A COLORIMETRIC METHOD FOR THE ASSAY OF STREPTOMYCIN

A Thesis

Presented to

the Faculty of the Department of Biology

UNIVERSITY OF HOUSTON

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Jean J. Szafir

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Chemical methods for the assay of streptomycin and dihydrostreptomycin were investigated and some that have survived trial were described. Due to their inadequacies it seemed feasible to investigate the possibilities of the Voges-Proskauer method for the assay of these antibiotics. Some modifications of the Voges-Proskauer method are rapid and simple to perform and should any of these modifications prove adaptable for the assay of streptomycin and dihydrostreptomycin, a cheaper, simpler, and faster method of assay than any now known would be available. The Voges-Proskauer method of producing a color complex with free guanido groups seemed very promising because free guanido groups are present in streptomycin and dihydrostreptomycin.

The modification of the Voges-Proskauer method developed in the course of these experiments did produce color with streptomycin and dihydrostreptomycin. This color was proportional to concentration, fulfilling Beer's Law. Therefore, a quantitative test has been developed that can be read in ten minutes after adding the reagents. The sensitivity of this modification is limited to 25 gamma/ml. of streptomycin and dihydrostreptomycin in a total volume of 5 ml. The usefulness of this method is that it adapts itself to the assay of the streptomycin calcium chloride complex and to the assay of dihydrostreptomycin sulphate with equal facility by a rapid and simple procedure.

The function of potassium hydroxide in this reaction is usually discussed in the literature according to its merits as an oxidizing

agent. In the light of this investigation it seems that a further role of this alkali should be investigated. Since no color production is apparent in this reaction without the addition of alkali, it should be worth investigating to determine whether or not potassium hydroxide is a catalyst to the formation of color in this reaction.

The procedure according to the Barritt modification of the Voges-Proskauer method was followed in the final tests of this work, and a modification of the Barritt method was developed for the assay of streptomycin and dihydrostreptomycin.

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CHAPTER I

INTRODUCTION

At the present time there are several chemical methods for assaying streptomycin and dihydrostreptomycin, each possessing certain limitations which make them unadaptable for general use. The principal chemical methods that have survived trial according to Waksman[1] are the tests of Sullivan and Hilmer[2], Boxer, Jelinek and Leghorn[3], and Marshall[4].

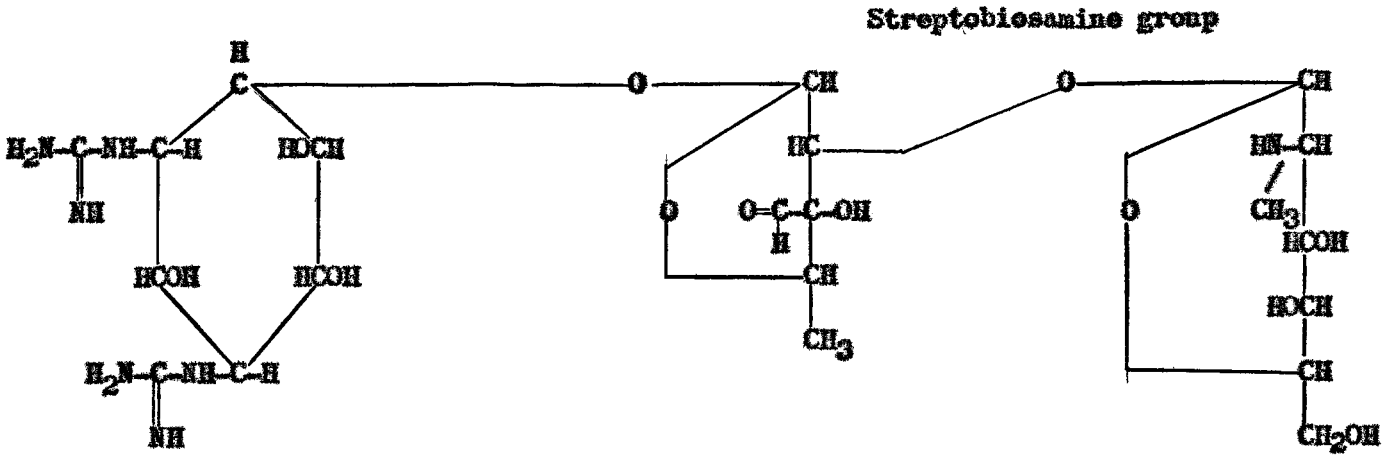
The test of Sullivan and Hilmer is one that involves the portion of the streptomycin molecule containing guanido groups (Fig. 1, page 3). The values obtained are too high due to certain breakdown products, and sensitivity is only as low as 100 mg./ml. The maltol method of Boxer, et al, and Marshall's semicarbizide method are excellent chemical methods, but these methods only react with the streptobiosamine portion of the streptomycin molecule (Fig. 1, page 3). Neither is adaptable for the assay of dihydrostreptomycin (Fig. 1, page 3). In the course of this investigation, a method employing the Voges-Proskauer reaction for the assay of streptomycin was published. However, the production of color was very slow. Due to their inadequacies, none of the aforementioned methods is used to any great extent in the chemical assay of streptomycin.

