THE IDENTIFICATION OF STREPTOMYCIN ON PAPER STRIP CHROMATOGRAMS

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During the course of an investigation into the antibiotic activities of a considerable number of strains of *Streptomyces* it became apparent that a simple and rapid test for streptomycin would save much time and effort. The paper strip chromatogram method of Consden, Gordon, and Martin (1944) seemed to offer interesting possibilities. This method was originally developed for the identification of amino acids but has been extended by Partridge (1946) and Flood, Hirst, and Jones (1947) to sugars; by Lugg and Overell (1947) to organic acids; and by Nord and Vitucci (1947) to creatine. It has been used also by Goodall and Levi (1946) and by Winsten and Spark (1947) for the identification of analogues of penicillin.

METHODS

Pilot experiments using commercial streptomycin and also the culture broth from one of the proved strains of *Streptomyces griseus* soon demonstrated that the solvent systems used by the authors cited were not sufficiently selective for streptomycin. It was eventually found that a 3 per cent solution of NH_4Cl would move the streptomycin in a sharp band near the advancing solvent front. The mechanism of this action was investigated by excising sections of the strip, leaching them in water, and determining the relative concentrations of salt by Nesslerization using a Klett-Summerson photoelectric colorimeter to determine the ammonia content. The streptomycin is deposited at a critical salt dilution which is in the neighborhood of 0.5 per cent. This was proved by running strips in a series of dilutions until the streptomycin band became diffuse.

Neither ninhydrin nor Somogyi's reagent (1945) were satisfactory to develop a colored band on the paper strip. Silver nitrate was, of course, useless with culture filtrates. It was found, however, that a slight modification of the Sakaguchi reaction (1925) gave excellent results. The strip is removed from the chamber and dried on a stainless steel screen over a hot plate. It is then sprayed with N/2 NaOH and immediately with 0.25 per cent α-naphthol. After standing 2 minutes it is sprayed with NaOCl prepared according to the original directions of Sakaguchi (1925). A brilliant red band is formed with streptomycin. The band is usually sharply outlined with a center at an R_f value of 0.80 to 0.86 depending upon temperature. The R_f value is the ratio of movement of the band to the total movement of the solvent. This can be confirmed by excising sections from a parallel strip and testing them on plates of *Bacillus subtilis* or other susceptible organisms. Figure 1 shows the NH_4Cl concentration and appearance of the strip.

During the pilot runs it was found that the apparatus and manipulation could be considerably simplified by running the strip against gravity rather than from a suspended solvent container as described by Consden *et al.* (1944). The simplified apparatus is shown in figure 2. The strips used were no. 2 Whatman paper cut to 12 mm in width. The average length of run from the point where the substance to be distributed was applied was 170 mm. A pencil mark was drawn 65 mm from the bottom, and one drop of the solution to be investigated was applied at that

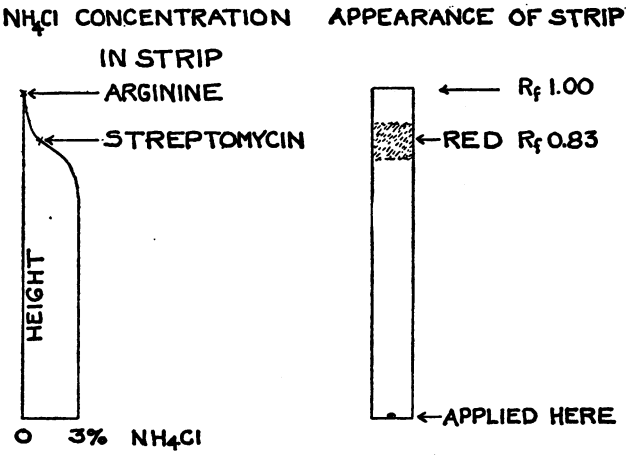

FIG. 1. *Left:* LEVELS TO WHICH ARGININE AND STREPTOMYCIN ARE CARRIED BY SOLVENT IN PAPER STRIP. *Right:* STREPTOMYCIN ZONE IN DEVELOPED PAPER STRIP

