Biosynthesis of the Monoguanidinated Inositol Moiety of Bluensomycin, a Possible Evolutionary Precursor of Streptomycin*

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SUMMARY

The general pattern of biosynthesis of the bluensidine (1D-1-O-carbamoyl-3-guanidino-3-deoxy-scyllo-inositol) moiety of bluensomycin, a monoguanidinated analogue of dihydrostreptomycin, has been studied in extracts of Streptomyces hygroscopicus forma glebasus ATCC 14607 (S. glebasus). Our results are consistent with the following biosynthetic pathway: myo-inositol \( \rightarrow \) keto-scyllo-inositol \( \rightarrow \) aminodeoxy-scyllo-inositol \( \rightarrow \) 1-amino-1-deoxy-scyllo-inositol-4-P \( \rightarrow \) 1D-1-O-carbamoyl-3-amino-3-deoxy-scyllo-inositol-6-P \( \rightarrow \) bluensomycin.

A major uncertainty concerns the step (U) at which the carbamoyl group is introduced. Carbamoylation might occur, for example, prior to phosphorylation or following transamidination.

myo-Inositol:NAD oxidoreductase activity (C) is reported for the first time in extracts of Streptomyces; activity was assayed by two radiochemical methods, both involving coupling with aminotransferase (D). L-Glutamine:keto-scyllo-inositol aminotransferase (D) was also found to catalyze aminodeoxy-scyllo-inositol:keto-scyllo-inositol, amino-deoxy-scyllo-inositol:pyruvate, and 1,3-diamino-1,3-dideoxy-scyllo-inositol:keto-scyllo-inositol transaminations. L-Arginine:inosamine-P amidotransferase (F) catalyzed transamidinations with the following compounds as amidino acceptors: NH20H, 1-amino-1-deoxy-scyllo-inositol-4-P, 1D-1-guanidino-3-amino-1,3-dideoxy-scyllo-inositol-6-P, 1D-1,3-diamino-1,3-dideoxy-scyllo-inositol-6-P, and a compound present in S. glebasus extracts believed to be 1D-1-guanidino-1-deoxy-scyllo-inositol-4-P, and a compound present in S. glebasus extracts believed to be 1D-1-guanidino-3-amino-3-deoxy-scyllo-inositol-6-P, and a compound present in S. glebasus extracts believed to be 1D-1-guanidino-1-deoxy-scyllo-inositol-4-P, and a compound present in S. glebasus extracts believed to be 1D-1-guanidino-3-amino-3-deoxy-scyllo-inositol-6-P, and a compound present in S. glebasus extracts believed to be 1D-1-guanidino-1-deoxy-scyllo-inositol-4-P phosphohydrolase activity; neither this enzyme nor the corresponding enzyme from streptomycin producers can dephosphorylate the transamidination product presumed to be bluensidine-6-P. Acid hydrolysis of the latter compound gave a compound which, unlike the unhydrolyzed compound, was converted to 1D-1-guanidino-3-amino-1,3-dideoxy-scyllo-inositol by enzymes from a streptomycin producing strain. S. glebasus cannot carry out the above conversion since it apparently lacks at least two enzymes which occur in streptomycin producers: guanidinodeoxy-scyllo-inositol dehydrogenase and L-alanine:1D-1-guanidino-3-keto-1-deoxy-scyllo-inositol aminotransferase.

It is suggested that streptomycin producing strains might be descendents of an ancestral strain which, like S. glebasus, produced the monoguanidinated inositol derivative, bluensomycin. It is further suggested that gene duplication and subsequent evolutionary divergence resulted in biosynthesis of the diguanidinated inositol derivative, streptomycin, which is 10 times more effective than bluensomycin as an antibiotic and inhibitor of protein biosynthesis.

