Streptomycin Biosynthesis

SEPARATION AND SUBSTRATE SPECIFICITIES OF PHOSPHATASES ACTING ON GUANIDINO-DEOXY-SCYLL0-INOSITOL PHOSPHATE AND STREPTOMYCN-(STREPTIDINO)PHOSPHATE*

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SUMMARY

Two distinct guanidinocyclitol phosphate phosphatase activities have been detected in extracts of streptomycin-producing strains of Streptomyces and separated on a Sephadex G-200 column. Both enzymatic activities appear following a phase of rapid mycelial growth on complex media, and the enzymes thus are idiosynthetic enzymes. Both enzymes require Mg2+ for activity, and both are inhibited by inorganic phosphate. Guanidinodeoxy-scyllo-inositol phosphate phosphatase catalyzes an essential reaction in the biosynthesis of streptidine, a component of streptomycin. 2-Guanidino-2-deoxy-neo-inositol phosphate and N'-amidinostreptamine phosphate can also serve as substrates. Activity is inhibited by sulfhydryl reagents and stimulated by dithiothreitol. Periodate degradation studies suggest that substrate phosphate is esterified at position 4, para to the guanidino group. Streptomycin-(streptidino)phosphate phosphatase hydrolyzes phosphate esterified with streptidino, either free or combined in streptomycin derivatives, such as dihydrostreptomycin-(streptidino)phosphate and phosphoryldihydrostreptomycin-(streptidino)phosphate. Tris competes effectively with water as an acceptor of phosphate from these compounds; O-phosphoryl-Tris, once formed, is only slowly hydrolyzed. Activity is not inhibited by sulfhydryl reagents. This enzyme also acts to a lesser extent on N-amidinostreptamine phosphate and N'-amidinostreptamine phosphate. Periodate degradation studies on the latter two compounds have suggested that the natural N isomer has its amino group para to the phosphate, whereas the unnatural N' isomer has its guanidino group para to the phosphate. A current concept of the pathway of streptidine biosynthesis is presented.

The antibiotic streptomycin (Fig. 1) is synthesized by certain strains of Streptomyces following a period of rapid mycelial growth. The biosynthesis of streptomycin has been studied by a number of investigators, and their principal findings have recently been reviewed by Demain and Inamine (1). Our laboratory has been primarily concerned with biosynthesis of streptidine, a component of streptomycin. Our current scheme for biosynthesis of streptidine (XI) from myo-inositol (I) is given in Fig. 2. It can be seen that five enzymatic reactions are required to convert a cyclitol—OH group to a —NH-C(=NH2)NH2 group; a dehydrogenation, transamination, phosphorylation of an hydroxyl group, transamination, and dephosphorylation. After the first guanidino group is synthesized, an analogous sequence of five enzymatic reactions, in the same order, functions in biosynthesis of the second guanidino group (2-4). Many details of the scheme of Fig. 2 remain to be elucidated, but the general pattern appears to be correct.

It was of particular interest to determine whether corresponding reactions of the same type were catalyzed by the same or different enzymes. No evidence has yet been obtained that the two transamidination reactions are catalyzed by substantially different enzymes; the same is true for the first two phosphorylation reactions. On the other hand, the two transamidination reactions have been shown to be catalyzed by different enzymes, although their substrate specificities show considerable overlap (4). Studies on the two dephosphorylation reactions are complicated by the fact that it is not known with certainty at what stage in the biosynthesis of streptomycin the phosphate group esterified with streptidine is removed. It is possible that phosphate is removed from the streptidine moiety at more than one stage during biosynthesis, being introduced at an intervening step by the action of streptidinokinase, an enzyme which occurs in streptomycin producers (5). Characterization of these dephosphorylations is of practical importance because of the pronounced inhibitory effect of excess inorganic phosphate on streptomycin formation (1), an inhibition which was recently shown to result in accumulation of streptomycin-(streptidino)phosphate in the culture medium (6).