It has been shown by many workers that the Voges-Proskauer reaction produces a color complex when free guanido groups are present in a compound. The generally accepted theory is that acetyl methyl carbinol is oxidized to diacetyl in the presence of alkali and in some unknown

manner produces a red color when free guanido groups are present. This color complex has not been purified nor has its structure been determined. The Harden and Norris[6] modification of the Voges-Proskauer test produces color reactions only with substances containing guanido groups however, since all guanidine-containing substances do not give the reaction, it was the conclusion of those authors that the color was somewhat dependent on the remaining structure of the substance to which the guanidine group was attached as to whether or not a reaction would take place.

Since streptomycin and dihydrostreptomycin contain free guanido groups, it is the purpose of this thesis to investigate whether or not the Voges-Proskauer reaction could be adapted to their quantitative assay in small concentrations. If the test is adaptable, the method would be cheaper and faster than any now known assay method and might be used to determine therapeutic levels of streptomycin and dihydrostreptomycin in the blood of patients.

STREPTOMYCIN



Streptidine
(1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane)

Streptose

2-methyl amino glucose

DIHYDROSTREPTOMYCIN

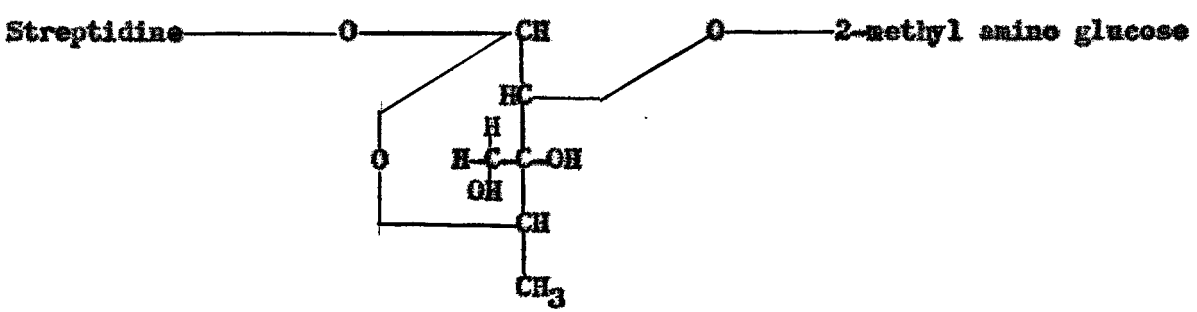


Figure I
Structures of Streptomycin and Dihydrostreptomycin

CHAPTER II

METHODS AND MATERIALS

Many modifications of the Voges-Proskauer test were investigated to determine their desirability for use with streptomycin. The only methods that seem adaptable are the following qualitative methods, which are commonly employed for the detection of acetyl methyl carbinol in bacterial cultures:

Hardin and Norris[6] (by producing color with substances which contained guanido groups, as mentioned in the Introduction)

Barritt[7] (by use of alpha naphthol which increases the sensitivity of the test for more compounds containing guanido groups and develops a greater intensity of color than in the Hardin and Norris modification)

O'Meara[8] (by employing the use of sodium hydroxide and creatine)

Coblentz[9] (by employing alkaline creatine as a reagent composed of .3% creatine in 40% potassium hydroxide)

Liefson[10] (by using a cupric sulphate reagent which is a compound not commonly used in the other modifications)

In the tests that were subsequently attempted, these procedures were followed. Modifications of these procedures were made in an effort to produce a quantitative test.

The color readings were made with a Klett-Summerson photoelectric colorimeter. Various filters were used; these were the #42 blue filter with a spectral range of 400-465 millimicrons, the #54 green filter with a range of 500-570 millimicrons, and the #66 red filter

with a range of 640-700 millimicrons. The chemicals used were sodium hydroxide, cupric sulphate, potassium hydroxide, creatine, alpha naphthol, acetyl methyl carbinol, and diacetyl. The last two compounds were obtained from the Matheson Company and the Eastman Distillation Products Industries, respectively. The streptomycin calcium chloride complex and streptomycin hydrochloride were obtained from Merck and Company, and dihydrostreptomycin sulphate from the Eli Lily Company.

CHAPTER III

EXPERIMENTAL PROCEDURES

Preliminary tests employing Liefson's technique were carried out. The reagent was prepared by dissolving 1 gram of cupric sulphate in 40 ml. of ammonia water and adding 10% sodium hydroxide in enough water to make 1000 ml. of solution. To 2.5 ml. of streptomycin hydrochloride containing 50,000 gamma/ml., was added 0.5 ml. of acetyl methyl carbinol and an equal amount of Liefson's reagent. Blanks were run for all tests. Several other tests employing varying dilutions of streptomycin and different concentrations of acetyl methyl carbinol were attempted with this technique. This is shown in Table I, page 10.

