UDP-*N*-METHYL-D-GLUCOSAMINE-PHOSPHATE — A POSSIBLE INTERMEDIATE OF *N*-METHYL-L-GLUCOSAMINE MOIETY OF STREPTOMYCIN

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The formation of *N*-methyl-L-glucosamine moiety of streptomycin from D-glucosamine by *Streptomyces griseus* was studied. The addition of thymine to the culture medium stimulated the formation of streptomycin and the incorporation of D-glucosamine into *N*-methyl-L-glucosamine moiety. During a study of sugar nucleotides in the mycelia, a novel UDP-amino sugar was isolated. The compound was formed before the maximum production of streptomycin. It was UDP-*N*-methyl-D-glucosamine-phosphate.

The synthesis of the carbon chain of *N*-methyl-L-glucosamine of streptomycin has been studied.[1~4] We reported previously the possibility that the OH residue attached to C-3 of D-glucose might be inverted through oxidation and reduction without scission of the carbon skeleton.[4] BLUMSON and BADDILEY[5] suggested that *N*-methyl-L-glucosamine would occur as part of a nucleotide sugar at some stage in the biosynthetic route. AKAMATSU[6] isolated an UDP-*N*-acetyl-amino sugar from growing mycelia of *Streptomyces griseus* before streptomycin production became maximal; the sugar portion was *N*-acetyl-D-glucosamine. The possibility that D-glucosamine might be involved in the synthesis of *N*-methyl-L-glucosamine has been studied.[2,3] In the present paper, we describe the occurrence of a novel sugar nucleotide identified as UDP-*N*-methyl-D-glucosamine-phosphate, the formation of which correlated with the production of streptomycin.

Materials and Methods

Materials

N-Methyl-L-glucosamine hydrochloride was prepared from streptomycin sulfate by the procedure of SILVERMAN and RIEDER.[1] Diaminopimelic acid, muramic acid and UDP-*N*-acetyl-D-glucosamine were purchased from Sigma. Alkaline phosphatase from calf intestinal mucosa was from Miles-Seravac. D-[1-^{14}C]Glucosamine (60.8 mCi/mmole) and D-[6-^{3}H]glucosamine (38 Ci/mmole) were purchased from Radiochemical Centre. Other chemicals were of commercial origin.

Microorganisms

S. griseus ME 936-B3, from Institute of Microbial Chemistry, Tokyo, was used for streptomycin production. *Bacillus subtilis* IAM 1069, a streptomycin-sensitive strain, was supplied by the Institute of Applied Microbiology, University of Tokyo.

Culture Conditions

S. griseus was grown in a medium containing 0.5% D-glucose, 0.5% D-glucosamine, 0.5% meat extract, 0.5% Polypeptone, 0.5% NaCl, with or without 0.04% thymine. Fifty ml of the medium was inoculated with 1 ml of a vegetative culture and shaken vigorously at 28°C for indicated times.

In the isotopic experiments, D-[1-^{14}C]glucosamine (10 μCi, 0.16 μmole) was administered to thymine-supplemented medium and D-[6-^{3}H]glucosamine (10 μCi, 0.26 nmole) was administered to the control medium at indicated times. Incubation was carried out further for 6 hours.

Determination of Streptomycin

Streptomycin was assayed by the agar diffusion method with *B. subtilis* as the test organism.

Isolation and Degradation of Streptomycin

Streptomycin was isolated from the culture media by the method of HUNTER and HOCKENHULL.[7] After addition of carrier streptomycin sulfate (1.0 g), streptomycin was adsorbed on Amberlite IRC-50 (200~400 mesh, Na^+ form) colum (1.2×15 cm) and eluted with 0.5 M HCl. *N*-Methyl-L-glucosamine was then prepared as described above.[1]

Paper Chromatography

Descending paper chromatography was carried out for 18 hours on Toyo No. 51 paper using the following solvents: (a) isobutyric acid - 0.5 M NH_4OH (5: 3, v/v), (b) 95% ethanol - 1 M ammonium acetate, pH 7.5 (5: 2, v/v), (c) 1-butanol - acetic acid - water (3: 1: 1, v/v), (d) isopropanol - water (4: 1, v/v), (e) 1-butanol - pyridine - water (6: 4: 3, v/v). Nucleotides were detected with ultraviolet light. Aniline hydrogen phthalate reagent was used for the detection of reducing sugars.[8]

