A NEW METHOD OF STREPTOMYCIN CHROMATOGRAPHY AND ITS USE IN THE EXAMINATION OF THE REACTION BETWEEN STREPTOMYCIN AND AMMONIA

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A new paper chromatographic method for streptomycin and similar compounds is described. It is used to detect the products of reaction between streptomycin and ammonia in low purity streptomycin; the presence of such compounds causes the microbiological and maltol methods of streptomycin assay to give different results.

A NUMBER of solvent systems have been described for the paper chromatography of streptomycin and that of Winsten and Eigen (1948) appears to have been given the greatest attention. Its use, however, suffers from "double spotting" caused by the salt content of the sample (Consden, 1944; Peterson and Reineke, 1950). The present paper describes a solvent system which is unaffected by salt content. It is used to examine the reaction between streptomycin and ammonia first mentioned by Solomons and Regna (1950).

EXPERIMENTAL

Qualitative Chromatography

Solvent system. Following the use of pentachlorophenol for the extraction of streptomycin-like compounds (Yabuta, 1955), we find that the solvent system of Winsten and Eigen (1948) is best replaced as follows: Sodium hydroxide (15 g.) in water (150 ml.) is shaken for 1 hr. with pentachlorophenol (30 g.) and n-butanol (850 ml.). Any lower aqueous phase is discarded and the upper phase filtered through glass wool.

Whatman No. 4 paper buffered to pH 7·0 with 2 per cent phosphate buffer was used. Sample aliquots within the range 10 to 800 units streptomycin were placed on the paper and developed for about 18 hr. (descending technique). This produced a movement of the streptomycin zone of about 12 in. Equilibration of the papers in the chromatography tank before development was not necessary. The papers were then washed by drawing them through ether to remove the pentachlorophenol, and so assist detection. Two methods of detection were used: (i) The papers were dipped in 0·005M periodic acid and then in 0·01M benzidine, both solutions being in acetone, the components thus being revealed as yellow or white spots on a blue background (Gordon, 1956). (ii) This comprised dipping the papers in a mixture of diacetyl, alkali and α-naphthol (Foster and Ashton, 1953) which showed streptomycin and associated compounds as red spots on a white background. Both reagents were sensitive to as little as 10 μg. streptomycin.

Quantitative Chromatography

For an initial separation 600–900 units streptomycin were applied to a sheet of Whatman's No. 4 paper and chromatographed as above. The required zones, indicated by tracer strips treated with one of the colorimetric indicators, were cut out. These were then macerated with 10 ml. of pH 4·4 McIlvaine buffer to extract the streptomycin, after which the solution was filtered and assayed by a sensitive method already described by Savitskaya and Kartseva (1953).

This consists in forming the 2,4-dinitrophenylhydrazone in aqueous solution and in measuring the extinction of this at 430 mμ. The method was used satisfactorily with streptomycin concentrations of 10–150 μg./ml. and about ten equivalents of the reagent. Formation of the 2,4-dinitrophenylhydrazone by heating in a boiling water-bath for 2½ min. gave results similar to those obtained by standing at room temperature for 2 hr., as recommended by Savitskaya and Kartseva, or by standing for periods up to 48 hr. The shorter reaction time at high temperature was therefore adopted. Excess reagent was removed by washing with n-butyl acetate. This caused no measurable loss of 2,4-dinitrophenylhydrazone, but several washings were required to rid the solution of excess reagent and give a constant extinction value which was measured at 430 mμ.

Streptomycin of known purity was used as a standard and treated similarly. Tests with solutions of pure streptomycin showed an error of about ±3 per cent between replicates.

Electrophoresis. This was carried out using Shandon apparatus and Whatman 3MM paper with a buffer of M/15 pH 5·6 potassium phosphate (Foster and Ashton, 1953).

Other analyses. Microbiological assays were carried out using the cavity plate method described by Brownlee and his co-workers (1948) with *Bacillus subtilis* NCTC 8236 as test organism. The method described by Grove and Randall (1955) was used for maltol assays.

