Enzymic Studies on the Biosynthesis of Streptomycin

TRANSMIDINATION OF INOSAMINE AND STREPTAMINE DERIVATIVES*

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SUMMARY

1. Heat extracts of mature mycelia of streptomycin-producing strains of Streptomyces contain two compounds which, when incubated with arginine-guanidino-14C plus a crude enzyme preparation from S. griseus ATCC 12475 containing amidinotransferase, give products detected as radioactive Peaks I and II. Heat extracts from S. bluensis var. bluensis, which synthesizes bluensomycin, a monoguanidinated analogue of streptomycin, give only Peak I. A number of Streptomyces strains which do not produce streptomycin and which normally give neither Peak I nor Peak II give Peak II after exposure during growth to added streptomycin and which normally give neither Peak I nor Peak II after exposure during growth to added streptomycin. Evidence is presented that Peak I is N-amidino-scyllo-inosamine-P and Peak II is streptidine-P.

2. Purified amidinotransferase preparations from derepressed S. griseus ATCC 12475 and S. bikiniensis, both streptomycin producers, and a crude enzyme preparation from S. bluensis catalyze the following transamidinations with chemically phosphorylated acceptor compounds: arginine : scyllo-inosamine-P, arginine : streptamine-P, and arginine : 2-deoxystreptamine-P. In each case only one amino group is transamidinated, with the respective formation of N-amidino-scyllo-inosamine-P, N-amidinostreptamine-P, and N-amidino-2-deoxystreptamine-P. Location of the single phosphate group is unknown. Treatment with alkaline phosphatase gives the corresponding dephosphorylated derivatives.

3. Crude enzyme preparations from S. griseus and S. bikiniensis catalyze incorporation of radioactivity from arginine-guanidino-14C into streptidine-P after a lag period which is eliminated following preincubation in the absence of arginine. Preincubation presumably transforms streptidine-P into the actual amidine acceptor, which can now react more rapidly with purified amidinotransferases and the S. bluensis enzyme.

4. A scheme for biosynthesis of the streptidine moiety of streptomycin, the bluensidine moiety of bluensomycin, and the deoxystreptamine moieties of kanamycin and neomycin is presented.

In contrast to enteric bacteria, which excrete very little material (1), the more complex Streptomyces strains synthesize and excrete a variety of unusual compounds during specific stages in their life cycles (2, 3). Certain of these compounds, the antibiotics, modulate metabolic processes in sensitive cells in a manner reminiscent of the inter- and intracellular control processes responsible for differentiation and function of higher organisms. One might speculate that a Streptomyces strain selected for high antibiotic production is actually a variant capable of increased formation of one of its physiological regulator compounds, with a decreased sensitivity to the regulatory action of that compound. A detailed study of the formation of an antibiotic may therefore shed light on a number of fundamental biological problems.

Streptomycin is a basic oligosaccharide antibiotic (4) and Fig. 1) with interesting regulatory properties (7, 8). Only fragmentary knowledge is available concerning the metabolic pathways specifically involved in streptomycin biosynthesis. Experiments in vivo with radioactive compounds have helped delineate the problem by showing that carbon dioxide and the guanidino carbon of arginine are preferentially incorporated into the guanidino carbons of the streptidine moiety (9, 10); the N-methyl group of the L-glucosamine moiety is derived from methionine (11); and exogenous myo-inositol can serve effectively as a precursor of the ring carbons of the streptidine moiety (12-14). However, further progress on this problem has awaited the development of suitable cell-free systems. Recently we reported the detection in cell-free mycelial extracts of trace amounts of compounds believed to be phosphorylated precursors of streptomycin (15). The purpose of this paper is to present the results of experiments designed to characterize further these compounds, and to relate our findings to the biosynthesis of streptomycin and its monoguanidino analogue, bluensomycin (16, 17).

