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Enzymatic Synthesis of Streptidine from *scyllo*-Inosamine*

James B. Walker and Margaret S. Walker

ABSTRACT: Cell-free preparations from post-log-phase mycelia of *Streptomyces bikiniensis* ATCC 11062 catalyzed the conversion of *scyllo*-inosamine (aminodeoxy-*scyllo*-inositol) to streptidine (all *trans*-1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane). Evidence has been obtained consistent with the following biosynthetic pathway: myoinositol $\xrightarrow{C}$ *scyllo*-inosose $\underset{D1}{\rightleftarrows}$ *scyllo*-inosamine $\xrightarrow{D2}$ *scyllo*-inosamine-P $\underset{E}{\rightleftarrows}$ *N*-amidino-*scyllo*-inosamine-P $\xrightarrow{F1}$ *N*-amidino-*scyllo*-inosamine $\xrightarrow{F2}$ *N*-amidino-3-keto-*scyllo*-inosamine $\underset{F3}{\rightleftarrows}$ *N*-amidinostreptamine $\xrightarrow{F4}$ *N*-amidinostreptamine-P $\underset{G}{\rightleftarrows}$ streptidine-P $\xrightarrow{W}$ streptidine. A free inosamine, apparently of the *scyllo* configuration, was detected for the first time in a biological system, in mycelia of *Streptomyces griseus* ATCC 12475 fed myo[^{14}C]-inositol. Enzymatic activities detected for the first time included: (i) *scyllo*-inosamine kinase (D2), which required adenosine 5′-triphosphate and Mg^{2+} and did not react with physiological concentrations of myoinosamine-2, DL-myoinosamine-4, or neoinosamine-2; (ii) a kinase which phosphorylates 2-deoxystreptamine and streptamine (1,3-diamino-1,3-dideoxy-*scyllo*-inositol) with adenosine 5′-triphosphate; (iii) *N*-amidinostreptamine–pyruvate transaminase (F3), which also reacts with α-ketoglutarate; and (iv) *scyllo*-inosamine–pyruvate transaminase (D1), which also reacts with α-ketoglutarate. In the forward direction of reaction F3, L-alanine and L-glutamate can serve as amino donors, but D-alanine, D-glutamate, L-glutamine, L-aspartate, and glycine are inactive. We conclude that amino groups of both inosamines and inosadiamines can be derived by transamination of nonphosphorylated precursors with certain amino acids.

A number of inosamine derivatives have been implicated in the biosynthesis of streptidine from myoinositol[1] (Walker and Walker, 1966, 1967a,b). The principal uncertainties in the biosynthetic sequences proposed earlier involved (i) configurations and state of phosphorylation of early intermediates in the pathway, and (ii) mechanisms by which amino groups were substituted for hydroxyl groups on the cyclitol ring. In this paper evidence will be presented consistent with all *trans-scyllo* configurations of early intermediates, and formation of cyclitol amino groups by transamination of nonphosphorylated inosose derivatives with L-alanine or L-glutamate. Phosphorylation steps appear to be required primarily for formation of the guanidino moieties of streptidine by transamidination of the amino groups of suitable phosphorylated-inosamine precursors. Our findings and hypotheses have been summarized in Scheme I.

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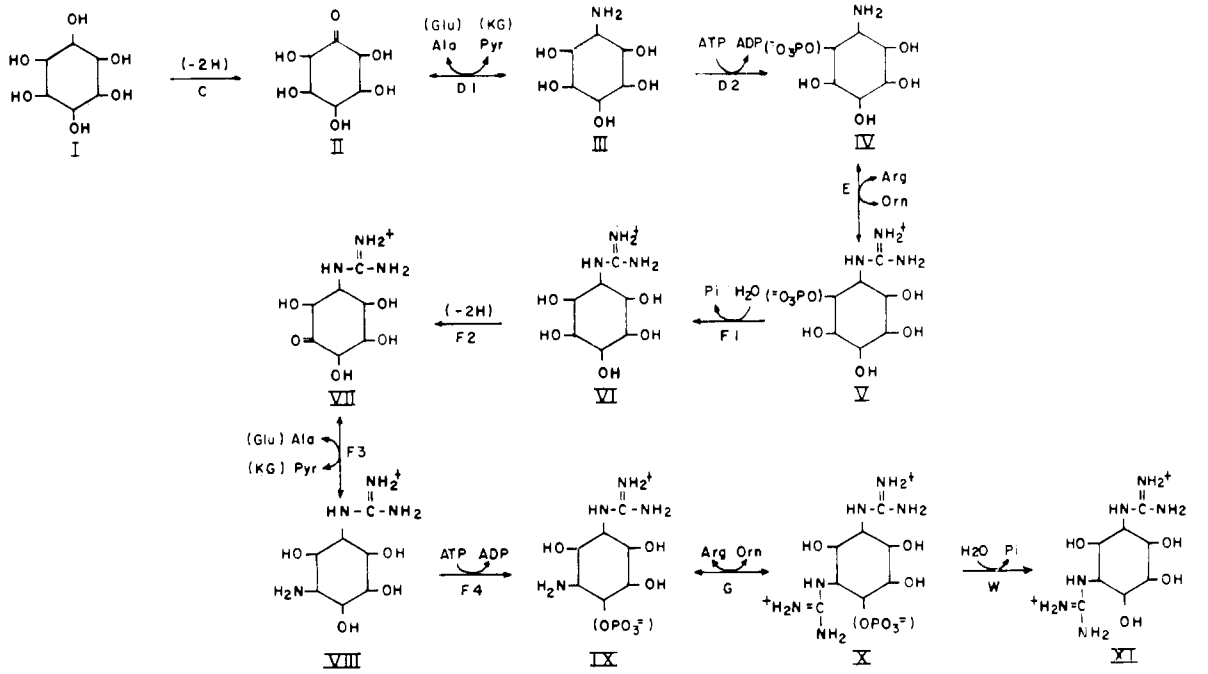
[1] Trivial names and abbreviations used: streptidine, all *trans*-1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane; *scyllo*-inosamine, aminodeoxy-*scyllo*-inositol; DL-myoinosamine-4, DL-4-amino-4-deoxymyoinositol; neoinosamine-2,2-amino-2-deoxy-neoinositol; streptamine, 1,3-diamino-1,3-dideoxy-*scyllo*-inositol; ATP, adenosine 5′-triphosphate; NAD^+ and $NADP^+$, oxidized nicotinamide–adenine dinucleotide and its phosphate; orn, ornithine; pyr, pyruvate; KG, α-ketolgutarate.

