STREPTOMYCIN BIOSYNTHESIS: PARTICIPATION OF A PHOSPHATASE, AMINATING ENZYME, AND KINASE IN CELL-FREE SYNTHESIS OF STREPTIDINE-P FROM INOSAMINE-P.

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Received December 20, 1966

Aminocyclitol derivatives are components of a number of antibiotics, including streptomycin, kanamycin, neomycin, hygromycin, actinospectacin, and bluensomycin (Dutcher, 1963). Consequently the enzymic reactions by which amino groups are substituted for hydroxyl groups on the cyclitol ring are of considerable interest. The first such amination to be reported is described here: addition of the second amino group in the biosynthesis of the streptidine moiety of streptomycin. We have found that cell-free preparations of Streptomyces bikiniensis catalyze the following reactions: inosamine-P $\xrightarrow{E}$ N-amidinoinosamine-P $\xrightarrow{F1}$ N-amidinoinosamine $\xrightarrow{F2}$ $\xrightarrow{F3}$ N-amidinostreptamine $\xrightarrow{F4}$ N-amidinostreptamine-P $\xrightarrow{G}$ streptidine-P. Reactions E and G are transamidination reactions, apparently catalyzed by the same amidinotransferase (Walker and Walker, 1966, 1967). Reactions F1 through F4 have not previously been described.

Materials and Methods -- Mycelia of S. bikiniensis ATCC 11062 were grown at room temperature on a rotary shaker for 3 days on 2% peptone-0.2% yeast extract, harvested by filtration, and frozen. Extracts were prepared by adding an equal weight of water to minced frozen mycelia and sonicating the chilled suspensions with a Biosonik II, in 1-min bursts to avoid overheating, followed by centrifugation for 30 min at 30,000 x g. Reactions F1 through F3 were carried out with non-dialyzed supernates of 14-min sonicates. Reaction F4 was performed with dialyzed supernates of 2-min sonicates. Labelled compounds were isolated by adsorption on 1 x 22 cm columns containing Dowex-50(H^+), 200-400 mesh, followed by elution with HCl solutions; 5 µl aliquots of the ca. 3 ml fractions

were spotted on paper and counted with a liquid scintillation system. Labelled compounds were characterized before and after treatment with Escherichia coli alkaline phosphatase by means of high voltage paper electrophoresis, 30 volts per cm, at pH 3.6 (NH_4formate) and pH 10.4 (NaOH-glycine), and by paper chromatography with a solvent of 80% phenol-20% water, NH_3 atmosphere (Walker and Walker, 1966, 1967), as shown in Table I. Labelled inosamine-P was isolated from mycelia fed myo-inositol-2-^{14}C (Walker and Walker, 1967). "Natural" N-amidino(^{14}C)-inosamine-P was prepared by enzymic transamidination, with L-arginine-guanidino-^{14}C as donor, of hot-water extracts of mature mycelia, followed by column isolation. "Synthetic" N-amidino(^{14}C)-inosamine-P was prepared by chemical phosphorylation (Walker and Walker, 1966) of inosamines obtained by reduction of scyllo-inosose oxime with sodium amalgam (Anderson and Lardy, 1950), followed by enzymic transamidination and column isolation as before.

TABLE I

Mobilities of Various Aminocyclitol Derivatives

R_F values are for paper chromatograms with ammoniacal phenol solvent. Paper electrophoretic mobilities are distances traveled relative to migration of picric acid. A minus sign indicates migration toward the negative pole, a positive sign, migration toward the positive pole.

Compound	R_F	Electrophoretic mobility relative to picric acid	
		pH 3.6	pH 10.4
scyllo-Inosamine	0.43	-1.15	-0.60
scyllo-Inosamine-P	0.12	-0.31	+0.90
N-Amidino-scyllo-inosamine	0.67	-1.15	-1.31
N-Amidino-scyllo-inosamine-P	0.17	-0.31	+0.16
N-Amidinostreptamine	0.83	-2.05	-1.33
N-Amidinostreptamine-P	0.33	-0.91	+0.15
Streptidine	0.94	-1.98	-1.66
Streptidine-P	0.46	-0.83	-0.47

Transformations of Inosamine-P-- When labelled inosamine-P was incubated with a 14-min sonicate supernate containing added arginine, N-amidinoinosamine-P was formed, as reported previously. In addition, smaller amounts of compounds tentatively identified as N-amidinoinosamine and N-amidinostreptamine were also formed.

Transformations of N-Amidinoinosamine-P-- N-Amidino(^{14}C)-inosamine-P, natural or synthetic, was readily converted by the 14-min sonicate supernate to N-amidinoinosamine and N-amidinostreptamine, as shown in Fig. 1A. Incubation conditions were as follows: N-amidino(^{14}C)-inosamine-P (synthetic), 1.0 ml (6.6 x 10^6 c.p.m.); 14-min sonicate supernate, 1.0 ml; 1 M Tris buffer, pH 7.4, 0.2 ml; incubated in 50 ml beaker for 2.5 hr at 37°. The reaction was stopped with 0.2 ml of 30% trichloroacetic acid, and the supernate plus washings were chromatographed (Fig. 1A). Pooled fractions were evaporated to dryness, and mobilities of the isolated compounds were compared with model compounds as indicated in Table I (cf. Walker and Walker, 1967). N-Amidino(^{14}C)-neo-2-inosamine-P was not significantly dephosphorylated nor aminated by this preparation.