In this paper it is seen that two distinct guanidinocyclitol phosphate phosphatase activities occur in extracts of streptomycin-producing strains. These two phosphatases differ in their substrate specificities, pH optima, and reactivity toward sulfhydryl reagents. Evidence bearing on location of the phosphate groups on the cyclitol rings of the various substrates of these enzymes is described also.

MATERIALS AND METHODS

Cultures of Streptomyces strains came from the American Type Culture Collection. p-Nitrophenyl phosphate and purified alka-
line phosphatases from *Escherichia coli* (type VII) and calf intestinal mucosa (type VIII) came from Sigma. References for preparation of labeled substrates employed are as follows: \([\text{H}]\)dihydrostreptomycin-(streptidine)phosphate, labeled at position 3' of the dihydrostreptose moiety (7); phosphoryl\([\text{H}]\)dihydrostreptomycin-(streptidine)phosphate from *Streptomyces bikiinensis* (7); dihydrostreptomycin-(streptidine)\([\text{P}]\)phosphate (7); aminodeoxy-scyllo-\([\text{C}]\)inositol phosphate (3); \([\text{C}]\)guanidinoa

Cultures of *Streptomyces* were grown and harvested as described previously (7). Phosphohydrolase and phosphotransferase activities can be readily demonstrated with dialyzed lysozyme extracts of frozen mycelial pads. However, more purified preparations were employed here; all purifications were performed at 4° or less. Frozen mycelial pads of *S. bikiniensis* ATCC 11062, 18.7 g, were sonicated with an equal volume of water for a total of 15 min in 30-sc bursts, and centrifuged 20 min at 30,000 × g. To 20 ml of supernatant solution were added, dropwise with stirring, 6.7 ml of 10\% MnCl₂ . 4H₂O in 0.1 M Tris, pH 7.4. After 20 min the mixture was centrifuged. Powdered (NH₄)₂SO₄ was added to the supernatant solution to bring it to 20\% of saturation. After 15 min the mixture was centrifuged. The supernatant solution was brought to 65\% saturation with (NH₄)₂SO₄, while the pH was adjusted to neutrality with NH₄OH. After 15 min the mixture was centrifuged, and the precipitate was taken up in 5 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 5 mg per ml of EDTA, and dialyzed against 4 liters of 1 mM phosphate buffer, pH 7.4, containing 0.05 mg per ml of EDTA, and 0.1 ml of mercaptoethanol for 2.5 days with one change in external medium. A 0.5-ml aliquot of the dialyzed preparation, containing 17.5 mg of protein, was diluted with 1 ml of 0.1 \(\text{M} \)Tris, pH 7.4, and applied to a Sephadex G-200 column, 3 cm × 67 cm, previously equilibrated with 0.01 \(\text{M} \)Tris, pH 7.4, plus mercaptoethanol (10 \(\mu\)l/100 ml). The column was eluted at the rate of 1 drop per 37 sec with the equilibrating buffer; 2.5-ml fractions were collected, and 1.5 mg of serum albumin were added to odd-numbered tubes, starting with tube 61, to preserve activity of the guanidinoa- 

**Fig. 1.** Structure of streptomycin. In dihydrostreptomycin the aldehyde group of the streptose moiety has been reduced to a hydroxymethyl group.

**Fig. 2.** Our current concept of intermediates involved in the biosynthesis of streptidine (XI) from myo-inositol (I). GLN, glutamine; KGAM, 2-ketoglutaramate.
Guanidinocyclitol Phosphate Phosphatases

Separation of Phosphatase Activities on Sephadex Column—In our experiments, p-nitrophenyl phosphate was employed as a model substrate for the assay of relatively nonspecific alkaline phosphatases; the specific substrates employed were [3H]guanidino-2-scylllo-inositol phosphate (Compound V, Fig. 2) and [14C]guanidinocyclitol phosphate. When an ammonium sulfate fraction from an extract of S. bokiniensis was subjected to gel filtration on a Sephadex G-200 column and assayed for phosphatase activities with the substrates mentioned above, the separations shown in Fig. 3 were obtained. It can be seen that at least three phosphatase activities were detected: one reacting most readily with guanidino-2-scylllo-inositol phosphate, one reacting with p-nitrophenyl phosphate, and one reacting with both dihydrostreptomycin-(streptidino)phosphate and p-nitrophenyl phosphate. The latter two activities are similar to those described by Nimi et al. (12) for their strain of S. griseus; however, these authors did not further characterize the substrate specificity of their fraction reacting with streptomycin-(streptidino)phosphate.

