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ORDER OF ENZYMIC INCORPORATION OF *O*-METHYL GROUPS INTO THE *O*-METHYL-D-GLUCOSE-CONTAINING POLYSACCHARIDE OF *Mycobacterium smegmatis*. A TRITIUM-LABELLING STUDY

DONALD J. WALTON AND DAVID D. JORDAN
Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6 (Canada)
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ABSTRACT

The order of enzymic incorporation of *O*-methyl groups into the *O*-methyl-D-glucose-containing polysaccharide (MGP) of *Mycobacterium smegmatis*, 3MG(*J*)→G(*I*)→G(*H*)→G(*G*)→6MG(*F*)→$(GMG)_9$(*E*)→[G(*L*)→G(*D*)]→G(*C*)→[G(*K*)→G(*B*)]→G(*A*)→Ga, where G is D-glucose, 3MG is 3-*O*-methyl-D-glucose, 6MG is 6-*O*-methyl-D-glucose, and Ga is D-glyceric acid, was studied by incubating cultures of *M. smegmatis* with L-[^{3}H-*Me*]methionine for various times. MGP was then extracted from the cells, and relative radioactivities of residues *D*, $(E + F)_{average}$ and *J*, or of *D*, $E_{average}$, *F*, and *J*, were determined. Tritium-labelling of these residues increased in the reducing-to-nonreducing residue direction, the steepness of the gradient becoming more shallow with increasing incubation time. The results are consistent with a biosynthetic mechanism that involves sequential addition of *O*-methyl groups to residues of the pre-formed D-glucan, in the reducing-to-nonreducing residue direction.

INTRODUCTION

Mycobacterium smegmatis cells contain two type of cytosolic polymethylpolysaccharides that stimulate fatty acid synthesis by complexing with palmitoyl-CoA (ref. 1), thereby relieving product inhibition of the synthetase. One of them is an *O*-methyl-D-glucose-containing polysaccharide (MGP; **1**) that contains eleven internal, contiguous 6-*O*-methyl-D-glucose residues and a 3-*O*-methyl-D-glucose residue at the nonreducing terminus[2]. Although conclusive proof is lacking, the available evidence[3] suggests a biosynthetic mechanism for MGP which begins with the assembly of D-glucose residues, with the completed D-glucan being methylated by *S*-adenosylmethionine in the presence of a methyltransferase. After studying the substrate specificity of this enzyme, Grellert and Ballou[4] speculated that it recognizes residues *F* (five units from the nonreducing end) and *D* (a branch point) as start or stop signals for methylation. They also questioned whether methylation of the D-glucan is sequential, like the *in vitro* methylation of DNA[5], or random. We describe herein tritium-labelling studies in which this question has been addressed,

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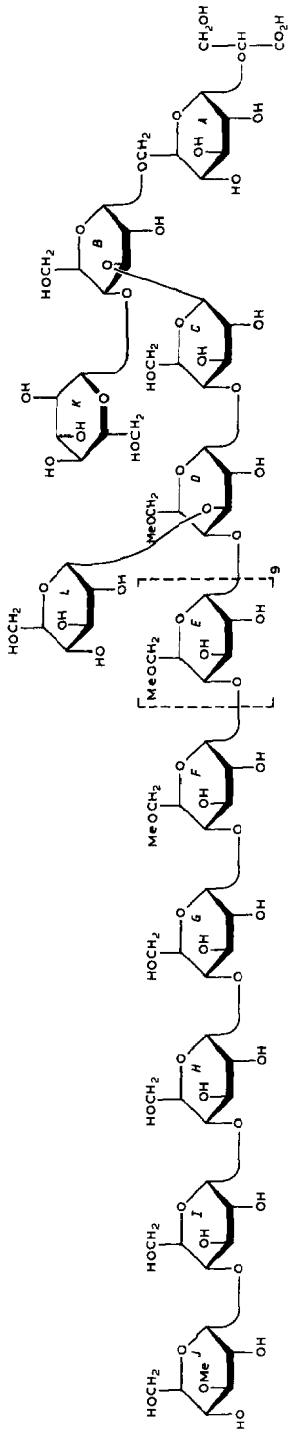
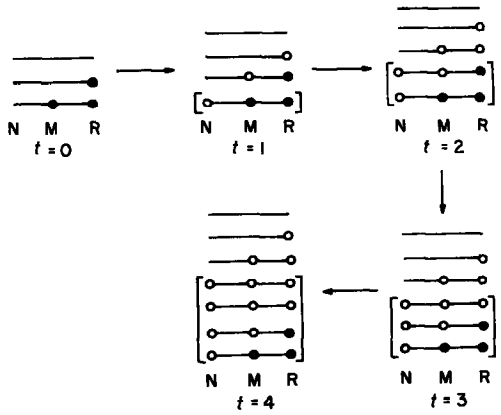




