Enzymic Studies on the Biosynthesis of Streptomycin

TRANSAMIDINATION 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 arginase: X amidinotransferase, give products detected as radioactive Peaks I and II. Heat extracts from S. bluensis var. bluensis, which synthesizes bluensomycin, a mono-guanidinated 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 streptidine. 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. bluensis, 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. bluensis catalyze incorporation of radioactive 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 Streptomycesstrain 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 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 agar eluate plus frozen vegetative mycelia of S. bluensis var. bluensis NRRL 2876 were kindly supplied by Dr. Brian Bannis-ter, The Upjohn Company. L-Arginine-guanidino-14C came from Calbiochem. Streptomyces 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. biki-

1 After this paper was submitted, evidence was presented that the l-streptose moiety is in the α, rather than β, configuration (6).
Streptidine sulfate was prepared by treatment of streptomycin or dihydrostreptomycin with 1 N H$_2$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 Ba(OH)$_2$. A solution of 1 N H$_2$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 acetonitrile 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 acetonitrile, 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 9 ml of this commercial preparation were slowly added, with stirring, 2.2 g of Ba(OH)$_2$; after standing for 15 min the mixture was centrifuged. Saturated 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 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 water 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 acetonitrile. Yield of deoxystreptamine dihydrochloride (21) was 500 mg.

Chemical phosphorylation was carried out by the nonspecific method of Plimmer and Burch (22). This method proved effective for phosphorylating streptidine, streptamine, 2-deoxystreptamine, and scyllino-insamine, 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 H$_2$PO$_4$ and, with a minimal exposure to the atmosphere, 5 g of K$_2$PO$_4$. 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 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 scyllino-insamine-P was obtained by heating a mixture of 250 mg of bluenosomycin, 0.5 ml water, and 2.0 ml of 2 N KOH at 100° for 6 hours; 2.5 ml of 12 N HCl were added and the mixture was heated for 2 hours at 100°. The mixture was dried, phosphorylated, treated with

![Diagram of streptomycin structure](http://www.jbc.org/)

**FIG. 1.** The structure of streptomycin (5). See also Footnote 1.

**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 hydroxyguanidine (Reaction 2).

Arginine + enzyme + ornithine + enzyme ++C(=NH)NH$_2$ (1)

Enzyme-C(=NH)NH$_2$ + HO-NH$_2$ →

enzyme + HO-NH$_2$-C(=NH)NH$_2$ (2)

Enzyme-C(=NH)NH$_2$ + X → enzyme + X-C(=NH)NH$_2$ (3)

X-C(=NH)NH$_2$ → 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-$^{14}$C 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 L-arginine-$^{15}$N and $^{14}$C-urea could not replace L-arginine-guanidino-$^{14}$C, 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-
tected only in strains possessing amidinotransferase activity: S. griseus ATCC 12475, S. griseus W4, S. biktinensis, and S. griseocarnicus ATCC 12628 (cf. Reference 18). However, S. kanamyceticus ATCC 12853, previously not believed to have amidinotransferase activity, was observed to accumulate substrates I and II after 3 or 4 days of growth and after any glucose in the medium was exhausted. Under these conditions amidinotransferase activity also appeared. For most strains of Streptomyces, and particularly S. kanamyceticus, the presence of myo-inositol in the growth medium enhanced accumulation of both substrates I and II (cf. Reference 12).

Systems Accumulating Only One of Two Peaks—Peak II was generally considerably higher than Peak I, except under certain conditions with S. biktinensis preparations. No evidence was obtained for the interconversion of the two peaks during incubation. As an aid in determining the relationship between Peaks I and II, we made an intensive search for systems in which only one of the peaks might be found. Since bluensomycin is a monoamino analogue of streptomycin (16, 17), S. bluenensis, the organism which synthesizes bluensomycin, was tested. A clean preparation of Peak I, with no Peak II, was obtained from heat extracts of mycelia of this organism grown in the presence of myo-inositol (Fig. 3A). Unfortunately, this organism proved not to be a reliable source, since neither amidinotransferase activity nor the ability to accumulate Peak I survived transfer of this strain on agar slants. Only vegetative cultures were active, and even that activity was soon lost.

