COUNTER-CURRENT DISTRIBUTION STUDIES ON
STREPTOMYCIN: THE TAUTOMERISM
OF STREPTOMYCIN

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The simplicity and reproducibility which characterize the conversion of
streptomycin to maltol (1) by heating in dilute alkali have made this reac-
tion the basis of a frequently used analytical method for streptomycin (2).
When used in these laboratories as a method of assaying streptomycin
preparations, however, the maltol reaction consistently gave lower yields
per unit of potency with pure streptomycin trihydrochloride or its calcium
chloride double salt than with impure preparations of lower activity. This
observation suggested the possibility that the cruder concentrates contained
other maltol precursors less active than streptomycin, a supposition which
has been confirmed by the isolation of mannosidostreptomycin¹ (5, 6).

In a preliminary communication (7) it was remarked that the broad dis-
tribution curves obtained by the application of the Craig counter-current
distribution technique (8, 9) to solid streptomycin preparations assaying
approximately 400 units per mg. could be indicated to indicate the pres-
ence of at least three maltol-producing entities (cf. Curve B (7) ). The
distribution studies herein described were originally undertaken as part of
the effort to isolate the hypothetical third streptomycin-like substance, but
they have now shown that this third fraction does not represent such an
entity and have instead yielded evidence that streptomycin exists in several
tautomeric modifications, the relative proportions of which depend on the
pH. These tautomers differ in the ratio of distribution between some
immiscible solvent pairs and are at least partially separable by adsorption
on alumina.

In order to concentrate the different components, the crude mixture of
streptomycin (400 to 500 units per mg.) was fractionated by liquid chroma-

¹ It has been suggested by Dr. Selman A. Waksman (Science, 107, 233 (1948)) that
the term streptomycin be reserved for the pure substance C₁₂H₁₄N₂O₁₂ described by
Peck et al. (3) and by Fried and Wintersteiner (4). The Squibb investigators (Fried,
Stavely, Titus, and Wintersteiner) have cooperated with Dr. Waksman by sub-
stituting the term mannosidostreptomycin for streptomycin B. The new name is
based on data to be published shortly by Dr. Stavely and Dr. Fried. Crude con-
centrates containing unknown proportions of the two streptomycins would be re-
ferred to under the revised nomenclature as "streptomycin complex."
tography with dry methanol and acid-washed alumina (10, 11). The solid residues obtained by lyophilization of each chromatographic fraction were then subjected to counter-current distribution in butanol and 5 per cent p-toluenesulfonic acid (7). Since for each constituent of a mixture examined by the latter technique the distribution curve will show a maximum, the location of which is a simple function of the partition coefficient (12), it proved convenient to refer to the various fractions obtained in terms of their distribution constants between the phases used. These constants, which may be readily calculated from the curves (12), represent the ratio of concentration in butanol over concentration in the aqueous phase, and are for the sake of brevity hereinafter referred to as $K$. The twenty-four plate distribution curves illustrated in this paper are all plotted so that fractions appear from left to right in the order of increasing distribution constants, substances with a $K$ of 1 having their maximum concentration in the center.

Distribution quickly revealed that chromatography had effected the separation of three types of material. That obtained in the first three or four fractions from the column contained 20 to 40 per cent of what appeared from the broad distribution curves to be an ill defined mixture of maltol-producing substances with $K$ ranging from 1 to 10. Streptomycin, with a $K$ of unity, accounted for the remainder of the first fractions and occurred practically pure in subsequent ones. This yielded place to mannosidostreptomycin (5, 6) as the more strongly adsorbed fractions emerged from the column, a process which was reflected in the distribution curves by the disappearance of the central band corresponding to a $K$ of 1 and the appearance of a peak corresponding to a $K$ of about 0.4. This paper will be concerned only with the early chromatographic fractions in which mannosidostreptomycin had not yet appeared.

Curve A in Fig. 1 illustrates the distribution in a 5 per cent $p$-toluenesulfonic acid system of a typical early chromatographic fraction. The broad curve obtained is open to two interpretations, the immediately obvious one being the presence of several substances with $K$ ranging between 1 and 10. Such curves would also result, however, if material moving far to the right of the distribution pattern because of its high $K$ was to be gradually converted during the course of the distribution to a substance of lower partition coefficient. In that event, part of the material would be left behind as the decomposing substance moved to the right, causing the sort of curve illustrated. Such a reaction would not be surprising in view of the high acidity of the system used.