METHODS

 Cultures of most of the Streptomyces were obtained from the American Type Culture Collection. Bluensomycin sulfate and n-Arginine-guanidino-14C came from Calbiochem. Streptomycetes cultures were grown and enzyme extracts were made and assayed for amidinotransferase activity as described previously (18). The procedure for purifying amidinotransferase 25-fold from derepressed mycelia of S. bikiniensis was described (19). The procedure for purifying amidinotransferase 25-fold from derepressed mycelia of S. bikiniensis was described (19). The procedure for purifying amidinotransferase 25-fold from derepressed mycelia of S. bikiniensis was described (19). The procedure for purifying amidinotransferase 25-fold from derepressed mycelia of S. bikiniensis was described (19). The procedure for purifying amidinotransferase 25-fold from derepressed mycelia of S. bikiniensis was described (19). The procedure for purifying amidinotransferase 25-fold from derepressed mycelia of S. bikiniensis was described (19).
Streptidine sulfate was prepared by treatment of streptomycin or dihydrostreptomycin with $1 \text{ N H}_2\text{SO}_4$ for 3 days (4) and recrystallized from hot aqueous solution. Streptamine sulfate (4) was prepared by refluxing for 25 hours a mixture of 25 g of streptidine sulfate and 1250 ml of saturated $\text{Ba(OH)}_2$. A solution of $1 \text{ N H}_2\text{SO}_4$ was slowly added to the hot mixture, with stirring, until the pH reached 5 to 6 (about 415 ml required). The mixture was filtered while still hot. To the chilled filtrate 0.4 volume of acetone was added and the suspension was filtered under suction. The precipitate was washed three times with a total of 30 ml of cold water, washed with acetone, and dried (yield, 11.5 g).

Kanamycin was selected as the source of 2-deoxystreptamine because it is more easily hydrolyzed (19) than is neomycin (20). Vials containing 1 g, as kanamycin, of kanamycin sulfate in 3 ml of water plus preservatives were purchased locally (Kantrex, Bristol Laboratories). To 0.6 ml of this commercial preparation were slowly added, with stirring, 2.2 g of $\text{Ba(OH)}_2$; after standing for 15 min the mixture was centrifuged. Saturated $\text{Ba(OH)}_2$ solution was added to the supernatant solution plus washings until no more precipitate formed. Following centrifugation the supernatant solution (25 ml) was refluxed with an equal volume of concentrated $\text{HCl}$ for 75 min. The hydrolysate was treated with acid-washed charcoal, filtered, and concentrated in a desiccator over NaOH pellets. Ethanol plus a small amount of methanol was added slowly until turbidity appeared. On chilling, an oil formed which was converted to a precipitate by the addition of methanol. The precipitate was washed twice with cold 75% methanol and then acetone. Yield of deoxystreptamine dihydrochloride (21) was 560 mg.

Chemical phosphorylation was carried out by the nonspecific method of Pflummer and Burch (22). This method proved effective for phosphorylating streptidine, streptamine, 2-deoxystreptamine, and $N\text{-methyl-L-}

\text{glucosamine}$. Pure or in mixtures. Moreover, streptidine-P could be obtained by direct treatment of dihydrostreptomycin sulfate, and deoxystreptamine-P from direct treatment of dried commercial kanamycin sulfate. Formation of streptidine-P will be used as an example. To 5 g of streptidine sulfate in a 50-ml round bottom flask were added 10 ml of concentrated $\text{H}_2\text{PO}_4$ and, with a minimal exposure to the atmosphere, 5 g of $\text{OsO}_4$. A loosely stoppered reflux condenser was immediately joined to the flask. The mixture was heated on a steam bath for 6 hours and left at room temperature overnight. A hot saturated solution of $\text{Ba(OH)}_2$ was added to the mixture, with thorough stirring, to a slightly alkaline pH, and the mixture was filtered. The precipitate was washed several times with hot water. The original filtrate and each wash were frozen for subsequent assay; all proved to have activity. A preparation containing scyllo-inosamine-P did not give an active preparation.

Results

Our experimental approach involved a search for the physiological substrate of an amidinotransferase found in strains of *Streptomyces* which produce streptomycin, since there was strong circumstantial evidence that such a substrate would be a specific precursor of streptomycin (18). Amidinotransferase activity can be detected by certain enzymic half-reactions common to both bacterial arginine:X amidinotransferases and animal arginine:glycine amidinotransferase (EC 2.1.4.1) (23). In most cases arginine or a derivative is the physiological amidine donor.

Reaction 1 shows enzymic activation of the amidino group and formation of an enzyme-amidine intermediate, originally termed "active urea" (24, 25), which can be trapped with hydroxylamine (26) to give hydroxycyanidine (Reaction 2).