In the next group of tests, the Coblents method was employed, using varying amounts of acetyl methyl carbinol in order to determine the optimum proportion necessary for the reaction. Streptomycin hydrochloride in 100 gamma/ml. and 50 gamma/ml. concentrations was used. This is shown in Tables II and III, pages 11 and 12. The order of adding the reagents was also varied, since negative results are obtained if these reagents are not introduced in the proper order.

Individual tests were made in order to determine the optimum proportions of alpha naphthol and alkaline creatine. These reagents were also introduced in varying concentrations and amounts. This is shown in Tables IV and V, pages 13 and 14. Separate tests were made to determine at what time after the reaction it was most desirable to read color and how long shaking was necessary for good color

development (Tables VI and VII, pages 15 and 16). No matter in what order the reagents were added, enough distilled water was included to make the total volume 5 ml. After the tubes were shaken they were read in the colorimeter.

Subsequent tests employed the use of diacetyl and the streptomycin calcium chloride complex. These tests were also performed by varying the concentrations and amounts of all compounds used and the order of adding them to the tubes was also varied. Preliminary tests were conducted according to the Harden and Norris[6] procedure and in the remaining tests the procedure according to Barritt[7], using alpha naphthol, was followed. It was necessary for separate groups of tests to be run for different concentrations of streptomycin in order to determine the optimum proportions of the reagents to be used to encompass the widest range of concentrations. Dihydrostreptomycin was tested by using the optimum proportions and concentrations found best for the streptomycin calcium chloride complex.

Once optimum conditions had been determined, tests were run with a wide range of concentrations of streptomycin and the resultant readings plotted against concentration to determine the nature of the curve. Most of this last group was run with concentrations of 250 gamma, 125 gamma, 50 gamma, and 25 gamma per ml. of streptomycin. In determining unknowns, readings were obtained in the colorimeter and plotted on a standard curve.

The best test that was developed above was used with streptomycin diluted in a protein-free filtrate which had been obtained by the method

of Somogyi. Further tests using trichloracetic acid for obtaining a protein free filtrate were attempted.

CHAPTER IV

RESULTS

Tests with Liefson Method[10]. An eosin color developed within thirty minutes by this method. An example of the results using this technique is recorded in Table I. All attempts to produce color with concentrations of streptomycin below 2500 gamma/ml. produced negative results. This test proved to be sensitive in dilutions of 2500 gamma/ml. of streptomycin and no better sensitivity could be obtained using the O'Meara method[7].

Tests with Coblentz Method[9]. The best results were obtained by the following procedure as shown in Table III. The amounts and concentrations of the reagents, in the order of their addition, were placed in the Klett-Summerson tubes thus:

1.	Streptomycin (50 gamma/ml.)	0.5 ml.
2.	Acetyl methyl carbinol	0.05 ml.
3.	Alpha naphthol (5% in 95% ethanol)	0.6 ml.
4.	Alkaline creatine (40% KOH containing 0.3 gram of creatine)	0.2 ml.

After all the reagents had been added, the tubes were shaken for ten minutes, water added to 5 ml., and read in the colorimeter. Results of these experiments are recorded in Tables II and III. Agreement was found with Barritt[6] that the color was not intensified if alpha naphthol was added after the alkaline creatine. The results of the tests determining the optimum proportions of alpha naphthol and alkaline creatine may be seen in Tables IV and V. As shown in Tables VI and VII, it was

TABLE I

COLOR PRODUCED WITH LIEFSON REAGENT

	TEST NO.	1	2	3	4	5	6*	7*	8*	9*	10*
1.	Concentration of streptomycin (in gamma/ml.)	20,000	10,000	5,000	2,500	2,000	200 100 50	200 100 50	200 100 50	200 100 50	200 100 50
2.	Streptomycin (in ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
3.	Liefson reagent (in ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
4.	Acetyl methyl carbinol (in ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.3	0.2	0.1
5.	Eosin color produced	+	+	+	+ weak	-	-	-	-	-	-

*Tests with different concentrations of streptomycin
Results were the same for the fifteen determinations

TABLE II

TESTS WITH VARYING AMOUNTS OF ACETYL METHYL CARBINOL

	TEST NO.	1	2	3	4	5	6
1.	Acetyl methyl carbinol (in ml.)	0.3	0.2	0.1	0.075	0.05	0.025
2.	Alpha naphthol (5% in 95% ethanol) (in ml.)	0.6	0.6	0.6	0.6	0.6	0.6
3.	Alkaline creatine (3% creatine in 40% KOH) (in ml.)	0.2	0.2	0.2	0.2	0.2	0.2
4.	Streptomycin, 100 gamma/ml. (in ml.)	1.0	1.0	1.0	1.0	1.0	1.0
5.	Shake						
6.	Water added to total volume of 5 ml.						
	Colorimeter readings (Each blank individually zeroed)	35	35	42	57	62	40
	Colorimeter readings (Zeroed with Test No. 1 blank)	0	0	85	106	135	141*

*Most intense test. The blank was also pink.