Evolutionary mechanisms for the acquisition of novel biosynthetic capabilities remain among the important unsolved problems in biology. It is particularly difficult to understand how biosynthetic pathways involving 20 or more specific enzymatic reactions could have arisen when neither intermediates nor the final end products appear to be required for growth. Such compounds might be termed idiolites, since they are synthesized during the idiophase of the growth cycle, and have a restricted biological distribution.

Our laboratory has been studying one such biosynthetic pathway, the biosynthesis of dihydrostreptomycin, an aminoacyclitol antibiotic (I, Fig. 1) isolated by certain strains of filamentous soil bacteria of the genus Streptomyces. Our current concept of the biosynthesis of the streptidine moiety of dihydrostreptomycin is summarized in Fig. 2. Two analogous sequences of five enzymatic reactions each, operating in series, appear to be involved in biosynthesis of the streptidine moiety from myo-inositol (V). Each sequence consists of a hydroxyl group to a guanidino group and involves, in order, a dehydrogenation (Reactions C

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Fig. 1. Comparison of the structures of dihydrostreptomycin (I) and bluensomycin (II). These compounds differ only in the substituent at position 1 (1-5). Dihydrostreptomycin is N-methyl-L-glucosamine (1→2)-α-L-dihydrostreptose (1→4)-streptidine. Streptomycin has an aldehyde group rather than an hydroxymethyl group at position 3α. Bluensomycin (2, 3), or globomycin (4, 6), is N-methyl-L-glucosamine (1→2)-α-L-dihydrostreptose (1→4)-bluensidine. Mild acid hydrolysis of these compounds gives streptidine or bluensidine plus the disaccharide, dihydrostreptobiosamine. Bluensidine has also been called glebisdine (4). None of the components of these antibiotics has so far been found elsewhere in nature.

and H), transamination (Reactions H and I), phosphorylation (Reactions E and J), transamidination (Reactions F and K), and dephosphorylation (Reactions G and N). For certain pairs of corresponding reactions in the two sequences it is known that different enzymes are involved, although some have overlapping substrate specificities (7-9).

An attractive hypothesis for later stages in the evolution of the streptidine biosynthetic pathway is that a segment of chromosomal or episomal DNA containing genes involved in one sequence of five reactions was duplicated in an ancestral strain, followed by independent evolution of the respective genes. The question then arises whether descendents of the hypothetical ancestral strain can be found which have not undergone duplication in this pathway, and which therefore catalyze only one sequence of five reactions. Likely candidates are strains which synthesize globomycin (5) or the apparently identical bluensomycin (II, Fig. 1), a monoguanidinated analogue of dihydrostreptomycin in which a carbamoyl group replaces the guanidino group at position 1 (3-5).

One of the purposes of the present investigation was to determine the general enzymatic pattern of biosynthesis of the monoguanidinated inositol (bluensidine) moiety of bluensomycin, and compare that pattern with the one proposed for biosynthesis of the diguanidinated inositol (streptidine) moiety of dihydrostreptomycin. Our findings are compatible with the general pattern of bluensidine biosynthetic pathway suggested in Fig. 2.

MATERIALS AND METHODS

Cultures of S. hygroscopicus forma gelobus ATCC 14607 (S. gelobus) and S. bikinniensis ATCC 11062 came from the American Type Culture Collection and were maintained on slants containing 1% maltose-0.5% tryptone-0.05% KH₂PO₄-0.05% NaCl-1.5% agar, plus a trace of Fe++. As a source of most enzyme preparations used in this paper, S. gelobosus cultures were inoculated from slants grown for 3 days at approximately 26° on a rotary shaker in 2-liter Erlenmeyer flasks containing 500 ml of medium composed of 0.06% glucose-1% soytone-1% tryptone-0.2% yeast extract-0.03% KH₂PO₄. When accumulation of amidino acceptor compounds was desired (Figs. 7 and 8), 1% myo-inositol was added to the growth medium. Mycelia were harvested by suction filtration on filter paper, blotted dry, and stored frozen. Extracts were prepared with a Branson Sonifier by sonication of 6 g of mycella in 12 ml of water for 4.5 min in 1.5-min segments separated by recooling periods. Sonicates were centrifuged at 30,000 × g for 20 min, and the supernatant solutions were stored frozen.

