MARTIN, Lawrence Leo, 1942-
BIOSYNTHESIS OF THE ANTIBIOTIC SPECTINOMYCIN.
The Ohio State University, Ph.D., 1971
Chemistry, pharmaceutical

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BIOSYNTHESIS OF THE ANTIBIOTIC SPECTINOMYCIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Lawrence Leo Martin, B.S.

* * * * *

The Ohio State University
1971

Approved by

[signature]
Adviser
College of Pharmacy
PLEASE NOTE:
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ACKNOWLEDGMENTS

The author wishes to express his gratitude to the many people who assisted him during the course of his studies. Dr. D. Feller, Dr. J. LaPidus, Dr. J. R. Martin, and Dr. L. Mitscher deserve thanks for their stimulating discussions, advice, and assistance during the course of this work. It is clear that the author has benefited, consciously and unconsciously, from the publications of many authors. It is hoped that adequate acknowledgment has been given in the references. The author also wishes to express his gratitude to the American Foundation for Pharmaceutical Education and to the Ohio State University for financial support during the course of his studies.
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FIELDS OF STUDY

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PUBLICATIONS

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INTRODUCTION

Literature Survey

Spectinomycin (1) is a broad spectrum antibiotic produced by various species of the genus Streptomyces. Mason et al.\(^1\) reported the isolation of spectinomycin from Streptomyces spectabilis NRRL 2792. Oliver et al.\(^2\) subsequently described an antibiotic produced by Streptomyces flavopersicus NRRL B-2820 which they demonstrated to be identical with spectinomycin. The identity of these materials was independently confirmed by Sinclair and Winfield\(^3\).

\[
\begin{align*}
\text{CH}_3\text{NH} & \quad \text{OH} \\
\text{HO} & \quad \text{NH} \\
\text{NH} & \quad \text{CH}_3
\end{align*}
\]

Details of the isolation procedures were reported by Sinclair and Winfield\(^3\) and by Bergy et al.\(^4\). Typically, the antibiotic was removed from the clarified fermentation liquor by cation exchange chromatography and eluted from the resin with dilute mineral acid. The eluents were neutralized to pH 6.5 and lyophilized. The crude antibiotic was chromatographed on a carbon column and eluted with 1% aqueous acetone. The eluents were concentrated and the antibiotic crystallized from
solution upon addition of acetone or ethanol. The amorphous free base was prepared when an aqueous solution of the hydrochloride or sulfate salt was passed through an ice-chilled column of Dowex 2-x8 (OH⁻) ion exchange resin and the effluent lyophilized. Jahnke developed a milder procedure for preparing and crystallizing the free base.

The structure of spectinomycin was elucidated by Wiley et al. Their studies demonstrated that spectinomycin is an aminocyclitol antibiotic composed of the aglycone actinamine (2) and a sugar moiety, actinospectose (3). The proposed structure (1) was partially confirmed by Chapman et al. The fused tricyclic ring system of this compound was novel and distinguished spectinomycin from other clinically available aminoglycoside antibiotics.

The chemistry of spectinomycin was largely explored in the degradative studies reported by Johnson and by Wiley et al. Since many of these reactions were suitable for work with isotopically labeled material, these reactions were employed for this study. In addition, the stereochemistry of actinamine (2) was determined by Slomp and MacKellar and by Colebrook and Courlay. From these studies it
was apparent that the A/B ring juncture of spectinomycin is trans; however, the stereochemistries of the B/C ring juncture and the C-methyl group were not assigned.

The nomenclature for spectinomycin varied over the years. Mason et al.\(^1\) originally called their material actinospectacin; Trobicin being Upjohn's trade name. Oliver et al.\(^2\) called their antibiotic "M-141" until its identity with actinospectacin was established. This name was subsequently changed to spectinomycin to conform with the nomenclature rules of the American Society for Microbiology. Chemical Abstracts currently lists the antibiotic as decahydro-4a,7,9-tri-hydroxy-2-methyl-6,8-bis(methylamino)-4H-pyranol2,3-b][1,4]benzodioxin-4-one and as spectinomycin. Although usage favors retention of the names actinamine, actinospectose, and actinospectinoic acid (5), consistency would support changing these names to spectinamine, spectose, and spectinoic acid, respectively. To avoid confusing the issue, the three traditional names will be retained in this dissertation.

Mason et al.\(^1\) discovered that spectinomycin was active \textit{in vitro} against a variety of gram-positive and gram-negative organisms, but failed to demonstrate significant antifungal or antiparasitic activity. Independent studies\(^2,5,14\) confirmed these observations. Martin et al.\(^15\) reported favorable activity \textit{in vitro} against \textit{Neisseria gonorrhoeae} and \textit{Neisseria meningitidis}, and emphasized the need for a new antibiotic for the treatment of gonorrhea. Lewis and Clapp\(^5\) found spectinomycin active against a variety of agents \textit{in vivo} (mice) and observed that the antibacterial response \textit{in vivo} was greater than what
might be expected from the in vitro data. They also observed that the antibiotic was more effective administered parenterally than orally. Subsequent reports\textsuperscript{2,16-18} confirmed these observations. Spectinomycin appears to be characterized by a poor correlation between in vitro tests and in vivo activity\textsuperscript{5,17}.

Hwang et al.\textsuperscript{16} performed absorption, distribution, excretion, and toxicity studies with spectinomycin in a variety of laboratory animals. Spectinomycin was not absorbed from the GI tract, which accounted for the diminished oral activity as previously mentioned. The dihydrochloride salt of spectinomycin was rapidly absorbed after intramuscular administration, with 80 to 90 percent of the dose recovered from the urine approximately four hours later. Intravenous administration yielded similar results. Plasma levels of the antibiotic were sustained several hours in bilaterally nephrectomized dogs; thus, renal clearance appears to be the major route of excretion. Toxicity studies suggested that a low acute toxicity for the dihydrochloride salt exists in a variety of animals. The results of subacute toxicity studies with rats and dogs showed no significant difference between treated and untreated animals. This observation of low toxicity was most significant in light of the characteristic nephro- and auricular toxicities caused by other effective aminoglycoside antibiotics.

Clinical trials with spectinomycin in 35 adults\textsuperscript{14} and 35 children\textsuperscript{19} displaying a variety of systemic infections were conducted. Response to spectinomycin was good in most cases and in a few cases of resistant infection spectinomycin was considered as life saving. For adults, intramuscular or intravenous administration of spectinomycin
(0.4-1.0 gm., base equivalent) at 4-6 hour intervals was recommended. Barry and Koch, however, concluded that spectinomycin did not appear to offer any advantage over other commercially available antibiotics and cautioned that spectinomycin might be nephrotoxic. Their observations on nephrotoxicity are inconclusive and have not been independently confirmed. Very favorable clinical trails employing spectinomycin for the treatment of gonorrhea were also reported. Indeed the need for new agents to treat gonorrhea was emphasized by Sparling et al. Spectinomycin appears suited for the treatment of gonorrhea because it is not cross-resistant nor cross-allergenic with other antibiotics and it is approximately 95 percent effective in a single intramuscular injection of the sulfate salt (2.0 gm., males; 3.0 gm., females). In April, 1971, the Upjohn Company announced its intention to market spectinomycin in the U.S. for the treatment of gonorrhea. The Food and Drug Administration approved the first commercial use of spectinomycin in humans in September, 1971; however, antibiotic therapy was limited to the treatment of resistant gonorrhoeal infections or for use in the treatment of gonorrhea with penicillin hypersensitive patients. Penicillin remained the drug of choice for the treatment of gonorrhea. Commercial interest for the use of spectinomycin as a veterinary feed supplement to enhance the rate and amount of weight gain in meat producing animals and to control certain diseases (Coccidiosis and Mycoplasmosis) of poultry was also announced.

The mechanism of action of the aminoglycoside antibiotics has not been completely elucidated. In general, these antibiotics appear to inhibit protein biosynthesis and cause misreading of the genetic code.
at the level of the 30S ribosomal subunit. For streptomycin these effects are presumably due to an antibiotic induced conformational change in the ribosome. This conformational change results in a decreased binding of the aminoacyl-tRNA molecules to the codons of the messenger RNA. The decreased binding, in turn, decreases the specificity of the messenger-RNA-aminoacyl-tRNA interaction causing misreading. Spectinomycin is an exception among these antibiotics and appears to inhibit protein synthesis without producing misreading. The antibiotic acts at the level of the 30S subunit and apparently interferes with the translocation processes (release of free tRNA, shift of peptidyl-tRNA, advancement of the messenger). Bollen et al. isolated a protein from the 30S subunit which they feel is responsible for spectinomycin sensitivity. Furthermore, they demonstrated that this protein is different from the protein responsible for streptomycin sensitivity. It is of interest to note that aminoglycoside antibiotics such as spectinomycin which do not produce misreading are not bactericidal.