Substrate Specificities of Three Phosphatase Fractions—The substrate specificities of certain Sephadex column fractions were tested with a number of phosphorylated guanidinocyclitol derivatives. The concentrations of these various labeled substrates were different, but for a given substrate the same concentration was employed for assay of each of the three enzyme fractions. The results are given in Table I. In these experiments incubations were performed at the pH optimum with the preferred substrate for each of the three enzyme fractions. Appropriate cross checks were made to ensure that the differences in substrate specificities of the fractions were not due primarily to differences in pH optima or sulfhydryl group activation. It can be seen that streptomycin-(streptidino)phosphate phosphatase hydrolyzes phosphate esterifled with streptidine, either free or combined in streptomycin derivitives; a general term for this enzyme would be streptidino-phosphate phosphatase. Guanidino-2-scylllo-inositol phosphate phosphatase also hydrolyzes two compounds not known to occur in nature, 2-guanidino-2-deoxy-neo-inositol phosphate and N'-amidinostreptamine phosphate.

The occurrence of dihydropyrophosphatase activity in dihydrostreptomycin, with our phosphate esterified with the streptidine group and one with the dihydrostreptosobiosamine moiety (7), has furnished another test of the substrate specificity of streptomycin-(streptidino)phosphate phosphatase. It can be seen from Fig. 4 that the latter enzyme preparation removed only one phosphate from the dihydropyrophosphorylated dihydrostreptomycin derivative formed from dihydrostreptomycin and ATP in S. bokiniensis extracts, as described previously (7). The fact that the phos-
Labeled compounds were incubated at 37° for 1 hour with the following Sephadex column fractions (Fig. 3): No. 54 for streptomycin-(streptidinophosphate) phosphatase activity (SMP Pase); No. 89 for p-nitrophenyl phosphate phosphatase (pNPP Pase); No. 109 for guanidinodeoxy-scyllo-inositol phosphate phosphatase (IGP Pase). Compounds were separated by paper chromatography.

<table>
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<tr>
<th>Substrate hydrolysed</th>
<th>SMP Pase</th>
<th>pNPP Pase</th>
<th>IGP Pase</th>
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</thead>
<tbody>
<tr>
<td>Aminodeoxy-scyllo-([^{14}C])inositol phosphate</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
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<td>1</td>
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<tr>
<td>2-(([^{14}C])Guadinine-2-deoxy-neo-inositol phosphate</td>
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<td>5</td>
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<tr>
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<td>2</td>
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<td>55</td>
</tr>
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<td>0</td>
<td>2</td>
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<tr>
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<td>30</td>
<td>1</td>
<td>37</td>
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<tr>
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<td>11</td>
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<td>0</td>
</tr>
<tr>
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<td>30*</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

*Percentage of streptidinophosphate moiety hydrolyzed (see Fig. 4).

Phosphate removed was that esterified with the streptidine moiety was shown by mild acid hydrolysis of the reaction product (Fig. 4B); this treatment separates the streptidine and dihydrostreptobiosamine moieties. The remaining phosphate was esterified with the labeled dihydrostreptobiosamine moiety (Fig. 4C). On the other hand, calf intestinal mucosa alkaline phosphatase preferentially hydrolizes phosphate esterified with the dihydrostreptobiosamine moiety; phosphate esterified with the streptidine moiety is hydrolyzed much more slowly (7).