Isolation of Nucleotides

The nucleotides were isolated from the mycelia by the method of BLUMSON and BADDILEY.[5] The ethanolic extract was applied on a Dowex 1×2 (200~400 mesh, Cl^- form) column (1.2×36 cm). Elution was carried out with a linear gradient with 400 ml of 0.01 M HCl in a mixing flask and 400 ml of 0.20 M LiCl in 0.01 M HCl in a reservoir. Four ml each of the eluate were collected and their absorbance at 260 nm was measured. The appropriate nucleotide fractions were combined and subjected to two dimensional paper chromatography for 18 hours in solvents (a) as first dimension, then in (b) as second dimension. The spots on the paper chromatogram were visualised by ultraviolet lamp. The radioactive spots were detected by Paper Radiochromatography Systems, Actigraph III (Nuclear Chicago). The samples were eluted from the spots with water.

Analytical Procedures

Reducing sugar was determined by the method of PARK and JOHNSON.[9] Total and acid-labile phosphorus were assayed by the method of FISKE and SUBBAROW.[10] Amino acids and muramic acid were analyzed with the Hitachi High Speed Amino Acid Analyzer Type 835 after hydrolysis of the samples in 6 M HCl at 110°C for 24 hours. Optical rotation of the amino sugar was measured with a JASCO J-20 Automatic Recording Spectropolarimeter. Periodate oxidation was performed essentially by the procedure of GUTHRIE,[11] and the uptake of periodate was determined by absorbance at 232 nm.

The radioactive samples were spotted on Whatman 3 MM filter papers (2.4 cm in diameter), and radioactivity was determined in 0.6% 2,5-diphenyl oxazole in toluene with a Beckman liquid scintillation counter.

Results

Effect of Thymine on the Formations of Streptomycin and its Intermediates

It has been suggested that streptose and *N*-methyl-L-glucosamine in the molecule of streptomycin might be present as nucleotide sugars at some stage in the biosynthetic pathway.[5] To increase the production of the intermediates and streptomycin, some purine and pyrimidines were added to the culture medium. The enhancement of streptomycin production was observed only with thymine addition (Table 1). Since the mycelial weight was almost the same in thymine-added and control groups (data not shown), the increase in streptomycin brought about by thymine was probably due to the increased formation of the antibiotic, not to an effect on growth.

As shown in Table 2, the incorporation of radioactive D-glucosamine into streptomycin and into *N*-methyl-L-glucosamine was increased by the addition of thymine to the culture media.

The stimulation of the formation of streptomycin (Table 1) correlates well with the increased incorporation of D-glucosamine into streptomycin and *N*-methyl-L-glucosamine (Table 2).

Table 1. Effect of thymine addition on the formation of streptomycin.

Time of incubation (hours)	Streptomycin (μg/ml)	
	No thymine	0.04% Thymine
24	—	14
48	90	135
72	135	150
96	145	155

Thymine was added to the media at 0 time, and streptomycin was assayed at 24, 48, 72 and 96 hours, respectively.

—: Not determined.

Table 2. Effect of thymine on the incorporation of D-glucosamine into streptomycin and *N*-methyl-L-glucosamine moiety.

Compound analyzed	$^{14}C/^{3}H$ Ratio
Streptomycin	1.45
N-Methyl-L-glucosamine	1.41

D-[1-^{14}C]Glucosamine (10 μCi, 0.16 μmole) and D-[6-^{3}H]glucosamine (10 μCi, 0.26 nmole) were administered to thymine-supplemented medium and the control medium respectively at 24 hours. After further incubation for 24 hours, both broths were combined. $^{14}C/^{3}H$ Ratio in the molecules of streptomycin and *N*-methyl-L-glucosamine were measured.

Sugar Nucleotides in the Mycelia of *S. griseus*

From the above results, we expected that the *N*-methyl-L-glucosamine and/or its precursor as sugar nucleotides would be increased in the mycelia at some stage in thymine-supplemented medium. Radioactive D-glucosamine was added at 40 hours to the culture media both supplemented and unsupplemented with thymine. Several nucleotides corresponding to ultraviolet absorption peaks in ion exchange chromatography of the mycelial extract were checked from their position in elution pattern (Fig. 1). From fractions I, III and IV, several radioactive spots of the sugar nucleotides were separated by two-dimensional paper chromatography. Paper chromatogram of the major sugar nucleotides, compound I-1, III-1, and IV-8 are shown in Fig. 2.