Resistance development to spectinomycin has been reported. Lewis and Clapp observed that spectinomycin "exhibits a streptomycin-like pattern of resistance development when Staphylococcus aureus is used as the test organism". They noted, however, that other test organisms did not develop resistance as rapidly as Staphylococci. Oliver et al. noted rapid resistance development with Salmonella enteritidis, S. typhimurium, Pseudomonas aeruginosa BMH 10, Staphylococcus aureus, and S. epidermidis ATCC 3519. The streptomycin-like pattern of resistance development is characterized by
the development or selection of a highly resistant mutant organism on a single exposure to an antibiotic. For spectinomycin the genetic determinant for such resistance is known to be associated with a protein of the 30S ribosomal subunit\(^{32}\) and to map near the streptomycin locus\(^{33}\).

The fact that cross resistance of spectinomycin with other antibiotics had not been observed, led Griffith et al.\(^{17}\) to conclude that spectinomycin could be a valuable therapeutic adjunct in cases of resistant infection. However, R-factor mediated cross resistance of spectinomycin with streptomycin, presumably by enzymatic adenylation of the C-9 hydroxyl group of spectinomycin, was recently reported\(^{34,36}\).

Several investigations have been directed toward the elucidation of structure-activity relationships among the aminoglycoside antibiotics. Davies\(^{38}\) recently summarized the results of these investigations and concluded that the aminocyclitol moiety of the aminoglycoside antibiotics is responsible for both translation errors (misreading) and inhibition of protein synthesis. However, he noted that the aminocyclitol moiety does not have sufficient activity by itself to account for the biological activity of the intact molecule. The importance of the nature of the substituent (H, OH) and the stereochemistry (OH series) at C-2 of the aminocyclitol moiety (2) was emphasized. Thus, in a series of antibiotics, little difference in activity was observed with the streptamine (4, R = OH) and deoxy-streptamine (4, R = H) containing members. In each of these series of antibiotics, both misreading and inhibition of protein synthesis was observed. However, with antibiotics containing epi-streptamine
(2 = actinamine) only inhibition of protein synthesis was observed.

\[ \begin{align*}
\text{H}_2\text{N} & \quad \text{OH} \\
\text{R} & = \text{OH}, \quad \text{I} = \text{streptamine} \\
\text{R} & = \text{H}, \quad \text{I} = \text{deoxystreptamine}
\end{align*} \]

These observations apparently extended to spectinomycin since it contained the actinamine moiety and inhibited protein synthesis without producing misreading\(^3\).

Little comparative information is available on structure-activity relationships between spectinomycin and its various derivatives. The free base and sulfate salt appear to have the same activities in vitro and in vivo. Reduction of the carbonyl group yields the dihydro-derivative which had one-tenth of the antibacterial activity of spectinomycin\(^3\). Various acyl derivatives of dihydrospectinomycin have been prepared and found to have activity in vitro, but comparative biological data were not reported\(^3\). Partial acylation of spectinomycin followed by counter-current distribution led to the recovery of the N,N'-diacyl- and N,N',O-triacyl-derivatives\(^7\). For these derivatives, biological data were only obtainable for the trifluoroacetyl spectinomycins which were active in vitro against a variety of gram-positive and gram-negative organisms\(^4\). Comparative data to spectinomycin were not presented. Various N,N'-bis-carbamoyl
derivatives of spectinomycin were also prepared but biological data were not reported.

STATEMENT OF PROBLEM

The purpose of this investigation is to study the biosynthesis of spectinomycin. The unusual structural features of the antibiotic (the fused tricyclic ring system) coupled with its interesting biological properties and the resurgence of commercial interest and support prompted our study. The problem was approached by selection of several potential precursors based on consideration of certain structural features of the antibiotic and on the proposed biosynthetic pathways for the neomycins and streptomycin (Figures 1 and 2).

Acid hydrolysis of spectinomycin yields the aminocyclitol actinamine. Since a similar myo-inositol-derived moiety, streptamine (H, R = OH), is present in streptomycin and since actinamine has the same configuration of the parent nucleus, myo-inositol was selected as a potential precursor. As a corollary, D-glucose must also be considered as a potential precursor since the conversion of D-glucose to myo-inositol by yeast has been demonstrated by Chen and Charalampous, and because D-glucose has been demonstrated to be a precursor of the corresponding moieties of streptomycin (Figure 1) and the neomycins (Figure 2).
Figure 1

Proposed Streptomycin Biosynthesis\textsuperscript{45,49-51}
The Neomycins

Figure 2

Proposed Neomycin Biosynthesis
Ring C of spectinomycin could be considered as arising from an acetate-derived polyketide as shown below. Ring C could also be considered as a dideoxy-sugar derived from a hexose precursor such as D-glucose as shown above. Thus, D-glucose could serve as the carbon source for both rings A and C.

Three observations cast doubt on the participation of D-glucose in the biosynthesis and instead suggest the participation of D-galactose. The first observation is that D-galactose stimulates the biosynthesis of spectinomycin$^{47}$. Secondly, D-galactose can be substituted for D-glucose as a precursor in the biosynthesis of streptomycin$^{52}$. Finally, Imai$^{48}$ has demonstrated the conversion of D-galactose to myo-inositol (but his studies were conducted with rats). Thus, D-galactose must also be considered as a potential precursor of spectinomycin.

The structural feature remaining to be considered is the origin of the N-methyl groups. The nitrogen atoms are undoubtedly derived by transamination. The N-methyl groups are probably derived from a C-1 donor such as methionine. Thus, D-glucose, D-galactose, myo-inositol, acetate, and methionine were selected for our preliminary feeding studies.
Incorporation of Labeled Precursors into Spectinomycin*

All precursors except actinamine-\(^{14}\)C were purchased from commercial suppliers and were used directly after determining purity by thin layer chromatography. Actinamine-\(^{14}\)C was obtained by degradation of spectinomycin isolated from fermentations supplemented with myo-inositol-2-\(^{14}\)C. All labeled precursors were added to actively spectinomycin producing *Streptomyces flavopersicus* cultures, Abbott strain 66-666, at 69 hours. The antibiotic was isolated from the beans 24 to 120 hours after addition of the label. The cells were separated by centrifugation and washed, and the supernatant and washings passed over an IRC-50 (Na\(^+\)) ion exchange column. Hydrochloric acid solution (1 N) was passed over the column. Crystalline spectinomycin was obtained by addition of acetone to the effluent containing antibiotic activity. The pooled active fractions were recrystallized to constant specific activity with the aid of non-radioactive carrier spectinomycin when necessary. The results of the feeding experiments are presented in Table 1. These experiments demonstrated that acetate and D-galactose were not significantly incorporated into the

* Feeding experiments were performed by Dr. J. R. Martin, Abbott Laboratories. Final purification, degradation, and determination of specific activity were performed by the candidate (at Ohio State University).
Table 1
Incorporation of Labeled Precursors into Spectinomycin

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Harvest Time (Hrs. after addition)</th>
<th>Quantity Administered (dpm x 10^-6)</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid-2-14C</td>
<td>24</td>
<td>22.20</td>
<td>0.26</td>
</tr>
<tr>
<td>D-galactose-U-14C</td>
<td>24</td>
<td>1.75</td>
<td>0.0003</td>
</tr>
<tr>
<td>D-glucose-6-3H</td>
<td>48</td>
<td>5.87</td>
<td>3.5</td>
</tr>
<tr>
<td>D-glucose-2-14C</td>
<td>24</td>
<td>2.55</td>
<td>1.05</td>
</tr>
<tr>
<td>L-methionine-S-14C-methyl</td>
<td>48</td>
<td>2.23</td>
<td>38.9</td>
</tr>
<tr>
<td>Myo-inositol-U-14C</td>
<td>30</td>
<td>2.24</td>
<td>37.1</td>
</tr>
<tr>
<td>Myo-inositol-2-14C</td>
<td>24</td>
<td>6.42</td>
<td>47.0</td>
</tr>
<tr>
<td>Myo-inositol-2-14C</td>
<td>120</td>
<td>6.42</td>
<td>46.8</td>
</tr>
<tr>
<td>Actinamine-14C</td>
<td>72</td>
<td>1.10</td>
<td>6.6</td>
</tr>
</tbody>
</table>
antibiotic, whereas D-glucose, myo-inositol, and methionine were significantly incorporated into the antibiotic. Assuming that the citrate cycle is operative, the small incorporation of acetate probably reflects gluconeogenesis. The very low incorporation of D-galactose was surprising since addition of D-galactose to the fermentation media stimulated antibiotic production\(^7\) and since D-galactose had been shown to substitute for D-glucose as the sole carbon source in the biosynthesis of streptomycin\(^2\). These results suggested that D-galactose spared utilization of D-glucose in other metabolic pathways and, consequently, more D-glucose was available for spectinomycin biosynthesis. An analogous relationship exists between phenylalanine and chloramphenicol\(^5\). The high incorporation of myo-inositol compared to that of D-glucose suggested that D-glucose was converted to myo-inositol which was subsequently incorporated into the antibiotic. These observations are analogous to the formation of streptidine in the biosynthesis of streptomycin\(^4\,5\) (Figure 1). The low incorporation of actinamine compared to that of myo-inositol suggested that actinamine was not a direct precursor of spectinomycin. However, with these data one cannot rule out the possibility of selective permeability problems or the operation of a minor pathway.

