Mycothiol Biosynthesis and Metabolism

CELLULAR LEVELS OF POTENTIAL INTERMEDIATES IN THE BIOSYNTHESIS AND DEGRADATION OF MYCOTHIOL IN MYCOBACTERIUM SMEGMATIS

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Mycobacterial mycothiol (MSH; 1-d-myo-inositol-2-(N-acetyl-L-cysteinylamido-2-deoxy-D-glucopyranoside (AcCys-GlcN-Ins))) is a novel thiol produced at millimolar levels by mycobacteria and other actinomycetes that do not make glutathione. We developed methods to determine the major components of MSH (AcCys, Cys-GlcN, AcCys-GlcN, Cys-GlcN-Ins, GlcN-Ins) in cell extracts. Mycobacterium smegmatis was shown to produce measurable levels (nmol/g of residual dry weight) of AcCys (30), Cys-GlcN-Ins (8), and GlcN-Ins (100) but not Cys-GlcN (<3) or AcCys-GlcN (<80) during exponential growth in Middlebrook 7H9 medium. The level of GlcN-Ins declined 10-fold in stationary phase and 5-fold in 7H9 medium lacking glucose. Incubation in 10 mM AcCys produced 50- and 1000-fold increases in cellular Cys and AcCys levels, respectively, a 10-fold decrease in GlcN-Ins and a transient 3-fold increase in Cys-GlcN-Ins. These results exclude Cys-GlcN and AcCys-GlcN as intermediates in MSH biosynthesis and implicate GlcN-Ins and Cys-GlcN-Ins as key intermediates. Assay of GlcN-Ins/ATP-dependent ligation activity with Cys and AcCys as substrates revealed that Cys was at least an order of magnitude better substrate. Based on the cellular measurements, MSH biosynthesis involves assembly of GlcN-Ins, ligation with Cys to produce Cys-GlcN-Ins, and acetylation of the latter to produce MSH.

Mycothiol (MSH)1 is a recently described novel cysteine derivative containing D-glucosamine and myo-inositol isolated from streptomycetes (1–3) and a mycobacterium (4) (see Structure I). It is produced at millimolar intracellular levels but only by actinomycetes, including pathogenic mycobacteria, none of which produce glutathione (5). Because mycothiol is more resistant to autoxidation than is glutathione, both of which are more resistant than cysteine, it may serve as a stable intracellular reserve of cysteine and also have protective functions analogous to that of glutathione (6). If so, then compounds that block the functions of MSH might be developed as effective drugs for use against pathogenic actinomycetes (5, 6). Elaboration of the biosynthetic and metabolic pathways for MSH is an important goal in this pursuit.

Relatively little is presently known about the biochemistry of MSH. It has recently been shown to be the cofactor for an NAD-dependent formaldehyde dehydrogenase found in actinomycetes, where it functions in a fashion analogous to that of GSH in the NAD/GSH-dependent enzyme (7). In another recent publication, Bornemann et al. (6) presented evidence derived from studies with partially purified extracts of Mycobacterium smegmatis, indicating that the final steps in the biosynthesis of MSH involve ATP-activated coupling of Cys to GlcN-Ins followed by acetylation of Cys-GlcN-Ins with acetyl-CoA to produce MSH. If MSH serves as an intracellular reserve of cysteine, then a pathway for regeneration of cysteine from MSH must exist and might, in principle, involve initial cleavage of any of the three links in MSH to produce acetate plus Cys-GlcN-Ins, AcCys plus GlcN-Ins, or AcCys-GlcN plus inositol.

The objective of the present study was to measure the cellular levels of potential intermediates in the biosynthesis and degradation of MSH. Methods for determination of GlcN-Ins, AcCys, Cys-GlcN, AcCys-GlcN, and Cys-GlcN-Ins are described and used to analyze cellular levels of these mycothiol components in M. smegmatis and other bacteria. Results with M. smegmatis under a variety of conditions provide insight into the pathways of MSH biosynthesis and degradation.

EXPERIMENTAL PROCEDURES

Organisms and Culture Conditions—M. smegmatis strains MC66 and MC2155 and Staphylococcus aureus RN450 were kindly provided by Julian Davies, Department of Microbiology and Immunology, University of British Columbia. Micromonospora echinospora 14847 was purchased from Northern Regional Research Center (NRRL B-12180), and Escherichia coli HB101 was purchased from Promega. M. smegmatis cultures were grown in Middlebrook 7H9 broth (0.05% Tween 80 and 0.4% glucose) at 37 °C. S. aureus and E. coli cultures were grown in Trypticase soy broth at 37 °C.