Dialyzed S. gelobus extracts employed for studies of Reactions C and D were prepared by thawing a supernatant solution from a sonicate and adding to 2 ml of such an extract 0.15 ml of a solution containing 0.7% potassium phosphate and 0.3% EDTA, pH 7.6, plus 0.04 mM pyridoxal-P; the solution was dialyzed 16 hours at 4° against deionized water.

myo Inositol preparations from a number of sources were employed with and without recrystallization for the experiments of Fig. 3. Purified alkaline phosphatase from Escherichia coli (type I), NAD, NADP, keto-scyllo-inositol, diethylaminoethylamino-P, and pyridoxal-P came from Sigma; L-1guanidino-3[14C]arginine, 46 Ci per mole, from Schwarz-Mann; [1-amino-1-deoxy-scyllo-1,3[14C]-inositol, 3.5 Ci per mole, from Mallinckrodt; Dowex-50(10)), 200 to 400 mesh, resin from BioRad; and D-chiro-inositol and L-chiro-inositol from Calbiochem. Amino-deoxy-scyllo-inositol was prepared by reduction of the oxime of keto-scyllo-inositol with sodium amalgam (10). Streptidine and streptamine were prepared from kanamycin (11). Aminocyclitols were chemically phosphorylated with P₃O₄ plus H₃PO₄, followed by treatment with Ba(OH)₂ (11). For these compounds to serve as amidinotransferase substrates, residual Ba²⁺ should be removed by addition of Na₂SO₄, and EDTA should be present in the incubation mixture. EDTA also serves to inhibit phosphatase action on amidino acceptors during transamidination.

Separations of labeled compounds were performed on Whatman No. 1 filter paper by (a) ascending paper chromatography, developed with 80% phenol-20% H₂O₂, ammonia atmosphere (provided by adding 1 ml of concentrated NH₄OH to the inside of the glass chromatography cylinder); and (b) high voltage paper electrophoresis with a refrigerated Savant horizontal plate apparatus. The effective path was 46 cm, 30 volts per cm. Ammonium formate buffer, pH 3.6, at 0.2 ionic strength was used. For both types of separations, strips were cut at 1-cm intervals, put in bottles containing Liquifluor in toluene, and counted with a Nuclear-Chicago liquid scintillation system. In the figures, gross counts per min are given, uncorrected for background.

RESULTS

The experiments which have been performed on biosynthesis of the bluensidine moiety of bluensomycin (II, Fig. 1) can be most conveniently described by referring to the reaction schemes of Fig. 2. Fig. 2 depicts our previously proposed scheme for biosynthesis of the streptidine moiety of dihydrostreptomycin (I) from glucose-6-P (III). The corresponding bluensidine moiety of bluensomycin contains one guanidino group and a carbamoyl group instead of two guanidino groups (2, 4); the location of these two groups was recently established by Barlow and Anderson (5). Fig. 2 also indicates one possible pathway for biosynthesis of the bluensidine moiety; in this scheme the single guanidino group is synthesized by the same enzymes which participate in biosynthesis of the first guanidino group introduced on the inositol ring of dihydrostreptomycin. However, this guanidino group ends up at position 3 of bluensomycin, rather than position 1 as in the case of dihydrostreptomycin in biosynthesis. An alternative scheme (not shown) for bluensidine biosynthesis was also considered. In the latter scheme, the single guanidino group is synthesized by the same enzymes which participate in biosynthesis of the second guanidino group introduced on the inositol ring of dihydrostreptomycin, but again ends up at position 3. In this
BIOSYNTHESIS OF I: \( R = -\text{OH} \)

BIOSYNTHESIS OF II: \( R = -\text{OH} \) OR \( -\text{O-!-NH} \)

