Streptomycin Biosynthesis. Transamination Reactions Involving Inosamines and Inosadiamines*

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ABSTRACT: In the biosynthesis of the streptidine moiety of streptomycin from *myo*-inositol, the two nitrogen atoms on the cyclitol ring are introduced by transamination of inosose derivatives. The two transaminase activities have been separated on a Sephadex G-100 column; pyridoxal phosphate must be present during the separation to retain activity. The first transaminase to emerge from the column was L-glutamine–*scyllo*-inosose transaminase, which catalyzes the first reaction unique to streptidine biosynthesis. *scyllo*-Inosamine, a known precursor of streptidine in cell-free preparations, is a product of this reaction. Enzyme preparations containing this activity also catalyze the following transaminations: *scyllo*-inosamine–*scyllo*-inosose, 2-deoxystreptamine–*scyllo*-inosose, *scyllo*-inosamine–*myo*-inosose-4(6), *myo*-inosamine-6–*scyllo*-inosose, streptamine–*scyllo*-inosose, L-alanine–*scyllo*-inosose, and *scyllo*-inosamine–pyruvate. The second amino group is added to the cyclitol ring in a reaction catalyzed by L-alanine–*N*-amidino-3- (or 5-) keto-*scyllo*-inosamine transaminase. *N*-Amidinostreptamine is a product of this reaction. L-Glutamate is the next most effective amino donor, followed by L-glutamine. Enzyme preparations containing this activity also catalyze the following transaminations: *N*-amidinostreptamine–pyruvate, *N*-amidinostreptamine–α-ketoglutarate, and *N*-amidinostreptamine–*N*-amidino-3- (or 5-) keto-*scyllo*-inosamine. At concentrations higher than those required for glutamine–*scyllo*-inosose transaminase, *scyllo*-inosose can serve as an amino acceptor from *N*-amidinostreptamine, and *scyllo*-inosamine as an amino donor to *N*-amidino-3- (or 5-) keto-*scyllo*-inosamine.

We have proposed that biosynthesis of the streptidine moiety of streptomycin from *myo*-inositol involves a sequence of ten enzymatic steps (Walker and Walker, 1967a). An unusual feature of this biosynthetic scheme is that it consists of two analogous sequences of five reactions; each sequence accomplishes the conversion of a cyclitol OH group into a $-NH(C{=}NH_2{}^+)NH_2$ group. The reactions of each sequence are, in order: a dehydrogenation, a transamination, phosphorylation of a neighboring hydroxyl group, a transamidination, and a dephosphorylation. As the biosynthetic pattern emerged, it became important to determine whether analogous steps in the two sequences are catalyzed by the same enzyme.

In this paper attention will be focused primarily on the two transamination reactions, which form an inosamine and an inosadiamine derivative, respectively. Evidence will be presented that two separate enzymes are involved, with distinct, but overlapping, substrate specificities. One of these enzymes, L-glutamine–*scyllo*-inosose aminotransferase, appears to be the first pathway-specific enzyme in the biosynthesis of streptidine. Preparations containing this activity also catalyze transaminations between certain inosamines or inosadiamines, and inososes. The other enzyme, L-alanine–*N*-amidino-3- (or 5-) keto-*scyllo*-inosamine aminotransferase, has a somewhat different substrate specificity, but reacts to a significant extent with certain of the cyclitol substrates of glutamine–*scyllo*-inosose transaminase. Structures of cyclitol derivatives which were found to participate in transamination reactions are included, along with some inactive isomers, in Figures 1 and 2.

Results

The two transamination steps involved in streptidine biosynthesis were first detected and assayed in the reverse directions, with pyruvate as the acceptor of an amino group from *scyllo*-[^{14}C]inosamine or *N*-[^{14}C]-amidinostreptamine, respectively (Walker and Walker, 1967a). When the corresponding labeled inosose substrates were prepared, and conditions established which retained enzymatic activity during dialysis, it became possible to evaluate further the substrate specificities in the forward directions and determine whether one or two enzymes were involved.

Effect of Pyridoxal Phosphate on Enzyme Activities. When mycelial extracts of *Streptomyces bikiniensis* were dialyzed against buffer overnight, neither aminocyclitol transaminase activity could be detected. Subsequent addition of pyridoxal phospate to the incubation mixture did not restore activity. However, if pyridoxal phosphate was present during dialysis, transaminase activities were retained (Walker and Walker, 1968). Pyridoxamine phosphate, pyridoxal, or alanine was ineffective in preventing loss of activity during dialysis; the presence of pyruvate enabled lower

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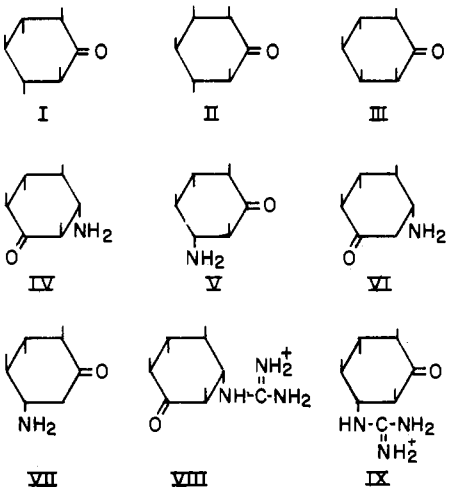