**Arginine + enzyme + ornithine + enzyme-C(=NH)NHz (1)**

**Enzyme-C(=NH)NHz + HO-NH2 -+ enzyme + HO-NH-C(=NH)NHz (2)**

**Enzyme-C(=NH)NH2 + X -+ enzyme + X-C(=NH)NH2 (3)**

**X-C(=NH)NH2 -++++ streptomycin (4)**

Reactions 3 and 4 depict the postulated role of the unknown physiological substrate of *Streptomyces* amidinotransferase.

**Biological Distribution of Substrates of *S. griseus* Amidinotransferase**—Our first successful experiments indicated the occurrence of not one, but two, physiological substrates of *S. griseus* amidinotransferase. Two radioactive compounds, designated Peaks I and II, are formed on incubation of L-arginine-guanidino-14C with heat extracts of mature mycelia of *S. griseus* ATCC 12475, in the presence of a crude preparation of arginine:X amidinotransferase (15). Similar results are obtained with heat extracts of *S. bikiniensis* ATCC 11062 (Fig. 2). Suitable control experiments established that these compounds are formed by transamidination reactions specific for certain *Streptomyces* strains, e.g. in the incubation mixture dl-arginine-5-14C and 14C-urea could not replace L-arginine-guanidino-14C, pancreatic arginine:glycine amidinotransferase could not replace *S. griseus* amidinotransferase, and the reaction was inhibited by specific inhibitors of transamidination, such as ornithine and cysteamine (15, 18).

Heat extracts from a number of strains of *Streptomyces* were tested for the accumulation of these substrates of amidinotransferase. In general, substrates of amidinotransferase were de-
Fig. 2. Characteristic radioactive peaks (I and II) obtained on incubation of L-arginine-guanidino-\(^{14}C\) with heat extract of 3-day mycelia of *S. bikiniensis* for 2 hours in the presence of a dialyzed amidinotransferase preparation from 2-day mycelia of *S. griseus* ATCC 12475. *S. bikiniensis* was grown on a 3% myo-inositol-2% peptone-0.1% yeast extract medium. Ordinate represents radioactivity for 20 \(\mu\)l of reaction mixture; separation was by ascending paper chromatography with 80% phenol-20% water, \(\text{NH}_3\) atmosphere. Peak II is characterized by this solvent system, with a shape determined in part by migration of other reaction mixture components (cf. Fig. 3B).

Fig. 3. Curves showing ability of various mycelial heat extracts and synthetic preparations to serve as amidine acceptors from L-arginine-guanidino-\(^{14}C\), when incubated for 2 hours with a dialyzed amidinotransferase preparation from *S. griseus* ATCC 12475. Ordinates represent radioactivity, usually for 20 \(\mu\)l of reaction mixture; separation was by ascending paper chromatography with aqueous phenol, \(\text{NH}_3\) atmosphere. A, curve showing formation of radioactive compound in Peak 1 position with a heat extract of 2-day vegetative mycelia of *S. bluenos* var. *bluenos* grown on 1% myo-inositol-2% peptone-0.1% yeast extract medium. Note the absence of Peak II activity in this organism which synthesizes bluensomycin. B, curves showing effect of prior exposure of mycelia to streptidine on formation of Peak II with heat extracts of mycelia of a non-streptomycin-producing strain, *S. griseus* ATCC 10971. This strain also lacks amidinotransferase activity (18). Mycelia were grown on 2% peptone-0.1% yeast extract medium for 3 days. The solid curve shows the effect of adding streptidine sulfate (SD, 1 mg per ml) to the growth medium 3 hours before harvest. Only Peak II, and no Peak I, is formed. A number of other *Streptomyces* strains show similar behavior. C, curves showing ability of chemically phosphorylated streptidine, isolated by Dowex 50(H\(^+\)) column chromatography (cf. Fig. 4), to incorporate radioactive activity from labeled arginine. Dashed curve: pattern obtained from spotting 10 \(\mu\)l of reaction mixture; note that some of the radioactivity is left at the origin. Solid curve: pattern obtained following treatment of reaction mixture with dilute HCl and spotting. D, curve showing ability of chemically phosphorylated streptidine to serve as an amidine acceptor. E, curve showing ability of a chemically phosphorylated *scyllo*-inosamine preparation to serve as an amidine acceptor. The source of *scyllo*-inosamine was bluensomycin sulfate successively subjected to alkaline and acid hydrolysis. When the alkaline hydrolysis step was omitted, no radioactive peak was formed; the curve (not shown) remained at the origin. F, curve showing ability of chemically phosphorylated 2-deoxystreptamine to serve as an amidine acceptor.