Results

Detection of Inosamine Derivatives in Mycelia Fed Myo[^{14}C]inositol. When myo[^{14}C]inositol was fed to mycelia of *Streptomyces griseus* ATCC 12475

SCHEME I: Our Present Concept of Reactions Involved in the Biosynthesis of Streptidine (XI) from Myoinositol (I).



for 1 hr, mycelial extracts were found to contain a number of radioactive inosamine derivatives, as shown in Figure 1. No additional radioactive peaks were obtained following further elution with 2.5 and 5 N HCl. Inosamine-P (peak 0.5A), *N*-amidinostreptamine-P (peak 2.0 A), and streptidine-P (peak 2.0 B) had been detected and characterized earlier in mycelia fed myo[2-^{14}C]inositol (Walker and Walker, 1967a). The labeled compound of peak 0.5B had mobilities on paper chromatograms and high-voltage paper electrophoresis identical with those of dephosphorylated inosamine-P (Walker and Walker, 1967a), indicating that it was an inosamine. Since no specific chemical or microbiological assays for trace amounts of inosamines were known, characterization procedures had to be devised. We reasoned that if the compound were a normal precursor of streptidine, and not merely a degradation product of inosamine-P, it might be converted enzymatically to known intermediates in the biosynthetic pathway. It can be seen from Figure 2 that the peak 0.5B compound can be enzymatically phosphorylated with ATP. The phosphorylated product had mobilities (Walker and Walker, 1967a,b) identical with those of an inosamine-P (IV). When arginine was added as an amidine donor, the phosphorylated product was enzymatically converted to an *N*-amidinoinosamine-P (V), as determined by its paper electrophoretic mobilities. Similar results were obtained when the inosamine-P isolated as peak 0.5A was dephosphorylated with alkaline phosphatase, and the result ing inosamine was enzymatically phosphorylated with ATP in the presence or absence of arginine (Figure 3). These reactions were consistent with steps D2 and E of Scheme I, with the peak 0.5B compound being inosamine (III), and the peak 0.5A compound, an *O*-phosphorylinosamine (IV).

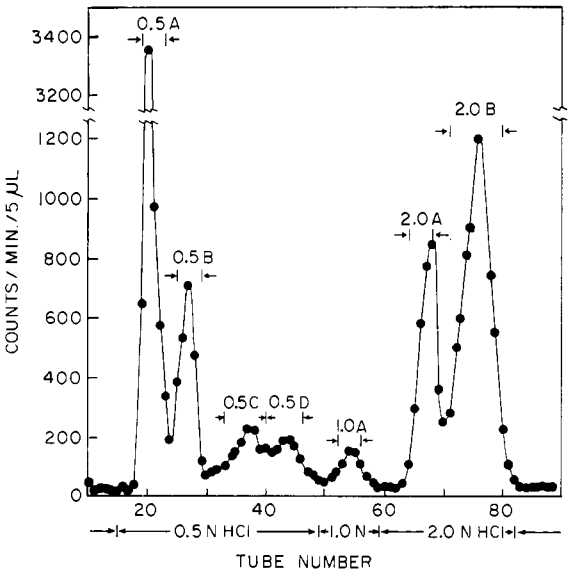


FIGURE 1: Column separation of radioactive, positively charged metabolites of myoinositol present in a hot-water extract of *S. griseus* mycelia fed myo[^{14}C]inositol for 1 hr. Compounds were adsorbed on a Dowex 50 (H^+) column, washed with H_2O, and eluted with HCl solutions.

Substrate Specificity of Inosamine Kinase. Attention was next directed to the configuration of the isolated inosamine. It can be seen from Figure 4 that dialyzed

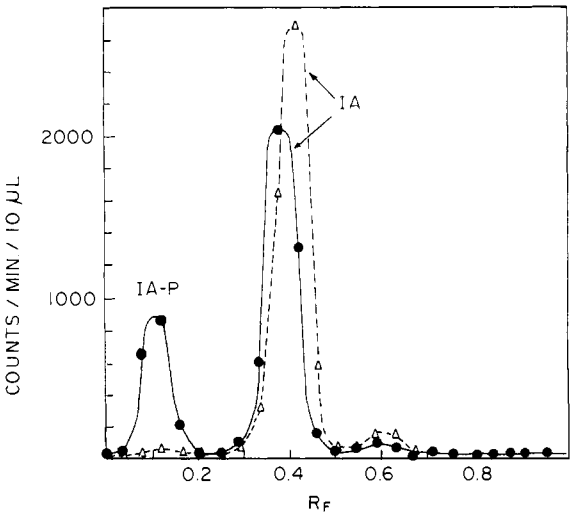


FIGURE 2: Enzymatic phosphorylation of peak 0.5B compound, an [^{14}C]inosamine (IA), with ATP, catalyzed by a dialyzed lysozyme extract of *S. bikiniensis*. The [^{14}C]inosamine-P (IA-P) formed has the same mobility on paper chromatography (80% phenol–20% H_2O, NH_3 atmosphere) as does the peak 0.5A compound. Ornithine was added to inhibit subsequent transamidination of the inosamine-P formed. Solid line: complete incubation mixture. Dashed line: ATP omitted.