FIGURE 1: Inosose derivatives, many of which participate in transamination reactions. I, *scyllo*-inosose (*myo*-inosose-2; 2,4,6/3,5-pentahydroxycyclohexanone). II, *myo*-inosose-4 (2,4,5,6/3-pentahydroxycyclohexanone; 2D-2,3,4,6/5-pentahydroxycyclohexanone). III, *myo*-inosose-6 (2,3,4,6/5-pentahydroxycyclohexanone; 2L-2,3,4,6/5-pentahydroxycyclohexanone). IV, 5-keto-*scyllo*-inosamine ((2,4,6/3,5)-5-amino-2,3,4,6-tetrahydroxycyclohexanone; 2D-(2,4,6/3,5)-3-amino-2,3,4,6-tetrahydroxycyclohexanone). V, 3-keto-*scyllo*-inosamine ((2,4,6/3,5)-3-amino-2,4,5,6-tetrahydroxycyclohexanone; 2L-(2,4,6/3,5)-3-amino-2,3,4,6-tetrahydroxycyclohexanone). VI, 6-deoxy-5-keto-*scyllo*-inosamine (2L-(2,4/3,5)-5-amino-2,3,4-trihydroxycyclohexanone). VII, 2-deoxy-3-keto-*scyllo*-inosamine (2D-(2,4/3,5)-5-amino-2,3,-4-trihydroxycyclohexanone). VIII, *N*-amidino-5-keto-*scyllo*-inosamine ((1D-(1,5/2,4,6)-2,4,5,6-tetrahydroxy-3-ketocyclohexyl)guanidine). IX, *N*-amidino-3-keto-*scyllo*-inosamine ((1L-(1,5/2,4,6)-2,4,5,6-tetrahydroxy-3-ketocyclohexyl)-guanidine).

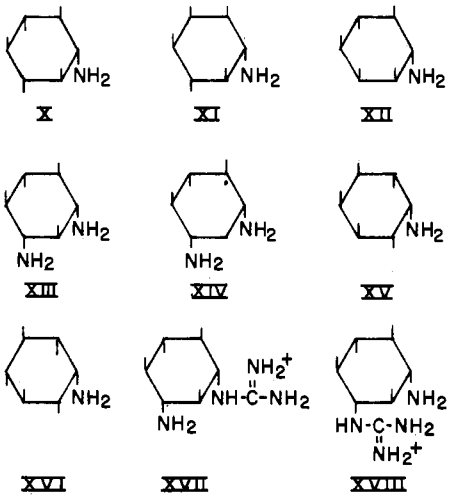

FIGURE 2: Inosamine derivatives, many of which participate in transamination reactions. X, *scyllo*-inosamine (amino-deoxy-*scyllo*-inositol). XI, *myo*-inosamine-4 (1L-4-amino-4-deoxy-*myo*-inositol). XII, *myo*-inosamine-6 (1D-4-amino-4-deoxy-*myo*-inositol). XIII, streptamine (1,3-diamino-1,3-dideoxy-*scyllo*-inositol; (1,3,5/2,4,6)-4,6-diaminocyclohexane-1,2,3,5-tetrol). XIV, 2-deoxystreptamine (1,3-diamino-1,2,3-trideoxy-*scyllo*-inositol; (1,3/2,4,6)-4,6-diaminocyclohexane-1,2,3-triol). XV, *neo*-inosamine-2 (2-amino-2-deoxy-*neo*-inositol). XVI, *myo*-inosamine-2 (2-amino-2-deoxy-*myo*-inositol). XVII and XVIII, *N*- and *N'*-amidinostreptamine isomers. XVII, (1L-(1,3,5/2,4,6)-5-amino-2,3,4,6-tetrahydroxycyclohexyl)guanidine. XVIII, (1D-(1,3,5/2,4,6)-5-amino-2,3,4,6-tetrahydroxycyclohexyl)guanidine. Streptidine is *N,N'*-diamidinostreptamine (1,1'-((1,3,5/2,4,6)-2,4,5,6-tetrahydroxy-1,3-cyclohexylene)diguanidine).

concentrations of pyridoxal phosphate to be employed. With pyridoxal phosphate present, enzyme purification could be undertaken.

Separation of Two Transaminase Activities. When an ammonium sulfate fraction containing both transaminase activities was applied to a Sephadex G-100 column equilibrated with pyridoxal phosphate, partial separation of the two activities was obtained, as shown in Figure 3. It would appear that each transamination is catalyzed by a different enzyme.

Reactions Catalyzed by Glutamine–scyllo-Inosose Transaminase. The first transaminase to emerge from the column utilized L-glutamine as the amino donor and *scyllo*-inosose as the amino acceptor, as shown in Figure 4. L-Alanine was active only at very high concentrations; L-glutamate was even less effective (Walker and Walker, 1968). L-Asparagine, L-aspartate, D-alanine, D-glutamine, L-isoglutamine, L-glutamate γ-hydroxamate, glycine, and a number of other amino acids were inactive as amino donors at the concentrations employed.

The requirement for pyridoxal phosphate indicated that the α-amino group, rather than the amide moiety, of glutamine was transferred, as depicted in reaction 1. To our knowledge no amide group transfer is known to require pyridoxal phosphate as a cofactor. Transamination reactions generally proceed by a double-displacement mechanism. Implicit in such a mechanism is the occurrence of the exchange reaction shown in reaction 2. It was found that this enzyme preparation

$$\text{L-glutamine} + \textit{scyllo}\text{-inosose} \rightleftarrows \alpha\text{-ketoglutaramate} + \textit{scyllo}\text{-inosamine} \quad (1)$$

$$\textit{scyllo}\text{-inosose} + \text{enzyme–pyridoxamine phosphate} \rightleftarrows \textit{scyllo}\text{-inosamine} + \text{enzyme–pyridoxal phosphate} \quad (2)$$

catalyzes an active *scyllo*-[^{14}C]inosamine–*scyllo*-inosose transamination, as shown in Figure 5A for synthetically prepared *scyllo*-[1-^{14}C]inosamine. The same exchange transamination occurred with *scyllo*-[^{14}C]-inosamine isolated following transamination of glutamine with *scyllo*-[^{14}C]inosose. Similarly, when *scyllo*-[^{14}C]inosose was the labeled substrate, *scyllo*-inosamine was an active amino donor (Table I). A number of other transaminations between inosamines and inososes were also catalyzed by preparations containing this activity. With *scyllo*-[^{14}C]inosose as acceptor, the following aminocyclitols can serve as amino donors at low concentrations, as shown in Table I: *scyllo*-inosamine (X), *myo*-inosamine-6 (XII), streptamine (XIII), and 2-deoxystreptamine (XIV). Inactive compounds include *myo*-inosamine-2 (XVI) and *neo*-inosamine-2 (XV). With *scyllo*-[1-^{14}C]inosamine as

