The spectrum of the unhydrolyzed material taken in the ultraviolet (Figure 2) indicates a peak at 248 mμ. The unhydrolyzed material was also subjected to ultracentrifugation at 60,000 r.p.m., but no peak was discernible, indicating that the material is of low molecular weight.

On paper electrophoresis in veronal buffer at 8.7 under the same conditions as for fraction Aa_1, the bulk of the ninhydrin-positive material moved toward the negative pole. A heavy spot appeared at 2.5 cm. from the origin and another at 1 cm. A very faint ninhydrin-positive spot moved 9 cm. toward the positive pole. When a duplicate strip was checked for carbohydrate, the bulk of the material also moved toward the negative pole in the same position as the ninhydrin-positive material. One faint sugar spot was located at 12.2 cm. toward the positive pole. When a duplicate strip was checked for phosphorus, the bulk of the material remained at the base line, but some of the material streaked toward the positive pole with a definite spot at around 13 cm.

When this fraction (Aa_2) was run in 0.1*M* sodium carbonate at pH 11, the ninhydrin-positive material moved toward the positive pole. Two spots appeared, one at 4.5 cm. and another at 7 cm. The bulk of the carbohydrate material again moved toward the negative pole in the same position as it did at pH 8.7, but two spots moved toward the positive pole, one at 5.5 cm. and another again at 13 cm. The phosphorus-containing material moved essentially the same as it did at pH 8.7. The fact that a pH of 11 was necessary to cause the ninhydrin-positive material to move to the positive pole, suggested a high content of basic amino acids. However, a Sakaguchi test for arginine was only faintly positive.

Fraction Aa_2, when heated with fat, produced an odor similar to that of broiled steak. However, when small aliquots of fractions Aa_1 (protein) and Ab_1 (carbohydrate) were added to Aa_2 and then heated, the odor, based on subjective evaluation, was equivalent to the odor observed for the original fraction A. At the present stage of this work it appears that fraction Aa_2 contains the material necessary for the "basic" odor of broiled steak, but other constituents—i.e., protein (fraction Aa_1) and carbohydrate (fraction Ab_1)—are necessary for the development of the cooked meat flavor and odor. Natural fat and hydrogenated fat, which were used interchangeably in heating the isolated material, also contribute something to the odor. The same basic odor can be produced if the three fractions are heated in mineral oil, but it is distinguishable from that when natural or hydrogenated fats are used.

The fractionation procedure was tried on chicken and pork loin. The material (fraction Aa_2) isolated in these experiments had the same basic odor as the material isolated from beef. When heated with fat, the odor was similar to that of broiled steak. However there were enough distinguishing characteristics to indicate which material had been isolated from chicken and which came from pork. The 248-mμ peak was present in both materials.

At present, attempts are in progress to identify the various components present in fraction Aa_2, and to attempt to determine the role of these constituents in the production of odor and flavor in meat.

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FEED ADDITIVES MEASUREMENT

Ultraviolet Method for Determination of Streptomycin in Feeds

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THE USE OF ANTIBIOTICS in animal feeds is constantly increasing and state feed control laboratories face problems in determining these drugs. As most feed control laboratories do not have facilities for biological assay procedures, the development of chemical methods for determining antibiotics in feeds is an urgent problem.

All current AOAC methods for antibiotics are microbiological assay procedures. When these AOAC procedures for antibiotics were being studied (*12*), streptomycin was not considered because it was not widely used. Now that its use in poultry and swine feeds is increasing, it is imperative that a suitable chemical method be devised.

The streptomycin molecule can be determined by any one of three basic ways: analysis for the streptose moiety by determining the alkaline degradation product, maltol; for the combined guanidine groups; and for the *N*-methyl-1-glucosamine fraction.

Schenck and Spielman (*14*) showed that heating streptomycin at 100° C. for 3 minutes with 1*N* sodium hydroxide converted to streptose moiety of the streptomycin molecule quantitatively to maltol (3-hydroxy-2-methyl-4-pyrone).