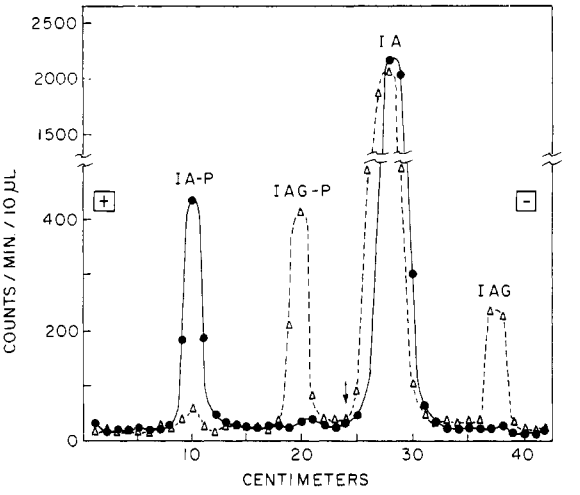


FIGURE 3: Enzymatic phosphorylation, with ATP as donor, of [^{14}C]inosamine (IA) formed by treatment of peak 0.5A compound with *E. coli* alkaline phosphatase, catalyzed by a dialyzed lysozyme extract of *S. bikiniensis*. In the presence of ornithine to inhibit transamidination, only an inosamine-P (IA-P) was formed (solid line). When arginine was added in place of ornithine (dashed line), the inosamine-P formed was transamidinated to *N*-amidinoinosamine-P (IAG-P); a portion of the latter was dephosphorylated (IAG). Compounds were separated by high-voltage paper electrophoresis at pH 10.4. Incubation mixtures were spotted at 24 cm; picric acid moved 12.8 cm. In the absence of ATP, the only peak above background counts was at 28 cm (IA).

lysozyme extracts of *Streptomyces bikiniensis* readily phosphorylated *scyllo*-inosamine at the physiological concentration of 3×10^{-4} M (Figure 4A), but not myoinosamine-2, DL-myoinosamine-4, or neoinosamine-2 (Figure 4B). At concentrations several orders of magnitude higher than this, the latter isomers, and even ethanolamine and 1-aminopropanol, had detectable activity. This enzyme preparation evidently could not convert *N*-acetyl-*scyllo*-inosamine to free inosamine, since no labeled product was formed when *N*-acetyl-*scyllo*-inosamine was added. Both ATP and Mg^{2+} were required, as shown in Table I. The assay involved subsequent transamidination of the enzymatically phosphorylated inosamine with L-[*guanidino*-^{14}C]arginine, catalyzed by the amidinotransferase present (step E). Chemically phosphorylated (Walker and Walker, 1966) neoinosamine-2 was transamidinated as readily as chemically phosphorylated *scyllo*-inosamine (R_F for *O*-phosphoryl-*N*-amidinoneoinosamine-2, 0.25; R_F for *N*-amidinoneoinosamine-2, 0.76), which suggested that the kinase might be somewhat more specific than the amidinotransferase. The inosadiamines, streptamine and 2-deoxystreptamine, both with *trans* configurations, were also readily phosphorylated and subsequently transamidinated with labeled arginine in the presence of dialyzed mycelial extracts (Figure 4C,D). It is possible that these inosadiamines were serving as analogs of *N*-amidinostreptamine (VIII) for the enzymes catalyzing steps F4 and G. Further enzyme purification is required to decide this point. The *N'* group of streptamine, rather than the *N* group (Walker and Walker, 1967b), was transamidinated under these conditions. Evidence for this was

TABLE I: Phosphate Donors for *scyllo*-Inosamine Kinase.

Phosphate Donor	*N*-[^{14}C]Amidino-*scyllo*-inosamine-P Formed (cpm/10 µl)[a]
None	24
Adenosine 5′-triphosphate	2196
Guanosine 5′-triphosphate	10
Uridine 5′-triphosphate	35
Cytidine 5′-triphosphate	45
Thymidine 5′-triphosphate	25
2′-Deoxyadenosine 5′-triphosphate	2090
Adenosine 5′-triphosphate, no Mg^{2+}	12

[a] Compounds were separated by paper chromatography (*cf.* Figure 4).

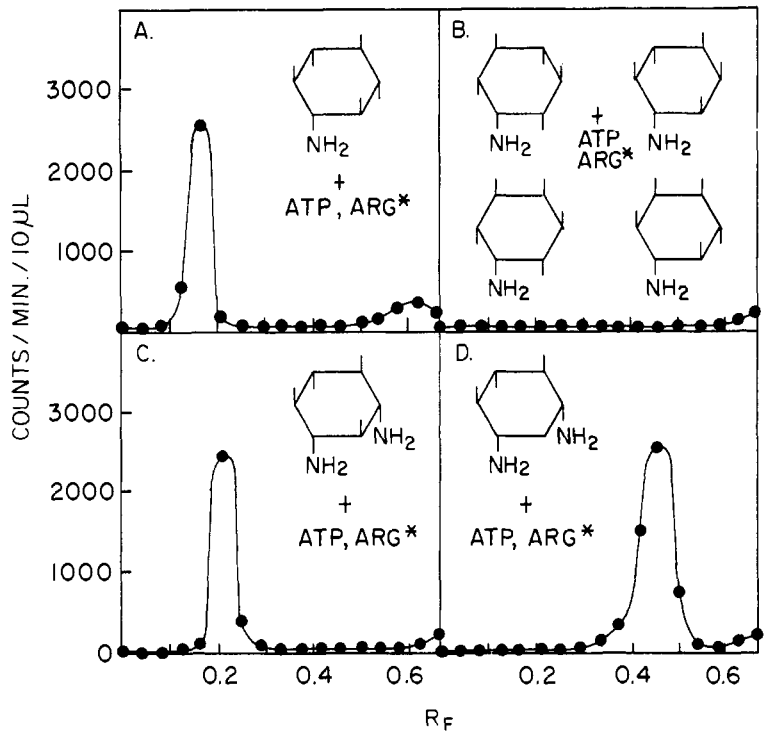


FIGURE 4: Curves showing ability of various inosamines and inosadiamines of known configurations to undergo, at physiological concentrations, a two-step enzymatic conversion catalyzed by a dialyzed lysozyme extract of *S. bikiniensis*. Compounds were first phosphorylated with ATP and then transamidinated with L-[*guanidino*-^{14}C]arginine. Reactants and products were separated by paper chromatography (80% phenol–20% H_2O, NH_3 atmosphere). (A) Formation of *N*-[^{14}C]amidino-*scyllo*-inosamine-P from synthetic *scyllo*-inosamine. (B) Curve showing lack of activity of myoinosamine-2 (upper left), neoinosamine-2 (lower left), and the D and L forms of myoinosamine-4 (right). (C) Formation of *N'*-[^{14}C]amidinostreptamine-P from streptamine. (D) Formation of *N'*-[^{14}C]amidino-2-deoxystreptamine-P from 2-deoxystreptamine.

