ON THE ROLE OF DIHYDROSTREPTOMYCIN IN STREPTOMYCIN BIOSYNTHESIS

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1. Introduction

We have recently described the NADPH-dependent formation of thymidine diphosphodihydrostreptose from dTDP-4-keto-6-deoxyglucose with a cell-free extract from a streptomycin producing strain of *Streptomyces griseus* [1]. No formation of dTDP-streptose could be detected under various conditions. We were, however, unable to find dihydrostreptomycin in the fermentation medium [1].

With an improved technique we have now been able to prove the formation of dihydrostreptomycin in the mycelium and in the fermentation broth of the streptomycin producer. We were also able to show that during all stages of fermentation dihydrostreptomycin but not streptomycin is present inside the mycelium.

2. Materials and methods

2.1. Materials

D-[U-^{14}C]-glucose, 221.2 μCi/μmol, was purchased from Radiochemical Centre, Amersham. Dihydrostreptomycin was obtained from Bayer AG, Leverkusen. Streptomycin and the other chemicals were purchased from Merck AG, Darmstadt.

2.2. *Analytical procedures*

Descending paper chromatography was carried out on Schleicher & Schüll 2043b with the following solvent systems: 1, *n*-butanol (water saturated) -piperidine-*p*-toluenesulfonic acid (96:2:2, v/v/w) [2]; 2,water-*n*-butanol-methanol-*p*-toluenesulfonic acid (1:4:2:0,1, v/v/v/w) [31]; 3,*n*-butanol (water saturated)-*p*- toluenesulfonic acid (98:2, v/w) [4].

Thin layer chromatography on silica gel plates (Woelm) was carried out with: 4,ammonium acetate–water (3.5:96.5, w/v); 5,*n*-propanol-pyridine-acetic acid–water (15:10:3:12, by vol.); 6, methylethylketone-acetic acid–water (6:1:3, by vol.).

For paper electrophoresis on Macherey and Nagel MN 214 the buffer 7, 0.05 M glycine–NaOH, pH 7.7, was used.

Streptomycin and dihydrostreptomycin were detected with the nitroprusside ferricyanide reagent [5].

For bioautography the dry chromatograms were placed for 15 min on an agar plate (15 × 30 cm, Standard Nährager I 'Merck') which had been seeded with about 10^7 spores/ml of *B. subtilis* (strain SP. 5832 from Merrell Iptor, Groß-Gerau). The agar plate was then incubated for 12 hr at 37°C. 5 μg of streptomycin or dihydrostreptomycin after paper chromatography gave an inhibition zone of about 1.8 cm diameter under these conditions.

2.2.1. Cultivation of *S. griseus*

S. griseus strain N 2-3-11 from Kaken Chem. Co., Tokyo, was grown in a synthetic medium [6].

2.2.2. Preparation of cell-free extract

The cell-free extract was obtained as described previously [1]. The mycelium was washed with 0.1 M Tris–HCl, pH 7.3. The protein in the cell-free supernatant was precipitated by addition of 10% fluoroacetic acid before chromatography.

3. Results and discussion

D-[U-^{14}C]-glucose was added in 3 portions of 10 μCi each after 50,70 and 90 hr fermentation to 100 ml of

a culture of a streptomycin-producing strain of *S. griseus*. After 159 hr fermentation a paper chromatogram in solvent 1 was prepared from the fermentation medium and from the cell-free extract of the mycelium. After 168 hr chromatography the radioactive zone which cochromatographed with dihydrostreptomycin (DHSM) was eluted with water and rechromatographed on paper with solvent 2. In the experiment with the medium a separation into 5 radioactive products was observed of which 4 showed antibiotic activity. The sample from the mycelium separated into 2 antibiotic-active substances. After elution of the zones corresponding to DHSM with water cochromatography with authentic DHSM was carried out on paper with