Eisenman and Bricker (*6*) reported the use of this reaction in a spectrophotometric method, in which the color reaction between maltol and ferric iron was employed to estimate the concentration of streptomycin. Boxer, Jelinek, and Leghorn (*4*) used the color reaction between maltol and the Folin-Ciocalteu phenol reagent, as well as the ferric iron–maltol complex, for streptomycin determinations. St. John, Flick, and Tepe (*13*) and Angeles (*1*) adapted the colorimetric maltol procedure for the assay of streptomycin in fermentation broths.

Various investigators determined streptomycin by means of the combined guanidine groups. The two general procedures used were colorimetric estimation with sodium nitroprusside and potassium ferrocyanide (*10*) and colorimetric estimation of the condensation product with biacetyl and 1-naphthol (*2, 3, 7*).

The ultraviolet method of determining streptomycin in feeds involves a dilute sulfuric acid extraction of the streptomycin from a ground feed, separation of the aqueous extract from the feed solids, neutralization of the extract, and further separation of the neutralized extract from the solids. This is followed by concentration and purification of the streptomycin with an ion exchange resin, elution of the streptomycin from the resin, conversion of the streptose moiety of the molecule to maltol, and the quantitative determination of the streptomycin by means of the ultraviolet absorbance of maltol. Recoveries at the recommended level averaged 98.4%, with a coefficient of variation of 4.8%.

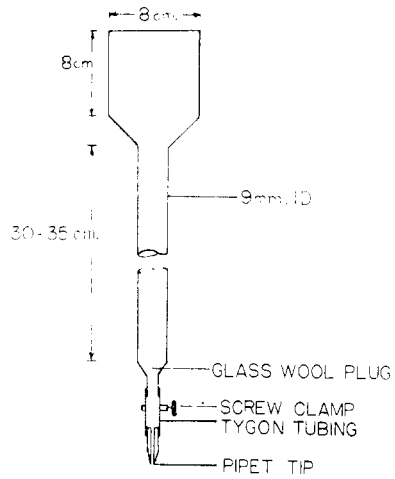

Figure 1. Amberlite IRC-50 Na form column

Determination of streptomycin using the *N*-methyl-1-glucosamine fraction of the molecule has not been widely employed. Kamata *et al.* (*8*) used the reaction between the *N*-methyl-1-glucosamine and acetylacetone, followed by the colorimetric reaction with *p*-dimethylaminobenzaldehyde, to estimate the streptomycin.

The separation of streptomycin from fermentation broths using ion exchange resins has been the subject of much discussion. Doery, Mason, and Weiss (*5*) as well as St. John, Flick, and Tepe (*13*) used Amberlite IRC-50 in their analytical procedures for the chemical assay of the streptomycin content of fermentation broths. Kotula (*9*) in his investigation of the adsorption and elution of streptomycin with five different cation exchangers, showed Amberlite IRC-50 to be the most suitable, with recoveries in the order of 93%.

Procedure

Preparation of Calibration Curve. Prepare a calibration curve by transferring 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 ml. of a standard streptomycin solution containing 100 μg. of streptomycin per ml. into 10-ml. volumetric flasks. Add 1 ml. of 1*N* sodium hydroxide and bring to volume with distilled water. This will yield standards from 50 to 600 μg. Place the 10-ml. volumetric flasks in a 90° C. hot water bath for 5 minutes. Cool the flasks by placing them in an ice water bath. Determine the absorbance of each standard with a Beckman DU spectrophotometer at 324 mμ in 1.0-cm. silica cells. Plot a standard curve from the observed absorbance values.

Standardization of Amberlite IRC-50 Resin. To minimize resin problems due to variations in available exchange resin batches, the following procedure was used.

Place 100 grams of Amberlite IRC-50, H form, 16 to 50 mesh, in a 1-liter Erlenmeyer flask with 250 ml. of 5% sodium hydroxide and shake mechanically for 1 hour. Wash the resin into a Salvarsan tube, or similar tube, plugged with glass wool at the constricted end to retain the resin. Wash the resin until the effluent is neutral to litmus.