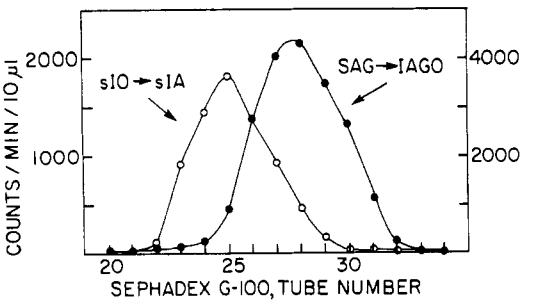

FIGURE 3: Separation of two transaminase activities on a Sephadex G-100 column. Fractions were incubated with the appropriate substrates, and compounds were separated by high-voltage paper electrophoresis at pH 3.6 and counted. (○—○) Activity converting *scyllo*-[^{14}C]inosose (sIO) into *scyllo*-[^{14}C]inosamine (sIA) with L-glutamine as amino donor; counts per minute in *scyllo*-[^{14}C]inosamine shown on left coordinate. (●—●) Activity converting *N*-[^{14}C]amidinostreptamine into *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine (IAGO) with pyruvate as amino acceptor; counts per minute in *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine shown on right coordinate.

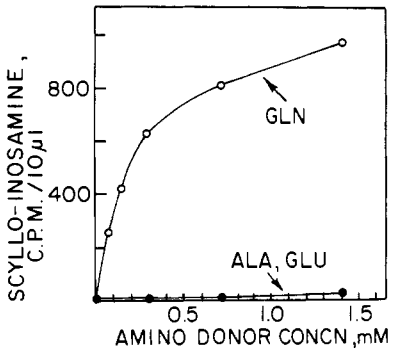

FIGURE 4: Comparison of ability of certain amino acids to serve as amino donors to *scyllo*-[^{14}C]inosose, in reaction catalyzed by first transaminase to emerge from Sephadex column. Enzyme was obtained from tube 24 (Figure 3). L-Glutamine was more active than L-alanine or L-glutamate at physiological concentrations. Compounds were separated by high-voltage paper electrophoresis at pH 3.6.

amino donor, both *scyllo*-inosose (I) (Figure 5A) and *myo*-inosose-4(6) (II, III) (Figure 5B) can serve as acceptors.

Enzymatic Conversion of scyllo-Inosose into N-[^{14}C]-Amidino-scyllo-inosamine Phosphate. The preceding experiments were performed with trace amounts of uniformly labeled *scyllo*-inosose of high specific activity. Independent confirmation of the glutamine–*scyllo*-inosose transamination was sought by employing as the amino acceptor commercially prepared, nonlabeled *scyllo*-inosose. Identification of the aminated reaction product was accomplished by means of coupled reactions with *scyllo*-inosamine kinase and *scyllo*-inosamine phosphate amidinotransferase (*cf.* Walker and Walker, 1967a). Two sequential incubations were performed. *scyllo*-Inosose was first transaminated with a dialyzed (*plus* pyridoxal phosphate) mycelial extract and glutamine as the amino donor (reaction 1). The positively charged product was adsorbed and eluted

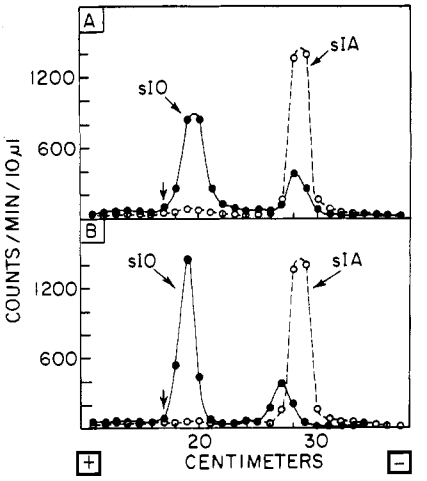

FIGURE 5: Transamination reactions between *scyllo*-[1-^{14}C]inosamine and inososes. The *scyllo*-[1-^{14}C]inosamine (sIA) substrate and *scyllo*-[1-^{14}C]inosose (sIO) product were separated by high-voltage paper electrophoresis at pH 3.6, as shown; samples were applied at 17 cm. Picric acid migrated 14.4 cm. Dashed curves: inosose acceptor omitted from incubation mixture. (A) *scyllo*-[1-^{14}C]Inosamine–*scyllo*-inosose exchange transamination. (B) *scyllo*-[1-^{14}C]Inosamine–*myo*-inosose-4(6) transamination.

TABLE I: Inosamines and Inosadiamines as Amino Donors to *scyllo*-[^{14}C]Inosose.