Column fractions containing streptomycin-(streptidino)phosphate phosphatase not only transfer phosphate from dihydrostreptomycin-(streptidino)phosphate or from streptidine phosphate to water, but also to Tris, as shown in Fig. 5. In experiments in which the phosphate group of the substrate is labeled, Tris buffer must be rigidly excluded from incubation mixtures. For this reason certain of our experiments have been conducted with glycylglycine buffer. The time course of Tris-phosphate formation is shown in Fig. 6. Tris-phosphate, once formed, is only slowly hydrolyzed by this preparation. Tris-phosphate formation could be detected at Tris concentrations as low as 1.6 mm. Comparison of electrophoretic mobilities of Tris-phosphate versus known compounds at pH 3.6 and pH 10.4 indicated that O-phosphoryl-Tris was the reaction product.

**Effects of Metal Ions, pH, and Sulphydryl Reagents on Guanidinocyclitol Phosphate Phosphatases—**Both phosphatases require Mg\(^{2+}\) for maximal activity. The following ions did not substitute for Mg\(^{2+}\): Mn\(^{2+}\), Zn\(^{2+}\), Ca\(^{2+}\), Cu\(^{2+}\), Fe\(^{3+}\), or K\(^{+}\). A requirement for Mg\(^{2+}\) is easily demonstrated with guanidinodeoxy-scyllo-inositol phosphate phosphatase. To show a marked requirement of streptomycin-(streptidino)phosphate phosphatase for Mg\(^{2+}\), repeated dialysis against EDTA is usually needed. Guanidinodeoxy-scyllo-inositol phosphate phosphatase has an optimal activity around pH 7.4. Streptomycin-(streptidino)phosphate phosphatase has a higher pH optimum, between pH 8.0 and 9.0. From Table II it can be seen that guanidinodeoxy-scyllo-inositol phosphate phosphatase is a sulfhydryl enzyme, being strongly inhibited by \(p\)-hydroxymercurobenzoate, with subsequent reversal by mercaptoethanol. Formamidine disulfide and cystamine also inhibit this enzyme, presumably by formation of mixed disulfides (cf. 13). Enzymatic activity during incubations was often higher in the presence of dithiothreitol. Our previously reported low activity of this enzyme on 2-guanido-2-deoxy-neo-inositol phosphate (8) can be explained by inhibitory contaminants in the substrate preparations available at that time. No inhibition of streptomycin-(streptidino)phosphate phosphatase by \(p\)-hydroxymercurobenzoate, formamidine disulfide, or cystamine has been observed.

**Inhibition by Inorganic Phosphate—**Most phosphatases are inhibited by inorganic phosphate, and the data of Table III show that guanidinocyclitol phosphate phosphatases are no exceptions. These experiments were performed because of the well known
Fig. 5. Experiment showing that column fraction containing streptomycin-(streptidino)phosphate phosphatase activity catalyzes transfer of phosphate from dihydrostreptomycin-(streptidino)phosphate to Tris. Compounds were separated by high voltage paper electrophoresis at pH 3.6. Picric acid migrated 16.9 cm. Sephadex Fraction 53 (Fig. 3) was the enzyme source. A, complete hydrolysis of dihydrostreptomycin-(streptidino)[32P]phosphate (DSMP) in the absence of Tris to give labeled inorganic phosphate (Pi). B, in the presence of 80 mM Tris, Tris-[32P]phosphate was formed, with one negative and one positive charge at this pH. Note that again all of the DSMP reacted, and that 80 mM Tris competed effectively with 80 mM water for the transferred phosphate.

Fig. 6. Experiment showing time course of formation of Tris-[32P]phosphate and inorganic [32P]phosphate (Pi) from dihydrostreptomycin-(streptidino) [32P]phosphate (DSMP) as catalyzed by Sephadex Fraction 52. Compounds were separated on amoniacal phenol paper chromatograms. By 30 min all of the phosphate had been transferred to the two acceptors, water and Tris (80 mM). Note that Tris-phosphate is relatively stable, but hydrolysis does occur at a very slow rate. Rf values were as follows: Pi, 0.02; Tris-P, 0.25; DSMP, 0.67.

inhibition of streptomycin production by an excess of inorganic phosphate (1). No marked repression of these phosphatases was detected when mycelia of a number of streptomycin producers were grown on peptone-yeast extract media with 10 mM inorganic phosphate added.