Air-dry the Amberlite IRC-50, Na form, for 24 hours and store the resin in a closed bottle. Prepare an ion exchange column containing 8 inches of resin. The dimensions of a convenient form of the column are shown in Figure 1. Backwash the resin and allow it to settle without a flow. Drain the column to within 1 cm. of the resin bed and wash the column with 100 ml. of distilled water. Never allow the liquid level to drain into the resin bed. After the column wash water has drained to within 1 cm. of the resin bed, add 500 μg. of streptomycin in 250 ml. of water. Pass the solution through the resin at the volumetric rate of 3 to 5 ml. per minute. Drain the liquid level to within 1 cm. of the resin bed. Wash the column with two 25-ml. portions of distilled water and drain to within 0.5 cm. of the resin bed. Elute the column with 2% (by volume) sulfuric acid at the volumetric rate of 1 ml. per minute. Collect 5-ml. fractions of the eluate in 10-ml. volumetric flasks containing 5 ml. of 1*N* NaOH. Twelve fractions are usually sufficient. Place the volumetric flasks in a 90° C. hot water bath and proceed as described under Preparation of Calibration Curve.

Determine the recovery and elution curve for the batch of resin. If the recoveries of streptomycin are 95% or greater, the resin is suitable for use. If recoveries are less than 95%, repeat this complete procedure.

Determination of Streptomycin in Feeds. Place a quantity of ground feed containing approximately 500 μg. of streptomycin in a 500-ml. Erlenmeyer flask, fitted with a ground-glass stopper. Add 200 ml. of 0.02*N* sulfuric acid and shake mechanically for 1 hour. Place the contents of the flask in a 250-ml. centrifuge bottle and centrifuge for 5 minutes at 2000 r.p.m. Filter the turbid solution through H. Reeve Angel No. 711 filter paper, or its equivalent. Measure the quantity of filtrate. This will yield the aliquot size for the calculations later in the procedure.

Neutralize the filtrate with 1*N* sodium hydroxide to a pH value of 7.0 ± 0.2. Place the filtrate in another 250-ml. centrifuge bottle and centrifuge for 5 minutes. Filter the clear supernatant through H. Reeve Angel No. 711 filter paper. Transfer the gelatinous precipitate onto the filter paper and wash several times with water. Discard the precipitate.

Pass the filtrate and washings through the Amberlite IRC-50, Na form, column at the volumetric rate of 3 to 5 ml. per minute. Permit the filtrate to flow to within 1 cm. of the resin bed. Wash the column with four 50-ml. portions of distilled water and drain each to within 1 cm. of the column before addition of the next wash. Drain the final wash to within 0.5 cm. of the column.

Elute with 2% (by volume) sulfuric acid at the volumetric rate of 1 ml. per minute. Discard the eluate before the appearance of the streptomycin and collect the fraction containing the streptomycin (determined from elution curve).

Pipet 9 ml. of the well-mixed eluate into two 10-ml. volumetric flasks. Bring to volume with 7*N* sodium hydroxide and shake well. Place one flask (I) directly in an ice water bath. Place the other flask (II) in a 90° C. hot water bath for 5 minutes and then cool in the ice water bath. Centrifuge the contents of each volumetric flask before determining the absorbance values of each in 1.0-cm. silica cells with water as a blank.

Subtract the absorbance of flask I from that of flask II and determine the quantity of streptomycin from a stand-

ard curve. Determine the total amount of streptomycin present from the ratio of the total volume of extracting liquid to the aliquot taken and from the ratio of the total volume of streptomycin-containing eluate to the 9-ml. aliquot. Total micrograms of streptomycin in the sample can be converted to grams per ton by using the appropriate factors.

Results

Determination of Streptomycin by Combined Guanidine Groups. SODIUM NITROPRUSSIDE–POTASSIUM FERROCYANIDE METHOD. The Monastero procedure (*10*) for the determination of combined guanidine groups, although sufficiently sensitive, proved to be too dependent upon pH for consistent results. In the elution of streptomycin from the resin, it was extremely difficult to adjust the pH of the eluate precisely to a value of 7.0 with no appreciable change in volume.

Biacetyl - Naphthol Condensation Method. The low sensitivity as well as the relatively high blank made the determination by the biacetyl-naphthol method (*2, 3, 7*) unsuitable for streptomycin determination in feeds.

Analysis by *N*-Methyl-1-glucosamine Fraction. Determining streptomycin by means of the *N*-methyl-1-glucosamine fraction was not attempted because the intensity of the color developed in the condensation with acetylacetone and *p*-dimethylaminobenzaldehyde was grossly insensitive (*8*) at the levels necessary in feed determinations.