TABLE III

TESTS WITH VARYING AMOUNTS AND CONCENTRATIONS OF ACETYL METHYL CARBINOL

	TEST NO.	1	2	3	4	5
1.	Streptomycin, 100 gamma/ml. (in ml.)	0.5	0.5	0.5	0.5	0.5
2.	Acetyl methyl carbinol (in ml. and concentrations)	0.05	0.025	0.1/1-100	0.05/1-100	0.1/1-1000
3.	Alpha naphthol (5% in 95% ethanol) (in ml.)	0.6	0.6	0.6	0.6	0.6
4.	Alkaline creatine (.3% in 40% KOH) (in ml.)	0.2	0.2	0.2	0.2	0.2
5.	Shake					
6.	Water added to total volume of 5 ml.					
	Colorimeter readings (colorimeter zeroed with water)					
	Blanks	122 (yellow)	171 (pink)	purple	purple	purple
	Tests	202 (pink)	283 (pink)	purple	purple	purple

TABLE IV

TESTS WITH VARYING AMOUNTS OF ALPHA NAPHTHOL AND ALKALINE CREATINE

	TEST NO.	1	2	3	4	5	6	7
1.	Streptomycin, 100 gamma/ml. (in ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2.	Acetyl methyl carbinol (in ml.)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
3.	Alpha naphthol (5% in 95% ethanol) (in ml.)	0.5	0.4	0.3	0.2	0.1	0.6	0.6
4.	Alkaline creatine (.3% creatine in 40% KOH) (in ml.)	0.2	0.2	0.2	0.2	0.2	0.15	0.1
5.	Shake							
6.	Water added to total volume of 5 ml.							
	Colorimeter readings (with the blank readings subtracted)	128	58	37	too low to read	too low to read	135*	118

*Most intense test

TABLE V

TESTS WITH VARYING AMOUNTS OF ALPHA NAPHTHOL AND ALKALINE CREATINE

TEST NO.	1	2	3	4	5	6	7	8	9	10	11
1. Streptomycin, 100 gamma/ml. (in ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2. Acetyl methyl carbinol (in ml.)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
3. Alpha naphthol (5% in 95% ethanol) (in ml.)	0.5	0.5	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1	0.6
4. Alkaline creatine (.3% in 40% KOH) (in ml.)	0.15	0.1	0.15	0.1	0.15	0.1	0.15	0.1	0.15	0.1	0.2
5. Shake											
6. Water added to total volume of 5 ml.											
Colorimeter readings (colorimeter zeroed with water)											
Blanks	90	85	87	80	50	45	51	14	25	0	92
Tests	214	170	196	113	147	134	68	43	13	0	250*

*Most intense test

TABLE VI

TRIALS TO DETERMINE TIME FOR GOOD COLOR TO BE PRODUCED

TEST USED

1. Streptomycin, 100 gamma/ml. 0.5 ml.
2. Acetyl methyl carbinol 0.05 ml.
3. Alpha naphthol (5% in 95% ethanol) 0.6 ml.
4. Alkaline creatine (.3% in 40% KOH) 0.2 ml.
5. Shake
6. Water added to total volume of 5 ml.

RESULTS

Time (in minutes)	3*	5*	10**	10*
Colorimeter readings (no blanks run)	130	140	160	170

*Continuous shaking
**Intermittent shaking

TABLE VII

TRIALS TO DETERMINE LENGTH OF TIME BEFORE COLOR FADES

TEST USED

1. Streptomycin, 100 gamma/ml. 0.5 ml.
2. Acetyl methyl carbinol 0.05 ml.
3. Alpha naphthol (5% in 95% ethanol) 0.6 ml.
4. Alkaline creatine (.3% in 40% KOH) 0.2 ml.
5. Shake
6. Water added to total volume of 5 ml.

RESULTS

Time (in minutes) (with continuous shaking)	10	30	45
Colorimeter readings (colorimeter zeroed with water)			
Blanks	135	140	129
Tests	172	182	160

determined by the time trials that a ten minute shaking period was necessary for good color development and fading was not observed until thirty minutes had elapsed.

The blank containing 0.05 ml. of acetyl methyl carbinol usually remained yellow; however, occasionally a pink tint was observed. The only chemical not present in the blank was the aqueous solution of streptomycin. Tests were run with the blanks only, adding an amount of water to the blank which was equivalent to the amount of streptomycin solution added to the test (Table VIII). With the water added, all of the blanks became a pink color. The creatine was the cause of the color, as determined by later tests without creatine. This color had not been observed until water was introduced into the blank therefore, in subsequent tests potassium hydroxide without creatine was employed for all tests and blanks.

It was observed that acetyl methyl carbinol, which was kept in the cold as well as at room temperature, changed into crystalline polymer form. According to Pound and Wilson[11] crystals are deposited in two to nine days at ordinary temperatures but, upon heating, assume the liquid state again. They determined that a temperature of at least 30 degrees C. should be maintained to keep the crystalline polymer from forming. Because of the fact that stable reagents are more desirable, diacetyl was used in later tests.

