Estimation of Streptomycin in Fermentation Broths

HAZEL M. DOERY AND E. C. MASON, *Commonwealth Serum Laboratories,* AND D. E. WEISS, *Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia*

An ion exchange procedure is presented for the chemical estimation of streptomycin in fermentation broths. The method is suitable for plant control purposes.

STREPTOMYCIN present in a fermentation broth cannot be assayed directly by the maltol assay method of Titus and Fried (*5*) because of the presence of interfering substances, but may be assayed biologically, or more rapidly by the sodium cotton succinate ion exchange method of McIntire and Schenck (*3*, *4*), or by the method of Boxer, Jelinek, and Leghorn (*1*), in which the maltol formed by degradation of the streptomycin is extracted with an organic solvent before colorimetric assay. [Attention has been drawn to an alternative maltol assay method which has been recently published by Eisenman and Bricker (*2*).] The authors have developed an assay method, suitable for plant control work, using the carboxylic-type cation exchange resin, Amberlite IRC-50 (a product of the Rohm & Haas Company, Philadelphia, Pa.) combined with the maltol assay procedure.

EXPERIMENTAL

Apparatus and Preparation. The apparatus used is shown in Figure 1.

Sufficient of the sodium form of the washed resin, prepared by equilibrating the hydrogen form of the resin with three of four successive lots of a saturated sodium bicarbonate solution, was introduced into an adsorption tube 4 mm. in diameter with sufficient saturated sodium bicarbonate solution to give a 1-cm. head of solution above a 12-mm. resin column. After the resin bed had been stirred thoroughly to remove air bubbles, a glass wool pad was placed above it and the funnel attachment was fitted. The streptomycin broth was diluted with a 0.2 *M* disodium hydrogen phosphate solution to a potency of 20 to 50 units per ml., adjusted to pH 8.5 to 9 with a 0.2 *N* sodium hydroxide solution, and clarified by centrifuging.

Adsorption. Distilled water (0.5 ml.) followed by the prepared broth (5 ml.) and wash water (1 ml.) were consecutively percolated through the resin bed at a flow rate not exceeding 0.3 ml. per minute. The streptomycin cations were quantitatively adsorbed by the resin at this pH.

Elution. Immediately after the wash water had drained from the funnel, 25 ml. of a 0.2 *N* hydrochloric acid solution were passed through the column at a flow rate of 0.5 ml. per minute. The first 20 ml. of effluent, which contained the eluted streptomycin free from interfering substances, were collected for chemical assay.

Chemical Assay. The streptomycin was hydrolyzed to form maltol by adding 0.2 ml. of a 4 *N* sodium hydroxide solution to 4-ml. aliquots of the acid eluate. These were heated in a boiling water bath for exactly 6 minutes, and then immediately cooled to room temperature in an ice bath. Evaporation losses were minimized according to the procedure of Titus and Fried. The ultraviolet absorption at 322 mμ of each aliquot before and after heating was measured in a 1-cm. cell in a Beckman spectrophotometer. The difference between the two readings, ΔD, was converted to the potency of the original streptomycin solution by referring to curve 4 in Figure 2.

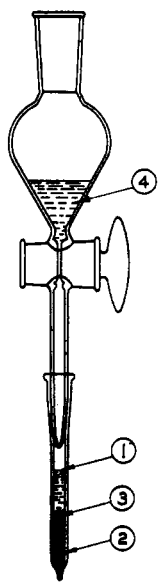

Figure 1. Resin Adsorption Column and Attached Reservoir

1. Liquid head
2. Resin bed
3. Glass wool pad
4. Reservoir

DISCUSSION

The slope of the linear relationship between ΔD and the potency of the streptomycin solution under examination depends on the strength of the alkali used for hydrolysis, and on the order of purity of the streptomycin in the original sample. This relationship was determined using Food & Drug Administration (F.D.A.) standard streptomycin containing 400 units per mg. and 2 *N*, 4 *N*, and 6 *N* sodium hydroxide solutions for the hydrolysis. The results are plotted in curves 1, 2, and 3 in Figure 2. Samples of broth at various concentrations were assayed by the above method using a 4 *N* sodium hydroxide solution for the hydrolysis. The eluates obtained were diluted to various concentrations and their ΔD values compared with the broth potency obtained by biological assay. Application of the method of least squares to the results from thirty samples examined yielded the following relationship between ΔD and the broth potency, *P*:

$$\Delta D = 0.0147\ P + 0.0178$$

which is shown as curve 4, Figure 2. This calibration curve gave more reproducible results than the corresponding curve