Amino Donor	*scyllo*-[^{14}C]Inosamine Formed[a] (cpm/10 µl)
None	66
scyllo-Inosamine, 0.2 mM	2694
myo-Inosamine-2, 0.2 mM	218
myo-Inosamine-6, 0.2 mM	2639
neo-Inosamine-2, 0.2 mM	224
2-Deoxystreptamine, 0.2 mM	2615
Streptamine, 0.2 mM	2520
scyllo-Inosamine, 0.8 µM	888
myo-Inosamine-6, 0.8 µM	568
myo-Inosamine-4(6) (racemic), 0.8 µM	365
Streptamine, 0.8 µM	446

[a] Compounds were separated by high-voltage paper electrophoresis at pH 3.6.

from a small Dowex-50 (H^+) column. This product was then incubated with ATP, L-[*guanidino*-^{14}C]-arginine, and a dialyzed mycelial extract containing *scyllo*-inosamine kinase and amidinotransferase activities (reactions 3 and 4). The results are given in Table II.

scyllo-inosamine + ATP
→ *scyllo*-inosamine phosphate + ADP (3)

scyllo-inosamine phosphate + L-arginine
⇄ *N*-amidino-*scyllo*-inosamine phosphate + L-ornithine (4)

When either *scyllo*-inosose, glutamine, or ATP was omitted, little or no *N*-[^{14}C]amidino-*scyllo*-inosamine

TABLE II: Multistep Enzymatic Conversion of *scyllo*-Inosose into *N*-[^{14}C]Amidino-*scyllo*-inosamine Phosphate.

Incubation Conditions	*N*-[^{14}C]Amidino-*scyllo*-inosamine Phosphate Formed (cpm/10 μl)
Complete	3501
Complete minus *scyllo*-inosose	71
Complete minus L-glutamine	228
Complete minus ATP	67

TABLE III: Enzymatic Phosphorylation of Glutamine–*scyllo*-[^{14}C]Inosose Transamination Product Isolated by Ion-Exchange Chromatography.

Incubation Conditions	Unchanged *scyllo*-[^{14}C]-Inosamine (cpm/10 μl)	*scyllo*-[^{14}C]-Inosamine Phosphate Formed (cpm/10 μl)
Complete	2604	2681
Complete minus ATP	5249	56

phosphate was formed. As a further check, *scyllo*-[^{14}C]inosamine was isolated by ion-exchange chromatography following transamination of glutamine with *scyllo*-[^{14}C]inosose, and then phosphorylated with ATP in the presence of *scyllo*-inosamine kinase (Table III). The foregoing results, taken together, confirm the occurrence of reaction 1, and hence the configuration of the inosamine product.

Reactions Catalyzed by Alanine–N-Amidino-3- (or 5-) *keto-scyllo-inosamine Transaminase*. The second transaminase to emerge from the Sephadex column (Figure 3) reacted with a number of amino acids at high concentrations, while at lower concentrations the most active donors were L-alanine, followed by L-glutamate and L-glutamine, as shown in Figure 6. The D analogs of these amino acids were inactive. This enzyme might be called alanine–*N*-amidino-3- (or 5-) keto-*scyllo*-inosamine aminotransferase until further information is available. The reverse reaction with pyruvate occurs quite readily (Figure 7A), and indeed was employed for assays of the Sephadex fractions (Figure 3); α-ketoglutarate is slightly less active than pyruvate.

There are two isomeric forms of monoamidinated streptamine (XVII and XVIII). We have designated the second nitrogen atom to be substituted on the cyclitol ring during the biosynthesis of streptidine as *N'*. *N'*-[^{14}C]Amidinostreptamine, prepared by enzymatic reaction of streptamine with ATP and L-[*guanidino*-^{14}C]-arginine (Walker and Walker, 1967a) followed by enzymatic dephosphorylation, is not a substrate for this transaminase, as shown in Figure 7B. It is not known which of the two structures (XVII or XVIII)

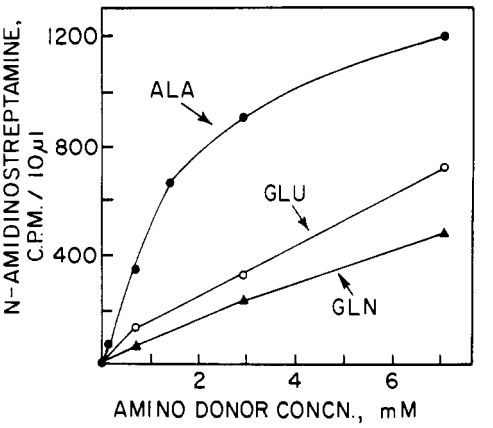

FIGURE 6: Comparison of ability of certain amino acids to serve as amino donors to *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine, in reaction catalyzed by second transaminase to emerge from Sephadex column. Enzyme was obtained from tube 30 (Figure 3). L-Alanine was somewhat more active than L-glutamate or L-glutamine at physiological concentrations. Compounds were separated by high-voltage paper electrophoresis at pH 3.6.

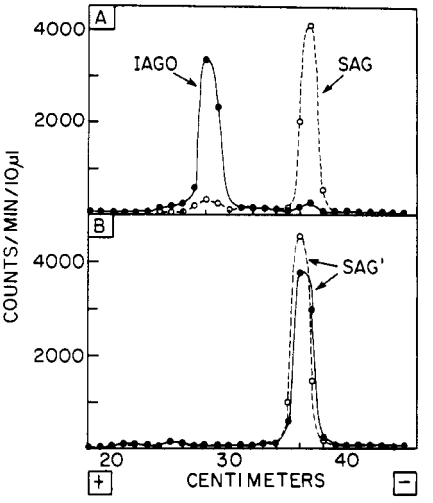

FIGURE 7: Comparison of two monoamidinostreptamine isomers as substrates for the second transaminase. Compounds were separated by high-voltage paper electrophoresis at pH 3.6; samples were applied at 13 cm. Picric acid migrated 14.5 cm. Dashed curves: pyruvate omitted. (A) *N*-[^{14}C]Amidinostreptamine- (SAG) pyruvate transamination, with formation of *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine (IAGO). (B) *N'*-[^{14}C]Amidinostreptamine (SAG') cannot serve as an amino donor to pyruvate.

corresponds to this compound; the same, of course, applies to the respective keto analogs, only one of which (VIII or IX) must be a substrate of this transaminase.

