Biosynthesis of Streptomycin

Part III. Liberation of Inorganic Phosphate from a Natural Precursor in Transformation of the Precursor to Streptomycin

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A natural precursor (L) of streptomycin which had no antibiotic potency was obtained from mycelium suspension of *Streptomyces griseus* in glucose solution and was transformed to streptomycin by H enzyme obtained from mycelium of the organism. This transforming reaction was carried out most effectively at slightly alkaline pH and inhibited by inorganic phosphate and ethylenediaminetetraacetate. L component was considered to be a phosphorylated compound and liberation of the phosphoric acid was essential for L component to be transformed to streptomycin. This transformation was performed not only by H enzyme but also by intestinal alkaline phosphatase, although some difference in the reaction mechanism was supposed to be between those two enzymes.

INTRODUCTION

In the previous papers,^{1,2)} it was reported that an active supernatant obtained from mycelium suspension of *Streptomyces griseus* in glucose solution contained a precursor (L) of streptomycin and an enzyme (H) transforming the precursor to streptomycin. The L component had no antibiotic potency and was isolated from the supernatant by CM-cellulose column chromatography or with active carbon. The H enzyme was obtained from cell-free extract as well as from active supernatant by DEAE-cellulose column chromatography. In this paper, some informations are presented on the reaction of those two components.

MATERIALS AND METHODS

The same materials and methods as those in the previous papers^{1,2)} were employed in the present in-

vestigation with respect to strain, inoculum, preparation of active supernatant, assay of antibiotic potency, preparation of cell-free extract and estimation of L and H components.

Preparation of L component. The L component was prepared from active supernatant on the basis of the data presented in the previous paper.²⁾ Active supernatant was applied to a column of CM-cellulose equilibrated previously with 0.05 M Tris-HCl buffer (pH 8.0). After being washed thoroughly with water, the column was eluted with the same buffer as that for equilibration, collecting fractions which gave positive Sakaguchi reaction. The fractions were pooled and adjusted at pH 2.0 with hydrochloric acid. To the pooled solution was added 0.2% (W/V) active carbon in order to adsorb impurity. After stirring for 30 min., the carbon was removed by filtration; the filtrate being adjusted at pH 7.0 with sodium hydroxyd. To this filtrate was added 0.5% (W/V) active carbon to adsorb L component on it. After stirring for 30 min., the active carbon was collected on filter paper. The filtrate was once more subjected to the same adsorption treatment. Active carbon which adsorbed L was washed with a small amount of water and methylalcohol. In order to liberate L component from active

1) R. Nomi, O. Nimi and T. Miyazaki, This Journal, **30**, 296 (1966).

2) R. Nomi, O. Nimi, T. Miyazaki, A. Matsuo and H. Kiyohara, This Journal, **30**, 1269 (1966).

carbon, 15 ml of 95% methylalcohol per one gram of active carbon employed was added and adjusted at pH 2.0, subsequently stirring for 30 min. For good liberation of L, the reaction of the suspension was readjusted, if necessary, at pH 2.0 during stirring. By filtration, L-containing methylalcohol solution was obtained. Carbon cake was once more eluted with 95% methylalcohol in the same way. Two acidic MeOH-eluates were pooled and concentrated in vacuo to a small amount at 40°C. By addition of acetone to the concentrated residue, L component precipitated as colorless oily substance. This precipitate is easily soluble in water. The aqueous solution was adjusted at pH 7.0 for use. This had no antibiotic potency by itself.

Preparation of H component. Cell-free extract²⁾ was adsorbed on a DEAE-cellulose column previously equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). After being washed with 0.1% NaCl solution in the same buffer, the column was eluted with 1% NaCl solution in buffer, collecting fractions which contained high concentration of H component. These fractions

were pooled, when necessary, for use in the following experiments. These had no antibiotic potency by itself.

Composition of reaction mixture of L and H components. L component was made to react with H component at 28°C for a given time in a reaction mixture composed of 1 part of L-containing solution, 1 part of H-containing solution, 0.2 part of 0.05 M Tris-HCl buffer (pH 8.5), 0.2 part of 10^{-2} M MgSO_4 aqueous solution and a small amount of toluene.

RESULTS

Gel Filtration of H Component with Sephadex G-25

For further fractionation of H-containing solution which was obtained by DEAE-cellulose column chromatography, it was subjected to gel filtration with Sephadex G-25. In each fraction obtained by the gel filtration, H enzymic activity transforming L to streptomycin, absorbancy at 260 m μ and protein

FIG. 1. Gel Filtration of H Component with Sephadex G-25

Column size: 2.7×62 cm. Applied H component: 30 ml. Eluting velocity: 0.25 ml/min. Fraction: 5 ml. Tris-HCl buffer (0.05 M, pH 8.0) was used for previous equilibration and elution of the column.