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Todd Hewitt broth (0.5% yeast extract and 0.25% sucrose) at 28 °C. All cultures were shaken at 220 rpm.

Reagents—AccQ-Fluor was purchased from Waters. ATP, AcCys, GlcNHCl, HEPES, inositol, NEM, sucrose, Tris, and yeast extract were obtained from Sigma. Cys and DTT were from Calbiochem. 3-C-inositol was from NEN Life Science Products, Inc., and Tween 20 was from Fisher. and acetonitrile were from Mallinckrodt. Atto-Tag CBQCA, dansyl chloride, fluorescamine, mBBR, and 12-(N-methyl-1H-1,3-diazol-2-oxa,1,3-diazol-4-yl) chloride were purchased from Molecular Probes. Methanesulfonic acid was supplied by Fluka. Cys-GlcN and AcCys-GlcN were prepared as described previously (8). Middlebrook 7H9, yeast extract, and Trypticase soy broth were purchased from Difco Laboratories, and Trypticase soy broth was purchased from BBL. All other reagents were of reagent grade or higher quality.

Preparation of 1-α-myoinositol-2-amino-2-deoxy-α-D-glucopyranoside (GlcN-Ins)—GlcN-Ins was purified from M. echinospora (NRRL B-12180) by a modification of the method of Maehr et al. (9). M. echinospora was cultured on 0.5% yeast extract, 3% Todd Hewitt media, and 0.25% sucrose to late log phase and collected by centrifugation. Bulk cell pellets were frozen at −70 °C until the material was processed. A 235-g pellet of M. echinospora was suspended in 1 liter of 50% acetonitrile-water (60 °C) containing 20 mM H2SO4 (pH 5 cell suspension). The suspension was adjusted to pH 2.5 with concentrated H2SO4 and then disrupted using a Branson sonicator at ~70% maximum power for 15 min without cooling. The cell extract was cooled on ice and the cell debris was removed by centrifugation at 6000 g for 15 min at 4 °C. The supernatant was reduced to 250 ml using a rotary evaporator and clarified by centrifugation as above. The supernatant contained 240 μmol of thiol by assay with 5,5′-dithiobis(2-nitrobenzoic acid) (10).

Next, MSH was recovered from the extract. DTT (250 μmol) was added, and the extract was adjusted to pH 7.9 with concentrated NH4OH. After clarification by centrifugation, the supernatant was passed over a 2-thiopyridine-activated thiolpropyl-agarose column (3), revealed that GlcN was a minor component and that another amine had been completely removed. The product gave a small peak at mass 341 (molecular ion) and a major peak at mass 364 (molecular ion plus 2 mass unit) by negative ion APCI mass spectrometry.

Assignment GlcN-Ins MSmB

| Assignment | GlcN-Ins | MSmB
|------------|----------|----------|
| 1-D-Gluosamine | 5.13 (d) | 5.12 (d, 3.5) |
| 2 | 2.76 (dd, 10.4, 3.8) | 3.96 (dd, 10, 3.5) |
| 3 | 3.76 (m) | 3.78 (t, 10) |
| 4 | 3.38 (t, 10) | 3.45 (t, 10) |
| 5 | 3.84 (m) | 3.86 (m) |
| 6 | 3.76 (m) | 3.79 (m) |
| 3.84 (m) | 3.88 (m) |

* Data are from Ref. 3.