Fig. 2. Our current concept of enzymatic steps involved in biosynthesis of the bluensidine moiety of bluensomycin (II) by S. glebasus, and the streptidine moiety of dihydrostreptomycin (I) by S. humnisus, starting from glucose-6-P (III). Participation of epimers of certain intermediates has not been excluded. Extracts of S. glebasus catalyze Reactions C, D, F, and G. Reaction E can be inferred from the presence of its product in mycelial extracts, and slight activity was detected in vitro. The step at which the carbamoyl group is added has not yet been established. Enzyme G apparently dephosphorylates only substrate molecules which have escaped carbamoylation. It is suggested that S. glebasus cannot synthesize dihydrostreptomycin (I) because it lacks enzymes H and I, possibly lacks enzyme J, and enzyme N’ (I) by S. humidus, starting from glucose-6-P (III). Participation of epimers of certain intermediates has not been excluded. Extracts of S. glebasus catalyze both Reactions F and K. Note that in these schemes, corresponding carbon atoms of the inositol moieties of dihydrostreptomycin (I) and bluensomycin (II) are derived from different carbon atoms of glucose-6-P (III).

Abbreviations: DSBA, dihydrostreptobiosamine; KGAM, \( \alpha \)-ketoglutaramate; ORN, ornithine; PYR, pyruvate; NADP-SUGAR, nucleosidediphosphate-sugar.

The occurrence of Reaction C in cell-free extracts of S. glebasus was confirmed by employing another version of the coupled dehydrogenase-transaminase assay system described above. In this assay, instead of the use of a low concentration of labeled \( \text{myo-inositol} \) and a high concentration of nonlabeled glutamine or aminodeoxy-scyllo-inositol as amino donor, a high concentration of nonlabeled aminodeoxy-scyllo-inositol was used. The most active amino donors tested were L-glutamine, aminodeoxy-scyllo-inositol, and streptamine, all of which are known to be able to serve as amino donors in Reaction D of streptomycin producers (7, 9). L-Alanine was relatively inactive as an amino donor, an indication that enzyme I was not involved.
myo-inositol was employed to help overcome the unfavorable equilibrium (15) of the dehydrogenation step, and the labeled reactant was the amino donor 1-amino-1-deoxy-scyllo-[1-14C]-inositol. The results are shown in Fig. 3. Both myo-inositol and NAD\(^+\) were required for conversion of labeled aminodeoxy-scyllo-inositol to labeled keto-scyllo-inositol by the sum of Reactions 1 and 2.

\[
\text{myo-Inositol} + \text{NAD}^+ \rightarrow \text{keto-scyllo-inositol} + \text{NADH} + \text{H}^+ \quad (1)
\]

\[
\text{Keto-scyllo-inositol} + 1\text{-amino-1-deoxy-scyllo-} [1-14\text{C}] \text{inositol}
\]

\[
\text{H-chiro-} \text{inositol and L-chiro-} \text{inositol could not substitute effectively for myo-inositol in this reaction.}
\]

Separate Assay for Reaction \(D\) in \(S.\) \(glebosus\)—The occurrence of Reaction \(D\) in \(S.\) \(glebosus\) has already been indicated by the previously described coupled reactions. We have suggested (7) that Reaction \(D\) is the sum of Reactions 3 and 4. This transaminase can therefore be assayed by the partial or half-reaction

\[
\text{L-Glutamine} + \text{pyridoxal-P-enzyme} \rightarrow \alpha\text{-ketoglutaramate}
\]

\[
+ \text{pyridoxamine-P-enzyme}
\]

\[
\text{Pyridoxamine-P-enzyme} + \text{keto-scyllo-inositol} \rightleftharpoons \text{pyridoxal-P-enzyme} + \text{aminodeoxy-scyllo-inositol}
\]

depicted in Reaction 4. Operationally this assay is carried out as indicated in Reaction 2. The results of such an experiment are given in Fig. 4. A role for L-glutamine rather than L-alanine as the physiological amino donor was suggested by the data of Table 1. These results confirm the presence of Reaction \(D\) in \(S.\) \(glebosus\). As in the case of streptomycin producers, high concentrations of pyruvate can serve as amino acceptor in the reverse reaction (7).