Biological Distribution and Appearance of Guanidinocyclitol Phosphatases—The biological distribution of streptomycin-(streptidino)phosphate phosphatase activity closely parallels the ability to produce streptomycin. Strains with activity include Streptomyces griseus ATCC 12475, S. griseus SC-2376 (Squibb), Streptomyces griseocarneus ATCC 12628, and Streptomyces galbus ATCC 14077. Relatively inactive strains include Streptomyces kanamyceticus ATCC 12853, S. griseus ATCC 10971, and Streptomyces radiae. Strains with this activity also transfer phosphate to Tris from streptidine phosphate or from dihydrostreptomycin-(streptidino)phosphate.

Both guanidinocyclitol phosphate phosphatases are idiophase enzymes, appearing only after the phase of rapid mycelial growth on complex media. As an illustration, in Fig. 7 guanidinocyclitol phosphate phosphatase activity of an extract of S. bikinisensis prepared from mycelia grown on a complex medium for 1 day is compared with an extract from mycelia grown for 3 days. No activity was detected in extracts of 1-day mycelia. Similar results have been obtained with other enzymes involved in biosynthesis of the streptidine moiety (cf. 13).

### Table II

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Substrate hydrolyzed</th>
</tr>
</thead>
</table>
| 1. [14C]Guanidinodeoxy-scyllo-inositol phosphate + Sephadex Fraction 108 | 87%
| 2. (1) + p-hydroxymercuribenzoate, 1 mM | 3%
| 3. (2) + mercaptoethanol, 17 mM | 89%
| 4. [3H]Dihydrostreptidine-(streptidino)phosphate + Sephadex Fraction 54 | 87%
| 5. (4) + p-hydroxymercuribenzoate, 1 mM | 88%

### Table III

<table>
<thead>
<tr>
<th>Inorganic phosphate</th>
<th>Substrate hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IGP Pase</td>
</tr>
<tr>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>46%</td>
</tr>
<tr>
<td>20</td>
<td>19%</td>
</tr>
</tbody>
</table>

* Guanidinocyclitol phosphate phosphatase (Sephadex No. 107).
* Streptomycin-(streptidino)phosphate phosphatase (Sephadex No. 54).
product of the latter reaction readily incorporates label into one phosphorylated in streptidine phosphate (Compound X, Fig. 2) since the same position is believed to be phosphorylated in streptidine phosphate (Compound X, Fig. 2). The product of the latter reaction readily incorporates label into one of its guanidino groups during an exchange transamidination in the presence of L-L[^14C]^guanidino)arginine and amidinotransferase (6); presumably N-amidinoamidinostreptamine phosphate is an intermediate in this incorporation. By this reasoning N-amidinoamidinostreptamine phosphate would have one of the structures shown in Fig. 9; the other isomer would be N'-amidinoamidinostreptamine phosphate. The latter isomer is formed by allowing amidinostreptamine (1,3-diamino-1,3-dideoxy-scyllo-inositol) to react with a dialyzed extract of S. bikiniiensis, ATP, and L-L[^14C]^guanidino)arginine (3). N designates the first nitrogen substituted on the cyclitol ring during streptidine biosynthesis, and N' designates the second nitrogen. These two isomers migrate differently on paper chromatograms (9), which, together with the above considerations, rules out the more symmetrical meta location for the phosphate group. The two compounds of Fig. 9 should react differently with periodate. It can be seen from Fig. 8C that the labeled unreacted isomer derived from amidinostreptamine, N'-[^14C]^amidinoamidinostreptamine phosphate, reacted with periodate similarly to[^14C]^guanidinoamidinostreptamine phosphate (Fig. 8A); phosphate was readily separated from the labeled guanidino group in both cases. On the other hand, reaction of N[^14C]^amidinoamidinostreptamine phosphate with periodate gave a labeled product which was eluted from a Dowex 50 column at the position expected for a reaction product containing one guanidino group and one phosphate group (Fig. 8B, 0.5 N HCl). High voltage paper electrophoresis of all reaction products of Fig. 8 before and after treatment with E. coli alkaline phosphatase confirmed that, of all the reaction products, only the 0.5 N HCl eluate of Fig. 8B contained phosphate associated with the labeled guanidino moiety. These results suggest that the first compound of Fig. 9 is N-amidinoamidinostreptamine phosphate, the precursor of streptidine phosphate. The second compound of Fig. 9 would then be N'-amidinoamidinostreptamine phosphate, which cannot be further converted enzymatically to streptidine phosphate (2).