Distribution of the Label

The second phase of this investigation was to determine the labeling pattern resulting from the feeding of various precursors. To gain preliminary insight into the labeling pattern, the degradative sequence shown in Figure 3 was employed. According to this sequence, spectinomycin (1) was treated with barium hydroxide solution (0.1 N) for
Figure 3

Base-Acid Hydrolysis of Spectinomycin

R = 2,4-Dinitrophenyl-
24 hours at room temperature. The reaction mixture was neutralized with sulfuric acid solution (1.33 N) and filtered to remove the barium sulfate. Actinospectinoic acid (2) was contained in the filtrate but was not isolated. The filtrate was immediately subjected to acid hydrolysis with steam distillation yielding carbon dioxide (6), actinamine (2), and 1-hydroxy-3-pentene-2-one (8). The carbon dioxide was trapped as barium carbonate (7). The 1-hydroxy-3-pentene-2-one was isolated from the distillate presumably as the corresponding 2,4-dinitrophenylsazone derivative (9). The distilland was concentrated and diluted with ethanol, whereupon actinamine separated as the crystalline hydrated sulfate salt. When 1-hydroxy-3-pentene-2-one no longer distilled from the reaction mixture, as determined by the absence of ultraviolet absorption at 227 nm, the hydrolysis was terminated. This sequence permits complete separation of rings A and C of spectinomycin and recovery of all carbon atoms.

Two mechanisms were suggested for the base catalyzed rearrangement of spectinomycin. Wiley et al.7 proposed that this reaction was a special case of the tertiary ketol rearrangement54,55 as illustrated in Figure 4. Johnson11 suggested an alternative mechanism which involved benzilic acid rearrangement of the diketo-form of spectinomycin as illustrated in Figure 5. If the tertiary ketol mechanism were valid, only the ester carbonyl group would be lost (as carbon dioxide) on acid hydrolysis of actinospectinoic acid. However, if the benzilic acid mechanism were valid, either ketone carbonyl group could be attacked and subsequently lost as carbon dioxide.

The identity of each product from base-acid hydrolysis of
Figure 4

Proposed Tertiary Ketol Rearrangement of Spectinomycin
Figure 5

Proposed Benzilic Acid Rearrangement of Spectinomycin$^{11}$
spectinomycin was confirmed by several methods. However, the nature of the osazone derivative is still the subject of some controversy. The melting point, infrared spectrum, and elemental analysis of our material agreed favorably with Wiley’s data; however, neither elemental analysis was in agreement with theory. In addition, isolation of the unsaturated osazone from aqueous acidic 2,4-dinitrophenylhydrazine solution is at variance with the published behavior of 1-hydroxy-3-pentene-2-one under similar conditions. Lettenbauer and Zamen reported that in aqueous solution 1-hydroxy-3-pentene-2-one (8) was rapidly converted to 1,4-pentanediol-2-one, and when treated with 2,4-dinitrophenylhydrazine yielded mainly the 2,4-dinitrophenylosazone (10) of the diol along with trace amounts of 1-(2,4-dinitrophenyl)-3-hydroxymethyl-5-methyl-Δ²-pyrazoline (11) as shown below. Mass spectral analysis of our material suggested that it was a mixture of both 2,4-dinitrophenylosazones (9,10) although infrared analysis failed to indicate the presence of a hydroxyl group. The 1,4-pentanediol-2-one-2,4-dinitrophenylosazone (10), obtained by room temperature acid hydrolysis of actinospectinoic acid in the presence of 2,4-dinitrophenyl-
hydrazine, displayed a weak hydroxyl absorption at 3540 cm⁻¹. The weak nature of this absorption may account for its absence in the infrared spectrum of the mixture. Our criteria for this phase of the study were the complete separation of rings A and C of spectinomycin and the isolation of all carbon atoms. To obtain more specific data on the labeling pattern of ring C, other degradative methods were applied which circumvented the difficulties in obtaining a pure osazone derivative. The results of this study are presented in Table 2.

The incorporation of D-glucose-6-³H into both halves of spectinomycin suggested that the antibiotic was derived from D-glucose. This conclusion was in agreement with the observation that streptomycin and the neomycins were derived from D-glucose⁵,⁶. The exclusive and high incorporation of myo-inositol into ring A suggested that D-glucose was cyclized to myo-inositol which was subsequently incorporated into the antibiotic. The incorporation of the ¹⁴C-label of L-methionine-S-¹⁴C-methyl into ring A of spectinomycin supported but did not prove our suggestion that the N-methyl groups were methionine-derived. Furthermore, the absence of radioactivity in the ring C fragments demonstrated that the C-methyl group of spectinomycin was not derived from the methylating pool. The low but significant incorporation of actinamine-¹⁴C compared to the incorporation of myo-inositol indicated that actinamine was not a direct precursor of spectinomycin. The incorporation of actinamine probably reflects degradation and resynthesis or the operation of a minor pathway. However, selective permeability problems cannot be ruled out with these data. The results with actinamine were analogous to the incorporation of streptamine into
<table>
<thead>
<tr>
<th>Precursor</th>
<th>Spectinomycin Sp. Act. (µCi/mM)</th>
<th>% Recovery</th>
<th>Actinamine</th>
<th>Osazone</th>
<th>BaCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myo-inositol-</strong>&lt;sup&gt;U-¹⁴C&lt;/sup&gt;</td>
<td>a) 0.1541</td>
<td>83.06</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>b) 0.1405</td>
<td>88.40</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Myo-inositol-</strong>&lt;sup&gt;2-¹⁴C&lt;/sup&gt;</td>
<td>a) 0.1631</td>
<td>90.55</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>b) 0.1523</td>
<td>89.20</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Myo-inositol-</strong>&lt;sup&gt;2-¹⁴C&lt;/sup&gt; (120)</td>
<td>a) 0.1319</td>
<td>91.05</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>b) 0.1195</td>
<td>88.03</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>L-methionine-S-</strong>&lt;sup&gt;¹⁴C-methyl&lt;/sup&gt;</td>
<td>a) 0.08276</td>
<td>94.16</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>b) 0.1062</td>
<td>91.05</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>D-glucose-6-³H</strong>&lt;sup&gt;(48)&lt;/sup&gt;</td>
<td>a) 0.4192</td>
<td>49.45</td>
<td>37.45</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b) 0.5088</td>
<td>46.11</td>
<td>36.91</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Actinamine-</strong>&lt;sup&gt;¹⁴C&lt;/sup&gt; (72)</td>
<td>0.02493</td>
<td>88.73</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td><strong>D-glucose-2-¹⁴C</strong>&lt;sup&gt;(24)&lt;/sup&gt;</td>
<td>a) 0.1083</td>
<td>50.83</td>
<td>7.52</td>
<td>24.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b) 0.09106</td>
<td>53.78</td>
<td>5.16</td>
<td>22.50</td>
<td></td>
</tr>
</tbody>
</table>
streptomycin. Hunter and Hockenhull administered streptamine-$^{14}$C to *Streptomyces griseus* and observed that the label was incorporated only to a small extent and that it was distributed throughout the streptomycin molecule. They suggested that streptamine undergoes degradation, including ring fission, followed by resynthesis. If degradation and resynthesis were responsible for the incorporation of actinamine into spectinomycin, then the degradation apparently did not extend beyond myo-inositol since no radioactivity was detected in the ring C fragments. However, due to the low specific activity of our sample, this point requires reinvestigation.

The determination of the specific labeling pattern employing more selective degradative methods was then investigated. Kuhn-Roth oxidation of spectinomycin derived from D-glucose-$^{6-3}$H, followed by steam distillation, yielded acetic acid (12) which was converted to the crystalline p-phenylphenacyl derivative (13) as shown in Figure 6. The results of this experiment are presented in Table 3. Since all the radioactivity present in the dinitrophenyllosazone derivative (Table 2) was also present in the acetic acid, this experiment demonstrated that the C-methyl group of spectinomycin was derived from C-6 of D-glucose. The choice of D-glucose-$^{6-3}$H obviated the necessity of performing a Schmidt degradation, which would have been required if D-glucose-$^{6-14}$C had been employed. This experiment strengthened our suggestion that ring C of spectinomycin is a dideoxy-sugar derived from a hexose precursor.