Assays for Reactions \(H\) and \(I\) in \(S.\) \(glebosus\)—Extracts of \(S.\) \(glebosus\) were not observed to catalyze either Reaction \(H\) or Reaction \(I\) of Fig. 2. These negative results argue against the involvement of enzymes with these same respective substrate specificities in biosynthesis of the bluensidine moiety of bluenosinomycin.

Assays for Amidinotransferase Activity in \(S.\) \(glebosus\)—Both \(L\)-arginine:glycine amidinotransferase from vertebrates (16) and \(L\)-arginine:inosamine-P amidinotransferase from \(S.\) \(glebosus\) (11, 17) have been found to catalyze transfer of an amidino group...
from arginine to hydroxyamine to form hydroxyguanidine, as indicated in Reaction 7. This reaction is believed to be the sum of Reactions 5 and 6 (18). As is the case for streptomycin producing strains (II), extracts of S. glebosus catalyzed L-arginine-:inosamine-P amidinotransferase occurring in streptomycin producing strains of *Streptomyces* has a substrate specificity which can be depicted in part as shown in Fig. 5 (18). Certain of these substrates were tested with S. glebosus extracts, with the results shown in Fig. 6. The following inosamine derivatives, prepared by nonspecific chemical phosphorylation (11), were found to serve as amidino acceptors with L-[guanidino-14C]arginine as donor: Compound XV (Fig. 6A); Compound XVb (Fig. 6B); Compound XVc (Fig. 6D). Fig. 6A corresponds to Reaction F of Fig. 2, and Fig. 6B corresponds to Reaction K of Fig. 2. The substrate specificity of S. glebosus amidinotransferase could not be distinguished by these experiments from the substrate specificity of amidinotransferase of streptomycin producing strains. The results of Fig. 6D suggest that streptidine phosphate, and presumably dihydrostreptomycin, could be produced by S. glebosus mycelia if Compound XIII could be synthesized. Evidently the absence of enzymes H and I preclude that possibility.

**Detection of Physiological Amidino Acceptors in S. glebosus**—A search for physiological amidino acceptors (X-NH2) in extracts of *S. glebosus* was next undertaken, employing Reaction 8 as an assay.

\[
\text{L-[guanidino-14C]arginine} + \text{X-NH}_2 \rightarrow \text{L-ornithine} + \text{X-NH-C(=NH_2^+)NH}_2
\]  

In these experiments the supernatant solutions from sonicated mycelia of *S. glebosus* were used as a source of both amidino acceptors and amidinotransferase activity. When such extracts were incubated with labeled arginine as amidino donor, a single peak containing radioactive products was obtained after paper chromatographic separation, as shown in Fig. 7. In contrast, it will be recalled that two distinct peaks were obtained with extracts of streptomycin producing strains, corresponding to Compounds IX and XIV of Fig. 9 (11). The presence of myo-inositol in the growth medium increased the concentrations of amidino acceptors in *S. glebosus* mycelia. Furthermore, when the radiometrical enzymatic assay for amidino acceptors (Reaction 8) was con-