○-----○: Optical density at 260 m μ . ●-----●: Optical density at 750 m μ given by protein according to Lowry's method. : Activity transforming L to streptomycin, being expressed with the difference of antibiotic potency between before and after 24 hr. reaction at 28°C of the reaction mixture composed of 0.5 ml portion of each fraction, 0.5 ml of L solution, 0.1 ml of Tris-HCl buffer (0.05 M, pH 8.5), 0.1 ml of 10^{-2} M MgSO_4 solution and 2 drops of toluene.

content by Lowry's method³¹ were estimated. The conditions and results are presented in Fig. 1. H-enzymic activity gave two peaks. Peak I in Fig. 1 which appeared at the elution volume corresponding to the void volume of the column was considered to contain major constituent of H enzyme. But this peak had a possibility still to be contaminated with other high molecular weight substance. However, the substance having absorbancy at 260 m μ was removed from H component, although this contamination was unavoidable in DEAE-cellulose column chromatography.²¹ Peak II appeared at the elution volume corresponding to considerably low molecular weight substances. It is not obvious whether the active substance in peak II is protein or not. The yield of the peak II substance considerably varied with the mycelium employed. Since there were several questions to be solved on peak II, peak I was used as H component in the following experiments.

Demonstration of Streptomycin Produced by the Reaction of H Component with L Component

The fraction of peak I in Fig. 1 was made to react with L component in the indicated reaction composition for 24 hr. at 28°C as is described in "Materials and Methods." The reacted solution was applied to a column of Amberlite IRC-50 for isolation of streptomycin as was described by Doery et al.⁴¹ The eluate with 0.2N HCl was neutralized with NaOH, then evaporated in vacuo at 45°C. The residue was extracted with methylalcohol. The extract was again evaporated in vacuo to remove methylalcohol, then dissolved in water. This was applied to one-way ascending paper chromatography on Toyo Roshi No. 50 paper. At the same time, authentic streptomycin was treated in the same way and used as control. Developing solvent was consisted of *n*-butanol-pyridine-2.5% NaCl aqueous solution (4:8:3)

FIG. 2. Bioautogram of Reaction Product of L Component with H Component

* Figures represent the number of drop of the sample applied on paper.

and 1% (W/V) of *p*-toluenesulfonic acid. After accomplishment of chromatography, the paper was dried thoroughly at room temperature, then was put on an agar plate for bioautography which had the same agar medium as was used for streptomycin assay in petri dish. The paper was removed after 8 min. contact with the plate which, then, incubated at 33°C over night. The results are shown in Fig. 2. The R_F value of the reaction product of L and H components agreed closely with that obtained with authentic streptomycin. Although only one antibiotic spot was detected by this procedure, the possibility for other antibiotic substance to be produced is not excluded. If it were the case, the other antibiotics would have been eliminated during the above isolation process.

Optimum pH for Reaction of L Component with H Component

Peak I fraction shown in Fig. 1 and L

3) O. H. Lowry, N. J. Rosenbraugh, A. L. Farr and R. T. Rondall, *J. Biol. Chem.*, **193**, 265 (1951).

4) H. W. Doery, E. C. Mason and D. E. Weiss, *Anal. Chem.*, **22**, 1038 (1950).

FIG. 3. Effect of pH on Reaction of L Component with H Component

component were mixed in the indicated reaction composition without buffer and adjusted at different pH as are shown in Fig. 3. After addition of buffer in respective pH, the mixtures were kept at 28°C for 24 hr. Tris-maleate buffer (1/3 M) and carbonate-bicarbonate buffer (1/3 M) were employed for pH 6 to 8.5 and for pH 9 to 11, respectively. Antibiotic potency produced at each pH were presented in Fig. 3 with relative potency, providing the highest production to be 100%. Optimum pH for reaction of L and H components was 8.5.

Effect of Inorganic Phosphate and Ethylenediaminetetraacetate on the Reaction of L Component with H Component

In the previous paper,¹⁾ potassium phosphate and ethylenediamine tetraacetic acid (disodium salt) (EDTA) were reported to inhibit the increase in antibiotic potency of active supernatant. If L and H components are the major components which are responsible for increase in antibiotic potency of active supernatant, the reaction of these two components should be inhibited by the same compounds. L and H components were made to react in the same way as is described above except that the portion of magnesium sulfate solution was replaced by a inhibitor solution. The concentrations of inhibitor employed and the results obtained are presented in Tables I and II. Potassium phosphate and EDTA inhibited the reaction of L and H components at the same levels as those in the case of active

TABLE I. INHIBITING EFFECT OF POTASSIUM PHOSPHATE ON THE REACTION OF L COMPONENT WITH H COMPONENT

Concentration of K_2HPO_4 (mole)	0	10^{-3}	5×10^{-4}	10^{-3}	5×10^{-3}	10^{-2}
Antibiotic potency*	100	100	100	90	70	43

* It was expressed with relative potency of streptomycin produced at 28°C for 24 hr.

TABLE II. INHIBITING EFFECT OF EDTA ON THE REACTION OF L COMPONENT WITH H COMPONENT

Concentration of EDTA (mole)	0	0.5×10^{-4}	10^{-4}	0.5×10^{-3}	10^{-3}
Antibiotic potency*	100	53	6.8	3.2	0

* Same as that in Table I.

supernatant.