Conversion of N-Amidinoinosamine to N-Amidinostreptamine-- N-Amidino(^{14}C)-inosamine, prepared by treatment of its phosphorylated derivative with E. coli

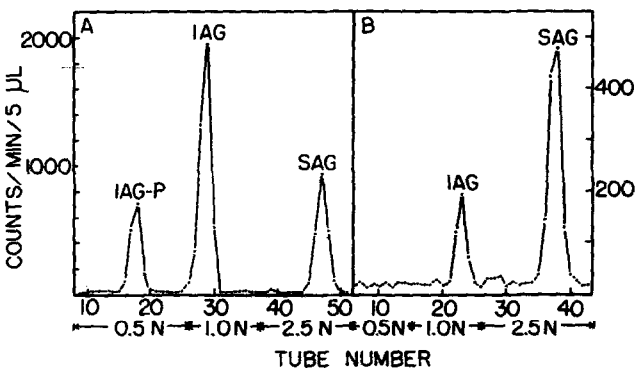

Fig. 1. Column separation of reactants and products in enzymic conversions of: A, 0-phosphoryl-N-amidinoinosamine (IAG-P) → N-amidinoinosamine (IAG) → N-amidinostreptamine (SAG); B, N-amidinoinosamine → N-amidinostreptamine. Water washes prior to elution with indicated concentrations of HCl. For details see text.

with antisera prepared against the S and D strains. This was also the case with cell walls of R- and R+ strains of *B. cereus T*; however, R- and R+ walls of *E. coli* could be so distinguished, the walls of R- *E. coli* agglutinating with a higher dilution of antiserum against Meg S walls and the walls of R+ *E. coli* agglutinating with a higher dilution of Meg D antiserum (Table 2).

Table 2

Agglutination titers of pooled anti-megaterium sera against fractions of streptomycin-sensitive and streptomycin-dependent strains of other species and genera.

Imm. Serum	Test antigens									
	B.cereus T pure walls†		S. aureus pure walls		S. aureus whole cells		S. lutea pure cells		E. coli pure walls	
	S	D	S	D	S	D	S	D	R(-)	R (+)
Meg S crude	x	x	x	x	32	<4	x	x	x	x
Meg D crude	x	x	x	x	8	128	x	x	x	x
Meg S pure	64	8	32	4	<4	<4	16	<4	32	4
Meg D pure	4	16	8	16	<4	256	8	64	16	256

x Not tested
S = fraction of streptomycin-sensitive strain.
D = fraction of streptomycin-dependent strain.
† = In addition to the pure walls of S & D strains of *B. cereus T*, the pure walls of R(-) & R(+) strains of this organism were tested against antisera to Meg S and Meg D pure walls. In each of these latter two cases, the titer was 1:8.

That this antigenic specificity crosses species and generic lines can be seen in Table 2. Here also common antigens were present, as has been found by several workers using cell wall preparations of various bacteria (Abdulla and Schwab, 1965; Wiseman, 1963; Cummins, 1962).

ornithine prevents transamidination to streptidine-P. ATP cannot be replaced by UTP, GTP, or CTP; dATP is as effective as ATP. The phosphorylation is rather specific for N-amidinostreptamine, since neither its positional isomer, N'-amidinostreptamine, derived from enzymic transamidination of streptamine-P (Walker and Walker, 1966, 1967), nor various N-amidinoinosamine's are phosphorylated. myo-Inositol is not inhibitory at 0.03 M.

Discussion-- Data presented in this paper are compatible with the biosynthetic scheme of Fig. 3. We have shown that each of the intermediates is a precursor of the following compound in cell-free extracts. Moreover the enzymes observed for the first time, i.e., the phosphatase, aminating enzyme, and kinase, were found to be relatively specific. However, configurations of a

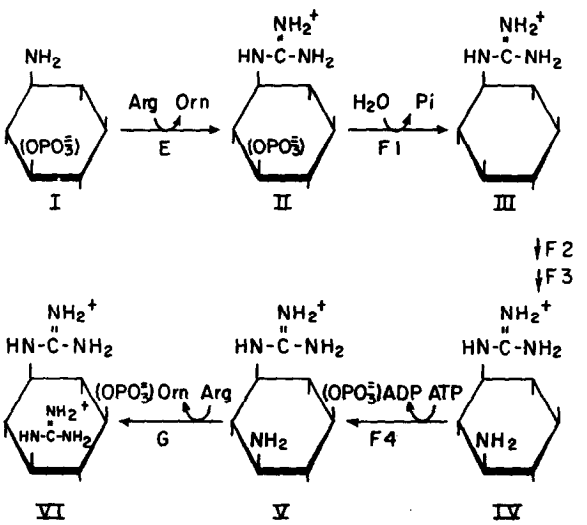

Fig. 3. Proposed scheme for synthesis of streptidine-P from inosamine-P by extracts of mature mycelia of streptomycin-producing strains of Streptomyces. I, scyllo-inosamine-P; II, N-amidino-scyllo-inosamine-P; III, N-amidino-scyllo-inosamine; IV, N-amidinostreptamine; V, N-amidinostreptamine-P; VI, N,N'-diamidinostreptamine-P (streptidine-P).

number of the intermediates have not yet been established with certainty. In particular, the position of the phosphate group is not known for any compound; the positions suggested in Fig. 3 are consistent with participation of a single amidinotransferase in Reactions E and G, but other assignments

are also possible. We have designated the first amino group to be transamidinated as N- and the second amino group as N'- ; the absolute assignments cannot yet be made. We have suggested that transamidination of chemically phosphorylated streptamine gives the N'-amidinostreptamine-P isomer (Walker and Walker, 1967). The fact that the dephosphorylated derivative of the latter compound is not a substrate for N-amidinostreptamine kinase supports this suggestion.

Acknowledgments-- This work was supported by grants from the National Institute of General Medical Sciences (GM-12807), USPHS, and the Robert A. Welch Foundation. We thank Miss I. Grups and Mrs. J. Collins for their aid.

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