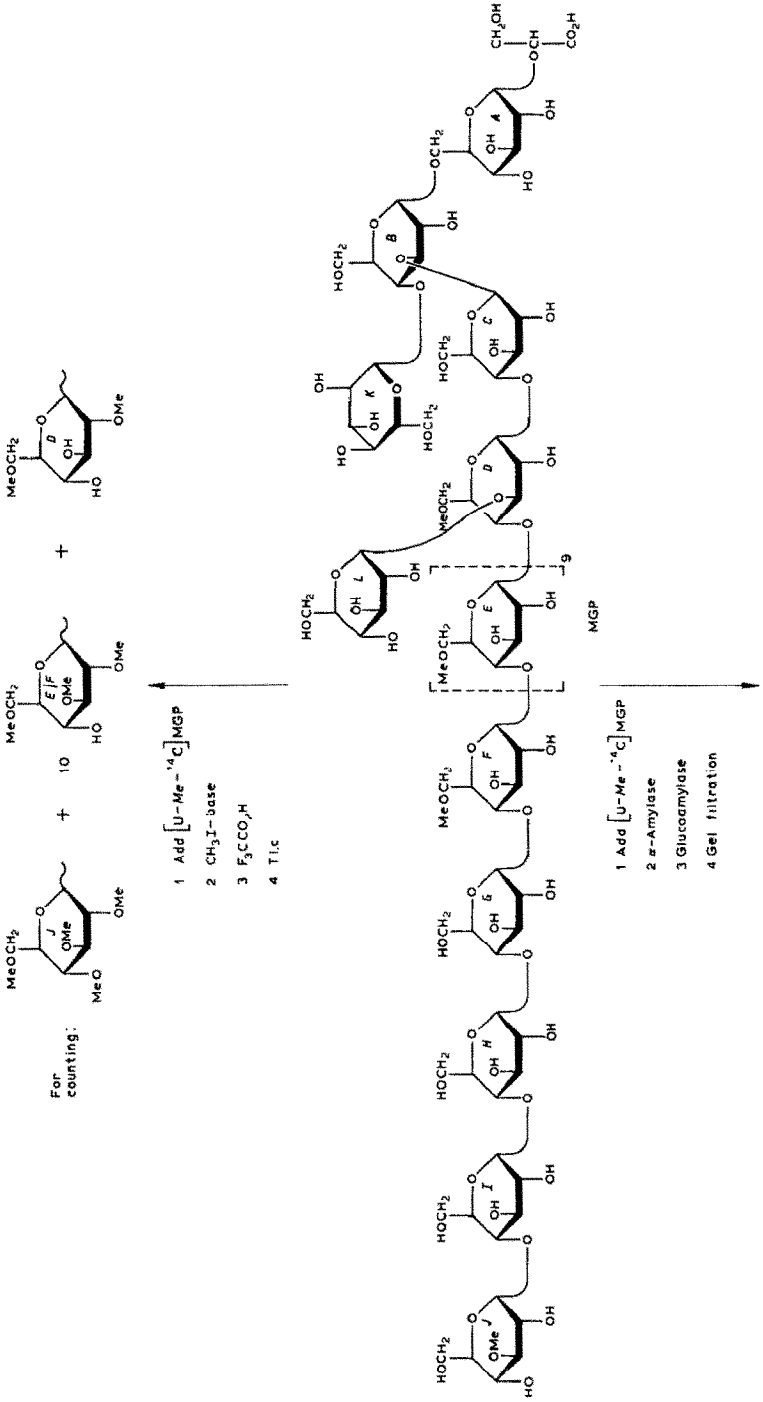
Scheme 1. Sequential model for methylation of a trisaccharide in a bacterial culture. The letters N, M, and R represent the nonreducing, mid-chain, and reducing residues of each trisaccharide molecule. Each residue can accommodate a single *O*-methyl group. Residues R, M, and N are always methylated in that order. Closed and open circles represent unlabelled and tritium-labelled *O*-methyl groups, respectively. One unmethylated chain and two partially methylated chains are shown at the start (t = 0), when L-[*Me*-^{3}H]methionine is added to the medium. During each time interval, a labelled methyl group is added to each chain, and a new, unmethylated trisaccharide is synthesized. The distributions of labelled methyl groups among residues N, M, and R of fully methylated chains (enclosed in square brackets) are as follows: at t = 1, only N labelled; at t = 2,3, and 4, labelling of N/M/R is 2:1:0, 3:2:1, and 4:3:2, respectively.