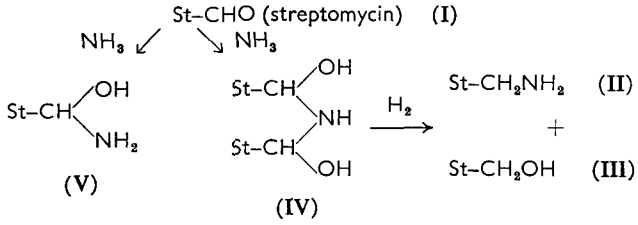
Results and Discussion

The Reaction Between Streptomycin and Ammonia

The reaction between streptomycin and ammonia yields bis-(α-hydroxystreptomycylamine) (IV) a highly toxic substance with a high maltol/microbiological streptomycin ratio (Solomons and Regna, 1950). These authors showed that in aqueous solution at pH 2·5 it reverts to streptomycin (I) in a few hours, and that upon hydrogenation at a platinum catalyst it produces streptomycylamine (II) and dihydrostreptomycin (III).

We find that bis-(α-hydroxystreptomycylamine) (IV) has an R_F value of 0·08 relative to streptomycin as 1, when chromatographed under the conditions described above. Its electrophoretic movement towards the cathode is about 0·3 relative to streptomycin as 1. The hydrogenation products streptomycylamine and dihydrostreptomycin have R_F values of 0 and 0·75 respectively. These values were checked by comparison with streptomycylamine prepared by the reduction of streptomycin

oxime and with dihydrostreptomycin prepared by hydrogenation of streptomycin. Both moved towards the cathode on electrophoresis giving values of 0·66 (streptomycylamine) and 1·0 (dihydrostreptomycin) relative to streptomycin as 1.



Mixtures of streptomycin sulphate and ammonium sulphate gave rise to three main components on chromatography: two major zones at R_F values 0·08 and 0·48 with a minor zone at R_F 0·23. The component of R_F 0·08 corresponds with bis-(α-hydroxystreptomyclamine) and the R_F 0·48 component is probably streptomycin monoaldehyde ammonia (V) because it is also produced by acid hydrolysis of bis-(α-hydroxystreptomycylamine). The zone at R_F 0·23 has not been identified.

The reaction between ammonium sulphate and streptomycin was examined for its effect on the maltol and microbiological assay methods. A solution of streptomycin containing 10 per cent by weight of ammonium sulphate, based on the weight of streptomycin, was made and portions of the solution were adjusted separately to pH 7·0 and pH 9·0. No reduction in the result from the maltol or microbiological assay over control values was observed in the experiment at pH 7·0. This suggests that no reaction had taken place between the streptomycin and ammonium sulphate. The experiment at pH 9·0 showed no reduction in the maltol assay but a loss of about 6 per cent of the microbiological activity after 2 hr., indicating that some reaction had now taken place.

The Examination of Low Purity Streptomycin

Streptomycin of less than 90 per cent purity nearly always gave a substantially higher assay result by the maltol or 2,4-dinitrophenylhydrazine methods than by the microbiological method, the difference often being as much as 10 per cent. Impure streptomycin was found to contain small proportions of the components of R_F 0·08, 0·48 and 0·23 shown above to result from the reaction of streptomycin with ammonia. Furthermore, when an eluate of the component of R_F 0·08, bis-(α-hydroxystreptomycylamine) (IV) was allowed to stand overnight in solution at pH 2·5 it gave some streptomycin and also some of the component of R_F 0·48 (streptomycin monoaldehyde ammonia, V) which did not further readily change to streptomycin. The presence of bis-(α-hydroxystreptomycylamine) in impure streptomycin was confirmed by electrophoresis.

The reactivities of these components with various indicators is shown in Table I, whilst the quantitative examination of several samples of

impure streptomycin is shown in Table II. The results in Table II illustrate the interference of the R_F 0·08 and 0·48 components with the maltol assay and show the relative specificity of the microbiological method for streptomycin.

