</i> grown in asparagine medium	2	12	24	26	4
	5	44	42	53	18
	10	50	50	54	58
	25	53	60	58	58
	50	58	68	60	60
<i>M. avium</i> grown in C57 Black mice	5	40	17	4	20
	10	43	40	10	24
	20	45	40	14	35
<i>M. leprae</i> isolated from armadillo liver	10	16	20	0	23
	20	60	62	7	24
	50	85	76	17	25

enzyme had a lower affinity for aspartate (apparent K_m 60 mM) and ATP (apparent K_m 26 mM) than the enzyme from *in vivo* grown cells and moreover, it was inhibited by threonine and homoserine while methionine had barely any inhibitory effect (Table 4).

Though aspartokinase activity was present in *M. leprae* at a specific activity of $[0.097 \text{ units (mg protein)}^{-1}]$ which was comparable to that of *M. avium* also grown *in vivo*, it had affinities towards aspartate (apparent K_m 25 mM) and ATP (apparent K_m 20 mM) which were similar to those of the enzyme from *M. avium* grown axenically rather than *in vivo*. However, its inhibition by threonine and homoserine and not, or only weakly, by methionine or lysine was similar to inhibition of aspartokinase activity from *M. avium* grown *in vivo*.

During determinations of K_m values, individual values for reaction velocities varied no more than 7% from the regression line in the Lineweaver-Burk plots. Thus the apparent K_m values for ATP are similar, but the K_m for aspartate of 60 mM for aspartokinase in *M. avium* grown *in vivo* appears distinct from the apparent K_m for aspartokinase in *M. avium* grown axenically and in *M. leprae*.

Homoserine dehydrogenase in extracts of M. avium and M. leprae

Cell-free extracts of *M. avium* grown on asparagine medium contained homoserine dehydrogenase at a specific activity of $1.4 \text{ units (mg protein)}^{-1}$ with apparent

K_m values of 40 mM and 0.32 mM for DL-homoserine and NADP⁺, respectively. Threonine and isoleucine inhibited the activity of this enzyme significantly while methionine or lysine had little effect (Table 5). Together at 1 mM each, threonine and isoleucine gave 60% inhibition of homoserine dehydrogenase activity from *M. avium*, a value greater than for either, individually, at 10 mM – this is concerted feedback inhibition (see Truffa-Bachi, 1973).

Homoserine dehydrogenase activity could not be detected in extracts of different isolates of *M. avium* grown in separate batches of mice. The limit of detection, using 8 mg protein ml⁻¹, was $0.00125 \text{ units (mg protein)}^{-1}$. However, extracts of *M. leprae* contained homoserine dehydrogenase at $0.72 \text{ units (mg protein)}^{-1}$, about half the activity which was present in axenically grown *M. avium*. Affinities for the substrates were also similar (apparent K_m 32 mM and 0.15 mM for DL-homoserine and NADP⁺, respectively) to those of the enzyme from *M. avium* grown axenically. During determinations of K_m values individual values for reaction velocities varied from the regression lines in Lineweaver-Burk plots as follows: with DL-homoserine, no more than 3%; with NADP, no more than 25%. Methionine was the most potent inhibitor of homoserine dehydrogenase activity through threonine and isoleucine also had some inhibitory effect (Table 5). When the effect of these inhibitors was tested in different combinations (each at 5 mM), the inhibition observed was additive.

Table 5. *Inhibition of homoserine dehydrogenase*

Homoserine dehydrogenase was assayed at pH 9 and 37 °C in cell-free extracts as in Methods; the assay mixture contained 20 mM-DL-homoserine and 4 mM-NADP. Values are means from four assays done with extracts prepared from three different isolates. The range of percentage inhibition values was the mean $\pm$ 5%. Values for 100% activity (with no inhibitor) were 1.4 units (mg protein)⁻¹ and 0.72 units (mg protein)⁻¹ for *M. avium* grown on asparagine medium and *M. leprae* respectively.

Organism/ source	Inhibitor concn. (mM)	Percentage inhibition of homoserine dehydrogenase activity			
		L-Threonine	L-Isoleucine	L-Methionine	L-Lysine
<i>M. avium</i> grown in asparagine medium	2.5	20	15	s*	s
	5.0	26	20	s	s
	10.0	40	20	2	s
	20.0	60	20	15	s
<i>M. avium</i> grown in C57 Black mice		Activity not detectable			
<i>M. leprae</i>	5	40	60	70	4
isolated from armadillo liver	10	50	65	85	5

* s, Slight stimulation (upto 8%)

Discussion

Mycobacteria harvested from host tissue and incubated in a medium which did not support growth synthesized the aspartate family of amino acids more slowly than axenically grown mycobacteria incubated in the same medium. It was important to use a medium which did not support growth of *M. avium* to determine any differences between bacteria grown *in vivo* and *in vitro*, so that one explanation for any differences between metabolism in *M. leprae* and the cultivable mycobacterium could be ruled out, i.e. that *M. leprae* was not growing but *M. avium* was. Furthermore, even though mycobacteria harvested from host tissue are subject to a lengthy purification procedure they appear to be metabolically intact: activities such as incorporation of hypoxanthine into nucleic acids are detected at levels similar to those found in axenically grown mycobacteria (Wheeler, 1987); the ATP content of *M. leprae* is comparable with the ATP content of rapidly growing mycobacteria (reviewed in Barclay & Wheeler, 1989); and in this study, uptake of lysine and methione – two of the aspartate family – was higher for host-grown *M. avium* than for axenically grown *M. avium* (Table 3). This final observation (Table 3) also suggests that the host-grown mycobacteria tend to scavenge amino acids of the aspartate family rather than synthesize them from aspartate.

