Dihydrostreptomycin Produced by Direct Fermentation

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Most of the streptomycin produced in the United States (342,000 kg in 1958) is sold as dihydrostreptomycin. Production of dihydrostreptomycin from streptomycin entails catalytic hydrogenation followed by a crystallization. Production of dihydrostreptomycin directly by fermentation would have the advantage of simplicity of purification with its attendant economic advantage. Our antibiotics screening group found two streptomycetes which produced an antibiotic chromatographically indistinguishable from dihydrostreptomycin. We obtained the organisms, increased their productivity, and identified the antibiotic.

While this work was in progress, Tatsuoka et al. (1957) reported the isolation of an antibiotic substance which had the physicochemical and biological characteristics of dihydrostreptomycin. They confirmed the identity by degradation products.

Extensive nutritional studies of the two cultures, strains M43-05026 and M43-05227, failed to provide a medium on which attractive yields of antibiotics were obtained. However, yields large enough so that material could be obtained for isolation and identification were easily achieved. All biological assays for concentration of antibiotic were by a modification of our turbidimetric assay for streptomycin in which dihydrostreptomycin was the standard and Micrococcus pyogenes var. aureus strain ATCC 9996 was the test organism. Results of assays were reported in terms of dihydrostreptomycin free base (DSM).

Materials and Methods

Fermentation. Fermentation studies were made in 500-ml wide mouth Erlenmeyer flasks. The flasks contained 100 ml of medium and were closed by two milk filter discs. The media were sterilized for 20 min at 121 C. Incubation was at 28 C for 7 days on a Gump shaker running at 245 rpm. The fermentor flasks were inoculated with 1 per cent by volume of a vegetative stage which was cultivated at 28 C for 24 hr. The vegetative stage was inoculated by spores obtained from an agar slant of the cultures.

Biosynthesis of streptomycin is profoundly influenced by composition of medium and balance among the various components of the medium. To learn whether biosynthesis of this antibiotic was similarly affected, kinds and amounts of carbon and nitrogen sources and the levels of calcium carbonate were varied.

The usual carbohydrates, glucose, sucrose, maltose, starch, dextrin, were tested singly and in combination. As was not unusual, production of the antibiotic was greater with mixtures than with single carbohydrates. One of the better mixtures was glucose and potato dextrin in a ratio of 1:2. This mixture at a total concentration of 6 per cent was used in most of the media.

The influence of concentration of corn steep solids was investigated in a basal medium containing 4 per cent potato dextrin, 2 per cent glucose, 0.75 per cent peptone 159 (Wilson2), and 1 per cent calcium carbonate in tap water (table 1). Since a concentration of corn steep solids of 1 per cent was optimal, it was used at this level in all corn steep containing media.

The corn steep solids supplied a large number of organic compounds and inorganic elements to the medium. The effective components of corn steep were not identified. Since corn steep supplied so many compounds and much ash to the medium, ease of purification usually would be improved by reducing it to the minimum. None of the following substances was a substitute for corn steep solids: distillers solubles, carob protein, soybean meal, corn gluten, and potassium phosphate.

Additions of crude sources of nitrogen to a basal medium, containing glucose, dextrin, corn steep solids (also a nitrogen source) and calcium carbonate, were made in an effort to increase the production of the antibiotic substance (table 2). Too much or too little of a nitrogen source was detrimental to formation of the antibiotic. This requirement for proper balance among the nutrients for good production of an antibiotic was observed in many other fermentations.

Although sodium chloride was added to the fermentation medium, its addition was not essential for good


Diagnosis of the antibiotic. Calcium carbonate was essential to production of the antibiotic. Very little antibiotic substance was found either in the absence of or with small amounts (0.25 per cent) of calcium carbonate. Good production was obtained with 0.5 and 1.0 per cent calcium carbonate. Presumably, pH regulation by the calcium carbonate was its essential property because calcium chloride and calcium sulfate did not substitute for the calcium carbonate.

**Chromatography.** Descending paper chromatography with a solvent system composed of a 2 per cent solution of p-toluene sulfonic acid dissolved in n-butyl alcohol saturated with water (Peterson and Reineke, 1950) separated mannosidostreptomycin from streptomycin and from their dihydro derivatives satisfactorily. The time of development, 24 hr, was so long that the solvent front dripped off the paper; consequently, Rf values could not be measured. The order of the spots on the paper reading from origin and their positions relative to streptomycin were: mannosidodihydrostreptomycin (0.25), mannosidostreptomycin (0.4), dihydrostreptomycin (0.7), and streptomycin (1.0). Bioautographs were made against the strain of *M. pyogenes* used for the turbidimetric assay.

**Biosynthesis of dihydrostreptomycin.** Mannosidostreptomycin usually can be demonstrated in streptomycin beers. Langlykke and Perlman (1950) state: "It has been found that: (A) in a growing culture of a streptomycin-producing strain of *Streptomyces griseus*, the originally and/or concurrently formed mannosidostreptomycin is at the same time being converted into streptomycin; (B) said culture is able to decompose added mannosidostreptomycin to streptomycin, and added dihydromannosidostreptomycin to dihydrostreptomycin; and (C) that an enzyme or enzymes may be obtained from said culture, which enzyme is capable of converting mannosidostreptomycin to streptomycin, and dihydromannosidostreptomycin to dihydrostreptomycin. These properties (the properties of a mannosidostreptomycinase) are lacking in cultures of strains of *Streptomyces griseus* which do not produce streptomycin, and in other actinomycete cultures which do not produce streptomycin."