Attempts were made to stimulate preferential accumulation of one of the substrates by adding various compounds and inhibitors to the growth media. Compounds such as glucosamine, streptomycin, and streptamine had no effect. The only effective compound of the large number tested was streptidine. Streptidine sulfate (1 mg per ml) added to the growth media after 3 days of growth caused the accumulation of large amounts of...
streptidine. One such strain is S. griseus ATCC 10971 (Fig. 3B). Peak I does not appear along with Peak II in these latter strains, nor does streptidine induce the appearance of amidinotransferase activity. This procedure proved extremely useful for providing a natural source of one of the amidinotransferase substrates (II), uncontaminated by the other (1).

Separation of Peaks I and II by Column Chromatography—In our early experiments, Peaks I and II were separated by thin layer chromatography on powdered cellulose, with the same solvent system (80% phenol-20% water, ammonia atmosphere) used for their detection on paper chromatograms (10). By this means enough material was obtained to determine that both I and II were phosphorylated derivatives (15). A more suitable column separation was next devised as shown in Fig. 4; here the source of substrates was a heat extract of S. kanamyceticus. Column fractions were combined and evaporated to dryness in vacuum desiccators over NaOH pellets. Although I and II are completely hydrolyzed by 6 N HCl at 100° in 24 hours, they appear relatively stable to lower acid concentrations at room temperature. The isolated compounds behaved similarly to those isolated by the thin layer technique; however, the possibility of some migration of the phosphate groups during column isolation cannot be excluded.

Characterization of Peak II—Paper chromatographic experiments established that neither Peak I nor Peak II was streptomycin or its guanidino-containing component, streptidine. Their mobilities were consistent with either more acidic compounds or compounds of much higher molecular weight. Mild acid treatment did not change the \( R_f \) values, but heating with 6 N HCl at 100° for 20 hours transformed each substance into a derivative of higher \( R_f \) value in the ammoniacal phenol solvent. Peak I was converted to Peak III (\( R_f \) 0.63) and Peak II to Peak IV (\( R_f \) 0.90). Incubation of Peaks I and II with purified alkaline phosphatases from *Escherichia coli* (Sigma Type III) and calf mucosa (Sigma Type VI), or acid phosphatase preparations from wheat germ and potato, gave similar results. It therefore appeared probable that Peaks I and II were phosphorylated derivatives of Peaks III and IV, respectively. It was noted that some migration of the phosphate groups during column isolation cannot be excluded.

Confirmation was obtained by synthesis of phosphorylated streptidine, with the use of concentrated \( H_3P_0_4 \) plus \( P_2O_5 \). The reaction mixture contained an active substrate of amidinotransferase (Fig. 3C), which proved to be streptidine-P (27). Appreciably 82 mg of a fraction containing the active synthetic compound was isolated by column chromatography and dephosphorylated. To\( 200 \mu \text{l} \) of the product (554,000 cpm) were added 100 \( \mu \text{l} \) of 4 N KOH plus 5-mg aliquots of S-methylisothiouronium sulfite added at zero time, and after 24 and 72 hours. After 11 days the reaction mixture was crystallized with authentic streptidine as the dipicrate and counted (27).

<table>
<thead>
<tr>
<th>Crystallization</th>
<th>Specific activity cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1a</td>
<td>Experiment 1b</td>
</tr>
<tr>
<td>1st</td>
<td>362</td>
</tr>
<tr>
<td>2nd</td>
<td>284</td>
</tr>
<tr>
<td>3rd</td>
<td>281</td>
</tr>
<tr>
<td>4th</td>
<td>278</td>
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<tr>
<td>5th</td>
<td>222</td>
</tr>
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<td>6th</td>
<td>212</td>
</tr>
<tr>
<td>7th</td>
<td>211</td>
</tr>
<tr>
<td>8th</td>
<td>208</td>
</tr>
</tbody>
</table>

* Dephosphorylated Peak II from streptidine-treated mycelia of non-streptomycin-producing *S. griseus* strain.
* Dephosphorylated transamidination product with streptamine-P as acceptor, following treatment with S-methylisothiourea.

Peak IV had a mobility similar to that of streptidine. The identity of Peak IV and streptidine was supported by repeated crystallization of Peak IV with authentic streptidine as the dipicrate derivative to constant specific activity (27).