Fig. 8. Experiment comparing products of periodate degradation of certain[^14C]^guanidinoamidinostreptamine phosphate derivatives. Labeled compounds were incubated in the dark with 0.05 M sodium periodate and 0.05 M sodium acetate buffer, pH 4.6, for 5 days at room temperature. Degradation fragments containing the labeled guanidino moiety were separated by chromatography on Pasteur pipette columns containing Dowex 50(H+)-X8, 200 to 400 mesh, and eluted with a total of 5 ml of H2O, then 10 ml of 0.5 N HCl, 10 ml of 1 N HCl, and 10 ml of 2 N HCl. Fragments containing one guanidino group and one phosphate group should be eluted by 0.5 N HCl; all other combinations of labeled fragments should be eluted only by higher concentrations of acid. A, elution pattern following periodate treatment of[^14C]^guanidinoamidinostreptamine phosphate (IGP). Phosphate group and guanidino group were readily separated. (Unchanged IGP would be eluted by 0.5 N HCl.) B, elution pattern following periodate treatment of N[^14C]^amidinoamidinostreptamine phosphate (SAGP), an isomer which cannot be converted enzymatically to streptidine phosphate. The phosphate group and guanidino group were readily separated, as shown by the elution behavior and paper electrophoresis.

Fig. 7. Experiment showing that guanidinoamidinostreptamine phosphate phosphatase activity is detectable only after a phase of rapid growth on complex media. Compounds were separated on ammoniacal phenol paper chromatograms. Enzyme sources were dialyzed lysozyme extracts from S. bikiniiensis grown on peptone-yeast extract medium for time indicated. Dashed curve shows unreacted[^14C]^guanidinoamidinostreptamine phosphate (IGP) following incubation with extract from 1-day mycelia. Solid curve shows complete hydrolysis to[^14C]^guanidinoamidinostreptamine following incubation with extract from 3-day mycelia.

Fig. 9. Suggested structures for N-amidinoamidinostreptamine phosphate (Compound 1) and N'-amidinoamidinostreptamine phosphate (Compound 2). Dashed lines show bonds attacked by periodate.
**DISCUSSION**

In any organism there are a large number of phosphatases of varying degrees of specificity. Purified nonspecific alkaline phosphatases from calf intestinal mucosa or *E. coli* can hydrolyze the phosphate linkages of guanidinoscyllo-inositol phosphate (9) and streptomycin-(streptidino)phosphate (7,12). Consequently, to establish the occurrence of phosphatases specific for each of these substrates, it was necessary to separate the respective activities (Fig. 3) and test their ability to hydrolyze a number of structurally related guanidinocyclitol phosphates (Table I and Fig. 4). Our data are consistent with the occurrence of a 1-guanidino-1-deoxy-scylllo-inositol 4-phosphate phosphatase and a streptomyacin-(streptidino)phosphate phosphatase. N°-Aminostreptamine phosphate, believed to have the structure of Compound 2 of Fig. 9, is acted upon almost equally well by both phosphatases, whereas its isomer, believed to be Compound 1 of Fig. 9, was not hydrolyzed by guanidinoscylllo-inositol phosphate phosphatase. The latter enzyme apparently prefers that a guanidino group be para to the phosphate group. It will be interesting to see if the phosphatase preparation (H enzyme) of Nimi et al. (12) has a substrate specificity similar to the streptomyacin-(streptidino)phosphate phosphatase preparation described here, or whether their incubation period of 24 hours reflected hydrolysis of streptomyacin-(streptidino)phosphate by relatively nonspecific alkaline phosphatases. One test would be to allow H enzyme to react with phosphoryl-[3H]dihydrostreptomycin-(streptidino)phosphate from *S. bikiniensis*. Nonspecific alkaline phosphatases react first with phosphate esterified with the dihydrostreptomycin moiety of this compound, whereas streptomyacin-(streptidino)-phosphate phosphatase hydrolyzes phosphate esterified with the streptidine moiety (Fig. 4 and Ref. 7).