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

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**Fig. 2.** Characteristic radioactive peaks (I and II) obtained on incubation of L-arginine-guanidino-\(^{14}\text{C}\) with heat extract of 3-day mycelia of S. biktinensis for 2 hours in the presence of a dialyzed amidinotransferase preparation from 2-day mycelia of S. griseus ATCC 12475. S. biktinensis was grown on a 3% myo-inositol-2% peptone-0.1% yeast extract medium. Ordinates represent radioactivity, usually for 20 \(\mu\)l of reaction mixture; separation was by ascending paper chromatography with 80% phenol-water, \(\text{NH}_3\) atmosphere. Peak II is characteristically broad with 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-\(^{14}\text{C}\), 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 80% phenol, \(\text{NH}_3\) atmosphere. A, curve showing formation of radioactive compound in Peak I position with a heat extract of 2-day vegetative mycelia of S. bluenensis var. bluenensis grown on 1% myo-inositol-2% peptone-0.1% yeast extract medium. Note the absence of Peak II activity in this organism which synthesizes a monoguanidinated analogue of streptomycin. 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 to serve as an amidine acceptor. The source of chemically phosphorylated streptidine, isolated by Dowex 50 column chromatography (cf. Fig. 4), to incorporate radioactivity from labeled arginine. Dashed curve: pattern obtained from spotting 10 \(\mu\)l of reaction mixture; note that some of the relatively insoluble material 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 streptamine to serve as an amidine acceptor. E, curve showing ability of a chemically phosphorylated scyllo-inosamine 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.

Peak II in all strains of Streptomyces tested. Accumulation reached a maximum after 3 hours. This accumulation was most dramatic in strains which lack amidinotransferase, do not produce streptomycin, and do not give either peak in the absence of
strep tidine. 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 (I).

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

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\text{Fig. 4. Separation of radioactive Peaks I and II following adsorption on a 200 to 400 mesh Dowex 50 (H\(^+\)) column, 1 \times 18 cm, by elution with HCl solutions. The heat extract was from S. kanamyceticus grown for 4 days on 1% glucose-1% peptone-0.1% yeast extract. Preparations from S. kanamyceticus grown on inositol rather than glucose have 5- to 20-fold greater amounts of Peaks I and II. The purpose of this experiment was to determine if peaks from S. kanamyceticus grown in the absence of myo-inositol are the same compounds; subsequent analysis of the isolated Peak II preparations showed their identity. Column fractions were 4 ml each; 5 \mu l were spotted on paper and counted.}
\]

<table>
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<th>Crystallization</th>
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<td></td>
<td>( \text{cpm/mg} )</td>
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<tr>
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\( ^a \) Dephosphorylated Peak II from streptidine-treated mycelia of non-streptomycin-producing \( S. \) griseus strain.

\( ^b \) Dephosphorylated transamidination product with streptamine-P as acceptor, following treatment with \( S. \) methylisothiourea sulfate.

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 \( \text{H}_4\text{PO}_4 \) plus \( \text{P}_2\text{O}_5 \). 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 often stayed at the origin of paper chromatograms (Fig. 3C). Addition of HCl to the reaction mixture before chromatography resulted in a normal migration.

Two methods were used to determine the number of phosphate groups on the streptidine ring. Direct analysis (28) of the iso-
Table II
High voltage paper electrophoresis of phosphorylated transamination products

A Savant horizontal plate apparatus and Whatman No. 1 paper were used. A negative number signifies centimeters of migration toward the negative pole, a positive number migration toward the positive pole. Experiments 1 and 2: ammonium formate buffer, pH 3.6, ionic strength 0.2, 800 volts, 2 hours. Experiment 3: glycine-NaOH buffer, pH 10.4, ionic strength 0.2, 800 volts, 2 hours.