Determination of GlcN and GlcN-Ins—AceQ-Fluor was dissolved in acetonitrile to 10 mM as recommended by the manufacturer. Standard solutions of 1-glucosamine-HCl and purified GlcN-Ins were prepared at 3.1 to 200 μM in water. For the derivatization of GlcN and GlcN-Ins, 6 μl of the standard amine was dilute to 30 μl in 200 μM HEPES (pH 8.0), 15 μl of acetonitrile; 15 μl of 10 mM AceQ-Fluor were added, and the mixture vortexed. After 1 min at room temperature, samples were heated for 10 min at 60 °C. The samples were diluted 4-fold with water and stored at −70 °C. To determine GlcN and GlcN-Ins levels in cells, a sample of cell suspension on ice and then disrupted using a Bransonic sonicator at 70% maximum power for 15 min at room temperature, the samples were heated for 10 min at 60 °C, diluted 4-fold with water, and stored at −70 °C. The cell pellet was dried in a tared tube to determine the residual dry weight (RDW) of the extract. The AceQ-Fluor-derivatized samples were analyzed by HPLC utilizing a Waters 600E solvent delivery system equipped with a Waters WISP Model 710B autoinjector, Laboratory Data Control Flurometer III, and a Nelson Model 444 data collection system. Separation was obtained on a Beckman Ultraphase IP (250 × 4.6 mm) analytical column equipped with a Brownley HPLC guard column containing an OD-GU 5-μm C18 cartridge using the following linear gradients: 0 min, 100% A (0.1% trifluoroacetic acid in water); 10 min, 100% A; 50% B (0.1% trifluoroacetic acid in methanol); 53 min, 100% B; 57 min, 100% B; 60 min, 100% A; 70 min, recombination. The flow rate was 1 ml/min, and the effluent was monitored by fluorescence with an excitation at 370 nm and emission at 418–700 nm. Determination of GlcN and GlcN-Ins by NMR was carried out as described previously (5). HPLC analysis of Cys, MSH, AcCys-GlcN, and H2S was performed using method 1A, a modification of the previously described method 1 (12), with the gradient as follows: 0 min, 10% B; 5 min, 10% B; 15 min, 18% B; 45 min, 37% B; 65 min, 70%; 67 min, 100% B; 68 min, 100% B; 70 min, 10% B; 80 min, 10% B (reinjection). For separation of Cys-GlcN-Ins and Cys-GlcN,
the HPLC conditions were identical to those of method 5 above with the following modifications: solvent B was 7.5% methanol in acetonitrile, and fluorescence detection was accomplished with a 370-nm excitation filter and a 418–700 emission filter (designated method 5A).

**Assay of ATP-dependent Ligase Activity with GlcN-Ins Plus Cys/AcCys**—A minor modification of the protocol described by Bornemann et al. (6) was used. *M. smegmatis* MC²155 was grown to mid-log phase in Middlebrook 7H9 medium with 0.4% glucose and 0.05% Tween. Cells were pelleted and washed with 50 mM sodium phosphate (pH 7.5) containing 1 mM DTT and resuspended in the same buffer at a concentration of 0.25 g of wet weight/ml. Cells were lysed by sonication on ice and pelleted by ultracentrifugation at 100,000 × g for 30 min in a Beckman tabletop ultracentrifuge. Assay of the supernatant was conducted in a final volume of 600 μl containing 60 μl of supernatant, 50 μM GlcN-Ins, 100 μM Cys or AcCys, 100 μM sodium acetate, 1 mM ATP, 1 mM MgCl₂, 50 mM sodium phosphate (pH 7.5), 1 mM DTT, and 35 μM each of the protease inhibitors phenylmethanesulfonyl fluoride, N-α-p-tosyl-l-phenylalanlychloromethyl ketone, and N-α-p-tosyl-l-lysinechloromethyl ketone. The mixture was incubated at 30 °C, and 100 μl samples were removed at 0 and 60 min for thiol analysis. One sample was mixed with 4 μl of 100 mM mBBr and allowed to react for 5 min at room temperature before acidification with 0.5 μl of 5 M methanesulfonylic acid to quench the reaction. A second control sample was reacted 5 min with 5 mM NEM before treatment with mBBr as above.

**RESULTS**

**Analysis of GlcN and GlcN-Ins**—Several standard methods of fluorescent amine labeling in conjunction with HPLC were tested initially with GlcN as potential ways to determine GlcN and GlcN-Ins. Reaction with dansyl chloride, fluorescamine, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)) chloride, and CBQCA (Molecular Probes) provided markedly poorer sensitivity than labeling with AccQ-Fluor, a reagent recently developed for analysis of amino acids (13). For labeling of amino sugars, a buffer of slightly lower pH than employed with amino acids was chosen so that the amino sugars, whose ammonium forms have lower pKa values than those of most amino acids, would have a competitive advantage during labeling of cell extracts. The chromatogram obtained with a standard GlcN-Ins sample and the corresponding NEM control sample showed a peak at approximately 60 min with a retention time of 60 min. A second control sample was reacted 5 min with 5 mM NEM before treatment with mBBr as above.