As shown in Table 3, the incorporation of radioactive D-glucosamine into compound I-1 and compound III-1 was increased by the addition of thymine to the culture medium. However, the incorporation into compound IV-8 was decreased. Incorporation into other sugar nucleotides changed only little (data not shown).

Fig. 1. Separation of nucleotides from mycelia by ion exchange chromatography.

D-[1-^{14}C]Glucosamine (10 μCi, 0.16 μmole) and D-[6-^{3}H]glucosamine (10 μCi, 0.26 nmole) were administered at 40 hours to thymine-supplemented medium and the controls, respectively. At 46 hours, both broths were combined. Nucleotides extracted from the mycelia were separated by Dowex 1×2 (Cl^- form) column as indicated in the text. Several fractions of the eluate were combined to the portions. Roman numerals indicate numbers of the portions.

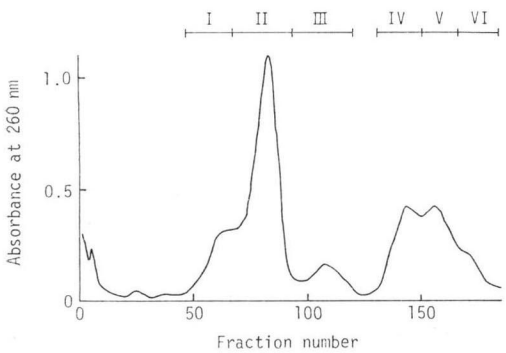


Fig. 2. Paper chromatogram of radioactive sugar nucleotides from *S. griseus*.

The solvents in the direction 1 and 2 were (a) and (b), respectively. Descending method for 18 hours each.

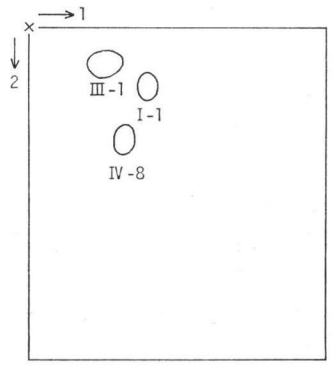


Table 3. Effect of thymine on the incorporation of D-glucosamine into sugar nucleotides.

Compound	$^{14}C/^{3}H$ Ratio
I-1	1.40
III-1	1.36
IV-8	0.75

D-[1-^{14}C]Glucosamine (10 μCi, 0.16 μmole) was administered to thymine-supplemented medium at 40 hours and D-[6-^{3}H]glucosamine (10 μCi, 0.26 nmole) was administered to the control medium at 40 hours. After further incubation for 6 hours, the culture media were combined. Sugar nucleotides were isolated from the mycelia. $^{14}C/^{3}H$ Ratio in the compounds was measured.

Characterization of Compound I-1

Compound I-1 had a characteristic spectrum of the uridine type at both pH 1.0 and pH 11.0. Phosphorus analysis showed the presence of 3 phosphorus atoms per molecule of uridine and 1 molecule of acid-labile phosphorus.

For the identification of the bound sugar, compound I-1 was hydrolyzed with 0.01 M HCl at 100°C for 15 minutes and evaporated *in vacuo*.

Table 4. Paper chromatography of the sugar from compound I-1.

	$R_{N\text{-Acetyl-D-glucosamine}}$		
	Solvents (c)	(d)	(e)
Authentic *N*-methyl-L-glucosamine[a]	0.64	0.81	0.71
Sugar from compound I-1[b]	0.34	0.60	0
Sugar from compound I-1 treated with alkaline phosphatase[c]	0.64	0.81	0.71

S. griseus was grown in thymine-supplemented medium, to which D-[1-^{14}C]glucosamine was added at 40 hours. After further incubation for 6 hours, compound I-1 was prepared from the mycelial extract as indicated in the text.

[a] Detected by aniline hydrogen phthalate reagent.[8]

[b] The sugar was liberated from the compound after hydrolysis in 0.01 M HCl at 100°C for 15 minutes.

[c] The sugar was incubated with 0.12 unit of alkaline phosphatase (from calf intestinal mucosa) in 0.1 ml of 0.02 M tris-HCl (pH 8.0) at 37°C for overnight.