TABLE I

DETECTION OF IMPURITIES IN LOW PURITY STREPTOMYCIN BY CHROMATOGRAPHY AND VARIOUS INDICATORS

Relative R_F values	Known or probable identity	Colour produced by indicator				
		Elson Morgan	Sakaguchi	Diacetyl	Periodate/ benzidine	Silver nitrate
0·08	Bis-(α-hydroxystreptomycyl-amine)	Blue	Pink	Pink	Yellow	—
0·23	?	—	—	Pink	White	—
0·48	Streptomycin monoaldehyde ammonia	Blue	Pink	Pink	White	—
1·00	Streptomycin	Blue	Pink	Pink	Yellow	Brown

Eluates of the R_F 0·08 and 0·48 component were next given an ion-exchange treatment to remove phosphate and then assayed by the maltol and microbiological methods. The maltol to microbiological assay ratios were found to be 3·2 and 1·3 for the respective components, showing them to be principal causes of the differences between the assay results by the two methods.

TABLE II

COMPARISON OF ASSAYS ON LOW POTENCY STREPTOMYCIN SULPHATE

Sample	Streptomycin (units/mg.)					
	Chromatographic assay				Maltol assay	Microbiological assay
	Bis-(α-hydroxy-streptomycylamine) R_F 0·08	Streptomycin monoaldehyde ammonia R_F 0·48	Streptomycin R_F 1·00	Total		
A	40	40	640	720	680	630
B	10	10	640	660	690	640
C	30	40	650	720	700	650
D	40	20	570	630	650	560
E	70	40	560	670	640	570
F	50	30	580	660	660	610

Note.—Pure streptomycin sulphate has a potency of 798 units/mg.

Hydrogenation of a solution of impure streptomycin often led to a rapid initial uptake of hydrogen although the reaction proceeded very slowly in the final stages. A long time was taken to lower the residual streptomycin, as measured by maltol assay, below about 3 per cent. The solution thus appeared to contain an impurity, representing up to about 3 per cent of the streptomycin, which assayed as streptomycin by the maltol method and which was not very readily reduced. Similar results had been reported by Kaplan, Fardig and Hooper (1954) who also attributed reduction difficulties to the presence of maltol-producing

impurities. Chromatographic examination of the course of hydrogenation showed the production of a component of R_F 0, probably streptmycylamine; electrophoretic examination confirmed this supposition.

Pereira (1961), in studying ion-exchange chromatography of streptomycin, had shown the presence of unidentified maltol-producing impurities at R_F 0·18, 0·32 and 0·58 relative to streptomycin as 1, using the Winsten and Eigen (1948) solvent system. These impurities had a maltol to microbiological assay ratio of 2·5, 1·9 and 1·5 respectively. They may well have been similar to those found in this present study or similar to those described by Winsten and Eigen (1948).

A comparison of the chromatographic and electrophoretic results on impure streptomycin and on the reaction products of streptomycin and ammonia is given in Table III.

TABLE III

CHROMATOGRAPHIC AND ELECTROPHORETIC SEPARATION OF LOW PURITY STREPTOMYCIN AND OF THE PRODUCTS OF THE STREPTOMYCIN/AMMONIA REACTION

Known or proposed identity	*Movement relative to streptomycin = 1*		Occurrence
	Chromatography	Electrophoresis	
Streptomycylamine	0	0·66	Found in hydrogenated bis-(α-hydroxystreptomycylamine) and hydrogenated impure streptomycin
Bis-(α-hydroxystreptomycylamine)	0·08	0·33	Produced by the streptomycin/ammonia reaction and found in impure streptomycin
?	0·23	—	,,
Streptomycin monoaldehyde ammonia	0·48	—	,,
Dihydrostreptomycin ..	0·75	1·00	Found in hydrogenated bis-(α-hydroxystreptomycylamine) and is produced by hydrogenating streptomycin
Streptomycin	1·00	1·00	—

The various experiments described above showed the similarity between two major impurities in low purity streptomycin and the products from the reaction between streptomycin and ammonia.

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