Our early preparations of *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine were quite impure (Walker and Walker, 1967a). Relatively clean preparations have now been obtained. These latter preparations can be readily converted into *N*-amidinostreptamine with L-alanine as amino donor, as shown in Figure 8A, corresponding to reaction 5. The transaminase catalyzing this over-all reaction should also catalyze the exchange reaction

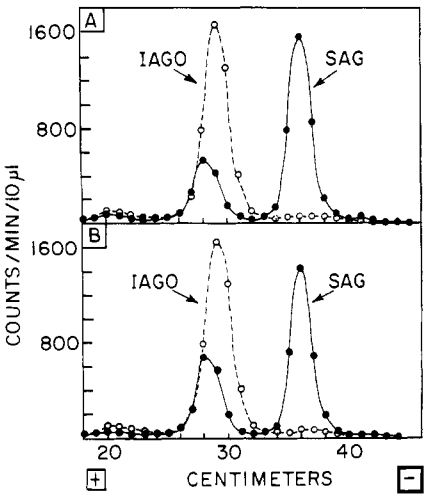

FIGURE 8: Transamination of *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine by certain amino donors. Compounds were separated by high-voltage paper electrophoresis at pH 3.6, as shown; samples were applied at 13 cm. Picric acid migrated 14.7 cm. Dashed curves: amino donor omitted. (A) L-Alanine–*N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine (IAGO) transamination, with formation of *N*-[^{14}C]-amidinostreptamine (SAG). This is the physiological reaction. (B) *N*-Amidinostreptamine–*N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine exchange transamination.

depicted in reaction 6. It was found that a mixture of the two monoamidinated streptamine isomers (XVII and XVIII), prepared by chemical amidination of streptamine with an equimolar amount of *S*-methylisothiouronium sulfate (Walker and Walker, 1967b), could serve at low concentrations as an amino donor to *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine, as shown in Figure 8B.

L-alanine + *N*-amidino-3- (or 5-) keto-*scyllo*-inosamine $\rightleftarrows$ pyruvate + *N*-amidinostreptamine (5)

N-amidino-3- (or 5-) keto-*scyllo*-inosamine + enzyme–pyridoxamine phosphate $\rightleftarrows$ *N*-amidinostreptamine + enzyme–pyridoxal phosphate (6)

As might be anticipated from consideration of the structures of the aminocyclitol substrates of the two transaminases, certain substrates for the first transaminase can serve as substrates for the second transaminase. However, higher substrate concentrations were required under our experimental conditions. At concentrations higher than those required for glutamine–*scyllo*-inosose transaminase, *scyllo*-inosose can serve as an amino acceptor from *N*-[^{14}C]amidinostreptamine, and *scyllo*-inosamine can donate an amino group to *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine, as shown in Table IV. The *myo*-inosose-4(6) and *myo*-inosamine-6 isomers are less effective relative to the *scyllo* isomers as substrates for the second transaminase.

TABLE IV: Cross-Reactivity between Substrates of the Two Transaminases.

Nonlabeled Substrate	Labeled Product Formed[c] (cpm/10 μl)
Expt 1[a]	
None	842
scyllo-Inosose (0.2 mM)	2100
scyllo-Inosose (2 mM)	5247
Expt 2[b]	
None	150
scyllo-Inosamine (0.8 mM)	363
myo-Inosamine-6 (0.8 mM)	148
scyllo-Inosamine (4 mM)	1438
myo-Inosamine-6 (4 mM)	369

[a] *N*-[^{14}C]Amidinostreptamine was the labeled amino donor. [b] *N*-[^{14}C]Amidino-3- (or 5-) keto-*scyllo*-inosamine was the labeled amino acceptor. [c] Compounds were separated by high-voltage paper electrophoresis at pH 3.6.

Discussion

Role of scyllo-Inosose in the Biosynthesis of Streptomycin. There are a number of reports in the literature which appear to argue against a role for *scyllo*-inosose in the biosynthesis of the streptidine moiety of streptomycin. Negative results obtained by feeding *scyllo*-inosose to mycelia (Hunter and Hockenhull, 1955; Majumdar and Kutzner, 1962) might be attributed to permeability restrictions or to enzyme inhibitions. However, the report of Heding (1964) that *myo*-[2-^{3}H]inositol is incorporated into streptidine in good yield cannot be so easily rationalized, since this label would presumably be removed on dehydrogenation to *scyllo*-inosose. We were aware of Heding's report at the time we first proposed *scyllo*-inosose as a precursor (Walker and Walker, 1966), and we considered an H-conserving epimerization to the symmetrical *scyllo*-inositol as a possible explanation. Another possibility is that Heding's sample of *myo*-[2-^{3}H]inositol also contained *scyllo*-[-^{3}H]inositol. *S. griseus* can interconvert *scyllo*-inositol and *myo*-inositol (Bruton *et al.*, 1967; Horner and Thaker, 1968). In any event, our experiments demonstrate for the first time that *scyllo*-inosose can serve as an excellent precursor of streptidine in cell-free preparations (*cf.* Table II). It should be noted, however, that our biosynthetic scheme derived from cell-free studies (Walker and Walker, 1967a, and earlier papers) has not yet been accepted by a number of workers in this field (*cf.* Bruce *et al.*, 1968).