The slow rate of biosynthesis of the aspartate family in host-grown mycobacteria, including *M. leprae* (Table 2) could be a result of repression or feedback inhibition of

the pathway. The relatively low specific activities of the first two enzymes of the pathway – aspartokinase and homoserine dehydrogenase – provides evidence for repression, as these activities were detected in desalted extracts from mycobacteria, from which any endogenous feedback inhibitors would be removed. Repression of these two enzymes also occurs in the saprophytic *M. smegmatis* grown in the presence of the aspartate family of amino acids (Sritharan *et al.*, 1989; see also Fig. 1). The failure to detect homoserine dehydrogenase activity at all in *M. avium* grown *in vivo* is somewhat puzzling. The finding that *M. leprae* incorporates 4 to 6% of ¹⁴C from aspartate into methionine and lysine as against 0.5 to 2% in *M. avium* grown *in vivo* is consistent with the failure to detect the enzyme in *M. avium* grown *in vivo*, but its low activity in *M. leprae*. However, *M. avium* grown *in vivo* incorporates ¹⁴C from aspartate into isoleucine, an activity which requires the participation of homoserine dehydrogenase.

Feedback inhibition of both aspartokinase (Table 4) and homoserine dehydrogenase (Table 5) provides a further mechanism for control of aspartate metabolism in *M. leprae* and *M. avium*. Differences in patterns of inhibition of aspartokinase activity between *M. avium* grown axenically and grown *in vivo* may be due to different proportions of isoenzymes being elaborated under different growth conditions, if each isoenzyme is affected as they are in *M. smegmatis* (Sritharan *et al.*, 1989) by different amino acids. These results show the overall effect of inhibitors on aspartokinase activity.

Fig. 1. Regulation of the aspartate pathway in mycobacteria. Feedback inhibition (I) and repression (R) of aspartokinase and homoserine dehydrogenase are shown in the scheme. →, Pathway; --→, regulatory effect.

* Inhibition by methionine of aspartokinase in *M. avium* (grown *in vitro*).

† Inhibition of homoserine dehydrogenase in *M. leprae*. Three aspartokinase isoenzymes occur in *M. smegmatis*.

Attempts to look for isoenzymes of aspartokinase in *M. avium* and *M. leprae* would require considerably greater amounts of bacteria, as demonstration of such isoenzymes would require their partial purification (Sritharan *et al.*, 1989). The feedback inhibition may have affected the activities shown in Table 2, though this is speculative without knowing the pool sizes for various amino acids in these difficult-to-obtain mycobacteria. What is known is that mycobacteria can accumulate amino acids to concentrations several times higher than in the environment (Sritharan *et al.*, 1987). This suggests that the inhibition effects, shown in Tables 4 and 5, could occur in mycobacteria growing *in vivo*, as amino acids of the aspartate family are readily available within mammalian tissues. Pathogenic mycobacteria are clearly capable of scavenging these amino acids from the environment as shown in this work: exogenously supplied methionine and lysine as well as aspartate are incorporated by *M. avium* grown *in vivo* (Table 3).

Aspartokinase and homoserine dehydrogenase in *M. smegmatis* were shown previously (Sritharan *et al.*, 1989) to be subject to complex regulation, with all the products of amino acid biosynthesis via aspartate, the aspartate

family of amino acids, being feedback inhibitors or repressors, or both, of one or both activities (see Fig. 1). Complex though the regulation was in *M. smegmatis*, additional control is shown here in *M. avium*, which exhibited feedback regulation of aspartokinase by methionine, and also in *M. leprae* in which homoserine dehydrogenase was strongly inhibited by methionine.

The additional complexities in the regulation of aspartate metabolism in the pathogenic mycobacteria, and differences between *M. leprae* and *M. avium* may reflect the greater problems of acquiring nutrients – in this case amino acids – which intracellular microbes encounter. Amino acids can be withheld from intracellular parasites by host cells in an attempt to achieve nutritional immunity (Byrne *et al.*, 1986). In this context the complex pattern of regulation of the aspartate pathway (Fig. 1) – perhaps reflected in the ability of *M. leprae* preferentially to synthesize methionine and lysine but of host-grown *M. avium* preferentially to synthesize isoleucine (shown in Table 2) – may play a vital role in the nutrition of pathogenic mycobacteria.

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