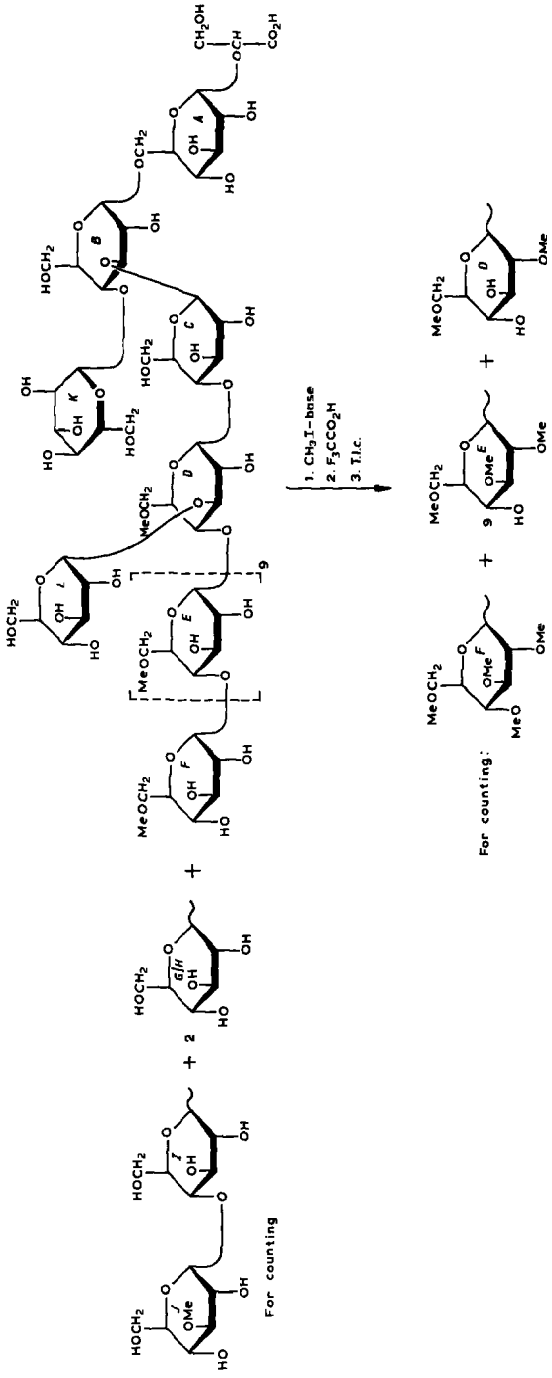
and present results to support a mechanism by which the incorporation of methyl groups into MGP is sequential.

RESULTS AND DISCUSSION

The experimental approach in this work resembled that of Dintzis[6], who demonstrated that, following an incubation of reticulocytes with [^{3}H]leucine, the radioactivity of leucyl residues of completed hemoglobin α- and β-chains increased along each polypeptide in the direction N-terminus to C-terminus. This result supported a model of polypeptide synthesis involving the sequential addition of amino acid residues in the same direction. To understand how similar principles were employed in our laboratory to study the timing of incorporation of methyl groups into MGP, it is useful to consider the model system shown in Scheme 1. Here, a trisaccharide undergoes sequential *O*-methylation in the reducing-to-nonreducing residue direction. After the addition of L-[*Me*-^{3}H]methionine to the medium, a gradient of radioactivity develops in the population of completely methylated trisaccharide molecules, the *O*-methyl group at the nonreducing residue being the most highly labelled. Note that the gradient is maximal after three methylation steps, lessens thereafter, and would be imperceptibly small after many steps.

With this model in mind, we examined the distribution of labelling in MGP


For counting:
1 Add [U-Me-^{14}C]MGP
2 CH_3I-base
3 F_3CCO_2H
4 Tlc
MGP
1 Add [U-Me-^{14}C]MGP
2 α-Amylase
3 Glucoamylase
4 Gel filtration



Scheme 2. Measurement of distribution of tritium in MGP isolated from cells grown in L-[*Me*-^{3}H]methionine. For explanation, see text. The fates of only those residues having labelled methyl groups, D, E, F, and J, are shown here.

