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N-Demethylstreptomycin

I. Microbiological Formation and Isolation

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Addition of methylation inhibitors to growing cultures of *Streptomyces griseus* decreased the production of streptomycin several fold. On a synthetic substrate a significant discrepancy between the yield estimated by the chemical analysis and the microbiological agar diffusion method was observed when ethionine was added as inhibitor. This indicated the formation of a hitherto unknown streptomycin-like antibiotic of low antimicrobial activity.

The new antibiotic was isolated from the fermentation broth by ion exchange and obtained as the hydrochloride. The NMR spectra of dihydrostreptomycin and the hydrogenated new antibiotic were almost identical except that the three proton band corresponding to the N-CH_3 group in dihydrostreptomycin was absent in the spectrum of the new compound. The new compound was thus assigned the name of N-demethylstreptomycin.

The pH-dependence of the activity of streptomycin was first observed by Waksman and his colleagues [1] who found that increasing the pH from 5 to 8 increased the antibacterial effect several fold. Eagle, Levy and Fleischman [2] have investigated the effect of the pH on the action of a number of antibiotics and have found that the highest activities, in most cases, were observed at pH-values at which the antibiotics are non-ionized.

However, pus from infections, for instance from a tuberculous lung, often has a pH between 6.0 and 6.5. At this pH the streptomycin has less than 25 % of its maximum activity, and remembering the toxic side effects of streptomycin this is by no means negligible.

Of the three basic groups in streptomycin, two guanidino groups and one N-methylamino group, the N-methylamino group has a pK_a value of 7.7. The striking increase in activity with increasing pH in the range 5 to 7.5 may thus be explained by the decharging of this N-methylamino group.

This fact prompted us to investigate if it would be possible to change the maximum activity towards a lower pH by introducing electronegative substituents at the secondary N-methylamino group. We have been able to show that all chemical modifications leading to a stable derivative with a tertiary

amino group simultaneously resulted in complete loss of antibacterial activity. Therefore, to prepare a modified streptomycin with maximum activity in the desired pH range, *i.e.* 6.0—7.0, the starting material should be *N*-demethylstreptomycin (NDMS) or dihydro-*N*-demethylstreptomycin (DH—NDMS) containing a primary amino group. Mono-substitution at the amino nitrogen atoms in these hypothetical compounds might yield streptomycins of higher therapeutical value.

It is known that in the tetracycline group of antibiotics the *C*-methyl and the two *N*-methyl groups are derived from L-methionine.[3,4] Furthermore, an inhibition of the natural methylation processes in the fermentation substrates leads to the formation of demethyltetracyclines.[5-8] The inhibition of the methylation processes responsible for the *C*-methyl and the two *N*-methyl groups can be accomplished either by adding folic acid antagonists, for example, aminopterin or sulfonamides, or by adding L-methionine analogues, such as ethionine or D-methionine to the substrates.

The demethyltetracycline has also been obtained by using methionine-blocked mutants of the producing strains.

It has recently been shown that the *N*-methyl group of streptomycin also has methionine as precursor.[9,10] We therefore decided to investigate the influence of methylation inhibitors on streptomycin fermentations.

MATERIALS AND METHODS

Culture methods and inhibitors. A high producing strain of *Streptomyces griseus*, No. 1280, was used in shake flask experiments. One hundred ml of a glucose-corn steep liquor-soy bean meal substrate in 500 ml flasks was seeded with spores from an agar slant culture. After 2 days' incubation on a rotary shaker at 26°C the cultures were used to inoculate the production substrates. For the production, a glucose-corn steep liquor substrate or a chemically defined substrate as described by Carvajal [11] was used. 5 % inoculum was used throughout the experiments and the incubation was performed as described above. Incubation times varied from 5 to 8 days.

Methylation inhibitors were added as sterile aqueous solutions: DL-ethionine (Koch-Light Laboratories Ltd., Colnbrook, Bucks., England), D-methionine (Hoffmann-La Roche & Co. Ltd., Basel, Switzerland), aminopterin (Sigma Chem. Comp. St. Louis, Mo., U.S.A.), and sulfadiazin (Northern Drug, Copenhagen, Denmark) were tested for inhibitory effect on the methylation process.

Recovery of streptomycins. The streptomycins were recovered by treating the filtered broth with Amberlite IR 50 in the sodium form. Subsequent elution with hydrochloric acid yielded an eluate containing the hydrochlorides of the streptomycins contaminated with sodium chloride. The eluates were decolorized with active carbon and evaporated to dryness on a rotary evaporator under vacuum at a temperature below 40°C. The residue was extracted with methanol and the insoluble sodium chloride removed by filtration. The extract was evaporated to dryness and the residue again extracted with methanol leaving a small amount of undissolved sodium chloride. The streptomycins were either precipitated as the hydrochlorides by addition of acetone or as sulphates by addition of triethylammonium sulphate. In the chemically defined substrates calcium was precipitated as the oxalate prior to the adsorption step.