Tests with Harden and Norris[6], and Barritt Methods[7]. In the tests with the Harden and Norris method a 0.05 ml. amount of a 1-100 dilution of diacetyl was found to give a light pink color with 5000 gamma/ml.

TABLE VIII

TESTS TO DETERMINE SOURCE OF INTERFERING COLOR USING BLANKS ONLY

TEST NO.	1	2	3	4	5
1. Water (in ml.)	0.5	0.5	0.5	0.5	0
2. Acetyl methyl carbinol (in ml.)	0.05	0.05	0.05	0.05	0.05
3. Alpha naphthol (5% in 95% ethanol) (in ml.)	0.6	0.4	0.3	0.2	0.6
4. Alkaline creatine (.3% in 40% KOH) (in ml.)	0.2	0.2	0.2	0.2	0.2
5. Shake					
6. Water added to total volume of 5 ml.					
Colorimeter readings (colorimeter zeroed with water)	210*	165*	150*	115*	193**

*Pink
**Yellow

of streptomycin, which faded rapidly. Adding streptomycin first or last to diacetyl, water, alpha naphthol, and potassium hydroxide in that order gave negative results, but the addition of streptomycin first and diacetyl last gave positive results. Alpha naphthol, as in the Barritt method, was used in the next group of tests in 0.5 ml. amounts with 0.5 ml. of potassium hydroxide, and 5 ml. of a 1-100 dilution of diacetyl. Streptomycin in a concentration of 5000 gamma/ml. was added first in 1 ml. amounts. These tests produced a light pink color which faded rapidly. Therefore, the amount of potassium hydroxide was reduced to .25 ml. and further tests run. Greater amounts of alpha naphthol had no appreciable effect on the intensity of color.

Best results were obtained by the following procedure, as shown in Table IX. The amounts and concentrations of the reagents, in the order of their addition, were placed in the Klett-Summerson tubes thus:

1.	Streptomycin (5000 gamma/ml.)	1.0 ml.
2.	Alpha naphthol (5% in 95% ethanol)	0.5 ml.
3.	Potassium hydroxide (40%)	0.25 ml.
4.	Distilled water	enough to bring total volume to 5.0 ml.
5. a.	Diacetyl (1-100)	0.2 ml.
	or	
b.	Diacetyl (1-1000)	0.5 ml.

The tubes were shaken for ten minutes, then read in the colorimeter.

It was determined in the best tests for concentrations of streptomycin of 2000 gamma/ml., 100 gamma/ml., 500 gamma/ml., and 250 gamma/ml. that all amounts and concentrations of reagents remained

TABLE IX

TESTS WITH 5000 GAMMA/ML. OF STREPTOMYCIN WITH VARYING CONCENTRATIONS AND AMOUNTS OF DIACETYL

	TEST NO.	1	2	3	4	5
A.	1. Streptomycin, 5000 gamma/ml. (in ml.)	1.0	1.0	1.0	1.0	1.0
	2. Alpha naphthol (5% in 95% ethanol) (in ml.)	0.5	0.5	0.5	0.5	0.5
	3. Potassium hydroxide (40%) (in ml.)	0.25	0.25	0.25	0.25	0.25
	4. Water added so total volume will be 5.0 ml.					
	5. Diacetyl, 1-100 (in ml.)	0.05	0.1	0.2	0.3	0.4
	6. Shake					
	Color produced	yellow	pink	red*	pink	copper
B.	Same test as above, using diacetyl, 1-1000 (in ml.)	0.5	1.0			
	Color produced	red*	red			
C.	Same test as above, using diacetyl, 1-2000 (in ml.)	0.5	1.0			
	Color produced	red*	red			

*Most intense tests

the same, with one exception, and that was in the tests for 5000 gamma/ml. For 250 gamma/ml. the stronger 1-100 dilution of diacetyl gave best results using .025 ml. This was so critical that the remaining tests were run with weaker dilutions of diacetyl.

The tests with streptomycin in a concentration of 50 gamma/ml. were also run to determine the optimum proportions of reagents for this concentration. Reagents were added in all orders possible. Best results were obtained by the following procedure (Table X). The amounts and concentrations of the reagents, in the order of their addition, were placed in the Klett-Summerson tubes thus:

1.	Streptomycin (50 gamma/ml.)	1.0 ml.
2.	Alpha naphthol (5% in 95% ethanol)	0.5 ml.
3.	Potassium hydroxide (40%)	0.1 ml.
4.	Distilled water	2.9 ml.
5.	Diacetyl (1-1000)	0.5 ml.

A concentration of diacetyl 1-2000 also gave positive results, but not quite so great an intensity of color was developed as with the 1-1000 dilution. Even weaker concentrations of diacetyl produced a light pink color but faded rapidly.

Tests with a 25 gamma/ml. concentration of streptomycin produced a light pink color with the amounts and concentrations found best for 50 gamma/ml. These amounts and concentrations of reagents could also be used with concentrations of streptomycin up to 5000 gamma/ml. Smaller concentrations of streptomycin gave negative results.