[b,c] Detected by paper radiochromatography systems.

As shown in Table 4, paper chromatographic analysis of the residue in solvents (c), (d) and (e) revealed a single spot. In addition, the sample was located at the origin with solvent (e). After alkaline phosphatase treatment, the Rf values of the sugar in the three solvent systems corresponded to those of authentic *N*-methyl-L-glucosamine.

The position of phosphorus on the sugar chain was studied by the periodate oxidation method.[11] Compound I-1 and UDP were separately oxidized with periodate at room temperature for 3 hours. Periodate uptake was 2.2 and 1.2 moles per mole of the samples, respectively. This result is consistent with the phosphorylation of compound I-1 on the OH group at C-4 of the *N*-methyl-glucosamine moiety. If the OH group at C-3 of the *N*-methyl-glucosamine is phosphorylated, 1 mole of periodate should be consumed, and if the OH group at C-6 is phosphorylated, 3 moles of periodate should be consumed. However, final proof of the position of the phosphorus must await the isolation of the product after periodate oxidation.

The specific rotation of the *N*-methyl-glucosamine prepared from compound I-1 was opposite in sign of that of authentic *N*-methyl-L-glucosamine. Therefore the *N*-methyl-glucosamine from compound I-1 was the D-form.

For the quantitative determination of UDP-*N*-methyl-D-glucosamine-phosphate, compound I-1 was mildly hydrolyzed with 0.01 M HCl at 100°C for 15 minutes. The mixture was neutralized with 0.01 M NaOH, and the sugar was determined as indicated in the text. The analysis showed the ratio of uridine: total phosphorus: acid-labile phosphorus: *N*-methyl-D-glucosamine-phosphate to be 1: 3: 1: 1 (Table 5). To summarize the above results, compound I-1 was identified UDP-*N*-methyl-D-glucosamine-

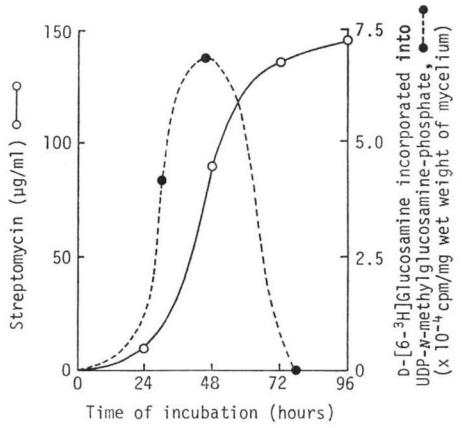
Table 5. Analysis of compound I-1 isolated from the mycelia of *S. griseus* cultured in thymine-supplemented medium.

	Found	Calculated
Uridine[a]	1.00	1.00
Total phosphorus[b]	2.87	3.00
Acid-labile phosphorus[c]	1.17	1.00
Reducing sugar after mild acid hydrolysis[d]	1.16	1.00

[a] Calculated from $\varepsilon_{260nm}=9.95\times10^3$.
[b] Determination by the method of FISKE and SUBBAROW.[10]
[c] Phosphorus liberated by hydrolysis in 1 M H_2SO_4 for 10 minutes was determined by the method of FISKE and SUBBAROW.[10]
[d] The sugar was determined by the method of PARK and JOHNSON[9] with *N*-methyl-L-glucosamine as a standard.

Fig. 3. Relationship between formations of UDP-*N*-methyl-D-glucosamine-phosphate (●) and of streptomycin (○).

D-[6-^{3}H]Glucosamine (10 μCi, 0.26 nmole) was administered to media at 24, 40 and 72 hours, respectively. After 6 hours, the radioactivity incorporated into UDP-*N*-methyl-D-glucosamine-phosphate determined as described in the text.



phosphate.

Characterization of Compounds III-1 and IV-8

The ultraviolet absorption spectra of compound III-1 and IV-8 at pH 1.0 and 11.0 indicated the presence of a uridine base.

The sugar portion of compound III-1 after hydrolysis in 0.01 M HCl at 100°C for 15 minutes showed positive reactions to the reagents of MORGAN and ELSON[12] and BARKER and SUMMERSON.[13] Another portion was hydrolyzed in 6 M HCl at 110°C for 24 hours, and then analyzed with the amino acid analyzer. The ratio of muramic acid - diaminopimelic acid - Glu - Ala was 1 : 1 : 1 : 3. Thus compound III-1 was identified as UDP-*N*-acetyl-muramyl pentapeptide.