The question next arose as to the relationship between the Peak II which occurs in streptomycin-producing strains and the Peak II which accumulates in nonproducing strains in the presence of added streptidine (Fig. 3B). Column isolation, followed by treatment with alkaline phosphatase and crystallization with authentic streptidine as the dipicrate, indicated that Peak II preparations from both sources are phosphorylated derivatives of streptidine (Table I).

Confirmation was obtained by synthesis of phosphorylated streptidine, with the use of concentrated \( H_3P_0_4 \) plus \( P_2O_5 \). The reaction mixture contained an active substrate of amidinotransferase (Fig. 3C), which proved to be streptidine-P (27). Approximately 82 mg of a fraction containing the active synthetic compound was isolated by column chromatography. This preparation was soluble at acid pH, but relatively insoluble at neutral and alkaline pH. The purer preparations were difficult to work with for this reason, and a portion of this portion was crystallized with authentic streptidine as the dipicrate. The isolated Peak II preparations showed their identity. Column fractions were 4 ml each; 5 \( \mu \text{l} \) were spotted on paper and counted.
Table II

<table>
<thead>
<tr>
<th>Radioactive preparation</th>
<th>Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1, pH 3.6</td>
<td></td>
</tr>
<tr>
<td>Peak I</td>
<td>-13</td>
</tr>
<tr>
<td>Peak II</td>
<td>-4.5</td>
</tr>
<tr>
<td>Streptidine-P (synthetic)</td>
<td>-13.5</td>
</tr>
<tr>
<td>N'-Amidoterpamine-P</td>
<td>-4.5</td>
</tr>
<tr>
<td>Experiment 2, pH 3.6</td>
<td></td>
</tr>
<tr>
<td>Peak I</td>
<td>-13</td>
</tr>
<tr>
<td>Peak II</td>
<td>-13</td>
</tr>
<tr>
<td>N'-Amidoterpamine-P</td>
<td>-13.5</td>
</tr>
<tr>
<td>N'-Amidoterpamine-P treated with HNO₂</td>
<td>-13</td>
</tr>
<tr>
<td>N'-Amidino-2-deoxyterpamine-P</td>
<td>-14</td>
</tr>
<tr>
<td>Pieric acid</td>
<td>+14</td>
</tr>
</tbody>
</table>

Characterization of Peak I—Its behavior on paper chromatograms and ion exchange resins (Fig. 4) indicated that Peak I was less basic than Peak II. High voltage paper electrophoresis experiments at acid and alkaline pH values have supported this concept (Table II). The migratory properties of Peak I are consistent with the presence of a single guanidino group and 1 phosphate group per molecule. If Peak I has only one guanidino group and it is a precursor of streptomycin, it is probably also a precursor of streptidine-P. We have, of course, always been aware of the possibility that one or both of the peaks are not on the direct pathway of streptomycin biosynthesis.

Electrophoresis experiments indicated that Peak I did not have a free amino group. However, further data were sought to clarify this point. Streptamine was phosphorylated in the same manner as streptidine and tested as a substrate for amidotransferase. Streptamine-P proved to be an excellent substrate, with an $R_f$ between Peaks I and II (Fig. 3D). The reaction product was eluted from Dowex 50 (H⁺) columns with 2 N HCl at approximately the same position as streptidine-P. Treatment of the isolated product with phosphatase gave a new compound which had an $R_f$ of 0.78, between that of Peak III and streptidine, and did not recrystallize with streptidine dipicrate (27). It therefore appeared that only one of the amino groups of streptamine-P received an amidino group. This was supported by the electrophoretic mobility of the radioactive phosphorylated product at various pH values, and particularly at pH 10.4, at which the amino group would not be charged (Table II). Confirmation was obtained by converting the radioactive dephosphorylated monooamidino derivative to streptidine with S-methylisothioura and crystallizing as the dipicrate with authentic streptidine (Table I). We were never able to form streptidine-P or streptidine enzymically from streptamine-P, despite the use of longer incubation times and higher concentrations of arginine. These results suggest that the immediate precursor of radioactive streptidine-P and the transamination product of streptamine-P are not identical.