<table>
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<tr>
<th>Experiment</th>
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<th>Migration</th>
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<td>1</td>
<td>3.6</td>
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</tr>
<tr>
<td>2</td>
<td>3.6</td>
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<td>Streptidine-P (synthetic)</td>
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<td>1</td>
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<tr>
<td>2</td>
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<td>N'-Amidinostreptamine-P treated with ( \text{HNO}_3 )</td>
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<td>-13.5</td>
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<td>N'-Amidino-2-deoxystreptamine-P</td>
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<tr>
<td>Pieric acid</td>
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</tr>
<tr>
<td>3</td>
<td>10.4</td>
<td>+8</td>
</tr>
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<td>4</td>
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<td>N'-Amidino-2-deoxystreptamine-P</td>
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<td>+18</td>
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Radioactive preparation and migration of synthetic fraction gave 1 phosphate residue per molecule. High voltage paper electrophoresis studies at various pH values also indicated that both the natural and synthetic phosphorylated streptamines had one phosphate group per molecule (Table II). Two positive charges would be contributed by streptidine at all pH values used. At pH 10.4 little or no migration occurs, which suggests that two negative charges from a phosphate group neutralize the two positive charges of the guanidino groups; this also accounts for the insolubility of streptidine-P in alkaline solutions.

At pH 3.6 each phosphate group contributes a single negative charge, and streptidine P with a net single positive charge migrates toward the cathode. Peak II is therefore an O-phosphoryl-N-amidino-scyllo-inosamine. How- ever, further data were sought to clarify this point. Streptidine was phosphorylated in the same manner as streptidine and tested as a substrate for amidinotransferase. Streptidine-P proved to be an excellent substrate, with an \( R_p \) 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 \( R_p \) 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 monoamidino 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 monoamidinoinosamine-P was a rather likely candidate for Peak I. Subsequent findings have supported this conjecture. Treatment of the monoamidino- streptamine-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-scyllo-inosamine (16, 17). Treatment of bluensomycin with alkali to hydrolyze the guanidino and carbamyl groups of the bluensomycin moiety (16), followed by acid treatment and phosphorylation gave a preparation which was an excellent substrate for amidinotransferases from \( S. \ griseus, S. \ bikinios, \) and \( S. \ bluenisi \) (Fig. 3E). 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 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...
streptamine 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 H$_3$PO$_4$ plus P$_2$O$_5$. 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 $R_p$ than streptidine-P and is eluted at a similar position from Dowex 50 (H$^+$) columns. Its dephosphorylation product has an $R_p$ of 0.92 in the ammoniacal phenol solvent.

**Question of One or Two Amidinotransferases**—Streptomyces has two guanidino groups (Fig. 1). 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 dispersed mycelia (18) of S. bikiniensis, to an activity of 15 to 25 $\mu$moles of deoxystreptamine-P, and streptidine-P. Purification of amidinotransferase activity 15- to 25-fold over that found in the extract of mycelia of S. griseus ATCC 10971 previously exposed to streptidine (Fig. 3B). The amidine acceptor for both curves was a heat extract of mycelia of S. griseus ATCC 10971 previously exposed to streptidine; note the lag period involved. The upper curve shows the time course of incorporation of radioactivity into streptidine-P; note the lag period involved. The lower curve shows the time course of incorporation of radioactivity into streptidine-P; note the lag period involved. The upper curve shows the effect of preincubating the heat extract with the enzyme preparation for 30 min prior to the addition of labeled arginine; the lag period is eliminated.

Preincubation of the heat extract of S. griseus ATCC 10971 with a crude S. bikiniensis enzyme preparation, followed by heat denaturation, also increases its reactivity with purified S. bikiniensis amidinotransferase and S. griseus amidinotransferase.