provided by the fact that, when the labeled product of Figure 4C was enzymatically dephosphorylated, it was not an active substrate for either *N*-amidinostreptamine kinase (step F4) (*cf.* Walker and Walker, 1967b) or *N*-amidinostreptamine–pyruvate transaminase (step F3) (see below).

Conversion of Synthetic scyllo-Inosamine to Streptidine. If the naturally occurring inosamine was indeed of the *scyllo* configuration, it should be possible to convert authentic *scyllo*-inosamine to streptidine by the enzymatic reactions of Scheme I. *scyllo*-Inosamine was first converted to *N*-[^{14}C]amidino-*scyllo*-inosamine-P (V) by steps D2 *plus* E, using L-[*guanidino*-^{14}C]-arginine to label the product (Figure 5A). Some *N*-amidino-*scyllo*-inosamine was also produced, owing to the presence of phosphatase (step F1). The isolated *N*-[^{14}C]amidino-*scyllo*-inosamine-P (V) was then essentially completely converted to *N*-[^{14}C]amidinostreptamine (VIII), as described previously (Walker and Walker, 1967b), by steps F1, F2, and F3, in a single incubation mixture fortified with L-alanine and pyridoxal-P (Figure 5B). The isolated product, *N*-[^{14}C]-amidinostreptamine (VIII), was next converted to [*N-amidino*-^{14}C]streptidine-P (X) by steps F4 and G, with ATP and nonlabeled arginine as donors of phosphate and amidino groups, respectively. Alkaline phosphatase from *Escherichia coli* was added to the incubation mixture to aid in its further conversion (step W) to [*N-amidino*-^{14}C]streptidine, which was adsorbed and then eluted from a Dowex 50 column with 5 N HCl (Figure 5C). Mobilities of the various compounds have been listed elsewhere (Walker and Walker, 1967a). Confirmation that the final product was streptidine was obtained by cocrystallization of a portion of the isolated labeled product with authentic streptidine (Walker and Walker, 1965) as the dipicrate (Table II).

Transamination Reactions of Cyclitol Amino Groups. Conversion of *N*-amidinoinosamine (VI) to *N*-amidinostreptamine (VIII) (steps F2 and F3) was favored by oxidizing conditions and was inhibited by dithionite. This suggested the oxidation of an hydroxyl group to a keto group, followed by an amination reaction. If glutamine were the amino donor, reversibility of step F3 would not be expected. However, if a transamination with glutamate, alanine, or aspartate were involved, the reaction might be reversible. We found that *N*-[^{14}C]amidinostreptamine formed a compound

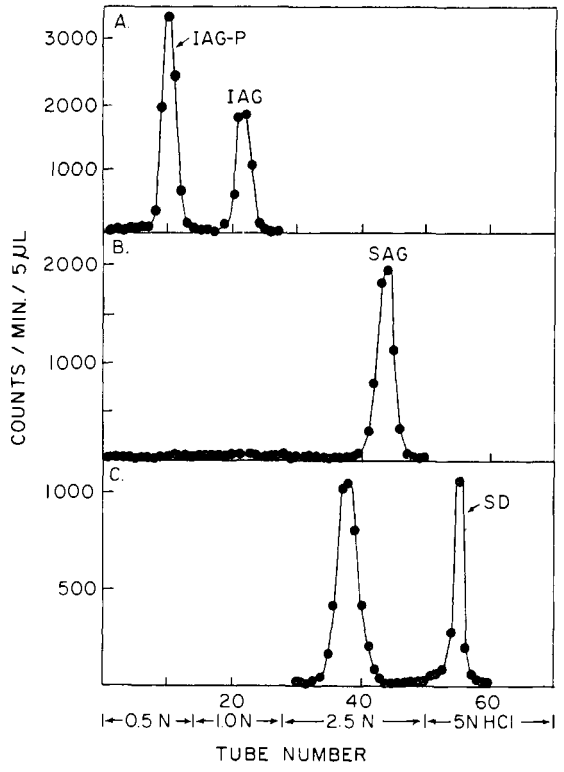


FIGURE 5: Isolation of intermediates and final product in the multistep enzymatic conversion, by extracts of *S. bikiniensis*, of synthetic *scyllo*-inosamine to [*N-amidino*-^{14}C]streptidine. Compounds were separated by adsorption on Dowex 50 columns and subsequent elution with HCl solutions. (A) Separation of *N*-[^{14}C]-amidino-*scyllo*-inosamine-P (IAG-P) and its dephosphorylated derivative (IAG) formed from *scyllo*-inosamine, ATP, L-[*guanidino*-^{14}C]arginine, and dialyzed lysozyme extract. (B) Separation of *N*-[^{14}C]amidinostreptamine (SAG) formed from *N*-[^{14}C]amidino-*scyllo*-inosamine-P, L-alanine, pyridoxal-P, and a 12-min sonicate supernatant. (C) Separation of [*N-amidino*-^{14}C]-streptidine (SD) formed from *N*-[^{14}C]amidinostreptamine, ATP, L-arginine, and a dialyzed lysozyme extract; subsequent incubation with *E. coli* alkaline phosphatase aided in formation of streptidine from its phosphate. Conditions were not optimal in this experiment for streptidine formation; the first peak contains *N*-amidinostreptamine and streptidine-P.