The biosynthesis of 4,6-dideoxyhexoses has not been investigated;
Figure 6

Selective Degradations of Spectinomycin
Table 3

Distribution of Label as Determined by Selective Degradations of Spectinomycin

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Spectinomycin Activity (µCi/mM)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose-6-³H</td>
<td>a) 0.4190</td>
<td>41.30⁸</td>
</tr>
<tr>
<td></td>
<td>b) 0.2325</td>
<td>41.39⁸</td>
</tr>
<tr>
<td>L-methionine-S-¹⁴C-methyl</td>
<td>0.08276</td>
<td>92.10⁷</td>
</tr>
<tr>
<td>Myo-inositol-²⁻¹⁴C</td>
<td>0.1061</td>
<td>36.60⁹</td>
</tr>
</tbody>
</table>

a) as p-phenylphenacyl acetate (13).
b) as N-methyl-3,5-dinitrobenzamide (15).
c) as N,N'-diphenylformamidine·HCl (17).
however, the biosynthesis of 6-deoxy- and 3,6-dideoxy-hexoses has been studied by several groups. The sequence shown below was observed for the biosynthesis of these deoxyhexoses from D-glucose. 4-Keto-6-deoxy-D-glucose was proposed as an intermediate in the biosynthesis of these deoxyhexoses and would be an attractive intermediate for the biosynthesis of 4,6-dideoxyhexoses such as actinospectose. A similar intermediate, 4-keto-6-deoxy-L-idose, was proposed as an intermediate in the biosynthesis of the streptose moiety of streptomycin. Melo et al. demonstrated that the formation of 4-keto-6-deoxy-D-glucose involved intramolecular transfer of hydrogen from C-4 to C-6 of D-glucose-dTDP. This observation suggested that further insight into the biosynthesis of actinospectose could be gained by feeding D-glucose-4-3H. If the acetic acid from a Kuhn-Roth oxidation of spectinomycin contained all the radioactivity present in ring C, then could be postulated as an intermediate in the biosynthesis of actinospectose. Failure of the label to appear on the C-methyl group would suggest that the pathway below is not involved.

The results obtained with D-glucose-2-14C (Table 2) confirmed that
the actinospectose moiety was a 1,6-dideoxyhexose derived from D-glucose as shown below. The numbers represent the corresponding carbon atoms of D-glucose. This experiment demonstrated that C-4α of spectinomycin was derived from C-2 of D-glucose. This information coupled with that derived from the study with D-glucose-6-³H confirmed that actinospectose (3) was derived from the intact D-glucose molecule. The small percentage of label present in the osazone derivative would be expected due to randomization caused by gluconeogenesis. This experiment supported the proposed structure of spectinomycin and the proposed tertiary ketol rearrangement previously described.

That the N-methyl groups are derived from methionine was proved by periodate oxidation of actinamine obtained by base-acid hydrolysis of methionine-labeled spectinomycin as shown in Figure 6. The periodate oxidation was terminated by addition of barium hydroxide solution. The mixture was filtered and the filtrate steam distilled, collecting the distillate in dilute hydrochloric acid solution. The distillate was evaporated to dryness and the methylamine hydrochloride (lit) was converted to N-methyl-3,5-dinitrobenzamide (15). The results of this experiment are presented in Table 3. This study demonstrated that 92% of the radioactivity of methionine-derived spectinomycin resided on the N-methyl groups. If the specific activity of actinamine
was employed, greater than 98% of the label resided on the N-methyl groups.

Periodate oxidation of spectinomycin derived from myo-inositol-2-\(^{14}C\) was attempted to investigate the labeling pattern of ring A. The assumption which prompted this study was that C-2 of myo-inositol (21) might correspond to C-2 of actinamine (2). As shown in Figure 6, periodate oxidation of spectinomycin should yield two moles of formic acid (16) per mole of spectinomycin. If our assumption were correct, then the specific activity of the formic acid would be half that observed for spectinomycin. Fleury-Lange\(^{6r}\) titration indicated rapid consumption of three moles of periodate per mole of spectinomycin within five minutes, followed by slow consumption of one mole of periodate per mole of spectinomycin during the next 55 minutes. Titration of the acid liberated indicated formation of 1.9 moles of acid per mole of spectinomycin in five minutes, followed by slow formation of an additional 0.35 mole of acid per mole of spectinomycin during the next 55 minutes. Model studies with glycerol indicated the feasibility of isolating the formic acid as \(N,N'\)-diphenylformamidine hydrochloride\(^{65}\) (17) in 38-40% overall yield. However, attempts to isolate formic acid as the formamidine derivative from spectinomycin at five to fifteen minutes failed, although the titration data indicated the presence of two moles of acid per mole of spectinomycin. Small amounts of the formamidine derivative were isolated after thirty minutes with yields increasing with longer reaction times. During work-up, copious quantities of iodine were liberated and so phenol was added to prevent iodine from distilling with the formic acid. Iodine libera-
tion was not noted during the studies with glycerol. These observations suggested an abnormal periodate oxidation.

Based on the above observations, a reaction time of one hour was selected. This reaction time was consistent with the acid formation data and theoretical cleavage pattern as indicated below. The results of this study are presented in Table 3 and suggest that the label is being scrambled during the biosynthetic sequence. Unfortunately, the same level of radioactivity could result from complete oxidation of the actinamine ring. Since the course of the periodate oxidation is uncertain, the results of this study are inconclusive. To obtain more specific information on the labeling pattern of ring A, periodate oxidation of N,N'-diacetylactinamine should be investigated. This approach should avoid the difficulties encountered with the previous periodate oxidation. Johnson described the preparation of N,N'-diacetylactinamine and the yields obtained are suitable for handling small quantities of material.

These experiments suggest something of the probable sequence of biochemical events as illustrated in Figure 7. Assuming the absence of permeability problems, the low incorporation of actinamine indicates that formation of 2 before completion of the glycosidic linkages probably does not occur. Consequently, the sequence suggested in
Figure 7

Proposed Biosynthesis of Spectinomycin
Figure 7c would not be valid. The sequence suggested by Figure 7a is probably not valid since studies of the biosynthesis of streptomycin and the neomycins suggest that the glycosidic linkages are formed after the biosynthesis of each component is fairly complete\(^{45,46}\). However, ethionine blockade of methylation by methionine led to accumulation of N-demethyl-streptomycin\(^{49}\). Thus, the biosynthesis of 1,3-\textit{myo}-inosadiamine (22) followed by formation of desmethylspectinomycin (24) and N-methylation by methionine as shown in Figure 7b is strongly suggested as the probable sequence of events in the biosynthesis of spectinomycin.

To conclude this investigation, a study as to the feasibility of synthesizing labeled 1,3-\textit{myo}-inosadiamine (22) was conducted. Nakajima et al.\(^{66}\) reported the synthesis of 1,3-\textit{myo}-inosadiamine hexaacetate as shown below. The length of this synthesis and the poor overall yields decreased the attractiveness of using a \(^{14}\text{C}\)-label. However,  

\[ R = \text{Ac} \]

\(^{15}\text{N}\)-hydroxylamine could be employed as this component is introduced late in the sequence. Incorporation of the label into spectinomycin
would be determined by mass spectrometry.

Lichtenthaler et al. \textsuperscript{67} devised a unique synthesis of 1,3-\textit{myo}-inosadamine from \textit{myo}-inositol as shown below. Although this sequence is short and labeled \textit{myo}-inositol is readily available, the extremely low yields immediately disqualify this synthesis from further consideration.

Suami et al. \textsuperscript{68,69} reported the synthesis of 1,3-\textit{myo}-inosadamine from 2,1,5,6-tetra-O-acetyl-1,3-di-O-tosyl-\textit{myo}-inositol (25) as shown below. This sequence is unsuitable for incorporation of a \textsuperscript{14}C-label due to the lengthy preparation of the starting material. However, the synthesis appears well suited for the incorporation of a \textsuperscript{15}N-label from \textsuperscript{15}N-hydrazine.

Ogawa et al. \textsuperscript{70} reported the synthesis of 1,3-\textit{myo}-inosadamine from epi-inositol through the diaza-intermediate shown on the next page. The length of this synthesis and the overall low yields disqualifies it for our proposed study.
All of the previous syntheses are characterized by starting with an intact cyclohexane ring. Hasegawa and Sable reported the synthesis of 1,3-myo-inosadamine hexaacetate by a double aldol-type condensation between a dialdehyde and nitromethane as shown below. Although a mixture of products was obtained, this synthesis was repeated with
$^{14}$C-nitromethane yielding a small amount of 1,3-myo-inosadimine-$^{14}$C. This synthesis did not appear suited to our needs due to the lengthy preparation of the starting material and the fact that the yield of 22 is reduced by formation of other products.

The synthesis of 1,3-myo-inosadimine reported by Suami et al. was selected for our labeling study. The synthesis of the starting material, 2,4,5,6-tetra-O-acetyl-1,3-di-O-tosyl-myo-inositol (25), shown in Figure 8 was reported by Angyal et al. Due to unforeseen difficulties, only the synthesis of 25 was achieved in time to be included in this dissertation.