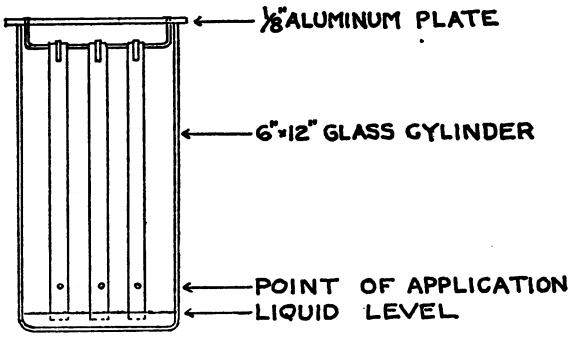

FIG. 2. APPARATUS FOR PAPER STRIP CHROMATOGRAMS AGAINST GRAVITY

point. The spot was then dried on a hot plate to prevent seepage back into the solvent solution. After drying, the strip was hung in the chamber with the end submerged in the solvent solution, and the chamber was sealed with vaseline to maintain a saturated atmosphere. The time to complete a run varied with the solvent used and the temperature, but was usually from 4 to 12 hours. Solutions containing 30 μg per ml of streptomycin gave excellent reactions.

When the solvent front had reached the desired height, the strip was dried to drive off water and solvent and to fix the constituents in the strip. Unknown

antibiotics were run with both NH_4Cl solutions as here described and usually with butyl alcohol and water mixtures as described by Consden *et al.* (1944).

Experiments on unknown broth cultures of various organisms were made on parallel strips with 3 per cent NH_4Cl. One strip was developed with Sakaguchi's reagent and the other cut into eight sections and tested on *Escherichia coli* test plates. In some cases three strips were run and tested on both *E. coli* and *B. subtilis* plates. At the same time two or three strips were run with butyl alcohol saturated with water. These were developed with ninhydrin and colored bands correlated with the activity shown on test plates.

EXPERIMENTAL RESULTS

1. Pilot runs on streptomycin using 2 μg of streptomycin calcium chloride complex.

a. Pure water showed no distribution. The streptomycin diffused in a wide band in both directions from the point of application.

b. Butyl alcohol and water and phenol water systems showed no distribution.

c. Pyridine and NH_4Cl showed a sharp band at an R_f value 0.83.

d. Three per cent NH_4Cl alone gave substantially as good results as it did with the addition of pyridine.

e. Other salts such as $(NH_4)_2SO_4$ and NaCl gave somewhat more diffuse bands but at the same R_f value.

2. A broth filtrate from a strain of *S. griseus* furnished through the courtesy of Merck and Company of Rahway, New Jersey, gave a band identical to that obtained with commercial streptomycin.

3. A run was made with arginine to eliminate the possibility of confusing free arginine with streptomycin. This gave an extremely sharp band at R_f 1.00.

4. Culture filtrates from *Streptomyces* 17C, isolated in this laboratory, showed good activity against *E. coli* and *B. subtilis*. No streptomycin could be demonstrated when run with NH_4Cl. A run with butyl alcohol and water developed with ninhydrin showed a pink band at R_f 0.2 that was confirmed on *B. subtilis*.

5. Culture filtrates from *Streptomyces* M17-N3, isolated in this laboratory, showed high activity against *B. subtilis* but none against *E. coli*. A run with NH_4Cl showed a diffuse pink band at R_f 0 that was confirmed on *B. subtilis*. A run with butyl alcohol and water gave a diffuse yellow band at R_f 1.00 that was confirmed on *B. subtilis*.

6. Culture filtrates from *Streptomyces* 2B, isolated in this laboratory, showed fair activity against *E. coli* and *B. subtilis*. A run with NH_4Cl gave the typical streptomycin reaction, which was confirmed on *E. coli* test plates. A run with butyl alcohol and water showed a pink band at R_f 0, which was also confirmed on *E. coli* test plates.

DISCUSSION AND CONCLUSIONS

The chromatographic method here described is apparently highly selective for streptomycin. The mechanism involved is obviously a salting-out process, but more detailed information concerning it is still under investigation. In the

meantime it is being used to advantage to detect streptomycinlike substances in culture filtrates. In connection with the other procedures described in the literature cited it is also used to indicate successful methods of extraction, since the behavior of unknown substances on paper strips with various solvents classifies them in this respect.

Streptomyces 2B, mentioned above, produces very small quantities of antibiotic in shake cultures and somewhat more in stationary broth cultures. On agar plates, however, it shows unusual activity with the typical streptomycin bacteriostatic spectrum. Work on it would have been abandoned had the chromatographic method not given evidence that it produces a streptomycinlike substance.

It would seem possible that the paper strip method of studying salting-out effects at low salt concentrations might be useful in biochemical fields other than antibiotics.

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