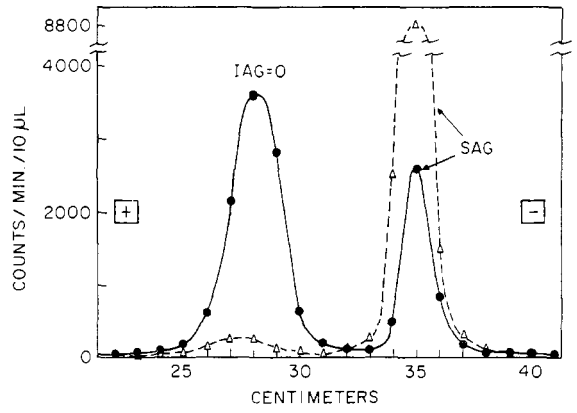


FIGURE 6: Enzymatic reaction of *N*-[^{14}C]amidinostreptamine (SAG) with pyruvate, catalyzed by a dialyzed $(NH_4)_2SO_4$ fraction from *S. bikiniensis*, with pyridoxal-P added. Solid line: complete incubation mixture, showing formation of a compound presumed to be *N*-[^{14}C]-amidino-3-keto-*scyllo*-inosamine (IAG=O). Dashed line: pyruvate omitted. Compounds were separated by high-voltage paper electrophoresis at pH 3.6. Incubation mixtures were spotted at 13 cm; picric acid migrated 11.9 cm.

with one less positive charge when incubated with pyruvate and certain enzyme preparations (Figure 6). This reaction was reasonably specific, since the closely related isomer, *N'*-[^{14}C]amidinostreptamine, had little or no activity; α-ketoglutarate was almost as effective as pyruvate. Pyridoxal-P in some preparations stimulated the reaction 25%. Presumably we were observing the reverse reaction of step F3. The isolated inosamine (peak 0.5B) labeled in the cyclitol ring reacted similarly with pyruvate or α-ketoglutarate, as shown in Figure 7A; this reaction would be the reverse of step D1. A similar transamination reaction was observed with the inosamine obtained by treatment of the peak 0.5A compound with alkaline phosphatase (Figure 7B).

Inosose derivatives are relatively unstable at neutral and alkaline pH values (Anderson and Lardy, 1950; Angyal and Anderson, 1959; Posternak, 1965). However, we were able to isolate the labeled product, together with some decomposition products and/or metabolites, following incubation of *N*-[^{14}C]amidinostreptamine (VIII) with pyruvate and an enzyme fraction from *S. bikiniensis* (Figure 8). This preparation, presumably containing *N*-[^{14}C]amidino-3-ketoinosamine (VII), could be reconverted in part to *N*-[^{14}C]amidinostreptamine by incubating with L-alanine or L-glutamate and the same enzyme preparation (Table III). This enzyme preparation could not catalyze step F2, and therefore could not convert *N*-amidino-*scyllo*-inosamine

TABLE II: Cocrystallization of Labeled Streptidine Formed from *scyllo*-Inosamine with Authentic Streptidine.

Crystzn No.	Sp Act. of Streptidine Dipicrate (cpm/mg)[a]
1st	600
2nd	620
3rd	610
4th	592

[a] By the method of Walker and Walker (1965).

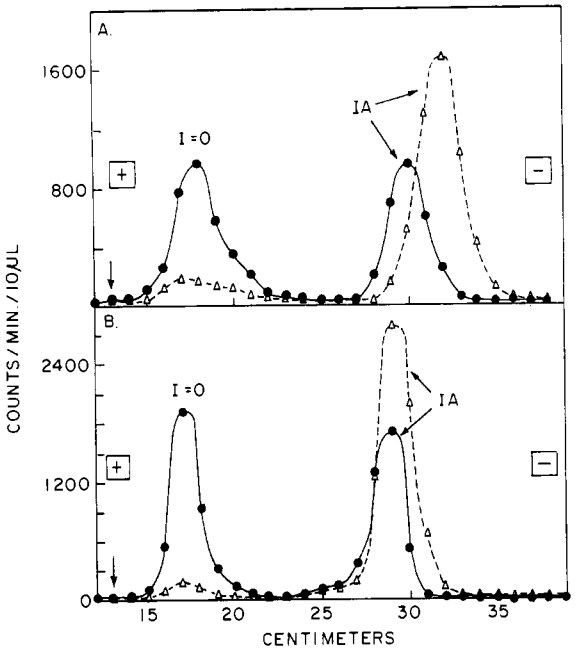


FIGURE 7: Enzymatic reactions of isolated inosamines with pyruvate, catalyzed by a 12-min sonicate supernatant from *S. bikiniensis*, with pyridoxal-P added. Compounds were separated by high-voltage paper electrophoresis at pH 3.6. Incubation mixtures were spotted at 13 cm; picric acid moved 11.3 cm. Solid lines: complete mixtures. Dashed lines: pyruvate omitted. (A) Reaction of [^{14}C]inosamine (IA) obtained from peak 0.5B with pyruvate to give a compound presumed to be an inosose (I=O). Displacement of IA in the control mixture was the spotting error. (B) Reaction of [^{14}C]inosamine (IA), formed by enzymatic dephosphorylation of the peak 0.5A compound, with pyruvate to give a compound as in A.

TABLE III: Amino Donors for the Enzymatic Synthesis of *N*-Amidinostreptamine.

Substrate	*N*-[^{14}C]Amidinostreptamine Formed (cpm/10 μl)[a]
N-[^{14}C]Amidino-*scyllo*-inosamine + L-alanine	0
IAG=O,[b] no added amino donor[c]	314
IAG=O + L-alanine	1419
IAG=O + D-alanine	310
IAG=O + L-glutamate	1163

[a] Compounds were separated by high-voltage paper electrophoresis at pH 3.6 (*cf.* Figure 6). [b] Compound isolated from reaction of *N*-[^{14}C]amidinostreptamine with pyruvate (*cf.* Figure 8). [c] *Streptomyces* are excellent sources of proteolytic enzymes.