Dihydrostreptomycin, which was tested by using the optimum

TABLE X

TESTS WITH 50 GAMMA/ML. OF STREPTOMYCIN, WITH VARYING CONCENTRATIONS AND AMOUNTS OF DIACETYL AND VARYING AMOUNTS OF POTASSIUM HYDROXIDE

	TEST NO.	1	2	3	4	5	6	7	8	9	10
A.	1. Streptomycin, 50 gamma/ml. (in ml.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	2. Alpha naphthol (5% in 95% ethanol) (in ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	3. Potassium hydroxide (40%) (in ml.)	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.4	3.0	0.05* 0.15*
	4. Water added so total volume will be 5.0 ml.										
	5. Diacetyl, 1-1000 (in ml.)	2.0	1.0	0.5	0.5	0.025	0.1	0.5* 0.25* 0.1* 0.05*	0.0	0.5	0.5
	6. Shake										
	Color produced	copper	copper	pink	copper	yellow	yellow	yellow	yellow	yellow	copper
B.	Same test as above, using diacetyl, 1-2000 (in ml.)	1.0**	0.5**	0.1	0.025	1.5	2.0				
	Color produced	Pink	pink	yellow	yellow	yellow	yellow				

C. Same test as above with diacetyl (in 0.5 ml. amounts), 1-3000, 1-4000 and 1-8000, produced a faint pink color momentarily.

*Results were the same for these different amounts

**Color fades rapidly

proportions and concentrations found best for the streptomycin calcium chloride complex, produced similar results.

The spectral transmission was not in the range of the blue filter in any reaction, but the red filter was useful in the reactions with streptomycin in concentration ranges of from one to two thousand gamma per ml. Dilutions of the reaction to 20 ml. had to be made in order to read in the colorimeter with the green filter in these stronger concentrations. The green filter was used in concentrations below 500 gamma/ml.

Tests to Determine the Nature of the Curve Produced. The results of these tests showed that in all concentrations of streptomycin and dihydrostreptomycin a straight line could be drawn, and therefore the reactions fulfilled Beer's Law. (Figs. 2 and 3)

Tests with Protein Free Filtrates. It was observed that the test using a protein free filtrate obtained by the Somogyi method[12] produced good color and fulfilled Beer's Law. By this method the test was sensitive only to 200 gamma/ml. of streptomycin. The filtrate by the trichloracetic acid method was adjusted to a pH of about 7 and tested. All blanks and tests were of about the same intensity and no differentiation could be made.