In paper chromatography, the Rf values of compound IV-8 in solvents (a) and (b) were those of UDP-*N*-acetyl hexosamine. The sugar portion was identified as *N*-acetyl-glucosamine as described in previous paper.[6]

Relationship between Syntheses of UDP-*N*-Methyl-D-glucosamine-phosphate and Streptomycin

Time course of the incorporation of [^{3}H]glucosamine into UDP-*N*-methyl-D-glucosamine-phosphate and the production of streptomycin are shown in Fig. 3. The formation of UDP-*N*-methyl-D-glucosamine-phosphate reached a maximum value at 46 hours, decreased and disappeared by 78 hours. The production of streptomycin reached a stationary phase at about 72 hours. From these results together with those described above, we conclude that UDP-*N*-methyl-D-glucosamine-phosphate may be one of the sugar nucleotide precursors which participates in the synthesis of streptomycin.

Discussion

D-Glucose is converted to the L-configuration during the synthesis of *N*-methyl-L-glucosamine.[1~4] KUMAGAI and AKAMATSU[4] administered a mixture of D-[1-^{14}C]glucose and D-[6-^{3}H]glucose to the cul-

ture medium of *S. griseus*. The $^{3}H/^{14}C$ ratio found in *N*-methyl-L-glucosamine moiety of streptomycin supports a mechanism involves the conversion of D-glucose to L-hexose without scission of carbon skeleton.

The possibility that D-glucosamine is involved in the synthesis of *N*-methyl-L-glucosamine has been studied. BRUTON *et al.*[2], AKAMATSU and ARAI[3] administered D-[1-^{14}C]glucosamine to the culture media and isolated radioactive streptomycin. Degradation studies revealed that as much as 64% of the isotope was located in the *N*-methyl-L-glucosamine moiety. These data suggest that D-glucosamine or a close derivative may be involved in the synthesis of *N*-methyl-L-glucosamine.

In this report, the formation of streptomycin and the incorporation of D-glucosamine into *N*-methyl-L-glucosamine were stimulated by the addition of thymine to the culture medium. Thymine is one of the components of dTDP-L-dihydrostreptose, an intermediate in the synthesis of streptomycin.[14] The formation of dihydrostreptomycin is carried out by a mycelial enzyme in the presence of the protein-free extract and dihydrostreptosyl streptidine 6-phosphate.[15] The latter compound is synthesized enzymatically from dTDP-L-dihydrostreptose and streptidine 6-phosphate.[14] Thus, thymine may stimulate the synthesis of dTDP-L-dihydrostreptose and then increase the amount of dihydrostreptosyl streptidine 6-phosphate, to which *N*-methyl-L-glucosamine is transferred to give dihydrostreptomycin 6-phosphate. In this way, thymine may stimulate the formation of streptomycin and the incorporation of D-glucosamine into *N*-methyl-L-glucosamine moiety of streptomycin.

BLUMSON and BADDILEY[5] suggested that *N*-methyl-L-glucosamine might be present as a sugar nucleotide at some stage in the biosynthetic pathway. Recently, WHAL and GRISEBACH (unpublished result in Ref. 15) reported that *N*-methyl-glucosamine occurs in the sugar nucleotide fraction of *S. griseus* mycelia. However, they did not isolate the nucleotide-linked *N*-methyl-glucosamine. In the present work, we aimed to obtain sugar nucleotides related to the synthesis of the *N*-methyl-L-glucosamine moiety. We administered labeled D-glucosamine to the culture medium and examined the labeled sugar nucleotides in the mycelia. We found one novel sugar nucleotide whose formation correlated with streptomycin production. The sugar nucleotide was increased by thymine addition, and characterized as UDP-*N*-methyl-D-glucosamine-phosphate. The correlation between the synthesis of UDP-*N*-methyl-D-glucosamine-phosphate and streptomycin production suggests that UDP-*N*-methyl-D-glucosamine-phosphate is involved in the synthesis of the *N*-methyl-L-glucosamine moiety of streptomycin. The sugar moiety of this sugar nucleotide may be converted to the L-configuration in a later step, and then incorporated into streptomycin.

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