The preparation of 1,2-O-cyclohexylidene-myo-inositol (26) as described by Angyal et al. is unsatisfactory. After several abortive attempts to prepare 26, a thorough literature survey revealed that a revised procedure had been published. The revised procedure worked and good yields of 26 were obtained. Difficulty was encountered in the hydrolysis of 27. Acid hydrolysis of 27 under a variety of conditions yielded a mixture of products or starting material. Due to difficulties encountered with purification of 28, preparation of the corresponding monoisopropylidene derivatives (29,30) as described by Gigg and Warren was investigated. Acid hydrolysis of the monoisopropylidene tetra-acetate derivative also produced a mixture of products; however, 28 was easily obtained by recrystallization of the mixture from water. The synthesis of 25 is now "in hand" and the synthesis of 1,3-myo-inosadimine (22) as described by Suami et al. is being pursued. This will complete the model preparation of unlabeled material on a "labeling" scale in preparation for subsequent isotope experiments.
Figure 8

Synthesis of 2,4,5,6-Tetra-O-acetyl-1,3-di-tosyl-myoinositol
EXPERIMENTAL

A. Instrumentation

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were obtained with a model 257 Perkin Elmer grating spectrophotometer. Ultraviolet spectra were obtained with a model 15 Cary recording spectrophotometer. The NMR spectra were recorded on a Varian A-60A spectrometer. Gas chromatographic analyses were performed with a F and M model 402 high efficiency gas chromatograph employing a column packed with 10% silicone gum XE-60 on Diatoport S, 80-100 mesh. Weighings of samples for scintillation analyses were performed with a model G Cahn electrobalance. Specific activity determinations were obtained with a model 3375 Packard Tri Carb liquid scintillation counter. The various scintillation solutions employed are described in appendix A. Microanalyses were performed by Midwest Micro-laboratories Ltd., Indianapolis, Indiana. All solvents employed were of analytical grade unless otherwise noted.

B. Feeding and Harvesting Procedures*

Synthetic fermentation media (100 ml.) in 500 ml. Erlenmeyer flasks were inoculated with an 8% volume of a 48 hr. seed culture grown with shaking in a complex medium. The fermentation medium was

*Performed by Dr. J. R. Martin, Abbott Laboratories.
incubated at $32^\circ$ with shaking (160 rpm). Each of the labeled precursors was dissolved in water (100 µl.) and added to the fermentation media. At the times indicated, the broth was separated from the mycelia by centrifugation. The mycellial pellet was washed with water (25 ml.) and the washings added to the broth. The combined washings and broth were passed through a column (1.2x10 cm.) of IRC-50 (Na$^+$) ion exchange resin. The column was washed with water and eluted with 1N hydrochloric acid, collecting 1.0 ml-fractions. The eluents were examined for antibiotic activity using the disc method with *Bacillus subtilis* as the test organism. Eluents containing antibiotic activity were pooled and the total radioactivity determined. The combined eluents were evaporated to dryness and the yellow residue extracted several times with methanol to remove the spectinomycin. The combined extracts were evaporated to dryness and the spectinomycin crystallized from water-acetone. If necessary, non-radioactive spectinomycin dihydrochloride pentahydrate (50 mg.) was added before crystallization.

C. Scintillation Counting

Three methods of sample preparation were employed. The first method involved simple solution counting of the sample in an appropriate scintillation solvent system. This method was applied to spectinomycin dihydrochloride pentahydrate, actinamine sulfate, N-methyl-3,5-dinitrobenzamide, and p-phenylphenacyl acetate. Two difficulties were encountered which necessitated application of other scintillation techniques. First, the amine salts including spectinomycin tended to separate from the various scintillation solvents employed.
Second, the dinitrophenylosazone derivative could not be determined by solution counting since it proved to be quite insoluble as well as a severe color quencher. The best data for the amine salts were obtained with scintillation solution A employing Cab-O-Sil as a suspending agent. In general, the amine salt (0.2–0.8 mg.) was washed from the weighing pan into the scintillation vial with 50 μl. of water. Sufficient Cab-O-Sil was added to loosely fill the vial and scintillation solution A (15 ml.) was added. The mixture was agitated until a clear gel was obtained. N-Methyl-3,5-dinitrobenzamide and p-phenylphenacyl acetate were counted by washing the sample from the weighing pan into the scintillation vial with scintillation solution A (15 ml.).

The difficulties encountered with the amine salts and the dinitrophenylosazone derivative led to the application of combustion analysis for determining these samples. The method of sample preparation was identical to that described by Ober et al.75. The sample (0.2–0.8 mg.) was placed in a cellophane bag containing a small amount of powdered cellulose (Cellulose Powder MN300; Macherey, Nagel and Co.; Germany) as fuel. The bag was supported in the combustion apparatus, the system flushed with oxygen and sealed, and the bag ignited by means of a high intensity lamp. The carbon dioxide (or water) was absorbed with methanol-phenethylamine solution (1:1 v/v) (or absolute ethanol for H₂O). An aliquot of the absorbing solution was transferred to a scintillation vial, the appropriate volume of scintillation solution B added, and the mixture counted. This technique was applied to spectinomycin dihydrochloride pentahydrate, actinamine sulfate, N,N'-diphenylformamidine hydrochloride, and to the dinitrophenylosazone
derivative. For the amine salts, the specific activities obtained by combustion analysis were consistently higher than those obtained by simple solution counting.

The third method of sample preparation was specifically applied to the carbon dioxide liberated during acid hydrolysis of actino-spectinoic acid. The carbon dioxide was trapped as barium carbonate which was suspension counted, employing scintillation solution B instead of the toluene-PPO system recommended by Cluley76.

Quenching was monitored in all samples by addition of internal standard (\(^{14}\)C-benzoic acid in toluene). Each sample was counted for thirty minutes before and after addition of the internal standard. Duplicate analyses were performed on each sample.

D. Preparation of Spectinomycin Dilutions

The specific activity of each spectinomycin sample received from Dr. Martin was determined by simple solution and/or combustion analysis. From this data dilutions with non-radioactive spectinomycin dihydrochloride pentahydrate were prepared to give the desired quantity of labeled material containing 200 to 500 observed counts per minute per milligram. The "desired quantity" was determined from experiments with non-radioactive spectinomycin and can be best described as that weight of spectinomycin dihydrochloride pentahydrate which yields two or four times the minimum weight of product required for scintillation analysis. In general, 1-3 mg of analytically pure product were required for this analysis.

Each dilution was prepared by placing the calculated quantities
of labeled and unlabeled antibiotic in a test tube. The mixture was dissolved in a few drops of water and the solution pressure filtered through a micro-filtration beaker (Pyrex 31025) into a Craig tube, washing through with two small portions of water. Acetone was added to the combined filtrates to the cloud point. Colorless needle crystals rapidly formed and the solution clarified. Addition of small portions of acetone was continued until further addition failed to produce a detectable cloudiness. The mixture was refrigerated four to six hours. The crystals were centrifuged to the bottom of the tube, the tube inverted, and the mother liquor removed by centrifugation. The crystals were washed with two small portions of acetone and dried with a gentle stream of filtered air. The specific activity was determined as previously described, the material being recrystallized until constant specific activity was obtained. Generally only three to four crystallizations were required since the non-radioactive spectinomycin employed for preparation of these dilutions had previously been recrystallized several times from aqueous acetone.

**Anal.** Non-radioactive sample. Calcd. for

\[ \text{C}_{14}\text{H}_{24}\text{N}_{2}\text{O}_{7}\cdot 2\text{HCl} \cdot 5\text{H}_2\text{O}: \quad \text{C}, 33.95; \text{H}, 7.33; \text{N}, 5.66; \text{O}, 38.76; \text{Cl}, 14.31. \]

**Found:** C, 34.16; H, 7.32; N, 6.00; O, 38.95; Cl, 14.28.

E. Base-Acid Rearrangement and Hydrolysis of Spectinomycin.

Barium hydroxide solution (3.4 ml., 0.1N) was added to labeled spectinomycin dihydrochloride pentahydrate (50 mg., recrystallized to constant specific activity) contained in a Craig tube and the solution allowed to stand 24 hours at room temperature. The reaction
was terminated by dropwise addition of sulfuric acid (1.33N) until the supernatant was neutral to pH paper. The barium sulfate was centrifuged to the bottom of the tube, the tube inverted, and the supernatant removed by centrifugation. The supernatant was used directly in the acid hydrolysis or could be stored for several days by evaporating to dryness. Final drying of the residue was achieved by azeotroping the last traces of water with absolute ethanol.

Sulfuric acid (1.5 ml., 1.33N) was added to the neutralized supernatant or to the dried residue. The last traces of barium sulfate were removed by filtration through moistened filter paper followed by filtration through a micro-filtration beaker, washing through with 1.5 ml. portions of sulfuric acid (1.33N) and boiled distilled water. The flask containing the combined filtrates was attached to a Claisen head equipped with a Liebig condenser, thermometer, and an adapter bearing a dropping funnel (not self compensating) and a gas inlet tube extending into the reaction vessel. The condenser was attached by means of a vacuum adapter to a cow receiver bearing four round bottom flasks. The vacuum adapter was attached to a small gas washing bottle equipped with a fritted disc. The gas inlet tube and washing bottle were connected to traps containing "Baralyme" granules to prevent carbon dioxide from entering the system. Boiled distilled water (10 ml.) was added to the contents of the reaction vessel and carbon dioxide-free air was drawn through the system with a controlled vacuum. After the system had been thoroughly flushed, saturated barium hydroxide solution was quickly added to the gas washing bottle and the contents of the reaction vessel were heated to boiling, while
carbon dioxide-free air was drawn through the system. When the precipitation of barium carbonate ceased, the vacuum line was disconnected and the reaction mixture distilled. Distillate was collected in 10 ml. increments, adding 10 ml. of water as each fraction collected. The hydrolysis was terminated when the ultraviolet absorption of the distillate at 227 nm. had almost ceased.