**FIG. 6.** Some reactions catalyzed by *S. glebosus* amidinotransferase with chemically phosphorylated aminocyclitols as amidino acceptors. In each case the enzyme selected the proper positional isomer from the mixture of phosphorylated derivatives, presumably according to the specificities indicated in Fig. 5 (cf. 11). Each incubation mixture contained: L-[guanidino-14C]arginine, 33 \( \mu \)Ci per ml, 5 \( \mu \)l; 0.5 M Tris, pH 7.4, containing 13 mM EDTA, 5 \( \mu \)l; 0.38 M mercaptoethanol, 1 \( \mu \)l; dialyzed *S. glebosus* extract, 10 \( \mu \)l; and 10 \( \mu \)l of a solution containing the indicated chemically phosphorylated aminocyclitol. After incubation at 35° for 190 min, 10 \( \mu \)l were spotted and separated on ammoniacal phenol paper chromatograms. A, chemically phosphorylated amino-deoxy-scyllo-inositol-1-P (IGP). B, chemically phosphorylated monomonominated streptamine isomers as acceptor, giving d-streptidine-6-P (GGP). C, chemically phosphorylated streptamine as acceptor, giving 1D-1-amino-3-guani-dino-1,3-dideoxy-scyllo-inositol-6-P (INGP). D, chemically phosphorylated 2-dioxystreptamine as acceptor, giving 1D-1-amino-3-guani-dino-1,2,3-trideoxy-scyllo-inositol-6-P (dINGP).

**FIG. 7.** Physiological amidino acceptors in extracts of *S. glebosus* grown in presence of myo-inositol. A nondialyzed supernatant solution from sonicated mycelia was the source of both amidinotransferase and amidino acceptors. More labeled products were formed when carbamoyl-P was present during incubation (solid curve) than when it was omitted (dashed curve). The single peak formed subsequently was shown to contain two different monoguanidinated, monophosphorylated compounds, 1-guanidino-1-deoxy-scyllo-inositol-4-P (IGP) and an unknown compound (XGP). The complete incubation mixture contained: L-[guanidino-14C]arginine, 33 \( \mu \) Ci per ml, 5 \( \mu \)l; 0.5 M Tris, pH 7.4, containing 13 mM EDTA, 5 \( \mu \)l; 33 mM dithiothreitol carbamoyl-P, 5 \( \mu \)l; nondialyzed *S. glebosus* extract, 10 \( \mu \)l. After incubation at 35° for 115 min, 10 \( \mu \)l aliquots were spotted and separated by paper chromatography with ammoniacal phenol.
Transamidination products were not separated from each other. With HCl solutions. Column fractions were 3 to 4 ml each (timed collection). XGP, was eluted first with 0.5 N HCl. Resin, 200 to 400 mesh, in a column (1 cm x 22 cm), and eluted from Compound IX when incubated with extracts containing XGP were a carbamoylated derivative of Compound IX. Such a compound would be difficult to distinguish from Compound IX not detected in the same extracts. Incubation mixture could be separated from other labeled compounds in a single peak on a Dowex 50(H+) column, as shown in Fig. 8A. The isolated radioactive compounds migrated similarly to Compound IX during paper chromatography with a number of solvents and during high voltage paper electrophoresis at pH 3.6 and pH 10.4. These compounds could be dephosphorylated by incubation with Escherichia coli alkaline phosphatase to give compounds with mobilities similar to Compound X. Evidently the labeled peak contained compounds possessing one guanidino group and one phosphate ester group. The occurrence of enzyme E in S. gleboua could be inferred from the fact that the amidine acceptors are phosphorylated. Weak enzyme E activity was occasionally detected in S. gleboua extracts, but enzyme J was not detected in the same extracts. A hint that the labeled peak of Fig. 8A contained a compound, XGP, different from Compound IX came from its slightly higher RF value on ammoniacal phenol paper chromatograms; its dephosphorylated derivative also had a slightly higher RF than Compound X. The question then arose whether the postulated XGP were a carbamoylated derivative of Compound IX. Such a compound would be difficult to distinguish from Compound IX by the methods described above, but it might react differently from Compound IX when incubated with extracts containing enzymes G, I, and I of Fig. 2.