The ability of preparations containing streptomyacin-(streptidino)phosphate phosphatase activity to transfer phosphate from dihydrostreptomycin-(streptidino)phosphate or from streptidine phosphate to Tris (Fig. 5) is of considerable interest. The O-phosphoryl Tris formed was only slowly hydrolyzed by this preparation (Fig. 6). This phosphotransferase activity, which appears to differ significantly from that of *E. coli* alkaline phosphatase (16), is currently under active investigation in our laboratory.

Although our streptomyacin-(streptidino)phosphate phosphatase preparation has not been extensively purified, this enzyme appears to be specific for streptomyacin biosynthesis or metabolism. Its biological distribution seems to be confined to streptomyacin producers, and its activity appears during the same stage in the life cycle at which enzymes involved in the biosynthesis of streptidine appear. Inorganic phosphate at a concentration inhibitory to the enzyme (Table III) is added to a culture actively synthesizing streptomyacin, production of free streptidine stops and accumulation of streptomyacin-(streptidino)phosphate begins (6). Streptomyacin-(streptidino)phosphate phosphatase might well act on a number of intermediates in streptomyacin biosynthesis, if these possess a phosphorylated streptidine moiety. Streptidinokinase might then serve a salvage function in restoring the phosphate group. A regulatory function for the latter two enzymatic activities has also been suggested (6).

It is of interest that a number of the enzymes involved in streptidine biosynthesis are strongly inhibited by sulphydryl reagents; this has been observed for the aminocyclitol kinase activities (10), the amidotransferase activities (13), and now guanidinoxy-scylllo-inositol phosphate phosphatase (Table II). These three enzymatic activities all function best at approximately pH 7.4. In contrast, both streptidinokinase (10) and streptidinophosphatase react optimally at more alkaline pH levels and are not inhibited by sulphydryl reagents. Whether these relationships reflect the evolutionary development of the respective enzymes is not known.

In the biosynthesis of streptidine (Fig. 2), analogous reactions of the same type have now been found to be catalyzed by different enzymes in the cases of the two dehydrogenations, the two transaminations, and the two dephosphorylations. Neither the two aminocyclitol kinase reactions nor the two transamination reactions have been shown to be catalyzed by different enzymes. It remains to be determined whether these exceptions are apparent or real. If our periodate studies are valid (Fig. 8), taking into account the liability of cyclitol phosphate groups to periodate (17), the long-standing problem of the location of the phosphate groups of key streptidine precursors has been solved. The two aminocyclitol kinase activities are now believed to transfer a phosphate group to the hydroxyl group para to the amino group which is to be transamidinated in the following reaction (Fig. 2). Recent work (18) comparing incorporation of label into streptotmycin from [1-¹H]glucose with that from [3-¹H]glucose would appear to be consistent with certain assignments in our proposed scheme. However, the latter experiments unfortunately lacked certain positive internal controls to establish that reduced incorporation of label from [3-¹H]glucose into all three moieties of streptomyacin was due to the mechanisms postulated (18), and not due to loss of label from that position in a reaction unrelated to streptotmycin biosynthesis (19). Further studies are, of course, necessary before the scheme of Fig. 2 can be regarded as conclusively established.

**REFERENCES**

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