The distillate was concentrated to approximately 2 ml. at 40° in vacuo and pressure filtered through a filtration beaker. Ethanol was added to the filtrate to the cloud point and the mixture refrigerated overnight. Addition of ethanol in small portions, with refrigeration, was continued until a total of 40 ml. of ethanol had been added. The crystals were isolated by suction filtration; washed with ethanol, acetone, and ether; and then dried in vacuo at room temperature. For scintillation analysis, anhydrous actinamine sulfate was obtained by drying in vacuo at 100° over P₂O₅.

Typical yield: 21.6 mg., 60.3% (sulfate trihydrate).

M.P.: 298° (decomp.).

Infrared (KBr, cm⁻¹, anhydrous sample): 3360 (s), 3280 (s), 3225 (s), 2930 (w), 2915 (w), 1583 (m), 1470 (m), 1460 (m), 1445 (w), 1410 (sh), 1390 (m), 1360 (w), 1338 (m), 1242 (w), 1195 (m), 1120 (s), 1108 (sh), 1095 (sh), 1048 (m), 1025 (m), 9410 (m), 872 (m), 834 (w), 720 (w).

Anal. Calcd. for C₆H₁₆N₂O₄·H₂SO₄·3H₂O: C, 26.81; H, 7.31; N, 7.82; O, 49.11; S, 8.95. Found: C, 26.95; H, 7.52; N, 7.96; O, 48.98; S, 9.10.
The distillate was added to an equal volume of 1% dinitrophenyl-hydrazine in 9N sulfuric acid and the mixture was incubated at 37° for 24 to 48 hours. The orange precipitate was isolated by centrifugation after siphoning off most of the supernatant. The precipitate was washed with two portions (30 ml.) of 9N sulfuric acid and then with distilled water until the washings were neutral to pH paper. The precipitate was dried in vacuo for 24 hours at room temperature over P₂O₅, then at 50° for 12 hours. Recrystallized from acetone.

Typical yield: 16.8 mg., 36.6%.


Infrared (KBr, cm⁻¹): 3290 (w), 3100 (w), 1610 (s), 1590 (s), 1515 (sh), 1500 (s), 1425 (w), 1330 (s), 1315 (s), 1270 (sh), 1220 (w), 1135 (m), 1082 (m), 1055 (sh).

Anal. Calcd. for C₁₇H₁₄N₂O₈: C, 44.55; H, 3.07; N, 24.45; O, 27.93.

Found: C, 44.45; H, 4.19; N, 22.17;

The barium carbonate was separated from the barium hydroxide solution by centrifugation. The precipitate was washed with boiled distilled water until the washings were neutral to pH paper and dried as described for the dinitrophenylosazone derivative.

Typical yield: 10.1 mg., 51.3%.

Infrared (KBr): Identical to authentic barium carbonate (Baker AR barium carbonate).

F. Kuhn-Roth Oxidation of Spectinomycin

A 40 mg. dilution of spectinomycin dihydrochloride pentahydrate derived from glucose-6-³H was prepared and recrystallized to constant
specific activity. The antibiotic (24.9 mg.) was dissolved in 3 ml. of oxidizing mixture (appendix B) and transferred to a round bottom flask, washing through with two additional portions of oxidizing mixture. The reaction mixture was refluxed for two hours and then steam distilled into water containing a few drops of 2N sodium hydroxide solution.

The distillate (65 ml., pH 10) was evaporated in vacuo to dryness. The residue was dissolved in water (3 ml.) and transferred to a small flask, washing through with two 1.5 ml. portions of water. The solution was adjusted to pH 5.0 with 5% hydrochloric acid. p-Phenylphenacyl bromide (400 mg., m.p. 125-126.5°) and carboxylic acid-free acetone \(^7\) (10 ml.) were added and the mixture heated to reflux. At reflux, just sufficient acetone was added to effect solution. The reaction mixture was refluxed for two hours and then allowed to stand for 24 hours at room temperature.

The excess p-phenylphenacyl bromide which crystallized on standing was removed by filtration. The filtrate was diluted with an equal volume of chloroform and water (165 ml.) was added. The organic phase was separated, the aqueous phase extracted with four 50 ml. portions of chloroform, and the combined extracts dried (Na\(_2\)SO\(_4\)) overnight. The mixture was filtered and the filtrate evaporated in vacuo to dryness. Thin layer chromatographic analysis of the residue on silica gel GF\(_254\) developing with chloroform-ether (29:1 v/v) and visualizing with iodine vapors revealed the presence of p-phenylphenacyl bromide (R\(_f\) 0.74), p-phenylphenacyl acetate (R\(_f\) 0.37), and p-phenylphenacyl alcohol (R\(_f\) 0.14).
Initial separation of the bromide from the ester and alcohol was achieved by passing a chloroform solution of the mixture through a column containing 3.2 gm. of silica gel (dry packed), eluting with chloroform. The ester and alcohol were then separated on an identically prepared column eluting with chloroform-ether (29:1 v/v). The ester was recrystallized from ethanol or isolated as an amorphous solid.

Yield: 5.76 mg., 44.9%.
NMR (CDCl₃, t): 7.61 (singlet, 3H), 4.64 (singlet, 2H), 2.25 (multiplet, 9H).
Infrared (CHCl₃, cm⁻¹): 3030 (m), 2932 (w), 1750 (s), 1700 (s), 1605 (s), 1580 (w), 1560 (w), 1485 (w), 1448 (w), 1420 (m), 1405 (m), 1372 (m), 1280 (m), 1250 (sh), 1230 (s), 1192 (m), 1118 (w), 1090 (m), 1050 (w), 1028 (w), 1010 (sh), 1000 (w), 975 (m), 830 (w). These properties are identical to those of authentic material prepared from sodium acetate (Baker AR sodium acetate) and p-phenylphenacyl bromide.

Gas Chromatography: Retention time identical to authentic p-phenylphenacyl acetate. No other peaks were observed except the solvent peak.

Found: C, 75.36; H, 5.80.

G. Periodate Oxidation of Actinamine Sulfate

Spectinomycin dihydrochloride pentahydrate, harvested from cultures fed S-¹⁴C-methyl methionine, was subjected to base-acid
rearrangement and hydrolysis. The actinamine sulfate was dried to constant weight in vacuo at 106-110° over P₂O₅. The anhydrous salt (19.98 mg.) was dissolved in a few drops of water and transferred to a flask containing sodium metaperiodate (185 mg.) in water (7.0 ml.), washing through with two portions of water. Sodium bicarbonate solution (10% v/v) was added to the reaction mixture in sufficient quantity to maintain pH 5.0. Acid formation was quite rapid during the first fifteen minutes and then appeared to cease. The reaction mixture was allowed to stand overnight at room temperature in a dark cupboard. The periodate and iodate were precipitated by addition of excess barium hydroxide solution (0.1N). The precipitate was removed by filtration, washed with two small portions of water, and the washings added to the filtrate. The filtrate (pH 10) was steam distilled until the distillate failed to give an alkaline test with pH paper. The distillate was collected in 5% hydrochloric acid solution (50 ml.), evaporated to dryness in vacuo at 50°, and the residue stored overnight under nitrogen.

The residue was dissolved in water (4 ml.) and transferred to a separatory funnel, washing through with two portions of water. Ether (30 ml.) was added to the contents of the separatory funnel and the funnel thoroughly chilled in an ice bath. Pyridine (0.5 gm.) and 3,5-dinitrobenzoyl chloride (0.5 gm. in 2 ml. benzene) were added and the reaction mixture was thoroughly agitated. Potassium carbonate (11.0 gm., anhydrous) was added in portions with agitation and cooling. The mixture was then allowed to stand 30 minutes in the ice bath with occasional agitation. The phases were separated and the aqueous
phase extracted with three 50 ml. portions of ether. The combined organic phases were extracted with three 25 ml. portions of 1% sodium bicarbonate and 1% sulfuric acid solutions, then dried by extracting with saturated saline solution followed by treatment with anhydrous sodium sulfate. The solution was filtered and the filtrate evaporated in vacuo to dryness. The residue was dissolved in acetone and the solution slurried with Woelm neutral aluminia (0.5 gm.). The acetone was evaporated and the powdered residue packed onto the top of a column of Woelm neutral aluminia (4.5 gm.) contained in a 5 ml. burette. The column was eluted with chloroform (25 ml.) and then with chloroform-ethyl acetate (4:1 v/v) collecting 4 ml-fractions. The fractions containing N-methyl-3,5-dinitrobenzamide were combined, evaporated to dryness, and the residue recrystallized from ethanol. This procedure is a modification of that reported by Randerath.