**Presence of Reaction G Activity in S. gleboua—Contrary to our expectations (9), extracts of S. gleboua actively dephosphorylated Compound IX, as shown in Fig. 9A. However, neither extracts of S. gleboua nor S. biktiniensis were able to dephosphorylate all of the components in the isolated peak of Fig. 8A, as shown in Fig. 9B for S. gleboua.** The isolated peak of Fig. 8A apparently contained Compound IX, which could be dephosphorylated by enzyme G, plus a component (XGP) of a closely related structure, possibly bluensidine-6-P, which is not a good substrate for enzyme G (Fig. 9B). The radioactive preparation of Fig. 8A was enriched in the XGP component by incubation with extracts of S. gleboua or S. biktiniensis to dephosphorylate the Compound IX component, followed by chromatography on a Dowex-50 column as before, as shown in Fig. 8B. The resulting XGP component was resistant to dephosphorylation by enzyme G, as shown in Fig. 9C. Alternatively, enrichment of the XGP component could be obtained by performing the initial transamidination reaction with S. gleboua and labeled arginine in the presence of Mg2+ and absence of EDTA to allow any Compound IX formed to be dephosphorylated by enzyme G present in the same extract. More Characterization of Unknown Physiological Transamidination Product (XGP)—When the labeled preparation (XGP) of Fig. 8B was dephosphorylated with E. coli alkaline phosphatase, the resulting compound (XGP) could not serve as a substrate for enzymes H plus I from a streptomycin producer, as shown in Fig. 10A. These results demonstrated that XG is not Compound X. XG was resistant to dephosphorylation by the methods described above, but it might react differently from Compound IX when incubated with extracts containing enzymes G, I, and I of Fig. 2.

**Discussion**

At the outset of this investigation, little was known of the enzymatic steps involved in biosynthesis of bluensomycin, although suggestions had been made on the basis of preliminary findings (11). Our experimental results are consistent with the general scheme shown in Fig. 2 for biosynthesis of the monoguanidinated inositol (bluensidine) moiety of bluensomycin (II).
The step at which carbamoylation occurs is not yet known. Extracts of S. glebosus have been shown in this paper to catalyze Reactions C, D, F, and G; these reactions are apparently catalyzed by enzymes similar to those involved in biosynthesis of the first guanidino group of the diguanidinated inositol (streptidine) moiety of dihydrostreptomycin (I), as depicted in Fig. 2. Since enzymes catalyzing Reactions H and I were not detected in S. glebosus, a possible alternate pathway for biosynthesis of the bluensidine moiety of bluensomycin, involving participation of enzymes utilized in biosynthesis of the second guanidino group of the streptomycin moiety of dihydrostreptomycin, appears to be ruled out. The latter scheme required that the substrate specificities of these enzymes, e.g., enzyme I, could be satisfied by either a guanidino or carbamoyl group at position 1. It is important to note that both of the above schemes for biosynthesis of the bluensidine moiety of bluensomycin predict a different labeling pattern in the end product, starting from specifically-labeled glucose-6-P (I1), from that observed for biosynthesis of the streptomycin moiety of streptomycin. In studies in vitro of streptomycin biosynthesis, Bruce et al. (20) found that position 5 of streptomycin was derived from C-1 of glucose as shown in Fig. 2. In our scheme, position 5 of bluensomycin would be derived from C-3 of glucose.

The biosynthetic scheme of Fig. 2 is consistent with the recent stereospecific assignments for the bluensidine moiety by Barkow and Anderson (5). Their assignments, arrived at by physicochemical means (5), are compatible with the substrate specificities of (a) enzyme F, assuming that R can also be a carbamoyl ester (Fig. 5); and (b) enzymes L and M, assuming that their substrates must be phosphorylated and at the position indicated in Fig. 2.