The first indication that the form of Curve A might be attributable to the interconversion of several modifications of the same substance was the striking similarity between fractions removed from various tubes of the distribution apparatus.
The concentrations of streptomycin in the run illustrated by Curve A were measured by the maltol test, by bioassay, and by the quantitative application of the Sakaguchi reaction. The modification of the latter suggested by Thomas, Ingalls, and Luck (13) was used as described in the literature, the more concentrated sample being diluted to approximately 100 units per ml. with the blank lower layer prior to the treatment with concentrated alkali. The correspondence of the maltol and Sakaguchi determinations is illustrated in Curve A in Fig. 1. Bioassay of these fractions against Klebsiella pneumoniae (14) revealed that the ratio of biological units per ml. to the optical density at 325 με of the maltol solution produced in the chemical determination was constant, regardless of the fractions
analyzed. The average deviation of ±5.3 from the value of 88.2 for the constant was within the limit of error of the microbiological assays, so that no difference in potency, streptidine content, or ability to produce maltol could be demonstrated between the fractions in the higher numbered tubes and those in the central band.

Further evidence was obtained in an attempt to isolate some of the material in the fraction of the higher partition coefficient. A twenty-four plate run with 5 per cent toluenesulfonic acid was made with material with the distribution pattern illustrated in Curve A, Fig. 2. The conditions were identical to those used in obtaining Curve A, but the run was made with 50-fold quantities in 1 liter separatory funnels. The lower layer in the separatory funnel corresponding to Tube 22 of the machine was neutralized with Ba(OH)$_2$ and the solution freeze-dried. The solid residue was transferred to a glass-stoppered centrifuge tube and triturated with twice its volume of anhydrous methanol to separate the readily soluble streptomycin trihydrochloride from the relatively insoluble (9.8 mg. per ml.) barium p-toluenesulfonate. After centrifugation and removal of the supernatant, the residue was triturated with another portion of methanol and centrifuged.
The combined methanol extracts were evaporated to dryness in the cold and the triturations with methanol were repeated. Approximately 10 per cent of the total streptomycin content was lost at each step. Evaporation of the final methanol solution left a residue of 37 mg. of solid material containing 22 per cent of streptomycin base, calculated from maltol analysis.

![Graph](http://www.jbc.org/)  
**Fig. 3.** Curve A, distribution of pure calcium chloride double salt of streptomycin hydrochloride; Curve B, distribution of the same material after standing at pH 7.42.

Redistribution of 20.9 mg. of the material gave Curve B, Fig. 2, practically identical with that of the starting material, a result which could only be explained by transformation of the high K material into a substance of lower partition coefficient.

In order to determine whether the high K fraction, which appeared from the above to be unstable in acid, could be reconstituted at higher pH, a
sample of 9.9 mg. of the pure calcium chloride double salt of streptomycin (11) was allowed to stand in solution at pH 7.42 for 30 hours. The solution was freeze-dried and the residue distributed. Fig. 3 illustrates the significant increase in material of high $K$ caused by this treatment, Curve A representing the distribution in 5 per cent toluenesulfonic acid of 10 mg. of the original sample and Curve B the same material after standing.

The ready interconvertibility of these fractions strongly suggested that streptomycin can exist in several tautomeric modifications, the forms of high $K$ being favored in alkaline and those of low $K$ in acid solutions. To determine quantitatively the relative proportions of such substances by distribution in a system in which one form is rapidly being converted to the other is obviously impossible. A qualitative demonstration of their existence is possible only when conversion proceeds slowly enough so that significant quantities of the forms with higher $K$ survive to be carried to the higher numbered tubes of the apparatus. The distorted distribution curves caused by this phenomenon likewise make it impossible to state with certainty the number of tautomeric forms.

These difficulties were partially surmounted by the use of less drastic conditions.

It was found possible to slow the rate of conversion of one form to the other by running distributions with 3 rather than 5 per cent toluenesulfonic acid, and to do so even more effectively by using the 3 per cent system at 5°. No other changes were made in the described procedure and the only effect was a lowering of the distribution constant. Distribution in these systems revealed that the early chromatographic fractions contained far higher proportions of the high $K$ forms than originally suspected. Curves B and C of Fig. 1 illustrate the patterns obtained when 12 mg. samples of the same material whose distribution is recorded in Curve A were run in the 3 per cent system at 22°, and in the 3 per cent system at 5°.

Use of these systems permitted a clearer demonstration of the interconvertibility of the tautomeric forms. A sample of 12 mg. of the early chromatographic fraction whose distributions are recorded in Fig. 1 was allowed to stand 24 hours in dilute sulfuric acid, neutralized, and freeze-dried. Distribution of the residue in 3 per cent toluenesulfonic acid is recorded as Curve D in Fig. 1, and comparison with Curve B in the same figure makes clear the decrease in the amount of material in Tubes 18 to 24, and the corresponding increase in the centrally located band.