Analytical. Chemical analyses of the streptomycins were preformed according to Boxer *et al.*[12] Streptomycin is degraded in alkaline solution to yield the γ-pyrone maltol which gives a purple colour with ferric ions in acid solution, measured at 540 mμ (Beckman model DU). The hydrogenation products, DHS (dihydrostreptomycin) and DH—NDMS, were obtained by low pressure hydrogenation at room temperature in the presence of a palladium catalyst. These compounds were assayed according to Monastero.[13] Mannosidostreptomycins were analysed by the anthron-sulfuric acid method

as described by Emery and Walker.[14] Microbiological activities were estimated by the agar diffusion method, using *Bacillus subtilis* ATCC 6633 as test organism.

Chromatography and NMR-spectra. Chromatography of the streptomycins was performed as described by Heding.[15] Nuclear resonance spectra were determined using a Varian A-60 instrument. Solutions of the streptomycins in deuterium oxide were lyophilized and the spectra determined of the compounds redissolved in deuterium oxide.

RESULTS AND DISCUSSION

Preliminary results showed that addition of aminopterin (0—1000 ppm) and D-methionine (0—1000 ppm) to the substrate had little or no influence on the formation of streptomycin. The yields obtained in the flasks containing the highest amounts of the inhibitors varied from 60 to 80 % of the control flasks. No modified streptomycin could, however, be detected.

Sulfadiazin (SDZ) addition had a pronounced effect on the streptomycin production. The yields in both the complex and the chemically defined substrates decreased considerably. Addition of *p*-aminobenzoic acid (PABA) could, as expected, partly neutralize the SDZ effect. Table 1 illustrates the influence of addition of SDZ and PABA on the streptomycin production on a chemically defined substrate. The yields were estimated by the maltol method and by the microbiological method. Good correlation between the two analytical methods was found, and all attempts to demonstrate the presence of modified streptomycins in the inhibited cultures failed.

Addition of DL-ethionine to the two production substrates also caused a decrease in the final yield of streptomycin. However, the microbiological activity in the synthetic substrate decreased more than the streptomycin content estimated by the maltol method.

Table 2 shows the relationship between the microbiological assays and the maltol assay on the two substrates with increasing amounts of DL-ethionine. The assays were performed after 7 days incubation.

The discrepancy between the two different methods of assay could be explained by assuming: 1) the formation of a simple phenolic substance giving a colour reaction with the ferric reagent used in the maltol assay, or 2) the formation of a condensation product between the carbonyl function of the streptomycin and a sulphydryl containing compound; such condensation

Table 1. Influence of addition of sulfadiazin (SDZ) and *p*-aminobenzoic acid (PABA) on the streptomycin yields on a chemically defined substrate after 7 days fermentation.

Supplement	pH	% Glucose	Yield, per cent of control
Control	7.9	0.35	100
2000 ppm SDZ	6.9	2.70	3
25 ppm PABA	7.4	0.35	98
100 ppm PABA	7.4	0.35	92
2000 ppm SDZ+25 ppm PABA	6.7	1.30	37
2000 ppm SDZ+100 ppm PABA	6.8	1.30	43

Table 2. Relationship between microbiological assays and maltol assays in cultures of *Streptomyces griseus* on a complex and a chemically defined medium with increasing amounts of DL-ethionine. Fermentation time 7 days.

	Complex	Defined
Ethionine added g/l	microbiol./maltol	microbiol./maltol
0	0.99	1.05
1.0	0.67	0.45
2.0	0.67	0.36
4.0	0.67	0.20

products are known to be inactive,[16] or 3) the formation of the *N*-ethyl analogue of streptomycin, or 4) finally the formation of NDMS.

The first possibility could be excluded readily. No free phenol was present in the substrate and furthermore the compound responsible for the low microbiological activity could be concentrated by treatment with a weak cation exchange resin followed by elution with dilute hydrochloric acid. This indicated the presence of at least one strong basic group in the molecule, for instance a guanidino group.

The second possibility was excluded because elemental analysis performed on the freeze dried eluate showed that the compound or mixture of compounds was sulphur-free.

To distinguish conclusively between the last two possibilities, the *N*-ethyl analogue and NDMS, a larger amount of the compounds was prepared.

The recovery of the streptomycins was performed as described above, maltol and microbiological assays performed after each purification step. During this work we observed that extracting the evaporated eluates, mentioned above, with methanol yielded an unexpected separation of streptomycin and NDMS. The hydrochloride of NDMS was less soluble in CH_3OH than streptomycin hydrochloride which is freely soluble in this solvent.