Analysis by the Alkaline Degradation Product, Maltol. COLORIMETRIC METHOD. With the procedure of St. John, Flick, and Tepe (*13*) (a colorimetric reaction between iron and maltol), the smallest quantity determinable was approximately 1 mg. Although extremely reproducible and relatively sensitive, this method was still insufficiently sensitive for determining streptomycin in feeds.

ULTRAVIOLET METHOD. This procedure is basically similar to that used in the colorimetric iron determination of maltol (*13*). Measurement of the ultraviolet absorbance of maltol rather than of the color complex proved to be a very sensitive, reproducible method of determining the streptomycin content in feeds.

A calibration curve in the range of 50 to 600 μg. was established according to the previously described procedure. Twenty-six determinations, all at different levels, from five separately prepared standard solutions were made. The coefficient of determination, r^2 (*11*), indicates the percentage of explainable variation in the concentration as determined by absorbance readings. The calculated r^2 value of 0.9918 indicates that 99.18% of the variation is accounted for and 0.82% is not. The 0.82% unaccounted for is considered the per cent error in pure solution.

Recoveries of Streptomycin from Feed. An unmedicated poultry feed, Rutgers' Ration 15, was blended with streptomycin at a level of 20 μg. per gram of feed (approximately 20 grams per ton of feed). The blended sample was ground prior to analysis. Recoveries in the 300- to 600-μg. level (Table I) ranged from 91.6 to 108.0% with an overall average of 96.5%. Six replications at the same level, 500 μg., yielded an average recovery of 98.4%. The coefficient of variation between replications at the 500-μg. level was 4.8%.

Because aliquots taken from the eluate containing streptomycin are approximately 1/3 to 1/4 of the total volume, a factor of 3 to 4 is necessary. This means at the 200-μg. level an aliquot would contain 50 to 65 μg. of streptomycin. These concentrations approach the lower limit of the calibration curve and are subject to greater errors. The aliquot of a 500-μg. sample would contain 125 to 165 μg., a more accurately determinable quantity. Sample size is strictly governed by the amount expected in the aliquot taken from the eluate fraction containing the streptomycin.

Several attempts to eliminate the need to aliquot the eluate containing the streptomycin were unsuccessful. Converting the streptose moiety to maltol and steam-distilling the maltol were not satisfactory; concentrating the acid eluate failed, and chloroform extraction of the maltol proved to be short of quantitative.

The measurement of a sample's absorbance before and after its conversion to maltol eliminates any interfering substances absorbing at 324 mμ. The increase in absorbance after heating is attributable to streptomycin alone. Determinations on the same feed with no streptomycin added indicated that other substances may absorb at 324 mμ, but heating does not alter these absorbance values. Some drugs commonly used with streptomycin, such as penicillin, furizolidone, sulfaquinoxaline, nicarbazine, and glycarbylamide, did not interfere. Other drugs were not studied for possible interference.

The binding of streptomycin by some of the feed components initially caused a problem. Preliminary studies of extraction showed that streptomycin was not quantitatively removed from the feed, and, as time progressed, less and less streptomycin was removed from the feed by extraction. By using a dilute aqueous acid extraction, the binding was broken and the streptomycin quantitatively recovered from the feed samples. It appears that grinding and/or storing the sample for a time caused the sulfate salt to revert to the free base, which is insoluble in water. The dilute acid extracted the free base as the soluble sulfate with a quantitative recovery.

Table I. Recovery of Streptomycin from a Laboratory-Prepared Feed

Feed Sample, Grams	Streptomycin, μg. Present	Streptomycin, μg. Found	Recovery, %
10	200	150	75.0
		170	85.0
		155	77.5
		160	80.0
		150	75.0
15[a]	300	275	92.1[a]
		280	93.6[a]
25[a,b]	500	490	98.0[a,b]
		480	96.0[a,b]
		472	94.4[a,b]
		510	102.0[a,b]
		463	92.6[a,b]
		540	108.0[a,b]
30[a]	600	580	96.6[a]
		550	91.6[a]
		Av.[a]	96.5
		Av.[b]	98.4

[a] Range in which quantitative recovery is possible.
[b] Recommended range.

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