At this point it appeared that a monooamidinoamines-P was a rather likely candidate for Peak I. Subsequent findings have supported this conjecture. Treatment of the monooamidinostreptamine-P preparation with nitrous acid gave a product which migrated to the same position as Peak I during paper electrophoresis, as would be expected if a primary amidino group was converted to a hydroxyl group (Table II). Treatment of streptamine with half the theoretical amount of nitrous acid, followed by phosphorylation and testing as a substrate, gave inconclusive results, possibly due to inversion of configuration. Our attention was next focused on a natural product, the antibiotic bluensomycin, which contained the desired moiety, N-amidino-scyllino-samine (16, 17). Treatment of bluensomycin with alkaline hydrolysis gave a product which migrated to the same position as streptidine-P during paper electrophoresis, as would be expected if a primary amidino group was converted to a hydroxyl group (Table II). Omission of the alkaline hydrolysis step gave negative results; this indicates that removal of the amidino group is required for activity. Behavior of the product on paper chromatograms and Dowex 50 (H⁺) columns was similar to that of Peak I. Its dephosphorylation product had the same $R_f$ (0.63) as the dephosphorylation product of Peak I. Since Bannister and Argoudelis (16, 17) have established that bluensidine is a derivative of scylyno-samine, we consider this good evidence that Peak I is an O-phosphoryl-N'-aminostreptamine-P.
streptidine in lacking an hydroxyl group between the two amino groups, and thus phosphate cannot be esterified at that position. Deoxystreptamine-P was prepared by treating either intact kanamycin sulfate, or deoxystreptamine dihydrochloride isolated from acid-hydrolyzed kanamycin, with concentrated H₃PO₄ plus P₂O₅. A component of the reaction mixture proved to be an active substrate for amidinotransferase (Fig. 3F). The enzyme has thus indicated one position at which the phosphate group is not located; however, this position is not ruled out for a possible second transamidination reaction. N-Amidino-2-deoxystreptamine-P has a slightly higher Rp than streptidine-P and is eluted at a similar position from Dowex 50 (H⁺) columns. Its dephosphorylation product has an Rp of 0.92 in the ammoniacal phenol solvent.

**Question of One or Two Amidinotransferases**—Streptomyces has two guanidino groups (29). Our data show that at least one group is synthesized by a transamidination reaction. If both groups undergo transamidination, the question arises whether one or two amidinotransferases are involved. When the source of amidinotransferase activity is a crude extract of *S. griseus* ATCC 12475 or *S. bikiniensis*, the pattern of formation of radioactive peaks depends entirely upon the nature of the heat extract, and not on the source of enzyme. Enzyme preparations from both sources react with scyllo-inosamine-P, streptamine-P, deoxystreptamine-P, and streptidine-P. Purification of amidinotransferase activity 15- to 25-fold over that found in derepressed mycelia (18) of *S. bikiniensis*, to an activity of 15 to 25 pmoles of deoxystreptamine-P, and streptidine-P. Purification of amidinotransferase activity 15- to 25-fold over that found in derepressed mycelia (18) of *S. bikiniensis*, to an activity of 15 to 25 μmoles of hydroxyguanidine formed from arginine plus hydroyxylamine per hour per mg of protein, results in a marked decrease in ability to incorporate radioactivity into streptidine-P. Activity of the purified enzyme with the other substrates is not impaired. Attempts to recombine fractions and restore normal rates of formation of radioactive streptidine-P have so far been unsuccessful. It is of particular interest that unpurified extracts of *X. bluensis* have a substrate specificity similar to that of the purified *S. bikiniensis* preparation.

All amidinotransferase preparations, crude or purified, from all three strains of *Streptomyces*, including *S. bluensis*, can catalyze weak transamidinations with radioactive streptidine-P as the amidine donor and glycylglycine as the acceptor. Reversibility of transamidinations with the monoamidinated inosamine-P derivative has not yet been demonstrated. However, amidinotransferase activity 15- to 25-fold over that found in derepressed mycelia (18) of *S. bikiniensis*, to an activity of 15 to 25 μmoles of hydroxyguanidine formed from arginine plus hydroyxylamine per hour per mg of protein, results in a marked decrease in ability to incorporate radioactivity into streptidine-P. Activity of the purified enzyme with the other substrates is not impaired. Attempts to recombine fractions and restore normal rates of formation of radioactive streptidine-P have so far been unsuccessful. It is of particular interest that unpurified extracts of *S. bluensis* have a substrate specificity similar to that of the purified *S. bikiniensis* preparation.