Yield: 1.43 mg., 4.89%.

M.P.: 146-147° (lit. 146-146.5°).

NMR (acetone-d₆, t): 7.08 (doublet, 3H), 2.50 (broad, 3H), 1.03 (multiplet, 3H).

Infrared (KBr, cm⁻¹): 3278 (s), 3080 (m), 2960 (w), 2925 (w), 2860 (w), 1640 (s), 1560 (sh), 1530 (s), 1510 (sh), 1460 (w), 1410 (m), 1350 (sh), 1340 (s), 1325 (s), 1310 (s), 1192 (w), 1150 (m), 1110 (w), 1075 (m), 920 (m), 910 (m), 820 (w), 715 (s). These properties are identical to those of authentic material prepared from methylamine hydrochloride (Matheson, Coleman and Bell, M.P. 230-232°).

Gas Chromatography: Retention time identical to authentic material. No other peaks observed except the solvent peak.
Anal. Calcd. for C₁₈H₂₇N₃O₅: C, 42.68; H, 3.13; N, 18.66; O, 35.53.
Found: C, 42.46; H, 3.21; N, 18.70;

H. Attempted Periodate Oxidation of Spectinomycin

Spectinomycin dihydrochloride pentahydrate obtained from a culture fed myo-inositol-2-¹⁴C was diluted with non-radioactive material and recrystallized to constant specific activity. The antibiotic (200 mg.) was transferred to a flask containing 1.08% sodium metaperiodate solution (40 ml.). The reaction mixture was maintained at pH 5.0 by addition of 10% sodium bicarbonate solution. The reaction was terminated at the end of one hour by addition of excess saturated barium hydroxide solution and the precipitate was removed by filtration, washing well with water. A test of the combined filtrate and washings (pH 10) with saturated barium hydroxide solution failed to produce additional precipitate. The solution was evaporated to dryness in vacuo at 45-50°C.

The residue was dissolved in water (20 ml.) and phenol (2.0 gm.) was added. The solution was acidified to pH 2.0 with 10% phosphoric acid solution. On addition of the acid, a brown color and precipitate formed. The precipitate dissappeared at pH 2.0. The solution was distilled to dryness under reduced pressure from an aspirator. The residue was slurried with water (20 ml.), adjusted to pH 2.0 with 10% phosphoric acid, and distilled to dryness under reduced pressure.

The combined distillates were adjusted to pH 7.0 with 5% hydrochloric acid solution and distilled to dryness in vacuo. The residue was dissolved in a little water and transferred to a small
round bottom flask, washing through with two portions of water. The solution was adjusted to pH 7.0 with 5% hydrochloric acid solution and evaporated to dryness in vacuo. The last traces of phenol were removed by holding the residue under high vacuum at 50° for several hours.

Aniline hydrochloride (0.2 gm., recrystallized from ethanol) was added to the residue and the two solids intimately mixed. Aniline (1.2 ml., redistilled) and xylene (2.0 ml.) were added and the mixture refluxed with water separation for 30 minutes. The mixture was cooled to room temperature and 2N hydrochloric acid solution (20 ml.) was added. The crude crystalline product was isolated by suction filtration, washed with several portions of ether, and dried in vacuo at room temperature for 12 hours. A portion of the product was recrystallized from 0.2 N hydrochloric acid solution (1.0 ml.), yielding N,N'-diphenylformamidine hydrochloride monohydrate. The monohydrate was converted to the anhydrous material by drying in vacuo at 80° over P₂O₅.

M.P.: 253° (decomp., sealed tube), (lit 251°, decomp., sealed tube).

Infrared (KBr, cm⁻¹, anhydrous sample): 3200 (w), 3090 (m), 3040 (sh), 2985 (sh), 2955 (m), 1708 (s), 1700 (sh), 1608 (m), 1572 (w), 1562 (w), 1500 (m), 1492 (sh), 1358 (m), 1342 (m), 1222 (w), 1182 (w), 1160 (w), 1030 (w), 1030 (w), 1015 (m), 920 (w), 900 (w), 842 (w), 810 (w), 750 (m), 690 (m). These properties are identical to those of authentic material prepared from sodium formate (Baker AR sodium formate).
Anal. Calcd. for C_{13}H_{13}N_{3}Cl: C, 67.10; H, 5.63; N, 12.04; Cl, 15.24. Found: C, 67.04; H, 5.57; N, 11.86; Cl, 15.48.

Scintillation Analysis:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectinomycin·2HCl·5H_{2}O</td>
<td>0.1064 μCi/mM</td>
</tr>
<tr>
<td>N,N’-diphenylformamidine·HCl</td>
<td>0.0194 μCi/mM</td>
</tr>
</tbody>
</table>

I. Attempted Synthesis of 1,3-Myo-inosadiamine. Synthesis of 2,4,5,6-Tetra-O-acetyl-1,3-di-O-tosyl-myo-inositol. (+)-1,2-O-Isopropylidene-myo-inositol (29)\textsuperscript{74}.

A mixture of powdered myo-inositol (40 gm.) and p-toluene sulfonic acid (0.60 gm.) suspended in anhydrous dimethyl sulfoxide (150 ml.) and 2,2-dimethoxypropane (150 ml.) was stirred at 110\degree for three hours with the exclusion of moisture. Methanol and 2,2-dimethoxypropane were allowed to distill slowly from the reaction mixture. The myo-inositol dissolved rapidly and at the end of the reaction time 80 ml. of distillate had collected. The remaining 2,2-dimethoxypropane was removed under reduced pressure. Myo-inositol (40 gm.) and anhydrous dimethyl sulfoxide (50 ml.) were added and the mixture was heated at 120\degree for 2 hours. Triethylamine (5 ml.) was added and the dimethyl sulfoxide was removed in vacuo. The brown syrupy residue was stirred with ethanol (300 ml.) for a few minutes and the mixture was refrigerated overnight. The solid was isolated by suction filtration, washed with ethanol, and suspended in ethanol (1 l.). The suspension was heated to boiling and filtered to remove the excess myo-inositol. The filtrate was concentrated to approximately 300 ml.
in vacuo yielding a heavy suspension of 29. The suspension was heated to boiling, adding just sufficient ethanol to effect solution, followed by refrigeration for 8 hours. The product was isolated by suction filtration, washed with ethanol, and dried in vacuo at 40°. A trace of triethylamine was added to the ethanol used throughout this procedure.

Yield: 35.0 gm., 35.6% (based on 80 gm. myo-inositol).

M.P.: 172-174° (lit74. 184-186°, from n-propanol).

(+)-1,4,5,6-Tetra-O-acetyl-2,3-O-isopropylidene-mylo-inositol (30)74.

1,2-Isopropylidene-mylo-inositol (32.0 gm.) was suspended in pyridine (200 ml., KOH dried). After one hour the mixture was strained through glass wool to remove a small quantity of insoluble material. The filtrate was chilled in an ice bath and acetic anhydride (200 ml.) was slowly added. After 15 minutes, the ice bath was removed and the mixture was allowed to stand overnight at room temperature. The mixture was poured into ice water (1 l.) with stirring whereupon the product separated as an oil which rapidly solidified. After 15 minutes, 30 was collected by suction filtration. The filter cake was washed with several portions of water and recrystallized from ethanol.

Yield: 44.5 gm., 79.3%.

M.P. 119-122° (lit74. 122-124°). Recrystallization from ethanol raised the melting point to 121-123°.

(+)-1,4,5,6-Tetra-O-acetyl-mylo-inositol (28)72.

Monoisopropylidene myo-inositol tetracetate (5.0 gm., m.p. 121-123°) was suspended in a mixture of glacial acetic acid (8.0 ml.) and water (2.0 ml.), and the suspension heated for 2.5 hours on a steam
bath. The solvents were removed \textit{in vacuo} and the residue examined on silica gel thin layer plates, developing with chloroform-isopropanol (9:1 v/v) and visualizing with hydroxylamine-ferric chloride sprays\cite{82}. A trace of starting material, 2 minor components, and 28 were detected. Three recrystallizations of the residue from boiling water afforded nearly pure 28. The product was dried \textit{in vacuo} over P$_2$O$_5$ for 3 days while gradually raising the temperature from 70° to 100°. 

\textbf{Yield:} 1.9 gm., 42.3%.

\textbf{M.P.:} 140-142° (lit\cite{83}, 139°).

\textbf{2,4,5,6-Tetra-O-acetyl-1,3-di-O-tosyl-myo-inositol (28)\cite{84}.}

A solution of 28 (3.0 gm., m.p. 140-142°) and p-toluenesulfonyl chloride (2.4 gm.) in dry pyridine (30 ml.) was heated at 100° for 3 hours. The mixture was poured into water and extracted with chloroform. The chloroform extracts were washed with 5% hydrochloric acid solution and water, and evaporated \textit{in vacuo} to dryness. The residue was recrystallized from ethanol yielding (\textsuperscript{+})-1,4,5,6-tetra-O-acetyl-3-O-tosyl-myo-inositol.