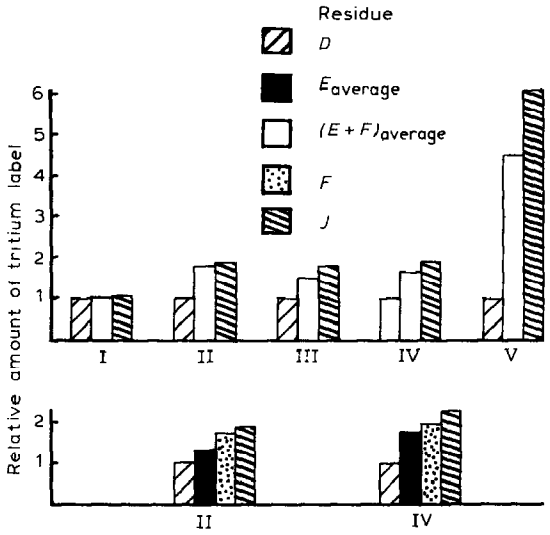


Fig. 1. Distribution of tritium-labelling in MGP isolated from cells grown in a medium containing L-[*Me*-^{3}H]methionine for 3 days (experiment I), 15 min (II, III, and IV), and 0.5 min (V). Specific activities of labelled methionine were 2.8 PBq/mol for experiment IV and 0.44 PBq/mol for all other experiments. Heights of bars represent relative magnitudes of the ratios, ^{3}H-d.p.m./^{14}C-d.p.m., for specific residues, or groups of contiguous residues. Bars representing residue *D* have been assigned an arbitrary height of 1 scale unit. Actual values of ^{3}H-dp.p.m./^{14}C-d.p.m. for residue *D* varied between 10.9 and 2.20 (for experiments I and V, respectively).

isolated from *M. smegmatis* cells that had been incubated with L-[*Me*-^{3}H]methionine for different times (see Scheme 2). The [*Me*-^{3}H]MGP was isolated and mixed with uniformly-labelled [*Me*-^{14}C]MGP, and the mixture was chemically methylated and hydrolyzed. The *O*-methyl derivatives of glucose were separated by t.l.c., and the radioactivity of those that contained tritium was counted. The magnitudes of the ratios, ^{3}H-d.p.m./^{14}C-d.p.m., for 2,6-di-*O*-methyl-, 2,3,6-tri-*O*-methyl-, and 2,3,4,6-tetra-*O*-methyl-D-glucose represented the relative amounts of tritium-labelling of the methyl groups at residues *D*, $(E + F)_{average}$, and *J*, respectively. A modification of the procedure involving chemical methylation of enzymically-degraded MGP (see Fig. 2) gave an additional ^{3}H/^{14}C ratio for residue *F*.

Significant differences were observed in the tritium-labelling of residues *D*, $(E + F)_{average}$, and *J* in MGP isolated after a 0.5-min incubation with L-[*Me*-^{3}H]methionine (see upper portion of Fig. 1). The differences were less after a 15-min incubation, and almost nonexistent after three days of incubation. In two cases, differential labelling was demonstrated at residues *D*, $E_{average}$, *F*, and *J* (lower part of Fig. 1). In all experiments, tritium-labelling increased in the reducing-to-nonreducing residue direction.

All of the observed labelling patterns are consistent with a process of the type illustrated in Scheme 1, in which methyl groups are added sequentially in the reducing-to-nonreducing residue direction. The diminution of the steepness of the

gradient with increasing incubation time can also be rationalized in terms of this mechanism. An alternative mechanism, in which the methyltransferase acts at random, but in which rates of methylation, fortuitously, decrease in the order $J > F > E > D$, cannot be excluded but is highly implausible, and we favour the sequential mechanism.

Finally, it was reasoned that if the concentration of the methionine in the medium was reduced, the cells might use it up in a shorter time than usual, which would result in the formation of a steeper gradient of tritium-labelling in the MGP produced. In fact, the steepness of the gradient was not increased significantly when L-[^{3}H]methionine of high specific activity (*i.e.*, smaller mass) was used (Fig. 1, experiment IV), presumably because the labelled methionine was rapidly diluted by a relatively large endogenous pool of the unlabelled compound.