Transamination of scyllo-Inosose. The enzyme *myo*-inositol dehydrogenase, which catalyzes the reversible formation of *scyllo*-inosose, is widely distributed in nature, occurring in animals (Posternak *et al.*, 1963; Candy, 1967), higher plants (Scholda *et al.*, 1964; Kindl, 1969), and bacteria (Posternak, 1942; Berman and Magasanik, 1966), including *S. griseus* (Horner and Thaker, 1968). It would therefore appear that transamination of *scyllo*-inosose is the first committed step unique to the biosynthesis of streptidine, and perhaps of streptomycin as well. In a biosynthetic se-

quence, it is often advantageous for the first pathway-specific step to be irreversible, for purposes of control. It is therefore appropriate that glutamine is the amino donor to *scyllo*-inosose (Figure 4) as depicted in reaction 1, since Meister (1954) has shown such transaminations to be physiologically irreversible in other systems because of enzymatic deamidation or non-enzymatic cyclization of the α-ketoglutaramate formed in the reaction. α-Ketoglutaramate, rather than glutamate, is presumed to be a product of the transamination because of: (i) the pyridoxal phosphate requirement for activity, (ii) the occurrence of a *scyllo*-inosamine–*scyllo*-inosose exchange reaction (Figure 5A), as depicted in reaction 2, and (iii) lack of evidence for the reductive step necessary if the amide group were transferred.

As for the other reaction product, transamination of *scyllo*-inosose (I) could theoretically result in the formation of either *myo*-inosamine-2 (XVI) or *scyllo*-inosamine (X), if no epimerase were present. Presence of an appropriate epimerase might result in the formation of *myo*-inosamine-4 (XI) or *myo*-inosamine-6 (XII). *myo*-Inosamine-2 was ruled out as a transamination product for two reasons: (i) it does not transaminate with *scyllo*-inosose (Table I), and (ii) it is not a substrate for *scyllo*-inosamine kinase (Walker and Walker, 1967a). *myo*-Inosamine-6 (XII) transaminates with *scyllo*-inosose (Table I), but it is not a substrate for *scyllo*-inosamine kinase (Walker and Walker, 1967a). On the other hand, *scyllo*-inosamine participates in an exchange transamination (reaction 2) with *scyllo*-inosose (Figure 5A and Table I), and the transamination product formed by reaction of glutamine with *scyllo*-inosose is a substrate for *scyllo*-inosamine kinase (Tables II and III). We conclude that the transamination product is *scyllo*-inosamine, which we had previously found to be a precursor of streptidine in cell-free preparations (Walker and Walker, 1967a; Walker, 1969).

Transaminations between Various Cyclitol Isomers. It is of interest that a number of inososes, inosamines, and inosadiamines which are not involved in streptidine biosynthesis can participate in transamination reactions catalyzed by extracts of streptomycin-producing strains of *Streptomyces*. These reactions appear to be catalyzed by glutamine–*scyllo*-inosose transaminase, but this has not been established with certainty. When *myo*-inosose-4(6) is the amino acceptor (Figure 5B) one might expect that *myo*-inosamine-6 (XII), and possibly its enantiomer (XI), is formed, although this has not been established. The discovery that 2-deoxystreptamine (XIV) is a transaminase substrate (Table I) is of particular interest, in view of the presence of this inosadiamine in numerous antibiotics; which of the two possible ketodeoxyinosamine isomers (VI or VII) is formed in reaction 7 is not known. We favor compound VII at present. Perhaps such a transaminase participates in the biosynthesis of 2-deoxystreptamine, or in its catabolism. Streptamine (XIII) is also a transaminase substrate (Table I); again, it is not known which ketoinosamine isomer (IV or V) is formed in reaction 8. We currently favor compound V because

2-deoxystreptamine + *scyllo*-inosose
⇄ 2- (or 6-) deoxy-3- (or 5-) keto-*scyllo*-inosamine + *scyllo*-inosamine (7)

streptamine + *scyllo*-inosose
⇄ 3- (or 5-) keto-*scyllo*-inosamine + *scyllo*-inosamine (8)

myo-inosamine-6 can serve as a substrate. The occurrence of transaminations between other combinations of the above inosose and inosamine derivatives can be predicted. It will be interesting to determine which of the inosamine and inosadiamine isomers not yet tested can also participate in transamination (and phosphorylation) reactions. A number of these isomers have been chemically synthesized in recent years in the laboratories of Anderson, Wolfrom, Sable, Umezawa, Ogawa, Lichtenthaler, and Suami (for earlier references, see Posternak, 1965).

Genetic and Evolutionary Implications. In the biosynthesis of streptidine (Walker and Walker, 1967a), no evidence has yet been obtained that the two phosphorylation steps are catalyzed by more than one enzyme, or that the two transamidination steps are catalyzed by different enzymes. It was therefore of interest to find that each of the two transamination steps is catalyzed by a different enzyme (Figure 3). These two enzymes have distinct but overlapping substrate specificities (Figures 4 and 6; Tables I and IV). It will be important to determine whether the similarities in catalytic behavior reflect corresponding similarities in primary structures of the two enzymes. Both transaminase activities are low or absent during the early phase of rapid mycelial growth on complex media. The subsequent developmental increases in enzymes of the streptidine pathway provide a convenient experimental system for studying differentiation in a procaryote (Walker and Hnilica, 1964). A careful study of the molecular biology of streptidine biosynthesis, with its two analogous sequences of five enzymatic reactions, might also provide evidence relating to the widely held hypothesis that acquisition of biosynthetic capabilities results from gene duplication followed by independent changes in base sequence with time (*cf.* Watts and Watts, 1968). The diverse biosynthetic capabilities of the *Streptomyces* group might well provide present-day examples of evolution in action (*cf.* Walker, 1965, 1969).