The NMR-spectra of DHS and the DH—NDMS were determined. Our interpretation of the DHS-spectrum is in good agreement with the data published by Aronson, Meyer and Brock.[17] The two acetal protons show resonance in the region 6 to 5 ppm. In the region 5 to 3.5 ppm the CHO and CHN protons show resonance. The sharp three proton band at 3.18 ppm must be attributed to the *N*-CH_3 group and finally the doublet at 1.5 ppm corresponds to the CH—CH_3 group. The spectrum of the derivative shows many similarities. Although the quality of the spectrum is not good it is evident that the resonance from the *N*-CH_3 group has almost disappeared. Furthermore, the peaks corresponding to the two acetal protons are not resolved (see Fig. 1).

This strongly indicated that inhibition of the methylation processes in streptomycin fermentations by adding DL-ethionine led to the formation of NDMS.

Electrometric titration showed that the amino group of NDMS has a pK_a value of 7.7.

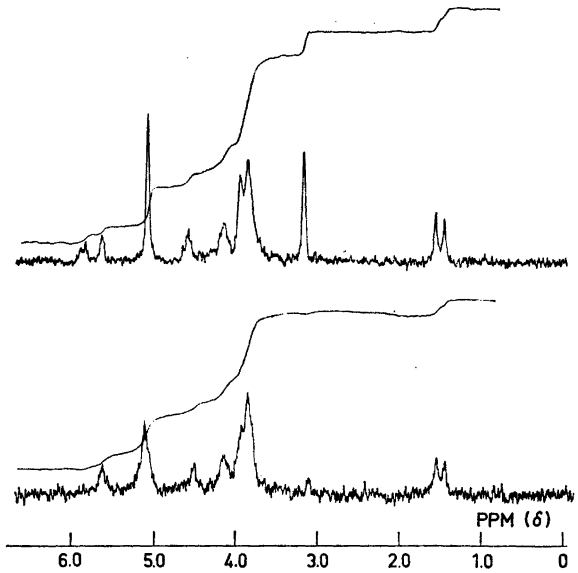

Fig. 1. 60 Mc NMR-spectra of DHS and DH—NDMS dissolved in deuterium oxide.

Microbiological assays performed at pH 5, 6, 7, 8 showed, in all four cases, an activity of about 10 % compared to streptomycin.

We must conclude that not only are streptomycin derivatives containing a tertiary amino group inactive, NDMS containing a primary amino group also has a very low microbiological activity. However, the activity shows the same pH dependence as streptomycin and DHS. In agreement with our assumption the activity of NDMS also showed a sharp increase at pH 7.7, *i.e.* at the pH corresponding to the pK_a value of the amino group.

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REFERENCES

1. Waksman, S. A., Bugie, E. and Schatz, A. *Proc. Staff. Meet. Mayo Clin.* **19** (1944) 537.
2. Eagle, H., Levy, M. and Fleischman, R. *Antibiot. Chemotherapy* **2** (1952) 563.
3. Miller, P. A., McCormic, J. R. D. and Doerschuk, A. P. *Science* **123** (1956) 1030.
4. Snell, J. F., Birch, A. J. and Thomson, P. L. *J. Am. Chem. Soc.* **82** (1960) 2402.
5. Goodman, J. J. and Matrishin, M. *J. Bacteriol.* **82** (1961) 615.
6. Niedleman, S. L., Bienstock, E. and Bennet, R. E. *Biochim. Biophys. Acta* **71** (1963) 199.
7. Perlman, D., Heuser, L. J., Semar, J. B., Frazier, W. R. and Boska, J. A. *J. Am. Chem. Soc.* **83** (1961) 4481.

8. Goodman, J. J. and Miller, P. A. *Biotechnol. Bioeng.* **4** (1962) 391.
9. Bruton, J. and Horner, W. H. *J. Biol. Chem.* **241** (1966) 3142.
10. Candy, D. J. and Baddiley, J. *Biochem. J.* **96** (1965) 526.
11. Carvajal, F. U.S. patent 2.808.364.
12. Boxer, G. E., Jeninek, V. C. and Leghorn, P. M. *J. Biol. Chem.* **169** (1947) 153.
13. Monastero, F. *J. Am. Pharm. Assoc.* **41** (1952) 322.
14. Emery, W. B. and Walker, A. D. *Analyst* **74** (1949) 455.
15. Heding, H. *Acta Chem. Scand.* **20** (1966) 1743.
16. Bondi, A., Dietz, C. C. and Spoulding, F. H. *Science* **103** (1946) 399.
17. Aronson, J. N., Meyer, W. L. and Brock, T. D. *Nature* **202** (1964) 555.

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