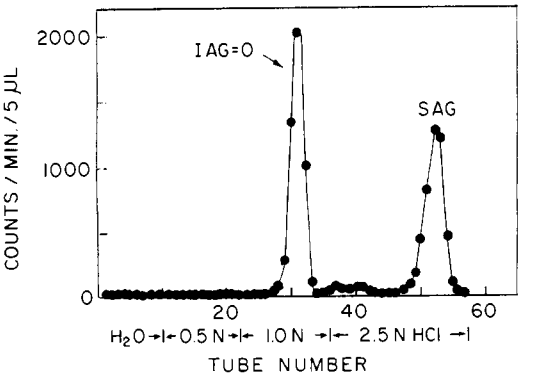


FIGURE 8: Column separation of product formed (IAG=O) by the transamination reaction between *N*-[^{14}C]amidinostreptamine (SAG) and pyruvate. Reaction conditions were as in Figure 6, suitably scaled up. Compounds were adsorbed on Dowex 50 column and eluted with HCl solutions.

to *N*-amidinostreptamine (Table III); consequently the compound designated IAG=O cannot be *N*-amidino-*scyllo*-inosamine. As indicated in Table III, L-alanine was the most effective amino donor tested, followed by L-glutamate. D-Alanine, D-glutamate, L-glutamine, L-aspartate, glycine, and several other compounds, were inactive. Pyridoxal-P stimulated the reaction significantly.

Discussion

Inosamine derivatives are components of a number of antibiotics such as streptomycin, kanamycin, neomycin, paromomycin, hygromycin, and actinospectacin (Angyal and Anderson, 1959; Posternak, 1965; Dutcher, 1963). However, until recently free inosamines or inosamine derivatives had not been detected in biological systems, and almost nothing was known of their biogenesis or metabolism (Mendicino and Picken, 1966). Our current concept of the biosynthesis of streptidine, an *N,N'*-diamidinoinosadiamine, is summarized in Scheme I. We had previously reported detection of compounds IV, IX, and X in mycelia of *S. griseus* fed myo[2-^{14}C]inositol (Walker and Walker, 1967a). In this paper, a free inosamine (III) was detected in a biological system for the first time, in a hot-water extract of mycelia of *S. griseus* fed myo[^{14}C]inositol for 1 hr. This inosamine was assigned a position in the main biosynthetic pathway between myoinositol and streptidine because it underwent both a transamination with pyruvate as acceptor (step D1) and a phosphorylation with ATP as donor (step D2). Moreover, the enzymatically phosphorylated inosamine was readily transamidinated with arginine as donor (step E).

The configuration of the isolated inosamine was studied indirectly by testing the ability of a number of synthetic inosamine isomers to serve as substrates for inosamine kinase (step D2) and inosamine-P amidinotransferase (step E) acting in sequence, employing L-

[*guanidino*-^{14}C]arginine to label the final product. *scyllo*-Inosamine was the most active inosamine isomer tested, being maximally active at 3×10^{-4} M. DL-Myoinosamine-4, myoinosamine-2, and neoinosamine-2 were inactive at this concentration. Assuming a minimal amount of epimerization of precursors during biosynthesis of the all-*trans*-streptidine, *scyllo*-inosamine remained the most likely candidate for III; the next most likely candidates on this assumption, the D and L isomers of myoinosamine-4, were inactive in the above 2-enzyme test. The ability of synthetic *scyllo*-inosamine to be converted *in vitro* all the way to streptidine, reported here, satisfied the presumably sterner specificity requirements of seven enzymes acting in sequence. Comparative biochemical support for the role of *scyllo*-inosamine is given by the fact that bluensomycin, a monoguanidinated analog of dihydrostreptomycin, is a derivative of *scyllo*-inosamine (Bannister and Argoudelis, 1963).

The enzymatic phosphorylations of *scyllo*-inosamine and the inosadiamines, streptamine and 2-deoxystreptamine, were described here for the first time. After transamidination of the enzymatically phosphorylated inosadiamines, the final products were *N'*-amidinostreptamine-P and *N'*-amidino-2-deoxystreptamine-P, respectively, analogous to steps F4 *plus* G, where *N'* designates the second amino group added to the cyclitol ring in the biosynthesis of streptidine. Chemically phosphorylated inosadiamines behaved similarly (Walker and Walker, 1966). Although absolute assignments cannot yet be made, the two isomeric forms of monoamidinated streptamine can be distinguished by the relative lack of activity of the *N'* isomer with *N*-amidinostreptamine kinase (step F4) (Walker and Walker, 1967b) and transaminase (step F3).

The location of the phosphate group is not known for any of the compounds; phosphorylation is evidently required for transamidinations to occur. We have previously depicted the phosphate group *meta* to the primary amino group to be transamidinated (Walker and Walker, 1967b); here we depict it *ortho* to the amino group (Scheme I), since ethanolamine (as well as 1-aminopropanol) at high concentrations can be phosphorylated and subsequently transamidinated. In addition, the greater asymmetry of the *ortho vs.* the *meta* positions might better explain the marked differences in R_F values observed for *N*-amidinostreptamine-P and *N'*-amidinostreptamine-P on paper chromatograms employing 80% phenol–20% H_2O, NH_3 atmosphere (Walker and Walker, 1967a). Further work on this problem is planned.