The formation of the high $K$ modifications in alkaline solution was strikingly illustrated when distributions were run at 5° with 3 per cent toluenesulfonic acid systems. Curve A in Fig. 4 represents such a distribution of 19.8 mg. of the same sample of calcium chloride double salt which was used for Curve A of Fig. 3. A 24.6 mg. sample of this salt was allowed to stand
in solution at pH 7.52 at room temperature for 24 hours, after which the solution was freeze-dried and the residue distributed as before, with the result shown in Curve B of Fig. 4.

More nearly complete conversion was noted when another sample was dissolved in water, brought to pH 8.77, and allowed to stand for 72 hours, during which time 16 per cent decomposed, as measured by the loss in ability to produce maltol. An aliquot of this material was freeze-dried and the residue distributed at 5° with the 3 per cent system, as shown in Curve B of Fig. 4.

The presence of the aldehyde group in the streptose portion of the mole-

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Distribution in butanol and 3 per cent toluenesulfonic acid at 5°. Curve A, the pure calcium chloride double salt of streptomycin trihydrochloride; Curve B, the same material after standing at pH 7.52; Curve C, the same material after standing at pH 8.77.

cule is apparently essential for these interconversions, since reduction to the dihydro derivative eliminated the tautomerism. The dihydro derivatives of either the early chromatographic fractions or the pure calcium chloride double salt, when distributed in butanol and 5 per cent sulfonic acid, gave identical curves in good agreement with the theoretical distribution of a homogeneous substance. Distributions of 40 mg. samples of these substances are illustrated in Fig. 5.

It seems plausible, then, to suggest that, although the phenomena might
at first glance be taken as evidence for the existence of a third substance related to streptomycin, they are more probably an indication of a tautomeric mixture, possible components of which might be the aldehydo structure proposed by Kuehl and coworkers (15), and a ring form obtained by the reaction between the aldehyde group and a guanidine group from the streptidine moiety.

There is some evidence for the existence of similar modifications of mannosidostreptomycin since samples of the latter give curves of the same general form as the early chromatographic fractions of streptomycin. Be-
cause of the distribution constant of 0.44 for this substance in the 5 per cent toluenesulfonic acid system, most of the material appears in a band with maximum concentration at Tube 7, while that portion of the curve assumed to represent the form with higher $K$ occurs in the region of Tube 12. The possibility that this fraction indicates contamination of these samples with streptomycin has been eliminated by distribution in other two-phase systems which differentiate between the streptomycins, but do not distinguish between tautomeric modifications. These systems are described elsewhere by others in these laboratories.²

EXPERIMENTAL

Counter-Current Distribution—The immiscible liquid pairs used for these studies were prepared by mutually saturating $n$-butanol and water. Eastman C.P. $p$-toluenesulfonic acid monohydrate was made up in the water to the desired concentration, usually 3 or 5 gm. per 100 ml., and this solution was then shaken for several minutes with an equal volume of the butanol.

For the preliminary determination of distribution constants in these systems, streptomycin hydrochloride, assaying at 750 units per mg., which had been purified by the chromatographic procedure of Carter et al. (10), recrystallization of the helianthate (11), and conversion to the hydrochloride, was made up to 100 $\gamma$ per ml. in the aqueous phase. Equal volumes of this solution and the butanol upper layer were shaken together for 2 minutes and allowed to separate. The streptomycin concentration in the aqueous layer was determined by the maltol reaction before and after shaking and the distribution constant calculated as the difference in the two readings divided by the final concentration.

To determine streptomycin concentrations, 0.2 ml. of 4 N NaOH was added to 4 ml. of the aqueous phase in a test-tube. The contents were mixed well, heated in a boiling water bath for exactly 10 minutes, and brought to room temperature by cooling in an ice bath. Loss of volume by evaporation during the heating was prevented by suspending a small funnel in the mouth of each test-tube to provide a surface for condensation and blowing a stream of air against the exposed upper portion of the test-tube. The ultraviolet absorption at 325 m$\mu$ of each solution was measured before and after heating, with 1 cm. cells in the Beckman quartz spectrophotometer, and the difference between the two readings, referred to as $\Delta D$, was taken as a measure of the streptomycin concentration. Values of $\Delta D$ obtained in these toluenesulfonic acid systems are shown in Table I to be proportional to the streptomycin concentration down to levels of approximately 25 units per ml. Below this, the increasing yield of maltol necessitates the use of correction factors to retain the proportionality.