EXPERIMENTAL

General methods. — L-[*Me*-^{3}H]- and L[*Me*-^{14}C]-methionine were obtained from Amersham (Amersham, U.K.). Bio-Gel P-2 (200–400 mesh), Sephadex media and pancreatic α-amylase were products of Bio-Rad (Richmond, CA), Pharmacia (Montreal), and Worthington (Freehold, NJ), respectively. *A. niger* glucoamylase I was a gift from Dr. J. Pazur. Radioactive samples were dissolved in Aquasol scintillator solution (10 mL; New England Nuclear, Boston, MA) and the radioactivity was counted with a Beckman LS 3100 liquid scintillation spectrometer. Channel ratios were used to establish counting efficiencies. Effluents from chromatography columns were monitored by the phenol–H_2SO_4 colorimetric method[7]. For t.l.c. of hydrolyzates of permethylated [^{3}H, ^{14}C]MGP, layers of Silica Gel G, 0.25-mm thick, were developed twice with 15:1 (v/v) chloroform–methanol. Areas containing labelled 2,6-di-*O*-methyl-, 2,3,6-tri-*O*-methyl-, and 2,3,4,6-tetra-*O*-methyl-D-glucose were located by chromatographing a reference mixture in the outer lanes. The latter were sprayed with 50% aqueous H_2SO_4 while central lanes were protected with polyethylene, and the plate was heated until spots were formed. The *O*-methyl derivatives had R_F 0.12, 0.42, and 0.75, respectively. Hydrolyzates also contained 2,3,4-tri-*O*-methyl-D-glucose, R_F 0.33, but this was ignored, as it was not tritium-labelled.

Unlabelled MGP. — *M. smegmatis* cells (ATCC 346) were grown in a glycerol–Casamino acids medium[8] (200 L) in a fermenter at the National Research Council of Canada, Ottawa. The acylated form of MGP was extracted with 70% ethanol and then saponified, as described by Saier and Ballou[2]. The resulting MGP was purified by chromatography on DEAE-Sephadex, followed by Bio-Gel P-2. Pure MGP (250 mg) was obtained from a 700-g batch of packed, wet cells.

[U-Me-^{14}C]MGP. — Cells were grown in glycerol–Casamino acids medium (50 mL) containing L-[*Me*-^{14}C]methionine (3.7 MBq; 1.85 TBq/mol) for 3 days at 37°. Extraction and purification, as described above, gave 0.2 mg (0.118 MBq) of product, stored as a frozen solution.

[Me-^3H]*MGP for tritium distribution studies.* — Cultures of *M. smegmatis* were grown in glycerol–Casamino acids medium (50 mL) for 3 days at 37°. The cells were collected by centrifugation, and resuspended in a glycerol medium (50 mL) containing methionine-free Difco "Methionine Assay Medium", instead of Casamino acids, together with L-[*Me*-^{3}H]methionine (11–15 MBq). Cultures were shaken at 37° for various times up to 3 days, during which the growth rate was 67% of that occurring in the Casamino acids medium. [*Me*-^{3}H]MGP was isolated and purified as described above, except that the extract was supplemented with unlabelled MGP (3 mg) just before the two chromatographic steps.

Measurement of tritium distribution in [Me-^3H]*MGP.* — Sufficient [U-*Me*-^{14}C]MGP was added to the [*Me*-^{3}H]MGP to give a ratio, ^{3}H-d.p.m./^{14}C-d.p.m., of 8–15:1. The double-labelled MGP was methylated twice by the Hakomori procedure[9]. The product was purified on a column of Sephadex LH-20 packed in chloroform, and was then hydrolyzed with 2M trifluoroacetic acid (1 mL) for 2 h at 110°. The hydrolyzate was subjected to t.l.c.; the areas containing 2,6-di-*O*-methyl-, 2,3,6-tri-*O*-methyl-, and 2,3,4,6-tetra-*O*-methyl-D-glucose were scraped from the plate and extracted with methanol (1 mL), and the radioactivity of the methanol extracts was counted. In two cases, double-labelled MGP (2–3 mg) was degraded with α-amylase, followed by glucoamylase I, according to published methods[10]. The hydrolysis products (see Scheme 2) were fractionated by gel filtration on a column (2 × 90 cm) of Bio-Gel P-2. The radioactivity of disaccharide *IJ* was counted, and the degraded, double-labelled MGP was subjected to the same processing (permethylation, hydrolysis, t.l.c., and counting) as that described for the undegraded compound.

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