**Discussion**

Prior to the experiments reported here, arginase: 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 aminoseryl groups, the soluble ribonucleic acids, were eventually discovered (30). Similarly, we have looked for a natural acceptor of the enzyme-activated amino group of ar-

**Figure 5.** Curves comparing time courses of incorporation of radioactivity from l-arginine-$\gamma$H$^3$C into nonamidinated and amidinated substrates in the presence of a crude dialyzed enzyme preparation from S. bikiniensis. A. Upper curve (left ordinate) shows time course of formation of N-amidinostreptidine-P (SAG-P) from chemically phosphorylated streptidine. Lower curve (right ordinate) shows time course of formation of N-amidino-scyllo-inosamine-P (SIAG-P) from a reaction mixture containing scyllo-inosamine-P (Fig. 3E). Both transamidinations occur with no time lag. The amidine acceptor for both curves was a heat extract of mycelia of S. griseus ATCC 10971 previously exposed to streptidine (Fig. 3B). The lower curve shows the time course of incorporation of radioactivity into streptidine-P; note the lag period involved. The upper curve shows the effect of preincubating the heat extract with the enzyme preparation for 30 min prior to the addition of labeled arginine; the lag period is eliminated.
FIG. 6. Suggested metabolic relationships of various inosamine derivatives involved in biosynthesis of streptomycin, bluensomycin, kanamycin, and neomycin.  

Glucose-6-P  

Exogenous myo-inositol \(\xrightarrow{B}\) myo-inositol(P)  

Bluensidine  

\(\xrightarrow{J}\) N-amidino-scyllo-inosamine(P)  

Bluensomycin  

N-amidinostreptamine(P) \(\xrightarrow{M}\) streptamine(P)  

Exogenous streptidine \(\xrightarrow{I}\) streptidine(P) \(\xrightarrow{H}\) streptomycin  

\(\xrightarrow{O}\) kanamycin, neomycin  

(NDP-streptose,  
NDP-N-methyl-L-glucosamine)

The biosynthesis of streptomycin involves the conversion of glucose-6-P to myo-inositol, which is then phosphorylated to myo-inositol-1-P. This is followed by the conversion of myo-inositol-1-P to myo-inositol-1-P by another enzyme. The conversion of myo-inositol-1-P to myo-inositol-1-P is catalyzed by an amidinotransferase. The amidino group is then transferred to ornithine to form N-amidino-myoinosamine-1-P. This compound is then converted to N-amidino-scyllo-inosamine-P, which is further converted to N-amidinostreptamine-P. Streptidine-P is then formed by the transfer of an amidino group from L-arginine to 2-deoxystreptamine-P. The resulting compound is then converted to streptamine-P and finally to streptomycin. This process is catalyzed by a series of enzymes, including amidinotransferase, which transfer the amidino group from L-arginine to other acceptors, such as myo-inositol, to form the various inosamine derivatives involved in biosynthesis of streptomycin, bluensomycin, kanamycin, and neomycin.
Step D—Little is known of the biosynthesis of inosamines. The chemistry of inosamines, inososes, and inositols has been reviewed (35, 36).

Step E—Evidence for this step (Reaction 5) was presented in Fig. 3C.

Step F—This postulated two-step enzymic conversion can be reversed chemically by treatment of the product with nitrous acid. The deaminated compound has the electrophoretic mobility of N-amidino-scylla-inosamine-P (Table II), but inversion of configuration is probable (35).

Step G—At present we do not know whether the second amidino group of streptidine is normally derived from a transamidination reaction or is synthesized by two or more steps involving carbon dioxide and an amino donor. Stated in another way, it is not known whether the amidino group incorporated in Reaction 6 (Fig. 3C) is different from that involved in Reaction 5 or that in Reaction 6. The position of the phosphate group may be a deciding factor, and this may be subject to enzymic manipulation. No evidence was obtained for an enzymic condensation of streptidine-P with fumarate, in a reaction analogous to that catalyzed by argininosuccinase (37).

Step H—These postulated reactions can be studied more readily now that a number of radioactive precursors are available. Biosynthesis of the streptose moiety has recently been investigated by Candy and Baddiley (38). It is not known at what stage the aglycone, streptidine, loses its phosphate group. Alkaline phosphatase from S. griseus has been found to have a developmental time course similar to that of amidinotransferase, and it acts on all the phosphorylated derivatives shown here, reacting particularly well with N-amidino-streptamine-P.

The deaminated compound has the electrophoretic reversal chemically by treatment of the product with nitrous acid. The deaminated compound has the electrophoretic reversed chemically by treatment of the product with nitrous acid. The deaminated compound has the electrophoretic reversed chemically by treatment of the product with nitrous acid.

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