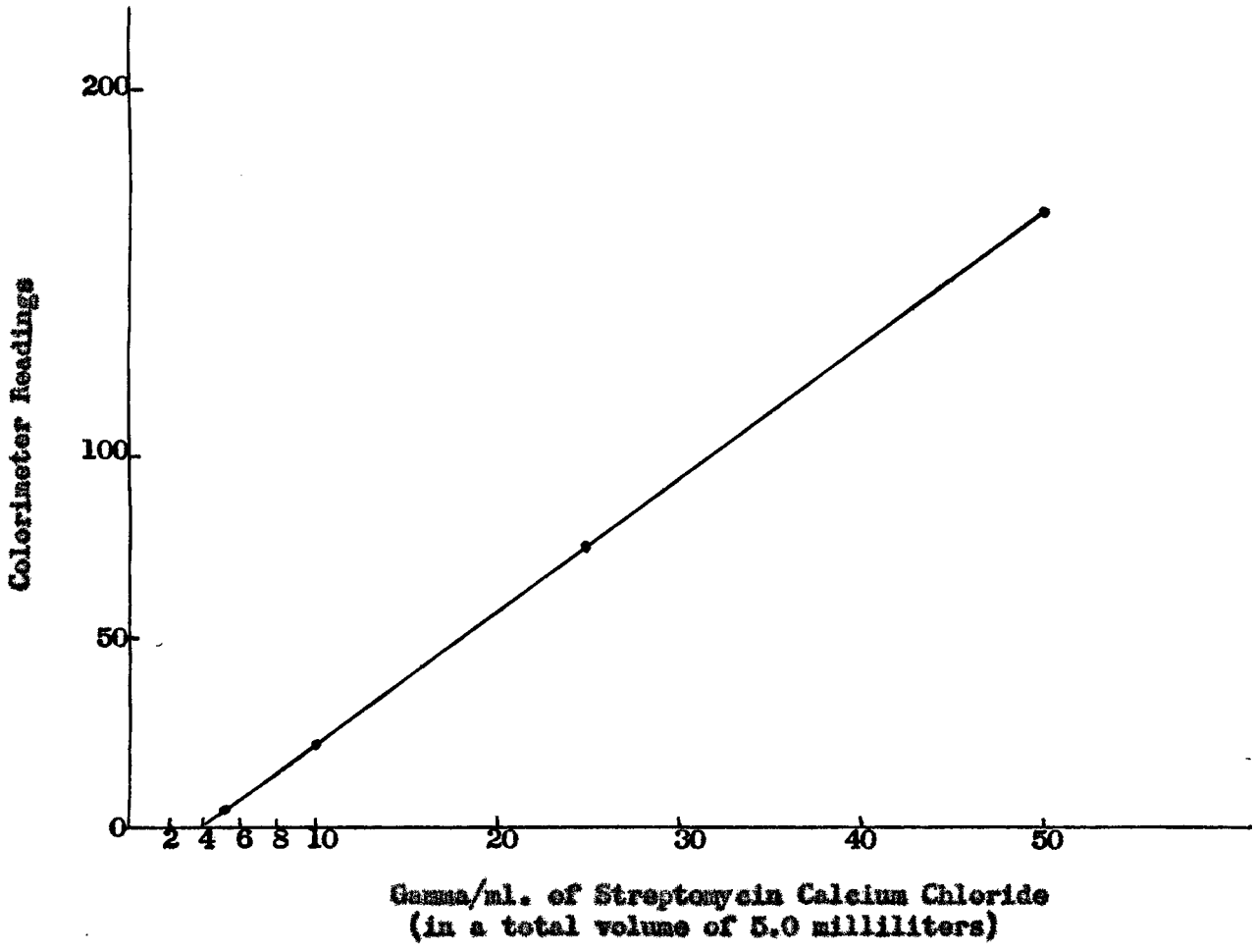


Figure 2

Curve Obtained with 25 gamma/ml., 50 gamma/ml., 125 gamma/ml. and 250 gamma/ml. of Streptomycin

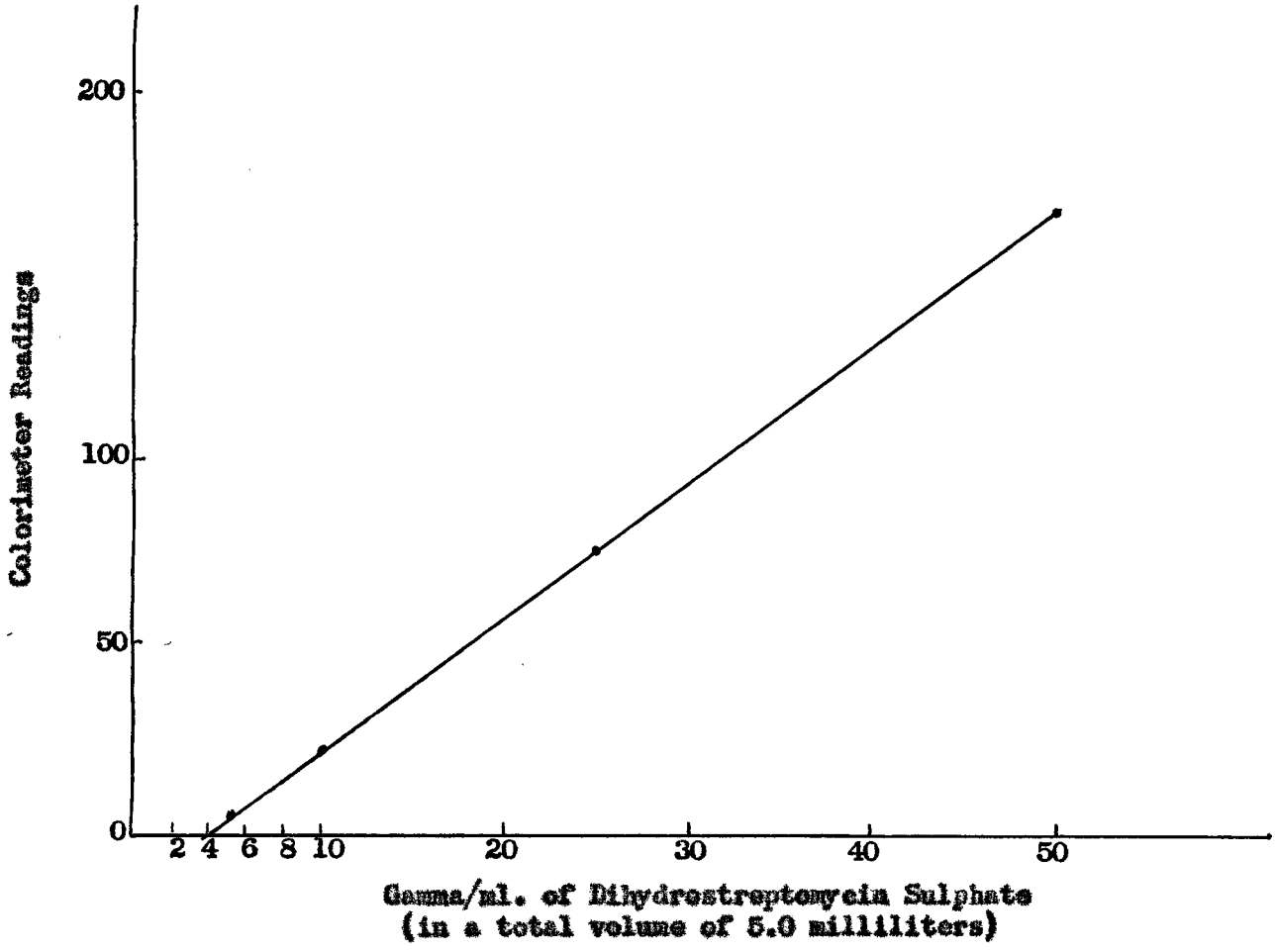


Figure 3

Curve Obtained with 25 gamma/ml., 50 gamma/ml.,
125 gamma/ml. and 250 gamma/ml. of Dihydrostreptomycin