**Fig. 1.** HPLC chromatograms (HPLC method 5) for analysis of amino sugars as their AccQ-Fluor derivatives. A, GlcN standard; B, GlcN-Ins standard; C, extract of *M. echinospora*.

**Fig. 2.** Calibration curves for analysis of GlcN (●) and GlcN-Ins (○).

**Fig. 3.** HPLC chromatograms (method 5A) for determination of Cys-GlcN-Ins and Cys-GlcN after labeling with mBBr. A, 1st panel, mB-Cys-GlcN-Ins standard; 2nd panel, mBBr-labeled extract of log phase *M. smegmatis* MC²155 cells; 3rd panel, NEM-treated and mBBr-labeled extract of log phase *M. smegmatis* MC²155 cells showing nonthiol fluorescent components; 4th panel, mB-CG standard. R denotes peaks produced in controls and derived from the reagent (mBBr).

**Fig. 4.** Expanded and amplified (50×) portion of the HPLC chromatogram for an mBBr-labeled sample with a Cys-GlcN-Ins content of 0.07 μmol/g RDW (solid line) and the corresponding NEM control sample (dotted line).
the end of the published isolation protocol our GlcN-Ins sample, although apparently pure by TLC, proved to be substantially contaminated by another amine. Pure GlcN-Ins was obtained by adding an additional purification step; the overall yield was poor but adequate for the present purposes.

The chromatogram obtained from a standard solution of GlcN-Ins after labeling with AccQ-Fluor is shown in Fig. 1 (middle panel), and a representative standard curve is included in Fig. 2. Fig. 1 also presents the chromatogram obtained from the labeling of a sample of a 50% acetonitrile extract of the *M. echinospora* used for the purification of GlcN-Ins. The peak for GlcN-Ins corresponds to a content of \(0.8\) \(\mu\)mol/g of RDW. From this value it can be calculated that the recovery in our purification of GlcN-Ins was only 8%. No doubt this can be improved with suitable modifications of the purification protocol and the use of AccQ-Fluor labeling with HPLC analysis to more closely monitor the purification process.

**Analysis of the Thiol Components of Mycothiol**—Cellular thiol levels were analyzed by HPLC after fluorescent labeling of the thiol moiety with mBBr (14). Quantitative values for the cellular thiol levels were obtained after correction for any non-thiol fluorescent background identified in control samples in which thiols were blocked by reaction with NEM before treatment with mBBr. Standards were prepared by labeling of the commercial (Cys, AcCys) or synthetic (Cys-GlcN, AcCys-GlcN) thiols or of isolated MSH (3). A standard for labeled Cys-GlcN-Ins was obtained by partial hydrolysis of MSmB, purification of CySmB-GlcN-Ins by preparative HPLC, and quantitation of the standard based upon the absorbance of the bimane label. All of these bimane derivatives could not be separated from each other, from reagent-derived components, and from fluorescent cellular materials using a single HPLC protocol, and many different HPLC separations were tested before two methods were found that provided analyses for the thiols of interest without major coeluting peaks in the NEM control sample. Fig. 3 illustrates the analysis used to obtain values for Cys-GlcN-Ins and Cys-GlcN. In cell extracts, no peaks were observed for Cys-GlcN, and reported values represent the limits of detection (Fig. 3B). Peaks for Cys-GlcN-Ins were observed with cell extracts as illustrated in Fig. 3B. An unidentified thiol peak of comparable size to the Cys-GlcN-Ins peak and eluting at 26.7 min is also apparent in Fig. 3B. Fig. 4 illustrates the analysis for Cys, AcCys, AcCys-GlcN, MSH, and H\(_2\)S; the Cys-GlcN-Ins and Cys-GlcN derivatives were only slightly retained and could not be separated under these conditions. The peaks for Cys and MSH have small coeluting peaks present in the NEM control (Fig. 4, lower panel), which amounted to 10 and 2%, respectively, of the Cys and MSH peaks measured in the sample (Fig. 4, middle panel). In calculating the Cys and MSH content, small corrections for these control values were incorporated.