**DISCUSSION**

Prior to the experiments reported here, arginine-X amidinotransferase from *Streptomyces* was an enzyme the function of which was only suspected, not known (18). The situation with respect to streptomycin biosynthesis was similar to that of protein synthesis a decade ago; amino acid-activating enzymes had been detected by means of a radioactive exchange reaction analogous to Reaction 1, or by use of hydroxylamine as a non-physiological trapping reagent, similar to Reactions 1 plus 2. With these half-reactions as probes, the physiological acceptors of the activated aminoscyI groups, the soluble ribonucleic acids, were eventually discovered (30). Similarly, we have looked for a natural acceptor of the enzyme-activated amidino group of ar-
Biosynthesis of Streptomycin

Glucose-6-P

\[ \text{Exogenous myo-inositol} \stackrel{B}{\longrightarrow} \text{myo-inositol(P)} \stackrel{C}{\longrightarrow} \text{scyllo-inosose(P)} \]

Bluensidine(P) \[ J \rightarrow \text{N-amidino-scyllo-inosamine-P} \leftarrow \text{scyllo-inosamine-P} \]

Bluensomycin \[ F \rightarrow \text{N-amidinostreptamine-P} \rightarrow \text{streptamine-P} \rightarrow \text{2-deoxystreptamine(P)} \]

Exogenous streptidine \[ I \rightarrow \text{streptidine-P} \rightarrow \text{streptomycin} \rightarrow \text{kanamycin, neomycin} \]

\( \text{(NDP-streptose, NDP-N-methyl-L-glucosamine)} \)

Fig. 6. Suggested metabolic relationships of various inosamine derivatives involved in biosynthesis of streptomycin, bluensomycin, kanamycin, and neomycin. NDP, nucleoside diphosphate.

ginine (Reaction 1), believing that such an acceptor would be a precursor of streptomycin (Reactions 3 plus 4).

However, not one, but several acceptors of the amidino group have been found. We have presented evidence that crude or purified amidinotransferase preparations from \( S. \) griseus ATCC 12475, \( S. \) bikinensis, and \( S. \) bluensis var. bluensis can catalyze Reactions 5 to 7.

\[
\text{Arginine + scyllo-inosamine-P} \rightarrow \text{ornithine + N-amidino-scyllo-inosamine-P} \quad (5)
\]

\[
\text{Arginine + streptamine-P} \rightarrow \text{ornithine + N-amidinostreptamine-P} \quad (6)
\]

\[
\text{Arginine + 2-deoxystreptamine-P} \rightarrow \text{ornithine + N-amidino-2-deoxystreptamine-P} \quad (7)
\]

Crude enzyme preparations from \( S. \) griseus ATCC 12475 and \( S. \) bikinensis can also catalyze the incompletely characterized over-all reaction shown in Reaction 8.

\[
\text{Arginine-guanidino-^{14}C} + \text{streptidine-P} \rightarrow \text{streptidine-P-guanidino-^{14}C} \quad (8)
\]

Purified amidinotransferase from \( S. \) bikinensis and unpurified enzyme preparations from \( S. \) bluensis are less active in catalyzing Reaction 8. Purified amidinotransferase from \( S. \) bikinensis and unpurified enzyme preparations from \( S. \) bluensis are less active in catalyzing Reaction 8.

We suggest that Reaction 5 represents the formation of naturally occurring Peak 1. Peak 11 has been identified as streptidine-P, and therefore it must be formed by transamidination of an \( N \)-amidinostreptamine-P derivative. The location of the single phosphate group of the various acceptors is not yet known, although the existence of Reaction 7 eliminates one potential site.

Our early term for the enzyme-amidine intermediate, “active urea,” (24, 25), has recently been given increased substance by Grazi, Coneoni, and Vigi (31) who obtained urea on heating an enzyme-amidine compound from a purified amidinotransferase preparation from hog kidney.

Only one amidino group is present in the products of Reactions 5 to 7. Neither streptidine-P nor streptidine is formed with synthetic streptamine-P as the acceptor (Reaction 6), regardless of the arginine concentration or duration of incubation. Reactions 5 to 7 proceed without a lag period (Fig. 5A), indicating that these compounds are the actual receptors. Reaction 8 has a lag period which can be eliminated by preincubation with a crude dialyzed \( S. \) bikinensis enzyme preparation prior to the addition of arginine (Fig. 5B). This lag period is apparently required for formation of the actual acceptor. The question of whether the amidino group newly incorporated into streptidine-P by Reaction 8 is at the same position involved in Reaction 5 has not been resolved. Since several compounds can serve as amidino acceptors, it is difficult to assign a precise name to this amidinotransferase. One designation might be \( L \)-arginine: \( scyllo \)-inosamine-P amidinotransferase; an alternative is \( L \)-arginine: \( N \)-amidinostreptamine-P amidinotransferase. Amidinotransferases have recently been shifted from the EC 2.6.2 class to the EC 2.1.4 class, since a 1 carbon group is the moiety transferred.