Inososes have not been detected in tissue extracts to our knowledge, although the occurrence of *scyllo*-inosose can be inferred wherever *scyllo*-inositol is found (Posternak *et al.*, 1959; Posternak, 1965; Kindl *et al.*, 1966; Candy, 1967). Our earlier suggestion of the involvement of *scyllo*-inosose in streptidine biosynthesis (Walker and Walker, 1966) has been given subsequent support by the experiments of Bruton *et al.* (1967), which showed that *scyllo*-inositol as well as myoinositol can serve as a precursor of the streptidine moiety of streptomycin *in vivo*. Conversion of *N*-amidinoinosamine (V) to *N*-amidinostreptamine (VII) appeared to require oxidizing conditions; in many incubation mixtures not fortified with L-alanine and pyridoxal-P, a compound accumulated which showed the same behavior on ammoniacal–phenol paper chromatograms as did the product formed by transamination of *N*-amidinostreptamine and pyruvate, presumably VII. Further purification of the transaminases reported here should help clarify the nature of the physiological reactants and products. We think it likely that the amino groups of inosamines and inosadiamines which occur in other antibiotics such as neomycin, kanamycin, and hygromycin are formed by similar transamination reactions.

The enzymatic reactions of Scheme I should provide useful genetic markers for studies of DNA transfer from streptomycin producers to other strains or streptomycin-negative mutants. The distribution of individual enzymes among various strains of *Streptomyces* should also prove of interest. Factors governing differentiation of mycelia to the antibiotic-synthesizing state could be conveniently studied using enzymes of streptidine biosynthesis as markers. From Scheme I an over-all equation for biosynthesis of streptidine can be derived.

$$\text{myoinositol} + 2\text{A} + 2\text{L-alanine} + 2\text{L-arginine} + 2\text{ATP} \longrightarrow \text{streptidine} + 2\text{AH}_2 + 2\text{pyruvate} + 2\text{L-ornithine} + 2\text{ADP} + 2\text{P}_i$$

For biogenesis from glucose, one more ATP would be required (*cf.* Chen and Charalampous, 1965). The nature of the primary hydrogen acceptor (A) is not known, but presumably is NAD^+ or $NADP^+$ (*cf.* Candy, 1967; Berman and Magasanik, 1966); the ultimate acceptor is probably oxygen. It will be instructive to compare these findings with commercial fermentation media found by empirical means to be favorable for streptomycin production.

Materials and Methods

Myo[^{14}C]inositol (150 mc/mmole) came from Nuclear-Chicago Corp. L-[*guanidino*-^{14}C]Arginine·HCl (25 mc/mmole) came from Calbiochem. *scyllo*-Inosamine, *N*-acetylmyoinosamine-2, and *N*-acetyl-DL-myoinosamine-4 were kindly supplied by Dr. Laurens Anderson, University of Wisconsin. The *N*-acetyl groups were removed from the latter two preparations by heating with a large excess of 6 N HCl for 6 hr at 100°, followed by evaporation to dryness *in vacuo*. This procedure was checked by hydrolyzing *N*-acetyl-*scyllo*-inosamine, prepared from inosose–oxime by the method of Anderson and Lardy (1950), and testing both reactant and product for substrate activity with *scyllo*-inosamine kinase. Neoinosamine-2 was a gift to Dr. A. D. Elbein from Eli Lilly Co. Streptidine, streptamine, and 2-deoxystreptamine were prepared as described previously (Walker and Walker, 1966).

Ring-Labeled Inosamine Derivatives. The procedure with uniformly labeled myo[^{14}C]inositol was similar to that described for myo[2-^{14}C]inositol (Walker and Walker, 1967a) except that 48 μc of myo[^{14}C]inositol (6×10^7 cpm) was incubated with 250 ml of a 3-day culture of *S. griseus* ATCC 12475 on a shaker for 1 hr, and the washed mycelial pad weighed 3.3 g. A hot-water extract was made and chromatographed on a Dowex 50 (H^+) column (Walker and Walker, 1967a).

Separation Methods. Column chromatography, paper chromatography, and high-voltage paper electrophoresis procedures were carried out as described previously (Walker and Walker, 1967a). The occurrence of amino, guanidino, and phosphate groups in the various intermediates greatly aided in their characterization. More conventional assays were not available for these compounds.

Enzyme Preparations. Cultures of *S. bikiniensis* ATCC 11062 were grown on a 2% peptone–0.2% yeast extract medium, and harvested as described earlier (Walker and Hnilica, 1964). Three types of enzyme preparations were used. Dialyzed lysozyme extracts were employed as a source of kinases and amidinotransferase (steps D2, E, F4, and G). Frozen 2.5-day mycelial pads were shaved with scissors and extracted for 1 hr at room temperature with three volumes of 0.1 M potassium phosphate buffer (pH 7.4), containing EDTA (5 mg/ml) and crystalline egg white lysozyme (1 mg/ml). Following centrifugation at 30,000*g* for 30 min at 4°, the supernatant solution was dialyzed for 48 hr against two changes of 0.001 M phosphate buffer–EDTA *plus* 0.1 ml of 2-mercaptoethanol/4 l. followed by dialysis against 2.5 mM Tris (pH 7.4) *plus* 2-mercaptoethanol. As a source of inosamine transaminases and dehydrogenase (steps D1, F2, and F3), 12-min sonicates of 3-day mycelial pads were prepared according to Walker and Walker (1967b). As a cleaner source of *N*-amidinostreptamine–pyruvate transaminase (step F3), the 12-min sonicate supernatant was treated with Mn^{2+} and then fractionated with $(NH_4)_2SO_4$ as described elsewhere (Walker and Walker, 1967c); a dialyzed 30–70% $(NH_4)_2SO_4$ enzyme fraction was used.

Conversion of Inosamine (Peak 0.5B) to Inosamine-P. Incubation conditions were [^{14}C]inosamine (peak 0.5B), 10 μl (23,000 cpm); 0.5 M Tris (pH 7.4) containing 0.04 M $MgCl_2$ and 0.083 M L-ornithine·HCl, 5 μl; 0.29 M 2-mercaptoethanol, 1 μl; 0.036 M ATP, 5 μl; dialyzed lysozyme extract of *S. bikiniensis*, 10 μl; H_2O, 5 μl; incubated 90 min at 37°.