In some respects streptidine formation might be considered as a bacterial analoge of the mammalian urea cycle. Proteins are preferred substrates for *Streptomyces*, and cultures increase in pH with time (Waksman, 1959; Hockenhull, 1960); suspensions of mycelia starved in saline also become alkaline. It might therefore be advantageous for these bacteria to be able to convert excess NH_3, formed from catabolism of either exogenous or endogenous proteins, into a less metabolically active compound such as streptidine. The ratio of N:C in streptidine is 6:8, compared with 4:5 in uric acid, and 2:1 in urea. The formation of streptidine might be summarized as shown in reaction 9,

glucose + O_2 + $6NH_3$ + $2CO_2$ → streptidine (9)

neglecting water balance and ATP requirements. Enzymes of the ornithine–arginine cycle participate in the biosynthesis of both urea and streptidine. With the discovery of the transaminases described here, the problem of the immediate origins of the nitrogen atoms attached to the cyclitol ring (*cf.* Tovarova *et al.*, 1966) appears to be solved. It is probable that incorporation into streptidine of both CO_2 molecules *plus* two NH_3 molecules occurs by way of carbamoyl phosphate; one NH_3 molecule is incorporated by way of the α-amino moiety of glutamine, two NH_3 molecules by way of aspartate, and the sixth NH_3 *via* alanine, glutamate, or glutamine. Further studies on the enzymatic pathways of nitrogen metabolism in this organism are planned. It is hoped that such studies will complement the interesting findings of Thoai (1965) and his collaborators concerning arginine metabolism in *S. griseus*.

Materials and Methods

Sources of many of the compounds employed have been described (Walker and Walker, 1967a). *scyllo*-Inosose (*myo*-inosose-2) came from Sigma Chemical Co. *N*-Acetyl-*myo*-inosamine-6, like most of the inosamines, was a gift from Dr. Laurens Anderson, University of Wisconsin. *scyllo*-[1-^{14}C]Inosamine came from Calbiochem as an impurity in *myo*-[2-^{14}C]-inositol; it was isolated on a Dowex 50 (H^+) column. *scyllo*-[^{14}C]Inosose was prepared by shaking in air 7 μCi of *myo*-[^{14}C]inositol (324 mCi/mmole) in 0.2 ml of H_2O for 3 hr at room temperature in a large test tube with 2 mg of platinum black (5% on charcoal) from Matheson Co. (*cf.* Heyns and Paulsen, 1953; Posternak, 1952). The suspension was filtered with suction on a Büchner funnel; the filtrate plus washings were evaporated to dryness *in vacuo* and taken up in 0.4 ml of H_2O. The *scyllo*-[^{14}C]inosose formed was not separated from unreacted *myo*-[^{14}C]inositol. *myo*-Inosose-4(6) was prepared by treatment of *myo*-inositol with HNO_3 (Posternak, 1952).

Separation Methods. Ion-exchange chromatography, paper chromatography, and high-voltage paper electrophoresis procedures were carried out as described previously (Walker and Walker, 1967a,b). The Sephadex G-100 (40–120-μ beads) column (2 × 54 cm) was equilibrated with 1 mM potassium phosphate buffer, containing EDTA (0.1 mg/ml), and pyridoxal phosphate (0.1 mg/ml, pH 7.4); 40-drop fractions (2.4 ml) were collected at 3° at a rate of 1 drop/20 sec. Protein was first detected in tube 19.

Enzyme Preparations. Cultures of *Streptomyces bikiniensis* ATCC 11062 were grown for 2.5–3 days, harvested, frozen, and extracted with lysozyme as described previously (Walker and Walker, 1967a). As a source of *scyllo*-inosamine kinase and amidinotransferase activities, lysozyme extracts were dialyzed, with stirring, for 2 days as described (Walker and Walker, 1967a). As a source of transaminase activities, lysozyme extracts were dialyzed overnight with stirring against 4 l. of 1 mM phosphate buffer (pH 7.4) plus 0.1 ml of 2-mercaptoethanol; at the start of dialysis, 0.25 ml of neutralized pyridoxal phosphate (20 mg/ml) and 0.4 ml of EDTA (50 mg/ml) were added to 4 ml of the lysozyme extract in the dialysis bag. This latter preparation was designated as dialyzed lysozyme–pyridoxal phosphate extract. Dialyzed enzyme preparations were stored frozen. For application to the Sephadex column, an $(NH_4)_2SO_4$ fraction was prepared at 4°. To 30 ml of lysozyme extract was added 0.3 ml of pyridoxal phosphate (20 mg/ml), and then 2 ml of 10% $MnCl_2$ was added slowly with stirring; after 20 min the suspension was centrifuged. To 27 ml of the supernatant was slowly added 12 g of powdered $(NH_4)_2SO_4$, with stirring; the solution was kept approximately neutral with NH_4OH. After 20 min the suspension was centrifuged and the precipitate was taken up in 8 ml of 0.1 M phosphate buffer (pH 7.4) containing EDTA (5 mg/ml) and pyridoxal phosphate (1 mg/ml), and dialyzed overnight as before; 1 ml of this preparation, containing 19.6 mg of protein, was applied to the Sephadex column.

Transamination of scyllo-[^{14}C]Inosose with Amino Donors. Incubation conditions were *scyllo*-[^{14}C]inosose (plus *myo*-[^{14}C]inositol), 5 μl (69,200 cpm); 0.2 M potassium phosphate (pH 7.4) containing EDTA (5 mg/ml), 5 μl; amino donor, 5 μl; and dialyzed lysozyme–pyridoxal phosphate extract, 10 μl; incubated 90 min at 37°. For assay of Sephadex column fractions this was modified: 20 μl of enzyme fraction; incubated 120 min; L-glutamine, 7 mM (final concentration 1.4 mM). For batch preparation: quantities were scaled up 60-fold; incubated 180 min; L-glutamine, final concentration 1.4 mM; the deproteinized solution was applied to a Dowex 50 (H^+) column and *scyllo*-[^{14}C]inosamine eluted with 0.5 N HCl and evaporated to dryness *in vacuo*.