In order to provide a framework for further discussion and future experiments, a tentative scheme for the biosynthesis of streptomycin and related basic oligosaccharide antibiotics is given in Fig. 6. Certain of the suggested steps warrant additional comment.

Step A—Chen and Charalampous (32) have recently demonstrated that glucose-6-P is converted to \( myo \)-inositol-1-P, followed by dephosphorylation to \( myo \)-inositol.

Step B—Exogenous \( myo \)-inositol favors accumulation of amidinotransferase substrates, although marked accumulation can occur in its absence. Heding (13) and Horner (14) have demonstrated that the streptidine ring carbons can be derived from \( myo \)-inositol added to culture media.

Step C—\( scyllo \)-Inosose can be formed by \( Acetobacter \) suboxydans ATCC 621 from \( myo \)-inositol (33, 34); however, the possibility exists that the reaction is also undergone by their phosphorylated derivatives in \( Streptomycetes \). Incorporation of radioactivity from \( myo \)-inositol-2-\( ^{3} \)H argues against a simple conversion (13).
The chemistry of inosamines, inososes, and inositols has been re-
lied now that a number of radioactive precursors are available.
manipulation. No evidence was obtained for an enzymic con-
may be a deciding factor, and this may be subject to enzymic
or that in Reaction 6. The position of the phosphate group
sion of configuration is probable (35).
acid. The deaminated compound has the electrophoretic
amidinat,ion reaction or is synthesized by two or more steps
amidino group of streptidine is normally derived from a trans-
Stage the aglycone, streptidine, loses its phosphate group. Al-
gated by Candy and Baddiley (38). It is not known at what
Biosynthesis of the streptose moiety has recently been investi-
rate of utilization. Excess streptidine-P may well precipitate
out in mycelia under certain conditions.
Step I—This step, of unknown physiological significance, oc-
curs in all strains of Streptomyces tested, including both strepto-
mycin producers and nonproducers (Fig. 3D), without an appreci-
able lag period. This reaction has not yet been observed in
mycelia from streptomycin producers.
Step M—This transamination occurs readily in vitro with
synthetic streptamine P (Fig. 3D), but the substrate has not yet
been observed to accumulate in mycelia and may not be a physi-
ological intermediate.
Step O—Added deoxystreptamine has been found to be in-
corporated into neomycin (39). The transamination shown in
Reaction 7 (cf. Fig. 3F) may actually occur in S. kanamyceticus.
This strain synthesizes kanamycin and under certain condi-
tions has amidotransferase activity, which would permit the bio-
synthesis of N-amidino-2-deoxystreptamine-P, and perhaps an
amidinated kanamycin. DiCuollo (40) has partially amidinated
kanamycin by chemical means and found the derivative to be
biologically active.
Step Q—Dehydration followed by amination is an attractive
possibility for this postulated conversion.
Future events will modify certain features of the proposed
scheme; its merit lies in suggesting experiments which need to be
done. Hopefully, further investigation will uncover some clue
to the physiological functions of streptomycin and its related
antibiotics.

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REFERENCES

1. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., and Britten, R. J., Studies of biosynthesis in Escher-
1962.
3. Miller, M. W. (Editor), The Pfzer handbook of microbial metabo-
4. LMEMIEUX, R. U., AND WOLPHROM, M. L., Advan. Carbohydrate
(1963).
9. Hunter, G. D., Herbert, M., AND Hockenhull, J. D. J.,
11. CANDY, D. J., Blumson, N. L., AND BADDILEY, J., Biochem.
12. MAJUMDAR, S. K., AND KUTZNER, H. J., Appl. Microbiol., 10,
157 (1962).
93, 201 (1964).
85, 234 (1963).
89, 475 (1964).
Y., Taylor, H. D., Whitehead, D. F., AND Hooper, I. R.,
395 (1927).
23. Walker, J. B., in G. E. W. Wolstenholme and M. P. Cam-
bridge (Editors), Goba Foundation Study Group on the Com-
parative Biochemistry of Arginine and Derivatives, Little,
97, 397 (1956).
(1925).
30. Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht,
2465 (1965).

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