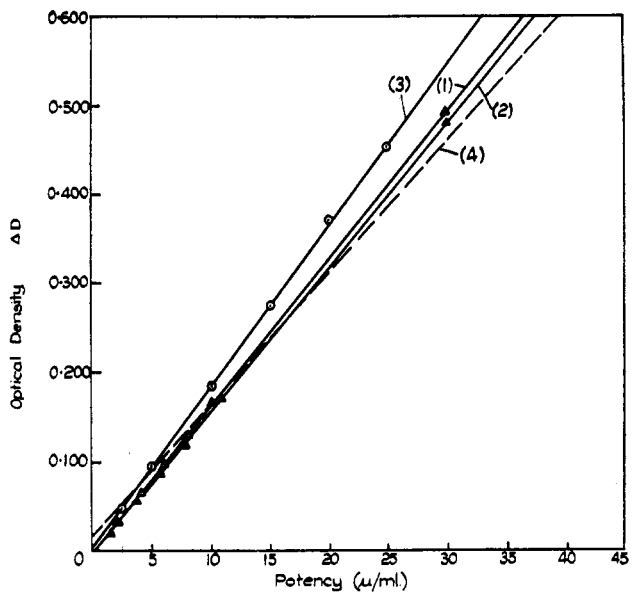

Figure 2. Variation of Optical Density with Potency of Streptomycin Sulfate Solutions

Various strengths of sodium hydroxide used for maltol reaction with aqueous solutions of F.D.A. streptomycin

1. 2 *N* NaOH
2. 4 *N* NaOH
3. 6 *N* NaOH
4. Best-fit line for broth eluate samples

obtained for the purer F.D.A. standard streptomycin (curve 2, Figure 2). If the method is to be applied to a variety of types of streptomycin broth individual calibration curves must be constructed for each system.

To determine the relative proportion of streptomycins A and B in a mixture, Schenck *et al.* (*4*) have utilized the fact that streptomycin B gives more maltol per unit of biological activity than streptomycin A. Because the above results show close

agreement between the chemical and biological methods, the broths used in the present work did not contain appreciable amounts of streptomycin B.

When the resin was prepared by equilibrating with phosphate buffer solution at pH 7 in place of the saturated sodium bicarbonate solution, the eluates from the streptomycin broth showed apparent recoveries of 130 to 170%. The recovery of streptomycin from the adsorption column was determined from a comparison of the potency of the original broth, determined biologically, with that derived from the maltol reaction of the eluate. Inasmuch as the ultraviolet absorption of each aliquot was measured at 322 mμ before and after heating, the high recoveries obtained indicate the presence of biologically inactive substances in the broth which hydrolyze to give an absorption at 322 mμ, and, as the results obtained with the sodium bicarbonate equilibrated resin at pH 9 showed recoveries of 100%, these substances have a lower basic strength than streptomycin A.

The assay method described, employing adsorption at pH 9 and hydrolysis with 4 *N* sodium hydroxide, yields results which agree with the biological assay method to within ±5%. The reproducibility of a single chemical assay is ±3%.

ACKNOWLEDGMENT

The authors wish to thank J. R. Schenck for kindly providing details of his sodium cotton succinate procedure. The work outlined here is part of a research program of the Division of Industrial Chemistry, C.S.I.R.O., Melbourne, and the Commonwealth Serum Laboratories.

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RECEIVED October 20, 1949.