Conversion of Inosamine from Dephosphorylated Inosamine-P (Peak 0.5A) to Inosamine-P and N-Amidinoinosamine-P. Incubation conditions to form inosamine-P were [^{14}C]inosamine (derived from peak 0.5A), 10 μl (26,000 cpm); 0.5 M Tris (pH 7.4) containing 0.04 M $MgCl_2$ and 0.083 M L-ornithine·HCl, 5 μl; 0.29 M 2-mercaptoethanol, 1 μl; 0.036 M ATP, 5 μl; dialyzed lysozyme extract of *S. bikiniensis*, 10 μl; H_2O, 5 μl; incubated 90 min at 37°. Incubation conditions to form *N*-amidinoinosamine-P were the same as above except that 0.067 M L-arginine·HCl was substituted for ornithine.

Phosphorylation of Synthetic Inosamines and Inosadiamines of Known Configurations. Incubation conditions were 1.1 mM inosamine or inosadiamine (2.2 mM for DL preparation), 10 μl; 0.5 M Tris (pH 7.4) containing 0.04 M $MgCl_2$, 5 μl; 0.036 M ATP, 5 μl; 0.29 M 2-mercaptoethanol, 1 μl; dialyzed lysozyme extract of *S. bikiniensis*, 10 μl; 33 μc/ml of L-[*guanidino*-^{14}C]arginine·HCl, 5 μl; incubated 60 min at 37°. The phosphorylated aminocyclitol formed was labeled by transamidination with arginine, catalyzed by the amidinotransferase present. For determination of specificity of the phosphate donor, 0.018 M solutions of nucleoside triphosphates were used.

Conversion of scyllo-Inosamine to [N-amidino-^{14}C]-Streptidine. This conversion occurred in three stages. *Stage 1: scyllo*-inosamine → *scyllo*-inosamine-P → *N*-[^{14}C]amidino-*scyllo*-inosamine-P. The incubation mixture of the preceding section, using *scyllo*-inosamine, was scaled up 400-fold, incubated 120 min at 37°, deproteinized with 0.4 ml of 30% trichloroacetic acid, and chromatographed on a Dowex 50 column. *Stage 2: N*-[^{14}C]amidino-*scyllo*-inosamine-P → *N*-[^{14}C]amidino-*scyllo*-inosamine → → *N*-[^{14}C]amidinostreptamine. Incubation conditions were *N*-[^{14}C]amidino-*scyllo*-inosamine-P, 0.72 ml (5.27×10^6 cpm); 0.5 M Tris (pH 8.8), 0.3 ml; 0.20 M L-alanine, 0.2 ml; 12-min sonicate supernatant from 3-day *S. bikiniensis*, 0.72 ml; incubated 180 min at 37° in a 100-ml beaker, then deproteinized with 0.2 ml of 30% trichloroacetic acid, and chromatographed on a Dowex 50 column. *Stage 3: N*-[^{14}C]amidinostreptamine → *N*-[^{14}C]amidinostreptamine-P → [*N-amidino*-^{14}C]streptidine-P → [*N-amidino*-^{14}C]streptidine. Incubation conditions were *N*-[^{14}C]amidinostreptamine, 0.65 ml (3.6×10^6 cpm); 0.5 M Tris (pH 7.4) containing 0.04 M $MgCl_2$, 0.30 ml; 0.036 M ATP, 0.30 ml; 0.29 M 2-mercaptoethanol, 0.05 ml; 0.095 M L-arginine·HCl, 0.10 ml; dialyzed lysozyme extract of *S. bikiniensis*, 0.60 ml; incubated 120 min at 37°, then 0.2 ml of 1 M Tris (pH 8.8) and 0.1 ml of *E. coli* alkaline phosphatase were added, and incubated 120 min more. The mixture was deproteinized with 0.2 ml of 30% trichloroacetic acid and chomatographed on a Dowex 50 column. An aliquot of the labeled streptidine product, calculated to give a specific activity of 600 cpm/mg, was cocrystallized with authentic streptidine as the dipicrate (Walker and Walker, 1965).

Reaction of N-Amidinostreptamine with Pyruvate. For high-voltage paper electrophoresis studies, incubation conditions were *N*-[^{14}C]amidinostreptamine, 10 μl (99,000 cpm); 0.5 M Tris (pH 7.4), 5 μl; 0.22 M sodium pyruvate, 5 μl; 0.02 M pyridoxal-P, 1 μl; 30–70% $(NH_4)_2SO_4$ enzyme fraction from *S. bikiniensis* sonicate, 10 μl; incubated for 120 min at 37°. For column isolation, incubation conditions were *N*-[^{14}C]amidinostreptamine, 0.70 ml (6.9×10^6 cpm); 0.5 M Tris (pH 7.4), 0.70 ml; 0.22 M sodium pyruvate, 0.70 ml; 30–70% $(NH_4)_2SO_4$ enzyme fraction, 1.4 ml; incubated 140 min at 37° in a 250-ml beaker, deproteinized with 0.15 ml

of 30% trichloroacetic acid, and chromatographed on a Dowex 50 column.

Reaction of Inosamine with Pyruvate. Incubation conditions were [^{14}C]inosamine, 10 μl (23,000 cpm for peak 0.5B compound, and 26,000 cpm for dephosphorylated peak 0.5A compound); 0.5 M Tris (pH 7.4), 5 μl; 0.22 M sodium pyruvate, 5 μl; 0.02 M pyridoxal-P, 1 μl; 12-min sonicate supernatant from 3-day *S. bikiniensis*, 10 μl; incubated for 120 min at 37°.

Reaction of Presumed N-Amidino-3-keto-scyllo-inosamine with Amino Acids. Incubation conditions were "*N*-[^{14}C]amidino-3-keto-*scyllo*-inosamine," 10 μl (27,800 cpm) (or for a control, *N*-[^{14}C]amidino-*scyllo*-inosamine, 66,000 cpm); 0.5 M Tris (pH 7.4), 5 μl; 0.02 M pyridoxal-P, 1 μl; 0.20 M amino acid, 5 μl; 30–70% $(NH_4)_2SO_4$ enzyme fraction from *S. bikiniensis* sonicate, 10 μl; incubated for 120 min at 37°.

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