They found mannosidostreptomycinase in the filtrate from a 9-day-old culture of a streptomycin-producing strain of *S. griseus*. Severin et al. (1957) found that washed mycelium of *S. griseus* converted added mannosidostreptomycin to streptomycin. Thus, the enzyme seemed to be in the culture medium and on or in the mycelium.

If our cultures produced streptomycin by the above mechanism and then caused its reduction to dihydrostreptomycin, we would expect to find at least traces of mannosidodihydrostreptomycin and streptomycin in the medium at some time before production of dihydrostreptomycin ceased. We would also expect the organism to form enzymes capable of converting mannosidostreptomycin into streptomycin and of reducing streptomycin to dihydrostreptomycin. There was no chromatographic evidence for mannosidodihydrostreptomycin, mannosidostreptomycin, or streptomycin, although the system was capable of resolving traces of them from dihydrostreptomycin. Mannosidostreptomycin added to the make-up or on the third day of the fermentation was not converted into streptomycin. Nor was streptomycin when added under similar conditions reduced to the dihydro form. Whatever may be the route of biosynthesis of dihydrostreptomycin, it appears not to be through mannosidostreptomycin and streptomycin by means of extracellular enzymes. Lack of conversion of mannosidostreptomycin to dihydrostreptomycin was not conclusive evidence for lack of enzymes; permeability barriers could have prevented access of substrate to enzyme.

These organisms probably make dihydrostreptomycin by pathways differing considerably from those followed by *S. griseus* in making streptomycin. Comparison of the two routes of synthesis should be of value in elucidating the pathways to the two end products which differ only in the oxidation state of one carbon of a sugar (aldehyde group in streptomycin and a primary alcohol group in dihydrostreptomycin).

**Isolation of the antibiotic.** The isolation procedure was

| TABLE 2 |
|-----------------|-----------------|
| **Crude source of nitrogen and production of dihydrostreptomycin** |
| **Additions to Basal Medium** | **Dihydrostreptomycin (μg/ml)** |
| None | 500 |
| Wilson peptone 150: | |
| 0.25% | 650 |
| 0.5% | 850 |
| 0.75% | 700 |
| 1.0% | 450 |
| Soybean oil meal: | |
| 1.0% | 700 |
| 1.5% | 600 |
| 2.0% | 450 |
| Soy peptone, 1.0% | 400 |

**Figure 1.** Infrared spectra of dihydrostreptomycin obtained by direct fermentation (**heavy line**) and by reduction of streptomycin (**light line**).
based upon the assumption that the antibiotic was dihydrostreptomycin as indicated by chromatographic evidence. Material for isolation of the antibiotic substance was prepared in shaken flasks. Pooled filtrates of from 5 to 9 L were processed at a time. The filtrates contained antibiotic substance equivalent in antibacterial activity to 300 to 550 μg of streptomycin base per ml. The filtrates were concentrated to ⅔ volume (to give a more concentrated feed solution for the adsorption column). The solution was decalcified with sodium oxalate. The neutral, decalcified concentrate was passed downflow through an IRC-50 (sodium form) column, and the column was washed with distilled water. The antibiotic was eluted batchwise by mixing the resin with dilute hydrochloric acid until the pH of the eluate was constant at 2 for ½ hr. The eluate was neutralized to pH 6.5 with sodium hydroxide, concentrated, and dried in vacuo. The powder was suspended in absolute methanol and filtered to remove sodium chloride. Triethylamine sulfate was added to precipitate the antibiotic as the sulfate. The white precipitate was dissolved in water, and the antibiotic was crystallized from an aqueous methanol solution. Rosettes typical of dihydrostreptomycin sesquisulfate formed; they were washed with methanol and dried in vacuo.

Characterization. Crystalline antibiotic M43-05026 assayed as follows (oxygen by difference):

Found for M43-05026:
C 34.04, H 6.44, N 12.85, S 6.15, O 40.52

Calculated for C 34.50, H 6.05, N 13.40, S 6.50, O 39.40

${\frac{3}{2}}$H$_2$SO$_4$;

The crystals of antibiotic M43-05026 had an X-ray diffraction pattern and an infrared spectrum (figure 1) identical with that of an authentic sample of crystalline dihydrostreptomycin sesquisulfate. A sample of crystalline antibiotic M43-05026 was assayed as dihydrostreptomycin and gave the following results:

- Guanidine assay (Hiscox)........... 750 μg per mg
- UV (Hiscox)......................... 760 μg per mg
- Biological (Bacillus subtilis)..... 755 μg per mg
- Ash.................................. 1.8 per cent
- Streptidine sulfate............... 1.5 per cent
- Moisture.......................... 1.5 per cent

The maltol assay for streptomycin gave negative results. These values are in the same range as those of crystalline dihydrostreptomycin sesquisulfate produced by the catalytic hydrogenation of streptomycin. When corrected for ash, streptidine, and moisture, the purity of the crystalline sulfate salt of antibiotic M43-05026 approximates the theoretical purity of dihydrostreptomycin sesquisulfate. Chromatography in five solvent systems routinely used for streptomycin antibiotics showed only a single component; this had an R$_f$ the same as dihydrostreptomycin.

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Summary

Two unidentified isolates of Streptomyces were shown to produce dihydrostreptomycin. No other antibiotic substance was found in the culture filtrates. Dihydrostreptomycin was isolated as the crystalline sesquisulfate and was shown to have the same X-ray diffraction pattern, infrared spectrum, chemical properties, antibacterial activity, and chromatographic behavior as dihydrostreptomycin sesquisulfate made from streptomycin.

References