Identification of Coupling Components in *p*-Toluene-Azoaryl Amide Dyes of Naphthol AS Series

LOUIS KOCH AND ROBERT F. MILLIGAN
H. Kohnstamm Research Laboratories, Brooklyn, N. Y.

A simple method has been developed for the identification of aminonaphthol AS type reduction products, by hydrogenating the pigment in dioxaneacetic acid with zinc dust, isolating the desired compound, and converting it to the *N*-benzoyl derivative.

THE amides resulting from the condensation of 2-hydroxy-3-naphthoic acid with aromatic primary amines constitute a very important group of coupling components known as the Naphthol AS intermediates. When treated with a diazonium salt, they give rise to a class of dyes which finds wide application in the textile and printing ink industries.

Unknown colors falling in this category are usually analyzed by reductive fission of the azo bond, followed by isolation and identification of the resulting products. This reaction normally splits the monoazo pigment into two fractions, the original amine and the amino derivative of the aryl amide, or their respective diamines or possibly triamines, if nitrated.

Procedures for the characterization of the former compounds are fairly numerous (*2–4, 6–9*), but except for the work of Battegay, Langjahr, and Rettig (*1*) on the synthesis of 1-aminonaphthol AS from 1-nitrosonaphthol AS and from phenylazonaphthol AS, and by Koch and Milligan (*4*) on the identification of unsulfonated dyes made with Naphthol AS, the chemical journals are practically devoid of analytical data regarding the latter products.

This paper, in an effort to bridge this gap, describes the hydrogenation of these colors with zinc dust, in dioxaneacetic acid, followed by isolation of the aminoaryl amides, which are subsequently converted into their *N*-benzoyl derivatives. Confirmation of the aminonaphthol AS group of scission products will be found in another report (*5*) which is based on the work of Battegay and co-workers (*1*). By a modification of their method, the 1-nitroso compounds of the known commercial Naphthol AS intermediates were successfully synthesized, and these gave, on reduction, amines identical with those obtained in this paper.

GENERAL PROCEDURE

Preparation of Dyes. Diazotized *p*-toluidine was coupled to twelve Naphthol AS intermediates, obtained from E. I. du Pont de Nemours & Company, and the resulting pigments were dried without further purification.

Preparation of Reduction Products. A finely ground 2-gram sample of coloring matter and 10 grams of zinc dust are placed in a 250-ml. Erlenmeyer flask, and the solids are suspended in a solution of 50 ml. of dioxane plus 10 ml. of glacial acetic acid. Heat is applied, and the mixture is refluxed gently until the dye particles are decolorized. The hot reaction mixture is filtered to remove insoluble matter, which is then washed with 25 ml. of dioxane, and the filtrate is acidified with 50 ml. of hydrochloric acid to precipitate the aminonaphthol AS hydrochloride.

Maximum yield of the addition product is achieved by cooling overnight, and the solid is then collected on a Büchner funnel, washed with a small volume of dioxane, and transferred to a 500-ml. extraction funnel with approximately 50 ml. of ethyl alcohol. Buffering the suspended hydrochloride with 10 ml. of a 10% sodium acetate solution liberates the free amine, which may wholly or partially dissolve in the alcohol. The mixture is diluted with 250 ml. of ether, and any precipitate that may remain is solubilized by further buffering with 200 ml. of a 2.5% sodium acetate solution. Several water washings are applied to the ether layer, which is then dried with anhydrous sodium sulfate, filtered, and evaporated to near dryness.

Resolution of the ether residue is effected with 50 ml. of hot benzene, and incipient crystallization or cloud formation of the aminoaryl amide is attained by dilution with petroleum ether. Cooling the solvent mixture overnight precipitates the desired compound as yellow to greenish crystals, which are collected and purified by dissolving in benzene and precipitating again in the afore-mentioned manner.

Occasionally, the aminonaphthol AS product cannot be dissolved in ether. When this occurs, 100 ml. of benzene and 50 ml. of xylene are added to the ether layer, after the water wash, and