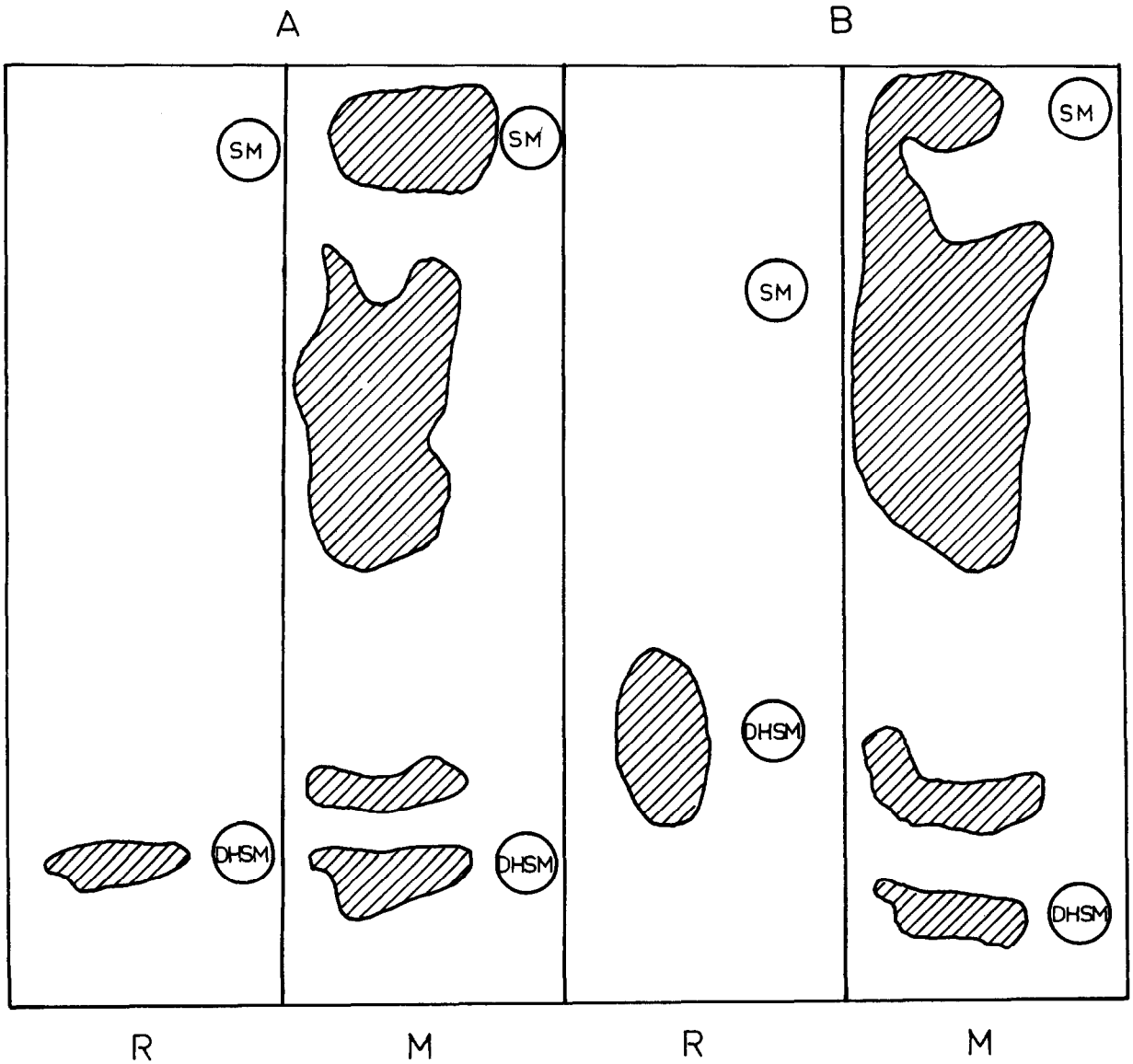

Fig. 1. Drawings of autobiograms from medium (M) and mycelium extracts (R) after 96 hr (A) and 120 hr (B) of fermentation. SM=reference sample of streptomycin; DHSM = reference sample of dihydrostreptomycin.

solvent 3 and on silica gel plates with solvents 4, 5 and 6. In each case the radioactive zone corresponded exactly to the position of DHSM. *p*-Toluenesulfonic acid eluted from the paper chromatograms had to be removed by stirring with Dowex 30 before rechromatography because its presence influenced the chromatographic behaviour of DHSM in solvent 6. The identity of the radioactive product with DHSM was further established by paper electrophoresis (2800 V, 1 hr) in buffer 7 and by recrystallization to constant specific activity after addition of carrier DHSM (208, 190, 173 and 168 counts $\times$ min^{-1} $\times$ mg^{-1} DHSM).

A radioactive compound which cochromatographed with streptomycin (SM) could be found on the chromatogram from the medium but not on the chromatogram from the mycelium extract. In order to investigate the localization of SM and DHSM further the following experiments were carried out. Autobiograms were prepared from chromatograms in solvent 1 of medium and of a cell-free extract from mycelium which had been washed 5 times with 3% acetic acid before the cells were broken. After 48 hr fermentation no SM or DHSM was present. After 96 hr only DHSM was present in the mycelium extract, whereas the medium contained DHSM and SM besides other unknown antibiotically active products (fig. 1A). The same result was obtained with autobiograms after 120 hr (fig. 1B) and 168 hr of fermentation.

Production of DHSM directly by fermentation has been found before in two unidentified isolates of *Streptomyces* [7] and in a species of *S. humidus* [8]. No streptomycin production by these organisms was observed, and Kavanagh et al. [7] assumed that they make DHSM 'by pathways differing considerably from those followed by *S. griseus* in making streptomycin'.

However, we have now shown that DHSM is a normal product of a streptomycin producing strain of *S. griseus*. From the fact that at all stages of fermentation only DHSM is found inside the mycelium whereas DHSM and SM are present in the medium, we can draw the conclusion that DHSM is the primary product in SM biosynthesis and that DHSM is oxidized to SM at the outer cell membrane or by an exoenzyme in the medium. The conclusion that DHSM is the precursor of SM is also in agreement with the fact that only dTDP-dihydrostreptose could be detected as reaction product from dTDP-4-keto-6-deoxyhexose with the enzyme preparation from *S. griseus* [1].

Work on the enzymatic oxidation of DHSM to SM is currently in progress.

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