Although myo-inositol dehydrogenase have been studied in a number of organisms (15, 21, 22), the enzymatic experiments described in Table I and Fig. 3 have provided the first evidence for Reaction C obtained in cell-free extracts of Streptomyces. It was necessary to couple Reaction C with Reaction D to demonstrate this dehydrogenation. Our findings in vitro thus complement the extensive studies in vivo by Horner and others on the roles of myo-inositol (23-25), keto-scyllo-inositol (13, 26), and scyllo-inositol (12, 13) as precursors of the streptidine moiety of streptomycin. It is still not known why this particular myo-inositol dehydrogenation reaction has been so difficult to demonstrate in vitro in Streptomyces which produce the streptomycin family of antibiotics. myo-Inositol dehydrogenases from other sources can readily be assayed in the reverse direction i.e., reduction of keto-scyllo-inositol by NADH (15, 21), but this reverse assay has not so far proved useful with Streptomyces extracts. Further studies will be necessary to determine whether this myo-inositol dehydrogenase, as an early enzyme in a biosynthetic pathway, has an important regulatory function; regulatory enzymes often present assay or stability problems. The details of its linkage with electron transport enzymes should also prove of interest.

Although the stage in bluensomycin biosynthesis at which carbamoylation occurs is not yet known, despite our efforts in that direction, the unknown transamination product (XGP) obtained on incubation of S. glebosus extracts with labeled arginine (Figs. 7 to 10) has many of the properties expected for bluensidine-6-P. The substrate specificity of enzyme F (Fig. 5) would probably permit a carbamyl group at the indicated position. Any intermediates which escaped carbamoylation would probably be enzymatically converted to Compound X, in view of the presence of enzyme G activity in S. glebosus (Fig. 9). No kinase activity with Compound X as acceptor has been detected in S. glebosus or S. bikijensis.

The mechanism of the marked enhancement of transamination reactions by carbamoyl-P observed in vivo with nondialyzed mycelial extracts (Fig. 7) remains unknown. Enhancement might result from: (a) carbamoylation of an inosaminic-P derivative to give a substrate with a lower K or higher V max with amidinotransferase; (b) carbamoylation of ornithine, a strong inhibitor of amidinotransferase, to form noninhibitory citrulline; or (c) an allosteric activation of amidinotransferase. This phenomenon will be examined further.

It is too early in our investigation to draw definitive conclusions concerning the evolutionary relationships between the respective enzymatic pathways for biosynthesis of bluensomycin and dihydrostreptomycin. However, two possibilities will be briefly considered as a framework for future experiments.

1. One possibility is that bluensomycin producers resemble an ancestral strain, or contain an ancestral epoxide, which has not undergone gene duplication in the guanidinocyclitol biosynthetic pathway. Bluensomycin producers can add only one guanidino group to the inositol ring, because they lack genes coding for enzymes II and I. If it turns out that enzymes E and J are coded by different genes, gene J would be missing. Bluensomycin producers presumably require an additional gene which codes for a carbamoylation enzyme. In this scenario, dihydrostreptomycin producing strains are descendents of the above ancestral strain, or contain epoxides, which have undergone duplication and subsequent independent mutation of genes coding for Reactions C and D, and possibly E, F, and G. The nature of any selection pressure is not known since the physiological functions of these idiolites have not been established (9). However, dihydrostreptomycin is approximately 10 times more effective than bluensomycin as an antibiotic and inhibitor of protein synthesis (6), and therefore might represent a later evolutionary product.

2. Another possibility is that bluensomycin producers are derived from dihydrostreptomycin producers. In this scenario,
Addition of a gene coding for a carbamoylation enzyme resulted in synthesis of bluensidine-6-P, which cannot be dephosphorylated by enzyme G. Bluensidine-6-P reacts in the presence of enzyme L and subsequent enzymes to form bluensomycin rather than dihydrostreptomycin. There would be no further need for enzymes H through K, since their substrates would no longer be formed. Genes coding for these latter enzymes could then be lost, further mutated to serve new functions, or be repressed. Again, the selection pressures are not known.

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Biosynthesis of the Monoguanidinated Inositol Moiety of Bluensomycin, a Possible Evolutionary Precursor of Streptomycin
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