CHAPTER V

DISCUSSION

In the determinations with the Liefson technique, twenty tests were run. The apparent lack of sensitivity for concentration of streptomycin of less than 200 gamma/ml. was the reason for discarding this method.

In the eighty determinations with acetyl methyl carbinol and the other reagents of the Coblentz method, two important facts were observed that demand particular attention:

1. Creatine was an interfering substance and detrimental to the effectiveness of this method.
2. Acetyl methyl carbinol was not stable under normal conditions and a stable reagent is certainly more desirable.

The many tests with diacetyl and reagents of the Barritt method showed that 40% potassium hydroxide was the best concentration for this compound. Variations in this concentration produced results of an inferior nature. Furthermore, a deviation of .05 ml. from the optimum amount of .1 ml. found best for use with the smaller concentrations of streptomycin would not produce positive results.

The completed and final test is conducted with 1 ml. of streptomycin or dihydrostreptomycin, .5 ml. alcoholic alpha naphthol, .1 ml. of potassium hydroxide and .5 ml. of 1-1000 diacetyl in a total volume of 5 ml., then shaken for ten minutes and read in the colorimeter. It was determined that fifteen minutes is the maximum time to be allowed

for reading the reaction after shaking. This final test is effective over the complete range of concentrations tested. However, the concentrations from 1000 gamma/ml. to 5000 gamma/ml. required dilution to a total volume of 20 ml. with water in order to be read with the green filter. All lower concentrations were within the range of the green filter.

It was also determined that the 1-1000 concentration of diacetyl was the most desirable for use. The 1-100 concentration produced best results with the small .025 ml. amount when testing 250 gamma/ml. of streptomycin, and it would become necessary to measure even smaller quantities for lesser concentrations of streptomycin, which is an unnecessary and undesirable feature with this substance since it is so viscous in the concentrated form. Diacetyl was found to be stable for months at room temperature and in a refrigerator. It could also be prepared in a concentration of 1-100, stored in the refrigerator, and still remain effective.

Eggleton, Elsden and Gough[13] found that amino acids interfere with color production in the Barritt production. Amino acids were certainly some of the interfering substances in the tests with the trichloracetic acid blood filtrates. However, no further tests were made with blood filtrates, or original samples of blood, because the sensitivity of this assay method was not of an order found in therapeutic levels of streptomycin.

This method for the assay of streptomycin produced a color proportional to concentration fulfilling Beer's Law. This was determined

with fifty tests after optimum conditions had been developed. Reproducible results were obtained when fresh alpha naphthol was used. It was found that pigmentation will form in this compound after several days and seriously interefere with readings in the colorimeter. Therefore, a fresh preparation of alpha naphthol should be made every two or three days.

Dihydrostreptomycin may be assayed as well as the streptomycin calcium chloride form of the antibiotic.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Chemical methods for the assay of streptomycin and dihydrostreptomycin were investigated and some that have survived trial were described. Due to their inadequacies it seemed feasible to investigate the possibilities of the Voges-Proskauer method for the assay of these antibiotics. Some modifications of the Voges-Proskauer method are rapid and simple to perform and should any of these modifications prove adaptable for the assay of streptomycin and dihydrostreptomycin, a cheaper, simpler, and faster method of assay than any now known would be available. The Voges-Proskauer method of producing a color complex with free guanido groups seemed very promising because free guanido groups are present in streptomycin and dihydrostreptomycin.

The modification of the Voges-Proskauer method developed in the course of these experiments did produce color with streptomycin and dihydrostreptomycin. This color was proportional to concentration, fulfilling Beer's Law. Therefore, a quantitative test has been developed that can be read in ten minutes after adding the reagents. The sensitivity of this modification is limited to 25 gamma/ml. of streptomycin and dihydrostreptomycin in a total volume of 5 ml. The usefulness of this method is that it adapts itself to the assay of the streptomycin calcium chloride complex and to the assay of dihydrostreptomycin sulphate with equal facility by a rapid and simple procedure.

The function of potassium hydroxide in this reaction is usually discussed in the literature according to its merits as an oxidizing agent. In the light of this investigation it seems that a further role of this alkali should be investigated. Since no color production is apparent in this reaction without the addition of alkali, it should be worth investigating to determine whether or not potassium hydroxide is a catalyst to the formation of color in this reaction.

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