Conversion of scyllo-Inosose into N-[^{14}C]Amidino-scyllo-inosamine Phosphate. For the transamination step, incubation conditions were *scyllo*-inosose (4 mg/ml), 0.2 ml; 0.2 M phosphate (pH 7.4) containing EDTA (5 mg/ml), 0.2 ml; L-glutamine (7.3 mg/ml), 0.2 ml; and dialyzed lysozyme–pyridoxal phosphate extract, 0.4 ml; incubated 180 min at 37°. In two control tubes either *scyllo*-inosose or glutamine was omitted, respectively. The mixtures were deproteinized with 0.1 ml of 30% trichloroacetic acid and centrifuged; the supernatants were neutralized with 2 N KOH. The supernatants were adsorbed on small Dowex 50 (H^+) columns consisting of Pasteur pipets, and the resin was washed well with H_2O. The inosamine fraction was eluted with 5 ml of 0.5 N HCl into evaporating dishes, and evaporated to dryness *in vacuo* over NaOH pellets; the residues were taken up in 0.2 ml of H_2O. For the combined phosphorylation and transamidination steps, incubation conditions were: reconstituted 0.5 N HCl eluate, 15 μl; 0.5 M Tris (pH 7.4) containing 0.04 M $MgCl_2$, 5 μl; 0.3 M 2-mercaptoethanol, 1 μl; 0.036 M ATP, 5 μl; dialyzed lysozyme extract, 10 μl; and 33 μCi/ml of L-[*guanidino*-^{14}C]arginine-HCl (25 mCi/mmole), 5 μl; incubated 120 min at 37°. Compounds were separated on paper chromatograms with 80% phenol–20% H_2O, NH_3 atmosphere (1 ml of concentrated NH_4OH on inside wall of jar), and counted.

Phosphorylation of scyllo-[^{14}C]Inosamine. Incubation

conditions were *scyllo*-[^{14}C]inosamine (from batch transamination of *scyllo*-[^{14}C]inosose with glutamine), 5 μl (16,000 cpm); 0.5 M Tris (pH 7.4) containing 0.04 M $MgCl_2$, 5 μl; 0.036 M ATP, 5 μl; 0.08 M L-ornithine-HCl (to prevent subsequent transamidination), 5 μl; and dialyzed lysozyme extract, 10 μl; incubated 120 min at 37°. Compounds were separated on paper chromatograms as above.

Transamination of scyllo-[1-^{14}C]Inosamine with Inososes. Incubation conditions were *scyllo*-[1-^{14}C]-inosamine, 2 μl (12,300 cpm); 0.2 M phosphate (pH 7.4) containing EDTA (5 mg/ml), 5 μl; *scyllo*-inosose (0.2 mg/ml) or *myo*-inosose-4(6) (0.4 mg/ml), 5 μl; and dialyzed lysozyme–pyridoxal phosphate extract, 10 μl; incubated 120 min at 37°.

Transamination of N-[^{14}C]Amidinostreptamine to Form N-[^{14}C]Amidino-3- (or 5-) keto-scyllo-inosamine. Incubation conditions were *N*-[^{14}C]amidinostreptamine, 5 μl (24,700 cpm); 0.2 M phosphate (pH 7.4) containing EDTA (5 mg/ml), 5 μl; sodium pyruvate (18 mg/ml), or other acceptor, 5 μl; and dialyzed lysozyme–pyridoxal phosphate extract, 10 μl; incubated 90 min at 37°. For assay of Sephadex column fractions this was modified: 20 μl of enzyme fraction; incubated 120 min. For batch preparations of *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine, the following was typical: *N*-[^{14}C]amidinostreptamine, 0.8 ml; 0.2 M phosphate (pH 7.4) containing EDTA (5 mg/ml), 0.2 ml; sodium pyruvate (18 mg/ml), 0.3 ml; and dialyzed lysozyme–pyridoxal phosphate extract, 0.8 ml; incubated 90 min at 37°. Alternatively, 3.2 mg of *scyllo*-inosose could be substituted for pyruvate. The mixture was deproteinized with 0.05 ml of 30% trichloroacetic acid and centrifuged; the supernatant was adsorbed on a Dowex 50 (H^+) column, washed with H_2O then 0.5 N HCl, and eluted with 1 N HCl. The tubes containing the labeled product were evaporated to dryness *in vacuo* over NaOH pellets and taken up in 0.2–0.5 ml of H_2O.

Transamination of N-[^{14}C]Amidino-3- (or 5-) keto-scyllo-inosamine with Amino Donors. Incubation conditions were *N*-[^{14}C]amidino-3- (or 5-) keto-*scyllo*-inosamine, 5 μl (15,000 cpm); 0.2 M phosphate (pH 7.4) containing EDTA (5 mg/ml), 5 μl; L-alanine (0.9 mg/ml) or *N*-amidinostreptamine plus *N'*-amidinostreptamine (0.5 mg/ml), 5 μl; dialyzed lysozyme–pyridoxal phosphate extract, 10 μl; incubated 120 min at 37°.

Counting Procedures. After components of incubation mixtures were separated by paper chromatography or high-voltage paper electrophoresis, the dried paper strips were cut into segments 1 cm wide along the direction of migration. Each segment was cut into two or three *ca.* 1.5 × 1.0 cm rectangles, deposited on the bottom of a liquid scintillation vial, and counted with a Nuclear-Chicago liquid scintillation counter. Usually 10 μl of incubation mixture was applied at the origin prior to separation and, where applicable, counts per minute were summed over the several segments representing a separated peak of activity. Actual counts per minute observed, corrected for background, were recorded; all experiments were repeated *in toto* a number of times to assure validity of results.

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