\textbf{Yield:} 2.95 gm., 68.2%.

\textbf{M.P.:} 190-192° (lit\cite{84}, 195-197°).

Further recrystallization did not raise the melting point. Thin layer analysis as described for 28 revealed the presence of starting material and a minor impurity.

(\textsuperscript{+})-1,4,5,6-Tetra-O-acetyl-3-O-tosyl-myo-inositol (950 mg., m.p. 190-192°) and p-toluenesulfonyl chloride (0.8 gm.) were dissolved in dry pyridine and heated at 120° for 18 hours. The mixture was poured into water and extracted with chloroform. The chloroform
extracts were washed with 5% hydrochloric acid solution and water, and dried (Na₂SO₄). The mixture was filtered and the filtrate evaporated to dryness in vacuo. The residue was dissolved in hot ethanol; the solution decolorized with activated carbon, filtered, concentrated, and refrigerated. The crystals of 25 were isolated by suction filtration, washed with ethanol, and dried in vacuo at room temperature.

Yield: 0.5 gm., 40.3%.

M.P.: 208-214° (lit4, 216-221°).

Thin layer analysis as described for 28 revealed the presence of 25 and four minor impurities. Column chromatography on silica gel eluting with chloroform-isopropanol (9:1 v/v) failed to resolve the mixture.

J. Attempted Isolation of 1,3-Butanediol from Dihydrospectinomycin and Assignment of Configuration.

Preparation of Dihydrospectinomycin

Adam's catalyst (305 mg.) and spectinomycin dihydrochloride pentahydrate (4.0 gm.) in 50% aqueous ethanol (30 ml.) were hydrogenated in a Parr apparatus at 40 lbs. for 3 days at room temperature. The catalyst was removed by filtration, the filtrate evaporated to dryness in vacuo, and the residue recrystallized from aqueous acetone.

Yield: 2.5 gm.


Chromatography on Eastman cellulose coated strips (2.5 x 20 cm.), developing with methyl ethyl ketone-isopropanol-6.5N ammonium hydroxide
solution (80:20:30 v/v) and visualizing with sodium nitroprusside spray\textsuperscript{BS}, indicated that this material was composed of two major components ($R_f$ 0.49, 0.62).

Reduction of Aldol. Isolation of 1,3-Butanediol.

Aldol (1.7 gm.) was dissolved in water (10 ml.) and the solution added to an ice chilled solution of sodium borohydride (420 mg.) in water (10 ml.). The mixture was allowed to stand overnight at room temperature, then acidified to pH 1.0 with 5\% hydrochloric acid solution. The solution was neutralized to pH 7.0 with 2N sodium hydroxide solution, diluted to 200 ml. with water, saturated with sodium chloride, and subjected to continuous extraction with ethyl acetate for 24 hours. The organic phase was dried ($\text{Na}_2\text{SO}_4$), filtered, and the filtrate evaporated \textit{in vacuo} to dryness yielding 0.8 gm. (46.0\%) of 1,3-butandiol. Infrared analysis (neat) indicated the presence of some boric acid.

Preparation of 1,3-Butanediol-bis-phenylurethane.

Dry toluene (15 ml.) containing phenylisocyanate (2.20 gm., redistilled) was added to the butanediol obtained from the borohydride reduction. The solution was refluxed one hour, cooled, and the volatile components removed by reduced pressure distillation. The residual oil was dissolved in chloroform (5 ml.) and the solution filtered, washing through with an additional portion of chloroform. Hexane was added to the cloud point followed by refrigeration. The product oiled out of solution. The solvents were evaporated \textit{in vacuo}. Thin
layer analysis of the residue on silica gel, developing with chloro-
form-ether (4:1 v/v) and visualizing with iodine vapors, indicated
the presence of several minor impurities. The residue was column
chromatographed on silica gel (75 gm.), eluting with chloroform-ether
(4:1 v/v) and collecting 3 ml-fractions. The product-containing
fractions were combined to yield 0.14 gm. (4.7%) of the bis-phenylure-
thane, m.p. 118-119°C, lit86. 118.5-119°C (from 80% aqueous ethanol).
The infrared spectrum was identical to authentic material prepared
from commerically obtained 1,3-butanediol. Lukes et al.87 reported
preparation of L(+)-1,3-butanediol-bis-phenylurethane from L(+)-β-
hydroxybutyric acid by lithium aluminum hydride reduction and treatment
of the diol with phenylisocyanate.

Periodate Oxidation of Dihydrorspectinomycin. Attempted Isolation
of 1,3-Butanediol.

Anhydrous dihydrospectinomycin dihydrochloride (4.07 gm.) was
added to 0.05M sodium metaperiodate solution (1.6 l.) and the mixture
was allowed to stand at room temperature in a dark cupboard. The
consumption of periodate was monitored by Fleury-Lange titration.64
After 94 hours, 7.0 moles of periodate per mole of dihydrospectinomycin
had been consumed. Barium chloride (17.7 gm.) was added to the reaction
mixture and the precipitate removed by filtration. Sodium sulfate
(0.86 gm., anhydrous) was added to the filtrate and the precipitated
barium sulfate removed by filtration. The filtrate was adjusted to
pH 8.0 with 20% sodium hydroxide solution, sodium borohydride (6.0 gm.)
was added, and the mixture was allowed to stand five hours at room
temperature. On addition of the sodium hydroxide solution, a white
precipitate formed. The precipitate was removed by filtration and dried. The infrared spectrum of this material was devoid of significant absorptions over the range 625-4000 cm\(^{-1}\). The reaction mixture was acidified to pH 3.0 with concentrated hydrochloric acid, then neutralized to pH 7.0 with 20% sodium hydroxide solution. The solution was saturated with sodium chloride and subjected to continuous extraction with ethyl acetate for 24 hours. The organic phase was dried (Na\(_2\)SO\(_4\)), filtered, and evaporated to dryness in vacuo. Infrared analysis of the residue indicated only the presence of boric acid.

The aqueous phase was acidified to pH 3.0 with concentrated hydrochloric acid, heated to 91°C for 20 minutes, cooled, neutralized, and extracted continuously with ethyl acetate for 24 hours. The organic phase was dried (Na\(_2\)SO\(_4\)), filtered, and evaporated in vacuo to dryness. Infrared analysis of the residue indicated only the presence of boric acid.
SUMMARY

Spectinomycin is an aminocyclitol antibiotic which is derived from D-glucose. D-Galactose and acetate were not significant precursors of spectinomycin, although addition of D-galactose to the fermentation media stimulated antibiotic production. The C-methyl group and carbon 4a of spectinomycin are derived from carbons 6 and 2 of D-glucose, respectively. These observations suggested that the actinospectose moiety of spectinomycin is a 4,6-dideoxyhexose derived from the intact D-glucose molecule. The N-methyl groups of spectinomycin are derived from methionine; however, the origin of the nitrogen atoms was not investigated. Studies with labeled D-glucose and myo-inositol demonstrated that the carbon skeleton of the actinamine moiety is derived from D-glucose. These studies suggested that D-glucose is cyclized to myo-inositol which is biologically transformed into the actinamine moiety. The low incorporation of actinamine into the antibiotic suggested that actinamine is not a direct precursor. The results of this study support the accepted structure for spectinomycin and suggest something of the biosynthetic sequence. D-Glucose appears to be converted to actinospectose and to 1,3-myoinosadiamine, which are joined to form desmethylspectinomycin. Methylation of the amino groups by methionine yields spectinomycin. Suggestions for further study of the biosynthesis are presented.
APPENDIX A

Liquid Scintillation Counting Systems

Scintillation Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPO</td>
<td>5.0 gm.</td>
</tr>
<tr>
<td>Dimethyl POPOP</td>
<td>0.2 gm.</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>400.0 ml.</td>
</tr>
<tr>
<td>Toluene</td>
<td>600.0 ml.</td>
</tr>
</tbody>
</table>

Scintillation Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPO</td>
<td>5.0 gm.</td>
</tr>
<tr>
<td>Dimethyl POPOP</td>
<td>0.1 gm.</td>
</tr>
<tr>
<td>Toluene</td>
<td>1000.0 ml.</td>
</tr>
</tbody>
</table>

Cab-O-Sil (0.7 gm./15 ml.) was added to the contents of each vial for suspension counting of BaCO₃.
APPENDIX B

Kuhn-Roth Oxidizing Mixture

1) Dissolve 16.8 gm. of A. R. chromic anhydride in water (100 ml.) and cool.

2) Cautiously add concentrated sulfuric acid (20 ml.) with stirring and cooling.
BIBLIOGRAPHY


42. P. F. Wiley and H. Hoeksema, U. S. Pat. 3,176,043.