**TABLE II**

| Organism       | GlcN | GlcN-Ins | H\(_2\)S | Cys  | AcCys | Cys-GlcN | AcCys-GlcN | Cys-GlcN-Ins | MSH  |
|----------------|------|----------|----------|------|-------|----------|------------|--------------|------|
| *M. smegmatis* | 0.030| 0.10     | 1.0      | 0.09 | 0.077 | <0.01    | <0.01      | <0.002       | 7.4  |
| *M. smegmatis* | 0.013| 0.10     | 0.87     | 0.16 | 0.030 | <0.003   | <0.003     | <0.008       | 9.9  |
| *S. aureus*    | ±0.004| ±0.04 | ±0.05 | ±0.04 | ±0.004 | ±0.005 | ±1.6       |              |      |
| *E. coli*      | 0.027| <0.001  | 0.36     | 0.35 | 0.002 | <0.03   | <0.004     | <0.004       | <0.05| <0.04 |
|                | 0.024| <0.001  | 1.7      | 0.0069 | 0.004 | <0.004   | <0.004     | <0.004       | 0.1  |

Numbers designated with \(<\) represent detection limits where no discernable peak was present. Values designated with \(\pm\) represent measurement of a discernable peak at the retention time for the indicated component but for which independent verification of the structure was not available; the value represents an upper limit for the content.
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**Variation in Metabolite Levels During Growth**—Levels of potential MSH intermediates were determined for *M. smegmatis* MC2155 as a function of growth phase (Fig. 5). The MSH level remained remarkably constant through exponential growth and into stationary phase, as did the Cys level at about 1% the MSH level. However, in stationary phase, there did appear to be a significant drop in the GlcN and GlcN-Ins levels and an increase in the H$_2$S level.

**Effect of Nutrient Supplementation and Deprivation Upon Intermediate Levels**—To test whether an increase in cellular Ins content would influence the levels of MSH or potential MSH intermediates, we examined *M. smegmatis* MC2155 incubated with Ins. We initially tested Ins uptake by adding 37 μM [^{14}C]Ins to a culture at $A_{600} = 1.0$ and determining the loss of radioactivity from the medium and its appearance in cells. After 2.5 h, 77% of the counts had been lost from the medium to the cells, showing that *M. smegmatis* efficiently imports Ins. Next we harvested cells after growth to mid-log phase, resuspended them at $A_{600} = 1.05$ in 7H9 media with glucose and 1 mM Ins at 37 °C, and took samples for analysis at 0, 0.5, and 2.5 h (Fig. 6A). The GlcN-Ins content was initially more than 3-fold greater than normal but fell to about half normal at 0.5 h before returning to normal at 2.5 h. Smaller reciprocal changes occurred in the Cys levels, whereas the MSH and H$_2$S levels did not change to a significant extent. The $A_{600}$ value increased 40% over the 2.5 h incubation, a change comparable with that found for exponential cultures without added Ins.

To test whether changes in cellular Cys levels would influence MSH or MSH intermediates, we incubated cells in medium containing 10 mM AcCys. Because AcCys is readily taken up by passive diffusion, we expected this to produce markedly elevated levels of cellular AcCys and, through deacylation, Cys. The results (Fig. 6B) showed this to be the case; the cellular AcCys level was elevated 1000-fold over the normal level, and the Cys level was increased 50-fold. This was accompanied by a 5- to 10-fold decrease in GlcN-Ins content, whereas the H$_2$S level roughly doubled. The Cys-GlcN-Ins content was elevated 20-fold over normal immediately after the start of incubation but then fell a factor of 70 over the next 2.5 h. Least affected was the MSH content, which was elevated ~40% after 2.5 h. The $A_{600}$ value increased little (5%) over the 2.5-h incubation period, indicating that AcCys significantly inhibits growth under these conditions.

Incubation of cells in the absence of glucose (Fig. 6C) resulted in a decrease in GlcN-Ins level to values 13–30% of normal during the initial 2.5-h incubation, but continuation of the incubation resulted in a return to normal values by the end of 8 h (data not shown). The MSH level increased to 40% over normal by 2.5 h and remained at that value to 8 h. The value of $A_{600}$ declined slightly (10%) during the 2.5-h incubation period, showing that glucose deprivation arrested cell growth.