² Plaut, G. W., and McCormack, D. R., in press.
The distribution constant of streptomycin varied with the concentration of \( p \)-toluenesulfonic acid, values of 0.3, 1.0, and 1.8 being obtained with acid made up to 3, 5, and 10 per cent in the aqueous phase.

Partition coefficients in the 3 and 5 per cent systems proved independent of streptomycin concentration within the limits encountered in the distributions, as illustrated by the series of determinations summarized in Table II.

Distribution curves obtained with 10, 50, and 130 mg. samples of the same material proved superimposable, so that deviations of experimentally obtained curves from the theoretically predicted patterns were not attributable to variations in partition coefficient during the runs.

<table>
<thead>
<tr>
<th>Units per ml.</th>
<th>( \Delta D ) per unit per ml. ( \times 100 )</th>
<th>Correction, per cent of ( \Delta D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.92</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.99</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2.07</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2.18</td>
<td>-9.0</td>
</tr>
<tr>
<td>10</td>
<td>2.25</td>
<td>-11.0</td>
</tr>
<tr>
<td>5</td>
<td>2.54</td>
<td>-21.2</td>
</tr>
<tr>
<td>2.5</td>
<td>3.04</td>
<td>-34.0</td>
</tr>
<tr>
<td>1.25</td>
<td>4.24</td>
<td>-53</td>
</tr>
</tbody>
</table>

**Table II**

*Distribution Constant of Streptomycin in Butanol-6 Per Cent p-Toluenesulfonic Acid*

<table>
<thead>
<tr>
<th>Mg. per ml.</th>
<th>Distribution constant</th>
<th>Per cent recovery by benzene treatment</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>0.124</td>
<td>83.9</td>
</tr>
<tr>
<td>50</td>
<td>0.25</td>
<td>76.4</td>
</tr>
<tr>
<td>25</td>
<td>0.46</td>
<td>76.5</td>
</tr>
<tr>
<td>2.0</td>
<td>1.2</td>
<td>76.5</td>
</tr>
<tr>
<td>1.0</td>
<td>1.1</td>
<td>77.5</td>
</tr>
<tr>
<td>0.50</td>
<td>1.2</td>
<td>76.5</td>
</tr>
<tr>
<td>0.25</td>
<td>1.4</td>
<td>76.5</td>
</tr>
<tr>
<td>0.13</td>
<td>1.4</td>
<td>78.8</td>
</tr>
<tr>
<td>0.063</td>
<td>1.1</td>
<td>81.5</td>
</tr>
<tr>
<td>0.031</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>
In spite of the acidity of the systems, samples of streptomycin showed no loss in potency when allowed to stand for 45 hours in these solutions.

All distributions were made in a twenty-four plate Craig counter-current apparatus similar to a previously described model (8), and containing 8 ml. of each phase in each tube. Samples of 8 to 10 mg. were dissolved in 8 ml. of one phase and placed in the 0 tube of the machine. The phases were equilibrated by rocking the apparatus 2 minutes and were allowed to separate for 2 minutes before each movement of the upper half of the machine to a new position.

Although it would have been desirable to displace all of the streptomycin in each tube into the lower layer so that the maltol analysis of the aqueous phase would reveal the total material in each tube, this did not prove practicable. It was possible, however, by the addition to the upper layer of one-half its volume of benzene to displace 26 to 30 per cent of the streptomycin contained in the butanol into the aqueous phase. This was checked experimentally at the time the distribution constants were measured and Table II lists the per cent of streptomycin recovered when a solution in the lower phase was shaken with an equal volume of upper layer and half the volume of benzene.

The variations in K values listed for the lower streptomycin concentrations in Table II are attributable to the fact that the constant was computed from the formula \( K = \frac{(C_B - C_A)}{(C_A)} \) where \( C_B \) and \( C_A \) represent the concentrations of streptomycin in the lower phase before and after shaking with an equal volume of the upper. Errors in determining concentration thus enter the computation twice and are magnified in the value for \( K \). The partition coefficient calculated by the method of Williamson and Craig (12) from the distribution curves with the 5 per cent toluenesulfonic acid system averaged 0.97 in runs made over a period of some months, the most extreme variations being from 0.92 to 1.05.