Liberation of Inorganic Phosphate together with Generation of Antibiotic Potency

Inorganic phosphate showed inhibitory effect on the reaction of L and H components. If this inhibition is competitive, the reaction of the two components is supposed to liberate inorganic phosphate. Determination of inorganic phosphoric acid in the reaction mixture was carried out with Bartlett's method⁵⁾ before and after 48 hr. reaction at 28°C. The difference of the two values represents the liberated phosphoric acid. The results are shown in Table III. For control, L and H components were separately subjected to phos-

TABLE III. DEVELOPMENT OF ANTIBIOTIC POTENCY AND LIBERATION OF INORGANIC PHOSPHATE BY THE REACTION OF L COMPONENT WITH H COMPONENT

Experiment No.	Increased antibiotic potency as streptomycin ($\times 10^{-3}$ μ mole)	Released Pi ($\times 10^{-3}$ μ mole)	Pi/Streptomycin
1	128.4	70.2	0.545
2	132.2	58.0	0.438
3	99.9	47.7	0.483

5) G. R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).

phoric acid determination before and after standing for 48 hr. at 28°C, subtracting the values obtained from those of reaction mixture, although little changes were observed during the period. It is clear from Table III that inorganic phosphate increased during 48 hr. reaction, while simultaneous increase in antibiotic potency occurred. This may be taken to mean that generation of antibiotic potency accompanied liberation of inorganic phosphate very probably from L component.

Generation of Antibiotic Potency by Reaction of L Component with Intestinal Alkaline Phosphatase

Since inorganic phosphate was liberated when L component was transformed to streptomycin, there is a strong possibility for L to be a phosphorylated compound. If phosphoric acid is combined in ester linkage, it may be cleaved by an alkaline phosphatase. Furthermore, if the liberation of phosphate has some connection with generation of antibiotic potency, also the reaction of L component with an alkaline phosphatase may generate antibiotic potency. Four mg of intestinal alkaline phosphatase (Nutritional Biochemical Corporation) was dissolved in 10 ml of 0.05 M Tris-HCl buffer (pH 8.5). A 1.35 ml portion of this phosphatase solution was mixed with 1.35 ml of L component solution, 0.3 ml of 0.05 M MgSO₄ solution and a few drops of toluene. Before and after the mixture was kept at 37°C for 48 hr., antibiotic potency and inorganic phosphate was determined. For

control, the changes in inorganic phosphate content of L component and the intestinal alkaline phosphatase solution during 48 hr. standing were separately determined as above described and subtracted from the value in the reaction mixture. As is shown in Table IV, intestinal alkaline phosphatase could provide L component with antibiotic potency, accompanying simultaneous liberation of inorganic phosphate. It is noteworthy to indicate here that the intestinal alkaline phosphatase liberated more phosphate per one unit of streptomycin produced than H component.

DISCUSSION

Active supernatant was reported in the previous papers^{1,6)} to generate antibiotic potency without participation of mycelium. The antibiotic potency developed most effectively at pH 9.0, and it was inhibited by EDTA and inorganic phosphate. In the reaction of L and H components, optimum pH and inhibiting effect of EDTA and inorganic phosphate were shown in the present paper to be the same as those in active supernatant. Streptomycin formation by the reaction of L and H components was demonstrated by bioautography. Thus, it is reasonable to suppose that the development of antibiotic potency in active supernatant is brought about mainly by the reaction of L and H components.

Intestinal alkaline phosphatase liberated inorganic phosphate from L component, simultaneously giving rise to antibiotic potency. Also H component, when it transformed L component to streptomycin, liberated inorganic phosphate from L and reacted most effectively at slightly alkaline pH. These results suggest that L component is phosphoric ester and H component is one of alkaline phosphatase.

It is impossible with the data so far obtained to interpret the fact that intestinal alkaline

TABLE IV. DEVELOPMENT OF ANTIBIOTIC POTENCY AND LIBERATION OF INORGANIC PHOSPHATE BY THE REACTION OF L COMPONENT WITH INTESTINAL ALKALINE PHOSPHATASE

Experiment No.	Increased antibiotic potency as streptomycin ($\times 10^{-3}$ μ mole)	Released Pi ($\times 10^{-3}$ μ mole)	Pi/Streptomycin
1	130.3	241.9	1.86
2	81.7	162.5	1.99
3	49.0	88.5	1.81

6) R. Nomi, *J. Bacteriol.*, **86**, 1220 (1963).

phosphatase liberated more inorganic phosphate from L component than H component. Furthermore, the ratios of the liberated inorganic phosphate to streptomycin produced were not integral as are shown in Table IV and V. The detailed interpretation of these results will be presented in the following papers together with other experimental results. In the present investigation, it should

be pointed out that the materials employed were not highly pure. In spite of these shortages, it is almost sure that the precursor (L) of streptomycin which has no antibiotic potency and locates probably at the nearest position to streptomycin on biosynthetic pathway is a phosphorylated compound. The liberation of phosphoric acid is essential for L component to obtain antibiotic potency.