**Assay of Enzyme Activity for ATP-dependent Ligation of Cys and AcCys to GlcN-Ins**—Bornemann et al. (6) demonstrated the presence of enzyme activity in extracts of *M. smegmatis* capable of converting Cys plus GlcN-Ins to Cys-GlcN-Ins in the presence of ATP and the accompanying production of MSH, which was enhanced by acetate or acetyl-CoA. They were unable to assess AcCys as substrate in this reaction, because it was rapidly converted to Cys. We conducted analogous assays with an undialyzed supernatant fraction from extraction of *M. smegmatis*. Incubation of 100 μM Cys and 50 μM GlcN-Ins with 1 mM ATP in phosphate buffer (pH 7.5) containing 1 mM DTT after the addition of cell extract supernatant resulted in an increase in Cys-GlcN-Ins from <0.2 to 26 μM over 60 min, whereas the MSH content declined from 15 to 13 μM, corresponding to the MSH content present in the undialyzed cell.

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**Note:** The α and β epimers of bimane derivatives of Cys-GlcN and AcCys-GlcN interconvert to give an equilibrium mixture producing two partially resolved peaks with similar retention times (Figs. 3A and 4).

**Analysis of Selected Bacteria**—Results for analysis of the full range of potential MSH intermediates in several bacteria harvested in log phase growth are given in Table II. Analysis of extracts from *M. smegmatis* MC2155 revealed the presence of a measurable GlcN-Ins content. To check whether the values are sensitive to the sample size analyzed, possibly as the result of depletion of the labeling agent by the plethora of amines present in cells, we analyzed 30 μl of supernatant from extracts (1 ml volume) of *M. smegmatis* MC2155 with increasing sample size from 3.9 to 61 mg RDW of cell pellet residue. For samples sizes up to 30 mg of RDW, there is an apparent slight decrease in measured GlcN-Ins content with increasing sample size (0.13%/mg RDW), but the change is within the uncertainties in the measurements. However, above 30 mg RDW, a marked decrease in measured value was apparent. To prevent underestimates of GlcN-Ins content, all quantitative determinations were made with samples corresponding to ≤30 mg RDW.

*M. smegmatis* MC6, the parent strain of *M. smegmatis* MC2155, also produced GlcN-Ins in significant amount. Cys was also found, but neither strain appeared to produce comparable amounts of other potential intermediates of MSH metabolism, including Cys, AcCys, GlcN-Ins, or Cys-GlcN-Ins. It had been previously shown that *S. aureus* and *E. coli* did not produce MSH (5). These were reexamined using the present methods to ascertain whether they might produce one or more of the intermediates involved in mycothiol biosynthesis and especially GlcN-Ins. The results (Table II) indicate that neither of these bacteria produce any component of MSH at measurable levels, other than Cys and GlcN.

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**Fig. 6. Effect of nutrient supplementation and glucose deprivation upon levels of MSH precursors and intermediates in *M. smegmatis* MC2155 at 37 °C.** A, cells suspended in 7H9 medium with 0.4% glucose and 1 mM myo-inositol; B, cells suspended in 7H9 medium with 0.4% glucose and 10 mM N-acetylcysteine; C, cells suspended in 7H9 medium without glucose.

Note that the α and β epimers of bimane derivatives of Cys-GlcN and AcCys-GlcN interconvert to give an equilibrium mixture producing two partially resolved peaks with similar retention times (Figs. 3A and 4).

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The present results demonstrate that the MSH level is very tightly regulated in *M. smegmatis*. Only minor changes in concentration were observed in the transition from exponential to stationary phase (Fig. 5) during glucose deprivation (Fig. 6C) and during inositol or AcCys/Cys supplementation (Fig. 6, A and B). The steady-state level of MSH depends upon the net balance in its rate of production and its rate of degradation. These can be influenced at a variety of levels, from feedback inhibition/activation of key enzymes in the pathway to regulation of gene expression, and elaboration of the mechanisms involved will be of interest as more is learned about the enzymes of MSH metabolism.

In conclusion, the present studies have provided some important tools for the study of MSH metabolism. Their application to *M. smegmatis* has provided evidence that GlcN-Ins and Cys-GlcN-Ins are intermediates in the biosynthetic pathway and suggested that AcCys and GlcN-Ins may be involved in the degradative pathway. The HPLC analysis methods presented here complement our immunoassay methods for MSH (8), and together these methods provide important tools for identifying and analyzing mycobacterial mutants blocked in MSH biosynthesis, a topic of subsequent papers in this series.

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