Recoveries of streptomycin from the distribution apparatus were calculated by summation of the \( \Delta D \) values from each tube. These were usually between 75 and 80 per cent in agreement with Table II. In extreme cases in which most of the streptomycin was present as the modification with high partition coefficient (e.g. Curve C, Fig. 4) the recoveries were lower, 68 to 70 per cent, as would be expected from the fact that most of the material in the higher numbered tubes is contained in the upper phases, so that the benzene treatment displaces a smaller proportion of the total into the lower layers. The same reasoning accounts for the higher recoveries of 80 to 82 per cent at the other extreme (e.g. Curve D, Fig. 1), where most of the streptomycin is concentrated in the fractions of lower \( K \).

Because of the phenomena herein described the \( K \) values in the 3 per cent system could not be calculated as precisely from the curves, but the
best estimates were from 0.49 to 0.55 at room temperature (21 ± 2°), and from 0.29 to 0.39 at 5°.

**Chromatography of Crude Material**—Through a column 3.2 cm. in diameter containing 460 gm. of alumina was passed a solution of 10 gm. of streptomycin (450 units per mg.) in 100 ml. of methanol. 80 ml. fractions were collected and the solvent removed in vacuo. The residues were taken up in 10 ml. of water, filtered, and lyophilized. Table III summarizes a typical experiment. In order to indicate the changing proportions of the forms with low and high partition coefficients, the final column of Table III lists the ratio of material appearing in Tubes 0 to 17 to that found in Tubes 18 to 24 when these chromatographic fractions were distributed in butanol and 5 per cent toluenesulfonic acid.

**Table III**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight</th>
<th>Biopotency</th>
<th>Ratio of material in Tubes 1-17/Tubes 18-24</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.2160</td>
<td>495</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.3186</td>
<td>593</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.3862</td>
<td>612</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.3559</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.2628</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.2758</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.4701</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>0.3616</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.3882</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>0.2917</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>0.2049</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>0.2219</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Attempted Isolation of Material of High K**—A 40 mg. sample of an early chromatographic fraction was distributed as described in a 5 per cent toluenesulfonic acid system. The lower layers, after the conversion to maltol, were diluted 1:5 with 0.1 N NaOH to obtain the ΔD values plotted in Curve A, Fig. 2.

In order to duplicate this run with 40-fold quantities of streptomycin and solvents, the distribution was made in a series of 1 liter separatory funnels, as described elsewhere (9). Since after each equilibration in separatory funnels the lower layer is transferred to the next funnel, those substances of lowest partition coefficient are carried farthest along the line of funnels. This is the reverse of the situation in the Craig apparatus, where the upper layer moves. The two techniques give identical curves if the order of num-
bering the tubes is reversed. For this reason, the funnel to which the sample was added and in which the first equilibration occurred was Tube 24 and succeeding funnels were numbered in descending order. Since only the fractions of the higher distribution constant were desired, it was necessary to set up but three funnels, Tubes 24 to 22. Each contained 320 ml. of the upper layer. The sample was added to Tube 24 and equilibrated, and the lower layer drawn into Tube 23. The fresh lower layer was then added to Tube 24 and both vessels were shaken. The lower phase of Tube 23 was then drawn into Tube 22, that from Tube 24 into Tube 23, etc., the process being repeated until Tubes 24, 23, and 22 had had 24, 23, and 22 equilibrations respectively. The lower phase from Tube 22 was drawn off and discarded at each step. The contents of Tube 22 were shaken with benzene as usual, and the lower layers withdrawn, titrated to pH 6 to 7 with Ba(OH)$_2$, and freeze-dried. The residues were broken up in 15 ml. of dry methanol, shaken vigorously, and centrifuged. The supernatant was removed and the residue again extracted with 10 ml. of methanol.

The combined methanol solutions were evaporated to dryness, and the residue extracted as before with 1.0 and 0.5 ml. portions of methanol. Evaporation of the solvent left a residue weighing 36.6 mg. Over-all recovery of the streptomycin originally in the lower layer was 66 per cent, based on analyses of each fraction by conversion to maltol. 20.9 mg. of the material were distributed with 5 per cent toluenesulfonic acid (Curve B, Fig. 2).

The authors wish to acknowledge the assistance of Mr. Edward Paredes in the chromatographic separations and of Miss Antonine Hoffmann, Mr. Stanley Ulick, and Mr. Robert Matusow in the counter-current distributions.

**SUMMARY**

Evidence obtained by counter-current distribution indicates that streptomycin exists as a mixture of tautomeric modifications, the relative proportions of which depend upon pH. The tautomers are at least partially separable by chromatography.

**BIBLIOGRAPHY**

COUNTER-CURRENT DISTRIBUTION STUDIES ON STREPTOMYCIN: THE TAUTOMERISM OF STREPTOMYCIN
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